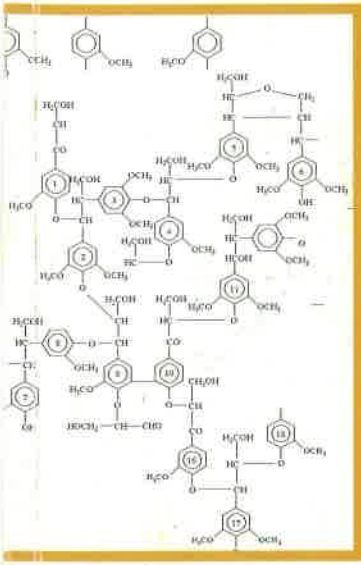

6th BSCLWC

6th Brazilian Symposium on the Chemistry of Lignins and other Wood Components – Volume VII (2001)

Proceedings

October 25-28, 1999, Clube dos 500 Hotel, Guaratinguetá, SP, Brasil

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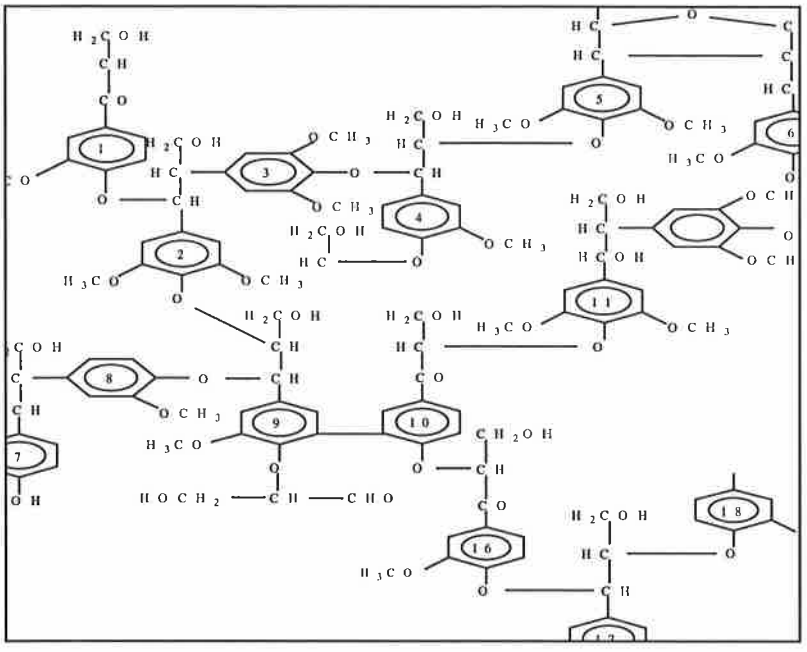

Volume VII (2001)

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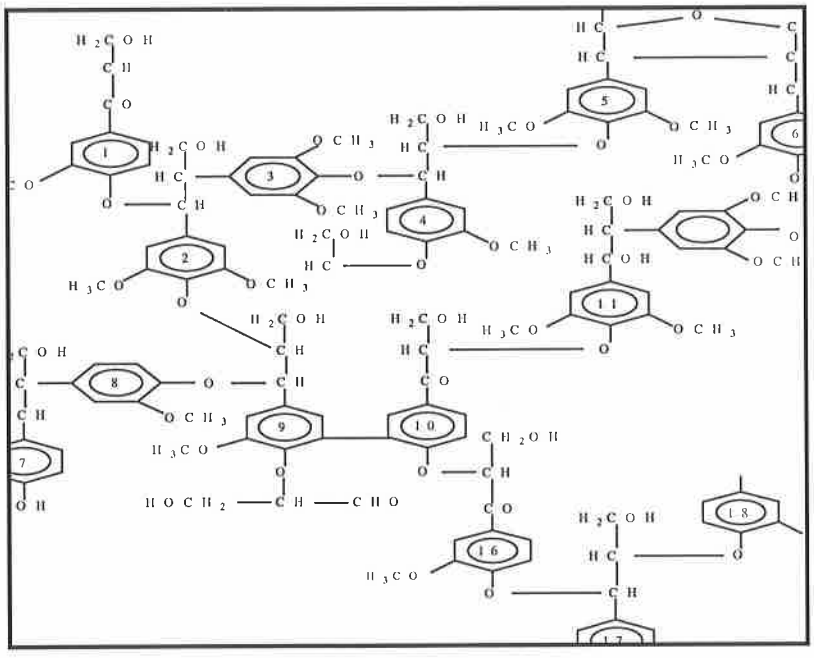

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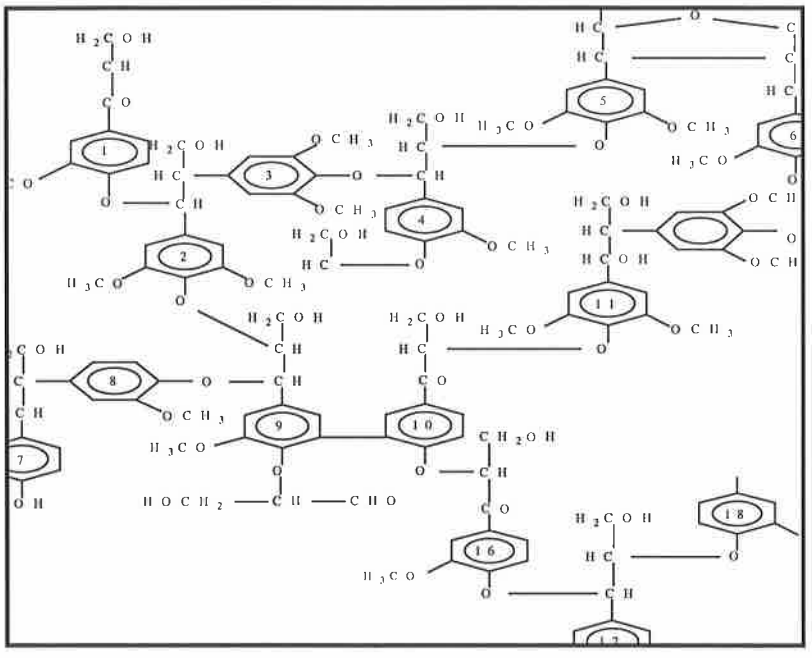

Opening Conference

Development of the lignocellulosics materials research in Brazil analyzed through the Brazilian Symposium on the Chemistry of Lignins and other Wood Components

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ABSTRACT

From the First Brazilian Symposium on the Chemistry of Lignins and Other Wood Components that was held in São Paulo-São Carlos in September 1989, other four Symposiun were organized. It will be done a statistical discussion related to participants' countries, authors, papers, conferences, posters and the development of the wood chemistry and biotechnology through all of these nine years. Some new aspects on biomass reutilization will be discussed on bases of the actual international symposia.

Keywords: Brazil, lignin, wood, lignocellulosic materials, cellulose, pulping, bleaching, ethanol production

GENERAL ASPECTS

The FIRST BRAZILIAN SYMPOSIUM ON THE CHEMISTRY OF LIGNINS AND OTHER WOOD COMPONENTS was held at the UNIVERSIDADE DE SÃO PAULO-SÃO CARLOS, SÃO CARLOS, SP. (1989) and the Proceedings edited by Prof. Drs. Robert R.A.M. de Groote and Antonio A.S. Curvelo in 1989. In that opportunity 6 different countries participating actively in the Symposium publishing their presentations. These participants were represented by 91 authors with 43 papers, distributed in 77% in wood chemistry and 33% in biotechnology (Table 1).

The SECOND BRAZILIAN SYMPOSIUM was held at the UNIVERSIDADE ESTADUAL DE CAMPINAS, CAMPINAS, SP (1991), and the Proceedings edited by Prof. Drs. Nelson Durán and Elisa Esposito in 1992, and this was the first Proceeding on the Symposium indexed in Chemical Abstract (Chem. Abstr. 117, B214785t (1992)). 104 authors representing 10 countries presented 46 papers distributed 65% wood chemistry and 35% in biotechnology (Table 1).

The THIRD BRAZILIAN SYMPOSIUM was held at the UNIVERSIDADE FEDERAL DE MINAS GERAIS, BELO HORIZONTE, MG (1993) and the Proceedings edited by Prof. Drs. Dorila Piló Veloso and Reinaldo Ruggiero in 1995 (Chem. Abstr. 124, 120459g (1996)). It was presented 65 papers by 146 authors, representing 12 countries. The papers distribution was 72% in wood chemistry and 28% in biotechnology (Table 1).

The FOURTH BRAZILIAN SYMPOSIUM was held at the UNIVERSIDADE FEDERAL DE PERNAMBUCO, RECIFE, PE, (1995) and the Proceedings edited by Prof. Drs.Eduardo H.M. Melo, Maria C.B. Pimentel, Alexandra A. Salgueiro and Cesar A.M. Abreu in 1996 (Chem. Abstr. 126, 90906 (1997)). In this opportunity a significant improvement in the area distribution was observed. A 59% of wood chemistry and 41% in biotechnology of the 51 papers presented. A 125 authors from 11 countries participated actively in the symposium.

The FIFTH BRAZILIAN SYMPOSIUM was held at UNIVERSIDADE FEDERAL DE PARANA, CURITIBA, PR (1997) and the Proceedings was edited by Prof. Dr.Luiz P. Ramos in 1997 (Chem. Abstr. 128, 4776 (1998)). In this opportunity were the largest countries and authors participating actively in the Symposium. A notorious enhancement in the biotechnology (48%) related to wood chemistry (52%) was observed.

SPECIFIC ASPECTS

Table 2 shows the active participant countries distribution. Besides the Brazilian researchers the most active countries in these five symposia were Chile, Uruguay, USA and Germany.

Table 1. Brazilian Symposium on the Chemistry of Lignins and other Wood Components. Summary of papers, conferences and participants

Symposium and year	Place	Countries	Authors	Papers	Authors per papers	Conferences	Posters	Wood Chemistry	Biotechnology
1º - 1989	USP São Carlos	6	91	43	2.1	11	32	33	10
2º - 1991	UNICAMP	10	104	46	2.3	14	32	30	16
3º - 1993	UFMG Belo Horizonte	12	146	65	2.3	18	47	47	18
4º - 1995	UFPE Recife	11	125	51	2.5	10	41	30	21
5º - 1997	UFPR Curitiba	14	173	75	1.4	7	68	39	36

All data were extracted from the published Proceedings

Table 2. Number of participants from different countries

Country	1989	1991	1993	1995	1997
Argentina	5	0	0	1	0
Australia	0	0	0	0	1
Brazil	48	49	77	67	122
Canada	0	0	0	0	4
Costa rica	0	0	3	0	0
Cuba	0	13	10	0	0
Chile	24	18	22	21	9
Denmark	0	0	0	0	7
England	0	1	1	1	2
Finland	0	0	0	6	0
France	2	1	1	0	2
Germany	11	7	6	3	3
Italy	0	0	0	0	5
Portugal	0	1	1	2	5
Russia	0	0	0	3	2
Spain	0	0	0	0	3
Sweden	0	1	5	0	0
Uruguay	0	9	10	7	1
USA	1	4	6	7	7
Yugoslavia	0	0	4	7	0
Total of participants (number of countries)	91(6)	104 (10)	146 (12)	125 (11)	173 (14)

Other aspect is the topic of interest in these symposia. Table 3 shows that the research topics in wood chemistry were characterization of lignocellulosics, organosolv pulping, utilization of biomass, chemical pulping and bleaching and remediation of waste materials from pulp and paper industry. The chemical pulping and bleaching and remediation were improved through these years. Utilization and characterization were maintained in the same level. However, organosolv pulping was significantly decreased in the last two years.

Table 4 shows that the main topics in the biotechnological area were bioconversion, bioremediation, enzymes utilization, biopulping, biobleaching and products production.

Table 3. Research topics on wood chemistry

Symposium	Year	Characterization	Organosolv	Utilization	Pulping and Bleaching	Remediation	Total of papers
1º	1989	13	13	7	0	0	33
2º	1991	6	10	9	5	0	30
3º	1993	7	12	21	0	7	47
4º	1995	5	4	9	6	5	30
5º	1997	9	5	9	11	5	39

Table 4. Research topics on biotechnology

Symposium	Year	Bioconversion	Bioremediation	Enzymes	Biopulping	Biobleaching	Products	Total of papers
1º	1989	3	0	6	1	0	0	10
2º	1991	2	7	4	1	1	1	16
3º	1993	0	6	7	3	0	1	18
4º	1995	4	8	2	3	4	0	21
5º	1997	8	4	17	3	1	3	36

Bioconversion, enzymes and biopulping have an increasement in these years. However, bioremediation and biobleaching the tendency is to stabilize at a 8 paper by symposium. We have to notice that a significant increase in the number of biotechnological papers and total percentage in the last two year was observed.

Table 5. Wood materials/Non-woody materials

Symposium	Year	Total of papers	Wood	Non-wood	Types of non-wood
1º	1989	43	38	5	Annual plants, bagasse, corn, mimosa
2º	1991	46	39	7	Bagasse, corn, orange residue
3º	1993	65	57	8	Annual plants, bagasse, corn, mimosa
4º	1995	51	45	6	Corn, bagasse, grass, straw
5º	1997	75	40	11	Bagasse, oil palm, wheat straw

Table 6. Alcohol production and related subjects

Symposium	Year	Total of papers	Alcohol related subjects	Comments
1º	1989	43	0	---
2º	1991	46	2	Steam explosion bagasse, simultaneous hydrolysis and fermentation, bagasse
3º	1993	65	1	Pre-treatment and enzymatic degradability
4º	1995	51	0	---
5º	1997	75	5	Bioconversion, ethanol-Canada, conversion of wheat straw to ethanol, enzyme digestibility, biotransformation of lignocellulosic materials

Table 5 shows the distribution in term of wood and non-woody materials in the 9 years period. Sugar cane bagasse appeared as the main subject, besides the other lignocellulosic as corn, cassava and wheat straw. Although the non-woody material was increased from 1989 still the number is below of the expected value. Worse is the case of alcohol production and related subjects (Table 6). Only 8 papers during 9 years were presented. A more significant enhancement in the 5th. Symposium in Curitiba was observed.

I think that our biomass researchers should be more concern with the new impact of alcohol production in USA, Asia and Europe. A good example was the 20th. Symposium on Biotechnology for Fuel and Chemical, published as Proceedings in Appl. Biochem. Biotechnol. Vol. 77-79, 1999. A number of Brazilian research groups have been involved with the development of bioprocesses related to either fermentation or biocatalysis. Some of these works was presented in that opportunity. It should also be noted that more than 25% of all the poster presentations were from Brazil. The centers were represented by Universidade de Maringá, Universidade Federal de Parana, Universidade Federal do Rio de Janiero, Universidade de São Paulo, Universidade Federal de são Carlos, Fundação Centro Tecnologico de Minas Gerais, Departamento de Biotecnologia-FAENQUIL and Universidade Estadual de Campinas.

Also we have to notice that research work related to bioresources utilization for chemicals and fuels is growing in importance in Brazil. Meetings on bioprocesses, bioresources and enzyme technology have been organized regularly beside our symposia. Most of the research work related to fermentation processes such as ethanol from sugar cane has been presented at the national Fermentation Symposium (SINAFERM-São Carlos-S.P.). Other important meeting is the Seminar on Enzymatic Hydrolysis of Biomass (SHEB-Maringa-PR)) which is mostly related to the biotransformation of lignocelllulose, starch and sucrose into fuels and chemicals. It also covers effluent treatment and biobleaching. The Brazilian Seminar on Enzyme Technology (ENZITEC-Rio de Janeiro, RJ.) and its aims is to improve enzyme technology in Brazil, i.e. production,

characterization and use of industrial enzymes. We have to notice that although Brazil is the "biomass and biodiversity" country it only contributes 3% to the world enzyme market.

FINAL REMAKS

In view of our long history in the Brazilian Symposium of the Chemistry of Lignin and Other Wood Components, and the increasing interest in the biomass utilization and alcohol production, I would like to comment the excellent view of a personal perspective of biofuels done by Prof. Elba Bom in "Liquid Biofuels in Brazil: Let's think about it" which appeared in New Lett-IEA Bioenergy, May 1998, which is absolute valid in this moment.

The 13 billion liters of alcohol produced by year in Brazil for the foreign countries it would seem that our country could easily implement a program that gradually could substitute the ethanol for gasoline. But this is not true, since there are some important aspects of biofuel production that must be addressed before this will come pass in Brazil.

a) Even though many industries produce both sucrose and ethanol is a sound long-term strategy? The current profitable sugar cane industry requires specific climate and soil conditions that are not found everywhere in the country, and then this is a limitation.

b) Can we afford to rely only on sugar cane and not make an effort to diversify our source of fermentable sugars to alternatives such starch and lignocellulose? The answer is obvious, of course not!

c) What is the next candidate? Starch from cassava, another traditional crop with fewer requirements to grow than sugar cane. Brazil is also one of the largest producers of cassava in the world. As starch is not directly fermentable it must be first hydrolyzed. The drawback is the need of alpha-gluco-amylases to hydrolize it. Although the alcohol from cassava is being produced in Brazil, it represents a minor contribution to the total ethanol production. We have to remeber that in USA the alcohol from corn starch is actually a reality.

d) What is the next in line? It is clear that the best source of lignocellulose in Brazil is sugar cane bagasse There are processing problems associated with sugar cane bagasse. However the processing problem is related to lignocellulosic nature and therefore the problems are not unique to Brazil. A lignocellulosic feedstock requires a pre-treatment plus hydrolysis (here is the enzyme issue again) to be fermentable. Pre-treatment it has been studied in Brazil and abroad and it seems it does not represent a real bottleneck. Hydrolysis is!

As conclusion, Profa. Bom pointed out that we must diversify the sources of fermentable sugar because it is wise and more logical to produce liquid fuel from agricultural waste rather than land suitable for food. She is convinced that still have the challenge of cheap industrial enzyme availability ahead of us.

Considering now the most vulnerable point of biotechnological processes, i.e. economical feasibility. These processes will always be a disadvantage when compared with traditional chemical processes due to its own nature, that you can improve but never change completely. Solutions? Put the price tag on the environmental recovery for each of the traditional chemical process and things will appear much better for biotechnology. She hopes that we can do it. Heavy chemical, highly polluting industries are presently operating or moving to Brazil. Prof. Bom finished her personal perspective with the following " The country is big, the environmental regulation of control can be loose and as the current pollution is increasing we can not forget that there are no winners in this game as we all live on the same planet".

Finally, I also hope that in the next Symposia the biomass reutilization and alcohol production (as of course the revival of alcohol chemistry) being important issues to discuss in the next years.

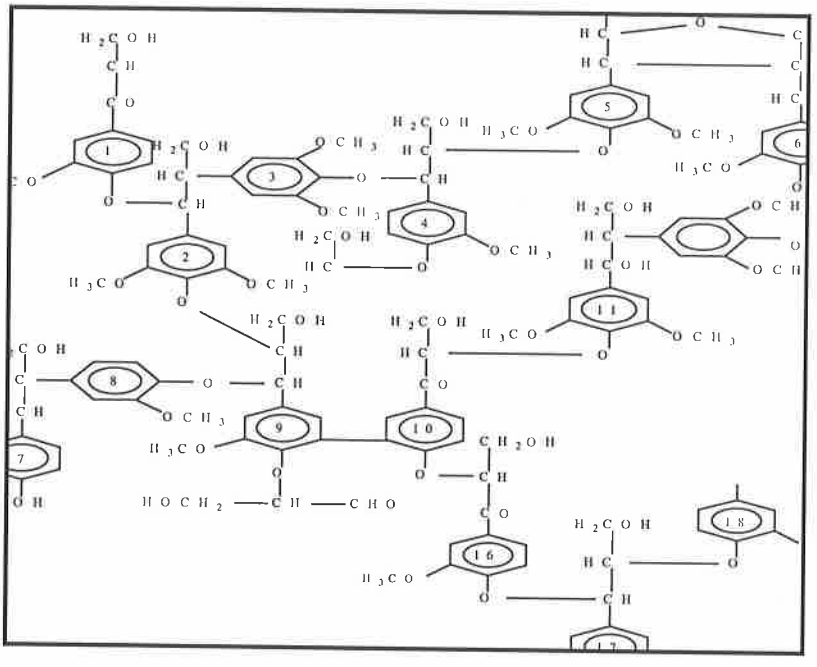
REFERENCES

Bom, E. (1998). Liquid biofuel in Brazil: Let's think about it. *Newsletter-IEA Bioenergy*. (2) May, 12-13.

Davison, B.H. & Finkelstein, M. Eds. (1999). 20th. Symposium on Biotechnology for Fuels and Chemicals. *Appl. Biochem. Biotechnol.* **77-79,** 1-631.

6th BS CLWC 6th Brazilian Symposium on the Chemistry of Lignins and other Wood Components

October 25-28, 1999, Clube dos 500 Hotel, Guaratinguetá, SP, Brazil



Oral Session

Acid-catalyzed steam explosion of *Pinus taeda* chips: II. Surface response in relation to the yield of water-insoluble fraction and alkali-soluble lignin

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ABSTRACT

Acid-catalyzed steam explosion of *Pinus taeda* was carried out under distinct pretreatment conditions to optimize lignin recovery. Apart from the water-soluble fraction, which contained a considerable amount of fermentable sugars, steam explosion allowed good recovery of a water-insoluble (WI) fraction mainly composed of cellulose and lignin. From this WI fraction, most of the lignin component was recovered by alkali extraction with NaOH 0.1 mol.l^{-1}. The remaining cellulosic fiber, which contained 10-15% of alkali-insoluble lignin, was considered a viable alternative for paper making, whereas the recovered lignin has a great potential as a chemical feedstock for several industrial uses (e.g., synthesis of polyurethanes). In this study, the optimal conditions for obtaining both fibers and alkali-soluble lignin (ASL) from *P. taeda* were determined through an experimental design. The data thus obtained was subsequently used to describe the surface response to the most important pretreatment parameters. In general, lignin yields decreased with increased residence time into the steam gun, which suggests condensation reactions followed by the reprecipitation of steam-exploded lignin onto the cellulose fibers. Therefore, the residence time into the steam gun was also considered one of the pretreatment parameters of greatest importance. Variations in experimental results revealed the difficulties in obtaining products quantitatively and process losses may have also interfered with the experimental data in some experiments.

Keywords: steam explosion, acid hydrolysis, *Pinus taeda*, lignin

INTRODUCTION

Steam explosion is a process whereby biomass is treated with high-pressure steam under optimal conditions and subsequently quenched to atmospheric by adiabatic expansion of the reactor content. This process has been widely applied to enhance bioconversion of lignocellulosics (Brownell and Saddler, 1984; Excoffier *et al.*, 1991; Ramos *et al.*, 1992a) and the optimal conditions usually used are those in which the best substrate for hydrolysis is produced with the least material lost due to decomposition reactions. Several advantages have been associated with steam explosion. They are: (a) the effective fractionation of wood into its three main components, cellulose, hemicellulose and lignin; (b) theoretical yields of cellulose conversion can be achieved through enzymatic hydrolysis; (c) after steam treatment, the lignin component is still suitable for conversion to chemical products; (d) hemicellulose can be fully utilised and converted to liquid fuels; (e) fermentation inhibitors are readily extracted by water-washing; (f) the method is effective for a wide variety of plant biomass, and (g) pretreatment conditions can be easily optimised to produce specific chemicals at high yield (Saddler *et al.*, 1993).

The major disadvantage is that, although steam explosion greatly enhances the enzymatic hydrolysis of hardwoods (Brownell and Saddler, 1984; Dekker *et al.*, 1987; Excoffier *et al.*, 1991; Ramos *et al.*, 1992a,b) and most agricultural residues (Dekker and Wallis, 1983) tested to date, it has not yet been successfully developed for the use of softwoods. Furthermore, steam explosion produces a relatively low bulk density substrate which has to be washed to remove fermentation inhibitors.

In this study, different conditions for obtaining both cellulose fibers (FIB) and alkali-soluble lignin (ASL) from *P. taeda* were used, based on an experimental design, to evaluate the process of acid-catalyzed steam explosion (Barros Neto *et al.*, 1995). The data thus obtained was subsequently used to describe the surface response to the most important pretreatment parameters.

EXPERIMENTAL

Pretreatment of *Pinus taeda* wood chips

Acid-catalyzed steam explosion of industrial chips derived from 4 year-old stems of *P. taeda* (Klabin Fabricante de Papel e Celulose Ltda., Telêmaco Borba, PR, Brazil) were steam-treated at the Pilot Plant Unit of the Federal University of Paraná using a 5L steam gun (Ramos *et al.*, 1995) under distinct pretreatment conditions to optimize lignin recovery.

Chips were impregnated with dilute sulfuric acid (H_2SO_4) and saturated steam temperatures ranging from 195 to 210°C were used over a range of residence times (1.7-5.9 min). Acid impregnation was carried out by soaking the wood chips in either 0.33 to 1.17 (w/v) H_2SO_4 overnight, at room temperature. The excess of H_2SO_4 solution was discarded. Acid-impregnated chips were shown to have approximately 50% (w/w) moisture content. Steam pretreatment was performed by loading the chips directly into the steam gun. After treatment for a given temperature and residence time, the steam treated pine (STP) was released from the steam-gun by rapid depressurization of the vessel, causing the material to expand (explosion) into a stainless steel cyclone. Solid and non-volatilized biomass were then recovered by water washing. The resulting STP was extracted twice with water at a 5% (w/w) solids concentration. The water insoluble fraction was termed STP-WI, whereas the water soluble fraction or liquor was termed STP-WS.

Lignin extraction by alkali washing

After water washing, STP-WI were extracted twice with alkali (NaOH, 0.4% (w/v), for 1 h with stirring at room temperature) at a 5% (w/w) solids concentration. The alkali-insoluble fraction was termed cellulose fibers (FIB) and its recovery yield was determined gravimetrically in relation to the dry weight of the untreated wood chips. The alkali-soluble lignin (ASL) was obtained from the extraction liquor by acid-precipitation. The liquor was acidified to pH 2 with sulfuric acid under continuous stirring at room temperature. The insoluble material was recovered by centrifugation and dried at 60°C.

Description of the experimental design.

Chips of *Pinus taeda* (near by 500 g) were pretreated under eight distinct conditions in two levels (Table 1) to estimate the influence of the reaction temperature, catalyst concentration and residence time into the steam reactor.

Table 1. Levels of the reaction temperature (**T**), sulfuric acid concentration (**H**) and residence time into the steam reactor (**t**).

Level	T (°C)	H (%)	t (min)
-1.0	195	0.50	2.50
0	202	0.75	3.75
+1.0	210	1.00	5.00

RESULTS AND DISCUSSION

Table 2 shows the yields of cellulose fibers (FIB) and yields of alkali-soluble lignin (ASL) used to estimate the influence of temperature (T), acid concentration (H) and time (t) on pretreatment efficiency. The 2^3 experimental design (Table 3) confirmed the importance of higher acid concentrations in predicting FIB yields, primarily due to the greater capability of pretreatment to hydrolyze glycosidic bonds in softwoods. There was a greater reduction in the FIB mean when high acid concentrations were used, as compared to the effect when lower acid concentrations were applied. On the other hand, time of steaming showed an opposite behavior. A considerable interaction of temperature with acid concentration and residence time into the steam reactor was also worth noticing. Once again, there was a positive effect on the FIB mean value (FIB average yield) and this suggested the occurrence and eventual supremacy of degradation reactions upon hydrolysis of glycosidic bonds. The complexity of this heterogeneous behavior can be best observed in Figure 1.

Compared to FIB, the yield of alkali-soluble lignin (ASL) showed an opposite behavior in relation to acid concentration and reaction time. ASL increased strongly in the experiments where higher acid concentration were used and decreased in the experiments on longer reaction times were applied (Figure 2). This fact suggested hydrolytic reactions prevailed over condensation reactions during pretreatment and this may have limited the previously described reprecipitation of steam-exploded lignin onto the cellulose fibers (Ramos *et al.*, 1999).

Table 2. Experimental design 2^3.

Exp.	Run	T	H	t	FIB, %	ASL, %
1	1	-1	-1	-1	85.27	4.16
2	16	+1	-1	-1	70.71	5.57
3	2	-1	+1	-1	69.21	8.37
4	17	+1	+1	-1	73.99	6.93
5	6	-1	-1	+1	87.86	3.05
6	3	+1	-1	+1	92.59	1.62
7	7	-1	+1	+1	80.87	3.90
8	4	+1	+1	+1	89.86	7.08

It has been reported that, during steam explosion, the lignin shield is chemically modified at temperatures usually beyond its vitreous transition and that lignin depolymerization occurs primarily through hydrolysis of β-arylether and other acid-labile linkages (Tanahashi, 1990). However, at exceedingly higher pretreatment severities, lignin condensation occurs yielding by-products which are likely to have very low solubility in both acid and alkali conditions (Ramos *et al.*, 1999). This observation has two very important implications. One is that the formation of these extraneous polymeric materials cause an apparent increase in the actual amount of acid-insoluble lignin (Klason lignin) in the steam-treated fiber. On the other hand, these by-products are likely to be enriched within the steam-treated fiber by alkali-washing, therefore increasing their detrimental effects on substrate accessibility. If one assumes that lignin condensation leads to a higher degree of hydrophobicity, the detrimental effects of these by-products on substrate accessibility would result from their redistribution onto the cellulosic fibers. This would not only restrict the available surface area of the substrate but also increase the relative amount of non-specific and/or non-productive binding of conversion catalysts (e.g., enzymes) onto the substrate (lignin coating of cellulosic fibers) (Ramos *et al.*, 1999).

Table 3. 2^3 experimental design.

Factor	factor name	FIB, %	ASL, %
Mean		81.30	5.08
T	Temperature	0.98	0.43
H	Acid	**-5.62**	**2.97**
t	Time	**13.00**	**-2.34**
TH		**5.90**	0.44
Tt		**5.88**	0.44
Ht		0.76	0.18
THt		-3.77	1.86

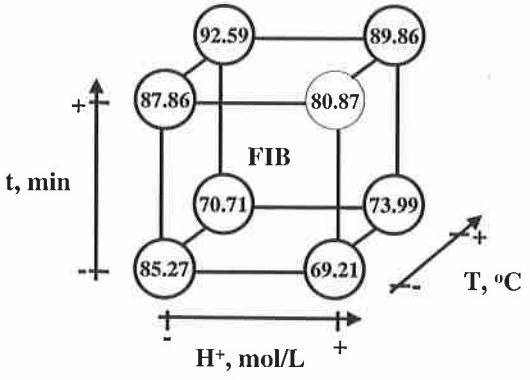

Figure 1. Trends on yield of cellulose fibers as a function of temperature, acid concentration and time of the reaction.

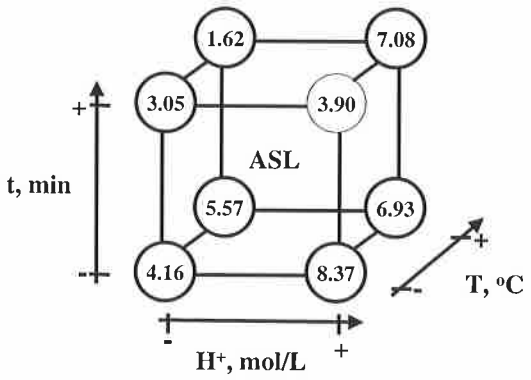

Figure 2. Trends on yield of alkali-soluble lignin as a function of temperature, acid concentration and time of the reaction.

CONCLUSIONS

Apart from the water-soluble fraction, which contained a considerable amount of fermentable sugars, steam explosion allowed good recovery of a water-insoluble (WI) fraction mainly composed of cellulose and lignin. From this WI fraction, most of the lignin component was recovered by mild alkali extraction. The remaining

cellulosic fiber, which contained 10-15% of alkali-insoluble lignin, was considered a viable alternative for paper making, whereas the recovered lignin has a great potential as a chemical feedstock for several industrial uses (e.g., synthesis of polyurethanes).

The 2^3 experimental design showed the importance of higher acid concentrations to hydrolyze glycosidic bonds in softwoods and allow for higher lignin yields by alkali washing. On the other hand, lignin yields decreased with increased of the residence time into the steam gun, which suggests the reprecipitation of steam-exploded lignin onto the cellulose fibers. Therefore, the residence time into the steam gun was considered one of the pretreatment parameters of greatest importance. The reproducibility of experiments carried out at the center point was low and this revealed the difficulty in obtaining products quantitatively, probably due to process heterogeneity and losses that may have interfered with the experimental data.

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REFERENCES

Barros Neto, B., Scarminio, I.S. & Bruns, R.E. (1995). Planejamento e otimização de experimentos. Unicamp, Campinas, pp 299.

Brownell, H. H. & Saddler, J. N. (1984). Steam-explosion pretreatment for enzymatic hydrolysis. *Biotechnol. Bioeng. Symp.*, **14**, 55-68.

Dekker, R. F. H. & Wallis, A. F. A. (1983). Enzymic saccharification of sugarcane bagasse pretrated by autohydrolysis-steam explosion. *Biotechnol. Bioeng.*, **25**, 3027-3048.

Dekker, R. F. H., Karageorge, H. & Wallis, A. F. A. (1987). Pretreatment of hardwood (*Eucalyptus regnans*) sawdust by autohydrolysis explosion and its saccharification by Trichodermal cellulases. *Biocatalysis*, **1**, 45-59.

Excoffier, G., Toussaint, B. & Vignon, M. R. (1991). Saccharification of steam-exploded poplar wood. *Biotechnol. Bioeng.*, **38**, 1308-1317.

Ramos, L. P., Breuil, C., Kushner, D. N. & Saddler, J. N. (1992a). Steam pretreatment conditions for effective enzymatic hydrolysis and recovery yields of *Eucalyptus viminalis* wood chips. *Holzforschung*, **46**, 149-154.

Ramos, L. P., Breuil, C. & Saddler, J. N. (1992b). Comparison of steam pretreatment of eucalyptus, aspen, and spruce wood chips and their enzymatic hydrolysis. *Appl. Biochem. Biotechnol.*, **34/35**, 37-47.

Ramos, L. P.; Mathias, A. L., Fontana, J. D. & Almeida, N. H. (1995). Development of a small pilot plant for pretreating lignocellulosic materials. *In Fourth Brazilian Symposium on the Chemistry of Lignins and Other Wood Components*, Proceedings.

Ramos, L. P., Mathias, A. L., Cotrim, A. R., Silva, F. T. & Chen, C.-L. (1999). Characterization of residual lignin after SO_2-catalyzed steam explosion and enzymatic hydrolysis of *Eucalyptus viminalis* wood chips. *J. Agric. Food Chem.*, **47**, 2995-2302.

Saddler, J. N.; Ramos, L. P. & Breuil, C. (1993). Steam pretreatment of lignocellulosic residues. In: *Bioconversion of Forest and Agricultural Plant Wastes*, ed. Saddler, J. N., C. A. B. International, London, pp. 73-92

Tanahashi, M. (1990). Characterization and degradation mechanisms of wood components by steam explosion and utilization of exploded wood. *Wood Res.*, **77**, 49-117.

Activity as function of pH and temperature and energy of activation of free and immobilized cellobiase Novozym 188

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ABSTRACT

Cellobiase from NOVO Nordisk has been immobilized in controlled pore silica particles by covalent binding with the silane-glutaraldehyde method and 74.88 mg protein/g dry support was fixed. The activity of the free and immobilized enzyme (IE) was determined with 0.2% (w/v) cellobiose, at 40 to 70°C, pH 3 to 7 for free enzyme and pH 2.2 to 7 for IE. At pH 4.8 the maximum specific activity for the free enzyme and IE occurred at 65°C: 17.0 and 2.2 μmol glucose/min.mg protein respectively. For all temperatures covered by this work, the optimum pH observed for free enzyme was 4.5 while for IE was 3.5. The Energy of Activation was 11 kcal/mol for free enzyme and 5 kcal/mol for IE, at pH values from 4.5 to 5.

Keywords: cellulases, cellobiase, enzyme immobilization

INTRODUCTION

The enzymatic hydrolysis of cellulose and cellulosic substrates to glucose is a multistep process catalyzed by the three major components of the cellulase complex, endoglucanases, exoglucanases, and β-glucosidases, working in a synergistic mechanism (Cantarella *et al.*, 1984; Woodward, 1991). The enzyme β-glucosidase (EC 3.2.1.21), also known as cellobiase, hydrolyses β-glucosidic bonds of cellobiose producing two molecules of glucose. In cellulose hydrolysis cellobiase is essential for the conversion of the intermediate cellobiose, which is a strong inhibitor of the other cellulase components. The action of β-glucosidase on cellobiose also shows substrate inhibition (Hsuanyu & Laidler, 1984; Aguado *et al.*, 1995). Since the substrate of cellobiase, that is cellobiose, is water soluble, this enzyme can be used for enhancement of cellulose hydrolysis, in the form of immobilized enzyme, usually in a column adjacent to the main reactor (Woodward and Capps, 1992).

The technology of immobilized enzyme offer technical and economical advantages, such as (Hartmeier, 1988; Zanin and Moraes, 1994):

- Reduction of enzyme usage, since once immobilized the enzyme can be used for a much longer period than in the soluble form;
- Immobilized enzyme can lead to preferred continuous processes that may use either fixed or fluidized bed reactors;
- It is possible to use higher enzyme dosage per volume of reactor than in the soluble enzyme process, and this contributes to high reaction rates and consequently, small reactor sizes;
- These technical advantages allow a reduction in the process operational and capital costs if the immobilized enzyme half-life is sufficiently long.

This article covers experimental determination of the activity and energy of activation, of free and immobilized NOVO Nordisk cellobiase.

Enzyme Activity as a Function of Temperature

The increase in activity as temperature is raised in an enzymatic reaction is modeled with Arrhenius equation:

$$A = A_0 \exp(-E_a / RT) \quad (1)$$

Where: A = enzymatic activity measured by the initial rate of reaction
A_0 = pre-exponential factor
E_a = energy of activation
R = universal gas constant (1.987 cal/mol K)
T = absolute temperature in Kelvin

This equation applies only for temperatures below that in which thermal denaturation of the enzyme becomes severe. From Eq. (1) it can be seen that the Arrhenius plot of log of activity (A) as a function of the inverse

of the absolute temperature (T), results in a straight line in which the angular coefficient multiplied by R gives the energy of activation (E_a). For the higher temperatures where thermal deactivation becomes too fast, the experimental points in the Arrhenius plot deviate from the straight line, giving lower activities and shaping the curve with a maximum, associated with the optimum temperature for maximum activity. At this point, the gains in activity given by higher temperatures are offset by decreased concentration in active enzyme, caused by thermal denaturation (Dixon and Weeb, 1979; Hartmeier, 1988; Ballesteros at al., 1994).

EXPERIMENTAL

Substrate

The substrate was cellobiose from Sigma (St. Louis, USA), and it contained a very low-level contamination of glucose (0.133% w/w).

Enzyme

Novozym 188, a β-glucosidase produced by the microorganism *Aspergillus niger*, containing 170 mg/mL of protein and a specific activity of 9.5 μmol glucose/min.mg protein at 50°C, pH 4.8, was kindly supplied by Novo Nordisk (Denmark).

Carrier

The carrier used for immobilization was controlled pore silica (CPS), a gift from Corning Glass Works, having mean pore size of 37.5 nm and average particle diameter of 0.351 mm.

Enzyme Immobilization

Cellobiase was immobilized in CPS by the covalent method of Weetall (1993) with the following steps: (a) silanization of the carrier, with a 0.5% (v/v) solution of γ-aminopropyltrietoxisilane, for 3h at 75°C; (b) washing with distilled water and drying for 15h at 105°C; (c) activation with a 2.5% (v/v) solution of glutaraldehyde, pH 7.0 for 45 min at 20°C; (d) wash with water, (e) contact the activated carrier with a solution of the enzyme for 15h at 20°C, and, (f) wash the immobilized enzyme with distilled water, and stock it under sodium acetate buffer (0.2M) pH 4.5 at 4°C.

Procedure for Assaying Enzymatic Activity

Activity tests were carried out using the method of initial rate (Dixon and Webb, 1979), using a jacketed glass batch thermo-controlled reactor, equipped with magnetic stirring. A volume of 20 mL of cellobiose solution, buffered with acetate buffer at 50 mM, pH 4.8 and containing 1 mg/mL of sodium benzoate was incubated at the test temperature with the enzyme β-glucosidase, at the concentration of 95 μL of enzyme/L of solution, whereas for the immobilized cellobiase, 0.06g dry weight immobilized enzyme was used inside a stainless steel screen basket. Half-milliliter samples were collected with an interval of 3 min, for a period of 18 min, boiled and stocked at 4°C for later glucose assay.

Analytical Methods

Glucose was assayed with the enzymatic method GOD-PAP (Trinder, 1969), and protein was measured according to Lowry *et al.* (1951) using BSA as protein standard.

Specific Activity as a Function of pH and Temperature

The specific activity of the free and immobilized enzyme (IE) was determined using a 0.2% (w/v) solution of cellobiose (5.85 mM), at 40 to 70°C, pH 3 to 7 for free enzyme and pH 2.2 to 7 for IE. This span of pH values was covered with the McIlvaine buffer (Morita and Assumpção, 1972) at the final concentration of 10 mM.

RESULTS AND DISCUSSION

Immobilization

During the immobilization, 67% of the offered enzyme was fixed on the support (CPS). The quantity of protein fixed was 74.88 mg protein/g dry support.

Activity as a Function of Temperature and pH

Figure 1 shows the specific activity of the NOVO free cellobiase as a function of temperature and pH. Figure 2 has the same kind of results, but for the immobilized enzyme.

In Figure 1 maximum activities were observed in the pH range 4.0 to 4.8. The maximum activity, 17.84 μmol glucose/min.mg protein, was found at 65°C, pH 4.5. These optimum conditions are in exact agreement with those of Woodward *et al.* (1993) and Dekker (1986). For the IE, Figure 2 shows that the optimum pH is observed at pH 3.5 for all temperatures studied in this experiment, and the optimum temperature was 65°C in the pH range 3.5 to 6.0. The activity at 65°C, was 6.10 μmol glucose/min.mg protein at pH 3.5. Disregarding the pH shift, the activity yield upon immobilization is low: 34.0%. For the same pH, namely pH 4.5, the activity yield is lower: 13.7%.

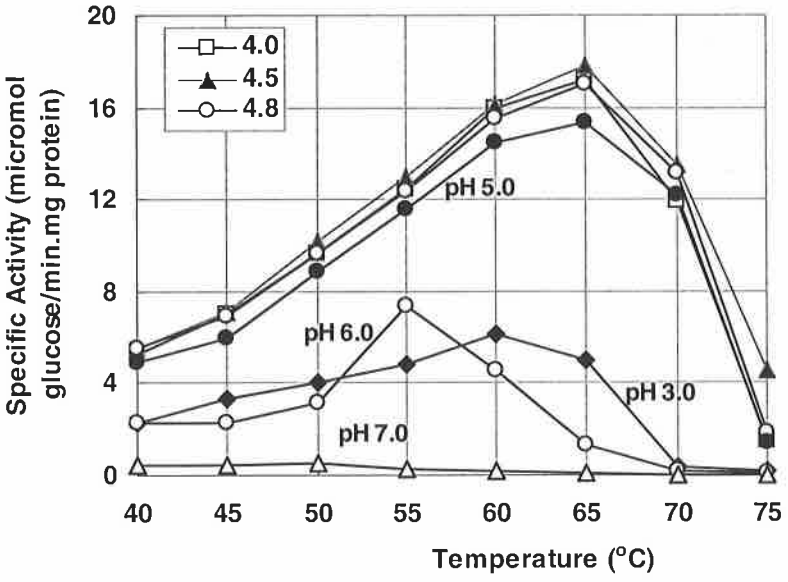

Figure 1. Specific activity as a function of temperature and pH for free Novozym cellobiase at the concentration of 95 μL/L in 2 g/L cellobiose.

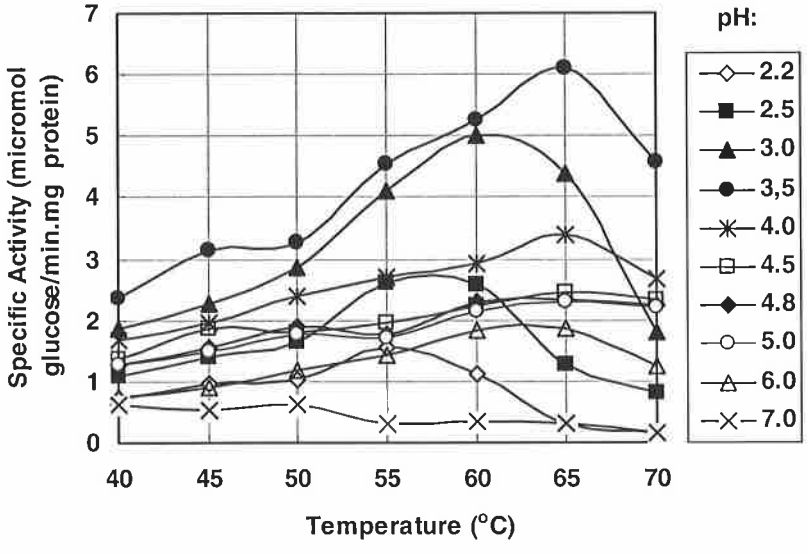

Figure 2. Specific activity as a function of temperature and pH for immobilized Novozym cellobiase at the concentration of 0.06g dry weight IE/20 mL substrate (2g/L cellobiose).

The comparison of Figures 1 and 2 shows that the immobilization of cellobiase in controlled pore silica by covalent method shifts the optimum pH of the enzyme about 1.0 point, from 4.5 to 3.5. Similar results were obtained by Bergamasco *et al.* (1997), for immobilized invertase in CPS: the pH optimum was shifted from 5.0 to 4.5.

The energy of activation was calculated by fitting Eq. (1) to the activity data of Figures 1 and 2, and the results are shown in Table 1.

The hydrolysis rate can be written in the Arrhenius form as presented in Table 1 for free and immobilized enzyme.

Figure 3 shows that the energy of activation for the hydrolysis of cellobiose is about 11 kcal/mol in the pH range 4.5 to 5.0, in accord with published data for the same enzyme (Beltrame *et al.*, 1983; Dekker, 1986; Alfani *et al.*, 1987), which show values for the energy of activation in the range of 10 to 12.5 kcal/mol. In the same range of values, other authors have found equivalent results for cellobiases produced by different microorganisms (Maguire, 1977; Bisset and Sternberg, 1978; Sundstrom *et al.*, 1981). In the same pH range, for the immobilized enzyme the energy of activation is about 5 kcal/mol. Bisset and Sternberg (1978) immobilized β-glucosidase of *Aspergillus phoenicis* on chitosan and obtained 11.95 and 7.93 kcal/mol for free and immobilized enzyme, respectively. The plot of E_a as function of pH in Fig. 3 reveals a dependence of the energy of activation on pH and shows that E_a for the immobilized enzyme is generally lower. The apparent lower energy of activation for the immobilized enzyme has been interpreted as an indication of diffusional limitation (Bisset and Sternberg, 1978).

Table 1. Energy of activation for the enzymatic hydrolysis of cellobiose with Novozym 188 β-glucosidase for different pH values calculated by fitting Eq. (1) to the activity data of Figures 1 and 2 (A_e = specific activity of the enzyme, A_r = Arrhenius constant, E_a = energy of activation of the enzyme).

PH	$A_e = A_r \exp(-E_a/RT)$	Energy of Activation (kcal/mol)
	FREE ENZYME	
3.0	$A_e = 1.6273.10^7 \exp(-9\,782/RT)$, r = 0.9917	9.78
4.0	$A_e = 6.9610.10^8 \exp(-11\,633/RT)$, r = 0.9993	11.64
4.5	$A_e = 5.2663.10^8 \exp(-11\,435/RT)$, r = 0.9972	11.44
4.8	$A_e = 2.6099.10^8 \exp(-11\,005/RT)$, r = 0.9985	11.01
5.0	$A_e = 7.7471.10^8 \exp(-11\,766/RT)$, r = 0.9948	11.77
6.0	$A_e = 1.2245.10^8 \exp(-15\,495/RT)$, r = 0.8830	15.50
7.0	$A_e = 3.1634.10^8 \exp(-7\,037/RT)$, r = 0.9806	7.04
	IMMOBILIZED ENZYME	
2.2	$A_e = 4.7516.10^6 \exp(-9\,773/RT)$, r = 0.9600	9.78
2.5	$A_e = 7.4223.10^6 \exp(-9\,790/RT)$, r = 0.9662	9.79
3.0	$A_e = 4.4190.10^7 \exp(-10\,588/RT)$, r = 0.9937	10.59
3.5	$A_e = 1.0581.10^6 \exp(-8\,086/RT)$, r = 0.9820	8.09
4.0	$A_e = 2{,}2004.10^4 \exp(-5\,889/RT)$, r = 0.9927	5.89
4.5	$A_e = 3.8238.10^3 \exp(-4\,931/RT)$, r = 0.9973	4.93
4.8	$A_e = 3.4547.10^3 \exp(-4\,881/RT)$, r = 0.9526	4.88
5.0	$A_e = 3.2202.10^3 \exp(-4\,861/RT)$, r = 0.9748	4.86
6.0	$A_e = 1.8636.10^6 \exp(-9\,192/RT)$, r = 0.9978	9.19

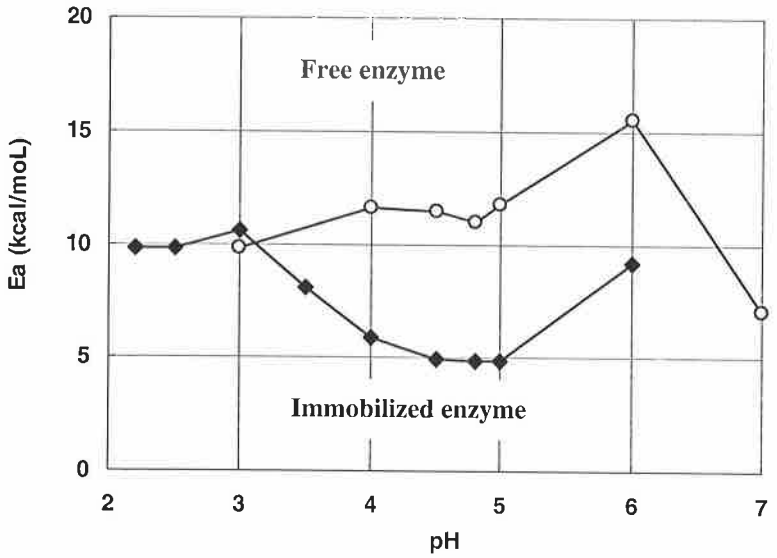

Figure 3. Comparison of the energy of activation as a function of pH, for free and immobilized Novozym cellobiase, cellobiose 2g/L, 95 μL enzyme/L for free and 0.06 g dry weight IE/20 mL substrate (2g/L cellobiose).

CONCLUSIONS

During the immobilization of the enzyme in CPS with the silane-glutaraldehyde method, 67% of the offered enzyme was fixed on the support. The method gives relatively low activity yield: 34% at 65°C.

For all temperatures the enzyme has shown optimum activities in the pH range of 4.0 to 4.8 for free enzyme and around pH 3.5 for IE.

Maximum activity was 17.84 μmol glucose/min.mg protein at 65°C and pH 4.5 for free enzyme and 6.10 μmol glucose/min.mg protein at 65°C and 3.5 for IE.

The energy of activation of the enzyme was 11.0 kcal/mol for free enzyme and 5 kcal/mol for IE, at pH values from 4.5 to 5.0.

Acknowledgments. We would like to thank Novo Nordisk (Denmark) for kindly providing the enzyme samples. We are also thankful to CAPES, CNPq, PADCT and the University for the financial support.

REFERENCES

Aguado, J., Romero, M. D., Rodríguez, L. & Calles J. A. (1995). Thermal deactivation of free and immobilized β-glucosidase from *Penicillinum funiculosum. Biotechnology Progress*, **11**, 04 –106.

Alfani, F., Cantarella, L., Gallifuoco, A., Pezzullo, L., Scardi, V. & Cantarella, M. (1987). Characterization of the β-glucosidase activity associated with immobilized cellulase of *Aspergillus niger. Annals of the New York Academy of Science,* **501**, 503-507.

Ballesteros, A., Boross, L., Buchholz, K., Cabral, J. M. S. & Kasche, V. (1994), In *Applied Biocatalysis*, Cabral, J. M. S., Best, D., Boross, L. and Tramper, J., Hardwood Academic Publishers, Switzerland, 237-278.

Beltrame, P. L., Carniti, P., Focher,B., Marzetti, A. & Sarto, V. (1983). Cellobiose hydrolysis by crude cellobiase. kinetics and mechanism. *La Chimica e L´Industria*, **65 (6)**, 398-401.

Bergamasco, R., Bassetti, F. J., Moraes, F. F. de & Zanin, G. M. (1997). Characterization of free and immobilized invertase regarding activity and energy of activation. *In Symposium on Biotechnology for Fuels and Chemicals*, Program and Abstracts, Poster 147.

Bisset, F. & Sternberg, D. (1978). Immobilization of aspergillus β-glucosidase on chitosan. *Applied and Environmental Microbiology,* **35 (4)**, 750-755.

Cantarella, M., Gallifuoco, A., Scardi, V. & Alfani, F. (1984). Enzyme stability and glucose inhibition in cellulose saccharification. *Annals of the New York Academy of Sciences*, **434,** 39-43.

Dekker, R. F. H. (1986). Kinetic, inhibition, and stability properties of a commercial β-d-glucosidase (cellobiase) preparation from *aspergillus niger* and its suitability in the hydrolysis of lignocellulose. *Biotechnology and Bioengineering*, **28**, 1438-1442.

Dixon, M. & Webb, E. C. (1979), *Enzyme*, 3rd Ed., Longman Group Limited, London, pp. 138-139; 164-168.

Hartmeier, W. (1988), *Immobilized Biocatalysts – an Introduction*, (Trans, J. Wieser), Springer – Verlag, Berlin, pp 16-17; 51-61.

Hsuanyu, Y. & Laidler, K. J. (1984). Kinetics of the hydrolysis of cellobiose catalysed by β-glucosidase. *Canadian Journal Biochemistry and Cell Biology*, **63**, 167-175.

Lowry, O. H. & Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *J. Biological Chemistry,* **193**, 265-275.

Maguire, R. J. (1977). Kinetics of the hydrolysis of cellobiose and *p*-nitrophenyl-β-glucoside by cellobiase of *Trichoderma viride. Canadian Journal Biochemistry*, **55**, 19-26.

Morita, T. & Assumpção, R. M. V. (1972). *Manual de Soluções Reagentes e Solventes – Padronização – Preparação – Purificação,* 2nd ed., ed. Edgard Blücher Ltda., São Paulo, pp.272 – 275.

Sundstrom, D. W., Klei, H. E., Coughlin, R. W., Biederman, G. J. & Brouwer, C. A. (1981). Enzymatic hydrolysis of cellulose to glucose using immobilized β-glucosidase. *Biotechnology and Bioengineering*, **23**, 473-485.

Trinder, P.(1969). Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor. *Annals of Clinical Biochemistry*, **6**, 24-27.

Weetall, H. H. (1993). Preparation of immobilized proteins covalently coupled through silane coupling agents to inorganic supports. *Applied Biochemistry and Biotechnology, **41***, 157-188.

Woodward, J. & Capps, K. M. (1992). Cellobiose hydrolysis by glutaraldehyde-treated β-glucosidase entrapped in propylene glycol alginate/bone gelatin spheres. *Applied Biochemistry and Biotechnology*, **34/35**, 341-347.

Woodward, J. (1991). Synergism in cellulase systems. *Bioresource Technology*, **36**, 67-75.

Woodward, J., Koran Jr., L. J., Hernandez, L. J. & Stephan, L. M. (1993). Use of immobilized β-glucosidase in the hydrolysis of cellulose. *American Chemical Society*, **533**, 240-250.

Zanin, G. M. & Moraes, F. F. de (1994). Performance of fixed and fluidized bed reactors with immobilized enzyme. *Applied Biochemistry and Biotechnology*, **45/46**, 627-639.

Advanced oxidation of pulp mill wastewater

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ABSTRACT

Advanced oxidation processes are capable to remove organic charge of bleaching effluents and degrade chlorinated compounds. Mineralization of the organic matter, AOX and TOC reduction are important findings. The effluent biodegradability enhancement was determined by BOD/COD ratio increases and activated sludge growing. Ozone/UV system is more efficient to remove acute toxicity (MICROTOX) from *P. radiata* ECF effluent and photocatalysis seems better to reduce colour and COD. Photocatalytic systems using supported TiO_2 on glass Rashig rings was very efficient to degrade both standard *E. globulus* BKME and a chlorinated model compound (6-chlorovanillin).

Keywords: bleaching effluents, effluent treatment, advanced oxidation systems, lignin, photocatalysis

INTRODUCTION

Physicochemical, biological and combined systems have been extensively studied to treat bleaching kraft mill effluent (BKME), and recently the use of Advanced Oxidation Processes (AOPs) has been included in the list of possibilities (Kahmark and Unwin, 1998). These oxidation systems can be used as a pre-treatment step for biological conventional treatments when dissolved molecules in the effluent are recalcitrant, toxic or inhibitory to the normal microorganism's growth. Cellulose pulp bleaching effluents contain high molecular weight chlorinated lignins, which are colored and recalcitrant to biological degradation and low molecular weight chlorophenols, which are toxic. It is well known that AOPs are capable to oxidize a wide range of organic compounds by generation *in situ* of highly oxidative species, such as hydroxyl radicals ($E° = 2.8$ eV). In the best of the hypothesis, the organic matter can be completely converted into CO_2, water and inorganic acids. Between these oxidation systems, photocatalysis (O_2/semiconductor/UV) present better possibilities of application because it is the only system that can work with solar light as radiation source, instead of other systems, such as ozone or H_2O_2, that need UV light to produce the reactive radicals. Great efforts to degrade toxic compounds by solar photocatalysis (Malato *et al.*, 1998; Herrmann *et al.*, 1998) have been successfully done.

Degradation of chlorinated phenols (Wang *et al.*, 1999) and kraft lignins by semiconductor photocatalysis, principally with TiO_2, in the presence of an irradiation source (Ohnishi *et al.*, 1989; Kobayakawa *et al.*, 1989), has been studied. Oxidation with ozone (Mao and Smith, 1995; Rodríguez *et al.*, 1998) and advanced oxidation with H_2O_2/UV (Prat *et al.*, 1988) or ozone/UV (Çeçen *et al.*, 1992) have been studied as alternative treatments for different cellulose bleaching effluents.

This report deals with the use of AOPs, particularly photocatalysis, focused to the reduction of colour, TOC, AOX and acute toxicity and the biodegradability enhancement of chlorinated bleaching effluents.

Oxidation of *Pinus radiata* ECF - BKME by AOPS

The oxidation of an ECF effluent was carried out using homogeneous (O_3/UV) and heterogeneous (O_2/sem/UV and O_3/sem/UV) advanced oxidation systems, being the semiconductors were ZnO and TiO_2 (Mansilla *et al.*, 1997; Reyes *et al.*, 1998; Yeber *et al.*, 1999a). The colour of the effluent was reduced from their initial value (4150 mg Pt L^{-1}) in around 40 % and 15 % and 5 %, for O_2/sem/UV, ozone/UV and ozone alone, respectively, after one min of reaction. Also, the initial pH (10.8) decreases in 3 units after 1 min of photocatalysis. In homogeneous systems the pH reduction was almost one unit. COD removal was considerably highest with heterogeneous systems (4 times) after one min of treatment. However, the homogeneous systems are more efficient in transforming the organic matter into more biodegradable compounds, as shown in Figure 1. After 5 min of reaction with O_3 and O_3/UV a significant increases in the biodegradability of 2.7 and 3.3 times, with respect to initial value (0.3) was observed. These results agree with the TOC and acute toxicity removal (Figures 2 and 3). A great reduction of TOC, which is indicative of

mineralization reaction, was observed when the effluent was submitted to one min of treatment on all AOPs systems. Also, the toxicity was reduced being the photocatalysis with TiO_2 the most efficient.

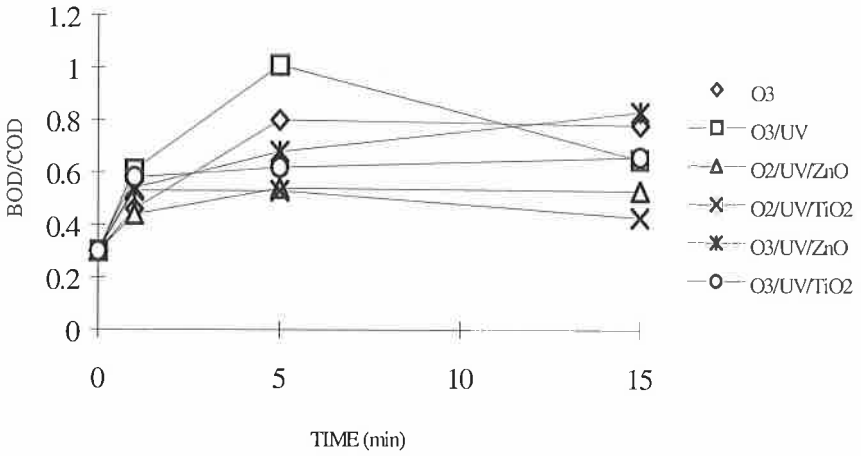

Figure 1. Effluent biodegradability (BOD_5/COD) change during treatment with AOPs.

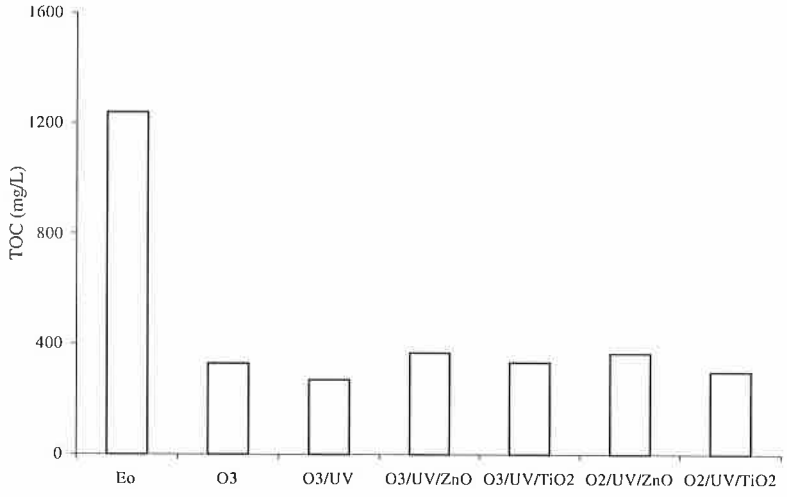

Figure 2. Total Organic Carbon Removal in the ECF effluent after treatment (1 min) with the AOPs.

All the results described concern to reactions where the catalyst was added in suspension in the effluent, which was treated without modification, and irradiated with an HP mercury lamp ($\lambda > 254$ nm). Samples of treated effluents were taken at different periods of time.

Kraft black liquor oxidation by ZnO - photocatalysis

ZnO-catalyzed photodegradation of kraft black liquor, used as model for effluent from the pulp and paper industry, shows a first order mineralization which is dependent on the oxygen concentration, increasing the degradation in the presence of Pt-impregnated ZnO (Mansilla *et al.*, 1994; Villaseñor and Mansilla, 1996). Also, high molecular weight compounds were converted into small molecules and all the process is dependent on the temperature. This means that the potentially temperature dependent–steps, such as adsorption, desorption, surface migration and rearrangements, appears to be rate determining.

Eucalyptus globulus standard BKME oxidation by supported ZnO and TiO_2 photocatalysts

Catalyst recovery is a key issue in the industrial implementation of photocatalytic systems. This problem can be solved, in part, if the catalyst is immobilized on inert surfaces without the loss of activity. Effluent coming

from conventional pulp bleaching (CEop) was treated for two h with the catalyst supported on glass Rashig rings (Yeber *et al.*, 1999b). Photocatalysis using TiO_2 remove the initial TOC (980 ppm) by 55 % whereas ZnO treatment reached only 31 % (Figure 4). That is, TiO_2 is more efficient as catalyst to induce the mineralization of the organic matter than TiO_2, when immobilized on glass. Initial colour (2933 mg Pt L^{-1}) was completely removed after 90 min of reaction with both catalysts. The total phenol content decreases with a similar profile than that of the colour reduction.

The AOX, related with the amount of chlorinated compounds and toxicity, was also partially removed by photocatalysis (Figure 5). Degradations of 85 % and 90 % of the initial AOX (73 mg L^{-1}) were reached after 60 and 120 min, respectively. On the other hand, a decrease in 2 units in pH (from 10.3 to 8.2) was observed after 60 min of reaction, which is an indication of the mineralization of the organic chlorine to chloride ions. These results, together with the colour reduction, total phenols, COD and TOC values indicate that photocatalysis, using impregnated catalysts, present better application possibilities for highly chlorinated effluent treatment.

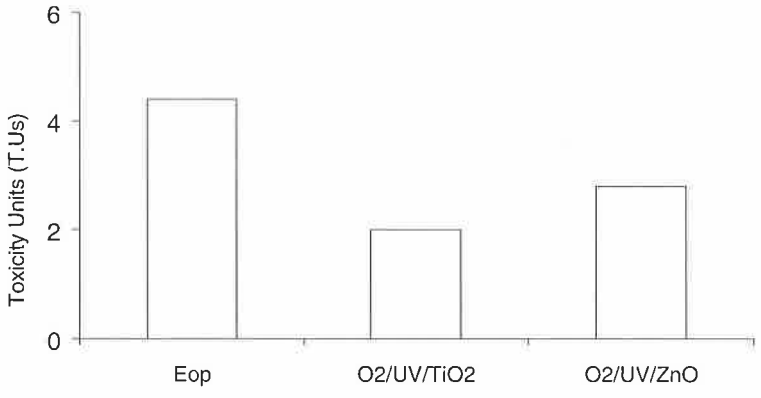

Figure 3. MICROTOX acute toxicity of the ECF effluent treated with photocatalysis.

As it was pointed out above, ZnO and TiO_2 photo assisted catalysis degrades efficiently the organic matter dissolved in an ECF effluent, reducing the toxicity and improving the biodegradability significantly. Those assays were carried out with the catalyst suspended in the effluent. When the catalyst was immobilized on Rashig rings the toxicity was reduced by almost 50 % for both catalysts. This means that the residual lignin derivatives are difficult to be completely degraded and they are responsible for the remaining toxicity. In the study of molecular mass changes in the treated effluent it was observed a significant decrease in high molecular weight compounds forming new low molecular species. In spite of the increase on the treatment time with immobilized catalyst this system can be used under flow conditions, avoiding the catalyst-recovering step.

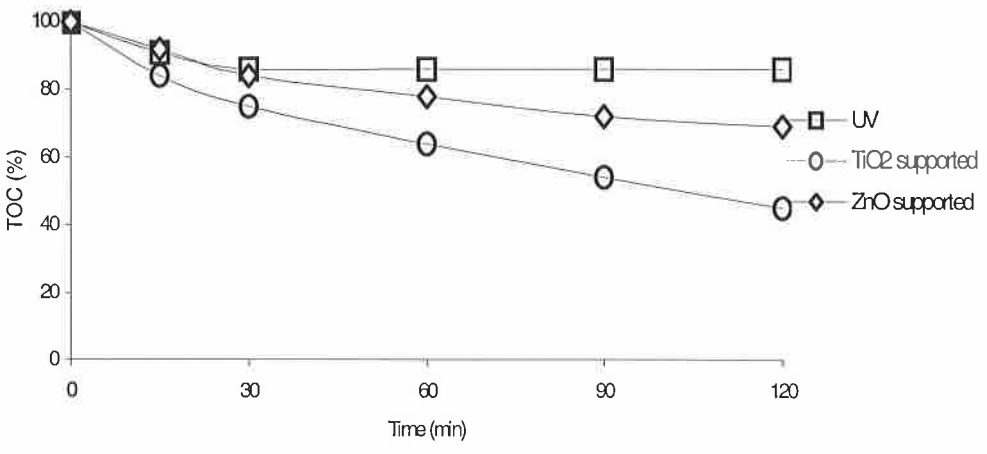

Figure 4. Photocatalytic TOC removal of *E. grandis* E_1-effluent.

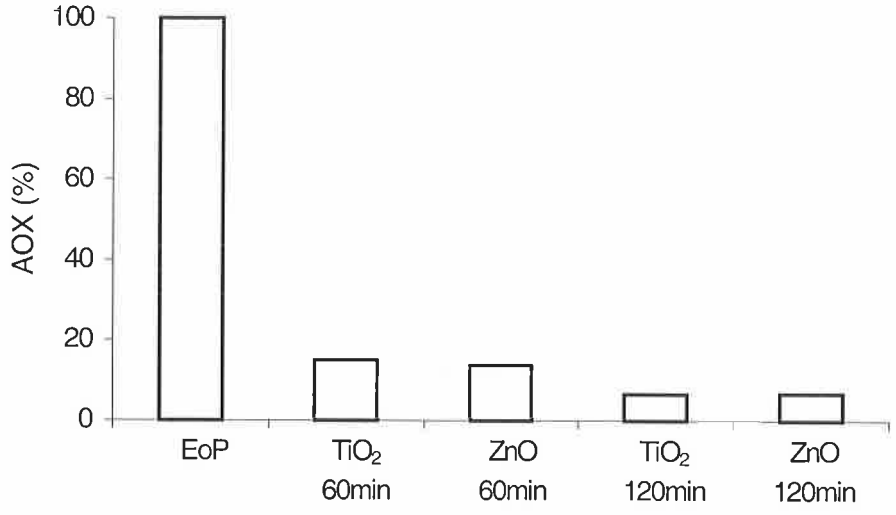

Figure 5. AOX removal of the E_1 effluent after photocatalysis.

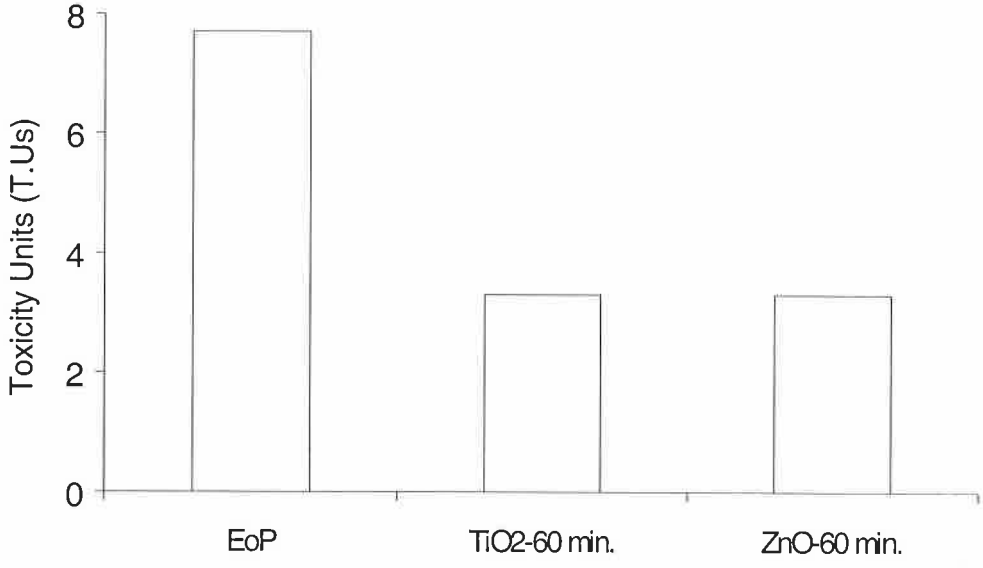

Figure 6. Acute toxicity removal by supported TiO_2 and ZnO photocatalysis on 60 min treated samples.

CONCLUSIONS

Homogeneous and heterogeneous advanced oxidation processes are very efficient in the degradation of high and low molecular weight compounds present in cellulose effluents. Chlorinated lignin derivatives are mineralized reducing their toxicity and improving the biodegradability.

Photocatalysis with ZnO and TiO_2 either in suspended solutions or immobilized on glass surfaces is capable to oxidize chlorolignins and chlorophenols to less toxic compounds, and to remove AOX, TOC and colour from pulp mill wastewaters.

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REFERENCES

Çeçen, F., Urban, W. & Haberl, R. (1992). Biological and advanced treatment of sulfate pulp bleaching effluents. *Wat. Sci. Technol.*, **26**, 435-444.

Herrmann, J.M., Disdier, J., Pichat, P., Malato, S. & Blanco, J. (1998). TiO_2-based solar photocatalytic detoxification of water containing organic pollutants. Case studies of 2,4-dichlorophenoxyacetic acid (2,4-D) and of benzofuran, *Appl. Catal. B: Environ.*, **17**, 15-23.

Kahmark, K.A. & Unwin, J.P. (1998). Pulp and paper effluent management. Literature Review. *Water Environ. Res.*, **70**, 667-690.

Kobayakawa, K., Sato, Y., Nakamura, S. & Fujichima, A. (1989). Photodecomposition of kraft lignin catalysed by titanium dioxide, *Bull. Chem. Soc. Jpn.*, **62**, 3433-3436.

Malato. S., Blanco, J., Brun, B. & Maldonado, M. I. (1998). Enhancement of the rate of solar photocatalytic mineralization of organic pollutants by inorganic oxidized species, *Appl. Catal. B: Environ.*, **17**, 347-356.

Mansilla, H.D., Villaseñor, J. Maturana, G., Baeza, J., Freer, J. & Durán, N. (1994). ZnO-catalysed photodegradation of kraft black liquor, *J. Photochem. Photobiol. A: Chem.*, **78**, 267-273.

Mansilla, H.D, Yeber, M.C., Freer, J., Rodríguez, J. & Baeza, J. (1997). Homogeneous and heterogeneous advanced oxidation of a bleaching effluent from the pulp and paper industry, *Wat. Sci. Technol.*, **35**, 273-278.

Mao, H. & Smith, D.W. (1995). Toward elucidating mechanism and kinetics of ozone decolorization and dechlorination of pulp mill effluents. *Ozone Sci. Engrg.*, **17**, 419-448.

Ohnishi, H., Matsumura, M., Tsubomura, H. & Iwasaki, M. (1989). Bleaching of lignin solution by a photocatalysed reaction on semiconductor photocatalysts, *Ind. Eng. Chem. Res.*, **28**, 719-724.

Prat, C., Vicente, M. & Esplugas, S. (1988). Treatment of bleaching waters in the paper industry by hydrogen peroxide and ultraviolet radiation, *Wat. Res.*, **22**, 663-668.

Reyes, J., Dezotti, M., Mansilla, H.D., Villaseñor, J., Esposito, E. & Durán, N. (1998). Biomass Photochemistry-XXII: Combined photochemical and biological process for treatment of kraft E1 effluent, *Applied Catal. B: Environ.*, **15**, 211-219.

Rodríguez, J., Fuentes, S., Freer, J., Mansilla, H.D., Ferraz, A. & Baeza, J. (1998). Response to ozonation of different cellulose pulp bleaching effluents, *Environ. Technol.*, **19**, 75-81.

Villaseñor, J. & Mansilla, H.D. (1996). Effect of temperature on kraft black liquor degradation by ZnO-photoassisted catalysis, *J. Photochem. Photobiol. A: Chem.*, **93**, 205-209.

Wang, K-H., Hsieh, Y-H., Chou, M-Y & Chang, C-Y. (1999). Photocatalytic degradation of 2-chloro and 2-nitrophenol by titanium dioxide suspensions in aqueous solution, *Appl. Catal. B: Environ.*, **21**, 1-8.

Yeber, M.C., Rodríguez, J. Freer, J., Baeza, J., Durán, N. & Mansilla, H.D. (1999a). Advanced oxidation of pulp mill bleaching wastewater, *Chemosphere*, in press.

Yeber, M.C., Rodríguez, J., Baeza, J., Freer, J., Zaror, C, Durán, N. & Mansilla, H.D. (1999b). Toxicity abatement and biodegradability enhancement of pulp mill bleaching effluent by advanced chemical oxidation. *In 6th IAWQ Symposium on Forest Industry Wastewaters, Finland,* Proceedings, pp 355-362.

An overview of biopulping and biobleaching

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ABSTRACT

To keep up with the increasing demand for pulp and paper and to meet increasing stringent environmental regulations, the industry has been constantly looking towards technological improvements. During the past 20 years, research efforts in laboratories around the world have sought to apply biotechnology in industrial wood applications of microbes and their enzymes in the pulp and paper industry that have been developed or are under development. In this review article, we will focus on biopulping and biobleaching.

Keywords: biopulping, wood biodegradation, lignin, white-rot fungi

INTRODUCTION

The pulp and paper industry utilizes mechanical or chemical pulping methods to produce pulps with desired characteristics. Mechanical pulping involves the use of mechanical force to separate the wood fibers. Mechanical processes are high yield (up to 95%) and produce paper with high bulk, good opacity, and excellent printability. However, these processes are electrical energy-intensive and produce paper with lower strength, higher pitch content, and higher color reversion rate compared to chemical processes. Chemical pulping involves the use of chemicals to degrade and dissolve lignin from the wood cell walls, releasing high-cellulose fibers. Chemical pulping processes yield pulps with higher strength; however, these processes are low yield (about 40% to 50%) and are very capital-intensive.

BIOPULPING

Biopulping, which is defined as the treatment of lignocellulosic materials with lignin-degrading fungi prior to pulping, appears to have the potential to overcome some problems associated with the above pulping methods. The following text summarizes biomechanical and biochemical pulping research and describes key findings.

Biomechanical pulping with fungi

Background research

In the 1970s, Eriksson and co-workers at the Swedish Pulp and Paper Research Institute (STFI) Stockholm, launched a fairly comprehensive investigation which demonstrated that fungal pretreatment could result in significant energy savings and strength improvements for mechanical pulping. Although this research met with limited success—encountering difficulties in scale up—it provided valuable insights; details can be found in a recent review article (Akhtar *et al.*, 1998). The subsequent effort to research and develop biomechanical pulping at the Forest Products Laboratory, Madison, Wisconsin has been a unique collaboration among a diverse group of government bodies, research institutions, and private companies. Beginning in April 1987, a biopulping consortium was formed with the overall goal of evaluating the commercial and economic feasibility of biomechanical pulping.

The two-week environmentally friendly process saves at least 30% electrical energy, and also improves paper strength properties. Investigations on a laboratory scale have sorted through the more than 30 variables associated with biopulping, including species and strains of fungi, inoculum form and amount, species of wood, wood chip size, environmental factors, effect of added nutrients, and need to sterilize the chips (Akhtar *et al.*, 1998). Some of the variables keys to the economic viability of biopulping are summarized below.

Selection of a suitable fungus

We identified a fungus, *Ceriporiopsis subvermispora*, that performs biopulping very effectively on both hardwood and softwood species. This fungus is a selective lignin degrader and was chosen after screening several hundred species of fungi and their strains.

Chip surface decontamination

Wood chip surfaces normally are contaminated with cells and spores of many fungi and bacteria. These unwanted microorganisms can hamper biopulping fungus, making decontamination desirable. We discovered that a brief atmospheric steaming of chips (as short as 15 seconds) decontaminates the surfaces of wood chip and allows the biopulping fungus to outcompete unwanted microorganisms and perform biopulping effectively, uniformly, and economically. A recent article published in Tappi Journal (Diehl, 1998) focuses on respiratory health problems associated with routine exposure of workers to the spores of miscellaneous fungi that inhabit wood chips in a normal wood yard operation. Some of these unwanted fungi also produce cellulolytic enzymes and thus would have an adverse effect on paper strength properties. Biopulping fungus is non-sporulating and is a selective lignin degrader, which would preclude such problems.

Inoculum

We reduced the required amount of inoculum from 3 kg to 5 g or less per ton of wood, which is well within a commercially attractive range. This was achieved by adding an inexpensive and commercially available nutrient source, unsterilized corn steep liquor, to the inoculum suspension (Akhtar *et al.*, 1998). This additive apparently "kick-starts" the fungal growth, making it possible to use a much lower inoculum level. Since corn steep liquor is produced widely in the United States and abroad, pulp and paper companies should be able to obtain a regular supply from the nearest location with minimal transportation cost.

Mill-scale evaluation of fungus-treated spruce chips

In an effort to scale-up biomechanical pulping to an industrial level, fifty tons of wood chips (dry weight basis) were inoculated with *C. subvermispora*, in a continuous operation and stored in the form of an outdoor chip pile for two weeks. The pile was ventilated with conditioned air to maintain the optimum growth temperature and moisture throughout the pile (Scott *et al.*, 1998) . The control and fungus-treated chips were refined through a thermomechanical pulp (TMP) mill. The fungal pretreatment saved 33% electrical energy and improved paper strength properties significantly compared to the control. Since biofibers were stronger than the conventional TMP fibers, we were able to reduce the amount of bleached softwood kraft pulp by at least 5% in the final product (Table 1).

Table 1. Strength and other physical properties comparison.

Parameters	Control (50%TMP + 50% kraft)	Treatment (55%TMP + 45% kraft)
Burst index (kN/g)	2.29	2.39
Tear index (mNm^2/kg)	9.92	10.0
Tensile index (Nm/g)	38.9	41.2
Brightness (%)	73.0	69.8[a]
	85.2	73.0[b]
Opacity (%)		86.4
Light scattering coefficient (m^2/kg)	46.2	47.7
Drainage time (sec)	8.5	9.9
Density (kg/m^3)	689	610

[a]Same amount of hydrogen peroxide was applied on the control and the treatment.
[b]Sixty percent more hydrogen peroxide was applied on the treatment.

Process economics

The economics of biomechanical pulping look attractive. A preliminary economic evaluation has been performed for a 250-ton/day TMP mill. The storage time was two weeks in a flat-pile geometry. Capital costs to incorporate biopulping technology into this paper mill are estimated to be about $ 6 to $ 8 million. Based on 33% energy savings and a 5% reduction in kraft in the final product, a savings of about $ 5 million each year can be realized. Based on this attractive economics, the above mill is seriously considering implementation of biopulping technology at one of its TMP mills. One of the largest producers of newsprint in Europe and others in the U.S. have expressed interest in conducting large-scale biopulping trials at their mill sites.

Biomechanical pulping with enzymes

Because of the low accessibility of wood chips for enzymatic modification, incorporation of an enzymatic step into a mechanical pulping process can be expected to be successful only after the primary refining. The effects of enzymatic modification of coarse mechanical pulp with monocomponent *Trichoderma* cellulases and hemicellulases were thus studied prior to secondary refining. Experiments with different enzymes

demonstrated that a slight modification of cellulose by CBH I gave rise to an energy saving of 20% in laboratory-scale disk refiner (Pere *et al.,* in press). Treatment with EG I decreased the energy consumption slightly but only at the expense of pulp quality. Interestingly, no positive effect on energy consumption was detected with a cellulase mixture. When the refining was performed with a low-intensity refiner (wingdefibrator), the positive effect of CBH I in reducing the energy consumption was further enhanced. At a freeness level of 100 ml the energy consumption in the secondary refining was reduced by 40% with CBH I, as compared with the untreated reference (Pere *et al.*, 1996). The results obtained with the laboratory refiners have been further verified in pilot-scale experiments. In a two-stage secondary refining an energy saving of 10-15% with CBH I was obtained (Pere *et al.,* 1996). These results clearly confirmed the previous results in smaller scale.

Neither the cellulase mixture nor CBH I induced evident morphological modifications in the coarse and rigid TMP fibers during a short incubation. Therefore, the possible loosening and unraveling of fiber structure induced by the action of the enzymes was analyzed as fibrillation index in the samples of different freeness levels after secondary refining. Significantly more intensive fibrillation was observed in the CBH I treated pulp than in that treated with the cellulase mixture and in the untreated control. This could be an indirect implication of decreased interfibrillar cohesion inside the fiber wall due to the action of CBH I.

The cellulase treatment with CBH I did not have any detrimental effects on pulp quality. In fact, the tensile index was even higher for the CBH I treated pulp than for the reference. The increase in tensile index could be explained by the intensive fibrillation induced by the CBH I treatment. The good optical properties were also maintained after the CBH I treatment. The method is further being studied.

Biochemical pulping with fungi

Fungal pretreatment of wood chips removes some of the lignin and modifies other lignin; these changes might make it easier to remove lignin in the subsequent pulping process. Also, the fungal treatment causes softening and swelling of wood cells. It is possible that these fungus-induced changes and the removal/modification of lignin may result in improved chemical penetration during pulping operations, which could result in more easily bleachable, lower kappa pulps; reduced cooking times and temperatures; reduced pulping chemical needs; and reduced effluent waste load.

Bio-kraft pulping

Very few studies have shown that fungal pretreatment is effective for increasing pulp yield and strength and for reducing kappa number and cooking time during kraft pulping (Messner *et al.*, 1998). A study done in collaboration with Pramod Bajpai and Pratima Bajpai of Thapar Research Group, India, on bio-kraft pulping of eucalyptus chips demonstrated some of the benefits of fungal pretreatment. For example, the fungal pretreatment reduced the cooking time from 90 to 30 min. without affecting the quality of the final product (Table 2).

Table 2. Bio-kraft pulping of eucalyptus chips with *C. subvermispora* (2-week treatment)[a].

Parameters	Control (cooking time 90 min)	Treated pulp (cooking time 30 min)
Pulp yield (%)	46	46
Brightness (%)	88.6	90.5
Burst index (kN/g)	4.6	4.8
Tear index (mNm^2/g)	7.8	8.0
Tensile index (Nm/g)	68.9	70.5
Breaking length (m)	7026	7193

[a]17% active alkali; 23% sulfidity.
Ramp time, 90 min.

Organosolv pulping

Technologies are currently in development that would reduce the environmental impact of kraft pulping. Organosolv pulping circumvents the environmental problems related to sulfur emissions, and it has been found to be effective on several wood species with a broad range of organic solvents in acid or alkaline media (Ferraz *et al.*, 1998). In addition to the high delignification efficiency of several organic solvent systems, the acidic conditions can also result in carbohydrate degradation. As a result, pulps with low papermaking quality are obtained. Therefore, selective delignification without carbohydrate degradation is required. With the use of selective lignin-degrading fungi, increases in delignification rates have been noted even at low pulp weight

losses in organosolv pulping (Ferraz *et al.*, 1998). Alternatively, the same residual lignin contents could be achieved in shorter reaction times with fungus-pretreated samples, thus providing energy savings in the organosolv process.

Dissolving pulp production

Dissolving pulp is a low-yield chemical pulp (30%–35%) with high cellulose content (95%–98%) and relatively low hemicellulose (1%–10%) and lignin (< 0.05%) contents. Dissolving pulp is manufactured by both prehydrolysis–kraft and acid sulfite methods. The end uses of dissolving pulp include cellophane and rayon, cellulose esters, cellulose ethers, graft derivatives, and cross-linked cellulose derivatives. The bleaching of both sulfite and prehydrolysis–kraft pulps is achieved by removing residual lignin to increase final brightness and α-cellulose content of the dissolving pulp. Although bleaching chemicals are selective and cost-effective, they cause environmental problems. The use of fungal pretreatment might result in savings of chemicals in pulping and bleaching and improvement of the quality of dissolving pulps. Biobleaching of sulfite pulp from eucalyptus wood chips with *C. subvermispora* enhanced brightness significantly, but it reduced the cellulose content of the resulting dissolving pulp. A combined fungus and xylanase pretreatment of sulfite pulps led to the production of dissolving pulp using 63% less active chlorine and having a brightness of over 93% ISO (Ferraz *et al.*, 1998). Subsequent studies showed that *C. subvermispora* pretreatment of eucalyptus wood chips not only increased lignin removal during sulfite pulping and increased brightness of the dissolving pulp, but also improved the selectivity of the bleaching process, thereby increasing the final pulp yield (Christov *et al.*, personal communication).

BIOBLEACHING

The main aim in chemical pulping is to remove lignin and to separate the wood fibers from each other in order to render them suitable for the papermaking process. In the pulping process, the lignified middle lamella located between the wood fibers is solubilized by various chemicals. Today, the predominant pulping method is the kraft process, where the cooking liquors are incinerated and the cooking chemicals recycled.

In bleaching, the primary goal is to remove the low amount of residual lignin, present in the pulp after cooking, without decreasing the molecular weight of cellulose. Lignin in unbleached pulps represents typically only about 1% of the dry weight. During pulping, however, lignin is chemically modified and condensed, resulting in poorly degradable structures. Bleaching is generally composed of at least five sequences. Previously, the bleaching of chemical pulps was carried out with elemental chlorine and chlorine dioxide. Today, in Europe, pulp is bleached by ECF (Elemental Chlorine Free) or TCF (Totally Chlorine Free) bleaching processes, where oxygen, ozone or peroxide are used.

The effect of hemicellulases in bleaching is based on the modification of pulp hemicelluloses, xylan and glucomannan, enhancing the removal of lignin in chemical bleaching (Viikari *et al.*, 1994). It has been proposed that the action of xylanases is due to the partial hydrolysis of reprecipitated xylan or to removal of xylan from the lignin-carbohydrate (LC) complexes. Both mechanisms would lead to enhanced diffusion of entrapped lignin from the fiber wall. In softwood kraft fibers, removal of xylan by xylanases was found to uncover lignin (Buchert *et al.*, 1996). Thus, it can be expected that removal of xylan improves the extractability of lignin by exposing lignin surfaces. The partial hydrolysis of xylan or glucomannan may also degrade and improve the extractability of lignin-carbohydrate complexes (LCC). The action of xylanases on both reprecipitated and LC-xylan in enhancing bleachability suggests that it is probably not only the type but also the location of the xylan that is important in the mechanism of xylanase-aided bleaching.

The use of xylanases in different bleaching sequences uniformly leads to a reduction in chemical consumption. The benefits obtained by enzymes are dependent on the type of bleaching sequence used as well as on the residual lignin content (measured as the kappa number) of the pulp. Originally, xylanases were studied in order to reduce the consumption of elemental chlorine. Later on, xylanases were combined to various ECF and TCF bleaching sequences to improve the otherwise lower brightness of pulp or to decrease the bleaching costs. In chlorine bleaching an average reduction of 25% in active chlorine consumption in prebleaching or a reduction of about 15% in total chlorine consumption has been reported both in laboratory scale and in mill trials. As a result, the concentration of chlorinated compounds, measured as AOX (adsorbable organic halogen), in the bleaching effluent during mill trials was reduced by 15-20% (Viikari *et al.*, 1994). Today, xylanases are industrially used both in ECF and TCF sequences. In ECF sequences the enzymatic step is often implemented due to the limiting chlorine dioxide production capacity. The use of

enzymes allows bleaching to higher brightness values when chlorine gas is not used. In TCF sequences, the advantage of the enzymatic step is due to improved brightness, maintenance of fiber strength and savings in bleaching costs.

The enzymatic pretreatment has been shown to be fully compatible with existing industrial equipment, which is a considerable advantage of this method, especially when compared with some other competing technologies. Xylanases are typically mixed with water before being added to the unbleached pulp by a shower bar and allowed to react in the high density storage tank for one or two hours before the subsequent chemical bleaching steps. Presently a number of mills in Northern America and Scandinavia use enzymes continuously.

The approximate price of xylanase in 1998 was less than 2 USD per ton of pulp. Estimations for the capital cost of enzyme delivery and pH adjustment varied from USD 10,000 to 1,000,000 in 1995. Calculation of the relative economic benefits in an ECF sequence reveals, that the reduction of the chlorine dioxide consumption leads to savings of at least 2 USD per ton of pulp (Table 3). The costs of oxygen based chemicals (ozone, peroxide) are even higher and the respective savings even more pronounced. The potential economic benefits of enzyme bleaching are thus significant to the pulp and paper industry.

Table 3. Chemical and cost savings with xylanases in bleaching (from Roehm Enzymes Finland).

Chemical consumption:	
Normal ClO_2 consumption	65 kg active chlorine/t
Saving of ClO_2 with enzyme, 15 %	10 kg active chlorine/t
Savings in chemicals:	
ClO_2 price: 0.4 USD/kg act. Cl	4 USD/t
Costs:	
Enzyme	1.5 USD/t
Acidification	0 - 0.2 USD/t
NET SAVINGS	2.3 - 2.5 USD/t pulp

Ligninases, especially manganese dependent peroxidase and laccase have also shown potential in pulp bleaching, but have not yet been commercialized. In the laccase-mediator concept, the enzyme oxidized mediator acts directly on lignin and results in efficient delignification. The common substrate of laccases, ABTS was the first mediator studied (Bourbonnais and Paice, 1992). Search for a more suitable mediator resulted in the use of 1-hydroxybenzotrizole (HBT) in laboratory and pilot scale tests. This delignification procedure is commonly referred to as the Lignozym or the LMS (laccase-mediator-system) process. Although the HBT mediator is very efficient, it suffers from some drawbacks, such as the high production cost and limited biodegradability. The most effective new mediators in delignification usually contain N-OH functional groups (Freudenreich *et al.*, 1998). So far, the most promising ones include violuric acid and N-hydroxy-N-phenylacetamide, NHA. Especially the latter mediator results in extremely fast delignification with no significant impact on the cellulose structure. The delignification degree after an alkaline extraction is reported to be high, up to 40%. The LMS system has been shown to be able to replace either the oxygen delignification or ozone stages (Poppius-Levlin *et al.*, 1998). It has been shown that the beneficial effects of xylanase and laccase mediator treatments can be supplementary, if the two methods are carried out simultaneously or successively, thus increasing the overall efficiency (Viikari *et al.*, 1999).

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REFERENCES

Akhtar , M., Blanchette , R.A., Myers, G.C., & Kirk, T.K. (1998). An overview of biomechanical pulping research. In: *Environmentally Friendly Technologies for the Pulp and Paper Industry*, ed. R. A. Young & M. Akhtar, John Wiley & Sons, Inc. New York, pp. 309-340.

Bourbonnais, R., & Paice M. (1992). Demethylation and delignification of kraft pulp by *Trametes versicolor* laccase in the presence of 2.2'-azinobis-(3-ethylbenthiazoline-6-sulphonate). *Appl. Microbiol. Biotechnol.*, **36**, 823.

Buchert, J., Carlsson, G., Viikari, L. , & Ström, G. 1996. Surface characterization of unbleached kraft pulps by enzymatic peeling and ESCA, *Holzforschung,* **50,** 69-74.

Diehl, S.V. (1998). Repiratory health problems associated with worker exposure to fungi on wood and wood chips. *Tappi J.* **81**, 115-118.

Ferraz, A., Christov , L.P., & Akhtar, M. (1998). Fungal pretreatment for organosolv pulping and dissolving pulp production In: *Environmentally Friendly Technologies for the Pulp and Paper Industry*, eds. R. A. Young & M. Akhtar, John Wiley & Sons, Inc., New York, pp.421-447.

Freudenreich, I., Amann, M., Fritz-Langhals, E., & Stohrer, J. (1998). Understanding the lignozym®-process. *In International Pulp Bleaching Conference*, Proceedings, pp 71-76.

Messner, K., Koller, K., Wall, M.B., Akhtar, M., & Scott, G.M. (1998). Fungal pretreatment of wood chips for chemical pulping In: *Environmentally Friendly Technologies for the Pulp and Paper Industry*, ed. R. A. Young & M. Akhtar, John Wiley & Sons, Inc., New York, pp. 385-419.

Pere, J., Liukkonen, S., Siika-aho, M., Gullichsen, J., & Viikari, L. (1996) Use of purified enzymes in mechanical pulping, *In Tappi Pulping Conference*, Proceedings, Nashville, TN.

Pere, J., Siika-aho, M., & Viikari, L. (1999) Biomechanical pulping with enzymes; susceptibility of coarse mechanical pulp to enzymatic modification and secondary refining, *Tappi J.*, in press.

Poppius-Levlin, K., Wang, W., & Ranua, M. (1998). TCF bleaching of laccase/mediator-treated kraft pulps. *In International Pulp Bleaching Conference*, Proceedings, pp 77-85.

Scott , G.M., Akhtar , M., Lentz, M.J., & Swaney, R.E. (1998) Engineering, scale-up and economic aspects of fungal pretreatments of wood chips. In: *Environmentally Friendly Technologies for the Pulp and Paper Industry*, ed. R. A. Young & M. Akhtar, John Wiley & Sons, Inc., New York, pp.341-384.

Viikari, L., Kantelinen, A., Sundquist, J. and Linko, M. (1994). *FEMS Microbiol. Rev.*, **13,** 335-350.

Viikari., L., Oksanen, T., Buchert, J., Amann, M., & Candussio, A. (1999). Combined action of hemicellulases and oxidases in bleaching, *ISWPC*, Proceedings, **Vol I**, pp 504-507.

BEM programme – Biomass – Energy – Materials

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ABSTRACT

A new reactor for acidic prehydrolysis has been developed with an external steel vessel (10 mm in thickness) lined with titanium sheet (2 mm in thickness), with vacuum between the vessels allowing helium leak detection. The technology is called Fail Safety Reactor. Due to its low weight the reactor can be moved to the source of biomass eliminating freight of the raw material. It operates with reversing rotation that desrupts the laminar layer between the biomass and the acid solution. The biomass is compacted up to 300 kg/m^3 (liquid/solid ratio L/S = 2) and prehydrolysis reaches brix 10 in sugar concentration in less than 10 min. Electron microscopy characterization of the cellulignin shows that the prehydrolysis of the hemicellulose is an explosive process mainly from the second layer of the secondary wall of the biomass cell to the cytoplasm cavity. Because of the high porosity of the cellulignin catalytic combustion takes place for particle size less than 250 μm. Burning timing is less than 10 ms similar to gas combustion (3 ms). Ash and K_2O typical contents in the cellulignin are respectively 0.20% and 75 ppm when prehydrolysis is processed with potable water and 0.10% and 5 ppm when the reaction is processed with deionized water. In this case cellulignin is classified as clean fuel for gas-type turbine.

Keywords: acid hydrolysis, titanium, fuel, biomass, lignin

INTRODUCTION

The BEM Programme (Biomass-Energy-Materials) has developed technology to convert any biomass in to a catalytic cellulignin fuel (CCF) a fine powder that burns like gas and meets the specifications for gas turbines. The process used an acidic prehydrolysis in a titanium-lined reactor at 160°C and 0.68 MPa, H_2SO_4 concentration of 1.7% of dry biomass and 30 min reaction time. The critical goals were to increase the internal surface of the powder fuel, reduce the ash content, especially K+Na, and maintain the cristallinity of the cellulose fiber in order to be ground in powder form with grain size <250 μm and low energy consumption. A second product is the prehydrolysated (10% sugar solution, mainly xylose) used for production of chemicals from biomass (furfural, alcohol and xylitol).

PREHYDROLYSIS OF BIOMASS

Figure 1 shows a mobile prehydrolysis reactor. It has the following characteristics: a) The reactor is made of 10 mm high strength low alloy carbon steel lined with 2 mm titanium sheet. Due to the low weight the mobile reactor goes to the source of biomass allowing its complete utilization (trunks and branches) and eliminating cost of transportation through medium distance (85 km); b) Table 1 shows the main consumption of energy in the production of short rotation biomass (David, 1981); the BEM Programme eliminates the expenses with fertilization (it returns the nutrients from the process to the field) and medium distance transportation, reducing consumption of energy to only 2.5% of biomass contained energy; c) Vacuum is kept between the steel vessel and the titanium (0.1 mm Hg) which avoids implosion and allows sporadic helium leak detection of the lining. Due to this characteristic the technology has been nominated "Fail Safety Reactor"; d) The feeding device compacts the biomass up to 300 kg/m^3 allowing to work with a liquid/solid (L/S) ratio of 2, decreasing considerably the process energy consumption.

The prehydrolysis is a batch process composed of the following steps: feeding (15 min), degassing and flooding (15 min), heating (30 min), prehydrolysis (15 min), discharge of prehydrolysate (10 min) and discharge of cellulignin (5 min). Energy is recovered in the discharge of the prehydrolysate. Table 2 shows the process energy consumption in the order of 6% of the biomass compared with the conventional consumption of 30% in tower reactors (L/S = 12) and 50% in percolation reactors (L/S = 12). Crystallinity of

cellulose is maintained to allow grinding below 250 μm of particle size and drying with low energy consumption (12 kWh/t from the chimney heat lost at 125°C).
Two products emerge from the prehydrolysis: catalytic cellulignin and prehydrolysate. The first is used as fuel (catalytic cellulignin fuel – CCF) and as the energy food component for animal feed. The second is used for production of chemicals from biomass (furfural, ethanol and xylitol).
Water from cellulignin washing meets potable water specification after conventional simplified water treatment. It is recycled and not discharged to the environment. In fact the technology of the BEM Programme does not discharge any residues to the environment either solid, liquid or gaseous.

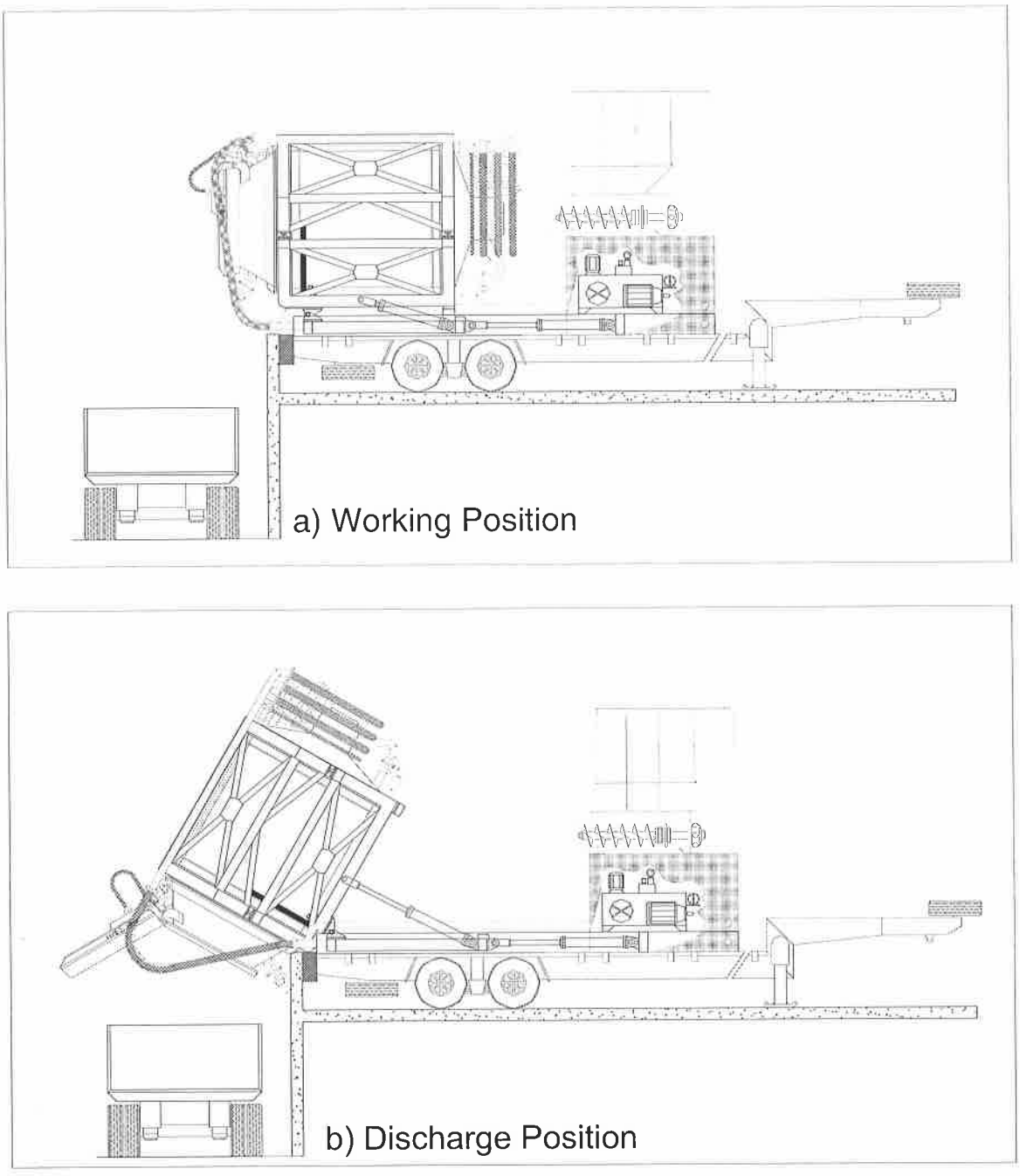


Figure 1. Mobile Prehydrolysis Reactor.

Figure 2 shows the increase in the Brix grade of the prehydrolysate as a function of the heating time. Figure 3 shows the microstructure of Eucalyptus grandis and cellulignin obtained by SEM – Scanning Electron Microscopy and Figure 4 shows the microstrucutre obtained by EMPA – Electron Microprobe Analyser with the fragile fracture of crystalline cellulose (3.a – 4.b), the ductile frature of eucalyptus (4.a), the globular-

shaped lignin in middle lamella (3.b) and the "volcanoes" in the inner surface of cytoplasm cavity (3.c – 4.d). These volcanoes are explosions of material due to formation of liquid and vapor during decomposition of the high concentration of hemicellulose in the inner layer of the secondary wall of the cell (3.d – 4.c).

Table 1. Energetic Analysis of Short Rotation Biomass Production.

Operation	Consumed energy per ton of dry wood (MJ//t)		Percentage of consumption	
	Conventional	BEM Programme	Conventional	BEM Programme
1 Production				
• Plantation and culture	9.3	9.3	0.8	3.3
• Fertilization	804	Null	52.1	---
• Harvest	87	87	7.5	31.0
• Transportation to trucks	115	115	9.9	41.0
• Loading of trucks	55	Null	4.7	---
• Transportation to the user (85 km)	221	Null	19.0	---
• Discharge	Null	Null	---	---
• Auxiliaries	69	69	5.9	24.6
Total	1160	280.3	99.9	99.9
2 Containing energy in raw biomass (11 MJ/kg)	11000	11000		
Efficiency in the energy production (%)	89.5	97.5		
Production ratio to net energy	9.48	39.2		

Table 2. Energy Consumption per Ton of Dry Biomass (TDB).

- Biomass heating (35% humidity, specific heat – C = 2.345 MJ/TDBºC.

MCΔT = 1 x 2.345 x (160 - 20) = 328.50 MJ/TDB

- Heating of 2 t of water (L/S = 2)

h^a_{20} = 83.96 KJ/kg (water)

h^a_{80} = 334.91 KJ/kg (water)

h^a_{100} = 418.94KJ/kg(water)

h^a_{160} = 675.55 KJ/kg (water)

h^v_{160} = 2,758.10 KJ/kg (vapour)

H_{CL} = 20 MJ/kg (heat of consumption of cellulignin)

Starting with water at 80ºC

$$m\left(h^a_{160} - h^a_{80}\right) = 2 \times 10^3 (675.55 - 334.91) = 681.28 MJ/TDB$$

Percentage of energy contained in the cellulignin

$$\frac{328.30 + 681.28}{10^3 \times 0.8 \times 20} = 0.0631 = 6.31\%$$

- Vapor consumption

$$\frac{(328.50 + 681.28) MJ/TDB}{(2{,}758.10 - 418.94) KJ/kg} = 431.68 \text{ kg of vapor/TDB}$$

- Boiler efficiency = 90%
- Cellulignin consumption for vapor production

$$\frac{484.9(2{,}758.10 - 418.94) KJ/TDB}{0.9 \times 0.8 \times 20 MJ/kg} = 70 \text{ kgCL/TDB}$$

CATALYTIC CELLULIGNIN FUEL (CCF)

The consequence of this process is the increase in the surface porosity from 0.46 m2/g for wood to 2.2 g/m^2 in the microporous surface (porous diameter < 2.0 nm – BET absorption of N2) plus another 2.2 g/m3 in the microporous surface (porous diameter > 50 nm – mercury porosimetry) totalizing 4.4 g/m2 for cellulignin, i.e., 10 times bigger than natural wood. Table 3 shows the physical characterization of microporous (BET - N2 adsorption) and Table 4 shows the physical characterization of meso and macroporous (mercury porosimetry) (Soares, 2000). Application of Thiele's theory (Essenhigh, 1989) results in catalytic combustion through the inner surface (Boudouard mechanism C + O2 → 2 CO) with ignition time less than 10 ms for particle diameter less than 250 μm, characteristic of gas combustion, instead of 100 ms for liquid and 300 ms for mineral coal that burns by the much smaller outside surface. Figure 5 shows the specific power irradiated by combustion of one cellulignin particle as a function of its diameter. The conventional combustion (smaller outside surface) takes place for particle diameter > 250 μm, and for particle diameter < 250 μm catalytic combustion takes place (ten times larger inner surface) (Vieira, 2000). Figure 6 shows the open-air flame geometry for an 83 kW axial combustor. With axial swirler the flame length is reduced to a one third.

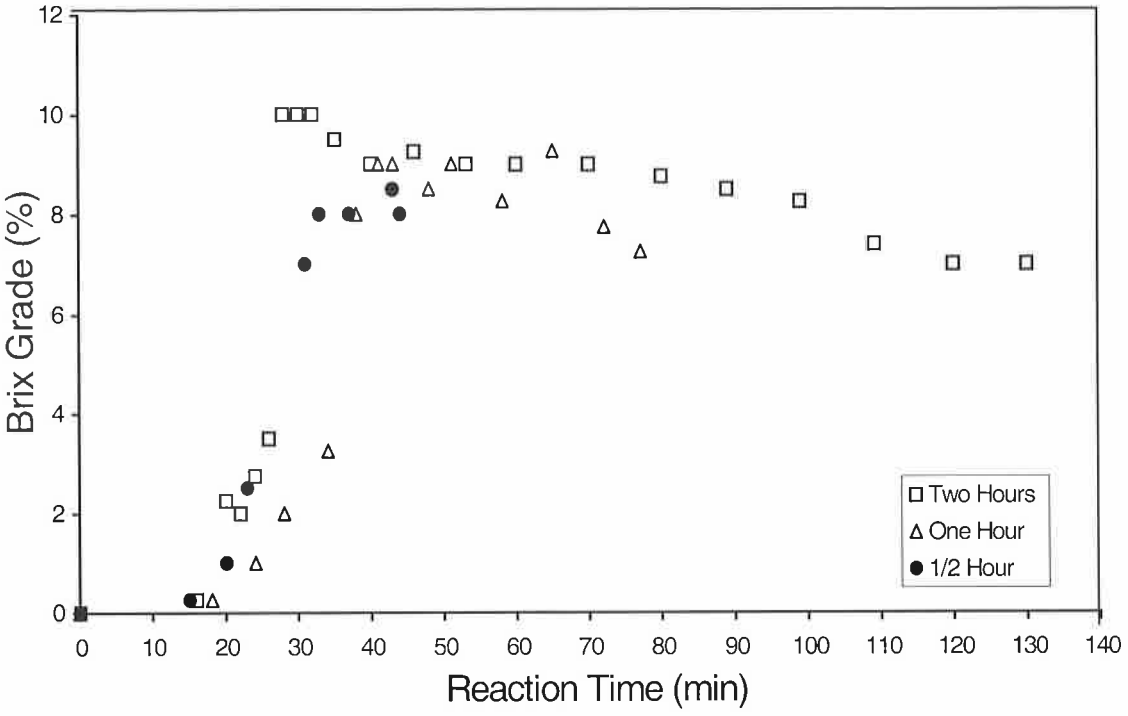
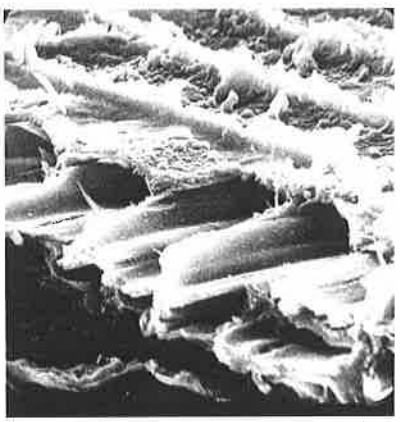
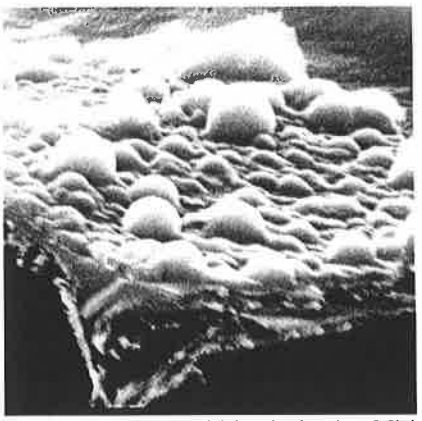


Figure 2. Acidic Prehydrolysis of Wood.

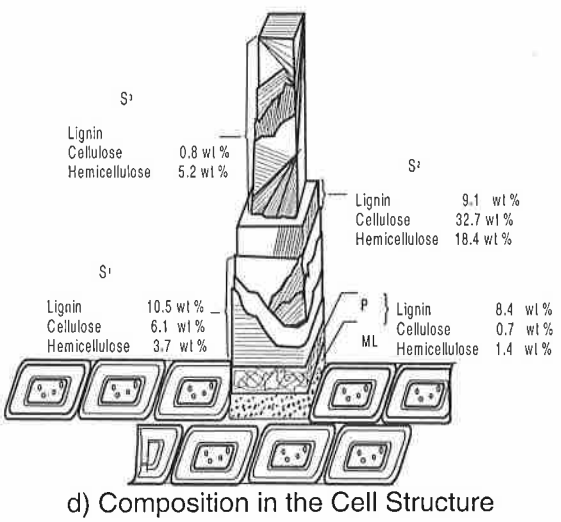


a) Fragile Fracture of the Cellulose



b) Globular-Shaped Lignin in the Middle Lamella of the Cell

c) "Volcanoes" in the Cytoplasm Inner Wall



d) Composition in the Cell Structure

Figure 3. SEM – Scanning Electron Microscopy of Cellulignin (Cambridge model S4-10).

Table 3. Physical Characterization of Microporous (Active Sites - $\phi < 2.0$ nm).

Samples	Crystalline Density (1) (g/cm^3)	Specific Surface (2) (m^2/g)	Microporous Radius (2) (nm)	Microporous Volume ($x\ 10^3\ cm^3/g$)
Wood	1.284	0.459	0.948	0.217
Cellulignin without grinding, prehydrolysis time				
• 0.5h (shaking)	1.331	0.756	0.980	0.371
• 1.0h (shaking)	1.337	1.463	0.905	0.662
• 1.0h (static)	1.314	1.342	0.970	0.651
• 2.0h (shaking)	1.351	2.249	0.964	1.080
Grinding Effect				
• Cellulignin without grinding	1.252	2.483	1.197	1.496
• 297μm < ϕ < 354 μm	1.353	2.758	0.997	1.375
• 177 μm < ϕ < 210 μm	1.368	2.013	1.135	1.143
• 125 μm < ϕ < 149 μm	1.375	2.114	1.032	1.090
• 88 μm < ϕ < 105 μm	1.372	1.915	0.962	0.921
• ϕ < 354 μm	1.346	3.179	0.914	1.454
Wood and carbonized cellulignin				
• Carbonized wood	1.314	0.965	1.024	0.494
• Cellulignin of 0.5h	1.292	2.474	1.014	1.254
• Cellulignin of 1.0h	1.327	1.452	1.002	0.727
• Cellulignin of 2.0h	1.371	1.932	1.009	0.974
• Double carbonization	1.421	2.497	1.000	1.248

(1) Helium Picnometry; Equipment used – Ultrapicnometer Quantachrome Model 1000 – Version 1.62
(2) BET – N_2 Adsorption; Equipment used – Adsortometer – New Quantachrome Model – Version 3.70

Table 4. Distribution of Meso (2nm<ϕ<50nm) and Macro (ϕ>50nm) porous (cm^3/g).

Samples	Mercury Porosimetry (%) D_p= Porous Diameter (nm)							Porous Cylindrical Geometry(2) (m^2/g)
	Medium Radius (nm)	D_1(1)	D_2(1)	D_3(1)	D_4(1)	D_5(1)	Total	
Cellulignin without grinding								
ϕ > 2mm (coarse)	631.2	0.016	0.078	0.233	0.038	0.056	0.421	2.668
ϕ > 354μm (fines)	1025.2	0.004	0.022	0.084	0.039	0.048	0.197	0.758
Grinding effect								
297μm<ϕ<354μm	893.0	0.006	0.033	0.095	0.052	0.046	0.232	2.080
210μm<ϕ<250μm	987.2	0.009	0.027	0.084	0.048	0.051	0.219	1.775
177μm<ϕ<210μm	—	—	—	—	—	—	—	—
149μm<ϕ<177μm	845.8	0.003	0.018	0.094	0.062	0.035	0.212	2.004
125μm<ϕ<149μm	—	—	—	—	—	—	—	—
105μm<ϕ<125μm	1180.6	0.007	0.025	0.090	0.058	0.049	0.229	1.553
88μm<ϕ<105μm	—	—	—	—	—	—	—	—
74μm<ϕ<88μm	1003.2	0.009	0.025	0.091	0.051	0.042	0.218	1.739
ϕ<74μm	1715.4	0.008	0.023	0.106	0.163	0.530	0.830	3.876

(1) $D_1 \equiv D_p < 10$ $D_2 \equiv 10 < D_p < 100$ $D_3 \equiv 100 < D_p < 1000$ $D_4 \equiv 10^3 < Dp < 2\times10^3$ $D_5 \equiv 2\times10^3 < D_p < 5\times10^3$
(2) r = 2l; $S/V = (2\pi r^2 + \pi rl)/(\pi r^2 l)$; $S/V = (2/r)$; $S = 2V/r$ S = porous surface; V = porous volume; r = porous radius; l = porous length.

Table 5 shows the composition of CCF and its precursor, Eucalyptus grandis. Table 5.a refers to the C, H, N, O, N and ash contents and Table 5.b refers to the concentration of some inorganic constituents (Conte, 1999). Prehydrolysis can be made with potable water, deionized water, non-washed and intensive washed cellulignin. Combination of these process conditions leads to a combustion gas (after cyclone cleaning of the cellulignin) with purities ranging from wood grade to natural gas grade.

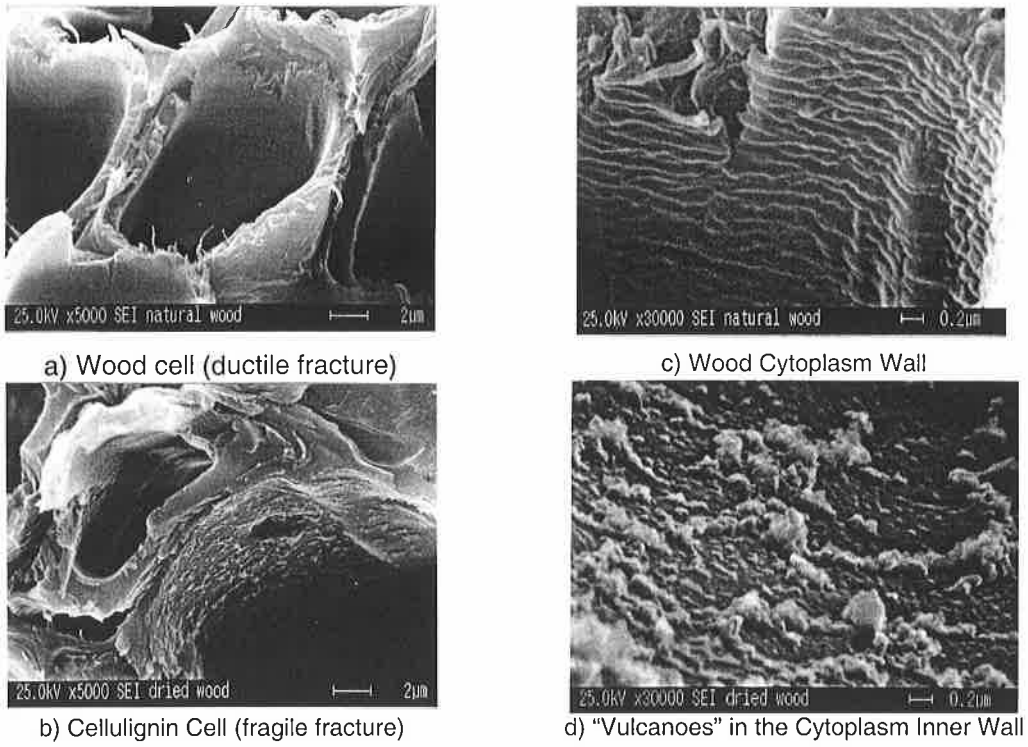


Figure 4. Microstructure of Wood and CCF. Porosity and Expelled Material. (EPMA Shimadzu 1660).

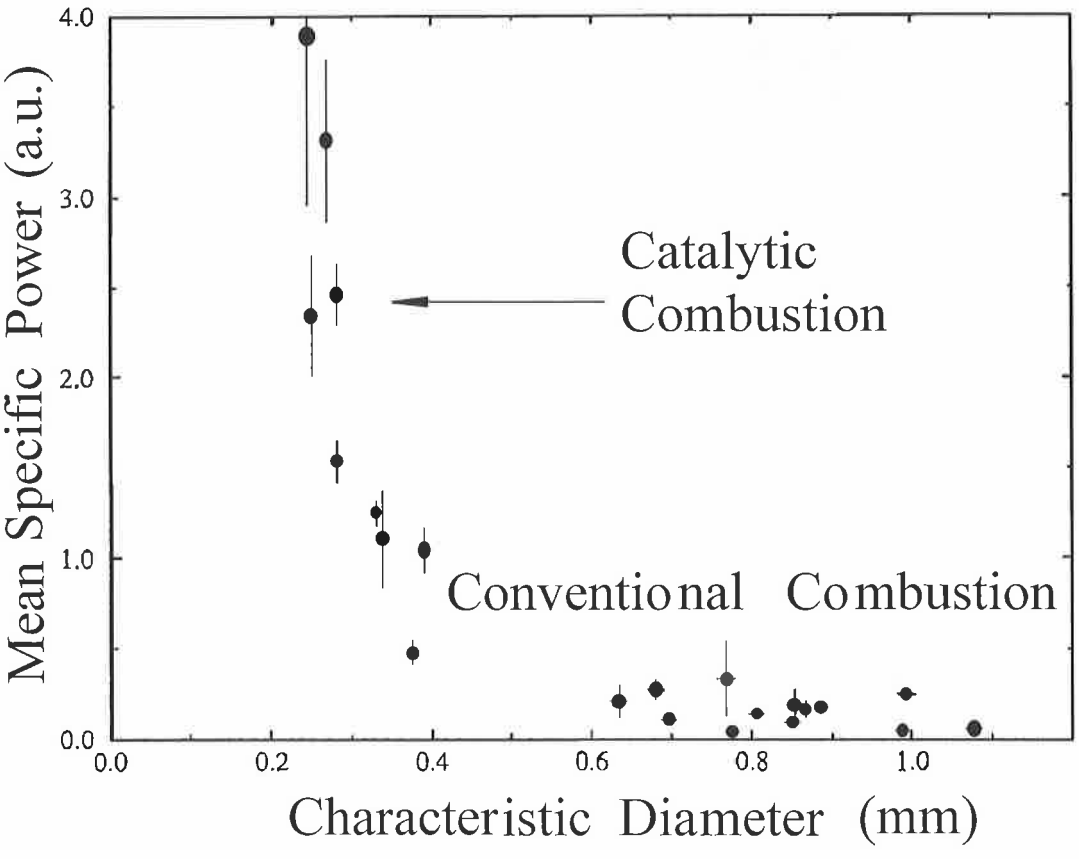


Figure 5. Mean Specific Irradiated Power in the Combustion of a Particle of Catalytic Cellulignin.

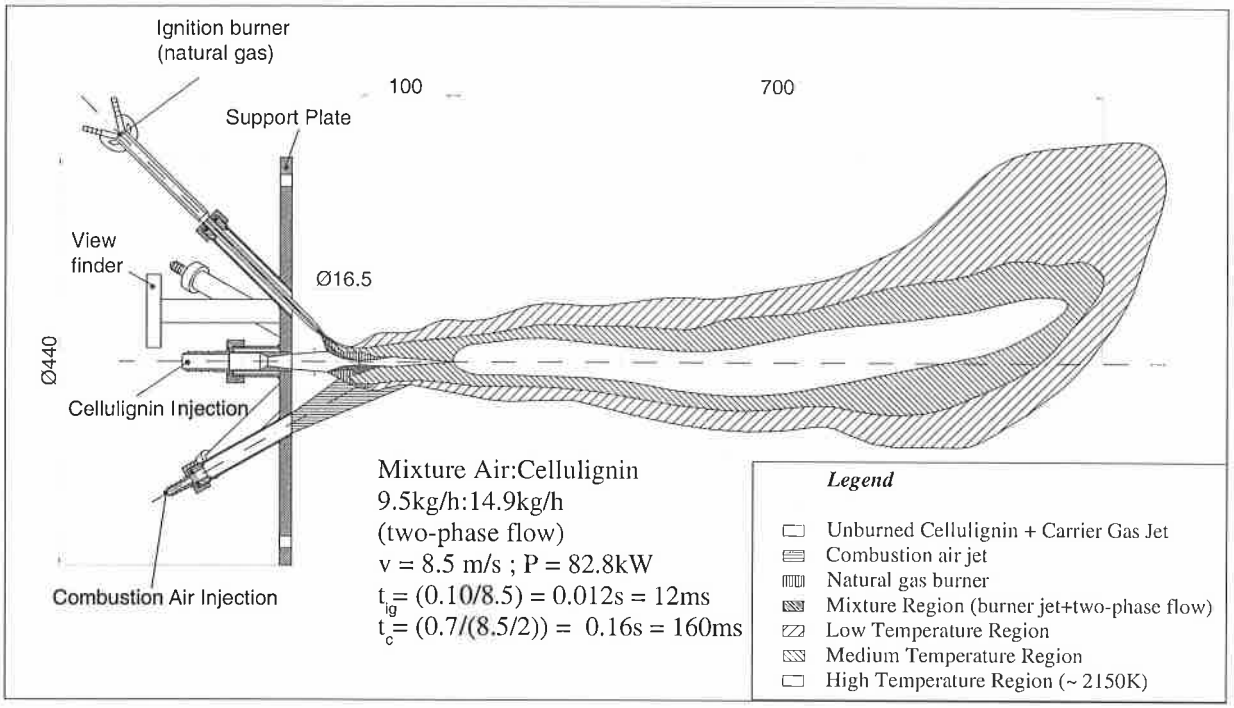


Figure 6. Axial Combustor with Cellulignin Flame in Open Air (dimensions in mm).

Table 5a. Biomass Fuel Analysis.

Proximate Analysis	Alder Fir (USA)	Eucalyptus grandis	Cellulignin Fuel		
			Potable Water (PW)	Deionized Water (DW)	
			Washed Cellulignin	Non-Washed Cellulignin	Washed Cellulignin
Fixed Carbon	19.31	27.23	31.42	31.42	31.42
Volatile Matter	75.56	72.38	68.41	68.37	68.50
Ash	4.13	0.39	0.17	0.21	0.08
Moisture	Oven Dry	Oven Dry	Oven Dry	Oven Dry	Oven Dry
Ultimate Analysis					
Carbon	51.02	—	66.23	66.21	66.30
Hydrogen	5.8	—	4.27	4.26	4.27
Oxygen	38.54	—	29.32	29.32	29.36
Nitrogen	0.46	—	—	—	—
Sulfur	0.05	0.014	0.008	0.02	0.008
Ash	4.13	0.39	0.17	0.21	< 0.08
Moisture	Oven Dry	Oven Dry	Oven Dry	Oven Dry	Oven Dry
LHV,BTU/#	7415	4729	8599	8599	8599

Table 5b. Concentration of Inorganic Constituents in Eucalyptus grandis and Cellulignin (μg/g).

Biomass Fuel	Ca	K	Na	Mg	P	Al	Si	Mn	Fe	Zn	S
Eucalyptus grandis	510	635	100	255	120	240	135	25	40	10	140
Washed Cellulignin (PW)(1)	500	< 60	< 140	< 40	10	< 40	< 120	< 4	< 10	< 4	< 80
Non-Washed Cellulignin (DW)(2)	120	35	55	30	<15	220	320	1	15	8	200
Washed Cellulignin (DW)(3)	<53	<5	<1	<60	<2	<40	<120	<2	<7	<4	<80

(1) Semiquantitative XRFS analysis.
(2) Quantitative ICP-AES analysis.
(3) Semiquantitative XRFS analysis, except for Na (AAS-flame) and K (ICP-AES), both referring to the limits of detection.

Table 6 shows the microanalysis parameters and composition by EPMA – Electron Probe Microanalyser (Shimadzu 1600). For Eucalyptus grandis the contents of Ca and K are higher than the values obtained by ICP-AES. The same behavior is found for S in the CCF. Elements like Na, Mg, P, Al and Si were not detected by EPMA. These differences are explained because EPMA makes a local analysis and ICP-AES

makes a volumetric analysis. For macroelements (like C, O), the agreement of EPMA results and elemental analysis is reasonable good.

Table 6. Microanalysis parameter and composition made by EPMA.

a) Wood Eucalyptus

*Ratio [Coating Element = Au] [Correction = ZAF4]

Nº	ELE.		Crystal	W.L.(A)	PKI-BGI	STD(1)	I-Ratio	Wt(%)	Mol(%)	ELE.
1	C	Ka	PBST	44.7000	2365.04	803.50	2.9434	86.084	88.404	C
2	O	Ka	RAP	23.6200	5.00	44.32	0.1128	13.358	10.415	O
3	K	Ka	PET	3.7414	25.65	3376.48	0.0076	0.226	0.072	K
4	Ca	Ka	PET	3.3584	27.94	3824.55	0.0073	0.214	0.066	Ca
5	Cl	Ka	PET	4.7278	10.00	2514.40	0.0040	0.119	0.042	Cl
TOTAL								100.000		

b) Catalytic Cellulignin Fuel (CCF)

*Ratio [Coating Element = Au] [Correction = ZAF4]

Nº	ELE.		Crystal	W.L.(A)	PKI-BGI	STD(1)	I-Ratio	Wt(%)	Mol(%)	ELE.
1	C	Ka	PBST	44.7000	2838.71	803.50	3.5329	76.880	81.207	C
2	O	Ka	RAP	23.6200	14.59	44.32	0.3292	23.489	18.733	O
3	S	Ka	PET	5.3721	17.32	2295.07	0.0075	0.150	0.060	S
TOTAL								100.000		

CONCLUSION

The correct use of refractory metals (Ti) in the construction of an acidic prehydrolysis reactor has allowed the production of a catalytic cellulignin fuel (CCF) with purity and combustion characteristics close to natural gas (< 10 ms ignition time). The new reactor rises the efficiency in biomass production to 97.5% (mobile characteristic) and 92% in the biomass to cellulignin conversion given a total of 90% of energy recovery. Cost analysis described elsewhere (Pinatti, 1997) shows that the technology of BEM Programme yield electric energy from biomass at competitive cost to hydro and fossil fuel energy.

REFERENCES

Conte, R.A. (1999). Pos Doctorate. Final Report, Delft University of Technology, Delft, The Netherlands.

David, A.T., Amadeo, J.R. & William, D.K. (1981). Wood Combustion, Principles, Process and Economics. Academic Press, New York.

Essenhigh, R.H. Misra, M.K. & Shaw, D.W. (1989). Ignition of Coal Particles: A Review. Combustion and Flame 77, pp 3-30.

Pinatti, D.G., Vieira, C.A., Cruz, J.A. & Conte, R.A. (1997). Cellulignin: a new thermoelectric fuel. In Latin-American Congress – Electricity Generation and Transmission, Proceedings, pp. 95-100.

Soares, A.G. (in execution, 2000). Carvão Ativado de Celulignina. PhD. Thesis, Instituto de Física, Universidade Estadual de Campinas.

Vieira, C.A. (in execution, 2000). Processos Fundamentais em Combustão de Celulignina. PhD. Thesis, Instituto de Física, Universidade Estadual de Campinas.

Binuclear manganese complex-catalysed oxidation of lignin with hydrogen peroxide – Kinetic, reaction mechanisms and application

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ABSTRACT

The Kinetics of delignification of a kraft-AQ pine pulp with hydrogen peroxide catalyzed by $[LMn(IV)(\mu\text{-}O)_3Mn(IV)](ClO_4)_2$ (**1**), where L = 1,2-bis(4,7-dimethyl-1,4,7-triazacyclonon-1-yl)ethane was studied. The pulp was bleached for 2 hours at 80°C, in 10 % consistency with 2 % NaOH, 4% H_2O_2 and 60 ppm catalyst charges on pulp (O.D.). Kappa number of the pulp was reduced from 31.6 to 16.8 corresponding to a degree of delignification of approximately 46%, while GE brightness was increased from 24.2 to 44.7. At the same time, viscosity of the resulting pulp was reduced moderately from 30.2 mPa.s to 20.1 mPa.s indicating that the degradation of the carbohydrates was rather moderate. The delignification was found to follow pseudo first order kinetics with respect to kappa number, i.e., residual lignin, in the initial phase and quickly slowed down after 30 min (residual phase) under all the reaction temperatures investigated. The delignification rate constants in the initial phase were 0.17, 0.18, and 0.21 min^{-1} at 50, 60, and 80°C, respectively. Degree of delignification at the delignification time of 30 min is approximately 40% at 80°C. The possible delignification mechanism was discussed on the basis of the kinetic studies and lignin model compound experiments.

Keywords: bleaching, delignification, cellulose pulp, manganese complexes

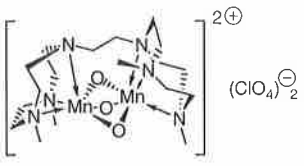
INTRODUCTION

It has been well established that the effluents from the conventional pulp bleaching processes, which employ elemental chlorine and/or chlorine containing chemicals, are contaminated by polychlorinated phenols, such as dioxins and other toxic compounds (McBrid *et al.*, 1988). The public awareness of this issue grew rapidly and had become global during the past few decades. Consequently, the environmental regulations on the pulp and paper industry implemented by the government became dramatically stricter (Unites States Environmental Protection Agency 1993). Driven mainly by the public concern of clean environment, the development of environmentally friendly bleaching technologies utilizing oxygen-based chemicals has been and will continuously be one of the major technical progresses in the pulp and paper industry.

Among the technologies aiming at "elementary chlorine free" (ECF) or "totally chlorine free" (TCF) bleaching, hydrogen peroxide bleaching was developed and has been widely used by the industry. However, hydrogen peroxide has long been used in alkaline condition only as a bleaching agent to improve the brightness of pulp, mainly mechanical pulp, rather than as a delignifying agent to remove residual lignin from chemical pulp. In fact, in alkaline condition, dissociated hydrogen peroxide, i.e. hydroperoxide anion, reacts with residual lingins in pulps mainly as a nucleophile instead of an oxidant. The hydroperoxide anion attacks carbonyls conjugated with aromatic rings in the residual lignin rather than oxidatively degrads the lignin (Pan *et al.*, 1993 and Pan *et al.*, 1994). Although there have been numerous efforts to improve the capability of hydrogen peroxide as a delignifying agent, it has been used as a delignifying agent only at high temperatures, frequently above 100°C in alkaline solution (Lanchenal *et al.*, 1980 and Alfthan *et al.*, 1977). Under these reaction conditions, the stability of hydrogen peroxide becomes so doubtful that certain chelants had to be added to suppress the decomposition of hydrogen peroxide. Moreover, the improvement in the degree of delignification is largely compromised by the rather higher energy cost. Until now, it is still a technological challenge to achieve a relatively high degree of delignification of kraft pulp with hydrogen peroxide at relatively low temperatures such as at temperature range of 75-80°C at which pulp comes out of the brown stock washer.

The objective of this investigation is thus to improve the reactivity of hydrogen peroxide as an oxidant to such an extent that it is capable of oxidatively degrading residual lignin remaining in the kraft pulp effectively at relatively low temperatures in presence of a binuclear manganese complex

$[LMn(IV)(\mu\text{-}O)_3Mn(IV)](ClO_4)_2$ (**1**) where L = 1,2-bis(4,7-dimethyl-1,4,7-triazacyclonon-1-yl)ethane, (abbreviated as Mn(IV)-Me_4DTNE hereafter) or one of the related compounds as a catalyst. In addition to the decoloration of the pulp, a relatively high degree of delignification is expected to achieve without severely damaging the pulps.



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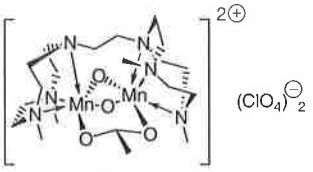

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As a matter of fact, the synthesis and characterization of this type of binuclear complexes had been extensively studied, however, mainly motivated by the theoretical significance (Wieghardt *et al.*, 1985, Wieghardt *et al.*, 1988). We began our investigation on the chemistry of Mn(IV)-Me_4DTNE (**1**) as a catalyst for delignification of chemical pulp with hydrogen peroxide shortly after Patt and Mielisch (1997) reported that Mn(IV)-Me_4DTNE (**1**) is an excellent catalyst in hydrogen peroxide bleaching of pulp, because the kinetics and reaction mechanism of this process were not well understood at that time. As reported in the previously papers by this research group (Cui *et al.*, 1998, Cui *et al.*, 1999a, Cui *et al.*, 1999b), Mn(IV)-Me_4DTNE (**1**) was capable of catalyzing oxidation of 1-(3,4-dimethoxy-phenyl)ethanol (**3**; L_1=H, L_2 = CH_3) to 3,4-diemthoxy-acetophenone (**4**; L_1=H, L_2 = CH_3) (Scheme 1), even at relatively low temperatures.

Scheme 1. The oxidation of α-hydroxyl group in residual lignin with hydrogen peroxide in the presence of Mn(IV)-Me_4DTNE (**1**) under alkaline condition.

In addition, Mn(IV)-Me_4DTNE (**1**) is also capable of catalyzing epoxidation of C-C double bonds conjugated with aromatic rings by hydrogen peroxide. In the case of **E**-stilbene, the reaction yielded only **E**-stilbeneoxide as product. In the case of 1-(3,4-dimethoxyphenyl)propene (a mixture of **E**, **Z** isomers), however, the resulting epoxide underwent further hydrolysis and oxidation give *trans*-1-(3,4-dimethoxylphenyl)-1,2-pantodiol and 3,4-dimethoxybenzaldehye, respectively. All the reactions studied showed that the reactions proceed at relatively high reaction rates at relatively low temperatures. In the light of the results from the model compound studies, we expected that Mn(IV)-Me_4DTNE-catalyzed oxidation with hydrogen peroxide is capable of oxidizing the α-hydroxyl groups and conjugated C-C double bonds in the residual lignin of chemical pulps. The resulting carbonyls and epoxides would then undergo further nucleophilic attack in alkaline solution by oxidants acting as nucleophiles. This would lead to further degradation and fragmentation of the residual lignin and would render it more soluble in the reaction medium under the condition of the reaction. A relatively high degree of delignification would be achieved, hopefully at relatively low temperatures.

EXPERIMENTAL

Reagents and Equipment

The kraft-AQ southern pine pulp was supplied by Covington mill of Westvaco Co., Covington, VA. Hydrogen peroxide was purchased from Fisher in the form of 30% aqueous solution and diluted to appropriate concentration for experiment. Mn(IV)-Me_4DTNE was supplied by Deguasse AG., Hanau, Germany.

Acid Treatment

Pulp (3% consistency) was placed in a plastic bag, then immersed in a constant temperature water bath. The pH of the pulp slurry was adjusted to 2.0 by sulfuric acid then the temperature was raised to 70°C. After 30 min, the pulp was removed from the bath and the resulting pulp was dewatered to about 33% consistence for the subsequent experiments.

Mn(IV)-Me_4DTNE-Catalyzed Delignification

In an Erlenmeyer flask, was placed 3g OD pulp 0.06g NaOH (2% on O.D. pulp) and 0.12 g H_2O_2 (4% on dry pulp) were added to the flask in the form of a mixed solution simultaneously with 0.45mL 0.4mg/mL Mn(IV)-Me_4DTNE solution (60 ppm on O.D. pulp). Depend on the starting consistency of the pulp to be delignified, an appropriate amount of DI water was added immediately to bring the final consistency to 10% under vigorous mechanical stirring. The delignification was allowed to proceed for designated periods of time then taken out of the flask to be washed with 3 x 50mL deionized water. After being dewatered, the pulp was ready for handsheet making.

Determination of Kappa Number, Viscosity, and GE Brightness

Handsheets were made from the pulp obtained using the procedure described above and conditioned at 23±1°C and 50±2% relatively humidity overnight. Then the GE brightness of the handsheets was measured according to Tappi Standard T 452. The kappa number and the viscosity were measured according to Tappi Standard T236 and T230 respectively.

RESULTS AND DISCUSSION

A kraft-AQ southern pine pulp was used in this study. The kappa number, viscosity and GE brightness of the pulp were 31.6, 30.2 mPa.s, and 24.2 after the acid treatment, respectively. This pulp was then subjected to delignification by hydrogen peroxide with and without the catalyst Mn(IV)-Me_4DTNE (**1**). The degree of delignification was determined by change in kappa number as a function of reaction time. The effects of pulp consistency and alkaline charge on pulp (O.D.) were studied on the degree of delignification as well as on some physical properties of the resulting pulp.

Comparison of Mn(IV)-Me_4DTNE-Catalyzed and Non-catalyzed Delignification

The physical properties of pulps obtained from Mn(IV)-Me_4DTNE-catalyzed and non-catalyzed delignification with hydrogen peroxide are summaried in Table 1. In the absence of Mn(IV)-Me_4DTNE, the pulp was treated with 2% NaOH and 4% hydrogen peroxide at 10% consistency and at bleaching temperature of 80°C for 120 mins (two hours). As expected, the degree of delignification was modest (Table 1). The kappa number of the pulp dropped from 31.6 to 23.6 (the degree of delignification: approximately 25%), and the brightness of the pulp increased from 24.2 GE to 40.8 GE (brightness increase: approximately 67%). Upon the addition of Mn(IV)-Me_4DTNE at 60 ppm on pulp (O.D.) under the same reaction conditions, the degree of delignification was appreciably improved, from 25% to approximately 46%.

The kappa number was brought down to 16.8, almost further decrease of 7 units compared to non-catalyzed delignification under the same reaction condition. Furthermore, in the presence of Mn(IV)-Me_4DTNE (**1**), the kappa number dropped to about 18 only in 20 min. The kappa number then decreased very slowly and eventually leveled off. It is noteworthy that the application of the catalyst also resulted in a moderate increase in GE brightness of approximately 4 points with a total brightness increase of approximately 85%. Considering much higher extent of decrease in the kappa number, it can be postulated that the catalyst mainly improves the reactivity of hydrogen peroxide as an oxidant, while retains its reactivity as a nucleophile. The increase of the brightness can be attributable to the higher degree of delignification that in turn results in more degradation of chromophoric groups in the residual lignin. As a consequence, the brightness is considerably improved. In addition, the degree of carbohydrate degradation was evaluated by determination of viscosity of the pulp before and after both the catalyzed and the non-catalyzed delignification. In general, the viscosity of the pulps from the catalyzed delignification was 7-11.5% lower than that of the corresponding pulps obtained by non-catalyzed delignification, 21.5 mPa.s *versus* 24.3 mPa.s at 50°C and 20.1 mPa.s *versus* 21.6 mPa.s at 80°C. Thus, the difference in the viscosity between the catalyzed and non-catalyzed pulp under the same delignification condition decreases with increasing reaction temperature. The viscosity of the pulps from the catalyzed delignification also decreses slightly (approximately 6.5%) with increasing reaction temperature, from 21.5 mPa.s to 20.1 mPa.s when temperature

increased from 50°C to 80°C. In contrast, the viscosity of the pulps from the non-catalyzed delignification decreases from 24.3 mPa.s to 21.6 mPa.s (approximately 11%) when the reaction temperature increased from 50°C to 80°C. The pulps from the catalyzed delignification have appreciably lower kappa number than the corresponding pulps from the non-catalyzed delignification. The rather slight difference in the viscosity among the pulps from the catalyzed delignification then implies that hydrogen peroxide is more active towards oxidative degradation of residual lignin in the presence of the catalyst.

Table 1. The comparison of physical characteristics among pulps obtained from the uncatalyzed and the Mn(IV)-Me_4DTNE-catalyzed delignification of pulps with hydrogen peroxide [1-6]

Pulp Samples	Delignification		Viscosity	GE Brightness
	Kappa Number	Degree of Delignification	(mPa.s)	
Original Pulp	31.6	----	31.1	24.2
Uncat. Delig. (50°C)	26.3	16%	24.3	38.1
Cat. Delig. (50°C)	19.9	37%	21.5	45.1
Cat. Delig. (60°C)	19.2	39%	21.4	46.4
Cat. Delig. (80°C)	16.8	46%	20.1	44.7
Uncat. Delig. (80°C)	23.6	25%	21.6	40.8

[1] Pulp consistency 10% in alkaline solution
[2] NaOH charge: 2% NaOH on pulp (O.D.)
[3] Starting pH 11.5, ending pH 10.9
[4] H_2O_2 charge: 4% on pulp (OD)
[5] MN (IV)-Me_4DTNE catalyst charge: 60 PPM on pulp (OD)
[6] Reaction time: 120 min.

Kinetics and Mechanism of Mn(IV)-Me_4DTNE-Catalyzed Delignification

As described in the previous papers (Cui *et al.*, 1998, Cui *et al.*, 1999), the precatalyst $[LMn(III)(\mu\text{-}O)_2(\mu\text{-}OAc)Mn(IV)](ClO_4)_2$ (**2**) undergoes nuclephilic attack by hydroperoxide anion to yield the actual catalyst $[LMn(IV)(\mu\text{-}O)_3Mn(IV)](ClO_4)_2$ (**1**) immediately after addition of **2** to hydrogen peroxide in alkaline solution (See Scheme 2 for reaction mechanism). Two mole equivalents of precatalyst **2** are oxidized by one mole equivalent of hydrogen peroxide to give two mole equivalents of the catalyst **1** and two mole equivalents of acetic acid.

Preliminary experiments established that the optimum condition for bleaching of pine kraft pulp is pulp consistency of 10% in an alkaline solution, NaOH charge of 2% on pulp (O.D.), H_2O_2 charge of 4% on pulp (O.D.), catalyst charge of 60 ppm on pulp (O.D.) and reaction temperature of 80°C. Therefore, kinetics of the delignification was carried out under these conditions with reaction temperatures at 50, 60, and 80°C. At these reaction temperatures the delignification procceeded readily at reaction time of up to 30 min (initial phase). The delignification rate was then quickly slowed down and eventually leveled off (residual phase) (Figure 1).

As expected, the delignification followed the pseudo first order rate kinetics with respect to kappa number, i.e., residual lignin in pulp in the initial phase. The delignification rate constants in the initial phase were 0.17, 0.18, and 0.21 min^{-1} at 50, 60, and 80°C, respectively. The delignification rate was then slowed down after 30 min at all the temperatures studied (Figure 1). These results were in consistence with the results of the model compound studies. This suggests that delignification proceeds in the similar ways as in the oxidation of lignin model compounds. Thus, it is likely that the residual lignin in pulps undergoes Mn(IV)-Me_4DTNE-catalyzed oxidation with hydrogen peroxide oxidizing the α-hydroxyl groups (see Scheme 1) and C-C double bonds conjugated with the aromatic rings in the residual lignin. The oxidation and the subsequent fragmentation were almost complete within 30 min (Figure 1), and the subsequent degradation of the lignin fragments was responsible for the further slower Delignification afterwards. The activation energy of the delignification was 7.16 kJ/(mol.K), calculated according to Arrhenius Equation,

suggesting that the dependence of the rate of Delignification on temperature was moderate because the activation energy of the delignification decreased considerably upon the addition of the catalyst.

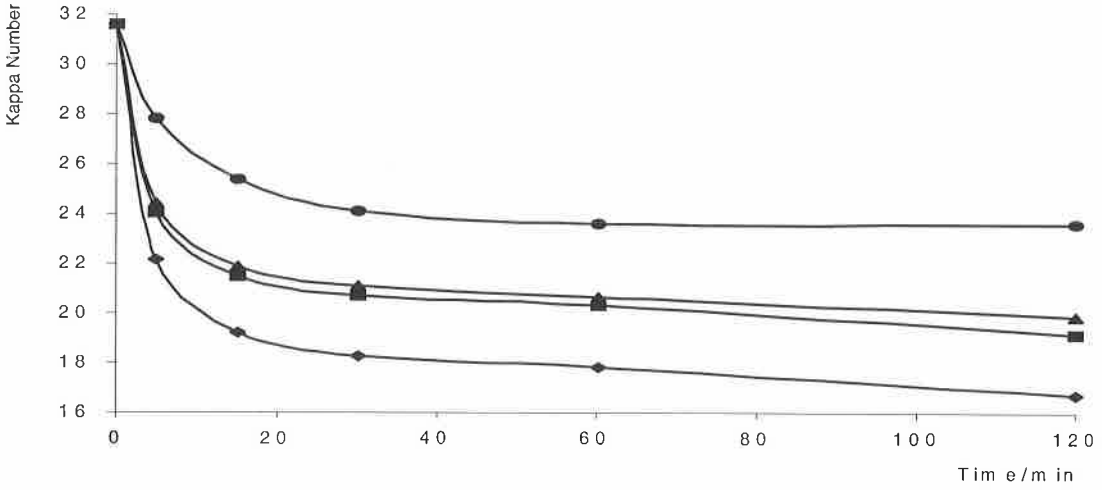

Figure 1. Comparison of uncatalyzed delignification at 80°C with Mn(IV)-Me_4DTNE-catalyzed delignification at 50, 60, and 80°C in alkaline solution (Uncatalyzed delignification at ● 80°C. Consistency, 10% in alkaline solution; NaOH charge, 2% on pulp (O.D.); H_2O_2 charge, 4% on pulp (O.D.). Catalyzed delignification at ▲50°C, ■ 60°C, ◆ 80°C. Consistency, 10% in alkaline solution; NaOH charge, 2% on pulp (O.D.); H_2O_2 charge, 4% on pulp (O.D.); Mn(IV)-Me_4DTNE catalyst charge, 60 ppm on pulp (O.D.).

Effect of Varying Alkaline Charge or Consistency on Delignification, GE Brightness, and Viscosity

Increasing the initial concentration of NaOH solution would increase the concentration of the dissociated hydrogen peroxide, i.e. hydroperoxide anion, the nucleophile responsible for the brightness improvement. Consequently, it is expected that the higher initial concentration of NaOH solution would lead to higher final brightness. Indeed, the final GE brightness was 46.4 if the initial NaOH charge was 2% on pulp (O.D.), while the final GE brightness was 41.6 if the initial NaOH charge was 1% on pulp (O.D.). The increasing alkaline charge also had a slight effect on delignification and viscosity.

Pulp consistency had a slight effect on the degree of delignification. After 120 mins (2 hours) of delignification with 2% NaOH, 4% hydrogen, and catalyst charge of 60 ppm/OD g pulp at 60°C, the final kappa numbers were 20.8, 21.4, and 21.8 for the pulp with the initial consistency of 5, 10, and 15%, respectively. The brightness improvement appeared lower at higher consistency, despite the slightly higher degree of delignification. The reason might be the solubility of the lignin fragments from the oxidative degradation. The effects of chromophoric groups became important at higher consistency because smaller amount of liquor is available for solublizing the lignin fragments.

CONCLUSIONS

The reactivity of hydrogen peroxide towards residual lignin in chemical pulp as an oxidant was significantly improved upon the catalysis of Mn(IV)-Me_4DTNE (**1**). The selectivity of hydrogen peroxide delignification was also considerably improved, indicated by the viscosity data. The Mn(IV)-Me_4DTNE-catalyzed delignification followed the pseudo first order rate kinetics and leveled off after 30 min, and had the optimum reaction temperature of 80°C. These are attractive characteristics of this process. The rate of delignification was not very sensitive to the change of temperature because of the decrease in activation energy of deligniifcation due to the effect of catalyst. Consequently, the degree of delignification was appreciable even at relatively low temperatures within a relatively a short period of time. At the same time, hydrogen peroxide remained as an excellent nucleophile for the brightness improvement of the pulp.

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REFERENCES

Alfthan, C., Andersson., E., Andersson., S. & Hook, J.E. (1977). High temperature peroxide bleaching of sulfate pulp. *Svensk Papperstidn.* **15**, 480-482.

Cui, Y., Chen, C.-L., Gratzl, J.S. & Patt, R. (1998). Hydrogen peroxide oxidation of lignin model compounds catalyzed by a manganese(IV)-Me_4DTNE complex. *5th European Workshop on Lignocellulosics and Pulp*, Proceedings, pp 389-392.

Cui, Y., Chen, C.-L., Gratzl, J.S. & Patt, R. (1999a). A manganese(IV) complex catalyzed oxidation of lignin model compounds with hydrogen peroxide. *J. Mol. Cat. A: Chem.* **144**, 411-417.

Cui, Y., Puthson, P., Chen, C.-L., J.S. Gratzl, J.S., Kirkman, A.G. & Patt, R. (1999b). Hydrogen peroxide bleaching of pine pulp catalyzed by a binuclear manganese complex. *10th International Symposium on Wood and Pulping Chemistry*, Proceedings, **Vol 1**, pp 256-261.

Lachenal, D., de Choundens, C. & Monzie, P. (1980). Hydrogen peroxide as a delignifying agent. *Tappi* **63 (4)**, 119-122.

McBrid, A.C., Amendola, R.O., Blosser, R.C., Whittermore, F., LaFleur, L.E. & Tiernan, T.O. (1988). *U.S. EPA/Paper industry cooperative dioxin screening study*. **EPA-440**/1-88-025.

Pan, X., D. Lachenal, D., Lapierre, C. & Monties, B. (1993). Structure and reactivity of spruce mechanical pulp lignins. Part III. Bleaching and photoyellowing of isolated lignin fractions. *J. Wood Chem. Technol.* **13 (2)**, 145-165.

Pan, X., D. Lachenal, V. Neirinck & D. Robert. (1994). Structure and reactivity of spruce mechanical pulp lignins. Part IV. ^{13}C-NMR spectral studies of isolated lignins. *J. Wood Chem. Technol.* **14 (4)**, 483-506.

Patt, R. & Mielisch, H.-J. (1997). Application of a catalyst in TCF pulp bleaching. *5th. Brazilian Symposium on the Chemistry of Lignin and Other Wood Components*, Proceedings, pp 44-58.

United States Environmental Protection Agency. (1993). U.S. EPA Proposal: Effluent limitations and pretreatment guildlines, and maximum achievable control techology (MACT). *Federal Register*, **58 (241)**, 66078-66216.

Wieghardt, K., Tolksdorf, I. & Herrmann, W. (1985). Coordination chemistry of the bimarocyclic, potentially binucleating ligand 1,2-bis(1,4,7-triaz-1-cyclonyl)ethane (dtne). Electrochemistry of its first transition series metal(II,III) complexes. Characterization of the new hemerythrin model complex $[Fe_2(dtne)(\mu\text{-}O)(\mu\text{-}CH_3CO_2)_2]Br_2H_2O$. *Inorg. Chem.* **24**, 1230-1235.

Wieghardt, K., Bossek, U., Nuber, B., Weiss, J., Bonviosin, J., Corbella, M., Vitol, S.E. & Girerd J.J. (1988). Synthesis, crystal structures, reactivity, and magnetochemistry of a series of binuclear relevance, the crystal structure of $[L'Mn(IV)(\mu\text{-}O)_3Mn(IV)L'](PF_6)_2$ containing an unprecedented short Mn-Mn distance of 1.296 A. *J. Am. Chem. Soc.*, **110**, 7398-7411.

Bio-oils as a renewable source of phenolics for production of resol wood adhesives

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ABSTRACT

Eucalyptus wood slow pyrolysis bio-oil (tar) was used to replace part of the petroleum-derived phenol used for preparation of phenol formaldehyde resins (resoles). Large quantities of this crude bio-oil are currently produced by the Brazilian charcoal industry and are available as a low cost feedstock. In the local market this bio-oil is priced at US $ 200 per ton while phenol is priced in the range of US $ 750 to 900 per ton. This phenolic rich lignin derived pyrolysis oil is recovered from the charcoal kilns and isolated by decanting it from the aqueous acidic fraction. A series of bio-oil modified phenol formaldehyde resins were prepared using different resin formulations. The levels of phenol replacement (25, 50 and 75%), the formaldehyde to phenol ratio, (F/P = 1.0 and 1.2) and the base to phenol ratio (N/P = 0.3 to 0.5) were all studied. The resins were prepared using a standard temperature profile and cooked to a target viscosity. Small wood composites were used to test the curing and performance properties of these resins before and after accelerated aging. Both the bond strength and the wood failure values were measured. These results indicate that phenol formaldehyde resins containing up to 50% eucalyptus bio-oil can be used to prepare resins with commercially attractive properties. The molecular details of the bio-oil and several of the resins were characterized using GPC, pyrolysis-GC and pyrolysis molecular beam mass spectroscopy (MBMS).

Keywords: wood adhesives, Eucalyptus, pyrolysis, phenol formaldehyde resins

INTRODUTION

Phenolic resins are relatively old synthetic polymers discovered at the beginning of this century, and the first industrial plant started to produce early in the 10's (Knop and Pilato, 1985). The present world annual production is 2,460 thousands of metric tons consuming 30% of the all petrochemical phenol produced, about 1,600 thousands of metric tons. There is a wide range of applications as coatings, abrasives, plywood and other wood materials foundry, insulation, rubber processing, molding, etc. The wood composites including plywood is far the biggest application consuming more than 30% of the resole production (Camara, 1999a, 1999b).

In terms of cost, phenol is the most expensive material used in the manufacturing of resoles. It is produced primary via cumene peroxidation from petrochemical benzene, thus the price of phenol depends on the price and supply of the crude oil. The substitution of synthetic phenol by a renewable source of phenolics has a long been sought. Cracked lignin, bark tannins and pyrolytic tar from wood are among the materials tested as replacements for synthetic phenol. The interest in phenol replacements increased during the 1970's oil crises, and development of a thermochemical conversion process that transform wood, agricultural and forestry residue into a valuable phenolic rich oil (lately named bio-oil) has revigorated the possibilities for this application (Lewis and Lantzy, 1987; Chum *et al.*, 1987).

Fast pyrolysis, a recent innovation, is a high temperature process in which feedstock is rapidly heated without oxygen producing condensable vapors, some gases and charcoal. To carry out this conversion process many different technologies have been investigated. Some of them are now commercial, for example, fluidized bed, circulating fluid bed, transported bed, and some are still in lab bench or pilot scale and been testing like ablative pyrolysis (Diebold and Bridgwater, 1999).

Slow pyrolysis or carbonization is not a novel process but it is able to generate bio-oils that are similar to those produced by fast processes. As a traditional technology carbonization has disappeared in the industrialized countries due the availability of coal to coke and difficulties to keep wood supplies to charcoal production. Brazil and Australia are still using charcoal industrially as an iron reductor and for other alloys. The annual production of Brazilian charcoal is around 7 million tones. Some 2,400 tones of bio-oil a year are currently recovered from the kilns using cyclones and pumps. These production levels can grow rapidly with

a minimal investment if more addition markets are open for use of bio-oil (Resende *et al.*, 1994). In this work a decanted heavy phase bio-oil was tested in phenolic resin formulations and the results are present.

EXPERIMENTAL

The eucalyptus oil sample used in this research was supplied by Biocarbo Ind. Com. Ltda in Brazil. It was recovered from the mansory kilns during the slow carbonization of wood from different eucalyptus species. This sample is the water insoluble organic fraction that was decanted from the aqueous components. The as received sample had the following properties: pH 2.75 at 25°C, viscosity of 132 cP at 25°C and 43.5 cP at 40°C (Brookfield), water content of 13.7% and M_n = 260, M_w = 470 by GPC.

The formaldehyde-phenol mole ratio (F/P) varied from 1.0 to 1.2, sodium hydroxide-phenol mole ratio (N/P) varied from 0.3 to 0.5 and the percentage of phenol substitution by the bio-oil ranged from 25 up to 75%. In calculating the mole ratios the bio-oil was treated as phenol. The resin solids were 45%, which is typical for a PF plywood resin. Sodium hydroxide (50% wt solution) was used to neutralize the acidic functionality in the bio-oil and raise the pH of the mixture to around 11. High purity paraformaldehyde and phenol were used as received. All components were charged to a 500-ml three-neck round bottom flask equipped with a reflux condenser, a thermometer, and a mechanical stirrer. The heat was supplied through an external heating mantle connected to the thermocouple. The temperature profile was the same for all samples. The mixture was heated to 70°C for 90 min, and then to 90°C until the solution viscosity was equivalent to stages to U-V as measured with Gardner-Holt viscosity tubes, about 1,000-1,200 cP. The resin was then quenched and stored in a freezer until needed for preparation of the wood composites.

The wood composites used to measure the resin quality were made according to the European Standard EN 301 for adhesives, phenolic and aminoplastic, using hard maple as the wood substrate. Between 100-200 mg of resin were applied to a 25.4x25.4 mm area at one end of each stick. After an open assembly time of 10 min at ambient conditions, the sticks were lapped over the length of their coated ends. The over-lapped stick assembly was then hot pressed at 200 psi and 160°C for 2, 2.5, 3.5 and 6 min, using a Carver Laboratory Press, Model-C 12 ton capacity. Five wood composite samples were prepared for the shorter press times and 10 composite samples were prepared for the 6 min press time. The samples pressed for 6 min were separated into two groups, one of that was tested dry the second was subjected to an accelerated aging sequence. After bonding, all specimens that were tested "dry" were reconditioned at 51% relative humidity for at least 24 h prior to mechanical testing. Samples that were tested "wet" were boiled in water for 4 h, followed by drying for 20 h at 60°C and 15 mm Hg and boiled again for 4 h and kept in plastic bag until the mechanical tests. Mechanical properties were measured with an Instron Testing machine. Maximum tensile load was recorded for each specimen and averaged for the five specimens prepared for each resin press time combination. Commercial plywood PF resin obtained from Georgia Pacific Resins (GPR) was used as a standard.

The wood failure was visually estimated based on the ASTM D 5266-92 standard procedure. The final viscosity, and for some samples, the viscosity in all measurement during the cooking time, were made with a Brookfield viscometer using appropriate spindle. The pH was for each sample measured in an Orion Research 601A pH meter.

The resins were characterized using pyrolysis-GC and molecular beam mass spectroscopy (MBMS). A HP 5890 Series II gas chromatograph connected to a CDS Analytical Pyroprobe 2000 with helium as a carrier gas and a HP 3365 GC computer program were used to produce and record the chromatograms. The pyroprobe program was set at 700°C for 10 seconds and the GC temperature program start at 45°C for 5 min then heat to 285°C at 5^0Cmin^{-1} and kept at that temperature for an additional 5 min. The mass spectrograms were produced using a MBMS developed at NREL described elsewhere (Evans and Milne, 1987). A sample of about 20 mg was pyrolysed at 600°C and the vapors analyzed. Char formation was observed for all experiments.

RESULTS AND DISCUSSION

Many formulations were tested varying the parameters described above, several of them failed due to phase separation of the sample during the reaction. The main cause of the phase separation appeared to be related to the low solubility of the phenolic polymers as the molecular weight increased. Some of the resins did not phase separate but they did not give good mechanical property after curing. Initial tests showed that high

quality resins could be prepared using 50 % bio-oil, and F/P ratios of 1.0 to 1.2. The N/P ratio was also an important factor that influenced the quality of the bonded composite and needed to be between 0.4 and 0.5.

In Figure 1 and 2 it is showed the shear strength and wood failure respectively for the GPR (control, commercial), BC-01 (3:1 phenol:bio-oil), BC-05 (1:3 phenol:bio-oil) and BC-09, BC-10 and BC-11 (1:1 phenol:bio-oil).

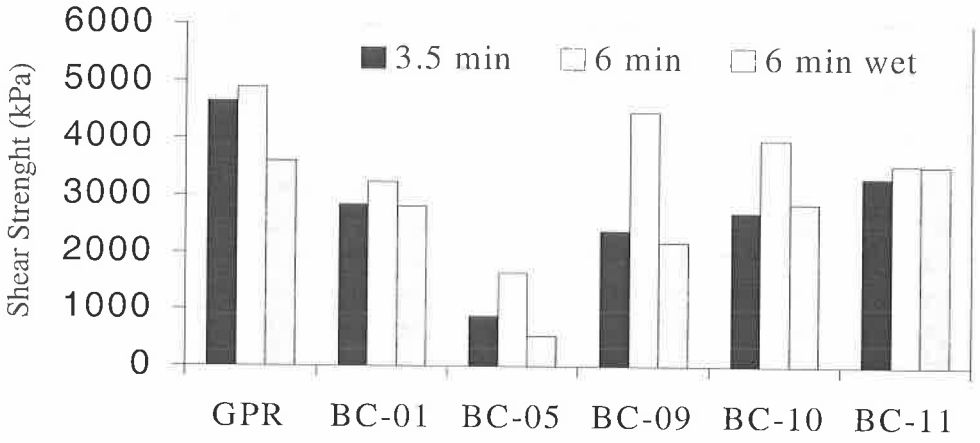

Figure 1. Shear Strength in kPa for 5 different eucalyptus oil resins and GPR.

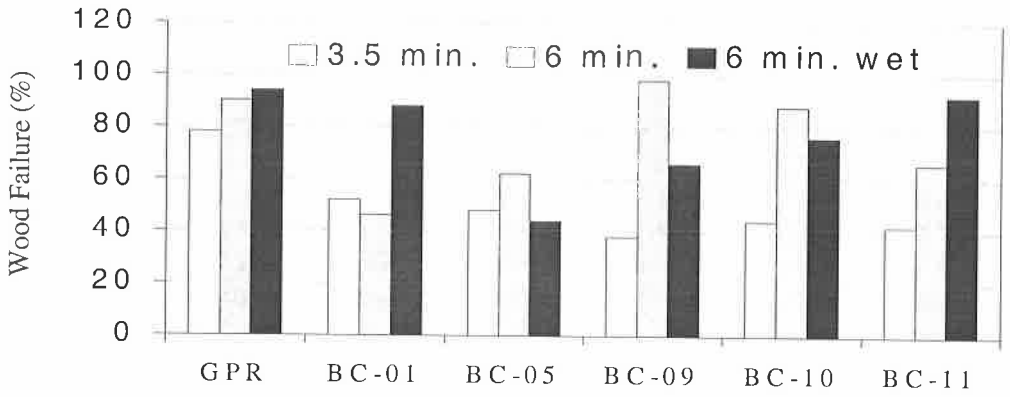

Figure 2. Wood failure for GPR and five eucalyptus oil resins.

In general the mechanical strength and wood failure values for resins with 50% wt of bio-oil are comparable to the commercial control. The exception to this trend is the wood failure values for the samples that were pressed for 3.5 min. For this one measure all of the bio-oil samples are lower than the commercial control. However, for the key set of samples that was subjected to the critical boil-dry-boil test, the mechanical strength and wood failure properties lap-shear samples bonded with the bio-oil resins are as good or even better than the commercial control.

Figure 3 shows a plot of shear strength as a function of press time for four different resin formulations. GPR and three samples using 50% wt of bio-oil added in different steps during the cooking time. Not too surprisingly the shear strength for all of the samples increases as the press time increases from 2 min to 6 min. The shear strength for all of the samples also decreases following exposure to the accelerated aging conditions. There are no statistical differences between any of the three bio-oil based PF resin formulations. While the commercial control has higher shear strength values at 2 and 2.5 min, there are no statistical differences between the control and BC-06. The most promising results for the bio-oil PF resin formulations are seen following the accelerated aging. While the shear strength of the commercial control decreases significantly, the shear strength of all three bio-oil formulations remains virtually unchanged. The bottom line

result is that there are no statistically significant differences between the shear strengths of the four resin formulations following the critical boil-dry-boil test.

These results highlight the importance of understanding the subtle chemical differences between phenol and bio-oil that control the formation of the polymeric network. Since the bio-oil has different functionality than phenol the amount of formaldehyde and NaOH must be adjusted to allow the polymerization reactions to proceed in a controlled manner. These results also highlight the importance of how and when the bio-oil molecules are incorporated into the polymer network. Since they have different functionality and reactivity they can not simple be added to a standard PF resin recipe, but the recipe has to be modified to accommodate these differences in pH and functionality.

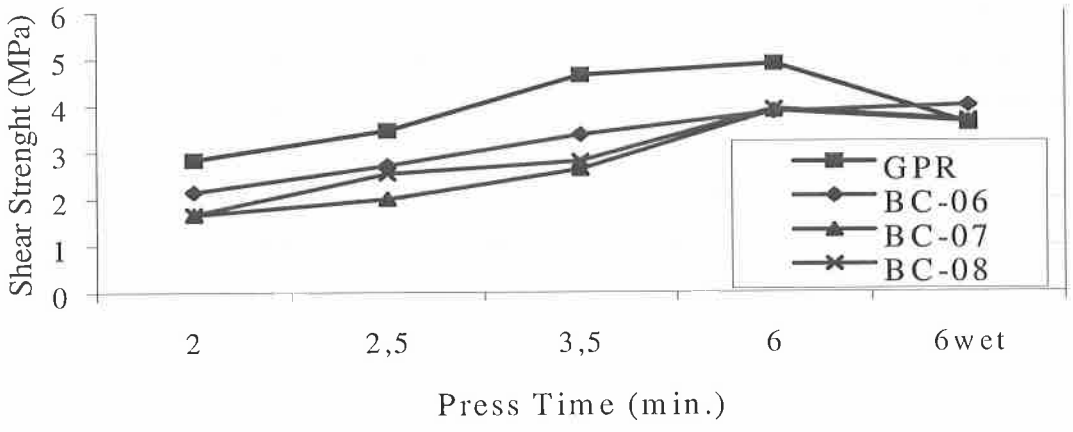

Figure 3. Shear Strength in MPa versus press time for GPR and 3 eucalyptus oil resins.

Figure 4 presents a mass spectrogram for a resin with 50% wt of bio-oil without previous curing. The biggest peaks are 94 and 108 representing phenol and cresol (orto, meta, para) respectively as major degradation products. For the Py-GC cromatograms phenol is also the main peak followed by separated cresois (orto and para) and 2,4-dimethylphenol and 2,6-dimethylphenol as main products after degradation.

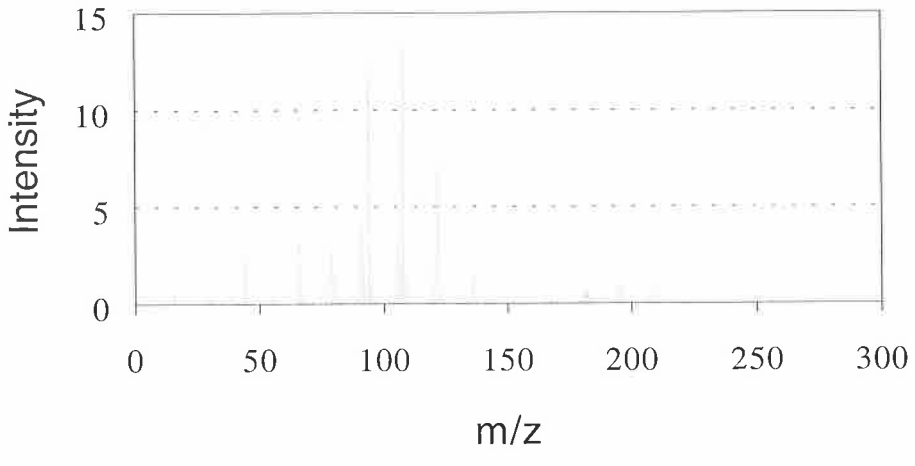

Figure 4. Mass spectrogram for a PF resin with 50% in bio-oil.

CONCLUSIONS

Phenolic oils produced from the slow pyrolysis of biomass can be an alternative feedstock for phenolic resin production. The resin formulation and cooking profile must be modified to account for the chemical differences between phenol and the bio-oil but substitution in the range of 50% it is feasible. Biomass oil resins worked better in severe conditions like in wet tests. The price for pyrolysis oil is extremely attractive.

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REFERENCES

Abiquim (1998). Anuário da Indústria Química Brasileira, SP, Ano 21, pp 248.

Abiquim (1999). Guia da Indústria Química Brasileira, SP, Ano 13, pp 370.

Camara, E. (1999a). Phenol. Chemical Economics Handbook Marketing Research Report, pp 33.

Camara, E. (1999b). Phenolic Resins. Chemical Economics Handbook Marketing Research Report, pp32.

Chum, H.L., Diebold, J.P., Scahill, J., Johnson, D., Black, S.K, Schroeder, H.A. & Kreibich, R.E. (1987). Biomass pyrolysis oil feedstocks for phenolic adhesives, in Adhesives from renewable resources. *In 194th Meeting of the ACS, Cellulose, Paper, and Textile Division,* ed. R.W. Hemingway, A.H. Conner & S.J. Branham, , pp 135-151.

Diebold, J.P. & Bridgwater, A.V. (1999). Overview of Fast Pyrolysis of Biomass for the Production of Liquid Fuels. *In Fast Pyrolysis of Biomass: A Handbook.* CPL Press, UK, pp 14-32.

Evans, R.J. & Milne, T.A. (1987) Molecular Characterization of the pyrolysis of biomass. 1. Fundamentals. Energy & Fuels, pp 123-137.

Knop, A. & Pilato, L.A., (1985) Phenolic Resins, Chemistry Applications and Performance, Springer-Verlag, pp 313.

Lewis, N.G. & Lantzy, T.R. (1987). Lignin in adhesives: Introduction and Historical Perspective, in Adhesives from renewable resources. *In 194th Meeting of the ACS, Cellulose, Paper, and Textile Division,* ed. R.W. Hemingway, A.H. Conner & S.J. Branham, , pp 13-26.

Resende, M.E.A., Pasa, V.M.D. & Lessa, A. (1994). *In Advances in Thermochemical Biomass Conversion*, pp.1289-1298.

Carbohydrates and lignin degradation during continuous kraft pulping of eucalyptus wood

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ABSTRACT

Eucalyptus urograndis wood was used for this study. Removal of wood chemical constituents was monitored during conventional kraft continuous cooking with and without addition of anthraquinone (AQ) and polysulfide (Sn). Cooking was carried out in a laboratory system to simulate a continuous pulping process. Samples were obtained by interrupting several cooks at different cooking times. Carbohydrate analysis (qualitative and quantitative) was accomplished by HPLC technique. Addition of anthraquinone and polysulfide resulted in improved retention of galactans, xylans, arabinans and uronic acids. Mannans were shown to be quite resistant during conventional cooking. Delignification rate was more affected by alkali charge than by presence of AQ. Pulping selectivity presented the highest values after the impregnation stage, when maximum temperature was reached. Delignification rate was considerably affected by alkali charge mainly in impregnation and concurrent stages. Use of additives (AQ and Sn), although required lower alkali charge to obtain the same kappa number, consumed more alkali per ton of pulp.

Keywords: kraft pulping, *Eucalyptus*, continuous cooking process, delignification, cellulose, hemicelluloses, lignin, anthraquinone, polysulfide.

INTRODUCTION

Pulping simulation studies are usually related to effect of cooking conditions on yield and to pulp quality for batch processes. A relatively small number of papers have been published on laboratory simulation of continuous pulping processes. Even more difficult to obtain are data concerning behavior of specific wood species, such as Eucalyptus, in continuous kraft pulping. Detailed information on wood behavior during pulping could favor process modifications aiming to establish the best pulping conditions for a specific wood species and also for quality optimization of the end product. Analysis of wood chemical composition as the cook progresses can be very important to establish the carbohydrates and lignin stability as wood chips passes throught the different cooking zones in a continuous digester. Such information could be used to determine the best cooking condition to minimize cellulose and hemicelluloses degradation and to intensify delignification.

Eucalyptus wood evaluation studies in batch cooks have been treated by several authors (Busnardo, 1981; Barrichello et al., 1984; Gomide and Colodette, 1983, etc). On the other hand, laboratory simulation of continuous cooking is much less frequent probably due to difficulties to precisely simulate industrial conditions, as related by Tikka and Kovasin (1994). However, some specific studies can be found in the literature. For example, Pasco et al. (1997) carried out a study about fiber characteristics and lignin degradation using Modified Continuous Cooking (MCC) and also conventional continuous cooking.

Carbohydrate degradation during kraft pulping has been quite intensively investigated but not as much as lignin degradation and removal (delignification). Usually carbohydrate degradation and dissolution is analyzed by indirect means such as yield or viscosity drop. According to Axegard and Wiken (1981) only a limited number of papers are available on carbohydrate behavior along a kraft cook. Degradation and removal of hemicelluloses during pulping is very important not only because of yield but also because of pulp quality. Carbohydrate degradation reactions in kraft pulping have been established several years ago, including chemical mechanisms and physical and structural aspects (Rydholms, 1965; Grace et al., 1989; Agarwal et al., 1991; Gierer, 1980; Olm and Tistad, 1979).

It has been established that kraft delignification of softwood occurs according to three well-defined phases: initial, bulk and residual (Kleppe, 1970, cited by Fengel and Wegener, 1989). This same delignification pattern has also been demonstrated for Eucalyptus wood (Fantuzzi Neto, 1997).

Studies conducted by Almeida (1999) aiming to increase pulping yield demonstrated that among different continuous cooking processes investigated, simultaneous addition of anthraquinone and polisulfide resulted in the highest yield for continuous cooking. Therefore, it was decided to monitor this process in order to analyze dissolution of wood constituents during pulping. Besides the Kraft/AQ/Polisulfide process (K/AQ/Sn) the conventional continuous process was also analyzed for comparison purposes.

EXPERIMENTAL

For this study a hybrid of Eucalyptus grandis and Eucalyptus urophylla wood, known as Eucalyptus urograndis, was used. Samples from several trees were debarked and chipped in laboratory. Chips were screened, air dried and stored in polyethylene bags.

Pulping Apparatus and Cooking Conditions

A laboratory apparatus that allowed use of the basic principles of modern modified kraft processes, as shown in Figure 1, was used for all cooks.

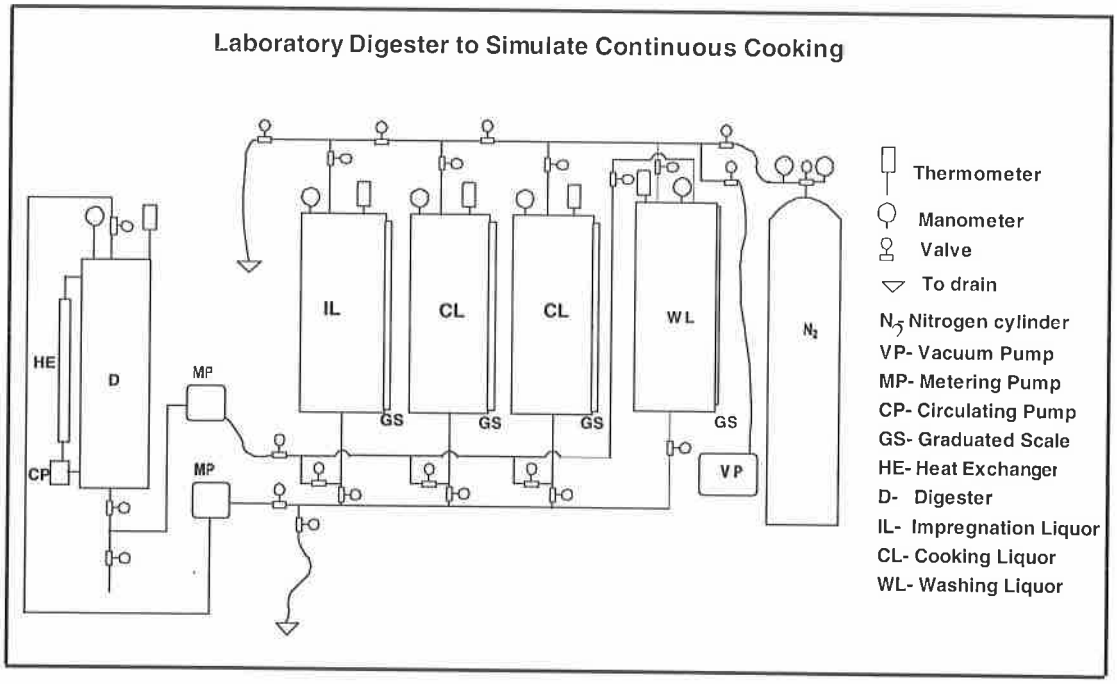

Figure 1. Schematic diagram of laboratory digester system to simulate continuous kraft pulping.

Preliminary cooks were carried out to establish the necessary alkali charge to obtain pulps having kappa number in the range of 17±1. Alkali concentration and alkali charge used for different cooking stages are presented in Table 1. Detailed information on laboratory pulping methodology is presented elsewhere (Almeida, 1997).

Table 1. Alkali concentration and alkali charge.

Pulping Stage	Effective Alkali, g/L NaOH		Effective Alkali Charge, %	
	Kraft	Kraft/AQ/Sn	Kraft	Kraft/AQ/Sn
Impregnation	35.0	27.5	14.0	11.0
Concurrent	30.0	30.0	5.0	3.0
Countercurrent	30.0	30.0	5.0	3.0
Washing	7.7	7.7	2.0	2.0
Total	---	---	26.0	19.0

Cooking Conditions and Monitorship

Several cooks were carried out to obtain wood/pulp samples at pre-established cooking times for both conventional and additivated (AQ/Sn) cooks. Samples were obtained by terminating different cooks at pre-

established cooking times. Samples were obtained after impregnation stage, after concurrent stage, after countercurrent stage, in the middle and end of washing stage.

Cooking conditions to obtain kappa number 17±1 were: chips = 1000 grams (b.d.), L/W ratio = 4/1, sulfidity = 32%, AQ = 0,05%, Sn = produced in situ by adding 2% elemental sulfur to white liquor. Temperature profile for different cooking stages was: pre-steaming = 5 min at 110°C, Sn treatment = 30 min at 85°C, heating = 15 min to 125°C, impregnation = 125°C, 10 min, 9Kg/cm2, heating = 15 min to 145°C, concurrent stage = 60 min at 145°C, countercurrent stage = 60 min at 145°C, washing stage = 180 min at 145°C. Pulp characterization was done according to Tappi standards and HPLC technique was used for carbohydrate analysis.

RESULTS AND DISCUSSION

In order to monitor wood dissolution during pulping it was necessary to know the chemical composition of wood at the beginning of cook. Chemical analysis of Eucalyptus wood used for this study is presented in Table 2. As it can be seen in this table, 4-0-methylglucuronoxylan is the main hemicellulose of Eucalyptus comprising 71% of all hemicelluloses.

Table 2. Chemical composition of *Eucalyptus urograndis* wood.

Chemical Constituents*	Amount
Extractives	3.1%
Lignin: soluble	1.2%
insoluble	25.1%
total	26.3%
Cellulose (glucans)	55.5%
Hemicelluloses	18.6%
Xylans	9.3%
Arabinans	0.6%
Galactans	2.1%
Mannans	2.7%
Acetyl Groups	1.5%
Methylglucuronic acids	2.4%

*Extractive free wood

Conventional and Additivated Kraft Pulping

Kappa number and screened yield for conventional and additivated kraft cooks are presented in Table 3. It was necessary to use a much higher alkali charge for conventional kraft cooking to obtain the same kappa number. The lower alkali charge and presence of additives resulted in a much higher yield for kraft/AQ/Sn cooking.

Table 3. Alkali charge and pulping results for kraft and kraft/AQ/Sn cooks (145°C).

Pulping Results	Kraft	Kraft/AQ/Sn
Effective alkali, %	26.0	19.0
Kappa number	17.1	17.1
Screened yield, %	54.5	57.4

Delignification

Delignification results obtained are presented in Table 4. At the beginning of concurrent stage, when cooking temperature was reached, 18.5% and 23.4% of lignin initially present in wood had already been removed for kraft and kraft/AQ/Sn cooks, respectively. Delignification was more intense during concurrent stage for both processes. In this stage 38.5% and 28,5% of the original amount of lignin in wood was removed during kraft and kraft/AQ/Sn pulping, respectively.

Pulping Selectivity

Pulping selectivity (% lignin removal/yield drop) during cooking for the two continuous pulping processes is presented in Table 5. Selectivity was lower at the beginning of cooks and similar for both processes during impregnation and heating stages. The lower selectivity can be explained by intense carbohydrate removal and minimum lignin degradation during the initial delignification phase. Even using higher alkali charge for

conventional kraft process, lignin removal was not improved during the initial stage of cooking. On the other hand, there was a considerable increase in selectivity for both processes during concurrent stage and a higher selectivity for conventional kraft. The higher selectivity of conventional kraft cooking can be explained by more intense lignin removal due to higher alkali charge used. During subsequent cooking stages (countercurrent and washing) the kraft/AQ/Sn cooking process presented better selectivity.

Table 4. Delignification and pulping yields during kraft and kraft/AQ/Sn cooks.

Pulping Stages	Kraft		Kraft/AQ/Sn	
	Delignification, %	Yield, %	Delignification, %	Yield, %
Impregnation	18.3	90.3	23.4	87.4
Concurrent	56.8	78.5	51.9	77.1
Countercurrent	82.9	62.8	68.9	67.5
Middle of washing	90.8	55.5	76.2	62.1
End of washing	93.8	54.5	93.0	57.4

Table 5. Selectivity of conventional kraft and kraft/AQ/Sn pulping.

Cooking stages	Kraft	Kraft-AQ/Sn
Impregnation	1.89	1.86
Concurrent	3.26	2.77
Countercurrent	1.66	1.77
Middle of washing	1.08	1.36
End of washing	3.01	3.58

Alkali Consumption

Although total alkali charge was higher for conventional kraft process, alkali consumption was lower when compared with kraft/AQ/Sn (Table 6). For both processes alkali consumption was much higher during impregnation stage, a well-known phenomenon for alkaline pulping (Rydholm, 1965). Alkali usage to generate polysulfide and a longer time could explain the higher alkali consumption during the impregnation stage of kraft/AQ/Sn process. In concurrent and countercurrent stages alkali consumption was lower for kraft/AQ/Sn process probably due to carbohydrate stabilization by additives and to the lower alkali concentration used in this process.

Table 6. Alkali consumption during kraft and kraft/AQ/Sn cooks.

Cooking	EA Consumption (Kg/Ton of Wood)					
	Impregnation	Concurrent	Countercurrent	Middle of washing	Second part of washing	Total AE (Kg/Ton)
Kraft	77.4	27.7	29.3	2.7	1.7	139
Kraft/AQ/Sn	88.8	24.9	20.4	2.7	7.5	144

Dissolution of Wood Constituents During Conventional Continuous Kraft Pulping

Dissolution of wood constituents during conventional kraft pulping is presented in Figures 2 and 3. Galactans were the most unstable hemicelluloses. During impregnation and heating stages 60% of galactans was dissolved and at the end of countercurrent stage those hemicelluloses had been completely removed. Also during these stages 48% of mannans was dissolved but only 8% of xylans, 2% of arabinans and 1% of cellulose (glucans) were removed. Acetyl groups were very unstable and were completely hydrolyzed and removed in the impregnation stage. Also 25% of uronic acids was dissolved during impregnation.

Lignin was strongly removed during concurrent stage (38.5%). During this stage removal of arabinans was more intense (17.8%) and dissolution of mannans (2.7%), uronic acids (1.2%) and galactans (10.4%) decreased.

Lignin dissolution was intense in countercurrent stage (26.1%), uronic acids degradation became more intense than in the preceding stage (38.7%) and galactans were completely removed. Xylans removal was more intense (17.3%) and glucans (6.4%), mannans (1.4%), and arabinans (2.8%) were degraded in less extension.

Although cooking conditions used for washing were quite mild, during the first half of this stage there was a considerable removal of arabinans (43.5%), lignin (7.7%), uronic acids (17%) and xylans (8.9%).

During the second half of the washing stage removal of cellulose (0.5%), mannans (3.7%), arabinans (4.6%), uronic acids (0.9%) and lignin (5.9%) tended to level off.

At the end of the cook 20% of glucans was dissolved. However, most of the glucose units should be related to hemicelluloses and not cellulose. Lignin removal occurred in three different phases: initial delignification (18% during impregnation), bulk delignification (65% during concurrent and countercurrent stages) and residual delignification (11% during washing stage).

Galactans were the most unstable hemicellulose. Sixty percent of galactans was dissolved during impregnation and heating stages and at the end of the cook 82% was removed. In impregnation and heating stages it was removed 32% of mannans, 16% arabinans, 12% xylans and 2% glucans (cellulose). Acetyl groups were very sensitive to alkaline hydrolysis and were completely dissolved in impregnation and heating up stages. Approximately 32% of uronic acids was removed during these stages.

During the concurrent stage there was an intensive removal of lignin (28.5%), a small decrease on arabinans (11.6%), galactans (11.6%), mannans (5%) and uronic acids (11.1%) dissolution and a slight increase on xylans (14.3%) removal. Glucans degradation was quite small (1.8% removal).

Removal of lignin (17%) and galactans (5.1%) was less intense in the countercurrent stage of additivated kraft continuous cooking. There was a considerable increase on arabinans (27.6%) and glucans (6%) degradation but xylans were quite stable.

Even using mild alkali conditions, there was some dissolution of lignin (7.3%), arabinans (7.9%), mannans (11.7%), galactans (3.4%), xylans (4.4%) and also glucans (4.3%) during the first half of the washing stage. During the second half of washing removal of glucans, mannans, xylans and galactans presented a tendency to level off.

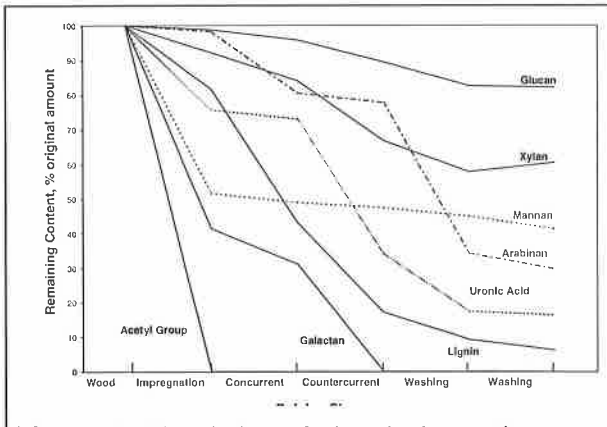

Figure 2. Dissolution of chemical constituents of *Eucalyptus urograndis* wood during conventional continuous kraft cooking.

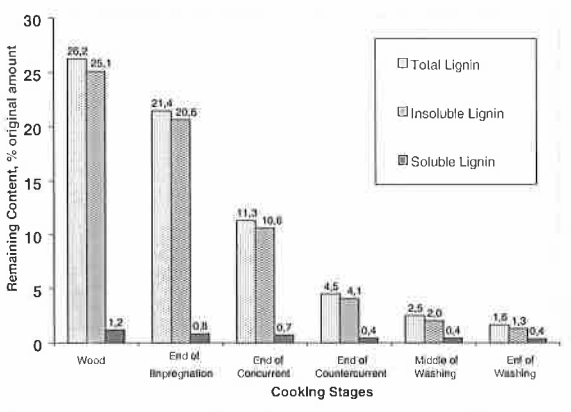

Figure 3. Remaining lignin content in *Eucalyptus urograndis* wood during conventional continuous kraft cooking.

Dissolution of Wood Constituents During Continuous Kraft/AQ/Sn Pulping

Figure 4 shows carbohydrate dissolution along the Kraft/AQ/Sn cooking.

Delignification of *Eucalyptus urograndis* wood followed the typical pattern (Figures 4 and 5). During impregnation occurred the initial delignification phase (23% removal). Bulk delignification (45% removal) occurred during concurrent and countercurrent stages and in the washing stage happened the residual delignification (24%). Removal of lignin during the last part of washing was unusually high (16.8%).

A Comparative Analysis of Conventional and Additivated Continuous kraft Cooking

In Table 7 is presented a summary of wood constituents removal during kraft and kraft/AQ/Sn continuous cooking.

A general analysis of Table 7 indicates that dissolution of glucans was 17.8 and 15%, respectively, for Kraft and Kraft/AQ/Sn cooks. However, it should be considered that most of the glucose units determined as glucans came from hemicelluloses and not from cellulose. Hemicelluloses removal was 60.8% for conventional continuous kraft process and 54.8% for continuous Kraft/AQ/Sn. Chemical constitution of kraft pulp was 83.4% cellulose, 13.4% hemicelluloses and 2.9% lignin and Kraft/AQ/Sn pulp presented 82.2% cellulose, 14.6% hemicelluloses and 3.1% lignin. Although the difference in chemical composition was small it should favor Kraft/AQ/Sn pulp for paper manufacture. Kraft/AQ/Sn pulping resulted in a considerably higher yield (57.4%) than conventional kraft. This superior yield can be explained by higher retention of cellulose (glucans) and hemicelluloses and also by the slightly higher lignin content (0,2%).

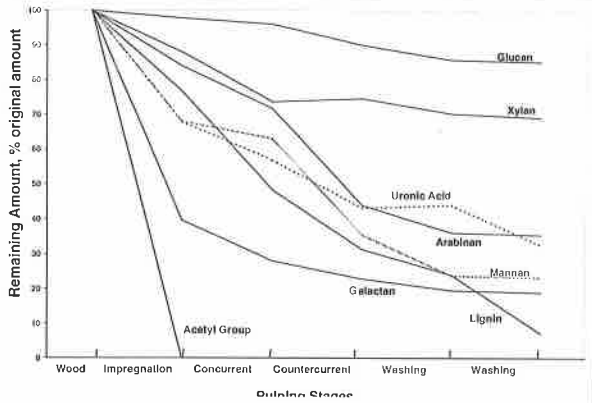

Figure 4. Dissolution of *Eucalyptus urograndis* chemical constituents during continuous kraft cooking using AQ and Sn as additives.

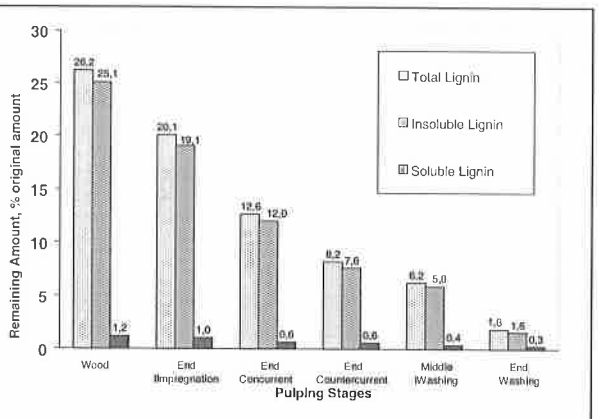

Figure 5. Delignification of *Eucalyptus urograndis* wood during continuous kraft cooking using AQ and Sn as additives.

Table 7. Effect of cooking process on removal of wood constituents.

Constituents	Wood Composition, %*	Constituents Remaining in Pulp	
		Kraft	Kraft/AQ/Sn
Glucans (cellulose)	55.5	45.6	47.2
Hemicelluloses	18.6	7.3	8.4
Xylans	9.3	5.6	6.4
Mannans	2.7	1.1	0.6
Galactans	2.1	0	0.4
Arabinans	0.6	0.2	0.2
Uronic Acids	2.4	0.4	0.8
Acetyl Group	1.5	0	0
Lignin	26.3	1.6	1.8

* Based on extractive free wood.

REFERENCES

Agarwal, N., Mckean, W. T.& Gustafson, R. R. (1991). Cellulose degradation kinetics in alkaline pulping. *International Synposium on Wood and Pulping Chemistry*, Proceedings, **Vol. 1**, pp. 213-220.

Almeida, J.M. (1999). Estudos de maximização de rendimento e monitoramento da degradação dos constituintes da madeira de *Eucalyptus* em processo kraft contínuo. PhD Thesis, Department of Forest Engineering, Federal University of Viçosa.

Axegard, P.& Wiken, J. E. (1981). Kinetics of kraft pulping including the final phase. *International Synposium on Wood and Pulping Chemistry*, Proceedings, **Vol. 2**, pp. 22 - 25.

Barrichelo, L. E. G, Nariyoshi, A. B., Beig, O. *et al.* (1984). Variação das características da madeira de eucalipto para diferentes espécies, idade e locais. *ABTCP Pulp and Paper Conference*, Proceedings, **Vol. 1,** pp. 385-399.

Busnardo, C. A. (1981). Estudos sobre a deslignificação da madeira de *Eucalyptus urophilla* de origem híbrida, pelo processo kraft, para produção de celulose. MSc Thesis, Department of Forest Engineering, Federal University of Viçosa.

Fantuzzi Neto, H. (1999). Dissolução de constituintes químicos da madeira de *Eucalyptus grandis* durante a polpação kraft convencional e modificada. MSc Thesis, Department of Forest Engineering, Federal University of Viçosa.

Fengel, D.& Wegener, G. ed. (1989). *Wood: chemistry, ultrastructure, reactions*. Walter Gruyter, Berlin, 613p.

Gierer, J. (1980). Chemical aspects of kraft pulping. *Wood Science and Technology*, **14**, 241-266.

Gomide, J.L. & Colodette, J.L. (1983). Avaliação estatística da otimização de parâmetros da polpação kraft de eucalipto. *Latin American Pulp and Paper Congress*, Proceedings, **Vol. 1**, pp.317-327.

Grace, T. M.& Leopold, B.& Malcolm, E. W. ed. (1989). Chemical reactions of wood constituents. In: Grace, T. M. Leopold, B. & Malcolm, E. W. ed. *Pulp and paper manufacture, Volume 5: Alkaline Pulping*, Tappi Press, Atlanta, pp. 23-44.

Olm, L.& Tistad, G. (1979). Kinetics of the initial stage of pulping. *Svensk Papperstidning*, **82**(15): 458-464.

Pasco, M.F., Suckling, I.D., Allison, R.W. (1997). Changes in lignin during laboratory MCC pulping. *Appita Journal*, **50**(1): 149-155.

Rydholm, S.A. (1965). *Pulping processes*. John Wiley & Sons, New York, 1269p.

Tikka, P.O.& Kovasin, K.K. (1994). Laboratory simulation of modified kraft cooking system – a new cooking system enables independent temperature and liquor transfer programs. *Tappi Pulping Conference*, Proceedings, **Vol. 3**, pp. 6-10.

Chemistry of bleaching with peroxy acids

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ABSTRACT

Reactions of three peroxy acids, peracetic, peroxymonosulfuric and peroxymonophosphoric acid, with lignin model compounds were investigated under bleaching conditions. While all three peroxy acids reacted via identical pathways, the reaction rates varied depending on the type of reaction and the reaction conditions. The initial reactions of lignin with these three peroxy acids can be classified into three main types, namely, aromatic hydroxylation, addition to carbocation followed by side-chain displacement and Baeyer Villiger Rearrangement. We propose that the rates of these reactions are determined by two main factors, the leaving ability of the conjugate acid, and the electrophilicity of the peroxy oxygen in these peroxy acids. The relative importance of these two factors in each type of reaction can be used to explain the observed rate differences among the three peroxy acids. While the electrophilicity of the peroxy oxygen is the dominant factor in Baeyer Villiger rearrangement, the leaving ability of the conjugate acid is the dominant one in aromatic hydroxylation and sidechain displacement after addition to carbocation.

Keywords: bleaching, lignin, peroxy acids, kraft pulp

INTRODUCTION

During the past two decades, environmental concerns regarding the impact of bleach plant effluents have resulted in major modifications to the bleaching processes for kraft pulps. Conventional bleaching sequences using chlorine and chlorine dioxide are being replaced by elemental chlorine-free (ECF) and total chlorine-free (TCF) sequences. This trend is expected to continue into the next millenium as new technologies continue to evolve. Peroxy acids, including performic, peroxymonosufuric (Px), peracetic (Pa), and peroxymonophosphoric (Pp) acids have been proposed as both delignifying and bleaching agents in various bleaching sequences. While the reactions of Pa with lignins have been studied since the 60's and early 70's, as summarized by Johnson (1977), there has not been a systematic study concerning the mechanism and relative reactivities of these peroxy acids with lignin. In the early 90's, we conducted comparative studies on the kinetics and reaction pathways of Pa and Px with various lignin model compounds (Kawamoto *et al.*, 1994, 1995). The results demonstrated that both Pa and Px react with lignins via identical pathways, but the reaction kinetics were quite different and were dependent on the type of reaction and the reaction conditions. The difference in leaving group stability between the two peracids was proposed as the contributing factor, however this alone could not satisfactorily explain the observed kinetic differences.

Pp has recently been shown to be a very effective delignifing agent for both aspen wood and northern pine kraft pulp at room temperature and low pH (Springer, 1997, Zhu *et al.*, 1999a). Pp, H_3PO_5, is the phosphorus analog of the better known Px (Caro's acid), H_2SO_5. Although extensive literatures exist for the preparation and chemistry of Px, very little has been reported for Pp. Of what has been reported, Pp has been shown to be a better oxidant than Px. For example, Pp oxidizes Mn(II) to MnO_4^- whereas Px does not (Schmidlin and Massini, 1910). Although its reactivity appears promising, the preparation of Pp has been problematic, being too costly or giving low yields. Recently, we were able to develop a new, less expensive and high yield alternative for the preparation of Pp, using a two-phase system (Zhu *et al.*, 1999a).

The new method for preparing Pp in high yield also enabled us to compare the reaction pathways and kinetics of Pp and Px with three simple non-phenolic lignin model compounds (Zhu, *et al.*, 1999b). The results of this study, when combine with those of our earlier studies on Pa and Px (Kawamoto, *et al.*, 1994, 1995) provide a new insight into the mechanisms of these peroxy acids with lignins. This paper will review pertinent results from these studies and elaborate on the mechanisms. The emphasis will be on the reactions with non-phenolic lignin structures since these structures may be the key to deligninfication in bleaching reactions.

RESULTS AND DISCUSSION

Reaction Pathways

The reaction pathways of lignin model compounds with Pa have been well studied (Johnson, 1977). The reactions involved include aromatic hydroxylation; ring opening via o- and p-quinone formation, side chain oxidation, side chain displacement and Baeyer Villiger reactions. We found that Px and Pp react with lignin via these identical pathways (Kawamoto, *et al.*, 1994, 1995; Zhu, *et al.*, 1999a, 1999b). In general, the initial reactions of peracids with lignin can be classified into three main types:

1. Electrophilic substitution resulting in ring hydroxylation,
2. Nucleophilic addition to a benzylic carbocation followed by side chain displacement, and
3. Nucleophilic addition to a α-carbonyl followed by Baeyer Villiger Rearrangement.

1. Electrophilic Aromatic Substitution

Aromatic hyroxylation is the dominant reaction for lignin structures with saturated side-chains. As shown in Figure 1, hyroxylation could occur either ortho (o-) and/or para (p-) to the phenolic hydroxyl or methoxyl groups. From the reaction products, p-hydroxylation is predominant. Both the hydroxylated products and the resulting quinones are more reactive towards the peracids (Kawamoto, *et al.*, 1994, 1995; Zhu, *et al.*, 1999a, 1999b), resulting in ring opening products (Figure 1).

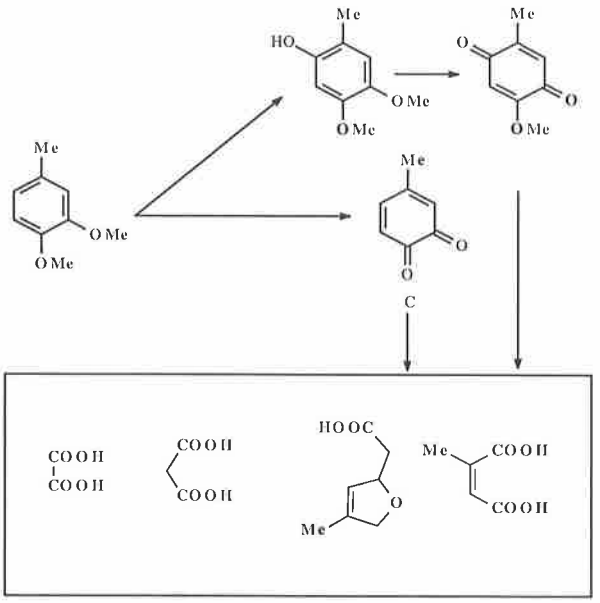

Figure 1. Electrophilic Aromatic Substitutio

2. Nucleophilic Addition to Benzylic Carbocation Followed by Side-chain Displacement

Peroxy acids are good nucleophiles and as such, add readily to carbocations or quinone methides. Under acidic conditons, carbocations can be formed from either α-carbinol or α-ethers of both phenolic and non-phenolic units, though the α-ether requires much lower pH. Nucleophilic addition of the peroxy acid gives a peroxy intermediate (1), which may rearrange via two different pathways, both resulting in side-chain displacement. In pathway I (Figure 2), rearrangement gives the α-carbonyl product, which is further degraded through the Baeyer-Villiger oxidation. In pathway II, the side-chain is displaced through epoxide formation, which is subsequently hydrolyzed to form 3,4-dimethoxy phenol and acetaldehyde via an unstable hemiacetal intermediate.

Previously, we reported that oxidation of phenolic and non-phenolic α-carbinol lignin model compounds proceeded through direct hydroxylation at the 1-position of the aromatic ring followed by side-chain displacement (Figure 3, Kawamoto, *et al.*, 1994). However, our recent studies on Px and Pp (Zhu, *et al.*, 1999b) and on hydrogen peroxide at low pH (Kishimoto, *et al.*, 1999) support that nucleophilic addition to the carbocation intermediate as described above is the correct reaction pathway.

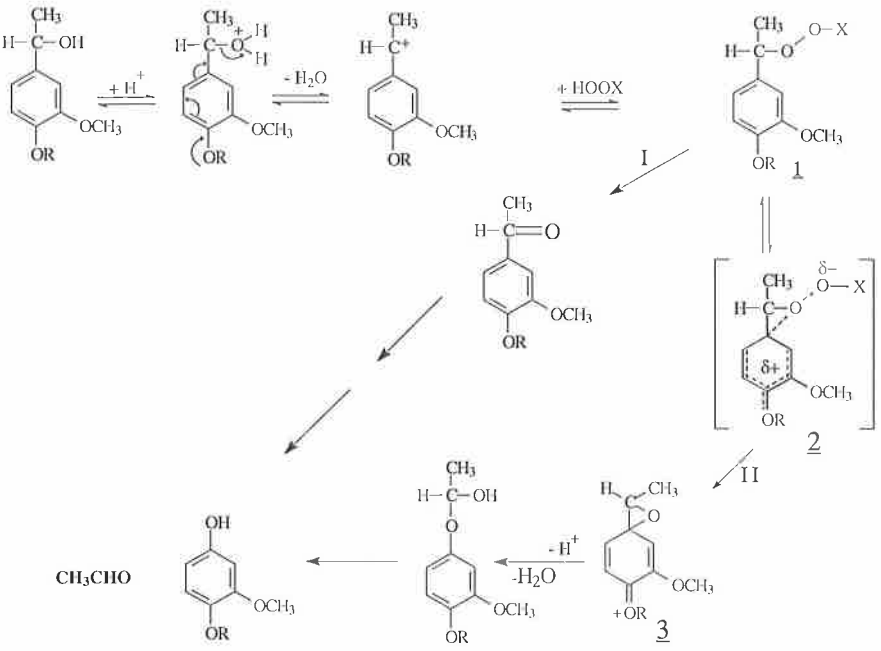

Figure 2. Nucleophilic Addition to a Benzylic Carbocation Followed by Side-chain Displacement.

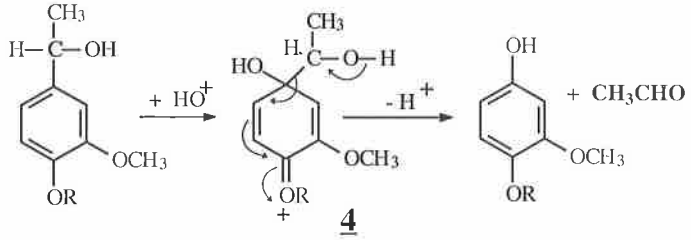

Figure 3. Sidechain Displacement by aromatic hydroxylation.

3. Nucleophilic Addition to a α-Carbonyl Followed by Baeyer Villiger Rearrangement

The reaction of ketones with peracids is well known to go through the Baeyer Villiger Rearrangement (Hassall, 1957). The initial step is nucleophilic addition of the peracid to the ketone followed by a rate-determining rearrangement of the peroxyester intermediate (5) via a concerted aryl migration to form the acetate ester, as shown in Figure 4.

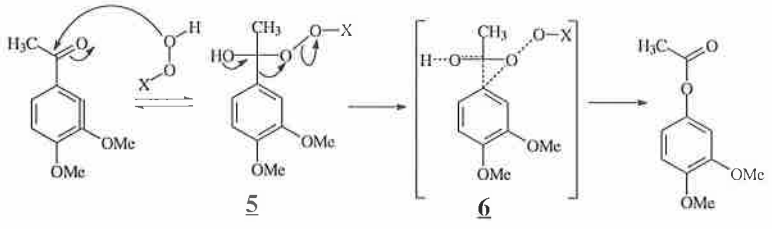

Figure 4. Baeyer-Villiger Rearrangement.

Reaction Kinetics

While Pa, Px and Pp react with lignin and lignin model compounds via identical reaction pathways, differences did exist in the respective reaction rates. In our earlier studies, we found that Pa was faster than Px for Baeyer Villiger and ring opening reactions, whereas Px was faster than Pa for hydroxylation and side-chain displacement (Kawamoto, *et al.*, 1994, 1995). These differences were explained by the assumptions that Pa is a better nucleophile than Px due to the lower pka of the peroxy acid anion, and that Px is a better electrophile than Pa due to the resulting acid being a better leaving group. However, as a result of

our recent studies on the reactions of Pp with lignin model compounds (Zhu, *et al.*, 1999b) the foregoing explanation was an over simplification.

The reactions of three non-phenolic lignin model compounds with Pp were studied at 70°C and 90°C and compared to those with Px. The three model compounds, as shown in Figure 5, were selected to represent the three types of reactions described above, namely, aromatic hydroxylation, nucleophilc addition to a carbocation follow by sidechain displacement and Baeyer Villiger rearrangement. Analogous to Px and Pa, Pp appears to follow the identical reaction pathways, however, the reaction kinetics are significantly different. Especially noteworthy is the sensitivity of the reaction rates of Pp towards changes in pH. While the reaction rates of Px increase only slightly as the pH decreases from 4 to 1, those of Pp increase drastically over the same pH change as shown in Figure 6-8. Thus, compared to Px, Pp reacts faster at pH below 2, about the same at pH 2, and slower as the pH is increased above 2. At pH above 7, Pp did not react with the lignin model compounds under the conditions investigated. The effect of pH on the reactivity of Pp can be explained by the fact that Pp is a weak acid (Zhu, *et al.*, 1999b). The pKa values of Pa, Px and Pp and their corresponding acids are giving in Table 1. Under normal bleaching conditions, pH 1-6, only one active species, HSO_5^-, is present in Px as a result of the extremely low pKa1 value. On the other hand, Pp has three active species, H_3PO_5, $H2PO_5^-$, and $HPO_5^=$, of which two may be present at any time over the above pH range. While $HPO_5^=$ does not react at all, H_3PO_5 is a more reactive than HSO_5^-, which is in turn better than $H_2PO_5^-$.

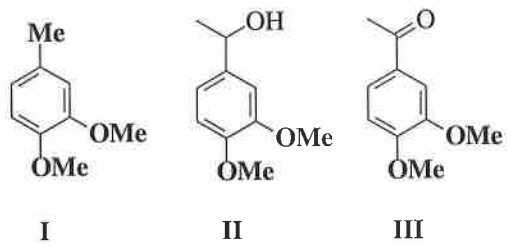

Figure 5. Non-phenolic model compounds

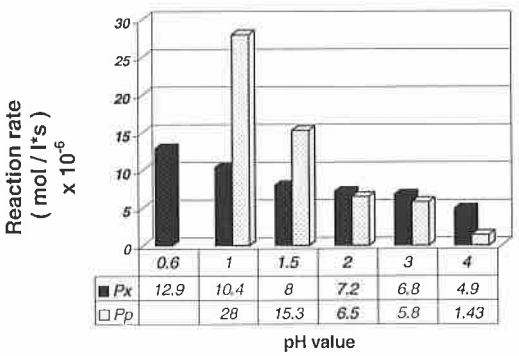

Figure 6. Initial Reaction Rates of Model Compound I

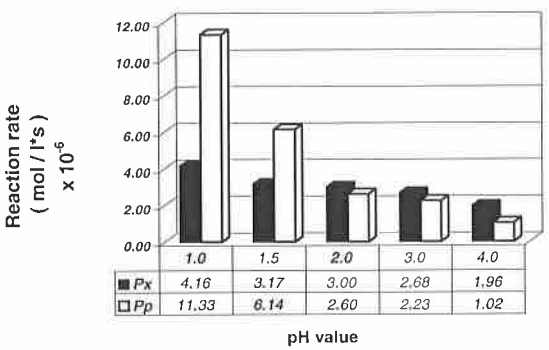

Figure 7. Initial Reaction Rates of Model Compound II

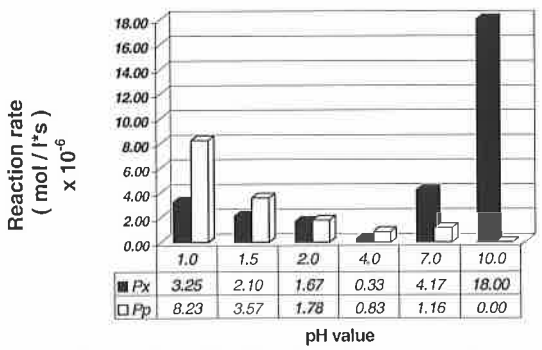

Figure 8. Initial Reaction Rates of Model Compound III

Table 1. pKa Values of the Different Acids.

	H_3PO_5	H_3PO_4	H_2SO_5	H_2SO_4	CH_3CO_3H	CH_3CO_2H
pKa_1	1.1	2.1	<-8	-8	7.2	4.8
pKa_2	5.5	7.1	9.4	1.99	N/A	N/A
pKa_3	12.8	12.3	N/A	N/A	N/A	N/A

Reaction Mechanisms

The fact that Pp reacts faster than Px at low pH clearly indicates that factors in addition to the leaving ability of the corresponding acids ($SO_4^=$ for Px and $H_2PO_4^-$ for Pp) are operative. We proposed that there are two main factors that affect the reactivity of these peroxy acids:

1) the leaving ability of XO^-,and

2) the electrophilicity of the peroxide oxygen O*.

If we let XOO*H represent a generic peroxy acid structure, the leaving ability of XO^- of the peroxy acid can be estimated by the pKa values of the conjugated acids, that is XOH (Table 1). The relative rank of the three peracids with their active species in parenthesis is Px (HSO_5^-) > Pp (H_3PO_5) > Pa (CH_3CO_3H) > Pp ($H_2PO_5^-$) >> Pp ($HPO_5^=$). The electophilicity of the peroxide oxygen O*, or the ability of the peroxide oxygen bond to become polarized and form a partial positive charge on the peroxide oxygen O* is dependent on the inductive effect of the XO group. Since O in the XO group is common to all peracids, the inductive effect of the X group will influence the electrophilicity of the peracids. The relative rank of the electrophilicity of these peracids is Pp (H_3PO_5) > Pa (CH_3CO_3H) > Px (HSO_5^-) > Pp ($H_2PO_5^-$) >> Pp ($HPO_5^=$). Since only the active species of Pp are affected by pH changes in the range of 1-6, one may simplify the relative rankings at a given pH:

At pH 1 or below

Leaving ability Px > Pp > Pa
Electrophilicity Pp > Pa > Px

At pH 4-6

Leaving ability Px > Pa > Pp
Electrophilicity Pa > Px > Pp

As the relative rankings are different between the two factors, leaving ability and electrophilicity, the relative effect of the two factors may vary depending on the type of reactions and the reaction conditions. We can now summarize the main factor that affecting the three types of reaction of peracids.

1. Electrophilic Aromatic Substitution

This reaction involves the initial formation of a π-complex followed by a rate-determining σ-complex formation as shown in Figure 9. The rate-determining step is affected mainly by the ability to stabilize the developing positive charge in the transitional state 7 leading to the formation of the σ-complex. Consequently, the rate of the reaction is mostly affected by the leaving ability of XO^-. Thus, Pp and Px are better than Pa at low pH, whereas Px is better than Pa and Pp at pH above 4.

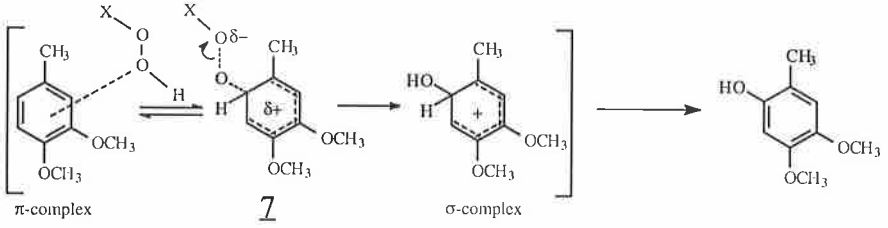

Figure 9. Mechanism of Aromatic Hydroxylation.

2. Nucleophilic Addition to a Carbocation Followed by Side-Chain Displacement

Under acidic conditons, carbocations can be formed from either an α-carbinol or an α-ether, in which the latter requires a much lower pH. Normally, the formation of the carbocation is the rate-determining step, however, for the α-carbinol compound, α-methylveratryl alcohol, the rearrangement of the adduct (1) to the positively charged epoxide intermediate (3) as shown in Figure 2 appears to be the rate-determining step. The transitional state (2) again involves the development of a positive charge and it is likely that the leaving ability is the main factor determining the rate. Thus, the rate of the reactions among the three peracids follows the same order as the electrophilic substitution reaction described above.

3. Baeyer Villiger Rearrangement

The reaction of ketones with peracids is well known to go through the Baeyer Villiger Rearrangement (Hassall, 1957). The initial step is a nucleophilic addition of the peracid to the ketone followed by the rate-determining rearrangement of the peroxyester intermediate (5) via a concerted aryl migration to form the acetate ester, as shown in Figure 4. The transitional state (6) of the rate-determining step likely involves neither the development of carbocation nor the tentative lost of the aromatic structure as the other two reactions described above. The rates of the reactions at pH 1 and 4 follow the order Pp > Pa > Px and Pa > Px > Pp, respectively. These results are consistent with the rate-determining step conceivable being influenced more by the electrophilicity than the leaving ability of the peracid adduct (5).

REFERENCES

Hassall, C. H. (1957). The Baeyer-Villiger oxidation of aldehydes and ketones, Org. React. 9, 73.

Johnson, D. C. (1977). Lignin reactions in delignification with peroxyacetic acid, In Chemistry of Delignification with Oxygen, Ozone and Peroxides, ed. J. S. Gratzl, J. Nakano & R. P. Singh, Uni Publishers Co., Ltd., Tokyo, Japan, pp. 217-228.

Kawamoto, H., Chang, H-m. & Jameel, H. (1994). Reaction of lignin model compounds with peracetic acid or peroxymonosulfuric acid. In International Symposium of Fiber science and Technology, Proceedings, pp 230.

Kawamoto, H., Chang, H-m. & Jameel, H. (1995). Reaction of peroxyacids with lignin and lignin model compounds. In 8th International Symposium on Wood and Pulping Chemistry, Proceedings, Vol. I, pp 383-390.

Kishimoto, T., Chang, H-m., Jameel, H. & Kadla J. F. (1999). The Reactions of Hydrogen Peroxide in Caro's Acid Bleaching at Low pH. In 10th International Symposium on Wood and Pulping Chemistry, Proceedings, Vol. II, pp. 386-389.

Schmidlin, J. & Massini, P. (1910). Phosphormonopersaure und uberphosphorsaure. Ber., 43, 1162.

Springer, E.L. (1997). Delignification of wood and pulp with peroxymonophosphoric acid. J. Pulp and Paper Science, 23 (12), 582

Zhu, T., Geng, Z. P., Kadla, J. F., Chang, H-m., & Jameel, H. (1999a). Preparation of Peroxymonosulfuric Acid and Its Use as a Delignification Agent for Kraft Pulps. In 10th International Symposium on Wood and Pulping Chemistry, Proceedings Vol. II, pp. 402-406.

Zhu, T., Kadla, J. F., Chang, H-m., & Jameel, H., (1999b) Reactions of Lignin Model Compounds with Peroxymonophosphoric Acid. In 10th International Symposium on Wood and Pulping Chemistry, Proceedings, Vol. II, pp 396-401.

Continuous xylitol production from barley bran hydrolyzates by *Debaryomyces hansenii*

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ABSTRACT

The continuous fermentation of xylose into xylitol was assayed with the yeast Debaryomyces hansenii without or with cell recycle. In the first case the dilution rate was varied between 0.026 and 0.088 h^{-1}. In media containing 3-4 g cells/L xylose was almost completely depleted at the lowest dilution rate assayed but the volumetric productivity was only 0.41 g/L.h. On the contrary, most of the xylose remained in the fermentation broth at the highest dilution rate considered, the volumetric productivity being only 0.21 g/L.h. The maximum volumetric productivity (0.61 g/L.h) was achieved with a dilution rate of 0.048 h^{-1}. The low volumetric productivity could be due to both the low cell and substrate concentration. In fact, when the continuous fermentation was performed with cell recycle the biomass concentration increased up to 20 g/L meanwhile the volumetric productivity raised up to 1.31 g/L.h.

Keywords: *Debaryomyces hansenii*; xylitol production; continuous fermentation; cell recycle

INTRODUCTION

Lignocellulosic materials (LCM) are largely available in the North-West country of Spain, constituting an abundant and inexpensive source of vegetable biomass. LCM have been considered as alternative raw materials for industry, for their low cost, large availability and offer a great potential for transformation into chemical feedstocks or fuels (Vázquez *et al.*, 1991). In conventional pulp processes, the lignin and hemicelluloses are wasted or used in low value-added applications (specially heat recovery). Remarkable improvements could be obtained if the raw materials were selectively "fractionated" into their main components (cellulose, hemicelluloses and lignin) and used for different products applications (Parajó *et al.*, 1994).

A mild acid hydrolysis allows the solubilization and depolymerization of hemicelluloses to give liquors containing extractives and products from the hemicelluloses (mainly xylose). This sugar can be used to produce xylitol, a naturally occurring five carbon polyalcohol with interest for the food industry due to its high sweetening power and because it is completely safe for the teeth, it has anticaries properties, it is tolerated by diabetics and its high negative heat of solution. For these reasons it is desirable for sugar-free confections and food (Emodi, 1978; Pepper and Olinger, 1988).

Xylitol was found in certain fruits and vegetables, but in amounts so small than quantitative extraction was difficult and uneconomical. Traditionally it is produced chemically by hydrogenation of D-xylose. However, new fermentative and biocatalytic processes have been investigated to produce xylitol with many yeasts species, in particular the yeast Debaryomyces hansenii was widely studied (Girio *et al.*, 1990; 1994; Parajó *et al.*, 1996; 1997a; 1997b; Domínguez *et al.*, 1997).

This work deals with the optimization of the prehydrolysis of barley bran and its subsequent continuous bioconversion into xylitol using the yeast Debaryomyces hansenii. First of all, the fermentation was performed with free cells and further experiments were performed using cell recycle.

EXPERIMENTAL

Raw material

The barley bran obtained from a local brewery industry was dried, milled to a particle size less than 1 mm and stored until use. Its composition was determined using standard methods containing 23.0 % of cellulose, 26.6 % of hemicellulose and 21.4 % of lignin.

Acid Hydrolysis
Acid hydrolyzates were obtained after treating 10 g of barley bran with 1% to 5% H_2SO_4 in autoclave at 130 °C for 5 min to 3 hours with a solid/liquid ratio of 1/8 g/g. The liquid phase from the acid hydrolysis was neutralized with $CaCO_3$ to a final pH of 6.5 and the $CaSO_4$ precipitate was separated from the supernatant by filtration.

Microorganism
The yeast Debaryomyces hansenii NRRL Y-7426 was used in this work.

Culture media
Freeze-dried cells were grown in a culture medium containing per liter: 10 g pure xylose, 3 g yeast extract, 3 g malt extract and 5 g peptone. Microorganisms were maintained in agar slant tubes containing a medium formulated with the same components and concentrations as the previous one plus 20 g agar. Adapted yeasts (maintained in agar slants made from hydrolyzates instead of commercial xylose solutions) were used for fermentation of barley bran hydrolyzates.

Fermentation conditions
The medium was supplemented as described by Parajó *et al.*, 1998 and fermented in a 2 L Braun Biostat B fermenter with 1.4 L working volume at 30 °C, pH = 5.5 and 250 rpm under microaerobiosis.

Analytical Methods
At given fermentation times, samples from the fermentation media were taken, centrifuged, filtered through 0.45 μm membranes and analyzed by HPLC using two Shodex SH columns (mobile phase, H_2SO_4 0.01 M; flow rate, 0.4 mL/min; IR and UV detection). This method allowed the determination of glucose, xylose, arabinose, acetic acid, ethanol, xylitol and furfural. The corresponding biomass concentrations were calculated by dry weight determinations.

RESULTS AND DISCUSSION

Optimization of the hydrolysis conditions
In lignocellulosic residues, like barley bran, the hemicellulose and cellulose portions can be converted to fermentable sugars, meanwhile the third major component, lignin, can not be converted to fermentable sugars due to its polyphenolic structure. The hydrolysis of hemicelluloses (a polymer of primarily pentoses with some hexoses and sugar acids) is easier than cellulose hydrolysis, since cellulose (a linear polymer of 1,4-beta linked anhydro glucose units) is resistant to saccharification because of its relatively crystalline structure and its close association with lignin, making difficult the access of catalyst (Conner, 1984).

The highest xylose concentration (35 g/L) was reached working with 3% of sulfuric acid during 15 min. Increasing the acid concentration from 3% to 5% as well as increasing the time of hydrolysis, the xylose produced was dehydrated to furfural, an inhibitor of the fermentation, reducing considerably the xylose concentration. These conditions (3% H_2SO_4 and 15 min) were selected to prepare the hydrolyzates for the bioconversion.

Continuous xylitol production with free cells
Figure 1 shows the global volumetric productivities (g/L.h) achieved in the continuous fermentation of xylose into xylitol using the yeast Debaryomyces hansenii without cell recycle at different dilution rates. At the highest dilution rate considered (0.088 h^{-1}) most of the xylose remained in the fermentation broth without being consumed leading to a low volumetric productivity (0.21 g/L.h). Under the lowest dilution rate (0.026 h^{-1}) the maximum xylitol concentration was achieved (16.2 g/L) but the prolonged times provoked a low volumetric productivity (0.41 g/L.h). The maximum volumetric productivity (0.61 g/L.h) was achieved with an intermediate dilution rate (0.048 h^{-1} to 0.063 h^{-1}).

Continuous xylitol production with cell recycle
The low volumetric productivities achieved in the previous case could be explained for the low cell concentrations employed (3-4 g cells/L). In order to increasing the biomass additional fermentations were carried out in the fermentor using cell recycle which increased the cell concentration up to 20 g/L. In these conditions, using the optimum dilution rate found without cell recycle (D = 0.063 h^{-1}) the volumetric productivity raised from 0.58 g/L.h up to 0.72 g/L.h. Notwithstanding, the higher cell concentration also represented that the optimum dilution rate was higher (D = 0.167 h^{-1}). In this case the maximum volumetric

productivity was achieved 1.31 g/L.h. This value represents an important improvement in relation to batch fermentations, where the maximum volumetric productivity reached was 1.1 g/L.h (Parajó *et al.*, 1996; 1997b).

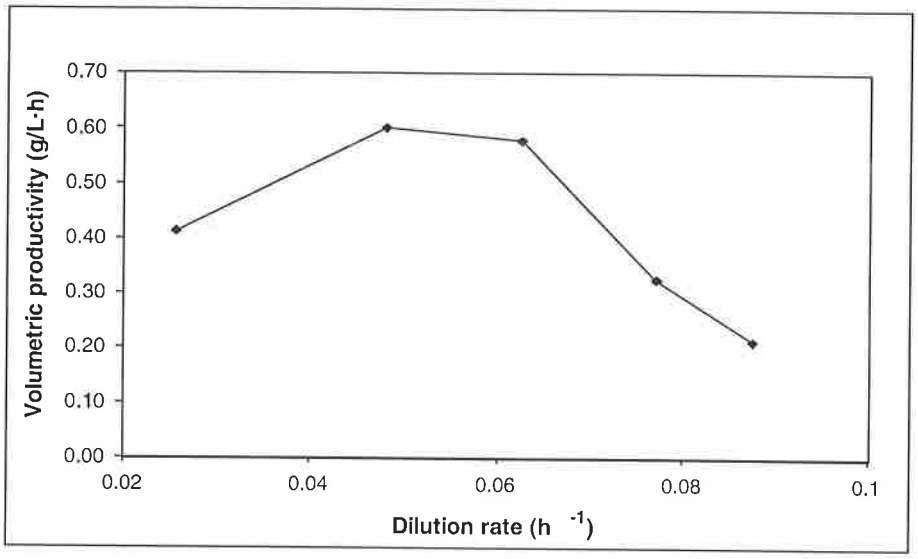

Figure 1. Xylose fermentation into xylitol in chemostast.

REFERENCES

Conner, A.H. (1984). Kinetic modeling of hardwood prehydrolysis. Part I. Wood Fiber Sci. 16, 268-277.

Domínguez, J.M., Gong, C.S. & Tsao, G.T. (1997). Production of xylitol from D-xylose by Debaryomyces hansenii. Appl. Biochem. Biotechnol. 63/65, 117-127.

Emodi, A. (1978). Xylitol. Its properties and food applications. Food Technol., 1, 28-32.

Girio, F.M., Peito, M.A. & Amaral-Collaço, M.T. (1990). In: Biomass for Energy and Industry, ed. Grassi, G., Gosse, G., & dos Santos, G. Elsevier Appl. Science. Vol. 2.

Girio, F.M., Roseiro, J.C., Sá-Machado, P., Duarte-Reis, A.R. & Amaral-Collaço, M.T. (1994). Effect of oxygen transfer rate on levels of key enzymes of xylose metabolism in Debaryomyces hansenii. Enzyme Microb. Technol. 16, 1074-1078.

Parajó, J.C., Vázquez, D., Alonso, J.L., Santos, V. & Domínguez, H. (1994). Prehydrolysis of Eucalyptus wood with dilute sulphuric acid: operation in autoclave. Holz Roh Werkstoff. 52, 102-108.

Parajó, J.C., Domínguez, H. & Domínguez, J.M. (1996). Production of xylitol from concentrated wood hydrolysates by Debaryomyces hansenii: effect of the initial cell concentration. Biotechnol. Lett. 18, 593-598.

Parajó, J.C., Domínguez, H. & Domínguez, J.M. (1997a). Improved xylitol production with Debaryomyces hansenii Y-7426 from raw or detoxified wood hydrolyzates. Enzyme Microb. Technol. 21, 18-24.

Parajó, J.C., Domínguez, H. & Domínguez, J.M. (1997b). Xylitol production from Eucalyptus wood hydrolyzates with organic solvents. Process Biochem. 7, 599-604.

Parajó, J.C., Domínguez, H. & Domínguez, J.M. (1998). Biotechnological production of xylitol. Part2: Operation in culture media made with commercial sugars. Bioresource Technol. 65, 203-212.

Pepper, T. & Olinger, P.M. (1988). Xylitol in sugar-free confections. Food Technol., 42, 98-106.

Vázquez, D., Lage, M.A., Parajó, J.C. & Vázquez, G. (1991). Transformación de materiales lignocelulósicos: composición, fraccionamiento y aprovechamiento. Rev. Agroq. Tecnol. Alim. 31, 143-164.

Developments in pulping and bleaching

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ABSTRACT

The development of new pulping processes based on organic solvents has slowed down. None of the new processes has been successfully introduced into the industry. Jakko Pöyry evaluated the potential of these new processes and concluded that only ASAM is real alternative to kraft pulping. Most of the research efforts in pulping were focused on kraft pulping. Really impressive improvements were achieved by modifying this process. The four principles of modified kraft cooking are: 1. Levelling out the alkali profile throughout the cook, 2. High sulfidity in the initial phase of the cook, 3. Concentration of dissolved lignin and sodium ions in the liquor as low as possible, in particular in the final cooking phase, 4. Cooking temperature as low as possible. The application of these principles in industrial kraft cooking has created various continuous and batch cooking modifications, such as MCC, EMCC, ITC, Superbatch, RDH and Enerbatch. All these modifications are accomplishing the above mentioned principles to a limited extent. The application of these modifications together with cooking additives such as anthraquinone and polysulfide improve further the selectivity of kraft cooking. ECF and TCF bleaching of kraft pulps has also made great progress. These improvements can be used to stop pulping earlier and start oxygen delignification and bleaching at higher unbleached pulp kappa number. This contributes to higher pulp yield and strength. Improvements in bleaching can be attributed to oxygen as well as peroxide containing stages. Two stage oxygen bleaching enhances delignification and its selectivity. Laccase-mediator oxygen delignification is highly selective. Activated oxygen bleaching can be performed using polyoxometallates and polypyridines. Cyanamide and discyanamide activate peroxide and improve and improve delignification in peroxide stages. Polypyridines are able to do so as well, and the application of manganese and iron based complexes in peroxide stages provide and efficient and highly selective bleaching. The combined action of different activated and catalyzed oxygen and peroxide containing bleaching stages result in high brightness and strong pulps. Our own research work is focused on ASAM pulping and chlorine free bleaching of ASAM as well as kraft pulps. Using catalyzed peroxide stages both pulp types can be bleached to high brightness in short sequences using just oxygen and peroxide as bleaching agents. Pulp yield, strength and bleachability of ASAM pulps are superior to kraft, and a closed ASAM mill is easier achievable.

Keywords: pulping, kraft pulp, bleaching, organosolv processes, ASAM

INTRODUCTION

During the last two decades a lot of research work has been performed on new pulping processes. There was a strong tendency to use organic solvents for lignin dissolution. The main objectives of these developments were:

- reduced pollution,
- simple recovery of cooking chemicals,
- lower investment costs,
- alternative use of dissolved wood components.

The original idea of solvent pulping includes a solvent wash of the pulp to separate pulp and dissolved wood components and a direct recovery of the solvent by distillation. The dissolved organic wood components should be separated into a lignin and a carbohydrate fraction and used as chemical feedstock or as fuel to generate energy.

The most serious problems of organosolv pulping are as follows:

1. limited lignin dissolution by the solvent, in particular for raw materials which are difficult to delignify, e. g. softwoods. This has to be compensated by the addition of inorganic catalysts or pulping chemicals like sodium hydroxide,
2. high consumption of solvents by solvent degradation or reaction of solvents with wood components,

3. problems associated with the recovery of the solvents, e.g. formation of azeotropic mixtures with water,
4. non-competitive pulp qualities,
5. the bleaching filtrates can not be integrated into the recovery system,
6. corrosion problems, in particular when organic acid, like formic acid or acetic acid are used as the solvent.

All these reasons, in particular the poor strength properties of organosolv pulps have more or less stopped the development of organosolv processes. This tendency was reinforced by low pulp prices, which did not cover production costs, and by stagnating pulp production figures throughout the last ten years, which means that there are no needs for new pulping capacities. The growing fiber demand of the paper industry was completely covered by increasing secondary fiber recycling rates. The present status of the development of new pulping technologies has been recorded by Young and Akhtar (1998). Jaakko Pöyry (1997) evaluated the potential of new pulping processes compared to the world wide leading kraft process. It was concluded that ASAM pulping might be the only alternative to kraft pulping.

The present research in pulping and bleaching is focused more on improving the kraft process than on developing new processes. In pulp bleaching, however, we are faced with really new developments, which aim at the improvement of chlorine free bleaching and finally the closure of the water cycle of a pulp mill.

DEVELOPMENTS IN KRAFT PULPING

Environmental pressure and competition caused by the development of the above mentioned new pulping processes have prompted intensive research efforts on improving the kraft process. We will try to compile the actual state of this development and identify further needs in this area.

It is well known that the bottleneck of kraft pulping is the low selectivity of lignin dissolution, in particular in the final phase of cooking. The two main reactions of degrading wood carbohydrates during alkaline pulping are alkaline peeling and alkaline hydrolysis. They cause intensive carbohydrate degradation, particularly in cooking of softwood, resulting in low pulp yield, viscosity and strength. This is the reason why a conventional softwood kraft cooking has to be stopped at a kappa number around 30. The most serious carbohydrate degradation reaction is alkaline peeling. This reaction can be stopped by several means. In all cases the alkali unstable carbohydrate aldehyde end group has to be transformed in an alkali stable configuration. This can be achieved either by reducing it to an alcohol or to oxidize it to a carboxyl group. End groups can be reduced to alcohol with sodiumborohydride or to thiols with hydrogen sulfide. They can also be oxidized to carbonyls with polysulfide or anthraquinone. These stabilizing reactions have to be accompanied by measures generally known as principles of modified cooking. These principles can be summarized as follows:

1. Levelling out the alkali profile throughout the cook.
2. Adjusting a high sulfidity in the initial phase of the cook.
3. Keeping the concentration of dissolved lignin and sodium ions in the liquor as low as possible, in particular in the final cooking phase.
4. Keeping the cooking temperatures as low as possible (Herschmiller, 1998)

Most of the alkali consumed during kraft cook is required to neutralize carbohydrate degradation products, such as saccharinic and acetic acids. In particular in the starting phase of a kraft cook the alkalinity should be kept as low as possible to prevent excessive carbohydrate degradation and dissolution. On the other hand, high sulfidity benefits lignin dissolution as well as carbohydrate stabilization. An increased sulfidity improves selectivity of the cook, particularly in the beginning. Alkali is required for neutralization of dissolved carbohydrates and for keeping the dissolved lignin. This means that the removal of dissolved organic material from the cook decreases alkali consumption. This particularly holds true for the final phase of a cook where the concentration of dissolved wood components is high. Cooking temperature and alkali concentration are the most important parameters of carbohydrate degradation and dissolution. Especially, in the initial phase of a cook the carbohydrate dissolution rate depends on temperature and alkali concentration. Even at temperatures below 100°C, low molecular carbohydrates like hemicelluloses will be dissolved. With increasing reaction temperature and alkali concentration, carbohydrates with increasing molecular weight will be dissolved. This effect is intensified when alkaline hydrolysis occurs forming alkali soluble degradation products. The activation energy for lignin dissolution is lower than that for cleavage of the β-1-4 cellulose

bonds. So it is advisable to keep the cooking temperature on a level which is required to dissolve lignin, but prevent cellulose degradation as far as possible.

Several attempts have been made to apply these principles in different modified kraft cooking processes. Continuous as well as batch processes have been developed. The most frequently applied processes are:

- modified continuous cooking (MCC),
- extended modified continuous cooking (EMCC),
- isothermal cooking (ITC),
- super batch,
- rapid displacement heating (RDH).

All these modified kraft pulping techniques try to apply the principles of extended delignification, but they are not able to fulfill all these principles.

In modified continuous cooking, the total white liquor is split into three sequential applications, and the final cooking zone is operated countercurrently. These process conditions verify the first and the third rule of the principles. Sulfidity of the liquor is not enhanced in the initial cooking phase, but in fact, is decreased due to the lower cooking liquor concentration.

In the extended modified continuous cooking, the white liquor is split into about 75 % for the main cooking zone and the remaining 25 % into the wash liquor circulation, where countercurrent cooking is applied. The temperature in this last cooking phase is reduced, which contributes to milder cooking conditions at the expense of longer cooking time. This process modification fulfil the principles of the extended delignification to a higher extent, as principle 4 is also accomplished. However, the actual kraft black liquor recovery limits variations of white liquor sulfidity in different cooking phases.

Isothermal cooking operates under similar conditions using a forced liquor circulation. The cooking temperature is kept on a constant low level in all cooking phases, which normally does not exceed 160°C.

The batch systems are even more flexible with regard to cooking conditions. They start with a black liquor impregnation procuring a very low alkali level in the chips and homogeneous impregnation at moderate temperature. The impregnation liquor is displaced by hot white liquor before cooking is started. In the final cooking phase, the cooking liquor is displaced by wash liquor, which can be reinforced with some white liquor. The main differences between the various batch systems are the way in which the black liquor impregnation is performed. Despite the higher flexibility of these systems, they are not able to completely fulfil the earlier mentioned principles of modified delignification.

In addition to these process modifications, kraft pulping can be improved by the above mentioned pulping additives. Olm *et al.* (1998) performed mixed spruce/pine softwood ITC-cooks and applied different additives. The reference was a standard ITC-cook yielding a kappa number around 24. 2 % polysulfide on wood was charged to the impregnation liquor. In all cases the cooking temperature was kept at 160°C. Cooking was performed in 120 minutes in the co-current and 180 minutes in the countercurrent zone. In addition to the polysulfide charge, 0.1 % AQ on wood was charged. Another cook was carried out using green liquor in presence of carbon dioxide in the pretreatment stage. This was followed by a standard ITC cook. The last modification worked with green liquor and hydrogen sulfide pretreatment of the chips, followed by an ITC cook. The results of these cooks are listed in Table 1.

All cooks resulted in kappa numbers between 23 and 24. The use of 2 % polysulfide increased yield by 2 % from 46.5 to 48.7. The addition of 0.1% AQ improved yield by another 1% to 49.9 %. While the green liquor/CO_2 pretreatment resulted in the same yield as the polysulfide cook, the green liquor/H_2S cook resulted in the highest total yield of this set of cooks reaching 51.8 %. Polysulfide improves the yield by end-group stabilization. The additive effect of polysulfide and anthraquinone reinforces this reaction. Furthermore, anthraquinone contributes to enhanced delignification. The green liquor/CO_2 pretreatment primarily improves glucomannan stabilization, whereas the green liquor/H_2S pretreatment preserves the glucomannan in the pulp and additionally stabilizes the reducing end groups of the carbohydrates.

These modifications would be less effective in hardwood pulping, in particular eucalyptus. Of course, stabilization of the carbohydrate end-groups would increase yield to some extent, but the main hemicellulose in hardwoods is xylan. Due to its 4-O-methylglucuronic acid group, xylan is more stable against alkaline peeling. Especially eucalyptus requires a higher alkali charge because of its high tannin content.

Table 1. Pulping results and carbohydrate composition, according to Jansson.

Pulp	Kappa no.	Viscosity (mL g^{-1})	Total yield (on wood)	Brightness (% ISO)	Cellulose (% on pulp)	Glucomanan (% on pulp)	Xylan (% on pulp)
Reference	23.8	1200	46.5	36.0	79.6	8.5	8.3
PS	24.5	1190	48.7	34.8	77.5	11.2	7.6
PS/AQ	23.6	1180	49.9	34.9	76.9	12.0	7.5
GL/CO_2	23.0	1260	48.6	35.9	78.4	10.2	7.9
GL/H_2S	23.0	1180	51.8	35.9	74.3	16.0	6.1

The increased pulp yield has an impact on strength properties of the resulting pulps. This is shown in Figures 1, 2. All pulps had excellent viscosities, but at higher pulp yield the tear index is reduced. It is well known that an increasing hemicellulose content improves tensile and reduces tear strength. This, however, is only true within certain limits. The presented pulps do not show this tendency, but higher pulp yield means a reduced number of fibers in the sheet and this reduces the strength of the fiber net.

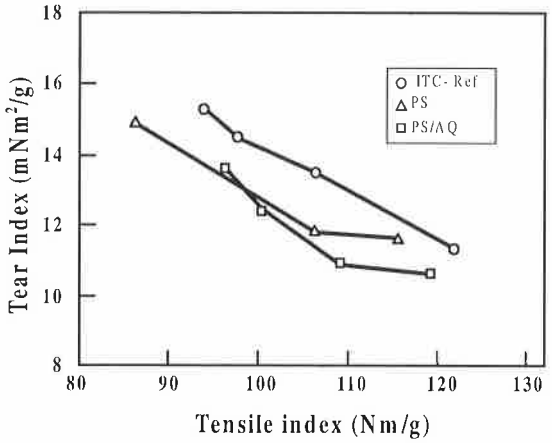

Figure 1. Tear-tensile plot of OS, OS/AQ and the reference pulp (Olm *et al.*, 1998).

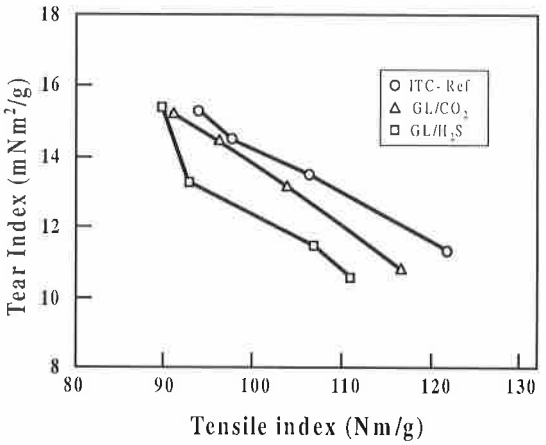

Figure 2. Tear-tensile plot of Gl/CO_2, GL/H_2S and the reference pulp (Olm *e al.*, 1998).

The conditions applied in these laboratory trials can only partially be applied to commercial cooks. The main reason for this is the missing flexibility of the conventional recovery system, which is not able to separate sodium from su1fur compounds.

PULP BLEACHING

In the past, the main objective of extended delignification was to facilitate bleaching in ECF and in particular in

TCF sequences. Therefore extended delignification aimed at removing as much lignin as possible without degrading cellulose to an unacceptable extent. Today, however, we have to state that all these carbohydrate preserving modifications, are not good enough to keep pulp viscosity and yield on an acceptable level. On the other hand, pulp bleaching has made great progress, particularly in ECF bleaching. The present tendency is to stop pulping at a higher kappa number, then start ECF bleaching. This strategy results in a higher yield and a better pulp strength. TCF bleaching, however, still requires lower unbleached kappa number pulps, and the fully bleached pulps show, reduced strength at lower yield. Bleaching costs of TCF sequences are higher. So TCF bleaching has to be improved to become competitive to ECF bleaching. From the environmental point of view, the burdens, of ECF and TCF filtrates are equivalent. This can not be the reason to favor TCF bleaching. But the final goal of pulp production must be a closed mill, and we think this is more easily achievable with a TCF sequence. In this paper we would like to limit the discussion to TCF bleaching.

1. Oxygen delignification

Two-stage delignification

One stage oxygen bleaching is limited to 30 - 50 % delignification. In general, the oxygen delignification rate in commercial plants is lower than in laboratory scale. The main reasons for that are the limited oxygen

availability in a medium consistency tower and the lower mixing intensity compared to a laboratory oxygen delignification. A two stage oxygen delignification is able to increase lignin removal to up to 70 % for softwoods. For hardwoods around 60 % might be achievable. This permits the starting of delignification at fairly high kappa number which improves pulp yield and strength (Chirat *et al.*, 1998). Two stage oxygen delignification does not require an intermediate wash. The temperature in the first stage should be lower than in the second stage (Bokström *et al.*, 1998). Intermediate washing requires higher investment, but it saves some alkali (Allison *et al.*, 1998).

Laccase-mediator

Lignin oxidation by laccase requires a mediator which is able to penetrate the fiber wall and to perform an electron transfer from the substrate to the enzyme. In presence of oxygen, this system can reduce the lignin content of the pulp up to 40 % in a very selective way. The pH of the system is weakly acid. At an operation temperature of around 45°C, 1 and 4 hours retention time are needed. It was possible to replace the oxygen stage by a laccase mediator stage with subsequent alkaline extraction. The high selectivity of this delignification results in higher viscosity and strength properties of the fully bleached pulps (Poppius-Levlin *et al.*, 1998). The main problem of laccase mediator bleaching is the cost of the mediator. The most common one is 1-hydroxybenzotriazole (HBT). Many others have been tested (Jacob *et al.*, 1999), but it was concluded that, for economic reasons, none of them would be applicable in commercial scale.

Polyoxomethalates

Intensive studies have been performed to activate oxygen delignification with polyoxomethalates. These mainly tungstates, molybdates, vanadates and manganese based oxidized heteropolyanions can be used for lignin oxidation under anaerobic as well as aerobic conditions. This is done in the neutral to acid pH range at similar temperature as for conventional oxygen bleaching. Compared to oxygen bleaching, delignification is slightly enhanced at the expense of some viscosity losses. Pulp degradation can be reduced by running this treatment in an alcohol/water mixture. The polyoxomethalate compounds have to be re-oxidized. The required POM-charges per pulp are still high, and it will take further research efforts to make this system competitive with conventional oxygen bleaching (Evtuguin *et al.*, 1998 a,b)

Activators and catalysts

It seems that alkali oxygen delignification can be catalyzed with cupric(1,10)-phenanthroline. The addition of this compound accelerates delignification to very low kappa numbers, but the conditions employed are still far from commercially applicable (Germer 1998).

In the area of oxygen delignification we are still searching for an activator or catalyst, which enhances selectivity of the delignification, process and which could be applied in an existing bleach plant.

2. Ozone Bleaching

Today, ozone bleaching is a well established technology. Its application in industry, however, has shown the limits of ozone bleaching. The main drawback of ozone is its poor selectivity. The consequence is that the ozone charge has to be restricted to what is absolutely necessary. It should not exceed 0.5 % per pulp. In many cases ozone is used more for lignin activation than for lignin removal. The selectivity of ozone can be improved when it is applied in presence of alcohol, for instance methanol. But in industrial operations, this can not be tolerated. The conditions of ozone bleaching are far from ideal. Prior to the treatment the pulp has to be acidified, and heavy metals have to be removed as far as possible. The temperature in ozone bleaching should be as low as possible. The upper limit is in the area of 55°C. The conditions of ozone bleaching do not fit very well into a closed mill system. From the actual state of development it cannot be concluded that the selectivity in ozone bleaching can be improved to such extent that ozone could take over a major part of the total delignification in pulp bleaching.

3. Peroxide bleaching

Peroxide is a very good lignin retaining bleaching agent, but drastic conditions have to be applied for lignin dissolution. Under these conditions, peroxide does not react very selectively. Many attempts have been made to improve the delignifying action of peroxide without degrading the carbohydrates excessively, or to use other more powerful peroxide-derived bleaching agents.

Peracetic acid

Peroxy acid, in particular peracetic acid, can be used in pulp bleaching as strong oxidizing agents. Peracetic acid (PAA) is able to substitute ozone in a TCF sequence, and it is used in some mills. In most of its

application, PAA is used more as an activator to provide the lignin with an improved reactivity in subsequent bleaching stages. A slightly acid pH (pH 4-4.5) is more favorable than an alkaline pH, which rather has in a brightening than a lignin dissolving effect. Especially under acidic conditions PAA should be peroxide-free to avoid the formation of OH radicals. This means that distilled peracetic acid should be used. The applied temperatures and reaction times are similar to those in alkaline peroxide stages. Compared with ozone, the chemical costs of PAA bleaching are higher, but no additional equipment is required. PAA bleaching can be performed in existing towers (Tibbling *et al.*, 1993).

Caroic acid

The ozone stage can also be replaced by application of caroic acid or better still its potassium salt. The pH of such a stage can be varied from acid to alkaline. The best brightness gain in a subsequent P-stage can be reached when the CA stage is performed under acid conditions. But the bleaching response in the stage itself is better when neutral or slightly alkaline conditions are applied. Neutral conditions improve in particular the selectivity of the reaction. A CA stage with 2 % caroic acid (calculated as H_2O_2), at 70°C for 2 h increased brightness of kraft softwood pulp by 10 brightness points. Pulp viscosity was more or less maintained. Due to the high costs of such a stage caroic acid is not used for commercial pulp bleaching.

DMD

Caroic acid can be used to produce dimethyldioxirane (DMD) in a reaction with acetone (Basta *et al.*, 1994). DMD is a very selective delignification agent, which is able to attack ring structures and remove up to 80 % of the lignin present in unbleached pulps. In reaction with lignin the peroxo structure of DMD will be reduced and acetone is formed. The technical application of this bleaching system fails because of the high chemical costs and the risk caused by the presence of acetone. Bleaching itself can be performed under very mild conditions, but this system has been buffered with $NAHCO_3$. It is not very probable that further improvements will render possible the technical application of this bleaching agent.

Cyanamide/Dicyandiamide

Cyanamide and dicyandiamide were used to activate peroxide bleaching. In spruce sulfite pulp bleaching cyanamide improves delignification and pulp brightness and stabilizes pulp viscosity. Similar results could be achieved in softwood kraft pulp bleaching. In peroxide reinforced oxygen stage, cyanamide activated in the same way. The kappa number, of the stating softwood kraft pulp was reduced from 18.3 to 9.4 when 2.5 % sodium hydroxide, 1 % peroxide and 0.33 % cyanamide were charged at 95°C. Pulp viscosity dropped from 980 to 903 mL/g, which can be regarded as a very selective delignification. The weight ratio peroxide to cyanamide should be 3: 1 (Sturm *et al.*, 1993).

Dicyandiamide seems to be even more efficient. We added dicyandiamide in a peroxide stage of an oxygen prebleached kraft pulp. Kappa number was 8.4, brightness 35 % ISO, and viscosity 763 mL/g. After the Q-stage the pulp was bleached charging 2 % peroxide, 1.7 % sodium hydroxide and 1 % dicyandiamide. Kappa number dropped to 2.7, and brightness was raised to 73.8 %, while viscosity dropped to 685 mL/g. The reference trial without dicyandiamide addition resulted in a kappa number of 4.6 and 65 % ISO brightness. This shows that dicyandiamide is a very effective activator in peroxide delignification and bleaching. But the required charges are fairly high and it is an open question whether this is economically viable.

Polypyridines

Polypyridines are known to activate peroxide bleaching. We tested different derivatives in conventional P- and OP-stages and fond that 4-ethyl-1,10-phenanthroline and 1,10-phenanthroline were the most effective. But activation of these compounds requires fairly high temperatures between 90 and 120°C, and the viscosity drop of the pulps during bleaching is quite drastic. So we do not believe that polypyridines are able to overcome the actual problems in TCF bleaching (Jaschinski *et al.*, 1998).

Catalysts

The best way to overcome these problems would be a catalyst which is able to reduce the activation energy for lignin degrading reactions of peroxide. Recently, different catalysts that act in this way were revealed. Collins *et al.* (1998) presented a paper on the action of an iron-containing activator in conventional peroxide stages. It is possible to perform a very intensive delignification at short reaction time, but obviously the kappa/viscosity ratio is the same as in conventional peroxide delignification at high temperature. It seems that the catalyst is not very stable, because in a run of 75 minutes it was replaced five times, and this means that the stability of this catalyst has to be improved to become applicable in a commercial peroxide stage. For

about 4 years now, we have been working on catalyst peroxide bleaching as well and we will present details later in this paper.

ASAM PULPING AND TCF BLEACHING

As mentioned in the beginning, it is generally accepted that ASAM is an alternative to kraft pulping because this process is able to delignify any raw material to low kappa numbers and fairly high unbleached pulp brightness, which renders this pulp easily bleachable. We would like to present just as an example the results of ASAM pulping of the most abundant Middle European tree species, which are beech and spruce. Table 2 shows the conditions applied in ASAM pulping and the corresponding results. It can be seen that delignification proceeds to very low kappa numbers for both raw materials. The high pulp viscosity means that even final delignification is performed in a very selective way. Despite the low kappa number, the yield is high compared with kraft pulping, especially for the softwood pulp.

Table 2. ASAM pulping of beech and spruce.

	Chemicals (% as NaOH)	Na_2SO_3/NaOH ratio	MeOH (% v/v)	Temp. (ºC)	Time (min)	Total yield (%)	Kappa no.	Viscosity (ml/g)
Beech	25	80/20	20	177	150	51.3	11.8	1167
Spruce	25	80/20	15	175	180	49.7	19.9	1321

Constant: 0.1 % AQ on wood

For pulp bleaching we selected TCF sequences using only oxygen and peroxide (Table 3). This leads to more or less homogeneous pH and temperature profiles and permits consequent countercurrent washing. These are important prerequisites for operating a bleach plant in a closed system. Two years ago, on the occasion of this symposium, we presented first basic reaction parameters of the bi-nuclear manganese based complex which we are using in peroxide contained bleaching stages to activate peroxide delignification (Patt *et al.*, 1997). In the meantime, we optimized the application conditions for this catalyst and both ASAM pulps were bleached with and without use of the catalyst.

The low kappa number of the unbleached pulp and its high brightness are excellent starting conditions for TCF bleaching of these pulps. The unbleached beech pulp was pretreated in a chelating stage sing 0.4 % EDTA. Bleaching was performed in only two stages. In the first peroxide reinforced oxygen stage 5 ppm of the catalyst was charged. Then a final P-stage was carried out. The bleaching response of the pulp was very good. A final brightness of 89.7 % ISO brightness was attained at a viscosity of 981 mL/g. The reference sequence without application of catalyst resulted in 88 % ISO brightness and a viscosity of 951 mg/I. These results show that even very low catalyst charges have a positive effect on residual lignin content, brightness and viscosity.

Due to the higher unbleached kappa number the spruce pulp required first an oxygen delignification, followed by the same bleaching sequence applied to the beech pulp. In the OP-stage 20 ppm of the catalyst was charged. The brightness target of 88 % ISO was reached at a still high pulp viscosity of 981 mL/g. Bleaching of this pulp without catalyst required quite drastic conditions. The final P-stage had to be replaced by an OP-stage and temperature had to be increased to110°C. The resulting brightness was good, but it could only be reached at the expense of drastic viscosity losses. This, of course, had a negative impact on the pulp properties. The high viscosities of the other fully bleached pulps indicate their good pulp properties. The applied TCF-sequences - in particular when the catalyst is used - are highly selective and can compete with ECF-sequences.

The beneficial effect of the catalyst on the selectivity of TCF-bleaching is even more pronounced in softwood kraft pulp bleaching. Table 4 shows the results of two TCF sequences for an oxygen delignified industrial softwood kraft pulp. The sequence Q(OP)Q(OP) is in operation in a Scandinavian mill. We applied the catalyst in the final OP-stag. It has a tremendous effect on viscosity and strength of the final bleached pulp as well as on the COD of the filtrate. With addition of the catalyst the temperature could be reduced from 110°C to 95°C, and bleaching time from 3 h to 1.5 h.

Table 3. TCF bleaching of ASAM pulps.

	Cat. (ppm)	Kappa no.	Brightness (% ISO)	Viscosity (mL/g)
Beech				
unbleached	-	11.8	47.2	1167
Q $(OP)_{CAT}$ P	5	3.1	89.7	981
Q (OP) P	-	3.4	88.0	951
Spruce				
unbleached	-	19.9	47.1	1321
O Q $(OP)_{CAT}$ P	20	4.8	88.0	981
O Q (OP) (OP)	-	4.3	88.7	782

Table 4. Properties of fully bleached ASAM spruce pulps.

Bleaching sequence	Q/OP/Q/OP_{cat}	Q/OP/Q/OP
Kappa no.	2.6	2.6
Viscosity (mL/g)	674	563
Brightness (% ISO)	88.8	88.4
COD (kg/t)	29.2	38.2
Tensile index at 25 ºSR (Nm/g)	89.6	83.4
Tear index at 25 ºSR (mNm^2/g)	12.8	11.8

Conditions applied in the final stage: 3.5 % H_2O_2, 2.5 % NaOH, 0.2 % $MgSO_4$, 0.05 % DTPMPA; 180 min at 105ºC uncatalyzed, 90 min at 95 ºC with 20 ppm catalyst

Closed Mill ASAM Pulping

Closed bleach plant operation is an important step towards a closed mill operation. Figure 3 shows the flow sheet of a closed ASAM mill. The bottleneck of sodium based sulfite mills is the recovery of the cooking chemicals. At present, sodium sulfite mills are using the carbonation process to convert Na_2S into Na_2SO_3. This is a fairly complicated recovery, which however, is able to separate non-process element from the loop very efficiently and to generate bleach alkali as well. But we believe that this recovery system has to be simplified to facilitate the implementation f the ASAM process in industrial scale. Recent developments in sodium based black liquor gasification give rise to hope that this technology could be a convincing alternative to the carbonation process as well as to the conventional kraft recovery. The ASAM flow sheet considers black liquor gasification. Cooking is performed with the ASAM cooking chemicals. Then the pulp is washed and the black liquor goes to a stripper where ethanol and other volatile compounds are stripped off. Methanol purification is performed in a rectification stage. Low boiling organic compounds other than methanol are separated in the rectification stage and burnt in a waste burner. The purified ethanol is recycled to the cooking department. No methanol make-up is required. Bleaching of the pulp is performed in a TCF sequence with countercurrent washing. The bleach filtrate from the alkaline stages is used for pulp washing. The Q-filtrate is partly recycled and partly used for dissolving sodium carbonate formed in the gasification stage. The water supply for the bleach plant comes from the evaporation stages where a clean condensate is produced, because volatile compounds have been separated from the black liquor in the stripper. Gasification will be performed at temperatures below the melting point of inorganic sodium compounds, e.g. at 600°C. Black liquor is converted into a gas fraction and a solid fraction. The solid fraction contains sodium carbonate, carbon and inorganic non-process elements. This fraction goes to a dissolving tank and then to a filter where carbon and non-process elements are filtered out. They are burnt in a waste burner. Most of the dissolved sodium carbonate goes directly to the absorption stage where it is reacted with sulfur dioxide to sodium sulfite. A minor part of the sodium carbonate has to be causticized to form sodium hydroxide, which is used as additional alkali for cooking and to supply the alkaline bleaching stages. Not more than 1/3 of the total chemicals have to be causticized. The gas fraction from the gasifier containing mainly H_2S, carbon monoxide, hydrogen and volatile hydrocarbons, is oxidized in a gas burner to generate energy and sulfur dioxide, and this sulfur dioxide goes to the absorption tower.

The installation- of a pyrolysis process in a sodium based sulfate mill or a kraft mill would facilitate chemical recovery by splitting cooking chemicals into a sodium and sulfur fraction. For kraft pulping, this would create the chance to apply all principles of modified cooking and generate bleach alkali at the same time. The existing experience in black liquor gasification is very encouraging. It seems that this technology is not only

able to solve environmental problems of pulping but can reduce production costs as well (Rockvam *et al.*, 1995).

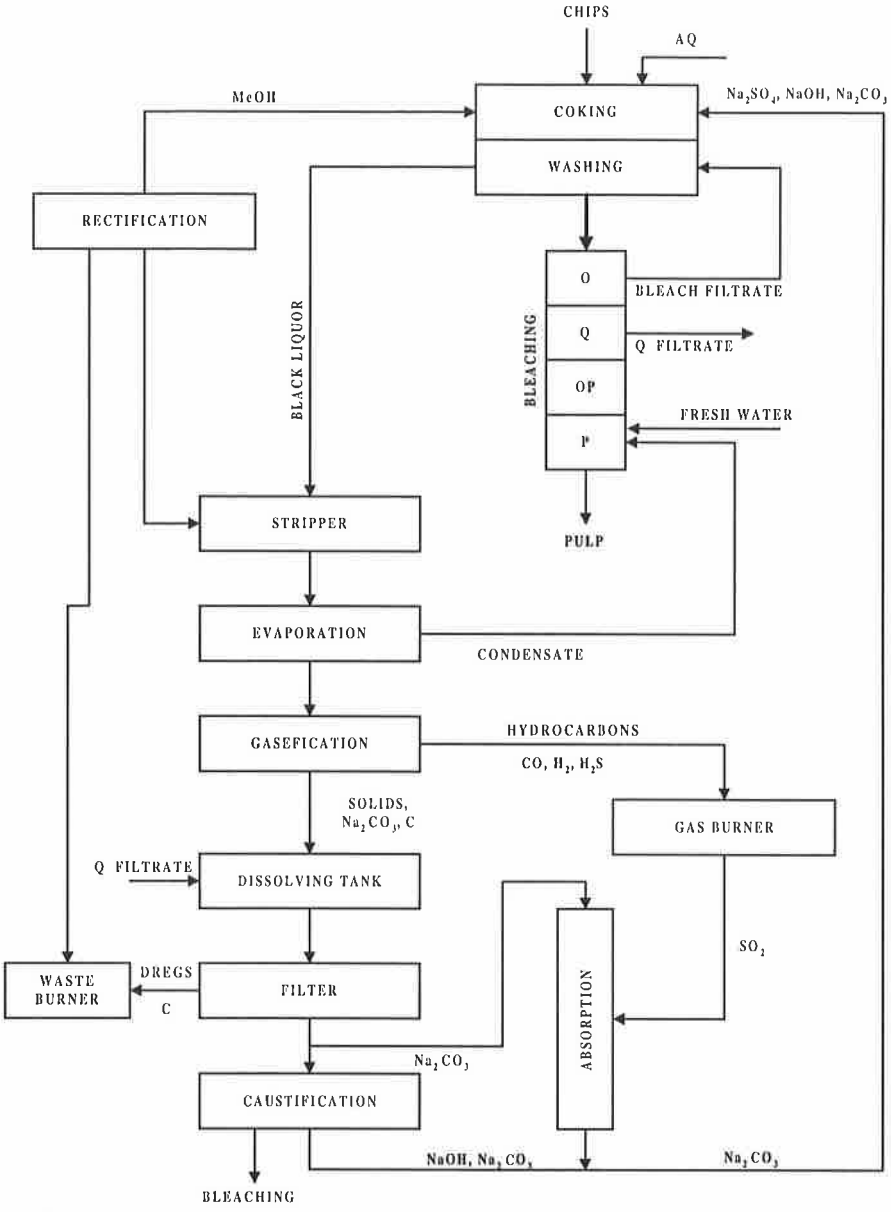

Figure 3. Flow sheet of an ASAM with recovery based on black liquor gasification.

As the ASAM technology offers better pulp yield, pulp strength and bleachability compared with kraft, we believe that the implementation of the gasification process into the ASAM process will be a decisive step on the way to applying this technology in industrial operation.

CONCLUSIONS

The optimism, which has accompanied the development of new pulping processes, has diminished. Most of these developments have been stopped for various reasons. One of them is the large amount of progress which kraft pulping has made by different process modifications. Today it is possible to produce kraft pulps with a good yield and excellent strength properties. This requires a multi-stage process, which is much more complicated than the standard kraft procedure. In particular, varying sulfur to sodium ratios in different phases of the cook are difficult to establish in industrial scale. The actual kraft recovery is not able to supply alkali for peroxide-containing bleaching stages.

Many efforts are dedicated to improving TCF bleaching, which is required to become competitive with ECF bleaching. Oxygen as well as peroxide bleaching can be improved, but there is little hope of impeding

carbohydrate degradation reactions in ozone bleaching. It can be assumed that the actual research in TCF bleaching will render possible a competitive process. The application of manganese based catalysts in peroxide-contained stages improves TCF bleaching to such an extend that corresponding TCF sequences are competitive to ECF bleaching.

The ASAM process has clear advantages over kraft pulping regarding pulp yield, strength and bleachability, but the bottleneck is the rather complicated chemical recovery. This problem can be overcome by application of a black liquor gasification process. This renders possible an environmentally friendly and cost competitive pulp production in a closed mill.

REFERENCES

Allison, R.W. & Wrathall, S.H. (1998). Optimizing Two-Stage Oxygen Delignification: Effects of Interstage Washing and Metal Ion Control. *In Intern. Pulp Bleaching Conf.*, Proceedings, pp 41-48.

Aurell, R. & Hartler, N. (1963). Sulfate Cooking with the Addition of Reducing Agents, Part III: The Effect of Added Sodium Borohydride, *Tappi J.*, **46 (4),** 209-215.

Basta, J.; Holtinger, L.; Hallisträm, A.-S. & Lundgren, P. (1994). Peroxo Compounds in TCF-Bleaching. *In TAPPI Pulping Conf.*, Proceedings, pp 953-956.

Bokström, M. & Norden, S. (1998). Extended Oxygen Delignification. *In Intern. Pulp Bleaching Conf.*, Proceedings, pp 23-31.

Chirat, C. & Lachenal, D. (1998). Limits of Oxygen Delignification. *In TAPPI Pulping Conf*, Proceedings, pp 619-624.

Collings, T.J.; Fattaleh, N.L.; Vuocolo, L.D.; Horwith, C.P.; Hall, J.; Wrightlan, L.J.; Suckling, D.; Allison, R.W. & Fullerton, T.J. (1998). New Efficient Selective TCF-Pulp Bleaching. *In TAPPI Pulping Conf.*, Proceedings, pp 1291-1340.

Evtuguin, D.V. & Pascoal Neto, C. (1998). *In Intern. Pulp Bleaching Conf.*, Proceedings, pp 487-492.

Evtuguin, D.V.; Pascoal Neto, C.; Marques, V.M. & Furtado, F.P. (1998). Polyoxometallates Promoted Bleaching of Kraft Pulp with Oxygen, Ozone and Hydrogen Peroxide. A Comparative Study. *In Intern. Pulp Bleaching Conf.*, Proceedings, pp 493-498.

Germer, E.I. (1998). Chemistry and Mechanism of Catalysis of Oxygen - Alkaline Delignification by Some Complex Compounds. *In 5th European Workshop on Lignocellulosics and Pulp*, Proceedings, pp 33-38.

Herschmiller, D.W. (1998). Kraft Cooking with Split Sulfidity - A Way to Break the Yield Barrier? *In TAPPI: Breaking the Yield Barrie Symposium*, Proceedings, pp 59-68.

Holton, A. (1977). Soda Additive Softwood Pulping: A Major New Process. *Pulp Paper Can.*, **78 (10)**, 19-24.

Jaakko Pöyry Deutschland GmbH, (1997). Umweltverträgliche Holzaufschlußverfahren, Schriftenreihe, Nachwachsende Rohstoffe" Bd. 8, Landwirtschaftsverlag GmbH, Münster, pp 1-532.

Jacob, H.; Del Grosso, M.; Küver, A.; Nimmerfroh, N. & Süss, H.U. (1999). Delignifizierung von Zellstoff mit Laccase und Mediator - Ein Konzept mit Zukunft?, *Papier*, **53(2)**, 85-95.

Jaschinski, T. & Patt, R. (1988). Effects of Polypyridines as Peroxide Activators in TCF-Bleaching of Kraft Pulps.*In Intern. Pulp Bleaching Conf.*, Proceedings, pp 417-422.

Kühne, L.; Kordsachia, O.; Odermatt, J. & Patt, R. (1999). Application of a Manganese Based Peroxide Catalyst in Kraft Pulp Bleaching. *In TAPPI Pulping Conf.*, Proceedings, in Print.

Olm, L.; Tormund, D. & Bergner Gidnert, E. (1998). Possibilities to Increase the Pulp Yield in a Kraft Cook of ITC-Type. *In TAPPI: Breaking the Pulp Yield Barrier Symposium*, Proceedings, pp 69-78.

Patt, R. & Mielisch, H.-J. (1997). Application of a Catalyst in TCF Pulp Bleaching. *In 5th Brazilian Symposium on the Chemistry of lignins and Other Wood Components*, Proceedings, pp 44-58.

Poppius-Levlin, Wag, W. & Rauna, M. (1998). *In Intern. Pulp Bleaching Conf.*, Proceedings, pp 77-85.

Rockvam, L.N. & Tenore, F. (1995). Spent Liquor Steam Reforming and Recovery. *In Intern. Chem. Recovery Conf.*, Proceedings.

Sturm, W.G.I. & Kuchler, J.G. (1993). The Nitrilamine Reinforced Hydrogen Peroxide Bleaching of Kraft Pulps. *In Non-Chlorine Bleaching Conf.*, Proceedings, pp 1-7.

Teder, A. (1969). Some Aspects of the Chemistry of Polysulfide Pulping, *Svensk Papperstid.* **72 (9)**, 294-303.

Tibbling, P. & Dillner, B. (1993). TCF Bleaching Can Be Carried out with Different Bleaching Systems. *In EUCEPA Conf.*, Proceedings, pp 65-83.

Vinje, M.G. & Worster, H.E. (1969). Hydrogen Sulfide Alkaline Pulping - A New Method for Increasing Pulp Yield, I, Process Principles, *TAPPI J.*, **52 (7)**, 1341-1345.

Young, R.A. & Akhtar, M. (1998). Environmentally Friendly Technologies for the Pulp and Paper Industry, John Wiley & Sons, Inc., New York, pp 1-577.

Effect of pH on xylose bioconversion into xylitol using the yeast *Pachysolen tannophilus*: Carbon material balance

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ABSTRACT

The data of batch fermentations performed at different pH on detoxified hemicellulose hydrolyzate have been used for a material balance of consumed carbon and to study the pH influence on *Pachysolen tannophilus* behavior under microaerophilic conditions. The metabolism of this versatile yeast seems to be preferentially addressed towards xylitol or ethanol, according to conditions. At pH close to the optimum (6.0-7.5), about 90% of xylose is utilized for xylitol production, the rest being devoted to alcohol fermentation. At extreme pH values (3.0 and 12.0), respiration becomes progressively more significant consuming up to 30% of xylose.

Keywords: *Pachysolen tannophilus*; microaerophilic conditions; xylitol production; pH variation

INTRODUCTION

The fermentation of hydrolyzates with yeasts is hindered by inhibitors present in the raw material or produced during chemical processing, such as furfural and hydroxymethylfurfural (generated by degradation of sugars) and acetic acid (liberated from the acetyl groups of the raw materials), lignin degradation products and compounds derived from wood extractives (primarily composed of phenolic compounds), and inhibitors derived from the metals or minerals in wood, soil or hydrolysis equipment (Parajó *et al.,* 1997). In order to minimize their effects, several technologies have been employed, including overliming (Roberto *et al.,* 1991) and charcoal adsorption (Parajó *et al.,* 1996).

The yeast *Pachysolen tannophilus* is a versatile microorganism whose metabolism can be preferentially addressed towards xylitol or ethanol productions depending on the xylose reductase (XR), the enzyme responsible for xylitol or ethanol production. In particular, four different possible metabolic pathways can be considered: reductive production of xylitol, ethanol fermentation, respiration and cell growth (the last being negligible under microaerophilic conditions).

Xylitol has found increasing use in the food industry as a sweetener because it has a sweetness equal to sucrose and several advantages including: it is tolerated by diabetics, it has been recommended for parenteral nutrition, it does not cause acid formation and has low viscosity and negative heat effect when dissolved in a solution (Pepper and Olinger, 1988).

This work deals with the biotechnological conversion of xylose into xylitol under microaerophilic conditions using detoxified hydrolyzate as substrate. In order to maximize the xylitol production the effect of the pH was considered in the range 3-12. Its influence on the metabolic pathway was studied simultaneously.

EXPERIMENTAL

Substrate preparation

The hemicellulose acid-hydrolyzate employed in the present work was prepared by the Tennessee Valley Authority with a final counter-current washing scheme, containing the following composition: 106 g xylose/L, 13.2 g glucose/L, 26.6 g acetic acid/L, 8.6 g galactose/L, 6.8 g mannose/L, 4.2 g furfural/L and some other minor components. After an adequate dilution with water (1:1), the hydrolyzate was detoxified by overliming (Perego *et al.,* 1990). In order to remove acetic acid and furfural as much as possible the hydrolyzate was then boiled for 160 min at 105°C (Converti *et al.*, 1999) and finally treated with activated charcoal for 1 h at room temperature in the ratio 1/10 g/g (Parajó *et al.,* 1996).

Microorganism

The yeast *Pachysolen tannophilus* NRRL Y-2460 was used in this work. The yeast was maintained on agar slants containing yeast extract (0.1%) and detoxified hemicellulose hydrolyzate (50%) through periodic

transfers and subcultures. The cells used for inocula were grown for 72 h on the same medium and then centrifuged.

Fermentation conditions
Batch fermentations were performed in an orbital shaker at 200 rpm and 30 ± 0.1°C under microaerophilic conditions, using 100-ml Erlenmeyer flasks with 50 ml of fermentation broth.

Analytical procedures
Sugar components of the hydrolyzate, as well as acetic acid and ethanol, were determined before and during each fermentation by HPLC using two Shodex SH columns (mobile phase, H_2SO_4 0.01 M; flow rate, 0.7 ml/min; IR and UV detectors). Biomass concentration was determined by dry weight filtering known volumes of samples on 0.45 μm membrane filters.

Carbon material balance
Carbon material balance has been performed on the basis of the productions of xylitol, ethanol, and CO_2, on the consumption of xylose, and biomass determinations.

RESULTS AND DISCUSSION

Several strategies were employed in the present work in order to diminish the inhibitory effect provoked by the presence of some inhibitory compounds:

1) dilution up to one half of the starting concentration,

2) neutralization by overliming, this technique is well-known to ensure several benefic effects, including a partial removal of acids (acetic and tannic acids) and phenolic compounds (Roberto *et al.*, 1991), precipitation of heavy metal ions (Strickland and Beck, 1984) as well as conversion of furfural into furfuryl acid (Perego *et al.*, 1990; Strickland and Beck, 1984; Tran and Chambers, 1985). In our case, the overliming removed 61.15 % of the lignin-derived compounds (LDC).

3) In order to overcome the inhibitory effect of the lignin-derived compounds, the hydrolyzate was treated with activated charcoal as described by Parajó *et al.* (1996). The activated charcoal/hydrolyzate ratio 1/10 g/g allowed eliminating up to 95.40 % of the LDC.

4) Finally, due to LDC were not the only inhibitors present in the hydrolyzate able to affect the yeast fermentation (Converti *et al.*, 1999) the influence of the acetic acid concentration was also studied. The acetic acid inhibitory action depends on the concentration of the undissociated form, which is a function of both concentration and pH. The sensitivity of yeasts to acetic acid depends on the microorganism considered. For the yeast *Pachysolen tannophilus* Watson *et al.* (1984) reported that acetic acid concentrations higher than 1.45 g/L inhibit completely its growth. In order to remove acetic acid from the fermentation broth, the hydrolyzate was boiled for 160 min decreasing the acetic acid concentration from 31.2 to 1.0 g/L, that is below the inhibition threshold mentioned by Watson *et al.* (1984) for this yeast. In addition, the furfural concentration decreased from 1.2 g/L to less than 0.5 g/L.

Four different possible metabolic pathways are considered to be responsible for xylose consumption: reductive production of xylitol, ethanol fermentation, respiration and cell growth. This last one has not been considered because it was always negligible under the conditions selected for this study (high starting biomass level and microaerophilic conditions). Figure 1 shows the carbon material balance for the fermentation of hemicellulose acid-hydrolyzates performed at different pH values in the range 3 – 12.

As it can be seen in Figure 1, the results indicate pH = 6.0 - 7.5 as the optimum pH range for xylitol production by *Pachysolen tannophilus*. This value is slightly different from the pH = 8.0 reported for the optimal growth of this yeast (Parajó *et al.*, 1998). This suggests that the optimum pH for xylose reductase activity in *Pachysolen tannophilus* is likely included in this range, outside of which the reaction rate decreases and alternative metabolic routes (respiration and alcohol fermentation) become relatively more important.

A progressive increase in pH affects xylitol production allowing alternative metabolic routes to be followed and increasing the pH over 6.0 - 7.5 tends to decelerate xylitol production and favors simultaneously the alcohol fermentation, which should anyhow occur at a rate decreasing with increasing pH. This means that,

although both processes decelerate with increasing pH, ethanol production, which competes with xylitol formation, gains relative significance.

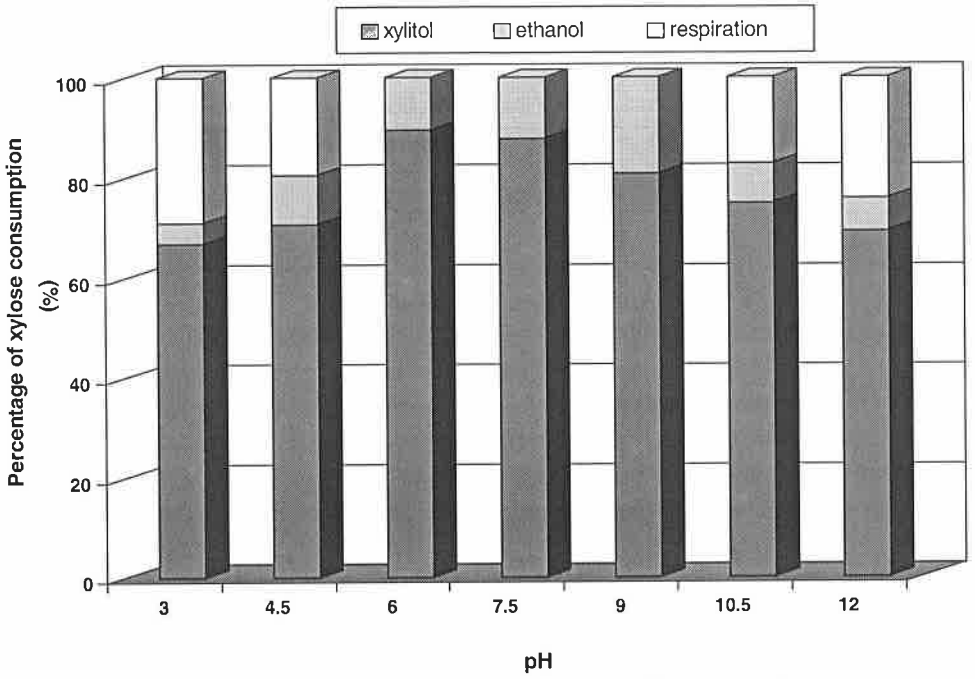

Figure 1. Material balance of carbon in microaerophilic metabolism of *Pachysolen tannophilus*.

It should finally be worth noticing that the biomass growth is practically negligible, because the process is carried out under conditions able to stimulate xylitol production and to prevent, at the same time, cell growth (microaerophilic conditions in the presence of high cell concentration).

Finally, at extreme pH values (3.0 and 12.0) both these processes are affected to such an extent that a further metabolic pathway, the respiration, becomes progressively more important, consuming up to 30% of the carbon source. In fact, although strongly limited by oxygen availability, the aerobic respiration is always possible even under microaerophilic conditions.

REFERENCES

Converti, A., Perego, P. & Domínguez, J.M. (1999). Xylitol production from hardwood hemicellulose hydrolyzates by *Pachysolen tannophilus*, *Debaryomyces hansenii* and *Candida guilliermondii*. *Appl. Biochem. Biotechnol.* In press.

Parajó, J.C., Domínguez, H. & Domínguez, J.M. (1996). Production of xylitol from concentrated wood hydrolysates by *Debaryomyces hansenii*: effect of the initial cell concentration. *Biotechnol. Lett.* **18,** 593-598.

Parajó, J.C., Domínguez, H. & Domínguez, J.M. (1997). Improved xylitol production with *Debaryomyces hansenii* Y-7426 from raw or detoxified wood hydrolyzates. *Enz. Microbiol. Technol.* **21**, 18-24.

Parajó, J.C., Domínguez, H. & Domínguez, J.M. (1998). Biotechnological production of xylitol. Part2: Operation in culture media made with commercial sugars. *Bioresource Technol.* **65**, 203-212.

Pepper, T. & Olinger, P.M. (1988). Xylitol in sugar-free confections. *Food Technol.*, **42,** 98-106.

Perego, P., Converti, A., Palazzi, E., Del Borghi, M. & Ferraiolo, G. (1990). Fermentation of hardwood hemicellulose hydrolysate by *Pachysolen tannophilus*, *Candida shehatae* and *Pichia stipitis*. *J. Ind. Microbiol.* **6,** 157-164.

Roberto, I.C., Lacis, L.S., Barbosa, M.F.S., & de Mancilha, I.M. (1991). Utilizaton of sugar cane bagasse hemicellulosic hydrolysate by *Pichia stipitis* for the production of ethanol. *Process Biochem.*, **26,** 15-21.

Strickland, R.C. & Beck, M.J. (1984). Effective pretreatments and neutralization methods for ethanol production from acid-catalyzed hardwood hydrolysates using *Pachysolen tannophilus*. *Proc. VI Int. Symp. Alcohol Fuels Technology*, **2,** 220-226.

Tran, A.V. & Chambers, R.P. (1985). Red oak wood derived inhibitors in the ethanol fermentation of xylose by *Pichia stipitis* CBS 5776. *Biotechnol. Lett.* **7,** 841-846.

Watson, N.E., Prior, B.A. & Lategan, P. M. (1984). Factors in acid treated bagasse inhibiting ethanol production from D-xylose by *Pachysolen tannophilus*. *Enzyme Microb. Technol.* **6,** 451-456.

Effect of variables on the kinetics of oxidative ammonolysis of technical lignins

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ABSTRACT

The effect of temperature, oxygen pressures and ammonia hydroxide concentration on the rate of oxidative ammonolysis of organosolv (Repap) lignins was studied. The kinetic experiments were conducted in 0.5 L Parr's reactor in ammonium hydroxide solutions of 0.4 – 1.6 M at the reaction temperature of 70 – 130°C under oxygen pressure of 0.5 – 1.2 MPa. The reaction mechanism of nitrogen incorporation does not change during entire course of oxidative ammonolysis. Most of nitrogen is incorporated into the lignin via cleavage of aromatic rings. The kinetics of nitrogen incorporation has two phases due to different reactivity of phenolic and non-phenolic lignin moieties and follows a pseudo-first order rate law in the both phases. The activation energy has been found to be rather low, about 35-40 kJ/mole. The rate of nitrogen incorporation is directly proportional to oxygen pressure indicating the reaction is first order with respect to oxygen concentration. The reaction order with respect to ammonium hydroxide concentration is 0.5 suggesting possible participation of NH_4OH in reactions inhibiting nitrogen incorporation. The reactivity of technical lignins of different origin (hardwood organosolv lignin, wheat straw organosolv and soda lignins, *Eucalyptus globulus*, *Eucalyptus grandis* and pine kraft lignins) is proportional to S/G ratio of C_9-units. Linear relationships between nitrogen incorporation and oxygen uptake and incorporation, CO_2 formation, decrease in methoxyl group content and carbon loss were found. Except for CO_2 formation, these relationships do not depend on the reaction conditions in the range studied. The mechanism of nitrogen incorporation is generally the same under the conditions studied. However, minor changes in the relation between different reaction pathways are possible.

Keywords: ammonolysis, lignin, lignin reactivity

INTRODUCTION

Oxidative ammonolysis is a promising method for utilization of technical lignins. Oxidation of lignin with molecular oxygen in ammonium hydroxide solution results in nitrogen incorporation into the lignin structure. These *N*-modified lignins can be used as effective, slow releasing fertilizers. However, industrial application of this process requires better understanding of the underlying reaction mechanism. Kinetics studies allow not only optimizing the reaction conditions, but also provide important information on the reaction mechanism. The aim of the present work was to study the effect of variables on the kinetics and the reaction mechanism of oxidative ammonolysis.

EXPERIMENTAL

Oxidative ammonolysis was conducted in a 300 mL Parr reactor. 1g of lignin and 50 ml of NH_4OH solution were placed to the reactor. Oxygen was supply to the reactor at room temperature. Time to temperature was 15 mins. After the reaction, the reactor was cooled in icy water to room temperature. The reaction mixture was filtered, and the precipitate was washed with distilled water. A part of the solution was analyzed for CO_2. The remaining solution of soluble lignin was freeze-dried. The soluble and insoluble lignins then were dried in a vacuum oven at 45-50°C to constant mass and weighted.

Oxygen uptake and CO_2 formation were determined by simplified procedure of Deineko and Balin (1981) described earlier (Capanema *et al.*, 1999).

RESULTS AND DISCUSSION

The original Repap lignin is only partially soluble in the ammonium hydroxide solution. However, the solubility of the lignin increases with the reaction time. The increase in the solubility of lignin indicates that

oxidative degradation of the lignin results in molecular mass and/or introduction of hydrophilic functional groups into the lignin moieties. The kinetics of lignin solubilization follows a pseudo-first order reaction law.

Nitrogen is incorporated in both soluble and insoluble lignin fractions. To estimate the process of nitrogen incorporation in general, so-called "average nitrogen content" was calculated as follows:

$$N_a = (N_s L_s + N_i L_i)/(L_s + L_i), \qquad (1)$$

where N_a is average nitrogen content, N_s and N_i are the nitrogen content in soluble and insoluble *N*-modified lignin fractions, respectively, and L_s and L_i are the amounts of soluble and insoluble lignins, respectively.

The maximum percentage of incorporated nitrogen (N_∞) was estimated from the experiments with the reaction time of 24 hours. The kinetics of nitrogen incorporation has two phases and follows a pseudo-first order reaction law in both phases. Kinetic parameters (Table 1) show that the reaction rate in the second phase is 2.3-4 times slower than that in the first phase. The concentration of incorporated nitrogen corresponded to the end of the first phase is different at different reaction temperature, and the reaction time corresponding to the transitional points were different at different reaction conditions. However, all these transitional points correspond to about 60% of maximum value for nitrogen incorporation (N_∞) at a particular reaction condition. This indicates that the transitional point does not depend on the reaction condition, but is determined by the structure of the organosolv lignin. Functions derived from kinetic parameters given in Table 1 show good correlation with the experimental points (Figure1 and 4).

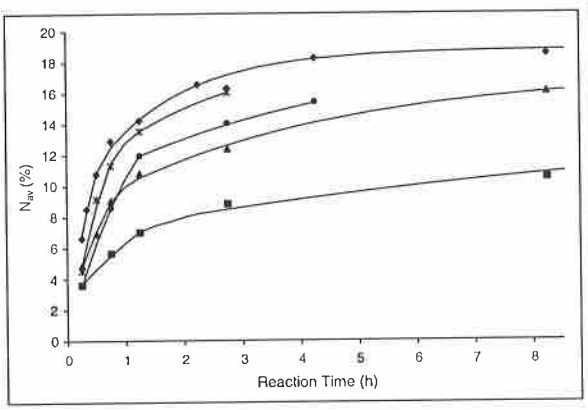


Figure1. Effect of the reaction temperature (at 12 bar) and oxygen pressure (at 130°C) on the nitrogen incorporation. (■) 70°C, (▲) 100°C, (♦) 130°C, (●) 5 bar, (*) 8 bar.

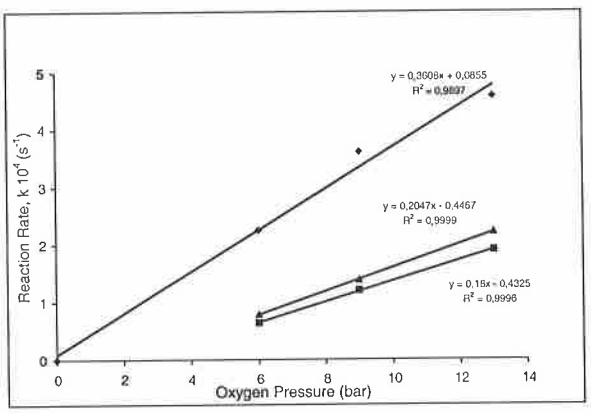


Figure 2. Effect of oxygen pressure on the rate of nitrogen incorporation in the first (♦) and second (■) phases, and lignin solubilization (▲).

Table 1. Kinetic Parameters of Oxidative Ammonolysis of Organosolv Lignin.

Variable	Nitrogen incorporation (N_a)					Lignin dissol. k_S 10^4 s^{-1}	CO_2/N_{inc} Mole/mole
	N_∞ %	1st phase		2nd phase			
		k_N 10^4 s^{-1}	v 10^3 s^{-1}	k_N 10^4 s^{-1}	v 10^3 s^{-1}		
70°C[1]	14.4	1.05	1.51	0.25	0.36	0.23	0.75
100°C[1]	17.2	2.34	4.02	0.70	1.21	0.78	0.92
130°C[1]	18.8	4.58	8.62	1.90	3.57	2.22	1.40
8 bar[2]	18.8	3.60	6.76	1.20	2.26	1.39	1.10
5 bar[2]	18.5	2.24	4.21	0.64	1.20	0.79	1.05
0.4 N NH_4OH[3]	14.5	1.64	2.38	0.61	0.88	0.39	0.91
0.8 N NH_4OH[3]	16.1	2.22	3.58	0.73	1.18	0.48	0.99
1.6 N NH_4OH[3]	17.8	2.65	4.69	1.01	1.80	0.68	0.72

[1] 12 bar, 0.8 N NH_4OH, [2] 130°C, 0.8 N NH_4OH, [3] 100°C, 8 bar

Effect of Oxygen Pressure on the Reaction Rate

Oxygen pressure does not affect maximum content of nitrogen in *N*-lignins. An increase in oxygen pressure increases the rate of nitrogen incorporation (Table 1, Figure 1). The reaction constants in the first phase correlate linearly with oxygen pressure (Figure2) with equation:

$$k_N = k' P_{abs}, \quad k' = f([Lig], [NH_4OH]) = 3.61x10^{-5}, \quad P_{abs} = P + 1,$$

where P_{abs} is absolute oxygen pressure.

Linear correlation between the reaction constant and oxygen pressure indicates that the reaction is first order with respect to oxygen concentration. The line correlates with a very low reaction rate in the absence of oxygen. The reaction constants in the second phase also linearly correlate with oxygen pressure according to the following equation:

$$k_N = (1.80\ P_{abs} - 4.325)\ x10^{-5}$$

In contrast to the first phase, this equation (or extrapolation of the experimental line to k=0) (Figure2) shows that incorporation of nitrogen in the second phase requires oxygen pressure higher than 2 bar.

The increase in oxygen pressure also increases the rate of lignin solubilization (Table 1), indicating higher rate of lignin degradation at higher oxygen pressure. The reaction constants (Table 1) show that the increase in the oxygen pressure from 5 to 8 and 12 bar increases the reaction rate about 1.75 and 2.81 times, respectively. The dependence of the reaction rate of lignin solubilization on the oxygen pressure is also linear and rather close to the corresponding effect of oxygen pressure on the rate of nitrogen incorporation in the second phase of the reaction (Figure2).

Effect of Temperature on the Reaction Rate

An increase in the reaction temperature results in increases in the rate of nitrogen incorporation (Figure 1) and the maximum amount of nitrogen incorporated into lignin (N_∞) (Table 1). Since the maximum amount of nitrogen incorporated is different at the different temperatures, the effect of the reaction temperature on the rate of nitrogen incorporation was estimated by reaction rate (v) that is calculated as followed:

$$v = k_N N_\infty \qquad (2)$$

There is good Arrhenius correlation for the dependence of the reaction rate on the reaction temperature (Figure 3). The activation energy calculated from the slope of these lines was 34 and 43 kJ/mole for the first and second reaction phase, respectively. The rather low values of the activation energy could be a result of competitive reactions. The second stage of the reaction is more sensitive to the reaction temperature resulting in about 1.3 times higher value of the E_a than that for the first phase.

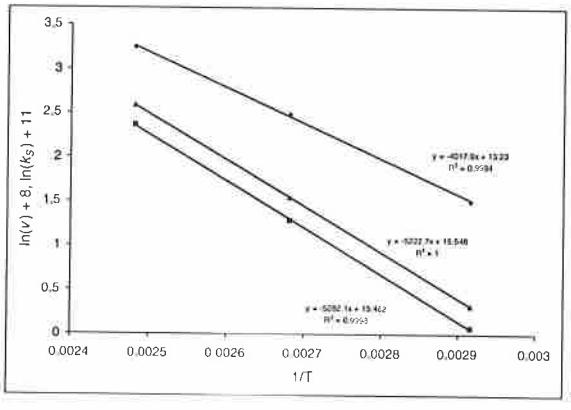


Figure 3. Arrhenius' plot for nitrogen incorporation in the first (♦) and second (■) reaction phase and for lignin dissolution (▲).

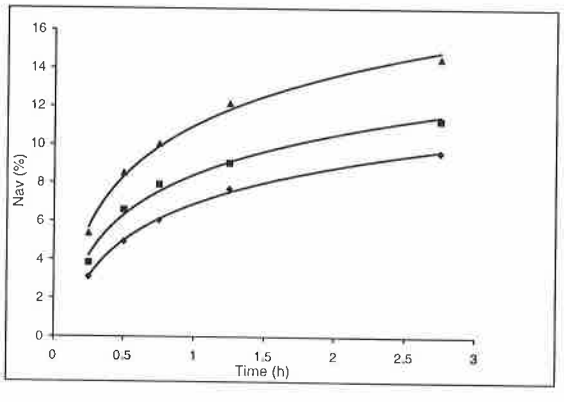


Figure 4. Effect of ammonium hydroxide concentration on the rate of nitrogen incorporation. (♦) 0.4 N, (■) 0.8 N, (▲) 1.6 N.

The reaction rate constants for lignin solubilization indicate that the reaction rate increases approximately 3 times with a 30°C increase in the reaction temperature. Similarly, the value of the activation energy, calculated from Figure 3 is also low, 40 kJ/mole. The activation energy for lignin dissolution is very close to that for nitrogen incorporation in the second phase of the reaction. This could indicate similarity in the rate-determining step for those processes.

Effect of ammonium hydroxide concentration

The maximum content of nitrogen in *N*-lignin increases from 14.3% to 17.8% with increase in ammonium hydroxide concentration from 0.4M to 1.6M. Increasing the ammonium hydroxide concentration 4 times, from 0.4 to 1.6 M results in about two-fold increases in the reaction rate in both phases (Table 1, Figure 4). A plot log (v) versus log ([NH_4OH]) (Figure 5) shows a linear correlation with the slope of about 0.5, corresponding to the reaction order with respect to ammonium hydroxide concentration.

The ammonium hydroxide concentration increases the rate of lignin solubilization. This effect is especially strong on the solubility of the original lignin. At the reaction time of 15 minutes, i.e. just after reaching the reaction temperature, 60% of lignin was insoluble in 0.4 M NH_4OH, 50% in 0.8 M NH_4OH and only 32% in 1.6 M NH_4OH. The higher solubility of lignin in more concentrated ammonium hydroxide solution implies that the solubility of lignin increases with increasing pH value. During the course of oxidative ammonolysis, the effect of the ammonium hydroxide concentration on the rate of lignin solubilization is not large. The reaction rate estimated through a pseudo-first order rate constant (Table 1) increases less than 2 times with 4 fold increase in the ammonium hydroxide concentration. The plot log (v) versus log ([NH_4OH]) (Figure 5) shows that the experimental value for the reaction order is 0.4 with respect to ammonium hydroxide concentration.

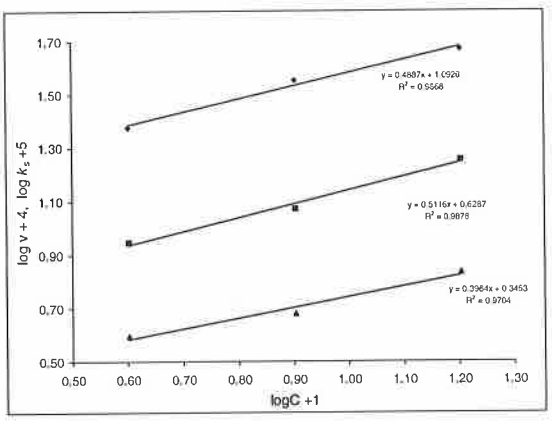


Figure 5. Plot log [NH_4OH] versus log (v) for nitrogen incorporation on the first (■) and second (▲) phases and lignin solubilization (♦).

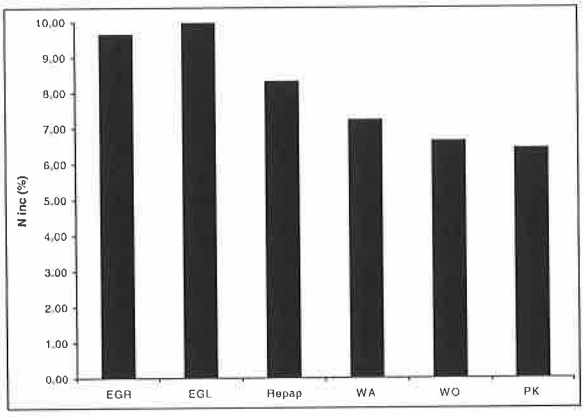


Figure 6. Reactivity of lignins of different origins. Reaction Conditions: 100°C, 12bar, 0.8M NH_4OH.

Correlation of nitrogen incorporation with others parameters of oxidative ammonolysis

It was of interest to find a correlation between the percentage of nitrogen incorporated (N_{inc}) and other parameters in the oxidative ammonolysis of lignin, oxygen uptake, CO_2 formation and changes in the lignin structure (oxygen incorporated, carbon loss, OMe content). Almost in all reaction conditions investigated, these correlations are linear. In addition, correlation between nitrogen incorporation and oxygen uptake, oxygen incorporation, MeO group content in soluble and insoluble lignins, and total carbon loss do not depend on the reaction conditions.

About 3 moles of oxygen are consumed and about 0.6 moles of oxygen (20% of uptake) are incorporated per incorporation of mole of nitrogen. In fact, incorporation of oxygen is higher, because of about 0.3 moles of oxygen per mole of nitrogen incorporated is eliminated with OMe groups. The relation between N and OMe percentage does not depend on the reaction conditions, but it is different for soluble and insoluble lignin. For soluble *N*-modified lignin, approximately 1.42 moles of nitrogen are incorporated into the lignin per mole of OMe lost, whereas for the insoluble *N*-modified lignin, this relationship is 0.73 mole/mole, which is two-fold lower. The appreciable amount of oxygen incorporated into lignin and the correlation N_{inc}- OMe content are in agreement with structural studies of the corresponding lignins (Capanema *et al.*, 1999) and literature data

(Potthast *et al.*, 1996). Accordingly, nitrogen is incorporated into lignin mainly in form of ammonium salt, amides and urea type structures but not amine or heterocyclic.

The nitrogen incorporation is accomplished with loss of carbon of about 1.1 atoms per atom of incorporated nitrogen. Elimination of carbon occurs in forms of CO_2 and other volatile product like formic and acetic acids, methanol and others. The reaction conditions do not noticeably affect the amount of total carbon loss per mole of nitrogen incorporated, but affect the relation between the volatile products. The reaction temperature has the strongest effect on the relation N_{inc} - CO_2. The amount of CO_2 formed increases from 0.75 moles per mole of nitrogen incorporated to 1.40 mole/mole at 70°C and 130°C, respectively (Table 1). The effect of the reaction temperature on the CO_2/N_{inc} relationship could be explained by stronger decarboxylation at higher reaction temperatures. Increase in oxygen pressure from 5 to 8 bar almost does not change the relation between nitrogen incorporated and CO_2 formed. However, at 12 bar more CO_2 is formed per mole of nitrogen incorporated. The relationship CO_2 formed – nitrogen incorporated shows that the values for oxidative ammonolysis of lignin in 0.4 M and 0.8 M NH_4OH solutions are rather close (Table 1). Increase of ammonium hydroxide concentration to 1.6M results in decrease of CO_2/N ratio to 0.72 mole/mole.

Reactivity of technical lignins of different origins

Technical lignins from different plant species and different pulping process have differences in structure. Therefore, comparing the reactivity of different substrates one can get useful information on the mechanism of the oxidative ammonolysis of lignins, particularly on the role of different lignin moieties. The reactivity of different lignins estimated by the rate of nitrogen incorporation could be presented as followed (Figure 6):

Eucalyptus kraft > Repap > Wheat > Pine kraft

The reactivity of *Eucalyptus globulus* (EGL) is slightly higher than that of *Eucalyptus grandis* (EGR), and the reactivity of wheat straw alkaline lignin (WA) is slightly higher than that of wheat straw organosolv (WO). It is obvious that the reactivity of hardwood lignins toward nitrogen uptake is appreciably higher than that of softwood and wheat straw. This indicates higher reactivity of syringyl moieties compared to guaiacyl ones. The higher reactivity of *Eucalyptus* lignin than the reactivity of another hardwood lignin, Repap, correlates with higher S/G ratio. It was reported that *Eucalyptus* MWL contains about 50% of syringyl moieties (Veloso *et al.*, 1991), whereas content of syringyl units in Repap lignin is only 25-35% (Faix *et al.*, 1994). Wheat straw lignin also contains appreciable amount of syringyl units. But at the same time it contains p-hydroxyphenyl units with rather low reactivity as compared to G and S types. This could result in close reaction rate for pine (PK) and wheat straw lignins.

Further kinetic investigations on the oxidative ammonolyis (100^0C, 8 bar, 0.8M NH_4OH) of EGL kraft and pine kraft lignins were undertaken to compare with that of Repap lignin. It is noteworthy that the maximum content of nitrogen in the final product does not depend on the origin of lignin and is about 16%. However, the rates of nitrogen incorporated in the first reaction phase estimated via pseudo-first order reaction constants are quite different. The rate of nitrogen incorporation in EGL kraft lignin ($k = 2.92 \times 10^{-4}$ s^{-1}) was two-fold higher than that for pine kraft lignin ($k = 1.39 \times 10^{-4}$ s^{-1}), and about 30% higher than for organosolv lignin ($k = 2.22 \times 10^{-4}$ s^{-1}). Considering G/S ratio for EGL and Repap lignin, the reactivity of S-units has been roughly estimated to be three-fold higher than that of G-units.

General Discussion

The occurrence of the two phases in the kinetics of nitrogen incorporation implies changes in the reaction pathway and/or in the rate-determining step. Linear correlation between nitrogen incorporation and oxygen uptake, oxygen incorporation, CO_2 formation and *O*-demethylation through the whole reaction period indicates that the reaction proceeds via the same reaction pathway in the different kinetic phases. Our NMR (Capanema *et al.*, 1999) and FTIR (Capanema, 1999) studies did not show qualitative differences between spectra obtained at different reaction time. The differences appear only in the intensities of signals, confirming the postulation that the reaction pathway does not change during course of reaction. Thus, the different kinetic phases are likely associated with the change in the rate-determining step, not the changes in the reaction pathway.

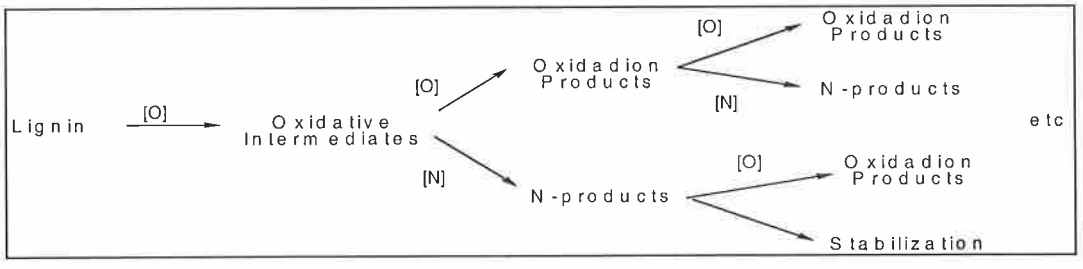
The correlation between the amount of nitrogen incorporated into lignin with *O*-demethylation of the lignin, high oxygen uptake and CO_2 formation suggests that incorporation of nitrogen in lignin occur predominantly

after oxidative cleavage of aromatic ring. This is in agreement with strong degradation of the aromatic ring observed by spectroscopic studies of the corresponding lignins (Capanema *et al.*, 1998).

The first phase of the oxidative ammonolysis is likely associated with the reactions of phenolic moieties of the original lignin, because their reactivity is much higher than etherified moieties (Kratzl *et al*, 1974, Gierer *et al.*, 1977). About 60% of maximum nitrogen uptake, incorporated in the first reaction phase, correlates with amount of phenolic hydroxyl groups (70%) in Repap lignin reported by Faix (Faix *et al.*, 1994).

The second phase of the reaction is likely associated with original non-phenolic moieties. This is in agreement with low OMe group content at the end of the reaction, which shows that demethylation occurs not only in original phenolic moieties. The original non-phenolic moieties could participate in the oxidative ammonolysis by transformation to phenolic moieties via oxidative cleavage of side chain and cleavage of alkyl-O-aryl bonds (see for example Gierer *et al.*, 1977). The reactions of side chain cleavage do not directly reflect on the nitrogen incorporation, but they could be associated with lignin solubility due to decrease in the molecular mass of the polymer. Thus, they could be monitored by kinetics of lignin solubilization. In contrast to the process of nitrogen incorporation, the kinetics of lignin solubilization has only one phase in the whole reaction. The similarities in the kinetics of lignin solubilization and nitrogen incorporation in the second reaction phase (effect of temperature and oxygen pressure) imply possibility of common rate-determining step for these processes. It could be cleavage of side chains in the lignin, which will determine the rate of lignin solubilization and the oxidation rate of newly formed phenolic moieties, and the corresponding rate of nitrogen incorporation in the second phase.

Possible mechanism for oxidative ammonolysis of lignins consists of complex net of consecutive and competitive reaction (Nekrasov *et al.*, 1980, Deineko and Balin, 1981). In general, we can present the reaction scheme could be presented as followed:



The kinetic data show that the rate of nitrogen incorporation is directly proportional to oxygen pressure (concentration) in the range of 5-12 bars. This indicates that a) oxygen directly participates in the rate-determining step and b) reactions of oxygen with the substrate do not inhibit nitrogen incorporation. The fractional reaction order with respect to ammonium hydroxide concentration could be due to participation of ammonium hydroxide in reactions inhibiting the oxidative ammonolysis of lignin and further nitrogen incorporation. It could be condensation reactions (Nekrasov *et al.*, 1980) or/and formation of *N*-containing products with lower rate of oxidation. Relatively low effective activation energy could be a result of this competition.

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REFERENCES

Capanema, E. C. (1999). Utilização dos Sub-produtos Provenientes dos Processos de Polpação e Carvoejamento. Belo Horizonte, UFMG. PhD Thesis. Departamento de Química-ICEX, Universidade Federal de Minas Gerais.

Capanema, E. C.; Chen, C-L.; Gratzl, J. S.& Kirkman, A. G. (1998). Study on the mechanism of oxidative ammonolysis of technical lignin. *In 5th European Workshop on Lignocellulosics and Pulp*, Proceedings, pp 397-400.

Capanema, E. A.; Balakshin, M.; Chen, C-L.; Gracz, H. S.; Gratzl, J. S. & Kirkmam (1999). Effect of temperature on the rate of oxidative ammonolysis and structure of *N*-modified lignins. *In 10th Int. Symp. On Wood and Pulp. Chem.*, Proceedings, **Vol. III**, pp. 404 – 409.

Deineko, I.P. & Balin N.B. (1981). On the role of hydroxylation reaction in oxyammonolysis of lignin. *Khimiya Drevesiny*, **N.5**, 53-59.

Faix, O.; Argyropoulos, D. S.; Robert, D. & Neirinck, V. (1994). Determination of hydroxyl Groups in Lignins Evaluation of ^{1}H, ^{13}C, ^{31}P NMR, FTIR and Wet chemical Methods. *Holzforschung*, **48**, 387-394.

Gierer, J., Imsgard, F. & Noren, I. (1977). Studies on the Degradation of Phenolic Lignin Units of the □Aryl Ether Type with Oxygen in Alkaline Media. *Acta Chem. Scand.*, **B 31 (7)**, 561-572.

Kratzl, K., Claus, P., Lonsky, W. & Gratzl, J. (1974). Model Studies on Reactions Occurring in Oxidation of Lignin with Molecular Oxygen in Alkaline Media. *Wood Science and Technology*, **8 (1)**, 35-49.

Nekrasov, S.V., Ioffe, L.O., Zakharov, V.I. & Zelikman, P.I. (1980). On the Mechanism of Formation of Strongly Bonded Nitrogen during Delignification by Oxidative Ammonolysis. Part 1. Formation of Aromatic Amino Groups. *Khimiya Drevesiny*, **N.2**, 48-53.

Potthast, A. Scheine, & Fischer, K. (1996). Structural investigation on *N*-modified lignins by ^{15}N-NMR spectroscopy and possible pathways for formation of nitrogen containing compounds related to lignin. *Holzforschung*, **50**, 554-562.

Veloso, D. P., Morais, S. A. L. & Nascimento, E. A. (1991). Nitrobezene Oxidation of both *Eucalyptus grandis* and Milled Wood Lignin. *In 1st European Workshop on Lignocellulosics and Pulp*, Proceedings, pp. 358-366.

Enzymatic delignification and bleaching with "green" components

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ABSTRACT

A new enzymatic bleaching system is presented which delignify and bleach all tested kinds of pulps, like e.g. softwood and hardwood sulfate or sulfite pulps. The main active components of this new system are lipases, peroxides, fatty acids and ketones, the latter are used e.g. as food or cosmetic additives. With aid of this new system delignification rates of more than 40% can be reached in one-step and short retention times. The system could be, due to the low cost and low toxic potential of its components, an attractive alternative to conventional chemical bleaching steps. Additionally it can be shown that also the strength properties of the treated pulps are comparable with untreated samples. Some detailed results referring the possible mechanisms and improvement strategies will be summarized.

Keywords: bleaching, cellulose pulp, lipases

INTRODUCTION

Since the discovery of the lignolytic enzymes in 1983 (Lignin peroxidases (LiP) and later Manganese peroxidases (MnP)) a lot of papers were published which deal with lignin degradation and bleaching of wood pulp.

Nevertheless both possible ways to degrade and remove lignin from wood fibers 1) with aid of lignolytic microorganisms (Paice, 1990; Fujita, 1993) or 2) with isolated (single) enzymatic catalyst systems were not successful so far.

The main reason for the lack of technically feasible fungal systems (mainly white rot fungi) for lignin removal e.g. for biobleaching in spite of considerable delignification (kappa reduction of more than 50% are reachable) is the long treatment time of several days or weeks, in order to reach these mentioned lignin degradation rates which does not fit into common bleaching processes.

With aid of isolated lignolytic enzymes like LiP, MnP or laccases and with isolated lignin as substrate a significant degradation could only be achieved under special circumstances e.g. low lignin contents or special solvent systems while with pulp as substrate with exception of Manganese peroxidase a polymerization of the lignin occurs rather than a depolymerization or degradation and bleaching effect.

Beside the relative weak performance, the critical concentration of the necessary H_2O_2 and the availability and costs of the enzyme are the main drawback of these Manganese peroxidase systems (Paice, 1997).

Therefore since several years the concept of a mediated enzymatic process was discussed keeping in account the fact that all known lignolytic enzymes seem to be to large for penetrating into the fiber walls.

In the meantime a lot of papers and patents were published which favoured this idea (Call, 1986; Bourbonnais, 1990; Call, 1991).

0wn first and second generation delignification and/or bleaching systems were based on the peroxidases from *Phanerochaete chrysosporium* and phenolic and nonphenolic redox mediators or in modified systems a combination of these enzymes with a redox cascade often in the presence of chelating agents were the delignifying agent (Call, 1986; Call, 1990; Call, 1991).

Due to the fact that these mentioned systems need for their optimal performance a low consistency (1-2%) of the pulp a technical realization was difficult.

With the finding (in 1993) of a new class of potent mediator substances (NO-, NOH-compounds) in combination with laccases for degradation and/or bleaching of lignolytic material mainly pulp called Ligozym^R - process, Laccase-Mediator-System [(LMS^R)-system] a commercialization of a biotechnological bleaching system could be realizable (Call, 1993; Call, 1994; Call, 1994a; Call, 1997).

In the meantime further groups of this very potent class of compounds were claimed by several patent applications (Call, 1996; Call, 1996a; Stohrer, 1996; Call, 1997a).

The driving force for the discovery of the presented new delignification system was to look for mimicking a known chemical pulping and/or bleaching system by an enzymatic way.

The solution was the finding that it seemed to be possible to generate activated oxygen, possibly e.g. dioxirane, which is a well known, selective and mild bleaching agent described in a lot of patents and papers (Lee, 1992; Murray, 1989; Adam, 1989; Ragauskas, 1993; Lee, 1993).

Though the preferred pathways of Baeyer-Villiger reactions (mainly also for enzymatically mediated reactions) - e.g., oxidation of cyclohexanone to ε-caprolacton- are the generation of a "Criegee" intermediate and do not proceed via dioxirane formation (dioxiranes are only formed with peroxides derived from very strong acids like Caro's acid etc. and enzymatic reactions do not appear to play any part in this kind of reactions (Adam, 1993)) it is not unlikely that dioxiranes are involved in the presented enzymatic delignification reactions.

The presented new enzymatic delignification and/or bleaching system contains a combination of hydrolases, especially lipases, special carboxylic acids, e.g. fatty acids, special ketones and peroxides.

The main advantages of this new delignification and/or bleaching concept are the very low dosage level of the system components, the very low toxic potential of the used fatty acids and ketones which are normally employed as food additives, the low-cost and established enzymes and the very good performance.

The possibility to reach delignification rates of more than 40% (with most of the tested pulps) within less than 2 h makes this system very attractive and competitive concerning the environmental and economic demands and requirements of the pulp industry also in respect to revive the TCF bleaching.

EXPERIMENTAL

Chemicals/enzymes

All chemicals were purchased from Aldrich, Sigma, Fluka or Merck and enzymes from Sigma or from Amano.

The pulps were obtained from Scandinavian or North American mills. Kappa Number and viscosity of the respective pulps were determined according TAPPI or SCAN methods.

Delignification experiments

The standard conditions were as follow:

Pulp (10% to 12.5% consistency) was treated in a vessel (without pressure) at 45°C, pH 4 (adjusted with sulfuric acid, acetic acid, sodium acetate, NaOH or sodium carbonate) for 1 to 4 h.

The system components like a) lipase (activity measurements were performed according different methods which are described in literature (Stellmach, 1988)), in the range of 5-20 g pure protein per ton of pulp, b) fatty acids and c) ketones in the range of 10 g to 1 kg per ton of pulp and d) H_2O_2 in the range of 100 g to 3 kg per ton of pulp were applied to the pulp. After pH adjustment a consistency range of 10 to 12.5% was adjusted.

After the enzyme-system treatment and washing with water an alkaline E-stage at 2% consistency with 8% NaOH was carried out for 1 h and at 70°C.

After an additional washing step handsheets were made for determination of the ISO brightness in %.

RESULTS AND DISCUSSION

It is certainly a fact that the commercialization of the LMS^R-system seems to be further proising.

Nevertheless the presented new delignification and/or bleaching concept may have some additional advantages because it combines well known enzymes like lipases, ketones, fatty acids as environmentally save compounds due to their use as food and/or cosmetic additives and peroxides which are also established in the pulp and paper industry.

As above mentioned the dosages of both chemicals and enzymes are rather low which is a strong economical benefit of this system.

The normal treatment conditions of this enzymatically produced oxidation catalyst system: EPOC-system are as follows:

Pulp consistency of 10 to 12.5%
pH-range depending on enzyme from 3 to 9*
temperatures range of 20 to nearly 80°C*
retention time of 30 minutes to 4 h.
(no O_2 - pressure is necessary)

(* not all datas are shown)

The respective results which were obtained referring consistency, pH-range, temperature-range, retention time were already published elsewhere (Call, 1999).

One can resume that for the momentary favorite lipase from *Aspergillus* an acid pH-range from pH 3 to 5, a temperature range from 40 to 50°C, a pulp consistency from 10 to 12.5% and a retention time from I to 2 h show the best delignification rates.

Table 1 shows the influence of selected lipases from different fungal or bacterial sources on the system. The lipase of *Aspergillus* shows the best performance at pH 4 while the lipases from *Rhizopus delamar* and *Pseudomonas sp* demonstrate the highest delignification activity at ca. pH 7 or higher (datas not shown).

Table 1. Influence of selected Lipases on the new delignification system. (Scandinavian softwood pulp)*. Reaction conditions: 4h; 45°C; 10% consistency (without pressure); Lipase: different sources; Ketone: aliphatic No. 5; H_2O_2 (600g/to pulp); Fatty acid: C16-Na-salt; * Pulp: oxygen pretreated.

Lipase type	Delignifcation (%)	
	PH4	pH7
Humicola lumigiosa	25.9	15.5
Aspergillus niger	42.7	26.0
Candida lipolytica	19.1	15.5
Rhizopus oryzae	16.5	17.2
Pseudomonas spec.	19.9	31.6
Geotrichum candidum	13.7	19.3
Rhizopus delamar	19.6	40.1
Candida antarctica	23.6	23.6
Chromobacterium viscosum	25.9	19.8
Rhizomucor miehei	19.6	40.1
Hog prancreas	29.3	20.0
Candida rugosa	21.6	19.4

Table 2 demonstrates the influence of the system components on the system.

It is known that lipases can under certain circumstances enzymatically attack some special wood compounds like fatty acid containing compounds (pitch control etc.).

It is also an hypothesis that lipases could facilitate and improve subsequent bleaching steps by increasing the chemical bleaching performance and lignin removal rate (like xylanases or other hemicellulases) through their, ability to increase the accessibility of the residual lignin structures (Vaheri, 1991).

In order to prove if the presented system is an oxidizing system or if the measurable effects are caused by hydrolytic activities the influence of the different system components on the system performance was tested.

The results in table 2 clearly indicate that the addition of all components is necessary for reaching the highest performance. The results with (only) purified enzyme (without any hydrolytic side activities like xylanases or cellulases) but also the result: crude enzyme (only traces of xylanase, no cellulase activity) plus (only) H_2O_2 demonstrate that there is no hydrolytic activity responsible for the main delignification effect.

Additionally some dye compounds like e.g. Azure B dye, Remazole Brilliant Blue, Poly R-478 and Rhodamine B, etc. (Archibald, 1992; Palma, 1998; Bourbonnais, 1990) - which are known that they can be oxidized by e.g. LiP, MnP, laccases or other chemical oxidants - were used in special photometric tests in

combination with the new system in order to demonstrate the oxidation properties of the system. It could be shown that most of the treated dye compounds cold be changed (datas not shown).

Table 2. Influence of different system compounds on the new delignification system. (Scandinavian softwood pulp/Kappa 16.6 untreated)*. Reaction condition: 4h; pH 4; 45ºC; 10% consistency (without pressure); Lipase: *Aspergillus* (1x=20g/to pulp; 10x=200g/to pulp); Ketone: aliphatic No. 5; H_2O_2 (~600g/to pulp); * Pulp: oxygen pretreated; ** Fatty acid: C16-Na-salt.

Treatment conditions	Delignification (%)
pulp untreated (K 16,6)	0
pulp untreated 4h, pH 4.5, 45ºC	4.5
pulp + Ketone 5; fatty acid**; H_2O_2; without enzyme	7.5
only lipase (crude) (lx)	12
only lipase (crude) (l0x)	19.2
lipase (crude) (lx) + H_2O_2	14.7
lipase (crude) + Ketone 5 + H_2O_2 without fatty acid**	12.6
lipase (crude) (lx) + fatty acid** + H_2O_2; without Ketone 5	14.2
lipase (crude) (lx) + Ketone 5 + fatty acid + H_2O_2 = complete system**	**41.3**
only lipase (purified) (lx)	8.1
lipase (purified) (lx) + Ketone 5 + fatty acid + H_2O_2 = complete system**	**30.7**

From the results in Table 3 one can see that the ketone compound is essential for the performance of the system. Similar results are obtained with different fatty acid compounds (datas here not shown) though this influence is less pronounced.

Table 3. Influence of selected Ketone compounds on the new delignification system. (Scandinavian softwood pulp)*. Reaction conditions: 4h; pH 4; 45ºC; 10% consistency (without pressure); Lipase: *Aspergillus;* Ketone: different compounds; H_2O_2 (~600g/to pulp); *Pulp: oxygen pretreated; Fatty acid: C 16-Na-salt.

Ketones (type)	Delignification (%)
1) aliphatic	23.6
2) aliphatic	38.4
3) cyclic	35.6
4) cyclic	37.8
5) aliphatic	40.1
6) cyclic	42.5
7) cyclic	54.7

The results in Figure 1 are a clear indication for the ability of the system to delignify different pulps like different softwoods, hardwood and sulfite softwood pulps considerably. The respective viscosity loss is rather low (in the range of 2 to 5% dependent on pulp and extraction procedure), see Table 4.

Figure 2 shows the influence of different treatment conditions on the system:

A) Chemical and enzyme charge only at the beginning of the treatment

B) Step by step (4 portion) chemical and enzyme charge (only 1/4 of the total charge in A) at time 0, 30 min, 60, min, 120 min.

The procedure B) without any intermediate washing shows a significant higher performance, which could be probably caused by the inactivating influence of the peroxide on the enzyme in experiment A.

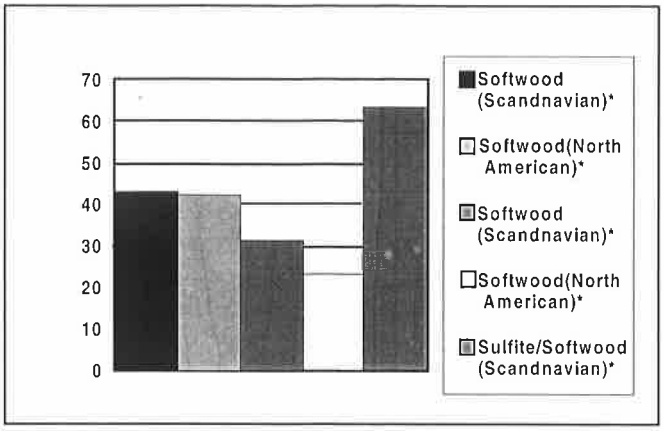

Figure 1. Influence of different pulp on the delignification system. Reaction conditions: 4h; pH 4; 45ºC; 10% consistency (without pressure); Lipase: *Aspergillus niger;* Ketone: aliphatic No. 5; H_2O_2 (~600g/to pulp); * Pulp: oxygen pretreated (diff. Sources); fatty acid: C16-Na-salt.

Table 4. Influence of the new delignification and bleaching system on the viscosity and bleaching behavior of the treated pulp (Scandinavian softwood pulp)*. Reaction conditions: 4h; pH 4; 45ºC; 10% consistency (without pressure); Lipase: *Aspergillus;* Ketone: different compounds; H_2O_2 (~600g/to pulp); *Pulp: oxygen pretreated; Fatty acid: C-16-Na-salt.

pulp treatment	viscosity (ml/g)	delignification (%)	ISO-brightness gain (%)
Untreated	880	/	/
Complete system(without extraction)	838 (-4.8%)	21.2	n.d.
Complete system (+extraction)	820 (-6.8%)	42.5	11.2

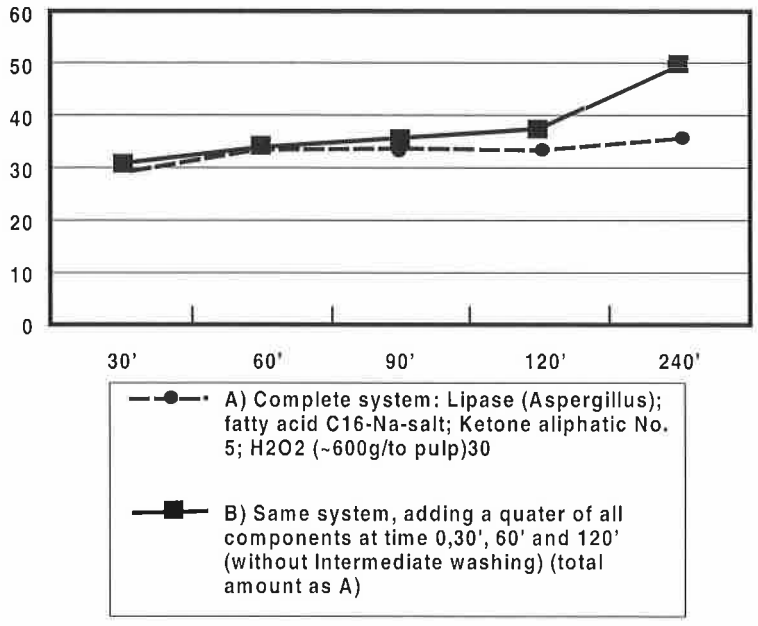

Figure 2: Influence of different treatment conditions during residence time on the new delignification system (Scandinavian softwood pulp)*. General reaction conditions: 4 h; 45ºC; 10% consistency (without pressure); * Pulp: oxygen pretreated.

CONCLUSION

The presented new enzymatic bleaching system (EPOC-system) has all properties becoming an alternative economically and environmentally to existing chemical bleaching procedure due to its main advantages summarized as follows:

competitive performance (mostly >40% delignification) with short residence time
no pressure necessary (low capital cost)

low chemical and enzyme dosages (low total system costs)
very low toxic potential --> system chemicals are used as food additives or cosmetic substances

REFERENCES

Adam, W. R., Curci, R. & Edwards, J.O. (1989). Dioxiranes: a new class of powerful oxidants. Acc. Chem. Res. **22,** 205-211.

Adam, W., & Hadjiarapoglou, L. (1993). Dioxiranes-oxidation chemistry made easy. Top. Curr. Chem., **164,** 45-62.

Archibald, F.S. (1992). Lignin peroxidase activity is not important in biological bleaching and delignification of unbleached kraft pulp by *Trametes versicolor*. Applied and Environm. Microbiol., **58 (9),** 3101-3109.

Bourbonnais, R. & Paice, M.G. (1990). Oxidation of non-phenolic substrates, an expanded role for laccase in lignin biodegradation. FEBS, **267,** 99-102.

Bourbonnais, R. & Paice, M.G. (1992). Demethylation and delignification of kraft pulp by *Trametes versicolor* laccase in the presence of 2,2-azinobis-(3-ethylbenzthiazoline-6-sulphonate). Appl. Microbiol. Biotechnol., **36,** 823-827.

Call, H.P. (1986). Process for producing cellulose from lignin containing raw materials using an enzyme or microorganism while monitoring and maintaining the redox potential. WO 88/ 03 190.

Call, H.P. (1990). Process of enzymatic bleaching of cellulose. WO 91/14823.

Call, H.P. (1991). Process for the delignification of material containing lignocellulose, bleaching, and treatment of waste water by means of laccases with extended efficiency. WO 92/20857.

Call, H.P. (1993). Process for changing, breaking down or bleaching lignin, material containing lignin or similar substances. WO 94/29510.

Call, H.P. (1994). Multicomponent system for modifying, decomposing or bleaching lignin, lignin containing materials or similar substances and method of using this system. WO 96/18770.

Call, H.P., Mücke, I. (1994a). State of the art of enzyme bleaching and disclosure of a breakthrough process. In: Proc. Int. Non-Chlorine Bleaching Conf., Amelia Island, Fl, USA.

Call, H.P., I. Mücke, I. (1997). History, overview and application of mediated lignolytic systems, especially laccase-mediator-systems (Lignozym^{R}-process). Journal of Biotechnology, **53,** 163-202.

Call, H.P. (1996). Multicomponent system for changing, reducing or bleaching lignin, lignin-containing materials or similar substances as well as processes for its application. WO 97/60041.

Call, H.P. (1996a). A Multicomponent system for changing, degrading or bleaching lignin, lignin-containing materials or similar substances and processes for its use. WO 97/60039.

Call, H.P. (1997a). Multi-component system for modifying, decomposing or bleaching lignin materials containing lignin or similar substances and methods for producing the same. WO 98/55684.

Call, H.P. (1999). Enzymatically mediated delignification and bleaching systems. In: ISWPC Proc. Yokohama, 540-545.

Fujita, K. (1993). Biobleaching of softwood kraft pulp with white-rot fungus IZU- 154. Tappi Joumal, **76 (1),** 81-84.

Lee, C-L., Kondo, R., Sakai, K., Kashina, Y., Nishida, T., Takahara, Y. (1992). Bleaching of lignocellulosic material with activated oxygen. WO 92/13993.

Lee, C-L., Hunt, K., Murray, R.W. (1993). Dimethyldioxirane as a nonchlorine agent for chemical pulp bleaching. Tappi Journal, 76(11), 137-140.

Murray, R.W. (1989). Dioxirane. Chem. Rev., **89**, 1167-1201.

Paice, M.G. (1989). Direct biological bleaching of hardwood kraft pulp with the fungus *Coriolus versicolor*. Tappi Joumal, **72,** 217-221.

Paice, M.G. (1997). Manganese peroxidase catalyzed bleaching of kraft pulp. In: Tappi Proc. Biological Science Symp., 343-345.

Palma, C., Moreira, M.T, Feijoo, G., Lema, J.M. (1998). In vitro degradation of the dye Poly R-478 by crude and semi purified *Manganese peroxidase*. In: Proc. Biotechn. in Pulp and Paper Industry, Vol. B, Vancouver, 103-105.

Ragauskas, A.J. (1993). Investigation of dimethyldioxirane as a bleaching reagent for kraft pulp. Tappi Journal, **76** (7), 87-90.

Stellmach, B. (I 988). Bestimmungsmethoden Enzyme, Steinkopf Verlag, Darmstadt.

Stohrer, J., Call, H.P., Amann, M., Freudenreich, J., Müller, R. (1996). Mehrkomponentensystem zum Verändern, Abbau oder Bleichen von Lignin, ligninhaltigen Materialien oder ähnlichen Stoffen sowie Verfahren zu seiner Anwendung. DE 19632623 Al.

Vaheri, M. (1991). A process for the bleaching of chemical pulp. WO 91/15627.

General and specific acid catalysis in buffered organosolv delignification

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ABSTRACT

The organosolv delignification processes have been studied as feasible alternatives for the treatment of lignocellulosic materials. Among these processes, the ethanol-water mixture has been particularly considered as it combines high efficiency and simple solvent recycling system. The catalysis in the organosolv processes can be performed by the acetic acid liberated from the hydrolysis of acetyl groups present in the wood tissues. In this case, the pH of the reaction mixture changes during the pulping process. The change of the pH can produce low pulp yields and the occurrence of undesirable side reactions. The utilization of buffered processes allows the optimization of the organosolv delignification processes. The occurrence of acid catalysis involving carboxylic acids can be attributed to the no dissociated acid (general catalysis) and the proton (specific catalysis). The determination of the rate constant correspondent to each type of catalysis will contribute to a better control of the delignification process. This work is usually done by using different buffer systems (each one at different pH) and different buffer concentrations. In this paper, we report on the delignification of *Eucalyptus urograndis* wood using buffered ethanol-water mixtures. The reactions were performed in temperatures ranging from 140 C to 200 C with four different buffer systems (pH range of 2.7 to 5.8) and four buffer concentrations (0.1 to 0.5 M) at each pH. The results indicate clearly the meaningful effect of the pH control, which compensates the irregular release of the acid catalyst, found in the self-catalyzed organosolv process. The pulp yields and the residual lignin contents were strongly affected by both pH and buffer concentration. The performance of the buffered organosolv delignification can be attributed to the occurrence of both general and acid catalysis.

Keywords: lignin, organosolv delignification, Eucalyptus, acid catalysis

INTRODUCTION

Chemical pulping processes degrade the polyoses and lignin in order to liberate the cellulose fibers from the lignocellulosic materials. Among the chemical pulping processes the organosolv method have been seen as an alternative to the more conventional ones. This process uses organic solvent associated with a certain water content (5-50%) and, in many cases, an acid catalyst. These processes have been studied under several different conditions comprising solvents, temperatures and catalysts. Three revisions published can give a very good overview about this process (Aziz and Sarkanen 1989; Paszner and Cho 1989; Sarkanen, 1990). Ethanol/water is the most used solvent system in organosolv delignification process. In this system the water content vary between 60 and 40%, operating at temperatures ranging from 120 to 240 °C, with or without the addition of an external catalyst (Johansson *et al.*, 1987).

In delignification process, the hydrolysis of acetyl groups of some polysaccharides produces acetic acid. This is responsible to the acid catalysis process when there is no addition of an external catalyst. The acetic acid liberation is not constant during the delignification and it is more efficient at higher temperatures. The effect of addition of an external acid catalyst is reflected on a higher delignification rate and on the reduction of the temperature needed to reach an adequate degree of delignification. On the other hand, the acid media in some cases can lead to condensation reactions between lignin fragments and/or to cellulose degradation if the experimental conditions were not strictly controlled. Thus, a detailed study of the acid catalysis effect on the delignification process could be of great importance in the control of the chemical pulping process.

The acid catalysis is classified, generally, in specific and general catalysis. When the reaction rate increases with the concentration of the protons present in the reaction media, the catalysis is of the specific type. When the reaction rate increases with the concentration of any kind of acid present in the reaction media, the catalysis is of the general type. The specific acid catalyst is most always indicative of a mechanism, which some initial reactant is rapidly and reversibly converted to its conjugate base, which undergoes slow conversion by one or more steps to the product. The general acid catalysis is most always indicative of a

mechanism involving a proton transfer in the rate determination step of the reaction. For general acid catalysis, any Bronstead acid may transfer the proton to the reactant. In buffered systems, there are at lest three acids in a buffer solution: the proton, the water and the buffer acid and there will be at least three elementary steps that contributes to the rate of reaction. (Ritchie, 1990.) The observed rate constant will be the sum of the rate constants of the elementary steps, which in principle can be determined with the proper use of different buffers.

In some delignification processes, which no external catalyst is added, the acetic acid liberated by the polysaccharides can lead to a general acid catalysis process in predominance. In process, which the external catalyst is added, the specific acid catalysis process can be more significant, together with the general catalysis promoted by the acetic acid. In these cases, the extent of the contribution of each of these types of catalysts in the delignification rate is unknown.

Although there are several papers reporting on organosolv process with or without the addition of an external acid catalyst, only few papers concerning buffered systems can be found in the literature (Delange *et al.*, 1991; Kitsos *et al.*, 1992; Faass *et al.*, 1989). Performing delignification processes using buffered solvents can lead not only to a more controlled pulping process, but also can be useful to understand the acid catalysis mechanisms that are leading to the delignification reactions.

This paper reports on the delignification processes of *Eucalyptus urograndis*, using buffered ethanol/water organosolv system. The effect of pH, buffer concentration and temperature on the values of observed rate constants are analyzed as well as the influence of a general and/or specific catalysis in the delignification process.

EXPERIMENTAL

Eucalyptus grandis and *Eucalyptus urophylla* hybrid, named *Eucalyptus urograndis*, was supplied as sawdust from Aracruz Celulose S/A. The sawdust was sifted and the fraction with 20/60 meshes was used in the delignification processes. The *Eucalyptus urograndis* presented 29.9% of lignin and 67.5% of holocellulose.

The sawdust was pre-extracted with cyclohexane and ethanol and with water. Stainless steel reactors (maximum capacity 200mL) containing 5 grams of pre-extracted material and 50 mL of solvent mixture were placed in an oil bath heated 3 degrees above the set temperature. The system took approximately 3 min to equilibrate the reactors temperature. The reactors were removed from the oil bath in different periods and cooled. The cooling time did not exceeded 2 min. Ethanol/water mixture was added to the liquor and the pulp dispersed using mechanical stirring. The dispersed pulp obtained was separated from the liquor by filtration, washed with ethanol/water 1:1 (v/v), dried in an oven at 105 °C for 6 h and kept in a vacuum dessicator. The reactions were carried out in temperatures ranging from 140 to 200 °C.

The solvent mixtures used consist in mixtures 1:1 (v/v) of ethanol and an aqueous buffer solution. Four buffered acids were used: formic, acetic, chloroacetic and dichloroacetic. The standard solution of the acid was partially neutralized (50%) by standard solution of sodium hydroxide. All solutions were prepared with water free of carbon dioxide. The final pH was measured with a glass electrode modV620 and an Analion mod PM608 pHmeter. The buffer solutions were mixed with ethanol and the apparent pH was measured. The final acid concentration ranged from 0.1 to 0.5 M, but only the minimum and maximum concentrations will be reported here.

The residual lignin content was determined for each sample by the Klason method. All samples were correct for the moisture content.

RESULTS AND DISCUSSION

The reactions were carried out in temperatures ranging from 140 to 200 °C and buffer concentration ranged from 0.1 to 0.5 M. Only the data obtained at 170 °C for all buffers at minimum and maximum concentration will be shown in this paper.

The pulp yields and the residual lignin yield, calculated in initial wood basis, for minimum (0.1 M) and maximum concentrations (0.5 M), for all buffer systems are shown in figures 1 to 4.

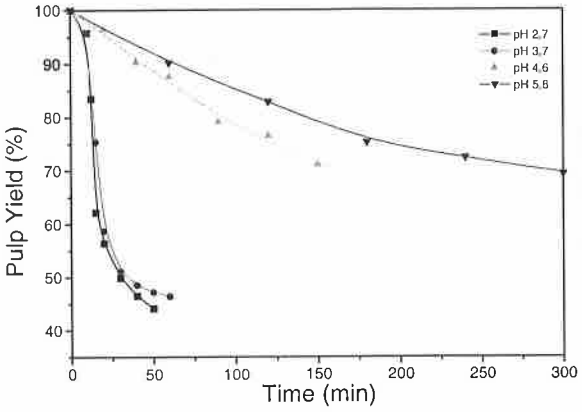

Figure 1. Pulp yields for the minimum buffer concentration. (0.1 M).

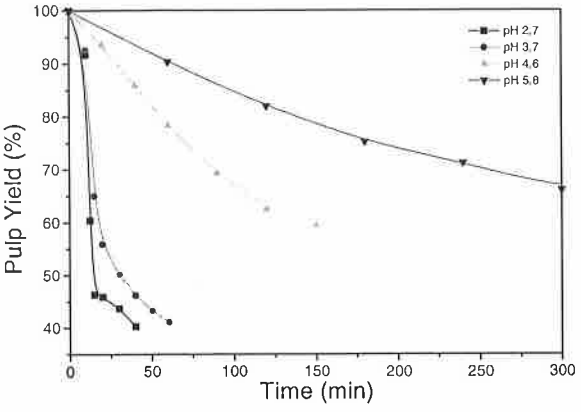

Figure 2. Pulp yields for the maximum buffer concentration. (0.5 M).

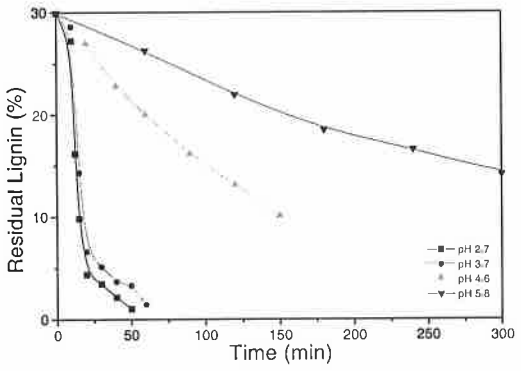

Figure 3. Residual lignin (initial wood basis) for the minimum buffer concentration. (0.1 M).

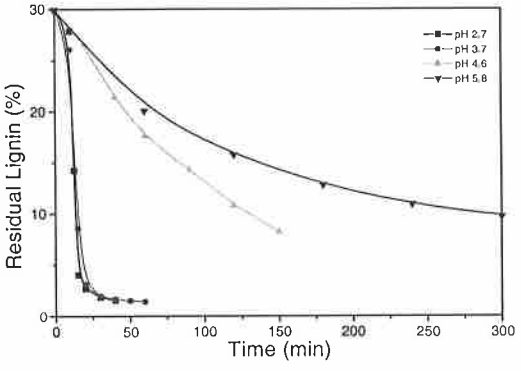

Figure 4. Residual lignin (initial wood basis) for the maximum buffer concentration. (0.5 M).

As expected, as lower the pH, higher the delignification extent (reflected by the lower residual lignin yield) and higher de polysaccharide dissolution (reflected in the pulp yields). The pulp yields obtained in the processes performed with higher buffer concentrations are remarkably lower, due to higher dissolution of lignin and polysaccharides.

The obtained pulps were analyzed by the Klason method to determine the residual lignin yield on the initial wood basis. When the logarithm of residual lignin yield (on wood basis) is plotted against time (pseudo-first order kinetics plot) two distinct straight lines could be calculated by least square minimum with good correlation coefficients for all buffers. These two lines correspond to two delignification phases: principal, at lower times; and residual phase, at higher times. This kinetic behavior is similar to the kinetic pattern presented by other processes. The phase transitions occurred at lower residual lignin contents and lower reaction times for the buffered system compared to the values of ethanol/water systems encountered in the literature (Araujo 1995, Goyal and Lora, 1991).

The results showed an effect of the temperature on the delignification degree and pulp yields (results not shown here). As expected, as higher the temperature higher the delignification extent and lower the pulp yields when all other conditions were kept constant.

The data showed in Table 1 correspond to the observed rate constants calculated from the angular coefficient of the first straight line, corresponding to the principal phase.

The rate constants values depend on the buffer concentration and increase at higher concentrations. The effect of the pH on the rate constants is much more significant. Thus, one can infer that the both general and acid catalysis mechanisms are occurring at the buffered delignification process reported here, but it seems that the specific catalysis influence is more accentuated.

Table 1. Observed rate constants for the delignification process performed at 170 °C for all buffered systems.

Buffer (Acid/Salt)	Apparent pH	Buffer Concentration (M)	Observed Rate Constant ($x 10^{-3}$)
Dichloroacetic/Dichloroacetate	2.7	0.1	179.4
		0.5	257.1
Chloroacetic/Chloroacetate	3.7	0.1	140.7
		0.5	213.4
Formic/Formiate	4.6	0.1	7.4
		0.5	8.8
Acetic/Acetate	5.8	0.1	2.9
		0.5	3.9

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REFERENCES

Araujo, E. S.(1995). Cinética de deslignificação Organossolve. MSc thesis, Instituto de Química de São Carlos – USP.

Aziz, S. & Sarkanen, K. V. (1989). Organosolv pulping: a review. *Tappi J.*, 169 - 175.

Delange, M.; Trummer, J.A.; Roberts, R.S.; Muzzy, J.D. & Gelbaum, L.T. (1991). C-13 NMR characterization of tulip poplar lignin solubilized by buffered solvent pulping. *Holzforschung,* **45**, 361-365.

Faass, G. S.; Roberts, R. S. & Muzzy J. D. (1989). Buffered solvent pulping. *Holzforschung,* **43,** 245 – 250.

Goyal, G.C. & Lora, J.H. (1991). Kinetics of delignification and lignin characteristics in autocatalyzed organosolv pulping of hardwoods. *In* 6th *Int. Symp. Wood Pulping Chem.*, Proceedings, pp 205- 207.

Johansson, A; Aaltonen, O. & Ylinen, P.T.I. (1987). Organosolv pulping - methods and pulp properties. *Biomass,* **13**, 45- 65.

Kitsos, H.M.; Roberts, R.S. & Muzzy, JD (1992). Normal-propylamine pretreatment of buffered solvent pulp to enhance enzymatic-hydrolysis. *Bioresource Technology,* **39**, 241-247.

Paszner, L. & Cho, H. J. (1989). Organosolv pulping: acidic catalysis options and their effect on fiber quality and delignification. *Tappi J.*, 135 – 142.

Ritchie, C.D. (1990). Physical Organic Chemistry-The Fundamentals Concepts - Marcel Dekker - New York.

Sarkanen, K. V. (1990). Chemistry of solvent pulping. *Tappi J.,* 125 - 219.

Lignin degradation by enzymatic mediators and iron chelating compounds

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ABSTRACT

Lignin biodegradation by fungi, beside enzymes, involves the participation of low molecular mass enzymatic mediators, manganese chelators and iron reducing compounds. Brown rot fungi degrade cellulose and modify lignin through a non-enzymatic Fenton-type reaction. Ferric chelators of low molecular mass may serve as oxygen-reducing agents, and redox-cycling molecules, acting as electron transport carriers in Fenton's reactions. In white rot fungi, low molecular mass peptides, manganese chelating organic acids and laccase mediators have been identified and attributed participation in lignin degradation. Attempts to achieve biomimetic processes using synthetic chelators such as dihydroxybenzoic acid have been published. One of the most interesting topics in this area is the elucidation of the nature of the chelators and the mechanisms used by white-rot fungi to degrade lignin selectively. In this paper we present our latest results, a review of the literature and a discussion about the mechanism of chelant-driven Fenton reaction.

Keywords: lignin biodegradation, mediators, iron, white-rot fungi, brown-rot fungi, wood decay

INTRODUCTION

As a result of intensive research to elucidate the lignin degradation mechanism, Tien and Kirk (1983) and Glenn *et al.* (1983) isolated, in 1983, an enzyme that they called, respectively, ligninase and diarylpropane oxygenase, which is now currently called Lignin-peroxidase (LiP). Since then lignin biodegradation was considered to be an enzymatic process caused by oxidative enzymes, such as LiP, Mn-peroxidase (MnP) and laccase, although reports on lignin polymerization and depolymerization *in vitro* using ligninolytic enzymes were contradictory. Hammel *et al.* (1993) presented conclusive evidences on lignin depolymerization *in vitro* but the understanding of lignin degradation in wood matrix still presents several unsolved problems.

Different reports have clearly demonstrated that lignin biodegradation in wood cell wall and middle lamella occurs before the penetration of enzymes as small as 5,760 Da (Blanchette *et al.*, 1997). The involvement of low molecular mass mediators in this process has been postulated to be indispensable for the increase in pore size of wood cell walls, what would permit the enzymes to penetrate the wood matrix (Messner and Srebotnik, 1994).

Several small molecules have been postulated to participate in lignin degradation. These involve LiP mediators, Mn(III) chelators, laccase mediators and iron reducing compounds. In this work the participation of low molecular mass compounds (LMMC) in lignin degradation is reviewed and new information is provided.

ENZYME MEDIATORS

LiP Mediators

Veratryl alcohol (3,4-dimethoxybenzyl alcohol-VA), a secondary metabolite produced by *Phanerochaete chrysosporium*, is a LiP substrate. Harvey *et al.* (1986) postulated that VA also stimulated the oxidation of monomethoxy-substituted aromatic substrates and that LiP oxidizes VA to an aryl cation radical that acts as a radical mediator oxidizing recalcitrant substrates. After that, Valli *et al.* (1990) suggested that VA stimulates the oxidation of monomethoxylated aromatic compounds by preventing the enzyme inactivation rather than by acting as a radical mediator. The body of evidences suggests that VA does play a role in LiP action in a number of ways; it now seems that VA reacts with LiP to form a cation radical that can act as a redox mediator toward non-phenolic substrates. Although VA has the potential of diffusing up to a distance of 7 μm, it may be present in an enzyme-bound form that confers it more stability (Zapanta and Tien, 1997).

Furthermore, it was demonstrated that 2-chloro-1,4-dimethoxybenzene, a secondary metabolite produced by the white rot fungus *Bjerkandera adusta*, can also act as a redox mediator in LiP catalyzed oxidations (Teunissen and Field, 1998).

Manganese Chelators

Manganese peroxidase (MnP) is a heme-containing peroxidase that oxidizes Mn^{2+} to Mn^{3+} in the presence of H_2O_2 and organic acid chelators. Therefore, it was proposed that the organic acids chelate and stabilize Mn^{3+} produced in the catalytic cycle of MnP. Malate showed to be the best stimulator for the activity of MnP (Glen and Gold, 1985). The stable Mn^{3+} complexes could act as diffusible oxidants of substrates away from the enzyme active site.

Laccase Mediators

Many white-rot fungi produce laccase, even though the redox potentials of known laccases are too low to directly oxidize non-phenolic components of lignin, they may play a role in lignin degradation since some laccase producing fungi degrade lignin very efficiently. Bourbonnais and Paice (1990) demonstrated that the synthetic laccase substrate ABTS (2,2'-azino-bis[3-ethylthiazoline-6-sulfonate]) could act as a redox-mediator enabling laccase indirectly to oxidize non-phenolic lignin model compounds and partially delignified wood pulp. ABTS must function as a diffusible electron carrier since laccase is a large molecule (70,000 D) and therefore can not enter the secondary wood wall to contact the lignin substrate directly. The most effective mediators discovered to the moment are N-OH containing compounds and one the most studied is 1-hydrobenzotriazole (HBT). The application of a very effective hydroxamate mediator is discussed in the section 3.3. Call (1994) patented the application of mediator in combination with laccase of *Trametes versicolor* in an efficient delignification system for chemical pulps. HBT is converted by the enzyme into the active intermediate by consuming oxygen (Call and Mücke, 1997).

To overcome the redox potential barrier, *Pycnoporus cinnabarinus* produces the metabolite 3-hydroxyantranilate that can mediate the oxidation of non-phenolic substrates by laccase acting as a laccase mediator in lignin degradation (Eggert *et al.*, 1996).

Recently, over 55% delignification of kraft pulps has been achieved with laccase in presence of HBT, or by repeated treatment with laccase and ABTS followed by alkaline extraction. Furthermore, application of laccase mediators system (LMS) has recently been described for degradation of policyclic aromatic hydrocarbons, chemical synthesis and bleaching of textile dyes (Bourbonnais *et al.*, 1998).

IRON CHELATING COMPOUNDS

The Fenton Reaction and Wood Biodegradation

The first study in iron binding compounds produced by wood-decaying basidiomycetes was reported by Fekete *et al.* (1989). After that, Jellison *et al.* (1991) reported the production of low molecular weight iron-binding compounds by the brown-rot fungus *Gloeophyllum trabeum*. Fenton type mechanisms were proposed to explain wood biodegradation involving the participation of iron chelators. Siderophore type compounds were suggested to be the iron chelators in wood biodegradation (Jellison *et al.*, 1991; Rodriguez 1990; Durán, 1995, Rodríguez, 1997). Siderophores are mainly classified as hydroxamates or catecholates based compounds. At non-acid pH values, catechol is a more powerful ligand for iron (III) than hydroxamates and also catechols are susceptible to oxidation (Raymond *et al.*, 1994).

Fenton reaction with chelated iron presents an interesting chemistry explored mainly in medicine to avoid damage by oxygen activated species. According to Winterbourn (1995), an inner sphere mechanism is the best representation of the Fenton reaction. The iron complexed to a ligand (L) forms a transient specie that can be regarded as a ferrous peroxide complex. The transient species could be break down in several pathways to give hydroxyl radical, or iron (IV) species or by oxidizing directly a substrate. A similar mechanism could be used by fungi to selective and non selective lignin degradation

Peptides

In 1990, Enoki *et al.* (1990) reported the isolation of low molecular weight extracellular glycoproteins (~1,600-2,000 Da) from *G. trabeum* and *Tyronyces palustris*. The peptides contain iron (Fe^{+2}) and are able to oxidize KTBA in presence of H_2O_2 through one electron transference process. The Fe^{+2} in the glycopeptide can be oxidized to Fe^{+3} by H_2O_2. It was proposed that in the early stages of wood decaying, the hyphae located into the lumen of the vegetal cell, excretes glycopeptides that combine with the iron present in wood. In the S2 layer, glycopeptides catalyze oxidation of the electron donors under O_2 atmosphere producing activated oxygen species like $O_2^{\cdot-}$ and H_2O_2. The produced H_2O_2 is reduced to OH radical by the ferrous ion attached to the glycopeptide and promotes the splitting of cellulose as well as lignin into fragments. The

glycopeptide of *T. palustris*, has a molecular weight between 1,500-5,000 Da and contains 0.12% of Fe, 13% of protein and 22% of carbohydrates. This compound shows ability to reduce Fe^{+3} to Fe^{+2} and absorbed Fe^{+2} strongly (Hirano *et al.*, 1995). Glycopeptide compounds with properties similar to the described above, were also isolated from the white rot fungus *Irpex lacteus*. In *I. lacteus*, the amount of extracellular glycopeptides produced in wood-containing cultures was higher than cultures containing glucose as carbon source (Tanaka *et al.*, 1993).

Hirai *et al.* (1997) reported the purification of iron-binding compounds from the white rot fungus *Phanerochaete sordida* YK-624. These compounds were secreted to extracellular medium, and binded to iron or cobalt, particularly Fe^{+3}. The Fe^{+3} complexes are incorporated to the fungal cell, and reduced to Fe^{+2} complexes. The authors suggested a probable role of iron binding complexes in the reduction of manganese dioxide, in the production of hydrogen peroxide, and in Fenton´s reaction (Hirai, 1998).

An extracellular low molecular weight substance (1,000-5,000 Da), composed of protein (18 %), neutral carbohydrates (20 %) and iron (0.28 %), that catalyzes redox reactions was isolated from *P. chrysosporium* (Enoki *et al.*, 1997). This substance catalyzes redox reaction between an electron donor, such as NADH, and O_2 to produce H_2O_2 and it acts further to reduce H_2O_2 to OH radical.

Our latest results present evidences that *P. chrysosporium* produces four chelators (PcChel) with molecular weight around 1,210 Da and they contain iron and aromatic aminoacids as part of the peptide structure. The iron chelators were able to reduce Fe^{+3} to Fe^{+2} forming very stable complexes. These compounds were also able to oxidize substrates such as ABTS, 2,6-dimethoxyphenol, veratryl alcohol and chlorophenols in presence or absence of H_2O_2 (Oses *et al.*, 1998).

Hydroxamate Siderophores and Hydroxamic Acid

Desferrioxamine B, a bacterial hydroxamate based siderophore was used by Santiago *et al.* (1997) to degrade Kraft black liquor mineralizing 10 % of the organic material. Parra *et al.* (1998a) reported lignin degradation by acetohydroxamic acid (AHA)/Fe(III) complex. The complex showed the highest phenoloxidase-like activity at pH 7 and it modified the fluorescence spectra of dioxane lignin and bleaching effluent, decreasing their fluorescence intensity in 65 % and 85 %, respectively.

The siderophore desferrioxamine was found to act as laccase oxidizable mediator in bleaching (Viikari *et al.*, 1998). It decreased the Kappa number of softwood kraft pulp from 25 to 20 and increased the brightness from 27.1 to 29.0.

The hydroxamate compound N-hydroxy-N-phenylacetamide (N-OH-acetanilide - NHA), was proposed as the most efficient laccase mediator combining good delignification capabilities and, low enzyme damage. The lacasse-NHA process provides additional benefits to pulp bleaching by requiring a low dosage of enzyme, by the low cost of the mediator and by presenting fast reaction kinetics (Pfaller *et al.*, 1998). The delignification degree after an alkaline extraction is reported to be high, up to 40 %. It is carried out at 10 % consistency in a pressurized vessel with 10 bar O_2, 45 °C, at pH 4.5 by 2 hours (Amann, 1997).

An extracellular LMMC with catalytic properties was isolated from liquid cultures of the thermophilic ascomycete *Thermoascus aurantiacus*. The partially purified LMMC oxidized typical phenoloxidase substrates in the absence of hydrogen peroxide at acidic pH (2.8). The LMMC contains hydroxamic acid and iron and presents a molecular mass of the 530 Da (Machuca *et al.*, 1999).

Dihydroxybenzenes

It has been demonstrated that catechol-iron-chelators are involved in brown-rot decay (Jellison *et al.*, 1991; Goodell *et al.*, 1997a). Recently, Paszczynski *et al.* (1999) isolated 4,5-dimethoxycatechol (DMC) and 2,5-dimethoxyhydroquinone (DMH) from the brown rot fungus *G. trabeum*. DMC and DMH may serve as ferric chelators, oxygen-reducing agents, and redox-cycling molecules which act as electron transport carriers to Fenton's reactions. Evidence for an extracellular hydroquinone-driven Fenton reaction in the biodegradative mechanism of the brown rot basidiomycete *G. trabeum* was reported by Kerem *et al.* (1999).

As an approach to the real system displayed by fungi, we studied the Fenton-driven reaction catalyzed by 1,2-benzenediol (CAT), 2,3-dihydroxybenzoic acid (2,3-DHBA) and 3,4-dihydroxybenzoic acid (3,4-DHBA). These compounds reduce Fe^{+3} to Fe^{+2} promoting the oxidation of veratryl alcohol (VA) in the presence of H_2O_2. In similar conditions, the iron-free Fenton reaction degrades VA at insignificant amount. It has been reported previously that catechols lead to an enhancement of the formation of hydroxyl radical in

the ferric ions - hydrogen peroxide reaction (Iwahashi *et al.*, 1989). A mechanism was proposed in which DHBA/Fe(III) reacts first by substitution on free iron (III) followed by two reversible electron–transfer steps to give the quinone product (Xu and Jordan, 1988). Therefore an activated Fe-catechol complex could be responsible by the enhanced oxidative activity.

A *Ganoderma applanatum* chelator (GaChel) was partially purified and characterized as a catecholate compound. When it was used to bleach cellulose pulp, a benefic protector effect on the pulp viscosity was observed. Beside the beneficial effect on viscosity in the cellulose pulp bleaching process by the fungal chelator (GaChel), attempts to use simple models such as catechol and 2,3-DHBA in the presence of iron and hydrogen peroxide, resulted not only in the reduction of Kappa number but also in the loss of viscosity (Parra *et al.*, 1998c).

Parra *et al.* (1998b) reported the oxidation of both lignin and pulp bleaching effluent by 2,3-DHBA and 3,4-dihydroxyphenylacetic acid (DHPAA) complexed with Fe(III). The 2,3-DHBA/Fe^{3+}/H_2O_2 and DHPAA/Fe^{3+}/H_2O_2 systems modified the fluorescence of dioxane lignin decreasing their intensity in 100% and 85%, respectively. A bleaching kraft effluent (first alkaline extraction) had its fluorescence intensity decreased in 80% after 2,3-DHBA/Fe(III) treatment.

Catechol, 2,3-DHBA and 3,4-DHBA chelated with iron, and with hydrogen peroxide were used in the pulp bleaching effluent treatment (Rodríguez *et al.*, 1999). The 2,3-DHBA/Fe^{3+}/H_2O_2 treatment at pH 4.0 decreased AOX in 95 % when compared to the 65 % decrease by free iron (Fe^{3+}/H_2O_2). This clearly shows an enhancement of the activity of the Fenton reaction. An expressive reduction in toxicity was observed with 2,3-DHBA at pH 4.0 treated samples (90 %) when compared to the iron free Fenton reaction (70 %). These promising results suggest that an improvement on Fenton process using chelated-driven Fenton reaction can be reached.

Delamination of the S1-compound middle lamellae interface was observed when wood was treated with *G. trabeum* chelator under the proper conditions. The authors suggest that using appropriated chelating agents, in bleaching systems requiring hydrogen peroxide, the trace amounts of iron in pulp, may not be deleterious to fiber integrity. Under appropriated conditions, chelating systems may be employed to promote delignification, for improvement of pulp properties or potentially in bleaching applications (Goodell *et al.*, 1997b).

CONCLUDING REMARKS

The understanding of the wood degradation mechanism by brown rot fungi has received an important advance during the last year. However, the selective delignification system displayed by white rot fungi which integrates LMMC and enzymes, still remain to be elucidated. It could be hypothesized that synergistic oxidation effects between the LMMC and enzymes could improve redox mechanisms early proposed. Application of these systems to pulp bleaching, effluent treatment and bioremediation certainly will continue to be improved in the near future.

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REFERENCES

Amann M. (1997). The Lignozym® Process – Coming Closer to the Mill. *In 9th International Symposium on Wood and Pulping Chemistry*, pp F4-1-F4-5.

Blanchette, R.A., Krueger, E.W., Haight, J.E., Akhtar, M. & Akin, D.E. (1997). Cell wall alterations in loblolly pine wood decayed by the white-rot fungus *Ceriposiopsis subvermispora*. *J. Biotechnol.* **53**, 203-213.

Bourbonnais, R., Leech, D. & Paice, M. (1998). Electrochemical analysis of the interactions of laccase mediators with lignin model compounds. *Biochim. Biophys., Acta* **1379**, 381-390.

Bourbonnais R. & M.G. Price (1990). Oxidation of non-phenolic substrates. An expanded role for laccase in biodegradation. *FEBS Lett.*, **267**, 99-102.

Call, H.P. (1994). PCT, World patent application WO 94/29510.

Call, H.P. & Mucke I. (1997). History, overview and applications of mediated lignolytic systems, especially laccase-mediator-system (Lignozym-process). *Journal of Biotechnology* **53**, 163-202.

Durán, N. (1995). Siderophores on wood degradation: reduction of molecular oxygen to hydroxyl radical and ferric iron to ferrous iron. *Fourth Brazilian Symposium on the Chemistry of Lignins and other Wood Components.* Proceeding **5,** 164-169.

Eggert, C., Temp, U., Dean, J. & Eriksson, K. (1996). A fungal metabolite mediates degradation of non-phenolic lignin structures and synthetic lignin by laccase. *FEBS Letters* **391**, 144-148

Enoki, A., Yoshioka, S., Tanaka, H. & Fuse, G.(1990). Extracellular H_2O_2-Producing and One-Electron Oxidation system of Brown-Rot Fungi. The International Research Group on Wood Preservation Doc.N°:IRG/WP/1445.

Enoki A., Itakura, S. & Tanaka, H. (1997). The involvement of extracellular substances for reducing molecular oxygen to hydoxyl radical and ferric iron to ferrous iron in wood degradation by wood deacay fungi. *J. Biotechnol.* **53**, 265-272.

Fekete, F.A., Chandhoke, V. & Jellison, J. (1989). Iron-binding compounds produced by wood-decaying basidiomycetes. *Appl. Environ. Microbiol.* **55**, 2720-2722.

Glen, J.K., Morgan, M.A., Mayfield, M.B., Kuwahara, M. & Gold, M.H. (1983). An extracellular H_2O_2-requiring enzyme preparation involved in lignin biodegradation by the white-rot basidiomycete *Phanerochaete chrysosporium. Biochem. Biophys. Res. Commun.* **114**, 1077-1083

Glen, J.K. & Gold, M.H. (1985). Purification and characterization of an extracellular Mn(II)-dependent peroxidase from the lignin-degrading basidiomycete *Phanerochaete chrysosporium. Arch. Biochem. Biophys.* **242**, 329-341.

Goodell, B., Jellison, J., Liu, J., Daniel, G., Paszczynski, A., Fekete, F., Krishnamurthy, S., Jun, L. & Xu, G. (1997a). Low molecular weight chelators and phenolic compounds isolated from wood decay fungi and their role in the fungal biodegradation of wood. *J. Biotechnol.* **53**, 133-162.

Goodell, B., Jellison, J., Daniel, G. & Yuhui, Q. (1997b). Redox cycling chelators isolated from *Gloeophyllum trabeum* and their effect on wood fibers. *TAPPI Biological Sciences Symposium and Pulping Symposium.* October, San Francisco, USA.

Hammel, K.E., Jensen, K.A., Mozuch, M.D., Landucci, L.L., Tien, M. & Pease, E.A. (1993). Ligninolysis by a purified lignin peroxidase. *J. Biol. Chem.* **268**, 12274-12281.

Harvey, P.J., Schoemaker, H.E. & Palmer, J.M. (1986). Veratryl alcohol as a mediator and the role of radical cation in lignin degradation by *Phanerochaete chrysosporium. FEBS Letters* **195**, 242-246.

Hirai, H., Kondo, R. & Sakai, K. (1997). A model system for NAD(P) H-dependent reduction of manganese dioxide mediated by ferrous chelate in white-rot fungus, *Phanerochaete sordida* YK-624. *Mokuzai Gakkaishi.* **43**, 247-253.

Hirai, H., Hirosue, S., Onitsuka, A., Kondo, R., Sakai, K. & Kurane, R. (1998). Involvement of ferrireductase and iron-binding compounds on the degradation of wood components by white-rot fungus *Phanerochaete sordida* YK-624. *7th International Conference on Biotechnology in the Pulp and Paper Industry.* Vancouver, Canada. Proceedings **Vol B**, 241- 244.

Hirano, T., Tanaka, H. & Enoki, A. (1995). Extracellular substance from the brown-rot basidiomycete *Tyromyces palustris* that reduces molecular oxygen to hydroxyl radicals and ferric iron to ferrous iron. *Mokuzai Gakkaishi* **41,** 334-341

Iwahashi, H., Morishita, H., Ishii, T., Sugata, R. & Kido R. (1989). Enhancement by catechols of hydroxyl-radical formation in the presence of ferric ions and hydrogen peroxide. *J. Biochem.* **105**, 429-434.

Jellison, J., Chandhoke, V., Goodell, B., & Fekete, F.A. (1991). The isolation and immunolocalization of iron-binding compounds produced by *Gloeophyllum trabeum. Appl. Microbiol. Biotechnol.* **35**, 805-809.

Kerem, Z, Jensen, K.A. & Hammel, K.E. (1999). Biodegradative mechanism of the brown rot basidiomycete *Gloeophyllum trabeum*: evidence for an extracellular hydroquinone-driven Fenton reaction. *FEBS Letters* **446**, 49-54.

Machuca, A., Aoyama, H. & Duran,N. (1999). Isolation and partial characterization of an extracellular low-molecular mass component with high phenoloxidase activity from *Thermoascus aurantiacus. Biochem. Biophys. Res. Commun.* **256**, 20-26.

Messner, K. & Srebotnik, E. (1994). Biopulping: An overview of developments in an environmentally safe paper-making technology. *FEMS Microbiol. Rev.* **13**, 351-364.

Oses, R., Rodriguez, J., Tatsumi, K., Freer, J. & Baeza J. (1998). Chlorophenols degradation by catalytic siderophores from white-rot fungi *Phanerochaete chrysosporium* ME 446". *7th International Conference on Biotechnology in the Pulp and Paper Industry.* Vancouver, Canada. Proceedings, **Vol C**, 227-230.

Parra, C., Santiago, M., Rodriguez, J. & Durán, N. (1998a). Hydroxamate iron complex with phenoloxidase activity acting on lignin and chlorolignin. *Biophys. Res. Commun.* **249**, 719-722.

Parra, C., Rodriguez, J., Baeza, J., Freer, J. & Durán, N. (1998b). Iron binding catechols oxidizing lignin and chlorolignin. *Biophys. Res. Commun.* **251**, 399- 402.

Parra, C., Oses, R., Ruiz, J., Urtubia, I., Baeza, J., Freer, J. & Rodriguez, J. (1998c). Kraft pulp bleaching by dihydroxybenzenes and siderophores from white rot fungi. *7th International Conference on Biotechnology in the Pulp and Paper Industry.* Vancouver, Canada. Proceedings, **Vol A**, 161-167.

Paszczynski, A., Crawford, R., Funk, D. & Goodell, B. (1999). De novo synthesis of 4,5-dimethoxycatechol and 2,5-dimethoxyhydroquinone by the brown rot fungus *Gloeophyllum trabeum. Appl. Environ. Microbiol.* **65**, 674-679.

Pfaller, R., Amann M. and freudenreich J. (1998). Analysis of Laccase and mediator interactions in the LMS. *7th International Conference on Biotechnology in the Pulp and Paper Industry.* Vancouver, Canada. Proceedings, **Vol A**, 99-102.

Raymond, K. (1994). Recognition and transport of natural and synthetic siderophores by microbes. *Pure Appl. Chem.* **66**, 773-781.

Rodriguez, J. (1990). Estudo do sistema ligninolítico do ascomycete C*hrysonilia sitophila*". PhD thesis, Chemistry Institute, Universidade Estadual de Campinas, Campinas, Brazil.

Rodriguez, J., Oses, R., Parra, C., Freer, J. & Baeza, J. (1997). Siderophore production by lignin degrading fungi. *5th Braz. Symp. Chem. Lignin and Other Wood Comp.* (Ramos, L.P. Ed.) Proceeding, **Vol 6**, 601-604.

Rodríguez, J., Contreras, D., Parra, C., Freer, J., Baeza, J. & Durán, N. (1999). Pulp mill effluent treatment by Fenton-type reactions catalyzed by iron complexes. *6th Symposium on Forest Industry Wastewater*, Tampere, Finland. Proceedings, 371-376.

Santiago, M.A.. Peralta-Zamora, P. & Durán, N. (1997). Polyhydroxamate/iron(III) complex as a mimetic of phenoloxidase and its application in bioremediation of pulp and paper residues. *5th. Braz. Symp. Chem. Lignin and Other Wood Comp.* (Ramos, L.P. Ed.), Proceeding,**Vol 6**,567-575.

Tanaka, H., Hirano, T. & Enoki, A. (1993). Extracellular substance from the white-rot basidiomycete Irpex lacteus for production and reduction of H2O2 during wood degradation. *Mokuzai Gakkaishi* **39**, 493-499.

Teunissen, P.J.M. & Field, J.A.(1998). 2-Chloro-1,4-dimethoxybenzene as a mediator of lignin peroxidase catalyzed oxidations. *FEBS Letters* **439**, 219-223.

Tien, M. & Kirk T.K. (1983). Lignin degrading enzyme from the hymenomycete *Phanerochaete chrysosporium* burds. *Science* **221**, 661-663.

Viikari, L., Harkki, T., Niku-Paavola, M.L., Buchert, J. & Poppius-Levin, K. (1998). Oxidative Enzymes for Fibre Modification. *7th International Conference on Biotechnology in the Pulp and Paper Industry.* Vancouver, Canada. Proceedings, **Vol A**,121-124.

Valli, K., Wariishi, H. & Gold, M.H. (1990). Oxidation of monomethoxylated compounds by lignin peroxidase: role of veratryl alcohol in lignin biodegradation. *Biochemistry* **29**, 8535-8539.

Winterbourn, C. (1995). Toxicity of iron and hydrogen peroxide: the Fenton reaction. *Toxicol. Letters* **82/83**, 969-974.

Xu, J. & Jordan, R.B. (1988). Kinetics and mechanism of the oxidation of 2,3-dihydroxybenzoic acid by iron(III). *Inorg. Chem.* **27**, 4563-4566.

Zapanta, L.S. & Tien, M. (1997). The roles of veratryl alcohol and oxalate in fungal lignin degradation. *J. Biotechnol.* **53**, 93-102.

Lignin structure: recent developments

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ABSTRACT

Studies of lignin structure and reactivity have been re-energized by the emergence of modern technologies, new analytical methods, and by exciting recent findings. The structural aspects which were considered complex but well understood some years back have seen revisions that add to the complexity in some ways but are clarified by the logical chemistry in others. One of the most significant recent findings has been the elucidation of dibenzodioxocins as significant components of natural lignins. Now found in lignins from all classes of plants which have a guaiacyl lignin component, they are new structures derived from 5–5-coupling of lignin oligomers followed by coupling with a new lignin monomer; other lignification reactions then follow to fully incorporate these structures into the polymer. Another new structural unit, aryl-isochromans, expose a logical new pathway following β–1-coupling. These new structures and others have been revealed by modern analytical and instrumental methods, particularly NMR. The now common use of 2D and 3D NMR, using pulsed field gradients, inverse detection, and modern digital instruments allows ever more sensitive detection and, more importantly, diagnostic interpretation. Because it is now possible to reveal a given unit in the complex polymer in a diagnostic way, structures are being suggested directly from spectra of the polymer (although confirmation and authentication using model compounds remains crucial). Studying lignins from non-woody plants has also provided significant new knowledge. Acylated (e.g. acetylated, *p*-coumaroylated, *p*-hydroxybenzoylated) lignins are now better understood thanks to NMR methods, as well as new and established degradative methods (e.g. the "DFRC" method, and thioacidolysis) combined with mass-spectrometry. Acylated units can be a significant component of lignins; for example, lignins in the bast fibers of kenaf are more than 50% acetylated. Cell wall cross-linking in grasses by ferulates has also recently been clarified. Mutant and transgenic plants in which steps in the monolignol biosynthetic pathway are down- or up-regulated are also providing a rich source of insight into the processes of lignification. Massive compositional shifts, significantly beyond traditional bounds, are found in a variety of mutants/transgenics. In some cases, units that are minor in "normal" lignins become major components. This facilitates their identification and may elucidate their radical-coupling reactions. Pathways interacting with lignification become identified, and the very definition of lignin itself becomes clouded or simplified depending on ones perspective. This paper explores recent advances in understanding lignin structure, and provides applications that show the breadth and plasticity of the lignification process.

Keywords: lignin, lignin biosynthesis, NMR, transgenic plants

INTRODUCTION

Lignification is the polymerization process in plant cell walls that takes phenolic monomers, produces radicals, and couples them with other monomer radicals (only during initiation reactions), or more typically cross-couples them with the growing lignin polymer/oligomer, to build up a phenylpropanoid polymer (Harkin, 1967; Freudenberg and Neish, 1968; Harkin, 1973; Brunow, 1998). As addressed below, there is current controversy over just what constitutes a lignin monomer or a lignin polymer. In the purest sense, lignins arise from radical coupling reactions of three primary precursors, the monolignols *p*-coumaryl, coniferyl and sinapyl alcohols, Figure 1. Since this process produces a polydisperse polymer with no extended sequences of regularly repeating units, its composition is generally characterized by the relative abundance of *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units (derived from each of the 3 primary monolignols) and by the distribution of interunit linkages in the polymer (e.g. β-aryl ether or β–O–4, phenylcoumaran or β–5, resinol or β–β, biphenyl or 5–5, diphenyl ether or 4–O–5). There are also hydroxycinnamyl alcohol endgroups from the few initial dimerization reactions, structures such as non-cyclic α-aryl ethers (which arise from addition of a phenol to an intermediate β-aryl ether quinone methide) and various other structures.

Often-overlooked Concepts; Differences Between Lignin and Lignans

Lignans are dimeric products from radical coupling of monolignols. As such, they have structures analogous to their counterparts in the lignin polymer. However, comparisons of the relative amounts of linkages that arise during dimerization and those that occur during lignification overlooks the fact that dimerization is minimal in lignification where fewer products are possible, as depicted in Figure 1. A simple illustration should convince us that dimerization reactions are not terribly significant when looking at lignin structure. For example, in a softwood there are two possible outcomes of coniferyl alcohol dimerization reactions that can be approximately quantified. Either the dimer is a β–β-dimer, i.e. pinoresinol, or the dimer bears a cinnamyl alcohol sidechain that must remain following further lignification. The total amount of dimerization reactions can therefore be measured from the sum of the resinol products and the unsaturated sidechains in lignins. Estimates in softwoods for resinol structures are low, typically less than 2% (Adler, 1977; Chen, 1998). Unsaturated sidechains run typically less than 1%. Thus, monomer-monomer coupling accounts for considerably less than 5% of lignification in typical softwoods. Hardwoods have higher amounts, but still only ~3% (Adler, 1977), of β–β-coupled units (resinols), mainly syringaresinol units from sinapyl alcohol, but barely detectable levels of cinnamyl alcohol end-units. Again therefore, monomer-monomer coupling accounts for considerably less than 5% of lignification in typical hardwoods. Grasses and legumes are similar to hardwoods in this regard.

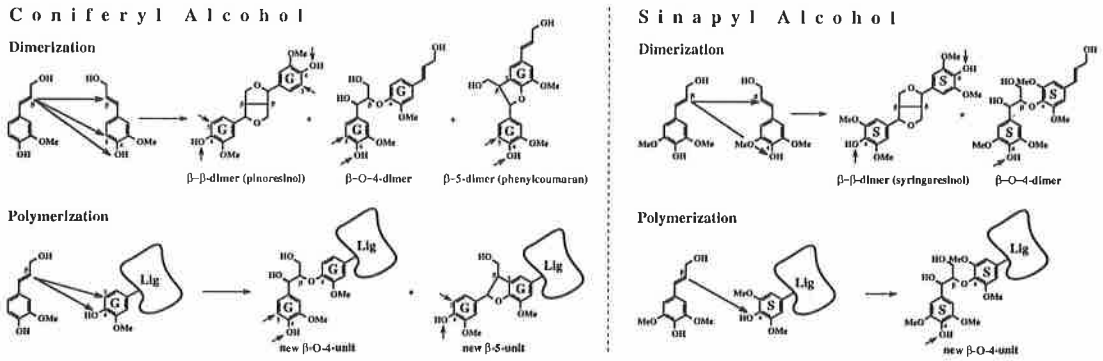

Figure 1. Radical coupling of monolignols, and differences between dimerization and lignin polymerization. During lignification, dimerization reactions are rare — the major linkages come from coupling of a monolignol radical with a radical of the growing lignin oligomer/polymer.

Lignins are Optically Inactive

Lignans, monolignol dimers, are often optically active. That is, they are produced by reactions in which the stereochemistry of the product is strictly controlled. Lewis' group have recently documented a fascinating protein that specifically facilitates the coupling of two coniferyl alcohol radicals to produce a single optical isomer of pinoresinol (Davin *et al.*, 1997). Such a protein is a significant finding in lignan chemistry/biochemistry. The group have further boldly extrapolated their findings to the process of lignification, suggesting that lignins also must be formed by carefully controlled processes (Lewis and Davin, 1998). However, there is currently no evidence for such a view. Lignins have always been considered optically inactive. Accommodating a lack of optical activity in lignins by the "dirigent protein array" theory of carefully controlled coupling unfortunately requires convoluted arguments (Lewis and Davin, 1998) that bring considerable conceptual difficulties, at least to this author (Ralph *et al.*, 1999c; Sederoff *et al.*, 1999). We and other groups have recently provided more significant evidence that lignins are indeed optically inactive. Matsumoto *et al.* examined ozonolysis products to demonstrate that β-aryl ethers are not optically active (Matsumoto *et al.*, 1999). We showed that phenylcoumarans and resinols weren't either, and that β–aryl ethers likely weren't (Ralph *et al.*, 1999c). These data complement early findings that resinol products from lignins had no detectable optical activity. Finally, we showed that lignins, isolated by means that could not affect the radically coupled centers, showed no detectable optical activity (Ralph *et al.*, 1999c).

New Analytical Method: The "DFRC" Method

There are many good analytical methods for analysis of the composition and structure of lignins, but lignins are enormously complex. No one method can answer all questions. New methods continue to be developed and established methods continue to evolve. We recently developed an efficient approach to cleaving β-ether units in lignins (Lu and Ralph, 1997b; Lu and Ralph, 1997a; Lu and Ralph, 1998b) — a method which

differed from other established methods such as the acidolytic methods (e.g. acidolysis, thioacidolysis) and high-temperature base. Due to unique features of the method, it has provided new insights into structural features.

The method has been dubbed the "DFRC" method as an acronym for the chemistry involved (Derivatization Followed by Reductive Cleavage) and because it was developed at the US Dairy Forage Research Center (for which DFRC is also the acronym). As seen in simplified form in Figure 2, the method is firstly based on the solubilization of cell wall material by acetyl bromide. Under sufficiently mild conditions, AcBr cleanly acetylates alcohols and phenols, and brominates the benzylic positions of β-aryl ethers (Lu and Ralph, 1996). The key feature then is the cleavage of the resulting β-bromo-ether by (formally) two-electron reducing reagents, in this case zinc in acetic acid. The two reactions effect the cleavage of etherified and free-phenolic β-ethers and proceed in very high yield on model compounds. On lignin, cleavage of β-ethers results in the liberation of monomers, dimers and higher oligomers; the monomers and dimers are suitable for analysis by GC (or HPLC) and GC-MS methods. Perhaps the most novel aspect of the reaction is that the primary monomeric products of this depolymerization are the lignin monomers themselves (acetylated). Another group has independently developed a related method, the "TIZ" method, based on similar chemistry (Nakatsubo *et al.*, 1997).

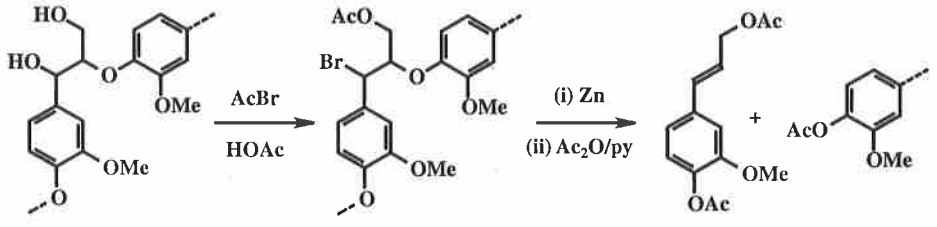

Figure 2. Basic DFRC Reaction on guaiacyl β-ether units. AcBr effects cell wall solubilization while acetylating and brominating lignin units. The resulting bromo-ethers are reductively cleaved to yield hydroxycinnamyl acetates, which can be quantified by GC.

The development and application of the DFRC method has already provided useful data. For example, a new β–1-coupling pathway in lignin has been elucidated (see below) (Ralph *et al.*, 1998c; Peng *et al.*, 1999a), the issue of lignin optical activity could be conveniently addressed (above) (Ralph *et al.*, 1999c), structures incorporated into lignins in plants deficient in enzymes in the lignin-biosynthetic-pathway can be nicely revealed (below) (Meyer *et al.*, 1998; Marita *et al.*, 1999; Peng and Ralph, 1999; Ralph *et al.*, 1999a), dimers provide a wealth of structural detail (Peng *et al.*, 1998; Peng *et al.*, 1999b), and minor structures in lignins can be studied (Lu and Ralph, 1998a; Lu and Ralph, 1999b). Because γ-esters are not cleaved by the reactions, determining lignin acylation details has been possible (below) (Ralph and Lu, 1998; Lu and Ralph, 1999a). Chen *et al.* used the method to elegantly establish the direct biosynthetic conversion of coniferyl to sinapyl alcohol without reversion to the aldehyde (Chen *et al.*, 1998; Chen *et al.*, 1999).

New Structural Features and New Mechanisms in Lignification

Despite the maturity of lignin chemistry, there are still many puzzling aspects of NMR spectra and other data. There has been a stimulating resurgence of interest in lignin structure and some rather novel components and mechanisms have been recently unearthed. By far the most important of these is the discovery of dibenzodioxocins in lignin (Karhunen *et al.*, 1995b; Karhunen *et al.*, 1995a).

Dibenzodioxocins

5–5-Coupled units react with new monolignols to produce cyclic ether structures, dibenzodioxocins as shown in Figure 3. The previously unknown component in lignins is strikingly prevalent. Dibenzodioxocins are readily apparent in NMR spectra, particularly from 2D ^{13}C–^{1}H-correlation (HMQC or HSQC) experiments where the Hα–Cα and Hβ–Cβ correlations are unique. NMR was crucial to the identification of the novel units and to the unambiguous demonstration of their presence, at significant levels, in (isolated) lignins. A survey of plants reveals that dibenzodioxocins are present in all lignin classes (from softwoods and hardwoods as well as grasses and legumes, as seen here) (Galkin *et al.*, 1997; Ralph *et al.*, 1999b). Representative spectra from the major classes, with the dibenzodioxocins highlighted in red, are shown in

Figure 4. Our group was originally concerned that such units could be artifacts of acetylation since they were difficult to find in unacetylated samples, yet readily showed up in acetylated samples. The reason appears to be that the chemical shifts are more variable in unacetylated samples, smearing out the required correlation peaks. They sharpen a little in DMSO (Ralph 1998, unpublished). Despite being ethers, such structures cannot necessarily be fully released by solvolytic methods (Karhunen *et al.*, 1999).

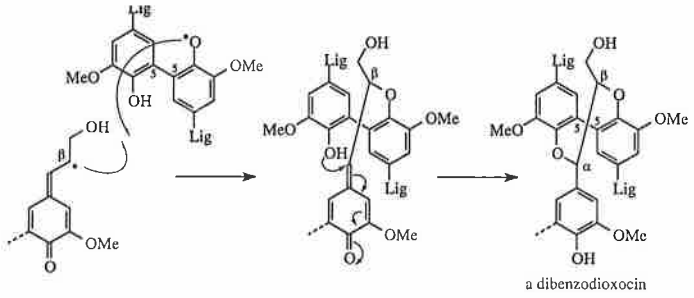

Figure 3. Biosynthesis of dibenzodioxocins in lignin. Radical coupling between a 5–5-unit radical and a monolignol radical (at β) forms the β–O–4-bond as usual. Internal trapping of the quinone methide by the other phenol completes re-aromatization — the process is similar to the formation of phenylcoumarans following β–5-coupling.

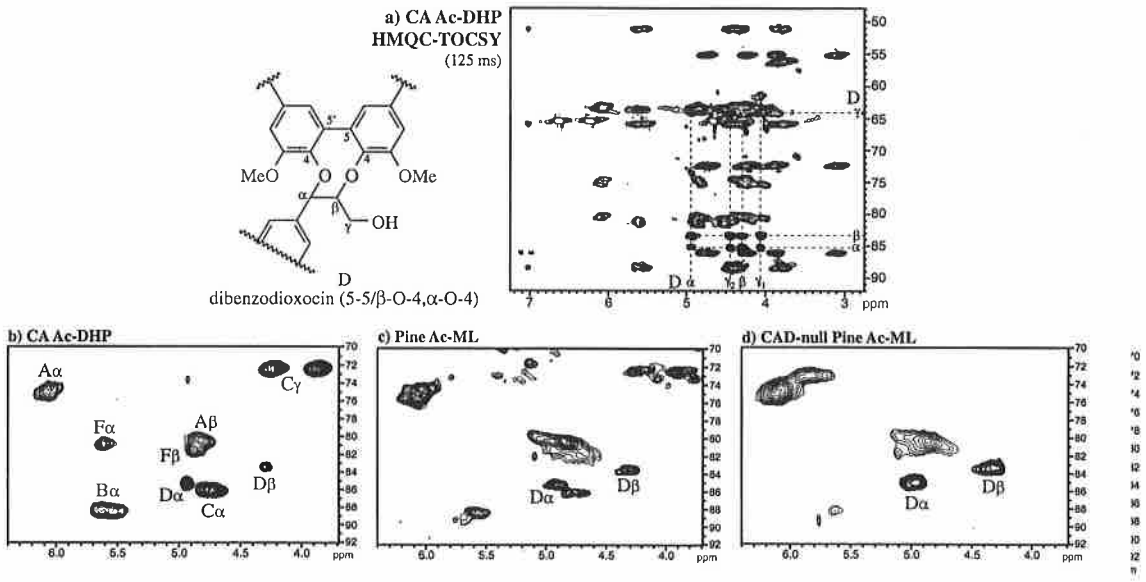

Figure 4. Dibenzodioxocins D are easily detected in HMQC or HSQC NMR spectra. The Cα–Hα and Cβ–Hβ correlations are unique and readily identified. Figures b-d are HSQC spectra from: b) an acetylated DHP (derived from coniferyl alcohol in which the methoxyl group is deuterated and ^{13}C-depleted; c) a *Pinus taeda* acetylated milled wood lignin; d) a CAD-deficient *Pinus taeda* mutant — the enhanced dibenzodioxocin levels are due to the high levels of 5–5-linked DHCA units in this lignin. Due to diverse proton-proton coupling constants, the full sidechain correlation matrix does not always show up well in HSQC-TOCSY experiments with typical (80-100 ms) TOCSY mixing times. Figure 4a shows an HSQC-TOCSY acquired using a 125 ms TOCSY mixing time, which beautifully emphasizes the full D coupling array. A = β-ethers, B = phenylcoumarans, C = resinols, D = dibenzodioxocin, E = α,β-diethers.

Arylisochromans

Recently we discovered a similar, although less prominent, product with interesting implications for the β–1 coupling pathway (Ralph *et al.*, 1998c; Peng *et al.*, 1999a). Aryl isochromans (**h**, Figure 5) can be reasonably easily identified in some softwood isolated lignins by their diagnostic HMQC or HSQC correlation at δ_C 41.3, δ_H 3.60 (acetylated units, in acetone). The complete sidechain of the crucial unit is seen in TOCSY spectra.

Correlations from an authenticated compound containing the isochroman trimeric structure (Ralph *et al.*, 1998c; Peng *et al.*, 1999a), isolated from DFRC degradation, confirm the assignment. Whether arylisochromans are present as such in native lignins is not yet clear, but even if not the internal trapping of a β–1 quinone methide intermediate (Figure 5) is presumably operating *in vivo*. The structure differs from structures previously assigned as β–6/α–1-isochromans — the regiochemistry assigned to those structures identified in hydrogenolysis and thioacidolysis products may be incorrect, i.e. they are probably β–1/α–6-compounds (Peng *et al.*, 1999a). The rational mechanism for formation of such aryl isochromans is given in Figure 5 (Peng *et al.*, 1999a).

Although the identification of the aryl isochroman structure in an isolated milled wood lignin can be made firmly, the apparently low amount visible in spectra does not account for the significant amounts of derived β-1 products that arise from DFRC-degradation, or various other acidolytic methods. The possibility remains that it is a product of isolation and that its precursor **d**, for example, may be the *in situ* product, as has been proposed (Brunow *et al.*, 1995; Brunow, 1998). Either way, however, structure **h** provides compelling evidence for the internal cyclization pathway from β–1 intermediate **c**.

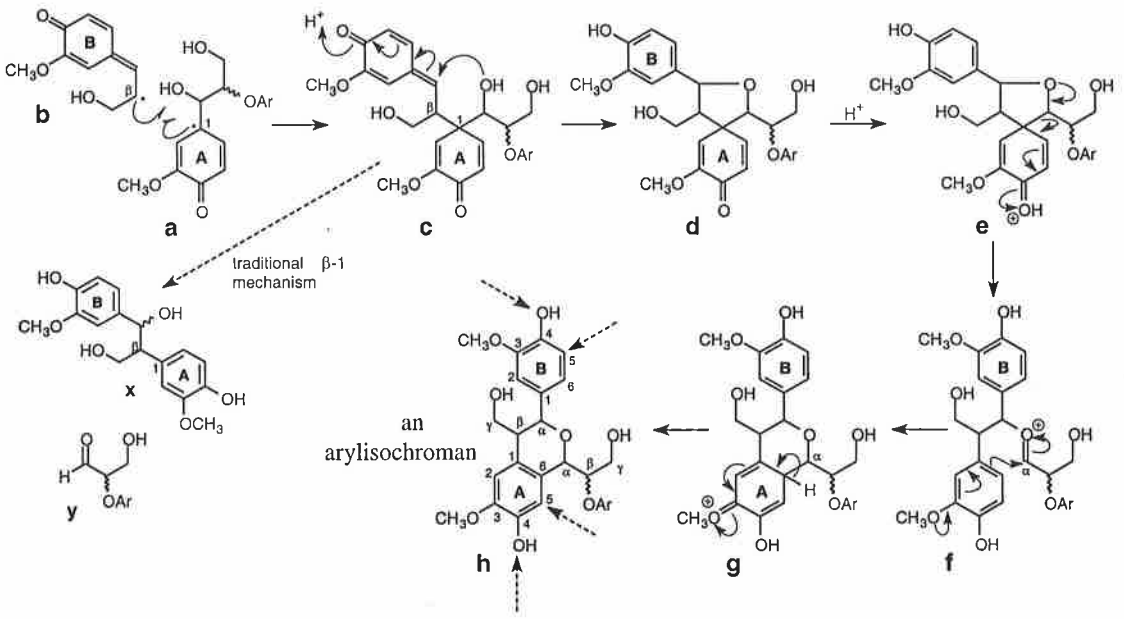

Figure 5. Proposed lignification mechanisms from β-1-coupling. The sidechain of the A-unit migrates to carbon-A_6 eventually forming arylisochroman h. (Dashed arrows indicate sites for further radical coupling in lignification).

Naturally Acylated Lignins

As alluded to in the introduction, lignins do not arise solely from polymerization of the three monolignols. Many plants have significant quantities of other conjugates incorporated into their lignins. Acyl components include acetate, *p*-hydroxybenzoate, and *p*-coumarate.

Acetylated lignins in Kenaf and Hardwoods

Perhaps the most striking example to date of a highly acylated lignin is in kenaf. The bast fibers have a lignin that is well over 50% acetylated. This was established initially by NMR (Ralph, 1996). However, a general disbelief by researchers required an independent method that could be used on whole cell wall materials. A minor modification was made to the basic DFRC method — all acetate-based reagents were replaced with their propionate analogues (Ralph and Lu, 1998). It is then a simple matter to differentiate fully propionylated monomers **7** (which come from normal unacetylated lignin units) from any γ-acetylated monomers **6** which must come from γ-acetylated units in the native lignin, Figure 6. The right-hand figure shows the monomeric products **6**, **7** that resulted from the modified DFRC procedure applied to isolated lignin from kenaf bast fiber; this lignin was never subjected to acetyl reagents in any form. The predominant 4-propoxy-sinapyl-γ–acetate peak **S-6** proves that syringyl units were highly acetylated in the isolated lignin. The same procedure applied to whole cell walls from kenaf bast fiber produced the central chromatogram.

This sample had been extracted only with toluene:ethanol to remove extractives. Obviously, the non-isolated cell wall lignin also had syringyl units which were heavily acetylated. In both cases, only minor amounts of acetate were on guaiacyl units, **G-6**. Although the guaiacyl component of kenaf lignins is strikingly low, the preference for acetylation of syringyl components suggests a specific enzymatic process (although chemical acetylation may also be selective). Presumably this is the acetylation of sinapyl alcohol **S-1** via a transferase and activated (e.g. S–CoA) acetic acid.

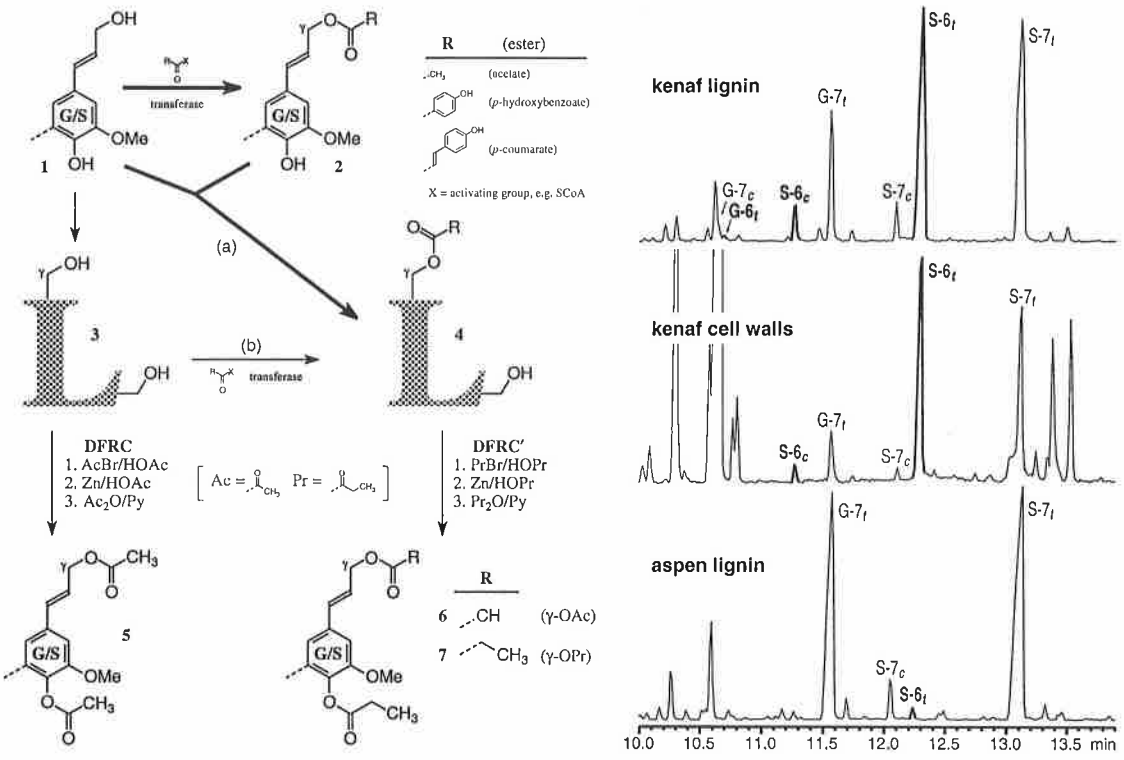

Figure 6. Formation and degradation pathways for acylated lignins 4. Monolignols **1** can be acylated by suitable activated acids (acetic, *p*-hydroxybenzoic, *p*-coumaric, and others are known in nature) via transferase enzymes to monolignol esters **2**, presumably before diffusing into the cell wall. Esters **2**, along with monolignols **1**, can then be incorporated via radical coupling reactions into lignins **4** with partial γ-acylation (pathway a). Alternatively, normal lignins **3** can be subsequently acylated (pathway b). The normal DFRC protocol cleaves α- and β-ethers (in un-acylated lignins **3**) releasing, following acetylation, quantifiable monomers **5**. Lignin γ-esters (such as in **4**) are not cleaved during the normal DFRC protocol and the method can be used for identifying *p*-coumarate, *p*-hydroxybenzoate (and presumably other) esters (not specifically shown in this figure). A modification to the DFRC protocol, substituting all acetate-based reagents with their propionate analogues (DFRC' protocol), allows determination of acetates that are naturally on lignin γ-positions. Right: GC-MS total-ion chromatograms of monomers from the modified DFRC procedure applied to a) isolated Tainung kenaf lignin, b) Tainung kenaf whole-cell walls, c) isolated aspen lignin. Components **S-6** and **G-6** (bold peaks) were from acetylated units in the original lignin; **S-7** and **G-7** were from normal (un-acylated) units. *c = cis, t = trans.* The peak labeled **S-6t** in c) contains a significant co-eluting component that was not identified. GC-MS is therefore crucial for compound authentication.

The modified DFRC procedure also allowed detection of minor acetate components in hardwood lignins (Ralph and Lu, 1998). The chromatogram of DFRC monomers from isolated aspen lignin (Figure 6, bottom) also showed a small 4-propoxy-sinapyl-γ-acetate **S-6** peak, confirmed by MS. The analogous guaiacyl **G-6** peak could not be detected. The methods applied here do not exclude acetylation at other positions in hardwoods, but apparently, as in kenaf, a similarly selective transferase enzyme exists in aspen for acetylating sinapyl alcohol prior to its export to the wall for lignification.

***p*-Coumaroylated Lignins in Grasses**

All grasses have lignins that are acylated by *p*-coumaric acid. Early attempts at establishing the acylation regiochemistry (the site on lignin where the acylation occurs) utilized UV and concluded that *p*-coumarates were mainly at the γ-position of lignin sidechains (Shimada *et al.*, 1971; Nakamura and Higuchi, 1976). NMR work on corn lignin, and subsequently on wheat (Crestini and Argyropoulos, 1997), and other grasses (Ralph *et al.*, 1999b) showed that the acylation was exclusively at the γ-position, implicating enzymatic processes in the formation of the ester. Thioacidolysis confirmed the presence of γ-acylation, although esters are partially cleaved (Grabber *et al.*, 1996). The DFRC method, which leaves such γ-esters intact, further established the γ-acylation and, as for acetates, indicated that *p*-coumarates were predominantly on syringyl units (Lu and Ralph, 1999a).

When Does Acylation Occur?

Does the acylation occur at the monomer stage or following lignification coupling reactions (See Figure 6)? From examination of naturally acetylated, *p*-coumaroylated, and other acylated lignins, there is reasonable, but not conclusive, evidence that acylated lignins are created from pre-acylated monomers. Perhaps the most compelling argument is that all types of units in corn are *p*-coumaroylated; this includes both β-aryl ether isomers, β–5 units, and even hydroxycinnamyl alcohol endgroups (Quideau, 1994; Ralph *et al.*, 1994a). It is unlikely that the specific enzyme reaction required for this acylation would be non-specific enough to acylate such a variety of structures without also acylating α-OHs or even polysaccharide primary alcohols. However, unambiguous proof of monomer pre-acylation does not currently exist. Our group is currently examining an approach using the DFRC method to provide such proof.

Although not unambiguously established, it is logical that acylated lignin monomers can also couple and cross-couple with normal (unacylated) monolignols to form a lignin polymer in which some units are acylated. Thus sinapyl acetate and sinapyl *p*-coumarate are examples of possible "monomers" for lignification. Here we will use the term "monolignol conjugate" to describe these entities, since calling sinapyl *p*-coumarate a monomer can lead to confusion. The ability of lignification to incorporate monomers (or conjugates) other than the three primary monolignols is examined further below.

One initially puzzling feature of grass lignins is that the *p*-coumarate units remain free-phenolic. That is, the *p*-coumarate unit does not appear to enter into the radical-coupling lignification reactions. It is a phenol, and it is possible to make polymers from *p*-coumarate using radical-generating conditions such as peroxidase/H_2O_2. Furthermore, *p*-coumarates will cross-couple into a coniferyl alcohol DHP as might be expected. The reasons for its non-incorporation into natural grass lignins are now becoming clear. *p*-Coumarate radicals are particularly good at radical transfer reactions. Thus, although *p*-coumarate radicals are readily formed by peroxidase, they will undergo radical transfer with sinapyl alcohol or free-phenolic syringyl groups on a growing lignin oligomer regenerating *p*-coumarate and producing the sinapyl/syringyl radical (Takahama and Oniki, 1994; Hatfield *et al.*, 1997; Hatfield *et al.*, 1999a). This helps to explain how corn can make a syringyl-rich lignin despite having peroxidases which oxidize sinapyl alcohol only extremely poorly. Unless the system is therefore overabundant in radical producing capability (as is a normal DHP system), the *p*-coumarate is likely to remain free phenolic and uncoupled. It is suspected that *p*-hydroxybenzoates (in some grasses, notably bamboo, and some hardwoods, notably willow and aspen) may function similarly.

Ferulate Incorporation into Grass Lignins

A great deal is now known about ferulates in grasses (Ishii, 1997; Ralph *et al.*, 1998a; Hatfield *et al.*, 1999b). Dimerization of ferulate-polysaccharide esters produces a whole range of diferulates (not just the 5-5-diferulate that was the only one characterized for some 20 years) and results in polysaccharide-polysaccharide cross-linking (Ralph *et al.*, 1994b). Cross-coupling of ferulates with lignin monomers (and possibly oligomers) incorporates ferulates intimately into lignin in a variety of structures (from which ferulate cannot be fully released or quantified), and results in lignin-polysaccharide cross-linking (Ralph *et al.*, 1992; Ralph *et al.*, 1998a). There is growing evidence that ferulates act as nucleation sites for lignification in grasses (Ralph *et al.*, 1995). Beyond that, diferulates also incorporate into lignins via radical coupling processes forming extensive lignin-polysaccharide-polysaccharide cross-linking (Quideau and Ralph, 1997). Each of these cross-linking mechanisms negatively impacts the availability of polysaccharides for utilization by, for example, ruminant microorganisms in ruminant animals (Grabber *et al.*, 1998a; Grabber *et al.*, 1998c; Grabber *et al.*, 1998b).

Low levels of ferulates in dicots may function in a similar way, but this has not been directly proven. Other ferulates, such as tyramine ferulate, can be incorporated into wound-response lignins, in tobacco for example (Negrel and Martin, 1984; Negrel and Jeandet, 1987; Ralph *et al.*, 1998b). Tyramine ferulate is found associated with lignins in healthy tobacco plants and may therefore be a product naturally incorporated into the lignin polymer by radical coupling processes. Its content is markedly increased in cinnamoyl-Co-A deficient transgenics, although these plants have markedly reduced vigor (see below). Whether it is a true component of cell wall lignin remains to be determined.

Are ferulates (and diferulates) then to be considered lignin "monomers"? Obviously ferulate is not one of the three accepted monolignols. Yet it behaves like a monomer, radically cross-coupling with monolignol radicals and fully and inextricably incorporating into the phenylpropanoid polymer. In grasses, it is likely that most lignin molecules have ferulate incorporated into them. Ferulates incorporated this way therefore analyze as lignin and there is no way to isolate or separate lignins from them. We suggest therefore that ferulates are a natural component of lignins in grasses.

Expanding the Definition of Lignins

It is important to note that not every entity that is "attached" to lignin should be considered a lignin component. The central precept of lignification is radical coupling of phenols. This concept must remain. One group has recently tried to attribute to us the absurd notion that anything that is attached to lignin should be called lignin (Kasahara *et al.*, 1999). The idea that xylans, for example, are lignins just because they are attached is clearly ludicrous; xylans are not phenols and are not incorporated into lignins by radical coupling reactions. Although the concept of lignification must be expanded from the strict definition of lignins as polymers derived solely from the three hydroxycinnamyl alcohol monolignols, as recognized in the early treatises on lignification (Sarkanen and Ludwig, 1971), the core concept must remain. Lignins are also typically referred to as phenylpropanoid polymers as this is more encompassing. We would like to suggest that hydroxyphenylpropanoids, and their conjugates, that can incorporate as "monomers" into phenylpropanoid polymers, via radical coupling reactions might be considered lignin components. This will be addressed again below, following a discussion of lignins in lignin-biosynthetic-pathway mutants and transgenics.

Lignins in Lignin-Biosynthetic-Pathway Mutants and Transgenics

Researchers have recently been interested in understanding how plants respond to deviations in the normal lignification process caused by natural or biogenetic down- or up-regulation of key genes responsible for enzymes in the monolignol biosynthetic pathway. As recently stated by Stuart L. Schreiber (Anonymous, 1999); "If you want to understand something it's very useful if you can perturb it. Complex biological systems can be thought of as complex circuitry. You need ways to break circuits, turn them on, turn them off, modulate them, in order to understand them." As we are learning, however, the lignification system has considerable metabolic plasticity. Breaking circuits does not necessarily turn off the process.

The potential to improve plant utilization by ruminants and in various other natural and industrial processes (such as chemical pulping) by engineering the amount, composition, and structure of lignin is currently attracting considerable interest. The major approach along these lines is to target enzymes of the monolignol biosynthetic pathway, as recently reviewed (Boudet *et al.*, 1993; Halpin *et al.*, 1994; Boudet and Grima-Pettenati, 1996; Boudet, 1998; Whetten *et al.* 1998). Various plants with deficiencies in lignin-biosynthetic-pathway genes (and consequent enzymes for monolignol synthesis) provide insights into the flexibility of the lignification process and aid in our understanding of "normal" lignification.

Aspen Pt-4CL Transgenics. Chiang's group have generated transgenic aspen with suppressed expression of 4-coumaric acid:coenzyme-A ligase (Pt4CL1) (Tsai *et al.*, 1994; Hu *et al.*, 1998). The enzyme functions early in the monolignol pathway and the researchers reasoned that this was a good target for effecting general lignin down-regulation. The downregulation of lignin was not only striking (up to a 45% reduction), but was also accompanied by a 15% increase in cellulose — the cellulose:lignin ratio nearly doubled (Hu *et al.*, 1999). The benefits of vigorous plants with these properties for pulp and paper production and in forages are obvious.

Milled wood lignins from a control (42% of the total Klason lignin) and the most heavily down-regulated transgenic (28% of the total original Klason lignin) were examined by NMR (Hu *et al.*, 1999). The lignin in the transgenic was not significantly different from that in the control, validating the choice of this strategy for effecting a general reduction in the amount of lignins in plants (without massively affecting the composition).

The plant appears to be supplanting its reduced lignin with additional cellulose. If the required defense, water transport, and other properties survive, as they appear to in these particularly vigorous plants, the potential for improving plant utilization is enormous.

Arabidopsis F5H-Mutants and Transgenics. An illustration of the massive compositional changes that plants can tolerate is strikingly revealed in mutants and transgenics of ferulate 5-hydroxylase (F5H) from Chapple's group (Meyer *et al.*, 1998; Marita *et al.*, 1999; Ralph *et al.*, 1999b). Ferulate 5-hydroxylase (enzyme F5H) is the first enzyme that controls the production of sinapyl alcohol and therefore syringyl units (Meyer *et al.*, 1996; Meyer *et al.*, 1997; Chapple, 1998; Meyer *et al.*, 1998). An F5H-deficient mutant, produced by chemical mutagenesis, is almost totally devoid of syringyl components, producing a guaiacyl lignin that would be typical of a softwood (rather than the dicot it is). More impressively, upregulating F5H in the mutant produces transgenics that are almost 100% syringyl (and devoid of guaiacyl units). Thus the lignin composition can be altered from essentially 100%G/0%S to 0%G/100%S in a single generation, without the benefit of evolutionary adaptation. Both lignins are otherwise "normal." The syringyl content of these transgenics is significantly higher (by a factor of 4!) than in any plant reported to date (Marita *et al.*, 1999; Ralph *et al.*, 1999b). The up-regulation is therefore strikingly effective at diverting the monolignol pool into almost entirely sinapyl alcohol. This example is one in which massive compositional shifts have been effected, but in the traditional lignin components. As will be seen, compositional shifts can range considerably wider.

Pine CAD-deficient Mutant. A CAD-deficient pine mutant was identified and characterized by workers at North Carolina State University (MacKay, 1997; MacKay *et al.*, 1997). The pine, despite a reduction in CAD levels to less than 1% of normal levels, appeared to be producing normal levels of lignin (Klason lignin values were almost identical). A possible interpretation then is that the CAD-deficiency is not sufficient to change the system and that there is still sufficient flux through the residual enzyme levels that normal lignification can proceed. The lignin in the CAD-deficient pine mutant however was highly colored and stained strongly with phloroglucinol, suggesting a higher aldehyde component. From NMR studies on isolated lignins, it was clear that aldehydes were substantial components of that lignin (Ralph *et al.*, 1997). New aldehyde components were misidentified originally (Ralph *et al.*, 1997), but corrected in subsequent publications (Ralph *et al.*, 1998b; Ralph *et al.*, 1999b). A book chapter on NMR even details how the incorrect assignment was made (Ralph *et al.*, 1999b). The aldehyde components now clearly all stem from incorporation into the "lignin" of more significant quantities of coniferaldehyde (and its product vanillin) than in normal pine. Is this aldehyde component part of the lignin? The low levels seen in normal lignins could easily result from post-lignification oxidation, but the levels seen in this and other CAD-deficient plants (see tobacco below) suggest that incorporation of hydroxycinnamaldehydes (and hydroxybenzaldehydes) into lignins is a normal process that becomes more significant when the levels of monolignols are insufficient for lignification.

Even more striking, however, was the observation (Ralph *et al.*, 1997) of substantial levels of dihydroconiferyl alcohol (DHCA) in the mutant's lignin. Lewis claimed that DHCA was not a monomer and only resulted from post coupling ancillary lignan reactions (Gang *et al.*, 1998; Lewis *et al.*, 1998). Our evidence that DHCA had to be a monomer in the production of the phenolic polymer we isolated was several-fold. Firstly, DHCA monomer was released in considerable amounts in extracts from the wood of the mutant pine. Secondly, degradation by our DFRC method (Derivatization Followed by Reductive Cleavage) produced significant quantities of DHCA proving that they were etherified. Even though they are likely to more normally couple in ways that cannot release the monomer (5–5 and 5–O–4 or 4–O–5) the amount released was still comparable to the (lower level of) coniferyl alcohol that was released. And the amounts released were significant whether the sample was the isolated lignin, the lignin residue following isolation, or the whole wood. These observations have recently been independently borne out by thioacidolysis (Lapierre *et al.*, 1999). Thirdly, and far more compellingly, NMR showed that about half of the DHCA units were involved in 5–5-coupled structures (Ralph, 1997; Ralph *et al.*, 1997). As anyone who has studied dimerization of coniferyl alcohol knows, the coupling almost entirely involves at least one coniferyl alcohol coupling at its β-position; the observed dimers are the β–β- (pinoresinol), the β–5- (phenylcoumaran), and the β–O–4-dimers. Under no conditions reported to date have 5–5-coupled dimers been reported as substantial products. How then can 50% of the DHCA units be 5–5-coupled if they arise post-dimerization? Obviously they do not. The logical conclusion is that it is DHCA monomer that is undergoing the radical coupling reactions. As we will show below, there is now even more evidence for this assertion. It is also logical, since

DHCA is known to prefer coupling reactions with radicals of its own kind (rather than coniferyl alcohol radicals), that much of the 5–5-DHCA units likely arise from such homo-coupling reactions when the levels of DHCA are so significant. It should be noted that evidence for DHCA units has been provided in numerous normal lignins (Lundquist and Stern, 1989; Fukagawa *et al.*, 1991; Brunow *et al.*, 1998; Ralph *et al.*, 1999b).

GC-MS of DFRC dimers (Peng *et al.*, 1998) from the mutant vs a normal pine reinforces the striking differences, Figure 7. The dimers, like thioacidolysis dimers, don't represent a huge fraction of the lignin, just those units that can be released by ether-cleavage reactions. But they can be used as diagnostic fingerprints of the polymer structure. In the normal lignin, we can identify and recognize anticipated dimers from a softwood lignin. These dimers are present in small amounts in the mutant's product, but are overwhelmed by a series of new dimers. From the fingerprint alone, it is obvious that these two lignins must differ greatly. The new dimers arise from DHCA (and DHCA-derived) units and aldehyde units in various combinations. Incidentally, the same products in similar distributions arise when whole-cell-wall samples, rather than simply the isolated lignins, are subjected to DFRC.

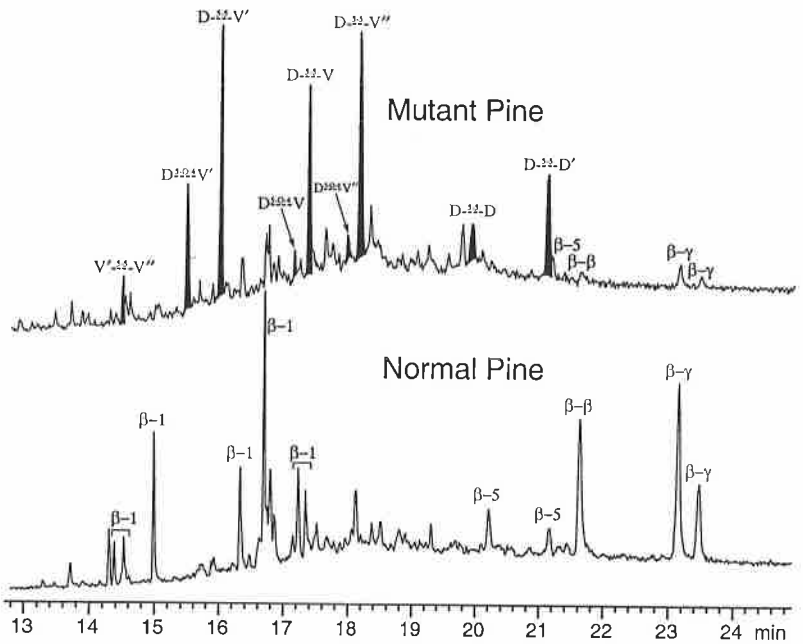

Figure 7. DFRC dimers from normal and mutant pines. DFRC dimers from normal vs mutant pine lignins.

Although the normal dimers can be detected in the CAD-deficient mutant, they are overwhelmed by new products that are insignificant in normal pine. These new dimers are all various combinations of DHCA (D) (or its derived guaiacylpropane-1,3-diol (D')) and vanillin (V) or its DFRC-derived products (V', V''). The same products appear in similar distributions whether the DFRC-degradation is performed on the isolated lignins or whole-cell-wall materials. From the fingerprint alone, it is obvious that the two lignins are significantly different.

Newly identified components of the mutant pine's lignin. Recently we have been able to identify another major new component in the lignin of the mutant pine. Again, this 'new' component is seen at trace levels in the normal pine. The units are arylpropane-1,3-diols, Figure 8. At first it is difficult to understand how these can arise, but in fact they stem directly from peroxidase reactions of DHCA. Figure 8 shows the NMR proof of the presence of these structures in the mutant's isolated lignin. This new unit provides additional proof that DHCA is a monomeric component of the phenolic polymer. Some of the new peaks in the DFRC dimer fraction from the mutant are attributable to coupling products of this new arylpropane-1,3-diol unit with DHCA and aldehyde units. Finding the oxidized α-keto-product (in collaboration with Jay Scott, NC State) provided further validation of the production of arylpropane-1,3-diols.

Is this polymer lignin?? The mutant and the normal pine have similar lignin levels as measured by Klason analysis. If lignins cannot, by definition, contain substantial portions of DHCA, DHCA-derived products, and aldehydes, then the mutant obviously has very little, if any, lignin. That is great news! It is one of the prime

objectives of biogenetic engineering work in this area to reduce the level of lignin. But if the lignin reduction comes *only* because of semantics, because of the definition of lignin, and not by a real reduction in the phenolic polymer component (that may be functioning as lignin in the plant!), then obviously no real benefits (for pulping, for example) have been gained. The question of whether the plant utilizes this polymer as lignin awaits further debate and experimentation. At this point, since these DHCA and aldehyde components appear to be likely components of normal lignins, we think this is merely another example of a broad compositional shift.

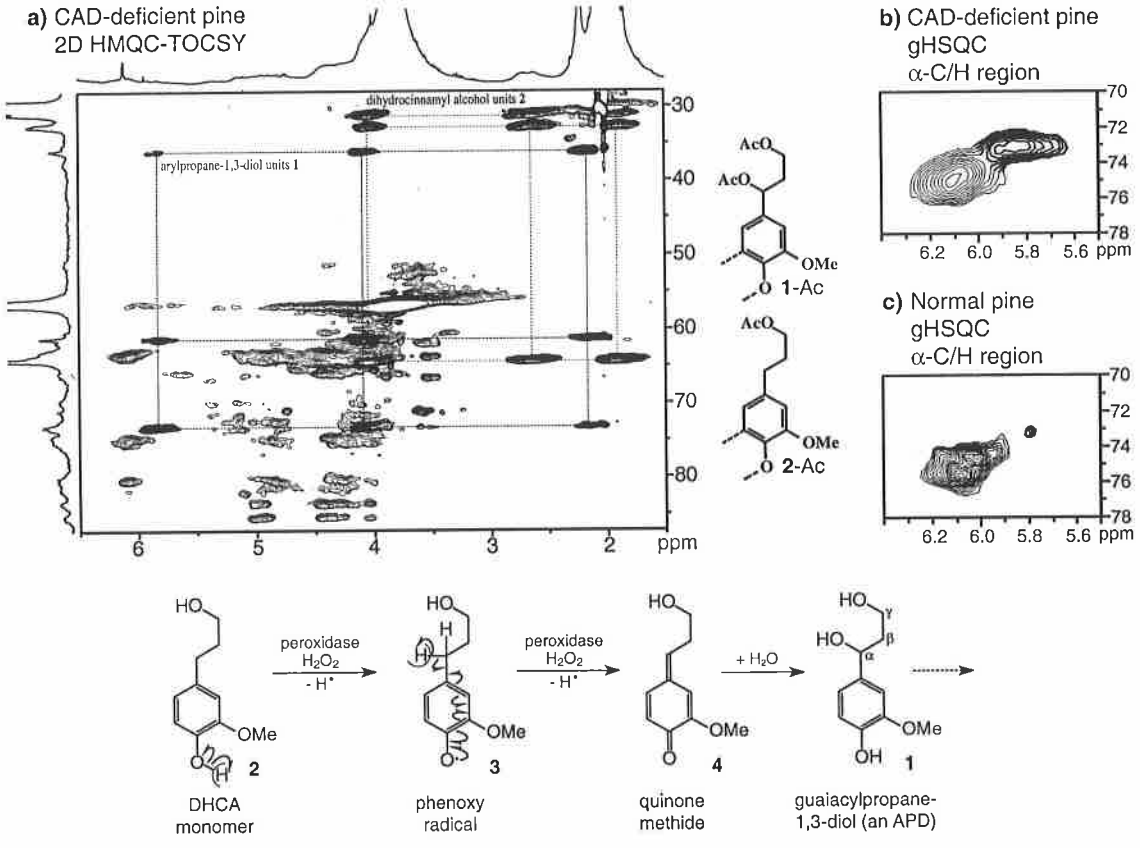

Figure 8. NMR spectra showing new APD structures. Left: HMQC-TOCSY showing APDs (bold), along with DHCA units previously identified. Figs. **b-c** are expansions from HSQC spectra showing the APD units' Hα/Cα□correlations in normal and mutant pine.

Tobacco CAD- and CCR-Down-regulated Transgenics. Tobacco CAD-deficient transgenics were produced in Alain Boudet's group (Piquemal et al., 1998; Yahiaoui et al., 1998). Again, the lignin levels in a CAD-downregulated transgenic were similar to the control, and fibers had the brown coloration. And again, the CAD-down-regulated transgenics showed a significant buildup of aldehyde components. New products due to homo- and hetero-coupling reactions of coniferaldehyde/sinapaldehyde are prevalent and are analogous to the coniferaldehyde products seen in the CAD-deficient pine mutant. No DHCA units were found, but there may be no pathway for their upregulation — DHCA is seen less commonly in NMR spectra of lignins of plant materials other than softwoods, and is not at detectable levels in any of the tobacco isolated lignins.

Transgenics with CCR (cinnamoyl-CoA reductase) downregulated (Piquemal *et al.*, 1998) by antisense methods had significantly lower (~50%) lignin contents in plants that were clearly less vigorous. The isolated "lignin" showed a striking increase in tyramine ferulate, which was shown to be incorporated in as an integral component of the polymer (Ralph *et al.*, 1998b). Tyramine ferulate is a logical sink-product for feruloyl-CoA when CCR is down-regulated in tobacco. However, it is a normal wounding response product and there are again issues of whether this component is truly incorporated into structural lignin, into suberized components (Bernards and Lewis, 1998), or simply represents polymerized secondary metabolites similar to those observed in a wounding response. However, the aliphatic components normally associated with suberins are not significant in these extracted lignins. Also, tyramine components are not elevated in the antisense-CAD

tobacco which was presumably also stressed. The dilemma of deciding whether tyramine ferulate is therefore a lignin component or not remains. (Is wound-response lignin still considered lignin?).

OMT-Deficient Plants. Naturally occurring mutants (*e.g.*, the brown-midrib (bm3) mutants of maize and sorghum and transgenic plants deficient in *O*-methyl transferase (OMT) contain significant amounts of units derived from 5-hydroxyconiferyl alcohol, as revealed in beautiful thioacidolysis studies (Lapierre *et al.*, 1988; Lapierre, 1993; Atanassova *et al.*, 1995; Doorsselaere *et al.*, 1995). We have not yet looked at such plants (by NMR) but it seems logical that the unmethylated precursors to sinapaldehyde could be incorporated into lignins if they were to build up in the system due to reduced OMT activity. Is it also reasonable that 3,4-dihydroxycinnamyl alcohol could similarly incorporate.

Components in Lignins, Summary

There are many known lignins that are derived from incorporation of precursors or derivatives of the three monolignols, i.e. from components other than the monolignols themselves. The idea that other components are incorporated into lignins is nothing new. If they are truly foreign components from other pathways that are not laid down during lignification, then they should be distinguished. But where the polymer contains intimately incorporated components that can only arise from radical coupling reactions, particularly involving monomers, then nature may be defining them as lignins even though some of us can not. Many lignins, for example, are biosynthesized by incorporating esterified monolignols into the lignification scheme. As detailed above, grasses utilize *p*-coumarates, hardwoods and some dicots such as kenaf utilize acetates, and some plants, notably bamboo, aspen and willow, use *p*-hydroxybenzoates as 'monomers' for lignification. Ferulates and diferulates are found intimately incorporated into all grass and some dicot lignins, where they are equal partners in the free-radical polymerization process and may even be nucleation sites for lignification. If aldehydes, tyramine hydroxycinnamates, dihydro-monolignols, 5-hydroxyconiferyl alcohol, and other precursors from the studies on mutants and transgenics are considered, it becomes clear that a general definition of lignin must include more than the traditional three hydroxycinnamyl alcohols, Figure 9. If not, the phenolic polymers serving the structural and functional role of lignin in many plants, *e.g.*, in grasses, might not be considered lignin.

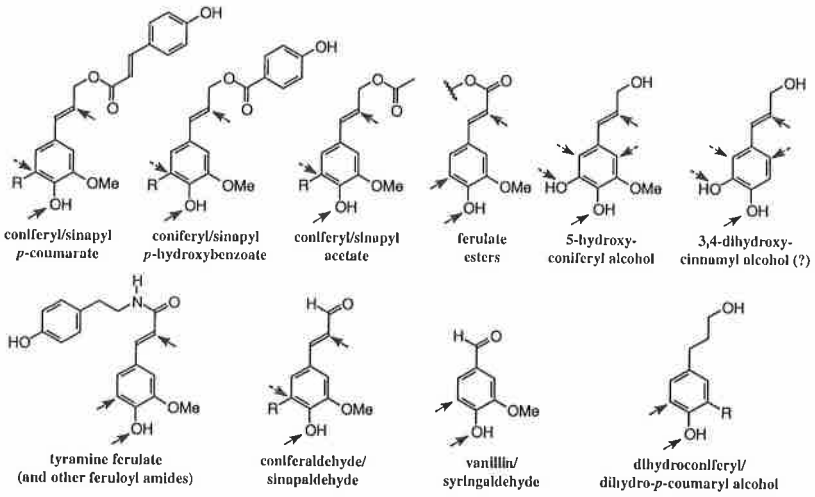

Figure 9. Compounds which function like "monomers" in free-radical coupling reactions that typify lignification. Arrows show sites available for radical coupling.

CONCLUSIONS

Lignin remains a complex enigma, and structural details of the phenylpropanoid polymers in plants are still emerging. Applications of new and traditional methods continue to reveal a fascinating complexity. The number of "monomers" or "monomer conjugates" that undergo radical coupling reactions with typical monolignols to generate complex phenylpropanoid polymers continues to grow. Whether these are true lignins or not continues to be debated. However, components that act as monomers, coupling and cross-coupling with monolignols and oligolignols to become intimately incorporated into the structure are obviously important components of the plant's architecture. These components will always analyze as lignin and for some industries such as the pulp and paper industry, will remain the troublesome polymeric phenolic

components that must be removed during pulping or bleaching to produce high-end products such as white paper. Structural analysis of normal and perturbed plant systems reveals a lot about the phenylpropanoid component and interacting biochemical pathways, providing a basis for in-depth biochemical studies. Structural elucidation remains a key entry point into understanding these complex plant systems. Instrumental and degradative methods continue to evolve to provide increasingly sophisticated probes to detail structural and mechanistic aspects of lignification.

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REFERENCES

Adler, E. (1977). Lignin chemistry — past, present and future. *Wood Sci. Technol.*, **11**, 169-218.

Anonymous (1999). Using synthesis to explain life's unsolved mysteries. *Chem. and Eng. News*, 44-46.

Atanassova, R., Favet, N., Martz, F., Chabbert, B., Tollier, M.-T., Monties, B., Fritig, B. & Legrand, M. (1995). Altered lignin composition in transgenic tobacco expressing O-methyltransferase sequences in sense and antisense orientation. *Plant J.*, **8**, 465-477.

Bernards, M.A. & Lewis, N.G. (1998). The macromolecular aromatic domain in suberized tissue: a changing paradigm. *Phytochem.*, **47**, 915-933.

Boudet, A.-M. (1998). A new view of lignification. *Trends in plant science*, **3**, 67-71.

Boudet, A.M. & Grima-Pettenati, J. (1996). Lignin genetic engineering. *Mol. Breed.*, **2**, 25-39.

Boudet, A.M., Inze, D.G. & Schuch, W.W. (1993). Modification of lignin synthesis in plants with sense and biosynthesis. *PCT Int. Appl.*, **68**.

Brunow, G. (1998). Oxidative coupling of phenols and the biosynthesis of lignin. In Lignin and Lignan Biosynthesis, ed. Lewis, N.G. & Sarkanen, S. American Chemical Society, Washington, DC, pp. 131-147.

Brunow, G., Ämmälahti, E., Niemi, T., Sipilä, J., Simola, L.K. & Kilpeläinen, I. (1998). Labelling of a lignin from suspension cultures of *Picea abies*. *Phytochem.*, **47**, 1495-1500.

Brunow, G., Sipilä, J., Syrjänen, K., Karhunen, P., Setälä, H. & Rummakko, P. (1995). Oxidative coupling of phenols and the biosynthesis of lignin. *8th International Symposium on Wood and Pulping Chemistry,* Proceedings, **Vol. I**, pp 33-35.

Chapple, C. (1998). Molecular genetic analysis of plant cytochrome P450-dependent monooxygenases. *Ann. Rev. Plant Phys. and Plant Mol. Biol.*, **49**, 311-343.

Chen, C.-L. (1998). Characterization of milled wood lignins and dehydrogenative polymerisates from monolignols by carbon-13 NMR spectroscopy. In *Lignin and Lignan Biosynthesis*, ed. Lewis, N.G. & Sarkanen, S. American Chemical Society, Washington, DC, pp. 255-275.

Chen, F., Fukushima, K. & Yasuda, S. (1998). New pathway on monolignol biosynthesis. *48th annual meeting of the Japan wood research society,* Proceedings, pp 397.

Chen, F., Yasuda, S. & Fukushima, K. (1999). Evidence for a novel biosynthetic pathway that regulates the ratio of syringyl to guaiacyl residues in lignin in the differentiating xylem of *Magnolia kobus*. *Planta*, **207**, 597-603.

Crestini, C. & Argyropoulos, D.S. (1997). Structural analysis of wheat straw lignin by quantitative ^{31}P and 2D NMR spectroscopy. The occurrence of ester bonds and α-O-4 substructures. *J. Agric. Food Chem.*, **45**, 1212-1219.

Davin, L.B., Wang, H.-B., Crowell, A.L., Bedgar, D.L., Martin, D.M., Sarkanen, S. & Lewis, N.G. (1997). Stereoselective biomolecular phenoxy radical coupling by an auxiliary (Dirigent) protein without an active center. *Science*, **275**, 362-366.

Doorsselaere, J.V., Baucher, M., Chognot, E., Chabbert, B., Tollier, M.-T., Petit-Conil, M., Leplé, J.-C., Pilate, G., Cornu, D., Monties, B., Van Montagu, M., Inzé, D., Boerjan, W. & Jouanin, L. (1995). A novel lignin in poplar trees with a reduced caffeic acid/5-hydroxyferulic acid O-methyltransferase activity. *Plant J.*, **8**, 855-864.

Freudenberg, K. & Neish, A.C. (1968). Constitution and Biosynthesis of Lignin. Springer-Verlag, Berlin-Heidelberg-New York.

Fukagawa, N., Meshitsuka, G. & Ishizu, A. (1991). A two dimensional NMR study of birch milled wood lignin. *J. Wood Chem. Technol.*, **11**, 373-396.

Galkin, S., Ämmälahti, E., Kilpeläinen, I., Brunow, G. & Hatakka, A. (1997). Characterization of milled wood lignin from Reed Canary grass (*Phalaris aruncinacea*). *Holzforschung*, **51**, 130-134.

Gang, D.R., Fujita, M., Davin, L.D. & Lewis, N.G. (1998). The 'abnormal lignins': mapping heartwood formation through the lignan biosynthetic pathway. In *Lignin and Lignan Biosynthesis*, ed. Lewis, N.G. & Sarkanen, S. Amer. Chem. Soc., Washington, DC, pp. 389-421.

Grabber, J.H., Hatfield, R.D. & Ralph, J. (1998a). Diferulate cross-links impede the enzymatic degradation of nonlignified maize walls. *J. Sci. Food Agric.*, **77**, 193-200.

Grabber, J.H., Quideau, S. & Ralph, J. (1996). *p*-Coumaroylated syringyl units in maize lignin; implications for β-ether cleavage by thioacidolysis. *Phytochemistry*, **43**, 1189-1194.

Grabber, J.H., Ralph, J. & Hatfield, R.D. (1998b). Ferulate cross-links limit the enzymatic degradation of synthetically lignified primary walls of maize. *J. Agric. Food Chem.*, **46**, 2609-2614.

Grabber, J.H., Ralph, J. & Hatfield, R.D. (1998c). Modeling lignification in grasses with monolignol dehydropolymerisate-cell wall complexes. In Lignin and Lignan Biosynthesis, ed. Lewis, N.G. & Sarkanen, S. American Chemical Society, Washington, DC, pp. 163-171.

Halpin, C., Knight, M.E., Foxon, G.A., Campbell, M.M., Boudet, A.-M., Boon, J.J., Chabbert, B., Tollier, M.-T. & Schuch, W. (1994). Manipulation of lignin quality by downregulation of cinnamyl alcohol dehydrogenase. *Plant J.*, **6**, 339-350.

Harkin, J.M. (1967). Lignin – a natural polymeric product of phenol oxidation. In *Oxidative Coupling of Phenols*, ed. Taylor, W.I. & Battersby, A.R. Marcel Dekker, New York, pp. 243-321.

Harkin, J.M. (1973). Lignin. In *Chemistry and Biochemistry of Herbage*, ed. Butler, G.W. Academic Press, London, pp. 323-373.

Hatfield, R.D., Grabber, J. & Ralph, J. (1997). A potential role of sinapyl *p*-coumarate in grass lignin formation. *Annual Meeting of the American Society of Plant Physiologists,* Proceedings, **Vol. 114**, pp #346, p. 86.

Hatfield, R.D., Grabber, J.H. & Ralph, J. (1999a). A potential role of sinapyl *p*-coumarate in the formation of sinapyl alcohol-enriched lignins of grasses. *Plant Physiology*, in preparation.

Hatfield, R.D., Ralph, J. & Grabber, J.H. (1999b). Cell wall cross-linking by ferulates and dehydrodiferulates in grasses. *J. Sci. Food Agric.*, **79**, 403-407.

Hu, W.-J., Kawaoka, A., Tsai, C.-J., Lung, J., Osakabe, K., Ebinuma, H. & Chiang, V., L. (1998). Compartmentalized expression of two structurally and functionally distinct 4-coumarate:CoA ligase genes in aspen (Populus tremuloides). *Proc. Nat. Acad. Sci.*, **95**, 5407-5412.

Hu, W.-J., Lung, J., Harding, S.A., Popko, J.L., Ralph, J., Stokke, D.D., Tsai, C.-J. & Chiang, V.L. (1999). Repression of lignin biosynthesis in transgenic trees promotes cellulose accumulation and growth. *Nature Biotechnol.*, **17**, 808-812.

Ishii, T. (1997). Structure and functions of feruloylated polysaccharides. *Plant Science*, **127**, 111-127.

Karhunen, P., Mikkola, J., Parjunen, A. & Brunow, G. (1999). The behaviour of dibenzodioxocin structures in lignin during alkaline pulping processes. *Nordic Pulp and Paper Research Journal*, **14**, 123-128.

Karhunen, P., Rummakko, P., Sipilä, J., Brunow, G. & Kilpeläinen, I. (1995a). Dibenzodioxocins; a novel type of linkage in softwood lignins. *Tetrahedron Lett.*, **36**, 169-170.

Karhunen, P., Rummakko, P., Sipilä, J., Brunow, G. & Kilpeläinen, I. (1995b). The formation of dibenzodioxocin structures by oxidative coupling. A model reaction for lignin biosynthesis. *Tetrahedron Lett.*, **36**, 4501-4504.

Kasahara, H., Gang, D.R., Xia, Z.-Q., Jiao, Y., Bedgar, D.L., Davin, L.B. & Lewis, N.G. (1999). Monolignol partitioning contradicts any notions beyond randomness. *Tenth International Symposium on Wood and Pulping Chemistry,* Proceedings, **Vol. 2**, pp 22-27.

Lapierre, C. (1993). Application of new methods for the investigation of lignin structure. In Forage Cell Wall Structure and Digestibility, ed. Jung, H.G., Buxton, D.R., Hatfield, R.D. & Ralph, J. ASA-CSSA-SSSA, Madison, WI, pp. 133-166.

Lapierre, C., Pollet, B., MacKay, J.J., Dimmel, D.R. & Sederoff, R.R. (1999). Lignin structure in a mutant pine deficient in cinnamyl alcohol dehydrogenase. *Tenth International Symposium on Wood and Pulping Chemistry,* Proceedings, **Vol. 1**, pp 520-523.

Lapierre, C., Tollier, M.T. & Monties, B. (1988). A new type of constitutive unit in lignins from the corn bm3 mutant. *C. R. Acad. Sci., Ser. 3*, **307**, 723-728.

Lewis, N.G. & Davin, L.B. (1998). The biochemical control of monolignol coupling and structure during lignan and lignin biosynthesis. In *Lignin and Lignan Biosynthesis*, ed. Lewis, N.G. & Sarkanen, S. Amer. Chem. Soc., Washington, DC, pp. 334-361.

Lewis, N.G., Davin, L.B. & Sarkanen, S. (1998). Lignin and lignan biosynthesis: distinctions and reconciliations. In *Lignin and Lignan Biosynthesis*, ed. Lewis, N.G. & Sarkanen, S. Amer. Chem. Soc., Washington, DC, pp. 1-27.

Lu, F. & Ralph, J. (1996). Reactions of lignin model β-aryl ethers with acetyl bromide. *Holzforschung*, **50**, 360-364.

Lu, F. & Ralph, J. (1997a). Derivatization followed by reductive cleavage (DFRC method), a new method for lignin analysis: protocol for analysis of DFRC monomers. *J. Agric. Food Chem.*, **45**, 2590-2592.

Lu, F. & Ralph, J. (1997b). The DFRC method for lignin analysis. Part 1. A new method for β-aryl ether cleavage: lignin model studies. *J. Agric. Food Chem.*, **45**, 4655-4660.

Lu, F. & Ralph, J. (1998a). The DFRC method for lignin analysis. Part 2. Monomers from isolated lignins. *J. Agric. Food Chem.*, **46**, 547-552.

Lu, F. & Ralph, J. (1998b). Efficient ether cleavage in lignins: the "DFRC" method as a basis for new analytical methods. In Lignin and Lignan Biosynthesis, ed. Lewis, N.G. & Sarkanen, S. American Chemical Society, Washington, DC, pp. 294-322.

Lu, F. & Ralph, J. (1999a). Detection and determination of *p*-coumaroylated units in lignins. *J. Agric. Food Chem.*, **47**, 1988-1992.

Lu, F. & Ralph, J. (1999b). The DFRC method for lignin analysis. 7. The behavior of cinnamyl end-groups. *J. Agric. Food Chem.*, **47**, 1981-1987.

Lundquist, K. & Stern, K. (1989). Analyisis of lignins by ^{1}H NMR spectroscopy. *Nordic Pulp and Paper Res. J.*, 210-213.

MacKay, J.J. (1997). A mutation in lignin biosynthesis in loblolly pine: genetic, molecular and biochemical analyses. Ph.D. thesis, North Carolina State, Raleigh.

MacKay, J.J., O'Malley, D.M., Presnell, T., Booker, F.L., Campbell, M.M., Whetten, R.W. & Sederoff, R.R. (1997). Inheritance, gene expression, and lignin characterization in a mutant pine deficient in cinnamyl alcohol dehydrogenase. *Proc. Natl. Acad. Sci. U. S. A.*, **94**, 8255-8260.

Marita, J., Ralph, J., Hatfield, R.D. & Chapple, C. (1999). NMR characterization of lignins in Arabidopsis altered in the activity of ferulate-5-hydroxylase. *Proc. Natl. Acad. Sci. USA*, in press.

Matsumoto, Y., Akiyama, T., Ishizu, A., Meshitsuka, G. & Lundquist, K. (1999). Proof of the presenced of racemic forms of arylglycerol-b-aryl ether structure in lignin — studies on stereo structure of lignin by ozonization. *10th International Symposium on Wood and Pulping Chemistry,* Proceedings, **Vol. 1**, pp 126-129.

Meyer, K., Cusumano, J.C., Ruegger, M.O., Bell-Lelong, D.A. & Chapple, C.C.S. (1997). Regulation and manipulation of lignin monomer composition by expression and over-expression of ferulate 5-hydroxylase, a cytochrome P450-dependent monooxygenase required for syringyl lignin biosynthesis. *Second Internat. Wood Biotechnol. Symp., Canberra, Australia*, paper 9.

Meyer, K., Cusumano, J.C., Somerville, C. & Chapple, C.C.S. (1996). Ferulate-5-hyrdoxylase from Arabidopsis thaliana defines a new family of cytochrome P450-dependent monooxygenases. *Proc. Nat. Acad. Science*, **93**, 6869-6874.

Meyer, K., Shirley, A.M., Cusumano, J.C., Bell-Lelong, D.A. & Chapple, C. (1998). Lignin monomer composition is determined by the expression of a cytochrome P450-dependent monooxygenase in Arabidopsis. *Proc. Natl. Acad. Sci. USA*, **95**, 6619-6623.

Nakamura, Y. & Higuchi, T. (1976). Ester linkage of *p*-coumaric acid in bamboo lignin. *Holzforschung*, **30**, 187-191.

Nakatsubo, F., Ujihara, M. & Katahira, R. (1997). Novel aelective degradation method of the β-O-4 substructure in lignin without any secondary side reactions — TIZ method. *9th International Symposium on Wood and Pulping Chemistry,*Proceedings, **Vol. 1**, pp B3 (1-3).

Negrel, J. & Jeandet, P. (1987). Metabolism of tyramine and feruloyltyramine in TMV inoculated leaves of Nicotiana tabacum. *Phytochemistry*, **26**, 2185-2190.

Negrel, J. & Martin, C. (1984). The biosynthesis of feruloyltyramine in Nicotiana tabacum. *Phytochemistry*, **23**, 2797-2801.

Peng, J., Lu, F. & Ralph, J. (1998). The DFRC method for lignin analysis. Part 4. Lignin dimers isolated from DFRC-degraded loblolly pine wood. *J. Agric. Food Chem.*, **46**, 553-560.

Peng, J., Lu, F. & Ralph, J. (1999a). The DFRC method for lignin analysis. Part 5. Isochroman lignin trimers from DFRC-degraded *Pinus taeda*. *Phytochem.*, **50**, 659-666.

Peng, J. & Ralph, J. (1999). Unique dimers from a CAD-deficient Loblolly pine mutant. *J. Agric. Food. Chem.*, in preparation.

Peng, J., Ralph, J. & Lu, F. (1999b). The DFRC method for lignin analysis. Part 8. Rapid detection of lignin dimers released from DFRC-degraded wood by GC-MS following solid-phase extraction. *J. Agric. Food Chem.*, in preparation.

Piquemal, J., Lapierre, C., Myton, K., O'Connell, A., Schuch, W., Grima-Pettenati, J. & Boudet, A.-M. (1998). Down-regulation of cinnamoyl-CoA reductase induces significant changes of lignin profiles in transgenic tobacco plants. *Plant J.*, **13**, 71-83.

Quideau, S. (1994). Incorporation of *p*-hydroxycinnamic acids into lignins *via* oxidative coupling. Ph.D. thesis, University of Wisconsin-Madison, USA, Madison.

Quideau, S. & Ralph, J. (1997). Lignin-ferulate cross-links in grasses. Part 4. Incorporation of 5–5-coupled diferulate into lignin. *J. Chem. Soc., Perkin Trans. 1*, 2351-2358.

Ralph, J. (1996). An unusual lignin from Kenaf. *J. Nat. Prod.*, **59**, 341-342.

Ralph, J. (1997). Recent advances in characterizing 'non-traditional' lignins and lignin-polysaccharide cross-linking. *9th International Symposium on Wood and Pulping Chemistry,* Proceedings, **Vol. 1**, pp PL2 (1-7).

Ralph, J., Grabber, J.H. & Hatfield, R.D. (1995). Lignin-ferulate crosslinks in grasses: active incorporation of ferulate polysaccharide esters into ryegrass lignins. *Carbohydr. Res.*, **275**, 167-178.

Ralph, J., Hatfield, R.D., Grabber, J.H., Jung, H.G., Quideau, S. & Helm, R.F. (1998a). Cell wall cross-linking in grasses by ferulates and diferulates. In *Lignin and Lignan Biosynthesis*, ed. Lewis, N.G. & Sarkanen, S. American Chemical Society, Washington, DC, pp. 209-236.

Ralph, J., Hatfield, R.D., Piquemal, J., Yahiaoui, N., Pean, M., Lapierre, C. & Boudet, A.-M. (1998b). NMR characterization of altered lignins extracted from tobacco plants down-regulated for lignification enzymes cinnamyl-alcohol dehydrogenase and cinnamoyl-CoA reductase. *Proc. Nat. Acad. Sci.*, **95**, 12803-12808.

Ralph, J., Hatfield, R.D., Quideau, S., Helm, R.F., Grabber, J.H. & Jung, H.-J.G. (1994a). Pathway of *p*-coumaric acid incorporation into maize lignin as revealed by NMR. *J. Amer. Chem. Soc.*, **116**, 9448-9456.

Ralph, J., Helm, R.F., Quideau, S. & Hatfield, R.D. (1992). Lignin-feruloyl ester cross-links in grasses. Part 1. Incorporation of feruloyl esters into coniferyl alcohol dehydrogenation polymers. *J. Chem. Soc., Perkin Trans. 1*, 2961-2969.

Ralph, J., Kim, H., Peng, J. & Lu, F. (1999a). Arylpropane-1,3-diols in lignins from normal and CAD-deficient mutant pines. *Organic Lett.*, **1**, 323-326.

Ralph, J. & Lu, F. (1998). The DFRC method for lignin analysis. Part 6. A modified method to determine acetate regiochemistry on native and isolated lignins. *J. Agric. Food Chem.*, **46**, 4616-4619.

Ralph, J., MacKay, J.J., Hatfield, R.D., O'Malley, D.M., Whetten, R.W. & Sederoff, R.R. (1997). Abnormal lignin in a loblolly pine mutant. *Science*, **277**, 235-239.

Ralph, J., Marita, J.M., Ralph, S.A., Hatfield, R.D., Lu, F., Ede, R.M., Peng, J., Quideau, S., Helm, R.F., Grabber, J.H., Kim, H., MacKay, J.J., Sederoff, R.R., Chapple, C. & Boudet, A.M. (1999b). Solution-state NMR of lignins. In *Progress in Lignocellulosics Characterization*, ed. Argyropoulos, D.S. & Rials, T. TAPPI press, Atlanta, GA, pp. 55-108.

Ralph, J., Peng, J. & Lu, F. (1998c). Isochroman structures in lignin: a new b-1 pathway. *Tetrahedron Lett.*, **39**, 4963-4964.

Ralph, J., Peng, J., Lu, F. & Hatfield, R.D. (1999c). Are lignins optically active? *J. Agric. Food Chem.*, **47**, 2991-2996.

Ralph, J., Quideau, S., Grabber, J.H. & Hatfield, R.D. (1994b). Identification and synthesis of new ferulic acid dehydrodimers present in grass cell walls. *J. Chem. Soc., Perkin Trans. 1*, 3485-3498.

Sarkanen, K.V. & Ludwig, C.H. (1971). Definition and nomenclature. In Lignins, Occurrence, Formation, Structure and Reactions, ed. Sarkanen, K.V. & Ludwig, C.H. Wiley-Interscience, New York, pp. 1-18.

Sederoff, R.R., MacKay, J.J., Ralph, J. & Hatfield, R.D. (1999). Unexpected variation in lignin. *Current Opinion in Plant Biology*, **2**, 145-152.

Shimada, M., Fukuzuka, T. & Higuchi, T. (1971). Ester linkages of *p*-coumaric acid in bamboo and grass lignins. *Tappi*, **54**, 72-78.

Takahama, U. & Oniki, T. (1994). Effects of ascorbate on the oxidation of derivatives of hydroxycinnamic acid and the mechanism of oxidation of sinapic acid by cell wall-bound peroxidases. *Plant Cell Physiol.*, **35**, 593-600.

Tsai, C.-J., Podila, G.K. & Chiang, V.L. (1994). Agrobacterium-mediated transformation of quaking aspen (*Populus tremuloides*) and regeneration of transgenic plants. *Plant Cell Rep.*, **14**, 94-97.

Whetten, R.W., MacKay, J.J. & Sederoff, R.R. (1998). Recent advances in understanding lignin biosynthesis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **49**, 585-609.

Yahiaoui, N., Marque, C., Myton, K.E., Negrel, J. & Boudet, A.-M. (1998). Impact of different levels of cinnamyl alcohol dehydrogenase down-regulation on lignins of transgenic tobacco plants. *Planta*, **204**, 8-15.

Mechanical properties of lignocellulosics/bioplastic composites

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ABSTRACT

In this work the mechanical properties of composites made of lignocellulosics fibers and bioplastic have been investigated. Several lignocellulosics, such as sisal, sugar cane bagasse, wood flour (*Pinus elliottii*), cellulose pulp, rice husk, vegetal sponge (*Luffa cylindrica)* and lignin were tested. Poly-D-(-3-hydroxybutyrate) (PHB) is a bioplastic, a thermoplastic polyester, produced by microorganisms, so-called polyhidroxyalkanoates (PHAs), which constitute a class of natural polymers with physical characteristics close to those of synthetic polymers, with the additional property of being completely biodegradable to carbon dioxide and water through natural microbiological mineralization, although it is water-resistant. Such polyesters are synthesized by bacteria from saccharides or other carbon sources and stored as intracellular inclusion bodies. Among these polymers, the best know PHA is the PHB. The preparations of lignocellulosics/PHB composites was done by mixing the components and were molding in specimens by injection molding technique according to ASTM standard. It was used a load of lignocellulosics fibers in ratio of 15% by weight. Dimensional stability, density, behavior toward some chemical reagents (solubility %) and tests of mechanical properties, such as elongation at break, tensile and flexural strength, tensile and flexural modulus of the composites with different lignocelullosics fibers were evaluated. The results indicate that dimensional stability (immersion in water and oven drying at 100°C, both for 24 hours), density (≈ 1.20 g/cm^3) and mechanical properties of composites were similar compared to pure PHB. Bagasse/PHB composite showed decrease in the mechanical properties compared to pure PHB and the other composites. The composites showed good resistance to attack by chemical reagents (NaOH, HCl, NaCl and CH_3COOH immersed separately in aqueous solution (1.0 mol/L) of the reagents for 24 hours at 25°C using a composite-liquor ratio 1:100), with exception bagasse and rice husks/composites, which had low resistance to NaOH. The results showed that these lignocellulosics fibers could be used as excellent reinforcing materials for low cost composites and are able to satisfy economical as well as ecological interests.

Keywords: lignin, sugar cane bagasse, wood, composites, bioplastic

INTRODUCTION

The natural polymers, in general, have received an increasing interest in the last years at a global level, mainly under environmental concerns related to products that are harmless to the environment. Several studies based on biodegradable polymers derived from lignin, cellulose, starch and others, have been developed due to the fact that this group of materials are made of renewable sources, biodegradable, low cost, as well its good specific mechanical properties (Peijs, 1999; Hänggi, 1998; Narayan, 1999; Shiraishi, 1999; Braunegg, 1998).

Among the natural polymers, there is a class with increasing development in research and production, the bioplastics or biodegradable plastics known as polyhydroxyalkanoates (PHAs) (Braunegg, 1998; Hankermeyer and Tjeerdema, 1999; Leaf and Srienf, 1998; Rehm and Steinbüchel, 1998). The PHAs are biopolyesters stored as reserve polymers by a great number of prokarytotes. The first and best known PHA is polyhydroxybutyrate (PHB). Lemoigne studied its structure in 1926 after he isolated it from a culture of *Bacillus megaterium* (Braunegg, 1998). The PHB is a biodegradable substitute to fossil raw materials plastics, which have begun to encroach on the conventional polymer market, and whose role is bound to acquire increasing importance. PHB can be produced from renewable raw materials such as saccharides, alcohols and low-molecular-weight fatty acids, and are completely degradable to carbon dioxide and water through natural microbiological mineralization (Braunegg, 1998). Consequently, neither there production nor their use or degradation has a negative ecological impact. By keeping closed the cycle of production and re-use, PHB can enable at least part of the polymer-producing industry to switch from ecologically harmful end-of-the-pipe production methods towards sounder technologies. The PHB homopolymer can be produced in high quantities from cheap raw materials, but there are limits to its industrial and commercial applications because of its less-than-perfect physical properties (namely its brittleness and high glass transition point).

Lignocellulosic fibers are inexpensive abundant product available annually from corn and other annual crops. It is totally biodegradable in a wide variety of environments and can be used as a raw material for the development of totally degradable products. In this work composites were made using as fillers lignocellulosic fibers aiming at the enhancement of mechanical and physical properties as well as modifying the surface appearance, the processing characteristics and reducing costs.

Lignocellulosic fibers are a kind of biopolymer composite, with the following components, in different proportions, depending on the specie considered: cellulose, hemicellulose, and lignin, and others in small proportions (extractives). These polymers are the basic constituents for the cell wall and are responsible for most of the physical and chemical properties, such as dimensional instability to moisture, biodegradability, flammability, thermoplasticity, and degradability by ultraviolet light, acids, and bases. All of these characteristics will result in specific end-use of lignocellulosics in composite formulation. Lignocellulosics can be put together with one or more substances, using some kind of a matrix that would hold the components together, resulting in a composite material. However, according to our knowledge, there are very few reports about the effect and feasibility of injection molding technique for lignocellulosic fiber/PHB composites systems. The objectives of the present work were to evaluate the mechanical properties of the Lignocellulosics/PHB composites and the influence of the reinforcement level over the mechanical properties.

EXPERIMENTAL

Materials

The materials used in this work were PHB and lignocellulosics fibers. The PHB was supplied by COPERSUCAR, and its characteristics are: purity (> 99.5%), ash content (< 0.25%), crystallinity (70%), molecular weight (250.000-600.000 D), and melting point (169-172°C).

Several lignocellulosics fibers, such as sisal, sugar cane bagasse, wood flour (*Pinus elliotti*), cellulose pulp from sugar cane bagasse, rice husk, vegetal sponge (*Luffa cylindrica)* and lignin were used. The chemical compositions of these materials are listed in Table 1.

Table 1. Chemical compositions of lignocellulosics fibers (% Wt –water free).

Lignocellulosics	% Cellulose	% Lignin	% Polyoses	% Ash
Sisal	64.3	13.2	27.4	1.4
Sugar cane bagasse	44.1	23.7	33.3	1.3
Wood flour	53.3	28.0	21.3	0.5
Cellulose pulp	94.6	< 0.3	5.0	0.4
Rice husk	38.5	21.1	22.0	13.6
Vegetal sponge	59.6	22.0	21.2	0.2

Methods

The preparations of lignocellulosics/PHB composites were done by mixing the components and molding the testing specimens suitable for mechanical properties testing according to ASTM standard by injection molding (tensile and flexural strength) at molding temperature 160°C in a Semeraro Injection Molding Machine, model Micro 65. The ratio of lignocellulosics fibers and PHB was 15:85 by weight.

The mechanical properties of the composites were measured with an EMIC 10,000 Universal Testing Equipment. All the results were average values of five measurements. Dimensional stability, water absorption, density, behavior toward some chemical reagents (solubility %) of the composites with different lignocelullosics fibers were also evaluated.

RESULTS AND DISCUSSION

The results of mechanical, physical and chemical properties for the different lignocellulosics/PHB composites are presented in Table 2 and 3, Figure 1 to 5. Those values correspond to the average of measurements made in 5 test pieces.

Physical Properties

Water absorption and density of lignocellulosic/PHB composites are important characteristics that determine end use application of these materials. The composites were oven-died for one hour at 105°C. Dry weight of the composite was measured. Composites were immersed in water at 25°C for 24 hours. Wet weight of each composite was then determined. Moisture content of each sample was calculated (%).

Composites were moisture resistant. In general, they tend to absorb moisture from 0.3 to 1.0%. Water absorption values are shown in Table 3. Their values were higher with increasing in cellulose content of lignocellulosics fibers.

The densities of lignocellulosics/PHB composites were lower than the mineral/PP composites systems (Table 3). Therefore, the density lignocellulosics/PHB composites compare favorably to other commercially filled composites.

Chemical Properties

The chemical behavior (% solubility) of the pure PHB and composites toward HCl, NaOH, NaCl, H_2O and CH_3COOH were tested by determining the percent solubility and dimensional stability. For this purpose, samples were immersed separately in aqueous solution (1.0 mol/L) of the reagents for 24 hours at 25°C using a sample-liquor ratio of 1:100. The results of solubility were recorded in Table 2. The data in Table 2 indicates good resistance to attack by chemical reagents (NaOH, HCl and NaCl) and solvents (H_2O and CH_3COOH), with exception of sugar cane bagasse and rice husks/composites, which had low resistance to NaOH.

Table 2. Effect of chemical behavior and water absorption of lignocellulosics/PHB composites.

Sample	% Water Absorption	Percent Solubility				
		NaOH	HCl	NaCl	CH_3COOH	H_2O
PHB	< 0.1	0.3	0	0	0	0
Sisal/PHB	0.70	3.6	0	0	0	0
Vegetal sponge/PHB	0.81	5.6	0	0	0	0
Wood flour/PHB	0.33	1.1	0	0	0	0
Rice husk/PHB	0.40	24.2	0	0	0	0
Cane bagasse/PHB	0.64	15.3	0	0	0.26	0
Cellulose pulp/PHB	0.96	2.3	0	0	0.62	0

Mechanical properties

The resulting mechanical properties are presented in Table 3 and Figure 1 to 5.

Table 3. Mechanical properties of lignocellulosics/PHB composites and comparison with data on commercially used composites.

Materials	d (g/cm^3)	Tensile Properties			Flexural Properties	
		Tensile Strength (MPa)	Tensile Modulus (GPa)	Elongation (%)	Flexural Strength (MPa)	Flexural Modulus (GPa)
PHB	1.20	18.97	3.394	0.63	45.09	3.417
Sisal/PHB	1.19	19.67	3.015	1.73	34.37	3.886
Vegetal sponge/PHB	1.19	17.31	3.348	0.74	37.02	3.598
Wood flour/PHB	1.17	18.79	3.860	0.79	39.47	3.309
Rice husk/PHB	1.20	19.09	3.862	0.73	40.65	3.800
Sugar cane bagasse/PHB	1.18	15.20	3.341	0.96	32.15	3.275
Lignin/PHB	1,19	18.42	4.217	0.50	---	---
Cellulose pulp/PHB	1.30	18.51	3.118	1.5	---	---
Polypropylene (PP)	0.90	26.83	1.514	>10	40.77	1.300
PP/Talc 20%	1.03	39	7.6	3.02	36.80	3.427
PP/Talc 40%	1.20	110	9.0	2.77	38.34	1.966
PP/$CaCO_3$ 40% *	1.25	25	3.5	--	48	3.1
PP/Talc 40% *	1.27	35	4.0	--	63	4.3
PP/Mica 40% *	1.26	39	7.6	2.3	62	6.9
PP/Glass 40% *	1.23	110	9.0	2.5	131	6.2

* Data from Modern Plastics Encyclopedia and Materials selector 1994.

Regarding the tensile strength at break, in general, the addition of lignocellulosics fibers did not affect significantilly, except for sugar cane bagasse, that reduced the tension resistance and lignin that resulted in higher material stiffness (Figure 1 and 2). For the flexural properties, the incorporation of lignocellulosics fibres showed a small reduction for flexural resistance (Figure 3). For flexural modulus there was no changes (Figure 4).

Values determined for elongation at break are presented in Table 3 and Figure 5. Observing the results of elongation, it can be noticed that as the tested fibers showed an improvement related to pure PHB, except for lignin.

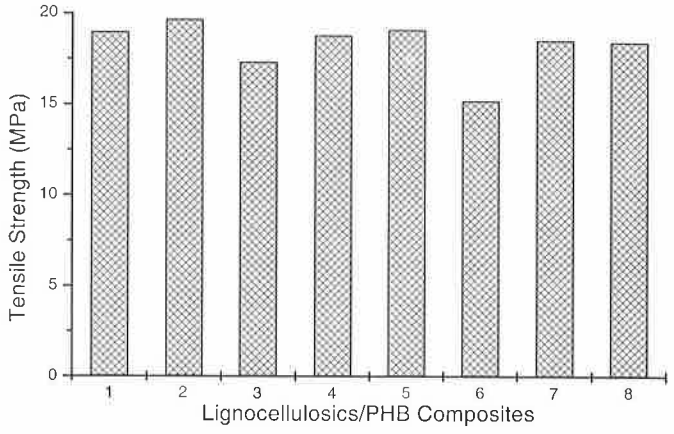

Figure 1. Comparison of tensile strength (MPa): 1. Pure PHB, 2. Sisal/PHB, 3. Vegetal sponge/PHB, 4. Wood flour/PHB, 5. Rice husk/PHB, 6. Sugar cane bagasse/PHB, 7. Cellulose pulp/PHB, and 8. Lignin/PHB.

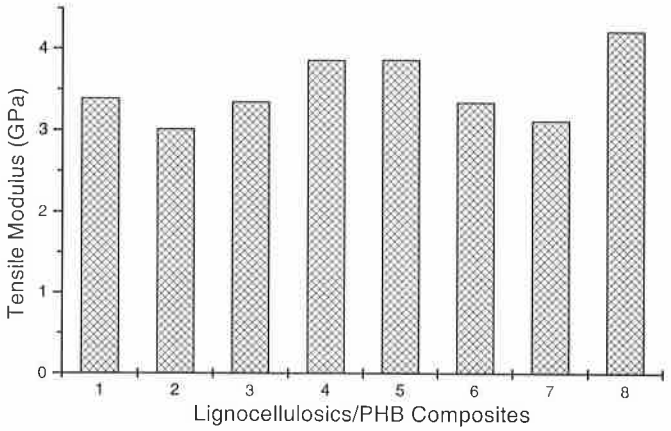

Figure 2. Comparison of of tensile modulus (GPa): 1. Pure PHB, 2. Sisal/PHB, 3. Vegetal sponge/PHB, 4. Wood flour/PHB, 5. Rice husk/PHB, 6. Sugar cane bagasse/PHB, 7. Cellulose pulp/PHB, and 8. Lignin/PHB.

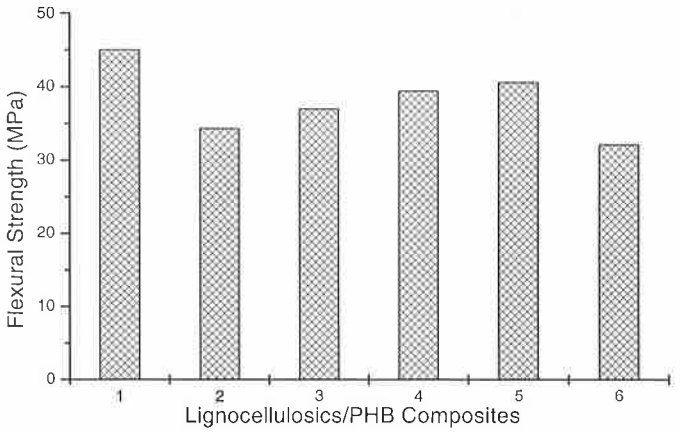

Figure 3. Comparison of of flexural strength (MPa): 1. Pure PHB, 2. Sisal/PHB, 3. Vegetal sponge/PHB, 4. Wood flour/PHB, 5. Rice husk/PHB, and 6. Sugar cane bagasse/PHB.

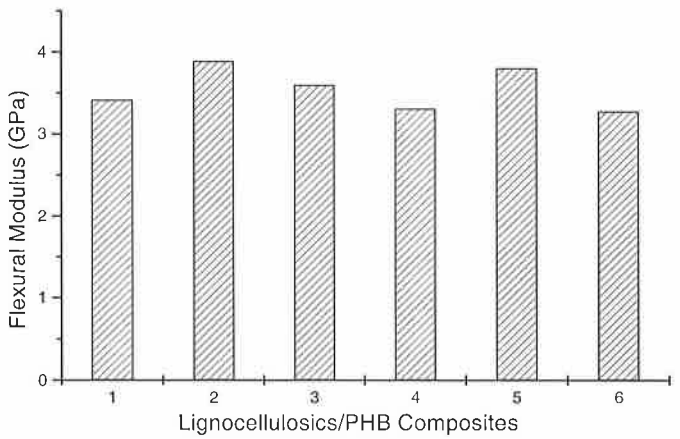

Figure 4. Comparison of of flexural modulus (GPa): 1. Pure PHB, 2. Sisal/PHB, 3. Vegetal sponge/PHB, 4. Wood flour/PHB, 5. Rice husk/PHB, and 6. Sugar cane bagasse/PHB.

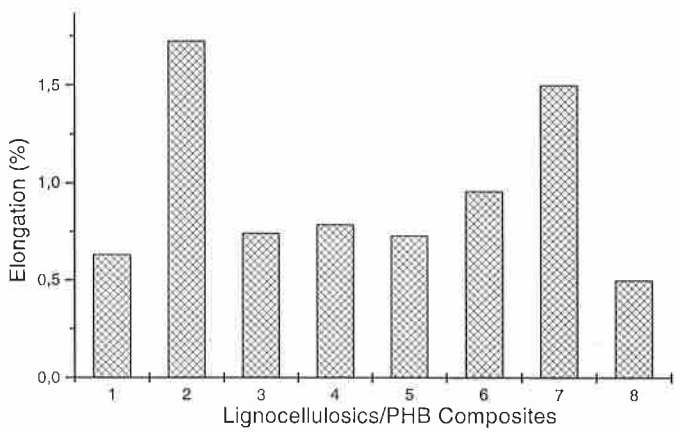

Figure 5. Comparison of percent elongation: 1. Pure PHB, 2. Sisal/PHB, 3. Vegetal sponge/PHB, 4. Wood flour/PHB, 5. Rice husk/PHB, 6. Sugar cane bagasse/PHB, 7. Cellulose pulp/PHB, and 8. Lignin/PHB.

CONCLUSION

It was showed in this article that the composites with lignocellulosics fibers provide a promising way to improve the pure PHB stiffness and lower the production costs. Results indicate that sisal, wood flour and sponge have equivalent mechanical properties to commercially available composites. Continuing research and development of lignocellulosics/PHB composites may show that these material systems are viable alternatives to commercially available PP composites. From the results obtained it is possible to conclude that lignocellulosics fibers can be used in satisfactory way as reinforcing agent of PHB due to the fact that its incorporation resulted in promising products regarding to the enhancement of mechanical, physical and chemical properties. The PHB composites reinforced with lignin and sugar cane bagasse showed lower strength compared to the others regarding its evaluated mechanical properties.

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REFERENCES

Peijs, T. (1999). New Developments in Eco-Composites. *In 2nd International Wood and Natural Fibre Composites Symposium*, Proceedings, pp 28 (1-2).

Hänggi, U.J. (1998). Market opportunities for bioplastics in 1998 to 2002. *In 2nd International Symposium on Natural Polymers and Composites (ISNaPol 98)*, Proceedings, pp 309-310.

Narayan, R. (1999). Emerging Plastic Products from starch. *In 5th International Conference on Frontiers of Polymers and Advanced Materials*, Proceedings, pp 18-20.

Shiraishi, N. (1999). Biodegradable plastics from cellulose. *In 5th International Conference on Frontiers of Polymers and Advanced Materials*, Proceedings, pp 15-17.

Braunegg, G. (1998). Polyhydroxyalkanoates: Environmentally Degradable Plastics from Renewable Resources. *Enviromentally Degradable Polymers*, Proceedings, pp 26-33.

Hankermeyer, C.R. & Tjeerdema, R.S. (1999). Polyhydroxybutyrate: plastic made and degraded by microorganisms. *Rev. Environ. Contam Toxicol*, **159**, 1-24.

Leaf, T.A. & Srienf, F. (1998). Metabolic modeling of polyhydroxybutyrate biosynthesis. *Biotechnol. Bioeng.*, **57**, 557-570.

Rehm, B.H.A. & Steinbüchel, A. (1998). Biotechnological Production of polyester: Novel Production Organisms and metabolic routes to novel polyester. *In 2nd International Symposium on Natural Polymers and Composites (ISNaPol 98)*, Proceedings, pp 315-320.

Pretreatment of bagasse pulp with ß-xylanase from *Thermomyces lanuginosus* SSBP

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ABSTRACT

A locally isolated thermophilic fungus *Thermomyces lanuginosus* SSBP produced a high level of extracellular ß-xylanase (1565 IU/ml) under shake-flask conditions. This enzyme was purified to homogeneity by DEAE-A25 and QAE-A25 Sephadex column chromatography. ß-Xylanase pretreatment of bagasse pulp was carried out at 60°C, at a solid consistency of 10% and at concentrations of 5 IU/g; 50 IU/g and 150 IU/g dry weight pulp. Spectrophotometric analyses of the bleach filtrate revealed a significant release of chromophoric material and reducing sugars from ß-xylanase pretreated bagasse pulp. The application of purified ß-xylanase to bagasse pulp did not produce distinct differences in reducing sugar release in comparison to the crude enzyme. ß-xylanase pretreatment was followed by chemical bleaching with the sequence D_1ED_2. The purified enzyme, however, produced a 4.5% increase in brightness in comparison to the 3.4% achieved with the crude enzyme after the chemical bleach sequence. This result correlated positively with a greater amount of chromophoric material released with the application of the purified enzyme in comparison to the crude enzyme. A ß-xylanase concentration of 5 IU/g dry weight pulp increased the brightness by 3% indicating the effectiveness of the enzyme at low concentrations. After the XE treatment the kappa number was reduced by 3.1 points with purified ß-xylanase at 150 IU/g in comparison to the kappa number reduction of 0.05 and 0.015 units after the XD_1E and XD_1ED_2 sequence, respectively. Scanning electron microscopy revealed a great degree of surface modification of bagasse pulp fibres after ß-xylanase pretreatment in comparison to the control sample. Examination of the pulp fibres at higher magnifications indicated a "tearing-off" effect after enzyme pretreatment with higher enzyme doses. These results emphasize the importance of enzyme application at the initial stages of the bleach sequence.

Keywords: xylanase, bagasse pulp, bleaching

INTRODUCTION

Xylan is the major component of the plant cell wall and the most abundant renewable hemicellulose (Whistler and Richards, 1970). Because of the complex nature of xylans, it is not surprising that the enzymatic degradation of this substrate to its monomer, xylose, is a complex process involving a battery of enzymes (Biely, 1985, Zimmerman, 1992). However, endoxylanases possess the most important activities among the xylanolytic enzymes, and one of the potential application areas of endoxylanases is in the pulp and paper industry.

Organic chlorine compounds formed during the bleaching of chemical pulps have probably attracted most attention in recent years (Buchert *et al.*, 1994). These compounds arise mainly from the reactions between residual lignin present in wood fibres causing the brown colour of unbleached pulp and the chlorine used for bleaching (Viikari *et al.*, 1994). In response to environmental concerns and stringent emission standards modification of the production process in the pulping and bleaching stages has been developed (Christov and Prior, 1998). Among the new bleaching technologies, xylanases have already shown their potential to alleviate the heavy chemical loads during the pulping and bleaching process (Koponen, 1991; Lavielle *et al.*, 1992). The bleach boosting impact of xylanase pretreatment of kraft pulps was first demonstrated by Viikari *et al.* in 1986 and since then, the efficacy of cellulase-free xylanase preparations has been studied by many research groups (Garg *et al.*, 1998). The commercial potential of these enzymes is indicated by the incorporation of xylanase pretreatment in ten paper mills (six in Europe and four in Canada (Jurasek and Paice, 1992).

Since its inception SAPPI Fine Papers (Stanger, South Africa) has been using chlorine to bleach bagasse pulp. The problems encountered with the use of chlorine are corrosion of storage tanks, high losses in pulp

yield and extensive emissions of chlorine in the bleach plant effluent. Extensive research on biobleaching with ß-xylanases on bagasse pulp has been conducted in our department (Bissoon, 1996; 1998). The main aim of our biotechnology programme was to optimize conditions for ß-xylanase pretreatment of bagasse pulp, and to evaluate the effects of the enzyme activity on the pulp.

EXPERIMENTAL

Isolate

A thermophilic hyphomycete, *Thermomyces lanuginosus* SSBP was isolated from soil (Singh *et al.*, 1995). The strain has been classified by the Council for Scientific and Industrial Research (CSIR).

Growth and maintenance of culture

T. lanuginosus SSBP was maintained on potato dextrose agar (PDA) slants and grown at 50 °C. The culture was subcultured every 2 weeks on PDA plates (pH 6.5).

Culture medium for xylanase production

T. lanuginosus SSBP was grown in shake-flask cultures incubated at a temperature of 50 °C at 150 rpm using an optimal corn cobs medium. (Purkarthofer *et al.,* 1993). The culture filtrates were filtered through whatman filter paper and centrifuged, and the supernatants analysed for xylanase activity.

Enzyme assay

ß-xylanase was assayed according to Bailey *et al.* (1992).

Purification of ß-xylanase

Purifications procedures were carried out at 4°C according to the protocol of da Silva *et al.* (1994) with a few modifications.

ß-Xylanase pretreatment of bagasse pulp

The pulp sample was autoclaved at 121 °C for 15 min and transferred into sterile plastic bags containing crude enzyme diluted in citrate buffer (pH 6.5) at concentrations of 150 IU/g; 50 IU/g; and 5 IU/g oven dried pulp. Each sample was then incubated in a waterbath for three hours at a solid consistency of 10%, at 60°C and aliquots removed every 30 min. The control sample was carried out in the same manner with inactivated enzyme being added. After the incubation period the pulp samples were washed in distilled water, and pulp sheets were prepared with a buchner funnel for further analyses.

Release of chromophoric material and reducing sugars

During pulp treatments enzyme mediated release of chromophoric material was monitored in filtrates by measuring the absorbance at a wavelength of 465 nm (Wong *et al.*, 1997). Reducing sugar concentrations in the pulp filtrate was determined by the DNS method (Miller, 1959).

Chemical Bleaching

ß-xylanase pretreatment and chemical bleaching conditions are summarized in Table 2.

Pulp properties

The kappa number of bagasse pulp was determined by SAPPI official test methods while the brightness and was determined by the Elrepho 2000 system.

RESULTS AND DISCUSSION

ß-Xylanase production and properties

T. lanuginosus SSBP produces a range of thermostable extracellular enzymes which includes ß-xylanase, ß-mannanase, ∝-galactosidase, ∝-L-arabinofuranosidase and esterases (Singh, 1998). In the present study this organism produced up to 1565 IU/ml of ß-xylanase activity in shake-flask cultures at 50°C after a 7 day incubation period. However, ß-xylanase activity of 3600 IU/ml was previously reported for this strain (Singh *et al.*, 1995). These results correlate closely with a recent report by Bennett *et al.* (1998) were a high production level of ß-xylanase (2840 IU/ml) by *T. lanuginosus* ATCC 56882 was achieved when grown on corncobs. ß-xylanase from *T. lanuginosus* SSBP has been reported to be extremely stable over a wide pH range (pH 5.5-9.5) at an optimum temperature of 70°C (Singh 1998). These two characteristics allow the enzyme to be a suitable alternative for enhancing lignin removal from bagasse pulp and improving pulp properties.

β-xylanase purification

Table 1 summarizes the properties of β-xylanase from *T. lanuginosus* SSBP after each purification step. Purity of the β-xylanase protein obtained during the final purification step was examined by SDS-PAGE. The purified protein appeared homogenous since the enzyme migrated as a single sharp band (data not shown). During the purification steps 65% of the β-xylanase activity from *T. lanuginosus* SSBP was lost. These results correlate with that of Bennet *et al.* (1998) where a 62 % yield loss was reported. The highest specific activity obtained on birchwood xylan for β-xylanase was 1357 IU/ml. This value remains the highest among purified β-xylanases from *T. lanuginosus* strians.

Table 1. Properties of β-xylanase after each purification step

Purification step	Total volume (ml)	Total protein (mg/ml)	Total activity (IU)	Specific activity (IU/mg)
Crude filtrate	30	7.6	1113	146
80% $(NH_4)SO_4$ precipitation	12	2.89	752	621
DEAE Sephadex A25, pH 6.0	8.4	0.53	547	1032
QAE Sephadex A25, pH 7.1	5.2	0.28	380	1357

Effect of ß-xyanase pretreatment on the bleach boosting of bagasse pulp

The bleach boosting effects of ß-xylanase pretreatment of kraft pulps (Kantelinen *et al.*, 1992; Hortling *et al.*, 1993, and Viikari *et al.*, 1994), and dissolving pulps (Christov and Prior, 1995; Buchert *et al.*, 1995, and Scott *et al.*, 1998) are well documentated. However, ß-xylanase pretreatment of non-woody material such as cereal straw (Sabharwal and Young, 1996) and bagasse pulp (Bissoon *et al.*, 1996) has only recently received attention. Preliminary studies with the crude ß-xylanase from *T. lanuginosus* SSBP has shown to significantly improve the brightness of bagasse pulp with a concomitant release of reducing sugars as well as chromophore material.

Table 2. Summary of ß-xylanase pretreatment and chemical bleaching conditions applied to bagasse pulp

	X	D_1	E	D_2
Pulp mass (g)	20	19.5	18.81	17.93
Time (min)	180	60+15	120+15	180+15
Charge	5-150 IU/g	3% active Cl	2.5% NaOH	0.75% active Cl
Consistency (%)	10	10	10	10
Temperature (°C)	60	70	70	70

X - ß-xylanase; D_1 - chlorine dioxide; E - NaOH extraction; D_2 - chlorine dioxide

The application of purified ß-xylanase from *T. lanuginosus* SSBP to bagasse pulp produced virtually the same extent of hemicellulose solubilization as did the crude enzyme (Table 3). This phenomenon was observed with all three-enzyme doses applied to the pulp. However, there was a corresponding increase in reducing sugar release with higher enzyme doses of the crude and purified ß-xylanase applications.

Table 3. Bleach filtrate analysis

Enzyme dose	Reducing sugar release (mg/g)	Chromophore release Abs at 465 nm
Control	0.116	0.981
P 5 IU/g	0.352	1.078
P 50 IU/g	0.601	1.165
P 150 IU/g	1.092	1.220
C 5 IU/g	0.33	1.087
C 50 IU/g	0.57	1.141
C 150 IU/g	1.095	1.179

P: Purified ß-xylanase, C; Crude ß-xylanase

These results indicated that the accessory enzymes present in the crude extract do not contribute significantly to the removal of xylan from bagasse pulp. A report by Bajpai and Bajpai (1998) revealed that the application of accessory enzymes was not effective in removing xylan when used alone, but when used in combination with ß-xylanases, solubilization of xylan increased moderately. Therefore an evaluation of purified accessory enzymes together with ß-xylanase would have investigated to confirm a synergistic effect of these enzymes. However, it has been suggested that the biobleaching effect maybe strongly dependant on the properties of the particular xylanase used, and secondly, since the xylan structure is inherently different in different pulp samples its response to enzyme degradation would differ as well (Suurnakki *et al.*, 1997).

The chromophore release was more significant with the purified ß-xylanase than the crude enzyme. Liberation of these coloured compounds, presumably lignin, by ß-xylanase pretreatment is supported by the observed reduction in kappa number by both the purified and crude enzyme. Since the crude and purified enzyme did not produce a significant difference in reducing sugar release but did so at the brightness level, it can be concluded that the purified ß-xylanase had a higher specific activity and removed a greater portion of the colour material, thereby enhancing the brightness of the pulp. It also suggests that the bleach boosting effect of the enzyme on bagasse pulp is not limited to xylan removal, but also involves the extraction of other material, which contributes to the low brightness of the untreated pulp as well.

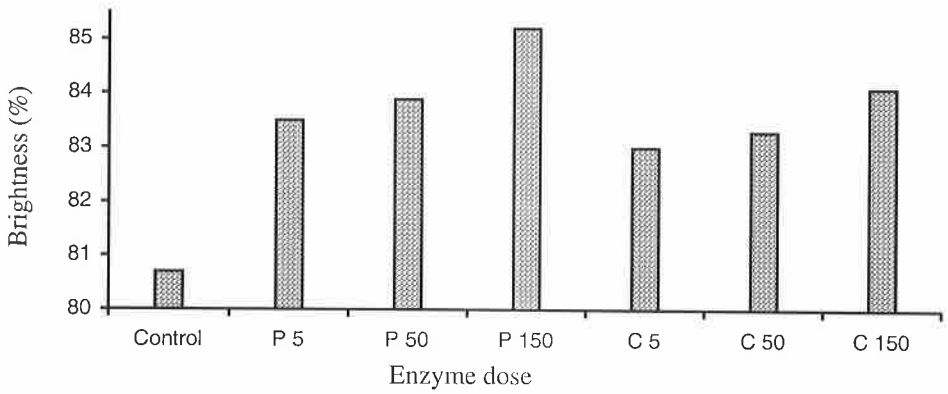

Figure 1. Brightness of bagasse pulp after bleach sequence XD_1ED_2 with crude (C) and purified (P) ß-xylanase.

The brightness increase on bagasse pulp after the application of purified ß-xylanase and crude enzyme at 150 IU/g pulp was 4.5% and 3.4%, respectively (Figure 1). It was also observed that lower ß-xylanase doses (5 IU/g) improved the brightness by more than 3% thereby indicating the effectiveness of the enzyme on bagasse pulp. Laboratory trials carried out by Allison *et al.* (1993) with a thermophilic ß-xylanase, Cartazyme HT, on kraft pulp in a conventional bleach sequence (D_1ED_2) improved the brightness by 3.5 points, which reduced the chlorine consumption by 19-24%. However, the final brightness was unaffected by the initial enzyme pretreatment, showing that it may only be effective prior to acidic delignification treatments such as chlorine. On the contrary, the ß-xylanase from *T. lanuginosus* SSBP produced a much higher final brightness in comparison to the initial enzyme treatment on bagasse pulp.

The reduction in kappa number of bagasse pulp monitored during the chemical bleach sequence is given in Table 4. The influence of ß-xylanase pretreatment in kappa reduction appears to be less significant as more chemicals are introduced into the bleach sequence. A reduction of 3.1; 0.5, and 0.015 kappa points was observed after the application of the bleach sequence XE; XD_1E, and XD_1ED_2, respectively. There were no distinct differences in kappa number of the xylanase pretreated and control pulp samples after the final chemical bleaching. This explains the similar kappa number values obtained after the final chemical treatment. These results confirm the hypothesis that an increase in the chemical bleaching steps results in a less pronounced biobleaching effect (Balakrishna, 1998).

Although kappa reduction has been attributed to lignin removal, hexeneuronic acid (Hex A) has been shown to account for a significant fraction of the oxidizable components in kraft pulps (Gellerstedt, 1996). Jeffries and Davis (1998) showed that there is an excellent correlation between ß-xylanase activity and Hex A release, however, the kappa reduction did not correlate directly with either of these factors. These findings

indicate that xylanase pretreatment results in the release of other materials which also contribute to kappa reduction.

ß-xylanase from *T. lanuginosus* SSBP appears to be very effective on bagasse pulp, even at low enzyme doses. The significant increase in brightness and reduction in kappa number observed with the enzyme pretreatment, provides a solid basis for its application on a larger scale. This study indicates that the enzyme is more effective at the pretreatment stage rather than at a later stage in the bleach sequence, allowing for minimal process changes when introduced on a mil scale. In this respect ß-xylanase pretreatment of bagasse pulp could play an important role in the bleach boosting process at SAPPI Fine Papers (Stanger).

Table 4. Kappa number reduction during the XD_1ED_2 bleach sequence.

Treatment	Kappa number after XE	Kappa number after XD1E	Kappa number after XD1ED2
Control	6.9	2.4	0.475
P : 150 IU/g	3.8	1,9	0.460
P : 50 IU/g	4.4	1.92	0.460
P : 5 IU/g	4.7	2.07	0.445
C : 150 IU/g	4.1	1.85	0.470
C : 50 IU/g	4.3	2.10	0.465
C : 5 IU/g	5.1	2.21	0.450

P : Purified ß-xylanase, C : Crude ß-xylanase

REFERENCES

Allison, R. W., Clark, T. A. & Wrathall, S. H. (1993). Pretreatment of radiata pine kraft with a thermophillic enzyme, Part I. Effect on conventional bleaching, *Appita,* **46**, 269.

Bailey, M.J., Biely, P. & Poutanen, K. (1992). Interlaboratory testing of methods for assay of xylanase activity. *Journal of Biotechnology.*, **23**, 257-270.

Bajpai, P. & Bajpai, P. K. (1998). Impact of xylanolytic enzymes in bleaching of dissolving pulp. *7th International Conference on Biotechnology in the Pulp and Paper Industry*, Proceedings, **Vol C**, pp 3-6.

Balakrishna, H., Szakas, G. Rele, M. V. Prior, B. A. & Christov, L. P. (1998). Screening of cellulase-free fungal xylanases and evaluation of their performance on sulfite pulp. *7th International Conference on Biotechnology in the Pulp and paper Industry*, Proceedings, **Vol C**, pp 7-10.

Bennett, N. A., Ryan, J., Biely, P., Vrsanska, M., Kremnicky, L., Macris, B. J., Kekos, D., Christakopoulos, P., Katapodis, P., Claeyssens, M., Nerinckx, W., Ntauma, P. & Bhatt, M. K. (1998). Biochemical and catalytic properties of an endoxylanase purified from the culture filtrate of *Thermomyces lanuginosus* ATCC 46882. *Carbohydrate Research,* **306,** 445-455.

Biely, P., Puls, J., & Schneider, H. (1985). Acetyl xylan esterases in fungal xylanolytic systems. *FEBS Letters*. **186**, 313-312.

Bissoon. S. Synergistic effect of xylanases and ligninases in biobleaching. BSc (Hons) dissertation, University of Durban Westville, Durban, South Africa.

Bissoon, S. (1998). ß-xylanase pretreatment of bagasse pulp. *In 7th International Conference on Biotechnology in the Pulp and Paper Industry.*

Buchert, J., Tenkanen, M., Kantelinen, A. & Viikari, L. (1994). Application of xylanases in the pulp and paper Industry. *Bioresource Technology*, **50**:65-72.

Buchert, J., Teleman, A., Harjunpaa, V., Tenkanen, M., Viikari, L. & Vuorinen, T. (1995). Effect of cooking and bleaching on the structure of xylan in conventional pine kraft pulp. *Tappi Journal*, **78**, 125-130.

Christov, L. P. & Prior, B. A. (1995). Role of hydrolysis conditions in the enzyme-mediated release of reducing sugars from sulphite pulp. *Biotechnology Letters*, **17**, 821-826.

Christov, L. P. and Prior, B. A. (1998). Research in biotechnology for the pulp and paper industry in South Africa. *South African Journal of Science*, **94**, 195-200.

Garg A. P., Roberts, J. C. & McCarthy, A. J. (1998). Bleach boosting effect of cellulase-free xylanase of *Streptomycetes thermoviolaceus* and its comparison with two commercial enzyme preparations on birchwood kraft pulp. *Enzyme and Microbial Technology*, **22**, 594-598

Gellerstedt, G. & Lindfors, E.-L. (1991). On the structure and reactivity of residual lignin in kraft pulp fibres. Protocol of the International pulp Bleaching Conference., Stolckholm, SPCI. **1**, 73-88.

Gellerstedt, G. (1996). An HPLC method for the quantitative determination of hexeneuronic acid groups in chemical pulps. *Carbohydrate Research*, **294**, 41-51.

Hortling, B., Korhonen, M., Buchert, J., Sundquist J. & Viikari, L. (1994). Leachability of lignin from kraft pulp after xylanase treatment. *Holzforschung*, **56**, 245-249.

Jeffries, T. W. & Davis, M. (1998). Mechanisms for kappa reduction and colour removal. *In 7th International Conference on Biotechnology in the Pulp and Paper Industry*, Proceedings, **C**:41-43.

Juracek, L. & Paice, M. (1992). Saving bleaching chemicals and minimizing pollution with xylanase. *In International Symposium of Pollution Prevention in the Manufacture of Pulp and Paper - Opportunities and Barriers*, Proceedings.

Kantelinen, A., Sundquist, J., Linko, M. & Viikari, L. (1992). The proposed mechanism of enzymatic bleaching of kraft pulps with xylanases. *Holzforschung*, **47**, 318-324.

Koponen, R. (1991). Enzymes systems prove their potential. *Pulp and Paper International*. **33**, 20-25.

Lavielle, P., Koljonen, M., Piiroinen, P., Koponen, R., Reid, D. & Fredriksson, R. (1992). Three large scale uses of xylanases in kraft pulp bleaching. *In 4th International Conference. New Available Techniques and Current Trends*. Protocols, pp 203-215.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*. **31**, 238-244.

Purkarthofer, H., Sinner M. & Steiner, W. (1993). Cellulase-free xylanase from *Thermomyces lanuginosus* : optimization of production in submerged and solid state culture. *Enzyme and Microbial Technology.*, **15**, 677-681.

Scott, G. M., Akhtar, M., Lentz, M. J. & Swaney, R. E. (1998). Engineering aspects of fungal pretreatment for wood chips. *In Environmental Friendly Pulping and Bleaching Methods*, New York.

Singh, S., Pillay, B. & Prior, B. A. (1995). Characterization of high activity xylanase from a thermophilic *Thermomyces lanuginosus*. *In 6th International Conference on Biotechnology in the Pulp and Paper Industry*, abstracts, pp 238.

Singh, S. (1998). Production and properties of hemicellulases by *Thermomyces lanuginosus* strains. PhD thesis, University of Durban Westville, Durban, South Africa.

Suurnakki, A., Li, T. Q., Buchert, J., Tenkanen, M., Viikari, L., Vuorinen, T. & Odberg, L. (1997). Effects of enzyme removal xylan and glucomannan on the pore size distribution of kraft fibres. *Holzforschung*, **51**, 27-33.

Viikari, L., Ranua, M., Kantelinen, K. & Linko, M. (1986). Bleaching with enzymes. *In 3rd International conference Biotechnology in the pulp and Paper Industry*, Proceedings, pp 67-69.

Viikari, L., Kantelinen, A., Sundquist, J. & Linko, M. (1994). Xylanases in bleaching: From an idea to the industry. *FEMS Microbiological Reviews*, **13**, 335-350.

Whistler, R .L. & Richards, E. L. (1970). Hemicelluloses. *In The Carbohydrates,* ed. Pigman, W. & Horton, D., Academic press, New York, **Vol 2a**, pp 447-469.

Wong, K. K. Y., de Jong, E., Saddler, J. N & Allison, R. W. (1997). Mechanisms of xylanase-aided bleaching of kraft pulp. *Appita*, **50**, 415-422.

Zimmerman, W. (1992). Bacterial degradation of hemicelluloses. *Metal Ions Biological Systems*. **28**, 357-398.

Quality and aerobic treatment of pulp and paper mill effluents

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ABSTRACT

Treatment efficiency and optimisation of wastewater treatment plants is usually based on parameters such as chemical (COD) or biochemical (BOD) oxygen demand and the amount of suspended solids (SS). However, to really judge the impact of effluents on the environment, detailed information on the wastewater constituents like high molecular weight lignin and extractives, toxicity a.s.o. are needed. Thorough Norwegian studies of the effluents of an integrated newsprint mill (TMP) and a neutral sulphite semichemical plant (NSSC) after treatment in a high-efficiency compact reactor (HCR) plant revealed reductions in COD of 80 and 65 %, removal of identified extractives of > 99 %, of acetate of 99 and 93 %, of carbohydrates of 89 and 80 % and 45 and 15 % removal of the lignin-like material, respectively. In both effluents the resin acids and the toxicity werde reduced by 98 and 93 %, respectively, whereas DTPA showed no significant removal. The characterization method used gave valuable information about removal mechanisms and analyses of the main organic compounds made it possible to explain the treatability of the effluent.

Keywords: cellulosic pulp effluents, effluent treatment, wastewater

INTRODUCTION

Wastewater from the pulp and paper industry contains organic material originating from the wood, such as carbohydrates, lignin and extractives, as well as chemicals added during the production processes. The organic material is found either in its original form, such as fibres or fines, in colloids or as dissolved material. The amounts and the solubilities of the organic compounds in the wastewater are dependent on the wood species, the chemicals used and the production conditions.

As a consequence of the demands from authorities and the public for reduced discharges of organic matter and toxic compounds, the industry has installed biological wastewater treatment plants (Springer 1993; Saunamäki 1997). Aerobic biological treatment, either in aerated lagoons or using the activated sludge process, has for a long time been used to treat various pulp and paper mill effluents with adequate removals (Springer 1993; Kantardjieff *et al.*, 1995). Generally these systems use long hydraulic retention times (HRT) and therefore occupy large areas. In the High-efficiency Compact Reactor (HCR) activated sludge system, the reactor combines efficient oxygen transfer with turbulent mixing (Yenkie *et al.*, 1992). This gives shorter retention times (within hours) and as well as reduced reactor volume requirements.

Treatment efficiency and optimisation of treatment plants are usually evaluated by parameters such as COD (chemical oxygen demand), BOD (biochemical oxygen demand) and SS (amount of suspended solids). These parameters were originally intended for use in municipal wastewater treatment, to describe the relationship between the loading and their effect on the recipient. The relationship between BOD in the effluent and the oxygen demand in the recipient is well established, but COD does not necessarily correlate with the environmental impact. To study the removal mechanisms of the organic material during the biological treatment, detailed information about the constituents is needed, both concerning the chemical composition as well as the physical state of the substances. Organic material in circulating water, e.g. water from thermochemical pulping (TMP), have been well characterised in several studies (Sjöström 1990; Nyland *et al.*, 1993; Thornton 1993; Jacobs 1995), since many of the substances are detrimental both to the paper quality and the process efficiency. Wastewater throughout biological treatment plants has first been characterisesd only recently (Magnus, 1998).

In production of newsprint, grinding and refining is used to mechanically separate the fibres in the wood chips. TMP, predominantly using softwood, usually gives pulp yield of 91-98 % of the raw material. The residual material is released into the process water. In neutral sulphite semi-chemical (NSSC) pulping there is a chemical treatment preceding the mechanical refining step. NSSC pulps are mainly based on hardwood and the chemical treatment is used to reduce the amount of lignin in the pulp. Normal pulp yields are 65 to 85 %. For both mill types the organic component groups released into the process water are carbohydrates from

cellulose and hemicellulose, lignin as well as lipophilic and polar extractives. The pulps are often bleached to improve the optical properties of the final product. Alkaline peroxide bleaching is frequently used and causes increased release of organic material, such as acetic acid, from the pulp (Sjöström 1990; Thornton *et al.*, 1991; Jour *et al.*, 1992).

The main removal mechanisms for organic material in wastewater during aerobic treatment are biodegradation and adsorption to the sludge, dependent on the molecular size distribution and the chemical characteristics of the compounds. To be biodegradable the compounds must become available to the bacteria and penetrate the cell membrane. Dissolved organic compounds with molecular weight less than 10^3 are easily transported through the cell membrane provided the correct permease is present (Horan 1991). Passive hydrophobic transportation through the cell membrane is, however, generally limited to molecules with molecular weight less than 500 (Schwarzenbach *et al.*, 1993). Consequently small saccharides from cellulose and hemicellulose, like glucose, as well as small organic acids like acetate, are easily biodegraded. This is also the case for soluble polysaccharides, but they are dependent on hydrolysis by extracellular enzymes into smaller units for transportation across the cell membrane and metabolism in the bacteria. Studies of molecular size versus biodegradability in bleached wastewater have shown that the majority of the removed organic material had molecular weight less than 960, and the upper limit for the most efficient degradation was 300 Da (Sonnenberg *et al.*, 1995). High molecular weight lignin is known to be recalcitrant in biological treatment plants, like humic acids are in municipal wastewater treatment plants (Levine *et al.*, 1985; Rintala and Lepistö 1992). In nature the only known microorganism able to degrade polymeric lignin completely are white-rot fungi (Boman *et al.*, 1988; Crestini *et al.*, 1996).

Several studies reported in the literature demonstrate that the amount of fatty and resin acids are removed by 80 to 99 % from pulp and paper effluents during aerobic treatment (Servici and Gordon 1986; Kantardjieff *et al.*, 1990; Schnell *et al.*, 1990; Gibbons *et al.*, 1992; Lo *et al.*, 1994a; LaFleur and Barton 1997). Liu *et al.* (1996) found that extractives were removed by three mechanisms: bio-oxidation, adsorption onto biosludge and air oxidation. Bio-oxidation was identified as the main removal mechanism, but adsorption was regarded as more important at short treatment times. The bioavailability of resin acids has been suggested to be dependent on the compound's distribution between the colloidal or dissolved phases of the effluent (Werker and Hall 1997). They found lowest residual concentration of resin acids after biological treatment above pH 7.

Extensive research has been performed during the last 10 years on the biological impact of pulp and paper industry effluents (Hall 1997; Lethinen 1997; Priha 1997). Even though most mills have biological treatment systems, wastewater toxicity is important to monitor since extractive material like resin acids are known to be toxic to aquatic organisms at low concentrations (Leach and Thakore 1977; Holmbom and Lethinen 1980). Toxicity of effluents towards a wide range of organisms has been studied both in natural environments and in laboratory conditions (Kovacs and Megraw 1996; Swanson *et al.*, 1996).

This paper gives an update of recent studies of wastewater treatment of pulp and paper mill effluents using the HCR process (Magnus *et al.*, 2000A, B and C).

QUALITY OF NSSC AND INTEGRATED NEWSPAPER MILL EFFLUENTS

The main characteristics of the untreated mill effluents studied here are shown in Table 1 (Magnus 1998). Whereas the NSSC mill effluent show about 4 times higher levels of COD, TOC and BOD than the effluent from the integrated newsprint mill, the toxicity of the former is 20 times higher showing that this parameter correlates with specific effluent compounds rather than the organic material content in general. For further details reference is made to Magnus (1998), and Magnus *et al.* (2000A, B and C), which also gives the characterisation methods used. The amounts of organic material found in the effluents were at the same level as previously reported for similar mills (Carlberg *et al.*, 1993; Broch-Due *et al.*, 1994).

The concentrations of the organic compounds in the two effluents were divided into three phases (particulate, colloidal and dissolved phases) by centrifugation and membrane filtration and the results are given in Table 2.

The total concentrations of all the organic compounds were highest in the NSSC mill effluent, in accordance with the highest COD in this effluent. In the effluent from the integrated newsprint mill, the carbohydrates and the lignin-like material show an even distribution between the dissolved and colloidal phases, while the

main part of these compounds are found in the dissolved phase of the NSSC mill effluent. The identified extractives were primarily found in the particulate and colloidal phases in the NSSC mill effluent but predominantly in the dissolved phase of the integrated newsprint mill effluent.

Table 1. The main characteristics of the total mill effluents (samples taken at reactor inlet).

		NSSC mill effluent	Integrated newsprint mill
COD	g/L	14.2	3.3
TOC	g/L	4.3	1.1
BOD_5	g/L	4.7	1.2
BOD_{28}	g/L	9.2	not available
Microtox	15 min, EC_{50}, vol %	0.24	4.9
Daphnia magna	48 hr, EC_{50}, vol %	2.8	not available

The higher portion of dissolved lignin-like material in the NSSC mill effluent may, apart from the pulping conditions, also be explained by the different raw materials used. Lignin derived material that is found in effluents from kraft pulping, has been reported to have lower molecular weight when originating from hardwood as compared to softwood (Dahlman *et al.*, 1995).

Table 2. The analysed organic material in the mill effluents divided into the different phases.

	NSSC mill effluent				Integrated newsprint mill effluent			
	g/L				g/L			
	Total	Partic.	Colloid.	Dissolv.	**Total**	Partic.	Colloid.	Dissolv.
	sample	phase	phase	phase	**sample**	phase	phase	phase
Carbohydrates	**3.10**	0.05	1.10	1.95	**1.04**	0.19	0.45	0.40
Lignin-like material	**3.49**	0.35	0.50	2.64	**n.a.**	n.a.	0.27	0.25
Acetate	**2.50**	0.00	0.10	2.40	**0.34**	0.00	n.a.	0.34
Identified extractives	**0.96**	0.42	0.50	0.04	**0.16**	0.04	0.02	0.10
DPTA	**n.a.**	n.a.	n.a.	n.a.	**0.04**	0.00	0.01	0.03

n.a. not available

The compositions of the organic material in the centrifuged effluent samples are shown in Figure 1. These compositions are expected to be the most influential factor for the treatability of the effluents. The carbohydrates play a more dominant role in the effluent from the integrated newsprint mill (46 %) than in the effluent from the NSSC mill (33 %). The compounds expected to be most readily reduced in the biological treatment plant, the acetate, carbohydrates and extractives, amount to nearly the same percent in both effluents (66 and 70 % in the NSSC mill effluent and integrated newsprint mill effluent, respectively).

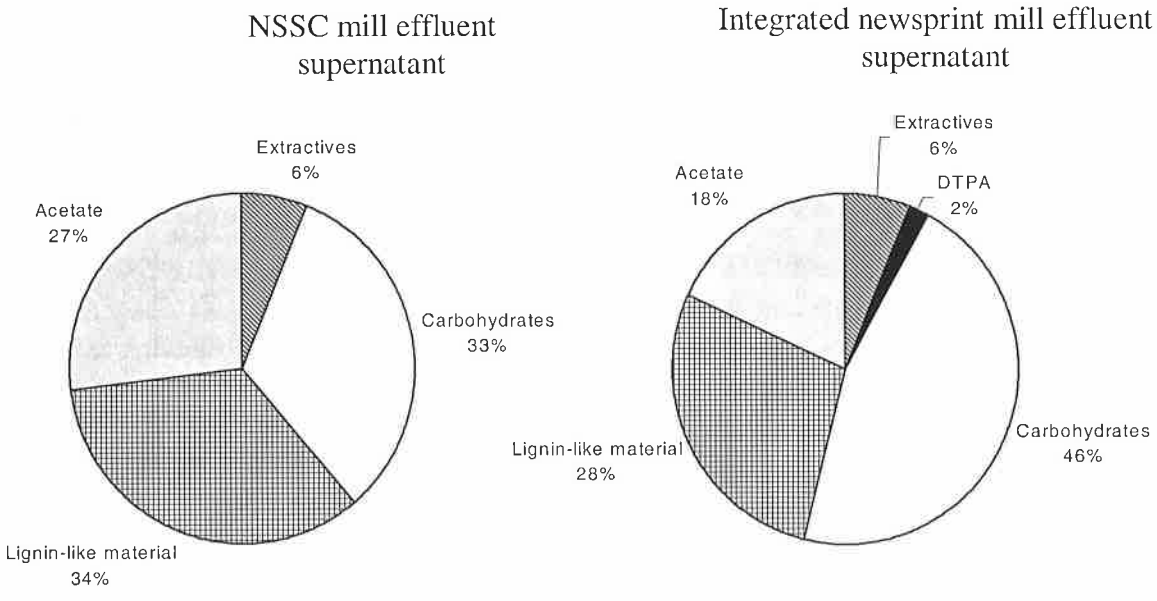

Figure 1. Composition of the organic material in the centrifuged samples of the untreated mill effluents.

HIGH-EFFICIENCY COMPACT REACTOR (HCR)

The first processes different from the conventional Activated Sludge Process and the Trickle Bed Reactor installed around the turn of the century were developed in the chemical industry in the seventies. The main objective of these developments was to minimize the amount of air passing through the wastewaster in order to reduce the costs for posttreatment of this air due to odour or solvents stripped from the wastewater. This goal was achieved by using closed vessels and increasing the height of the wastewater by up to 30 m in the so-called Tower-Technology or even to more than 100 m in the Deep-Shaft-Process (Zlokarnik 1982). Whereas the increased height of wastewater results in a reduction of oxygen in the exhaust air to less than 50 % and thus a significant reduction in the amount of air required, the space-time yield of these reactors is comparable to the conventional activated sludge process and trickle bed reactor.

A dramatic increase in space-time-yield was achieved by pulsing the fluid in a reactor via a periodic up-and-down movement of a stack of sieve plates as realized by Brauer in the so-called Hubstrahl-Reaktor (Brauer 1986) or by recirculating the fluid within a loop as done in our laboratory leading to the HCR-Reactor (Vogelpohl 1991, 1996). The HCR-Reactor as shown in Figure 2 consists of a cylindrical vessel with a height-to-diameter ratio of about 7:1 and carries inside a draft tube open at both ends. The two-phase jet (see insert in Figure 2) located at the top of the reactor creates a downward directed two-phase flow inside the draft tube and at the same time disperses the air sucked in through the gas tube located within the liquid jet.

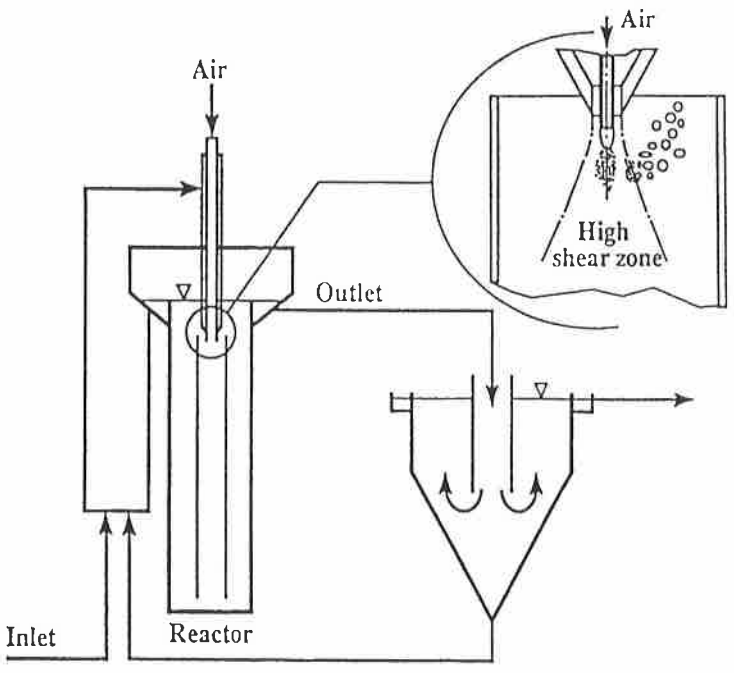

Figure 2. Diagram of the High-efficiency Compact Reactor (HCR) Wastewater Treatment Plant.

Due to the momentum of the liquid jet, the liquid and the gas inside the draft tube are flowing downwards and after reflection at the reactor bottom rise within the annulus between the wall of the reactor and the draft tube. At the upper end of the draft tube, a part of the fluid is recycled into the draft tube by the sucking action of the two-phase jet resulting in a redispersion of the bubbles and the biomass produced in the biological reaction. The remainder of the fluid is discharged from the reactor and the exhaust air is vented to the atmosphere.

The excellent performance of the HCR-reactor in wastewater treatment results from the homogeneous flow distribution and high recirculation rate of the loop and the very effective dispersion and redispersion of the gas bubbles and the biomass by the two-phase jet resulting in a large mass transfer area and high turbulence and thus an intensive oxygen transfer. In more than twenty industrial applications, the HCR-Reactor has proven its outstanding performance both with respect to space-time-yield and energy requirements. Figure 3

shows typical performance data from treating effluents from an Integrated newsprint mill by the HCR-Process (Kvaerner 1998). A further advantage of the HCR-Process is a low sludge volume index and a low production of surplus sludge.

Figure 4 shows as an example two reactors with a volume of 250 m³ each treating the wastewater of a NSSC mill. Almost 70 % of the COD-load of this wastewater with an initial concentration of about 14 g of COD /m³ is removed at a volume loading of 70 kg COD/m³ d and at an energy consumption of about 0.4 kWh per kg COD_{elim}.

As the reaction rate in wastewater treatment is directly proportional to the biomass concentration, a high concentration in biomass will result in a reduction of reactor volume and thus investment costs. As conventional sedimentation does not allow for a high biomass concentration, the application of membranes has been studied in our laboratory and the results have led to the development of the HCR/Membrane-Process, an advanced treatment process for wastewaters difficult to degrade biologically (Lübbecke *et al.*, 1993, 1995).

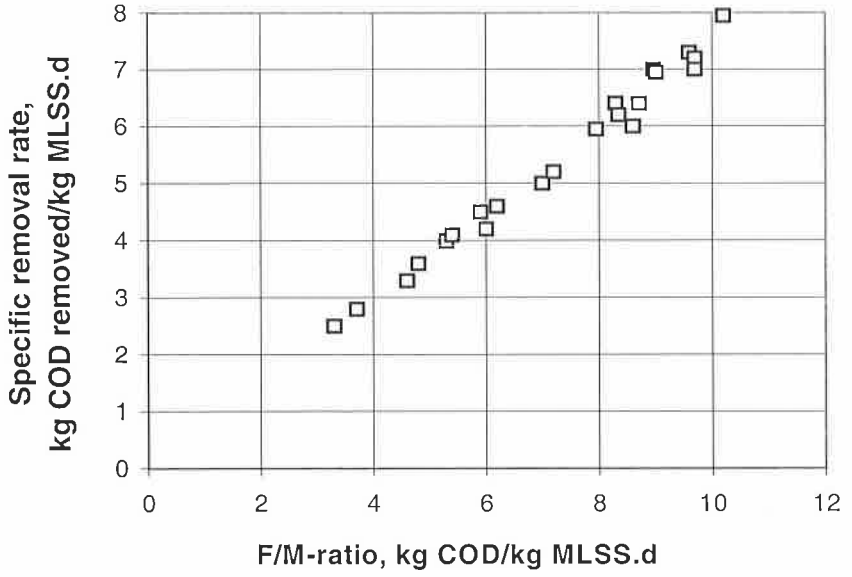

Figure 3. COD-removal of the HCR-Process treating integrated newsprint mill effluents.

Due to the development of improved and more versatile membranes in the last decade, membrane separation is now a predictable and reliable operation proven in many applications. The advantage of a membrane separation is that the wastewater is completely free of any solids or even infectous organisms. In addition, by choosing the proper cut-size of the membrane, it is possible to decouple the residence time of the wastewater from the residence time of the higher molecular weight compounds of the wastewater.

Thus, these are retained in the system until special microorganisms have developed which are able to degrade the large molecules to molecules sufficiently small to pass the membrane. These unique properties of a membrane process are of particular interest for the treatment of wastewaters with a high COD/BOD-ratio. Figure 5 gives the % elimination achieved by the HCR/Membran-Process treating leachate from landfills with an inital COD of approx. 3.5 g/L and an initial COD/BOD-ratio of ≅ 15. As shown in Figure 5, more than 60 % of the COD are removed and even the Adsorbable Organic Halogens (AOX), which are not degraded in conventional treatment plants, are reduced to an appreciable amount (Lübbecke S. (1994).

Treating a so-called „Foul Condensate" from a papermill with an initial COD of ≅ 3.5 g/l by the HCR/Membrane-Process with the objective of recycling this condensate to the mill, more than 95 % of the COD and up to 90 % of the ammonia and the primary amines could be removed. The color of the condensate was removed by 70 % and the effluent was completely free of solids and microorganisms (Schories 1999).

EFFICIENCY OF HCR AEROBIC BIOTREATMENT

Removal efficiencies of the treatment plants treating effluents from the NSSC and Integrated newsprint mills are shown in Table 3. The results show high removal efficiencies (between 65 and 98 %) in the HCR treatment plants with short retention times as compared to conventional activated sludge treatment plants (Wilson *et al.*, 1987; Lo *et al.*, 1994:b; Brender *et al.*, 1995; Elefsiniotis *et al.*, 1995). Pilot plant studies on other high rate aerobic treatment systems have shown similar large reductions in COD and BOD (Shere and Daly 1982; Schnell *et al.*, 1990). Comparisons of treatment efficiencies between different treatment plants are of course accompanied by uncertainties, as different mills have different effluent characteristics and because there are seasonal and daily variations in COD, BOD, SS and toxicity levels (Lo *et al.*, 1994:a). Different studies also use various techniques for fraction preparation.

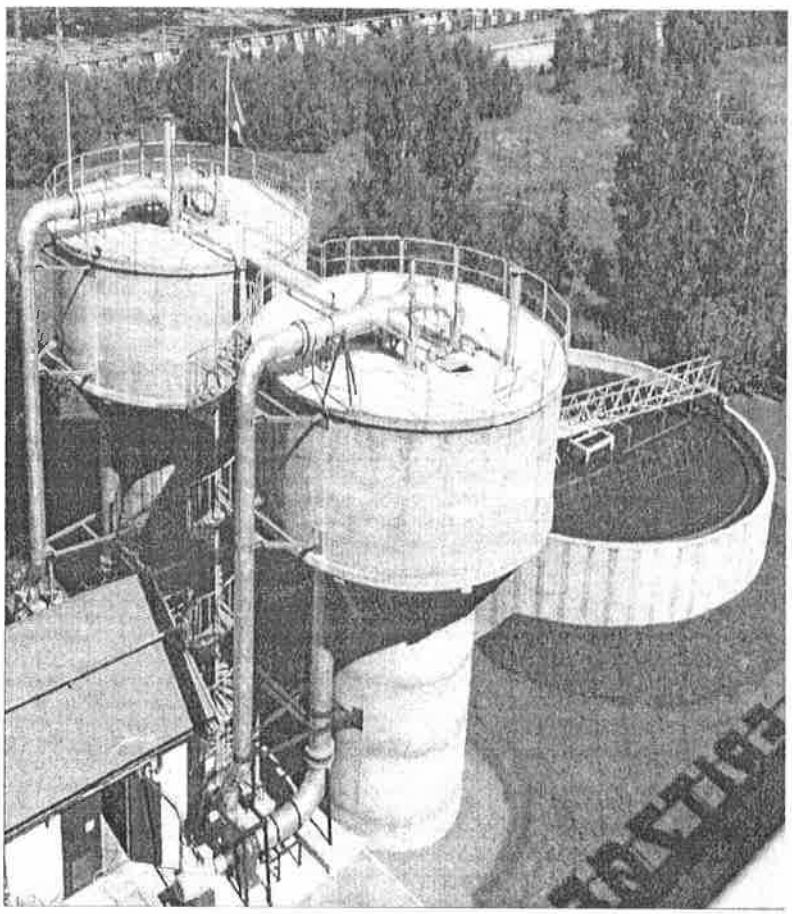

Figure 4. 250 m^3 HCR-reactors of a NSSC mill.

The HCR plant removal efficiencies at the NSSC mill was lower than at the integrated newsprint mill, which was in accordance with reports on treatability of effluents from these types of pulp mills. Results from aerobic treatment of effluents from NSSC mills has previously only been reported in pilot plant and laboratory scale (Wilson *et al.*, 1987; Broch-Due *et al.*, 1994). The HCR plant showed reduced removal efficiency for BOD_5 at the NSSC mill as compared to at the integrated newsprint mill, indicating a potential for further biodegradation of organic material in the NSSC mill effluent. The difference in reduction of COD between the two mill effluents is expected to be mainly due to the sulphite/bisulphite treatment in the NSSC mill. Some of the differences may arise from the wood species used. A study of aerobic treatment of CTMP effluents showed reduced treatability of an effluent based on aspen compared to an effluent based on spruce (Jour *et al.*, 1992).

The removal efficiencies of the HCR plant (including the secondary clarifier) were more prominent than the removal efficiencies of the bioreactors, as to be expected. As seen in Table 4, the reductions of the organic

compounds in the dissolved and colloidal phases can be ranked in the following order for both mills: identified extractives > acetate > carbohydrates > lignin-like material > DTPA.

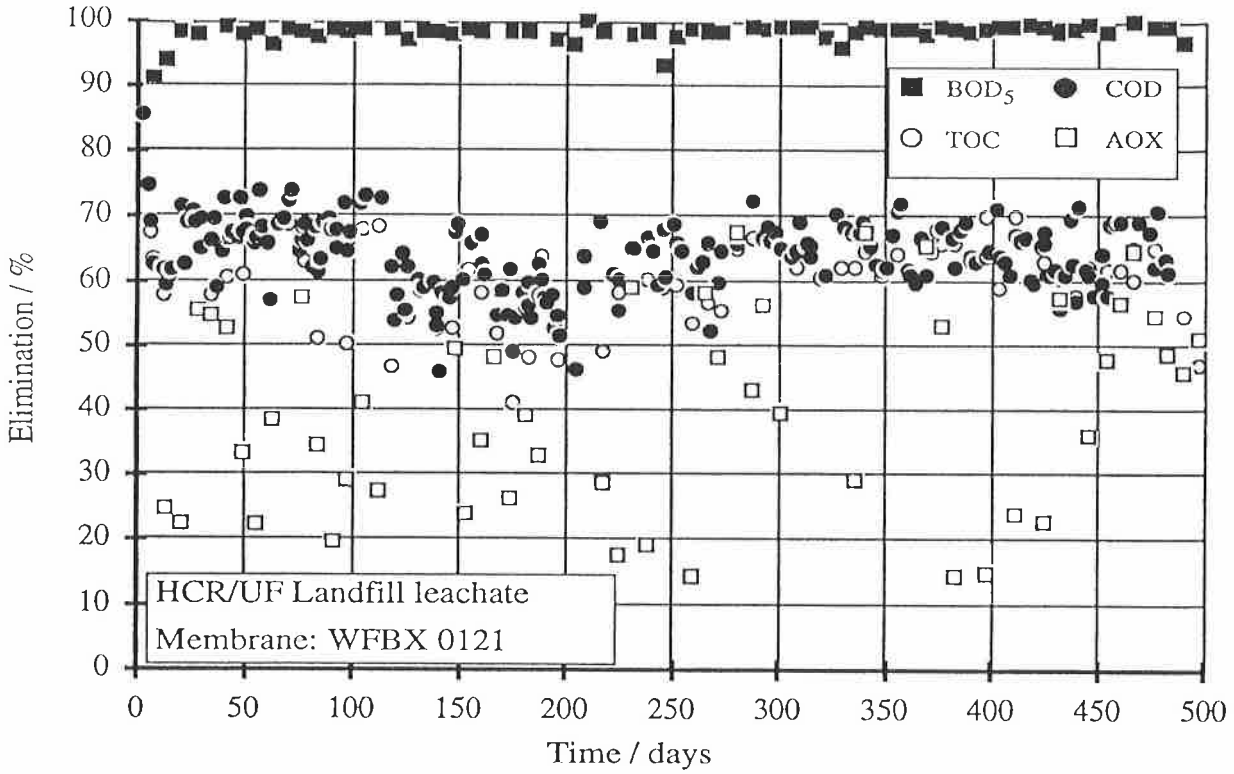

Figure 5. Removal of COD and organic halogens in leachate from landfills by the HCR/Membrane Process.

Table 3. Removal efficiencies of the treatment plants in % reduction.

	NSSC mill		Integrated newsprint mill	
	HCR plant	HCR reactor	HCR plant	HCR reactor
COD	65	59	79	74
TOC	-	63	77	62
BOD_5	93	84	98	70

The high removal efficiencies for carbohydrates and acetate at the short retention times of the HCR reactor confirm that these compounds can easily be biodegraded in effluents from pulp and paper mills. The highest bioreactor removal efficiency for lignin-like material was found at the integrated newsprint mill, being slightly lower than results reported in the literature (Liu *et al.*, 1993; Schnell *et al.*, 1997). The low reduction of the lignin-like material in the NSSC mill effluent indicates low adsorption to the sludge, as previously also reported for lignosulphonates (Liu *et al.*, 1993).

Table 4. Removal of organic compounds in the wastewater samples, due to treatment in the bioreactors. The residual concentrations were measured in the supernatants from reactor samples.

	NSSC mill		Integrated newsprint mill	
	Removal	Resid. concentr.	Removal	Resid. concentr.
	%	mg/L	%	mg/L
Carbohydrates	80	600	89	100
Lignin-like material	15	2660	45	290
Acetate	94	150	99	4
Identified extractives	99	8.5	>99	0.2
DPTA	-	not analysed	0	50

The highly dissolved chelating agent DTPA identified in the treatment plant from the integrated newsprint mill showed no significant removal in accordance with other studies (Dahlman *et al.*, 1995; Hinck *et al.*, 1997). Micro-organisms often lack the necessary enzymes for conversion of xenobiotic chemicals and

negatively charged chemicals experience electrostatic repulsion that limit their ability to enter the cell membrane.

The total amounts of carbohydrates, extractives and acetate in the colloidal and dissolved phases were reduced by 88 % in the bioreator at the NSSC mill and by 90 % in the bioreactor at the integrated newsprint mill. As seen in Table 5, these compounds contribute to nearly the same percent of the dissolved and colloidal organic material in both mill effluents. Together with the different degree of reduction of the lignin-like material, this demonstrates that the lignin-like material was the main cause of the differences in COD reductions found at the two mills. It also shows that simple analyses of the main organic compounds are useful for understanding the HCR's treatment efficiency and that the treatability of the effluent can be explained by reductions of the individual organic compounds.

All the identified wood extractives were found to be reduced by more than 96 % in the bioreactors. The resin acids in the integrated newsprint mill effluent, showed 99 % reduction, and the low content of resin acid found on the sludge indicated that the resin acids were nearly completely biodegraded (98%). The high degree of biodegradation of the resin acids shows that absorption to the biomass was not an important removal mechanism at short retention time, as suggested by Liu *et al.* (1996). Resin acids have also been found to biodegrade when they are the sole carbon source for the bacteria (Bicho *et al.*, 1995; Werker and Hall 1997).

Table 5. Removal of wood extractives (%) in the wastewaters calculated from the amount of extractives in the total samples prior to the bioreactors to the reactor sample at the NSSC mill and to the total sample taken after the secondary clarifier at the integrated newsprint mill.

	NSSC mill	Integrated newsprint mill
Resin acids	not identified	>99
Free and bound ß-sitosterol	98	96
Free and bound fatty acids	99	>99
Phenolic compounds	97	>99
Total identified extractives	99	>99
Unidentified MTBE extractables	not analysed	37

The results show that resin acids are reduced from all phases in the mill effluent, and that resin acids attached to colloidal and particulate material in the inlet to the bioreactor had sufficient accessibility to the microorganisms to be biodegraded in the reactor. One explanation for the high biodegradability may be that resin acids are surface-active compounds and tend to be adsorbed on interfaces. This is seen in conventional biological treatment systems where resin acids accumulate in surface foams (Werker *et al.*, 1996). In the HCR, with a high dispersion of air and biomass, a congregation of resin acids at the interfaces is expected to give a good availability of the resin acids to the micro-organisms.

β-sitosterol showed >96 % removal at both mills, and was removed by both adsorption to sludge (46%) and biodegradation/transformation (50 %), Magnus *et al.* C. Sterols are generally reported to have somewhat lower removal efficiencies (62 – 99 %) than resin and fatty acids (Stömberg *et al.*, 1996; Cook *et al.*, 1997; Kaplin *et al.*, 1997). The unidentified MTBE extractable material passing the biological treatment plant was mainly found in the membrane filtrate, showing that this recalcitrant material was water-soluble.

The toxicity towards the Microtox bacteria of the effluent from the integrated newsprint mill was completely reduced by the biological treatment. This corresponds with the removal of the wood extractives. The results show that the concentration of DTPA in the treated wastewater (≅ 40 mg/L) gave no contribution to the toxicity. DTPA has, however, been reported to be toxic to algae and bacteria at low levels (Lee *et al.*, 1996). The wastewater from this mill was not tested for toxicity towards the waterflea *Daphnia magna.*

Also the toxicity in the NSSC effluent was to a large degree reduced by the biological treatment plant. The toxicity towards the Microtox bacteria and the waterflea *Daphnia magna* was reduced by 99 and > 93 %, respectively. The colloidal phase of the treated wastewater was found to have the highest residual toxicity.

CONCLUSIONS

The NSSC mill effluent studied had a total COD of 14.2 g/L, 4 times higher than the total concentration of COD in the effluent from the integrated newsprint mill. For both mill effluents more than 2/3 of the organic

material in the dissolved and colloidal phases were carbohydrates, acetate and wood extractives, compounds known to be readily removed in aerobic biological treatment plants. The remaining organic material was mainly lignin-like material. The complexing agent DTPA was found in small concentrations in the effluent from the integrated newsprint mill (≅ 40 mg/L). A higher part of carbohydrates and lignin-like material were found in the dissolved phase of the effluent from the NSSC mill as compared to the integrated newsprint mill.

The High-efficiency Compact Reactor (HCR) showed high removal efficiencies for the organic material in the integrated newsprint and NSSC mill effluents, with 79 and 65 % COD removals, respectively. The reductions of the organic compounds due to treatment in the bioreactors can be ranked in the following order for both mills: identified extractives > acetate > carbohydrates > lignin-like material > DTPA.

The difference in COD reduction between the effluents are explained by the different reductions of the lignin-like material. The low reduction of lignin-like material from the effluent at the NSSC mill can be due to sulphonation of the lignin during the sulphite/bisulphite cooking. More detailed analyses of the lignin-like material in both effluents would be of interest to study the removal mechanisms and to study the potentiality of further reductions by tertiary treatments. Analyses of the main organic compounds in the different phases of pulp and paper mill effluents and their reductions in full-scale aerobic treatment plants have not previously been reported and made it possible to explain the treatability of the total effluents.

REFERENCES

Bicho P. A., Martin, V. & Saddler, J, N. (1995). Growth, Induction and Substrate Specificity of Dehydroabietic acid-degrading Bacteria Isolated from a Kraft Mill Effluent Enrichment. *Appl. Environ. Microbiol.,* **61**, 3245- 3250.

Boman B., Frostell, B., Ek, M. & Eriksson, K.-E. (1988). Some aspects on biological treatment of bleached pulp effluents. *Nord. Pulp Pap. Res. J.,* **3**, 13-18.

Brauer, H. (1986). Stoffaustausch-Maschinen, Chancen für den Gerätebau und die stoffwandelnde Industrie. *Chem.-Ing.-Tech..,* **58**, 97-107.

Brender M. J., Goodman, C. V., Richardson, D. E., Harden, P, E., Parsons, T., Ray, D. & Crockett, J. (1995). Activated sludge pilot treatment of mechanical pulp and paper mill effluent. *Proc. 49 Appita Annual Conf.,* 509-516.

Broch-Due, A., Andersen, R. & Kristoffersen, O. (1994). Pilot plant experience with an aerobic moving bed biofilm reactor for treatment of NSSC wastewater. *Wat. Sci. Tech.,* **29**, 283-294.

Carlberg G. E., Hoel, H., Aarsand, R. & Broch-Due, A. (1993). Chemical/biological characterization of discharges from integrated newsprint mills. *In 18th Inter. Mech. pupl. Conf., EUCEPA*, pp 418-427.

Crestini C., Bernini, R., Porri, A. & Giovannozzi-Sermanni, G. (1996). Biodegradation of Monomeric, Dimeric and Polymeric Lignin Models by *Lentinus edodes. Holzforschung,* **50**, 193-200.

Cook D. L., LaFleur, L., Parrish, A., Jones, J. & Hoy, D. (1997). Characterization of plant sterols from 22 US pulp and paper mills. *Wat. Sci. Tech.*, **35**, 297-303.

Dahlman O., de Sousa, F. & Ljungquist, P. (1995). Analysis of organic compounds in effluents from TCF-bleaching of softwood and hardwood kraft pulps. *In Int. Symp. Wood and Pulping Chem.*, **Vol. I**, pp 729-736.

Elefsiniotis P., Hall, E. R. & Johnson, R. M. (1995). Contaminant removal from recirculated white water by ultrafiltration and/or biological treatment. *In TAPPI Int. Environ. Conf. Proc. TAPPI PRESS Atlanta*, pp 861- 867.

Gibbons J. S., Kovacs, T. G., Voss, R. H., O'Connor, B. I. & Dorica, J. G. (1992). An appraisal of the effect of biological treatment on the environmental quality of high yield mechanical pulping effluents. *Wat. Res.,* **26**, 1425-1432.

Hall T. (1997) A review of recent studies in North America using integrated monitoring approaches to assessing bleached kraft mill effluent effects on receiving waters. In: Environ. Fate and Effects of Pulp and Paper Mill Effluents, ed. Servos M., Munkittrich K., Carey J. & Van der Kraak G., St. Luis Press, Delray Beach, Florida, pp 599-612.

Hinck L. M., Ferguson, J. & Puhaakka, J. (1997). Resistance of EDTA and DTPA to Aerobic Biodegradation. *Wat. Sci. Tech.,* **35**, 25-31.

Holmbom B. & Lehtinen, K.-J. (1980). Acute toxicity to fish of kraft pulp mill waste waters. *Paper Puu,* **62**, 673- 684.

Horan N. J. (1991). Biological Wastewater Treatment Systems. John Wiley &. Sons, Chichester, England, 125.

Jacobs F. Alvarado (1995). Extractives in Process Waters from Newsprint Papermaking (TMP). PhD thesis, Royal Institute of Technology, Stockholm.

Jour P., Wackerberg, E. & Wallin, S. (1992). The generation, identification and treatability of COD from CTMP production. *In TAPPI Int. Environ. Conf. Proc. TAPPI PRESS Atlanta*, pp 1161-1166.

Kantardjieff, A., Zaloum, R., Jones, J. P. & Araneda, L. (1990). Degradation of toxic compounds in TMP mill effluents by biological aerated filter. *In TAPPI Int. Environ. Conf. Proc. TAPPI PRESS Atlanta*, pp 251-259.

Kaplin C., Holmbom, B. & Huhtiniemi, M. (1997). Removal and degradation of wood resin components in an activated sludge treatment plant. *In preprint: 3th Int. Conf. on Environ. Fate and Effects of Pulp and Paper Mill Effluents*, Proceedings, pp 146-152.

Kovacs, T. G. & Megraw, S. R. (1996). Laboratory responses of whole organisms exposed to pulp and paper mill effluents. In: Environ. Fate and Effects of Pulp and Paper Mill Effluents, ed. Servos, M., Munkittrich, K., Carey, J. & Van Der Kraak, G. St Luis Press, Delray Beach, Florida, pp 459-472.

Kvaerner Water Systems a.s., Norway (1998), unpublished data.

LaFleur, L. E. & Barton, D. A. (1997). Pulp mill effluent characterization and treatment: an update. *In preprint: 3th Inter. Conf. On Environ. Fate and Effects of Pulp and Paper Mill Effluents*, New Zealand.

Leach, J. M. & Thakore, A. N. (1977). Compounds toxic fish in pulp mill waste streams. *Prog. Wat. Tech.*, **9**, 787-798.

Lee, H.-B., Peart, T. E. & Kaiser, K. L. E. (1996). Determination of nitrilotriacetic, ethylenediaminetetraacetic and diethylenetriaminepentaacetic acids in sewage treatment plant and paper mill effluents. *J. Chromatogr. A* **738**, 91-99.

Lethinen, K.-L. (1997). Biochemical responses in organisms exposed to effluents from pulp production: are they related to bleaching. *In: Environ. Fate and Effects of Pulp and Paper Mill Effluents*, ed. Servos, M., Munkittrich, K., Carey, J. & Van Der Kraak, G., St Luis Press, Delray Beach, Florida, pp 359-368.

Levine, A. D., Tchobanoglous, G. & Asano, T. (1985). Characterization of the size distribution of contaminants in wastewater: treatment and reuse implications. *J. WPCF* 57, **7**, 805-816.

Liu, H. W., Lo, S. N. & Lavallée, H. C. (1996). Mechanisms of removing resin and fatty acids in CTMP effluent during aerobic biological treatment. *Tappi J.*, **79**, 145-154.

Liu, H. W., Lo, S. N. & Lavallée, H. C. (1993) Secondary Treatment of CIMP Effluent. *J. Pulp Pap. Sci.*, **19:5**, J219-J224.

Lo, S.N., Lavallée, H. C., Rowbottom, R. S., Meunier, M. A. & Zaloum, R. (1994:a). Activated sludge treatment of TMP mill effluents. Part 1: Effluent characterization and treatability study. *Tappi J.*, **77**, 167-178.

Lo, S. N., Lavallée, C. H., Rowbottom, R. S., Meunier, M. A. & Zaloum, R. (1994:b). Activated sludge treatment of TMP mill effluents. Part 2: Biokinetic parameters and effluent treatment strategies. *TAPPI J.*, 77, 179-184.

Lübbecke S. (1994). Biological wastewater treatment in a combined biorector/membrane unit. PhD thesis, Faculty of Mechanical Engineering and Chemical Engineering, University of Clausthal, Germany.

Lübbecke, S., Geißen, S.-U. & Vogelpohl, A. (1993). Nitrifikation in einem Hochleistungssystem. *Chem.-Ing.-Tech.*, **65**, 1341-1343.

Lübbecke, S., Vogelpohl, A. & Dewjanin, W. (1995). Wastewater Treatment in a Biological High-Performance System with a High Biomass Concentration. *Wat. Res.*, **29**, 793-802.

Magnus, E. (1998). Characterisation of pulp and paper mill effluents and their treatment by full scale, aerobic High-efficiency Compact Reactors. PhD thesis, Faculty of Chemistry, University of Oslo, Norway.

Magnus, E., Hoel, H. & Carlberg, G.E. (2000) A. Organic components and toxicity of an aspen NSSC effluent and their removals in a biological High-efficiency Compact reactor. To be printed in *TAPPI J.* **83 (1)**.

Magnus, E., Carlberg, G.E. & Hoel, H. (2000) B. TMP wastewater treatment including biological High-efficiency Compact Reactor, Part 1: Characterisation and removal of organic components. To be printed in *NPPRJ.*

Magnus, E., Hoel, H. & Carlberg, G.E. (2000) C. TMP wastewater treatment including biological High-efficiency Compact Reactor, Part 2: Toxicity reduction and removal of extractives. To be printed in *NPPRJ.*

Nylund, J., Byman-Fagerholm, H. & Rosenholm, J.B. (1993). Physico-chemical characterization of colloidal material in mechanical pulp. *Nord. Pulp Pap. Res. J.*, **8**, 280-283.

Priha, M. H. (1997). Exotoxicological impacts of pulp mill effluents in Finland. In: Environ. Fate and Effects of Pulp and Paper Mill Effluents, ed. Servos M., Munkittrich, K., Carey, J. and Van Der Kraak, G., St Luis Press, Delray Beach, Florida, 637-650.

Rintala, J. A. & Lepistö, S. S. (1992). Anaerobic treatment of thermomechanical pulping whitewater at 35-70 ^{0}C. *Wat. Res.*, **26**, 1297-1305.

Saunamäki, R. (1997). Activated sludge plants in Finland. *Wat. Sci. Tech.*, **35**, 235-243.

Schnell, A., Dorica, J., Ho, C., Ashikawa, M., Munnoch, G. & Hall, E. R. (1990). Anaerobic and aerobic pilot-scale effluent detoxification studies at an integrated newsprint mill. *Pulp Paper Can.*, 91, 75-80.

Schnell A., Sabourin M. J., Skog S. & Garvie M. (1997) Chemical characterization and biotreatability of effluents from an integrated alkaline-peroxide mechanical pulping/machine finish coated (APMF/MFC) paper mill. *Wat. Sci. Tech.* **35**(2-3) 7-14.

Schories G. (1999) Unpublished Pilot Study. Mass Transfer Laboratory, Technical University of Clausthal, Germany.

Schwarzenbach, R. P., Gschwend, P.M. & Imboden, D. M. (1993). Environmental organic chemistry. John Wiley & Sons, New York, 523.

Servizi, J.A. & Gordon, R. W. (1986). Detoxification of TMP and CTMP effluents alternating in a pilot scale aerated lagoon. *Pulp Paper Can.*, **87**, T405-T409.

Shere, S. M. & Daly, P. G. (1982). High rate biological treatment of TMP effluent. *Pulp Paper Can.*, **83**, C 62-66.

Sjöström, J. (1990) Detrimental substances in pulp and paper production. Approaches to chemical analysis of deposits and dissolved organic matter. PhD thesis, Abo Akadami University, Finland.

Sonnenberg, L. B., Ard, T. A. & Wimer, P. (1995). Transformation of wastewater during biological treatment. TAPPI Int. Environ. Conf. Proc. TAPPI PRESS Atlanta, 219-231.

Springer, A. M. (1993). Bioprocessing of pulp and paper mill effluents - past, present and future. *Paper Puu*, **75**, 156-161.

Strömberg, L., Mörch, R., de Sousa, F. & Dahlman, O. (1996). Effects of internal process changes and external treatment on effluent chemistry. In: Environ. Fate and Effects of Pulp and Paper Mill Effluents, ed. Servos, M., Munkittrich, K., Carey, J. & Van Der Kraak, G., St Luis Press, Delray Beach, Florida, 3-19.

Swanson, S. M., Schryer, R. & Firth, B. (1996). In: Environ. Fate and Effects of Pulp and Paper Mill Effluents, ed. Servos, M., Munkittrich, K., Carey, J. & Van Der Kraak, G., St Luis Press, Delray Beach, Florida, 557-569.

Thornton, J. (1993). Dissolved and colloidal substances in the production of wood-containing paper. PhD thesis, Abo Akademi University, Finland.

Thornton, J., Eckerman, C. & Ekman, R. (1991). Effects of peroxide bleaching of spruce TMP on dissolved and colloidal organic substances. Proc. Appita, sixth Int. Symp. Wood Pulping Chem., 571-577.

Vogelpohl, A. (1991). The HCR-Process for Compact, High Performance Wastewater Treatment. *Pollution and Waste Management*, 140-145.

Vogelpohl, A. (1996). New Processes for the Removal of Carbon and Ammonia from Wastewater. *Journal of Energy and Mass Transfer,* **18**, 267-274.

Werker, A. G. & Hall, E. R. (1997). The influence of pH on resin acid solubility related to biodegration kinetics of resin acid in pulp mill effluents. TAPPI Int. Environ. Conf. Proc. TAPPI PRESS Atlanta, 19-26.

Werker, A. G., Bicho, P. A., Saddler, J. N. & Hall, E. R. (1996). Surface tension changes related to the biotransformation of dehydroabietic acid by *Mortierella isabellins.* In: Environ. Fate and Effects of Pulp and Paper Mill Effluents, ed. Servos, M., Munkittrich, K., Carey, J. & Van Der Kraak, G., St Luis Press, Delray Beach, Florida, 139-150.

Wilson, R. W., Murphy, K. L. & Frenette, E. G. (1987). Aerobic and anaerobic treatment of NNSC and CTMP effluent. *Pulp Pap. Can.*, **88**, T4-T8.

Yenkie, M. K. N., Geissen, S. U. & Vogelpohl, A. (1992). Biokinetics of wastewater treatment in the high performance compact reactor (HCR). *Chem. Eng. J.*, **49**, B1-B12.

Zlokarnik. M. (1982). Verfahrenstechnik der aeroben Abwasserreinigung, Entwicklungen und Trends. *Chem.-Ing.-Tech.*, **54**, 939-952.

Semiconductor-assisted photochemical degradation of paper effluents and chemical compounds of environmental importance by using Ag-doped ZnO photocatalyst

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ABSTRACT

This work reports a study of semiconductor-assisted photochemical degradation of lignin, Remazol Brilliant Blue R and Kraft E1 paper effluent by using ZnO and Ag-doped ZnO photocatalysts. The doped semiconductor was prepared in the reaction media by photoreduction of silver nitrate. With the use of 100 mg of ZnO or 15 mg of Ag-ZnO almost total decolorization of the dye and lignin samples in reaction times lower than 60 min were observed. Extending the photochemical reaction up to 120 min, the total organic carbon content (TOC) was reduced by 90%. For the paper effluent, a fast decolorization was obtained for relatively short reaction times. However, the TOC reduction was negligible (near of 10%) up to high reaction times (300 min). By using the Ag-ZnO photocatalyst, the toxicity of lignin and Kraft E1 effluent toward *E. Coli* was completely removed. For the dye, the formation of transient toxic species was observed.

Keywords: photocatalysis, effluent treatment, kraft pulp

INTRODUCTION

In view of the critical problem associated with the industrial activities, mainly related to the high environmental impact of their residues and the relatively low efficiency of the current remediation process, many efforts have been dedicated to the study of new and more efficient degradation alternatives. Among others, the semiconductor-assisted photochemical process occupies a prominent place, mainly because its high degradation capacity toward toxic and recalcitrant chemical species through relatively simple and low-cost procedures (Lisenbigler *et al.*, 1995, Hoffmann *et al.*, 1995, Mills and Le Hunte, 1997).

One of the main problems associated with the viability of the photochemical process is represented by the high tendency of the hole-electron pair to recombine, which implicates deactivation of the photocatalyst. The use of electron scavengers like oxygen is a good alternative to overcome this problem. However, many studies have demonstrated that the presence of transition metals (like platinum, silver and iron) improved the charge-transfer and consequently the performance of the photochemical degradation process (Hirano *et al.*, 1996, Ranjit and Viswanathan, 1997).

Although some works have been demonstrated a high photocatalytic activity of ZnO for degradation of some industrial residues and organic species, its application is absolutely insignificant when compared with the traditional anatase TiO_2. Likewise, studies related to the use of coupled semiconductor systems involving ZnO are also scarce.

The main objective of this work was investigating the photochemical efficiency of ZnO and Ag-doped ZnO semiconductors, using lignin, a reactive dye (Remazol Brilliant Blue R) and an effluent from the paper industry (Kraft E1) as substrates.

EXPERIMENTAL

Reagents

The reactive dye (Remazol Brilliant Blue R, C.I. 61200) was purchased from DYSTAR Ltda. (São Paulo, Brazil) while the Lignin-sulfonate was from Aldrich. The substrates were prepared in concentrations of 62.5 and 200 mg L^{-1}, respectively, by direct dissolution in distilled water. The final pH of these solutions was 7.0.

Zinc oxide (Nuclear, 99%) and Silver nitrate (Merck) were used without any pre-treatment. Kraft E1 effluent (pH: 3.3; absorbance at 465 nm: 0.200, TOC: 971 mgL^{-1}), was obtained from a pulp and paper plant in the Campinas' region (São Paulo, Brazil).

Preparation of the Ag-ZnO photocatalyst and photochemical treatment

The preparation of the Ag-doped ZnO photocatalyst was carried out according to the procedure reported by Chen and Nickel (1996), while the photochemical treatment of the substrates were consecutively carried out in the same photochemical reactor. 15 mg of ZnO and 20 mL of aqueous $AgNO_3$ solution of variable concentration were placed in a 150 mL reactor equipped with water refrigeration and magnetic stirrer. The suspension was irradiated from the top during 20 min with a 125 W Philips medium pressure mercury lamp without the glass cover (fluence rate: 15 $Jm^{-2}s^{-1}$ at λ>254 nm) at a distance of 12 cm from the solution surface. Subsequently, 80 mL of the dye or lignin aqueous solution was placed in the reactor and the photochemical process extended for a convenient time, bubbling commercial oxygen through a sintered glass placed in the bottom of the reactor at flows of about 10 $mLmin^{-1}$.

For analytical control, samples were taken at convenient times and filtered through a 0.45 μm Millipore filter.

Analytical control

The efficiency of the process was evaluated by monitoring the decolorization (at 592 nm for the dye and 465 nm for lignin), with a Hitachi U-2000 spectrophotometer. The total organic carbon content was measured with a TOC-5000 Shimadzu Total Organic Analyzer. Acute toxicity determinations were carried out by measuring the respiration inhibition in *Escherichia coli* cultures, by using a FIA-conductometric methodology (Jardim *et al.*, 1993). X-ray diffractometric analysis were carried out in a Rigaku instrument, using Si as internal standard (Instrumental conditions: reflection mode, 40 kV, 20 mA, CoKα=1,7902A).

RESULTS AND DISCUSSION

Ag-ZnO characterization

The deposition of metallic silver on the ZnO surface was monitored by powder X-ray diffraction analysis (Figure 1). The absence of silver in the final solution was confirmed by negative reaction with chloride.

When exposed to atmospheric conditions for some minutes, the photocatalyst lost its photochemical activity by probable formation of a thin film of Ag_2O (Cheng and Nickel, 1996) not detectable by powder X-ray diffraction. However, when resubmitted to a photochemical reduction process for 30 min, the photocatalytic activity was completely recovered.

The characterization by electronic scanning microscopy had shown almost spherical ZnO particles of about 2000 A with a deposit of silver particles smaller than 100 A (detection limit of the equipment).

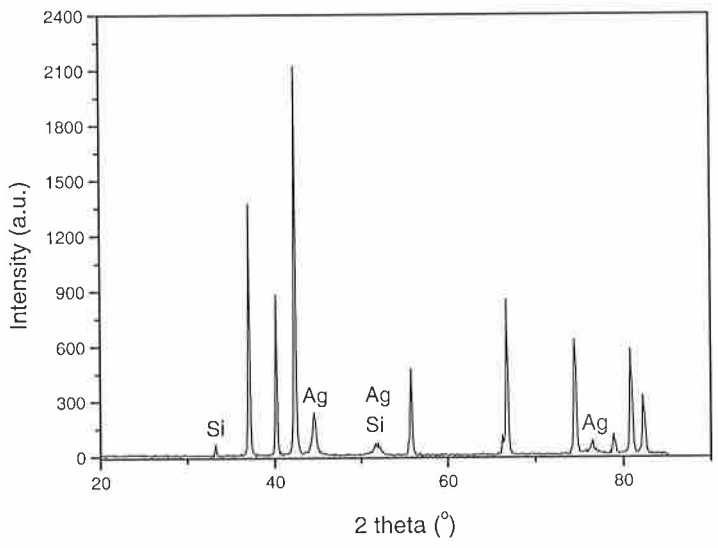

Figure 1. Typical X-ray diffractogram of Ag-doped ZnO. The unidentified peaks are attributed to ZnO.

Lignin degradation

Monitoring the degradation process by UV-VIS spectrophotometry (Figure 2) was possible to verify a fast photochemical breakdown of the chromophoric groups present in the lignin molecule. As these groups are

responsible for the characteristic dark coloration of the lignin solution, the color of the samples (expressed as absorbance at 465 nm) decreased progressively reaching a decolorization ratio of about 50% at the first 15 min, and near of 99% after 60 min. Moreover, the degradation process induces a very significant modification of the chemical structure of lignin, modification that implies an almost total suppression of the absorbance signals on the ultraviolet region (290 nm), mainly related to benzenoid compounds. The observed strong absorbance at wavelengths lower than 250 nm, inclusively for reaction times higher than 180 min, necessarily implies that the fragmentation of the lignin molecule leads to formation of transient species whose degradation require an higher reaction time (ex. methanol, ethanol, formaldehyde, formic acid, oxalic acid, etc. (Kobatakawa *et al.*, 1989).

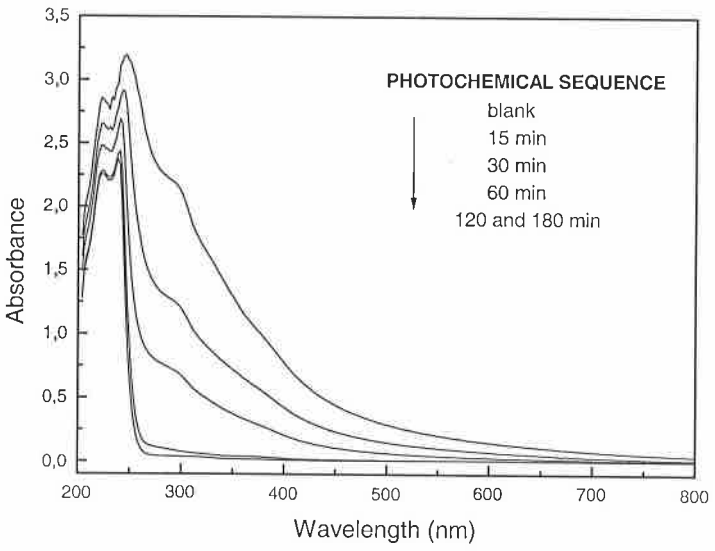

Figure 2. Spectrophotometric evaluation of the Ag-ZnO-assisted photochemical degradation of lignin. Lignin concentration: 160 mgL^{-1}; pH: 7.0; Ag-ZnO: 15 mg.

The efficiency of the photochemical process to effectively degrade the lignin molecule was confirmed by the significant reduction of the total organic carbon content (Figure 3). Observing the tendency of the reduction curve, it is possible to assume that for a time of about 90 min the mineralization of the substrate reached a atio higher than 90%, for both semiconductors.

Remazol Brilliant Blue R degradation

Applying the photochemical procedure at the same experimental conditions, a very resembling result was observed. The chemical structure of the chromophoric group can be modifies with high efficiency, resulting in an almost total decolorization for reaction times near of 60 min (Figure 4). The signals in the ultraviolet region were more persistent to the photochemical process, remaining practically unchanged up to reaction times of 180 min. However, the fact that the TOC reduction was higher than 90% for reaction times of 90 min (Figure 5) implies that the residual UV-signals corresponde to a low concentration of recalcitrant structures.

Paper effluent degradation

When the photochemical process was applied to the kraft E1 effluent, almost total decolorization was reached in around 120 min for both semiconductors. However, the TOC reduction was practically negligible, reaching a highest value of 7% and 15%, for ZnO and doped-ZnO, respectively, in reaction times of about 360 min. This result is not surprising, mainly due to the recalcitrant character of the chemical species derived from the pulp bleaching process, particularly those presented in the first alkaline extracting stage.

Residual acute toxicity

Due to the real possibility to generate during the photochemical process some molecular fragments that can be more toxic than the parent compound (Jardim *et al.*, 1997), the residual acute toxicity was measured. The results (Table 1), expressed as inhibition of the bacterial respiration, indicated that the application of the photochemical process with the Ag-doped photocatalyst removes all the acute toxicity associated to the lignin

solution and the kraft E1 effluent. For the dye, both photocatalysts show behaviors that characterize the photochemical generation of toxic transient species. That is, the toxicity is completely removed at the first reaction times, but almost totally recovered with the progress of the photochemical process. A similar behavior was observed on the lignin degradation study using the ZnO photocatalyst.

The fact that both residual organic carbon and acute toxicity have been observed for reaction times higher than 180 min, necessarily implicate photochemical formation of recalcitrant and toxic molecular fragments. However, the complete mineralization of molecules such as lignin and reactive dyes can be achieved by extending the photochemical process up to 360 min.

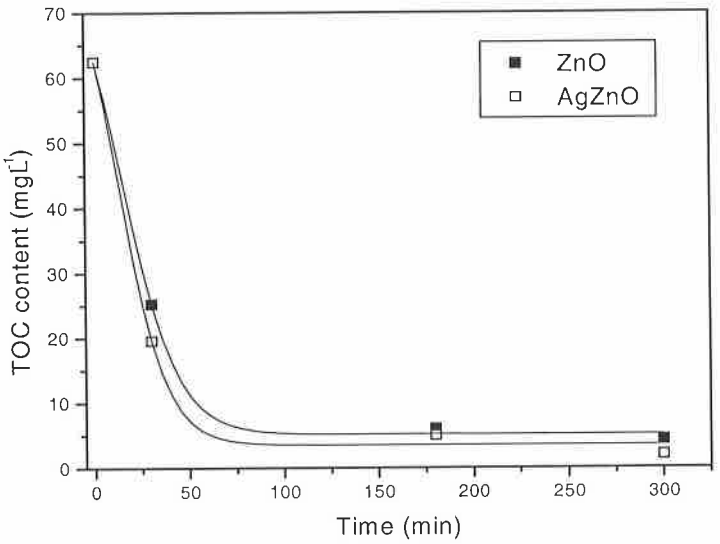

Figure 3. Reduction of the total organic carbon content by the lignin photochemical degradation process. Lignin concentration: 160 mgL^{-1}; pH: 7.0 (Ag-ZnO), pH 9.0 (ZnO); Ag-ZnO: 15 mg; ZnO: 100 mg.

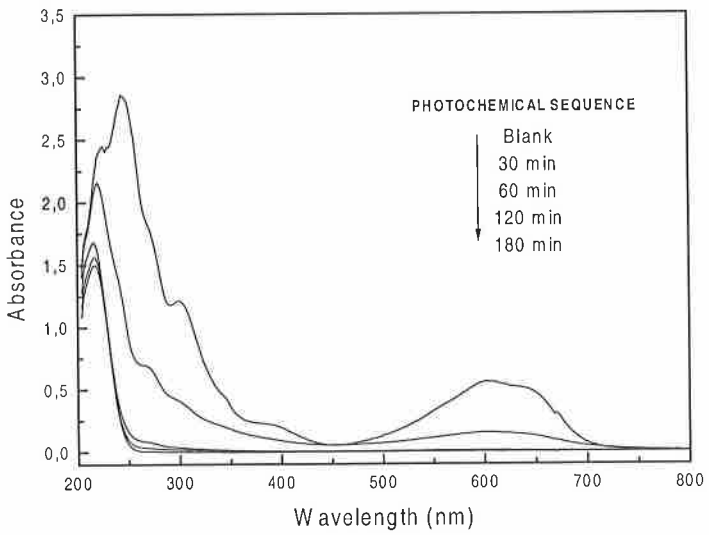

Figure 4. Spectrophotometric evaluation of the Ag-ZnO-assisted photochemical degradation of the dye. Dye concentration: 50 mgL^{-1}; pH: 5,5; Ag-ZnO: 15 mg.

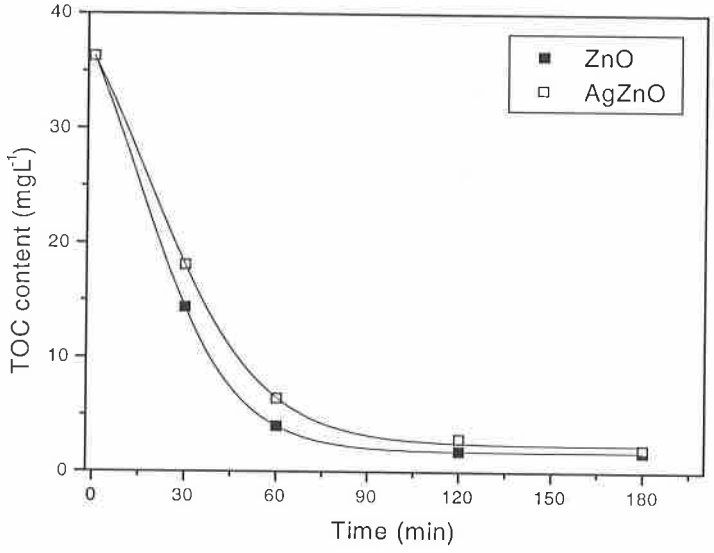

Figure 5. Reduction of the total organic carbon content by the dye photochemical degradation process. Dye concentration: 50 mgL^{-1}; pH: 7.0 (Ag-ZnO), pH 9.0 (ZnO); AgZnO: 15 mg; ZnO: 100 mg.

Table 1 – Photochemical reduction of the acute toxicity. Dye concentration: 50 mgL^{-1}; Lignin concentration: 200 mgL^{-1}; pH: 7.0 (dye) and 9.0 (lignin); photocatalyst: ZnO (100 mg); Ag-ZnO (15 mg).

SUBSTRATE (reaction time, min)	ACUTE TOXICITY (Relative respired CO_2, %)	
	ZnO	Ag-ZnO
Lignin	pH: 9.0	pH: 7.0
T0	10	10
T30	0	0
T180	28	0
Remazol brilliant blue R	pH: 9.0	pH: 5.5
T0	28	28
T30	0	0
T60	0	6
T180	20	22
Kraft E1 effluent	pH: 9.0	pH: 7.0
T0	80	80
T30	10	10
T360	0	0

FINAL REMARKS

Considering the recalcitrant character of the dye and lignin molecules and some results reported in the earlier literature (ex. total decolorization of lignin aqueous solutions by TiO_2 with reaction times higher than 8h (Kobayakawa et al., 1989), and almost total decolorization of acid orange 7 in reaction times of about 60 min by using a SnO_2-doped TiO_2 semiconductor assisted by an external anodic bias (Vinodgopal and Kamat, 1995)), the use of Ag-ZnO appears as a better new alternative. As commented previously, the achievement of a more efficient photocatalyst could be the first step for the definitive consolidation of the photochemical process as an effluent remediation alternative.

In this work, similar results were observed for ZnO and Ag-ZnO semiconductors. However, it is necessary to point out that in the experiments with ZnO a mass of 100 mg was used, while in the case of Ag-ZnO the mass was only 15 mg.

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REFERENCES

Cheng, S. & Nickel, U. (1996). Controllable exciton bleaching and recovery observed in ZnO-Ag hybrid nanometre-sized particles, *Chem. Commun.*, **2**, 133-134.

Hirano, K., Asayama, H., Hoshino, A. & Wakatsuki, A. (1996). Metal powder addition effect on the photocatalytic reactions and the photo-generated electric charge collected at na inert electrode in aqueous TiO_2 suspensions, *J. Photochem. Photobiol. A: Chem.*, **110**, 307-311.

Hoffmann, M., Martin, S.T., Choi, W. & Bahnemann, W. (1995). Environmental Applications of Semiconductor Photocatalysis, *Chem. Rev.*, **95**, 69-96.

Jardim, W.F., Gimenez, S.M.N., Canela, M.C. & Moraes, S.G. (1993). Acute toxicity of Hg and Hg^{2+} ions to *Escherichia coli*, *Chem. Spec. Bioavail*, **5**, 97-100.

Jardim, W.F., Moraes, S.G. & Takiyama,M.M.K. (1997). Photocatalytic degradation of aromatic chlorinated compounds using TiO_2: Toxicity of intermediates, *Wat. Res.*, **31**, 1728-1732.

Kobayakawa, K., Sato, Y., Nakamura, S. & Fujishima, A. (1989). Photodecomposition of kraft lignin catalized by titanium dioxide, *Bull. Chem. Soc.Jpn.*, **62**, 3433-3436.

Lisenbigler, A.L., Lu, G. & Yates, J.T. (1995). Photocatalysis on TiO_2 surfaces: principles, mechanism, and selected results, *Chem. Rev.*, **95**, 735-758.

Mills, A. & Le Hunte, S. (1997). An overview of semiconductor photocatalysis, *J. Photochem. Photobiol. A: Chem.*, **108**, 1-35.

Ranjit, K.T. & Viswanathan, B. (1997). Synthesis, charcaterization and photocatalytic properties of iron-doped TiO_2 catalysts, *J. Photochem. Photobiol. A: Chem.*, **108**, 79-84.

Vinodgopal, K. & Kamat, P.V. (1995). Enhanced rates of photocatalytic degradation of na azo dye using SnO_2/TiO_2 coupled semiconductor thin films, *Environ. Sci. Technol.*, **29**, 841-845.

Studies on loblolly pine lignin from the heterozygote genotype of the cinnamyl alcohol dehydrogenase (*CAD-N1*) mutation

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ABSTRACT

Lignin from the *cad-n1* heterozygote genotype loblolly pine was characterized to determine whether a 50% reduction in cinnamyl alcohol dehydrogenase (E.C. 1.1.1.195) activity had any phenotypic consequences. The lignin from the *cad-n1* heterozygote was compared with normal lignin and lignin from the *cad-n1* homozygote. The chemical analysis by DFRC showed that the *cad-n1* heterozygote had an intermediate level of guaiacyl subunits compared with normal and *cad-n1* homozygote lignins. A 2D NMR HMQC experiment did not reveal any meaningful differences between the *cad-n1* heterozygote lignin and normal lignin. These results suggest that there could be some subtle structural or compositional differences between normal and *cad-n1* heterozygote lignin. Thus, the *cad-n1* mutation appeared not to be completely recessive.

Keywords: cinnamyl alcohol dehydrogenase, lignin biosynthesis

INTRODUCTION

MacKay *et al,* (1997) described a naturally occuring loss-of-function mutation for the gene encoding cinnamyl alcohol dehydrogenase (E.C. 1.1.1.195) in loblolly pine (*Pinus taeda* L.). The expression of CAD enzyme activity in *cad-n1* homozygote seedlings was only 1% of the normal level, and the expression of CAD enzyme activity in the *cad-n1* heterozygote seedlings was 50% of the normal level. Heterozygotes have only one copy of the functional gene per diploid cell while normal genotypes have two. The wood from a 12-year-old *cad-n1* homozygote tree was shown to have a substantially altered lignin composition (MacKay *et al.,* 1997 and Ralph *et al.,* 1997). The *cad-n1* mutation demonstrated the large impact that variation in a single gene could have on the properties of wood. *Cad* gene expression has also been altered in genetic engineering experiments (Whetten *et al.,* 1998). Reduced CAD activity can increase the extractability of lignin and has economic implications for the production of paper (reviewed by Baucher *et al.,* 1998).

Is the *cad-n1* mutation completely recessive? In this paper we address the phenotypic consequences of reduced expression of CAD in heterozygotes for *cad-n1*. Lignin variation can be analyzed by chemically degrading lignin polymers and characterizing chemical structures of the resulting lignin fragments. Here, we used Derivitization Followed by Reductive Cleavage (DFRC, Lu and Ralph, 1997) an approach that systematically breaks α- and β-aryl ether bonds under mild reaction conditions. The guaiacyl and p-hydroxyphenyl subunits were quantified by gas chromatography. We also prepared Milled Wood Lignin (MWL) for a normal homozygote and a *cad-n1* heterozygote representative wood sample. A 2D NMR HMQC experiment was carried out on these samples and a sample of the MWL preparation used to originally characterize the *cad-n1* homozygote lignin. The objective of these experiments was simply to determine whether the lignin from the *cad-n1* heterozygote was identical with the normal lignin sample or intermediate between the normal and the *cad-n1* homozygote lignin samples.

EXPERIMENTAL

An open-pollinated family of loblolly pine accession 7-1037 (NCSU: Industry Cooperative Tree Improvement Program) provided wood for the *cad-n1* heterozygote and for the normal homozygote. These wood samples were obtained from a test planting site located in Lumberton, North Carolina. The trees were 4 years old and the wood samples were taken from a 50 cm length of stem just above DBH, which was approximately 6 cm. The wood samples were collected from approximately 12 trees per plot per genotype, chipped, and the chips bagged by plot and genotype. The samples for *cad-n1* heterozygote and normal homozygote wood were obtained by sampling approximately 50 chips per plot from four plots. A small amount of wood was shaved from each chip and pooled to constitute the two genotype samples from family

7-1037. A similar sample was obtained from chips of the *cad-nl* homozygote genotype reported by MacKay *et al.* (1997 PNAS). The trees from the 7-1037 OP family and the *cad-n1* homozygote tree shared a common grandparent, accession 7-56, which has a heterozygote genotype for the *cad-n1* gene.

A Wiley mill was used to grind the wood samples to pass through a 0.5 mm mesh screen. The wood was sequentially extracted using water, methanol, acetone and chloroform. Lignin was analyzed by Derivitization Followed by Reductive Cleavage (DFRC, Lu and Ralph, 1997). This method quantifies lignin monomer subunits released by cleaving α- and β-aryl ether bonds. A stock solution of internal standard was added to a 20 mg sample of ground, extracted wood. In this case the standard used was 4,4'-ethylidenebisphenol. A solution of AcBr in AcOH was added to the sample and stirred at 50°C for three hours. The solvent was then evaporated off under vacuum at 40°C. Then a solution of dioxane/AcOH/H_2O was added and 100 mg of Zn powder was stirred in. After stirring for 30 min. the zinc was filtered out and the solvent was evaporated. Finally the sample was acetylated using acetic anhydride and piridine for one hour. After the final evaporation the sample was dissolved in chloroform and quantified on a Hewlett Packard GC, using a Supelco column, 0.20 mm X 30m SPB-5. GC parameters: He gas at 1 ml/min, injector at 250°C, initial column temperature 140°C, ramped at 3°C/min to 300°C and held for 5 mins, FID at 300°C. MWL samples were then obtained following Björkman (1956) as modified by Chang *et al.* (1975). Samples were ball-milled under N_2 absent of toluene. The finished samples were analyzed by NMR. Spectra was taken on a Bruker AVANCE 500 MHz (1996 model)

RESULTS AND DISCUSSION

Representative wood samples for the three *cad* genotypes (*cad-n1* homozygote, *cad-n1* heterozygote, normal homozygote) were analyzed by DFRC. The gas chromatograms exhibited clearly resolved peaks corresponding with guaiacyl lignin subunits. The identity of these peaks was confirmed by comparison with standard compounds and GC-MS. The sample from the *cad-n1* homozygote showed no measurable guaiacyl peak. The samples from the two other genotypes both had pronounced guaiacyl peaks. The amount of guaiacyl subunits released from the normal heterozygote genotype was approximately double the amount released from the *cad-n1* heterozygote. A t-test rejected the null hypothesis of no difference in standardized peak area for the wood samples of the two genotypes ($P < .0001$).

Table 1. Standardized Peak areas of Guaiacyl Monomer for 14 DFRC Reactions from Normal and *cad-n1* Heterozygote Wood Samples.

cad-n1 heterozygote	normal
0.23	0.54
0.20	0.50
0.20	0.49
0.24	0.53
0.34	0.48
0.30	0.60
0.22	
0.25	
mean 0.25 ± 0.02	Mean 0.52 ± 0.02

Non-acetylated MWL samples from the three *cad* genotypes were analyzed by 2D NMR using the HMQC experiment. The normal homozygote and the *cad-n1* homozygote genotype differed substantially. The mutant homozygote showed signals in the aliphatic region corresponding to dihydroconiferyl alcohol (Ralph, 1997). The *cad-n1* homozygote spectra also showed increased intensity for vanillin and aldehyde signals, and to a decreased intensity for coniferyl alcohol signals. The spectra for the *cad-n1* heterozygote were very similar to the normal homozygote, with the exception of two different signals in the phenolic region. The structures giving rise to these signals have yet to be determined.

DFRC analysis of lignin from the different cad genotypes suggested that the cad null mutation is codominant. DFRC analysis of the lignin from the *cad-n1* heterozygote yielded approximately 50% of the amount of guaiacyl subunit as the normal homozygote. DFRC analysis of the *cad-n1* homozygote yielded no measurable amount of guaiacyl subunit. Normal pine wood has lignin composed primarily of guaiacyl subunits (approximately 95%, Adler, 1977). The amount of lignin in the normal homozygote and *cad-n1* heterozygote was approximately equal, but the *cad-n1* homozygote had a slightly smaller proportion of lignin (91%, Klason method, MacKay *et al.*, 1997). The *cad-n1* heterozygote could be comprised of less guaiacyl subunit than normal lignin (i.e., modified subunit composition), or the guaiacyl subunits were not released by DFRC (e.g., β - O - 4 linkages), or both. We observed no difference in the amount of p-hydroxyphenol subunit, but we could not separate and measure dihydroconiferyl alcohol subunits by GC of DFRC monomer products. Ralph *et al.* (1997) proposed that the lignin from the *cad-n1* homozygote was comprised of 15% guaiacyl subunits and 30% dihydroconiferyl alcohol subunits. An increase in the amount of dihydroconiferyl alcohol subunits in the lignin from the *cad-n1* heterozygote could result in a decrease of both β - O - 4 linkages and the guaiacyl subunits.

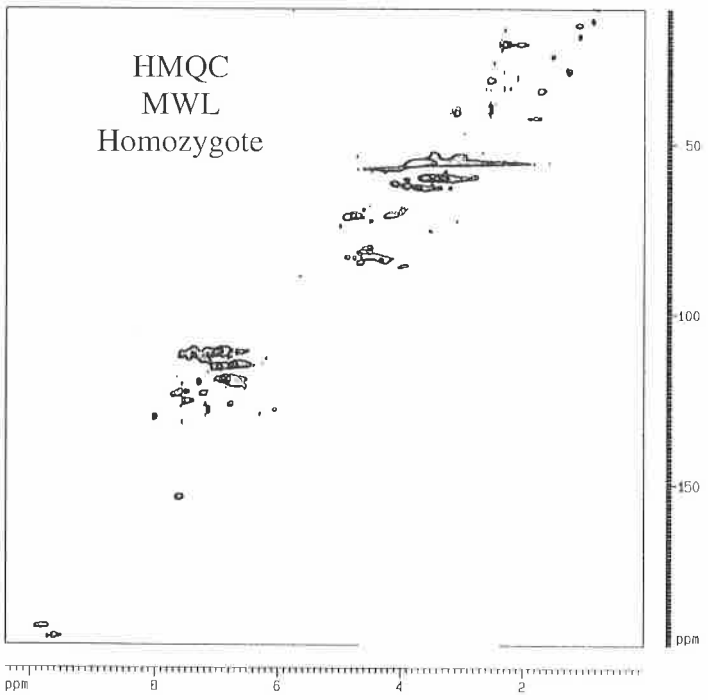
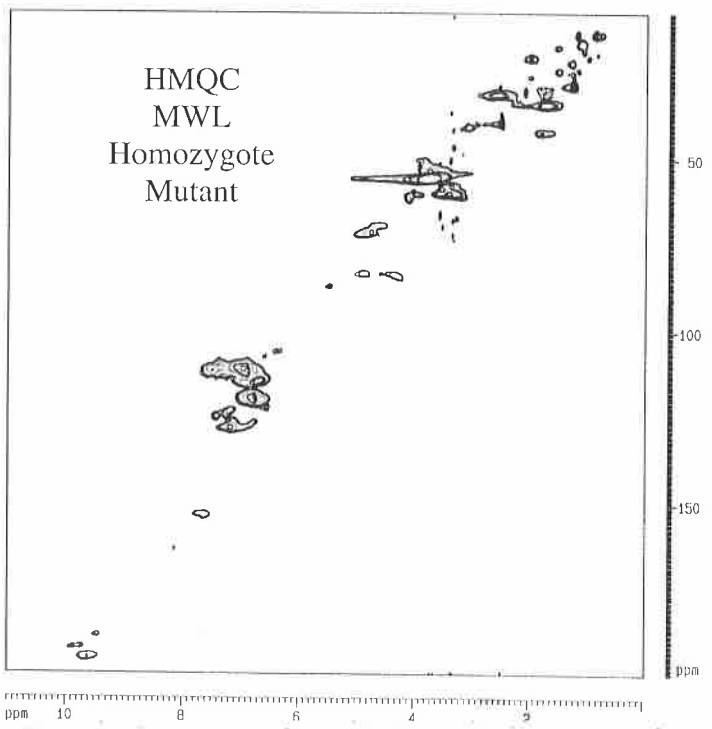


Figure 1a



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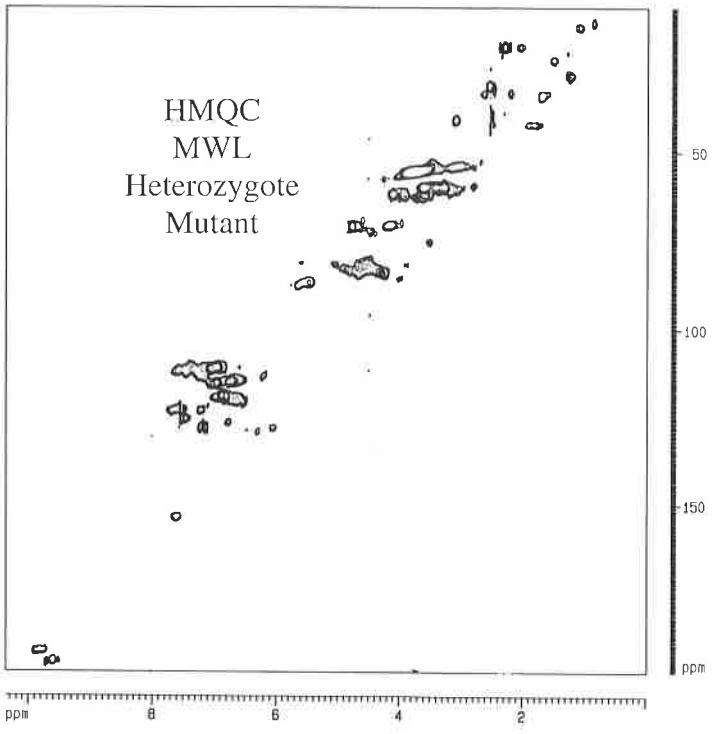

Figure 1. 2D NMR Spectra for MWL from 1a: Normal, 1b: *cad-n1* Homozygpte, 1c: *cad-n1* Heterozygote.

A 2D NMR HMQC experiment showed that the lignin from the *cad-n1* heterozygote more closely resembled the lignin from the normal homozygote, suggesting that the cad-n1 mutation is recessive. Ralph *et al.* (1997) carried out a 2D NMR HMQC-TOCSY experiment using a MWL preparation from the *cad-n1* homozygote and we used a sample of the same MWL preparation for our experiment. The strong signals present in the aldehyde and aliphatic regions of the spectrum of the *cad-n1* homozygote were not present in the *cad-n1* heterozygote genotype. The increased signal in the aliphatic region was interpreted as evidence for dihydroconiferyl alcohol subunits incorporated in the lignin of the *cad-n1* homozygote by Ralph *et al.* (1997). The spectra from the *cad-n1* homozygote showed a signal corresponding with 2-methoxybenzaldehyde (Ralph *et al.*, 1997) that was not present in the specta from the *cad-n1* heterozygote. However, the spectra from the *cad-n1* heterozygote showed signals in 2 regions that were not observed for the spectra from the normal homozygote. We were unable to identify any chemical structures that would be expected to correlate with signals in this region. The possibility exists that these signals either correlate to coniferyl aldehyde units or are simply artifacts. The 2D NMR HMQC experiment did not confirm the differences between the homozygote normal and *cad-n1* heterozygote detected by the DFRC method, but 2D NMR may not be sensitive enough to detect a small quantitative difference in lignin composition or structure.

We detected a statistically significant difference in lignin composition by DFRC that suggests that the cad null mutation is codominant. Most loss-of-function mutations are completely recessive, but a small proportion of genes are categorized as haploinsufficient. A 50% reduction in gene expression could produce a limitation in lignin biosynthesis that has phenotypic consequences, considering the large amount of photosynthate converted to lignin in the production of wood. We are pursuing further experiments to validate the DFRC monomer results. Analysis of the dimers and trimers from DFRC analysis of wood samples could be a more sensitive means to show small changes in lignin composition and bonding patterns. We are also pursuing an experiment to correlate the segregation of the cad mutation with the phenotypic difference in the abundance of guaiacyl monomer revealed by DFRC analysis. Our results suggest that the screening of large numbers of lignin samples by DFRC could uncover evidence of underlying mutations in the genes that encode lignin pathway enzymes even though they are in the heterozygous state. Very few mutant genes will occur in homozygous genotypes in forest trees due to the severe inbreeding depression and reduced viability. Most mutations in outbred species (e.g., forest trees and humans) occur in heterozygote genotypes and

appear recessive unless analyzed carefully by quantitative methods. Our work shows one way that DNA sequence variation could be associated with phenotypic differences in wood properties.

Acknowledgements. We are grateful to John Ralph for consultation on DFRC and NMR analysis, Hou-min Chang for expertise and direction on MWL preparation, Ewelyn Capanema and Mikhail Balakshin for consultation and skillful technical assistance, Kenji Kanazawa for advice and help with DFRC, Derek Harkins for help with computer graphics, Ron Sederoff and John MacKay for discussions and information on lignin, and Pioneer Hybred International for financial support.

REFERENCES

Adler, E. (1977). Lignin chemistry- past, present and future. *Wood Sci. and Technol.*, **11**, 169-218.

Baucher, M., Christenson, J.H., Meyermans H., Chen CY, Van Doorsselaere, J., Leple, J.P., Pilate, G., Petit-Conil, M., Jouanin, L., Chabbert B., Monties, B., Van Montagu, M. & Boerjan, W. (1998). Applications of molecular genetics for biosynthisis of novel lignins. *Polymer Degradation and Stability*, **59**, 47-52.

Björkman, A. (1956). Studies on Finely divided Wood. Part I. Extractions of Lignin with neutral Solvents., Svensk Pappers-tid., **59**, 477-485.

Chang, H.M., Cowling, E.B. & Brown, W. (1975). Comparative studies on Cellulolytic Enzyme Lignin and Milled Wood Lignin of Sweetgum and Spruce. Holzforchung, **29**, 153-159.

Lu, F. & Ralph, R. (1997). Derivitization Followed by Reductive Cleavage, a new method for Lignin Analysis, J. Agric. Food Chem., **45**, 2590-2592.

MacKay, J.J., O'Malley, D.M., Presnell, T., Booker, F.L., Campbell, M.M., Whetten, R.W. & Sederoff, R.R. (1997). Inheritance, gene expression, and lignin characterization in a mutant deficient in cinnamyl alcohol dehydrogenase, *Proc. Natl. Acad. Sci. USA*, **99**, 8255-8260.

Ralph, J., MacKay, J.J., Hatfield, R.D., O'Malley, D.M., Whetten, R.W. & Sederoff, R.R. (1997). Abnormal lignin in a loblolly pine mutant. *Science*, **277**, pp 235-239.

Whetten, R.W., MacKay J.J. & Sederoff, R.R. (1998). Recent advances in understanding lignin biosynthisis. Annu. Rev. Plant Physiol., *Plant Mol. Biol.*, **49**, 585-609.

Sugar cane bagasse utilization into fiber materials and value-added chemicals by steam explosion with the zero emissions target

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ABSTRACT

The steam explosion (SE) autohydrolysis treatment has been proposed as a method to obtain various value-added products from sugar cane (*Saccharum Officinarum*) bagasse. The changes occuring during SE treatment were evaluated using chemical composition analysis, solubility data, SEM micrographs and X-ray diffraction data. Both two-stage extractions (with hot water and subsequently with 90% ethanol) and one-stage extraction with 90% ethanol allowed separation of one quarter of the material obtained after SE treatment (235° C, 3 min). The destruction of the submicroscopical structure of bagasse and the damage of fibers as a result of SE was observed. The extraction procedure allowed removing the largest part of the formed fines and receiving a partially destructed fiber material. The bagasse plastic prepared using the solubles formed during SE treatment and the carbonized plastic produced from bagasse plastic were studied by X-ray diffraction. The transformation of bagasse under a simultaneous shear deformation under the high pressure (SDHP, 4 GPa) process was also estimated.

Keywords: sugar cane bagasse, steam explosion, hydrolysis

INTRODUCTION

Bagasse is usually burned to supply the energy required for evaporation of water during crystallization of sugar. However, a large amount of surplus bagasse still remains. Hence, it is significant to develop new environmentally friendly methods of bagasse processing into other value-added chemicals. Bagasse, owing to its worldwide availability and large quantities, will be a significant non-wood biomass resource to satisfy mankind demands for chemicals and materials. The Zero Emissions Research Initiative (ZERI), launched by the United Nations University (UNU) in 1994, proposes a concept and a methodology of structuring diverse industries into integrated clusters with Zero Emissions - no waste in the air, water, and soil. The aim that the waste from one industry becomes a raw material for other industries allows moving from the "cradle to grave" concept to the "cradle to cradle" one. It reflects positive changes toward a sustainable industry, symbolizes a shift from fossil resources to renewable sources and will change the focus from the consumption of natural forest to an alternative utilization of plantation or agricultural waste materials (Gravitis, Della Senta & Williams, 1997). ZERI is currently a project of the UNU Institute of Advanced Studies (UNU/IAS), Tokyo. The feasibility of bagasse utilization to produce charcoal and charcoal briquettes had been studied (Zandersons *et al.*, 1999a; 1999b; Zandersons *et al.*, 1999) in cooperation between the Latvian State Institute of Wood Chemistry (LSIWC), UNU/IAS and the University of Campinas.

As a method to obtain various value-added products from bagasse, the steam explosion (SE) autohydrolysis treatment has been proposed. Our previous investigations had shown that SE treatment allowed obtaining fibers and a variety of value-added biochemicals from wood and non-wood (bamboo, oil palm, parts of pineapple plant, corn cobs and kernels) biomass (Kokorevics *et al.*, 1998; Gravitis *et al.*, 1999). Simultaneous shear deformation under the high pressure (SDHP) action on bagasse will also be an interesting object of the study. Changes can be realized without any use of reagents, solvents and high temperatures, thereby SDHP will be regarded as an environmentally friendly technology. From the technological viewpoint, SDHP is a solid state extruding model process. The SDHP action on wood cell wall macromolecular components had been studied (Teeaar *et al.*, 1994). However, until the present time, the transformation of non-wood biomass under SDHP has not been studied. Both the SE and SDHP action on wood and non-wood biomass will be interpreted as a "self-sufficient" treatment method: the system components or conversion products start to act as physical and/or chemical factors necessary for further transformations during the process. The present article describes preliminary data of the SE and SDHP treatment of bagasse studies and briefly outlines the

possibilities to obtain potential value-added products. The research has been undertaken in the framework of the Zero Emissions Biorefinery approach developed at the UNU/IAS.

EXPERIMENTAL

Materials

Bagasse Brazilian origin, obtained after processing sugar cane in a sugar mill was used with the following composition (percentage from oven-dried material): ash – 3.2 %; acetyl groups – 3.2 %; protein – 2.4 %; ether extractives – 0.3%; 80% ethanol extractives – 1.9 %; easy hydrolyzable polysaccharides – 25.1 % (pentosans in easy hydrolyzable polysaccharides – 14.6 %); hard hydrolyzable polysaccharides – 36.7 %; Klason lignin – 25.4 % (OCH_3 groups in Klason lignin – 15.9 %). The scheme and methods of analysis has been described in detail (Zandersons, Kokorevics & Gravitis, 1999a). In the case of SDHP treatment, the bagasse was extracted subsequently by ether, 80% ethanol and water in a Soxlete type apparatus (6 h in each case) to remove extractives.

Steam Explosion (SE) treatment

A home-built batch-type SE device was used. Equipment includes electrically heated steam generator, 0.5 L reactor, pulp collector and condenser. Reactor was heated to desired temperature by electrical heater. Reactor was filled through the top trap and discharged through the ball valve on the bottom. Before the input in the SE device, the bagasse had been mechanically pressed into 15×15×50 mm briquettes to provide more rapid and thorough possible filling of the reaction chamber. The SE experiments conditions were characterized by the treatment temperature and duration using the so-called_severity factor or the reaction ordinate $\mathbf{R_0}$ (Overend & Chornet, 1987): $\mathbf{R_0} = \mathbf{t} \times \exp[(\mathbf{T}\text{-}100)/14.75]$ in units of time, where: **t** – treatment time (min); **T** – temperature (° C). In the present study, the following SE conditions were chosen: **T** - 235° C, **t**– 3 min, log $\mathbf{R_0}$ = 4.45. 145 g of air-dry bagasse specimen was loaded in SE reactor. Relative moisture content before SE treatment was 8.8 %. Two separation variants of the obtained materials were used, i.e. one and two stage extracted bagasse. For the former, batch extraction with 90% ethanol was done (72 h, twenty-fold excess of solvent, 20° C). According to two-step extraction, batch extraction with water was done (72 h, twenty-fold excess of solvent, 60° C), with subsequent filtration and washing with water (60° C). The second stage was the same as in the one-stage extraction.

Shear Deformation under High Pressure (SDHP) treatment

SDHP experiments were performed using a home-built "Bridgman anvils" device at room temperature and air atmosphere. Before the treatment, the bagasse had been milled into powder by a ball mill (particle size <0.2 mm). SDHP action conditions were characterized by pressure, the rotation angle of anvils (which played the role of time in the SDHP process kinetics) and the diameter of the anvils working surface. In the present study, the following SDHP conditions were chosen: pressure – 40 000 atmospheres (4 GPa); rotation angle – 360°, diameter of the anvils working surface – 9.1 mm. Relative moisture content before SDHP treatment – 7.8 %. The used SDHP device and treatment procedure had been described in details (Teeaar, Lippmaa, Gravitis et al., 1994). The water soluble fraction of the obtained material was separated by batch extraction with water (24 h, fifty-fold excess of solvent, 20° C), with subsequent washing with water (20° C).

Scanning electron microscopy (SEM)

Air dried samples were mounted on a stub using a double sided-adhesive tape and coated with gold in a T 1200 ion sputter and examined in scanning electron microscope JSM 840A at 15 kV.

X-ray diffraction (XRD)

Analysis was performed in a DMAX-2200 system (Rigaku International Corporation), using CuKα radiation (40 kV and 20 mA), in the range 2θ = 10° to 90°, with a variable slit in order to keep the irradiated area of 10 mm^2 on the specimen constant. The angular step was 0.1° in 2θ, with a counting time of 10 sec per step. The crystalline identification of XRD profiles was performed by searching a JCPDS data bank (Morales *et al.*, 1998).

RESULTS AND DISCUSSION

A loose homogeneous dark brown mass was obtained after the SE treatment (235° C, 3 min, log $\mathbf{R_0}$ =4.45) of bagasse. Relative moisture content after SE treatment – 47 %. Extraction with hot water allowed separating 5.2% (from oven-dried material), but subsequent extraction with 90% ethanol – 21.5%. In one-step extraction

with 90% ethanol, 26.6% of solubles was received. Both the methods give practically the same amount of soluble products. The composition of soluble fractions and the insoluble part will be an object of the further study.

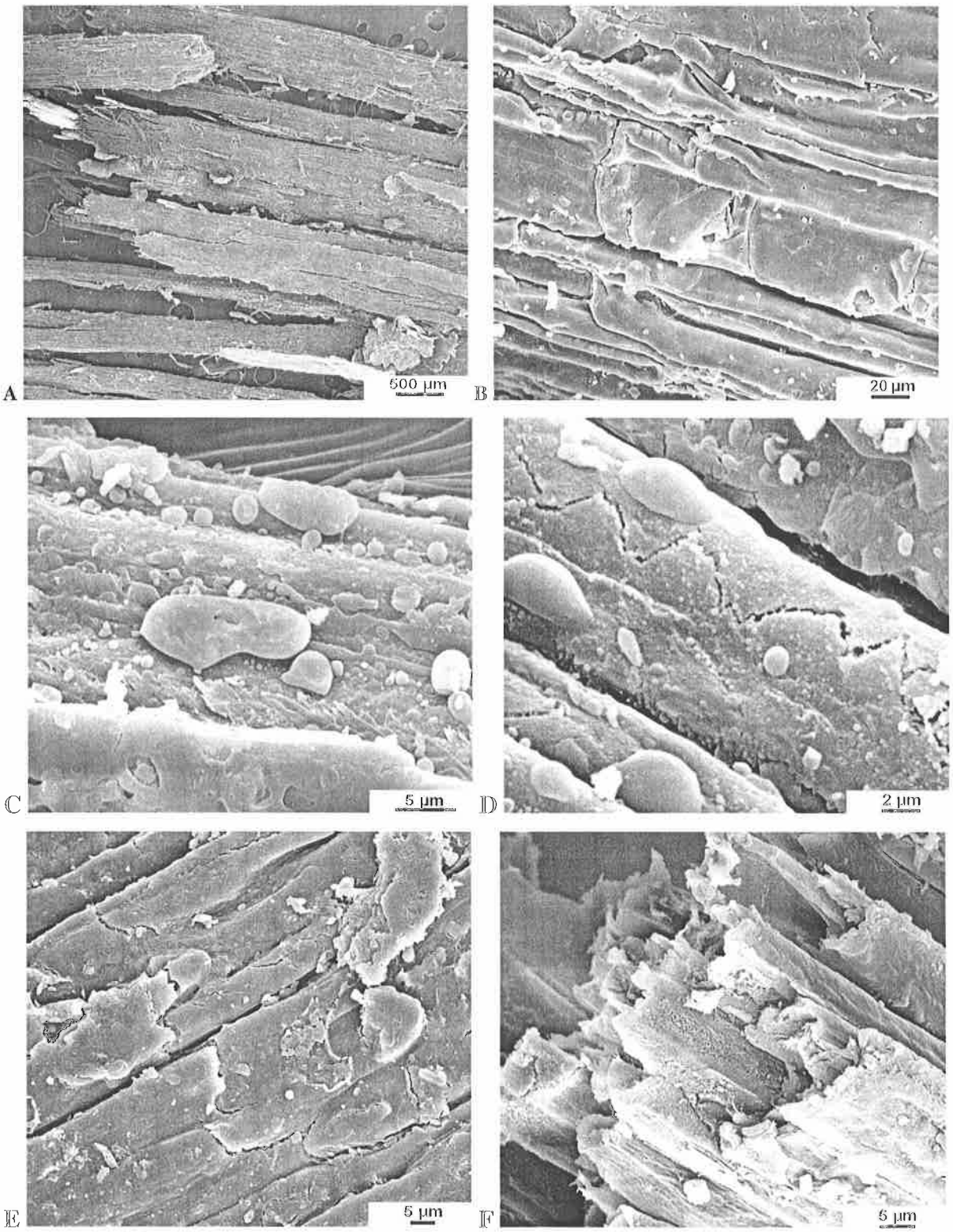

Figure 1. SEM micrographs of sugar cane bagasse: before SE treatment (A - overview of initial bundles of fibers, B - the centre parenhyma cells); after SE treatment (C, D); after SE treatment and hot water extraction (E); after SE treatment and 90% ethanol extraction (F).

Scanning electron microscopy (SEM) was used to detect changes in the cell and submicroscopical levels of the structure, and to evaluate the quality of fibers (Figure 1). In bagasse initial bundles of fibers (Figure 1A), vessel elements and parenhyma cells (Figure 1B) were detected. After SE treatment, a deeper destruction of

submicroscopical structure was observed. Few bundles of fibers are left, the sample consisted mostly of more or less rounded fractions. Broken vessel elements, fines consisting of pieces of broken cells, differently sized globules (Figure 1C), and broken fibers, also with a zigzag broken line (Figure 1D) and globules were detected. It is interesting that the globules attached to the surface were not adhesive to the surface and small sized globules tended to form microscopic droplets (Figure 1C, ID). After hot water extraction, a deeper destruction of fiber was detected, i.e. a cross-sectional breaking of fiber bundles (Figure 1E). The surface of fibers was covered with fines and amorphous substances which remained attached to the fiber bundles after hot water extraction. Two-stage extraction with hot water and ethanol produced a sample with smooth surfaces and free from fines. After one-stage extraction with 90% ethanol, the surface of fibers was less folded and more smooth. The bundles of fibers were slightly folded and covered with sediments (Figure 1F – the end of the disrupted bundle of fibers). It should be noted that neither one- nor two-stage extraction chosen in our investigation removed all the sediments and small particles attached to the fibers surface. The foldering surface was more clear after hot water and ethanol extraction. It was only ethanol extraction that modified slightly the surface, which seemed swollen even after drying.

Figure 2 shows a typical XRD halos (fiber structure) of initial bagasse as a raw material (Figure 2A), and after SE treatment (Figures 2B - 2E). It is interesting to observe the appearance of SiO_2 profiles due to SE.

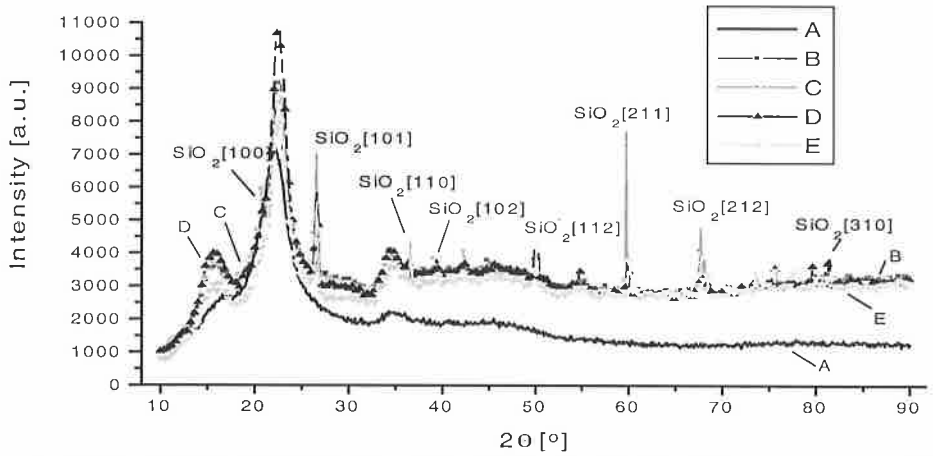

Figure 2. XRD of initial bagasse (A), after SE treatment (B), SE + water extraction (C), SE + water extraction and 90% ethanol extraction (D), and SE + 90% ethanol extraction (E).

Solubles extracted by 90% ethanol were used as a binder to produce a bagasse plastic: 75% of milled bagasse (the same as that used in SE treatment) was mixed with 25% of the binder and subsequently exposed to the pressure and temperature action. The obtained bagasse plastic was used to produce a carbonized plastic. The bagasse plastic and carbonized plastic were produced according to an original method, which is under development at the LSIWC. The producing of such new materials will be one of the ways of a sustainable utilization of bagasse. Bagasse plastic and the carbonized plastic produced using the solubles obtained by SE treatment as a binder were compared with the materials produced from bagasse by the same method, both using synthetic phenolic alcohols as a binder and without the use of any binders.

The XRD patterns of bagasse plastic are shown in Figure 3, where: Figure 3A – without use of any binder, Figure 3B – with binder of SE lignin of bagasse, Figure 3C – phenolic alcohol binder, Figure 3D – the binder itself, extracted with water acetone mixture. The crystalline structure evolution by increasing diffraction intensity and line broadening, and by the angular shifting of the lines through the graphite characteristic values (Emmerich *et al.*, 1987) takes place from Figures 3A to 3C. The binder itself shows a completely amorphous structure (Figure 3D). These samples after carbonization at 900°C (carbonized plastic) are presented in Figure 4. It is interesting to observe the structural evolution of carbon random structure into graphite-2H structure (g-C (002)), as well as the two dimensional hk (10) and hk (11) reflections for the specimens prepared without binder and SE binder (Figures 4A and 4B). For phenolic alcohol binder (Figure 4C), there is a substantial decrease of the graphite structure evolution.

After the SDHP action (360° rotation angle, 40 000 atm) on bagasse powder a light brown glass-like mass was received. The material was similar to that obtained after SDHP treatment of birch wood and cellulose

(Teeaar *et al.*, 1994). Extraction with water solubilized 41 % of substances from the initial mass. This solubilization was a substantially higher than the amount of solubles obtained after SDHP treatment of birch wood under the same conditions - 24% (Teeaar *et al.*, 1994). It can be explained by the fact that bagasse has a higher content of easy hydrolyzable polysaccharides. As shown in our previous investigations (Teeaar *et al.*, 1994), the water-soluble fraction consists of a mixture of mono- and oligosaccharides formed during depolymerization of cellulose and hemicelluloses in the solid state. Detailed investigations regarding the polysaccharides and lignin transformation in the non-wood plant cell wall under the SDHP action will be an object of the further studies.

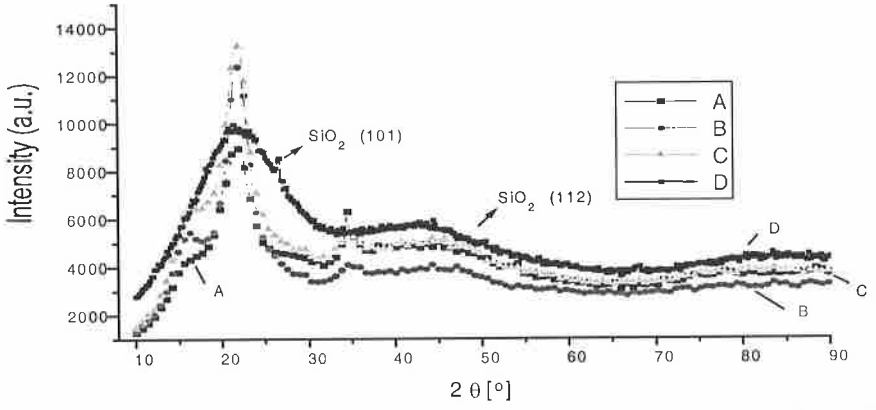

Figure 3. XRD of bagasse plastic: A – without use of any binder, B – with binder of SE lignin, C – phenolic alcohol binder, D – binder itself.

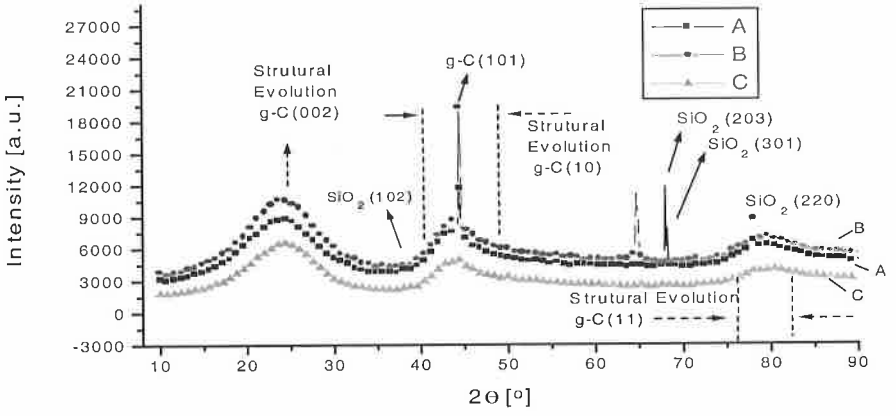

Figure 4. XRD of carbonized plastic samples: A – without binder, B – SE binder, and C – phenolic alcohol binder.

The effect of sample amorphyzation by SDHP action can be observed in Figure 5. The XRD patterns before (Figure 5A) and after SDHP action (Figure 5B) shows a transformation of semi-crystalline structure line into a completely amorphous structure (broad halo).

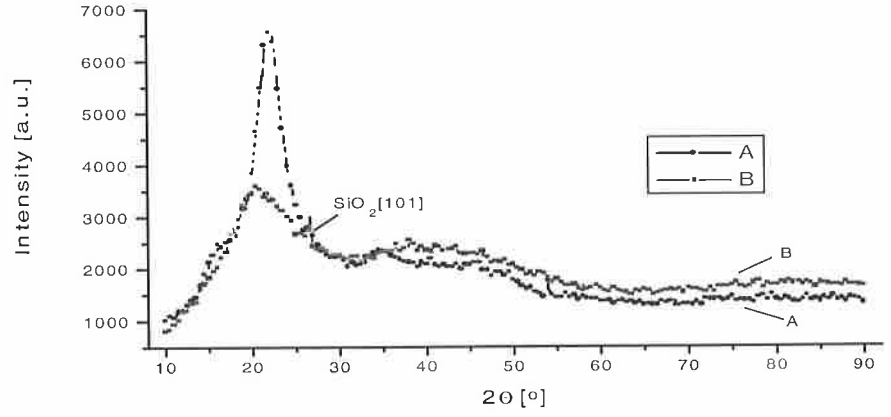

Figure 5. The effect of amorphyzation by SDHP observed by XRD.

Our preliminary studies have demonstrated that SE and SDHP will be effective methods of bagasse processing into potential value-added products. SE will play a role of one of the core industries around which supplementary industries will be integrated, according to the Zero Emissions Biorefinery approach. Bagasse based plastic and carbonized plastic materials are an attractive example. Sulfur-free SE lignin will be used as a binder for fiberboard and particleboard production instead of conventional materials based on formaldehyde synthetic binders. The processed fibers can be used as a blending material to produce composite materials. Water-soluble sugars and oligosaccharides will be promising raw materials for subsequent microbiological treatment after SE (including production of protein, bioethanol, citric acid, etc.).

REFERENCES

Emmerich, F.G., De Souza, J.C., Torriani, I.L. & Luengo, C.A. (1987). Applications of a granular model and percolation theory to the electrical resistivity of heat treated endocarp of babassu nut. *Carbon*, **25**, 417-424.

Gravitis, J., Della Senta, T. & Williams, E.D. (1997). The Conversation of biomass into fuels, fibers and value added chemical products from the perspective of the Zero Emissions concept. *In Symposium of the Biomass Conversion*, Proceedings, pp. 1-18.

Gravitis, J., Kokorevics, A., Bikovens, O., Kallavus, U. & Teeaar, R. (1999). Steam exploded bamboo fibers structure studies. *In 10th International Symposium on Wood and Pulping Chemistry,* Proceedings, **Vol 1**, pp. 656-661.

Kokorevics, A., Gravitis, J., Bikovens, O., Kallavus, U. & Teeaar, R. (1998). Tropical biomass conversion into value added products using steam explosion. *In 12th International Congress of Chemical and Processing Engineering CHISA'98,* CD-ROM of full texts. Report P5.65, pp. 1-20.

Morale, R.C., Godoy, P.H., Shinohara, A.H., Suzuki, C.K., Kokorevics, A., Zandersons, J. & Gravitis, J. (1998). *In 13th Brazilian Conference on Engineering and Materials Science,* CD-ROM of full texts, pp. 4609-4616.

Overend, R.P. & Chornet, E. (1987). Fractionation of lignocellulosics by steam-aqueous pretreatments. *Phil. Trans. R. Soc. Lond.*, **A321**, 523-536.

Teeaar, R., Lippmaa, E., Gravitis, J., Kokorevics, A., Kreituss, A. & Zharov, A. (1994). Structural changes of cellulose, wood, and paper under shear deformation and high pressure. *J. Appl. Polym. Sci.,* **54**, 697-708.

Zandersons, J., Kokorevics, A. & Gravitis, J. (1999a). Reduction of Amazon and Cerrado deforestation using sugar cane bagasse as an alternative wood source for charcoal production. Technological feasibility studies. *UNU/IAS Working Paper*, **N 57**, 1-44.

Zandersons, J., Kokorevics, A. & Gravitis, J. (1999b). Studies of bagasse charcoal briquetting, reduction of the ash content in charcoal, and preliminary material and energy estimations for the design of a pilot plant of bagasse charcoal. *UNU/IAS Working Paper*, **N 58**, 1-33.

Zandersons, J., R.C., Zhurinsh, A., Kokorevics, Gravitis, J., Morales,R. C., Godoy, P.H., Shinohara, A.K. & Suzuki, C.K. (1999). Some characteristic features of dispersed lignocellulosic biomass carbonization chemistry. *In 10th International Symposium on Wood and Pulping Chemistry,* Proceedings, **Vol 3**, pp. 398-403.

Textile effluent remediation using the sequence ozone-*Phanerochaete chrysosporium*

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ABSTRACT

Textile effluent normally is not efficiently treated by conventional treatment methods. The principal problem associated to the treatment of this kind of effluent is due to the presence of recalcitrant compounds like dyes that are wasted during the dyeing of textile fiber. This generates a highly colored effluent that, when released without treatment, can cause serious environmental problems. The use of ozone to treat this effluent has been shown to be efficient mainly in the degradation of the dyes. However, this shows some limitations like low mineralization ratios and some times an increase in the toxicity during ozonization. The biological treatment with *P. chrysosporium*, generally decrease the toxicity and degrade significantly the effluent but show a low degradation kinetic. In this work a combined method was tested using a chemical method (ozone) followed by the biological one (*P. chrysosporium*) to degrade a textile effluent. Some chemical parameters (decoloration, total organic carbon, effluent molecular mass distribution and total phenols) and toxicity using two different toxicity tests to evaluate the process were performed. The pre-treatment with ozone showed a fast and good decoloration, a considerable decrease in the molecular mass although significant reduction in total organic carbon were not reached and the toxicity was increased during ozonization. The post-treatment with the fungus did not reduce significantly the physico-chemical parameters but remove the toxicity that was initially increased by ozonization process.

Keywords: textile effluents, ozone, *Phanerochaete chrysosporium*

INTRODUCTION

Textile effluent are characterized by high pH values, a high organic matter concentration and intensely coloured. The effluent coloration is due to the presence of dyes that are released during the dying process of textile being normally recalcitrant to conventional biological treatment systems. The textile sector, in Brazil, waste around 20 ton/year of dyes and about 20 % of the total amount used are released to the effluent and normally discharged to the receptor bodies (Copper *et al.*, 1994; Furtado, 1996).

Nowadays, there are a lot of physical, chemical and biological processes that can be used in water and wastewater treatment. Combined processes combination shows some advantages and disadvantages mainly associated with some practical parameters like efficiency, versatility and cost of the process, being necessary to analyze each matrix to be treated.

In textile effluent, between the chemical processes, the pre-treatment with ozone is a good alternative when compared to other treatments due to that process is very efficient in the effluent decoloration, which is the main problem in textile effluent treatment (Churchley, 1994; Tzitzi *et al.*, 1994; Peralta-Zamora *et al.*, 1999).

Between the biological processes used in the degradation of recalcitrant compounds the basidiomycete fungi has been spared a great atention specially the white rot fungus *Phanerochaete chrysoporium* that has demonstrated very promissing results in this field (Haimann, 1995; Reddy, 1995; Swammy *et al.*, 1999).

In this work, textile effluent treatment was evaluated combining a chemical process (ozone) and a biological one (*P. chrysosporium*). The effluent was previously ozonized and after that submitted to a treatment with *Phanerochaete chrysosporium*. Some physico-chemical parameters and toxicity (*Scenedesmus subspicatus* and *Escherichia coli*) during both treatments were measured.

EXPERIMENTAL

Textile effluent

The textile effluent used in this study was obtained from a textile industry in Campinas region (São Paulo - Brazil). The effluent was stored at 4 ^{0}C and used without previous treatment.

Treatment with ozone

Generation and measurement of ozone

Ozone was generated from pure oxygen using a OZOCAV ZT-2 (Inter Ozone Ingenieria Ecologica, Santiago-Chile) equipment. This apparatus has a capacity of ozone generation in a range between 0.20 and 0.70 g h^{-1}. The produced ozone was determinated spectrophotometrically at 258 nm by passing the gas phase, which contains the mixture of oxygen and ozone, through a flow cell (Chieu *et al.*, 1995).

Ozonization

Samples of textile effluent were submitted to ozonization at pH 11 and room temperature, using a tubular reactor of 500 mL with a syntered glass dispersing that releases the gas from the bottom to the top of the reactor. The ozonized sample volumes were 400 mL, the oxygen flow rate was adjusted to 15 (±1) L h^{-1}, obtaining an ozone production of 14 mg L^{-1}, reaching the ozone steady-state production in 10 min (Kunz *et al.*, 1999).

Treatment with *P. chrysosporium*

Spores suspension preparation

The lyophilized *P. chrysosporium* (ATCC 24725) was rehydrated and seeded in solid medium in malt extract agar (2,5%) and incubated at 37 ^{0}C for five days. After the fifth day the solid medium was washed with sterilized water and the resulted suspension filtered with glass wool. The spores suspension concentration was determined spectrophotometrically at 650 nm (Linko, 1986).

Spores suspension inoculation in textile effluent

The spores suspension were inoculated in Erlenmeyers with 75 mL of textile effluent (with the pH previously adjusted between 4.5 – 5.0) and 1.7 g of nylon (support) cuted at shape of 0.5 x 1.0 x 1.0 cm. The inoculated spores suspension volume was corresponding to 2.2 x 10^5 spores mL^{-1}. The Erlenmeyer were incubated under agitation (120 rpm) at 37 ^{0}C. After reduction and stabilization of reducing sugars, veratryl alcohol and Tween-80 were added until final concentration of 0.6 mmol $L^{-1}$and 0.13 % respectively and agitation reduced to 60 rpm. Daily oxygen pulses (flow rate between 10 – 15 L h^{-1}) during 2 min were given to the samples with the fungus and effluent after veratryl alcohol addition. Control samples were constituted by effluent (without the fungus spores addition) and support.

Analytical control

Color determinations were performed by previous sample centrifugation at 3500 rpm during 5 min at pH 11, sample pH adjusted at pH 7.6 (with a phosphate buffer) and the absorbance measured at 400, 500, 550 and 600 nm using a Hitachi U-200 spectrophotometer The total organic carbon (TOC) reduction were measured using a Shimadzu TOC 5000A analyzer. The molecular mass distribution of effluent was determined by gel permeation chromatography (GPC) using a sephadex G-50 (1.5 x 60 cm). The mobile phase was 0.1 mol L^{-1} NaOH-LiCl adjusted at 0.66 mL min^{-1} (Ferrer *et al.*, 1991).Total phenols were determined by Folin-Ciocalteous method (APHA, 1992). Enzymatic activities were determined spectrophotometrically at 310 nm for lignin peroxidase (Tien at al., 1984), at 610 nm for manganes peroxidase (Kuwahara *et al.*, 1984) and at 525 nm for laccase (Szklarz *et al.*, 1989).

Acute toxicity tests

Algae test

The samples were incubated to 23 ^{0}C and under constant agitation to 100 rpm for 72 h under irradiation (approximately 70 mmol. m^{-2} s^{-1}). Measurements of the algae *S. subspicatus* concentration were determined at the begining and at the end of the test by *chlorophyll a* fluorescence measurements (ISO, 1989).

Bacterium test

The respirometric test of the effluent was evaluated by measuring the inhibition of the respiration of *Escherichia coli* (ATCC 25922) by a modified method as described in Durán *et al* (1998) using 3 mL of samples.

RESULTS AND DISCUSSION

Pre-treatment with ozone

The Figure 1a shows the textile effluent decoloration after different ozonization times. Around 50 % decoloration was reached after sixty min of treatment. The molecular mass distribution (Figure 1b) decreased

significantly during the ozonization speciallly until sixty min of treatment, showing that the treatment can be interrupted after this time.

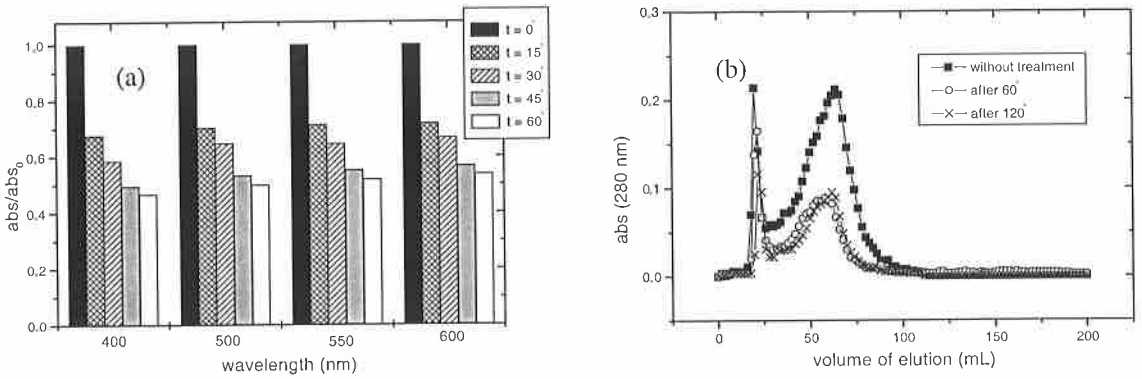

Figure 1. Decoloration of textile effluent ozonized at pH 11 (a) and molecular mass distribution before and after ozonization (b).

The Figure 2 shows the ozone consumed during the treatment, its possible to observe that after sixty min, the ozone consumption decrease that is in acording the datas showed above.

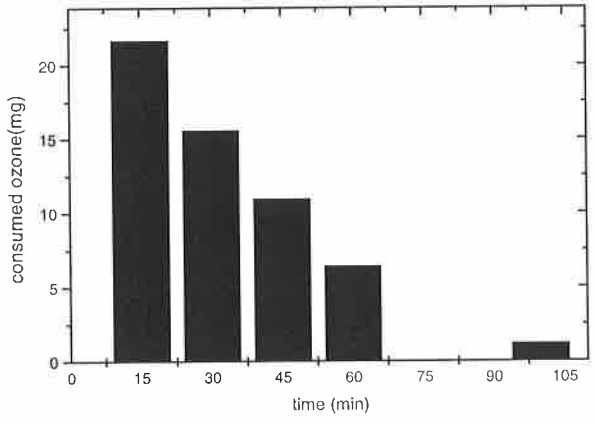

Figure 2. Consumed ozone at different ozonization times.

The total phenol reduction of the ozonized sample (FIGURA 3) was around 50 % after sixty min of ozonization. The degradation of this kind of compounds by ozone is reported in the literature showing that ozone is efficient in the degradation of phenolic compounds (Rice, 1981; Qieu *et al.*, 1999). Nevertheless, the reduction of total organic carbon (Figure 3) was not significantly removed. The same trend were observed with phenols and molecular mass reduction suggesting that the of utilization of a complementary method to mineralize the effluent is recommended.

The acute toxicity experiments using the two test organisms showed that it increase with the ozonization process, indicating that although degradation are occurring more toxic intermediates are formed, compared with the initial compounds (Table 1 and Figure 4).

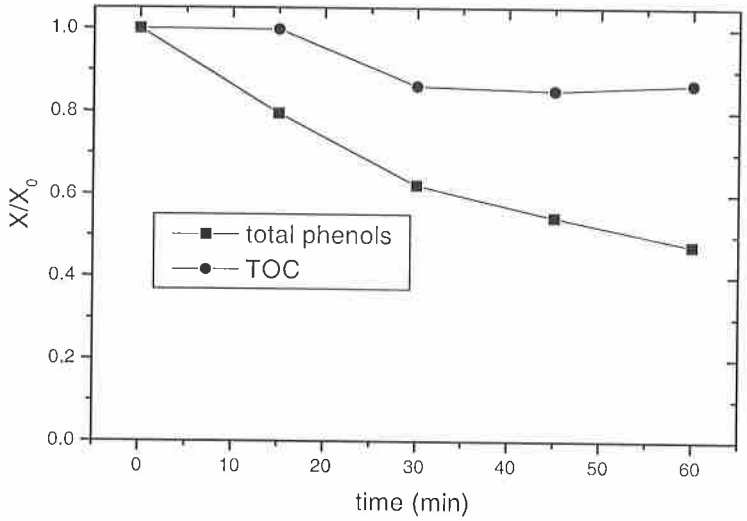

Figure 3. Total phenols reduction (X_0 = 16 mg L^{-1}) and TOC (X_o = 770.50 mg L^{-1}) of textile effluent after some ozonization time.

Table 1. Toxicity of textile effluent to *E. coli*, without ozonization and after 60 and 120 min of treatment.

Time of ozonization (min)	inhibition (%)
0	10.9
60	61.5
120	45.0

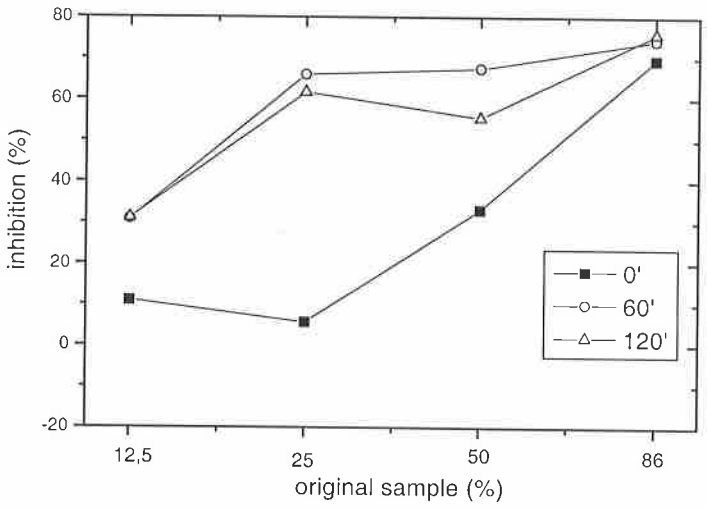

Figure 4. Textile effluent toxicity before and after ozonization determined by *S. subspicatus*.

Post-treatament with *P. chrysosporium*

The Figure 5a shows the color changes of textile effluent after some days of treatment. It is possible to observe that no effluent decolouration is verified with the treatment with the fungus, being detected an increase in the effluent color. The molecular mass distribution (Figure 5b) show that the treatment with the fungus do not degrade significantly the effluent, the third peak appearing in the nine day of treatment refers to Tween-80 added to the medium after the sixth day.

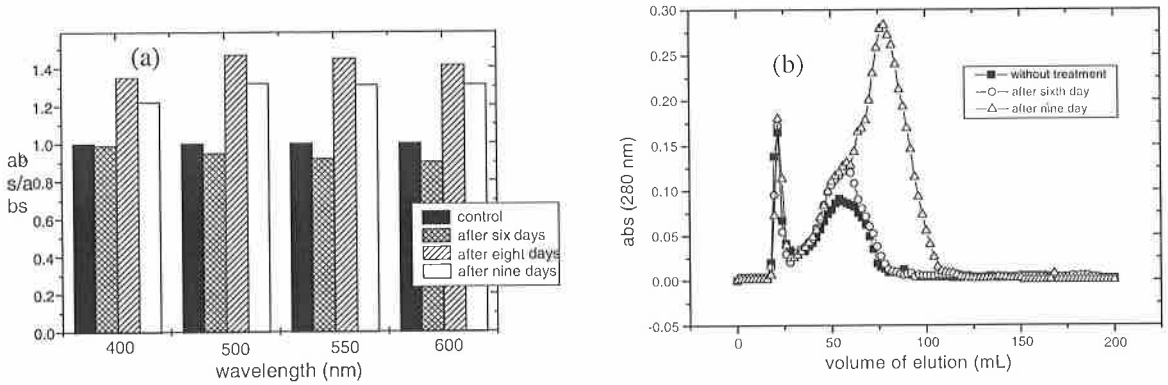

Figure 5. Change in textile effluent color (pre-ozonized) after some days of treatment with *P. chrysosporium* (a) and effluent molecular mass distribution (pre-ozonized) treated with *P. chrysosporium* (b).

A low lignin peroxidase activity as detected in the effluent (Table 2) after the adition of veratryl alcohol but this cause no decoloration of the effluent. Laccase and manganes peroxidase activities were not observed in these experimental conditions.

Table 2. Lignin peroxidase activity in textile effluent (pre-ozonized) after some days of treatment with *P. chrysosporium*.

Day	6	8	9
LiP ($U\ L^{-1}$)	0	29.4	17.7

Apparently the pre-ozonized effluent treatment with *P. chrysosporium* do not show a significant effect in the degradation but the effluent toxicity was reduced to both test organisms (S. *subspicatus* and *E. coli*) (Figure 6 and Table 3).

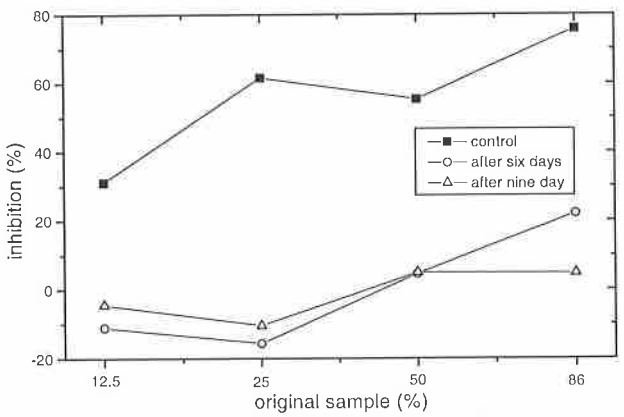

Figure 6. Textile effluent toxicity (pre-ozonized) treated with *P. chrysosporium* presented to *S. subspicatus*.

Table 3. Toxicity of textile effluent (pre-ozonized), before and after treatment with *P. chrysosporium*, presented to *E. coli*.

Days of treatment	Inhibition (%)
initial	42.36
six days	without inhibition
nine days	without inhibition

In conclusion, the pre-treatment with ozone showed a fast and good decoloration, a significant decrease in the molecular mass although considerable reduction in total organic carbon was not reached and the toxicity was increased during ozonization. The post-treatment with the fungus did not reduce significantly the physico-chemical parameters but remove the toxicity that was initially increased by the ozonization process.

Acknowledgements. FAPESP.

REFERENCES

APHA (1992). Standard methods for examination of water and wastewater, 18th edition. Ed. Amer. Publ. Health Ass. Nº 5550, pp. 5.41 - 5.42.

Chiou, C.; Mariñas, B.J. & Adams, J.Q. (1995). Modified indigo method for gaseous and aqueous ozone analyses. *Ozone Scie. & Eng.* **17**, 329 - 344.

Churchley, J. H. (1994). Removal of dyewaste colour from sewage effluent - The use of a full scale ozone plant. *Wat. Sci Technol.* **30**, 275-284.

Copper P.; Hinchcliffe C. & Churchley J. (1994). Proc. of intern. sem. - Textile industries Trade Effluents. Rochdale february.

Durán, N.; Sato, L.M.; Jardim, L.S.A.; Moraes, S.G. & De Conti, R. (1998). Nuevo metodo de sintesis de N-Indol-Glicosidos con actividad Antimicrobiana. *Bol. Soc. Chil. Quim.* **43**, 091-095.

Ferrer, I; Dezotti, M. & Durán, N. (1991). Decolorization of Kraft effluent by free and immobilized lignin peroxidases and horseradish peroxidase. *Biotechnol. Lett.* **11**, 577 – 582.

Furtado, M. R. (1996). Têxtil, beneficiamento lucra com alta tecnologia. *Quím. Der.*, 10 - 17.

Haimann, R.A. (1995). Fungal technologies for the treatment of hazordous waste. *Environ. Progr.*, **14**, 201 - 203.

ISO 8692 (1989). Water quality – Fresh water algal growth inhibition test with *Scenedesmus subspicatus* and *Selenastrum capricornutum.*

Kunz, A.; Freire, R.S.; Rohwedder, J.J.R; Durán, N.; Mansilla, H. & Rodriguez, J. (1999). Construção e otimização de um sistema para produção e aplicação de ozônio em escala de laboratório. *Química Nova* **22,** 425 – 428.

Kuwahara, M.; Glenn, J.K.; Morgan, M.A. & Gold, M.H. (1984). Separation and characterization of two extracellular H_2O_2-dependent oxidases from lignolytic cultures of *P. chrysosporium. FEBS J.* **169**, 247 - 250.

Linko, Y-Y (1986). Continuous production of lignin peroxidase by *P. chrysosporium. J. Biotechnol.*, **4**, 283 - 291.

Peralta-Zamora, P.; Kunz, A.; Moraes, S.G.; Pelegrini, R.; Moleiro, P.C.; Reyes, J. & Durán, N. (1999). Degradation of reactive dyes I. A comparative study of ozonation, enzymatic and photochemical processes. *Chemosphere,* **38**, 835 – 852.

Qiu, B.Y.; Zappi, M.E.; Kuo, C. & Fleming, E.C. (1999). Kiinetic and mechanistic study of ozonization of three dichlorophenols in aqueous solutions. *J. Environ. Eng.*, 441 – 450.

Reddy, C.A. (1995). The Potential for white-rot fungi in the treatment of pollutants. *Cur. Oppinium Biotechnol.* **6 (3)**, 320 - 328.

Rice, R. G. (1981). Ozone for the treatment of hazordous materials. *AICHE Symp. Series,* **209 (77)**, 79 - 107.

Swamy, J. & Ramsay, J. A. (1999). The evaluation of the white rot fungi in the decoloration of textile dyes. *Enz. Microb. Technol.* **24**, 130 – 137.

Szklarz, G.D. & Antibus, R.K. (1989). Production of phenol oxidases and peroxidases by wood-rooting fungi. *Mycologia,* **81**, 234 - 240.

Tien, M. & Kirk, T.K. (1984). Lignin-degradation enzyme from *P. chrysosporium* : purification, characterization and catalytic properties of a unique H_2O_2-required oxigenase. *Proc. Nat. Ac. Sci. USA*, **81**, 2280 - 2284.

Tzitzi, M.; Vagenas, V. & Lyberatos. G. (1994). Pretreatment of textile industry wastewaters with ozone. *Wat. Sci. Technol.,* **29(9)**, 151 - 160.

Understanding lignin condensation reactions during pulping and oxygen delignification

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ABSTRACT

This paper describes our efforts focused at defining the formation of diphenylmethane moieties in lignin as they emerge during conventional and EMCC7 kraft and soda pulping conditions. In addition it describes the fate of the condensed structures present in softwood residual kraft lignin during oxygen delignification. Overall, a new perspective with regard the abundance of such units, their formation profiles and their reactivity is presented. Diphenylmethane phenolic moieties when determined in isolated residual lignins from two western hemlock kraft pulp samples produced *via* an EMCC7 mill protocol and a laboratory batch digester, respectively, were found to prevail amongst the condensed phenolic units by 33% in the conventional cook. This information provided additional evidence supporting the fact that modern modified cooking technology after the structure of residual kraft lignin beneficially. Detailed quantitative ^{31}P NMR measurements have also provided three-dimensional plots describing the actual kinetics of formation and/or elimination of the condensed phenolic units present in residual kraft lignin under oxygen delignification conditions. A large portion of the free phenolic units (40-50%) present in residual kraft lignin was found to be fairly resistant to degradation by oxygen. 5-5´ biphenolic condensed moieties that originate from protolignin, were found to be fairly resistant to oxygen delignification conditions and dominated (>55%) the condensed phenolic units after extensive oxygen treatment. Therefore, despite their phenolic nature, diphenylmethanes and 5-5´ biphenols may be key inert units contributing to the resistance of residual kraft lignin toward oxygen delignification.

Keywords: lignin, kraft pulp, bleaching, NMR, ^{1}P-NMR

INTRODUCTION

While the ether bonds of lignin cleave efficiently under alkaline pulping conditions, the formation of condensed structures decreases its reactivity and diminishes the solubility of the residual lignin (Marton, 1971). This phenomenon seriously interferes with the complete removal of lignin and the rate of delignification, especially during the final stages of most pulping processes.

Amongst the reactions that are known to interfere with the process of alkaline delignification are those involving the formation of carbon-carbon bonds. Formaldehyde is known to be liberated as early as 100°C, as a result of Cγ elimination from phenyl- coumaran (Adler *et al.,* 1964; Gierer and Söderberg, 1959) and from guaiacylglycerol-β-aryl ether structures, both abundant in native lignin (Adler *et al.,* 1964; Adler *et al.,* 1961; Gierer *et al.,* 1965). The released formaldehyde is known to cause the substitution of hydroxymethyl groups at positions *ortho* to the phenolate moieties (Adler *et al.,* 1964). This potentially represents a very important reaction since one-third of the phenolic nuclei in softwood kraft lignin (Marton *et al.,* 1966) and about one-half of the phenolic and non-phenolic units in softwood milled wood lignin contain free C5 positions (Adler *et al.,* 1961). The methylolated phenols may undergo further reaction to yield diphenylmethane moieties (Marton, 1971; Chirkin and Tishschenko, 1962; Harkin, 1966).

Although several studies by Chiang and Funaoka (1988; 1990 a; b) have claimed to show convincing qualitative and quantitative evidence for the presence of diphenylmethanes in residual kraft lignin, a number of questions in relation to the method of detection have recently surfaced (Chan *et al.,* 1995). Since the development of ^{31}P NMR spectroscopy for probing lignin structure (Archipov *et al.,* 1991a; b; Argyropoulos *et al.,* 1992; 1993; Granata and Argyropoulos, 1995), the technique has been successfully implemented for the absolute determination of the various phenolic and non-phenolic structures in several native and technical lignins (Argyropoulos *et al.,* 1995; Sun and Argyropoulos, 1995; Froass *et al.,* 1996; Smit *et al.,* 1977). The availability of a phosphitylating reagent (Granata and Argyropoulos, 1995) that permits an excellent resolution and the quantitative spectroscopic detection of several phenolic moieties with varying substitution

patterns (Smit *et al.*, 1997; Jiang and 1995; Jiang and Argyropoulos, 1997), provides an excellent opportunity for systematically exploring the complex reactions that occur in lignin during alkaline pulping.

As mills move toward elemental chlorine free bleaching (ECF) the negative impact of condensed lignin structures on final pulp brightness ceiling provides additional incentives to minimize condensation reactions from occurring in the digester and during oxygen delignification. The precise identification of such structures and their formation kinetics could be a major step forward toward understanding and minimizing their detrimental effects.

In this paper we aim to describe our efforts that are focused at defining the formation of diphenylmethane moieties in lignin during conventional kraft and soda pulping conditions. This objective was realized by initially confirming the assignment of a ^{31}P NMR signal as being due exclusively to the presence of phosphitylated diphenylmethane phenolic hydroxyl groups. We then accumulated kinetic data for the formation of these species under soda and kraft pulping conditions and correlated this information to known chemistry. In addition the chemical changes that occur within the residual kraft lignin during oxygen delignification were explored with emphasis on the role and fate of the condensed units. More specifically, residual kraft lignin was isolated and systematically subjected to oxidative degradation as a function of time and temperature. Detailed quantitative ^{31}P NMR measurements on the oxidized residual lignins provided, for the first time, three-dimensional plots describing the formation and/or elimination of the condensed functional groups. Information regarding the nature of the somewhat unreactive fraction of the residual kraft lignin, thought to be responsible for the slow phase of oxygen delignification, has also been obtained.

EXPERIMENTAL

Treatment of Milled Wood Lignin under Homogeneous Kraft or Soda Pulping Conditions in Presence and Absence of Formaldehyde

The procedure described in our earlier effort that caused the definition of the fundamental thermodynamic parameters that govern the stereoselective degradation of the arylglycerol-β-aryl ether bonds in milled wood lignin under kraft conditions was used (Ahvazi and Argyropoulos, 1997).

Preparation of Kraft Pulps and Residual lignin Isolation

A conventional western hemlock kraft pulp was prepared at Paprican in a laboratory batch digester using conditions described previously (Jiang and Argyropoulos, 1997). The western hemlock EMCC® (extended modified continuous cooking) pulp was supplied by a Canadian mill. The isolation of residual lignin was carried out from pre-extracted pulp meal following a procedure (Jiang and Argyropoulos, 1997) described previously.

Isolation of Residual Kraft Lignin and Oxidation Treatment

The residual lignin was isolated from a softwood kraft pulp (kappa number, approximately 30) by using a slightly modified acidolysis procedure described elsewhere (Jiang and Argyropoulos, 1994). It was then oxidized with pressurized oxygen at 80, 95, 110, 125 and 140°C for 20, 40, 60 and 80 min respectively, using a one-liter Parr reactor. The precise treatment and isolation conditions have been reported by Asgari and Argyropoulos (Asgari and Argyropoulos, 1998 & 1997).

Purification of Oxidized Lignins

The recovered oxidized lignins were then suspended in acidified water (pH=4) and stirred for 24 h at room temperature. The remaining solid was centrifuged, freeze-dried and stored in a desiccator prior to quantitative ^{31}P NMR analyses.

GC/MS Analyses

The washing liquors obtained from the above purification procedure after ethyl acetate extraction were subjected to GC/MS analyses. The GC/MS analyses were carried out on a Hewlett Packard 5972 mass spectrometer interfaced to a Hewlett Packard 5890-A gas chromatograph equipped with a DB-5 30m x 0.25mm packed silica capillary column. The injection port temperature was 280°C and the oven temperature was raised from 70°C to 250°C with a gradient of 15°C/min. The products were analysed after being silylated using N,O-bis (trimethylsilyl) acetamide. The compounds were identified by comparing their fragmentation patterns with those of commercially available or independently synthesized authentic samples.

The major MS fragmentation patterns for trimethylsilylated compounds detected and of relevance to this effort were as follows: *-m/z* (relative intensity %): **4-hydroxy-5-methoxyisophthalic acid:** 413(M-15,85%), 309(6), 281(12), 251(48), 207(13), 159(23), 147(15), 73(100); **3-carboxy-4-hydroxy-5-methoxy-benzaldehyde**: 325(M-15,54%), 313(69), 159(50), 145(27), 117(52), 73(100).

Quantitative ^{31}P NMR Analyses of the Oxidized Lignins

Quantitative ^{31}P NMR analyses were carried out on the starting and the oxidized lignins using published procedures (Asgari and Argyropoulos, 1998 &1997; Argyropoulos, 1994; Granata and Argyropoulos, 1995). The procedure used in reference 27 was found particularly useful for oxidized lignins.

RESULTS AND DISCUSSION

Diphenyl Methane Formation During Pulping

In our earlier work the phosphorus NMR spectra of solubilized kraft lignin contained, amongst others, a signal centred around 143.5 ppm (Granata and Argyropoulos, 1995). On the basis of model compound work and calculations based on the Hammett principles (Jiang *et al.,* 1995), this signal was tentatively assigned to be due to diarylmethane structures possessing free phenolic hydroxyl groups (Granata and Argyropoulos, 1995). In an effort to confirm the validity of our earlier work, the interaction of milled wood lignin with formaldehyde under kraft pulping conditions was clarified by conducting experiments in which different amounts of formaldehyde were introduced in the reaction mixture prior to the cook. More specifically, two different sets of experiments were carried out with four samples of milled wood lignins being subjected to kraft pulping at 120°C with the addition of formaldehyde accounting for 50 and 100% of the total aryl ether units of lignin, respectively (Adler, 1977). After quantitative recovery of the lignin, the ^{31}P NMR spectra were recorded and the spectra critically examined.

When the ^{31}P NMR signal in the region 142.8-144.3 ppm, was integrated, the determination of the actual concentrations of diphenylmethane structures in milled wood lignin in the presence and absence of formaldehyde under kraft pulping conditions as a function of reaction time at 120°C was made possible (Table 1). The quantitative data thus collected revealed that under isothermal conditions (120°C) the addition of formaldehyde to the kraft reaction medium causes the build up of DPM structures within lignin, while in the absence of externally added formaldehyde, these structures predominate after three to four h of reaction.

In an effort to further substantiate our conclusions we then carried out two series of isothermal (120°C) kraft and soda pulping experiments followed by quantitative determinations of the DPM moieties and correlated our findings with the differences in chemistry known to occur between the two processes. The data of Table 2 clearly demonstrates that the formation of diphenyl- methane moieties is a considerably more facile reaction under soda pulping conditions than under kraft pulping conditions. Sulphide ions present in kraft liquors are responsible for promoting and accelerating lignin fragmentation reactions as well as suppressing undesirable condensation reactions (Marton, 1971; Hägglund, 1949; Enkvist and Hägglund, 1950).

Table 1. Showing the actual amounts of DPM(1) structures present in MWL(2) before and after the addition of formaldehyde(3) under homogeneous kraft pulping conditions(4).

Reaction Time (min)	Control Kraft	DPMx10^4 (mol/g)	
		100%(3)	50%(3)
60	0.8	1.6	4.0
120	1.4	2.2	4.2
180	2.4	3.0	4.4
240	4.1	4.1	4.6

(1) Diphenylmethane structures
(2) Milled wood lignin (softwood, *Picea mariana*)
(3) Amount of formaldehyde added, expresses as % of the total amount of aryl ethers present in MWL
(4) The temperature of the kraft pulping experiment was 120°C

The presence of sulfide ions within sulfate liquors has been suggested to preclude the liberation of formaldehyde from the lignin biopolymer (Ekman, 1965). Alternatively, the absence of sulfide, i.e soda pulping, allows the intermediate quinonemethides to lose formaldehyde, causing the formation of undesirable alkaline stable enol ethers (Marton, 1971; Gierer, 1980).

Amongst the various principles that operate during modified pulping, the control of alkalinity is of prime importance. Maintaining hydroxide concentration at a uniformly low level offers distinct advantages as far as carbohydrate degradation is concerned. In this context, maintaining a uniformly low alkalinity may also have an impact on the actual amount of diphenylmethane structures that form within the residual kraft lignin. For this reason residual lignin was isolated from two hemlock kraft pulp samples (kappa number 26.0) produced via an EMCC® mill protocol and a laboratory batch digester, respectively. The data of Table 3 is in accord with the previous observations. While in the conventional kraft pulp the overall amount of C-5 substituted phenolic environments is higher by 11%, the diarylmethane structures prevail amongst them by 37%.

Table 2. Showing the actual amounts of DPM(1) structures present in MWL(2) under homogeneous kraft and soda(3) pulping conditions(4).

Reaction Time (min)	DPMx10^4 (mol/g)	
	Kraft pulping	Soda pulping
60	0.83	1.60
120	4.41	2.20
180	2.41	3.10
240	4.10	4.30

(1) Diphenylmethane structures
(2) Milled wood lignin (softwood, Picea mariana)
(3) Amount of sodium sulphide was replaced with NaOH
(4) The temperature of the pulping experiments was set at 120°C.

During the 1960s Kratzl and his team (Kratzl *et al.,* 1966) showed that 5-5′ biphenolic units may degrade during oxygen delignification resulting in the formation of guaiacyl phenols with a carboxyl group *ortho* to the phenolic hydroxyl. Such moieties can also be generated by the oxidative degradation of other condensed phenolic units (Gellerstedt and Heuts, 1997; Gellerstedt *et al.,* 1986).

The Fate of the Condensed Phenolic Units under Oxygen Delignification Conditions

When a model 5-5' biphenolic structure was subjected to an oxidation treatment (125°C for 40 min) a sharp peak at 141.4 ppm was apparent. The same signal overlapping with a broad signal was also present when a residual kraft lignin sample was oxidized at the same conditions. A signal at 141.4 ppm due to the phenolic group of 3-carboxy-4-hydroxy-5-methoxy aromatic moieties overlapping with the condensed phenolic structures present in residual kraft lignin was obvious (Argyropoulos, 1994; Granata and Argyropoulos, 1995; Jiang *et al.,* 1995). Therefore, when quantifying condensed phenolic units present in oxidized kraft lignin samples, these signals should be taken into account. Ways to differentiate between the phenols of 3-carboxy-4-hydroxy-5-methoxy moieties and other condensed phenolic structures should thus be devised. Detailed solubility studies showed that 3-carboxy-4-hydroxy-5-methoxy toluene dissolved easily into acidified water (pH=4) and could be separated from various substituted guaiacols. As such, all oxidized lignins were suspended and stirred into acidified water for 24 h at room temperature.

Table 3. Showing the actual distribution of condensed and diphenylmethane structures present in conventional and EMCC® kraft pulps of similar kappa number.

Pulp Sample Identity	Total Condensed Phenolic Structures (mmole/gr)	Diphenylmethane Phenolic OH (mmole/gr)
Conventional Kraft (k=26.8)	1.7	0.3
EMCC® Kraft (k=26.0)	1.5	0.2

The profiles of the condensed phenolic group as a function of time and temperature (Figure 1) indicate that the degradation of these structures proceeds in two distinct phases: an initial rapid decrease within 20 min, followed by a significantly slower phase. Similar observations have also been reported by Asgari and Argyropoulos (1998 & 1997), who studied the elimination of uncondensed guaiacyl phenols and the formation of carboxylic acids during the oxidation of softwood residual kraft lignin. These data, which are in agreement with those of Gellerstedt and Heuts (1997), show that even after an oxygen delignification using

extreme conditions, the residual lignin still contains phenolic structures. The nature and reactivity of these somewhat less reactive phenolic structures will be investigated further in subsequent parts of this effort.

At the extreme end of our conditions (80 min at 140°C only about 20% of the original guaiacyl phenolic units remained in the oxidized residual lignin, while over 50% of the condensed phenolic units survived the oxidation. Therefore, the main reactions that occur in softwood residual kraft lignin during oxygen delignification are those that cause the oxidative degradation of the guaiacyl phenolic units. These findings agree with earlier observations (Gellerstedt *et al.*, 1986) that implied that during oxygen delignification, the uncondensed guaiacyl phenolic units seemed to be more easily degraded than the condensed units, resulting in a lignin with a more condensed structure. Furthermore, when the ratio of the condensed phenolic units to the total phenolic groups is plotted versus the oxidation time (Figure 2), it becomes apparent that temperature plays an important role in the development of the distribution of condensed to uncondensed phenolic structures.

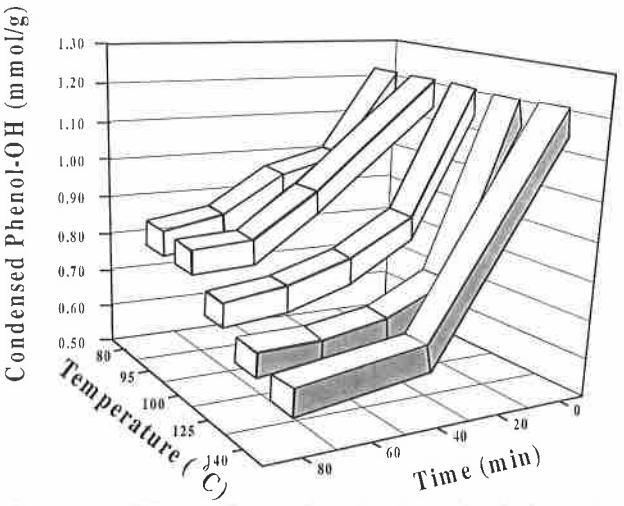

Figure 1. Three-dimensional plots depicting the oxidative elimination of condensed phenolic units within residual kraft lignin plotted as a function of time and temperature under oxygen delignification conditions.

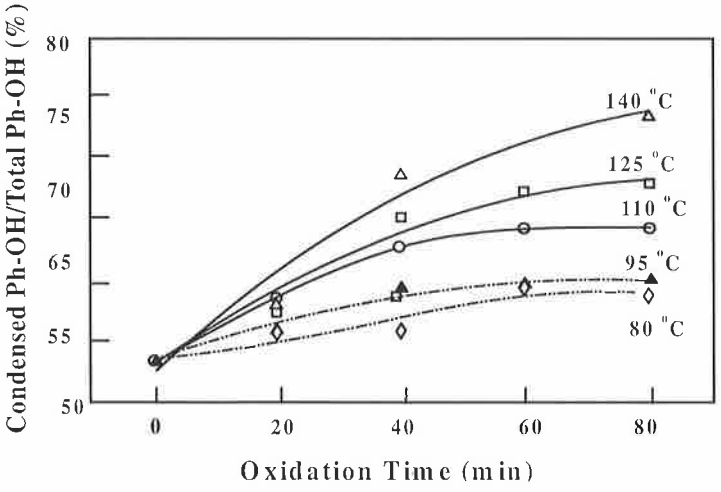

Figure 2. The ratio of condensed phenols to total phenols present within oxidized residual kraft lignins during an alkaline oxygen degradation.

While the condensed phenolic units account for 50% of the total phenolic units in the starting residual kraft lignin they are seen to increase by 75% after 80 min of oxidation at 140°C. The homogeneous nature of our experiments may have promoted the occurrence of condensation reactions and the formation of radical coupling products, which is unlikely to occur on real fibers. Alternatively, one may argue that despite the homogenous and idealized conditions of this work, the condensed phenolic structures survived the oxidation.

This implies that at lower temperatures and under less than ideal conditions of mixing and oxygen penetration inherent in current industrial practices, the condensed structures would most certainly survive. A deeper insight into the precise nature of the remaining condensed structures is needed to further clarify these issues.

On the Nature of the Condensed Phenolic Units

In 1995, Brunow and his coworkers identified the dibenzodioxocin structure as being present in softwood lignin and estimated that these moieties may account for as high as 19-26% of the phenylpropane units (Gierer and Nilvebrant, 1986; Karhunen *et al.,* 1995; Pew, 1963; Drumond *et al.,* 1989; Karhunen *et al.,* 1995). The new octagonal moiety when subjected to soda and kraft pulping conditions was shown to release significant amounts of 5-5N biphenolic hydroxyl groups (Karhunen *et al.,* 1997). In addition, recent evidence has shown that kraft pulping conditions cause the occurrence of similar reactions, releasing 5-5′ biphenolic hydroxyl groups which remain in the unbleached pulp lignin (Ahvazi *et al.,* 1998). Diphenylmethane structures are also known to be present in residual kraft lignin and are considered to be formed during the kraft pulping process (Gierer, 1970; Ahvazi *et al.,* 1998). Consequently, the percentage of condensed phenolic structures to the total phenolic units in unbleached residual kraft lignin reaches and exceeds 50%. Such structures become even more predominant after oxygen delignification (Figure 2).

Model compound studies accompanied by detailed theoretical calculations have shown that the ^{31}P NMR chemical shifts of the phenolic hydroxyl groups of 5-5′ biphenols give rise to signals ranging between 141.7-140.2 ppm (Granata and 1995; Jiang, *et al.,* 1995; Ahvazi *et al.,* 1998). This assignment was also confirmed by Smit and co-workers (Smit *et al.,* 1997). Consequently, the spectral data of Figure 2 was integrated accordingly to show the relative amounts of 5-5′ biphenols to the total condensed phenolic units present within oxidized residual kraft lignins (Figure 3).

Apparently, the 5-5′ biphenolic units represent over 55% of the total condensed phenolic units in oxidized residual kraft lignin (Figure 3). The sharp increase in the ratio of 5-5′ biphenols to the total condensed phenolic units, may be due to the formation of these structures *via* radical coupling reactions and/or the simultaneous faster elimination of other condensed structures. Once again, the homogeneous nature of our experiments cannot preclude these coupling reactions from taking place. However, the significant temperature dependence (Figure 3) most likely signifies that the enrichment in 5-5′ biphenols could be due to the faster degradation of other condensed structures and not due to radical coupling reactions, which are expected to have a rather low energy of activation with a smaller temperature dependence (if any) than that shown in Figure 3. Furthermore, if radical coupling reactions were solely responsible for the enrichment in 5-5' moieties, the 5-5' biphenolic portion would have monotonously increased in Figure 3. Instead it is seen to level off after about 40 mins. This is because the phenolic character of the lignin was still abundant even after extreme oxidation (Figure 2).

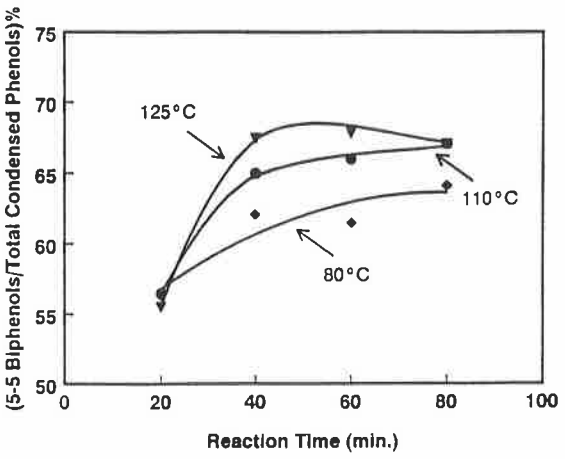

Figure 3. The portion of 5-5′ biphenols to the total condensed phenols present in oxidized residual kraft lignin during oxygen degradation as a function of time and temperature.

CONCLUSIONS

By using quantitative ^{31}P NMR spectroscopy it is possible to identify, quantify and follow the formation of phenolic hydroxyl groups that belong to diphenylmethane units under kraft and soda pulping conditions. These units exhibit a ^{31}P chemical shift signal that is confined between 142.8 to 144.3 ppm. Data derived during the course of this work implied that diphenylmethanes are formed in higher quantities during a conventional cook as opposed to its EMCC® counterpart, at the same kappa number.

Although the free phenolic units of residual kraft lignin play a dominant role in defining the efficiency of oxygen delignification, about 50% of these units were fairly resistant to it. The homogeneous oxidation of residual kraft lignin caused the dramatic elimination of the uncondensed guaiacyl phenolic units while the condensed phenolic units were eliminated more gradually. Consequently, the proportion of condensed phenolic units to the total phenolic units increased within the oxidized lignin. Despite the idealized and homogeneous conditions of these experiments, 5-5′ biphenyl phenolic moieties were fairly resistant to oxygen delignification. It is therefore thought that the biphenyl structures are key contributing phenolic moieties which are relatively unreactive under oxygen delignification conditions.

REFERENCES

Adler, E. & Lundquist, K. (1961). *Acta Chem. Scand.*, **15**, 223.
Adler, E. (1977). *J. Wood Sci. Technol.*, **11**, 169.
Adler, E., Falkehag, S.I., Marton, J. & Halvarson, H. (1964). *Acta Chem. Scand.*, **18**, 1313.
Adler, E., Marton, J. & Falkehag, S.I. (1964). *Acta Chem. Scand.*, **18**, 1311.
Ahvazi, B.C. & Argyropoulos, D.S. (1997). *Nord. Pulp Pap. Res. J.*, **12**, 282.
Ahvazi, B.C., Pageau, G. & Argyropoulos, D.S. (1998). *Can. J. Chem.*, **76**, 506-512.
Archipov, Y., Argyropoulos, D.S., Bolker, H.I. & Heitner, C. (1991). *J. Wood Chem. Technol.*, **11**, 137.
Archipov, Y., Argyropoulos, D.S., Bolker, H.I. & Heitner, C. (1991). *Carbohyd. Res.*, **220**, 49.
Argyropoulos, D.S. (1994) J. *Wood Chem. Technol.*, **14**, 45.
Argyropoulos, D.S., Archipov, Y., Bolker, H.I. & Heitner, C. (1992). *Holzforschung*, **47**, 50.
Argyropoulos, D.S., Bolker, H.I., Heitner, C. & Archipov, Y. (1993). *J. Wood Chem. Technol.*, **13**, 187.
Argyropoulos, D.S., Sun, Y. & Mazur, M. (1995). *Nord. Pulp Pap. Res. J.*, **10**, 68.
Asgari, F. & Argyropoulos, D.S. (1998 &1997). *Can. J. Chem.*, **76** , 1998 and *Tappi Pulp. Conf.*, Proceddings, **Vol 2**, pp 951.
Chan, F.D., Nguyen, K.L. & Wallis, A.F.A. (1995). *J. Wood Chem. Technol.*, **15**, 473.
Chiang, V.L. & Funaoka, M. (1988). *Holzforschung*, **42**, 385.
Chiang, V.L. & Funaoka, M. (1990). *Holzforschung*, **44**, 147.
Chiang, V.L. & Funaoka, M. (1990). *Holzforschung*, **44**, 309.
Chirkin, G. & Tishschenko, D. (1962). *Zhurn. Priklad. Khim.*, **35**, 153.
Drumond, M., Aoyama, M., Chen, C.-L. & Robert, D. (1989). *J. Chem. Wood Technol.*, **9**, 421.
Ekman, K.H. (1965). *Tappi*, **84**, 398.
Enkvist, T. & Hägglund, E. (1950). *Svensk. Papperstidn.*, **53**, 85.
Froass, P.M., Ragauskas, A.J. & Jiang, J. (1996). *J. Wood Chem. Technol.*, **16**, 347.
Gellerstedt, G. & Heuts, L. (1997). *J. Pulp Paper Sci.*, **23 (7)**, 335-340.
Gellerstedt, G., Gustafsson, K. & Lindfors, E.-L. (1986). *Nord. Pulp and Paper Res. J.*, **1 (3)**, 14-17.
Gierer, J. & Nilvebrant, N.-O. (1986). *Holzforschung*, **40** (Suppl.), 107.
Gierer, J. & Söderberg, S. (1959). *Acta Chem. Scand.*, **13**, 127.
Gierer, J. (1970). *Svensk Papperstid.*, 571-596.
Gierer, J. (1980). *J. Wood Sci. Technol.*, **14**, 267.
Gierer, J., Lenz, B. & Wallin, N.H. (1965). *Tappi*, **48**, 402.
Granata, A. Argyropoulos, D.S. (1995). *J. Agric. Food Chem.*, **43 (6)**, 1538-1544.
Hägglund, E. (1949). *Tappi*, **32**, 241.
Harkin, J.M. (1966). *Adv. Chem. Series*, **59**, 65.
Jiang, Z. & Argyropoulos, D.S. (1994). *J. Pulp Paper Sci.*, **20 (7)**, J183-188.
Jiang, Z.H. & Argyropoulos, D.S. (1995). *In 8th Int. Symp.on Chemistry*, Procedings, **Vol I**, pp 511-525.
Jiang, Z.H. & Argyropoulos, D.S. (1997). *In 9th Int. Symp. on Wood and Pulping Chem.*, Procedings, pp J2-1-J2-6.
Jiang, Z.H., Argyropoulos, D.S. & Granata, A. (1995). *Mag. Res. Chem.*, **33**, 375-382.
Karhunen, P., Mikkola, J. & Brunow, G. (1997). *In 9th Int. Symp. Wood & Pulp Chem.*, Procedings, pp R11-3.
Karhunen, P., Rummakko, P., Sipilä, J. & Brunow, G. (1995). *Tetrah. Letts.*, **36 (1)**, 169.

Karhunen, P., Rummakko, P., Sipilä, J., Brunow, G. & Kilpelainen, I. (1995). *Tetrah. Letts.*, **36 (1)**, 4501.
Kratzl, K., Gratzl, J. & Claus, P. (1966). *Adv. Chem. Series*, **59**, 157.
Marton, J. (1971). In Lignins: Occurrence, Formation, Structure, and Reactions, ed. KV. Sarkanen & C.H. Ludwig, Wiley Intersci, New York, pp 639.
Marton, J., Marton, T. & Falkehag, S.I. (1966). *Adv. Chem. Series*, **59**, 125.
Pew, J.C. (1963). *J. Org. Chem.*, **28**, 1048.
Smit, R., Suckling, I.D. & Ede, R.M. (1997). *In 9th Int. Symp. on Wood and Pulping Chem.*, Proceedings, pp L4-1.
Sun, Y. & Argyropoulos, D.S. (1995). *J. Pulp Paper Sci.*, **21**, 185.

Wood and pulp carbohydrates analysis using HPLC and electrochemical detection

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ABSTRACT

A method for wood and pulp carbohydrates quantitative analysis was developed using HPLC and pulsed amperometric electrochemical detection after acid hydrolysis. This method does not require derivative reactions with chemicals and has a high sensitivity and very efficient chromatographic separation between glucose, galactose, arabinose, rhamnose, xylose and mannose peaks in a single run using water as eluent, an ion-exchange column and a 300 mmol/L NaOH post column addition. The complete run including column cleaning takes about one h. A faster method was developed only for *Eucalyptus* pulp samples using 13 mmol/L NaOH as eluent without post column addition and two guard columns. It was good chromatographic separations for galactose, glucose and xylose. The complete run takes 35 min including column cleaning. The calibration procedure for both methods was done using inositol as internal standard and quality control monosaccharide standards in order to have yield before and after each wood and pulp sample injection.

Keywords: wood analysis, cellulose pulp, carbohydrates, Eucalyptus

INTRODUCTION

Wood and pulp carbohydrates quantitative analysis have been of interest to research and productive pulp areas for a long time. According to a didactic point of view we can divide the quantitative methods between classics and modern. Classic methods mainly use wet end chemistry extractive techniques followed by non-selective quantification and modern methods are based in separative techniques with specific components detection. The main problem to both methods is the distinction between cellulose and the different hemicellulose components.

Classic methods have been used since the twenties when Dore (1920) used 5% NaOH extraction to estimate hemicellulose concentration on wood as weight loss. Preece (1931) used a similar method to determine hemicellulose concentration in hardwoods with 4% NaOH solution. The prevailing method for the next years was extraction with different alkali concentrations followed by weight. In the fifties the classic methods using alkali extraction were improved with carbohydrates oxidative reactions using $K_2Cr_2O_7$ followed by titration with ammonium ferrous sulfate. Visible spectrometry also was be used for pentosan quantification after HCl digestion and paper chromatography methods were developed to identify monosaccharides resulted from polysaccharides acid hydrolysis. Hamilton and Thompson (1959) studied the carbohydrates composition for hardwood and softwood after alkaline extraction and acid hydrolysis using paper chromatography quantification. The development of chromatographic techniques was the main effort towards modern methods.

In the sixties and for the subsequent years there were chromatographic techniques development applied to wood and pulp sugars composition like thin layer chromatography (TLC), gas chromatography (GC) and adsorption, partition and ion-exchange liquid chromatography (LC). Methods using TLC have efficient separation for low monosaccharides quantities when a good combination between stationary and mobile phases are obtained, however a reliable quantification is not practical. Gas chromatography using flame ionization detection is a reliable quantification method but is limited to sugars that can be converted to volatile compounds. Liquid chromatography has very good selectivity on the monosaccharides separation and permits the usage of higher sample amounts than GC methods; however, it suffers limitations from the detection system available. Capillary electroforetic methods also were developed for sugar analysis with fast component separation but these methods depend on the formation of complexes between carbohydrates and borate and the compatibility behavior between solid support materials and chemical reagents.

Nowadays the analytical method recommended by the Technical Association of the Pulp and Paper Industry (Tappi T249, 1985) is based on gas chromatography of monosaccharide derivatives. The analytical procedure

requires acid hydrolysis followed by neutralization and sugar reduction to alditols with sodium borohydrate. The alditols yielded are then acetylated and extracted with methylene chloride prior to gas chromatography. This method is time consuming because requires few days for sample preparation, resulting in a poor separation between xylose, mannose, galactose and arabinose at trace quantitites. Studies using 1-methylimidazole as a catalyst for acetylation of alditols were carried out to reduce derivative reaction time (Cao *et al.*, 1997) for the TAPPI method, but the procedure complexity remained the same. Acid methanolysis and gas chromatography (Sundberg *et al.*, 1996) also can be used to quantify hemicellulose and pectins for wood and pulp samples after silylation, but this method also is time consuming and requires extreme care with moisture.

High performance liquid chromatography have improved applications in carbohydrates analysis in the nineties with the development of electrochemical detectors using pulsed amperometry and anion exchange columns that permits to operate at high pH, where neutral sugars are at least partially ionized like weak acids. Pulsed amperometric detection (PAD) is based on monosaccharide oxidizing reactions at the surface of a golden working electrode in a flow cell during a cycle of three potentials. The first potential oxidizes the sugar and measures the generated current, the second and third potential levels are employed for cleaning oxidation products from the electrode surface (Edwards *et al.*, 1987). Suzuki *et al.* (1995) and Laver and Wilson (1993) published successful results for wood pulps using HPLC-PAD with addition of carbonate and acetate as pusher ions without pre-column derivatization or sugar neutralization. Both methods require 75 and 60 min respectively for a complete run including column cleaning and stabilization. A method for *Eucalyptus* woods and pulps was published (Wright *et al.*, 1996) using two guard columns and 2 mmols/L NaOH as eluent, followed by 350 mmols/L NaOH as post column addition. The complete run took 80 min. Pettersen and Schwandt (1991) reported the use of water as eluent in a run that took 75 min. In this work we present a method that does not use pusher ions and takes 60 min to complete a run for wood and 35 min to *Eucalyptus grandis* kraft pulp samples.

EXPERIMENTAL

Wood samples

Two *E. grandis* wood samples were milled in a laboratory Willey mill using a screen of 40 meshes. The resulted powder was extracted to have extractive free wood (Tappi T264, 1988) and this was used for acid hydrolysis.

Pulp samples

Nine *E. grandis* kraft pulp samples with different carbohydrate composition were obtained in a 20 L laboratory reactor and conditioned at 50 +/- 2 % relative humidity and a temperature of 23 +/- 1° C, to maintain the moisture as low as possible.

Sample hydrolysis

Wood and pulp samples were hydrolyzed with sulfuric acid in two steps, a primary hydrolysis with H_2SO_4 72% for 1 h in a water bath at 30° C and a secondary hydrolysis with 3% diluted acid in an autoclave at 120° C for 1 h. After the secondary hydrolysis all samples were cooled in ice bath and the hydrolyzed volume was measured.

Hydrolyzed sample neutralization

Hydrolyzed samples were neutralized with barium hydroxide to pH 4.3 +/- 0.3 using a pH meter, a magnetic stirrer and a buret to have the volume measured. After neutralization the precipitated material was removed from the hydrolyzate by centrifugation. The clear supernatant was pipeted to a volumetric flask and inusitol was added as the internal standard.

Liquid chromatography

High performance liquid chromatography was carried in a Dionex DX 500 instrument using a CarboPac PA 1 column, water as eluent in a flow rate of 1.5 mL/min and NaOH 300 mmols/L as post-column addition in a flow rate of 2.0 mL/min for the first 30 min. The column cleaning was done using 1.5 mL/min of NaOH 200 mmols/L for 20 min and the last 10 min with water for column conditioning. The complete run including cleaning took 60 min. All the NaOH solutions were made using low carbonate reagent and with careful handling procedure to avoid carbonate contamination from laboratory environment.

A modified method for *Eucalyptus* pulp was developed using two CarboPac PA1 guard columns, NaOH 13 mmols/L as eluent in a flow of 0.7 mL/min without post-column addition and a short cleaning with NaOH 500 mmols/L in 5 min at 2.0 mL/min. The detection settings for both methods are described on Table 1.

Table 1. Pulsed amperometry detector settings for carbohydrates.

Time (sec)	Potential (V)	Integration
0.00	0.05	no
0.20	0.05	begin
0.40	0.05	end
0.41	0.75	no
0.60	0.75	no
0.61	-0.15	no
1.00	-0.15	no

Reagents

β-D(+)Glucose, D(+)xylose, D(+)galactose, D(+)mannose, L(+)arabinose, L(+)rhamnose and myo-inositol were obtained from Sigma Chemical. NaOH low carbonate grade was purchased from Fisher. Eluent and standard solutions were prepared using chromatographic grade water.

Calibration

Instrument calibration was done using myo-inusitol as the internal standard and yields were determined after injections using a quality control check sample made with the same components of the hydrolyzed sample. The injection sequence for all samples was the following: check (1)⇒sample (X1)⇒check (2)⇒sample (X2). The yield that was used to correct the result for sample X1 was obtained by the mean value between check 1 and 2.

RESULTS AND DISCUSSION

Validation Method

The validation method was performed the evaluating the correlation coefficient for multicomponent monosaccharide standards calibration curves and the concentration/response data of the calibration check standards. Figure 1 shows results obtained in calibration and the correlation value for each analyte. Table 2 shows the results for calibration check standards.

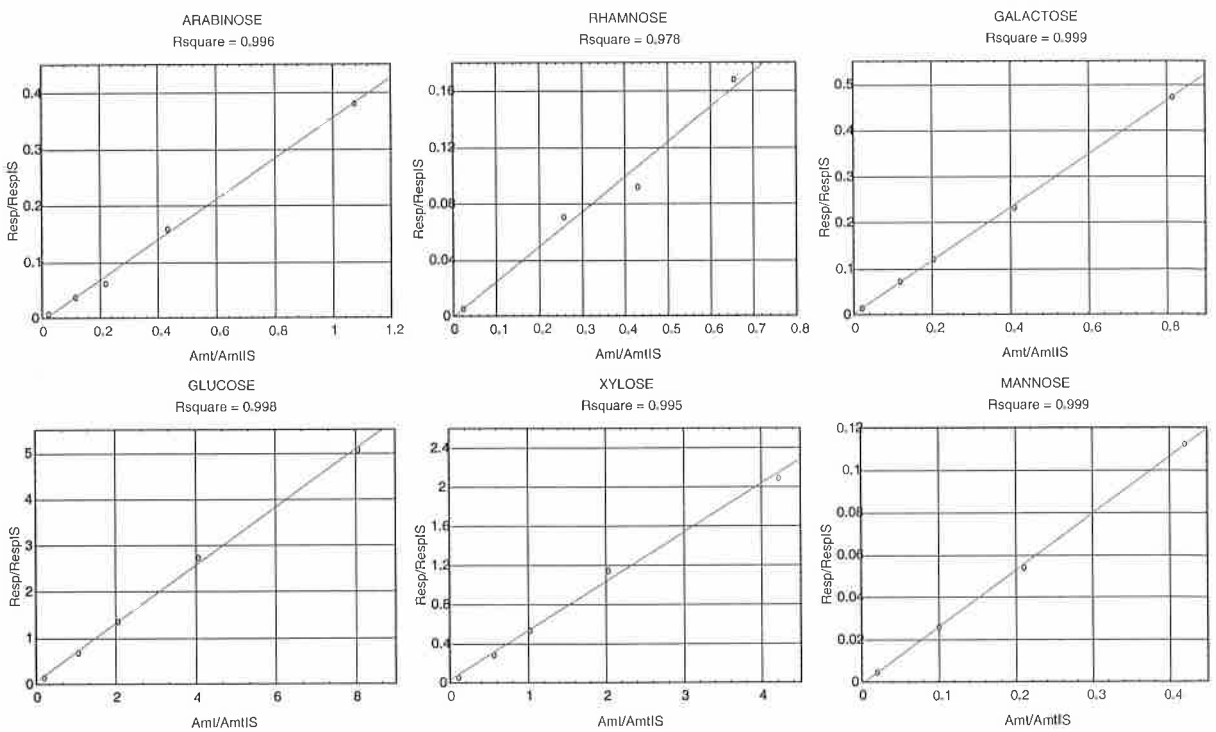

Figure 1. Calibration plots for multicomponent monosaccharide standards.

The correlation coefficient for all investigated analytes shows that linear models can be safely adopted for calibration, indicating that the detection system has a response linearly sensitive to the analyte concentration.

Table 2. Results for the calibration check multicomponent standards.

Analytes	Specified concentration (mg/L)	Response concentration (mg/L)	Yield (%)
Rhamnose	22.30	22.26	99.82
Arabinose	8.50	8.49	99.88
Galactose	6.30	6.32	100.31
Glucose	57.60	57.64	100.07
Xylose	36.30	36.33	100.08
Mannose	10.40	10.39	99.90

The yields obtained for the multicomponent check standards show good agreement between specified concentration and the response concentration for all investigated analytes.

Wood carbohydrates composition

The chromatogram of the hydrolyzed and neutralized wood sample is shown in Figure 2. Monosaccharide peaks are well defined with good separation according to differences in their dissociation constants. The hard separation between glucose, xylose and mannose is solved with very good baseline stability. The use of pusher ions to reduce retention times was not necessary, the good resolution between minor components was easily achieved. Separation between minor components concentration are showed in detail. *E. grandis* wood sample shows glucose and xylose as main carbohydrate compounds originated from cellulose and hemicellulose respectively. The overall chemical composition of wood samples including lignin is shown in Table 3.

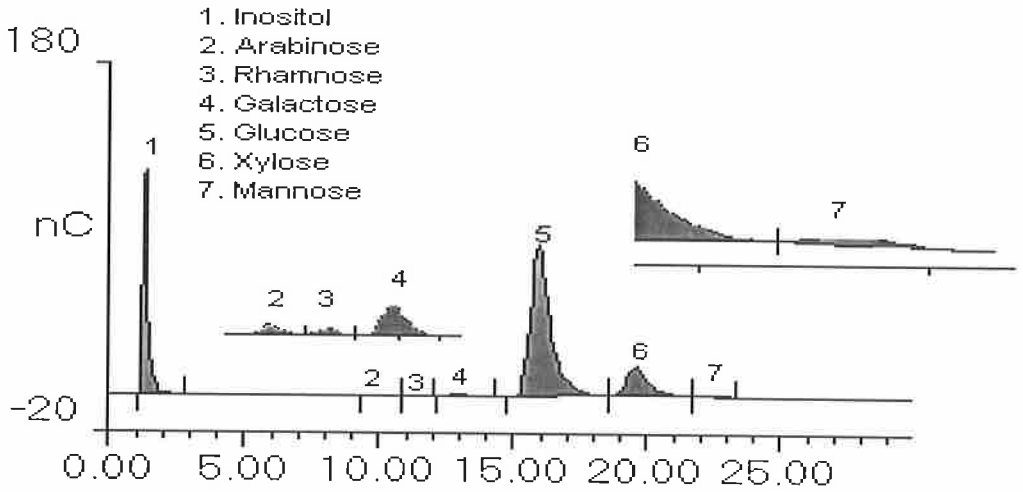

Figure 2. Chromatogram for a *E.grandis* wood hydrolyzate.

Table 3. *Eucalyptus grandis* wood carbohydrates and lignin composition.

Component	Wood sample A (%)	Wood sample B (%)
Total Lignin	26.10 ± 1.38	27.20 ± 1.14
Arabinose	0.20 ± 5.40	not detected
Rhmanose	0.21 ± 6.23	0.30 ± 5.77
Galactose	0.71 ± 3.50	0.80 ± 2.98
Glucose	51.10 ± 0.45	51.00 ± 0.61
Xylose	13.50 ± 1.80	13.60 ± 1.68
Mannose	0.40 ± 4.73	0.33 ± 4.92

The carbohydrates composition was expressed as percentage of anhydro sugar on ovendry basis. The conversion factors for anhydro sugars were 0.90 to hexoses and 0.88 to pentoses. Results are average values

for three determinations and relative standard deviations are in parentheses. The total yield for both samples were up to 92% and the remnant fractions are associated with uronic acids and acetyl groups from xylan and possible minor losses due to furfural and methylfurfural formation.

Modified method for kraft pulp samples

After testing the kraft pulp hydrolyzates with the previous method used for wood, it was possible to observe that mannose, rhamnose and arabinose were hydrolyzed by alkali during the pulping operation. Only galactose, glucose and xylose remained for these pulp samples; then, a modified method without post-column addition was developed and employed. This method is faster than the original and was turned valid by the same procedure. Table 4 shows the results obtained for kraft pulp samples and the respective kappa numbers for illustration. Figure 3 shows the chromatogram with good separation between the remained components, galactose, glucose and xylose.

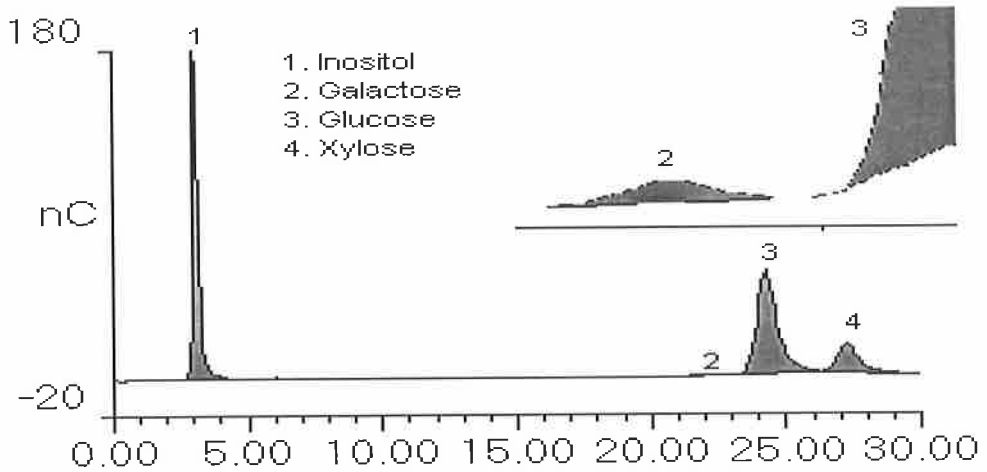

Figure 3. Chromatogram of a kraft pulp hydrolyzate.

Table 4. Kraft pulp carbohydrate composition.

Pulp sample	Kappa	Galactose (%)	Glucose (%)	Xylose (%)
1	23.7	2.49 ± 3.80	78.12 ± 0.12	12.87 ± 0.82
2	21.9	0.61 ± 3.40	79.59 ± 0.15	12.45 ± 1.00
3	18.3	0.85 ± 3.11	81.22 ± 0.11	12.33 ± 1.12
4	17.2	nd	82.83 ± 0.13	12.72 ± 0.93
5	16.2	nd	83.28 ± 0.18	12.26 ± 1.07
6	15.5	nd	82.67 ± 0.14	11.40 ± 1.24
7	14.6	nd	82.18 ± 0.08	10.29 ± 1.18
8	11.9	nd	82.31 ± 0.10	9.88 ± 1.22
9	11.6	nd	84.64 ± 0.11	8.98 ± 1.38

All the carbohydrates results are expressed as percentage of anhydro sugar on ovendry basis and are average values for three determinations. Relative standard deviations are in parentheses. Galactose is present only in samples obtained with low alkali charge that affects minor polysaccharides components by hydrolysis at high temperature in pulping conditions. Xylan chain also is broken by alkaline hydrolysis during pulping and the results show that glucan is more stable to the pulping conditions than others carbohydrates in pulp.

Injection of acid hidrolyzates without neutralization

When the injection of acid hydrolizates was performed as reported on previous publications, a large number of noise peaks were detected and this affected quantitation in our experiments. It is possible that the sulfate concentration from the sulfuric acid used for hydrolysis affected the amperometric cycle and the oxidative surface reactions on the electrode. Storage time also affected acidic samples showing new peaks at higher retention time that appears only when the hydroxide concentration was increased for column clean up. Acid

conditions are favorable to convert monosaccharides into disaccharides. Puls *et al.* (1995) also observed peak broadening and decreasing separation efficience for acid hydrolyzates in previous work.

CONCLUSION

A method to quantify carbohydrate component in wood and pulp was developed using HPLC-PAD system and an ion exchange column. This method is practical and do not require derivative reactions and turn possible sample quantification in a rapid way, with few preparation steps. A modified method for *Eucalyptus* kraft pulp was also developed without post column addition for galactose, glucose and xylose quantification. The validation procedure for both methods shows good performance for the quality control of multicomponent standards and for the calibration models using internal standards. This method can be improved in a future work to quantitation of 4-O-methylglucuronic acid and acetyl groups using proper eluent and gradient conditions.

A study using acid hydrolyzed pulps without neutralization with barium sulfate and the modified method described in this paper indicated that problems with detection and storage can occur and reliable quantification is not practical.

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REFERENCES

Cao, B., Tschirner, U., Ramaswamy, S. & Webb, A. (1997). A rapid modified gas chromatographic method for carbohydrate analysis of wood pulps. *Tappi J.*, **80 (9),**193-197.

Dore, W.H. (1920). The proximate analysis of coniferous woods. *J. Ind. Chem. Eng.*, **12**, 476-479.

Dore, W.H. (1920). The proximate analysis of hardwoods: studies on Quercus agrifolia. *J. Ind. Chem. Eng.*, **12**, 984-987.

Edwards, W.T., Pohl, C.A. & Rubin, R. (1987). Determination of carbohydrates using pulsed amperometric combined with anion exchange separations. *Tappi J.*, **70 (6),** 138-140.

Hamilton, J.K. & Thompson, N.J. (1959). A comparison of carbohydrates of hardwoods and softwoods. *Tappi J.*, **42 (9)**, 752-760.

Laver, M.L. & Wilson, K.P. (1993). Determination of carbohydrates in wood pulp products. *Tappi J.*, **76 (6)**, 155-159.

Pettersen, R. C. & Schwandt, V.H (1991). Wood sugar analyis by anion chromatography. *J. Wood Chemistry and Technology*, **11 (4)**, 495-501.

Preece, J.A. (1931). Studies on hemicelluloses: The proximate analysis of box wood and the nature of its furfuraldehyde yielding constituents (1931). *Biochemical J.*, **25**, 1304-1318.

Puls, J., Dokk, G., Herrman, A., Borchmann, A. & Saake, B. (1995). Comparative determinations for quantitative determinations of sugars. *In Symposium on Wood and Pulping Ch*emistry, Proceedings, **Vol 1**, pp 503.

Sundberg, A., Sundberg, K., Lillandt, C. & Holmbom, B. (1996). Determination of hemicelluloses and pectins in wood and pulp fibers by acid methanolysis and gas chromatography. *Nordic Pulp and Paper Research J.*, **4**, 216-226.

Suzuki, M., Sakamoto, R. & Aoyagi T. (1995). Rapid carbohydrate analysis of wood pulps by ion chromatography. *Tappi J.*, **78 (7)**, 174-177.

Tappi method T 233 cm-84 (1984). Pentosans in wood and pulp, Tappi Press, Atlanta.

Tappi method T249 cm-85 (1985). Carbohydrate composition of extractive-free wood and wood pulp by gas chromatography. Tappi Press, Atlanta.

Tappi method T264 om-88 (1988). Preparation of wood for chemical analysis. Tappi Press, Atlanta.

Wright, P.J, Wearne, R.H. & Wallis, A.F.A. (1996). Chemical analysis of polyssacharides in plantation eucalypt woods and pulps. *Appita J.*, **49 (4)**, 258-261.

Wood, biologically decayed wood and modified lignins as heavy metal ions sorbents

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ABSTRACT

Studies of the prospects of wastewater purification from heavy metal ions with low-cost and readily available sorbents such as undamaged softwood and hardwood, biologically decayed wood, commercially obtained hydrolysis lignin and modified hydrolysis lignin-aminolignins were carried out. It has been established that wood tends to adsorb copper and chromium ions in comparatively equal quantities (1.8 - 3.2 mg/g and 1.5 - 3.2 mg/g, respectively, depending on the wood species). The sorption capacity of wood damaged by white and brown rot fungi is approximately 2-3 times higher, while that for wood digested by grub is higher in terms of copper ions than chromium ions (3.0 mg/g, 1.0 mg/g, respectively). A similar selectivity has been observed for hydrolysis lignin samples (1.7 mg/g, 1.0 mg/g). Modification of hydrolysis lignin (by introducing the additional amino groups) provides an essential increase in heavy metal sorption capacity (26.4 mg/g, 11.5 mg/g). The explanation of the role of lignin in the ion binding process was based on the interpretation of water vapour sorption data. The chemical activity of the material surface - the level of lignification of the accessible surface plays an important role in the process of heavy metal sorption. The sorption occurs according to an ion exchange mechanism.

Keywords: heavy metals, wood decay, wood, lignin

INTRODUCTION

Due to the increasing environment protection requirements and the exhaustion of natural resources, the problem of the rational utilization of the existing raw materials becomes urgent. The principle that the waste of one production area is used in another production area and therefore would enable a simultaneous solution to the problem of waste and raw materials is the main idea of the Zero Emissions Research Initiative (ZERI) program (Pauli, 1995), which has been launched under umbrella of the United Nations University (UNU) and currently is the project of UNU Institute of Advanced Studies, Tokyo. Biodamaged low grade wood and industrial hydrolysis lignin are typical waste products. Their application for cleaning of polluted municipal and industrial wastewater is one such example for the ZERI approach. Clean water acts as a necessary element for integrated industries and sustainable development.

The existing methods which are used to remove toxic heavy metal ions (HMI) pollution from municipal and industrial waste water are based on binding of HMI within insoluble compounds and then separation of the sediments formed. The synthetic ions exchange resins, which provide effective HMI caption are still expensive for wide application. Experiments were carried out worldwide to develop cheap sorbents for HMI binding, using raw materials of the plant and animal kingdom. Good results were obtained by applying other materials with a high polyphenols content (tannins in nut waste and bark; humic substances in peat) to capture HMI. Taking into account these facts, it will be expected that lignin with a wide variety of functional groups is the most active wood component in the metal ions binding process. The aim of this work was to test the application potential of the large-scale waste such as sawdust, biodamaged wood and hydrolysis lignin, as well as chemical modification of the latter - aminolignin as Cu(II) and Cr(III) ions sorbents. The explanation of the role of lignin in the ion binding process was based on the interpretation of water vapour sorption data and Klason lignin content in the specimen.

EXPERIMENTAL

Materials

The wood of the typical Europe (the temperate climatic zone,) tree species such as white alder (*Alnus incana* Moench.), birch (*Betula Pubescens* Ehrh.), summer oak (*Quercus robur* L.), ordinary aspen (*Populus tremula* L.) and ordinary pine (*Pinus sylvestris* L.) was used. Summer oak and ordinary pine wood destroyed by brown rot and warty birch destroyed by white rot were collected in a native forest during the autumn period. Ordinary pine sapwood digested by grubs of the House Longhorn beetle (*Hylotrupes bajulus* L.) was prepared in laboratory conditions. The specimens were characterized by Klason lignin content (Table 1), detected according to the method (Zakis, 1994). Industrial hydrolysis lignin (the content of OCH_3 groups was 6.74%) was received from the Kedainiai hydrolysis plant (Lithuania). Aminolignins (the contents of nitrogen and OCH_3 groups were 2.91% and 7.39%, respectively) was synthesized at the Lignin Laboratory of the Latvian State Institute of Wood Chemistry (Riga, Latvia) from hydrolysis lignin according to the patented method (Zakis *et al.*, 1993).

Metal ions sorption: batch experiments

To provide rapid and uniform soaking of specimens, air-dry chips (0.6 g) were stored under vacuum during 15 min at a pressure of 0.7 kPa. Then the specimens were soaked into 50 ml of a $CuCl_2$ solution with a concentration of 0.01 mol/l (0.02 N) or a $CrCl_3$ chloride solution with a concentration of 0.0067 mol/l (0.02 N). The sorption proceeded for 24 hours at 22±1 °C. The determination of the Cr(III) and Cu(II) ions quantity was carried out using a flame atom absorption spectrometer in a decomposed filtrate. For this purpose, the chips after the batch experiment were filtered, dried to reach a constant mass and decomposed with a sulphuric and nitric acid mixture. All the sorption capacity data were calculated against to oven-dried sorbents.

The Cu(II) ions exchange capacity was determined in column experiments. An air-dry sorbent (5.0 g) was filled in glass columns (23 by 300 mm). The height of the filling depending on sorbent density was 5-10 cm. The sorption was carried out by pouring the Cu(II) chloride solution with a constant rate of 2-3 ml/min through a sorbent layer. The Cu(II) ions concentration in the effluent flow was monitored. The sorption was finished when the inlet and outlet solution concentrations became equal. The total dynamic ion exchange capacity $\mathbf{E_p}$ and the relative ion exchange rate **Z** (%) were calculated.

Water vapours sorption

Water vapours adsorption and desorption isotherms were recorded by a vacuum balance with quartz spirals according to method (Gregg & Sing, 1982). The equilibrium was reached at each point in 20-24 hours. The sorption isotherms were analyzed according to the comparative method along with the Brunauer-Emmet-Teller (BET) method (Chirkova *et al.*, 1995). The sorption in the monolayer $\mathbf{a_m}$ (mmol/g), the energy constant **C**, the specific water accessible surface **A** (m^2/g) and the concentration of hydrophilic centres α ($groups/nm^2$) were calculated. The water vapour sorption results are summarized in Table 1.

RESULTS AND DISCUSSION

Heavy metal ions sorption

The main attention has been turn to Cu(II) and Cr(III) ions sorption in batch conditions. Based on the sorption kinetics curves, it can be seen that the sorption equilibrium has not been reached within 48 hours. However, already in 2 hours, the adsorbed metal amount of Cu(II) and Cr(II) makes up 67% and 64%, respectively, from the adsorbed metal amount which was obtained in 48 hours. The 24 hours sorption duration was preferred to compare the sorption capacity of all the specimens. During this time, 88% of Cu(II) and Cr(III) ions was adsorbed as compared with that reached in 48 hours. We explain this phenomenon in the following way: already in the first hours of sorption, an equilibrium between the adsorbed metal amount on the wood cell wall surface and the metal ion amount in solution is established (a rapid process). Then HMI diffusion and sorption in the cell wall volume occurs (a slow process).

The Cu(II) and Cr(III) ions sorption results of the batch experiment are summarized in Table 1. Among the wood specimens, white alder chips exhibit the highest sorption capacity and adsorb both Cu(II) and Cr(III) in practically equal quantities. The sorption capacity of the rest of the wood samples is 1.5 to 2 times lower on

the average, and Cu(II) ions sorption is approximately 40% higher than that for Cr(III) ions on the average. Both white and brown rot biodamaged wood is capable of adsorbing approximately double to thrice larger quantities of metal ions comparing with undamaged wood of the same species. Among the biologically degraded wood specimens, ordinary pine wood digested by grub exhibit the lowest sorption capacity and the highest difference between Cu(II) and Cr(III) ions sorption, i.e. almost 3 times. When comparing the sorption capacity of the grub digested pine specimen with the initial sorption capacity of pine, it can be seen that the Cu(II) sorption capacity increases almost 1.7 times. In its turn, the Cr(III) sorption capacity decreases approximately 1.5 times. Hydrolysis lignin and the aminolignin are characterized by a capacity to adsorb more than twice as much Cu(II) ions as Cr(III) ions. Hydrolysis lignin exhibits rather a low capacity to adsorb metal ions. After the incorporation of aminogroups ($-N(C_2H_5)_2$) into hydrolysis lignin, its sorption capacity increased more than 15 times. A similar observation, that aminolignin is an effective sorbent of copper-containing compounds was described in the literature (Tzolova *et al.*, 1978): the sorption capacity of copper ions reaches up to 21 mg/g on the sorbent containing 1.92% N.

When comparing the capacity of different tested specimens to adsorb Cu(II) and Cr(III), the following row can be formed (without including a grub digested wood):

hydrolysis lignin < wood < white and brown rot damaged wood < aminolignin

Table 1. Wood, biodamaged wood, cellulose and lignin specimens: content of Klason lignin, water vapour sorption characteristics and heavy metal ions sorption capacity. **A** - water accessible surface; $\mathbf{a_m}$ - monolayer capacity; **C** - energy constant; α - concentration of hydrophylic centres, $\mathbf{a_{Cu(II)}}$ and $\mathbf{a_{Cr(III)}}$ - Cu(II) and Cr(III) ions sorption capacity, respectively.

Sample	Klason lignin, %	A, m^2/g	a_m, mmol/g	C	α, groups/nm^2	$a_{Cu(II)}$, mg/g	$a_{Cr(III)}$, mg/g
White alder (*Alnus incana* Moench.)	25.6	333±3	3.12	5.6	5.52	3.2	3.16
Birch (*Betula Pubescens* Ehrh.)	20.1	326±5	2.62	10	4.85	2.0	1.47
Summer oak (*Quercus robur* L.)	24.9	317±4	2.64	11	4.83	2.68	1.72
Ordinary aspen (*Populus tremula* L.)	18.2	258±8	2.03	13	4.74	2.23	1.52
Ordinary pine (*Pinus sylvestris* L.)	28.9	336±4	2.85	11	5.15	1.8	1.48
Summer oak wood damaged by brown rot	42.2	307±6	2.95	5	5.81	5.67	5.54
Ordinary pine wood damaged by brown rot	80.2	310±5	3.02	4	5.88	5.55	4.73
Birch wood damaged by white rot	32.5	360±8	3.29	5	5.49	6.35	5.55
Ordinary pine wood digested by grub	31.3	315±4	2.59	9	4.96	2.98	1
Hydrolysis lignin	52.3	200±4	2.09	5	6.29	1.67	0.79
Aminolignin	-	260±4	1.95	6	4.52	26.4	11.5

To investigate the Cu(II) ions sorption under dynamic conditions, two rot damaged wood specimens and aminolignin were chosen to investigations in column experiments. The subsequent values were established: brown rot damaged summer oak - the total dynamic ion exchange capacity $\mathbf{E_p}$ = 9.1 mg/g; white rot damaged warty birch - $\mathbf{E_p}$ = 11.0 mg/g; aminolignin - $\mathbf{E_p}$ = 44 mg/g and the relative ion exchange rate **Z** = 4.2%. Cu(II) ions appear in filtrate already at low volumes of the flowing out solution, and a plato is formed on the outlet chromatogram. After some period of time, the Cu(II) ions concentration in the outlet solution begins to increase until the inlet and outlet solution concentrations become equal. In our opinion, such a plato formation effect is connected with the morphology of wood and aminolignin material as well as the Cu(II) ions diffusion in these materials. At the first stage, rapid sorption proceeds on the wood cell wall surface partially damaged by rot or, in case of aminolignin, on lignin globules, which provides a complete capture of Cu(II) ions. After the moment, the surface linking capabilities are exhausted, and the ions appear in the outlet solution, because the capture in the cell wall or globule volume is a slower process, depending on diffusion. This is a longer stage, which will be recognized as a plato on the chromatogram. After exhausting of volume sorption capabilities, the ions concentration in the outlet solution begins to grow.

Water vapour sorption measurements and explanation of heavy metal ions sorption mechanism

Comparing the specimens sorption isotherms, we have observed that both brown and white rot damaged wood samples exhibit a wider hystheresis loop comparing to the untreated sample. For the grub digested pine wood, such a widening was not observed and its sorption isotherms are similar to those for untreated pine wood. As compared to hydrolysis lignin, aminolignin, which was produced from hydrolysis lignin by introducing hydrophile amino groups, showed a higher water vapour sorption capacity in the whole range of

sorption as well as a higher water accessible surface **A** value (Table 1). The data obtained reflect that, under the modification procedure, the more dense globules of hydrolysis lignin have been loosen and a more rare structure of aminolignin has been created. The fact that aminolignin has a lower concentration of the hydrophilic centres α value can be explained by the fact that a part of phenolic groups was involved into the crosslinking process with reagents to produce aminolignin.

To explain the Cu(II) and Cr(III) sorption capacity of various specimens, attempt has been made to find a correlation between the HMI sorption capacities on the one hand and the data of the chemical analysis of the specimens and the parameters of water vapour sorption on the other hand (Table 1). We did not prefer to include hydrolysis and aminolignin specimens into the correlation estimations, because both the separation process of hydrolysis lignin and its modification to aminolignin principally change its structure, and the HMI sorption process will be considered separately. The correlation coefficients $\mathbf{r}^2$ for wood and bio-damaged wood samples were calculated. The highest values of the correlation coefficient $\mathbf{r}^2$ were found between the Cu (II) and Cr (III) ions sorption capacities for the BET equation energy constant **C** (0.75 and 0.76, respectively), as well as for the concentration of hydrophilic centers α (0.67 and 0.77). In other cases, observed $\mathbf{r}^2$-values were principally smaller: Klason lignin content (0.41 and 0.34, respectively); water accessible surface **A** (0.08 and 0.09); and monolayer capacity $\mathbf{a}_m$ (0.44 and 0.51).

The BET equation energy constant **C** and the concentration of the hydrophilic centers α characterize well the structure and hydrophilic properties of the materials, as well as their surface (Gregg and Sing, 1982; Chirkova *et al.*, 1995). Obviously, the decisive role in HMI sorption could be played by the surface chemical activity. Rot biodamaged wood and white alder wood have values of **C** and α close to those observed for lignin specimens isolated in comparatively mild conditions, while the wood undamaged by rot exhibits values closer to those of cellulose samples. This can be expressed as follows. Lignin macromolecules contain approximately 30% of phenolhydroxyl groups (Sarkanen & Ludwig, 1971) whose interaction (hydrogen bonds) with water are weaker than the interaction between water and hydrohyl groups in cellulose and hemicelluloses. Hence, the decrease of the **C** constants exhibits an increase in the level of the phenolic groups of the surface accessible for water vapour. Obviously, these phenolic groups are very important for HMI sorption on wood sorbents.

Both brown and white root damaged specimens show a similar widening of the hystheresis loop and have close values of **C** and α (Table 1), although the interaction of white and brown rot with wood components differ principally (Fengel and Wegener, 1984). Brown rot decreases the polysaccharide content in wood, therefore substantially increasing the lignin content, while the white rot action affects mainly lignin and hemicelluloses, and the resulting content of lignin is lower, comparing with the brown rot damaged material. Hence, we can assume that an important role is played by the possible process: both brown and white rot destroy the naturally formed interaction between lignin and polysaccharides in wood, and, probably, these changes relate mainly to the wood cell wall surface. It creates an increase in the HMI sorption capacity, while, at the same time, an increase of lignin content will be principally different. As mentioned before, grub digested pine wood is rather similar to untreated wood. We can predict that, in this case, the nature of the interaction between wood components is not changed or these changes proceed only in the negligible part of the material and do not affect the common characteristics of the specimen.

The fact that no correlation exists among the HMI sorption capacity and the values of the water accessible surface **A** as well as the monolayer capacity $\mathbf{a}_m$ would indicate that the metal ion sorption in wood does not proceed according to the physical sorption mechanism. The correlation among sorption capacity and the energy constant **C** as well as the concentration of hydrophilic centres α indicates the role of phenolic groups in metal ion bonding. Namely, we can predict that sorption occurs in accordance with the ion exchange mechanism. Hydrolysis lignins and aminolignins tend to adsorb almost twice as much Cu(II) ions as Cr(III) ions. Perhaps, it is connected with the differences in the physical and chemical properties of these metal ions. The metal ion mechanical inclusion capacity in wood sorbents should be also taken into account. In lignins, such a metal ion binding is stronger than in carbohydrates, which have many intermolecular and intrachain H-bonds. The nature of the different functional groups presented in wood components (carboxyl groups, hydroxyl groups) or introduced during modification (aminogroups) on the metal ions sorption will be a separate object of the study.

REFERENCES

Chirkova, E.A., Andersone, I.B., Druz, N.I. & Gravitis, J.A. (1995). Sorption Properties of Wood Carbohydrates (Cellulose and Hemicellulose), Treated with Hexivalent Chromium Compounds. *Zh. Prikl. Khim. J. Appl. Chem.*, **68**, 1102-1106.

Fengel, D. & Wegener, G. (1984). Wood. chemistry, ultrastructure, reactions", Walter de Gruyter & Co, Berlin, pp 613.

Gregg, S.J. & Sing, W. (1982). Adsorption, surface area and porosity, Academic Press, London, pp 303.

Pauli, G.(1995). Zero Emissions. The new industrial clusters. *Ecodecission*, **6**, 26-30.

Sarkanen, K.V. & Ludwig, C.H. (1971). Lignins. Occurrence, formation, structure and reactions, Wiley Interscience, New York, pp 916.

Tzolova, E. & Christov, Z. (1978). Production of Ion Exchangers from Technical Lignin. *Holzforschung*, **32**, 142-144.

Zakis, G., Neiberte, B. & Meksha, M. (1993). Preparation method of a lignin's aminoderivative – acid sorbent. Latvian Republic Patent 5, 213.

Zakis, G.F. (1994). Functional analysis of lignins and their derivatives. *TAPPI Press*, 96.

Xylanase-ozone/solvent bleaching effect on the properties of softwood kraft pulp

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ABSTRACT

The physical properties of a bleached *Pinus radiata* kraft-oxygen pulp pre-treated with a commercial xylanase (Cartazyme 9704-E) in organic solvent were studied. The cellulose degradation during ozone bleaching was greatly reduced when the kraft-oxygen pulp from pine was pretreated with the xylanase in methanol (Xm) at 15% in a sequence XmZm (Z stage in methanol). The semi-bleached pulp was brightened with non-chlorine chemicals in a sequence XmZmEP to a brightness of 75%, with a selectivity of 2.2 and the following strength properties: tensile index 12.70 kNm/kg, tear index 9.81 mNm^2/g, burst index 1.27 $kPam^2/g$ at °SR 11.0 The refining pulp at 8000 rev. showed values of: tensile index 93.86 kNm/kg, tear index 8.98 mNm^2/g, and burst index 6.16 $kPam^2/g$ at °SR 31.0. The physical properties of the biobleached pulp were comparable to those of control pulps. The results are indicative that Cartazyme 9704-E-methanol mixture was important to improve the quality of the pulp in the XZEP bleaching sequence of *P. radiata* kraft-oxygen pulp.

Key words: Xylanase, cartazyme, methanol, organic solvent, biobleaching, TFC, Physical properties

INTRODUCTION

The importance of environmental aspects in pulp and paper manufacture processes has grown dramatically in recent years and has today a central role in the marketing of paper products (Viikari *et al.,* 1998). The pulp and paper industry has already modified the processes to reduce the formation of chlorinated organic compounds and other wastes. The beneficial effects of xylanase pre-treatment of pulps during ECF and TCF bleaching sequences are a reality. In search of new bleaching methods, enzymatic technologies have also been developed and are now used in the pulp mills (Eriksson, 1997), but very little is known about the direct use of xylanase in organic solvents and subsequent TCF bleaching. In recent years, the application of xylanase in the bleaching of *P. radiata* Kraft pulp has been described (Baeza *et al.,* 1999; Ruiz *et al.,* 1993, 1999, Curotto *et al.,* 1993, 1998; Kibblewhite and Clark, 1996; Aguirre *et al.,* 1995; Allison *et al.,* 1995; Vicuña *et al.,* 1995; Durán *et al.,* 1994, 1997; Angelo and Durán, 1998a,b). TCF bleaching on *Pinus radiata* D. Don pulps was improved by xylanases. Enzyme catalysis in aqueous-organic mixtures has wide applications (Gupta *et al.,* 1997); however, an inadequate attention has been paid to the issue of stability of enzymes in such media. Xylanase from *Aspergillus* 2M1 strain is stable in the presence of glycerol (Angelo *et al.,* 1997a). Xylanases are stable after precipitation with ethanol (Ganga *et al.,* 1997; Bakalova *et al.,* 1995; Tan *et al.,* 1993), and over 5% ethanol a significant decrease of its activity was observed (Herrmann and Kubicek, 1996). Xylanase from *Aspergillus niger* at 10% methanol or ethanol was inactivated (Sung *et al.,* 1996). The Cartazyme and Asperzyme 2M1 acting on *Pinus radiata* D. Don pulp, showed a good stability at 15% methanol aqueous mixture as a pre-treatment in a TCF bleaching (Baeza *et al.,* 1999; Ruiz *et al.,* 1999). A reduction of about 20% in kappa number and an improvement of the viscosity in 2.2 points were obtained.

The aim of this research was the study of the physical properties of a Kraft-oxygen pulp from pinus using methanol as non-aqueous solvent on an XmZmEP bleaching sequence. It is also presented a review of our previous work of solvent effect on xylanase activity.

EXPERIMENTAL

Pulp

Extended cooked oxygen-delignified softwood kraft pulp of *Pinus radiata* with Kappa number of 17.1, viscosity of 23.5 cps was employed.

Enzymatic Treatment

48 g (63% humidity) of pulps (10% consistency) were incubated with a commercial xylanase (Cartazyme 9704E) under the following conditions: 3 IU g^{-1}, 50°C, pH 6.0, and concentrations of methanol 15% vv^{-1}. The pH of the mixture was adjusted by adding 2 mol L^{-1} sulfuric acid. Incubation was conducted for 2 h in a rotavapor at 10% consistency. After the incubation step, pulps were immediately centrifuged and treated with a chemical bleaching sequence.

Chemical Bleaching

Ozone treatments at high pulp consistency (HC, 40%) were according to laboratory procedures employed previously (Ruiz *et al.,* 1997; 1999). Prior ozonation, pulps were soaked at pH 2.5 (adjusted with a sulfuric acid solution 2 M) for 1 hour, excess of water pressed out and the mixture centrifuged to obtain the desired pulp consistency. These impregnated pulps were fluffed and ozoned in a rotating round-bottomed flask equipped with a gas inlet system, using an ozone generator OZOCAV Mod. ZT-21. The ozone charge applied was of 0.7% /b.d.pulp.

The alkaline extraction stage was performed at 10% consistency in a polyethylene bag, at 60 °C, during 1 h, at pH 11 with a 2% NaOH solution.

The hydrogen peroxide stage was performed at 10% consistency in a polyethylene bag, at 70 °C during 3 h at pH 11.5, adjusted with 2% NaOH. Silicate, hydrogen peroxide and magnesium sulfate were added at 1%, 1.0% and 0.5%, respectively.

Pulp Analysis

Measurements of physical properties, Kappa number, viscosity and brightness of the pulps were performed according to TAPPI methods.

Xylanase activity

Methanol-aqueous (15 % v/v) solutions of Cartazyme 9704E (34 IU/mL) were prepared placing 15 µL of the Cartazyme 9704E stock solution (~23000 IU/mL) in different test tubes and adding 10 mL of methanol-water mixture to each. The pH was adjusted at 6.0 with 2% NaOH solution. The tubes were placed in a water bath at 50°C (± 0.5°C) and periodically shaken. The xylanase activity was measured following the standard Bailey's method (Bailey *et al.,*, 1992).

RESULTS AND DISCUSSION

The effect of pH and temperature on the xylanolytic activity of Cartazyme 7304-E in the presence of 15% methanol solution and their bleaching effect on *P. radiata* kraft pulp were previously studied (Ruiz *et al.,* 1999, Baeza *et al.,* 1999). The kinetic parameters were determined and the results are summarized in Table 1. The xylanase pretreated pulp bleached with an ozone stage showed a Kappa number decrease more significant in the presence of 15% methanol solution (Figure 1). The viscosity protection was also improved in the presence of methanol. The selectivity and brightness of Cartazyme pre-treated pulp in the presence of 15% methanol were also improved. The most significant results were observed when both, the xylanase pre-treatment and the ozonation stage were done in the presence of 15% aqueous-methanol. Further, at this condition the selectivity was very high compared with the control in methanol. In the sequence ZEP the same trend was observed. A high viscosity protection and very low kappa number were also found at the end of the bleaching sequence.

It was observed:

- The Cartazyme pre-treatment in 15% methanol and then ozonation and again Cartazyme post-treatment generated higher soluble lignin than in the other possible combinations. The reductant sugars followed the same trend.

- The Klason lignin analyses of the pulps do not show significant changes at the various conditions, however, the soluble lignin from the Klason process showed a better delignification when Cartazyme was in methanol than in pure water.

- The xylose retained in the pulp after enzymatic treatment showed a significant effect for X-water to X-methanol 15%, being 0.104% and 0.228%, respectively.

- In general, these results are indicative that the system Cartazyme 9704-E-methanol exerted an excellent treatment in the bleaching of pulp from *Pinus radiata* D. Don, giving a final product with excellent properties.

The results obtained for the physical properties of the xylanase pre-bleaching pulp obtained by a bleaching sequence XZP are shown in Table 2. It is interesting to note that physical properties, breaking index, tear index and burst index of the xylanase pretreated bleached pulps were comparable or slightly diferent to those of the control pulp (sequence ZEP). The observed reduction of the physical properties is owing to an ozonation stage, and not at the sequence or treatment applied. After refining at 8000 rev., the same trend was observed (Table 2). After bleaching with the same chemical loadings, most reports claim little change to papermaking properties, while some indicated an improvement in the tear index (Viikari *et al.*, 1998). However, some reports indicate that bleached pulps pretreated with xylanase are more difficult to refining, requiring more beating to achieve an equivalent tensile and freeness (Wong *et al.*, 1997).

The enzyme mechanisms involved in degradation of hemicellulose in wood are relatively well known (Bailey *et al.*, 1992); however, the effect of enzyme treatment on pulp fiber in organic solvents is not known. In kraft pulp, the residual hemicellulose have undergone drastic structural changes during the cooking, and the most of the substituents have been split off, facilitating the hydrolysis of the hemicellulose remaining in pulp fibers, and consequently permitting a better solvent extraction of lignin. A complete hydrolysis is very difficult to achieve using enzymes. One reason for this may be that new bonds are formed between the hemicellulose and lignin during the kraft cooking process. The inclusion of xylanase-methanol stage in the TCF bleaching sequence studied enhanced the quality of the pulp, also higher amounts of soluble lignin and reductant sugars were eliminated than in a similar xylanase-water treatment (Baeza *et al.*, 1999). The electron microscopy scanning of ozonated xylanase Cartazyme 9704-E treated pulps are showed in Figure 2. It is observed important changes, resulting from the xylanase acting on the fiber surface in relation of the untreated pulps. These morphological changes, which are favorable in the presence of the organic solvent, conduce at a better fiber-fiber contact that is reflected in the physical properties.

Table 1. Cartazyme Bleaching Experimental Conditions and Cartazyme kinetic parameters.

Bleaching stage	Parameter	Experimental value
XM15%	pH	6.0-6.5
	Temperature	50-55°C
	Charge	3 UI/g pulp
	Time	120 min
	Consistency	10%
KINETIC PARAMETERS (at 50°C)		
Aqueous medium	K_M	0.36 % pv^{-1}
	V_M	0.09 mmol l^{-1} min^{-1}
	E_{ac}	59.1 KJ mol^{-1} (14.13 Kcal mol^{-1})
Methanol 15%	K_M	0.38 % pv^{-1}
	V_M	1.20 mmol L^{-1} min^{-1}

Table 2. Physical properties of bleached pulps with Xylanase by the sequence XZEP.

Sequence	°SR	Tear Index (mNm^2/g)	Tensile Index (KNm/Kg)	Burst Index (Kpa m^2/g)
Unrefined				
Unbleached	12	11.82	15.19	1.53
ZEP	11	8.51	10.56	0.99
XZEP	11	8.47	12.55	1.23
XmZEP	11	9.81	12.70	1.27
MZEP	12	8.15	11.30	1.07
(Refined at PFI 8000 rev.)				
Umbleached	30	9.10	89.61	6.55
ZEP	29	8.77	86.69	6.55
XZEP	32	7.93	92.06	6.65
XmZEP	31	8.98	93.86	6.16
MZEP	31	8.46	87.83	6.84

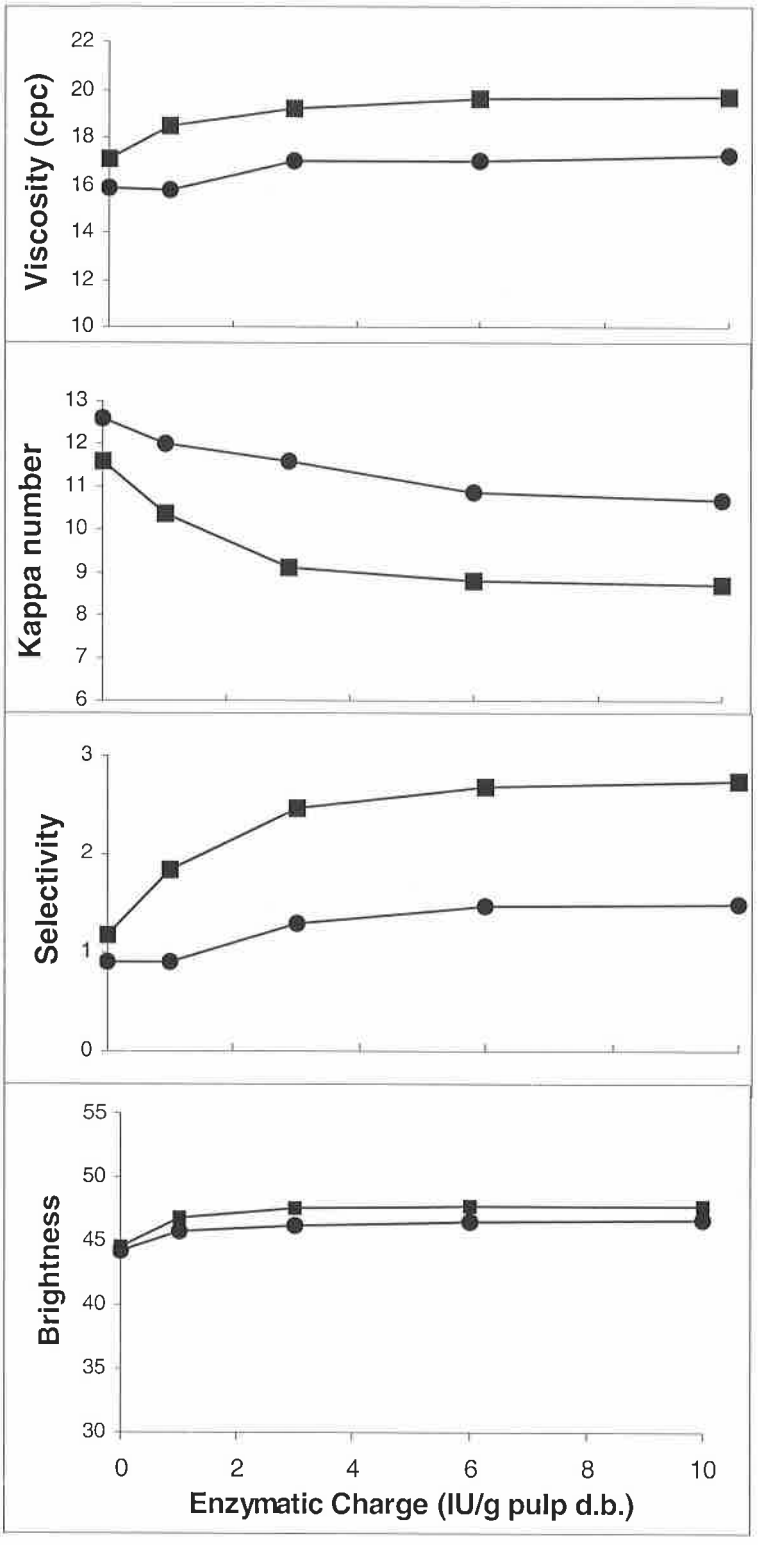
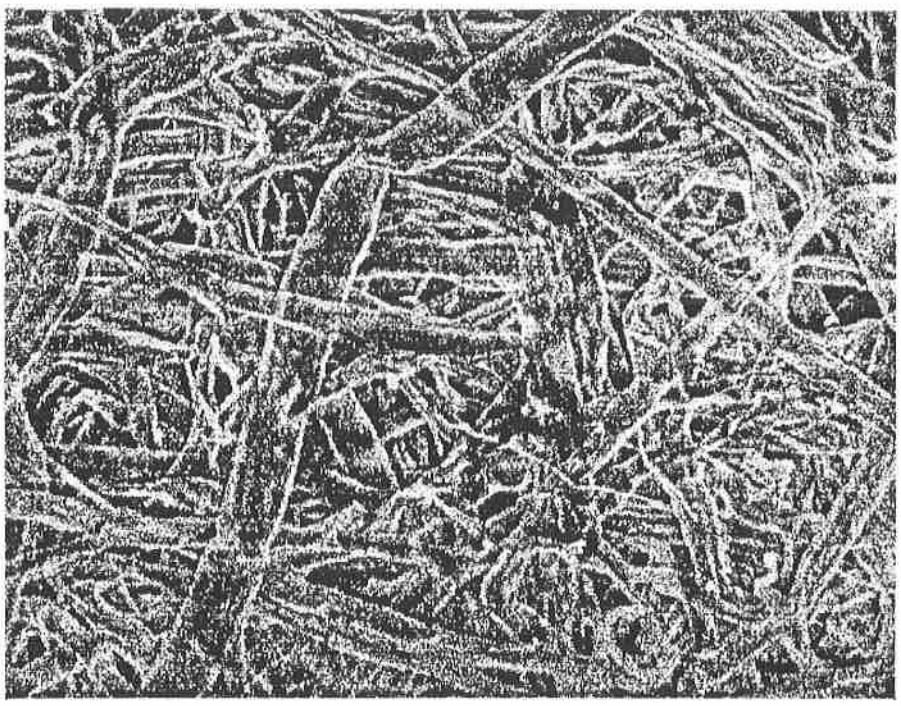


Figure 1. Effect of the Enzymatic Charge of Cartazyme 9704-E on the bleaching sequence OXZ. Cartazyme: • in water; ■ in methanol 15%. Incubation time: 120 min; Consistency: 10%; Ozone charge: 0.6%; Unbleached pulp: viscosity: 23.5 cps; Kappa number: 17.5; brightness: 30.8%.

A



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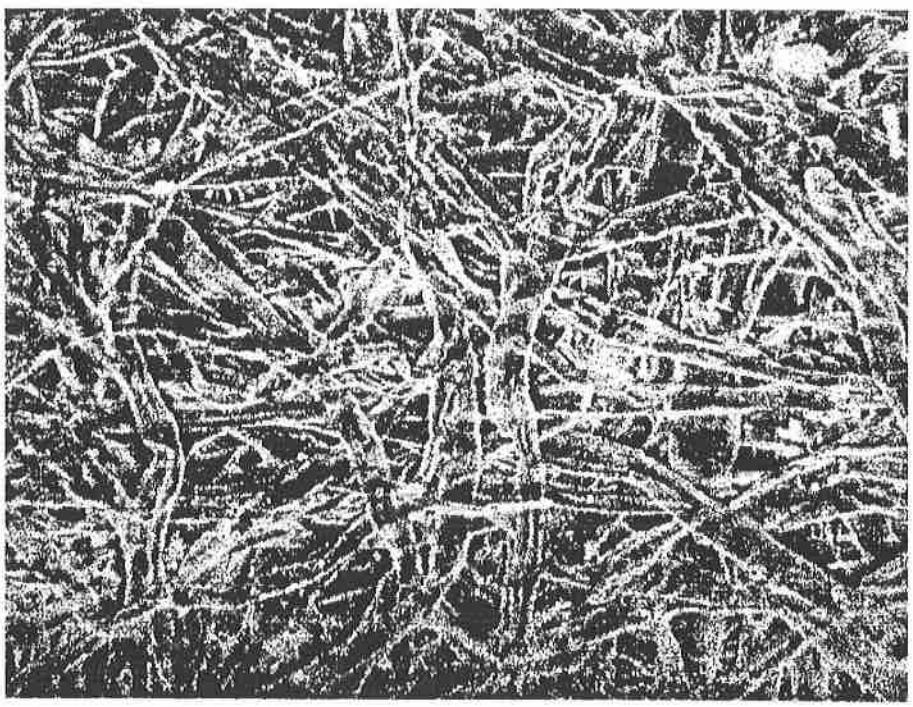

Figure 2. Electron micrograph of ozone bleaching *Pinus radiata* Kraft oxygen pulp pretreated with Cartazyme 9704 E. A) Incubated in water. B) incubated in methanol 15% v/v.

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REFERENCES

Aguirre, C., Ruiz, J., Castillo, C.,Nazal, A., Baeza, J., Durán, N. & Curotto, E. (1995). *4th Brazilian Symposium on the Chemistry of Lignins and Other Wood Components*, Proceedings, **Vol 5**, pp 170-183.

Angelo, R., Ruiz, J., Freer, J., Baeza, J. & Durán, N. (1997a). *5th Brazilian Symposium on the Chemistry of Lignins and Other Wood Components*, Proceedings, **Vol 6**, pp 537-546

Angelo, R., , Aguirre, C., Curotto, E., Esposito, E., Fontana, J.D., Baron, M., Milagres, A.M.F. & Durán, N. (1997b). *Biotechnol. Appl. Biochem.*, **25**, 19-27.

Angelo, R.S. & Durán, N. (1998a). *7th International Conference Biotechnology Pulp and Paper Industry*, Proceedings, **Vol 7C**, pp 19-22.

Angelo, R.S. & Durán, N, (1998b). *5th Europe Wokshop Lignocellulosics and Pulp. Advances in Lignocellulosics Chem. Ecol. Friendly Pulping Bleaching Technology*, Proceedings, pp 505-508.

Allison, R.W., Clark, T.A. & Ellis, M.J. (1995). *APPITA*, **48**, 201-206.

Baeza J., Freer J., Grandon, J., Rodriguez J., Curotto E. & Duran N. (1999). *10th International Symposium Wood and Pulping Chemistry*, Proceedings, **Vol. III**, pp 38-43.

Bailey, M.J., Biely, P. & Poutanen, K. (1992). *J. Biotechnol*, **23**, 257-270.

Bakalova, N., Petrova, S., Benadova, R. & Kolev, D. (1995). *Biotechnol. Biotechnol. Equip.*, **2**, 45-51.

Curotto, E., Aguirre, C., Concha, M., Nazal, A., Campos, V., Esposito, E. Angelo, R., Milagres, A.M.F. & Durán, N. (1993). *Biotechnol. Tech.*, **7**, 821-822.

Curotto, E., Nazal, A., Aguirre, C., Campos, V. & Durán, N. (1998). *Appl. Biochem. Biotechnol.*, **73**, 29-42.

Durán, N., Curotto, E., Esposito, E., Aguirre, C., Nazal, A. & Angelo, R. (1994). *In Advances in Bioprocesses Engineering*, ed. E. Galindo & O.T. Ramirez. Kluwer Acad. Publ., pp 489-494.

Durán, N., Toledo, A., Milagres, A., Esposito, E., Curotto, E. & Angelo, R. (1997). *5th Brazilian Symposium on the Chemistry of Lignins and Other Wood Components*, Proceedings, **Vol 6**, pp 490-437.

Eriksson, K.-E.L. (1997). Biotechnology in the Pulp and Paper Industry. *In Advances in Biochemical Engineering Biotechnology*, Proceedings, **Vol. 7**, pp.339.

Ganga, A., Gonzalez-Candelas, L., Ramon, D. & Perez-Gonzalez, J.A. (1997). *J. Agric. Food Chem.*, **45**, 2359-2362.

Gupta, M.N., Batra, R., Tyagi, R. & Sharma, A. (1997). *Biotechnol. Prog.*, **13**, 284-288.

Herrmann, M. & Kubicek, C.P. (1996). In Biotechnology in the Pulp and Paper Industry: Recent Advances in Applied and Fundamental Research, Facultas-Universitatsverlag, Austria, 103-106.

Kibblewhite, R.P. & Clark, T.A. (1996). *APPITA J.*, **49**, 390-396.

Miller, G.L. (1959). *Anal. Chem.*, **31**, 426-427.

Ruiz, J., Baeza, J., Freer, J., Curotto, E., Esposito, E. & Durán, N. (1993). *3th Brazilian Symposium on the Chemistry of Lignins and Other Wood Components*, Proceedings, **Vol 4**, pp 324-327.

Ruiz, J., Freer, J., Rodriguez, J. & Baeza, J. (1997). *Wood Sci. Technol.*, **31**, 217-223

Ruiz, J., Angelo, R., Freer, J., Baeza, J., Aguirre, C., Curotto, E. & Durán, N. (1999). *Biotechnol. Lett.*, **21**, 361-366

Norrstrom, H. & Teder, A. (1973). *Svensk. Paperstidn.*, **12**, 458-461.

Sung, C.-K., Lee, S.-W., Park, S.-K. & Chun, S.-S. (1996). *Han'guk Sikp'um Yongyang Kwahak Hoechi.*, **25**, 701-707.

Tan, S.K.H., Omar, O. & Kader, J.A. (1993). *18th. Malasya Biochem. Soc. Conf.*, Proceedings, pp 74-80.

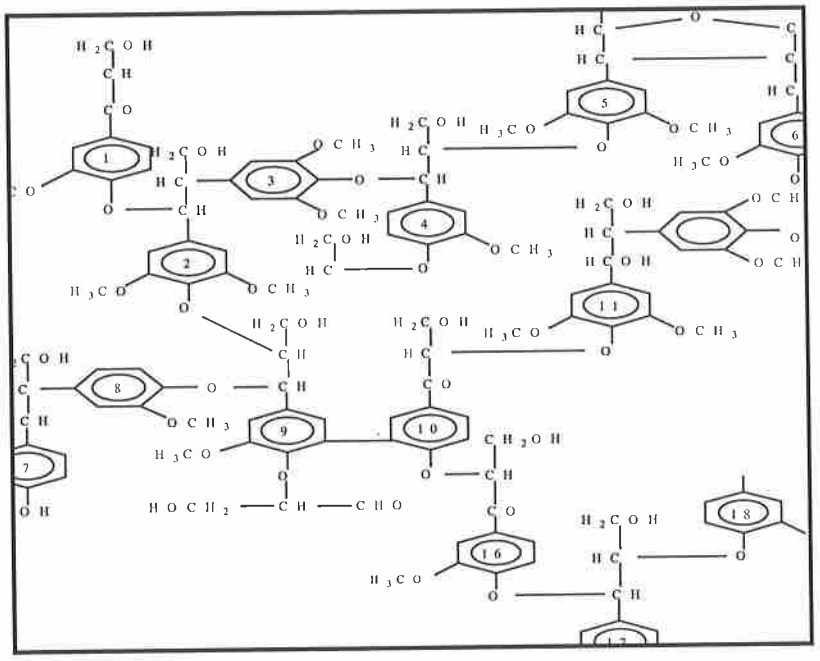
Viikari, L., Buchert, J. & Suurnakki, A (1998). In Forest Products Biotechnology, ed. A. Bruce & J.W. Palfreyman. Taylor & Francis Publ., pp 83-97.

Vicuña, R., Yeber, M.C. & Osses, M. (1995). *J. Biotechnol.*, **42**, 69-73.

Won, K., Jong, E., Saddler, J. & Allison, R. (1997). *Appita*, **50**(6), 509-518.

6th BS CLWC 6th Brazilian Symposium on the Chemistry of Lignins and other Wood Components

October 25-28, 1999, Clube dos 500 Hotel, Guaratinguetá, SP, Brazil



Poster Session

183A

A rigorous mathematical modeling of kinetics of herbicide controlled-release from lignin matrices

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ABSTRACT

Usually release kinetic data of active ingredients from matrices are modeled by the well-known equation $M_t/M_0=Kt^{1/2}$, which is valid only until 60% of release. A rigorous analysis based on the Fick's second law was used to develop a mathematical model able to describe the release kinetics in its all extension. The predictions of the developed model agreed well with release data of 2,4-D and diuron formulations employing several lignins as support. The effective diffusion coefficient of the rigorous model can be correlated to the K value obtained by the conventional approach and used for simulation of kinetic data for M_t/M_0 values above of 0.6. This result is extremely useful for kinetic studies of herbicide formulations having low release rates.

Keywords: controlled-release, herbicide, lignin, kinetics, mathematical modeling

INTRODUCTION

In the recent years, the controlled release (CR) of herbicides is a technology that is proving to be very attractive for the solution of the problems with defensive application and contamination. Polymeric and macromolecular matrices are often utilized as support for the herbicide and among the various materials used as support, lignin is an interesting alternative since it can be obtained from most agro industrial residues such as sugarcane bagasse, kraft liquor, rice and wheat straws (Wilkins, 1990).

In our research group, different CR formulations (CRFs) have been developed utilizing different herbicides (active ingredients AI) and lignins (macromolecular matrix MM) obtained by different processes. Release kinetics studies employing these CRFs were carried out monitoring the amount of AI released into water bath as a function of time (Cotrim *et al.*, 1994; Silva and Wilkins, 1992; Silva *et al.*, 1994; Souza, 1994; Ferraz *et al.*, 1997; Cotteril *et al.*, 1995; Pereira, 1995, Reis, 1999). The knowledge of release kinetics is very important to predict both dosage of AI used at time and the AI fate when applied in acceptors systems containing an aqueous phase, for example the soil. In previous studies, the main transport mechanism of AI from inside matrix to acceptor medium was demonstrated to be a diffusion mechanism where the release rate is dependent on the diffusion coefficient of AI inside the MM (Cotrim, 1994; Silva and Wilkins, 1992; Silva *et al.*, 1994; Souza, 1994; Ferraz *et al.*, 1997; Cotteril *et al.*, 1995; Pereira, 1995; Reis, 1999). In this way, the development of a suitable model to evaluate the diffusion coefficient is very useful to correlate this parameter as a function of structural characteristics of lignins employed as support (Ferraz *et al.*, 1997). Conventionally, the diffusion parameter is estimated from the linear regression between the amount of AI released and the square root of time. However, this approach is valid only until 60% of release. Above this value, the release kinetics have been described using an empirical model where the amount of AI released is proportional to an exponential decay in time (Zeoli and Kydonieus, 1983).

This article presents a rigorous mathematical modeling of the release kinetics using the Fick's law to describe the diffusion mechanism of AI through the matrix pores. The objective is to develop a model able to describe the release kinetics in all its extension.

EXPERIMENTAL

Release of Herbicides in Water Bath Experiments

A melting process prepared the formulations of herbicides with lignin extracted from several sources. After melting, the final formulations were ground and sieved to select a fraction granule size range from 0.71 to 1.00 mm. The main characteristics of CRFs obtained are presented in Table 1 (Souza, 1994; Pereira, 1995, Ferraz *et al.*, 1997).

The formulation granules (three replicates) were weighted to ensure a minimum amount of 25 mg of herbicide and kept immersed in 30 mL of deionized water. The flasks were closed and maintained at 30°C. In the first 10 days changing all the water contents of the flasks performed sampling daily. Further sampling was performed at intervals of 3-7 days. The concentrations of herbicide in samples were determined by HPLC (Souza, 1994; Pereira, 1995, Ferraz *et al.*, 1997). Released herbicide was expressed as a percentage of the initial amount added to the formulation: M_t/M_0.

Table 1. Characteristics of CRFs used in the experiments of herbicide release in water bath.

Symbol	Herbicide	Lignin Source	Lignin Production Process	Precipitant Agent	Herbicide Concentration
CRF1	2,4-D	Bagasse	Steam Explosion	HCl	45%
CRF2	2,4-D	Bagasse	Steam Explosion	H_2SO_4	45%
CRF3	2,4-D	Eucalyptus*	Kraft	H_2SO_4	45%
CRF4	2,4-D	Eucalyptus**	Kraft	H_2SO_4	45%
CRF5	2,4-D	Eucalyptus**	Kraft	HCl	45%
CRF6	2,4-D	Pinus	Kraft	***	45%
CRF7	Diuron	Bagasse	Steam Explosion.	HCl	50%

* from kraft liquor containing 14% solids,
** from kraft liquor containing 37% solids,
*** pinus kraft lignin (INDULIN AT, Westvaco Co.).

Release Kinetics Modeling

• Conventional Approach

Release kinetic data are often modeled by the following simplified equation:

$$\frac{M_t}{M_0} = Kt^{1/2} \qquad (1)$$

where: t is time (day), M_t is the cumulative amount of AI released at the time (g), M_0 is the initial amount of AI in the MM (g), K is the release rate constant ($day^{-1/2}$).

Equation (1) has shown to be valid to describe the release kinetics of herbicides from lignin matrices until about 60% of herbicide is released (Cotrim, 1994; Silva and Wilkins, 1992; Silva *et al.*, 1994; Souza, 1994; Ferraz *et al.*, 1997; Cotteril *et al.*, 1995; Pereira, 1995, Reis, 1999). For modeling the remaining kinetic data, the following empirical model was proposed by Zeoli and Kydonieus (1983):

$$M_t = M_0 - \frac{c_1}{c_2}\exp(-c_2 t) \qquad (2)$$

where: c_1 and c_2 are constants.

In order to describe the release kinetics in all its extension it is necessary to use a phenomenological approach discussed as follows.

• Rigorous Approach

In monolithic systems where the main interactions between AI and MM are physical interactions, the release rate of AI is controlled by a diffusion mechanism described by the Fick's law (Siegel, 1989).

Aiming the mathematical description of the diffusion mechanism, the following hypothesis are assumed (Siegel, 1989):

a) the matrix is an isothermal slab;

b) the AI is dissolved in the solvent (water) contained in the matrix pores at concentration less than the saturation concentration (c_s);

c) the release occurs through the two faces of the slab;

d) the total sum of the individual volumes of the pores is $\varepsilon A\lambda$;

e) the AI concentration in the matrix pores (c_p) varies with depth (x) into the matrix and with time (t): $c_p=c_p(x,t)$;

f) the initial concentration of AI in the pores is $M_0/\varepsilon A\lambda$;

g) perfect sink conditions at x=0 and x=λ (two situations where this hipothesis may not be valid are the release into a relatively small volume, or when a stagnant unstirred layer exists at the interface between the monolith and the release medium).

Considering the previous assumptions, the following equation (Fick's second law):

$$\frac{\partial c_p}{\partial t} = D_{ef}\frac{\partial^2 c_p}{\partial x^2} \qquad (3)$$

subject to the following initial and boundary conditions:

$$c_p(x,t=0) = \frac{M_0}{\varepsilon A\lambda} \qquad (4)$$

$$c_p(0,t) = c_p(\lambda,t) = 0 \qquad (5)$$

describe the spatial and temporal profiles of the herbicide concentration in the matrix pores. In Eqs. (3), (4) and (5) c_p is AI concentration in the pores (g cm^{-3}), D_{ef} is effective diffusion coefficient ($cm^2 day^{-1}$), ε is matrix porosity, A is slab area (cm^2) and λ is matrix thickness (cm).

The solution of the Eq. (3) satisfying the initial and boundary conditions is given by:

$$c_p(x,t) = \frac{4M_0}{\varepsilon A\lambda\pi}\sum_{n=0}^{\infty}\left\{\frac{1}{2n+1}exp\left[-\frac{(2n+1)^2\pi^2 D_{ef}t}{\lambda^2}\right]sin\left[\frac{2n+1\pi x}{\lambda}\right]\right\} \qquad (6)$$

The mass flow rate (Q_t) of herbicide at time through the face of the slab at x=λ may be evaluated using the following equation:

$$Q_t = -A\varepsilon D_{ef}\left.\frac{\partial c_p}{\partial x}\right|_{x=\lambda} \qquad (7)$$

Since release occurs through both faces of the slab, the cumulative amount of AI released at time t is given by the equation:

$$M_t = -\int_0^t 2A\varepsilon D_{ef}\left.\frac{\partial c_p}{\partial x}\right|_{x=\lambda} dt \qquad (8)$$

Solving the integral in Eq. (8), Eq. (9) is obtained

$$M_t = M_0\left\{1-\frac{8}{\pi^2}\sum_{n=0}^{\infty}\left\{\frac{exp\left[-\frac{(2n+1)^2\pi^2 D_{ef}t}{\lambda^2}\right]}{(2n+1)^2}\right\}\right\}. \qquad (9)$$

At early times, Eq. (9) may be replaced by the simpler equation (Dahiyat et al., 1995; Zeoli and Kydonieus, 1983; Scher 1977):

$$M_t = \frac{4M_0}{\lambda}\sqrt{\frac{D_{ef}t}{\pi}} \qquad (10)$$

From the Eqs. (1) and (10) the following relationship can be established between the K and D_{ef} parameters:

$$K = \sqrt{\frac{16D_{ef}}{\pi\lambda^2}} \qquad (11)$$

• Parameter Estimation

The K and D_{ef} parameters in Eqs. (1) and (9) were respectively estimated by linear and non-linear regression. For estimation of D_{ef} by non-linear regression was used the Marquardt's method (Marquardt, 1963). Since

granule size was the same for all formulations used in the release experiments, the value of slab thickness (λ) can be assumed to be constant and the D_{ef} parameter in Eq. (9) can be indirectly estimated as D^*_{ef} which incorporates the following constants:

$$D^*_{ef} = \frac{\pi^2 D_{ef}}{\lambda^2} \qquad (12)$$

RESULTS AND DISCUSSION

Table 2 presents the values of K obtained by fitting Eq. (1) to the experimental data until 60% of release, the D^*_{ef} values calculated from the K values using Eqs. (11) and (12), the D^*_{ef} values estimated from Eq. (9), and the R^2 values for both conventional and rigorous approaches.

Table 2. Parameter estimation results for conventional and rigorous mathematical modeling.

CRF	Conventional Approach			Rigorous Approach	
	K ($day^{-1/2}$)	D^*_{ef} (day^{-1})	R^2	D^*_{ef} (day^{-1})	R^2
CRF1	0.133 ± 0.003	0.035 ± 0.002	0.9976	0.037 ± 0.001	0.9983
CRF2	0.107 ± 0.003	0.022 ± 0.001	0.9935	0.023 ± 0.001	0.9960
CRF3	0.168 ± 0.005	0.055 ± 0.003	0.9982	0.058 ± 0.002	0.9901
CRF4	0.218 ± 0.008	0.092 ± 0.007	0.9837	0.107 ± 0.005	0.9911
CRF5	0.20 ± 0.01	0.078 ± 0.009	0.9891	0.097 ± 0.005	0.9913
CRF6	0.145 ± 0.006	0.043 ± 0.003	0.9956	0.047 ± 0.002	0.9908
CRF7	0.0452 ± 0.0005	0.0040 ± 0.0001	0.9976	0.0039 ± 0.0001	0.9978

Small standard deviations are observed for the values of K and D^*_{ef} estimated from the conventional and rigorous approach. Thus, the parameter estimates can be assumed to have a high level of statistical significance for all formulations studied.

Different D^*_{ef} values can be observed for 2,4-D formulations obtained at different processing conditions. This suggests that such conditions must be optimized in order to achieve a desirable release rate. The diuron formulation (CRF7) presented a D^*_{ef} value smaller than that observed for a 2,4-D formulation obtained at a similar processing condition (CRF1).

The D^*_{ef} values calculated from the K values agreed well with the D^*_{ef} values estimated from the rigorous approach. This result suggests that for the knowledgement of release kinetics in all its extension it is only necessary to obtain experimental data until 60% of herbicide is released. The data obtained in this range can be then treated using the conventional approach for K parameter estimation, which can be used for estimating the D^*_{ef} parameter from the Equations (11) and (12). The D^*_{ef} value thus obtained can be employed to simulate the remaining kinetic data from the Equation (9). This procedure is extremely useful in kinetic studies using CRFs having low release rates which requires a long monitoring time (625 days for diuron formulation, see Figure 1). However, a very important prerequisite for the success of this methodology is to dispose of a suficient number of experimental data to obtain a precise estimate of K parameter.

Comparison between experimental data and rigorous model predictions for 2,4-D and diuron formulations is shown in Figure 1. According to this figure, a good fit of the model to the experimental data was observed for all analyzed CRFs (high R^2 values). However, better simulation results could be obtained by taking in account the release mechanism by direct solubilization of herbicide present in the matrix surface. The consideration of this phenomenon is the main potential of further development of the rigorous model here discussed.

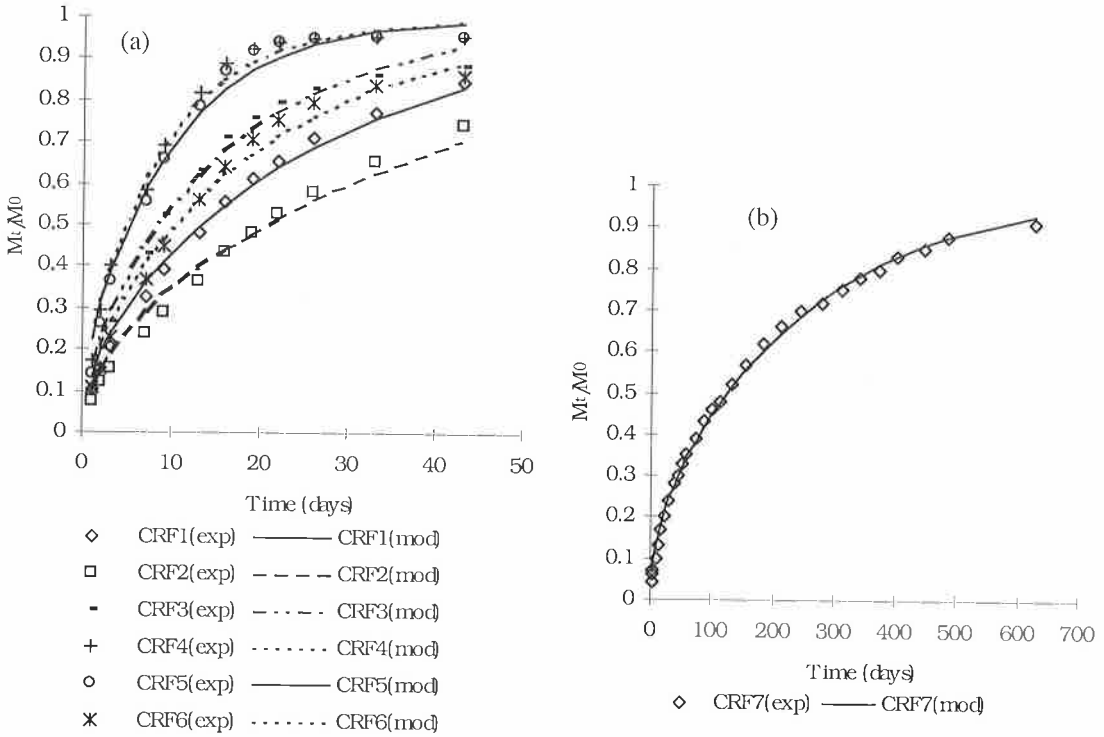

Figure 1. Comparison between experimental data and modified model predictions for herbicide formulations: (a) 2,4-D and (b) diuron.

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REFERENCES

Cotrim, A. R., Souza, J. A., Ferraz, A., Silva, F. T. & Wilkins, R. M. (1994). The Influence of Different Lignins on the Controlled Release Formulations. *In 3rd Brazilian Symposium on the Chemistry of Lignins and Other Wood Components*, Proceedings, **Vol. 4**, pp. 278-281.

Cotteril, J. V., Wilkins, R. M., & Silva, F. T. (1995). Controlled-Release of Diuron from Granules Based on a Lignin Matrix System. *J. Controlled Release*, **40**, 133-142.

Dahiyat, B. I., Richards, M. & Leong, K. W. (1995). Controlled Release from poly(phosphoester) Matrices. J. Controlled Release, 33, 13-21.

Ferraz, A., Souza, J.A., Silva, F.T., Gonçalves, A.R., Bruns, R.E. & Cotrim, A.R. (1997). Controlled Release of 2,4-D from Granule Matrix Formulations Based on Six Lignins. *J. Agriculture and Food Chemistry*, **45**, 1001-1005.

Marquardt, D. W. (1963). An Algorithm for Least-Squares Estimation of Nonlinear Parameters. *J. of the Society for Industrial and Applied Mathematics*, **11 (2)**, 431-441.

Pereira, C.A. (1995). Obtenção e Avaliação de Formulações de Liberação Controlada de Diuron/Lignina. MSc. thesis , Departamento de Biotecnologia, Faculdade de Engenharia Química de Lorena.

Reis, M. F. (1999). Estudo da Movimentação dos Herbicidas em Colunas de Solo. MSc. Thesis, Departamento de Biotecnologia, Faculdade de Engenharia Química de Lorena.

Siegel, R. A. (1989). Modeling of Drug Release from Porous Polymers. In *Controlled Release of Drugs: Polymers and Aggregate Systems*, ed. Morton Rosoff, VCH Publishers, New York, pp. 1-9.

Silva, F. T. & Wilkins, R. M. (1992). Use of Lignins as Support for Herbicides in Controlled Release Formulations. *In 2nd Brazilian Symposium on the Chemistry of Lignins and Other Wood Components*, Proceedings, **Vol. 3**, pp. 165-172.

Silva, F. T., Pereira C. A., Cotrim, A. R., Ferraz, A., & Wilkins, R. M. (1994). The Studies of Processing Parameters on the Release Rates of Diuron in Controlled Release Formulations. *In 3rd Brazilian Symposium on the Chemistry of Lignins and Other Wood Components*, Proceedings, **Vol. 4**, pp. 265-268.

Souza, J. A. (1994). Utilização de Lignina para Obtenção de Formulações de Liberação Controlada do 2,4-D. MSc. Thesis, Faculdade de Engenharia Química, Universidade Estadual de Campinas.

Wilkins, R. M. (1990). Biodegradable Polymer Methods. In *Controlled Delivere of Crop-Protection Agents*, Taylor and Francis, London, pp. 149-165.

Zeoli, L. T. & Kydonieus, A. F. (1983). Physical Methods of Controlled Release. In *Controlled Release-Technology: Bioengineering Aspects*, ed. Das, K. G.. John Wiley & Sons, New York, pp. 61-120.

Acid-catalyzed steam explosion of *Pinus taeda* chips: I. Characterization of the water-soluble fraction

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ABSTRACT

Steam explosion of *Pinus taeda* was carried out under eight distinct pretreatment conditions (195-210°C, 2.5-5.0 min and 0.5-1.0% H_2SO_4) to evaluate how this plant biomass could be effectively fractionated into its three main components, cellulose, hemicellulose and lignin. This paper reports on the characterization of the water-soluble (WS) fraction derived from these acid-catalyzed steam treatments in relation to the release of dehydration by-products, lignin-derived WS chromophores and WS carbohydrates. Acid-catalyzed steam explosion allowed good recovery of hemicellulose sugars (70%, mostly as xylose) in the WS fraction. The overall mass balance of pretreated fractions revealed a typical recovery of over 90% of the chips dry weight, which corresponded to yields of 66% in water-insoluble fibers, 23% in water-solubles and 11% in mass lost as unidentified volatiles. In general, the effect of doubling the residence time into the steam gun reduced the acid requirement for optimal pretreatment by 50%. Compared to pretreatment at 195°C for 2.5 min with 1.0% H_2SO_4–impregnated chips, a 15°C increase in pretreatment temperature resulted in an equivalent hemicellulose recovery but the amount of glucan hydrolyzed as a result of pretreatment almost doubled in relation to that obtained at 195°C. The same trend was observed in relation to the accumulation of dehydration by-products in the WS fraction. For pretreatment of 1.0% H_2SO_4–impregnated chips for 2.5 min, an 1.92-fold increase in furfural was observed when the pretreatment temperature was raised from 195 to 210°C. Pretreatment at 210°C for 5 min with 1.0% H_2SO_4–impregnated chips (highest severity) resulted in considerable deacetylation of pine hemicelluloses but the residual hemicellulose found within the steam-treated fibers appeared to be highly acetylated. In addition, an increase in pretreatment severity also triggered a considerable accumulation of formic acid, probably derived from lignin degradation reactions such as demethoxylation. Nearly 1-2% of the chips dry-weight were recovered as WS chromophores that were presumably aromatic in nature. However, there was no clear trend between pretreatment severity and the yields of WS chromophores for the conditions tested in this study.

Keywords: steam explosion, *Pinus taeda*, acid hydrolysis

INTRODUCTION

Lignocellulosic materials are inexhaustible resources that can be directly or indirectly used for the production of biomolecules and commodity chemicals. However, the industrial utilization of these renewable resources has been compromised by several factors such as the close association that exists among the three main components of the plant cell wall (cellulose, hemicellulose and lignin) and the low efficiency by which lignocellulosic substrates are converted through biological processes such as enzymatic hydrolysis and fermentation.

Several pretreatment options have been suggested in the literature to fractionate and effectively recover fermentable sugars from plant biomass (Parisi, 1989; Saddler *et al.*, 1993). Among them, steam explosion has been indicated as one of the most effective pretreatment options to date (DeLong, 1981, 1983). Steam explosion is a process whereby biomass is treated with high pressure steam under optimal conditions and subsequently quenched to atmospheric by adiabatic expansion of the reactor content. Optimal conditions are defined as those in which the best substrate for hydrolysis is produced with the least material lost due to decomposition reactions (Ramos *et al.*, 1992a,b). Several advantages have been associated with steam explosion, such as the effective fractionation of wood into its three main components (cellulose, hemicellulose and lignin) and the theoretical yields of cellulose conversion that can be achieved through enzymatic hydrolysis (Saddler *et al.*, 1993). The major disadvantage is that, although steam explosion greatly enhances the enzymatic hydrolysis of hardwoods (Brownell and Saddler, 1984; Ramos *et al.*, 1992a) and most agricultural residues (Dekker *et al.*, 1987; Kling *et al.*, 1987; Fontana *et al.*, 1995; Deschamps *et al.*,

1996) tested to date, it has not yet been successfully developed for the use of softwoods (Clark and Mackie, 1987; Ramos *et al.*, 1992b).

The lower susceptibility of softwoods to steam explosion have been largely attributed to three different aspects: (a) morphologically, softwood tracheids are not as porous and easily accessible as hardwood vessels and libriform fibers; (b) the major hemicellulose components found in softwoods are hexosans such as gluco- and galactoglucomannans and these materials are far less amenable to acid hydrolysis than hardwood heteroxylans; and (c) softwoods are composed of a lignin type that is more difficult to pretreat than hardwood lignins and this is directly related to its chemical composition, formed primarily by interlinked guaiacyl units which may reach a considerable degree of condensation (Tanahashi, 1990; Ramos *et al.*, 1992b).

In this work, the susceptibility to steam treatment of a low-resin content pine species (*Pinus taeda*) has been investigated. For this purpose, the best pretreatment conditions were defined as those in which the best recovey yields were obtained with the least possible degradation of fermentable sugars (maximum recovery of water-soluble hemicellulose sugars). As the beneficial effects of acid-catalysis has been well documented in the literature (Torget *et al.*, 1991; Saddler *et al.*, 1993; Deschamps *et al.*, 1996) and for the reasons mentioned above, pretreatment in the absence of an acid catalyst was not considered in this study.

EXPERIMENTAL

Pretreatment of *Pinus taeda* wood chips

Acid-catalyzed steam explosion of industrial chips derived from 4 year-old stems of *P. taeda* (Klabin Fabricante de Papel e Celulose Ltda., Telêmaco Borba, PR, Brazil) were steam-treated at the Pilot Plant Unit of the Federal University of Paraná using a 5L steam gun (Ramos *et al.*, 1995) under distinct pretreatment conditions.

Chips were impregnated with dilute sulfuric acid (H_2SO_4) and pretreated with saturated steam temperatures ranging from 195 to 210°C over a range of residence times (1.7-5.9 min). Acid impregnation was carried out by soaking the wood chips in either 0.33 to 1.17 (w/v) H_2SO_4 overnight, at room temperature. The excess of H_2SO_4 solution was discarded. Acid-impregnated chips were shown to have approximately 50% (w/w) moisture content. Steam pretreatment was performed by loading 350 g of acid-impregnated chips directly into the steam gun. After treatment for a given temperature and residence time, the steam-treated pine (STP) was released from the steam-gun by rapid depressurization of the vessel, causing the material to expand (explosion) into a stainless steel cyclone. Solid and non-volatilized biomass were then recovered by water washing. The resulting STP was extracted twice with water at a 5% (w/w) solids concentration. The water insoluble fraction was termed STP-WI, whereas the water soluble fraction or liquor was termed STP-WS.

Table 1. Pretreatment conditions for the steam explosion of *P. taeda* chips.

Experiment	[H_2SO_4] (% p/v)	Temperature, ºC	Time, min
EXP1	0.50	195	2.50
EXP2	1.00	195	2.50
EXP3	0.50	210	5.00
EXP4	1.00	210	5.00
EXP5	0.50	195	5.00
EXP6	1.00	195	5.00
EXP7	0.50	210	2.50
EXP8	1.00	210	2.50

Chemical analysis

Chemical analysis of substrates and raw materials was performed by a modification of the TAPPI Standard Method T222 os-74 for Klason lignin (acid insoluble lignin). After swelling 1 g of wood chips (40 mesh) in 15 mL of 72% H_2SO_4 (w/w) for 2 h at 20 ± 1°C, distilled water was added until a 3% (w/w) acid concentration was reached and the suspension was treated for 70 min in an autoclave. Acid-insoluble lignin was measured gravimetrically and total carbohydrates were determined by HPLC (Irick *et al.*, 1988). Acid-soluble lignin was also determined in this study, using an absortivity of 110 L/g.cm for absorbance measurements performed at 205 nm (see below for details).

Hemicellulose hydrolysis occurring during steam treatment was determined by assaying the WS fraction by HPLC (Irick *et al.*, 1988). Recovery yields of the STP-WS fractions were expressed in terms of g/100 g of oven-dry wood. The HPLC analyses were obtained with a Shimadzu HPLC system, model LC10AD, provided with a SIL10A autosampler and two detectors arranged in series, model SPD10A for UV spectroscopy and model RID10A for refractive index. The HPLC analysis of water-solubles and Klason lignin hydrolysates was performed at 65°C in an HPX-87H column (Bio-Rad), eluted with 8 mM H_2SO_4 at a flow rate of 0.6 mL/min. All hydrolysate components with a retention time of 9.8 min were analyzed, as xylose because the HPLC system applied in this study could not resolve among xylose, mannose and galactose.

Steam-treatment hydrolysates were also analyzed by ultraviolet (UV) spectroscopy. UV spectra were generated from liquid extracts (WS fractions) within the range of 190 to 400 nm, using a HP 8452A diode-array UV spectrophotometer and at an equivalent dilution rate.

RESULTS AND DISCUSSION

Acid-impregnated chips of *P. taeda* were steam-treated over a range of temperatures and residence times into the steam gun to evaluate the effect of pretreatment on the recovery yield of water-insoluble fibers and water-soluble hemicellulose sugars. In general, there was a gradual decrease in the overal recovery yield of steam-treated fractions towards more drastic pretreatment conditions (particularly longer residence times), indicative of partial destruction of wood components including carbohydrates and their subsequent loss as unidentified volatiles (Table 2).

The highest recovery of water-soluble components was obtained when wood chips were impregnated with aqueous 1.0% H_2SO_4 and pretreated at 210°C for 2.5 min (EXP8, Table 2). Nearly 29.2% of the chips dry weight was recovered as HPLC-detectable analytes (mostly monosaccharides) while 58.5% remained in the water-insoluble material (fibers). However, these pretreatment conditions where also those in which the highest levels of sugar degradation were achieved (0.71% conversion of wood into furfural) (Table 3), together with a substantial loss of wood components as volatiles (nearly 11%) (Table 2).

Based on the data showed in Tables 2 and 3, the best compromise between recovery yields and accumulation of dehydration by-products appeared to be a result of experiments EXP2 and EXP5, in which chips were pretreated at 195°C (a) for 2.5 min after impregnation with 1.0% H_2SO_4 and (b) for 5 min after impregnation with 0.5% H_2SO_4, respectively. Since it is unquestionable that lower severities are preferable for the process economics, EXP2 was the pretreatment of choice because it had the added bonus of performing pretreatment at lower temperature and residence time into the steam gun. The overall mass balance at these pretreatment conditions (EXP2) revealed the best recovery of the chips dry weight, which corresponded to yields of 78% in water-insoluble fibers, 24% in water-solubles and very little mass lost as volatiles (if any). It is also worth noticing that, for the compromised pretreatment conditions at 195°C, the effect of doubling the residence time into the steam gun reduced the acid requirements by 50%.

Pretreatment at 210°C for 2.5 min with 1.0% H_2SO_4–impregnated chips (EXP8) also resulted in similar hemicellulose recovery (determined as xylose) but the amount of glucan (derived from cellulose and/or glucans in glucomannan) hydrolyzed as a result of pretreatment doubled in relation to the compromised conditions mentioned previously (Table 3). The same trend was observed in relation to the accumulation of dehydration by-products in the WS fraction. For pretreatment of 1.0% H_2SO_4–impregnated chips for 2.5 min, an 1.92-fold increase in furfural was observed when the pretreatment temperature was raised from 195 to 210°C.

Acid-catalyzed steam explosion allowed good recovery of hemicellulose sugars (mostly xylose) in the WS fraction. On the basis of the chips dry weight, a maximum of 12% recovery of polyoses (xylose, mannose and galactose, collectively) was obtained from pretreatment at 195°C for (a) 2.5 min with 1.0% H_2SO_4–impregnated chips (EXP2) and (b) 5 min with 0.5% H_2SO_4–impregnated chips (EXP5) (Table 3). This figure corresponded to an overall recovery of 70% of the hemicellulose (polyoses) present within the wood chips. In addition, less than 5% of the chips glucan content was hydrolyzed to glucose and/or lower cello-oligomers (mostly cellobiose) under these pretreatment conditions.

Table 2. Recovery yields of pretreatment fractions derived from *P. taeda* steam explosion.

Pretreatment	WI fraction, %	WS fraction, %		Total, %
		HPLC analytes	A(280)	
EXP1	76.59	17.85	0.95	95.40
EXP2	78.26	22.82	1.37	102.45
EXP3	61.24	22.02	1.58	84.84
EXP4	65.77	27.84	1.21	94.82
EXP5	67.49	22.19	1.26	90.94
EXP6	68.05	16.09	0.98	85.13
EXP7	65.75	20.70	1.31	87.75
EXP8	58.45	29.17	2.07	89.69

Table 3. HPLC characterization of water-soluble components derived from acid-impregnated, steam-exploded *P. taeda* chips. Figures are expressed in relation to 100 g of oven-dry chips.

	Glc	Xyl, Man, Gal (polyose sugars)	Cellobiose	Ara	Furfural	Acetic acid	Formic acid
EXP1	2.82	10.17	1.76	1.41	0.13	0.67	0.90
EXP2	4.64	11.92	1.97	1.56	0.20	1.32	1.22
EXP3	6.57	9.89	1.37	1.42	0.37	1.33	1.08
EXP4	9.21	11.00	1.59	1.54	0.41	2.53	1.57
EXP5	3.72	12.34	1.72	1.50	0.41	1.25	1.25
EXP6	2.91	8.89	0.87	1.08	0.34	1.14	0.86
EXP7	4.66	10.60	1.63	1.40	0.24	1.07	1.10
EXP8	8.72	12.37	1.86	1.66	0.71	2.18	1.68

Glc, glucose; Xyl, xylose, Man, mannose; Gal, galactose; Ara, arabinose

The amount of acetic acid release in the WS fraction increased with pretreatment severity, reaching nearly 2.5% in relation to chips dry weight (Table 3). Since the acetyl content of the wood chips amounted 2.4% in relation to its dry weight, pretreatment at 210°C for 5 min with 1.0% H_2SO_4–impregnated chips (EXP4; highest severity) resulted in a considerable degree of deacetylation of the hemicelluloses found in *P. taeda*. In fact, 1.05 to 1.37% of the steam-treated water-insoluble fractions derived from *P. taeda* still comprised of acetyl groups (Table 4), suggesting that the severities used in this study were not critical enough to promote complete deacetylation of pine hemicelluloses.

Increased pretreatment severities also triggered a considerable accumulation of formic acid, probably derived from lignin degradation reactions such as demethoxylation and hydrolysis and removal of side chains (such as in the case of C(in the phenyl propane units) (Tanahashi, 1990). However, the possibility of having formic acid released from carbohydrate by-products such as hydroxymethylfurfural (HMF) was not totally discarted (Ulbricht *et al.*, 1984; Fengel and Wegener, 1989), even though the pretreatment conditions applied in this study seemed to be not strong enough to justify it because no detectable accumulation of HMF was found in any of the WS fractions by HPLC. The highest level of formic acid in the WS fraction corresponded to 1.6% in relation to chips dry weight (Table 3).

Table 4. Chemical composition of *P. taeda* chips before and after acid-catalysed steam-explosion.

Pretreatment	Glucan	Polyoses	Arabinan	Acetyl	Lignin	Total
Untreated	42.07	18.64	1.81	2.36	34.59	99.46
EXP1	50.33	5.03	nd	1.19	38.58	95.14
EXP2	51.71	2.91	nd	1.14	40.39	96.15
EXP3	50.23	1.08	nd	1.05	45.91	98.28
EXP4	46.48	3.99	nd	1.11	46.45	98.03
EXP5	53.30	1.11	nd	1.23	42.06	97.70
EXP6	52.91	5.27	nd	1.30	39.63	99.11
EXP7	54.61	2.62	nd	1.37	41.90	100.50
EXP8	50.54	nd	nd	nd	48.82	99.36

nd = not detected

Release of lignin-derived chromophores was monitored in the WS fraction by UV spectroscopy at 280 nm (Table 2). To avoid interference of WS dehydration by-products (mostly furfural), the amount of furfural determined by HPLC was deduced from the absorption pattern of the WS fractions on the basis of its molar

absortivity. This allowed for the observation that 1-2% of the chips dry weight were recovered as WS chromophores that were presumably aromatic in nature. However, there was no clear trend between pretreatment severity and the yields of WS chromophores for the conditions tested in this study.

Acid-catalyzed steam explosion had a major influence on the chemical composition of the *P. taeda* wood chips (Table 4). The hemicellulose content (polyoses) of the wood chips decreased from 18.6% to less than 2% when 0.5% H_2SO_4-impregnated wood chips were steam-treated for residence times of 5 min (EXP3 and EXP5). However, there was no clear explanation why EXP4 and EXP6 resulted in fibers with higher hemicellulose contents. Nevertheless, it is widely accepted that the higher the pretreatment severity, the lower the hemicellulose content left within the steam-exploded fibers. One possible explanation for our preliminary results is that the HPLC analysis carried out in this study was not appropriate for softwood hemicelluloses and that the xylose peak, quantified by external calibration, was actually a combination of more than one monosaccharide commonly found in these type of hydrolysates. We are currently analyzing these sugar moieties by capillary gas chromatography of their corresponding alditol acetates in order to clarify this point.

It is interesting to observe that arabinose could not be detected in any of the Klason lignin hydrolysates described in Table 4, suggesting that, for all pretreatment conditions tested in this study, the arabinosyl side groups of pine hemicelluloses were completely hydrolyzed to soluble products. Likewise, there was a considerable amount of this monosacharide detected in the water-soluble fractions; in recovery yields ranging from 1.08 to 1.66% of the chips dry weight. It is widely accepted that arabinose is readily removed from wood by chemical means (pulping or acid hydrolysis) and that its recovery is compromised by a rather low stability in both acid and alkaline solutions. However, at the conditions described in this study, most of the arabinose found originally in the wood chips could still be recovered in solution and easily quantified after pretreatment.

Based on Table 4, the more drastic the conditions used for pretreatment, the higher the lignin content of the STP-WI fractions. On the other hand, the opposite trend was observed with regard to their cellulose content. These changes in chemical composition were basically a result of hemicellulose removal, even though other factors may have also influenced the overal mass balance including partial hydrolysis of more accessible regions of the cellulose component and lignin hydrolysis and degradation. In fact, by considering the lignin contents listed in Table 4 and the STP-WI recovery yields found in Table 2, it is easily recognizable that the guaiacyl lignin component found in pine chips suffered a considerable degradation during pretreatment and that this degradation was mostly due to hydrolytic reactions. This assumption is partially confirmed by the relatively high amount of water-soluble chromophores found in this study for each of the WS fractions analyzed by UV spectroscopy (Tanahashi, 1990). In an attempt to conclude this work, we are currently investigating the chemical composition of the alkali-soluble lignin derived from steam-exploded pine chips in order to identify possible explanations for this chemical behavior.

CONCLUSIONS

Although steam explosion greatly enhances fractionation and enzymatic hydrolysis of hardwoods and most agricultural residues tested to date, it has not yet been successfully developed for the use of softwoods. Many recent studies have indicated that higher pretreatment severities are required for optimal pretreatment of softwoods and that delignification, a must-do step towards efficient enzymatic hydrolysis of cellulose, is very complicated and hardly achievable when applied to these steam-treated materials (Clark and Mackie, 1987; Ramos *et al.*, 1992b). Our results have shown that pretreatment of pine chips, as far as fractionation, can be easily obtained at the same lower severities that are usually applied to hardwoods and agricultural residues. However, it is still not clear whether the same effect could be expected for the susceptibility of the pretreated materials to enzymatic hydrolysis. Therefore, for what bioconversion is concerned, this work still needs further investigation on enzymatic hydrolysis but the excelent data obtained on fractionation indicates that residues derived from the industrial utilization of *P. taeda* stems could be a suitable raw material for the production of fermentable sugars and lignin for further processing.

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REFERENCES

Brownell, H. H. & Saddler, J. N. (1984). Steam-explosion pretreatment for enzymatic hydrolysis. *Biotechnol. Bioeng. Symp.*, **14**, 55-68.

Clark, T. A. & Mackie, K. L. (1987). Steam explosion of the softwood *Pinus radiata* with sulphur dioxide addition. I. Process optimisation. *J. Wood Chem. Technol.*, **7**, 373-403.

Dekker, R. F. H., Karageorge, H. & Wallis, A. F. A. (1987). Pretreatment of hardwood (*Eucalyptus regnans*) sawdust by autohydrolysis explosion and its saccharification by Trichodermal cellulases. *Biocatalysis*, **1**, 45-59.

DeLong, E. A. (1981). Canadian Patent 1,096,374.

DeLong, E. A. (1983). Canadian Patent 1,141,376.

Fengel, D. & Wegener, G. (1989) *Wood - Chemistry, Ultrastruture and Reactions*; Walter de Gruyter: New York, pp. 275.

Irick, T. J., West, K., Brownell, H. H., Schwald, W. & Saddler, J. N. (1988). Comparison of colorimetric and HPLC techniques for quantitating the carbohydrate components of steam-treated wood. *Appl. Biochem. Biotechnol.*, **17/18**, 137-149.

Kling, S. H., Carvalho Neto, C., Ferrara, M. A., Torres, J. C. R., Magalhães, D. B. & Ryu, D. D. Y. (1987). Enhancement of enzymatic hydrolysis of sugar cane bagasse by steam explosion pretreatment. *Biotechnol. Bioeng.*, **29**, 1035-1039.

Parisi, F. (1989). Advances in lignocellulosics hydrolysis and in the utilization of the hydrolysates. In: *Advances in Biochemical Engineering and Biotechnology*, ed. A. Fiechter, Springer-Verlag, **38,** pp. 53-87.

Ramos, L. P., Breuil, C., Kushner, D. N. & Saddler, J. N. (1992a). Steam pretreatment conditions for effective enzymatic hydrolysis and recovery yields of *Eucalyptus viminalis* wood chips. *Holzforschung*, **46**, 149-154.

Ramos, L. P., Breuil, C. & Saddler, J. N. (1992b). Comparison of steam pretreatment of eucalyptus, aspen, and spruce wood chips and their enzymatic hydrolysis. *Appl. Biochem. Biotechnol.*, **34/35**, 37-47.

Ramos, L. P., Mathias, A. L., Fontana, J. D. & Almeida, N. H. (1995). Development of a small pilot plant for pretreating lignocellulosic materials. *In 4th Brazilian Symposium on the Chemistry of Lignins and Other Wood Components*, Proceedings.

Ramos, L. P., Mathias, A. L., Cotrim, A. R., Silva, F. T. & Chen, C.-L. (1999). Characterization of residual lignin after SO_2-catalyzed steam explosion and enzymatic hydrolysis of *Eucalyptus viminalis* wood chips. *J. Agric. Food Chem.*, **47**, 2995-2302.

Saddler, J. N., Ramos, L. P. & Breuil, C. (1993). Steam pretreatment of lignocellulosic residues. In: *Bioconversion of Forest and Agricultural Plant Wastes*, ed. Saddler, J. N., C. A. B. International, London, pp. 73-92.

Tanahashi, M. (1990). Characterization and degradation mechanisms of wood components by steam explosion and utilization of exploded wood. *Wood Res.*, **77**, 49-117.

Torget, R., Walter, P., Himmel, M. & Grohmann, K. (1991). Dilute acid pretreatment of corn residues and short rotation woody crops. *Appl. Biochem. Biotechnol.*, **28/29**, 75-86.

Ulbricht, R. J., Northup, S. J. & Thomas, L. A. (1984). A review of 5-hydroxymethylfurfural (HMF) in parenteral solutions. *Fund. Appl. Toxicol.*, **4**, 843-853.

Analysis of *Pinus oocarpa* milled wood lignin

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ABSTRACT

Pinus caribaea var. hondurensis and *Pinus oocarpa* wood grows very quickly in Brazilian cerrado (savanna). The latter specie is also interesting due to the great amount of carbohydrates it contains (80.27% of holocellulose, 59.05% of α-cellulose, and 21.22% of hemicelluloses) and an amount of Klason lignin of 25.28%. These data recommend *Pinus oocarpa* for cellulose production. In order to characterize its natural lignin, milled wood was extracted with dioxane: water (96:4, v/v) in a Soxhlet apparatus. The purified lignin shows NMR, FTIR spectra and molecular weight distribution typical of guaiacyl lignins. The CHNO elemental analysis gave 63.44, 6.33, 0.99 and 29.24%, respectively; and the methoxyl content was 13.61%. According to these results, the calculated C_9 formula was $C_9H_{9.2}O_{2.6}(OCH_3)_{0.8}$ that is very similar to that calculated for *Pinus caribaea var. hondurensis* obtained under the same conditions. On the other hand, both tropical milled softwood lignins have a considerable lesser amount of methoxyl groups than those *Pinus* specie cultivated in temperate zones (~ 15%).

Keywords: milled wood lignin, *Pinus oocarpa*

INTRODUCTION

Cellulose, hemicelulose and lignin are the three main macromolecular components of the terrestrial plant cell walls. They are found in different proportions in wood, depending on the species and even on the characteristics of the soil and climate. In a simplification, cellulose can be viewed as a skeleton, fulfilled by substances that function as stuffing, the hemicellulose. Involving both is the lignin, offering rigidity to the whole.

Lignins, after cellulose, are the most abundant material in the plant Kingdom. Besides protection against the action of microorganisms, in vascular plants, they are responsible for the mechanical resistance. Since the works of Payen in 1838, and specially after the development of pulping processes from wood initiated in 1860, different studies have been conducted in order to elucidate the chemical constitution, biosynthesis and distribution of lignins on vegetal tissues. The knowledge about their chemical structures is fundamental for the improvement of pulping and bleaching processes (Veloso *et al.*, 1993).

This current work intends to present some information about the macromolecule of lignin obtained from the wood of *Pinus oocarpa*. Starting from the extraction of the milled wood with a solution of dioxane-water (96:4, v/v), followed by purification, some analysis were carried out, like the determination of the carbon, hydrogen, nitrogen and oxygen content (CHNO elemental analysis) and other (methoxyl and carbonyl groups; phenolic and aliphatic hydroxyl). Some spectroscopic techniques were used, like Fourier transform infrared (FTIR), proton (^{1}H NMR) and 13-Carbon Nuclear Magnetic Resonance (^{13}C NMR) in order to compare the purity of the isolated lignin and to acquire important information about its structure.

Completing the study about *Pinus oocarpa* wood, the content of cellulose, hemicellulose and Klason lignin was determined by alkaline and acid hydrolysis, respectively.

EXPERIMENTAL

The wood of *Pinus oocarpa* was from Sacramento, MG, and was 8 years old. With the purpose to measure its content of holocellulose (hemicellulose and α-cellulose), an extractive-free wood mass was delignified using as delignifying agents chlorine dioxide, chlorine and chlorate. The holocellulose, then, was extracted under nitrogen in two steps with 5% and 24% potassium hydroxide. The portions soluble in the alkaline were precipitated from the neutralized solutions giving hemicelluloses A and B, respectively. The final fibrous residue constitutes the α-cellulose (Fengel and Wegener 1984).

For the determination of Klason lignin, or acid insoluble lignin, it was followed the ABCP M10/71 norm.

The wood was milled in a ball mill, passed through screens, and the portion minor than 35 mesh was separated. After a period of 4 hours under extraction with a mixture of toluene: alcohol (2:1, v/v) in order to eliminate the extractives from the wood, its lignin was extracted in a Soxhlet apparatus using a solution of dioxane: water (96:4, v/v), for 12 hours.

For the purification of the lignin, the dioxane: water extract was evaporated under reduced pressure. The resulting concentrated extract was dissolved in a mixture composed by pyridine: acetic acid: water (9: 1: 4, v/v/v). With the addition of chloroform, two phases are formed to which another mixture composed by pyridine: acetic acid: water: chloroform (9: 1: 4: 18, v/v/v/v) was poured. The organic phase was separated again and submitted to evaporation under reduced pressure. The resulting extract was dissolved in 1,2-dichloroethane: ethanol (2: 1, v/v). The lignin was precipitated from this last solution by addition of ether and separated by centrifugation. The lignin, then, was dried at 70 °C for 12 hours (Lundquist 1992).

After purifying the lignin according to the procedure described above, a FTIR spectrum was obtained. Some part of the sample was submitted to reaction with acetic anhydride-pyridine reagent in order to acetylate the lignin. Then, the ^{1}H NMR and ^{13}C NMR spectrums were acquired as well as the CHNO analysis. Another part of the sample was submitted to reaction with hydroxylamine hydrochloride and its CHNO analysis carried out (Chen 1992).

Methoxyl content of non-derivatized lignin was determined using the modified Vieböck and Schwappach procedure (Chen 1992).

For the GPC analysis, a solution of the acetylated sample (1 mg/mL of THF) was injected in a WATERS ASSOCIATES HPSEC chromatograph, equipped with three PL-gel columns (10 μm; 30 cm length; 7.8 "i.d."; 500 Å + 10^3 Å + 10^4 Å) connected in series to an HPLC pump (WATERS M45), in order of increasing porosity. Detection was carried out at 254 nm with an ultraviolet detector (MOD. 440/010 – WATERS ASSOCIATES). The columns were calibrated using polysterene standards in the range from 106 to 450,000 Da. The flow rate of THF (HPLC-grade) was 1 mL/min and the sample loading was 1.0 mg/mL and 1.5 mg/mL. The data was processed in a WATERS ASSOCIATES DATA MODULE.

RESULTS AND DISCUSSION

Pinus oocarpa wood has a great amount of carbohydrates. 59.05% of its mass is compound by α-cellulose, while 21.22% of its composition is due to the hemicelluloses A and B. The Klason lignin content is about 25.18%. These data indicate that the wood of *Pinus oocarpa* would be a good raw material for the cellulose production.

The sum of the values of polysaccharides and Klason lignin content is over 100%. An ideal delignification should result in a total removal of lignin without chemical attack on the polysaccharides. However, as the literature says, there is no delignification procedure that can satisfy this requirement. So, the methods used for isolation of polysaccharides might have retained a residual lignin, increasing the values of polysaccharides content of the wood.

The yield in the extraction of MWL from *Pinus oocarpa* wood, according to the procedure described, was 0.54% (crude MWL). For the purified lignin, the yield reached was 0,24%.

In the Table 1 is presented the values for the CHNO analysis done for the purified lignin (MWL), for the acetylated lignin (MWL_A) and for the lignin after reaction with hydroxylamine hydrochloride (MWL_H).

Table 1. CHNO analysis for the MWL, MWL_A and MWL_H of *Pinus oocarpa*.

	Content (%)		
	MWL	MWL_A	MWL_H
C	63.44	62.11	57.23
H	6.33	5.47	6.07
N	0.99	0.50	1.80
O	29.24	31.92	34.90

The modified Vieböck and Schwappach procedure indicated for *Pinus oocarpa* MWL a value of 13.61% for methoxyl content. According to this result and to the CHNO analysis, the minimum formula for the lignin, based on the arylpropanoid unit (C_9), is $C_9H_{9.2}O_{2.6}(OCH_3)_{0.8}$. From equations presented in the literature

(Morais 1992), and using those data expressed in table 1, 100 arylpropanoid units have 11 carbonyl groups and 93 total hydroxyl groups (phenolic and aliphatic). Another important information about *Pinus oocarpa* MWL was obtained from its acetylated lignin ^{1}H NMR spectrum, which is showed in Figure 1. The chemical shift (δ) assignments are listed in Table 2.

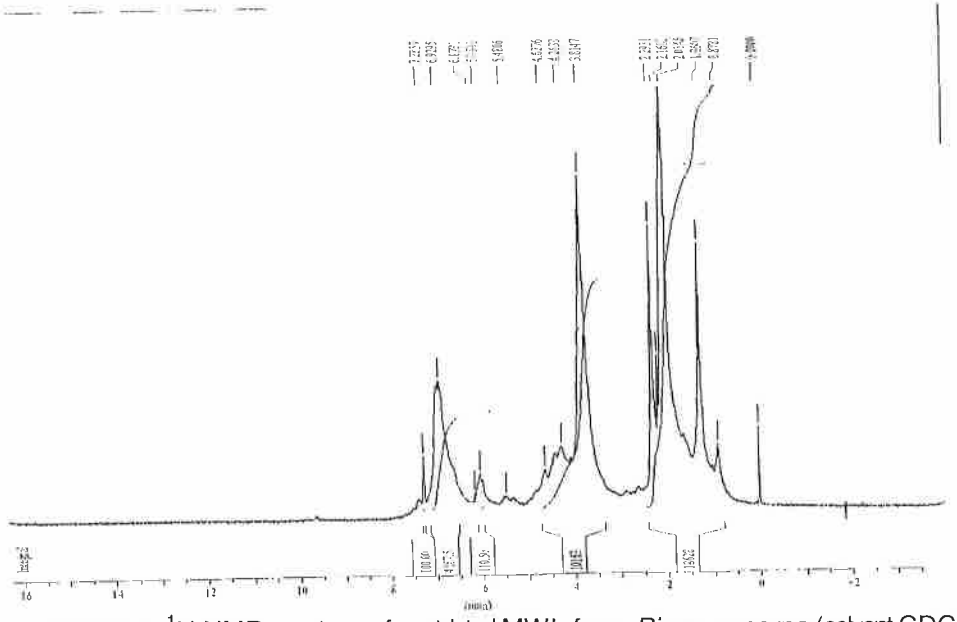

Figure 1. ^{1}H NMR spectrum of acetylated MWL from *Pinus oocarpa* (solvent $CDCl_3$).

Table 2. Assignment of signals in the ^{1}H NMR spectrum of acetylated *Pinus oocarpa* lignin.

δ (ppm)	Assignment
0.88; 1.26	Aliphatic hydrocarbon contaminant
2.01	Aliphatic acetate
2.16; 2.29	Aromatic acetate
3.81	Protons in methoxyl groups
4.26	H_γ in several structures
4.63	H_β in β-O-4 structures (methylene protons in cinnamyl alcohol units)
5.48	H_α in β-5 structures (H_α in noncyclic benzyl aryl ethers, H_β in 2-arylpropiophenones)
6.05	H_α in β-O-4 structures (H_α in β-1 structures, H_β in cinnamyl alcohol
6.18	units
6.93	Aromatic protons (from guaiacyl units)
7.29	Chloroform (solvent)

An important information that can be obtained from the Figure 1 concerns to the hydroxyl groups. The signals at 2.01, 2.16 and 2.29 ppm are due to hydrogen in the $OCOCH_3$, and then, related to the aliphatic and phenolic hydroxyl in MWL macromolecule. The intensities of these signals indicate a molar quantity for the aliphatic OH of 0.65, and for phenolic OH of 0.28.

The ^{1}H NMR spectrum shows that the chemical shift of hydrogen from methoxyl groups (3.81 ppm) is relatively intense when compared to the other types of protons. In addition, a great part of the hydrogen signals are due to aromatic protons in guaiacyl units. The hydrogen atoms whose abundance is relatively small are those from the aliphatic side chain, like H_α and cinnamyl hydrogen.

A ^{13}C NMR spectrum of acetylated MWL from *Pinus oocarpa* is presented in the Figure 2. The chemical shift (δ) assignments are listed in the Table 3.

The ^{13}C NMR spectrum also permits a general view at the lignin macromolecule of *Pinus oocarpa.* A prominent characteristic of this macromolecule that arises from the ^{13}C NMR spectrum is the presence of many signals due to guaiacyl carbons. The signal due to methoxyl groups was more intense, indicating a relative abundance of these structures.

At last, the FTIR spectrum of MWL from *Pinus oocarpa* is presented in the Figure 3 and the band assignments are listed in the Table 4.

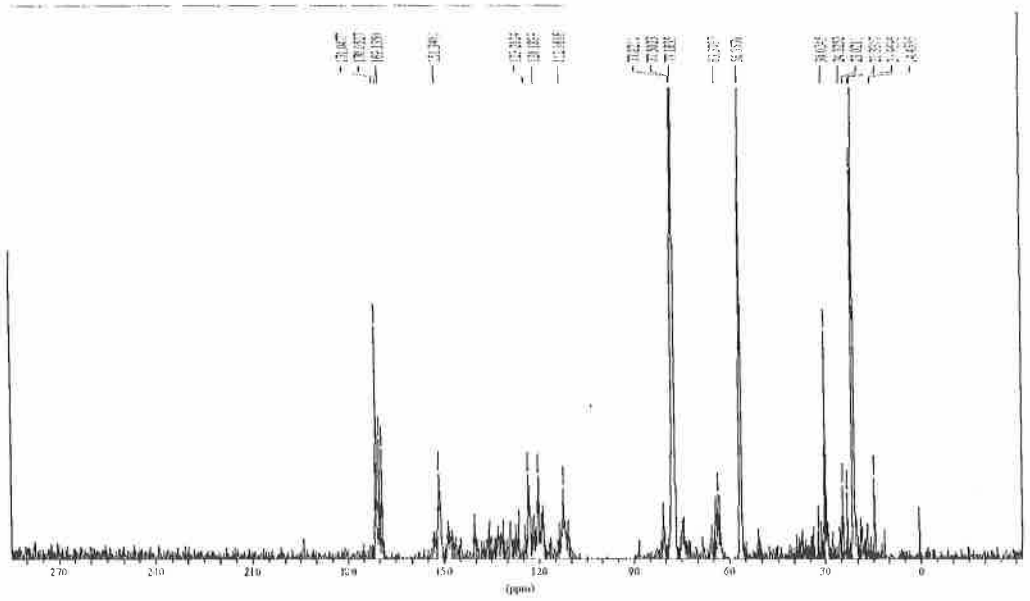

Figure 2. ^{13}C NMR spectrum of acetylated MWL from *Pinus oocarpa* (solvent $CDCl_3$).

Table 3. Signals assignment in the ^{13}C NMR spectrum of acetylated MWL from *Pinus oocarpa*.

δ (ppm/TMS)	Assignment
20.96 to 30.02	$-CH_3$ and CH_2 in saturated aliphatic chain
56.36	OCH_3
63.38	C_γ in β-O-4 and β-5 units
77.82;77.50;77.18	solvent
112.16	C_2 in guaiacyl and C_2 in acetylated guaiacyl (G-CH(OAc)-)
120.19	C_5 β-aryl ethers, etherified (*erythro*-form); C_6 G-CH(OAc)- β-aryl ethers
123.21	C_5 in acetylated guaiacyl unit (C_α-OAc and –OR)
151.39	C_3 in acetylated guaiacyl unit (C_α-OAc)
	C_4 in guaiacyl unit (C_α-OR and –OAc)
169.13	CO in Aromatic acetyl
170.05	CO in secondary aliphatic acetyl
171.05	CO in primary aliphatic acetyl

G = guaiacyl ; Ac = acetyl

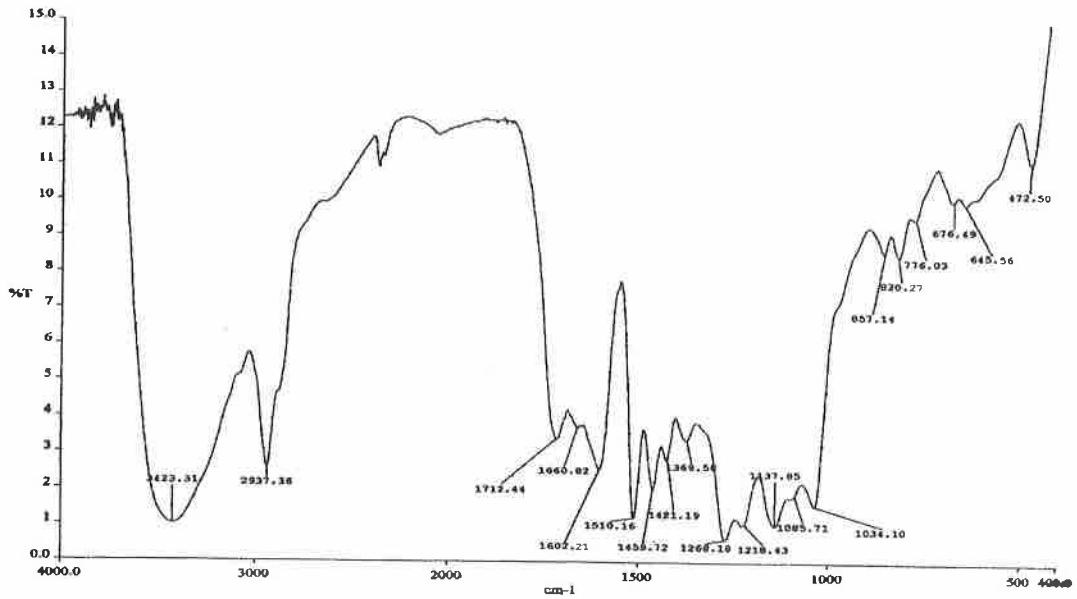

Figure 3. FTIR spectrum of MWL from *Pinus oocarpa*.

Table 4. Band assignments in the mid-infrared region of MWL from *Pinus oocarpa*.

Wavenumbers (cm^{-1})	Band origin
3423.36	O-H stretch
2937.38	C-H stretch in methyl and methylene groups
1715.00	C=O stretch in unconjugated ketones, carbonyls and in ester groups
1602.15; 1510.15	aromatic skeletal vibrations plus C=O stretch
1459.71	C-H deformation; asym. in $-CH_3$ and $-CH_2$
1268.17	C-O stretch (attached to a guaiacyl ring)
1137.80	aromatic C-H in-plane deformation; typical for guaiacyl units.

The Table 5 summarizes some important information about the macromolecule of MWL from *Pinus oocarpa* and presents the molecular weight of the acetylated milled wood lignin, obtained by GPC analysis.

Table 5. Minimum formula and important groups encountered for the MWL from *Pinus oocarpa*.

Minimum formula (C_9 unit)	$C_9H_{9.2}O_{2.6}(OCH_3)_{0.8}$
MW/C_9	183.6
C=O/C_9	0.11
OH (total)/C_9	0.93
OH (aliphatic)/C_9	0.65
OH (phenolic)/C_9	0.28
MW (Da)	3969

CONCLUSIONS

The analysis of *Pinus oocarpa* milled wood lignin confirmed that it is a typical softwood guaiacyl lignin.

Acknowledgments. FAPEMIG, UFU

REFERENCES

Chen, C. L. (1992). Determination of methoxyl groups. In *Methods in Lignin Chemistry*, ed. S. Y. Lin & C. W. Dence. Springer-Verlag, Berlin, pp. 465-472.

Chen, C. L. (1992). Determination of total and aliphatic hydroxyl groups. In *Methods in Lignin Chemistry*, ed. S. Y. Lin & C. W. Dence. Springer-Verlag, Berlin, pp. 409-422.

Fengel, D. & Wegener, G. (1984). Chemical composition and analysis of wood. In *Wood – Chemistry, Ultrastructure, Reactions,* ed. Walter de Gruyter, Berlin.

Lundquist, K. (1992). Isolation and Purification. In *Methods in Lignin Chemistry*, ed. S. Y. Lin & C. W. Dence. Springer-Verlag, Berlin, pp. 65-69.

Morais, S. A. L. (1992). Contribuição ao Estudo Químico e Espectroscópico da Lignina de Madeira Moída do *Euca.lyptus grandis*: Isolamento, quantificação e análise estrutural. PhD thesis, Departamento de Química , Universidade Federal de Minas Gerais.

Piló-Veloso, D., Nascimento, E. A. & Morais, S. A. L (1993). Isolamento e análise estrutural de ligninas. *Química Nova*, **16**, 435-448.

Autohydrolysis and acid-catalyzed steam explosion of sugarcane bagasse

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ABSTRACT

Steam explosion of sugarcane bagasse was carried out under both non-catalyzed (autohydrolysis) and acid-catalyzed (phosphoric acid, H_3PO_4) pretreatment conditions. Autohydrolysis was performed at 195 and 210°C for 2-4 min into the steam gun. Acid-catalyzed steam explosion was carried out at 195°C for 2-4 min after impregnation of sugarcane bagasse with dilute H_3PO_4 (0.02 and 0.1M). Autohydrolysis at 195°C for 4 min allowed for nearly 70% recovery of hemicellulose (mostly as xylose and xylo-oligomers) in the water soluble (WS) fraction. In addition, less than 4% of the cellulose component was hydrolyzed to glucose and/or its oligomers of lower molecular mass. Little dehydration by-products were detected in the WS fraction by high performance liquid chromatography. The overall mass balance of pretreated fractions for autohydrolysis at 195°C for 4 min revealed a recovery of 82% in relation to the dry weight of the starting material, which corresponded to yields of 15.2% in the water-soluble and 66.8% in water-insoluble fractions. Therefore, nearly 18% of bagasse was lost as volatiles and other inidentified compounds. The utilization of phosphoric acid improved pretreatment efficiency by recovering the entirety of the hemicellulose-derived sugars in their monomeric form. The highest recovery of oven-dried (45-50°C) alkali-soluble lignin was obtained from bagasse fibers that were pretreated at 210°C for 4 min and this corresponded to an yield of 17.8% in relation to the unextracted dry matter.

Keywords: sugar cane bagasse, acid hydrolysis, steam explosion

INTRODUCTION

The production of sugarcane in Brazil has expanded quite substantially during the last few years, rising from $240x10^6$ tons in the 1994/95-harvest season to nearly $300x10^6$ tons in 1997/98. According to recent surveys, this massive production corresponds to 25% of all sugarcane produced worldwide. A simple mass balance based on 1 ton of harvested sugarcane stalks points to a yield of 750 L of raw sugarcane juice and 250 kg of wet bagasse containing nearly 50% of its weight in moisture. In average, 125 kg of dry bagasse contain 53.8 kg of cellulose (43%), 31.3 kg of hemicellulose (25%) and 28.8 kg of lignin (23%), plus 4 kg of acetic acid (3% of acetyl groups in heteroxylans), 6 kg of extractives including residual sucrose (4%) and 3 kg of ashes (2%) (Silva, 1995). Therefore, considering that the amount of fermentable sugars found in bagasse could be recovered at a 90% efficiency after a two-stage acid treatment and/or enzymatic hydrolysis, the theoretical yield of fermentable sugars from whole sugar cane (juice plus bagasse) could be increased by nearly 50% if bagasse is converted to commodity chemicals such as ethanol and other biofuels. Nevertheless, most if not all bagasse produced in Brazil is used for purposes other than ethanol production (*e.g.*, cattle feeding and power co-generation) and this states to a need for the development of more noble uses for our most important secondary biomass feedstock.

Pretreatment of lignocellulosic materials is essential for their effective fractionation and enzymatic hydrolysis because of the various physical and chemical barriers that greatly inhibit the bioconversion of wood and non-woody fibers. One pretreatment method that several workers have used to enhance the bioconversion of lignocellulosics is high pressure steaming, with or without rapid decompression (explosion) (Brownell and Saddler, 1984; Morjanoff and Gray, 1987; Ramos *et al.*, 1992).

Steam explosion is a process whereby biomass is treated with high pressure steam under optimal conditions and subsequently quenched to atmospheric by adiabatic expansion of the reactor content. Optimal conditions are defined as those in which the best substrate for hydrolysis is produced with the least material loss due to decomposition reactions (Ramos *et al.*, 1992). Several advantages have been associated with steam explosion, such as the the effective fractionation of wood into its three main components (cellulose, hemicellulose and lignin) and the theoretical yields of cellulose conversion that can be achieved through

enzymatic hydrolysis (Saddler *et al.*, 1993). The major disadvantage is that, although steam explosion greatly enhances the enzymatic hydrolysis of hardwoods (Brownell and Saddler, 1984; Ramos *et al.*, 1992) and most agricultural residues (Dekker and Wallis, 1983; Kling *et al.*, 1987; Morjanoff and Gray, 1987; Fontana *et al.*, 1995; Deschamps *et al.*, 1996) tested to date, it has not yet been successfully developed for the use of softwoods. Furthermore, steam explosion produces a relatively low bulk density substrate, which has to be washed to remove fermentation inhibitors (mostly phenols and dehydration by-products derived from carbohydrates).

The aim of this study was to investigate the susceptibility of sugarcane bagasse to acid-catalyzed steam explosion while introducing dilute phosphoric acid as an acid catalyst for the effective fractionation of its three major components, cellulose, hemicellulose and lignin. For this purpose, the best pretreatment conditions were defined as those in which the best lignin yield was obtained through mild alkali extraction, with the greatest recovery of hemicellulose sugars in the water soluble fraction.

EXPERIMENTAL

Pretreatment

Sugarcane bagasse with typical moisture content of 10% (m/m) was steam-treated at the Pilot Plant Unit of the Federal University of Paraná using a 5L steam gun (Ramos *et al.*, 1995). Bagasse was impregnated with dilute phosphoric acid (H_3PO_4) and pretreated at saturated steam temperatures ranging from 190 to 210°C for a range of residence times (2-4 min) (Table 1). Acid impregnation was carried out by spraying the air-dried cane bagasse (5-8% moisture content) with 1 L of either 0.02 or 0.1 mol/L H_3PO_4 at room temperature (RT). Acid-impregnated fibers were shown to have approximately 50% (w/w) moisture content.

Table 1. Pretreatment conditions.

Conditions	Bagasse (g, dry basis)	Temperature (ºC)	Residence time (min)	[H_3PO_4] (mol/L)
1	273.0	195	2	--
2	307.9	195	4	--
3	341.6	210	2	--
4	291.2	210	4	--
5	266.1	195	2	0.02
6	253.9	195	4	0.02
7	272.3	195	2	0.1
8	273.6	195	4	0.1

Steam pretreatment was performed by loading the bagasse samples directly into the steam gun. After treatment for a given temperature and residence time, the steam treated bagasse (STB) was released from the steam-gun by rapid despressurization of the vessel, causing the material to expand (explode) into a stainless steel cyclone. Substrates were then recovered by water washing. The resulting STB was extracted twice with water at a 5% (w/w) solids concentration. The water insoluble fraction was termed WI, whereas the water soluble fraction or liquor was termed WS.

Chemical analysis

Chemical analysis of substrates and raw materials were performed by a modification of the TAPPI Standard Method T222 os-74 for Klason lignin (acid insoluble lignin). After swelling 1 g of milled bagasse (40 mesh) in 15 mL of 72% H_2SO_4 (w/w) for 2 h at 20 ± 1°C, distilled water was added until a 3% (w/w) acid concentration was reached and the suspension was treated for 70 min in an autoclave at 121°C. Acid-insoluble lignin was measured gravimetrically and total carbohydrates were determined by HPLC (Irick *et al.*, 1988). Acid-soluble lignin was not determined in this study.

Pentosan hydrolysis occurring during steam treatment was determined by assaying the WS fraction by HPLC (Irick *et al.*, 1988). Recovery yields of the STB-WS fractions were expressed in terms of g/100 g of oven-dry bagasse. The HPLC analyses were obtained with a Shimadzu HPLC system, model LC10AD, provided with a SIL10A autosampler and two detectors arranged in series, model SPD10A for UV spectroscopy and model RID10A for refractive index. The HPLC analysis of water-solubles and Klason hydrolysates was performed at 65°C in an HPX-87H column (Bio-Rad), eluted with 8 mM H_2SO_4 at a flow rate of 0.6 mL/min.

Hydrolysis factors were included in all calculations to account for carbohydrate destruction during acid hydrolysis (1.055 for hexoses and 1.155 for pentoses).

For pretreatments carried out by autohydrolysis (that is, without addition of an acid catalyst), a post-pretreatment hydrolysis step was included prior to HPLC analysis of the WS fraction to reduce the occurrence of hardly detectable water-soluble oligosaccharides. This additional hydrolysis step was carried out for 2 h at 105 ± 1°C after mixing 1 mL of the water-soluble fraction with 1 mL of 2 M H_2SO_4. Neutralization was not required in neither of the HPLC analysis carried out in this study.

RESULTS AND DISCUSSION

Steam explosion of sugarcane bagasse was carried out under both non-catalyzed (autohydrolysis) and acid-catalyzed (phosphoric acid, H_3PO_4) pretreatment conditions. Autohydrolysis was performed at 195 and 210°C for 2-4 min into the steam gun. Acid-catalyzed steam explosion was carried out at 195°C for 2-4 min after impregnation of sugarcane bagasse with dilute H_3PO_4 (0.02 and 0.1 M). Autohydrolysis at 195°C for 4 min allowed for an almost complete recovery of hemicellulose (mostly as xylose and related oligomers) in the water soluble (WS) fraction (Table 2). In addition, less than 4% of the cellulose component was hydrolyzed to glucose and/or lower cello-oligomers and very little dehydration by-products were detected in the WS fraction by HPLC (Table 3). The overall mass balance of pretreated fractions revealed a recovery of 83.9% in relation to the dry weight of the starting material, which corresponded to yields of 67.1% in water-insoluble fibers, 15.2% in water-solubles and 18% in mass lost as volatiles and/or other unidentified compounds.

Table 2. Mass balance and yields obtained after steam pretreatment.

Condition*	WS (g)	WS (%)	WI (g)	WI (%)	TOTAL (%)
1	23.02	8.4	172.8	63.3	71.7
2	46.79	15.2	204.8	66.5	81.7
3	39.05	11.4	218.7	64.0	75.4
4	48.68	16.7	195.5	67.1	83.9
5	20.91	7.9	172.2	64.7	72.5
6	23.95	9.4	183.5	72.3	81.7
7	32.74	12.0	166.6	61.2	73.2
8	40.81	14.9	153.7	56.2	71.1

* For pretreatment conditions, see Table 1.

Table 3. Total recovery of water-soluble components derived from steam-exploded sugarcane bagasse, as determined by HPLC analysis. The WS fraction derived autohydrolysis had to be post-hydrolyzed with dilute sulfuric to reduce the incidence of hardly detectable oligosaccharides.

Component (g)	Experiments*							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Glucose	0.86	4.07	3.77	4.33	1.99	1.85	3.33	3.64
Xylose	15.63	31.12	24.64	25.87	13.73	15.87	20.07	23.24
Arabinose	1.37	2.47	1.53	1.93	1.70	1.75	1.83	3.91
Acetic Acid	3.89	6.00	5.19	9.43	2.76	3.53	4.53	5.50
Formic Acid	0.99	1.91	1.91	3.64	0.38	0.60	0.56	0.81
Furfural	nd	1.01	1.57	3.01	nd	nd	2.14	3.21
Total	22.93	46.76	38.97	48.58	20.84	23.89	32.67	40.74

* For pretreatment conditions, see Table 1; nd = not detected

Interestingly, addition of phosphoric acid as a pretreatment catalyst did not improve the pretreatment profile of sugarcane bagasse. There was no substantial change in the overall recovery yield of pretreatment fractions which, in many cases, was lower than those obtained previously by autohydrolysis. However, the amount of WS oligosaccharides decreased with a concomitant increase in WS monosaccharides, thus avoiding the requirement for a further post-hydrolysis step of the WS after pretreatment. In this case, pretreatment at

195°C for 4 min of bagasse pre-impregnated with 0.1 M H_3PO_4 could be considered of technical interest. On the other hand, bagasse was impregnated with a fixed amount of dilute H_3PO_4 and this, of course, increased the moisture content of the raw material prior to pretreatment. As a result, the actual residence time at the reaction temperature might have been shorter than indicated. We are currently conducting a better control for these experiments, in which bagasse samples with the same moisture content have been applied for both autohydrolysis and acid-catalyzed steam treatments.

Hemicellulose recovery in the water soluble (WS) fraction is an important indicator for pretreatment efficiency and this value can be easily derived from Table 3 when the original mass of bagasse is considered in relation to its 15% content in arabinoxylan (4-*O*-methylglucuronic acid, acetic acid and other minor components not included). A typical mass balance based on both xylose and arabinose recovery in the WS fraction indicates that pretreatment at longer residence times of 4 min increased hemicellulose recovery in all cases.

The highest recovery obtained in steam explosion was associated with pretreatment at 195°C for 4 min (autohydrolysis #2) but, due to the absence of an exogenous acid catalyst, most of these sugars were found in the oligomeric form. On the other hand, pretreatment with phosphoric acid as the acid catalyst resulted in nearly 66% recovery of hemicellulose sugars (pretreatment #8), indeed a lower value but with the advantage of having all of the WS carbohydrates present in their monomeric form.

The amount of acetic acid release in the WS fraction increased with pretreatment severity, reaching nearly 3.2% in relation to bagasse dry weight (Table 3, experiment 4). Since the acetyl groups account for 3% of the bagasse dry weight (Silva, 1995), autohydrolysis at 210°C for 4 min (highest severity) resulted in a complete deacetylation of the hemicelluloses found in sugarcane bagasse. This result was in good agreement with the chemical composition of the fibers derived from this pretreatment condition, in which no acetyl groups were found as acetic acid by HPLC (Table 4). In any case, the acetic acid derived from the even complete deacetylation of hemicelluloses was not the only acid catalyst available for the autohydrolysis of cane bagasse (see below).

Klason lignin of steam-treated, water-insoluble (WI) bagasse varied according to pretreatment severity and, for the best autohydrolysis conditions shown above, the WI material contained 25.4% of steam-exploded lignin (Table 4). The concentration of furfural in the WS fraction increased with pretreatment severity, whereas hydroxymethylfurfural was never found in detectable amounts in any of the pretreatment hydrolysates.

Considering the amount of Klason lignin found in the starting material (23.1 ± 0.51%), the Klason lignin contents showed in Table 4 and the yields of water-insoluble fibers obtained for each pretreatment conditions (Table 1), there was an increase in the amount of lignin found within the steam-treated fibers when pretreatment was carried out for longer residence times. On the other hand, the utilization of phosphoric acid as a pretreatment catalyst decreased the amount of lignin that remained attached to the fibers and this could be attributed to the prevalence of hydrolytic reactions over condensation reactions.

Table 4. Chemical composition of steam-treated water-insoluble fibers.

Component (%)	Experiments*							
	1	2	3	4	5	6	7	8
Glucan	51.7	49.8	48.6	49.2	51.4	51.3	56.4	55.0
Xylan	12.9	14.0	10.4	6.8	17.4	11.6	10.2	6.3
Acetic Acid	0.7	0.8	0.2	nd	1.3	0.2	0.7	nd
Lignin	27.3	28.6	29.4	32.3	24.3	25.1	30.3	35.7
Total	92.6	93.2	88.6	88.3	94.4	88.1	97.6	97.1

* For pretreatment conditions, see Table 1; nd = not detected

The small yet considerable amount of formic acid found in the WS fractions can be an indication of two possible phenomena: lignin hydrolysis and carbohydrate degradation. Lignin hydrolysis yields formic acid through demethoxylation and hydrolysis and removal of side chains (such as in the case of C in the phenyl propane units), whereas this same acid can be produced from carbohydrates after they are dehydrated and/or

oxidized to by-products such as hydroxymethylfurfural and levulinic acid. In any case, the use of phosphoric acid decreased formic acid formation and, due to the apparent prevalence of hydrolytic reactions over condensation reactions at these pretreatment conditions, it is likely that this formic acid was arisen from carbohydrate degradation by-products.

The lignin component found in each WI fraction was then extracted with mild alkali (0.1 M NaOH, 1 h with stirring at room temperature) and recovered by centrifugation after acidification of the alkali extract with dilute H_2SO_4 (pH 2). FTIR spectroscopy allowed a direct observation of the chemical changes that were impaired to the raw material, particularly after alkali washing (data not shown). The highest recovery of oven-dried (45-50°C) alkali-soluble lignin was obtained from bagasse fibers that were pretreated at 210°C for 4 min and this corresponded to an yield of 17.8% in relation to the unextracted dry matter (Table 5). Therefore, pretreatment optimization will depend upon what is the most preferable by-product: lignin or hemicellulose-derived fermentable sugars.

Table 5. Recovery of alkali-soluble lignin from steam-treated bagasse.

Conditions	Temperature (°C)	Residence time (min)	$[H_3PO_4]$ (mol/L)	Lignin (%)
1	195	2	--	10.2
2	195	4	--	14.8
3	210	2	--	13.4
4	210	4	--	18.2
5	195	2	0.02	5.6
6	195	4	0.02	7.6
7	195	2	0.1	8.0
8	195	4	0.1	13.6

CONCLUSIONS

Pretreatment of sugarcane bagasse with phosphoric acid has been previously applied to the preparation of readily accessible substrates for cattle feeding (Fontana *et al.*, 1995; Deschamps *et al.*, 1996). Likewise, autohydrolysis and acid-catalyzed steam explosion (Dekker and Wallis, 1983; Morjanoff and Gray, 1987; Kling *et al.*, 1987) have been largely exploited to increase fractionation of sugarcane bagasse and the subsequent enzymatic hydrolysis of its cellulose component. However, there is no reference in the literature to the utilization of phosphoric acid in the steam explosion of sugarcane bagasse or any other wood or agricultural residue.

Addition of phosphoric acid as a pretreatment catalyst increased the amount of hemicellulose recovered from sugarcane bagasse as monomeric sugars (mostly xylose). At the pretreatment conditions tested in this study, impregnation with dilute phosphoric acid decreased both sugar and lignin yields in relation to autohydrolysis of air-dried bagasse. However, the actual residence time of pre-impregnated bagasse at the reaction temperature might have been shorter than indicated because bagasse picked up moisture after acid-impregnation. On the other hand, autohydrolysis provides the best possible results when applied to raw materials with low moisture content and this has been already shown for other feedstocks such as eucalyptus wood chips (Ramos *et al.*, 1992b).

Even though the effective pretreatment of sugarcane bagasse has been already described in the absence of any added catalyst (Dekker and Wallis, 1983), the utilization of inorganic acids (such as phosphoric and sulfuric acids) generally improves pretreatment efficiency. In this regard, when compared to stronger acids such as sulfuric, the utilization of phosphoric acid decreases the amount of inhibitors released as a result of pretreatment and indirectly increases fermentation yields (Fontana *et al.*, 1995). On the other hand, neutralization of lignocellulosic hydrolysates with ammonia generates ammonium phosphate, which in turn is a valuable micronutrient for the viability of the fermentation microorganism. Therefore, the utilization of phosphoric acid can be proven effective for the pretreatment of lignocellulosics, particularly if applied in low concentrations such as those used in this study but in slightly higher residence times to compensate for the moisture added during biomass impregnation with dilute acid.

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REFERENCES

Brownell, H. H. & Saddler, J. N. (1984). Steam-explosion pretreatment for enzymatic hydrolysis. *Biotechnol. Bioeng. Symp.*, **14**, 55-68.

Dekker, R. F. H. & Wallis, A. F. A. (1983). Enzymic saccharification of sugarcane bagasse pretrated by autohydrolysis-steam explosion. *Biotechnol. Bioeng.*, **25**, 3027-3048.

Fontana, J. D., Ramos, L. P. & Deschamps, F. C. (1995). Pretreated sugarcane bagasse as a model for cattle feeding. *Appl. Biochem. Biotechnol.*, **51/52**, 105-116.

Deschamps, F.C., Ramos, L.P. & Fontana, J.D. (1996). Pretreatment of sugarcane bagasse for enhanced ruminal digestion. *Appl. Biochem. Biotechnol.*, **57/58**, 171-182.

Irick, T. J., West, K., Brownell, H. H., Schwald, W. & Saddler, J. N. (1988). Comparison of colorimetric and HPLC techniques for quantitating the carbohydrate components of steam-treated wood. *Appl. Biochem. Biotechnol.*, **17/18**, 137-149.

Kling, S. H., Carvalho Neto, C., Ferrara, M. A., Torres, J. C. R., Magalhães, D. B. & Ryu, D. D. Y. (1987). Enhancement of enzymatic hydrolysis of sugar cane bagasse by steam explosion pretreatment. *Biotechnol. Bioeng.*, **29**, 1035-1039.

Morjanoff, P. J. & Gray P. P. (1987). Optimization of steam explosion as a method for increasing susceptibility of sugarcane bagasse to enzymatic saccharification. *Biotechnol. Bioeng.*, **29**, 733-741.

Ramos, L. P., Breuil, C. & Saddler, J. N. (1992a). Comparison of steam pretreatment of eucalyptus, aspen, and spruce wood chips and their enzymatic hydrolysis. *Appl. Biochem. Biotechnol.*, **34/35**, 37-47.

Ramos, L. P., Breuil, C., Kushner, D. J. & Saddler, J. N. (1992b). Steam pretreatment conditions for effective enzymatic hydrolysis and recovery yields of *Eucalyptus viminalis* wood chips. *Holzforschung*, **46**, 149-154.

Ramos, L. P., Mathias, A. L., Fontana, J. D. & Almeida, N. H. (1995). Development of a small pilot plant for pretreating lignocellulosic materials. *In 4th Brazilian Symposium on the Chemistry of Lignins and Other Wood Components*, Proceedings.

Saddler, J. N., Ramos, L. P. & Breuil, C. (1993). Steam pretreatment of lignocellulosic residues. In: *Bioconversion of Forest and Agricultural Plant Wastes*, ed. Saddler, J. N., C. A. B. International, London, pp. 73-92.

Silva, F. T. (1995). *Obtention of chemical goods from the processing and upgrading of whole sugar cane bagasse* (in Portuguese). Ph.D. Thesis, Institute of Chemistry, University of Campinas.

Biodegradation of sugarcane bagasse by different strains of *Panus tigrinus* as a pretreatment for kraft pulping

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ABSTRACT

Sugarcane bagasse was biologically pretreated with three strains of the white-rot fungus *Panus tigrinus* (FTPT-4741, FTPT-4742 and FTPT-4745). The semi-solid fermentation was carried out at 28°C with no additional carbon source in plastic bags for 10 and 30 days using 100 g of bagasse. After fermentation for 10 and 30 days, the liquid extract was filtered in membrane and the enzymatic activities were measured, before and after dialysis, in respect to lignin-peroxidase (LiP), manganese-peroxidase (MnP), laccase (Lac) and the hydrolytic enzyme xylanase (Xyl). The action of *P. tigrinus* was evaluated by chemical analysis and component losses of the decayed and undecayed (control) bagasse samples. Kraft pulping experiments were carried out using decayed and control samples in an 85 mL stainless steel reactor at 170°C, with 15% active alkali level, 20% sulfidity, using a liquor/bagasse ratio of 4:1 for 20, 40 and 60 min. The yields of kraft pulping were low, ranging from 20 to 45%. Kappa numbers were 0.9-10 and viscosity varied from 3.1 to 5.9 cP.

Keywords: *Panus tigrinus*, biodelignification, kraft pulping, sugarcane bagasse

INTRODUCTION

Lignocellulosics are the most abundant material on earth, comprising 50% of all biomass and with an estimated annual production of $50x10^9$ ton (Smith, 1996). Sugarcane bagasse is a type of lignocellulosic residue with a complex structure that can be efficiently decayed, specially by white-rot fungi (Zadrazil and Puniya 1995; Esposito *et al.*, 1995, 1996, Gao *et al.*, 1996; Breccia *et al.*, 1997). The production of sugarcane bagasse in Brazil has increased during the last decades and the integral use of its components is economically advantageous.

An alternative to use all those componentes is the biopulping, that is the fungal pretreatment of lignocellulosic materials, designed as a solid-state fermentation process, for production of mechanical or chemical pulps (Ferraz *et al.*, 1998).

White-rot fungi are able to break up the major structural polymers of wood and other lignocellulosics: lignin, cellulose, and hemicelluloses (Kirk e Cullen, 1998). The action of the white-rot fungi is focussed on the degradation of lignin. Because the macromolecule is large, ligninolytic systems have to be extracellular and have a degradative mechanism. They also need to be oxidative rather than hydrolytic, and because the polymer is stereoirregular, the ligninolytic agents have to be much less specific than other degradative enzymes. These challenges have resulted from the evolution of white-rot fungi, whose extracelular peroxidase and oxidase act nonspecifically via the generation of lignin-free radicals, which are unstable and undergo a variety of spontaneous cleavage reactions. The main enzymes acting directly or indirectly on lignin are lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac).

The introducton of these natural process into the pulp and paper industry as a pre-treatment previous to the pulping has a high economic potential and is harmless to the environment.

EXPERIMENTAL

Fungi

Three strains of *Panus tigrinus* (FTPT-4741, FTPT-4742, FTPT-4745) from a collection belonging to Fundação Tropical de Pesquisa e Tecnologia André Tosello (Campinas, SP, Brazil) were used in this study. These strains, which grown from a vegetative inoculum on malt extract agar (4%) at 28°C for five days, had been previously screened by Esposito *et al.* (1996) and Gonçalves *et al.* (1998).

Culture conditions

Semi-solid state fermentation cultures were grown in plastic bags at 28°C during 30 days. Samples of 100 g of depithed and sterilized sugarcane bagasse were supplemented only with 17 mL salts solution and 833 mL distilled water (see Figure 1). No additional carbon source was included.

Ligninolytic and Hydrolytic Enzymes

After fermentation, the extracellular enzymes present in the liquid extract were filtered through Millipore membranes (0.47 μm) and the activities of lignin peroxidase (LiP), manganese peroxidase (MnP), laccase (Lac) and xylanase (Xyl) were measured by standard methods, before and after dialysis (Tien and Kirk 1984; Kuwahara *et al.*, 1994 and Szklarz *et al.*, 1989; Bailey *et al.*, 1992).

Dialysis

The dialysis was performed with cellulosic membrane of cutoff 12 kDa and with acetate of sodium buffer at pH = 5.5.

Kraft pulping

The kraft pulping of the control sample and pretreated samples of sugar

Cane bagasse was performed in an 85-mL stainless steel autoclave under the following conditions: active alkali 15%; sulfidity 20%; liquor: bagasse ratio 4:1 (v:m); cooking temperature 170°C; cooking time 20, 40 and 60 min. The kappa number and viscosity of bagasse pulps were determined by TAPPI methods (TAPPI 1985).

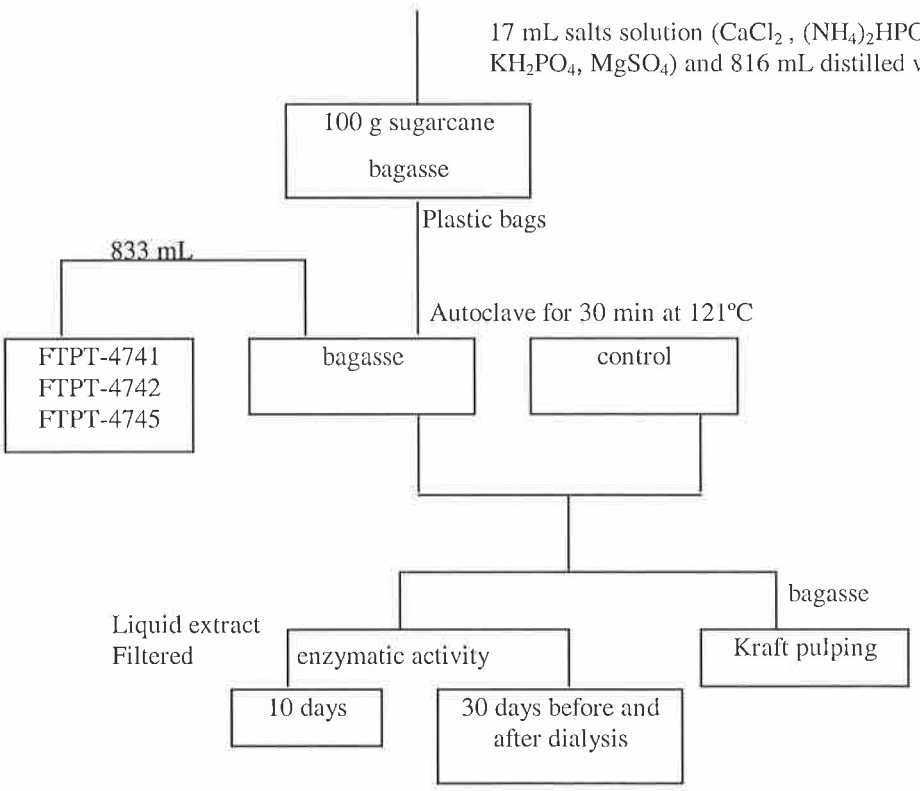

Figure 1. Scheme of the experimental procedure.

RESULTS AND DISCUSSION

Enzymatic activity

Table 1 shows the enzymatic activities of the three strains after growth in sugarcane bagasse. Three strains of *P. tigrinus* exhibited high MnP and Lac activities. MnP is an enzyme depending on H_2O_2 and Mn^{+2}. Maltseva et al. (1991) proved that MnP produced by *P. tigrinus* is able to degrade lignin models related to phenolic and non-phenolic structures. Lac is also involved in the degradation of lignin, since it totally reduces O_2 to H_2O with low specificity for electron-donor substrates. These two oxidative enzymes seem to be directly involved

in the removal of lignin, while LiP does not, since it was not detected in the strains. Xyl seems to play an important role in the degradation of xylan present in the bagasse fibers. Release of xylan gives rise to the most effective action of oxidative enzymes (MnP and Lac) on lignin.

Table 1. Enzymatic activities of *P. tigrinus* in sugarcane bagasse determined after 10 and 30 days of incubation, before and after dialysis.

strains	10 days	30 days before dialysis	30 days after dialysis
Lac (U/L)			
FTPT-4741	0.7 ± 0.2	0.2 ± 0.3	0.18 ± 0.03
FTPT-4742	0.2 ± 0.1	0.06 ± 0.04	0.16 ± 0.08
FTPT-4745	0.7 ± 0.5	0.06 ± 0.04	0.50 ± 0.05
MnP (U/L)			
FTPT-4741	28 ± 3	27 ± 5	43 ± 1
FTPT-4742	18 ± 4	33 ± 7	37 ± 3
FTPT-4745	13 ± 3	26 ± 1	28 ± 4
Xyl (U/mL)			
FTPT-4741	0.7 ± 0.6	1.4 ± 0.1	0.44 ± 0.01
FTPT-4742	2.2 ± 0.2	0.9 ± 0.3	0.54 ± 0.05
FTPT-4745	2.9 ± 0.1	1.2 ± 0.1	0.71 ± 0.20

After dialysis the enzymatic extract was concentrated and MnP and Lac activities increased. The Lac activity was 2 to 8 times higher for FTPT-4741, FTPT-4742 and FTPT-4745. As for MnP, the increase in activity was 59% smaller for FTPT-4741. Xylanase activity of the three strains decreased after dialysis, indicating that the membrane of cutoff 12 kDa may have been inadequate to concentrate xylanase in the extract. There is no information about the molecular weight of xylanase from *Panus tigrinus* but Kirk and Cullen (1998) reported that MM of endoxylanases from *Trametes reesei* are in the range of 16-75 kDa. Another possibility for this fact is the contamination of the enzymatic extract with xylan obtained from the polysaccharides, mainly at the beginning of the fermentation. This can be evaluated by comparing the Xyl activities after 10 and 30 days of incubation.

Kraft pulping

Table 2 shows the results of kraft pulping of decayed and undecayed sugarcane bagasse. The low viscosity value of samples of degraded bagasse is evident. After 40 and 60 min of pulping, the values of yields, kappa number and viscosity of bagasse kraft pulp were very low. All the pulps obtained from the decayed bagasse samples after 20 min of reaction presented a viscosity/kappa ratio higher than that of the control and a lower kappa number.

Table 2. Results of kraft pulping of treated bagasse and control in plastic bags after 20, 40 and 60 min reaction time.

Time (20 min)	yield (%)	Kappa number	residual Lignin* (%)	Viscosity (cP)	Viscosity / Kappa
CONTROL	46.9	10.2	1.5	5.9 ± 0.5	0.578
FTPT-4741	32.2	7.1	1.1	4.7 ± 0.4	0.662
FTPT-4742	33.0	7.9	1.2	5.5 ± 0.1	0.696
FTPT-4745	32.5	7.0	1.1	5.5 ± 0.1	0.786
Time (40 min)					
CONTROL	33.6	1.9	0.3	4.6 ± 0.2	2.421
FTPT-4741	30.5	5.0	0.8	3.5 ± 0.2	0.700
FTPT-4742	31.9	5.2	0.8	4.1 ± 0.2	0.788
FTPT-4745	30.0	4.0	0.6	4.3 ± 0.1	1.075
Time (60 min)					
CONTROL	30.3	0.9	0.1	3.5 ± 0.2	3.889
FTPT-4741	30.3	1.5	0.2	3.1 ± 0.4	2.067
FTPT-4742	30.6	2.0	0.3	3.2 ± 0.1	1.600
FTPT-4745	27.8	3.3	0.5	3.7 ± 0,2	1.121

* Calculate from of ratio LR = K X 0.15

CONCLUSIONS

The action of *Panus tigrinus* on the sugarcane bagasse fibers was evaluated by means of the enzymatic activity and the values of Lac and MnP were higher affter dialysis. The results show that this fungus is adequate to pretreat sugarcane bagasse, causing delignification and preserving the fibers properties, although the conditions used for kraft pulping seem to be very drastic, affecting yields, kappa number and viscosity. These conditions need to be optimized for sugarcane bagasse. The use of plastic bags to grow *P. tigrinus* strains represents an advantage/easiness in the industrial process.

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REFERENCES

Bailey, M.J., Biely, P. & Poutanen, K. (1992). Interlaboratory testing of method for assay of xylanase activity. *J. Biotechnol*, **32,** 257-270.

Breccia, J.D., Bettucci, L., Piaggio, M. & Siñeriz, F. (1997). Degradation of sugar cane bagasse by several white-rot fungi. *Acta Biotechnol*, **17**, 177-184.

Esposito, E., Speranza, M., Siñeriz, F. & Bettucci, L. (1995). Delignification of lignocellulosic materials with wood-rotting fungi. *In 4th Braz. Symp. Chem. Lignins Other Wood Compon.*, Proceendings, pp 121-125.

Esposito, E., Benar, P., Speranza, M &, Gonçalves, A.R. (1996). Growth of ligninolytic fungi on sugar cane bagasse as a pre-treatment for organosolv delignification. *In 3 th. Inter. Nonwood Fibre Pulping Papermaking Conf.*, Proceedings, pp 12-16.

Ferraz, A., Christov, L.P. & Akhtar, M. (1998). Fungal pretreatment for organosolv pulping and dissolving pulp production. *In Environmentally Friendly Technologies for the Pulp and Paper Industry*. ed. R. A. Young & M. Akhtar. John Wiley & Sons, New York , pp.421-447.

Gao, Y., Wang, S., Lin, L. & Chen, J. (1996). Studies on bagasse lignin biodegradation by white-rot fungi. *Ziran Kexueban,* **24,** 37-43.

Gonçalves, A.R., Esposito, E. & Benar, P. (1998). Evaluation of *Panus tigrinus* in the delignification of sugarcane bagasse by FTIR-PCA and pulp properties. *J. Biotechonol*, **66**, 177-185.

Kirk,T.K. & Cullen, D. (1998). Enzymology and molecular genetic off wood degradation by white-rot fungi. *In Environmentally Friendly Technologies for the Pulp and Paper Industry*. ed. R. A. Young & M. Akhtar. John Wiley & Sons, New , pp.273-301.

Kuwahara, M., Glen, J.K., Morgan, M.A. & Gold, M.H. (1994). Separation and characterization of two extracellular H_2O_2–dependent oxidases from ligninolytic culture of *Phanerochaete chrysosporium. FEBS Lett.* **169**, 247-250.

Maltseva, O.V., Niku-Paavola, M.-L, Leontievsky, A.A., Myasoedova, N.M. & Golovleva, L.A. (1991). Lygninolytic enzymes of the white rot fungus Panus tigrinus. Biotechnol. *Appl. Biochem.* **13**, 291-302.

Simith, J.F., Fermor, T.R. & Zadrazil, F. (1996). Pretreatment of lignocellulosic for Edible fungi. Treatment of lignocellulosic with white rot fungi . *Commission of European Communities*, F. Zadrazil and Reinigen, pp 3.

Szklarz, G., Antibus, R.K., Sinsabaugh, R.L. & Linkins, A E. (1989). Production of phenoloxidases and peroxidases by wood-rotting fungi. *Mycology*, **81**, 234-240.

TAPPI (1985)- Technical Association of the Pulp and Paper Industry , *TAPPI Standard Methods*, T 236 cm-85.

Tien, M. & Kirk, T.K. (1984). Lignin-degrading enzyme from *Phanerochaete chrysosporium*: purification, characterization, and catalytic properties of a unique H_2O_2–requiring oxygenase. Proceedings, Natl. Acad.Sci.USA, **Vol 81**, 2280-2284.

Zadrazil, F. & Puniya, A.K. (1995). Studies on the effect of particle size on solid-state fermentation of sugarcane bagasse into animal feed using white-rot fungi. *Biores. Technol*, **54**, 85-87.

Biolignin as a phenol alternative in the synthesis of phenol-formaldehyde resins

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ABSTRACT

The biolignin, a waste from the enzymatic hydrolysis of cellolignin was used for synthesis of lignin-containing phenol-formaldehyde resins (LPFR). This biolignin shows a fairly high reactivity and can contribute to copolycondensation with phenol and formaldehyde without extra activation. We managed to synthesize lignin-containing phenol-formaldehyde resins of resol type in which up to 50% of phenol was replaced by biolignin. The products obtained are low toxic and can be used as glues in woodworking industry.

Keywords: phenol-formaldehyde resins, lignin, enzymatic hydrolysis

INTRODUCTION

The use of cheap phenol raw materials provides an approach to cost reduction of phenol-formaldehyde resins. Lignin is of much interest in this respect since its molecules are like those of phenol-formaldehyde oligomers. Moreover, the utilization of industrial lignins is a serious ecological and economical problem. In Russia, the utilization of hydrolyzed lignin presents the most urgent question for wood industry enterprises. Any technological process of vegetable carbohydrate hydrolysis is profitable when lignin, a heavy-tonnage waste, is used as a raw material.

It is known that the low reactivity of highly condensed hydrolyzed lignin impedes its use as a chemical reagent. We have previously shown that mechanochemical activation of hydrolyzed lignin or its treatment with electrohydraulic effect allows using it successfully for the synthesis of liquid lignin-containing phenol-formaldehyde resins of resol type (Medvedeva *et al.,* 1996). Cellolignin, the lignocellulosic residue of furfural production, could be involved in this reaction only after alkaline activation under rigid conditions (Ladomerski *et al.*, 1989).

It was shown that one of advantageous pathways to making use of cellolignin is supplementary removal of glucose by hydrolysis with cellulolytic enzymes (Sinitsyn *et al.*, 1988; Vlasenko *et al.*, 1993). Thus, it may see that enzymatic saccharification of cellolignin is more effective than this one of natural cellulose-containing raw materials.

In this work, the synthesis of liquid lignin-containing phenol-formaldehyde resins of resol type using biolignin, representing a waste in cellolignin hydrolysis with an enzymatic agent celloviridin was studied.

EXPERIMENTAL

Hydrolysis of cellolignin was carried out in a batch reactor under stirring at 50°C and at celloviridin concentration 45 g/l in 0.1 M sodium acetate buffer solution (pH 4.5) during 8-12 h. Then the hydrolysate was micro-filtered. For the synthesis of LPFR we used a biolignin suspension, the residue of hydrolisate microfiltration and dried biolignin with 50-70 % and 3 %, respectively.

The synthesis of LPFR was carried out by our method (Medvedeva *et al.*, 1998). The combined phenol and lignin polycondensation with formaldehyde was carried out in water in the presence of 7-10 % (weight) of NaOH in molar ratio (phenol and biolignin):formaldehyde 1:(2.04-2.06) at 80-95°C for 90-110 min.

RESULTS AND DISCUSSION

We have found that the biolignin shows a fairly high reactivity and can contribute to copolycondensation with phenol and formaldehyde without extra activation. The enhanced reactivity of lignin is probably caused

by its partial hydrolytic depolymerization along ether alkyl - aryl bonds, which takes place in lignin bioconversion as have shown by Glasser *et al.* (1983).

We managed to synthesize liquid LPFR in which up to 30-50 % of phenol was replaced by biolignin saving consumer quantities of resins.

The results presented in the Table 1 show the characteristics of the suggested resins to be in accordance with Russian State Standard requirements to low-toxicity liquid resol phenol-formaldehyde resin 3013, used at present time in wood-working industry.

The negligible free monomers content characterizing toxicity, allows a conclusion that the products obtained are low toxic. Due to low toxicity, the synthesized liquid LPFR can be used as glues in production of multiply plywood and other glue-based products.

Table 1. Characteristics of reaction mixtures and liquid lignin-containing phenol-formaldehyde resins obtained.

N°	Initial biolignin humidity (%)	Ratio		Characteristics of resins				
		phenol: biolignin (w/w)	(phenol+lignin):formaldehyde (mol:mol)	Relative viscosity (sec)	Weight share of alkali (%)	Dry residue (%)	Content of free (%)	
							phenol	formal-dehyde
1	70	70:30	1:2.04	50	4.5	39.8	0.08	0.03
2	60	70:30	1:2.04	72	4.8	42.7	0.07	0.03
3	60	60:40	1:2.05	78	4.9	41.7	0.05	0.02
4	60	50:50	1:2.06	80	5.0	42.6	0.04	0.03
5	50	70:30	1:2.04	90	4.8	42.3	0.08	0.02
6	3	70:30	1:2.04	61	4.9	43.0	0.07	0.03
R-3013 by GOST 20907-75	-	-	1:2.11	40-130	4.5-5.5	39-43	<0.18	<0.18

CONCLUSION

The method of synthesis of liquid resol phenol-formaldehyde resins has been proposed, that allows a significant reduction in prices of resin due to the partial substitution of expensive phenol by cheaper raw materials. At the same time, the important problem of environment protection can be solved by biolignin utilization.

REFERENCES

Glasser W.G., Barnett C.A. & Muller P. (1983). The chemistry of several novel bioconversion lignins. *J. Agric. Food Chem.*, **31**, 921-930.

Ladomerski J., Wienhaus O. & Pecina H. (1989). Zur Nutzung von Lignocellulose Ruckstanden (LCR) aus der Furfuralerzeugung fur die Herstellung von Phenolharzklebstoffen *Holz als Roh- und Werkstoff.*, **47**, 47- 52.

Medvedeva E.N., Ivanova N.V., Gorokhova V.G. & Babkin V.A. (1996). Activated Lignins, the Substitutes of Phenol in the Synthesis of Phenol-formaldehyde Resins *Chemistry for Sustainable Development*, **4**, 355-359.

Medvedeva E.N, Babkin V.A., Popova N.N. & Sinitsyn A.P. (1998). The method of obtaining of liquid resol phenol-formaldehyde resins. RF Patent 2,123,503.

Sinitsyn A.P., Klesov A.A. & Rabinovitch M.L. (1988). *Itogi nauki i techniki. Ser. Biotechnologiya,* Moskwa,, **12**, 156.

Vlasenko E.Yu., Kasteliyanos O. & Sinitsyn A.P. (1993). Reactivity of celluloso- containing raw materials of different type in hydrolysis with cellulolytic enzymes. *Prikl. Biokhim. i Mikrobiol.*, **6**, 834-843.

Biological treatment of a TCF bleaching effluent in an airlift reactor with white-rot fungus immobilized on wood chips

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ABSTRACT

This work evaluates the performance of an airlift bioreactor for the treatment of a pulp mill TCF bleachery effluent with cells of *Lentinus edodes* immobilized on wood-chips. The treatments were carried out in a repeated batch operation mode using a 330 mL airlift reactor in the following conditions: temperature of 30^0C, air flow rate of 300, 400 and 600 mL/min, and initial pH of 5.0. Each batch operation was carried out for 5 days with total reutilization of the fungal biomass in the subsequent operations. The process was evaluated in terms of its efficiency in removing total phenols and hydrogen peroxide. Effluent color was also analysed. The results of the first batch operation showed that after 2 days of treatment all hydrogen peroxide was removed for all air flow rates used. However, increases in total phenols concentration and color values were observed at the end of the operation. In the second batch operation, the hydrogen peroxide concentration values were similar to those of the first operation, but accentuated increases in the color values were observed for all the air flow rates employed. Reductions in total phenols concentrations were also noticeable at the end of the second batch operation.

Keywords: effluent treatment, airlift reactor, white-rot fungi, lignin biodegradation

INTRODUCTION

Airlift bioreactors have been used for aerobic treatment of effluents owing to their high efficiency in oxygen transfer from gaseous to liquid phase (high k_La values) (Chisti, 1989; Siegel *et al.*, 1987). The utilization of airlift bioreactors for treatment of effluents has been discussed as an alternative to the conventional processes (Esposito *et al.*, 1991; Esposito *et al.*, 1993; Cammarota and Sant'Anna, 1992).

Totally chlorine-free (TCF) bleaching technologies can produce compounds of high toxicity. Despite the replacement of chlorine by oxygen, ozone and hydrogen peroxide as bleaching agents, few studies have reported the environmental impact of the effluents produced during these bleaching processes (Paiva *et al.*, 1998; Paiva *et al.*, 1997; Ristolainen and Alén, 1998; Dethlefs *et al.*, 1995).

On the other hand, as withe-rot fungi produce some ligninolytic enzymes, they have been used in degradation studies involving several compounds of environmental impact. This extracellular enzymatic system is able to degrade compounds of high toxicity, namely phenols and hydrogen peroxide, which are commonly found in effluents produced during kraft pulp EOP bleaching processes. The treatment of these effluents with *Lentinus edodes* in airlift reactor showed to be an efficient bioprocess for the removal of these pollutants (Esposito *et al.*, 1993; Paiva *et al.*, 1999).

In a previous paper, we have reported the performance of an airlift reactor for the treatment of a pulp mill EOP bleachery effluent with free cells of *L. edodes* (Paiva *et al*, 1999). Few applications of wood chips as fungus support to effluent (or pollutant) treatment have been reported (Yum and Peirce, 1998). In the present work we investigate the performance of the airlift reactor using cells of *L. edodes* immobilized on wood chips.

EXPERIMENTAL

Fungal strains

L. edodes (strain UEC-2019) was obtained from UNICAMP Culture Collection (Biol. Chem. Lab.). Stock cultures were maintained at 5°C in Petri dishes containing 1.25% malt extract medium.

Effluent

All experiments were carried out with EOP effluent generated in a kraft pulp mill, which uses *Eucalyptus urograndis* as the raw material. The effluent presented a pH value ranging from 9.5 to 11.5 and was stored at -18°C until it was used.

Inoculum preparation

Lentinus edodes was grown as previously described (Esposito *et al.*, 1991; Paiva *et al.*, 1999; Paiva *et al.*, 1997). Inoculum consisted of 12 pieces of 0.5 cm in diameter of cultures previously grown in agar plates in a 500 mL Erlenmeyer flask containing 2.5g of wood chips and 250 mL of 1.25% malt extract medium. The pH of the medium was adjusted to 5.0 with H_2SO_4 solution. Cultures were incubated at 28°C, for 20 days at stationary conditions.

Laboratory-scale airlift reactor

The reactor is composed of an internal (airlift) tube and an annular space. The reactor temperature was controlled by circulating water from a constant temperature bath through the annular jacket surrounding the reactional zone. The aeration rate was measured by a flow meter and controlled at the desired levels. Figure 1 shows a schematic diagram of the airlift reactor used in this work (Cammarota and Sant'Anna, 1992).

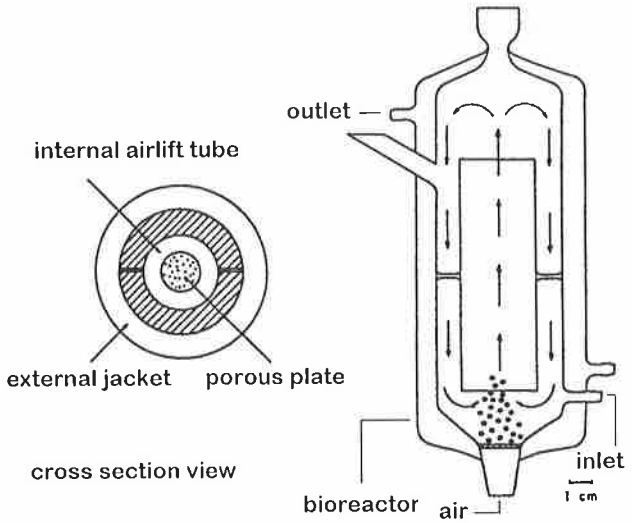

Figure 1. Schematic diagram of a fungal bioreactor.

Effluent treatment

After *Lentinus edodes* growth under standard conditions, all the inoculated chips contained in the Erlenmmeyer flask were removed and transferred to the 330 mL airlift reactor containing 250 mL of nonsterilized effluent, at a pH value previously adjusted to 5. The treatment was carried out at 28-30° C, air flow rates of 300, 400 and 600 $mL.min^{-1}$, over a period of 5 days.

Color, total phenols and hydrogen peroxide measurements

Color was measured as previously described (CPPA, 1975). In all cases the effluents were previously centrifuged for 15 minutes at 3,500 rpm. Total phenols were measured following the standard procedures (APHA, 1989). Hydrogen peroxide was determined by the iodinemetric method (Feffery *et al.*, 1992).

RESULTS AND DISCUSSION

Tables 1 and 2 show the chemical characteristics of the TCF effluent untretated and treated with immobilized cells of *L. edodes* using air flow rates of 300, 400 and 600 mL/min. In the first batch operation (Table 1), the hydrogen peroxide concentration was greatly reduced after five days of treatment using air flow rates of 300, 400 and 600mL/min. The total removal of hydrogen peroxide occurred after 48 hours for all the air flow rates employed (data not shown). In a previous study when free cells de *L. edodes* were used, the total removal of hydrogen peroxide occurred after 72 hours. The reduction in time was probably owing to an increase in the stability of peroxidase and/or catalase when cells of *L. edodes* are immobilized on wood chips (Paiva *et al.*, 1999).

A high increase in the total phenols content accompanied by an increase in the effluent color was observed at the end of the operation (Table 1). The high values of color and total phenols can indicate that a release of aromatic compounds from extractives and lignins probably occurred in the medium. Nevertheless, lowest values of color were observed when an air flow rate of 600 mL.min^{-1} was used.

In the second batch operation (Table 2), the values of hydrogen peroxide concentration were similar to those observed in the first operation. The total removal of hydrogen peroxide also occurred after 48 hours of treatment for all air flow rates employed.

Table 1. Chemical characteristics of the effluent before and after 5 days of biological treatment at the first batch operation.

	First batch Operation					
	air flow rates (ml.min^{-1})					
	300		400		600	
	NT	T	NT	T	NT	T
pH	5.0	7.0	5.1	7.5	5.0	7.6
Color (CU)	41.7	311	64.4	401	49.2	155
Total phenols (mg.L^{-1})	2.8	6.8	3.4	11.4	2.0	7.3
Hydrogen peroxide (mg.L^{-1})	40.0	0	43.0	0	40.0	0

NT: untreated; **T:** treated
CU: color units

A lower increase in total phenols content and in effluent color was observed in the second batch operation in comparison with the first one, when air flow rates of 300 and 400mL/min were used. However, for air flow rate of 600 mL/min, the color values in the second batch operation were similar to those observed in the first one.

Table 2. Chemical characteristics of the effluent before and after 5 days of biological treatment at the second batch operation.

	Second batch Operation					
	air flow rates (ml.min^{-1})					
	300		400		600	
	NT	T	NT	T	NT	T
pH	5.0	8.1	5.1	9.3	5.0	8.7
Color (CU)	37.9	155	41.7	280	45.5	167
Total phenols (mg.L^{-1})	2.6	3.9	2.3	5.2	2.5	5.8
Hydrogen peroxide (mg.L^{-1})	40.0	0	43.0	0	50.0	0

NT: untreated; **T:** treated
CU: color units

CONCLUSION

The performance of an airlift reactor for the treatment of EOP bleaching effluents using cells of *Lentinus edodes* immobilized on wood-chips was evaluated. The proposed process was capable to remove all hydrogen peroxide contained in TCF pulp mill effluents after 48 hours. However, accentuated increases in color values and total phenols concentration observed in both batch operations are problems whose solution require further studies. The color increase after the biotreatement will be better investigated by making a

control experiment with uninoculated wood chips. This control experiment can show how much color increase is related to extractives leaching from the wood chips.

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REFERENCES

APHA (1989).Standard Methods for Examination of Water and Wastewater, 17 ed. New York, Amer. Publ. Health Ass. N^0 5550B. pp 5.68-5.69.

Cammarota, M.C. & Sant'Anna (1992). Descolorization of kraft bleach plant E1 stage effluent in a fungal bioreactor. *Environ. Technol.*, **13 (1)**, 67-71.

Chisti,. M.Y. (1989). Airlift bioreactors. Elsevier Science, London, UK.

CPPA (1975). Technical Section Standard Method H5P.

Dethlefs, F.; Stan, H-J. & Kalnowski, G. (1995). Chemical and toxicological characterization of a pulp mill EOP bleachery effluent. *Vom wasser*, **85**, 149-165.

Esposito, E., Vanderlei, P. & Duran, N. (1991). Screening of lignin-degrading fungi for removal of color from kraft mill wastewater with no additional extr carbon-source. *Biotechnol. Lett.*, **13 (8)**, 571.

Esposito, E., Duran, N., Freer, J., Baeza, J. & Innocentini-Mei, L.H. (1993). Perfomance of a modified packed bed reactor to evaluate immobilized *Lentinus edodes* acting on kraft effluent. *In International Chemistry and Engineering Conference*, Proceedings, **vol 4**, pp 201-207.

Jeffery, G.H., Bassett, J., Mendham, J. & Denney, R.C. (1992). Vogel: Análise Química Quantitativa. 5. ed. Guanabara Koogan, Rio de Janeiro.

Paiva, T.C.B.; Ferraz, A.; Silva, F.T. & Durán, N. (1998). Toxicity and chemical characterization of a TCF bleaching effluent. *In 7th International Conference on Biotechnology in the Pulp and Paper Industry*, Proceedings, **vol C**, pp C211-214.

Paiva, T.C.B., Borlini, M.C., Castro, L.A.B.A., Oliveira, S.C. & Durán, N. (1999). Biotreatment of a pulp mill TCF bleachery effluent in a airlift reactors with the fungus *Lentinus edodes*. *In 10th International Symposium on Wood and Pulping Chemistry*, Proceedings, **Vol 2**, pp 110-113.

Paiva, T.C.P., Ferraz, A. & Durán, N. (1997). Biotreatment of a pulp mill EOP bleachery effluent with the fungus *Penicillium janthinellum* and *Lentinus edodes*. *In 5th Brazilian Symposium on the Chemistry of Lignins and Other Wood Components*, Proceedings, **Vol 6**, pp 391-396.

Ristolainen, M. & Alén, R. (1998). Characterization of effluents from TCF bleaching of hardwood kraft pulp. *J. Pulp and Paper Science*, **24 (4)**, 129-133.

Siegel, M.H., Hallaile, M. & Merchuk, J.C. (1987). Airlift reactors: Design, operation and applications. Advanced Biotechnology Processes, **Vol 7**, pp 79-124.

Yum, K.-J. & Peirce, J.J. (1998). Biodegradation kinetics of chlorophenols in immobilized cell reactors using a white-rot fungus on wood chips. *Water Environ. Res.* **70 (20)**, 205.

Bleaching of dissolving kraft pulp with xylanase enzyme

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ABSTRACT

A process has been developed for bleaching of dissolving pulp employing an enzymatic pretreatment with xylanase enzyme. Specially, this process consists in using mild prehydrolysis stage in order to have high pentosans in the unbleached pulp. The increase in pentosan content in the unblecahed pulp from mild water prehydrolysis and kraft cooking, results in high unbleached pulp yield at the same level of permanganate number. Bleaching of enzyme treated pulp in a CEHED sequence indicates reduction in bleach chemical requirement, improvement in the brightness of final bleached pulp by about 2 points, desired level of pentosans in the pulp (2.4 to 2.6%) and a significant increase in the bleached pulp yield as compared to control. The yield improvement is to the extent of 2.5%. Also, the rayon yield and reactivity are higher in case of enzyme treated pulp.

Keywords: bleaching, dissolving pulp, xylanase, kraft pulp

INTRODUCTION

Dissolving pulp is one of the products of pulp and paper industry used for manufacture of rayon, cellophane, carboxymethyl cellulose and other cellulose derivatives. It is produced from hard and soft woods by sulfite and sulfate processes. The removal of hemicellulose from wood fibre is a necessary step in the manufacture of dissolving pulp. In sulfite pulping, the acidic conditions used are responsible for removing most of the hemicelluloses while in sulfate process usually a prehydrolysis step is required to remove hemicelluloses represented in terms of pentosans. Pulp from sulfate cooking is of brown colour mainly due to residual lignin in the pulp. Bleaching of sulfate pulp is done using large amount of chlorine and chlorine compounds. These compounds are a cause for environmental concern since the process produces dioxins and other organochlorine compounds. Many of these compounds are toxic, mutagenic, persistent and/or bioaccumulative.

In the last few years, xylanase enzymes have been examined to reduce or eliminate the use of chlorine in the bleach plant. These enzymes are being used commercially since 1990 for prebleaching of paper grade pulp, (Suurnakki *et al.*, 1997) but have not been used commercially for prebleaching of dissolving pulp. Some researchers in South Africa have tried the enzyme on a lab scale for bleaching of dissolving sulfite pulp (Christov and Prior 1994, 1996). In Thapar Centre for Industrial Research & Development, we have carried an extensive work on the use of xylanolytic enzymes in bleaching of dissolving Kraft pulp. This paper reports on the development of a process for bleaching of dissolving kraft pulp employing an enzymatic pretreatment with xylanase enzyme.

EXPERIMENTAL

Unbleached pulps of varying levels of pentosan content (2.1 to 6.3%) were prepared by water prehydrolysis and kraft pulping from eucalyptus and casurina mixed chips (60:40). Mild prehydrolysis conditions were used to produce pulps of high pentosan contents. Tables 1 and 2 present the details of optimized conditions of prehydrolysis and kraft cooking, and characteristics of unbleached pulp.

Enzymes Used

A total of 6 commercial enzymes – Pulpzyme HB, Bleachzyme F, Irgazyme 10, Irgazyme 40S, VAI Xylanase and Cartazyme HS-10 were evaluated. These enzymes were gifted by M/s Novo Nordisk, Denmark; Biocon, India; Genencor, Finland; Voest Alpine, Austria and Sandoz Chemicals, U.K.

Enzyme Treatments

The unbleached pulps were treated with different enzymes in plastic bags under different reaction conditions (pH, temperature, enzyme dose) at 5-10% consistency for different lengths of time. The pH of the pulp was adjusted with H_2SO_4 before addition of the enzyme. The reference pulps were incubated at the same

conditions as the enzyme treated pulps prior to bleaching. After the enzyme treatment, the pulps were subjected to chlorination with or without washing.

Table 1. Details of optimized conditions of prehydrolysis and kraft cooking stages.

Particular	Test value
Prehydrolysis Stage	
Chips taken on o.d. basis (kg)	2.098
Moisture content (%)	26.7
Chips to liquor ratio	1:3.3
Steaming time (min)	90
Cooking temperature (°C)	161
Cooking time (min)	75
Kraft Cooking Stage	
Active alkali charged as Na_2O (%)	16.0
Chips to liquor ratio	1:3.3
Steaming time (min)	90
Cooking temperature (°C)	169
Cooking time (min)	75

Raw material: eucalyptus and casurina in 60:40 ratio

Table 2. Characteristics of unbleached pulp.

Particular	Test value
Unbleached pulp yield (%)	36.9
Permanganate no. of pulp	8.9
Pentosan content (%)	5.1
Unbleached pulp viscosity (cp)	36.5

Chemical Bleaching

After the enzyme treatment, the pulp was further bleached in a multistage bleaching process using CEHED sequence. Chlorine was applied at a kappa factor of 0.2 for 45 minutes at 30°C and 3% pulp consistency. The pulp was washed with water and extracted with NaOH (pH 11.2-11.5) at 10% consistency for 120 minutes at 65°C. The hypo treatment was done at 40°C with the pulp at 10% consistency. After hypo treatment, the pulp was again extracted with NaOH (pH 11-11.2) at 65°C for 60 minutes at 10% pulp consistency. The chlorine dioxide treatment was done at 75°C at 10% pulp consistency for 180 minutes. For comparison purpose, a control bleaching without enzyme was also carried out. The final bleached pulps were evaluated for various parameters like brightness, viscosity, bleached pulp yield, ash analysis, alkali solubility, pentosan and α-cellulose content and reactivity index.

Analytical Techniques

The presence of cellulase activity in the commercial enzymes was tested by the method of Mandels and Weber (1969). Xylanase activity was measured by reducing sugar production from oat spelt xylan (Bailey *et al.*, 1992). International enzyme units (IU) are defined as the amount of enzyme necessary for the production of 1 μmol product/minute.

Kappa number and P. No. were determined according to Tappi methods. Viscosity was determined according to Tappi method T206. Pentosans content and α-cellulose were determined by Tappi useful method 236 and T 203 om-88 respectively. Alkali solubility was determined by Tappi method T235 cm-85. Brightness of paper sheets was determined with a Technibrite instrument (Model TB 1c).

Effluents were analysed for COD and BOD following the standard methods (COD, 1985; BOD, 1985).

RESULTS AND DISCUSSION

All the six enzymes were found to be free of cellulase side activity and were highly specific for xylan containing polysaccharides. Pulp consistency of 5-10%, during enzyme treatment, was found to be suitable.

In order to test the effectiveness of different enzymes, several experiments were conducted to determine their optimum reaction conditions in bleaching. Unbleached pulp was treated with the enzymes under varying reaction conditions (pH, temperature, enzyme dose and reaction time) and the amount of released carbohydrates were determined. It was noted that each enzyme required special reaction conditions for optimum function. Bleachzyme F, Cartazyme HS-10 and Irgazyme 10 were found to be effective in the pH range of 4-6.5 and temperature of 40-55°C whereas Pulpzyme HB and Iragazyme 40S showed maximum activity at pH 7-8 and temperature 45-60°C. VAI xylanase could work over a wide pH range of 5 to 7.0 and tolerate temperatures of up to 50-60°C. Optimal enzyme dose was found to vary with different enzymes. Most of the enzyme effect was seen after 3 hours of treatment. Beyond that, very little enzyme action was seen.

It was observed that optimum level of pentosan content in the unbleached pulp should be around 5%, during enzyme treatment, in order to achieve desired level of pentosan content (2.4 to 2.6%) in the final bleached pulp (Table 3).

Unbleached pulp of high pentosan content, 5%, was treated with various enzymes at an equal xylanase activity level (30 IU/g o.d. pulp) at their optimum pH and temperature for 3 hours. The enzyme treated pulp was then subjected to CEHED bleaching and reduction in E stage P No., increase in final brightness of bleached pulp and final pentosan content in the bleached pulp were determined. It was observed that enzyme treatments led to a decrease in E stage P No. by about 0.9 to 1.15 units relative to untreated pulp. The gain in final pulp brightness under the same total bleaching chemical charge was 1.4 to 2.1 points with different enzymes. Maximum brightening effect was noted with Bleachzyme F, Cartazyme HS-10 and Irgazyme 40S (Table 4). Bleachzyme F also showed maximum reduction in pentosan level.

Table 3. Effect of pentosan content of unbelached pulp on pentosan content of bleached pulp.

Pentosan in unbleached pulp (%)	Pentosans in bleached pulp (%)	
	CEHED	Enz. CEHED
2.1	1.6	1.0
3.0	2.4	1.6
3.6	3.0	2.2
5.0	4.0	2.8
6.3	5.9	5.3

Table 4. Performance of various cellulase free commercial xylanases.

Enzyme	E stage P. No.	Brightness (% ISO)	Increase in brightness (points)	Final pentosans (%)
No enzyme (Control)	3.40	87.1	---	4.8
Bleachzyme F	2.40	89.2	2.0	2.6
Cartazyme HS-10	2.35	89.1	2.1	3.5
Irgazyme 10	2.20	89.0	1.8	3.1
Irgazyme 40S	2.25	89.1	1.9	3.2
VAI xylanase	2.25	88.9	1.7	3.5
Pulpzyme HB	2.50	88.6	1.4	3.8

Eucalyptus/casurina pulp (60:40) of Kappa No. 14.5
Pentosans in ubleached pulp – 5.2%
Bleaching sequence – CEHED
Enzyme dose – 30 IU/g pulp

The increase in pentosan content in the unbleached pulp using mild prehydrolysis conditions resulted in high unbleached pulp yield at the same level of permanganate no. The details of unbleached pulp characteristics are given in Table 5. The data show that unbleached pulp yield increased by 3% for the pulp of 5.1% pentosan content, compared to that of 2.4% pentosan content.

When the Bleachzyme F treated pulp was bleached in a CEHED sequence, a reduction in bleach chemical requirement was noted as compared to control. It was possible to save 0.25% elemental chlorine, 0.4% NaOH, 0.4% hypo and 0.1% ClO_2/ton of pulp (Table 6). There was significant improvement in the brightness of final bleached pulp after enzyme treatment. Final brightness was found to be about 2.1 points higher than that of control (Table 6). The viscosity of final bleached pulp was found comparable to control and the α-cellulose content and rayon yield of enzyme bleached pulp were higher than control (Table 6). The

pentosan content in the bleached pulp was found to be 2.6% after enzyme treatment indicating about 50% reduction in the pentosan content from the unbleached pulp (5% pentosans). There was significant increase in the bleached pulp yield by enzyme treatment as compared to control. Bleached pulp yield by enzyme treatment was 33.5% against the bleached pulp yield of 31.0% for control. There was only marginal improvement in final pulp brightness or reduction in bleaching chemicals, when the pulp was washed after the enzyme treatment prior to chlorination.

Table 5. Unbelached pulp characteristics.

Particular	Test value			
	1	2	3	4
Pentosan content (%)	2.4	3.9	4.5	5.1
Permangnate No.	11.5	10.6	11.4	8.9
Unbleached pulp yield (%)	33.6	34.8	36.2	36.9
Unbleached pulp viscosity (cp)	24.0	34.2	30.7	36.4

Table 6. Effect of Bleachzyme F treatment on

(a) Bleach chemical requirement

Chemical	Consumption (%)		Chemical savings (kg/TP)
	Control	Enzyme treatment	
Cl_2 as gas	2.75	2.50	2.50
Alkali in E_1 stage	1.50	1.25	2.50
Hypo in H stage	2.0	1.6	4.0
Alkali in E_2 stage	0.40	0.25	1.50
ClO_2 in D stage	0.50	0.40	1.00
O_2 in acid wash	0.51	0.51	--

(b) Final bleached pulp properties

Particular	Control	Enzyme
Brightness (% ISO)	89.1	89.2
Viscosity (cp)	11.8	12.4
Bleached pulp yield (% on o.d. wood basis)	31.0	33.5
Pentosan (%)	4.2	2.6
α-cellulose (%)	93.7	94.8
Rayon yield (%)	95.7	97.2
Reactivity index	17.2	13.6

Pentosan in unbleached pulp (%) = 4.5–5.0

With very high pentosan pulp, 6.3%, even by using very high enzyme loading and prolonged incubation period of 12 h, the pentosan level decreased to 4.8% only showing about 24% hydrolysis.

The total COD released from normal plant pulp (with 2.9% pentosans) was found to be -30 kg/TP in CEHED sequence whereas the total COD released from high pentosan pulp (5%) with enzyme was about 50 kg/TP in Enz.CEHED sequence. Most of the additional COD comes from the enzyme stage of bleaching, in case of high pentosan pulp.

Some researchers (Paice and Jurasek, 1984; Christov and Prior, 1993) have attempted to use xylanase enzymes for removal of pentosans from bleached dissolving grade pulp for quality upgradation. But, even with very high enzyme dosing and prolonged incubation, very limited hydrolysis of xylan was obtained. It appears that the remaining pentosans in the bleached pulp are very well shielded by other pulp components and, therefore, not recognized by enzyme or not easily susceptible to enzymatic attack although one would expect that residual xylan in bleached pulp, as compared to unbleached pulp, may be more accessible to enzymes due to partial removal of lignin and hemicelluloses. Therefore, interpretation of these results is controversial. Our work has shown that xylanase treatment of dissolving grade pulp prior to bleaching is more effective in removing pentosans because of the presence of more hemicellulose at this stage, accessible for enzymatic degradation. Xylanase pretreatment of dissolving grade pulp also reduces bleach chemical requirement and allows for a higher brightness ceiling to be reached.

The use of mild prehydrolysis stage to have high pentosans in the unbleached pulp, results in increased unbleached pulp yield at the same level of permanganate number. Some pentosans are removed by xylanase enzyme during the pretreatment and bleaching. Because enzymes are specific, cellulose is left intact. This contributes to an overall increase in bleached pulp yield. The use of mild prehydrolysis stage also leads to

steam conservation and formation of reduced quantity of pollutants in the prehydrolysate liquor. Xylanase treatment was not found to affect the viscosity of final bleached pulp. This could be due to the cellulase free nature of enzyme. Since, the pentosans are released from xylanase prebleaching stage, the COD of the effluent is rather high. For a mill, with no waste treatment, the effluent load can be reduced by recirculation of the water from the enzyme stage to recovery. Such effluents are more amenable to biological degradation due to higher proportion of degraded xylan as has been confirmed by Onysko (1993) and Senior and Hamilton (1992).

OUTCOME

This work has successfully demonstrated the applicability of xylanases in prebleaching of dissolving pulp. Various benefits of this novel process can be summarised as follows:

- Mild prehydrolysis stage to have high pentosans in the unbleached pulp (steam conservation and increased pulp yield)
- Reduced quantity of pollutants in the prehydrolysate liquor
- High brightness pulps of +92% due to increased brightness ceiling
- Increased bleached pulp yields
- Reduced bleach chemical consumption
- Environment friendly technology

Based on this work, a patent application has been filed with Indian Patent Office, New Delhi.

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REFERENCES

Bailey, M.J., Biely, P. & Poutanen, K. (1992). Interlaboratory testing of methods for assay of xylanase activity. *J. Biotechnol.* **23**, 257-270.

Biochemical oxygen demand method (1985) In *Standard Methods for the Examination of Water and Wastewater*, 16th Ed., APHA, AWWA, WPCF, Washington, p. 525.

Chemical oxygen demand method (1985) In *Standard Methods for the Examination of Water and Wastewater*, 16th Ed., APHA, AWWA, WPCF, Washington, p. 532.

Christov, L.P & Prior B.A. (1993). Xylan removal from dissolving pulp using enzymes of *Aureobasiduim pullulans. Biotechnol. Lett.* **15**, 1269-1274.

Christov, L.P. & Prior B. A. (1994). Enzymatic prebleaching of sulphite pulp. *Appl. Microbiol. Biotechnol.* **42**, 492-498.

Christov, L.P. & Prior B. A. (1996). Repeated treatments with *Aureobasiduim pullulans* hemicellulases and alkali enhance biobleaching of sulphite pulps. *Enz. Microbial. Technol.* **18**, 244-250.

Mandels, M. & Weber, J. (1969) The production of cellulases. *Adv. Chem.* **96**, 391-414.

Onysko, K.A. (1993) Biological bleaching of chemical pulps: a review. *Biotechnol. Adv.* **11**, 179-198.

Paice, M.G. & Jurasek, L. (1984). Removing hemicellulose from pulps by specific enzyme hydrolysis. *J. Wood Chem. Technol.*, **4**, 187-192

Senior, D. J. & Hamilton J. (1992). Use of xylanases to decrease the formation of AOX in kraft pulp bleaching. *J. Pulp Paper Sci.* **18 (5)**, J 165-168.

Suurnakki, A., Tenkanen, M., Buchert, J. & Viikari, L. (1997). Hemicellulases in the bleaching of chemical pulps. *Adv. in Biochem. Eng./Biotechnol.* **57**, 261-287.

Characterization of carbohydrates present in hydrolyzate obtained from sugar cane bagasse pretreated by steam explosion

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ABSTRACT

Sugar cane bagasse pretreated by steam explosion at 190°C for 15 minutes was separated from the hemicellulosic hydrolyzate fraction and both were chemically characterized. Free sugars and their oligomers were determined by HPLC in HPX-87H and HPX-42A columns, respectively, and lignin and ashes were determined by gravimetry. The pretreated bagasse, 34% of which was solubilized, contained 54% glucan, 6.4% xylan, 0.2% arabinan, 0.9% acetyl groups, 28.2% insoluble lignins, 0.87% soluble Klason lignin and 3.5% ashes. The hydrolyzate contained 1.25% glucose oligomers, 0.08% hydroxymethylfurfural, 10.67% xylose oligomers, 2.7% xylose, 0.6% furfural, 0.5% arabinose, 0.13% arabinose bonded to xylose oligomers, 1.06% acetyl groups, 0.2% acetyl groups bonded to xylan, 4.8% compounds detected as soluble lignin and 12% nonidentified compounds. The qualitative analysis of the hydrolyzate showed the presence of xylose oligomers with polimerization levels ranging from 1 to 6, mainly anhydroxylose, xylobiose and xylotriose. The molecular weight distribution of carbohydrates present in the hydrolyzate (134 to 1,364 $g.mol^{-1}$) was determined by HPSEC using a 320-H Asahipak column, and its average value was lower than that of the aromatic compounds.

Keywords: sugar cane bagasse, carbohydrates, steam explosion, hemicellulosic hydrolyzate, inhibitors

INTRODUCTION

Steam explosion is used in the treatment of lignocellulosic materials to enhance their susceptibility to biotechnological conversion. This process makes it possible to obtain a cellulose- rich material, susceptible to enzymatic hydrolysis, and a hydrolyzate with water-soluble carbohydrates, susceptible to fermentative process (Lawther *et al.*, 1996; Saddler *et al.*, 1983; Wilson *et al.*, 1989).

The substrate submitted to steam explosion suffers physical and chemical modifications. For example, in the case of grasses or hardwood, usually pretreated at temperatures around 200°C, the acetic acid released catalyzes the hydrolysis of the xylans, as well as, the dehydration of pentoses to furfural and of hexoses to 5-hydroxymethylfurfural, respectively. Additionally, lignin is partially degraded to low molecular weight aromatic compounds. Depending on their contents, these compounds can inhibit the enzymes or microorganisms involved in the biomass conversion (Puls *et al.*, 1985; Palmqvist *et al.*, 1996). It is, therefore, essential to determine their contents in each fraction, separately from the steam exploded lignocellulosic material.

The procedures used for characterization of sugar cane bagasse pretreated by steam explosion and for identification of the carbohydrates present in henicellulosic hydrolyzate are described in this paper.

EXPERIMENTAL

Steam pretreatment

Bagasse was directly loaded into a 0.65 L reactor for steam explosion pretreatment at 190°C (Silva, 1995). After 15 minutes, the material was released from the reactor by rapid despressurization, which caused its expansion into a collecting vessel. The resulting material was then filtered and washed with 2000 mL of water at 50°C. Next, the filtrate was separated from the bagasse and both were chemically analyzed to determine their contents of Klason lignin, hydroxymethylfurfural, furfural and carbohydrates. The xylose oligomers content in the water-soluble material and its molecular weight distribution were determined as well.

Chemical analysis

Determination of extractives from untreated bagasse

The content of total extractives in 20 g of sugar cane bagasse (Wiley-milled to pass through a 20 mesh screen) was determined by a treatment with 95% ethanol in a Soxhlet apparatus. After extraction, the bagasse was dried at 105°C and stored for further analysis (Silva, 1995).

Acid hydrolysis

The hydrolyzate, the extracted and the pretreated bagasse were chemically analyzed in triplicate. Extracted and pretreated bagasse samples (2 g, dry weight) were hydrolyzed with 10 mL of 72% sulfuric acid at 45°C for 7 min. The acid was diluted to a final concentration of 3% by the addition of 275 mL of water. Moreover, 250 mL hydrolyzate was transferred to a 500 mL erlenmeyer flask and acidified to pH 0.6 with 72% sulfuric acid. The acidified mixtures were heated to 121°C (1 atm) for 30 min. The residual materials were cooled and filtered through a paper filter. The filtrates were quantitatively transferred to 500 mL volumetric flasks and diluted with deionized water. All the solutions were analyzed by HPLC to determine their contents of carbohydrates, acetic acid, furfural and hydroxymethylfurfural. The solids were dried at 100°C to constant weight and calcined in porcelain crucible for 2 h, initially at 300°C and then at 800°C. The ash contents were determined gravimetrically and the Klason lignin was calculated by difference (Silva, 1995).

The hydrolyzate and the water-phase from acid hydrolysis of extracted and pretreated bagasse were analyzed by UV (Spectrophotometer Cintra 20) to determine the content of soluble lignin, using water solutions (pH 12.5) prepared from dilute filtrate samples (5 mL) and 6.5 $mol.L^{-1}$ NaOH solution (2 mL). The water solutions were introduced into 100 mL volumetric flasks and their absorbances were measured at 280 nm in triplicate, using the value of 25.2 $L.g^{-1}.cm^{-1}$ as absortivity of the soluble lignin (Silva, 1995).

Determination of carbohydrates, furfural and hydroxymethylfurfural by HPLC

The hydrolyzate and the water-phase from acid hydrolysis of the extracted and pretreated bagasse samples (40 mL of each) were eluted in Sep-Pak C18 (Waters) to remove aromatic compounds and analyzed by HPLC using Shimadzu CTO-6A equipment (Silva, 1995; Cunha, 1999). Sugars were separated on a Biorad Aminex HPX-87H column (300 x 7.8 mm) fitted with cationic and anionic de-ashing microguard precolumns (Biorad Laboratories, Richmond, CA). Degassed 0.005 $mol.L^{-1}$ H_2SO_4 was eluted at a flow rate of 0.6 $mL.min^{-1}$, at 45°C. Eluted carbohydrates and acetic acid peaks were monitored by an LC-10 refractive index detector. The elution time and the amount of each sugar and acetic acid were determined by calibration curves using analytical grade standard sugars.

Furfural e hydroxymethylfurfural present in the hydrolyzate and the water-phase from acid hydrolysis of the extracted and pretreated bagasse samples (5 mL of each) were determined by HPLC (Hewlett-Packard), using a LiChrospher 100 RP-18 (125 x 4mm) (Silva, 1995; Cunha, 1999). Degassed acetonitrile/water at a ratio of 1:8 (v/v) with 1% acetic acid was eluted at a flow rate of 0.8 $mL.min^{-1}$, at 25°C. Eluted compounds were monitored by a UV-VIS detector HP 79875 (Hewlett-Packard) at 276 nm. The elution time and the amounts of furfural and hydroxymethylfurfural were determined by calibration curves using analytical grade standard sugars. Chemical analyses were performed in triplicate.

Determination of xylose oligomers

Hydrolyzates samples (40 mL) were extracted in Sep-Pak C18 to remove aromatic compounds and the oligosaccharides present in the hydrolyzate were determined by HPLC using Shimadzu CTO-6A equipment. Xylan was hydrolyzed to xylose (monomer and oligomers) and used as standards. Hydrolysis was performed with 0.005 $mol.L^{-1}$ H_2SO_4 at 45°C for 7 min and the compounds were analyzed by HPLC. The sample was separated on a Biorad Aminex HPX-42A column (300 x 7.8 mm), eluted by degassed water at a flow rate of 0.6 $mL.min^{-1}$, at 45°C. Eluted sugar peaks were monitored by an LC-10 refractive index detector. The oligosaccharides were identified by comparing their retention times with the retention times of known oligomers (Cunha, 1999).

Molecular Weight Distribution

The apparent molecular weight distribution of the compounds present in the hydrolyzate was determined by HPSEC (Cunha, 1999). The hydrolyzate was extracted in a Sep-Pak C18 to remove aromatic compounds. The eluted fraction was analyzed by HPLC using Shimadzu CTO-6A equipament and eluted in Asahipak GS-320H column. The compounds were eluted by degassed water at a flow rate of 1.0 $mL.min^{-1}$, at 30°C. The peaks of the compounds were monitored by an LC-10 refractive index detector. The chromatographic column was calibrated with bovine albumin (66,000 $g.mol^{-1}$), carbonic anhydrase (29,000 $g.mol^{-1}$),

cytochrome c (12,400 $g.mol^{-1}$) and aprotinin (6,500 $g.mol^{-1}$). The column exclusion and total volumes were determined by elution of blue dextran (2,000,000 $g.mol^{-1}$) and acetone (58 $g.mol^{-1}$), respectively.

FTIR analysis

The samples were prepared by elution of 40 mL of hydrolyzate in Sep-Pak C18. The eluted carbohydrate solution was freeze-dried and the aromatic compounds retained by Sep-Pak C18 were desorbed with 25 mL of ethanol, which was removed by evaporation. The carbohydrate and aromatic fractions were dried under P_2O_5 /vacuum and analyzed by FTIR. FTIR spectra of samples were recorded in a Nicolet 520 spectrometer as KBr pellets at concentrations of 0.5%. Spectra were base-line corrected and converted to text files using OMNIC software (Nicolet, Inc.). The absorbances used were 400 to 4000 cm^{-1} (Cunha, 1999).

RESULTS AND DISCUSSION

The chemical composition of the sugar cane bagasse, pretreated bagasse and the hydrolyzate obtained from bagasse pretreatment is shown in Table 1.

The amount of sugar cane bagasse solubilized in the steam explosion pretreatment was 34%. Xylan and acetyl groups were hydrolyzed to the same extent (83%) and hydrolysis was followed by lignin (17%) and unidentified compounds (16.8%) solubilization. These data are similar that those observed by Silva (1995) and show the effectiveness of the steam explosion pretreatment.

The pretreated bagasse contained 54% glucan, 6.4% xylan, 0.2% arabinan, 0.9% acetyl groups, 28.2% insoluble lignin, 0.87% soluble Klason lignin and 3.5% ashes (Table 2). The hydrolyzate contained 1.25% glucose oligomers, 0.08% hydroxymethylfurfural, 2.7% xylose, 10.67% xylose oligomers, 0.6% furfural, 0.5% arabinose, 0.13% arabinose linked to xylose oligomers, 1.06% acetic acid, 0.2% acetyl groups bonded to xylan and 4.8% compounds considered as soluble lignin (Table 3).

The predominant carbohydrates in the hydrolyzate were xylose and their oligomers. The distribution of these sugars is shown in the chromatogram of Figure 1. G peak was attributed to arabinose and A to xylose, while the peaks from B to F were assigned to xylose oligomer with polimerization levels ranging from 2 to 6.

The HPSEC analysis of the hydrolyzate showed that the molecular weight of the compounds ranged from 85,000 $g.mol^{-1}$ to 500 $g.mol^{-1}$ (Figure 2a). The hydrolyzate presented a high molecular weight fraction (85,000 $g.mol^{-1}$ - 33,000 $g.mol^{-1}$) corresponding to the compounds eluted between 3.8 mL and 4.4 mL; an intermediate fraction (7,195 $g.mol^{-1}$ - 2,777 $g.mol^{-1}$) eluted between 6 mL and 7 mL and a low one (1,297 $g.mol^{-1}$ - 500 $g.mol^{-1}$) eluted between 7.9 mL and 8.8 mL.

The carbohydrate fraction (Figure 2b) eluted between 8 mL and 10 mL corresponds to sugars with molecular weight from 1,073 $g.mol^{-1}$ to 160 $g.mol^{-1}$, coinciding with the distribution of xylose and their oligomers in the chromatogram shown in Figure 1.

The fraction containing aromatic compounds (Figure 2c) eluted between 4 mL and 8 mL, corresponds to the components with molecular weight from 18,000 $g.mol^{-1}$ to 2,780 $g.mol^{-1}$. The ethanol used as a solvent for aromatic fraction eluted between 8.5 mL and 12 mL.

A comparison between the chromatograms in Figures 2a - 2c indicates that the compounds with molecular weight ranging from 85,000 $g.mol^{-1}$ to 33,000 $g.mol^{-1}$ correspond to those aromatics that were retained in Sep-Pak C18 and were not desorbed by ethanol, when the aromatic sampling was prepared.

The FTIR analysis confirmed that the aromatic compounds were absent from the carbohydrate fraction (Figure 3). The FTIR spectrum of the carbohydrate fraction (Figure 3a) showed bands at 3418 cm^{-1}, 2964 cm^{-1} and 1094-1044 cm^{-1}, attributed to O-H, C-H and C-O absortion groups, respectively, while the 1510 cm^{-1} band, representing lignin and their derivatives, was not detected. On the other hand, the FTIR spectrum of the aromatic fraction (Figure 3b) extracted from Sep-Pak C18 with ethanol showed peaks at 3417 cm^{-1}, 1607 cm^{-1} and 1094-1044 cm^{-1}, attributed to O-H, C=C and C-O absortion groups, respectively, besides absortions at 1050 cm^{-1} and 1510 cm^{-1} assigned to carbohydrates and lignin, respectively. This result indicates that the aromatic fraction was composed of lignin and lignin-carbohydrates complex.

Table 1. Chemical composition of the sugar cane bagasse, pretreated bagasse and hydrolyzate. Values corresponding to 100 g of dry bagasse.

Component	Sugar cane bagasse (%)	Pretreated bagasse (%)	Solubilized material (%)	Total recovered (%)
Glucan	39.2 ± 0.9	35.7 ± 0.1	1.25 ± 0.01	36.95 ± 0.01
Xylan	25.2 ± 0.6	4.2 ± 0.1	14.04 ± 0.06	18.3 ± 0.2
Acetyl groups	3.5 ± 0.2	0.5960 ± 0.0001	1.189 ± 0.001	1.785 ± 0.001
Total lignin	23 ± 2	19.1 ± 0.3	4.8 ± 0.1	23.9 ± 0.4
Extractives	2.0 ± 0.1	n.a	n.a	n.a
Ashes	3.1 ± 0.4	2.29 ± 0.01	n.a	2.29 ± 0.01
Total	96 ± 5	61.9 ± 0.5	21.2 ± 0.2	83.2 ± 0.6

n.a = not analyzed

Table 2. Chemical composition of pretreated sugar cane bagasse.

Component	Composition (%)
Glucan	54±0.2
Xylan	6.4±0.2
Acetyl groups	0.9057±0.0001
Soluble lignin	0.87±0.04
Insoluble lignin	28.2±0.4
Total lignin	29.1±0.4
Extractives	n.a
Ashes	3.48±0.02
Total	94.1±0.8

Table 3. Composition of water-soluble material obtained by steam explosion bagasse at 190ºC for 15 min.

Component	Composition (%)
Glucose Oligomers	1.25 ± 0.01
Hydroxymethylfurfural	0.077 ± 0.004
Xylose	2.70 ± 0.01
Xylose Oligomers	10.67 ± 0.03
Arabinose	0.543 ± 0.001
Arabinose Bonded to Oligomers	0.13 ± 0.01
Furfural	0.55 ± 0.03
Acetic Acid	1.063 ± 0.002
Acetyl Groups Bonded to Oligomers	0.2 ± 0.1
Lignin Soluble	4.75 ±0.01
Total	22.0 ±0.3

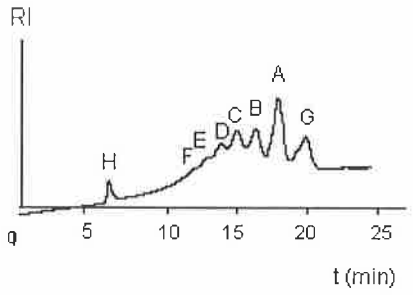

Figure 1. Chromatogram of the hemicellulosic hydrolyzate eluted in HPX-42A column.

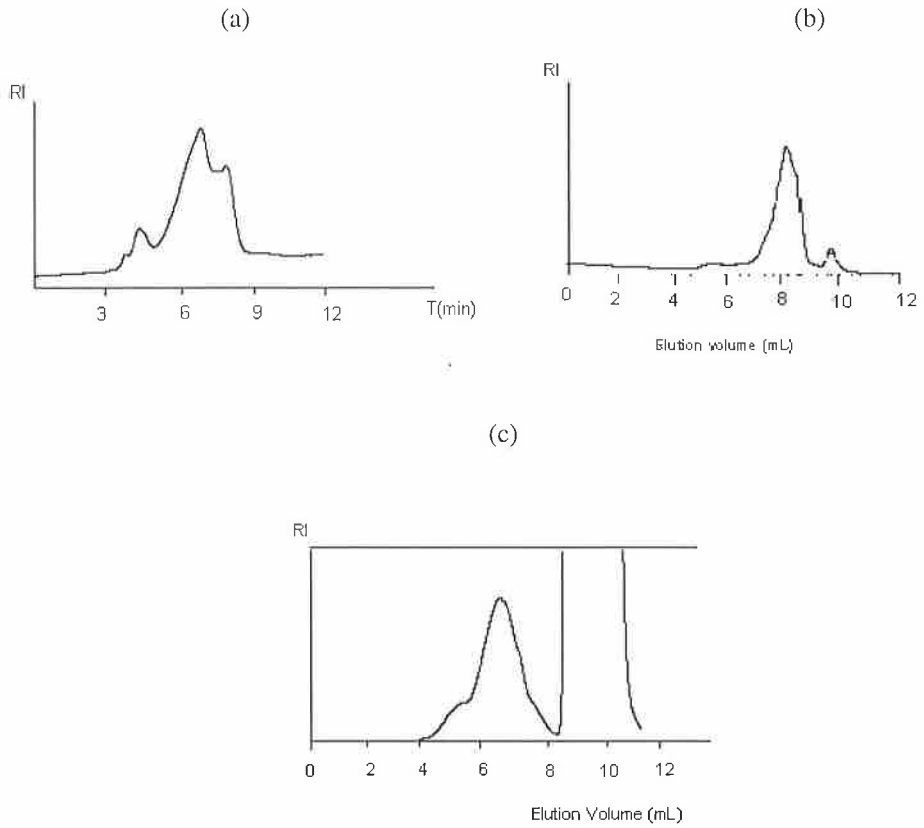

Figure 2. Molecular weight distribution of hydrolyzate (a), carbohydrates fraction (b) and aromatic compounds fraction (c).

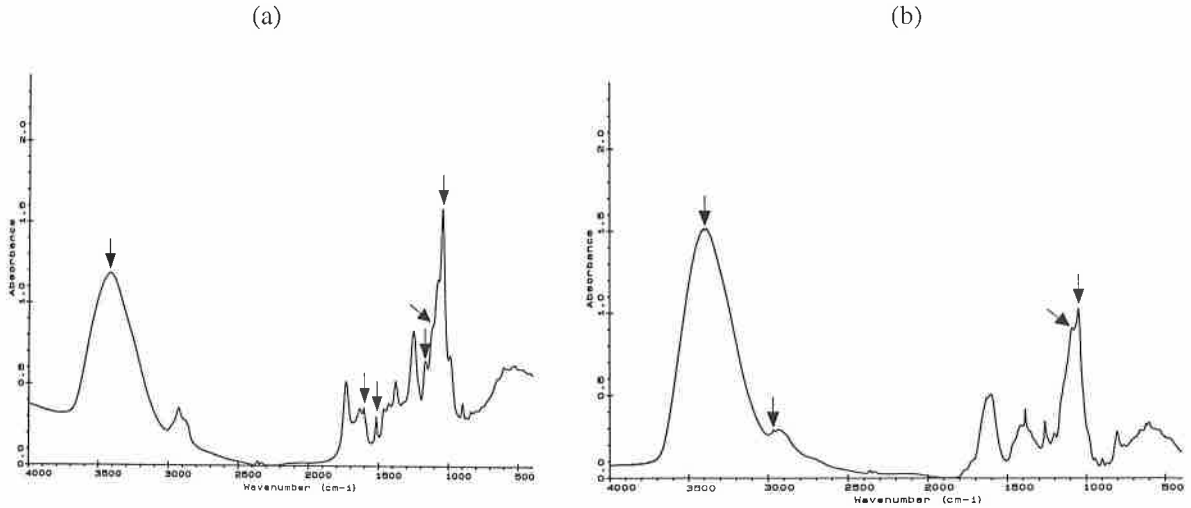

Figure 3. FTIR spectra of carbohydrate (a) and aromatic (b) fractions.

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REFERENCES

Cunha, H.C.M. (1999). Caracterização do bagaço de cana pré-tratado por explosão a vapor: identificação de inibidores potenciais de processos fermentativos e enzimáticos. MsC Thesis, .Faculdade de Engenharia Química de Lorena.

Lawther, J. M.; Sun, R. & Banks, W.B. (1996). Effect of Stem Treatment on the Chemical Composition of Wheat Straw. *Holzforschung*, **50**, 365-371.

Palmqvist, E.; Hahn-Hägerdal, B.; Galbe, M. & Zacchi, G. (1996). The effect of water-soluble inhibitors from steam-pretreated willow on enzymatic hydrolysis and ethanol fermentation. *Enzyme Microb. Technol.*, **19**, 470-476.

Puls, J.; Poutanen, K.; Körner, H.-U. & Viikari, L. (1985). Biotechnical utilization of wood carbohydrates after steaming pretreatment. *Appl. Microbiol. Biotechnol.*, **22**, 416-423.

Saddler, J.N.; Mes-Hartree, M.; Yu, E.G.C. & Brownell, H.H. (1983). Enzymatic hydrolysis of various pretreated lignocellulosic substrates and the fermentation of the liberated sugars to ethanol and butanediol. *Biotechnol. Bioeng. Symp.*, **13**, 225-238.

Silva, F.T. (1995). Obtenção de insumos químicos a partir do aproveitamento integral do bagaço de cana. PhD Thesis, Universidade Estadual de Campinas.

Wilson, J.J.; Deschatelets, L. & Nishikawa, N.K. (1989). Comparative fermentability of enzymatic and acid hydrolyzates of steam-pretreated aspenwood hemicellulose by *Pichia spitipis* CBS 5776. *Appl. Microbiol. Biotechnol.*, **31**, 592-596.

Characterization of insoluble polymers obtained from acid soluble lignin treated by oxidative enzymes

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ABSTRACT

A model effluent, containing acid soluble lignin was treated by a potato's extract containing polyphenoloxidase (PPO) activity and by the horseradish peroxidase/hydrogen peroxide system (HRP/H_2O_2). Gel permeation chromatography analysis of the soluble lignin fraction contained in the effluent indicated an average molecular weight of 365 ± 65 Da with polydispersity of 1.7 ± 0.3. Brown precipitates were obtained by one-way polymerization of the low molecular lignin fragments contained in this model effluent by PPO or HRP/H_2O_2 initiated reactions. The reaction conditions; time, pH, mg of soluble lignin/IU of enzyme activity and μmol of H_2O_2/IU of enzyme activity (only to HRP/H_2O_2 system), were previously optimized. Characterization of the macromolecules obtained at maximal yield values was performed. The overall structure of the insoluble lignin, as shown by GPC, U.V/visible, I.R. and elemental analysis were compared with the structure of the soluble lignin fragments present in the effluent. The analysis of these data allowed to conclude that the polymerization reaction occurred through the cleavage of α-β conjugated linkages followed by β-β, β-5 and 5-5 coupling. Additionally, the methoxyl group analysis indicated that p-hydroxycoumaryl units were easily oxidized by PPO. In contrast HRP oxidized preferentially guaiacyl and siringyl units.

Keywords: lignin, polyphenoloxidases, horseradish peroxidase, effluent treatment

INTRODUCTION

Enzymes are known to be highly selective catalysts and have been proposed as a means of removing target substances from wastewater (Aitken *et al.*, 1994). A number of investigators have demonstrated that several enzymes, including horseradish peroxidase (HRP) (Aitken *et al.*, 1994; Rocha, 1995; Guerra *et al.*, 1997; Guerra, 1998), laccase (Rocha, 1995; Karam and Nicell, 1997), lignin peroxidase from *Phanerochaete chrysosporium* (LiP) (Aitken *et al.*, 1994) and polyphenoloxidase (Atlow *et al.*, 1984; Guerra 1998; Guerra *et al.*, 1998; Guerra *et al.*, 1999) can catalyze the oxidation of phenolic pollutants. This oxidation generate the coupling of phenolic compounds resulting in water-insoluble polymers that can be removed, generating a less toxic waterwater.

Despite this enzymatic method is encouraging for treatment of effluent containing phenolic compounds, few efforts have been devoted to characterize the insoluble fraction produced and to evaluate the physical and chemical variables that affect the polymerization reaction (Aitken *et al.*, 1994; Guerra *et al.*, 1999). Actually, the polymerization reaction could minimize environmental problems, but it can also generate a new group of polymers with different physical and chemical properties as compared to natural and technical lignins.

The aim of this work is the characterization of end-products obtained by polymerization of acid soluble lignin contained in a model effluent by polyphenoloxidase from potato or horseradish peroxidase.

EXPERIMENTAL

Chemicals

Blue dextran (2,000,000 Da), albumin (60,000 Da), carbonic anhydrase (29,000 Da), cytochrome C (12,400) and aprotinin (6,500) were purchased from SIGMA. Steam-exploded sugar cane bagasse lignin (Mw = 4,000) was previously prepared (Ferraz *et al.*, 1997). All the other reagents were of analytical grade.

Enzyme source

A crude extract of horseradish peroxidase (EC 1.11.1.7) was purchased from SIGMA (P8000). This enzyme preparation had a specific activity of 10.0 IU. mg^{-1} of solid. One international unit activity was defined as the amount of enzyme that oxidized 1μmol of the syringaldazine per min at pH 5.0 (Szklarz *et al.*, 1989).

Potato-polyphenoloxidases were prepared by disrupting 50 g of washed potatoes cut into small pieces. Disruption was performed for 1 min in a blender containing 100 mL of 0.2 M KH_2PO_4 at pH 7.5 and 10 μmol of L-cysteine/g of potatoes. The resulting suspension was filtered through cheesecloth and centrifuged at 25°C and 4500 g for 20 min. Supernatant was dialyzed against distilled water for 12h at 4°C. Dialyzed solution was freeze-dried. Freeze-dried preparation presented a specific activity of 0.15 $IU.mg^{-1}$ of solids. Polyphenoloxidase activity was measured by chlorogenic acid oxidation monitored at 400 nm (Sánches-Ferrer *et al.*, 1993). One unit of enzyme activity was defined as the amount of enzyme necessary to produce 1μmol of quinone per min.

Effluent source and Polymerization Reaction

A liquor produced by soda delignification of steam-exploded sugar cane bagasse, which contains 22 g of $lignin.L^{-1}$ (Silva, 1995), was acidified with concentrated sulfuric acid under vigorous stirring until pH 2.0. Precipitated lignin was filtered-off and the filtrate containing the soluble fragments (5.1 $g.L^{-1}$) (effluent) was stored at 4°C. Before use, the effluent was filtered again through a Whatman-42 filter paper.

Polymerization reactions were performed in conditions optimized before (Guerra *et al.*, 1999). In a typical experiment, 10 ml of effluent was adjusted to pH 6.5 and placed into bottles containing 0.5 mg of soluble lignin/ IU of HRP or 3.4 mg of soluble lignin/ IU of PPO. The experiments with HRP started by one-way addition of 12 ml of 100 mM H_2O_2 solution. The reaction was conducted under stirring at 25-30°C, for 30 min. At the end of the reaction, the suspension was acidified to pH 2.0 and the insoluble material was recovered by filtration through a Whatman-42 filter paper. The insoluble residue was dried at 60°C to constant weight. The insolubilization yield was determined as the weight of insoluble polymer recovered divided by the initial amount of lignin in the soluble lignin fraction.

Characterization of the effluent and end-products

Molecular weight distribution was determined by gel permeation chromatography (GPC) on a 46.5 x 2.5 cm Sephadex G-50 column eluted at 0.4 $mL.min^{-1}$ with 0.5 M NaOH solution. About 2 mg of absorbed effluent (Guerra *et al.*, 1999) or end-products were dissolved in 1 mL of 0.5 M NaOH and a volume of 0.4 mL adjusted to pH 12 was directly injected into the column. The column system was calibrated with proteins of known molecular weight and steam exploded sugar cane bagasse lignin (Forss *et al.*, 1989). The void volume (V_0) and the total permeation volume (V_t) were determined by injection of blue dextran and acetone, respectively. Partition coefficients (Kd) were calculated as described elsewhere (Chum *et al.*, 1987).

UV spectra were recorded in a Cintra 20 spectrophotometer. The lignin sample was dissolved in 95 % dioxane-water solution. An aliquot of this solution was diluted and the absorbances between 240 nm and 400 nm were mensured.

FTIR spetra were obtained on a FTIR Nicolet 520 spectrophotometer using a KBr disk containing 0.5% of the sample. The absorbances were measured between 400 and 4000 cm^{-1}.

The elemental analysis and methoxyl contents were determined following the methods previously described (Ferraz *et al.*, 1997).

RESULTS AND DISCUSSION

Potato-polyphenoloxidase or horseradish peroxidase/hydrogen peroxide system was used to initiate polymerization reactions of lignin fragments contained in a model effluent. In both cases the polymerization reaction was evidenced by the formation of a brown precipitate. The reaction conditions that affect the precipitation yield were previously optimized (Guerra *et al.*, 1999) and the polymers obtained at maximal yields conditions were subjected to chemical and spectroscopic characterizations (46.0% and 46.8% for PPO and HRP/H_2O_2 initiated reactions, respectively).

Gel permeation chromatographic analysis (GPC) of the effluent and the end-products obtained by both polymerization systems are given in Figure 1. In both cases, the GPC of the brown precipitates presented a bimodal elution profile composed of a high molecular weigh fraction, which was almost excluded in G-50 Sephadex, and a permeated fraction. Weight average molecular weight (Mw), number average molecular weight (Mn) and polydispersidy (D) were calculated for the permeated fraction (Table 1). The percentage of excluded area (%A_e) in the chromatograms is also reported in Table 1.

The analysis of the chromatograms and the data reported in Table 1 confirmed that the insolubilization was a consequence of the increase in the average of molecular weight of the lignin present in the effluent. The Mw increased from 365 Da in the effluent to 800 and 2100 Da after polymerization initiated by PPO or HRP, respectively. The polydispersity of the end-products was also higher that that of the untreated effluent (Table 1). The GPC of the brown precipitates presented an excluded fraction (higher than 29 KDa), that afforded to 34 and 28% of the total chromatogram area in PPO and HRP-polymers, respectively. These high molecular weight fractions could be assigned to: (a) the presence of high molecular weight lignins and/or (b) the presence of enzyme residues that could be condensed to or adsorbed on the polymers obtained.

Table 1. Weight average molecular weigh (Mw), number average molecular weight (Mn) and polydispersity (D) of permeated fractions and the percentage of excluded area (%A_e) in the chromatograms of the effluent and the brown precipitates.

Sample	%A_e	Mw (Da)	Mn (Da)	D
Effluent	0	365 ± 65	198 ± 8	1.7 ± 0.3
PPO-polymer	34	800	367	2.2
HRP-polymer	28	2100	564	3.7

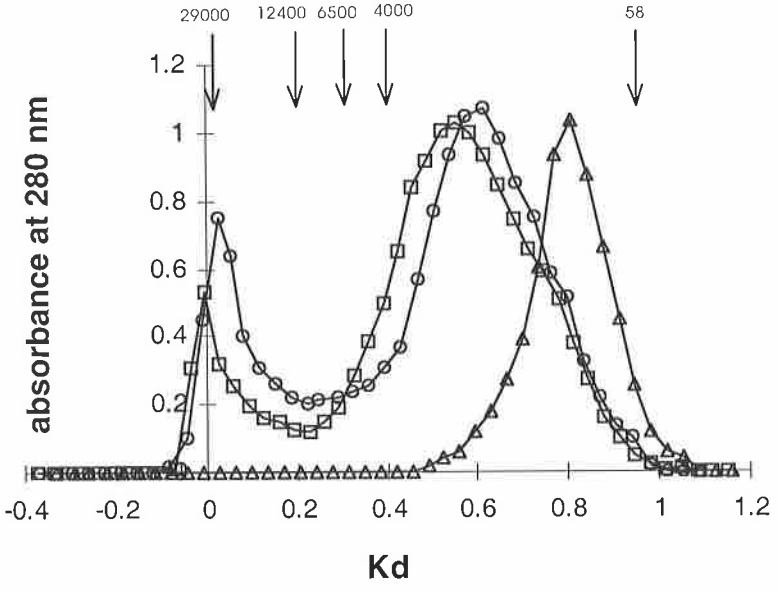


Figure 1. Gel permeation chromatography of the soluble fragments (Δ) and the brown precipitates obtained after polymerization reactions initited by PPO (--O--) and HRP/H_2O_2 system (--□--).

Further characterization of these polymers was carried out by UV (Figure 2) and FTIR spectroscopic methods and by C,H,N,O and methoxyl content determinations (Table 2). ^{1}H-NMR and ^{13}C-NMR characterization were also attempted, but the polymers were not susceptible to acetylation and insoluble DMSO.

The FTIR spectra (data not shown) of the brown precipitates showed some characteristic bands of proteins (1680 cm^{-1} related to C=O stretching in amide groups, and 3060 cm^{-1} and 1425 cm^{-1} assigned to free N-H and C-N stretching). These bands appeared overlaped to some bands characteristic of the lignin moieties.

Contamination of the polymers with enzyme residues was also confirmed by the high nitrogen contents detected (Table 2). Another data corroborating the contamination of the polymers with enzyme residues was the positive reaction of the GPC- excluded fractions with the Bradford reagent.

Considering that these lignins are contaminated with proteins their HGS classification based on the FTIR data can not be performed. However, a few inferences about the polymerization reactions are possible using the FTIR spectroscopic data. The characteristc C-H aromatic vibration in p-hydroxycoumaril units that appear at 834 cm^{-1} in the spectra of the effluent (Guerra *et al.*, 1999) disappeared in the PPO-polymer. This band absence could be related to the cresolase activity. The p-hydroxycoumaril untis, that occurs in considerable concentrations in the effluent (Guerra *et al.*, 1999), suffer hydroxylation generating 3,4 dihydroxycoumaril units by cresolase activity. The latter compounds should be polymerized originating a product containing a few residual p-hydroxycoumaril units. On the other hand, in the FTIR spectrum of the HRP-polymer the band

at 834 cm^{-1} is still present indicating the occurrence of p-hydroxycoumaril units. These data confirms different rotes of polymerization in PPO- and HRP-initiated reactions.

The methoxyl groups content of the PPO and HRP-polymers were (0.61 ± 0.01) and (2.6 ± 0.03), respectively. The comparison of these values with the methoxyl contents reported to the lignin contained in the effluent (1.2 ± 0.06) (Guerra *et al.*, 1999) suggests that the polymerization initiated by the PPO system occurred preferentially between p-hydroxycoumaril units. On the other hand, the polymerization initiated by the HRP/H_2O_2 system seems to be more effective in guaiacyl and syringyl units. This difference could be related to different enzymatic mechanisms presented by PPO and HRP. PPO tipically catalyzes two reactions:: the ortho-hydroxylation of phenolic compounds (cresolase activity) and the oxidation of catechols (catecolase activity) to ortho-quinones (Burton, 1994). These quinones are further coupled increasing the molecular weight of end products. In this way, syringyl units are not suitable to cresolase activity while the guaiacyl units are less reactive than p-hydroxycoumaril ones. On the other hand, in the polymerization initiated by the HRP/H_2O_2 system the end-product showed higher methoxyl content than the effluent. This result is in accordance with the data reported elsewhere. Hapiot *et al.* (1992) reported that phenolic compounds containing ortho substituted methoxyl groups are more susceptible to oxidation. Additionally, Jacquet *et al.* (1997) showed that in mixtures of guaiacyl, siringyl and p-hydroxycoumaril substructures, HRP polymerizes preferentially the two first ones.

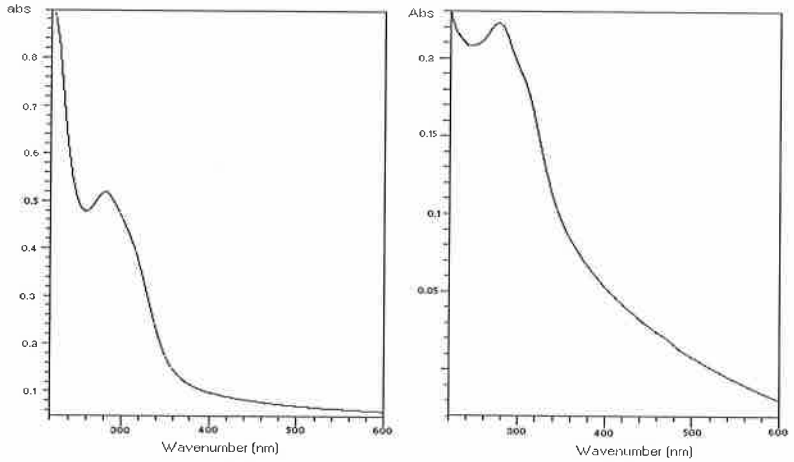
Table 2. Analytical composition of brown precipitated obtained by PPO and HRP/H_2O_2 polymerization.

Polymerized by	%C	%H	%O*	%N	% OCH_3
PPO	44.3	5.9	39.7	9.5	0.6
HRP/H_2O_2	50.9	5.2	35.5	6.0	2.6

* calculated by difference

Table 2 shows also that the C-content of the polymer obtained from the HRP/H_2O_2 initiated reaction is higher than the PPO end product whereas the hydrogen and oxygen contents are consistently lower. These changes in C, H and O contents suggest that the HRP-polymer is more condensed while the PPO-polymer is richer in oxygen. These results corroborate the differences in the polymerization reactions initiated by PPO and HRP. Once the HRP oxidizes the phenolic compounds without ortho-hydroxylation, the 5 position of the phenolic substrates are free and susceptible to condensation reactions.

The UV/Visible spectra of lignins are given in Figure 2. The UV spectra of C-18 retained material contained in the effluent showed the characteristic band of lignin near to 280 nm shifted to 310 nm, which is a characteristic of the occurrence of α-β unsaturated and α-carbonyl groups (Goldschimid, 1977). The UV spectra of the HRP and PPO-polymers showed only a shoulder at 310 nm and a strong absorption band at 274-290 nm, originating from non-conjugated phenolic groups in lignin (Goldschimid, 1977). The compilation of these data suggest that the polymerization reactions catalyzed by PPO and HRP occurred with cleavage of α-β unsaturated bonds and formation of β-5, β-β and β-1 linkages that are predominant in bulk polymerization (Tollier *et al.*, 1994).



(a) (b)

Figure 2. UV/visible spectra of the brown precipitates obtained in polymerization reactions by (a) PPO and (b) HRP/H_2O_2 systems.

CONCLUSIONS

The compilation of these results show that both PPO and HRP/H_2O_2 system are effective to remove phenolic compounds present in effluents by precipitation. This precipitation was a consequence of the increase in the molecular weight of the soluble lignin fractions. The polymerization reactions occurred with cleavage of the α-β unsaturated bonds and formation of β-5, β-β, β-1 linkages that are predominant in bulk polymerization. The polymerization reactions catalyzed by PPO and HRP occurred by different oxidation routes originating products more condensed in HRP system. However, the polymerization reactions were followed by protein linkage or adsorption on the lignin end-product.

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REFERENCES

Aitken, M., Massey, I., Chen, T. & Heck, P. (1994). Characterization of reaction products from the enzyme catalyzed oxidation of phenolic pollutants. *Wat. Res.*, **28**, 1879-1889.

Atlow, M., Bonadonna-Aparo, L. & Klibanov, A. (1984). Dephenolization of industrial wastewater catalyzed by polyphenoloxidase. *Biotechnology and Bioengineering*, **16**, 599-603.

Burton, S.(1994). Biocatalysis with poliphenoloxidase: a review. *Catalysis Today*,.**22**, 459- 487.

Ferraz, A., Souza, J., Silva, F., Gonçalves, A., Bruns, R., Cotrim, A. & Wilkins, R. (1997). Controlled released of 2,4-D from granule matrix formulations based on six lignins. *J. Agric. Food Chem.*, **45 (3)**, 1001-1005.

Forss, K., Kokkonnen, R. & Sagfors, P. (1988). Determination of the molar mass distribution of lignins by gel permeation chromatography. *ACS Sympsium. Ser.*, **397**, 124-133.

Goldshimid, O. (1977). Ultraviolet spectra. In: *Lignin-occurrence, formation, structure and reaction*, ed. K.V Sarkanen & L.H. Ludwid. Wiley/Interscience, New York.

Guerra, A. Ferraz, A., Cotrim, A.,Gonçalves, A. & Silva, F. (1997) The effect of four variables in the polymerization of acid soluble lignin by horseradish/hydrogen peroxide system as showed by statistic experimental design. *In 5th Brazilian Symposium on the Chemistry of Lignins and other Wood Components*, Proceedings, **Vol** 1, pp 293-298.

Guerra, A. (1998). Polimerização de lignina solúvel por polifenoloxidases e peroxidase/H_2O_2. MSc thesis, Departamento de Biotecnologia, Faculdade de Engenharia Química de Lorena.

Guerra A, Ferraz, A, Cotrim, A. & Silva, F. (1999). Polymerization of Lignin Fragments Contained in a Model Effluent by Polyphenoloxidases and Horseradish peroxidase/hydrogen Peroxide System. *Enzyme and Microbial Technology*, In Press.

Hapiot, P., Neta, P., Pinson, J., Rolando, C. & Shneider, S. (1993). Electrochemical and radiolytic studies on the primary polymerization step of coniferyl alcohol into lignin using model compounds. *New J. Chem.*, **17**, 211-224.

Jacquet, G., Pollet, B., Lapierre, C., Francesch, C., Rolando, C. & Faix, O. (1997). Thioacidolysis of enzymatic dehydrogenation polymers from p-hydroxyphenyl, guaiacyl, and syringyl precursors. *Holzforschung*, **51**, 349-354.

Karam, J. & Nicell, A. (1997). Potential applications of enzyme in waste treatment. *Journal of Technology and Biotechnology*, **69**, 141-153.

Rocha, G. (1995). Polimerização enzimática de lignina solúvel contida em um efluente proveniente da deslignificação alcalina de bagaço de cana. MSc. Thesis, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo.

Szklarz, G.D., Antibus, R.K., Sinsabaugh, R. & Linkins, A.E. (1989). Production of phenoloxidases and peroxidases by wood-rotting fungi. *Mycology*, **81**,234-240.

Sánches-Ferrer, A., Laveda, F. & García-Carmona, F. (1993). Partial purification of soluble potato polyphenol oxidase by partitining in an aqueous two-phase system. *Journal of Agricultural and Food Chemistry*, **41**, 1219-1224.

Silva, F.T. (1995). Obtenção de insumos químicos a partir do aproveitamento integral do bagaço de cana. PhD thesis, Instituto de Química, Universidade de Campinas.

Tollier, M., Lapierre, C. E. & Monties, B. (1994). Structural variations in synthetic lignins (DHPs) according to the conditions of their preparation. International Symposium on Wood and Pulping Chemistry, Proceedings, **Vol** 2, 35-40.

Characterization of TCF-bleached kraft pulps derived from an industrial bleaching sequence

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ABSTRACT

Kraft pulps derived from a specific TCF (Total Chlorine Free) bleaching sequence (Klabin Fabricadora de Papel e Celulose SA, Telêmaco Borba, PR) were characterized in relation to their carbohydrate, lignin and ash content. Pulps were derived from a kraft digestor, which processes a mixture of 80% softwood, and 20% hardwood chips for a TCF facility, which applies an O-Q-OP-ZQ-PO sequence (oxygen, quelation, oxygen/peroxide, ozone/quelation, peroxide/oxygen). Therefore, the resulting kraft pulp is heterogeneous in nature and requires a careful treatment to render good analytical data for both quality and process control. Pulp samples (± 92% in moisture) obtained from each bleaching stage were initially characterized for their Klason lignin content. The unbleached kraft pulp (UKP) was shown to contain 2.85% lignin and the remaining pulps, named O, Q, OP, ZQ and PO, had lignin contents of 0.90%, 0.85%, 0.31%, 0.21% and 0.21%, respectively. On the other hand, the ash content of these pulps was 0.98%, 1.32%, 0.98%, 0.81%, 0.71% and 0.77% (dry basis). The carbohydrate content of each pulp was characterized by HPLC of Klason lignin hydrolysates and the results indicated that the hemicellulose content of UKP remained relatively unmodified along the complete bleaching sequence and that lignin was the principal chemical target. Determination of the pulp weight-average degree of polymerization (DPw) by size exclusion chromatography revealed that treatment of UKP with oxygen increased cellulose DPw from 1144 to 1319 anhydroglucose (AnGlc) units and this was attributed to the selective removal of pulp lignin as well as lignin-carbohydrate complexes of low molecular mass. This higher DPw was also correlated with a shoulder of the HPSEC distribution profile at the higher molecular mass range. After that, quelation had no effect on pulp degree of polymerization (both DPw and DPn) but the following oxygen-peroxide (OP) step decreased DPw to 1284 AnGlc. The next step on the bleaching sequence (ZQ) further reduced pulp DPw to 1216 and the last bleaching stage (PO) had the most detrimental effect on DPw, bringing its average value to 938 AnGlc units. Interestingly, the DPn did not follow the same trend of DPw and this resulted in a gradual decreased in polydispersity towards the final bleaching stage.

Keywords: kraft pulps, bleaching, cellulose

INTRODUCTION

The industrial production of cellulose pulps for high quality paper making requires a sequence of bleaching steps to provide cellulose fibers with high brightness ceilings. Traditionally, the commercially viable bleaching sequences included pulp treatment with chemicals such as active chlorine, chlorine dioxide, alkali (sodium hydroxide), sodium hypochloride and types of oxygenated compounds such as oxygen itself, ozone, hydrogen peroxide (Aquino, 1995) and, more recently, enzymes (Buchert *et al.*, 1994). Many of the non-oxygenated chemicals listed above, particularly those containing active forms of chlorine, are nasty materials that have been gradually banished from industrial processes due to their aggressiveness to the environment. Therefore, more environmentally friendly technologies are required to substitute chlorine while providing the same pulp quality for paper making.

Recently, the utilization of multi-stage sequences of oxygenated compounds has been largely applied to the bleaching of kraft pulps (Aquino, 1995; Ruiz *et al.*, 1995; Sun and Argyropoulos, 1995, 1996). Oxygenated compounds allow for the elimination of chlorine from the industrial process in two possible ways: when active chlorine is banished but chlorine dioxide still remains in the process, the technology is called ECF, or elemental chlorine free, whereas when both forms of chlorine are eliminated, the technology is referred to as TCF, or total chlorine free.

This paper reports on the effect of a specific TCF bleaching sequence on the chemical composition and fine structure of kraft pulps. The sequence here described is practicized at the industrial facilities of Klabin

Fabricadora de Papel e Celulose SA in Telêmaco Borba, Paraná, Brazil, and the kraft pulp produced in their continuous digestor is derived from a mixture of hardwood and softwood chips at a 80:20 mass ratio.

EXPERIMENTAL

Pulps

The partially and fully bleached kraft pulps (± 92% in moisture) derived from a mixture of pine (*Pinus taeda*) and eucalypt (*Eucalyptus grandis*) chips were originally prepared and kindly supplied by the Klabin Fabricadora de Papel e Celulose SA (Telêmaco Borba, PR, Brazil), using an industrial kraft digestor and a TCF bleaching facility in which an O-Q-OP-ZQ-PO sequence (oxygen, quelation, oxygen/peroxide, ozone/quelation, peroxide/oxygen) is applied.

Pulp analysis

Chemical analysis of pulps was performed by a modification of the TAPPI Standard Method T222 os-74 for Klason lignin (acid insoluble lignin). After swelling 1 g of milled pulp (40 mesh) in 15 mL of 72% H_2SO_4 (m/m) for 2 h at 20 ± 1°C, distilled water was added until a 3% (m/m) acid concentration was reached and the suspension was treated for 70 min in an autoclave (Ona *et al.*, 1995). Acid-insoluble lignin was measured gravimetrically and total carbohydrates were determined by HPLC (Irick *et al.*, 1988). Acid-soluble lignin was not measured in this preliminary study.

The HPLC analyses were obtained with a Shimadzu HPLC system, model LC10AD, provided with a SIL10A autosampler and two detectors arranged in series, model SPD10A for UV spectroscopy and model RID10A for refractive index. The HPLC analysis of Klason lignin hydrolysates was performed at 65°C in an HPX-87H column (Bio-Rad), eluted with 8 mM H_2SO_4 at a flow rate of 0.6 mL/min (Irick *et al.*, 1988). All hydrolysate components with a retention time of 9.8 min were collectively analyzed as xylose because the HPLC system applied in this study could not resolve among xylose, mannose and galactose.

Pulp viscosity was measured with cupric ethylenediamine as the reaction solvent in a Viscomat Bertech apparatus, according to the Tappi T230 om-82 standard method. Kappa number was determined according to the Tappi T236 cm-85 standard method and ISO brightness was measured with a Collor Douch apparatus by light scattering.

Metals were determined in pulp by atomic absorption spectrometry using a AA 220 FS spectrometer. Approximately 0.5 g of pulp was mixed with 10 mL of 70% HNO_3 and submitted to a four-steps digestion procedure in a microwave oven at pressures ranging from 40 to 175 psi, from where the digest was transferred to a 100 mL volumetric flask and reserved for analysis.

Determination of the molecular weight distribution of cellulose

The molecular mass (MM) distribution of both the original substrate and partially hydrolyzed residues was obtained by gel permeation chromatography (GPC) of their tricarbamyl derivatives. Approximately 100 mg of the freeze-dried cellulosic material was transferred to a screw-cap assay tube and dried overnight against phosphorus pentoxide. To the dried material, 2 mL of anhydrous pyridine and 0.5 mL of phenyl isocyanate were added. The reaction was carried out for 48 h in a well of a heating block previously set at 80°C (Ramos *et al.*, 1999). The reaction was stopped by adding 1 mL of methanol to the mixture, when 1 mL was withdrawn and transferred to a round bottom flask containing 20 mL of methanol (Kossler *et al.*, 1981). The cellulose tricarbamylate was recovered after evaporation of the reaction solvents and co-evaporation with iso-octane was performed to facilitate the elimination of the residual pyridine. The pyridine-free cellulose derivative was subsequently treated with acetone, evaporated until dryness and solubilized in tetrahydrofuran (THF) at concentrations of about 0.2 mg.mL^{-1} for GPC analysis (Ramos *et al.*, 1993, 1999).

The remainder of the reaction mixture was precipitated in an excess of methanol containing few droplets of glacial acetic acid. The insoluble material was then washed with an aqueous methanol solution (80%, v/v) to remove pyridine, resuspended in water, freeze-dried and the extent of carbamylation was evaluated by Fourier-transformed infrared spectroscopy (FTIR). After milling, the tricarbamylate derivative was compacted into KBr disks (3 mg in 150 mg of KBr) for 2 min at 12 atm and analyzed using a Bomem MB-102 FTIR spectrophotometer. The spectra were recorded within the region from 500 to 4000 cm^{-1} with a resolution of 4 cm^{-1}, 64 scans and triangular apodization (Ramos *et al.*, 1999).

GPC of tricarbanyl derivatives was carried out on a Shimadzu HPLC system, model LC10AD, provided with a SIL10A autosampler and detection by UV spectroscopy, model SPD10A. Samples of cellulose tricarbamylate in THF were filtered through a Teflon membrane with a pore size of 0.45 μm and analyzed using a series of one guard column and four Tosoh TSK-GEL columns (7.8 x 300 mm) with exclusion limits of 4 x 10^7 (TSK 6000HXL), 4 x 10^5 (TSK 4000HXL), 6 x 10^4 (TSK 3000XHL) and 1 x 10^3 (TSK 1000HXL) units of molecular mass. THF was used as the eluting solvent at a flow rate of 1 mL min^{-1}. A minimal of three analyses were carried out for each replicate. The samples in the eluent were monitored by UV absorbance using a Shimadzu SPD-10A detector at the wavelength of 254 nm (Ramos *et al.*, 1999).

The GPC calibration curve was generated from the elution profile of polystyrene standards with narrow MM distributions. The universal parameters used for calibration (Mark-Houwink coefficients) were those reported by Valtasaari and Saarela (1975), $K_p = 1.18 \times 10^{-4}$ and $\alpha_p = 0.74$ for polystyrene in THF and $K_c = 2.01 \times 10^{-5}$ and $\alpha_c = 0.92$ for cellulose tricarbanylate in THF. The degree of polymerisation (DP) of cellulose was obtained by dividing the molecular mass (MM) of the tricarbanylated polymer by the corresponding MM of the tricarbanyl derivative of anhydroglucose (DP = MW/519). Pulp number average DP (DP_N), weight average DP (DP_W) and polydispersity ($P = DP_W/DP_N$) were calculated as described previously (Yau *et al.*, 1979).

RESULTS AND DISCUSSION

Pulp samples (± 92% in moisture) obtained from each bleaching stage were initially characterized for their lignin content, as determined by the classic Klason lignin procedure. The unbleached kraft pulp (UKP) was shown to contain 2.85% lignin and the remaining pulps, named O, Q, OP, ZQ and PO, had lignin contents of 0.90, 0.85, 0.31, 0.21 and 0.21, respectively (Table 1). When these data were compared to the Kappa numbers and ISO brightness obtained for each one of pulps, an interesting and somewhat expectable correlation was observed with lignin removal (Figure 1). Kappa number had a positive correlation with Klason lignin by linear regression ($y = 3.584 + 6.029*x$ for a R^2 of 0.9315 at a 95% level of confidence), whereas ISO brightness decreased exponentially with Klason lignin content ($y = 37.8 - 19.926*\ln(x)$ for a R^2 of 0.8679). However, it is important to emphasize that the Klason lignin procedure detects anything that is inherently insoluble in dilute sulfuric acid and this may also include other pulp components such as resin acids, pectin and lignin-carbohydrate complexes. On the other hand, a similar observation also applies to the determination of Kappa number in pulps and this are the reasons why small amounts of lignin are still found within pulps with high ISO brightness.

Table 1. Chemical characteristics of the kraft pulps before and after each sequential bleaching stage.

Sequential bleaching stages	Composition (%)			Kappa number (mL $KmnO_4$/g)	ISO Brightness (%)
	Glucan	Hemicellulose*	Klason lignin		
Kraft pulp	87.37	12.93	2.85	20.0	22.86
Stage O	88.35	12.36	0.90	11.6	32.85
Stage Q-1	86.22	12.42	0.85	8.95	38.84
Stage OP	85.57	11.53	0.31	6.28	55.67
Stage ZQ	87.10	12.48	0.21	4.25	64.25
Stage PO	88.75	11.98	0.21	2.56	82.33

* Hemicelluloses were quantified as xylan by HPLC.

The ash content of the TCF bleached pulps varied according to the pretreatment to which they were subjected and corresponded to 0.98, 1.32, 0.98, 0.81, 0.71 and 0.77% (dry basis), respectively. Since iron, copper and manganese are the most important metals to be monitored in this multi-stage process, each pulp was analyzed by atomic absorption spectrometry and the results are collectively shown in Table 2. As one could expect, there was no clear correlation between ash and metal content, mostly due to the inadequacy of the ash procedure for the quantitative determination of trace amounts of ash.

The carbohydrate content of each pulp was initially characterized by HPLC of Klason lignin hydrolysates. Based on these data, it seemed that the hemicellulose content of UKP remained relatively unmodified along the complete bleaching sequence and that lignin was the principal chemical target. This high selectivity is now being tentatively confirmed by capillary gas chromatography of the corresponding alditol acetates. As mentioned above, the HPLC system used in this study was not appropriate to resolve some of the pulp

hemicelluloses sugars such as xylose, mannose and galactose. Therefore, to overcome this transient limitation, the xylose peak was quantified by external calibration and used to express the total amount of hemicellulose sugars in pulp hydrolysates.

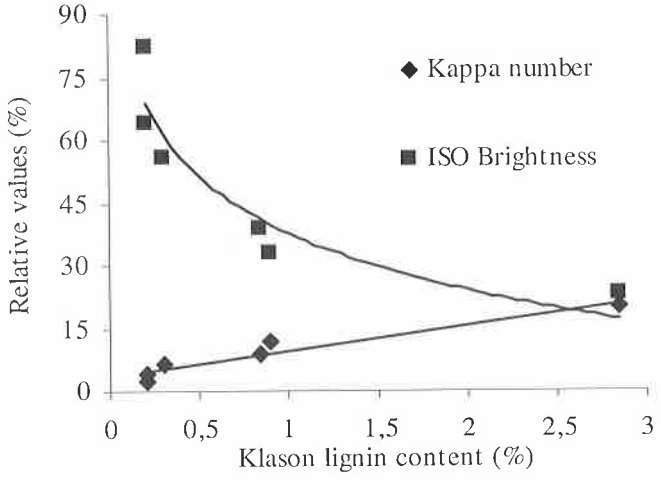

Figure 1. Correlation observed between Kappa number and ISO brightness with the Klason lignin content of pulps before and after each sequential bleaching stage.

Table 2. Ash and metal contents of the kraft pulps before and after each sequential bleaching stage.

Sequential bleaching stages	Ash content (%)	Copper (mg/L)	Manganese (mg/L)	Iron (mg/L)
Kraft pulp	0.98	2.07	3.73	10.64
Stage O	1.32	1.32	7.17	15.65
Stage Q-1	0.98	14.33	12.81	158.13
Stage OP	0.81	2.27	5.51	66.97
Stage ZQ	0.71	11.43	10.21	126.01
Stage PO	0.77	2.48	37.07	78.78

The effect of each bleaching sequence on the molecular mass (MM) distribution of cellulose was determined by high-performance size exclusion chromatography (HPSEC) of the per-carbamylated pulps in tetrahydrofuran (THF). The carbamylation reaction was monitored by FTIR spectroscopy (data not shown) through the appearance of absorption intensities typically attributed to C-N stretching in substituted urethanes (1330 cm^{-1}), N-ι stretchings (1650 cm^{-1}), symmetric (1050 cm^{-1}) and asymmetric (1230 cm^{-1}) C-O-C stretching in amides, skeletal vibrations (1420, 1505, 1600 cm^{-1}) and mono-substitution (690, 760 cm^{-1}) in aromatic rings, C=O stretching vibration in substituted urethanes (± 1730 cm^{-1}) and the N-H stretching of secondary amines (3325 cm^{-1}). There was a clear indication that most if not all cellulose hydroxyl groups had been successfully substituted at the conditions used and this was further confirmed by elemental analysis of the cellulose derivatives, which collectively showed an average nitrogen content of 8.3-8.5%.

Determination of the pulp weight-average degree of polymerization (DPw) by size exclusion chromatography revealed that treatment of UKP with oxygen increased cellulose DPw from 1144 to 1319 anhydroglucose (AnGlc) units and this was attributed to the selective removal of pulp lignin as well as lignin-carbohydrate complexes of low MM. This higher DPw was also correlated with a shoulder of the HPSEC distribution profile at the higher MM range. This shoulder did not respond for more than 3-5% of the whole population and yet had a considerable effect on DPw due to the range where it was located. After that, quelation had no effect whatsoever on pulp degree of polymerization (both DPw and DPn) but the following oxygen-peroxide (OP) step decreased DPw to 1284 AnGlc. The next step on the bleaching sequence (ZQ) further reduced pulp DPw to 1216 and the last bleaching stage (PO) had the most detrimental effect on DPw, bringing its average value to 938 AnGlc units. Interestingly, the DPn did not follow the same trend of DPw and this resulted in a gradual decreased in polydispersity towards the final bleaching stage. The final DP distribution of all pulps involved in this study is shown in Figure 2.

Changes in pulp DP followed the same trend and could be well correlated with pulp viscosity, as determined with cupric ethylenediamine as the reaction solvent (Table 3 and Figure 3). However, the average DP_W of pulp showed a much greater correlation with pulp viscosity than its average DP_N and this was readily attributed to the heterogeneity of the starting material, together with uncertainties in determining the exact location in which the distribution profile reached its unquestionable ending. GPC analyses of wide molecular mass ranges such as those exploited in this study are very difficult to validate because the baseline is not always reached at the same level of detector response. Thus, slicing at these not so well defined regions of the distribution profile may impair a significant influence on the number-average molecular mass or, in this specific case, cellulose DP_N.

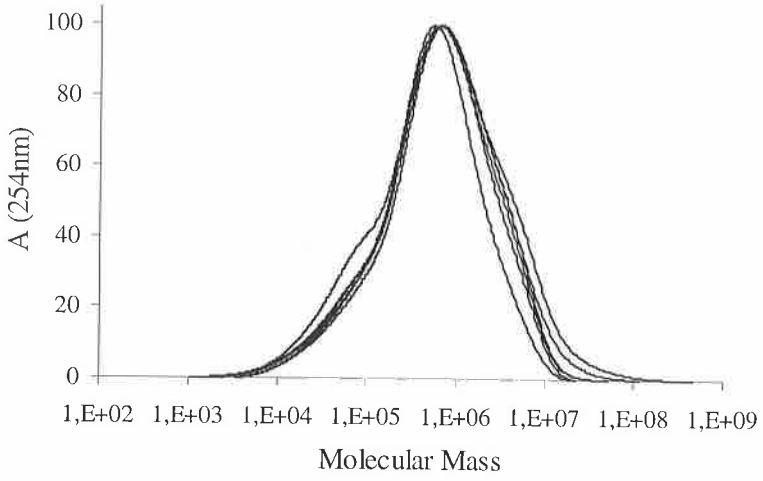

Figure 2. DP distribution of pulps before and after each sequential bleaching stage.

Table 3. Viscosity, average degrees of polymerization and polydispersity of the kraft pulps before and after each sequential bleaching stage.

Sequential bleaching stages	Viscosidade (cm^3/g)	DPw	DPn	DPw/DPn
Kraft pulp	685	1144	171	6.68
Stage O	673	1319	186	7.09
Stage Q-1	687	1320	185	7.15
Stage OP	682	1284	181	7.09
Stage ZQ	607	1216	183	6.69
Stage PO	508	938	158	5.96

When a linear regression analysis was applied to the correlation between the average DP_W and viscosity of partially bleached kraft pulps, a R^2 of 0.9534 was obtained (Figure 3, left), whereas much lower R^2 values were derived when pulp DP_N was considered for the same mathematical treatment (data not shown). Interestingly, an even better correlation was obtained when polydispersity was plotted against pulp viscosity (Figure 3, right). Pulp viscosity is a pulp property measured by capillary viscosimetry and, for this reason, the values assumed by pulps such as those included in this study are highly affected by polydispersity. Therefore, these lower polydispersities at latter stages of TCF bleaching indicated that the pulp DP distribution profile became sharper, an analytical observation that could not be easily detracted from direct viscosity measurements. This observation was apparently associated with two facts: initially, polydispersity decreased due to the removal of most of the pulp hemicelluloses, as indicated by the disappearance of the shoulder located at the lower molecular mass region of the distribution profile (Miller *et al.*, 1991) but, at the same time, cellulose molecules of higher molecular mass were also gradually converted to smaller oligomers. As shown in Figure 2, the former reactions predominated during the very first stages of bleaching, whereas the latter reactions prevailed at the last stages of the TCF sequence.

Both figures showed above have one experimental value that was not included in our linear regression analysis. These two points are those related to the kraft pulp as it was received in the TCF bleaching plant, therefore containing nearly 3% of lignin (Table 1). Therefore, both capillary viscosimetry and HPSEC of

cellulose tricarbamylate are severily influenced by residual lignin. To our understanding, calculation of the actual cellulose DP in pulps containing appreciable amounts of lignin is only possible is the lignin/hemicellulose contribution is removed from the distribution by deconvolution and this is not an easy task to pursue. Lignin and lignin-carbohydrate complexes, as well as any UV light-absorbing extraneous material that may be present within the pulp, are also characterized by different absortivities and may provide equally distinct response factors during HPSEC analysis. Therefore, the HPSEC method described in this paper can be considered a powerful technique for pulp characterization but its application to complex models such as partially lignified pulps must be handled with care.

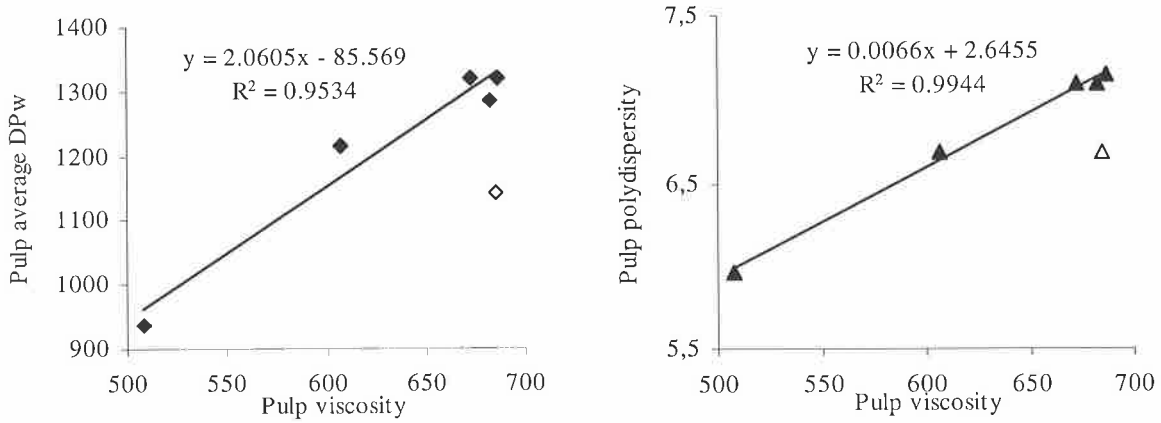

Figure 3. Linear correlations between pulp average DP_W and polydispersity with pulp viscosity.

We are currently extending this study to other types of TCF bleached kraft pulps to investigate whether the trends showed in this paper remain valid.

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REFERENCES

Aquino, F. J. T. (1997). Degradação da Lignina Organossolve do *Eucalyptus grandis* e Pré-Tratamento de Polpas de Celulose com Ozônio. M.Sc thesis, Universidade Federal de Minas Gerais, Brasil.

Buchert, J.; Tenkanen, M.; Kantelinen, A. & Viikari, L. (1994). Application of xylanases in the pulp and paper industry. *Biores. Technol.*, **50**, 65-72.

Kossler, I.; Danhelka, J. & Netopilik, M. (1981) The carbanilate method for determination of the degree of polymerization of cellulose. *Svensk. Papperstidn. Res.*, **18**, R137-R140.

Miller, D.; Senior, D. & Sutcliffe, R. (1991). Determination of the molecular weight distributions of xylanase-treated pulps. *J. Wood Chem. Technol.*, **11**, 23-32.

Ona, T.; Sonoda, T.; Shibata, M. & Fukazawa, K. (1995). Small-scale method to determine the content of wood components from multiple eucalypt samples. *Tappi J.*, **78**, 121-126.

Ruiz, J.; Freer, J.; Rodriguez, J. & Baeza, J. (1997) Ozone Organosolv Bleaching of Radiata Pine Kraft Pulp. *Wood Sci. Technol.*, **31**, 217-223.

Ramos, L. P.; Zandoná Filho, A.; Deschamps, F. C. & Saddler, J. N. (1999). The effect of *Trichoderma* cellulases on the fine structure of a bleached softwood kraft pulp. *Enzyme Microb. Technol.*, **24**, 371-380.

Ramos, L. P.; Nazhad, M. M. & Saddler, J. N. (1993). Effect of enzymatic hydrolysis on the morphology and fine structure of pretreated cellulosic residues. *Enzyme Microb. Technol.*, **15**, 821-831.

Sun, Y. & Argyropoulos, D. S. (1995). Fundamentals of high-pressure oxygen and low-pressure oxygen-peroxide (EOP) delignification of softwood and hardwood hraft pulp: a comparison. *J. Pulp and Paper Sci.*, **21**, J185-J190.

Sun, Y. & Argyropoulos, D. S. (1996). A comparison of the reactivity and efficiency of ozone clorine dioxide, dimethyldioxirane and hydrogen peroxide with residual kraft lignin. *Holzforschung*, **50,** 175-182.

Valtasaari, L. & Saarela, K. (1975). Determination of chain length distribution of cellulose by gel permeation chromatography using the tricarbanilate derivative. *Paperi ja Puu*, **57**, 5-10.

Yau, W. W., Kirkland, J. J. & Bly, D. D. (1979). *Modern Size-exclusion Liquid Chromatography*, John Wiley & Sons, New York. pp. 7-12.

Characterization of the building termite composite for ion exchange purposes

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ABSTRACT

The material produced by subway termites in their camera's construction has a complex character and is a composite mixture of earth, excrements and saliva of the termites. Literature shows that this materials, named "soil cemented" used in their house's manufacture are an organic/inorganic material and that the organic portion is basically a ligninic material allowed to argilous and inorganic substances. Their composition depends on the food habits of the termites, basically of wood origin. Investigations of this materials showed that it might act as an ion exchanger for cationic species. The exchange of alkaline and alkaline earth elements (M^{+n} = Na^+, K^+, and Ba^{+2}) is reversible and probably related to the acidic groups present in the ligninic part of this natural composite material. Characterization of this compound was performed by thermal analysis between 298 and 1073K results in a lost of 64.7 and 63.1%, respectively for original and water washed soil cemented samples. The original and water washed material present an amorphous behavior and the calcined material a poor pattern diffractogram distribution probably do to thermal treatment. The ion exchange capacities determined for studied ions were 0.4955; 0.6226 and 0.248 meq.g^{-1} for Na^+, K^+ and Ba^{+2} respectively.

Keywords: termite, composites, ion-exchange, lignin

INTRODUCTION

The interest in ion exchange materials grew after the the II World War when compounds having greater stability at high temperatures were needed, mainly for use in high radiation media in nuclear fields (Clearfield, 1988). The selectivity of these compounds towards cations has led to many applications in chromatograhic separations (Alberti *et al.,* 1962). The initial studies of ion exchangers were focused on hydrated oxides such as $ZrO_2.xH_2O$ and $SiO_2.xH_2O$. These compounds are normally prepared in amorphous or poorly crystalline forms and show amphoteric properties in sorbing or coprecipitating many ions from aqueous solutions. Zirconium phosphate played an important part in this field, being widely used as an ion exchanger material. It was initially isolated by coprecipitation at room temperature as an amorphous compound (Amphlett *et al.,* 1958). This material undergoes a complete change in properties when transformed into a crystalline form by refluxing with concentrated phosphoric acid (Hosley *et al.,* 1973; Inoue *et al.,* 1979). The resulting compound, often designated as the α form, behaves as a new exchanger.

Some natural clays, micas and zeolites, possessing ion exchange properties well defined (Bortun *et al.,* 1998c). Based on these and considering that soil cemented produced by termites is composed by a misture of organic/inorganic materials (excrements, saliva and soil), experiments were carried out to evaluate the potential of this material to act as ion exchanger. Soil cemented is a hard material with high mechanical and chemical resistance, which can be removed from the walls formed inside the subterranean galleries of termite's house.

According to literature data (Brian, 1978) the fecal materials produced by termites has a low levels of nitrogen (less than 1%), high carbon/nitrogen ratios (of 20-25% in grass feeeding species, more than 50% and approaching 100% in wood-feeding species), high lignin content (40 - 65%), low carbohydrate content (8-29%) and the lignin/cellulose ratio approaches 5:1.

Therefore, the lignin - a complex natural polymer having in its structure phenol and hydroxyl groups which are responsible for ion exchange properties - together with clay as the main constituent of the soil cemented-can be turned into a material quite promising material for ion exchange applications.

In literature very little are known concerning to the composition and chemical behavior of the soil cemented and it is just this that makes interesting this investigation.

This work study the soil cemented that was used as an ion exchange material. Their exchanger behavior was studied for sodium, potassium and barium íons as the cationic exchanged species.

EXPERIMENTAL

Reagents and materials

The material named soil cemented was obtained from a subway termite's house in their interior cameras. This material has a complex character and is a composite mixture of earth, excrements and saliva of the termites. Before use, it was powered and washed several times with destiled demineralysed water.

All reagents used, like alkaline chlorides, hydroxides and hydrochloric acid were of analytical grade and were used without purification.

Characterization of the soil cemented

The soil-cemented termite's material was characterized by thermal analysis and their crystallinity studied by X-rays powder diffractometry and by elemental analysis. Thermal analysis determinations were performed in a Shimadzu thermogravimetric instrument, TGA-50H model using about 9.00 mg of material that was heated at a 20 mL. min^{-1} rate in a dry nitrogen flux, betwen 298 and 1073 K . X-Rays diffractograms were obtained with a Rich Seifert equipament, model ISO-DEBYFLEX 100 using CuK α radiation, for 2θ values between 5 and 80. Elemental analysis was performed by a Perkin Elmer equipament, 2400 series.

Ion exchange behavior

The ion exchange of sodium, potassium and barium ions was studied in two different ways. By a titration procedure of a 0.2000 g sample of soil cemented mixed with 50.00 mL of MClx 0.100 eq.g.L^{-1} solution titred with a [MClx 0.0500+M(OH)x0.0500]eq.g.L^{-1} aqueous solutions until pH 11.0. The reverse reaction, the regeneration of the ion exchanger material, was performed in the same way by titration of the resulting solution with [HCl 0.100+MClx.0.0500] eq.g.L^{-1} until the final pH valor returned to the initial value.

A second procedure used to study the exchange process was performed in a batch way, using 0.2000 g individual samples with different OH- equivalent quantities. By mixing increase volumes of [MClx 0.0500+M(OH)x0.0500] eq.g.L^{-1} and enough mililiters of MClx 0,1000 eq.gL^{-1} up to a total volume of 50.00 mL, the pH is increased and the total metallic ion concentration kept the same at all prepared samples.The materials were stirred with aid of mechanical stirring at different times.and the curves pH x OH- miliequivalents obtained for each agitation time. By variation of the exchanger time is possible to determine the equilibrium exchanger capacity for each cation studied.

RESULTS AND DISCUSSION

The composite material produced termites is a hard dark brown product whose mechanical resistence is considerable and was collected in the internal cameras of the termite's house. It was pulverized and dried at 313 K at an electric furnace.

The powdered sample of soil cemented when washed with destiled and deionized produces a very little solubilization of the material as shown by the termal analysis results described below.

Two samples, the original and the washed ones were characterized by thermal analysis, by X-ray diffractometry and by elemental analysis(C, N and H).

The X ray results shown that both the two samples has amorphous characteristics, without any peak in the interval of 2θ between 5 and 80, confirm the results described in literature (Brian, 1978).

For the two analysed samples, the original soil cemented and the water washed soil cemented material were obtained three distint pathways corresponding to temperature ranges of 298 to 473 ; 474 to 723 and 723 to 1023 K. The material lost at the first step coresponding probably to dehydratation of interstitial water. The second and third steps that corresponding to the decomposition of the organic material of the composite mixture (cellulose, hemicellulose and lignins), contained in the fecal excrements of the termites (Brian, 1978).

Table 1 shows that the organic contribution of the soil cemented represents about 60% of the composite mixture and indicate that the losses by solubilization when the original sample were washed is very small as expected.

Elemental analysis results obtained for the washed material were C = 29.77%; H = 4.46% and N = 1.62%. These values are in accordance with the typical results of the fecal excrements of termites (Brian, 1978), with low nitrogen content and presenting a high carbon/nitrogen ratio approaches 18:1.

The ion exchange behavior

The ion exchange behavior was studied by two different procedures using titrimetric detection with aid of a glass-combined electrode measuring the pH of aqueous solutions containing the soil-cemented exchanger.

By the first procedure we may study the reversibility of the exchange by repetition of the exchanger and regenerations steps.

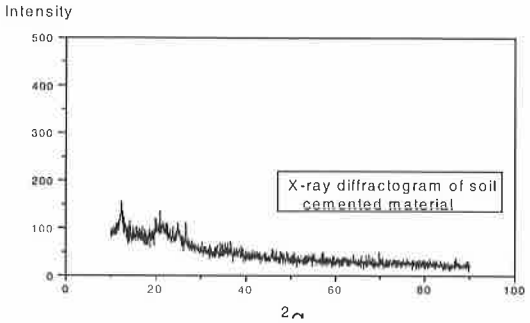

Figure 1. X-Ray diffractogram of soil cemented material.

Table 1. Experimental results obtained by thermal analysis of soil-cemented samples.

Temperature (K)	Weight loss (%)	
	Soil cemented	Washed soil cemented
298 - 473	9.2	8.4
473 - 723	50.5	49.6
723 – 1023	5.0	5.1

The second procedure determines ion exchange capacity for each ion studied at diferent agitation times. Figures 2 and 3 shows typical results obtained for the sodium ion respectively for the first and second procedures describe bellow.

By Figure 2 may be observed that this material is regenerable.

The results of ion exchange obtained shows of 0.4955 ; 0.6226 and of 0.248 meq.g^{-1}, from sodium, potassium and barium ions, respectively. These values are of the same order that those observed for synthetic inorganic exchangers (Bortun *et al.*, 1998a;b; c; Bortun *et al.*, 1997; Inoue *et al.*, 1996), which presents more chemical and thermal stability than the classical organic ion exchange (resins).

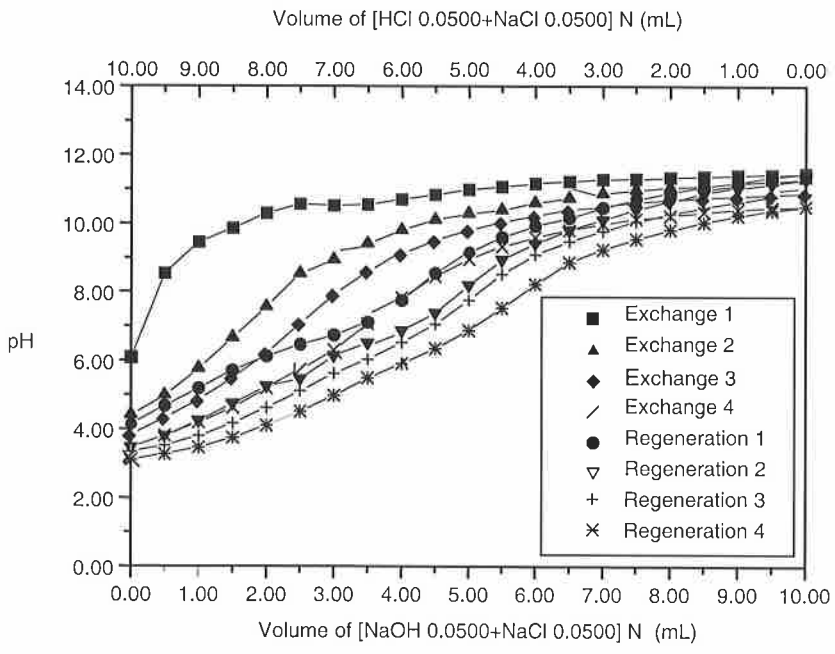

Figure 2. Reversibility study of ion exchange and regeneration process for sodium ions, using a 0.2000 g of soil cemented exchanger material.

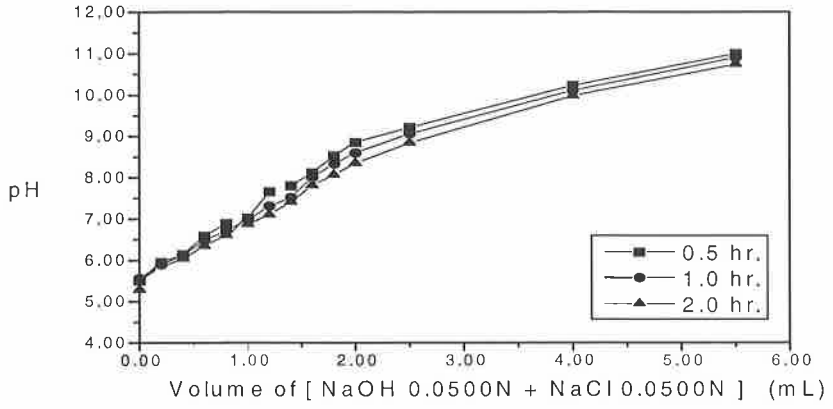

Figure 3. Typical ion exchange curves obtained for sodium exchange in order to determine the ion exchange capacities.

CONCLUSION

The soil cemented produced by subway termites can be used directly as ion exchanger for Na^+, K^+ and Ba^{+2} sorption. This material showed regenerable in the studied conditions.

Considering the soil cemented studied is a natural residue product of the termites relatively abundant in agricultural soils, therefore its uses as a sequestrant agent could be implemented at a low cost.

Ackowlegements. The authors gratefully ackowledge the financial support of this study to the Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP).

REFERENCES

Alberti, G.; Dobici, F. & Grassini, G. (1962). Chromatography on paper impregnated with inorganic ion exchangers:III Chromatography of inorganic ions zirconium phosphate paper with HCl, H2SO4, HNO3, HClO4 and CH3COOH at various concentrations, *J. Chromatography*, **8**, 103.

Amphlett, C.B.; McDonald, L.A. & Redman, M.J. (1958). Synthetic inorganic ion-exchange materials-I. Zirconium phosphate, *J.Inorg.Nucl.Chem.*, **6**, 220.

Bortun, A. I.; Bortun, L. N. & Clearfield, A. (1998a). Ion exchange behavior of the NH4 TiOPO4 based exchanger, *Solvent Extraction and Ion Exchange*, **16 (2)**, 669-681.

Bortun, A. I.; Bortun, L. N.; Khainakov, S.A. & Clearfield, A. (1998b). Ion exchange properties of the sodium phlogopite and biotite, *Solvent Extraction and Ion Exchange*, **16 (4)**, 1067-1090.

Bortun, A. I.; Bortun, L. N. ; Khainakov, S.A. & Clearfield, A. (1998c). Ion exchange properties of the sulfur modified biotite, *Solvent Extraction and Ion Exchange*, **16 (6)**, 1541-1558.

Bortun, A. I.; Bortun, L. N. ; Khainakov, S.A. ; Alonso, J.I.G. ; Garcia, J.R. & Clearfield, A. (1997). Modified titanium phosphates as cesium selective ion exchangers, *Solvent Extraction and Ion Exchange*, **15 (5)**, 895-907.

Brian, M.V. (1978). Production ecology of ants and termites, ed. Cambridge, University Press, London, pp 55-80.

Clearfield, A. (1988). Role of ion exchange in solid-state chemistry, *Chem. Rev.*, **88 (1)**, 125.

Horsley, S.E. & Nowell, D.V. (1973). The preparation and Characterization of crystalline zirconium phosphate, *J.Appl.Chem. Biotechnol.*, **23**, 215.

Inoue, Y. & Abe, M. (1996).Synthetic inorganic ion exchange materials .XLIII.Ion exchange mechanism of lithium ions on cubic tantalic acid , *Solvent Extraction and Ion Exchange*, **14 (2)**, 507-518.

Inoue,Y. & Yamada,Y. (1979). The synthesis of crystalline zirconium phosphate with large particle size by the direct precipitation methods, *Bull.Chem.Soc.Japan*, **12**, 3528.

Degradation of reactive dyes using free and immobilized laccase

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ABSTRACT

The decolorization of some reactive dyes was studied using free and immobilized laccase (produced by *Trametes villosa*), in the presence of 1-hydroxybenzotriazole (HOBT). Applying the enzyme in the free form the decolorization of reactive blue 19 (an anthraquinone dye) was higher than 50% at a reaction time of 60 min. However, for reactive black B (an azo dye), reactive red 251 and reactive orange 122 the decolorization ratios were negligible, even for higher reaction times. Several supports for enzyme immobilization were tested. Among others, the silica chemically modified with imidazole groups was the more efficient. The support permitted a complete laccase immobilization with nearly total retention of the enzymatic activity and low tendency to absorb colored substrates from the samples. When this supported enzyme was used, the decolorization of the reactive black B dye was almost complete, at reaction times lower than 120 min. This significant intensification of the enzymatic activity when the supported system was used can be explained in terms of enhancement of the enzyme stability towards chemical changes in the reaction medium.

Keywords: laccase, dyes, enzyme immobilization, effluent treatment

INTRODUCTION

The deleterious character of wastewater that contains high concentrations of reactive dyes is a well-known problem associated with dyestuff activities. In view of the relatively low fixation rates (ranging between 60 and 90%) and the low efficiency of the biological processes usually used for remediation of the effluents (activated sludge systems), about 20% of the unfixed dyes are discharged into the environment (Chao and Lee, 1994, Knapp *et al.*, 1995). Taking into account the carcinogenic or mutagenic character of some reactive dyes, the deleterious effect of the color in the receptor bodies, and the customary resistance of the effluents to biological degradation, the necessity to investigate new alternatives for the adequate treatment of this kind of residues is evident. Recently, some ligninolytic enzymes produced by white-rot fungi have been used in degradation studies involving several substrates of environmental importance. From these works (Khindaria *et al.*, 1995, Hammel, 1996, Dezotti *et al.*, 1995), it is possible to conclude that in order to increase the potential use of enzymes in wastewater treatment methods the enzymatic immobilization is absolutely necessary. The main objective of the present work was the investigation of the potential of free and immobilized laccase toward the decolorization of some reactive dyes.

EXPERIMENTAL

Reagents

The dyes were purchased from a textile mill located in Americana (São Paulo, Brazil). The chemical structure of Remazol Brilliant Blue R and Remazol Black B are presented in Figure 1. Information about the chemical nature of reactive red 251 and reactive orange 122 was not available from the current literature, presumably because of commercial confidentiality. Solutions of the dyes were prepared with distilled water in concentrations of 100 mgL^{-1}.

Laccase production

For laccase production *Trametes versicolor* CCT 4521 was maintained for 20 days at 28° C on malt extract agar media supplemented with ferulic acid. The enzyme was extracted in a 20mmol L^{-1} citrate-phosphate buffer solution (pH 5,0), filtered through a 0,45 μm Milipore sieve and lyophilized. The laccase activity was measured using syringaldazine as substrate.

Preparation of the support

Approximately 40 g of silica gel (Fluka, 70-230 mesh), previously activated for 4 h at 110 °C, was added to a solution containing 7.0 mL of 3-chloropropyltrimethoxyxilano (Fluka) and 2.4 g of imidazole (Fluka) dissolved in 300 mL of xylene. The system was maintained in reflux for 8 h at 80 °C with mechanic stirring. The functionalised silica was filtered, washing with dry ethanol and dried at vacuum. The material was characterized by elemental analysis (CHN), showing an equivalent functionalisation of 0.29 mol of imidazole groups per g of solid material.

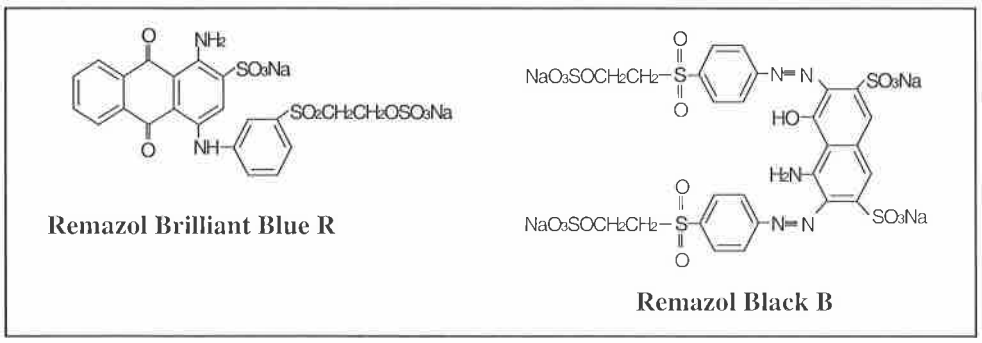

Figure 1. Chemical structure of the studied dyes.

Laccase immobilization

For enzymatic immobilization, 5.0 g of the supports were added to 20 mL of an enzyme aqueous stock solution (2000 UL^{-1}) and kept for 120 min at room temperature. The final solid material was thoroughly washed, separated by vacuum filtration and dried by suction. The amount of immobilized enzyme was determined by differential activity determinations in all the aqueous fractions obtained in the immobilization process (filtrate and washing), using syringaldazine as substrate.

Enzymatic treatment

When free laccase was used, the enzymatic reaction was conducted at 30°C in a spectrophotometric cubette using 2mL of aqueous dye solution, 50μL of laccase and 400μL of 1.0 $mmolL^{-1}$ HOBT. For immobilized laccase, the decolorization study was carried out by using 25 mL of aqueous dye solution, 0.15 g of supported laccase and 400μL of 1.0 $mmolL^{-1}$ HOBT. Samples were taken at convenient times, centrifuged at 5000 rpm for 15 min and analyzed by UV-VIS spectrophotometry (HP-8452-A), at the maximum absorption wavelength of the dye.

RESULTS AND DISCUSSION

Laccase was completely immobilized on the proposed support in contact times lower than 30 min (Figure 2). After immobilization the support was thoroughly washed with deionized water without releasing laccase from the support. Considering the volume of the laccase solution (20 mL), its activity ($2000UL^{-1}$), and the mass of support (5.0 g), it is possible calculating the activity of the supported enzyme as $8Ug^{-1}$.

Dye decolorization with free laccase

In a preliminary study, the HOBT volume was optimized approaching the maximal decolorization ratio of remazol brilliant blue R. The results (not shown) indicated that for the experimental conditions the volume of 400 μL induce the higher enzymatic decolorization ratio (approximately 40% for a reaction time of 30 min). After this preliminary optimization study, the decolorization of the four studied dyes was evaluated. The results, presented in Figure 3 indicated that the enzymatic process permits a decolorization ratio of 43% for remazol brilliant blue R, 15% for reactive red 251 and 10% for reactive orange 122, for reaction times of 30 min. For similar times, decolorization was not observed for remazol black B.

Dye decolorization with immobilized laccase

The results of enzymatic decolorization with immobilized laccase are presented in Figure 4. From these results, it is possible to observe that high decolorization ratios are obtained even in the absence of the mediator. If we consider that without the presence of HOBT the enzymatic reaction doesn't occur, we can assume that a great part of the decolorization process is due to adsorption of the substrates on the surface of the modified silica.

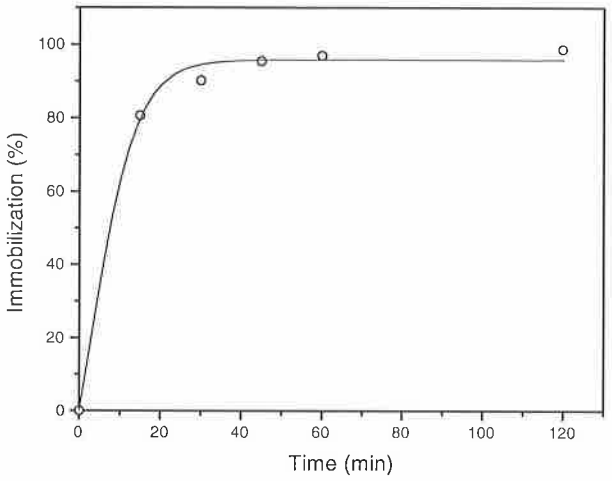

Figure 2. Immobilization of laccase on silica chemically modified with imidazole. Volume of enzyme: 20 mL; Initial activity: 2000 UL^{-1}; Mass of support: 5 g.

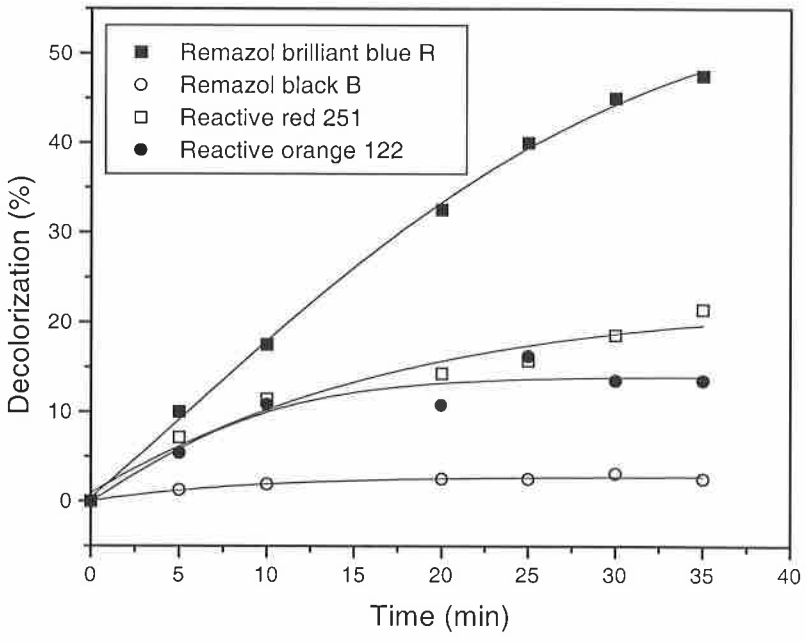

Figure 3. Dye decolorization with free laccase. Dye concentration: 100 mgL^{-1}; Laccase: 1,2 U (47,2 UL^{-1}).

In order to verify the existence of a decolorization process that combines an enzymatic reaction with adsorption of the dyes on the support surface, an experiment with successive additions of dye solution was carried out. The result (Figure 5) indicated that with the progressive saturation of the active sites of the support, the enzymatic process is manifested with higher intensity, representing in the second addition approximately 15% of the net decolorization process.

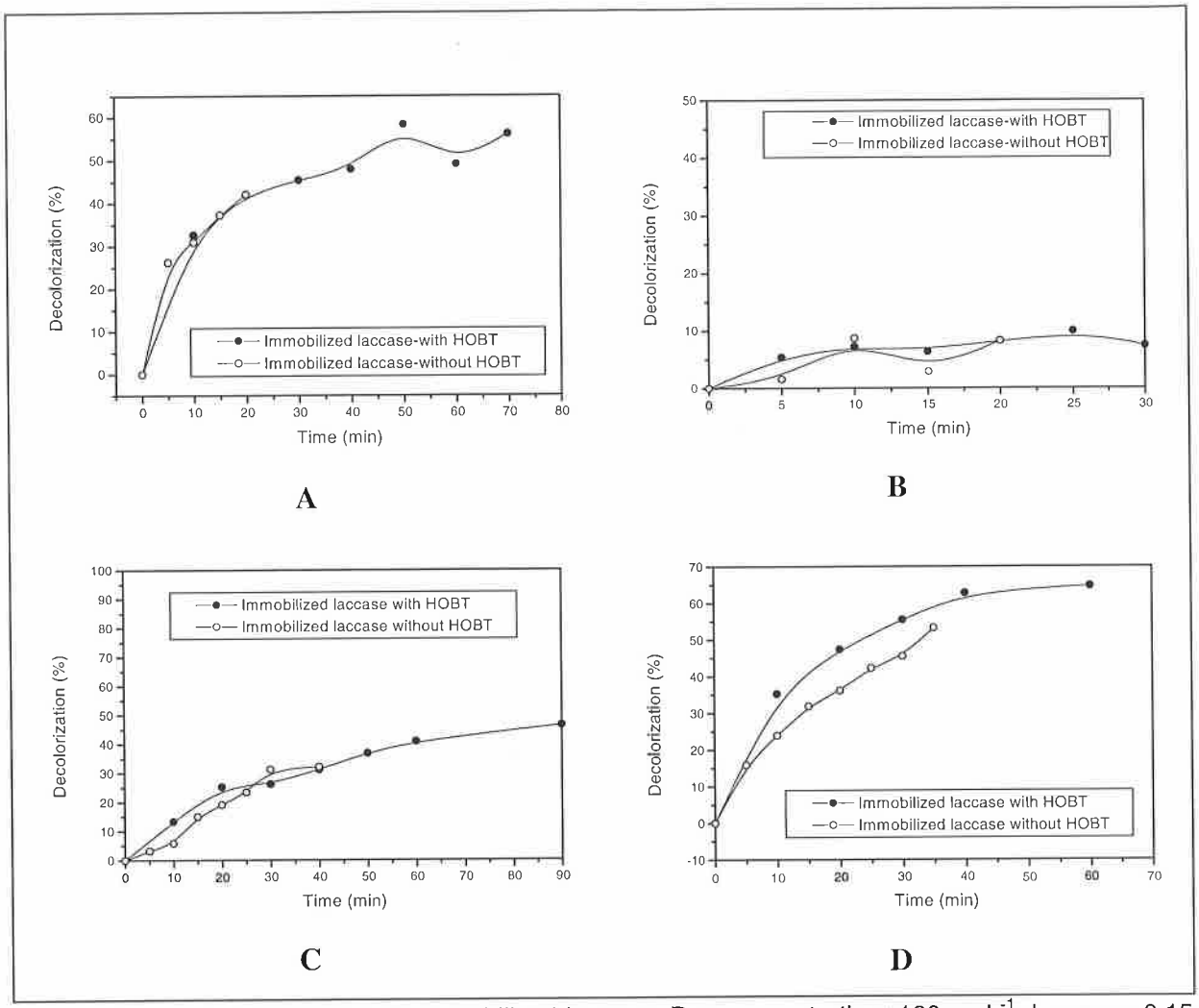

Figure 4. Dye decolorization with immobilized laccase. Dye concentration: 100 mgL^{-1}; Laccase: 0.15 g (48.0 UL^{-1}). A: Remazol brilliant blue R, B: Remazol black B, C: Reactive red 251, D: Reactive orange 122.

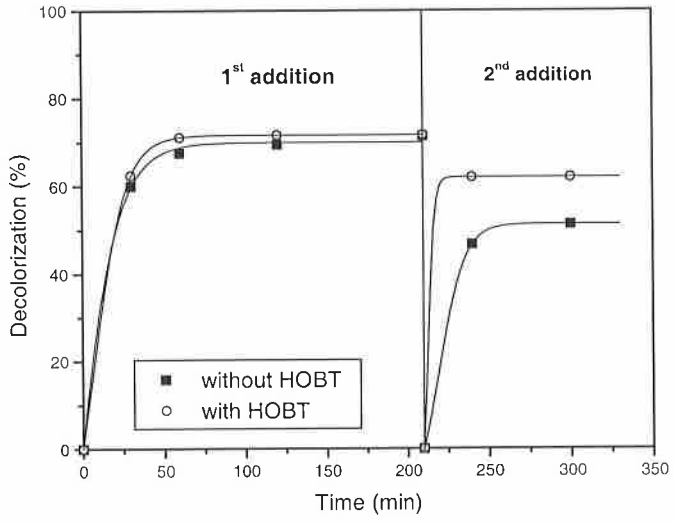

Figure 5. Remazol Brilliant Blue R decolorization with immobilized laccase (consecutive addition of dye). Dye concentration: 100 mgL^{-1}; Laccase: 0.15 g (48.0 UL^{-1}).

FINAL REMARKS

The use of ligninolytic enzymes to decolorize residues, which contain reactive dyes, is a very promissory alternative. However, the immobilized forms of the enzyme are absolutely necessary. Several supports can be used for fast enzymatic immobilization. Nevertheless, the chemical activity of the common supports is very high, which favor the decolorization by simple adsorption of the substrates. Complementary works has been demonstrated that with the progressive saturation of the active sites of the support, effective enzymatic decolorization is reached.

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REFERENCES

Chao, W.L. & Lee, S.L. (1994). Decoloration of azo dyes by three white-rot fungi: influence of carbon source, *World J. Microbiol. Biotechnol.,* **10**, 556-559.

Dezotti M., Innocentini-Mei L.H. & Durán N. (1995). Silica immobilized enzyme catalyzed removal of chlorolignins from eucalyptus Kraft effluent. *J. Biotechnol.*, **43**, 161-167.

Hammel K.E.(1996). Extracellular free radical biochemistry of ligninolytic fungi. New J. *Chem.*, **20**, 195-198.

Khindaria A., Grover T.A. & Aust S.D. (1995). Reductive dehalogenation of aliphatic halocarbons by lignin peroxidase of *Phanerochaete chrysosporium. Environ. Sci. Technol.*, **29**, 719-725.

Knapp, J.S., Newby, P.S. & Reece, L.P. (1995). Decolorization of dyes by wood-rotting basidiomycete fungi, *Enzyme Microb. Technol.,* **17**, 664-668.

Differential expression of proteins and mRNAS under iron limitation on wood degrading fungi

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ABSTRACT

Lignin biodegradation has been considered to be an enzymatic process caused by oxidative enzymes. However, the involvement of low molecular weight mediators has been postulated to be indispensable for the increase in the pore size of wood cell walls, to facilitate the enzyme penetration in the wood matrix. Low molecular weight iron-complexing agents (like siderophores) produced by fungi would mediate wood biodegradation. The mechanisms of iron-chelating compound biosynthesis and transport in wood degrading fungi has not been previously described. The objective of this study is to identify genes and proteins involved in the siderophore biosynthesis and/or transport in ligninolytic fungi by comparing the differential mRNA display and differential pattern of proteins of mycelia grown in liquid media in the presence or in the absence of Fe^{+3}. As a first screen, five species have been analyzed: *Phanerochaete chrysosporium*, *Lentinus edodes*, *Trametes versicolor*, *Laetiporus sulfureus* and *Poria cocos*. The pattern of proteins in both iron supplied or iron stress growth conditions was obtained in silver-stained SDS-PAGE gels. Proteins induced by iron restriction have been observed in all species, but *P. chrysosporium* amd *L. edodes* showed an increased number of differential bands. The size of those proteins varied from 5 to 70 KDa. In mRNA differential display experiments, a total of 57 bands for *P. chrysosporium* and 17 bands for *L. edodes* were obtained. In the *P. chrysosporium* pattern, thirty-six bands resulted from the induction of new transcripts or from the increase in the expression of existing bands in the iron(III) shortage culture and the other 21 bands were product of induction of new transcripts or increase in the expression of existing bands in the iron(III) supplied condition. Eighteen newly expressed bands from mycelia grown under iron stress have been isolated and reamplified for further characterization. The identification of genes involved in siderophore production are under investigation by cloning and sequencing those differential fragments. Our next steps will be to run a 2-D protein gel to isolate and characterize individual proteins and to sequence differential transcripts to identify iron stress expressed genes.

Keywords: wood decay, white-rot fungi, brown-rot fungi, lignin biodegradation, iron restriction

INTRODUCTION

The lignin biodegradation places a mechanistic problem to the fungal action because this amorphous heterogeneous polymer is not susceptible to normal hydrolytic mechanisms (Hammel *et al.*, 1993). With the isolation of lignin peroxidases (LiPs), which break carbon bonds in lignin model compounds, it had considered that the basic mechanism of lignin degradation by white rot fungi would have been completely understood (Glen *et al.*, 1983; Tien and Kirk, 1984). Nonetheless, given the complexity and heterogeneity of the lignin molecule, several works related to its degradation by microorganisms suppose that a relatively unspecific extracellular oxidative mechanism should exist. Actually, there are few evidences that LiPs can directly degrade lignin molecule (Sarkanen *et al.*, 1991). The main question that has been arisen against the direct action of enzymes is related to the small sized wood pore that would prevent the diffusion of LiPs (Carpita *et al.*, 1979; Flournoy *et al.*, 1991). In addition, the fact that other peroxidases (i.e., MnPs) are involved in lignin biodegradation, suggests that this is not a simple process involving only one enzymatic activity. Therefore, the theory that the process should be a result of combined action between ligninolytic enzymes and low molecular mass compounds has been reinforced (Paszczynski *et al.* 1988; Daniel, 1994).

Low molecular mass compounds with metal chelating properties are mainly considered (Enoki *et al.*, 1989; Hirano *et al.*, 1995; Goodell *et al.*, 1997). Recently, several papers describing the participation of low molecular mass compounds produced by fungi in the wood degradation have been published (Durán, 1995;

Durán and Machuca, 1995; Tanaka *et al.*, 1996; Goodell *et al.*, 1997, Kerem *et al.*, 1999, Paszczynski *et al.*, 1999). Among low molecular weight compounds, it has been proposed that the siderophores play a role in lignin biodegradation (Rodríguez *et al.*, 1997; Parra *et al.*, 1998a,b; Machuca *et al.*, 1999).

Siderophores are defined as low molecular weight chelating agents, specific for the ferric ion, produced by bacteria and fungi under iron stress (revised in Neilands, 1995). The main role of those compounds is to scavenge iron from the environment to turn it available to the microbial cell. Searches in this area have begun about five decades ago, but the interest for their isolation and characterization has been intensified with the verification that most of the aerobic and anaerobic facultative microorganisms synthesize at least one siderophore, usually related to their pathogenicity (Ecker *et al.*, 1982; Emery, 1980).

In natural environments, such as alkaline and calcareous soils or woods, the concentration of the free ferric ion is always very low. Solubilization of insoluble iron is the first step in iron assimilation. The two methods most commonly used by microorganisms for solubilization of iron are reduction of ferric iron to ferrous iron by enzymatic or nonenzymatic means and chelation. Under conditions of iron starvation, many fungi synthesize siderophores (Winkelmann, 1992; Konetschny-Rapp *et al.*, 1988; Huschka *et al.*, 1986). Two major responses to iron stress in fungi are siderophore synthesis and transport. Regulation of these two mechanisms at the molecular level has received attention.

A siderophore repressor gene designated *urbs*1 (Ustilago regulator of biosynthesis siderophore) was isolated from a genomic fragment of a mutant *U. maydis* defective in the regulation of the siderophore biosynthesis (Voisard *et al.*, 1993). The *sid*1 gene coding the enzyme that catalyzes the first step in the *U. maydis* siderophore biosynthesis was also cloned and characterized (Wang *et al.*, 1989). The expression of *sid*1 is regulated by *urbs*1 (An *et al.*, 1997). Recently, *urbs*1 homologous genes have been isolated from *Penicillium chrysogenum* (sreP) (Haas *et al.*, 1997) and *Neurospora crassa* (ser) (Zhou *et al.*, 1998) and *Aspergillus nidulans* (sreA) (Haas *et al.*, 1999). In addition, some knowledge on siderophore transport mechanism in fungi has been obtained with the isolation of the gene coding a permease involved in the uptake of ferrioxamine-type siderophores in *Saccharomyces cerevisiae* (Lesuisse *et al.*, 1998).

In this paper, we describe differential patterns of proteins and the isolation of differentially expressed mRNAs in wood degrading fungi grown under monitored conditions of presence or absence of ferric ions.

EXPERIMENTAL

Culture Conditions

The fungal growth was done in the Laboratório de Química de Lignocelulósicos no Departamento de Biotecnologia - FAENQUIL, SP. Two cultures were done: a wide scale mycelia growth for use in the molecular experiments and a small scale mycelia growth to obtaining the growth curve and measure of siderophore production. Five species of wood degrading fungi were used: *Trametes versicolor*, *Phanerochaete chrysosporium*, *Lentinus edodes*, *Poria cocos* e *Laetiporus sulfureus*. To establish a differential growth condition it was necessary to eliminate iron (III) traces from all glass flakes used and to standardize the culture conditions so that the only variable was the concentration of ferric ions in the iron supplied culture. Therefore, all material for mycelia growth was treated as follows: first, they were soaked in 1.2 M HCl for at least 48 hours, then washed in bidistillated water, autoclaved with a solution of 0.1% EDTA and rinsed in bidistillated water.

Mycelia were grown in liquid media contained 2% malt extract, with and without Fe^{3+} (80μM of $FeCl_3$). The cultures were incubated at 27 °C.

The mycelia were recovered by filtration and frozen in liquid nitrogen. The samples were lyophilized at -50°C and stored in freezer -70°C.

Total Protein Analysis

Total proteins were extracted as described by De Mot and Vanderleyden (1989) or, alternatively, it was used the method described by Kurtz and Champe (1981).

The extracted proteins were quantified by the method of Bradford (1976) and analyzed in SDS-PAGE (Laemmli, 1970). A molecular weight standard (10 kDa Protein Ladder, range 10,000-200,000 kDa) was used to estimate the apparent molecular weight of the proteins. Proteins were revealed by Coomassie blue and/or silver staining.

mRNA Differential Display

Total RNA was extracted using one of the following methods: Logemann *et al.* (1987); TRIzolTM (GibcoBRL); method for Yeast described by Windericks and Castro (1994); or, extraction of RNA from *Neurospora* developed by Sokolovsky *et al.* (1995).

For cDNA synthesis, approximately 30 µg of total RNA was treated with 10 U DNase I (Rnase-free) in a 100 µl final volume reaction containing 1.5 mM $MgCl_2$; 10 mM Tris-HCl, pH 8.0; 50 mM KCl. The solution was incubated at 37°C for 1 hour followed by extraction with phenol:chloroform and the RNA free from DNA was precipitated with sodium acetate/ethanol and stored in freezer at -70°C.

The cDNA was synthesized by reverse transcriptase using T12VN anchor primers (N = A, G or C). The reaction consisted of: 10 µM primer T12VN, 3 µl of RNA treated with DNase I in 10 µl solution were incubated at 68°C for 5-10 min, to the mixture was added 10 µl of a solution: 10 mM Tris-HCl, pH 8,3; 75 mM KCl; 3 mM MgCl2; 10 U Human Placental RNase inhibitor; 10 mM DTT; 100 mM dNTPs and 0,5 µl of Superscript II Reverse Transcritase (200U/µl) and the reaction was incubated at 37°C for 50 min. The cDNAs were stored at -20°C.

For the differential display, cDNAs of mycelia grown in the presence or in the absence of Fe^{3+} were amplified in a termociclador (Gene Amp PCR System 2400 - Perkin-Elmer) with anchor primers T12VN and the arbitrary primer OPJ-01 (sequence CCCGGCATAA, OPERON 10-Mer Kit J). Duplicate reactions consisting of: 1µg of the cDNA, 10 µM of the anchor primer, 10 µM of the arbitrary primer 20 mM Tris-HCl, pH 8,4; 50 mM KCl; 1.25 mM MgCl2; 2 µM dNTPs; 5 µCi α-^{33}P-dATP and 2.5 U Taq DNA polimerase. The amplification conditions were: 94°C (4 min), followed by: 40 cycles of 94°C (30s), 40°C (2 min) and 72°C (30 s). After the amplification, 7 µl of the PCR product was mixed to 5 µl of loading buffer: 88% (v/v) deionized formamide, 10 mM EDTA, 0,01% xylene cyanol and 0.01% bromofenol blue. The samples were denatured in 80°C for 3-4 min and loaded in 12% polyacrylamide denaturing gel. After electrophoresis, the gel was transferred to Whatman 3MM filter paper and vacuum dried and exposed to X-ray X-Omat AR films.

RESULTS AND DISCUSSION

Five different species of wood degrading fungi have been analyzed, three white rot species: *P. chrysosporium*, *L. edodes*, *T. versicolor*, and two brown rot species: *L. sulfureus* and *P. cocos*. The protein patterns of mycelia grown in iron supplied and iron stress conditions were obtained in silver-stained SDS-PAGE.

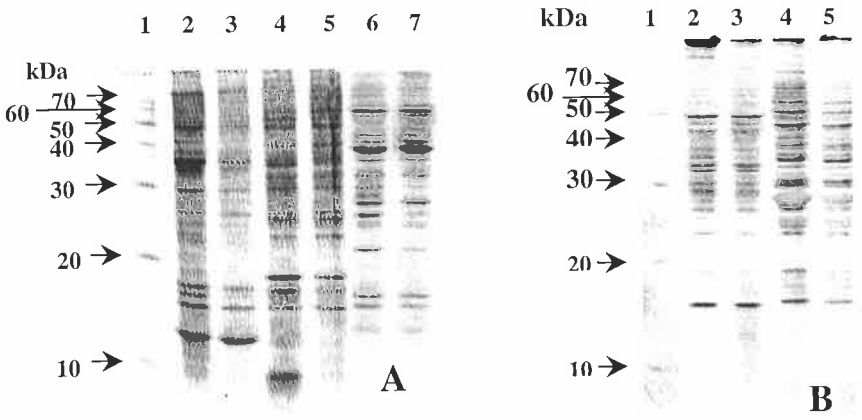

Figure 1. Differential patterns of total proteins obtained in silver-stained SDS-PAGE gels. Lane 1 in both Panels is the 10 kDa molecular weight marker. Panel A shows white rot fungi: *P. chrysosporium* under iron stress (lane 2) and in iron supplied (lane 3) conditions; *L. edodes* under iron stress (lane 4) and in iron supplied (lane 5) conditions; *T. versicolor* under iron stress (lane 6) and in iron supplied (lane 7) conditions. Panel B shows brown rot fungi: *P. cocos* under iron stress (lane 2) and in iron supplied (lane 3) conditions; *L. sulfureus* under iron stress (lane 4) and in iron supplied (lane 5) conditions.

Figure 1 shows that proteins induced by iron restriction have been observed in all species, but *P. chrysosporium* amd *L. edodes* showed an increased number of differential bands. The sizes of those proteins varied from 5 to 70 kDa.

The mRNA differential display of *Phanerochaete chrysosporium* obtained with random primer OPJ-01 and the anchor primers T12VA, T12VC and T12VG showed a total of 57 bands (Figure 2). Thirty-six bands were newly induced or they showed increased expression as a result of iron stress. The other 21 bands were newly induced or they showed increased expression in mycelia grown in iron supplied medium.

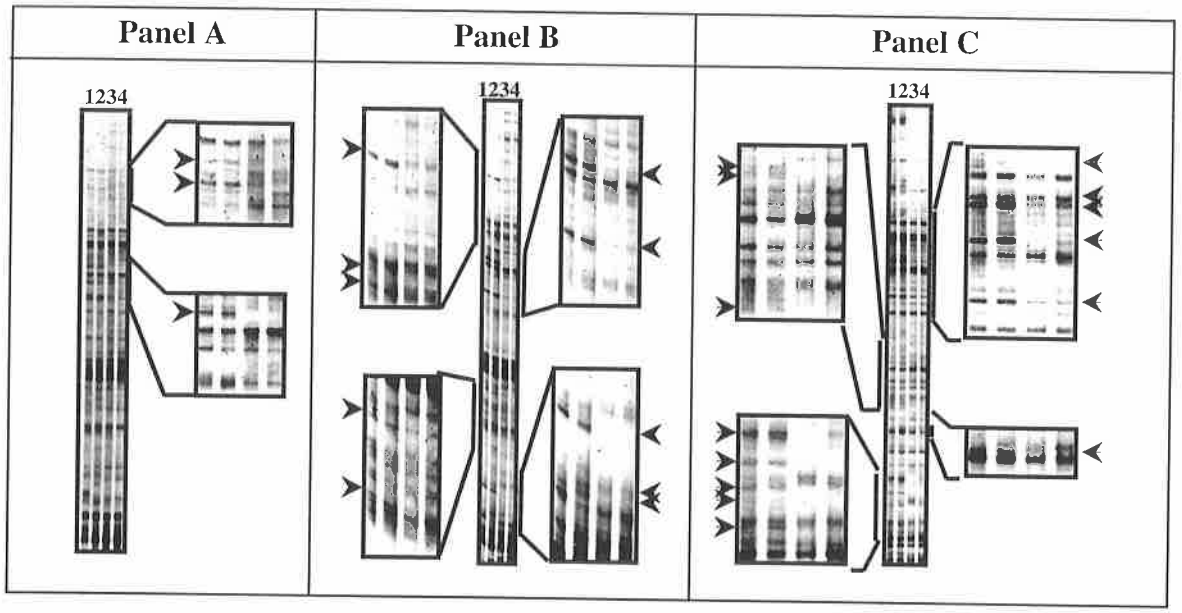

Figure 2. mRNA differential display for *P. chrysosporium* obtained with random primer OPJ-01 and anchor primers: Panel A, T12VA; Panel B, T12VC; Panel C, T12VG. Gels were loaded as follows: lanes 1 and 2, mycelia grown under iron stress conditions; lanes 3 and 4, mycelia grown in iron supplied medium. Arrows indicate bands induced by iron stress.

In the mRNA differential display of *Lentinus edodes* with the random primer OPJ-01 and the anchor primers T12VA, T12VC and T12VG it has been observed fewer differential bands being seventeen in total. The reproducibility of each display experiment was verified by comparison of different gels obtained with different mycelia samples (figures not shown). Due to the quite elevated number of bands that could be differentiated in each display, we should suppose that some of those bands can be involved in other metabolic steps influenced by iron, besides that related to the siderophore production. Some of those transcripts can be related to the high-affinity ferric iron reductase pathway, which is also iron regulated (Martins *et al.*, 1998). We are continuously isolating and sequencing differential bands to identify transcripts involved in siderophore expression/transport keeping in mind that, with this methodology, we have the possibility of isolated genes participating in other iron regulated pathways.

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REFERENCES

An, Z., Mei B., Yuan, W.M. & Leong, A.S. (1997). The distal GATA sequences of the sid1 promoter of *Ustilago maydis* mediate iron repression of siderophore production and interact directly with Urbs1, a GATA family transcription factor. *EMBO J.*, **16**, 1742-1750.

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantites of protein dye utilizing. *Anal. Biochem.*, **72**, 248-254.

Carpita, N., Sabularse, D., Montezinos, D. & Delmer, D.P. (1979). Determination of the pore size of cell walls of living plant cells. *Science,* **205**, 1144-1147.

Daniel, G. (1994). Use of electron microscopy for aiding our understanding of wood biodegradation. *FEMS Microbiol. Rev.*, **13**, 199-233.

De Mot, R. & Vanderleyden, J. (1989). Application of two-dimensional protein analysis for strain fingerprinting and mutant analysis of *Azospirillum* species. *Can. J. Microbiol.*, **35**, 960-967.

Durán, N. & Machuca, A. (1995). Kraft pulp bleaching by na extracellular low molecular weight metabolite from the ascomycete *Thermoascus aurantiacus*. *Holz Roh Werkstoff,* **53**, 346.

Durán, N. (1996). Siderophores on wood degradation: Reduction of molecular oxygen to hydroxyl radical and ferric iron to ferrous iron. In: 4th *Brazilian Symposiun on Chemistry of Lignin and other Wood Components*, Proceedings, **Vol 5** , pp.164-169.

Ecker, D.J., Passavant, C.W. & Emery, T. (1982). Role of two siderophores in *Ustilago sphaerogena.* Regulation of biosynthesis and uptake mechanisms. *Biochim. Biophys. Acta,* **720**, 242-249.

Emery, T. (1980). Iron deprivation as a biological defence mechanism. *Nature*, **287**, 776-777.

Enoki, A., Tanaka, H. & Fuse, G. (1989). Relationship between degradation of wood and production of H2O2-producing one-electron oxidases in brwon-rot fungi. *Wood Sci. Technol.*, **23**,1-12.

Flournoy, D.S., Kirk, T.K. & Highley, T. (1991). Wood decay by brown-rot fungi: changes in pore structure and cell wall volume. *Holzforsch*, **45**, 383-388.

Glenn, J.K., Morgan, M.A., Mayfield, M.B., Kuwahara, M. & Gold, M.H. (1983). An extracellular H2O2-requiring enzyme preparation involved in lignin biodegradation by the white rot basidiomycete *Phanerochaete chrysosporium. Biochem. Biophys. Res. Commun.*, **114**, 1077-1083.

Goodell, B., Jellison, J., Liu, J., Daniel, G., Paszczynski, A., Fekete, F., Krishnamurthy, S., Jun, L. & Xu, G. (1997). Low molecular weight chelators and phenolic compounds isolated from wood decay fungi and their role in the fungal biodegradation of wood. *J. Biotechnol.*, **53**, 133-162.

Haas, H., Angermayr, K. & Stoffler, G. (1997). Molecular analysis of a Penicillium chrysogenum GATA factor encoding gene (sreP) exhibiting significant homology to the *Ustilago maydis* urbs1 gene. *Gene* **184**, 33-37.

Haas, H., Zadra, I, Stoffler, G. & Angermay, R.K. (1999). The Aspergillus nidulans GATA factor SREA is involved in regulation of siderophore biosynthesis and control of iron uptake." *J. Biol. Chem.*, **274**, 4613-4619.

Hammel, K.E., Jensen, K.A. Jr., Mozuch, M.D., Landucci, L.L., Tien ,M. & Pease E.A. (1993). Ligninolysis by a purified lignin peroxidase. *J. Biol. Chem.*, **268**, 12274-12281.

Hatakka, A. (1994). Lignin-modifying enzymes from selected white-rot fungi: Production and role in lignin degradation. *FEMS Microbiol. Rev.*, **13**, 125-135.

Hirano, T., Tanaka, H. & Enoki, A. (1995). Extracellular substance from the brown-rot fungus *Tyromyces palustris* that reduces molecular oxygen to hydroxyl radicals and ferric iron to ferrous iron. *Mokuzai Gak.*, **41**, 334-341.

Huschka, H.G., Jalal, M.A., Van Der Helm, D. & Winkelmann, G. (1986). Molecular recognition of siderophores in fungi: role of iron-surrounding N-acyl residues and the peptide backbone during membrane transport in *Neurospora crassa. J. Bacteriol.*, **167 (3)**, 1020-1024.

Kerem, Z, Jensen, K.A. & Hammel K.E. (1999). Biodegradative mechanism of the brown rot basidiomycete Gloeophyllum trabeum: evidence for an extracellular hydroquinone-driven fenton reaction. *FEBS Lett.*, **446**, 49-54.

Konetschny-Rapp, S., Huschka, H.G., Winkelmann, G. & Jung, G. (1988). High-performance liquid chromatography of siderophores from fungi. *Biol. Met.* **1**, 9-17.

Kurtz, M.B. & Champe, S.P. (1981). Dominant spore color mutants of *Aspergillus nidulans* defective in germination and sexual development. *J. Bacteriol.*, **148**, 629-638.

Laemmli, V.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **247**, 37.

Lesuisse, E., Simon-Casteras, M. & Labbe, P. (1998). Siderophore-mediated iron uptake in Saccharomyces cerevisiae: the SIT1 gene encodes a ferrioxamine B permease that belongs to the major facilitator superfamily. *Microbiology*, **144**, 3455-3462.

Logemann, J., Fritsch, E.F. & Willmitzer, L. (1987). Improved Method for the Isolation of RNA from Plant Tissues. *Anal. Biochem.*, **163**, 16-20.

Machuca, A., Aoyama, H. & Duran, N. (1999). Isolation and partial characterization of an extracellular low-molecular mass component with high phenoloxidase activity from *Thermoascus aurantiacus. Biochem. Biophys. Res. Commun.,* **256**, 20-26.

Martins, L.J., Jensen, L.T., Simon, J.R., Keller, G.L. & Winge, D.R. (1998). Metalloregulation of FRE1 and FRE2 homologs in *Saccharomyces cerevisiae. J. Biol. Chem.*, **273**, 23716-23721.

Neilands, J.B. (1995). Siderophores: structure and function of microbial iron transport compounds. *J. Biol. Chem.*, **270**, 26723-26726.

Parra, C., Rodríguez, J., Baeza, J., Freer, J. & Durán, N. (1998). Iron-binding catechols oxidating lignin and chlorolignin. *Biochem. Biophys. Res. Commun.*, **251**, 399-402.

Parra, C., Santiago, M.F., Rodríguez, J. & Durán, N. (1998). Hydroxamate iron complex with phenoloxidase activity acting on lignin and chlorolignins. *Biochem. Biophys. Res. Commun.*, **249**, 719-722.

Paszczynski, A., Crawford, R., Funk, D. & Goodell, B. (1999). De novo synthesis of 4,5-dimethoxycatechol and 2,5-dimethoxyhydroquinone by the brown rot fungus *Gloeophyllum trabeum*. *Appl. Environ. Microbiol.*, **65**, 674-679.

Paszczynski, A., Crawford, R.L. & Blanchete, R.A. (1988). Delignification of wood chips and pulps using natural and synthetic porphyrins: models of fungal decay. *Appl. Environ. Microbiol.* **54**, 62-68.

Rodríguez, J., Oses, R., Parra, C., Freer, J. & Baeza, J. (1997). Siderophore Production by Lignin Degrading Fungi. In: 5th *Brazilian Symposiun on Chemistry of Lignin and other Wood Components*, Proceedings, **Vol 6**, pp. 601-604.

Sarkanen, S., Razal, R.A., Piccariello, T., Yamamoto, E. & Lewis N.G. (1991). Lignin peroxidase: toward a clarification of its role *in vivo*. *J. Biol. Chem.*, **266**, 3636-3643.

Sokolovsky, V., Kaldenhoff, R., Ricci, M. & Russo V.E.A. (1995). Fast and reliable mini-prep RNA extraction from *Neurospora crassa*., pp 3.

Tien, M. & Kirk T.K. (1984). Lignin-degrading enzyme from the *Phanerochaete chrysosporium*: Purification, characterization, and catalytic properties of a unique H2O2-requiring oxygenase. *Proc. Natl. Acad. Sci. USA*, **81**, 2280-2284.

Voisard, C., Wang, J., Mcevoy, J.L., Xu, P. & Leong A.S. (1993). Urbs1, a gene regulating siderophore biosynthesis in *Ustilago maydis*, encodes a protein similar to the erythroid transcription factor GATA-1. *Mol. Cell. Biol.*, **13**, 7091-7100.

Wang, J., Budde, A.D. & Leong, A.S. (1989). Analysis of ferrichrome biosynthesis in the phytopathogenic fungus *Ustilago maydis*: cloning of an ornithine-N5-oxygenase gene. *J. Bacteriol.*, **171**, 2811-2818.

Windericks, J. & Castro, I.M. (1994). Practical course in molecular biology of microorganisms. Universidade Federal de Ouro Preto-MG., pp59-63.

Winkelmann, G. (1992). Structures and functions of fungal siderophores containing hydroxamate and complexone type iron binding ligands. *Mycol. Res.* **96**, 529-234.

Zhou, L.W, Haas, H. & Marzluf, G.A. (1998). Isolation and characterization of a new gene, sre, which encodes a GATA-type regulatory protein that controls iron transport in *Neurospora crassa*. *Mol. Gen. Genet.*, **259**, 532-540.

Equilibrium study of humic substances model compounds and molybdenum

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ABSTRACT

Model compounds of humic and nitrohumic acids (phthalic and 3-nitrophthalic acids) have been complexed to molybdenum in aqueous solutions. Organic matter in soils and waters can arise from biodegradation of lignins among other organic materials. The main biological importance of molybdenum is its role in nitrogenase. By using potentiometric titrations, the formation constants of the complexed species among those ligands and molybdenum could be determined. The values of the constants showed that the complexation is more effective when the nitro- ligand was employed, due to the more acidity imparted by the delocalization effects of this nitro- substituent. Also the speciation according to the variation in the pH values is more effective with the nitro- ligand, since there is a bigger concentration of complexed species in the same pH value when compared to the non nitro- ligand. The humic acid and nitrohumic acids complexed to metal ions intend to be used as slow-release fertilizers.

Keywords: humic substances, lignin biodegradation, molybdenum

INTRODUCTION

The organic matter in the soil (OM - all carbon containing substances) influences plant growth, promotes good soil structure and its chemical function is seen by its ability to interact with metals, metal oxides and hydroxydes and clay minerals to form metal organic complexes and act as an ion exchanger and storehouse for N, P and S, paving way to its use as possible slow release fertilizers. The OM, a very heterogeneous system, is usually subdivided into nonhumic (carbohydrates, proteins, fats, waxes, etc) and humic structures.

Lignin is among many other compounds either from vegetal or animal a origin that by biodegradation gives way to the humic substances of soils and water. Humic acids are part of those humic substances that impart more or less fertility to soils. Metal ions are also important in this quantification due to the need in different ways and quantities for the perfect development of plantations.

Studies in the literature involving pyrolysis - mass spectrometry of humic acids (HAs) have revealed the existence of Lewis basic sites composed of -COOH and -OH groups (salicylic, phthalic and catechol derivatives) (Schulten and Schnitzer, 1993).

Further investigations (Schulten and Schnitzer, 1995) proposed a model structure for HAs which helped to understand and sometimes predict the trapping and binding of substances in soils.

Despite the great efforts in literature to clarify the complexation of metal ions with HA (Bryan *et al.*, 1998; Kim and Czerminski, 1996; Stevenson *et al.*, 1993; van Dijk, 1971), a good way still is to study model compounds of HA and then to compare the results obtained with a more intrincate chemical structure in the formation of complexed species and in the determination of binding constants (Mercê *et al.*, 1998; Mercê *et al.*, 1996; Mercê *et al.*, 1996a; Cruywagen *et al.*, 1995; Cruywagen *et al.*, 1994; Garret *et al.*, 1989).

Molybdenum and oxovanadium metal ions play an important role into a number of redox enzymes, being essential micronutrients for microorganisms, plants and animals (Dash, 1995).

Molybdenum in the soil presents a problem whenever it is deficient or excessive. The excess molybdenum in soils causes copper defficiency in animal grazing on such lands due to the metabolism of the anaerobic bacteria in the rumen of these kind of animals. Besides, as an essential micronutrient, its deficiency would also be a problem. The major molybdenum enzymes are nitrogenase, aldehyde oxidases, nitrate redutase, sulphite oxidase, xanthine oxidase and formate dehydrogenase. In the case of nitrogenase, which complexity is appaling, each molybdenum atom acts at a separate catalytic centre. Not only molybdenum atom is one of the first metals in the periodic table to undergo relatively easy M=O bond breakage (M + O) but also it presents the ability to go between oxidation states readily, and a small change is needed in the redox potentials from Mo(III) / Mo(IV) up to Mo(V) / Mo(VI), being these potentials already low values

themselves. Those characteristics have great importance when one considers all the enzyme reactions of molybdenum (Fraústo da Silva and Williams, 1998).

As the water soluble species of molybdenum is MoO_4^{2-} (molybdate), this oxoanion presents a variety of hydrolysis steps until it becomes Mo^{6+}, a cation. All these equilibria have to be considered when studying this metal ion (Fraústo da Silva and Williams, 1998; Prasad and Guimarães, 1998).

In order to continue previous studies involving Lewis basic sites derived from salicylic, phthalic and catechol compounds and some metal ions (Mercê *et al.*, 1998; Mercê *et al.*, 1996; Mercê *et al.*, 1996a) this present study shows the formation constants of the complexes between HA model compound - phthalic acid - and nitrohumic acid (NHA) model compound, 3-nitrophthalic acid (3-NPA) - with molybdenum. NHAs are laboratory artifacts obtained by the addition of nitric acid to HAs.

EXPERIMENTAL

All chemicals were used without further purification. KCl (J.T.Baker - USA) was the supporting electrolyte. Aqueous solutions ($0.1 mol.L^{-1}$) of the ligands phthalic acid (APA - Reagen - Brasil), 3-nitrophthalic acid (3-NPA - Sigma - USA), and $0.1 mol.L^{-1}$ KOH (Merck - Brasil) were prepared in double distilled and deionized carbonate - free water. The KOH solutions were standardized against potassium acid phthalate. The aqueous solution of Mo^{6+} were made from potassium molybdate hydrate (Merck - Brasil).

Three titrations were performed with the metal ion and each ligand. All the potentiometric studies were carried out in a Micronal (Brasil) model B-375 research pHmeter fitted with blue glass and calomel reference electrodes calibrated with standard HCl $10^{-2} mol.L^{-1}$ ($\mu = 0.100 mol.L^{-1}$ (KCl)) and KOH aqueous solutions to read -log $[H^+]$(pH) directly, under a stream of purified N_2 (White-Martins - Brasil). The temperature was maintained at $25 \pm 0.1°C$ and the ionic strenght adjusted to $0.100 mol.L^{-1}$ by the proper addition of KCl.

RESULTS AND DISCUSSION

The protonation constants of the ligands employed were reported earlier (Mercê *et al.*, 1998). The calculations employed the microcomputer program Best7 and the species distribution were calculated with the microcomputer program SPE (Mercê *et al.*, 1998; Mercê *et al.*, 1996; Mercê *et al.*, 1996a).

$$M + nL \leftrightarrows ML_n \quad \log \beta_n = [ML_n]/[M].[L]^n$$

Table 1. Protonation and formation constants calculated for APA and 3-NPA and Mo(VI) systems. (T=25°C; I=0.100 $mol.L^{-1}$; KCl).

log K	[HL]/[H].[L]	[H2L]/ [HL].[H]	Mo(VI) [ML]/[M].[L]	Mo(VI) [MHL]/ [ML].[H]	Mo(VI) [ML2]/ [ML].[L]	Mo(VI) [ML3]/ [ML2].[L]
3-NPA	4.00 ± 0.01	2.02 ± 0.01	5.5 ± 0.2	3.9 ± 0.2	2.9 ± 0.2	2.5 ± 0.2
APA	4.94 ± 0.01	2.75 ± 0.01	3.1 ± 0.2	n.d.	2.5 ± 0.2	2.0 ± 0.2

The potentiometric titration profiles obtained are in Figures 1 and 2. In those curves one can see an increase in the pH values in the titrations when the ligand is in the presence of the metal ion, just at the starting points compared to the ligand alone. After that, the profiles are moved to higher values of *a* as the pH values increases.

The complex formation constants as well as the protonation constants are in Table 1. These values have provided the distribution species diagrams (SPE) according to the variation of pH. Those diagrams showed a major formation of ML, ML_2 and ML_3 at pH values of 4.8, 5.5 and 6.3, respectively for APA and Mo^{6+}, and of 3.8, 4.0 and 4.6, respectively for 3-NPA and Mo^{6+}.

The chemistry of MoO_4^{2-} have shown that at the starting points of the potentiometric titrations (refer to Figures 1 and 2) the molybdenum acts as an oxoanion, taking up protons from the system, and gradually, as the pH value is increased, the oxoanion by getting rid of the oxygen atoms, becomes the cation final entity - Mo^{6+}.

So, since it is not possible to differentiate very similar species by the microcomputer calculations, the final values for the formation constant for the first complexed species (ML), which is expected in low values of pH, was calculated as average of a possible mixture of both MoL and MoO_xL, being x= 2 and 4.

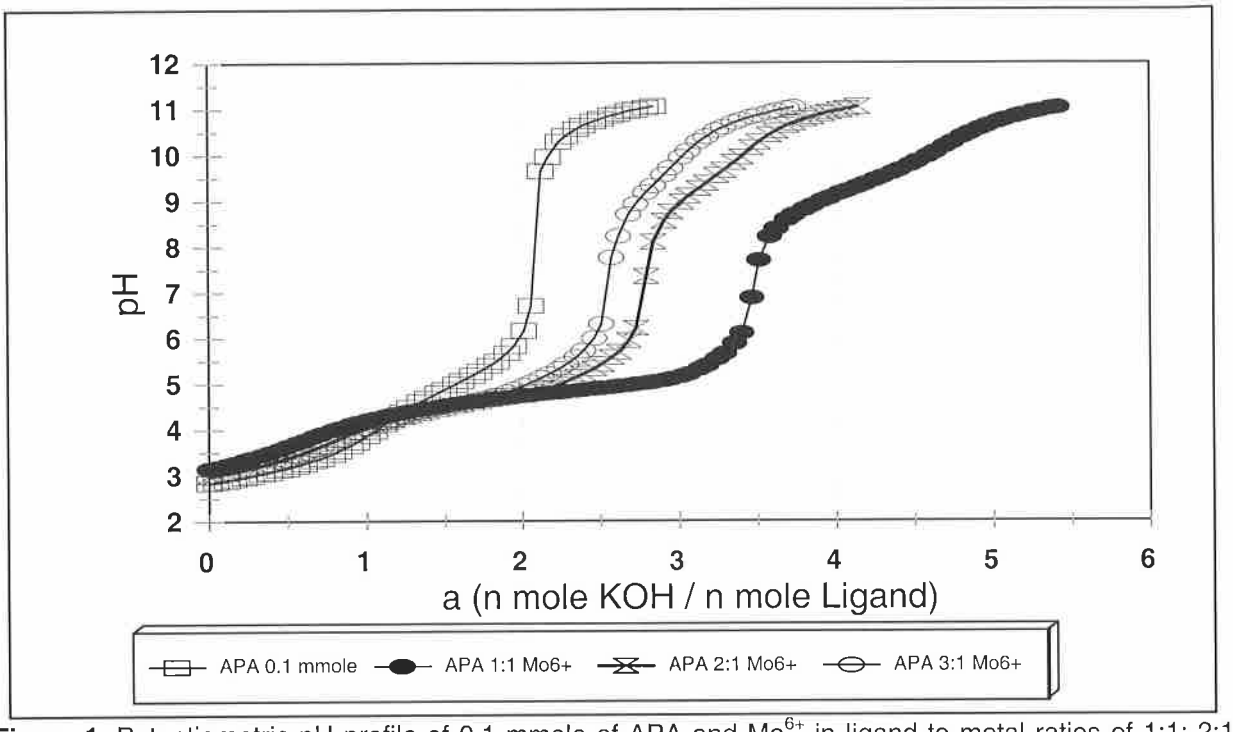

Figure 1. Potentiometric pH profile of 0.1 mmole of APA and Mo^{6+} in ligand to metal ratios of 1:1; 2:1 and 3:1.

The formation of the species with the ligand with the nitro- groups happens at early pH values than with the non nitro- ligand (phthalic acid). This effect of bringing the complexation to small values of pH is due to the delocalization of the electrons in the ligand imparted by the nitro- substituent, increasing the acidity of the nitro-compound.

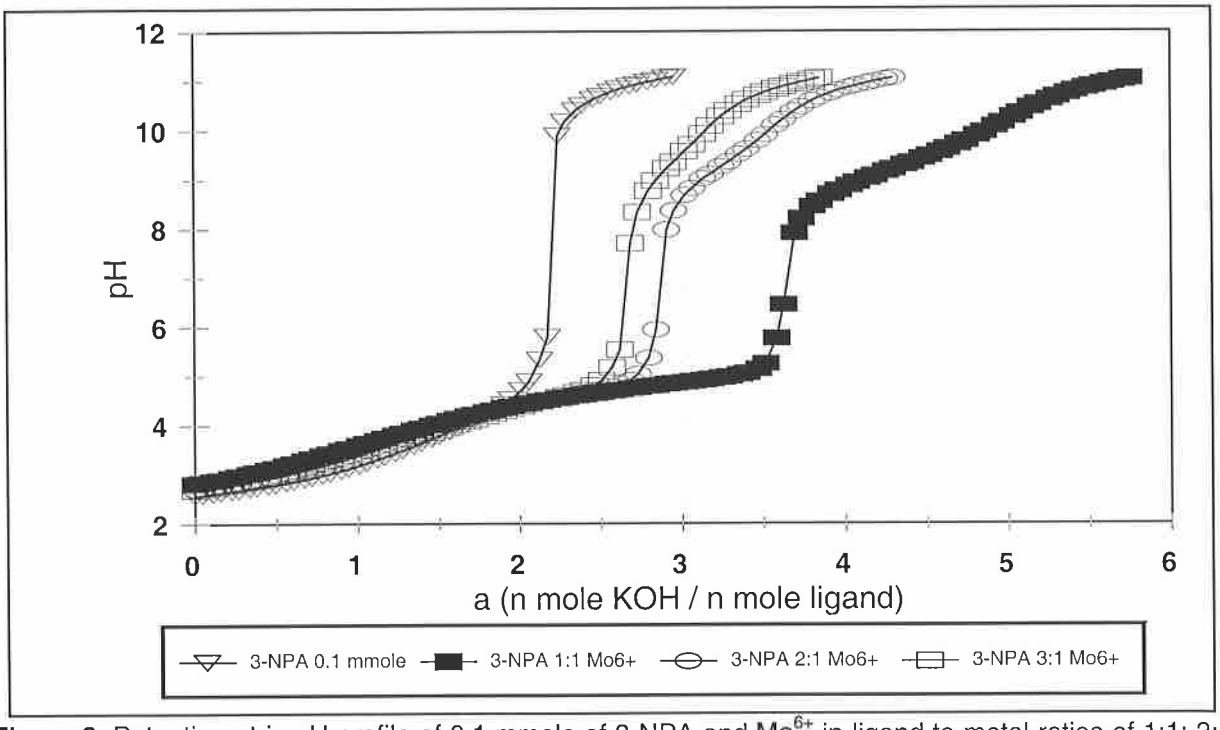

Figure 2. Potentiometric pH profile of 0.1 mmole of 3-NPA and Mo^{6+} in ligand to metal ratios of 1:1; 2:1 and 3:1.

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REFERENCES

Bryan, N.D., Hesketh, N., Livens, F.R., Tipping, E. & Jones, M.N. (1998) *J. Chem. Soc., Faraday Trans.*, **94 (1)**, 95-100.

Cruywagen, J.J., Heyns, B.B. & Rohwer, E.A. (1994). *J. Chem. Soc., Dalton Trans.*, **1994**, 45-49

Cruywagen, J.J., Rohwer, E.A. & Wessels, G.F.S. (1995). *Polyhedron,* **14**, 3481-3493.

Dash, K.C. (1995). *J. Ind. Chem. Soc.*, **72,** 359-374.

Fraústo da Silva, J.J.R. & Williams, R.J.P. (1998) *The Biological Chemistry of the Elements. The Inorganic Chemistry of Life.* Clarendon Press, Oxford, pp. 411-427.

Garrett, T.M., Miller, P.W. & Raymond, K.N.(1989). *Inorg. Chem.,* **28**, 128-133.

Kim, J.I. & Czerwinski, K.R. (1996). *Radiochim. Acta,* **73**, 5-10.

Mercê, A.L.R., Mangrich, A.S., Szpoganicz, B., Levy, N.M. & Felcman, J. (1996) *J. Braz. Chem. Soc.*, **7 (2)**, 97-102.

Mercê, A.L.R., Mangrich, A.S., Szpoganicz, B., Levy, N.M. & Felcman, J. (1996a) *J. Braz. Chem. Soc.*, **7 (4)**, 239-245.

Mercê, A.L.R., Lombardi, S.C., Mangrich, A.S., Levy, N.M. & Szpoganicz, B. (1998) *J. Braz. Chem. Soc.*, **9 (3)**, 231-242.

Prasad, S. & Guimarães, T.L.M. (1998). *J. Braz. Chem. Soc.*, **9 (3)**, 253-259.

Schulten, H.-R & Schnitzer, M. (1993). *Naturwissenschaften*, **80**, 29-30.

Schulten, H.-R & Schnitzer, M. (1995). *Naturwissenschaften*, **82**, 487-498.

Stevenson, F.J., Fitch, A., & Brar, M.S. (1993) *Soil Sci.*, **155 (2)**, 77-91.

Van Dijk, H. (1971). *Geoderma*, **5**, 53-67.

Eucalyptus tar constituents – an approach to the syntheses of potential dopaminergic compounds

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ABSTRACT

Chemical transformations of the wood tar constituents gave rise to 3,4,5-trimethoxybenzoic acid, which was converted into 3,4,5-trimethoxybenzyl chloride and it was allowed to react with 1-adamantanamine giving N-(3,4,5-trimethoxybenzyl)-1-adamantanamine. So this amine was allowed to react with different acyl chlorides affording four new amides.

Keywords: wood tar, trimethoxybenzyl-1-adamantanamine, N,N-disubstituted amides

INTRODUCTION

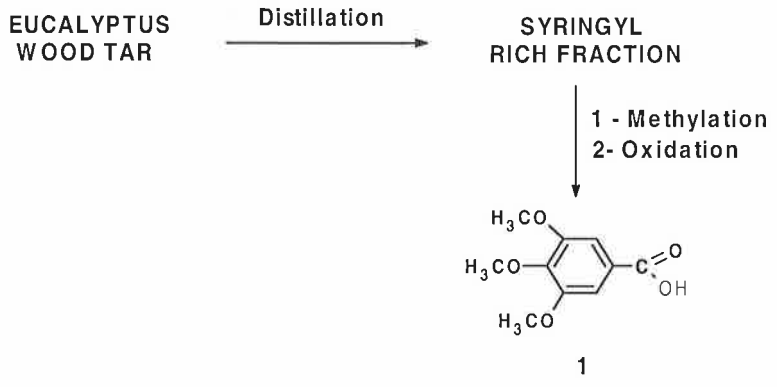
The thermal degradation of lignin results in the formation of mono, di, and trioxygenated phenols and their 4-alkyl derivatives (Carazza *et al.*, 1994). These compounds are found in different fractions of wood tar, but the 240-270 °C fraction turned out to the richest one in siringyl derivatives, which could be transformed into 3,4,5-trimethoxybenzoic acid (Carazza *et al.*, 1993).

In nature there are some substances containing methoxyphenyl groups that show pharmacological action on the central nervous system (CNS), such as the pharmacological important alkaloids including the phenylethylamines that show stimulant activity, as the 3,4,5-trimethoxyphenylethylamine, which is known for its hallucinogenic activity (Bailey *et al.*, 1972). On the other hand, amantadine HCl after clinical investigations was found as a therapeutic drug toward influenza virus strains (Aldrich *et al.*, 1971), and had also been demonstrated to benefit patients suffering Parkinson's disease (Florio, 1971; Grelak *et al.*, 1970)

Recently the peptide Cholecystokinin bearing 1-adamantylmethyl group was found to have a very good pharmacological profile and was recommended for exploratory development for the treatment of CNS disorders (Araldi *et al.*, 1998). Maniecka had investigated some potential dopaminergic compounds that bear the two moieties: methoxyphenyl group and adamantanamine in order to investigate their activity at the CNS (Maniecka *et al.*, 1987). Some of them were amides synthesized from the N-(3,4,5-trimethoxybenzyl)-1-adamantanamine **6** (Scheme 2).

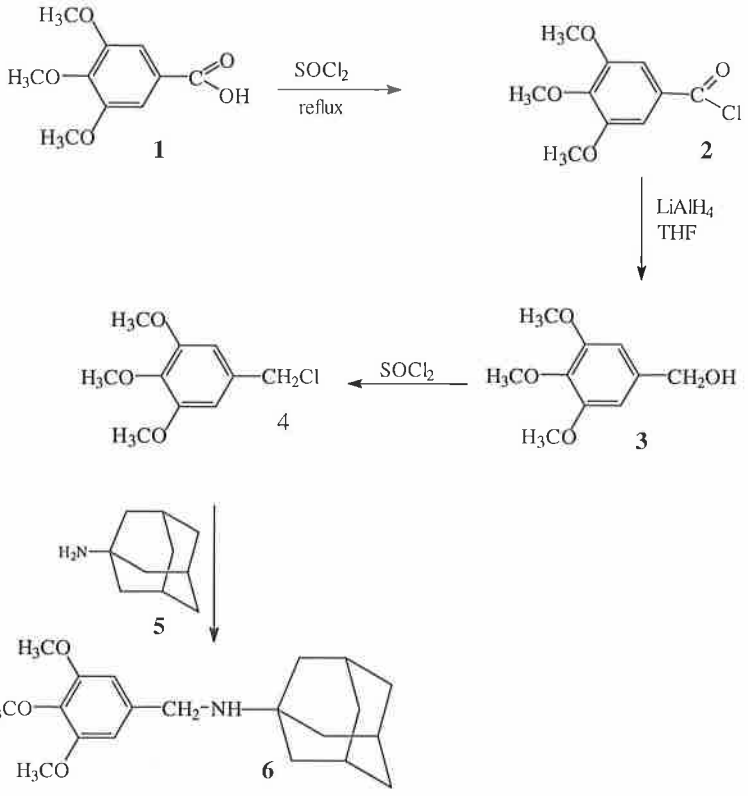
So, in the course of our studies towards the preparation of trimethoxyaryl derivatives from Eucalyptus wood tar constituents we prepared the N-(3,4,5-trimethoxybenzyl)-1-adamantanamine **6** (Carazza, F. *et al.* 1997), which was converted to novel N,N-disubstituted amides by using different acyl chlorides (Schemes 1, 2 and 3).

Scheme 1

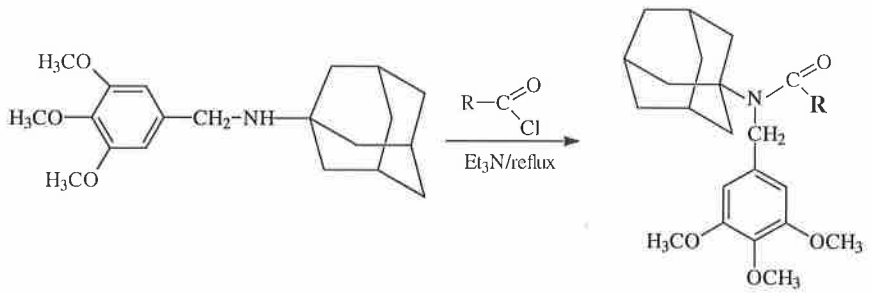


1

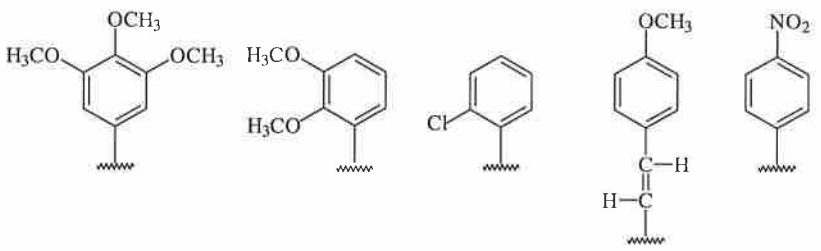
Scheme 2



Scheme 3



R



7 8 9 10 11

EXPERIMENTAL

All melting points are uncorrected. IR analyses were performed on a Shimadzu/IR-408 spectrometer; NMR spectra were recorded on a Brucker Avance DRX400 and Brucker AvanceDRX200 using TMS as the internal standard. HRGC-MS/C analyses were carried out with a HP 5989-A-System (70eV) column 25m; 0,2 mm i.d.; phase HP-1; 0,33 μm; using He as carrier gas. Oven 150 °C, 1min, 5 °C/min to 280 °C, 15 min

isothermal. Commercially available organic and inorganic compounds were used without further purification except for the solvent, which was distilled by standard methods before use (Perrin *et al.*, 1980).

General Procedure for Preparations of:

N,N-(3,4,5-trimethoxybenzyl)-R-1-adamantanamine (7 - 11): In a 50mL round bottomed flask, equipped with a reflux condenser, were placed 3 mmol of carboxylic acid and 9 mmol (0,6 mL) of thionyl chloride. The flask was heated to gentle reflux until TLC (3:1 hexane-ethyl acetate) showed no starting material (approximately 4-8 h). Excess $SOCl_2$ was removed under reduced pressure, and the residue rinsed twice with dry benzene, and evaporated, and the residue was taken up in $CHCl_3$. To N-(3,4,5-trimethoxybenzyl)-1-adamantanamine **6** (827 mg; 2,5 mmol) dissolved in anhydrous $CHCl_3$ (10mL) and placed under N_2, was added a solution of acid chloride. The reaction mixture was stirred at room temperature for 0,5 h. Then freshly distilled triethylamine (0,2 mL) was added and the temperature was elevated to 55 °C. After 1 h, more triethylamine (0,1mL) was added. The reaction mixture was stirred until the starting acid chloride was consumed (approximately 4 - 5 h, according to TLC using 3:1 hexane-ethyl acetate).

Compounds 7, 8 and 9: The product was extracted with $CHCl_3$ and dried over sodium sulfate, filtered, and concentrated to give a white solid. The crude material was purified by flash chromatography with ethyl acetate/hexane (1/3), and recrystallized from ethanol.

Compounds 10 and 11: The solution was washed successively with water, NaOH 5%, and water, dried over sodium sulfate and concentrated. The product was recrystallized from ethanol.

N,N-(3,4,5-trimethoxybenzyl)-(3,4,5-trimethoxybenzoyl)-1-adamantanamine 7: yield 74%, white solid, mp 132-139 °C (Maniecka *et al.*, 1987). IR (film, cm^{-1}, ν): 2900, 1640, 1580, 1500, 1450, 1410, 1380, 1340, 1320, 1230, 1130, 1000, 900. ^1H RMN ($CDCl_3$): δ 6,60 (s, 2H), 6,48 (s, 2H), 4,55 (s, 2H), 3,88 (s, 6H), 3,82 (s, 3H), 3,79 (s, 3H), 3,65 (s, 6H), 2,37 (m, 6H); 2,11 (br s, 3H), 1,69 (m, 6H). ^{13}C NMR ($CDCl_3$): δ 173,46; 153,35; 152,90; 138,66; 136,72; 136,66; 134,58; 103,50; 103,09; 60,93; 60,83; 59,85; 56,19; 55,91; 50,37; 3974; 36,46; 30,17. EIMS m/z (%): 525 (10) $M^{+\cdot}$, 390 (57), 195 (100), 181 (51),135 (23), 93 (12), 70 (16). Anal. Calcd for $C_{30}H_{39}O_7N$: C 68,57; H 7,43; N 2,66. Found: C 68,27; H 7,56; N 2,62.

N,N-(3,4,5-trimethoxybenzyl)-(2,3-dimethoxybenzoyl)-1-adamantanamine 8: yield 78%, white solid, mp 199-200 °C. IR (film, cm^{-1}, ν): 2900, 1640, 1580, 1500, 1450, 1400, 1260, 1230, 1130, 1070, 1000, 990. ^1H RMN ($CDCl_3$): δ 6,95 (t, 1H, J = 8 Hz), 6,82 (dd, 1H, J = 8 Hz, 1 Hz), 6,76 (dd, 1H, J = 8 Hz, 1 Hz), 6,43 (s, 2H), 4,53 (d, 1H, J = 17 Hz), 4,38 (d, 1H, J = 17 Hz), 3,90 (s, 3H), 3,84 (s, 9H), 3,81 (s, 3H), 2,34 (br s, 6H), 2,08 (br s, 3H), 1,65 (m, 6H). ^{13}C NMR ($CDCl_3$): δ 170,49; 153,07; 152,73; 144,20; 136,27; 134,64; 124,57; 118,63; 112,16; 102,94; 61,63; 60,88; 59,77; 56,03; 55,58; 50,10; 39,70; 36,29; 30,12. EIMS m/z (%): 495 (6) $M^{+\cdot}$, 360 (41), 181 (25),165 (100), 122 (10), 79 (20). Anal. Calcd. for $C_{29}H_{37}O_6N$: C 70,30; H 7,47; N 2,83. Found: C 70,12; H 7,40; N 2,76.

N,N-(3,4,5-trimethoxybenzyl)-(2-chlorobenzoyl)-1-adamantanamine 9: yield 81%, white solid, mp 170-172 °C. IR (film, cm^{-1}, ν): 2910, 2850, 1640, 1580, 1510, 1460, 1390, 1330, 1240, 1130, 1050, 1000, 990, 960, 820, 750. ^1H RMN ($CDCl_3$): δ 7,33-7,31 (m, 1H), 7,22-7,12 (m, 3H), 6,39 (s, 2H), 4,58 (d, 1H, 17,6 Hz), 4,30 (d, 1H, J=17,6 Hz), 2,37 (br s, 6H), 2,10 (br s, 3H), 1,72-1,63 (m, 6H). ^{13}C NMR ($CDCl_3$): δ 169,89; 153,28; 138,38; 136,72; 135,65; 129,80; 129,55; 129,43; 127,40; 126,95; 102,92; 61,81; 60,92; 60,40, 59,14; 55,88; 49,63; 39,80; 36,38; 30,32; 30,18; 30,01; 29,70. Anal. Calcd for $C_{27}H_{32}O_4NCl$: C 68,44; H 6,82; N 2,98. Found: C 68,77; H 6,73; N 2,82.

N,N-(3,4,5-trimethoxybenzyl)-(4-trans-methoxycinamoyl)-1-adamantanamine 10: yield 45%, white solid, mp 170-172 °C. IR (film, cm^{-1}, ν): 2980, 2980, 2850, 2820, 1650, 1600, 1570, 1550, 1500, 1450, 1400, 1375, 1340, 1270, 1250, 1225, 1200, 1175, 1130, 1050, 1000, 870, 800. ^1H RMN ($CDCl_3$): δ7,59 (d, 1H, J = 16 Hz), 7,29 (dd, 2H, J = 8,1Hz; 2,0Hz), 6,81 (dd, 2H, J = 8,1Hz; 2,0Hz), 6,52 (d, 1H, J = 16 Hz), 6,51 (s, 2H), 4,64 (s, 2H), 3,87 (s, 3H), 3,84 (s, 1H), 3,78 (s, 1H), 2,28 (br s, 6H), 2,09 (br s, 3H), 1,67 (m, 6H). ^{13}C NMR ($CDCl_3$): δ 169,08; 160,65; 153,57; 141,61; 136,79; 135,80; 129;19; 128,21; 119,53; 114,13; 102,70; 60,87; 59,28; 56,15; 55,27; 47,79; 39,79; 36,44; 30,12. EIMS m/z (%): 491 (19) $M^{+\cdot}$, 356 (13), 181 (22), 161 (100),135 (41), 133 (24), 118 (12), 107 (15), 105 (6), 94 (35), 92 (14), 91 (7), 80 (39), 78 (21), 68 (13). Anal. Calcd for $C_{29}H_{37}O_5N$: C 72,65; H 7,72; N 2,92. Found: C 72,87; H 7,83; N 2,56.

N,N-(3,4,5-trimethoxybenzyl)-(4-nitrobenzoyl)-1-adamantanamine 11: yield 88%, white solid, mp 176-178 °C. IR (film, cm^{-1}, ν): 2900, 1700, 1640, 1540, 1450, 1400, 1350, 1260, 1240, 1160, 1130, 1000, 960, 860,

830, 750, 700, 660. ^{1}H RMN ($CDCl_3$): δ 8,15 (dd, 2H, J = 8,5 Hz; 1,6Hz), 7,23 (dd, 2H, J = 8,5 Hz; 1,6Hz), 6,37 (s, 2H), 4,46 (s, 2H), 3,84 (s, 9H), 2,34 (br s, 6H), 2,12 (br s, 3H), 1,66 (m, 6H). ^{13}C NMR ($CDCl_3$): δ 171,39; 153,51; 147,76; 145,29; 137,21; 135,03; 126,82; 123,75; 103,07; 60,91; 60,33; 56,21; 49,99; 39,81; 36,30; 30,15. EIMS m/z (%): 480 (14) $M^{+\cdot}$, 346 (10), 345 (56), 329 (22), 328 (24), 181 (67),150 (69), 135 (100), 120 (53), 108 (30), 105 (58), 94 (71), 92 (44), 82 (21), 80 (79), 77 (23), 68 (34). Anal. Calcd for $C_{29}H_{37}O_6N$: C 67,50; H 6,67; N 5,83. Found: C 67,25; H 6,95; N 5,60.

RESULTS AND DISCUSSION

As described previously the 3,4,5-trimetoxybenzoic acid **1**, obtained by oxidation of the previously methylated fraction from *Eucalyptus* tar (Scheme 1), was converted to the correspondent acid chloride **2**. The acid chloride was reduced to the benzyl alcohol, which was converted to the benzyl chloride in presence of HCl. The benzyl chloride **4** was allowed to react with 1-adamantanamine affording the N-(3,4,5-trimethoxybenzyl)-1-adamantanamine **6** (Scheme 2). The Schotten-Baumann reaction, previously used by us (Carazza *et al.*, 1993) gave poor results, so the acylation of the N-(3,4,5-trimethoxybenzyl)-1-adamantanamine by benzoic acid chlorides proceeded on gentle reflux in chloroform in the presence of triethylamine. This method gave better results than the former. ^{13}C-NMR and Dept 135 spectra gave evidence of a carboxyl group δ 168-173 and a benzyl carbon at δ 50-47. High-resolution of ^{1}H-NMR spectra showed the singlet at δ 6,5-6,3 integrated for two hydrogens, showing the trimethoxybenzyl group to be symmetrically substituted. The spectrum of compounds **8** and **9** displayed a set of doublets at δ 4,53 and 4,38 (J=17,0 Hz) for compound **8**, 4,58 and 4,30 (J=17,6 Hz) for compound **9**, indicating a non-equivalence of the benzyl hydrogen. The spectrum of **10** showed two doublets at δ 7,59 and 6,52 (J=16 Hz) integrated for one hydrogen that is an indicative of a trans-cinnamoyl derivative. The synthetic route to tertiary amides **7-11** is outlined in Scheme 2.

This is the first report on the preparation of N,N-(3,4,5-trimethoxybenzyl)-(2,3-dimethoxybenzoyl)-1-adamantanamine **8**, N,N-(3,4,5-trimethoxybenzyl)-(2-chlorobenzoyl)-1-adamantanamine **9**, N,N-(3,4,5-trimethoxybenzyl)-(4-trans-methoxycinamoyl)-1-adamantanamine **10** and N,N-(3,4,5-trimethoxybenzyl)-(4-nitrobenzoyl)-1-adamantanamine **11**.

REFERENCES

Aldrich, P.E., Hermann, E.C., Meier, W.E., Paulshock, M.; Prichard, W.W.; Snyder, J.A. & Watts, J.C. (1971). Antiviral Agents. 2. Structure-Activity Relationships of Compounds Related to 1-Adamantanamine. *J. Med. Chem.*, **14**, 535-543.

Araldi, G.L.; Donati, D.; Tranquillini, M.E. & Ursini, A. (1998). Diastereomeric separation of 1,5-benzodiazepines due to the presence of a chiral center on the N-5 alkylic chain. *Il Farmaco*, **53**, 49-54,

Bailey, P.T. & Pradhan, S.N.(1972). Effects of Δ^9-Tetrahydrocannabinol and mescaline on self-stimulation *Neuropharmacology*, 831-838.

Carazza, F.; Alcântara, M.F.C. & Pereira, M.O.S. (1993). Synthesis of amides containing 3,4,5-trimethoxyphenyl groups from Eucalyptus wood tar. *3th Brazilian Symposium on the Chemistry of Lignins and other wood Components*, **Vol. 1**, pp 268-271.

Carazza, F.; Oliveira, R.A.; Alcântara, M.F.C. & Pereira, M.O.S. (1997). The utilization of methoxyaryl compounds from Eucalyptus wood tar in fine chemistry. *5th. Brazilian Symposium on the Chemistry of Lignin and Other Wood Components*, **Vol. 1**, pp 308-314.

Carazza, F.; Resende, M.E.A.; Pasa, V.M.D. & Lessa A. (1994). *Advances in Thermochemical Biomass Conversion*, Bridger, ed. A.V.; London, pp 1465-1474.

Florio, V. & Longo, V.G. (1971). Effects of amantadine and of some synthetic antiparkinsonian drugs on the behavioral response to L-dopa in mice. *Physiology and Behavior*, **6**, 465-466.

Grelak, R.P. (1970). Amantadine-dopamine interaction: Possible mode of action in Parkinsonism. *Science*, **169**, 203-204.

Maniecka, W. & Plachta, D. (1987). Poszukiwanie Zwiazkow o Dzialaniu Dopaminergicznym. *Acta Poloniae Pharmaceutica*, **44**, 305-308.

Perrin, D.D. & Armarego, W.L.F. (1980). *Purification of laboratory Chemicals*. 2nd. Ed. Pergamon Press, New York.

Evaluating lignocellulolytic enzymes secreted by *Poria medula-panis* during wood decay

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ABSTRACT

The lignocellulolytic enzyme profiles of the white-rot fungus *Poria medula-panis* grown on wood chips in solid medium were studied. The wood decay was conducted during 90 days and the proteins were recovered at different times. Hydrolytic and oxidative enzymes produced by *P.medula-panis* were detected in the protein extracts. Cellulase and xylanase enzymes were recovered in highest levels at 15 days of decay. β-glucosidase reached a maximum at 30 days, and remained almost constant until the end of decay period. High peroxidase activity was observed at 15 days whereas laccase activity was low at this time. A peak of laccase was observed at 30 days, after which the activity decreased significantly. The protein patterns present in the fungal extracts were analyzed using polyacrylamide gel electrophoresis (SDS-PAGE). The higher band numbers appeared at 60 days of decay. At this stage, protein bands exhibited the widest range of molecular weight (approximately 30-66 kDa). At the early and advanced stages few protein bands were observed. The SDS-PAGE results together enzyme activity assays, suggested the presence mainly of phenoloxidases and xylanases at the early decay stages. These enzymes were present during all decay period. However, bands corresponding probably to cellulolytic enzymes appeared only at advanced decay stages (60-90 days).

Keywords: wood decay, phenoloxidases, xylanases, white-rot fungi, *Poria medula-panis*

INTRODUCTION

In natural environments wood-rotting fungi grown on their substrate under conditions well different from those used in submerged cultures in laboratory studies. Since the production of lignocellulolytic enzymes is influenced by culture conditions, the enzyme patterns produced by fungi grown in liquids medium may differ widely from those found when fungi grown in solid wood (Datta *et al.*, 1991; Dawson-Andoh and Morrell, 1992; Zhao *et al.*, 1996).

The morphological aspects of wood decay process by wood-rotting fungi are well known, mainly through electronic microscopic studies (Eriksson *et al.*, 1990, Blanchette, 1991; Blanchette *et al.*, 1997). However, there is a lack of available information about fungal enzymes directly involved in wood decay. Such information will be useful in understanding the biochemical mechanisms of wood decay by fungi and consequently facilitate the fungal strain selection for biopulping and other industrial applications.

Several studies describe the production of extracellular ligninolytic enzymes by fungi belonging to basidiomycetes, mainly white-rot fungi. However, the production of hydrolytic enzymes (cellulases, xylanases, β-glucosidases) by these fungi has received little attention (Tuor *et al.*, 1995; Kirk and Cullen, 1998). The white-rot fungus *Poria medula-panis* was shown to selectively remove lignin from birch wood (Blanchette *et al.*, 1987; Eriksson *et al.*, 1990). Moreover, to our knowledge studies on lignocellulolytic enzymes produced by this fungus are nonexistent.

The aim the present work was to determine the profile of hydrolytic and ligninolytic enzymes produced by the white-rot fungus, *P. medula-panis*, during wood degradation in a solid-substrate system. Moreover, the protein patterns produced by fungus during wood decay were also studied using polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE).

EXPERIMENTAL

Microorganism and Culture Conditions

The white-rot fungus *Poria medula-panis* (ATCC 42463) was cultivated on 2% malt extract-agar (MEA) plates at 28°C during 5-6 days. To study the enzyme production by fungus, Erlenmeyers (2 L) containing 200

mL of 2% MEA were prepared. The flasks were inoculated with 4 plugs of agar-mycelium taken from MEA plates and incubated during 7 days (necessary time to the mycelium cover the agar surface) at 28°C.

Wood Decay Conditions

The *Eucalyptus grandis* chips (50 g air dried) were moistened with distilled water overnight and then the water excess was removed and the wood chips were autoclaved for 30 min at 121°C. After, the wood chips were cooled at room temperature and aseptically added on fungal cultures. The fungal wood decay proceeded at 28°C during 90 days. Control wood samples (not inoculated) were prepared by chips addition on MEA medium (without inoculum) in the same conditions above described. All cultures were carried out in triplicate. Mycelium was carefully removed from the surface of decayed chips; chips were washed with distilled water and then dried at 105°C and reweighed to determine weight loss (%).

Enzymes Extraction from Degraded Chips

After 15, 30, 60 and 90 days of incubation, the flasks were sequentially extracted twice in 100 mL of a solution containing 50 mM sodium acetate buffer, pH 5.4, plus 0.01% Tween 80. Each extraction was carried out at 20±2 °C on a rotary shaker (120 rpm) for 3 h. The content of each flask was filtered through fine filter paper. Crude extracts were maintained at 4°C and the lignocellulolytic enzymes were determined in the maximum period of 2 days after extraction. The filtered extracts corresponding to each decay time were freeze-dried for volume reduction. Subsequently, the extracts were concentrated by ultrafiltration (UF) using a 75 kDa cut-off membrane. The dark color of fractions > 75 kDa and the initial extract (before UF), was removed by adsorption on polyvinylpyrrolidone (PVP). When the proteins were analyzed under denaturing conditions, each fraction was precipitated with 100% thrichloroacetic acid (TCA) and redissolved in 0.1 M NaOH.

Enzyme Assays

The hydrolytic enzymes: total cellulase (filter paper activity) and xylanase were assayed with filter paper Whatman N° 1 and birch xylan, respectively (Mandels *et al.*, 1976; Bailey *et al.*, 1991). The reducing sugars were determined by using dinitrosalicylic acid (DNS) (Miller, 1959). β-glucosidase was determined by measuring of p-nitrophenol released from p-nitrophenyl-β-D-glucopyranoside (Tan *et al.*, 1987). Oxidase activity was determined by o-dianisidine oxidation at pH 3 (Machuca *et al.*, 1998). Total phenoloxidase and laccase were assayed by oxidation of syringaldazine at pH 5, with and without H_2O_2, respectively (Szklarz *et al.*, 1989). Peroxidase was calculated as the total phenoloxidase minus laccase activities. One unit of activity was defined as the amount of enzyme that release 1 μmol of product per min (IU). The enzyme activity was expressed in units recovered per volume of extraction buffer (U/100 mL), and the total enzyme activity was expressed as total units recovered from each culture after two extractions (Total Units). All enzyme activities and other values stated are the result of triplicate determinations. In all cases, standard errors were less than 10% of the mean values given.

Gel Electrophoresis and Staining

The patterns of extracellular proteins present in lyophilized extracts and concentrate fractions (> 75 kDa) were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Alfenas *et al.* (1991). The running gel was 10% acrylamide (pH 8.9), and the stacking gel was 4% acrylamide (pH 6.8). Protein bands were visualized by silver staining. Protein standards (Dalton Mark VII-L kit Sigma) were used to estimated the molecular mass of the fungal proteins under denaturing conditions (molecular mass range from 66 to 14.2 kDa).

RESULTS AND DISCUSSION

In this work, we described the enzymes production by a white-rot fungus, *P.medula-panis*, during the *E.grandis* chips decay under solid-substrate conditions. In the extracts recovered from decayed wood, the hydrolytic enzymes cellulase and β-glucosidase related to cellulose degradation and xylanase, related to polyoses degradation, were detected (Figure 1).

P.medula-panis produced low cellulase levels over all decay period. The maximal level of these enzymes was observed at beginning of decay, and after 90 days, a slight tendency of increase was detected. β-glucosidase activity was also detected at 15 days of decay increasing after this time and remaining almost constant until the end of decay. A peak of high xylanase activity was detected at 15 days after which the level of activity declined rapidly and low levels of this enzyme were observed until the end of decay.

The major enzymes related to lignin degradation, laccase and peroxidase, were detected in the *P. medula-panis* extracts throughout decay period. Total phenoloxidase, which can include laccase and several peroxidases, was detected using syringaldazine in the presence of H_2O_2. Although no manganese was added to syringaldazine oxidation assay, the peroxidase activity detected could correspond to manganese-dependent peroxidase (MnP), since the needful manganese for reaction can be present in wood (Eriksson *et al.*, 1990) and consequently in the extracts recovered of decayed wood.

The ligninolytic enzymes showed a different recovery profile from decayed wood as compared with hydrolytic enzymes. Peroxidase activity higher than laccase was detected in the *P.medula-panis* extracts at the beginning and at the end of decay period. At 30 days, the maximal laccase levels were detected, which were higher than peroxidase levels. The oxidase activity, assayed as o-dianisidine oxidation at pH 3, was found to be lower than laccase and peroxidase activities during all decay period showing a maximum between 30-60 days (Figure 1). This activity could be considered as a laccase activity oxidizing other substrate at low pH. In fact, the oxidase activity exhibited a similar profile to laccase activity during the decay, except at 60 days (Figure 1).

The degradative ability of *P.medula-panis*, determined as the weight loss of *E.grandis* chips after fungal treatment is show in the Figure 1. The weight loss produced by this fungus increased gradually reaching a maximum of 8.2 % at 90 days. The degradative capability seems to be not correlated with lignocellulolytic enzymes production because the maximal levels of hydrolytic and ligninolytic activities were observed at early decay stages when low weight loss values were observed. Thus, the analysis of individual components of decayed wood is necessary to attempt to correlate the enzyme production with the cellulose, hemicellulose or lignin loss.

The protein patterns recovered from decayed wood during different periods were analyzed by polyacrylamide gel electrophoresis (SDS-PAGE) (data not shown). The higher number of protein bands was observed at 60 days of decay. At this stage, the bands showed a wide range of molecular masses (20 - 66 kDa). Few protein bands appeared at early (15-30 d) and advanced decay stages, but differences in the range of molecular masses between these decay times were observed. Protein bands corresponding to approximately 50 and 66 kDa were detected during all decay periods. However, protein bands that exhibited low molecular masses (≤ 36 kDa) were only observed at 60 and 90 days of decay.

Recently, laccases and peroxidases produced by several white-rot fungi were characterized. Laccases produced by these fungi exhibited a molecular mass of approximately 60 kDa and MnP and other peroxidases varied between 39 and 45 kDa (Heinzkill *et al.*, 1998). Moreover, the molecular mass of endo-cellulase isolated from *Sporotrichum pulverulentum* varied between 28 and 37 kDa (Eriksson *et al.*, 1990) and the β-glucosidase isolated from the same fungus showed the higher molecular size (114 kDa) as compared with other lignocellulolytic enzymes (Lyman *et al.*, 1995). Xylanases from white-rot fungi have received less attention, however, xylanases isolated from non-wood decaying fungi usually have a molecular mass over the range from 16 to 75 kDa (Kirk and Cullen, 1998).

In accordance with enzyme activity results, electrophoresis observations and literature data above mentioned, we can suggests that protein bands appearing at early stages and remaining during all decay most likely represent laccase or peroxidase (bands at ~66 kDa) and xylanase (bands at ~50 kDa). Moreover, protein bands ≤ 36 kDa, which appeared at 60 days, could correspond to enzymes from cellulolytic complex. At 60 days also appeared bands > 66 kDa which could correspond to β-glucosidase.

P.medula-panis showed the capability to produce simultaneously hydrolytic and oxidative enzymes, which are needed to degrade complex lignocellulosic substrates such as wood. Among the lignocellulolytic enzymes assayed, xylanase was the enzyme recovered in highest quantity (IU/culture) from decayed wood. This enzyme together with peroxidase achieved a maximum at beginning of decay. According to Tuor *et al.* (1995) the close association of lignin and hemicelluloses suggest that the primary attack of the wood cell wall requires enzymatic degradation of the hemicelluloses prior to lignin. During wood decay by *Phanerochaete chrysosporium,* a xylanase was expressed at early decay stages as demonstred by inmunolabeling experiments (Joseleau and Ruel, 1992). These facts are in according with our results, which indicated the maximal xylanase production at beginning of decay by *P.medula-panis*.

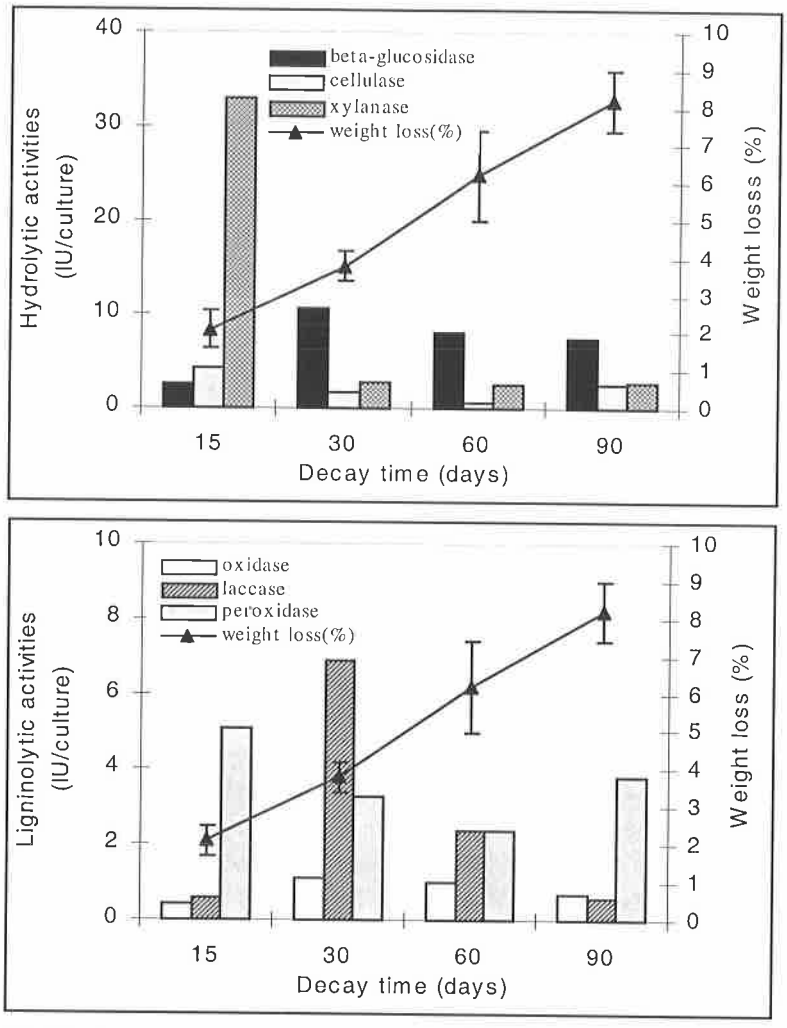

Figure 1. Recovery of hydrolytic and ligninolytic enzymes from *E.grandis* chips decayed by *P.medula-panis* in solid-substrate system and weight loss of decayed wood.

At 60 days of decay, protein bands spanning a wide range of molecular masses were visualized by SDS-PAGE. These bands indicated that a variety of extracellular proteins were present at this time. However, at early decay stages (15 days) the number of protein bands seems not correlated with the variety of enzyme activities detected in the fungal extracts. At this time, all lignocellulolytic activities were detected, however, only two weak protein bands were obtained through SDS-PAGE. It suggests that other lignocellulolytic systems of non-protein nature could be responsible by high hydrolytic activities observed at 15 days.

Recent theories have postulated the participation of several agents of low-molecular mass which catalyses redox reactions in the presence of iron in the early stages of wood decay by white and brown-rot fungi (Goodell *et al.*, 1997; Enoki *et al.*, 1997). These agents can be responsible by initial modification of the cell wall structure, depolymerizing the cellulose and lignin, when lignocellulolytic enzymes cannot penetrate in the cell wall because their large size. The production of iron-binding compounds siderophore-type by *P.medula-panis* grown on solid medium was previously reported (Milagres *et al.*, 1999). Thus, research are being development by us to investigate the presence of agents of low-molecular mass which could be responsible by part of the catalytic activity observed at beginning of decay by *P.medula-panis*.

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REFERENCES

Alfenas, A.C., Peters, I., Brune, W. & Passador, G.C. (1991). *Eletroforese de Proteinas e Isoenzimas de Fungos e Essências Florestais*. Universidade Federal de Viçosa, Brazil.

Bailey, M.J., Biely, P. & Poutanen, K. (1992). Interlaboratory Testing and Methods for Assay of Xylanase Activity. *J.Biotechnol.*, **23**, 257-270.

Blanchette, R.A. (1991). Delignification by Wood Decay Fungi. *Annu. Rev. Phytophatol.*, **29**, 381-398.

Blanchette, R.A., Otjen, L. & Carlson, M.C. (1987). Lignin Degradation in Cell Walls of Birch Wood Decayed by White Rot Basidiomycetes. *Phytopatol.,* **77,** 684-690.

Blanchette, R.A., Krueger, E.W., Haight, J.E., Akhtar, M. & Akin, D.E. (1997). Cell Wall Alterations in Loblolly Pine Wood Decayed by the White-rot Fungus, *Ceriporiopsis subvermispora. J. Biotechnol.* **53**, 203-213.

Daniel, G., Volc, J. & Kubatova, E. (1994). Pyranose Oxidase, a Major Source of H_2O_2 during Wood Degradation by *Phanerochaete chrysosporium*, *Trametes versicolor*, and *Oudemansiella mucida. Appl.Environ.Microbiol.*, **60**, 2524-2532.

Datta, A., Bettermann, A. & Kirk, T.K. (1991). Identification of a Specific Manganese Peroxidase among Ligninolytic Enzymes Secreted by *Phanerochaete chrysosporium* during Wood Decay. *Appl.Environ.Microbiol.*, **57**, 1453- 1460.

Dawson-Andoh, B.E. & Morrell, J.J. (1992). Extraction of Proteins from Wood Wafers Colonized by Decay Fungi. *Holzforschung.,* **46**, 117-120.

Enoki, A., Itakura, S. & Tanaka, H. (1997). The Involvement of Extracellular Substances for Reducing Molecular Oxygen to Hydroxyl Radical and Ferric Iron to Ferrous Iron in Wood Decay Fungi. *J.Biotechnol.*, **53**, 265-272.

Eriksson, K.-E., Blanchette, R.A. & Ander, P. (1990). *Microbial and Enzymatic Degradation of Wood and Wood Components*. Springer-Verlag, Berlin

Kirk, T.K. & Cullen, D. (1998). Enzymology and Molecular Genetics of Wood Degradation by White-rot Fungi. In *Environmentally Friendly Technologies for the Pulp and Paper Industry*, ed. R.A.Young & M. Akthar. John Wiley & Sons, New York, pp. 273-307.

Goodell, B., Jellison, J., Liu, J., Daniel, G., Paszczynski, A., Fekete, F., Krihnamurthy, S., Jun, L. & Xu, G. (1997). Low Molecular Weight Chelators and Phenolic Compounds Isolated from Wood Decay Fungi and their Role in the Fungal Biodegradation of Wood. *J.Biotechnol.*, **53**, 133-162.

Heinzkill, M., Bech, L., Halkier, T., Schneider, P. & Anke, T. (1998). Characterization of Laccase and Peroxidase from Wood-rotting Fungi (Family *Coprinaceae*). *Appl. Environ. Microbiol.*, **64**, 1601-1606.

Joseleau, J.P. & Ruel, K. (1992). Ultrastructural Examination of Lignin and Polysaccharide Degradation in Wood By White-rot Fungi. In *Biotechnology in the Pulp and Paper Industry*, ed. M. Kuwahara & M. Shimada. Uni Publishers Ltd., pp. 195-202.

Lyman, E.S., Li, B. & Renganathan, V. (1995). Purification and Characterization of a Cellulose-Binding β-Glucosidase from Cellulose-Degrading Cultures of *Phanerochaete chrysosporium. Appl. Environ. Microbiol.*, **61**, 2976-2980.

Machuca, A., Aoyama, H. & Durán, N. (1998). Production and Characterization of Thermostable Phenoloxidases of the Ascomycete *Thermoascus aurantiacus. Biotechnol.Appl. Biochem.* **27**, 217-223.

Mandels, M., Andreotti, R. & Roche, C. (1976). Measurement of Saccharifying Cellulose. *Biotechnol.Bioeng.Symp.* **6,** 2-34.

Milagres, A.M.F., Machuca, A. & Napoleão, D. (1999). Detection of Siderophore Production from Several Fungi and Bacteria by a Modification of Chrome Azurol S (CAS) Agar Plate Assay. *J. Microbiol. Methods*, **37**, 1-6.

Miller, G.L. (1959). Use of Dinitrosalicylic Reagent for the Determination of Reducing Sugar. *Anal. Chem.,* **31**, 426-428.

Tan, L.U.L., Mayers, P. & Saddler, J.N. (1987).Purification and Characterization of a Thermostable Xylanase from a Thermophilic Fungus *Thermoascus aurantiacus. Can.J.Microbiol.* **33**, 689-692.

Tuor, U., Winterhalter, K. & Fiechter, A. (1995). Enzymes of White-rot Fungi Involved in Lignin Degradation and Ecological Determinants for Wood Decay. *J.Biotechnol.*, **41**, 1-17.

Szklarz, G.D., Antibus, R.K., Sinsabaugh, R.L. & Linkins, A. (1989). Production of Phenoloxidases and Peroxidases by Wood-rotting Fungi. *Mycol.* **81**, 234-240.

Zhao, J., De Koker, T.H. & Janse, B.J.H. (1996). Comparative Studies of Lignin Peroxidases and Manganese-dependent Peroxidases Produced by Selected White-rot Fungi in Solid Media. *FEMS Microbiol.Lett.* **145**, 393-399.

Evaluation of the removal of heavy metals by kraft lignin considering coprecipitation, solubility and coordination capacity of the ions tested

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ABSTRACT

This work has as objective to test the chelating capacity of both kraft lignin and liquor in the removal of several metallic ions. 100 mg of lignin or 1 mL of kraft liquor was added to standard 0.01 mol.L^{-1} solutions of Pb^{+2}, Cu^{+2}, Cd^{+2} and Cr^{+3}. The mixtures were stirred during 60 min at 60°C, precipitated with H_2SO_4 at pH 1 (for kraft liquor only) and filtered. The filtrates were analyzed by ICP-AES (Inductively Coupled Plasma Atomic Emission Spectrometry), in order to verify the remained concentration of the metals to calculate the removal of the metallic ion. The precipitates were analyzed by infrared espectroscopy (FTIR) and the obtained spectra submitted to the statistical treatment by PCA (Principal Components Analysis). The removal of Pb^{+2} and Cu^{+2} reached 98 and 76% by kraft liquor, respectively, and 19 and 20% for the lignin, respectively. However Cr^{+3} and Cd^{+2} ions presented low removal values. A new phenomenon was also considered: the coprecipitation occured in kraft liquor due to the decreasing of pH, provided by the adsorption of the ions (as sulfate) on the lignin macromolecule. The coprecipitation and the coordination capacity of each metallic ion were analyzed towards the solubility of the respective sulfate. Relationships between the solubility and the removal capacity were studied. The more soluble the metallic sulfate, the minor the possibility of its removal by the kraft liquor. PCA of FTIR spectra of the precipitate showed evidences of displacement of bands and characteristic absorbances of carbon-metal and oxygen-metal bonds.

Keywords: removal of metals, kraft lignin, chelating capacity

INTRODUCTION

The chelation of kraft and oxidized lignins with metals has been recently studied by our research group (Gonçalves and Luz 1999). The insoluble complexes obtained from different conditions of preparation should be analyzed by spectroscopy in the infrared region, in order to verify the possible changes in the C=O and O-H linkages, due to interactions between lignin and metallic ions. New linkages can be generated between metallic ions and C, S or O atoms present in kraft lignin (Davidson and Ebsworth, 1988).

Infrared spectroscopy is used thoroughly for lignin characterization. On the other hand, changes in the spectra of lignins complexed with metals can be small and apparently insignificant. Such spectra can be analyzed by means of a statistical treatment called Principal Components Analysis (PCA), in order to obtain the largest possible number of information from the samples (Cotrim *et al.* 1999).

The amount of metallic ions in the filtrates and precipitate generated by the chelation processes can be determined by inductively coupled plasma atomic emission spectrometry (ICP-AES) using standard solutions (Ohlweiler, 1981; Pungor, 1995).

The aim of this work is the evaluation of the coprecipitation and adsorption phenomena occurring with lignin and metallic ions as an exclusive function of the change in the pH value (Baccan *et al.*, 1979). In the case of lignin, the adsorption of metallic ions is also a function of the electronic nature of the adsorbing groups. Another important fact to be considered is that the higher the coprecipitation the smaller the solubility of the metallic ions (Martínez and Mcbride, 1998).

EXPERIMENTAL

Chelation tests of lignin and kraft liquor with metallic ions

Lignin (100 mg) or kraft liquor (1 mL) was added to standard solutions (0.01 mol.L^{-1}) of Pb^{+2}, Cu^{+2}, Cd^{+2} and Cr^{+3} contained in 125-mL Erlenmeyer flasks. Precisely 0.4 mL of concentrated H_2SO_4 was added to the mixtures containing kraft liquor, in order to precipitate all the lignin. The mixtures were stirred for 1 h at 60°C and filtered, the filtrates being completed to 50 mL with distilled water. A chromium-platting effluent was also analyzed.

Characterization of the precipitates by Fourier-transformed infrared spectroscopy (FTIR)
The precipitates were ovendried and submitted to analysis by infrared (400-4000 cm^{-1}) in a Nicolet 520 spectrometer with Fourier transform, from pellets containing 1.5 mg of sample and 300 mg of KBr. The transmittance spectra were obtained with 4 cm^{-1} resolution and 64 scans. The spectra were normalized by the absorption 1516 cm^{-1}, corresponding to the aromatic structures of lignin. A statistical treatment of the variance of the intensities was performed by principal components analysis (PCA), using the softwares BIOTEC and FAEN2 developed by Bruns (1994). The variation of the spectra as a whole (values of the scores) as well as the weight of each band in the principal components (loadings) were determined.

Determination of the concentration of metallic ions in the filtrates obtained from the chelation tests
This analysis was performed by ICP-AES (Inductively Coupled Plasma Atomic Emission Spectrometry) in an ICP-AES 3410 ARL/Applied equipment. Standard solutions of the several metallic ions were prepared in HNO_3 3% (w/v). The emission lines used for detection of the metallic ions were selected in respect to a minimum interference (Boumans, 1980).

RESULTS AND DISCUSSION

The precipitates obtained after the addition of kraft lignin or liquor to the solutions of metallic ions were characterized by FTIR in the region from 400 to 4000 cm^{-1}. The spectra are very similar, with no remarkable differences. The region 400-1900 cm^{-1} is interesting for the present study, including the fingerprint region (900-1900 cm^{-1}) and the absorptions of carbon-metal and oxygen-metal bonds, observed in the regions of low energy (below 900 cm^{-1}) (Davidson and Elbsworth, 1988).

Table 1 shows the precipitates obtained with several metallic ions and kraft lignin or liquor. Sample numbers and codes were used in Figure 1.

Table 1. Description of the precipitates obtained

1	kraft liquor + Cd^{+2}	8	kraft liquor + Cr^{+3}
2	kraft lignin + Cd^{+2}	9	kraft lignin + Cr^{+3}
3	kraft lignin + Pb^{+2}	B1	kraft lignin + water
4	kraft liquor + Pb^{+2}	B2	kraft lignin + water
5	kraft liquor + Pb^{+2}	B3	kraft liquor + water
6	kraft liquor + effluent	B4	kraft liquor + water
7	kraft liquor + effluent		

The statistical treatment by PCA was applied to the FTIR spectra in order to obtain the largest possible number of information. The score values summarize all the information on the spectra in principal components. Different spectra are located in different regions of a cross-plot of the principal components. All the spectra were jointly analyzed by PCA since few spectra of each type (metal ion) were obtained.

Figure 1 shows the plot of PC2 x PC1 of the scores. The differences between the spectra were evalutated by separating the points in the regions. Area A contains the scores of samples 2, 3, 9, B1 and B2, showing that the precipitates formed when only lignin and metals were used can not be distinguished from blanks. On the other hand, the samples containing Pb^{+2} or effluent obtained from kraft liquor (region B and samples 4, 5 and 6) are well differentiated from the blanks, probably due to the chelation with lignin in the precipitate. These differences can be explained by the high removal of ions or by the presence of anionic species in the precipitate (for example, chromate present in the effluent). Area C is composed of 1, B3 and B4 (samples from kraft liquors). It can be seen that sample 1 is very similar to the blanks, possibly due to the small removal of the Cd^{+2} ions.

The loadings of Figure 2 show the contribution of the bands of the first three principal components to the formation of the scores. It can be seen that at 600 cm^{-1} there are some differences between the spectra related with some changes in the linkages, due to the incorporation of metallic ions in the lignin structure. Another evidence of the incorporation of metallic ions is the regions at 1000 and 1200 cm^{-1}, characteristic of C-O bonds, that can have been weak due to the formation of complexes.

The removal of Pb^{+2} and Cu^{+2} in the chelation tests with kraft liquor is very high, owing perhaps to the coprecipitation and adsorption phenomena. By comparing these results with those of the literature (Perry and Green, 1997), and considering that sulfate corresponds to the salt obtained in the precipitation with sulfuric

acid, the same order of solubility was found (measured at 30°C):

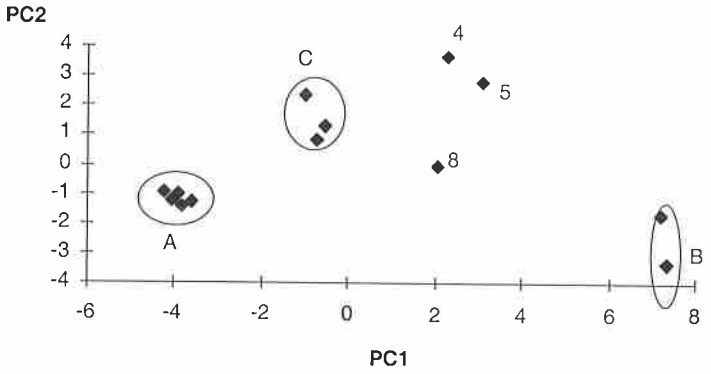

Figure 1. Scores of PC2 x PC1 related to the FTIR spectra of the precipitates obtained with kraft lignin or liquor and several metallic ions (region 400-1900 cm^{-1}, 779 variables). The numbers and codes refer to the samples listed in Table 1.

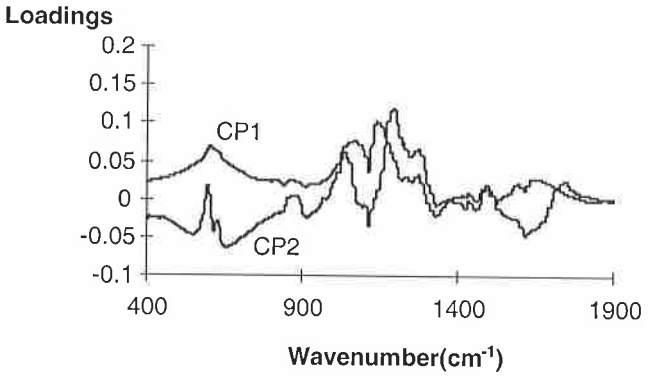

Figure 2. Loadings (importance of the variables) of the first and second principal components

Table 2. Percentage of removal of metallic ions (1 mL of kraft liquor or 100 mg of kraft lignin added to 10 mL of 0.01 $mol.L^{-1}$ solution of different metallic ions)

	Removal of metallic ions(%)	
Sample	Kraft Liquor	Kraft Lignin
Pb	98.10	18.84
Cr	5.76	6.99
Cu	76.24	20.24
Cd	0.46	0.00

$CdSO_4 = 3.72\ mol.L^{-1} > CuSO_4 = 1.57\ mol.L^{-1} > Cr_2(SO_4)_3 = 0.32\ mol.L^{-1} >> PbSO_4 = 1.38 \times 10^{-4}\ mol.L^{-1}$

According to Baccan *et al.*, (1979) the higher coprecipitation the lower the salt solubility. This rule, however, does not apply to Cu^{+2} ions, whose removal is more effective than the Cr^{+3} removal, since Cu^{+2} ions have more capacity for coordination, as a consequence of their electronic configuration.

In the case of kraft lignin the relationship between solubility and removal is not observed. As the pH of the medium is constant, only a difference in the tendency of each element to bind to the atoms of the lignin is observed. For both cases (chelation tests with lignin or liquor), the removal of cadmium is very high.

CONCLUSIONS

Besides having the same removal properties of kraft lignin, kraft liquor also has the power to remove metallic ions by coprecipitation, but not by chelation. The removal of metallic ions is intensified by the

coprecipitation of the salt formed together with the lignin.

Infrared spectroscopy is very important for the characterization of the chelates obtained. Infrared and Principal Components Analysis showed a separation between the lignins and the liquors precipitated with H_2SO_4.

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REFERENCES

Baccan, N., Andrade, J.C., Godinho, O.E.S. & Barone, J.S. (1979). *Química Analítica Quantitativa Elementar*. Edgard Blücher, São Paulo, pp. 35-36.

Boumans, P.W.J.M. (1980). *Line Coincidence Tables for Inductively Coupled Plasma Atomic Emission Spectrometry*. Pergamon Press, New York, pp. 150-152.

Bruns, R.E. (1994), *Implantação e Aplicações de Metodologia Quimiométrica no CEBIQ/FAENQUIL*. Projeto FAPESP, Processo 94/2280-8.

Cotrim, A.R., Ferraz, A., Gonçalves, A.R., Silva, F.T. & Bruns, R.E. (1999). Identifying the origin of lignins and monitoring their structural changes by means of FTIR-PCA and -SIMCA. *Bioresource Technol.*, **68**, 29-34.

Davidson, G. & Elbsworth, E.A.V. (1988). *Spectroscopic Properties of Inorganic and Organometallic Compouds*. Royal Society of Chemistry, London, pp. 299-301/320-325.

Gonçalves, A.R & Luz, S.M. (1999). Chelation of Copper (II) Ions with Kraft Lignin. *10th International Symposium on Wood and Pulping Chemistry*, Proceedings, **Vol. 3**, pp. 410-413.

Martínez, C.E. & Mcbride, M.B. (1998). Solubility of Cd^{+2}, Cu^{+2}, Pb^{+2}, and Zn^{+2} in Aged Coprecipitates with Amorphous Iron Hydroxides. *Environ. Sci. Technol.*, **32**, 743-748.

Ohlweiler, O.A. (1981). *Fundamentos de Análise Instrumental*. Livros Técnicos e Científicos editora, Rio de Janeiro, pp. 141.

Pungor, E. (1995). *A Practical Guide to Instrumental Analysis*. CRC Press, Boca Raton, pp. 139-145.

Perry, R.H. & Green, D.W. (1997). *Perry's Chemical Engineers' Handbook*. Mc Graw Hill, seventh edition, New York, pp. 2-11 - 2-14; 2-17.

Fungal treatment of eucalyptus with lignin-degrading fungi for bio-kraft pulping

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ABSTRACT

Pretreatment of eucalyptus wood chips with lignin-degrading fungi, *Ceriporiopsis subvermispora* was found to be suitable for bio-kraft pulping of eucalyptus. Fungal pretreatment reduced the pitch content in the wood chips and during kraft pulping, reduced the active alkali requirement upto 18% or reduced the cooking time upto 33% or reduced the sulfidity of the white liquor upto 30%. The quality of the resultant biopulps was found to be better than control (high brightness, better strength and improved bleachability). The bleached biopulps were easier to refine than the reference pulp. The beating time was reduced by 18-33% in different studies.

Keywords: biopulping, white-rot fungi, *Ceriporiopsis subvermispora*, kraft pulp, Eucalyptus

INTRODUCTION

Biotechnology offers potential solution to many of the problems faced by the paper industry due to economic conditions and environmental pressures (Bajpai and Bajpai, 1998). Biopulping, the pretreatment of wood chips prior to pulping, offers both economic and environmental benefits. Two sequential biopulping consortia (April 1987-June 1996) comprised of the USDA Forest Service, Forest Products Laboratory (FPL), the Universities of Wisconsin and Minnesota and industry have established the technical and economic feasibility of the mechanical pulping (Akhtar *et al.*, 1997,1998). Through the use of proper lignin-degrading fungi, at least 30% electrical energy is saved in mechanical pulping and paper strength properties are improved (Scott *et al.*, 1998: Scott and Swaney, 1998). Messner *et al.* (1992) and Scott *et al.* (1996) have demonstrated the great potential of using lignin-degrading fungi to reduce the kappa number in sulfite pulping. The effect of fungal pretreatment on kraft pulping has been investigated to a lesser extent (Wolfaardt *et al.*, 1993; Oriaran *et al.*, 1990 and 1991; Labosky *et al.*, 1991) and the benefits are not clear and need further research.

As no systematic work has been reported on bio-kraft pulping of eucalyptus which is one of the major raw materials for production of paper in many countries, studies on bio-kraft pulping of eucalyptus were initiated in our laboratory. The present paper deals with a method for bio-kraft pulping of eucalyptus (PCT patent pending). It is based mainly on the work from a collaborative project between Thapar Centre for Industrial R&D, India and Biopulping International Inc., USA.

EXPERIMENTAL

Wood Chips

The experiments were conducted with eucalyptus chips (hybrid variety) obtained from a large paper mill in Haryana, India.

Fungus Screening

Several lignin-degrading fungal cultures – *Ceriporiopsis subvermispora* L-14807 SS-3, CZ-3, L-9186-SP, FP-105752, FP-105752-SS-5, *Hyphodontia setulosa* FP-106976-SP, *Phlebia subserialis* RLG-6074-SP, *Phlebia brevispora* HHB-7099, *Phlebia tremellosa* FP-102557-OP and *Phanerochaete chrysosporium* BKM-F-1767 – were used in this study. All these cultures were obtained from the Center for Forest Mycology Research at Forest Products Laboratory, Madison, Wisconsin, USA. The cultures were maintained on potato dextrose agar (PDA) slants at 4°C until used.

PDA plates were inoculated from these slants and incubated at 27±1°C and 65±5% relative humidity. These plates were used to inoculate 1 litre Erlenmeyer flasks containing 100 ml of liquid medium which contained 36 g of potato dextrose broth and 10.91 g of yeast extract in 1500 ml water. The inoculated flasks were

incubated without agitation in an incubator at 27°C and 70-90% relative humidity for 10 days. The surface of the medium was covered with the fungus in the form of mat. The fungal mat was removed from the medium, washed with sterilized water on a sterilized Buchner funnel to remove all the medium. The fungal mat was transferred into a sterile Waring blender with sterile forceps. About 50 ml of sterile water was added to the blender and the mycelium was blended for 15 seconds. The fungal suspension was transferred to a beaker and diluted to 100 ml by adding sterile water. This suspension was used to inoculate wood chips. Fungal treatment of wood chips was carried in 20 litre aerated static bed bioreactor (made up of stainless steel 316). It was fabricated from stainless steel 316. For the fungal treatment, 1500 gm (o.d basis) of wood chips were put in the bioreactor and autoclaved at 121°C for 60 min and cooled to room temperature. About 7.5 mg (dry wt.) of the fungus (5 g dry wt. of the fungus per ton of material) and 15 g of corn steep liquor (0.5% CSL on dry wt. basis) were added to the 1500 gm of wood chips in the bioreactor and mixed thoroughly. The moisture content of the chips was adjusted to 55% (wet weight basis). The bioreactors were incubated in a room maintained at 27-32°C. The bioreactors were aerated with humidified air at a rate of 1 cubic ft per hour for 2 weeks. The humidified air was passed through air filter to eliminate the contaminants from the air. After incubation for two weeks, the fungus treated chips were removed from the bioreactors, weighed and sampled for moisture content and pulped in bomb digestors.

Kraft Pulping and Bleaching

The conditions for the kraft cooks were: 17% AA as Na_2O, 22.9% sulfidity, 3.1 liquor/wood ratio, 165°C cooking temperature, 90 min to cooking temperature and 90 min at cooking temperature. After the cooking, brightness, P. No., yield, viscosity and strength properties of the unbleached pulp were determined. The pulps were then bleached using a multistage bleaching process using CEHD sequence. Chlorine was applied at a kappa factor of 0.20 for 45 min at 30°C and 3% pulp consistency. The pulp was washed with water and extracted with NaOH (pH 11.2-11.5) at 10% consistency for 120 min at 65°C. The hypo treatment was done at 40°C with the pulp at 10% consistency for 150 min and the chlorine dioxide treatment was done at 75°C at 10% pulp consistency for 180 min. Brightness of the bleached pulp was also determined. The bleached pulps were beaten to 30-35° SR and the strength properties were determined.

Analytical Methods

Cellulose, hemicellulose and holocellulose were determined by the methods of Updegraff (1969), Deschatelets and Yu (1986) and Kurth (1948) respectively. Lignin and pitch were determined according to Tappi Test Methods T222 om-88, and T204 om-88 respectively. All assays were carried out in triplicate and average values are reported.

P. No., a measure of lignin content was determined by reaction of pulp samples with acidified potassium permanganate solution according to Tappi Test Method. Viscosity, an indicator of cellulose chain length, was determined by dissolving the pulp in cupri-ethylene diamine (CED) solution and measuring the viscosity of a 0.5% solution with an Ostwald Viscometer (Tappi method T230). Brightness of the paper sheets was determined with a Technibrite instrument (Model TB1c). Strength properties of handsheets were tested according to Tappi methods.

RESULTS AND DISCUSSION

Fungus Screening

All the lignin-degrading fungal cultures grew very well on eucalyptus chips in the bioreactor. Good growth was achieved even without addition of nutrients. The fungal treated chips appeared brighter than the control chips. Most of the biopulping effect was obtained in about 2 weeks time with 5 g of inoculum per ton of chips (data not shown). The weight loss of wood chips after treatment with various fungi was about 2-4%.

Detailed studies were carried out with *Ceriporiopsis subvermispora* L-14807 SS-3 as this fungus was found to be the best for biomechanical pulping (Akhtar *et al.*, 1997,1998).

Treatment with *Ceriporiopsis subvermispora* L-14807-SS-3

The fungus degraded the lignin selectively while the relative amount of cellulose and hemicellulose increased. The lignin content decreased from 28% to 27% in 2 weeks (Table 1). The pitch content in the treated wood chips also decreased. The reduction was of the order of 17% and 38% in the seasoned and fresh wood chips respectively (Table 2).

When the cooking was done at the same active alkali charge for reference chips as well as fungal-treated chips, the brightness and strength properties of unbleached biopulp were higher as compared to those of control. The unbleached yield of the biopulp was lower and there was no drop in the permanganate number (P. No.) of the biopulp. However, the lignin content in the biopulp was found to be lower as compared to control. The unbleached brightness of biopulp was higher by more than 2 points. Tensile index and breaking length of unbleached biopulp increased by 12.2%, and burst index and double fold increased by 35.7% and 66.6%, respectively. The unbleached yield of the biopulp decreased slightly. The bleaching response of biopulp was better than that of control. The final brightness of biopulp (CEHD sequence) was higher by 2 points. The bleached biopulp was easier to refine than the reference pulp. The beating time was reduced by 33.3%. The strength properties of bleached biopulp were better than those of control (Table 3).

Table 1. Effect of fungal treatment with *Ceriporiopsis subvermispora* L-14807-SS-3 on cellulose, hemicellulose and lignin content of eucalyptus wood chips.

Component (%)	Control	Fungal treated
Cellulose	41.10	43.00
Hemicellulose	20.83	22.24
Lignin	27.96	26.98
Holocellulose	68.60	69.80

Table 2. Effect of fungal treatment with *Ceriporiopsis subvermispora* L-14807-SS-3 on total extractives content of fresh and seasoned eucalyptus wood chips.

Type of wood	Pitch	
	Total (%)	Reduction (%)
Seasoned eucalyptus	2.08	
Fungal treated seasoned eucalyptus	1.72	17.3
Fresh eucalyptus	3.01	
Fungal treated fresh eucalyptus	1.85	38.5

Even when the fungal-treated chips were cooked at reduced active alkali charge i.e. 14% AA, the unbleached brightness of biopulp was better than that of the reference pulp (no fungal treatment) obtained by cooking the chips at 17% AA charge. The strength properties of biopulp (with 14% AA charge) were substantially higher and the bleaching response was better than those of reference pulp (with 17% AA charge). When the bleaching was done under identical conditions at the same total chemical charge, the final brightness of the biopulp was higher by about 1 point in a 4-stage (CEHD) bleaching sequence. The bleached biopulps were easier to refine than the reference pulp. The beating time was reduced by ~20%. Most of the strength properties of the bleached biopulp were better than those of control (Table 4).

Due to higher P No., 14% AA control required more chlorine for bleaching by 8.6 kg /ton of pulp as compared to 17% AA control to reach the same brightness. The unbleached yield and strength properties of 14% AA control were higher compared to 17% AA control due to lesser degradation of carbohydrates at lower active alkali charge.

Few cookings with the *C. subvermispora L-14807-SS-3* treated eucalyptus chips were also performed in which cooking time was reduced by 16.6, 25.0 and 33.3%. The time to cooking temperature was fixed at 90 min and time at cooking temperature was reduced. When the cooking time was reduced by 25% and 33.3%, the control wood chips after cooking remained partially cooked. On the other hand, the fungal-treated chips were still uniformly cooked even in 30 min cooking. In all the cases, the brightness and mechanical properties of unbleached biopulps were higher and the bleaching response was better as compared to control (untreated chips cooked for 90 min at 165°C). The final brightness of the biopulps in CEHD sequence was also higher as compared to control. When the cooking time was reduced by 16.6 and 25%, higher final pulp brightness was obtained at the same total chemical charge. The bleached biopulps were easier to refine than

the reference pulp. The beating time was reduced by 16-18%. There was no significant difference in the strength properties of bleached biopulps and reference pulp.

Few cookings with *C. subvermispora L-14807-SS-3* treated eucalyptus chips were also performed in which the sulfidity was reduced from 22.9% to 16%. The unbleached brightness and strength properties of the fungal-treated chips at 16% sulfidity level were found to be higher than those of reference chips cooked at 22.9% sulfidity (Table 5).

Table 3. Bio-kraft pulping of eucalyptus with *C. subvermispora* L-14807-SS-3 at same active alkali charge.

a. Pulp properties

Parameter	Treated	Control
P. No.	14.46	14.40
Lignin (%)	1.55	1.33
Unbleached brightness (% ISO)	29.1	27.0
Unbleached pulp yield (%)	45.80	46.10
Final brightness (% ISO)	87.2	85.2
Bleach chemical consumption (kg/ton)		
-Elemental Cl_2	40.0	40.0
-NaOH	18.8	18.8
-Hypo	12.3	12.3
-Chlorine dioxide	6.0	6.0

b. Strength properties

Parameter	Unbleached		Bleached	
	Control	Treated	Control	Treated
Wetness (°SR)	17.0	18.0	35.0	35.0
Beating time (min)	--	--	30.0	20.0
Tensile index (Nm/g)	42.15	47.98	75.51	82.30
Breaking length (m)	4299	4894	7702	8394
Burst index (kN/g)	1.93	2.62	4.59	5.14
Tear index (mNm^2/g)	5.66	5.48	6.92	7.20
Double fold (No.)	6	10	102	112

Treatment with Other Strains of *C. subvermispora* and Other Lignin-degrading Fungi

Other strains of *C. subvermispora* – CZ-3, L-9186-SP, FP-105752, FP-105752-SS-5 and other lignin-degrading fungi – *Hyphodontia setulosa* FP-106976-SP, *Phlebia subserialis* RLG-6074-SP, *Phlebia brevispora* HHB-7099, *Phlebia tremellosa* FP-102557-OP and *Phanerochaete chrysosporium* BKM-F-1767 were also found to be suitable for bio-kraft pulping of eucalyptus. The active alkali charge was reduced by 30 kg/T of wood chips in all the cases. The beating response and the quality of the biopulp in terms of brightness and strength were better than those of control.

Treatment of Eucalyptus Chips with C. subvermispora L-14807-SS-3 for 2 Weeks

These results clearly show that fungal pretreatment of eucalyptus offers benefits for kraft pulping. The fungal pretreatment is effective in reducing the pitch and improving the chemical pulping and bleaching efficiency and the pulp properties (higher brightness and higher strength). Treated wood chips could be pulped in a shorter cooking time or could alternatively be used to produce pulp using lower active alkali charge or sulfidity. The bleached biopulps are easier to refine than the reference pulps. Scale up of the process is now required to develop the basic technical and engineering knowledge needed for commercial utilization of fungi for kraft pulping.

Table 4. Bio-kraft pulping of eucalyptus with *C. subvermispora* at reduced active alkali charge.

a. Pulp properties

Parameter	AA Charge (%)			
	17	14	14	14
	Control	Treated	Control	Treated
P. No.	13.50	15.86	16.28	15.86
Lignin (%)	1.55	1.57	--	--
Unbleached brightness (% ISO)	27.3	28.3	25.9	28.3
Unbleached pulp yield (%)	45.67	45.53	47.15	45.53
Final brightness (% ISO)	87.0	88.3	87.6	89.1
Bleach chemical consumption (kg/ton)				
-Elemental Cl_2	37.5	37.5	46.1	46.1
-NaOH	19.1	19.1	18.9	18.9
-Hypo	13.5	13.5	12.8	12.8
-Chlorine dioxide	6.0	6.0	6.0	6.0

b. Strength properties

Parameter	Unbleached			Bleached	
	Control		Treated	Control	Treated
	17% AA	14% AA	14% AA	17% AA	14% AA
Wetness (°SR)	16.5	17.0	17.5	35.0	35.0
Beating time (min)	--	--	--	29.0	22.5
Tensile index (Nm/g)	33.68	34.10	40.75	66.25	72.26
Breaking length (m)	3435	3478	4157	6757	7364
Burst index (kN/g)	1.38	1.62	1.89	4.30	4.85
Tear index (mNm^2/g)	5.45	5.77	6.81	7.68	7.88
Double fold (No.)	5	8	10	58	80

Table 5. Effect of fungal treatment on sulfidity requirement in cooking.

Parameter	Control		Treated	
	22.9% S	16% S	16% S	22.9% S
P. No	13.71	14.16	14.16	13.82
Unbleached brightness (% PV)	28.9	28.0	30.3	31.2
Unbleached pulp yield (%)	46.20	45.60	45.40	45.10
Wetness (°SR)	17.5	17.5	18.0	18.0
Tensile index (Nm/g)	35.71	33.91	41.79	42.10
Breaking length (m)	3642	3458	4262	4293
Burst index (kN/g)	1.55	1.32	1.81	2.01
Tear index (mNm^2/g)	5.69	5.35	6.90	6.12
Double fold (No.)	5	4	8	9

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REFERENCES

Akhtar, M., Blanchette, R.A. & Kirk, T.K. (1997). Fungal delignification and biochemical pulping of wood. *Advances in Biochemical Engineering /Biotechnology*, **57**, 159-195

Akhtar, M., Blanchette, R.A., Myers, G. & Kirk, T.K. (1998). An overview of biochemical pulping research. In *Environmentally Friendly Technologies for the Pulp & Paper Industry,* ed. R.A. Young & M. Akhtar. John Wiley & Sons, New York, pp. 309-340

Bajpai, P. & Bajpai, P.K.(1998). *Biotechnology in Pulp and Paper Industry- A Route to Energy Conservation*, PIRA International, UK

Deschatelets, L. & Yu, E.K.C. (1986). A simple pentose assay for biomass conversion studies. *Appl. Microbiol. Biotechnol.*, **24**, 379-385

Kurth, E.F. (1948). Determination of holocellulose in wood. *Paper Trade J.*, **126 (Feb.)**, 56-58

Labosky Jr. P., Zhang J. & Royse D.J. (1991). Lignin biodegradation of nitrogen supplemented red oak wood chips with two strains of *Phanerochaete chrysosporium*. *Wood Fiber Science*, **23**, 533-542

Messner, K., Masek, S., Srebotnik, E. & Techt. G. (1992). Fungal treatment of wood chips for chemical pulping. In *5th Conference on Biotechnology in the Pulp and Paper Industry,* Proceedings, pp. 9-13

Oriaran, T. Ph., Labosky Jr. P. & Blankenhorn P.R. (1990). Kraft pulp and papermaking properties of *Phanerochaete chrysosporium* degraded aspen. *Tappi J.*, **73 (7),** 147-152

Oriaran, T. Ph., Labosky Jr. P. & Blankenhorn P.R. (1991). Kraft pulp and papermaking properties of *Phanerochaete chrysosporium* degraded red oak. *Wood Fiber Science*, **23**, 316 -327

Scott, G.M. & Swaney R. (1998). New technology for papermaking: biopulping economics. *Tappi J.*, **81 (12),** 153-160

Scott, G.M., Akhtar, M., Lentz, M.J., Kirk, T.K. & Swaney, R. (1998). New technology for papermaking: commercializing biopulping. *Tappi J.*, **81 (11),** 220-225

Scott, G.M., Akhtar, M., Lentz, M., Sykes, M. & Abubakr, S. (1996). Biosulfite pulping using *Ceriporiopsis subvermispora*. In *Biotechnology in the Pulp & Paper Industry: Recent Advances in Applied and Fundamental Research,* Proceedings, pp. 217-219

Updegraff, D.M. (1969). Semi-micro determination of cellulose in biological materials. *Anal. Biochem.*, **32**, 420-424

Wolfaardt, J.F., Bosman, J.L., Jacobs, A., Male, J.R. & Rabie, C.J. (1993). Biokraft pulping of softwood. In *Biotechnology in the Pulp and Paper Industry. Recent Advances in Applied and Fundamental Research,* Proceedings, pp. 211-216

Hydrogen peroxide bleaching of kraft pulps in the presence of polyoxometalates

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ABSTRACT

Negative environmental impact associated with chlorine in kraft pulp bleaching led to the development of new technologies, which eliminate the use of chlorine-based compounds. In this work we examine an alternative bleaching system employing H_2O_2 and Keggin-type polyoxometalates (POMs) for the selective removal of lignin from the pulps. We observed that some POMs act as a fiber protector, avoiding cellulose degradation, thus showing a higher selectivity for lignin oxidation. Experiments using the bleaching liquor show that it can be reused in more than one bleaching sequence, without loss of efficiency.

Keywords: kraft pulps, bleaching, polyoxometalates, lignin

INTRODUCTION

In the bleaching of kraft pulps, many cellulose mills employ chlorine and chlorine containing compounds to oxidize the residual lignin. The problem with this bleaching agent is that it produces chlorinated organic compounds, which are very toxic and highly resistant to biodegradation (Solomon, 1996). The ecological concerns are responsible for the high interest in new bleaching technologies, which could also produce good quality pulp paper (McDonough, 1995). Recently, a class of inorganic metal-oxygen cluster anions (polyoxometalates - POMs) was proposed as a high selective stoicheometric reagent or catalyst for the pulp delignification (Weinstock *et al.*, 1996, Evtugin *et al.*, 1997, Bianchi *et al.*, 1999). POMs are inorganic compounds consisting of transition metal cations M (W^{VI}, Mo^{VI}, V^{V}, etc.), a central heteroatom X (Si^{IV}, Ge^{IV}, P^{V}, etc.) and oxygen. The Keggin-type polyoxometalates, the mostly used in catalysis, comprise a central XO_4 tetrahedron surrounded by 12 metal oxygen octahedra, grouped to 4 M_3O_{10} edge sharing units (Figure 1) (Pope, 1983). Many POMs have the attractive property that, after reduction, they can be easily reoxidized by oxygen, peroxides or ozone (Jansen *et al.*, 1994). This means that POMs can be employed as reusable agents in repeated cycles of pulp bleaching.

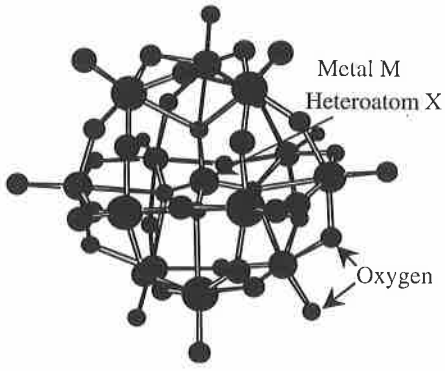

Figure 1. Keggin-type structure $[X^{n+}M_{12}O_{40}]^{(8-n)-}$ where X (central heteroatom) = P, Si, As, etc. and M = W, Mo, V, etc.

In the presence of H_2O_2, some POMs lead to the formation of a variety of peroxo complexes (or polyoxoperoxo species) (Dickman *et al.*, 1994). Some of these complexes are excellent epoxidation and oxidation catalysts (Venturello *et al.*, 1985).

In a previous work we showed that $H_3PMo_{12}O_{40}$ is the best catalyst, in combination with H_2O_2, for delignification of hardwood kraft pulps, giving low kappa numbers. However, this system is still inefficient for softwood, leading to a pulp with high kappa number and low viscosity (Bianchi *et al.*, 1998; 1999). In this work, in order to improve the quality of the bleached pulps (kappa number ~1 and viscosity >10 cP), we subjected the pulp (before bleaching) to chelation (using EDTA), for the removal of some heavy metals, such as iron, manganese and copper, which can catalyze the decomposition of H_2O_2. After bleaching, an alkaline extraction was carried out to remove some fragments of lignin, still remaining on the surface of the bleached pulp.

EXPERIMENTAL

Hardwood and softwood kraft pulps were, first of all, analyzed for their kappa number (Berzins, 1966) and viscosity (Tappi T230). The bleaching reactions were carried out in a 1000 mL stirred tightly closed glass

reactor. In the experiments, 15.0 g of oven-dried (o.d.) chelated pulp (using EDTA as a chelation agent), H_2O_2 30% w/w, $H_3PMo_{12}O_{40}$, $MgSO_4$ (5 mg/g of o.d. pulp) as an additive for the fiber protection and approximately 500 mL of water (to obtain a final consistency of 3%), were combined. The mixture was heated in the glass reactor at 70°C for 4 h. After bleaching, the pulp was filtered in a Büchner funnel and washed with hot water (approximately 60 to 70°C) and, then, with water at room temperature. The pulps were subjected to an alkaline extraction and analyzed for their kappa number and viscosity.

The filtrate (bleaching liquor) was used in other bleaching sequences under two different conditions: 1) without further treatment or 2) after being heated at 130°C for 3 h with 7 bar of O_2. After each bleaching reaction the kappa number, viscosity and H_2O_2 consumed were determined.

RESULTS AND DISCUSSION

Three kraft pulps from Brazilian mills were analyzed for their kappa numbers and viscosities (Table 1). We used softwood (pines) and hardwood (eucalyptus) pre-bleached pulps to verify the behavior of each one under the system POM/H_2O_2.

Table 1. Kappa number and viscosity of the kraft pulps.

Kraft pulps from different industries	Kappa	Viscosity (cP)
A (hardwood, pre-bleaching with O_2)	11.9	26.0
B (hardwood, pre-bleaching with O_2)	9.4	26.8
C (27% hardwood/73% softwood, pre-bleaching with O_2)	19.4	24.5

Figure 2 shows the results obtained with and without pulp treatment (chelation, $MgSO_4$ and alkaline extraction). In all cases, higher viscosities and lower kappa numbers was observed, indicating that the treatment is efficient. Pulp **C** is more influenced by the treatment than pulp **A** and **B**.

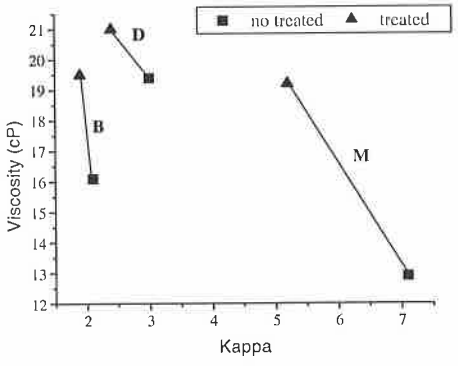

Figure 2. Influence of chelation, addition of $MgSO_4$ (75 mg) and alkaline extraction on kappa number and viscosity. Pulp **A**, **B** and **C**, 0.047 g of $H_3PMo_{12}O_{40}$, 1.65 g of H_2O_2 30% w/w, 70°C, 4 h, 3% consistency.

Using the pulp treatment we studied the influence of the amount of H_2O_2 and $H_3PMo_{12}O_{40}$ on kappa number and viscosity (Table 2). In the absence of $H_3PMo_{12}O_{40}$ (experiments 1, 2 and 3) the kappa number did not change significantly, but the viscosity decreased as the amount of H_2O_2 was raised. In the absence of H_2O_2 (experiments 4 and 7) we observed little changes in viscosities, indicating that $H_3PMo_{12}O_{40}$ degraded the fibers slightly. The increase in the amount of $H_3PMo_{12}O_{40}$ (0, 0.047 and 0.47 g) did not cause a significant increase in the H_2O_2 consumption. Moreover, the viscosities were higher in the presence of $H_3PMo_{12}O_{40}$ (comparing experiments 1, 5 and 8; 2 and 9; 3, 6 and 10). When 4.7 g of $H_3PMo_{12}O_{40}$ were used (experiment 11) we observed low viscosity and higher H_2O_2 consumption. It seems that a decrease in viscosity is related to the H_2O_2 consumption. The pH in experiment 11 was low, causing cellulose hydrolysis. The pulp yields did not undergo significant changes. Best results were obtained using 0.47 g of $H_3PMo_{12}O_{40}$ and 1.65 g of H_2O_2 (experiment 8). Experiments using pulps **B** and **C** (not reported here) showed the same behavior. The best results for **B** and **C** are showed in Table 2 (experiments 12 and 13 respectively). For **B** we obtained good results, however with pulp **C,** the delignification was not so effective.

Is interesting to note that experiment 1 (absence of $H_3PMo_{12}O_{40}$) and 7 (absence of H_2O_2) showed the same kappa number (~8), however, we obtained higher brightness (and higher viscosity) in the presence of $H_3PMo_{12}O_{40}$ (experiment 7). Improve in brightness was obtained when $H_3PMo_{12}O_{40}$ was used in combination with H_2O_2 (experiment 8).

Table 2. Influence of the amount of H_2O_2 and concentration of $H_3PMo_{12}O_{40}$ on kappa number, viscosity and yield.

Pulp	Exp.	$H_3PMo_{12}O_{40}$ (g)	H_2O_2 30% w/w added (g)	pH	Kappa	Viscosity (cP)	H_2O_2 consumed (g)	Yield[2] (%)	Brightness (%)
	1	0	1.65	7	8.1	17.4	0.43	95.7	61
	2	0	3.30	7	7.9	17.0	0.63	95.7	---
	3	0	16.5	7	6.4	13.1	1.7	96.6	---
	4	0.047	0	6	10.8	22.3	---	97.0	---
A	5	0.047	1.65	6	3.0	19.4	0.53	96.8	---
	6	0.047	16.5	6	2.8	15.0	1.5	96.2	---
	7	0.47	0	3	8.0	24.4	---	95.5	69
	8	0.47	1.65	3	2.1	20.6	0.61	95.4	80
	9	0.47	3.30	3	2.3	18.4	0.72	96.4	---
	10	0.47	16.5	3	2.0	17.6	1.4	96.2	---
	11	4.7	16.5	1	1.6	10.4	2.2	97.3	---
B	12	0.47	1.65	3	1.3	19.8	0.59	96.9	---
C	13	0.47	1.65	3	4.9	18.3	0.76	97.7	---

Conditions: 15 g of chelated pulp **A**, H_2O_2 30% w/w, 75 mg of $MgSO_4$, 70°C, 4 h, 3% consistency. Alkaline extraction after the bleaching reaction.

Our previous work (Bianchi *et al.*, 1998 and 1999) showed that, in opposition to $H_3PMo_{12}O_{40}$, $H_7PMo_8V_4O_{40}$ (in reactions using 4.7 and 4.1 g of POM respectively, 16.5 g of H_2O_2 30% w/w, 4 h, 60°C, 3% consistency) promotes high fiber degradation. The vanadium (on the structure of $H_7PMo_8V_4O_{40}$) in presence of oxygen and/or H_2O_2 produces radicals, which attack cellulosic fibers. Using milder conditions, we studied the influence of some catalysts (V_2O_5 and POMs containing different numbers of vanadium atoms) on the selectivity (Figure 3).

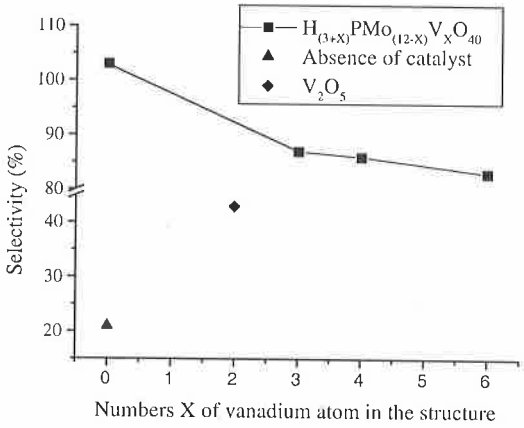

Figure 3. Influence of the catalysts on selectivity. 15 g of chelated pulp **B**, 0.025 mmol of POM or V_2O_5 (0.047 g $H_3PMo_{12}O_{40}$, 0.042 g of $H_7PMo_9V_3O_{40}$, 0.041 g of $H_7PMo_8V_4O_{40}$, 0.039 g of $H_7PMo_6V_6O_{40}$, 4.6 mg of V_2O_5), 1.65 g of H_2O_2 30% w/w, 75 mg of $MgSO_4$, 70°C, 4 h, 3% consistency. Alkaline extraction after the bleaching reaction. Selectivity = (Δkappa/Δviscosity) x 100, where Δkappa = kappa of the unbleached pulp - kappa of the bleached pulp and Δviscosity = viscosity of the unbleached pulp - viscosity of the bleached pulp.

We observed that V_2O_5 was not a good catalyst for the bleaching reaction. Raising the number of vanadium atoms in the structure of POM from 0 to 3 we observed a decrease in selectivity. Subsequent increase in the number of vanadium atoms from 3 to 4 and 6 did not promote significant changes in selectivity.

In order to check the possibility of recycling the bleaching liquor, we carried out many reactions using the same liquor, without previous treatment. After each reaction the amount of H_2O_2 consumed was determined and, for the following reaction, new charge of peroxide was added (an amount necessary to obtain 1.65 g of H_2O_2 in solution). Figure 5 shows a decrease in the selectivity and an increase in the H_2O_2 consumption after the first reaction (bleaching experiment 1). However, for the subsequent reactions (bleaching experiments 2, 3, 4, 5 and 6), the values of selectivity and H_2O_2 consumption fluctuate around 170 and 72%, respectively.

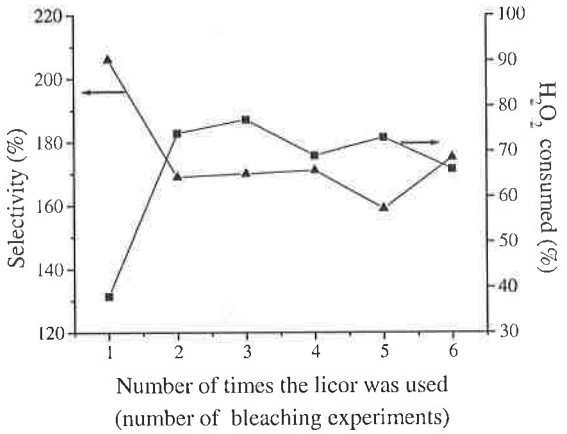

Figure 4. Selectivity and percentage of H_2O_2 consumed in each bleaching reaction using the "not treated" bleaching liquor. 15 g of chelated pulp **A**, 1.65 g of H_2O_2 30% w/w, 4 h, 70°C, 3% consistency, alkaline extractions after the bleaching reaction. First reaction: 0.47 g of $H_3PMo_{12}O_{40}$.

In order to oxidize the lignin fragments and some reduced POM presents in the bleaching liquor, we treated it with O_2 (3 h, 130°C, 7 bar of O_2), after each reaction. In this way, we tried to improve the efficiency for the subsequent reactions. We observed that, after the first experiment (Figure 5, bleaching experiment 1), H_2O_2 was completely consumed in all the subsequent reactions. The selectivity decreased more than in the last case (when the liquor was not treated), and remained at a value around 132%. The measures of kappa numbers and viscosities (not reported here) showed that the decrease in selectivity was due to an inefficient delignification, since the viscosities did not change significantly. This results suggest that POM has been decomposed in some species that consume the H_2O_2 and are not active in lignin oxidation.

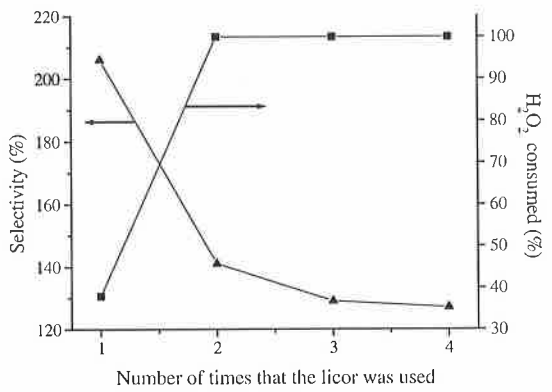

Figure 5. Selectivity and percentage of H_2O_2 consumed in each bleaching reaction using the "treated" bleaching liquor. 15 g of chelated pulp **A**, 1.65 g of H_2O_2 30% w/w, 75 mg of $MgSO_4$, 4 h, 70ºC, 3% consistency, alkaline extraction after bleaching. First reaction: 0.47 g of $H_3PMo_{12}O_{40}$.

In an attempt to verify what happens with the bleaching liquor we recorded a ^{31}P NMR spectrum of $H_3PMo_{12}O_{40}$ solution (in presence of H_2O_2) and compared with the spectra from the treated and not treated bleaching liquors. Figure 7 shows that the ^{31}P NMR spectra **a** and **b** are very similar, with signals at 0.8 and

1.6 ppm, indicating that the catalytic species are the same. These species, probably, are peroxo complexes, formed when POM and H_2O_2 are mixed.

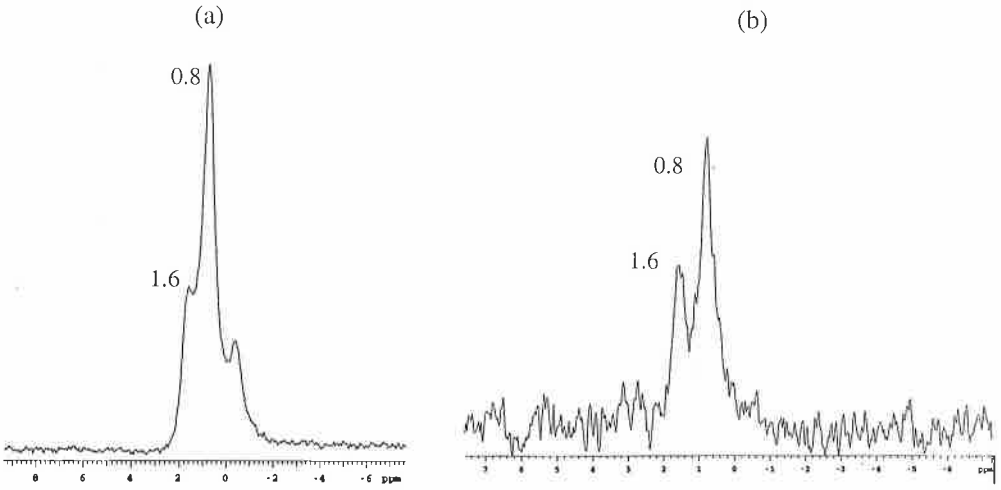

Figure 6. ^{31}P NMR of $H_3PMo_{12}O_{40}$. (a) in H_2O_2 solution, (b) "not treated" bleaching liquor (bleaching experiment 6, Figure 4). 85% H_3PO_4 as reference.

However Figure 7 shows, beyond the signal at 0.8 ppm, some more peaks, indicating that POM has been decomposed in other species.

Since we obtained better results with the "not treated" liquor, the treatment used seems to be inefficient to recuperate it.

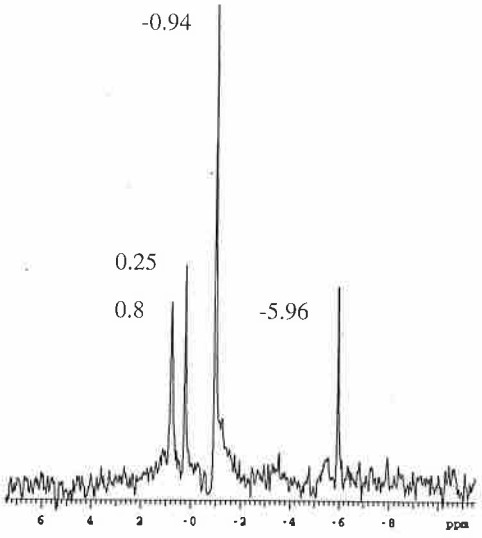

Figure 7. ^{31}P NMR of the "treated" bleaching liquor (bleaching experiment 4, Figure 5). 85% H_3PO_4 as reference.

CONCLUSION

The bleaching system POM/H_2O_2, used in this work, produced good quality pulps. The use of POM in combination with H_2O_2 promoted an effective pulp delignification and does not degrade the fibers significantly. Best results are obtained using 0.47 g of $H_3PMo_{12}O_{40}$ and 1.65 g of H_2O_2 30% w/w (15 g of chelated pulp, 75 mg of $MgSO_4$, 4 h, 70°C, 3% of consistency) producing pulps with a kappa number of 2, viscosity of 20 cP and a brightness of 80%. The system was more efficient for hardwoods pulps (**A** and **B**) but relatively good results were obtained with pulp **C** (hardwood/softwood kraft pulp). The use of POM improves the brightness, since brighter pulps were obtained in the presence of catalyst than in the control reactions (in the absence of POM).

Variation of the number of vanadium atoms in the structures of POM ($H_6PMo_9V_3O_{40}$, $H_7PMo_8V_4O_{40}$, $H_9PMo_6V_6O_{40}$) did not improve the selectivity (no significant changes in kappa number and viscosity values). Best results were still obtained in the absence of vanadium atoms.

Using the same bleaching liquor (without previous treatment) in several bleaching sequences, the selectivity decreased slightly and the H_2O_2 consumption increased after the first reaction. After the sixth experiment a good selectivity was still obtained. This indicate that the bleaching liquor can be reused in more than one sequence.

Contrary to our expectations, the treated bleaching liquor (3 h, 130°C, 7 bar of O_2) did not improve the selectivity and, after the first reaction, all the H_2O_2 added was consumed.

^{31}P NMR spectra suggest a decomposition of the POM, probably with the formation of other species that consume the H_2O_2 and are not active in lignin oxidation.

Finally the POM promoted the pulp delignification did not cause significantly damage on fibers and improved brightness. Moreover we have the possibility to reuse the bleaching liquor.

The next step will be the removal of lignin fragments dissolved and the recuperation of the bleaching liquor, using different methods.

Acknowledgments. The authors acknowledge financial support and fellowships from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

REFERENCES

Berzins, V. (1966). Micro kappa number. *Pulp Paper Mag. Can.*, **67**, T 206-T208.

Bianchi, M. L., Crisol, R. & Schuchardt, U. (1999). Bleaching of commercial pulps with H_2O_2 catalyzed by heteropolyacids. *Biores. Technol.*, **68**, 17-21.

Bianchi, M. L., Crisol, R. & Schuchardt, U. (1998). Hydrogen peroxide bleaching of commercial pulps in the presence of heteropolyacids. *Fifth European Workshop on Lignocellulosics and Pulp: In Advances in lignocellulosics chemistry for ecologically friendly pulping and bleaching technologies*, Proceedings, pp. 191-104.

Dickman, M. H. & Pope, M. T. (1994). Peroxo and superoxo complexes of chromium, molybdenum and tungsten. *Chem. Rev.*, **94**, 569-584.

Evtugin, D. V. & Pascoal Neto, C. (1997). A process of oxidative delignification of lignin cellulose materials in the presence of heteropolyanions. Pt patent 101857.

Jansen, R. J. J., van Veldhuizen, H. M., Schwegler, M. A. & van Bekkum, H. (1994). Recent (1987-1993) developments in heteropolyacids catalysts in acid catalyzed reactions and oxidation catalysis. *Recl. Trav. Chim. Pay-Bas*, **113**, 115-135.

McDonough, T. J. (1995). Recent advances in bleached chemical pulp manufacturing technology. *Tappi, J.*, **78**, 55-62.

Pope, M. T. (1983). Heteropoly and Isopoly Oxometalates, Springer-Verlag, New York, pp. 23-28.

Solomon, K. R. (1996). Chlorine in the bleaching of pulp paper. *Pure and Appl. Chem.*, **68**, 1721-1730.

Technical Association of the Pulp and Paper Industry, Viscosity of pulp (capillary viscometer method). TAPPI Standard Methods, T230, om-82.

Venturello, C., D'Aloisio, R., Bart, J. C. J & Ricci, M. (1985). A new peroxotungsten heterepoly anion with special oxidizing properties: Synthesis and structure of tetrahexylammonium tetra(diperoxotungsto) phosphate (3-). *J. Mol. Catal.*, **32**, 107-110.

Weinstock, I. A. & Hill, C. L. (1994). Oxidative bleaching of wood pulp by vanadium-substituted polyoxometalates. US Pat. 5,302,248.

Hydroxymethylation of lignins evaluated by PCA applied to FTIR data

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ABSTRACT

The first step in obtaining phenolic resin involves hydroxymethylation. This study focuses on hydroxymethylation of kraft and Alcell lignins in alkaline medium. The results of the reaction were evaluated by determining HCHO consumption in the reaction mixture and by analysing the lignin derivatives by infrared spectroscopy (FTIR). The FTIR spectra were evaluated by principal component analysis (PCA). The parameters for hydroxymethylation were: HCHO/lignin ratio of 2:1, NaOH/lignin ratio of 1:1 and 15% solids content. The lignin was solubilized in alkaline solution and the formaldehyde (as 37% solution) was slowly added to the mixture. The reaction was maintained for 360 min at 60°C under constant stirring. Every 30 or 60 min of reaction, 2 samples of the reaction mixture were collected. One was used to determine the residual HCHO content in the mixture, by reaction with Na_2SO_3/HCl and potentiometric back-titration with NaOH, whereas the other was used to precipitate hydroxymethylated lignin, which was analyzed by FTIR (from 400 to 4000 cm^{-1}), using KBr pellets. The evaluation of the FTIR data by PCA showed that PC1 describes 87% and 77% of the spectra variance for hydroxymethylated kraft and Alcell lignins, respectively. Plotting the PC1 scores as a function of the reaction time revealed stable values after 210 min for kraft lignin and after approximately 200 min for Alcell lignin. These results are in agreement with the HCHO consumption during the reaction, showing that FTIR/PCA method is adequate to determine the end of chemical modifications in lignin struture.

Keywords: hydroxymethylation of lignins, principal component analysis, FTIR of lignins, utilization of lignins, lignin modification

INTRODUCTION

Lignins can be used as components of phenol-formaldehyde (PF) resins. The first step in the synthesis of PF resins is the addition of formaldehyde to phenol or lignin (hydroxymethylation). This study evaluates the reactivity of different lignins with the hydroxymethylation time, by means of two analytical methods: determination of formaldehyde consumption in the reaction mixture (by volumetric analysis) and infrared spectra of the hydroxymethylated lignins produced.

FTIR spectroscopy is an instrumental method largely used for characterization of lignocellulosics and for analysis of their chemical modifications. Principal component analysis (PCA) is a mathematical methodology for data treatment, already adopted by researchers dealing with lignocellulosics (ACS Symposium, 1996). By means of PCA, the whole FTIR spectrum (from 400 to 4000 cm^{-1}; approximately 1800 points) can be reduced to a few parameters (principal components – PC) for which similar spectra (samples) will give similar scores (Beebe and Kowalski, 1987). This method is specially useful for classifying lignins and pulps (Angelo and Durán, 1998; Billa *et al.*, 1998; Fardim and Durán, 1998; Gonçalves *et al.*, 1998; Cotrim *et al.*, 1999) and for accompanying chemical reactions (Benar *et al.*, 1999).

The hydroxymethylation of Acetosolv lignin from sugarcane bagasse at 40°C has been already investigated by us (Benar *et al.*, 1999). By PCA treatment of FTIR spectra, the reaction time required is 4 h, which is confirmed by the formaldehyde consumption during reaction. After this time, condensation reactions occur. In our present work, we have studied the hydroxymethylation of kraft and Alcell lignins at 60°C, to verify the suitability of FTIR/PCA as a quantitative method for predicting the end of reactions in lignins.

EXPERIMENTAL

The kraft lignin was obtained by industrial pulping of eucalyptus and precipitated at pH 1. The Alcell lignin was obtained from a pilot plant by pulping of softwoods. Both lignins were filtered, dried at 60°C and sieved. Hydroxymethylation was performed with 10 g of lignin in 82.50 mL of water by addition of 2.22 g of NaOH

and 9 g of formaldehyde (8.33 mL of a 37%-aqueous solution) in a round bottom flask, which was kept at 60±2°C under constant stirring. For both lignins, 2 samples of the reaction mixture were taken every 30 or 60 min of reaction, during the whole hydroxymethylation time (360 min). In one sample the amount of residual formaldehyde in solution was determined by reaction with Na_2SO_3/HCl and potentiometric back-titration with NaOH (Peng *et al.*, 1993). The other sample of the mixture was added dropwise to water (pH 1). The solid obtained (hydroxymethylated lignin) was exhaustively washed with water, filtered, and dried over P_2O_5/ vaccum. FTIR spectra were recorded in a Nicolet 520, using pellets containing 1.5 mg of lignin and 350 mg of KBr.

All the spectra were recorded from 400 cm^{-1} to 4000 cm^{-1} and normalized for the absorption of the aromatic ring vibrations at 1510 to 1514 cm^{-1}. Spectra of kraft lignins and of Alcell lignins were converted into text files using OMNIC software (Nicolet). The normalized spectra were submitted to PCA calculations using the BIOTEC and FAEN programs compiled in FORTRAN, which were written in our laboratory and adapted from the literature (Scarminio and Bruns, 1989). Graphical presentations were made with Microsoft EXCEL 5.0.

RESULTS AND DISCUSSION

The FTIR spectrum of hydroxymethylated lignins were determined every 30 or 60 min, within a total reaction time of 360 min (= 12 spectra). Figure 1 shows FTIR spectra of the original lignin and samples of hydroxymethylated kraft (a) and Alcell (b) lignins (after 240 min of reaction). The spectra show that the bands presenting chemical modifications in lignin are: region at 3400 cm^{-1} (corresponding to O-H stretching), region at 2900 cm^{-1} (corresponding to C-H stretching); absorptions at 1680 cm^{-1} (related to C=O bands) and 1010 cm^{-1} (related to C-O stretching in primary alcohols). However, a univariate study of the absorptions of individual bands result in poor information about the reaction.

The PCA treatment of the whole spectra of both lignins shows that six principal components describe almost the total variance in the system (Table 1). The first principal component (PC1) describes the major variance for kraft (87.0%) and Alcell (77.0%) lignins.

Table 1. Percentages of Variance Captured by PCA Model of FTIR spectra for hydroxymethylated kraft and Alcell lignins.

Principal Component	% Variance	
	kraft lignin	Alcell lignin
PC 1	87.0	77.0
PC 2	6.5	18.9
PC 3	4.0	3.1
PC 4	0.8	0.4
PC 5	0.7	0.2
PC 6	0.5	0.1

It was observed that the PC1 scores correspond to the reaction times. This shows that PC1 is related to the chemical modifications in the lignin struture due to the global reaction. Figure 2 presents the plot of PC1 scores *versus* hydroxymethylation time and also the consumption of HCHO during the reaction time for each lignin.

In the case of kraft lignin the PC1 scores stabilize after 210 min, showing that with longer reaction times, the modifications in the lignin structure are smaller. The PCA results are in agreement with the results of HCHO consumption as a function of the reaction time. In the case of Alcell lignin, the plots of the PC1 scores and the results of HCHO consumption as a function of the hydroxymethylation time are also in agreement. In both cases the reaction occurs until approximately 200 min at 60°C, and the other principal components (PC2 to PC6) do not correspond to the hydroxymethylation time.

The PC1 loadings show IR bands with high significance for the hydroxymethylation of both lignins (Figure 3).

In regard to kraft lignin, the IR absorption with higher significance in PC1 loadings corresponds to O-H stretching. The other significant bands are related to C-O bands in alcohols and ether groups. As for Alcell lignin, not only the same bands observed for PC1 loadings of kraft lignin, but also the IR bands

corresponding to C-H stretching and C=C in aromatic rings have high significance. It can be concluded that structural chemical modification caused by hydroxymethylation is greater in Alcell lignin than in kraft lignin.

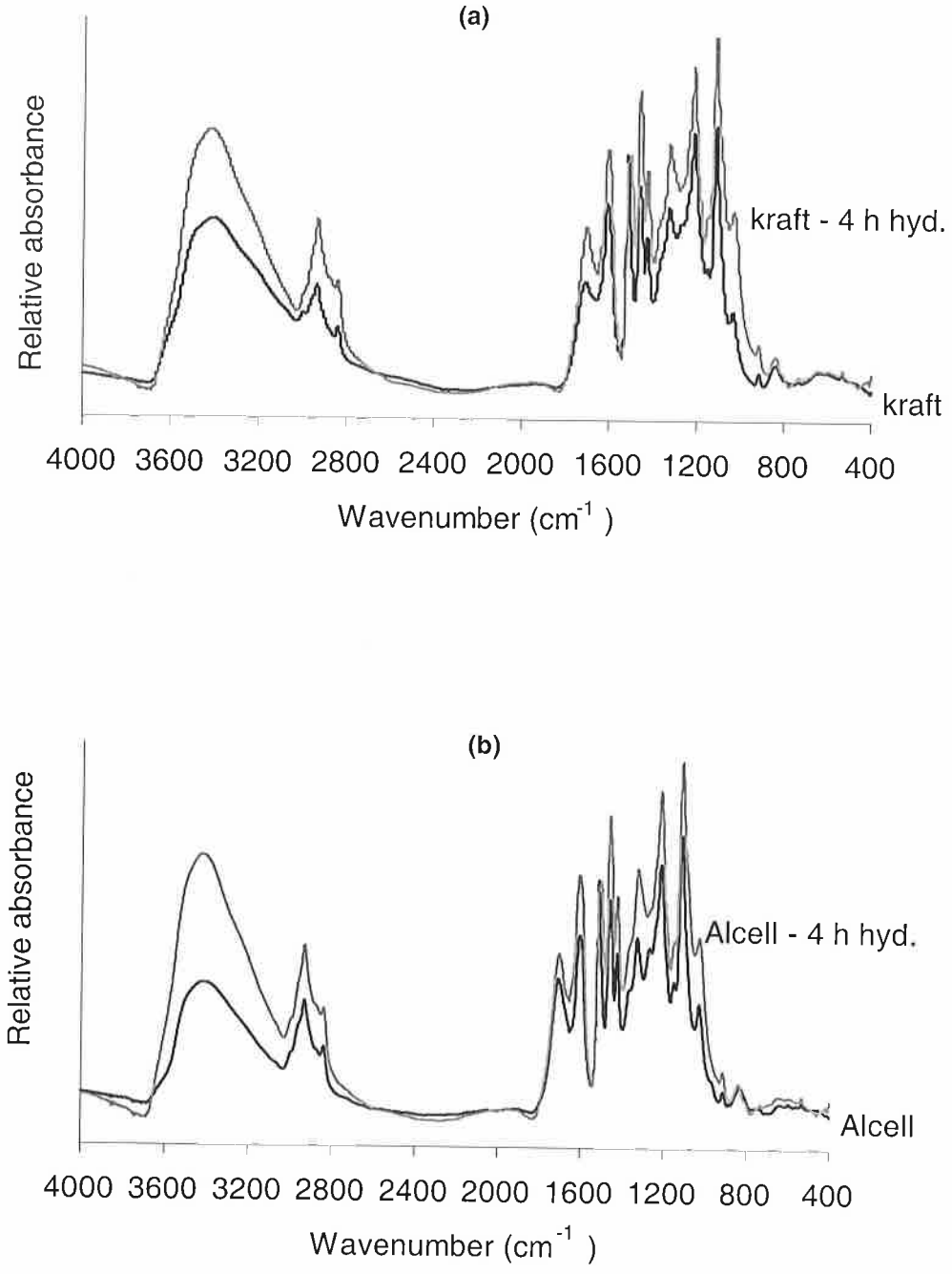

Figure 1: FTIR spectra of original and hydroxymethylated kraft (a) and Alcell (b) lignins.

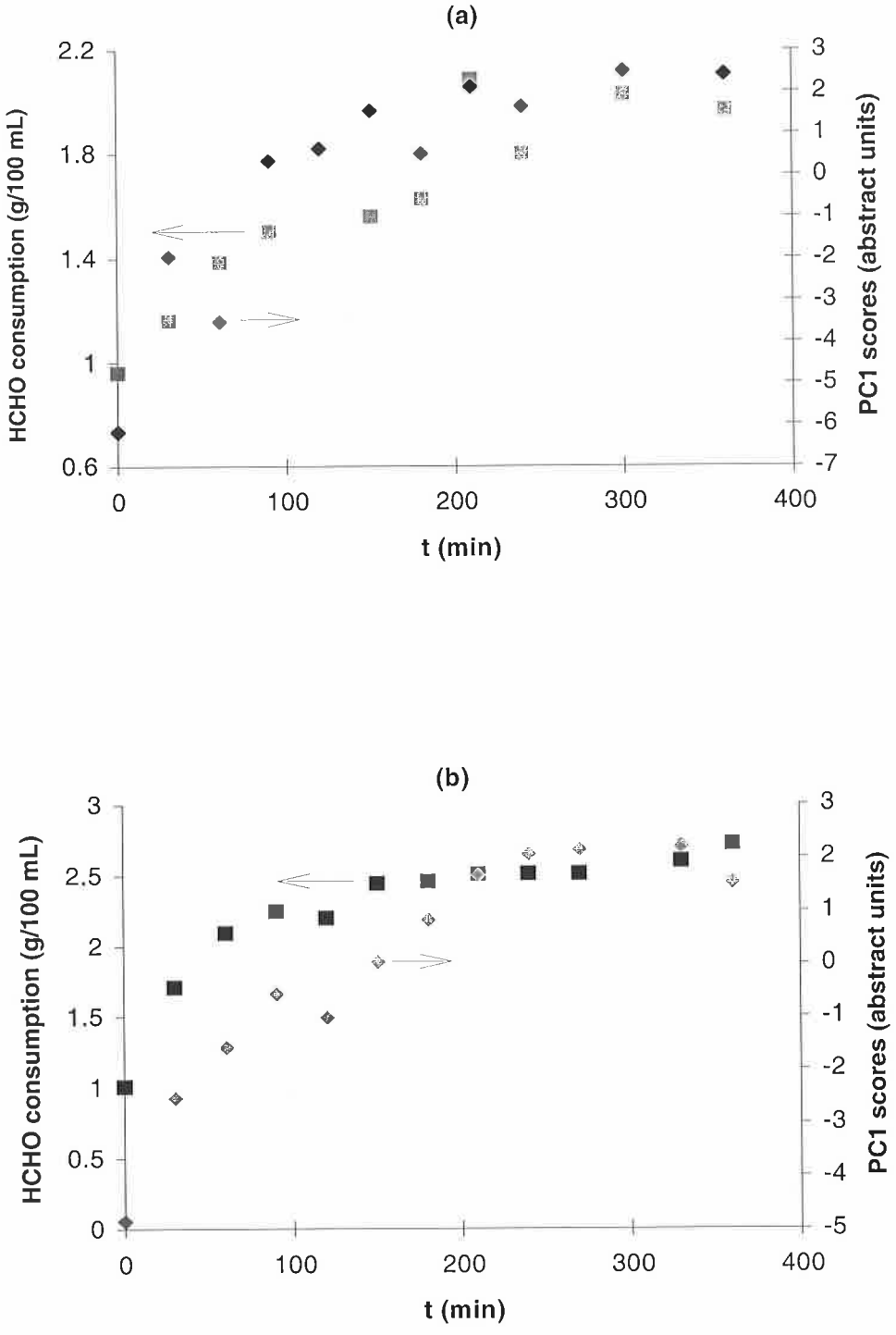

Figure 2. PC1 scores and HCHO consumption *versus* hydroxymethylation time of (a) kraft lignin and (b) Alcell lignin.

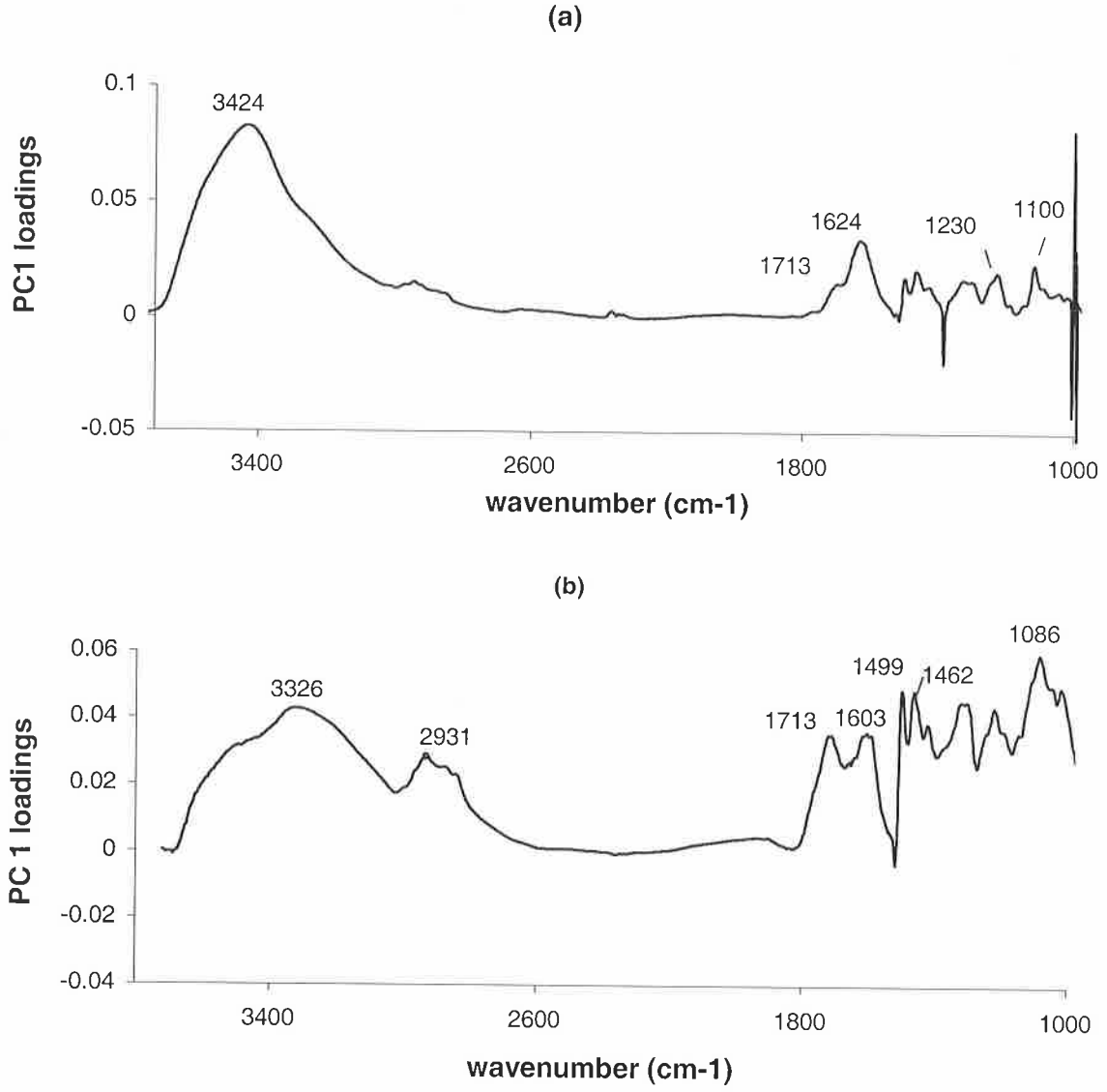

Figure 3. PC1 loadings *versus* wavenumber of the FTIR spectra of hydroxymethylated (a) kraft and (b) Alcell lignins.

CONCLUSIONS

The PCA treatment of FTIR data provides a good method to study the end of chemical modifications in lignin structure. For hydroxymethylation of lignins, the result of PC1 scores correspond to the reaction time and are in agreement with the HCHO consumption in the reaction medium.

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REFERENCES

ACS Symposium' 96, Cellulose, Paper and Textile Division; Chemometrics in Biomass Characterization and Biomass Conversion, Orlando/Florida, Aug. 28-29/1996.

Angelo, R.S. & Durán, N. (1998). Characterization of Xylanase-Aided TCF Bleaching of *Eucalyptus grandis* kraft pulps by XPS, FTIR and WIR spectroscopies. *Fifth European Workshop on Lignocellulosics and Pulp*, Proceedings , pp. 505-508.

Beebe, K.R. & Kowalski, B.R. (1987), *Anal. Chem.* **59**, 1007.

Benar, P. , Gonçalves, A.R., Mandelli, D., Ferreira, M.M.C. & Schuchardt, U. (1999). Principal component

analysis of the hydroxymethylation of sugarcane lignin: A time-depending study by FTIR. *J. Wood Chem. Technol.* **19**, 151.

Billa, E. , Koutsoula, E. & Koukios, E. (1998). Multivariate Chemometrics Analysis of Fluorescence Spectra of Paper Pulps from different biological sources. *Fifth European Workshop on Lignocellulosics and Pulp*, Proceedings, pp. 475-478.

Cotrim, A.R., Ferraz, A., Gonçalves, A.R., Silva, F.T. & Bruns, R.E. (1999). Identifying the origin of lignins and monitoring their structural changes by means of FTIR-PCA and -SIMCA. *Bioresource Technol.* **68**, 29.

Fardim, P. & Durán, N. (1998). Fearing strength and kraft pulp chemical composition: a principal components analysis study. *Fifth European Workshop on Lignocellulosics and Pulp*, Proceedings, pp. 87-90.

Gonçalves, A.R., Esposito, E. & Benar, P. (1998). Evaluation of *Panus tigrinus* in the delignification of sugarcane bagasse by FTIR-PCA and pulp properties, *J. Biotechnol.* **66**, 177.

Peng, W., Riedl, B. & Barry, A.O. (1993). Study on the Kinetics of Lignin Methylolation, *J. Appl. Polym. Sci.* **48**, 1757.

Scarminio, I.S. & Bruns, R.E. (1989). An adaptation of ARTHUR for microcomputers. *Trends Anal. Chem.*, **8**, 326.

Identification of low molecular weight aromatic compounds present in the hydrolyzate obtained from sugar cane bagasse pretreated by steam explosion

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ABSTRACT

Hydrolyzates obtained from steam exploded sugar cane bagasse contain several aromatic compounds, originated from lignin degradation, besides furfural and hydroxymethylfurfural, which are produced from xylan and cellulose, respectively. Some of them are toxic and can inhibit fermentative and enzymatic processes. In order to identify and isolate these compounds, sugar cane bagasse was pretreated at 190°C for 15 min by steam explosion and its water-soluble fraction was acidified with 2 mol.L^{-1} HCl (pH = 2) and extracted with ethyl acetate. Subsequently, the ethyl acetate fraction was extracted three times with saturated $NaHCO_3$ solution and three times with 2 mol.L^{-1} NaOH solution. Both the $NaHCO_3$ and the NaOH-soluble fractions were acidified with 2 mol.L^{-1} HCl and successively extracted with methylene chloride and ethyl acetate. Thus, the aromatic compounds present in the hydrolyzate were separated into four fractions: total, acid, phenolic and neutral. These fractions were analyzed by GC-MS and the identified compounds were quantified by GC/FID, using a DB-5 capillary column (30 m x 0.25 mm). In the acid fraction, cis- and trans-coumaric and trans-ferulic acids were found as major compounds. The products contained in the phenolic fraction were similar to those contained in the acid fraction, trans-coumaric acid being the major product in the latter. In the neutral fraction, no compound was identified. All of these compounds are potential inhibitors of many fermentative and enzymatic processes. Three of them, p-hydroxybenzoic and syringic acids and syringaldehyde, have already proven to be toxic to *Saccharomyces cerevisiae*.

Keywords: Sugar cane bagasse, lignin degradation products, steam explosion, inhibitors, hemicellulosic hydrolyzate

INTRODUCTION

Steam explosion pretreatment has been used to enhance the susceptibility of lignocellulosics to biotechnological conversion (Lawther *et al.*, 1996; Wilson *et al.*, 1989). However, during the pretreatment, water-soluble compounds such as acetic acid, sugar derived by-products and lignin degradation products are formed. Acetic acid is released through the partial hydrolysis of hemicellulose. Sugar-derived by-products, e.g., furfural and 5-hydroxymethylfurfural, are formed from carbohydrates dehydration. Lignin degradation products, including a wide range of aromatic and polyaromatic compounds with a variety of substituents, are originated from the clivage of β-O-4 lignin structures (McMillan, 1994; Fengel and Wegener, 1989).

Some of these substances can inhibit both hydrolysis and fermentation processes (Palmqvist *et al.*, 1996; Hahn-Hägerdal, 1996; Fenske *et al.*, 1998). Vanillin, syringaldehyde, acetic acid and furfural, for instance, can inhibit fermentation by *Pichia stipitis*. Syringaldehyde, vanillin and other lignin-derived compounds are potent inhibitors of ethanol production and cell growth. Also, vanillin, a guaiacyl-containing compound, was shown to be inhibitory than representative syringyl- and hydroxybenzyl- containing compounds (Delgenes *et al.*, 1996).

This paper reports the results obtained from the isolating and identifying of low molecular weight aromatic compounds, formed from lignin degradation during the steam explosion of sugar cane bagasse, in order to understand how these compounds are formed.

EXPERIMENTAL

Preparation of hemicellulosic hydrolyzate

Sugar cane bagasse was steam pretreated at 190°C for 15 min (Silva, 1995). The resulting material was

filtered and washed with 2000 mL water at 50°C and the filtrate was extracted by different organic solvents, according to the procedure desbribed below and outlined in Figure 1.

Extraction by solvents

The filtrate (580 mL) was acidified with 2 $mol.L^{-1}$ HCl (pH 2) and then extracted three times with ethyl acetate (OF1). The ethyl acetate solution was successively extracted with saturated $NaHCO_3$ solution and 2 $mol.L^{-1}$ NaOH. The $NaHCO_3$ and the NaOH soluble fractions were acidified with 2 $mol.L^{-1}$ HCl and extracted with methylene chloride (OF3 and OF5) and ethyl acetate (OF2 and OF4), respectively. The $NaHCO_3$ solution was extracted with NaOH 2 $mol.L^{-1}$ solution three times (OF6). The organic fractions were identified as total (OF1), acid (OF2 and OF3), phenolic (OF4 and OF5) and neutral (OF6) fractions. The solvents were removed from all the organic fractions by evaporation and their residues were dried under P_2O_5/vacuum, analyzed by GC/MS and quantified by GC/FID (Cunha, 1999).

GC/MS and GC/FID

Two mg of each organic fraction was dissolved in 100 µL pyridine and fluorantene was used as an internal standard (200 ppm). The samples were converted to their TMS derivatives with 500 µL of *N,O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) at 60°C for 1 h (Cotrim, 1990) and analyzed by GC/MS (Gas chromatography/ mass spectrometry) using a Finnigan MAT GCQ™ model on a DB-5 (30 x 0,25 m) capillary column. Mass spectra were obtained at 70 eV. The carrier gas was helium, using a split of 1:100. Weight fragmentation and retention time were employed for identification. To complete the analysis of each sample, three column-temperature-programs were utilized, as shown in Table 1. The quantification of low molecular weight aromatic compounds in each fraction was carried out by GC/FID (Gas chromatography/flame ionization detector) using a Varian CX-3400 model on a DB-5 (30 x 0.25 m) capillary column. The carrier gas was helium, using a split of 1:100. Fluoratene was utilized as the internal standard and the column-temperature-programs used were the same that were used for GC/MS. The peaks before 11 min and after 42 min were considered as impurity of the solvent.

Table 1. Column-temperature-programs of organic fractions utilized in GC/MS and GC/FID analyses (Cunha, 1999).

SAMPLES	INITIAL		INTERMEDIARY			FINAL		
	Temp (°C)	Time (min)	Temp (°C)	Time (min)	Rate (°C/min)	Temp (°C)	Time (min)	Rate (°C/min)
OF2, OF4, OF6	60	1	140	2	15	280	10	2
OF1	80	1	140	4	4	280	25	4
OF3, OF5	60	1	140	3	30	240	13.33	2

RESULTS AND DISCUSSION

The sugar cane bagasse, the pretreated bagasse, the hydrolyzate and the fraction weights obtained from hydrolyzate extractions using organic solvents are shown in Table 2.

The weight of OF1, obtained from hydrolyzate extraction using ethyl acetate, represented 2.67% of the amount of compounds present in the hydrolyzate. When ethyl acetate and CH_2Cl_2 were used for extraction of the hydrolyzate sequentially, in order to separate the acid (OF2 and OF3), phenolic (OF4 and OF5) and neutral (OF6) fractions, the total weight of extracted-compounds increased to 13%. However, the analyses of these fractions by GC/MS (TIC chromatograms shown in Figures 2-4) and by GC/FID indicated that the identified low molecular weight compounds were detected as minor components.

The OF1 fraction contained 2.12% p-hydroxybenzaldehyde [1], 0.09% p-hydroxybenzoic acid [2], 4.1% trans-coumaric acid [3], 0.17% trans-ferulic acid [4], 0.40% syringaldehyde [5], 0.74% vanillin [7], corresponding to 7.62% of the OF1 total weight.

In the OF2 and OF3 acid fractions (Figure and Table 3), besides the compounds [2-5] and [7] present in OF1 fraction, cis-coumaric [6], vanillic [8] and syringic [9] acids were also found. On the other hand, p-hydroxybenzaldehyde [1] was not detected. The results also showed that cis-coumaric [6] and trans-ferulic [4] acids, vanillin [7] and syringaldehyde [5] were more soluble in ethyl acetate, while p-hydroxybenzoic [2], trans-coumaric [3] and syringic [9] acids were more soluble in CH_2Cl_2. Moreover, the amount of identified compounds corresponded to 4.5% and 4.0% of the total weight of OF2 and OF3 fractions, respectively.

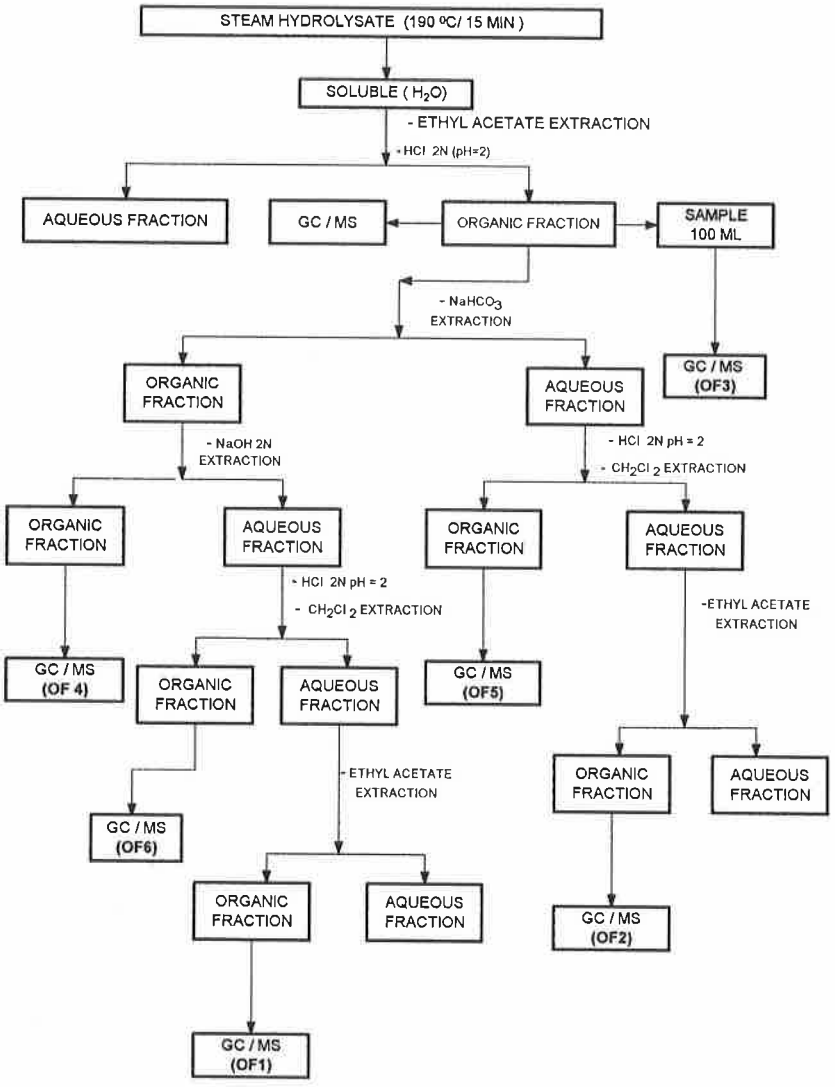

Figure 1. Scheme for hydrolyzate extraction with organic solvents.

Table 2. Sugar cane bagasse, pretreated bagasse, hydrolyzate and the fraction weights obtained from hydrolyzate extractions using organic solvents.

FRACTIONS		WEIGHT (g)	% YIELDS
Exploded bagasse (oven-dried)		10.0	100
Pretreated bagasse		6.6	66.0
Hydrolyzate		3.4	34.0
Total fraction	OF 1	0.0907	0.9
Acid fractions ($NaHCO_3$ ext.)	OF 2	0.1372	1.4
	OF 3	0.002	0.02
Phenolic fractions (NaOH ext.)	OF 4	0.087	0.87
	OF 5	0.002	0.02
Neutral fraction	OF 6	0.204	2.04

In the OF4 and OF5 phenolic fractions (Figure and Table 4), only vanillin [7], vanillic [9] and cis-coumaric [10] acids were not identified. The p-hydroxybenzaldehyde [1] (1.5%) and trans-coumaric acid [3] (2.99%)

were the main compounds extracted by CH_2Cl_2 and ethyl acetate, respectively. The other components were detected in less than 0.5% of the total amount of phenolic fractions. Additionally, the amount of identified compounds corresponded to 1.8% and 4.8% of the total weight of OF4 and OF5 fractions, respectively. The results also showed that syringaldehyde [5] and cis-coumaric [6] and vanillic [8] acids were not soluble in ethyl acetate, while p-hydroxybenzoic acid [2] and p-hydroxybenzaldehyde [1] were solubilized only by this solvent.

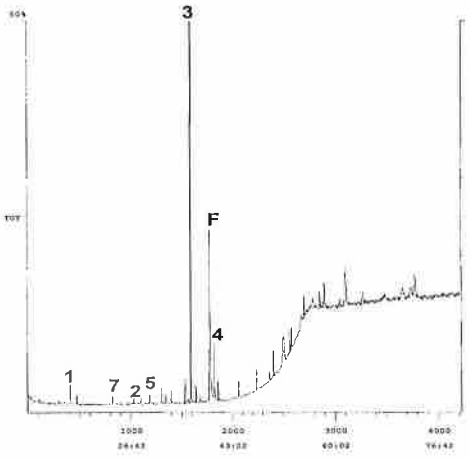

Figure 2. TIC chromatogram obtained from TMS-derivatized organic fraction total (OF1).

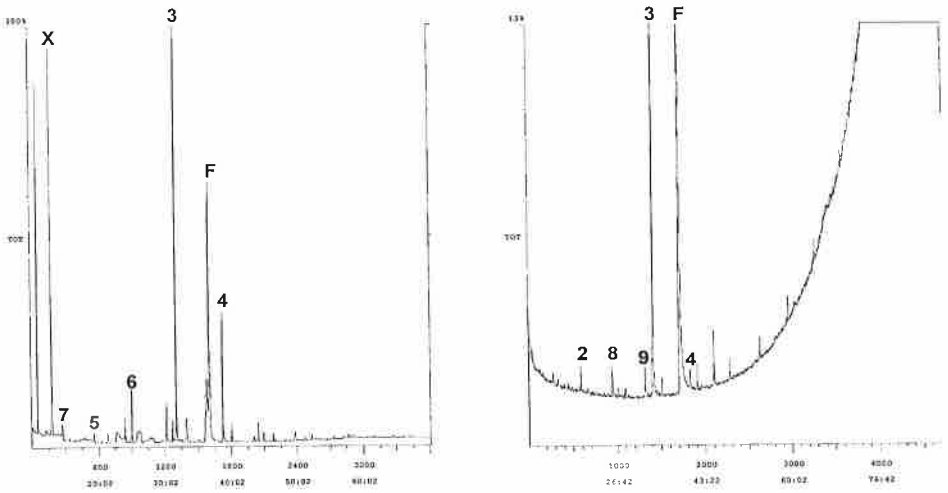

Figure 3. TIC chromatogram obtained from TMS-derivatized acid fractions (OF2 and OF3). (X= unidentified compound).

Table 3. Lignin-derived compounds identified and quantified in acid fractions obtained from hydrolyzate extraction with organic solvents. Percentage calculated in relation to total weight of each fraction.

COMPOUNDS	OF 2 (%)	OF 3 (%)
Trans-coumaric acid (3)	4.01	2.56
Cis-coumaric acid (6)	n.d.	0.51
P-hydroxybenzoic acid (2)	0.05	n.d.
Trans-ferulic acid (4)	0.04	0.32
Vanillin (7)	n.d.	0.40
Vanillic acid (8)	0.01	n.d.
Syringic acid (9)	0.39	n.d.
Syringaldehyde (5)	n.d	0.36

n.d. = not detected

No compounds were identified in the neutral fraction (OF6).

The aromatics identified were only 4.9% of the whole compounds in the hydrolyzate, which indicates that lignin was partially degraded to a low molecular weight compound and further repolymerized to a high molecular weight lignin derivative during the steam treatment (Ando, 1986).

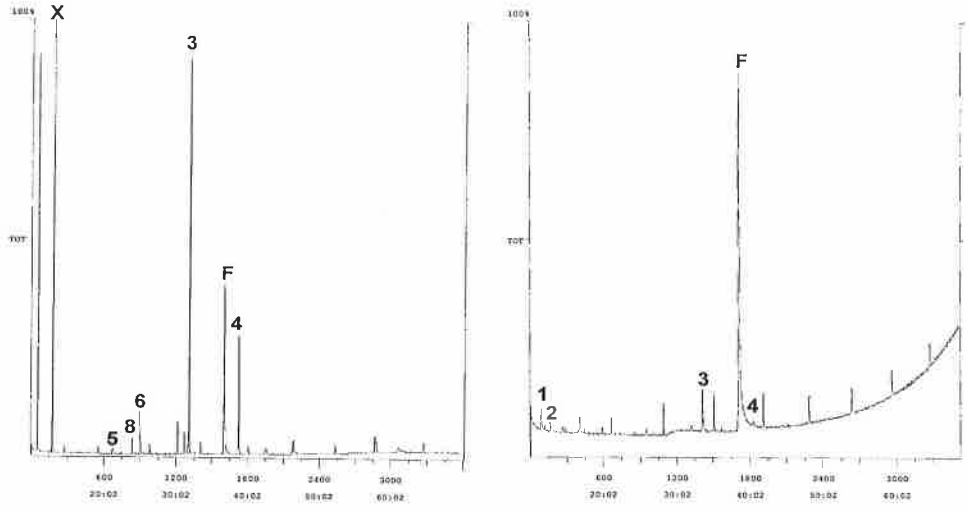

Figure 4. TIC chromatogram obtained from TMS-derivatized phenolic fractions (OF4 and OF5). (X= Unidentified compound)

Table 4. Lignin-derived compounds identified and quantified in the phenolic fractions obtained from hydrolyzate extraction with organic solvents. Percentage relative to total weight of each fraction.

COMPOUNDS	OF 4 (%)	OF 5 (%)
Trans-coumaric acid (3)	0.05	2.99
Cis-coumaric acid (6)	n.d.	0.64
P-hydroxybenzoic acid (2)	0.06	n.d.
Trans-ferulic acid (4)	0.14	0.45
Vanillic acid (8)	n.d.	0.012
p-hydroxybenzaldehyde (1)	1.5	n.d.
Syringaldehyde (5)	n.d	0.51

n.d. = not detected

The formation of these low molecular weight compounds during the biomass pretreatment and in hydrolytic process has been studied by different authors: Chua and Wayman (1979), Robert *et al.* (1988), Hishiyama and Sudo (1992), Bardet *et al.* (1985), Tanahashi *et al.* (1989, 1990) and Karina *et al.* (1990, 1992 a,b and 1994 a,b). Hishyama and Sudo (1992) detected Hibbert's ketones know as acidolysis monomers by gas chromatography-mass spectrometry (GC/MS) in the lignin fractions of steamed shirakaba wood (*Betula platyphylla* var. *japonica*) and concluded that lignin was decomposed by an acid-catalyzed hydrolysis during steaming. On the other hand, Tanahashi *et al.* (1989 and 1990) proposed a homolytic cleavage of the β-O-4 ether linkage of lignin by steam explosion and by high-temperature steaming, to form cinnamyl alcohol radicals that would be reduced to cinnamyl alcohol, oxidized to cinnamaldehyde, or coupled to dimers and higher compounds. Moreover, Iwanade *et al.* (1995) suggested that homolysis and acidolysis reactions should take place when guaiacylglycerol-β-guaiacyl ether was subjected to a steam treatment with holocellulose. In this work, acidolysis monomers (Hibbert's ketones) and cinnamyl alcohols were not detected in the reaction mixture obtained from the steam-treated sugar cane bagasse hydrolyzate, as expected from the cleavage of β-O-4 ether linkage by acidolysis or homolytic reaction mechanisms.

Thus, the proposed reaction mechanism of lignin degradation by steam explosion was not understood satisfactorily and does not explain the identified compounds shown in Figure 5, which are inhibitors of many fermentative and enzymatic processes.

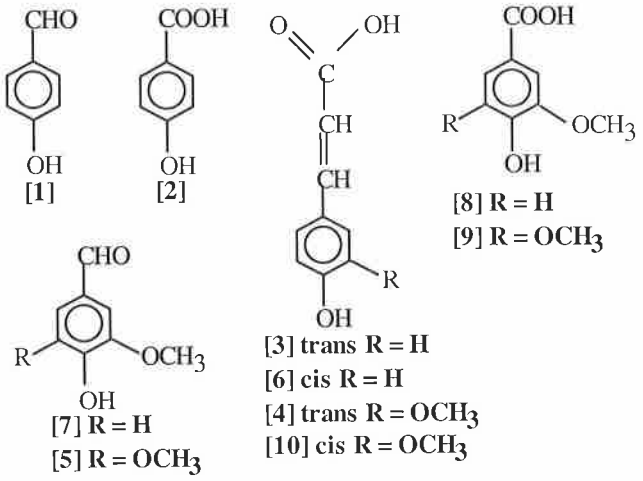

Figure 5. Identified degradation products in the hydrolyzate of pretreated sugar cane bagasse.

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REFERENCES

Ando, S.; Arai, I.; Kiyoto, K. & Hanai, S. (1986) Identification of aromatic monomers in steam-exploded poplar and their influences on ethanol fermentation by *Saccharomyces cerevisiae. J. Ferment. Technol.*, **64 (6)**, 567-570.

Bardet, M.; Robert, D. & Lundquist, K. (1985). On the reactions and degradation of the lignin during steam hydrolysis of aspen wood. *Svensk Papperstidn*, **6**, 61-67.

Chua, M.G.S. & Wayman, M. (1979). Characterization of autohydrolysis aspen (*P. tremuloides*) lignins. Part 1. Composition and molecular weight distribution of extracted autohydrolysis lignin. *Can. J. Chem.*, **57**, 1141-1149.

Cotrim, A.R (1990). Separação e caracterização de óleos provenientes da liquefação direta de ligninas da hidrólise ácida de eucalipto. PhD Thesis, Universidade de Campinas,.

Cunha, H.C.M. (1999). Caracterização do bagaço de cana pré-tratado por explosão a vapor: identificação de inibidores potenciais de processos fermentativos e enzimáticos. MsC Thesis, Faculdade de Engenharia Química de Lorena.

Delgenes, J.P.; Moletta, R. & Navarro, J.M. (1996). Effects of lignocellulosic degradation products on ethanol fermentations of glucose and xylose by *Saccharomyces cerevisiae, Zymomonas mobilis, Pichia stipitis,* and *Candida shehatae. Enzyme Microb. Technol.*, **19**, 220-225.

Fengel, D. & Wegener, G. (1989). *Wood-Chemistry, Ultrastruture and Reactions*. Walter de Gruyter, Berlin, New York.

Fenske, J.J.; Griffin, D.A & Penner, M.H. (1998) Comparison of aromatic monomers in lignocellulosic biomass prehydrolyzates. J. *Ind. Microbiol. Biotechnol.*, **20**, 364-368.

Hahn-Hägerdal, B. (1996). Ethanolic fermentation of lignocellulose hydrolyzates. *Appl. Biochem. Biotech.* **57/58**, 195-199.

Hishiyama, S. & Sudo, K. (1992). Degradation mechanism of lignin by steam-explosion. *Mokuzai Gakkaishi*, **38 (10)**, 944-949.

Iwanade, A.; Ohi, H. & Yamaguchi, A. (1995). Steam treatment of guaiacylglycerol-β-guaiacyl ether on a holocellulose support. *Mokuzai Gakkaishi*, **41 (7)**, 683-688.

Karina, M.; Tanahashi, M. & Higuchi, T. (1990). Degradation mechanism of lignin accompanying a steam explosion. III. Degradation of non-phenolic β-O-4 lignin substructure model compound by a steam treatment. *Mokuzai Gakkaishi*, **36 (6)**, 466-473.

Karina, M.; Tanahashi, M. & Higuchi, T. (1992a). Degradation mechanism of lignin by steam explosion. IV. Steam treatment of a dehydrogenative polymer of coniferyl alcohol. *Mokuzai Gakkaishi*, **38 (2)**, 159-165.

Karina, M.; Tanahashi, M. & Higuchi, T. (1992b). Degradation mechanism of lignin by steam explosion. V. Steam treatment of guaiacylglycerol-α-guaiacylpropyl-β-guaiacyl diether. *Mokuzai Gakkaishi*, **38 (2)**, 166-172.

Karina, M.; Tanahashi, M. & Kuwahara, M. (1994a). Degradation mechanism of lignin by steam explosion. VI. Steam treatment of milled wood lignin. *Mokuzai Gakkaishi*, **40 (9)**, 943-949.

Karina, M.; Ueno, N.; Tanahashi, M. & Kuwahara, M. (1994b). Degradation mechanism of lignin by steam explosion. VII. Steam treatment of syringyl type lignin model compounds. *Mokuzai Gakkaishi*, **40 (9)**, 950-957.

Lawther, J. M.; Sun, R. & Banks, W.B. (1996). Effect of Stem Treatment on the Chemical Composition of Wheat Straw. *Holzforschung*, **50**, 365-371.

McMillan, J. D. (1994) Conversion of hemicellulose hydrolyzates to ethanol, *In Enzymatic Conversion of Biomass for Fuels Production*, Himmel, M.E.; Baker, J.O.; Overend, R.P., eds, American Chemical Society Symposium Series 566, pp. 411-437.

Palmqvist, E.; Hahn-Hägerdal, B.; Galbe, M. & Zacchi, G. (1996). The effect of water-soluble inhibitors from steam-pretreated willow on enzymatic hydrolysis and ethanol fermentation. *Enzyme Microb. Technol.*, **19**, 470-476.

Robert, D.; Bardet, M.; Lapierre, C. & Gellerstedt, G. (1988). Structural changes in aspen during steam explosion treatment. *Cell. Chem. Technol.*, **22**, 221-230.

Silva, F.T. (1995). Obtenção de insumos químicos a partir do aproveitamento integral do bagaço de cana. PhD Thesis, Universidade de Campinas.

Tanahashi, M.; Karina, M. & Higuchi, T. (1990). Degradation mechanism of lignin accompanying steam explosions. II. High-pressure steam treatment of guaiacylglycerol-β-guaiacyl ether. *Mokuzai Gakkaishi*, **36 (5)**, 380-388.

Tanahashi, M.; Karina, M.; Tamabuchi, K. & Higuchi, T. (1989). Degradation mechanism of lignin accompanying steam explosions. I. Degradation products of lignin and β-O-4 lignin substructure model dimers. *Mokuzai Gakkaishi*, **35 (2)**, 135-143.

Wilson, J.J.; Deschatelets, L. & Nishikawa, N.K. (1989). Comparative fermentability of enzymatic and acid hydrolyzates of steam-pretreated aspenwood hemicellulose by *Pichia spitipis* CBS 5776. *Appl. Microbiol. Biotechnol.*, **31**, 592-596.

Identification of low molecular weight organic compounds in an effluent from TCF bleaching of hardwood kraft pulp before and after biotreatment

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ABSTRACT

Low molecular weight organic compounds present in a TCF (totally chlorine-free) bleaching effluent were investigated. After acidification to pH 2, the effluent was extracted with ethyl acetate. The ethyl acetate phase was derivatized with N, O-bis(trimetilsilil)trifluoroacetamida (BSTFA). TMS derivatives were analyzed by gas chromatography/mass spectrometry (GC/MS). GC/MS data revealed the occurrence of some compounds derived from sugars and lignin oxidation. Among theses compounds, butanedioic; methyl-, butanedioic; 2,3,-bis(oxy)-propanoic; oxy-butanedioic; oxy-propanedioic; 3-methoxy-4-(oxy)-benzoic and 3,4-dimethoxy-benzoic were detected. An effluent sample recovered after biological treatment with the fungus *Lentinus edodes* was analyzed. The biotreatment was carried out in a shaker at 150 rpm and at 28-30°C over a period of 5 days without any additional carbon sources. The characterization of this treated fraction by GC/MS indicated that methyl-, butanedioic and 3-methoxy-4-(oxy)-benzoic were recalcitrant compounds to the biological treatment with this white-rot fungus.

Keywords: kraft pulp effluent, effluent treatment, lignin

INTRODUCTION

The generation of effluents from totally chlorine-free (TCF) bleaching processes includes a new class of xenobiotics that has been extensively studied in order to evaluate the impact of their discharge on the environment (Folke *et al.*, 1996; O'Connor *et al.*, 1993; Verta *et al.*, 1996; Paiva *et al.*, 1998). Generally, these effluents reports low in COD, BOD and color values leading to the premature conclusion that these effluents have little toxicity. However, the characteristics and the environmental impact of the TCF effluents are not completely understood (Ristolainen and Alén, 1998).

Gas chromatography/mass spectrometry (GC/MS) has been applied to the identification of organic material in bleach plant effluents (Ristolainen and Alén, 1998; Dethlefs *et al.*, 1995; Dahlman *et al.*, 1995; Le Loacheur *et al.*, 1993). This work, deals with the identification of some low molecular weight organic compounds present in a TCF bleaching effluent.

EXPERIMENTAL

Fungal strain

L. edodes (strain UEC-2019) was obtained from UNICAMP Culture Collection (Biol. Chem. Lab.). Stock cultures were maintained at 5°C in Petri dishes containing 1.25% malt extract medium.

Effluent

All experiments were carried out with EOP effluent generated in a kraft pulp mill, which uses *Eucalyptus urograndis* as the raw material. The effluent presented a pH value ranging from 9.5 to 11.5 and was stored at -18°C until it was used.

Inoculum preparation

Lentinus edodes was grown as previously described (Esposito *et al.*, 1991; Paiva *et al.*, 1997). Inoculum consisted of 8 pieces (0.5 cm in diameter) of cultures previously grown in agar plates into 125 mL Erlenmeyer flasks containing 25 mL of 1.25% malt extract medium. The pH of the medium was adjusted to 5.0 with H_2SO_4 solution. Cultures were incubated at 28°C at 150 rpm for 5 days.

GC/MS analyses

The low molecular weight compounds present in the effluent (100 mL) were successively extracted (3x100 mL) with ethyl acetate. Organic extracts were evaporated and dried over P_2O_5. Around 2 mg of the

extract was dissolved in 100 μL of pyridine and 1 mL of BSTFA. Derivatization reaction was carried out at 60°C during 1 hour and the reaction mixture was analyzed immediately after derivatization. The GC/MS analysis was performed in a Finningan MAT GCQ™ instrument equipped with a DB-5 (30 m x 0.25 mm) column. Column temperature was initially maintained at 60°C for 1 minute and then heated up to 140°C at 30°C/min. This temperature was maintained for 3 minutes and then raised to 240°C at 2°C/min. The final temperature was maintained for 13 minutes. Helium at 33.0 cm/s was used as the carrier gas. Injector and transfer line temperatures were 170°C and 220°C, respectively. 1μL of the sample was injected using a split of 1:100.

RESULTS AND DISCUSSION

The low molecular weight fraction of the EOP effluent extractable with ethyl acetate at pH 2 was characterized by GC/MS. Figure 1 shows the ions chromatogram (TIC) for the ethyl acetate extract derivatized with BSTFA. The identification of the compounds was made by comparison between the mass spectra obtained and those of spectra libraries installed in the GC/MS and/or by propositions of logical fragmentations. The numbers of peaks related to the compounds found in the sample, its retention times, the molecular ions and the identification of the compounds are presented in Table 1.

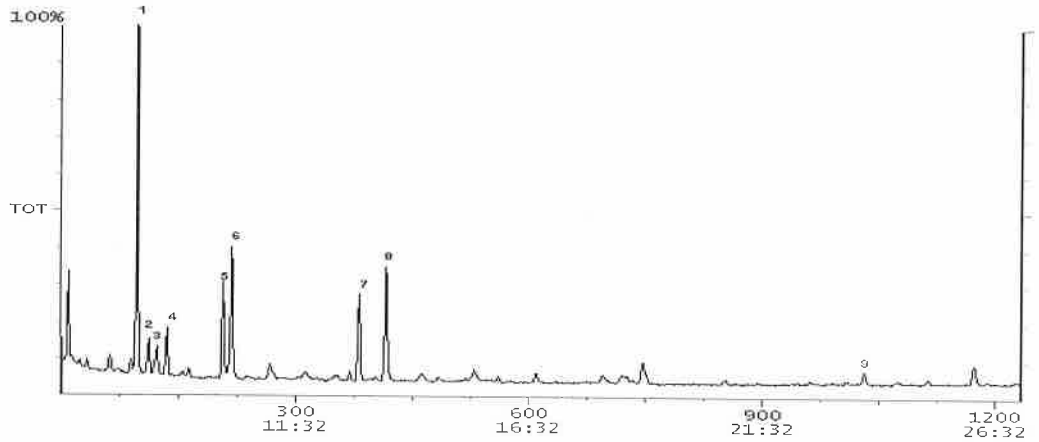


Figure 1. Ions chromatogram (TIC) of the original EOP effluent fraction extracted with ethyl acetate, evaporated at reduced pressure and derivatized with BSTFA.

Compound 1, responsible for the highest peak in the chromatogram was identified as silylated derived from butanedioic acid. The compounds responsible for peaks 2, 3, 5 and 9 were identified as silylated derived from methylbutanedioic; 2,3-bis (oxy) propanedioic; oxy-propanedioic and 3-methoxy-4-(oxy) benzoic acids, respectively. Compounds 4 and 7 presented different retention times; howeves, the fragmentation path presented by the mass spectra of both compounds was the same (compound 7 had a complete silylation) and was attributed to the silylated derived from the oxy-butanedioic acid. Peaks 6 and 8 also presented compounds identified, as silylated derived from carboxilic acids and their complete identification was not possible. The other peaks that appeared in the chromatogram after peak 9 were identified as silylated derived from sugars. The eluted peaks in the beginning of the chromatogram (until 7:40 min) were attributed to the decomposition of the BSTFA.

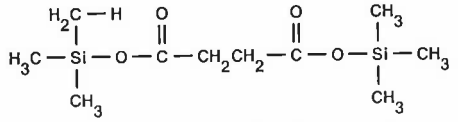
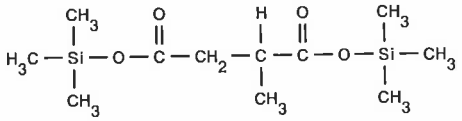
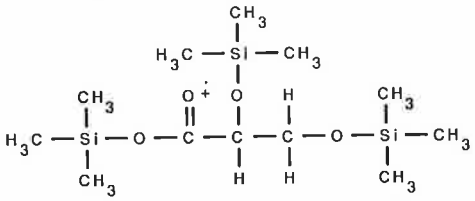
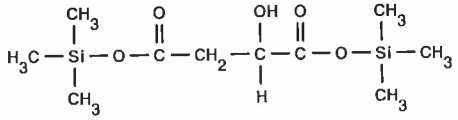
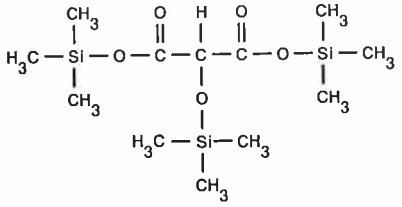
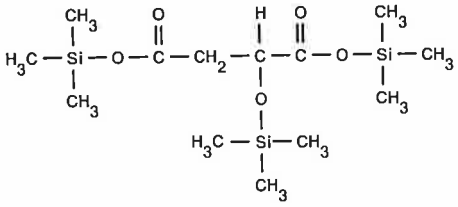
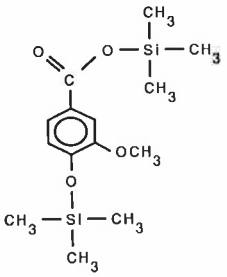
All these carboxilic acids of low molecular weight must have been generated as final products of sugar decomposition in basic medium, except for number 9, generated from lignin degradation (Fengel and Wegener, 1989). However, the toxicity of these compounds to aquatic life is still not very clear. Probably, a large part of these compounds is biodegraded by the microbiota existing in the environment. However, some compounds can be biodegraded very slowly, and for this reason they are bioaccumulative for the fish.

After biological treatment, the low molecular weight fraction of the effluent extracted with ethyl acetate at pH 2 was characterized by GC/MS. Figure 2 shows the ion chromatogram (TIC) for the effluent biologically treated and derivatized with BSTFA.

The results obtained by comparing the mass spectra of the treated effluent with those of the original effluent show that compounds 2 and 9 were recalcitrant to biological treatment. In addition to these compounds, other

compounds (N1, N2, N3 e N4) formed after the biotreatment were detected in the effluent. Compound N3 was identified as silylated derived from 3,4-dimethoxy-benzoic acid (Table 2). The other peaks that appeared in the chromatogram (N1, N2 e N4) were attributed to products of cell metabolism and their complete identification was not possible.

Table 1. Identification of the compounds present in the original EOP effluent.

Peak n^0	Retention time (min)	Molecular ion	TMS Derivate	Molecular formula
1	8 :08	262	butanedioic acid	
2	8:24	276	methyl-, butanedioic acid	
3	8:36	322	2,3,-bis(oxy)-propanoic acid	
4	8:47	278	oxy-butanedioic acid	
5	9:59	336	oxy-propanedioic acid	
6	10:09		non-identified	
7	12:53	350	oxy-butanedioic acid	
8	13:27	234	non-identified	
9	23:43	312	3-methoxy-4-(oxy)-benzoic	

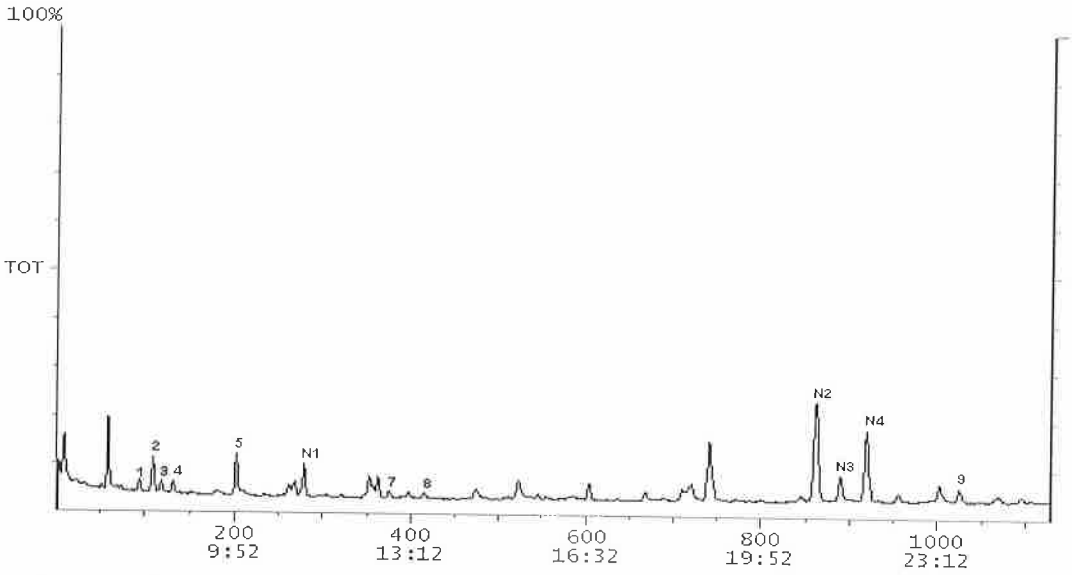
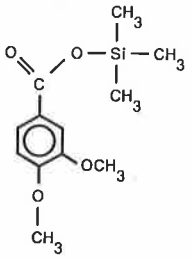


Figure 2. Ions chromatogram (TIC) of the fraction of the EOP effluent biologically treated, extracted with ethyl acetate, evaporated at reduced pressure and derivatized with BSTFA.

Table 2. Identification of the EOP effluent compounds formed after biological treatment.

Peak n^0	Retention time (min)	Molecular ion	TMS Derivate	Molecular formula
N1	11:12	318	non-identified	-
N2	20:53	258	non-identified	-
N3	21:21	254	3,4-dimethoxy-benzoic acid	
N4	21:53	295	non-identified	-

CONCLUSIONS

As the original EOP effluent contains carboxilics acids as products of the oxidation reaction with hydrogen peroxide, its composition is probably not changed by other oxydative processes.

The characterization of the low molecular weight fraction of the EOP effluent by GC/MS, after the biological treatment, showed the presence of some compounds recalcitrant to the microbial activity.

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REFERENCES

Dahlman, O.; Sousa, F. & Ljungquist, P. (1995). Analysis of Organic Compounds in Effluents from TCF Bleaching of Softwood and Hardwood Kraft Pulps. *In: 8th International Symposium on Wood and Pulping Chemistry*, Proceedings, pp 729-736.

Dethlefs, F.; Stan, H-J.& Kalnowski, G. (1995). Chemical and Toxicological Characterization of a Pulp Mill EOP Bleachery Effluent. *Vom Wasser*, **85**, 149-165.

Esposito, E.; Vanderlei, P.; Canhos, P.& Durán, N. (1991). Screening of Lignin-Degrading Fungi for Removal of Color from Kraft Mill Wastewater with no Additional Extr Carbon-Source. *Biotechnol. Lett.*, **13 (8)**, 571.

Fengel, D.& Wegener, G. (1989). Wood Chemistry, Ultrastructure, Reactions. Walter de Gruyter, Berlin, New York, pp 297.

Folke, J.F.; Renberg, L. & McCubbin, N. (1996). Environmental Aspects of ECF VS. TCF Pulp Bleaching. *Environmental Fate and Effects of Pulp and Paper Mill Effluents*, ed. M.R. Servos, K.R. Munkitrick, J.H. Carey & G. Van Der Kraak, St. Lucie Press, Delray Beach, FL., pp 681-691.

O'Connor, B.I.; Kovacs, T.G.; Voss, R.H.; Martel, P.H. & Van Lierop, B. (1993). A Laboratory Assessment of The Environmental Quality of Alternative Pulp Bleaching Effluents. *In Environmemtal Symposium EUCEPA*, Proceedings, **Vol 1**, 273. Centre Technique du Papier, Grenoble, France, **1**, pp 273-297.

Paiva, T.C.B.; Ferraz, A.; Silva, F.T. & Durán, N. (1998). Toxicity and chemical characterization of a TCF bleaching effluent. *In 7th International Conference on Biotechnology in the Pulp and Paper Industry*, Proceedings, **vol C,** pp C211-214.

Paiva, T.C.P., Ferraz, A. & Durán, N. (1997). Biotreatment of a pulp mill EOP bleachery effluent with the fungus *Penicillium janthinellum* and *Lentinus edodes*. *5th Brazilian Symposium on the Chemistry of Lignins and Other Wood Components*, Proceedings, **Vol 6**, pp 391-396.

Ristolainen, M. & Alén, R. (1998). Characterization of Effluents from TCF Bleaching of Hardwood Kraft Pulp. *Journal of Pulp and Paper Science*, **24 (4)**, pp 129-133.

Verta, M.; Ahtiainen, J.; Nakari, T.; Langi, A. & Talka, E. (1996). The Effect of Waste Constituents on The Toxicity of TCF and ECF Pulp Bleaching Effluents. *Environmental Fate and Effects of Pulp and Paper Mill Effluents*, ed. M.R. Servos, K.R. Munkitrick, J.H. Carey & G. Van Der Kraak, St. Lucie Press, Delray Beach, FL., pp 41-51.

Introduction of a solvent extraction stage before the eucalyptus kraft pulp bleaching

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ABSTRACT

The introduction of non-pollutant methods for bleaching cellulose pulps has grown in the last years due to environmental concerns. In this work we present the analysis of the residual lignins extracted from Eucalyptus kraft pulp using mixtures of ketone:water and dioxane:hydrochloric acid 0.1 N. The residual lignins are also submitted to oxidations with the current bleaching agents used in mills. The obtained results suggested us to introduce a ketone:water extraction stage previously to the pulp bleaching. The used sequence was ODED and the kraft pulp presented a kappa number = 14, viscosity = 45 mPa.s and brightness = 36% ISO. The bleached pulps presented following characteristics: a) using a previous extraction stage: viscosity = 19 mPa.s and brightness = 87% ISO and b) ECF sequence only: viscosity = 13 mPa.s and brightness = 81% ISO.

Keywords: bleaching, cellulose pulp, Eucalyptus

INTRODUCTION

The introduction of non-pollutant methods for bleaching cellulose pulps has grown in the last years due to environmental concerns. The generation of advanced bleaching technologies can result from a better understanding of residual lignin structure. Therefore, in regard to bleaching process, the newest studies on lignin reactions have also focused the residual lignin because it is relatively different as compared with the lignin precipitated from the pulping liquor used as standard before. We have not found any detailed study on the Eucalyptus kraft pulp residual lignin in the literature. Eucalyptus is the most employed wood for short fiber cellulose production in Brazil.

In this work we present the analysis of the residual lignins extracted from Eucalyptus kraft pulp using mixtures of ketone:water and dioxane:hydrochloric acid 0.1 N. The residual lignins were also submitted to oxidations with the current bleaching agents used in mills. The obtained results suggested us to introduce a ketone:water extraction stage previously to the pulp bleaching. An ECF bleaching sequence was used.

EXPERIMENTAL

Lignins isolation

Milled wood lignin (MWL) was obtained from ball-milled wood according to classical method adapted for tropical Eucalyptus wood (Veloso, 1993) . Kraft lignin (KL) was precipitated from pulping liquor with dilute hydrochloric acid (Nascimento, 1994). Organosolv lignin (OL) was also precipitated from the liquor resulted from the pulping of Eucalyptus chips using a mixture of acetic acid:ethyl acetate:water (4:4:2, v/v).

Residual lignins extraction

Eucalyptus kraft pulp (100 g, o. d.), kappa number 17, was extracted with ketone:water (9:1), in a Soxhlet, until the fresh extract remained colorless. The extract was filtered, the ketone evaporated and the water distilled with benzene. The resulting residue (RL1) was dried in a oven at 70 °C for 12 hrs (yield = 33.3 % of the total amount given by the formula: residual lignin = kappa number x 0.16). The ketone:water extracted pulp was, then, treated with dioxane:hydrochloric acid 0.1 N for 2 hrs in reflux (Pepper, 1959; Jiang, 1996). The extract was filtered, neutralized with sodium bicarbonate and the dioxane was evaporated. The residual lignin (RL2) was precipitated with water and adjusting the pH between 2-3. The suspension was centrifuged and RL2 was washed with water. The dryness was done at 70 °C for 12 hrs. Yield = 20.5 % of the total lignin. The dioxane:hydrochloric acid extracted pulp was air dried and milled in a ball-mill for 48 hrs. The residual lignin (RL3) was obtained as in the case before. Yield = 20.9 %.

^{13}C and ^{1}H NMR spectroscopies
Routine ^{13}C and ^{1}H NMR spectroscopies were carried out in a BRUCKER DRX 400 AVANCE spectrophotometer using DMSO-d_6 as solvent.

FTIR spectroscopy
FTIR spectra were recorded in a SPECTRUM 1000 (PERKIN ELMER). The samples (1%) were mixed with KBr, milled for 20 min and pressed.

Gas chromatography - mass spectrometry (GC-MS)
GC-MS analyses were carried out in a SHIMADZU QP 5000 using a capillary column DB5 (30 m; 0.25 mm i.d.; and film thickness 0.25 μm). Temperature program was: 60°C to 240°C (3°C/min) 240°C (15 min); and detector and injector temperatures of 240°C and 220°C, respectively. The samples were previously methylated with tetramethylammonium hydroxide. The impact energy was 70 eV and were collected fragment ions from 45 Da to 650 Da. Identification of unknown substances were based on mass spectra data (Wiley library containing spectra of 140.000 compounds) and Kovats indexes.

High performance liquid chromatography (HPLC)
HPLC analysis was carried out in a chromatograph equipped with a diode array detector, Hypersil ODS (200 x 4 mm i.d., particles diameter 5 μm) column, protected by a precolumn of the same material. For elution two solvents were used: A: MeOH:H_3PO_4 (999:1) and B: H_2O:H_3PO_4 (999:1) with the gradient profile: A:20 %; B:80 % (0 min); A:0 %; B:100 % (40 min); A:0 %; B:100 % (5 min); the flow rate was 1 mL/min and the column oven temperature was 30 °C. Detection was carried out at 325 ± 75 nm, 270 ± 15 nm and 365 ± 2 nm. For compounds identification, a private library of phenolics and flavanoids was used.

Bleaching of lignin solutions
The ozonolysis of lignin solution (70 % acetic acid as solvent) was carried out at room temperature using a mixture of ozone and oxygen (flow of 0.1 L/min, 1.0 % of ozone in weight) produced in a bench ozonizer (OREC VI). The initial absorbance was controlled at approximately 0.4 and the development of the reaction was monitored every 5 min by UV absorption at 400 nm (HITACHI UV-VI 2000 spectrophotometer).

Oxidations of residual lignins solutions with chlorine (C), ozone (Z) and chlorine dioxide (D) were carried out in acidic medium (pH 2.5 – 3.0) and with hypochlorite (H), hydrogen peroxide (P) and oxygen (O) in alkaline medium (pH 10.0 – 12.0). The initial absorbance was also near 0.4. The quantity of oxidation equivalents was proportional to that used in kraft pulp bleaching sequences. The UV absorption of the reaction solution was monitored every 5 min.

RESULTS AND DISCUSSION

Analysis of residual lignins
IR spectrum of RL1
Taking as reference lignins spectra, the typical band of lignins (near 1515 cm^{-1}) is very weak; the bands of aliphatic C-H (stretching: 2923 and 2854 cm^{-1}; bending: 1463 and 1373 cm-1) are very intense; the band due to carbonyl groups is also relatively intense.

^{13}C NMR spectrum of RL1
The presence of signals due to sp^3 carbons (15-35 ppm) is expressive; the signal of methoxyl carbon (56 ppm) is relatively weak and the presence of characteristic signals of lignins (56 - 155 ppm) is also discrete.

^{1}H NMR spectrum of RL1
Signals of saturated carbons (not characteristic of lignins) appear between 0.8 – 2.0 ppm, they are significant and confirm the data obtained earlier.

The elemental analysis of RL1 furnished a carbon content of 69 %, i. e., greater than that found in genuine lignins (about 60 %). This confirms once more the great presence of saturated aliphatics in RL1.

The liquid chromatogram of the solution, from which RL1 was precipitated, indicates the presence of several components but just one presented UV spectrum characteristic of lignin fragments. This solution was mildly

evaporated in a sand bath. The resulting residue was methylated and injected in a GC-MS spectrometer. Most of the components shown in the chromatogram were aromatic acids.

The residue obtained in the first extraction with dioxane:HCl 0.1 N (RL2) presented an IR spectrum similar to those of lignins, with exception of the methoxyl C-H bending band (1371 cm^{-1}) that is very weak, and the stretching C-O band (1110 cm^{-1}) is relatively intense.

The ^{13}C NMR spectrum of RL2, however, is not characteristic of lignins and confirms the discrepancies observed earlier: the presence of methoxyl groups (signal at 56 ppm) is low and there is a great amount of signals due to oxygenated sp^3-carbons (region from 60 to 80 ppm). In addition, the signals between 90 - 100 ppm and 10 – 35 ppm are also not characteristic of lignins.

The ^1H NMR spectrum of RL2 has not the feature of those shown by lignins.

The second extraction with dioxane:HCl 0.1 N furnished the RL3. The IR spectrum of this residue is characteristic of lignins but two details must be pointed out: the concentration of methoxyl groups is low (band at 1371 cm^{-1}) and the carbonyl absorption is relatively intense (band at 1714 cm^{-1}). The elemental analysis of RL3 gave the following composition: C = 58.5 %; H = 6.3 %; and O (by difference) = 35.2 %. These results confirm the IR spectrum and, on the other hand, suggest a small contamination by oxygenated compounds.

This contamination may be due to carbohydrates as can be inferred from the IR spectrum of the residue resulting after mild evaporation of the solution from which RL3 was precipitated. This residue exhibits bands characteristic of polysaccharides.

From the above study we can conclude that *Eucalyptus* kraft pulp contains lignin and other, precipitable or not, organic material. The precipitable part (RL1, RL2 and RL3) reached 74.7 % of the total lignin. The soluble fractions contain a lot of organic substances but most of them are aromatic acids and carbohydrates. These last substances turn also the solution brown.

Following study focuses the behavior of RL1, RL2, RL3, MWL, KL, and OL against bleaching agents.

Bleaching assays

Bleaching of RL1 solution with C, D, H, P, O and Z

The degradation of RL1 with these reagents is very difficult. Ozone was the best of all but had not very good performance because the absorbance at 400 nm of the initial solution dropped 65 %; chlorine dioxide and chlorine could reduce the absorbance only in 25 and 15 %, respectively; and the treatment with hypochlorite, oxygen and hydrogen peroxide increased the absorbance.

Bleaching of RL2 with O, P and Z

Ozone was very effective to degrade RL2 since in 10 min the absorbance practically felt down to zero; the absorbance remained practically the same with hydrogen peroxide and increased by the action of oxygen.

Bleaching of RL1, RL2, RL3, MWL, KL, and OL with ozone

Ozone has proved to be an excellent oxidant for all substrates excepting RL1. In 10 min the absorbance felt down; for RL1 solution, however, the absorbance could not be reduce to values beyond 0.18.

Bleaching a kraft pulp after remotion of RL1 component

As ketone:water residue (RL1) was resistant to oxidation, the introduction of a ketone:water extraction could reduce the chemical charge necessary to bleach the *Eucalyptus* kraft pulp. In view of that, an extraction stage using this solvent mixture was introduced before an ECF bleaching sequence and the results compared with those obtained without the extraction stage. The used sequence was ODED and the kraft pulp presented a kappa number = 14, viscosity = 45 mPa.s and brightness = 36 % ISO.

After both treatments, the bleached pulps presented following characteristics: a) using a previous extraction stage: viscosity = 19 mPa.s and brightness = 87 % ISO and b) ECF sequence only: viscosity = 13 mPa.s and brightness = 81 % ISO.

Table 1. *Eucalyptus* kraft pulp bleaching using an ODED sequence.

Conditions and Results	bleaching sequence			
	O	D	E	D
Consistency, %	10	10	10	10
Temperature, °C	90	70	60	70
Time, min	60	60	30	60
Initial Pressure, kPa	6.0	-	-	-
H_2O_2 , kg/t	5.0	-	-	-
O_2 , kg/t	-	-	-	-
O_3, kg/t	-	2.0	-	-
NaOH, kg/t	7.0	-	10.0	-
H_2SO_4, kg/t	-	4.0	-	4.0
ClO_2 , kg/t	-	10.4	-	
Initial Ph	10.2	2.48	10.3	2.5
Final pH	9.6	2.95	10.0	2.8
Reagent consumed, %	-	-	-	-
Brightness A.D. % ISO	41.55	66.52	-	81
Brightness O.D. % ISO	-	-	-	-
Kappa Number	10.0	-	-	-
Viscosity, mPa.s	-	-	-	13

Table 2. *Eucalyptus* kraft pulp bleaching using a sequence ODED preceded by a extraction with ketone:water (E_{kw}).

Conditions and Results	bleaching sequence				
	E_{kw}	O	D	E	D
Consistency, %	10	10	10	10	10
Temperature, °C	90	90	70	60	70
Time, min	60	60	60	30	60
Initial Pressure, kPa	-	6.0	-	-	-
H_2O_2 , kg/t	-	5.0	-	-	-
O_2 , kg/t	-	-	-	-	-
O_3, kg/t	-	-	2.0	-	-
NaOH, kg/t	-	7.0	-	10.0	-
H_2SO_4, kg/t	-	-	4.0	-	4.0
ClO_2 , kg/t	-	-	10.4	-	
Initial Ph	-	10.2	2.48	10.3	2.5
Final pH	-	9.6	2.95	10.0	2.8
Brightness A.D. % ISO	53.95	43.65	68.52	-	87.00
Kappa Number	4.0		-	-	-
Viscosity, mPa.s	45	-	-	-	19

CONCLUSIONS

The introduction of a ketone:water extraction previously to an ECF bleaching sequence reduced the chemical charge necessary for bleaching *Eucalyptus* kraft pulp.

Aknowledgments. FAPEMIG

REFERENCES

Jiang, Z. H. & Argyropoulos, D. S. (1996). The Stereoselective Degradation of Arylglyceros-beta-aryl Ethers During Kraft Pulping, *J. Pulp Paper Sci.*, **16 (4)**, j183-j188.

Nascimento, E. A., Morais, S. A. L., Veloso, D. P. & Menezes, S. M. (1994). Oxidative Ammonolysis of *Eucalyptus grandis* Kraft Lignin, *J. Braz. Chem. Soc.*, **5 (1)**, 5-14.

Pepper, J. M., Baylis, P. E. T. & Alder, E. (1959). The Isolation and Properties of Lignin Obtained by the Acidolysis of Spruce and Aspen Wood in Dioxane-Water Medium. *Can. J. Chem*, **37**, 1241.

Veloso, D. P., Morais, S. A. L. & Nascimento, E. A. (1993) Isolamento e Análise de Ligninas. *Química Nova*, **16 (5)**, 435-448.

Isolation and characterization of MWL and enzymatically-liberated lignin from loblolly pine (*Pinus taeda*)

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ABSTRACT

Milled wood lignin (MWL) and enzymatic-liberated lignin were extracted from ball-milled *Loblolly Pine* wood. MWL was extracted with 96 % dioxane from 5 days-ball milled wood. The residue of MWL-extraction was treated with comercial cellulases, and further extracted successively with 96 % and 50% dioxane aqueous solutions. The extractions yielded the enzymatic liberated lignins named here as ELL96 and ELL50, respectively. The lignin contents increased from 59.1% in ELL50 to 65.5% in ELL96 and reach a maximum of 83% in MWL. Olygomers of glucose (9–16%), xylose (3–4%) and manose (6–7%) composed the carbohydrates portions of both ELL50 and ELL96 whereas in MWL, glucose (1.6%) and arabinose (1.0%) were the main contaminants. Gel permeation chromatography analysis (GPC) of these lignins indicated an average of molecular weight (Mw) of 5.2 kDa with polydispersidy of 3.5 in the MWL fraction. Both ELL96 and ELL50 were excluded by GPC-system (Sephadex G-50) indicating a Mw higher than 29 kDa in these fractions. A comparative study of these three lignin preparations using FTIR, UV/visible, sugar analysis and quantification of GPC eluted carbohydrates, suggests that the main association between carbohydrates and lignin in MWL occurs by covalent bonds whereas in the ELL-fractions the physical interactions seems to be predominant.

Keywords: lignin, *Pinus taeda*, enzymatically-liberated lignin

INTRODUCTION

In recent years there is an increasing interest in the biopulping technology (Akthar *et al.*, 1995). Selective lignin biodegradation is essential in the fungal pretreatment, since cellulose degradation means losses in yield and pulp quality in the subsequent mechanical or chemical pulping. However, most of the data obtained in this area showed that there is no direct correlation between the extent of lignin biodegradation and the benefits observed in the subsequent pulping processes. This lack of correlation indicates that other wood modifications caused by the fungal pretreatment must be involved in the behavior of these wood samples in the subsequent pulping process. These results have corroborated the idea that partial lignin depolymerization with fungal pretreatment is enough to produce the benefits observed in the biopulping technology (Ferraz *et al.*, 1998)

The evaluation of this hypothesis imply in the isolation and characterization of residual lignin from biodegraded wood samples. In this work, we report the initial data on isolation and characterization of milled wood lignin (MWL) and enzymatically-liberated lignin (ELL) from undecayed Loblolly Pine that is the most used wood in biopulping technologies. This work is part of a continued research directed to understand the chemical basis of biopulping. Characterization of lignin preparations recovered from biodegraded wood samples is the subject of further research.

EXPERIMENTAL

Material

Blue dextran (2,000,000 Da), albumin (66,000 Da), carbonic anhidrase (29,000 Da), citochrome C (12,400 Da) and aprotinin (6,500 Da) were purchased from SIGMA. A crude extract of cellulase (1,4-[1,3;1,4]-β-D-glucan-4-glucano-hidrolase) (EC 3.2.1.4) was also purchased from SIGMA (SIGMA type I). This enzyme preparation had a specific activity of 11.8 IU. mg^{-1} of solid.

A freshly cut tree of 30 years-old Loblolly Pine wood (*Pinus taeda*) was obtained from "Parque Estadual de Campos do Jordão" (São Paulo, Brazil). Wood chips were prepared by hand and then milled in a laboratory

knife mill to pass in a 20-mesh screen. Previous to lignin extractions the wood sawdust was extracted with ethanol 95% for 6 h in Soxhlet apparatus.

Lignin isolation

Milled wood lignin and enzymatic liberated lignin were successively extracted from ground extractive-free wood, according to Lawther *et al.* (1996). After drying over P_2O_5, 90 g of the extractive-free wood was ground in a rotary porcelain ball-mill in toluene at room temperature for 130 h at 96 rpm using a porcelain ball/ground wood weight ratio of 20. The ball-milled samples were then extracted through two consecutive extractions with dioxane-water solution (96 v/v) in a shaker at 190 rpm for 24 h. The extractions were performed using 1 g of ball-milled wood to each 10 ml of solvent at room temperature in the darkness. The dioxane-water extracts were combined and the solvents removed at reduced pressure at 35°C and the solid extract was further dried over P_2O_5.

After extraction of the MWL, the dioxane-extracted residue was washed exhaustively with water and then treated at 50°C with 800 mL of a cellulase solution containing 1180 $IU.L^{-1}$ of 50mM acetate buffer at pH 4.5. The mixture was agitated in a shaker at 190 rpm and samples were taken at each 10 h for glucose determination (Mandels *et al.*, 1976). The shaking was interrupted when solubilized glucose raised to a constant concentration. The mixture was centrifuged at 2000 rpm for 30 minutes and the supernatant was discarded. The procedure was repeated and the residues of the two enzymatic hydrolysis were washed with water and yielded ELL96 and ELL50 by successive extractions with 95% and 50% dioxane-water solution (v/v), respectively.

Lignin characterization

Molecular weight distribution was determined by gel permeation chromatography (GPC) on a 46.5 x 2.5 cm Sephadex G-50 column eluted at 0.4 $mL.min^{-1}$ with 0.5 M NaOH solution. The samples were dissolved with NaOH 0.5 M in a concentration of 2 $g.l^{-1}$, and 0.4 ml were directly injected into the column. Elution was monitored by measuring absorbances at 280 nm for lignin (Atsushi *et al.*, 1984) and at 490 nm for carbohydrates after phenol-sulfuric acid reaction (Dubois *et al.*, 1976). The column system was calibrated with proteins of known molecular weight (Foors *et al.*, 1989). The void volume (V_0) and the total permeation volume (V_t) were determined by injection of blue dextran and acetone, respectively. Partition coefficients (Kd) were calculated as described elsewhere (Chum *et al.*, 1987).

UV spectra were recorded in a Cintra 20 spectrophotometer. The lignin samples were dissolved in 95% dioxane-water solution. A diluted sample of this solution was used to measure the absorbances from 240 nm to 400 nm.

FTIR spetra from 4000 to 400 cm^{-1} were recorded on a FTIR Nicolet 520 spectrophotometer using a KBr disk containing 0.5% of the sample.

The lignin and carbohydrate quantification was performed as described by Mendonça *et al.* (1999).

RESULTS AND DISCUSSION

Extraction yields for milled wood lignin (MWL) and enzymatically liberated lignin are reported based on the total amount of Klason lignin present in Loblolly Pine which is 28.2 % (w/w) (Table 1). The extraction yield for MWL (4%) was lower than those reported elsewhere (Pew, 1957; Chang *et al.*, 1975; Lundquist, 1992; Lawther *et al.*, 1996). This low extraction yield probably was related to small milling time (5 days) used for the pretreatment step. Considering that long milling time is known to diminish MWL molecular weight (Lawther *et al.*, 1996), further treatment of residual wood with cellulase was performed to increase the extraction yield instead of increasing milling time. After wood hydrolysis with cellulases the extraction yields increased: 10.5 % for ELL96 and 10 % for ELL50, reaching a total yield of approximately 25 %. The efficient extraction of lignin after hydrolysis of polysaccharides with cellulases can be related to easier solvent access to lignin in the cell wall (Pew, 1957; Lundquist, 1992).

Table 1. Extraction yields for recovering MWL and ELL from Loblolly Pine.

Lignin extracted	Yield (%) (g/100 g of klason lignin)
MWL	4.0
ELL96	10.5
ELL50	10.0

Molecular weight distribution of the three lignin preparations are shown in Figure 1. The elution profile of MWL showed a high polydispersity. Mw, Mn and D values calculated for MWL were 5.2 kDa, 1.4 kDa and 3.5, respectively. For ELL96 and ELL50 the estimated average of molecular weight was not calculated because these lignin preparations were excluded in the chromatographic system. However, the Figure 1 shows that ELL96 is more disperse than ELL50 and both fractions are higher than 29 kDa.

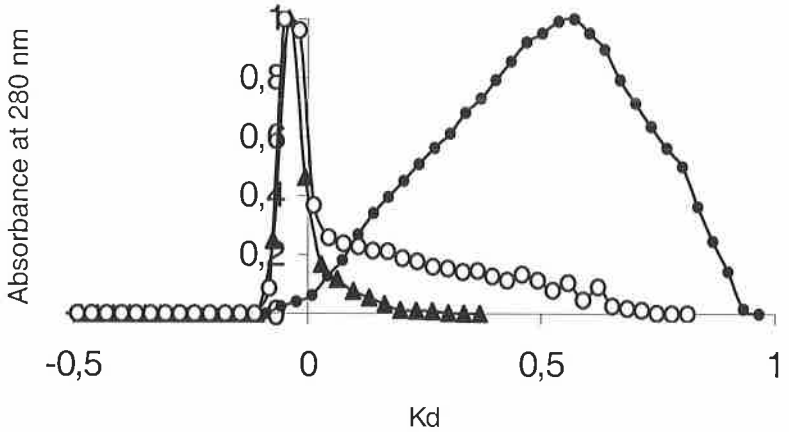

Figure 1. Gel permeation chromatography of MWL (•); ELL96 ( ) and ELL50 (♦).

Similarly to other softwood lignin preparations, the UV spectra of these fractions showed a characteristic strong absorption at 274-290 nm, originated from non-conjugated phenolic groups in lignin (Goldschimid, 1977). Additionally, only a weak absorption at 305–315 nm, originated from α-conjugated phenolic structures, were observed in these spectra. The absorptivity values at 280 nm decreased from 17.3 in MWL to 9.4 in ELL96 and reach a minimal value of 4.4 $L.g^{-1}.cm^{-1}$ in ELL50. Low 280 nm absorptivities for ELL96 and ELL50 indicated that these lignin preparations should contain non-phenolic contaminants.

The FTIR spectra of MWL, ELL96 and ELL50 are shown in Figure 2a-c. The characteristic band at 1140 cm^{-1} and the strong band at 1270 cm^{-1} confirmed that the MWL fraction is a G type lignin (Faix, 1991). However, the band at 1738 cm^{-1} (unconjugated carbonyl groups) was atypically intense in this spectrum. This high band intensity could be assigned to the presence of covalent ester bonds between carbohydrates and lignin (Faix, 1991).

Figure 2b and c show also that the characteristics of G type lignins are still present in the spectra of ELL96 and ELL50. On the other hand, the bands at 3500, 1031 and 1086 cm^{-1} (stretching vibration in OH, aromatic C-H plus C-O deformation in primary alcohols and C-O deformation in secondary alcohols, respectively) were intense in these spectra. The high intensity of these bands suggest the presence of high amounts of carbohydrates in these lignin fractions. However, the band assigned to unconjugated carbonyl groups is not intense in ELL96 and fades away in the ELL50 spectra. These data hinting to the presence of a few covalent bonds between lignin and polysaccharides in these preparations. These results analyzed altogether indicate that the association between lignin and carbohydrates has different origins in milled wood and enzymatic liberated lignin preparations. These aspects were further evaluated through the determinations of chemical compositions and the GPC profile of lignin and carbohydrates of these lignins preparations.

The level of contamination by carbohydrates increases from 4.7% in MWL to 20.2 % in ELL96 and reaches a maximal value of 30.2% in ELL50 (Table 2).

Table 2. Chemical composition of MWL, ELL96 and ELL50 and Loblolly Pine wood.

Component after acid hydrolysis (%)	Loblolly pine	MWL	ELL96	ELL50
Insoluble lignin	28.8 ± 0.15	82.8 ± 0.8	65.5 ± 0.8	59.1 ± 0.9
Soluble lignin	0.22 ± 0.03	1.00 ± 0.08	0.71 ± 0.05	0.74 ± 0.03
Glucuronic acid	0.7 ± 0.01	0.20 ± 0.03	0.32 ± 0.05	0.53 ± 0.02
Glucose	43.0 ± 0,17	1.55 ± 0.03	9.11 ± 0.02	16.2 ± 0.2
Xylose + manose	19.3 ± 0.2	0.9 ± 0.01	8.5 ± 0.1	10.6 ± 0.2
Arabinose	1.1 ± 0.01	1.03 ± 0.03	0.38 ± 0.01	0.81 ± 0.01
Acetic acid	2.5 ± 0.15	1.04 ± 0.07	1.93 ± 0.07	2.01 ± 0.02

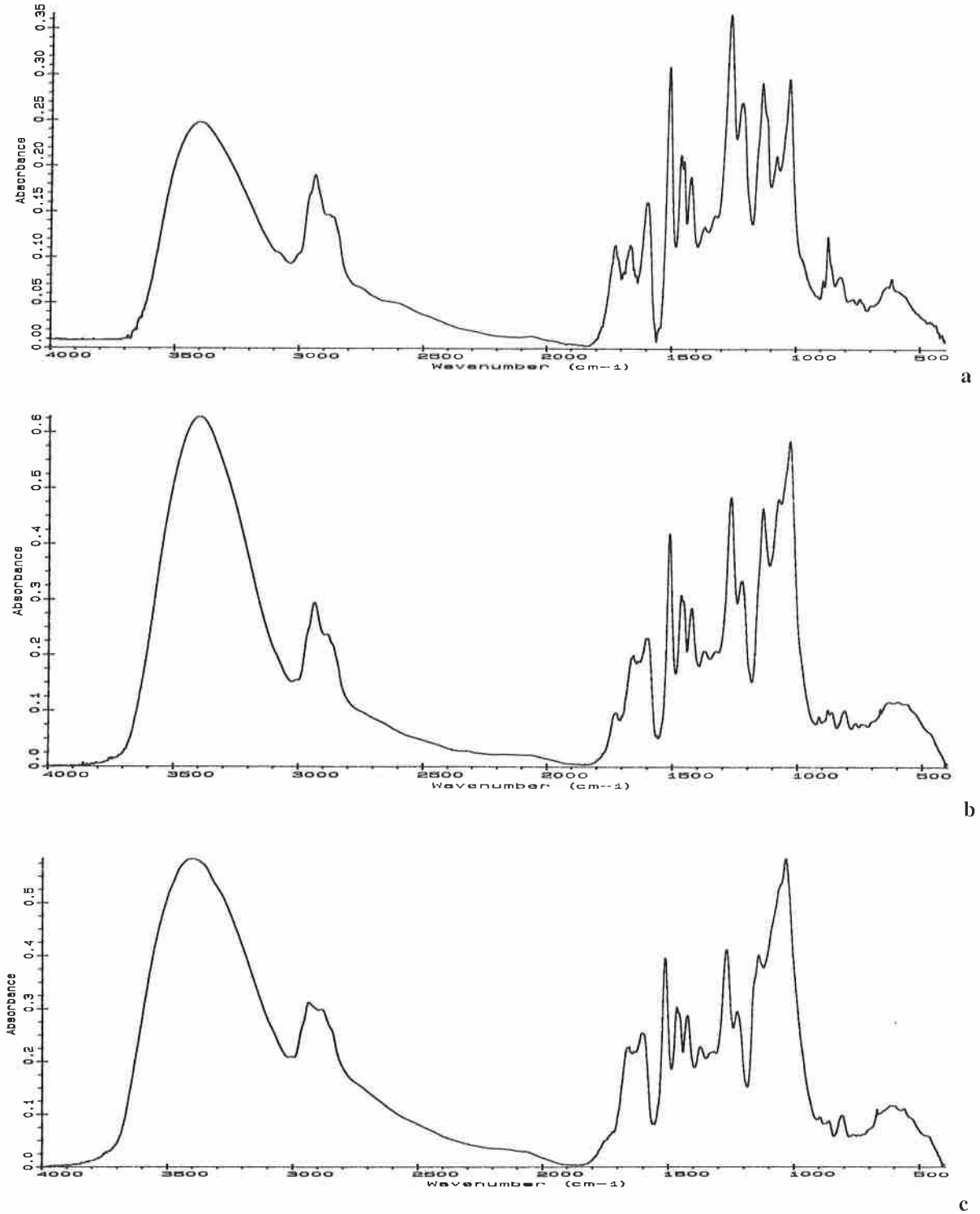

Figure 2. FTIR spectra of lignin preparations recovered from Loblolly Pine: a) MWL; b) ELL96 and c) ELL50.

Data shown in Table 2 confirms high amounts of cellulose and polyoses olygomers contamination in ELL50 and ELL96. Glucuronic acid/xylose ratio in ELL96 (1:9) and ELL50 (1:7) approximates the value observed in untreated *P. taeda* wood (1:9). On the other hand, the glucuronic acid/xylose ratio in MWL (1:1.5) was low which can indicates the presence of covalent bonds between polysaccharides and lignin in this fraction, as also observed by FTIR. It is known that ester linkages between carboxyl groups of glucuronic acids and hydroxyls in alpha carbons of lignins are typical association sites of these two components in wood materials (Fengel and Wegener, 1989).

The GPC profile of these lignin preparations detected for lignin (Goldshimid, 1977) and for carbohydrates (Dubois *et al.*, 1976) is shown in Figure 3. The elution of MWL clearly showed that the absorbance at

280 nm due to lignin molecules overlapped with the 490 nm absorbance assigned to carbohydrates. On the other hand, the ELL96 and ELL50 preparations showed carbohydrates eluting together lignin (excluded fraction) and also a significant amount of carbohydrates eluting at the low molecular weight range where no lignin absorption was observed.

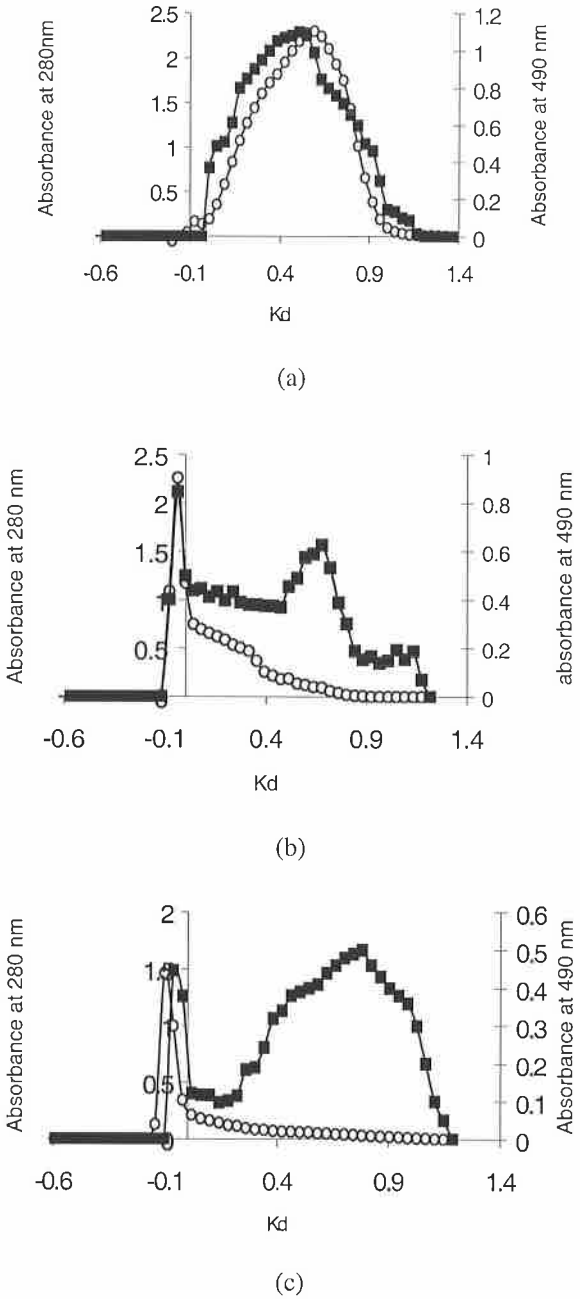

Figure 3. GPC of the three lignin preparations detected for lignin (O) and carbohydrates (■): a) MWL; b) ELL96 and c) ELL50.

The compilation of these data with those obtained by FTIR and chemical compositions led to the conclusion that the main association between carbohydrates and lignin in the MWL preparation occurs through covalent bond, while in ELL96 and ELL50 physical association seems to be preponderant.

Characterization of MWL, ELL96 and ELL50 recovered from biodegraded Loblolly Pine wood samples and the comparison of the data reported here with those obtained from biodegraded wood could increase the knowledge of the chemical basis of biopulping technologies.

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REFERENCES

Akthar, M., Kirk, T. & Blanchette, R.A. (1995). Biopulping: an Overview of Consortia Research. International Conference on Biotechnology in the Pulp and Paper Industry, Proceedings, **Vol** 6, 187-192.

Atsushi, K., Azuma, J. & Koshijima, T. (1984). Lignin-Carbohydrate Complexes and Phenolic Acids in Bagasse. *Holzforschung*, **38**, 141-149.

Chang, H., Cowling, & Brown, W. (1975). Comparative Studies on Cellulolytic Enzyme Lignin and Milled Lignin of Sweetgum and Spruce. *Holzforschung*, **29**, 153-159.

Chum, H. L., Johnson, D. K., Tucker, M. P. & Himmel, E. (1987). Some Aspects of Lignin Characterization by High Performance Chromatography Using Styrene Divinyl Benzene Copolymer Gels. *Holzforschung*, **41**, 97-108.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P & Smith, F. (1976). Colorimetric Method for Determination of Sugars and Related Substances. *Anal. Chem*, **28**: 350-356.

Faix, O. (1991). Classification of Lignins from Different Botanical Origins by FT-IR Spectroscopy. *Holzforschung*, **45**, 21-27.

Fengel, D. & Wegener, G. *Wood Chemistry, Ultrastructure and Reactions*, Walter de Gruyter, Berlin, pp 613.

Ferraz, A., Christov, L. & Akthar, M. (1998). Fungal Pretratement for Organosolv Pulping and Dissolving Pulp Production. In *Enviromentally friendly methods for the pulp and paper industry*, ed. M. Akhtar & R.A. Young. John Wiley & Sons, New York.

Forss, K., Kokkonnen, R. & Sagfors, P. (1988). Determination of the Molar Mass Distribution of Lignins by Gel Permeation Chromatography. *ACS Sympsium. Ser.*, **397**, 124-133.

Goldshimid, O. (1977). Ultraviolet Spectra. In: *Lignin-occurrence, formation, structure and reaction*, ed. K.V Sarkanen & L.H. Ludwid. Wiley/Interscience, New York.

Lawther, J., Sun, R. & Banks, W. (1996). Extraction and Comparative Characterization of Ball-Milled Lignin (LM), Enzyme Lignin (LE) and Alkali Lignin (LA) from Wheat Straw. *Cellulose Chemistry and Technology*, **30**, 395-410.

Lundquist, K. (1992). Wood. In: *Methods in lignin chemistry*, ed. S.Y. Lin & C.W. Dence. Springer-Verlag, New York, pp 65-70.

Mandels., M., Andreotti, R. & Roche, C. (1976). Measurement of Saccharifying Cellulase. *Biotechnology and Bioenginering*, **6**, 21-39.

Mendonça, R., Ferraz, A., Cotrim, A., Silva, F.T. & Oliveira, S. C. (1999). Development of Mathematical Models gor Organosolv Pulping Kinetcs of Fungally Pretreated Wood Samples. Journal of Wood Chemistry and Technology, **19**, 99-114.

Pew, J. (1957). Properties of Powdered Wood and Isolation of Lignin by Cellulytic Enzymes. *Tappi Journal*, **40**, 553-558.

Recovery of lignins from kraft liquors

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ABSTRACT

The utilization of Kraft lignin as raw material for different products has consolidated market in United States and in some countries of Europe. In our country the local production of lignins products are restricted to lignosulfonates recovered from sulfite pulping. The aim of this work was the study of different lignin recovering processes from Kraft liquors of *Eucalyptus urograndis* pulping, as well as the chemical characterization of these lignins.

Keywords: kraft liquor, lignin, Eucalyptus

INTRODUCTION

Lignins and lignosulfonates can be employed as co-reactant in many different adhesive systems, binders in animal feed, in the improvement of fertilizer applications; vanillin production, etc. The goal in lignin utilization is not only performance, but also reduction in costs by replacing more expensive petroleum based synthetic chemicals. All of these applications start with recovering lignins from spent industrial pulping liquors. The different recovery processes affect not only the yield but also the properties of technical lignins.

The Kraft lignin has been recovered conventionally from black liquor, in laboratory or industrial plants, by precipitation with mineral acids like sulfuric or hydrochloric acid under vigorous agitation and subsequent coagulation by heating. The acidification can be also performed by addition of carbon dioxide (Winhaus *et al.*, 1992; Uloth and Wearing, 1989; Alén *et al.*, 1985; Loutfi *et al.*, 1991). The isolation process consists essentially in three stages: acidification, filtration and washing. To prevent the located acidification and to guarantee the uniformity in the precipitation, it should be avoided high acid concentrations. Concentrations between 5 and 20% are preferred (Loutfi *et al.*, 1991). When the liquor is acidified until pH 2 practically the whole lignin is precipitated. The washing stage is critical for the obtaining a lignin with high purity.

The possibilities of commercial lignin based products can be subdivided in three groups: macromolecular products (Sarkanen *et al.*, 1995; Glasser, 1998), micromolecular products obtained by lignin cracking and uses that don't need special lignin treatments.

Another potential application of technical lignins is the preparations of novolac type resins using lignins as co-reactant (Keslarek, 1995; Santos, 1996; Santos and Curvelo, 1999). These resins are synthesized in acidic media employing molar excess of phenol relatively to formaldehyde. Novolac resins are an interesting alternative to introduce lignins in applications such as injection molding plastics (Santos, 1999; Kharade and Kale, 1998).

Most of the commercial lignin based products are derived of lignosulfonates. Approximately 95% of all lignin products use lignosulfonates and just 5% are based on non-sulfonated lignin (Glasser and Kelley, 1988). Part of these lignosulfonates is produced from sulfonation of Kraft lignins. The success of lignosulfonates application is due to its solubility in water and its surface-active properties.

EXPERIMENTAL

The black liquor of *Eucalyptus urograndis* wood supplied by ARACRUZ Celulose was analyzed with respect to total solid content and ash content in the dry solids. A small black liquor sample (3 to 6 g) was dried in a stove at 110°C for 2 h to determine the solid content. These solids were then submitted at 800°C for 2 h, being obtained the ash content in the sample.

The molar absortivity of lignin was determined in order to make possible the determination of the lignin concentration in the black liquor by ultraviolet spectroscopy. A lignin sample was precipitated with sulfuric acid and later purified. This sample was employed for preparation of standards solutions for ultraviolet spectroscopy. They were prepared 5 lignin NaOH 0.1N solutions in the concentration range of 15 to 35 mg/l.

These solutions were analyzed by UV spectroscopy (190 to 400nm), being determined the absorbance at 280nm (1cm cell).

The lignins were isolated from the Kraft liquors by filtration after different procedures of pH adjustment. Four different procedures were used in order to precipitate the lignins from the Kraft liquors (Figure 1). Process A: The pH was adjusted with sulfuric acid until pH 3. Process B: The pH was adjusted with sulfuric acid in two steps. In the first step the pH was adjusted until pH 11 - the precipitated lignin was filtered - and then to pH 3. Process C: The pH was adjusted with carbon dioxide until pH 10. Process D: The pH was adjusted with carbon dioxide and sulfuric acid in two steps. In the first step the pH was adjusted with carbon dioxide until pH 11 - the precipitated lignin was filtered - and then with sulfuric acid to pH 3. The procedures C and D were performed at 80°C and at room temperature.

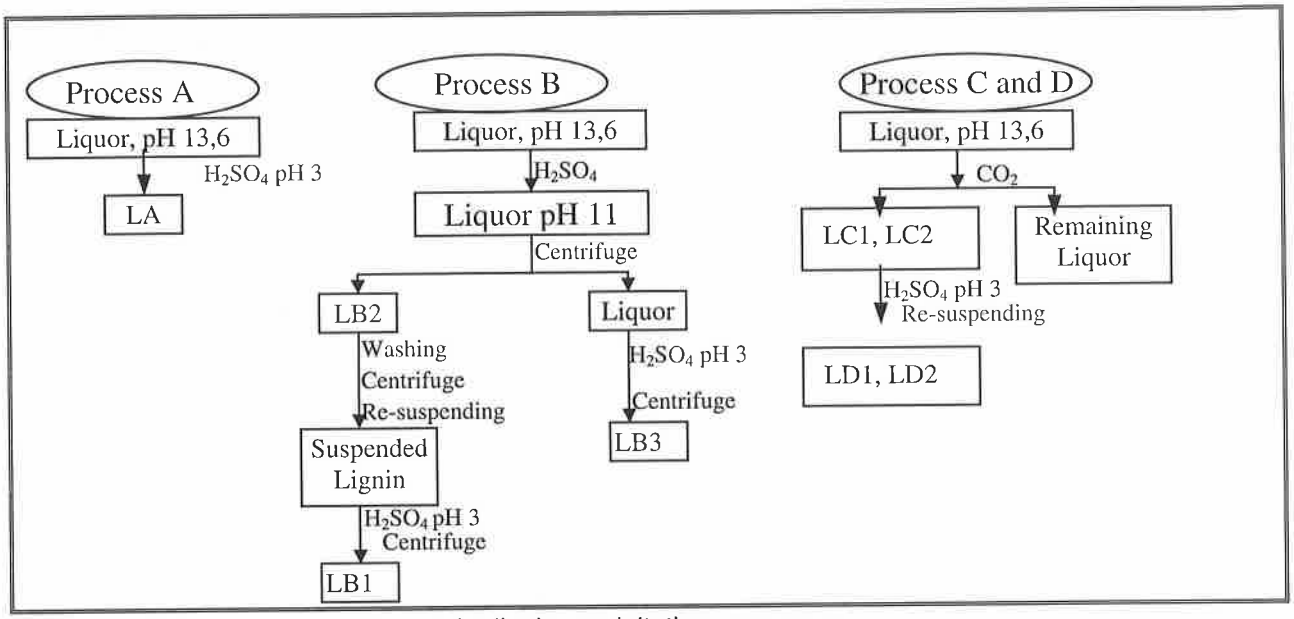

Figure 1. Schematic description of the lignin precipitation processes.

Acetylated lignins were prepared by dissolving 1g of lignin in 30ml of pyridine and 30ml of acetic anhydride. After 48 h of stirring at room temperature 60ml of ethanol was added to decompose the excess of anhydride. The ethyl acetate produced, ethanol and pyridine were evaporated under reduced pressure until dryness. The product was washed with ethanol (10 x 60ml) to ensure the total removal of pyridine. All lignins described in figure 1 were acetylated. The acetylated lignin samples were coded with a letter A after the previous code. For example, lignin LA after acetylation is called LAA.

The lignins were characterized by elemental analysis, Size Exclusion Chromatography (SEC), Thermogravimetric Analysis (TGA) and Atomic Absorption spectroscopy (sodium content determination).

The molar mass distribution was obtained using a Shimadzu equipment in the following conditions: pre-column PLGel followed by PLGel 500+103+104Å columns; temperature 30°C; pressure 69kgf/cm^2; THF as eluent; flow 1.0 ml/min; sample concentration 1.2 mg/ml; injection volume: 5μl; detector UV 254nm; polystyrene standards with molar weight of 450,000; 195,000; 68,000; 50,000; 28,000; 12,500; 7,600; 3,770; 1,800; 1,050 g/mol and ethylbenzene (106g/mol).

The phenolic hydroxyl content was determined by conductimetric titration. Lignin samples (0.5g) were dissolved in 30ml of acetone and under stirring. Next, 60ml of ethanol and 90ml of water were added dropwise, with constant stirring. For all lignins a well-known volume of hydrochloric acid 0.1N was added, aiming to certify the total protonation of the phenolic hydroxyl groups. The solution was titrated with NaOH 0.1N solution and the conductivity (μS) was determined at each added volume. The equivalence points were determined graphically.

Thermogravimetric analyses were accomplished in the following conditions: equipment Netzsch TG 209 and Shimadzu TG-50; mass of the sample: ≈25mg; argon atmosphere, flow 20 ml/min; temperature range: room temperature to 1000°C; heating rate 20°C/min.

The sodium content was determined by atomic absorption, using a Hitachi equipment, model Z-8100, in the following conditions: wavelength 589nm; flame: acetylene/air; flow: 2.2 l/min; sodium chloride standards (0.1 to 0.8 ppm).

RESULTS AND DISCUSSIONS

From the value determined for the lignin molar absortivity (21.5 l/g.cm) it was possible to determine the lignin concentration in the liquor that was of 177.8g/l. This value corresponds to 146.9 g/kg as the density of the black liquor is 1.21g/l. The total content in solids was also determined (364.4g/kg), thus 40.3% represents lignin, 31.1% are due to inorganic compounds and 28.6% are polysaccharides (by difference).

The lignin-recovered yields obtained in the different processes are showed in Table 1. The yields of process B (LB2 + LB3) although performed in two steps was higher than that obtained in process A. The purification of lignin LB2 decreased the yield for this fraction from ~54% to ~40%. The combined LB1 + LB2 fractions presented a recovery yield slight lower than LA yield. The processes that used an acidification step with CO_2 showed lower yields. The utilization of high temperature in these processes increased the recovery yield in approximately 8%. Process D lead to recovery yields lower than the observed for process C as consequence of loses that occurred in the dissolution performed before the acidification to pH 3 with sulfuric acid.

Table 1. Lignin recovery yields and analytical results obtained for the recovered lignins.

	pH	Recovered yield (%)	%C	%H	%O	%N	%S	OH_{ph} (%)	Ash (%)	%Na
LA	3	87.8 ± 2.0	61.73	6.02	29.12	0.13	2.99	5.87	3.12	1.78
LB1	3	39.6 ± 1.9	55.67	5.23	32.17	0.37	6.56	4.73	1.21	1.20
LB2	11	53.9 ± 3.0	43.77	4.84	49.34	0.64	1.40	6.50	7.19	6.62
LB3	3	39.5 ± 2.8	55.30	5.44	37.78	0.76	0.71	5.88	2.32	1.90
LC1	10	72.4 ± 2.1	51.19	5.36	40.83	0.30	2.31	6.68	8.44	6.66
LC2	9	80.6 ± 2.2	50.47	5.15	41.83	0.23	2.35	6.86	10.15	5.60
LD1	3	67.3 ± 2.5	63.05	6.19	28.80	0.72	1.24	5.10	3.10	1.33
LD2	3	75.9 ± 2.1	64.35	6.21	27.85	0.82	0.76	5.26	3.34	1.95

Elemental analysis, ash and phenolic hydroxyl determinations are also showed in Table 1. These data are the net result of both differences in lignin's chemical structure and the presence of contaminants (in this case polysaccharides and inorganic compounds). The phenolic hydroxyl contents determined by conductimetric titration revealed that lignins LC1, LC2 and LB2 (lignins isolated at highest pH) still contain non-protonated aliphatic hydroxyl groups that were quantified as phenolic hydroxyl groups. This assumption is confirmed by the lower volumes of sodium hydroxide solutions spent to neutralize the fixed amount of hydrochloric acid added for these determinations (see experimental procedure). The higher sodium contents of these lignins (LB2, LC1 and LC2) also confirm the presence of sodium lignate (salt form lignin's aliphatic hydroxyl groups). The low value of phenolic hydroxyl content obtained for LB1 is consistent with the high molar mass determined for this lignin (Table 2).

The amounts of ash and sulfur compounds must be considered in the further applications for these lignins. The samples submitted to more washing steps and/or precipitated with sulfuric acid presented lower amounts of these contaminants.

With respect to lignin's thermal stability it was observed that up to 200°C the weight loss corresponds to vaporization of low molar mass compounds and humidity. The lignins started to decompose between 200 and 600°C. It was also observed that LC1, which presents highest sodium content (Table 1), showed the greatest weight loss in the temperature range from 600 to 900°C. This weight loss can be attributed to the decomposition of sodium carbonate that was produced in the precipitation step by the reaction of sodium hydroxide and carbon dioxide.

Table 2 shows the values of average molar mass of acetylated lignin samples. Lignins LB2, LC1 and LC2 were not soluble in tetrahydrofuran (THF) due to the presence of sodium lignate. For this reason the molar mass determinations were performed with acetylated lignin samples. The recovery processes resulted in lignins with different molar mass distribution as measured by the dispersity's values. Lignins LAA, LB1A, LC1A and LC2A presented higher weight average and lower number average molar mass resulting dispersity's values in the range of 3.8 to 5.0. Lignins LB2A, LB3A, LD1A and LD2A exhibited opposite

behavior with respect to average molar mass values and as consequence the dispersity's values were in the range of 1.7 to 1.9. The recovery processes produced also a fractionation of the Kraft lignin that is composed of lignins with different molar mass. The molar mass is an important parameter in the choice of raw materials for production of lignin-based phenolic resins. The utilization of lignins with higher molar mass can be an advantage in the reaction with formaldehyde in the production of these resins (Santos, 1999).

Table 2. Number (Mn) and Weight (Mw) average molar masses and dispersity determined for the recovered lignins.

Acetylated Lignin	$\bar{M}n$	$\bar{M}w$	$\bar{D}$ispersity
LAA	900	3,960	4.4
LB1A	1,050	5,330	5.0
LB2A	1,200	2,290	1.9
LB3A	1,180	2,060	1.7
LC1A	950	3,660	3.8
LC2A	930	3,540	3.8
LD1A	1,270	2,300	1.8
LD2A	1,250	2,280	1.8

CONCLUSIONS

With respect to the choice of the best recovery process taking into account the preparation of lignin derivatives three main aspects must be considered: recovery costs; composition of the remaining liquor and the characteristics of the recovered lignins. Process A is the simplest one and the precipitated lignin is easily removed from the treated liquor. Otherwise this process consumes a large amount of sulfuric acid and liberates hydrogen sulfide. Process B consumes smaller amounts of sulfuric acid but it involves two acidification steps and the individual recovered yields are lower when compared with processes A, C and D.

The process that uses CO_2 (lignins LC1 and LC2) presents a good yield and it is a cleaner technique because the acid is generated in the breast of liquor. This process is also economically feasible. The utilization of high temperature (80°C) produces no significant differences in the recovered lignins and lead to an increase in the recovery costs. Therefore the utilization of room temperature seems to be more adequate. The main characteristic of lignins LC1 and LC2 to be considered is the presence of sodium lignate and sodium carbonate.

Process D that uses CO_2 to neutralize the solution to pH 9-10 and then sulfuric acid until pH 3 is also clean, safe and cheap. The recovered lignin is totally protonated (as in processes A and B) and should be the best choice considering further utilization in the production of lignin-based derivatives performed at lower pH as in the synthesis of Novolac resins.

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REFERENCES

Alén, R; Sjöström, E. & Vaskikari, P. (1985). Carbon dioxide precipitation of lignin from alkaline pulping liquors. *Cellulose Chemistry and Technology*, **19 (5)**, 537-541.

Glasser, W.G. (1998). Lignin utilization in thermosetting and thermoplastic polymers and materials. *In International Symposium on natural polymers and composites*, Proceedings, pp.33-39.

Glasser, W.G. & Kelley, S.S. (1988). Lignin. In: *Encyclopedia of polymer Science and Engineering.* New York, John Wiley&Sons, **8**, pp.795-852.

Keslarek, A. (1995). Preparação e Caracterização de Resinas Fenólicas (novolacas) a partir de ligninas Organossolve. MSc thesis, Instituto de Física e Química de São Carlos, Universidade de São Paulo.

Kharade, A. Y. & Kale, D. D. (1998). Effect of lignin on phenolic novolak resins and moulding powder. *Eur. Polym, J.*, **34 (2)**, 201-205.

Loutfi, H; Blackwell, B. & Uloth, V. (1991). Lignin recovery from black liquor – preliminary process design. *Tappi Journal*, **74 (1)**, 203-210.

Santos, F. (1996). Utilização de lignossulfonatos na preparação de resinas fenólicas tipo novolaca e pós de moldagem fenólicos. MSc thesis, Instituto de Química de São Carlos, Universidade de São Paulo.

Santos, F. & Curvelo, A.A.S. (1998). Utilização de lignina kraft na preparação de novolacas e pós de moldagem fenólicos. *In Simpósio Interunidades de Ciência e Engenharia de Materiais*, Proceedings, **1**, pp 3-4.

Santos, F. & Curvelo, A.A.S. (1999). Utilização de ligninas em resinas fenólicas. 1. Preparação de novolacas a partir de lignossulfonatos. *Polímeros: Ciência e Tecnologia*, **9 (1)**, 49-58.

Santos, F. (1999). Lignina kraft em novolacas e pós de moldagem fenólicos. Ph.D. Thesis, Instituto de Química de São Carlos, Universidade de São Paulo.

Sarkanen, S.; Yan L.I. & Mlynar, J. (1995). Towards a New Generation of Lignin-based plastics. *In International Symposium on wood and pulping chemistry*, Proceedings, pp.705-712.

Uloth, VC. & Wearing, JT. (1989). Kraft lignin recovery – acid precipitation versus ultrafiltration. 1.Laboratory results. *Pulp&Paper-Canada,* **90 (9)**, 67-71.

Uloth, VC. & Wearing, JT. (1989). Kraft lignin recovery – acid precipitation versus ultrafiltration. 2.Technology and economics. *Pulp&Paper-Canada,* .**90 (10)**, T357-T360.

Winhaus, O.; Bermaczyk, Z. & Pecina, H. (1992). Análises relativas à precipitação da ligina de licor negro sulfato especialmente com dióxido de carbono. *O Papel,* 45-51.

Laccase-mediators acting on E_1 bleaching effluent

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ABSTRACT

The effects of laccase-mediator system were studied in E_1 effluent. The effluent biotreatments were not efficient in decolorization. At high laccase activity the highest total phenol degradation was observed. The best value for total phenol reduction was around 23% in the absence of mediators. The presence of 1-hydroxybenzotriazole (HBT) did not increase the phenol reduction. The 3-hydroxyanthranilic acid (3-HAA) was rapidly metabolized by the laccase showing that this acid was not a good mediator for this system. However, the acetohydrozamic acid (AHA), which was not degraded by laccase, acted very efficiently on E_1 effluent, reducing 70% and 73% the total phenol and total organic carbon, respectively. The calculations considered the phenol and organic charge given by the controls. Then, AHA appears as the best mediator for laccase bioremediation in E_1 effluent and a more detailed study is in progress.

Keywords: laccase, effluent treatment, bleaching, kraft pulp

INTRODUCTION

Lignin is a complex aromatic biopolymer degradable by a few organisms, like white-rot basidiomycetes. Industry has an increasing interest in extracellular enzymes from white rot fungi, such as lignin and manganese peroxidases and laccases, due to their potential to degrade both highly toxic phenolic compounds and lignin (Mansur *et al.*, 1998).

Laccases (EC 1.10.3.1) are multi copper enzymes that catalyze the oxidation of a variety of phenolic compounds, with concomitant reduction of O_2 to H_2O. These polyphenol oxidases are widely distributed among plant and fungal species; however, their biological significance is unclear. Interest in laccases has been fueled by their potential uses in detoxification of environmental pollutants, wine stabilization, paper processing, enzymatic conversion of chemical intermediates, and production of useful chemicals from lignin (Berka *et al.*, 1997; Gianfreda *et al.*, 1999). Many producers of laccase secrete isoenzymes, which differ in stability and catalytic features (Heinzkill *et al.*, 1998). Laccase alone has a limited effect on bioremediation due to its specificity for phenolic subunits in lignin. Bourbonnais and Paice (1990) founded that the substrate range of laccase can be extended to nonphenolic subunits of lignin by inclusion of a mediator, such as 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS). Furthermore, the laccase-ABTS couple has also been shown to effectively demethylate and delignify kraft pulp (Bourbonnais and Paice, 1992). Since then, HBT has been found to mediate pulp delignification laccase (Call, 1994). Phenothiazines have been found to bleach and prevent redeposition of azo dyes in textile manufacturing (Schneider and Pedersen, 1995), and 3-hydroxyanthranilic acid oxidized nonphenolic lignin model compounds (Eggert *et al.*, 1996).

Laccase has been studied recently in both pure enzymatic, and 'enzyme plus mediator' systems described for the degradation of lignin in wood, pulp, and model compounds (Bermek *et al.*, 1998; Goodell *et al.*, 1998; Sealey and Ragauskas, 1998; Li *et al.*, 1999; Potthast *et al.*, 1999). Potthast *et al.* (1999) investigated the polymerizing and depolymerizing properties of laccase-mediator system. The polymerizing action of laccase and the ABTS cation radical on phenols was demonstrated in addition to the depolymerization effect of the system. The laccase-mediator system cause polymerization of phenolic substratesin the initial phase of the reaction, but then degrades the initially formed polymers. The extensive breakdown of this oligomeric and polymeric material occurs exclusively in the presence of boths components, mediator and laccase. The use of a laccase–mediator system is one of the promising possibilities for an environmentally benign pulp-bleaching process (Li *et al.*, 1998). The University of Georgia has recently opened a pilot plant for deinking of recycled paper. Pulp bleaching with a laccase mediator system has reached pilot plant stage and is expected to be commercialized soon (Bajpai, 1999).

Chlorine is an effective and widely used bleaching agent for chemically produced wood pulps. However, chlorination followed by alkaline extraction results in large volumes of effluents containing substantial levels

of adsorbable organic halogens (AOX), primarily in the form of chlorophenols, chloroguaiacols, chloroaliphatics, chlorocatechols, chlorosyringols, and large polymerized chloroaromatics.

The aim of this work was to study the effects of laccase-mediator system on the treatment of E_I effluent, since no study in this direction was published before.

EXPERIMENTAL

Enzyme Production

Laccase was obtained by *Trametes versicolor* CCT 4521 grown for 20 days, at 30°C and 240 rpm in a liquid medium containing (g/L): peptone, 10; malt extract, 5; $CuSO_4 .5H_2O$, 0.005; glucose, 20; pH 5.4. Laccase induction consisted in the addition of 0.5mmol L^{-1} 2,5-xylidine at 96 hours of growth. The laccase activity in a culture filtrate was around 10000 U/L.

Enzyme assay

Laccase activity was assayed by measuring oxidation of syringaldazine. The assay mixture contained 0.1 mL of 1.0 mmol L^{-1} syringaldazine, 0.3 mL of 50 mmol L^{-1} citrate-phosphate buffer (pH 5.0) and 0.6 mL of culture filtrate. Oxidation of syringaldazine was monitored by measuring the increase in A_{525} for 5 min. Enzyme activity was expressed in units; 1 U was defined as 1 μmol of syringaldazine oxidized per min.

Effluent Treatments

Laccase was added in different activities (25, 50 and 100 U/L) to the E_I effluent. The mediators used were 1-hydroxybenzotriazole (HBT), 3-hydroxyanthranilic acid (3-HAA) and acetohydroxamic acid (AHA) at 0.34 mmol L^{-1} concentration. Total phenols (APHA, 1989), total organic carbon and color (Livernoche *et al.*, 1983) were monitored from 0 to 5 h of treatment.

RESULTS AND DISCUSSION

The results of this work showed that the effluent biotreatments were not efficient in decolorization (data not shown). At higher laccase concentration, higher the total phenol degradation observed (Figure 1). The best value for phenol reduction was around 23% in the absence of mediators. The presence of HBT did not increase the phenol reduction. Bourbonnais *et al.* (1997) found significant differences in reactivity between laccases from different fungi when they were tested for pulp delignification in the presence of the mediators ABTS and HBT. A more detailed study of *T. versicolor* laccase with ABTS and HBT showed that HBT gave the most extensive delignification over 2 h but deactivated the enzyme, and therefore a higher enzyme dosage was required. They tested seven crude preparations of laccase from various white-rot fungi and found that they were all equally capable of delignifying kraft pulps when they were coupled to ABTS. However, their performances with HBT were much more variable, which can be explained in part by their rates of activity loss in the presence of this mediator. Xu *et al.* (1996) compared the reactivities and redox potentials of fungal laccases and concluded that higher redox potential correlates with higher activity. They concluded that structural differences in the substrate activation site (the blue, type I copper center) control the redox potentials, as well as the substrate specificities, of the laccases.

Kadhim *et al.* (1999) showed that culture filtrate extract from *Coriolus versicolor* grown on wheat bran supplemented with yeast extract was used to remove a range of phenolic compounds and the removal was due to the presence of laccase. The effect of the position of chlorine substitution on the benzene ring and its influence on the removal was examined. Phenolic compounds with chlorine substitutions in ortho and para positions were easily attacked by the enzyme unlike compounds with substitutions in the meta position. Jolivalt *et al.* (1999) also observed that the para- and ortho-hydroxyphenyl derivatives were enzymatically transformed, whereas the meta derivative was not. The performance of laccase-mediator depended on the pH, with an optimum value for the para-derivative degradation rate at pH 5.

We observed that the 3-HAA was rapidly degraded by the laccase showing that this acid was not a good mediator for this system. However, the AHA, which was not degraded by laccase, acted very efficiently on E_I effluent reducing in 70% and 73% of the total phenol and total organic carbon, respectively (Durán *et al.* 1999). The calculations considered the phenol and organic charge given by the controls (data not shown). These experiments are now under study by HPLC in order to corroborate them.

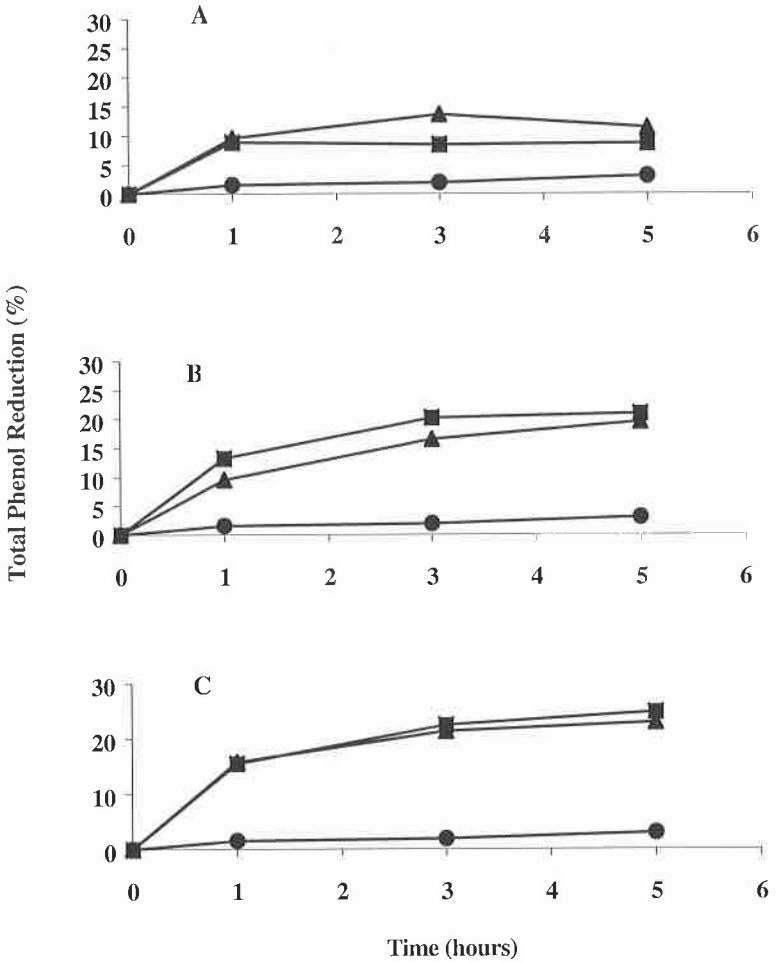

Figure 1: Total phenol reduction of E_1 effluent (control (•)) treated for 5 hours with laccase (▲), laccase and HBT (■). A) Laccase activity: 25 U/L; B) Laccase activity: 50 U/L; C) Laccase activity: 100 U/L.

CONCLUSION

Acetohydroxamic acid appears as the best mediator for laccase bioremediation in E_1 effluent and a more detailed study is in progress.

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REFERENCES

APHA (1989). Standard methods for examination of water and wastewater. 17 ed. American Publ. Health Ass., New York, pp. 568.

Bajpai, P. (1999). Application of enzymes in the pulp and paper industry [Review]. *Biotechnol. Prog.*, **15**, 147-157.

Berka, R.M., Schneider, P., Golightly, E.J., Brown, S.H., Madden, M., Brown, K.M., Halkier, T., Mondorf, K. & Xu, F. (1997). Characterization of the gene encoding an extracellular laccase of *Myceliophthora thermophila* and analysis of the recombinant enzyme expressed in *Aspegillus oryzae*. *Appl. Environ. Microbiol.*, **63**, 3151-3157.

Bermek, H., Li, K.C. & Ericksson, K.E.L. (1998). Laccase-less mutants of the white-rot fungus *Pycnoporus cinnabarinus* cannot delignify kraft pulp. *J. Biotechnol.*, **66**, 117-124.

Bourbonnais, R. & Paice, M.G. (1990). Oxidation of non-phenolic substrates. An expanded role for laccase in lignin biodegradation. *FEBS Lett.*, **267**, 99-102.

Bourbonnais, R. & Paice, M.G. (1992). Demethylation and delignification of kraft pulp by *Trametes versicolor* laccase in the presence of 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonate). *Appl. Environ. Microbiol.*, **36**, 823-827.

Bourbonnais, R., Paice, M.G, Freiermuth, B., Bodie, E. & Borneman, S. (1997). Reactivities of various mediators and laccases with kraft pulp and lignin model compounds. *Appl. Environ. Microbiol.*, **63**, 4627-4632.

Call, H.P. (1994). Process for modifying, breaking down or bleaching lignin, materials containing lignin or like substances. *PCT. World patent application* WO 94/29510.

Durán, N., Minussi, R.C. & Cordi, L. (1999). Process for bioremediation of of textile and pulp and paper effluents by the laccase/acetylhydroxamic acid system. *Brazilian Patent*. PIBr. Submitted.

Eggert, C.B., Temp, U. & Eriksson, K.-E. (1996). A fungal metabolite mediates degradation of non-phenolic lignin structures and synthetic lignin by laccase. *FEBS Lett.*, **391**, 144-148.

Ginafreda, L., Xu, F. & Bollag, J.-M. (1999). Laccases: A useful group of oxidoreductive enzymes. *Bioremediation J.* **3**, 1-25.

Goodell, B., Yamamoto, K., Jellison, J., Nakamura, M., Fujii, T., Takabe, K. & Hayashi, N. (1998). Laccase immunolabelling and microanalytical analysis of wood degraded by *Lentinus edodes*. *Holzforschung*, **52**, 345-350.

Heinzkill, M., Bech, L., Halkier, T., Schneider, P. & Anke, T. (1998). Characterization of laccases and peroxidases from wood-rotting fungi (family *Coprinaceae*). *Appl. Environ. Microbiol.*, **64**, 1601-1606.

Jolivalt, C., Raynal, A., Caminade, E., Kokel, B., Le Goffic, F. & Mougin, C. (1999). Transformation of N',N'-dimethyl-N-(hydroxyphenyl)ureas by laccase from the white rot fungus *Trametes versicolor*. *Appl. Microbiol. Biotechnol.*, **51**, 676-681.

Kadhim, H., Graham, C., Barrat, P., Evans, C.S. & Rastall, R.A. (1999). Removal of phenolic compounds in water using *Coriolus versicolor* grown on wheat bran. *Enzyme Microb. Technol.*, **24**, 303-307.

Li, K.C., Helm, H.F. & Eriksson, K.E.L. (1998). Mechanistic studies of the oxidation of a non-phenolic lignin model compound by the laccase/1-hydroxybenzotriazole redox system. *Biotechnol. Appl. Biochem.*, **27**, 239-243.

Li, K.C., Xu, F. & Eriksson, K.E.L. (1999). Comparison of fungal laccases and redox mediators in oxidation of a nonphenolic lignin model compound. *Appl. Environ. Microbiol.*, **65**, 2654-2660.

Livernoche, D., Jurasek, L., Desrocher, M. & Dorica, J. (1983). Removal of color from kraft mill wastewater with cultures of white-rot fungi and with immobilized mycelium of *Coriolus versicolor*. *Biotechnol. Bioeng.*, **25**, 2055-2060.

Mansur, M., Suarez, T. & Gonzalez, A.E. (1998). Differential gene expression in the laccase gene family from Basidiomycete I-62 (CECT 20197). *Appl. Environ. Microbiol.*, **64**, 771-774.

Potthast, A., Rosenau, T., Koch, H. & Fischer, K. (1999). The reaction of phenolic model compounds in the laccase-mediator system (LMS) – Investigations by matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). *Holzforschung*, **53**, 175-180.

Schneider, P. & Pedersen, A.H. (1995). Enhancement of laccase reactions. PCT. World patent application WO 98/01426.

Sealey J. & Ragauskas, A.J. (1998). Residual lignin studies of laccase-delignified kraft pulps. *Enzyme Microb. Technol.*, **23**, 422-426.

Xu, F., Shin, W., Brown, S.H., Wahleithner, J.A., Sundaram, U.A. & Solomon, E.I. (1996). A study of a series of recombinant fungal laccases and bilirubin oxidase that exhibit significant differences in redox potential, substrate specificity, and stability. *Biochim. Biophys. Acta*, **1292**, 303-311.

Langmuir films of sugar cane bagasse lignin

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ABSTRACT

The molecular organization of lignins at the air-water interface may bring important information about spatial arrangement and lignin characteristics at the molecular level. Conclusions drawn from monolayer studies using lignins isolated with distinct methods can be extrapolated, in principle, to the protolignin in terms of the influence of the extraction method. In a series of papers, Langmuir monolayers and Langmuir-Blodgett films of various lignins with different molecular weights were studied. Here we extend such studies by investigating effects from varying experimental conditions for Langmuir monolayers of a sugar cane bagasse lignin with low polydispersity. The experiments were carried out with the lignin dissolved in a mixture of N,N-dimethylacetamide (DMA) and chloroform. Surface pressure and surface potential isotherms were recorded for films obtained under a variety of conditions, e.g. changing spreading volumes, compression speed, subphase temperature, pH and ionic strength. We observed that films obtained with large volumes of DMA led to very low areas per molecule, thus indicating material loss to the subphase. In subsequent experiments, we therefore adopted a fixed, low amount of DMA (10% in volume) in the spreading solution. Hysteresis and stability experiments were also carried out where the time elapsed for the film to stabilize was varied. When left to accommodate for 10 min. or more, the films displayed considerable hysteresis, which could be attributed to their metastability. The mean area per molecule and the collapse pressure depend on the experimental parameters, such as compression speed, temperature, pH and ionic strength. This points to distinct spatial arrangements depending on the conditions employed in film formation.

Keywords: lignin, sugar cane bagasse, Langmuir films

INTRODUCTION

Lignins constitute the second most abundant renewable biomass resource and represent massive wastes of industrial processes, mainly related to papermaking and sugar production. Various efforts have been made to understand the structure-properties relationships of the lignins in order to find noble applications that would increase their added value (Glasser and Kelley, 1987). In this context, the Langmuir-Blodgett technique may prove to be an additional tool to investigate the lignin characteristics at the molecular level. For the formation of a Langmuir film, a small amount of molecules in diluted solution is spread on the clean surface of a liquid subphase, generally ultrapure water, contained in a recipient referred to as a Langmuir trough. The solvent is then allowed to evaporate, after which the film-forming molecules are compressed with moveable barriers. By recording the average area per molecule and the surface pressure, i.e. change in surface tension of the subphase owing to the film one obtains the pressure-area isotherm. Upon compression, the film evolves from a "gaseous" state, where the molecules do not interact with each other, to other states such as the liquid-expanded and liquid-condensed phases up to the condensed or solid phase, in which the molecules are closely packed. If the film is compressed beyond this point, the molecules arrange themselves in a disordered manner, in the so-called film collapse. Thus, basic relevant parameters in monolayer characterization are the collapse pressure and corresponding area per molecule, the extrapolated area per molecule, obtained by extrapolating the steepest part of the isotherm down to zero pressure, and also the monolayer surface potential which is related to the molecular dipole moments. Langmuir films can be transferred onto solid substrates, forming Langmuir-Blodgett films.

Langmuir films of various types of lignin have been studied by Luner *et al.* (1970,1986), Gilardi and Cass (1993), Baumberger *et al.* (1997), Chakrabarty *et al.* (1985) and in our own group (Barros *et al.*, 1999, Oliveira *et al.*, 1993, 1994; Constantino *et al.*, 1996, 1998). Among the several results derived from such studies are possible packing arrangements for the lignins and the influence from different functional groups, cross-linking, molecular weight and polydispersity, which may be used to infer the organization of lignins in the cell wall (Luner and Roseman, 1986). A key observation in practically all studies was the dependence of monolayer properties on the experimental conditions employed, which is characteristic of films formed from

macromolecular compounds. In order to better control such influence, in this study we have chosen a lignin from sugar cane bagasse with low polydispersity and varied the experimental conditions for film fabrication systematically.

EXPERIMENTAL

A lignin sample obtained from *Saccharum officinarum* (sugar cane bagasse) was supplied by Dedini S/A. Prior to use, the lignin was suspended in water and stirred for 1 hour, filtered and dried under reduced pressure. In order to have its polydispersity reduced, the lignin was submitted to a successive solvent fractionation with dichloromethane, acetone, tetrahydrofuran (THF), acetone/methanol (1/1 v/v), methanol and dioxane/methanol (1:3). In this fractionation process, the lignin was suspended in the solvent, stirred for 30 minutes, and filtered. The solid was resuspended in the same solvent, stirred for an additional 30 minutes and filtered. The filtrates were mixed and the solvent evaporated under reduced pressure yielding the soluble lignin fraction. The remaining solid was dried under reduced pressure and treated with the next solvent in the same way. In this work, results will be presented for the fraction obtained with acetone only. The molecular weight of the lignin and of the lignin fraction were determined by Size Exclusion Chromatography (SEC) using three Plgel columns (500, 1000 e 10000 Å), THF as solvent at 1 mL/min and polystyrene standards. Prior to fractionation the lignin presented Mn=1380 and Mw=2850 (Mw/Mn = 2.1), while the acetone fraction presented Mn=1280 and Mw=1890 (Mw/Mn=1.5).

Isotherm experiments were carried out in a KSV-5000 LB system placed in a class 10,000 clean room. Surface pressure isotherms were obtained with the Wilhelmy plate method and surface potential measurements were carried out with a Kelvin probe supplied by KSV. Ultra pure water supplied by a R060 Millipore filter followed by a Millipore-Q system was used as subphase. The temperature subphase was kept constant by a Nesslab circulating water bath. The spreading solutions (0.2 mg/mL) were prepared using mixtures of N,N-dimethylacetamide (DMA) and chloroform.

RESULTS AND DISCUSSION

The influence of various experimental parameters was investigated, based upon our previous experience with macromolecular compounds, such as conducting polymers and several types of lignin.

Solvent mixture, spreading volume and compression speed

Lignins are usually insoluble in volatile, water immiscible solvents such as chloroform, which requires a systematic search for solvents that will allow spreading of the molecules but at the same time prevent the solvent from affecting the film strongly. For instance, DMA is a good solvent for most lignins but is also water-soluble and has a high boiling point (165°C). Langmuir films spread from DMA solutions of the sugar cane bagasse lignin were highly unstable, since a considerable part of the lignin molecules are dragged by the solvent into the aqueous subphase. This is why the area per molecule is so small for the isotherm shown in Figure 1 (curve 100%). In order to have an adequate balance between these opposite tendencies, solvent mixtures may be employed. Indeed, this was tried with DMA and chloroform and the results are shown in Figure 1. For mixtures containing 20 and 50% (v/v) of DMA material loss to the subphase was still apparent, but when the amount of DMA was fixed in 10% more stable monolayers were obtained. Since experiments with less than 10% of DMA led to unstable solutions, with precipitation of the lignin, in all subsequent experiments we employed the 90:10 mixture of chloroform:DMA.

Traditional amphiphiles, such as stearic acid, display isotherms that do not depend on either concentration or volume of solution spread. This is expected insofar as the Langmuir film of stearic acid is a true monolayer, with each molecule in the condensed phase occupying an area that is not influenced by the amount of molecules spread. For materials that do not form true monomolecular films, as is the case of lignins, isotherms may indeed vary with the volume spread or the concentration of the spreading solution. Such dependencies may appear due to aggregation or even owing to the adoption of distinct molecular arrangements as the interaction between molecules vary with concentration. Ideally, the lowest possible concentration should be used in order to minimize aggregation. However, upon using very diluted solutions, experiments are only feasible if large volumes of spreading solution are utilized, which again may cause aggregation and/or formation of non-monomolecular structures. For the lignin investigated here, isotherms were essentially the same if the volume spread lied between 300 and 400 µL, but high surface pressures could

not be achieved with 300 μL, for compression had to be stopped before the condensed phase was reached. The spreading volume of 400 μL was therefore used in all further experiments.

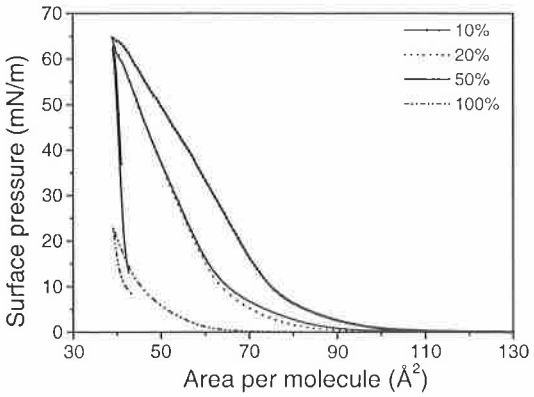

Figure 1. Isotherms obtained with various concentrations of chloroform and DMA as spreading solvents.

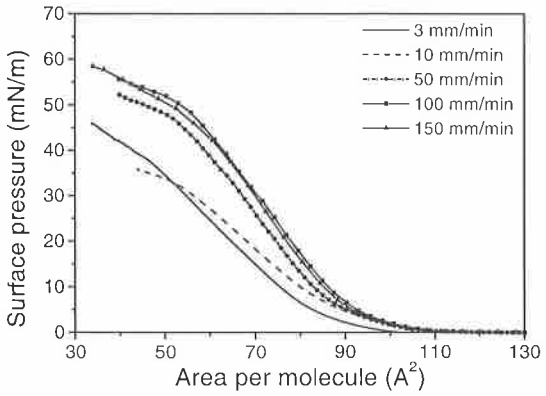

Figure 2. Isotherms obtained with different compression speeds.

When compressed by the barriers in a Langmuir trough, macromolecules such as lignins may take a reasonable amount of time to find the most stable arrangement, and therefore compression may be performed under non-equilibrium conditions. Hence, isotherms may depend on the barrier speed, which was observed to be the case for the lignin from sugar cane bagasse, as shown in Figure 2. In particular, the collapse pressure was lower for low speeds, which was to be expected owing to either material loss (as the monolayer is metastable) or to the possibility of the molecules having the time to find a more compact arrangement.

Subphase Temperature

Various subphase temperatures were employed, viz. 10, 15, 24 and 30 ^{0}C. Figure 3 shows that as the temperature increased, the mobility of the molecules also increased, leading to more compact films, for which the extrapolated area per molecule decreased from 97 to 75 Å^2 as the temperature increased from 10 to 30 ^{o}C.

Subphase pH and ionic strength

It is known that lignin monolayers spread onto pure water are neutral. They may be protonated upon decreasing the subphase pH from 6 (Milli-Q water) to 3 or 1, as we did through addition of hydrochloric acid. Figure 4 shows that the extrapolated area per molecule is slightly larger at low pHs (95 Å^2 as compared to 85 for pure water), being practically the same for subphases with pH=1 and 3. Protonation, probably of oxygen atoms in the lignin molecules, causes electrostatic repulsion, which does not occur at higher pHs. Also, upon protonation the monolayer surface potential increases, as observed in Figure 4, where the potential for a monolayer spread on a pH=1 subphase is considerably larger than for pure water. This increase appears due to the double-layer formed at the film/water interface when the film is at least partially charged.

We also investigated the effect of increasing the ionic strength of the subphase, for pH=6. Since at this pH the lignin monolayer is neutral, the monolayer surface potential should not be affected by changing the ionic strength. Indeed, as Figure 5 shows, the maximum surface potential for condensed monolayers is not significantly affected if a 0.1 M NaCl subphase is used. However, the surface potential as well as the surface pressure isotherms are more expanded for the salt-containing subphase, which is illustrated in Figure 5, where the extrapolated area per molecule increased from 85 to 102 Å^2 with addition of salt. This is a surprising result because the monolayer is not ionized and therefore no screening effect should be observed, which could change the molecular packing substantially.

Hysteresis and Stability

In hysteresis experiments, the Langmuir film is compressed to a certain surface pressure (lower than the collapse pressure) and decompressed at a fixed compression speed several times. If the film is completely stable, no changes in the area per molecule were observed. Figure 6a shows isotherms from three compression-decompression consecutive cycles, showing no significant hysteresis. When left to accommodate for 10 min. or more between the compression-decompression cycles, however, the films displayed considerable hysteresis as shown in Figure 6b, which could be attributed to their metastability. Such metastability was also observed in stability experiments, where the film is compressed to a surface

pressure below the collapse pressure and the area per molecule is monitored as time evolves (Figure 7). In the first 100 minutes the area decreased almost exponentially, stabilizing only after 400 minutes.

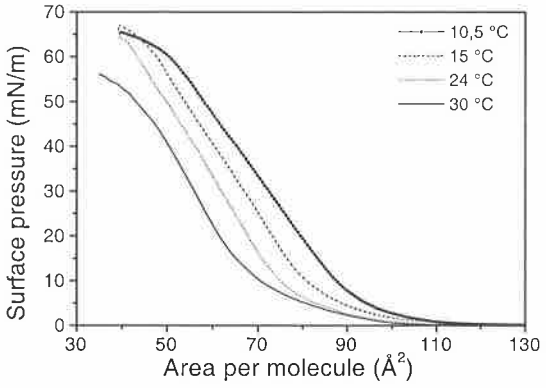

Figure 3. Isotherms obtained with different subphase temperatures.

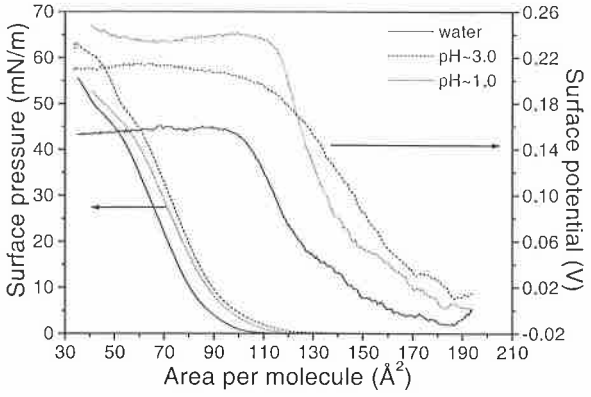

Figure 4. Isotherms for lignin spread on subphases with different pHs.

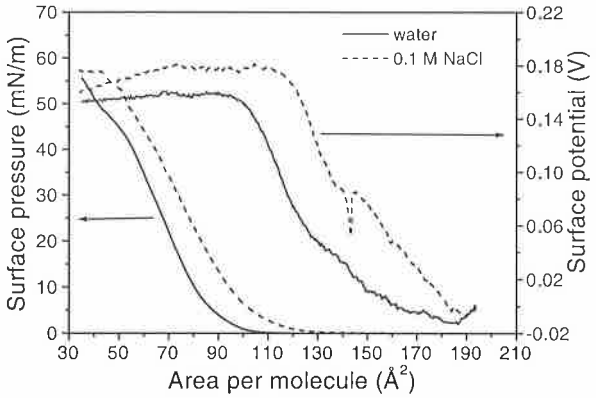

Figure 5. Isotherms obtained with different subphase ionic strengths.

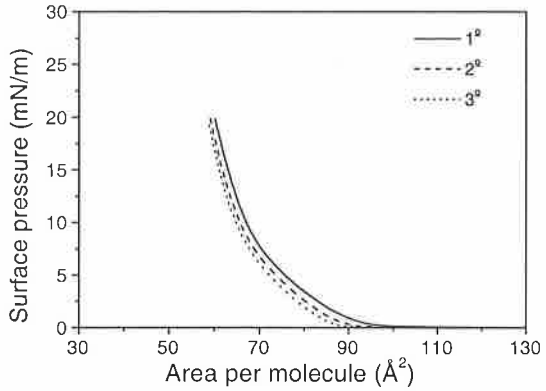

Figure 6a. Hysteresis curves with no waiting time for consecutive runs.

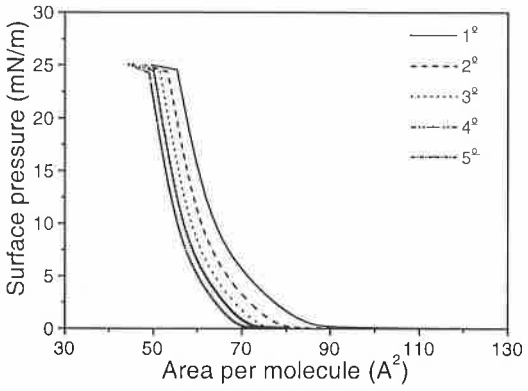

Figure 6b. – Hysteresis with waiting time between consecutive runs.

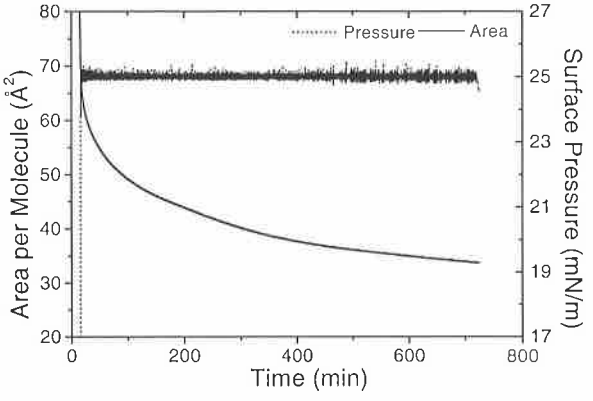

Figure 7. Langmuir film stability.

In summary, the influence of experimental parameters on monolayer characteristics of a lignin from sugar cane bagasse was investigated. The mean area per molecule and collapse pressure depend on compression speed, temperature, pH and ionic strength, which means that distinct spatial arrangements may be adopted by the lignin molecules depending on the conditions employed in film formation.

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REFERENCES

Barros, A.M., Dhanabalan, A., Constantino, C.J.L., Balogh, D.T. & Oliveira Jr., O.N. (1999). Langmuir monolayers of lignins obtained with different isolation methods. *Thin Solid Films* in press.

Baumberger, S.; Aguié-Béghin,V.; Douillard, R., Lapierre C. & Monties, B. (1997). Properties of grass lignin layers at air-water interface. *Ind. Crop Prod.* **6**, 259- 263.

Chakrabarty, R.R.; Neuman, R.D. & Sarkanen, S. (1986). The macromolecular configuration of lignins. *Int. Symp. Wood and Pulp. Chem.* , Proceedings, pp.35-36

Constantino, C.J.L.; Dhanabalan, A., Curvelo, A.A.S. & Oliveira Jr., O.N. (1998) Preparation and characterization of composite LB films of lignin and cadmium stearate. *Thin Solid Films*, **329**, 47- 51.

Constantino, C.J.L; Juliani, L.P., Botaro, V.R., Balogh, D.T., Pereira, M.R., Ticianelli, E.A., Curvelo A.A.S. & Oliveira Jr., O.N. (1996). Langmuir-Blodgett films from lignin.*Thin Solid Films*, **284-285**,191-194.

Gilardi, G. & Cass, A.E.. (1993). Associative and colloidal behavior of lignin and implications for its biodegradability. *Langmuir*, **9,** 1721-1726.

Glasser, W. & Kelley, S. S. (1987). *Encyclopedia of Polymer Science and Engineering*, ed. H.F. Mark, N.M. Bikales, C. G. Overberger and G. Menges, Wiley, New York, **Vol 8,** pp 795.

Luner, P. & Roseman, G. (1986). Monomolecular film properties of isolated lignins. *Holzforschung*, **40,** Suppl. 61-66.

Luner, P.& Kempf, U.(1970). Properties of lignin monolayers at air-water interface. *TAPPI*, **53,** 2069-2076.

Oliveira Jr., O.N.; Constantino, C.J.L., Balogh, D.T. & Curvelo, A.A.S. (1993). Monolayers of organosolv lignin. *In 3th brazilian symposium on the chemistry of lignins and other wood components*, Proceedings, pp.216-219

Oliveira Jr., O.N.; Constantino, C.J.L., Balogh, D.T. & Curvelo, A.A.S. (1994). Langmuir monolayers of lignins from *Pinus caribaea hondurensis*. *Cellulose Chem. Technol.* **28,** 541-549.

Membrane concentration of lignin peroxidase from *Streptomyces viridosporus* T7a by cross flow ultrafiltration

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ABSTRACT

Lignin peroxidase has a potential use on environmental biocatalysis due its ability to oxidize a large number of aromatic substances, including recalcitrant compounds such as chlorolignin, pesticides, and azo dyes. However the cost for its production, separation and concentration must be low to justify its use in the control of environmental pollution. Therefore membrane separation and concentration processes, that are low cost and low energy consumption technologies, are suitable for downstream enzyme processing. In this work a crude *Streptomyces viridosporus* T7A lignin peroxidase preparation was concentrated by ultrafiltration. Lignin peroxidase production was performed in 2 L agitated flasks (200 rpm) at 37°C, containing 400 mL of culture medium (0.65% yeast extract, 0.65% glucose plus mineral salts) that was previously optimised. Crude enzyme preparation was obtained through filtration of the fermentation broth in paper Whatman n° 1. After that, the culture supernatant was concentrated by cross flow ultrafiltration using distinct polysulfone membranes with 50,000, 20,000 and 10,000 $g.mol^{-1}$ cut off . The enzyme activity was measured, using 2,4-dichlorophenol as substrate, in feed solution, concentrate and permeate in order to evaluate the membrane rejection for lignin peroxidase and the activity loss during the process. The best results were obtained using the 10,000 $g.mol^{-1}$ cut off membrane with retention of 97% of enzyme activity in the concentrate. It was also observed that the use of low temperatures favours the enzyme stability during the ultrafiltration.

Keywords: lignin peroxidase, enzyme purification, *Streptomyces viridosporus*

INTRODUCTION

The biodegradation of lignin is an oxidative process involving extracellular enzymes produced by certain microorganisms. Lignin peroxidase, a component of the ligninolytic complex, is responsible for lignin degradation in the presence of hydrogen peroxide (Schoemaker and Leisola, 1990; Tien and Kirk, 1983; Ramachandra *et al.,* 1988). An important characteristic of lignin peroxidase is its ability to oxidize, besides lignin, a great variety of compounds such as chlororganics (Mileski *et al.*, 1988; Winter *et al.*, 1991), polycyclic aromatic hydrocarbons (Haemmerli *et al.*, 1986), phenolic compounds (Spiker *et* al., 1992), azo dyes (Pasti-Grigsby *et* al., 1992), lignosulfonated compounds (Hernandez Perez *et al.*, 1997) and pesticides (Gauger *et al.*, 1986). Such property makes lignin peroxidase potentially useful enzyme in industries that use lignocellulosic raw material, such as cellulose and pulp industries, in organopollutant's treatment systems and in lignin conversion for obtaining raw material for chemical industries (Odier and Artaud, 1992). Due to this potential use on environmental biocatalysis, the cost for its production must be low. Costs can be reduced by improving the technique employed for separation (Mulder, 1991).

From an economical point of a view worthless waste streams are hardly worthy of treatment, but environmental considerations and governmental regulations often determine that the separation must carried out. The economics of a separation process may be governed by product loss and damage. Damage to the product can occur particularly when heat-sensitive components are produce, like enzymes. Membrane technology is an emerging technology and because of its multidisciplinary character it can be used in a large number of separations process. The mainly benefits of membrane technology are: separation can be carried out continuously; energy consumption is generally low; separation can be carried out under mild conditions; up-scaling is easy; membrane properties are variable and can be adjusted; no additives are required and it can be easily combined with other separation process (Mulder, 1991). Ultrafiltration is often used for the fractionation and concentration of macromolecules where large molecules have to be retained by membrane while small molecules (and the solvent) should permeate through freely (Mulder, 1991).

According to the literature, lignin peroxidase produced by *Streptomyces viridosporus* had been concentrated by ultrafiltration in order to provide an enzymatic concentrate that can be used for posterior characterization, purification and evaluation of others properties of this enzyme (Ramachandra *et al.*, 1988; Deobald and Crawford, 1989; Lodha *et al.*, 1991; Magnuson *et al.*, 1991; Spiker *et al.*, 1992). However, in none of this works have been specified the operational conditions and the enzymatic activity loss during the process. Our research group has been studied *Streptomyces viridosporus* T7A lignin peroxidase by aiming at reducing the cost of enzyme production and separation. In this present work, it was investigated the membrane concentration of lignin peroxidase by cross flow ultrafiltration.

EXPERIMENTAL

Microorganism and media

S. viridosporus T7A (ATCC 39115) stock spores were maintained at -20°C in a 20% (w/v) glycerol aqueous solution (Hopwood *et* al., 1985). For LiP production the culture medium (6.5 g/L yeast extract, 6.5 g/L glucose, mineral salts and trace metal stock solution (McCarthy and Broda, 1984; Pometto III and Crawford, 1986)) was inoculated with a spore suspension to a final absorbance at 570 nm of 0.01. Cultures were incubated at five 2 L shake flasks containing 400 mL of the medium at 37°C and 200 rpm in a shaker (Tecnal BTC 9090). Peak enzyme production was observed upon glucose depletion, within 48 h of fermentation. The culture was filtered in Whatman n° 1 paper. This supernatant was used for posterior ultrafiltration.

Ultrafiltration apparatus and procedure

All ultrafiltration experiments were carried out with the equipment shown in Figure 1. The flat membranes were tested using a Rhône-Poulenc Ultrafiltration cell model Pleiade-UF2 shown in Figure 2. The filtration area of this cell is 0.0109 m^2. In this ultrafiltration system it is possible to adjust feed flow velocity and transmembrane pressure independently from each other. The temperature of the feed was maintained constant during all the process with the aid of a thermostatic bath. Three sets of experiments were performed with distinct cut-off polysulphone membranes: 50,000, 20,000 and 10,000 g.mol-1 (Type: GR51PP, GR61PP and GR81PP, Dow Danmark, Dennamark). The cut off is defined as that molecular weight which is 90% rejected by the membrane (Mulder, 1991). This mean that for a membrane with cut off of 50,000 $g.mol^{-1}$, 90% of molecules with molecular weight greater than 50,000 $g.mol^{-1}$ can not pass through this membrane, staying in concentrate. Molecules with a molecular weight lower than 50,000 $g.mol^{-1}$ can pass through membrane staying in permeate.

LiP enzyme assay

Lignin peroxidase activity was assayed by a modified method based on enzymatic oxidation of 2,4-dichlorophenol (2,4-DCP, Sigma) (Ishida *et* al., 1987). Reaction mixture contained 50 mM sodium phosphate buffer, (pH 7.0), 164 μM 4-aminoantipyrine (Sigma), 3.0 mM 2,4-DCP, 4.0 mM hydrogen peroxide and 200 μL enzyme preparation in a final volume of 1.0 mL. Reaction was initiated by the addition of hydrogen peroxide, and the increase in absorbance at 510 nm was monitored for 1 min at room temperature (about 29°C). One unit of enzyme activity was expressed as the amount of enzyme required for an increase of one absorbance unit per min. Lignin peroxidase activity was measured in the feed, in the permeate and the concentrate in order to obtain the percentage of enzyme in each fraction.

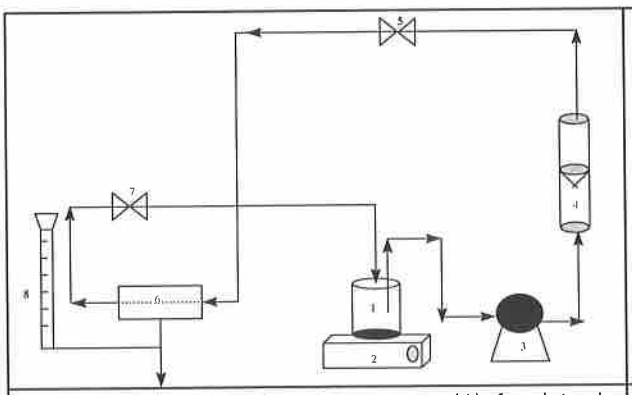

Figure 1. Ultrafiltration apparatus: (1) feed tank; (2) thermostatic bath; (3) recirculating pump; (4) feed flow meter; (5) and (7) pressure gauges; (6) ultrafiltration module; (8) permeate flow meter

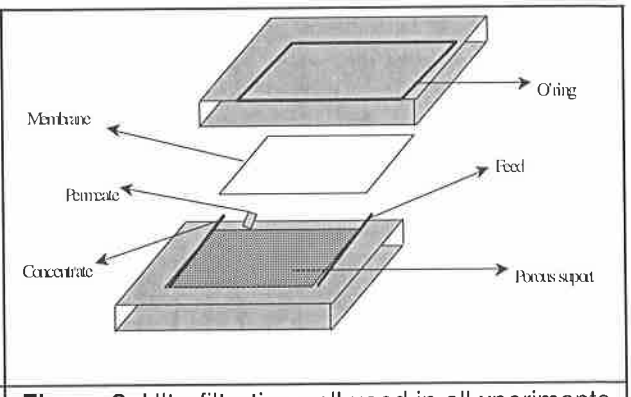

Figure 2. Ultrafiltration cell used in all xperiments

RESULTS AND DISCUSSION

In literature, there is not a consensus in relation to the lignin peroxidase molecular weight. A molecular weight of 17,800 g.mol^{-1} was estimated for the enzyme by SDS-PAGE (Ramachandra *et al.*, 1988). However different values were observed for the isoenzyme when it was purified by preparative polyacrilamide gel electrophoresis and electroelution using unboiled (75,800 g.mol^{-1}) and boiled (67,100 and 37,100 g.mol^{-1}) samples. Also the Fergusson plot for the native polyacrylamide electrophoresis indicated a molecular weight of 134,000 g.mol^{-1} (Magnuson *et al.*, 1991). On the other hand, the relationship between molecular weight and specie dimension of macromolecules that can suffer deformation depending of operation conditions may difficult the correct choice of the membrane cut off (Nobrega and Balmann, 1989). For this reason lignin peroxidase was concentrated using polysulphone membranes within cut off of 50,000, 20,000 and 10,000 g.mol^{-1}.

In all experiments were used the same applied pressure (2 bar) and cross flow velocity (0.20 m/s) in order to supply the same concentration polarization and fouling effects. There is a decrease in the permeate flux that is due to concentration polarization and fouling. This flux decline has a negative influence on the economics of a given membrane operation, and for this reason measures must be taken to reduce its incidence (Mulder, 1991). Fouling phenomena diminish as concentration polarization decreases. Concentration polarization can be reduced by increasing the mass transfer coefficient (high flow velocities) and using low(er) flux membranes.

Before ultrafiltration experiments for lignin peroxidase concentration, the membranes were washed to eliminate traces of solvent or impurities left over from the manufacturing process, then an ultrafiltration operation was carried out with microfiltered water under pressure of 0.5 to 4.0 bar and the permeate flux was measured in order to obtain the hydraulic permeability before each run (Figure 3). There is a linear relationship between the hydrodynamic pressure (ΔP) and the permeate flux (Jperm). The slope of the corresponding flux-pressure curve is the hydraulic permeability coefficient (Lp) and it can be obtained via equation: $Jperm = Lp.\Delta P$ (Mulder, 1991).

The 20,000 g.mol^{-1} cut off membrane was the most permeable with a permeability of 1.26×10^{-5} m^3/m^2.s.bar. The 10,000 g.mol^{-1} membrane presented a permeability almost two times lower than the 20,000 g.mol^{-1} cut off membrane (7.10×10^{-6} m^3/m^2.s.bar) as it was expected. Unexpectedly the permeability measured for the 50,000 g.mol^{-1} cut off membrane was the smallest off all membrane tested (6.83×10^{-6} m^3/m^2.s.bar). This probably occurred due its global porosity and porous size distribution (Figure 3).

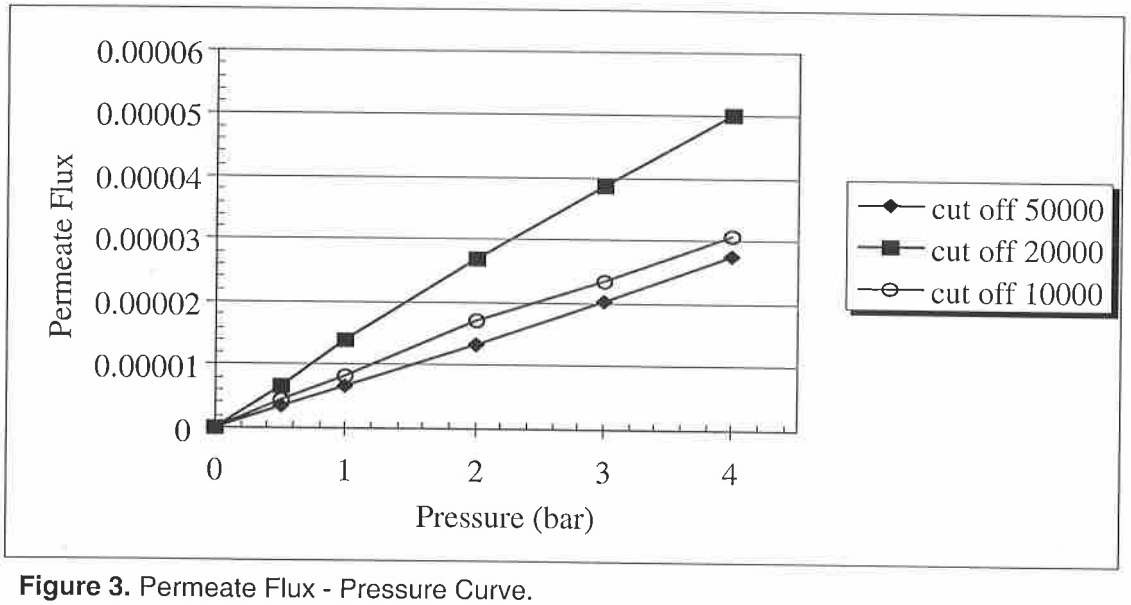

Figure 3. Permeate Flux - Pressure Curve.

In all experiments lignin peroxidase was concentrated approximately five times by cross flow ultrafiltration. The initial volume was around 1500 mL and the concentrate volume was approximately 300 mL. The enzyme activity was measured in the feed before starting the ultrafiltration process. At the end, LiP activity was measured in concentrate and in permeate in order to calculated the percentages of enzyme in each

fraction. The activity loss was calculated by comparison the total initial activity in feed and the total final activity presented in concentrate and permeate. The results were presented in Table 1.

The first set of experiments has been done with the polysulphone membrane with a cut off of 50,000 $g.mol^{-1}$. Even having the lowest permeability, 55% of total enzyme have been passed through membrane, remaining in the permeate. This result contradicts those founded by Magnuson *et al.* (1991) and suggested that the enzyme must have a molecular weight lower than 50,000 $g.mol^{-1}$.

Table 1. Comparison of % LiP in concentrate, % LiP in permeate, % LiP Activity loss, Process Time (h) and Temperature (°C) in all membranes tested.

Membrane	% LiP in Concentrate	% LiP in Permeate	% LiP Activity loss	Process Time (h)	Temperature (°C)
50,000	45	55	25	5	30
20,000	73	27	16	4	20
10,000	97	3	11	5	15

An improved result was obtained when the 20,000 $g.mol^{-1}$ cut off membrane was used. However 27% off enzyme was still present in the permeate. The best results for concentration lignin peroxidase were obtained within 10,000 $g.mol^{-1}$ cut off membrane, with retention of 97% of enzyme activity in the concentrate. This result was in agreement with Ramachandra *et al.* (1988) where a molecular weight of 17,800 $g.mol^{-1}$ was estimated for the enzyme by SDS-PAGE. This result corroborated a recently work where was found that a molecular weight of 13,500 $g.mol^{-1}$ obtained by SDS-PAGE, indicating a lignin peroxidase monomer based structure (Bon *et al.*, 1999).

It was also observed that the use of low temperatures favors the enzyme stability during the ultrafiltration. The mainly lignin activity loss (25%) occurred with 50,000 $g.mol^{-1}$ cut off membrane where the temperature was maintained in 30°C and the lowest activity loss (11%) occurred with 10,000 $g.mol^{-1}$ cut off membrane where the temperature was maintained in 15 °C. Another experiments must be made to confirm the temperature influence on lignin peroxidase concentration by cross flow ultrafiltration since the process time in all experiments was almost the same.

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REFERENCES

Bon, E.P.S., Nascimento, H.J., Macedo, J.M.B. & Silva Jr., J.G. (1999). Lignin peroxidase isoforms from *Streptomyces viridosporus* T7A: would they show a monomer based structure? *Biotechnol. Tech.*, **13 (5)**, 289-283.

Deobald, L.A. & Crawford, D.L. (1989). Lignin biotransformations by an aromatic aldehyde oxidase produced by *Streptomyces viridosporus* T7A. *Appl. Biochem. Biotechnol.*, **20/21**, 153-163.

Gauger, W.K., MacDonald, J.M., Adrian, N.R., Matthees, D.P. & Walgenbach, D.D. (1986). Characterization of *Streptomycetes* growing on organophosphate and carbamate insecticides. *Arch. Environ. Contam. Toxicol.*, **15**, 137-141.

Haemmerli, S.D., Leisola, M.S.A. & Fiechter, A. (1986). Oxidation of benzo(a)pyrene by extracellular ligninases of *Phanerochaete chrysosporium. FEMS Microbiol. Lett.*, **35**, 33-36.

Hernandezperez, G., Goma, G. & Rols, J.L. (1997). Degradation of lignosulfonated compounds by *Streptomyces viridosporus* strain T7A. *Biotechnol. Lett.*, **19**, 285-289.

Hopwood, D.A., Bibb, M.B., Chater, K.F., Kieser, T., Bruton, C.J., Kieser, H.M., Lydiate, D.J., Smith, C.P., Ward, J.M. & Schrempf. H. (1985). *Genetic Manipulation of Streptomyces: A Laboratory Manual.* The John Innes Foundation Norwich., pp 3-5.

Ishida, A., Futamura, N. & Matsusaka, T. (1987). Detection of peroxidase activity and its localization in the forespore envelopes of *Bacillus cereus. J. Gen. Appl. Microbiol*,. **33**, 27-32.

Lodha, S.J., Korus, R.A. & Crawford, D.L. (1991). Synthesis and properties of lignin peroxidase from *Streptomyces viridosporus* T7A. *Appl. Biochem. Biotechnol.*, **28/29**, 411-420.

Magnuson, T.S., Roberts, M.A., Crawford, D.L. & Hertel, G. (1991). Immunologic relatedness of extracellular ligninases from the actinomycetes *Streptomyces viridosporus* T7A and *Streptomyces badius* 252. *Appl. Biochem. Biotechnol.*, **28/29**, 433-442.

McCarthy, A.J. & Broda, P. (1984). Screening for lignin-degrading actinomycetes and characterization of their activity against [^{14}C]lignin-labelled wheat lignocellulose. *J. Gen. Microbiol.*, **130**, 2905-2913.

Mileski,G.J., Bumpus, J.A., Jurek, M.A. & Aust, S.D. (1988). Biodegradation of pentachlorophenol by the white-rot fungus *Phanerochaete chrysosporium. Appl. Environ. Microbiol.*, **54**, 2885-2889.

Mulder, M. (1991). In *Basic Principles of Membrane Technology,* ed. Kluwer Academic Publishers, Netherlands.

Nobrega, R. & Balmann, H. (1989). Transfer of dextran through ultrafiltration membranes: a study of rejection data analysed by gel permeation chromatography. *J. Memb. Science,* **45,** 17-36.

Odier, E. & Artaud, I. (1992). Degradation of Lignin, in *Microbial Degradation of Natural Products*, ed. Winkelmann, G. & Germany VCH, pp161-191.

Pasti-Grigsby, M.B., Paszczynski, A., Goszczynski, S., Crawford, D.L. & Crawford, R.L. (1992). Influence of aromatic substitution patterns on azo dye degradability by *Streptomyces* spp. and *Phanerochaete chrysosporium. Appl. Environ. Microbiol.*, **58,** 3605-3613.

Pometto III, A.L. & Crawford, D.L. (1986). Catabolic fate of *Streptomyces viridosporus* T7A-produced, acid-precipitable polymeric lignin upon incubation with ligninolytic *Streptomyces* species and *Phanerochaete chrysosporium. Appl. Environ. Microbiol.*, **51**, 171-179.

Ramachandra, M., Crawford, D.L. & Hertel, G. (1988). Characterization of an extracellular lignin peroxidase of the lignocellulolytic actinomycete *Streptomyces viridosporus. Appl. Environ. Microbiol.,* **54**, 3057-3063.

Schoemaker, H.E. & Leisola, M.S.A. (1990). Degradation of lignin by *Phanerochaete chrysosporium. J. Biotechnol.*, **13**, 101-109.

Spiker, J.K., Crawford, D.L. & Thiel, E.C. (1992). Oxidation of phenolic and nonphenolic substrates by the lignin peroxidase of *Streptomyces viridosporus* T7A. *Appl. Environ. Microbiol.*, **37,** 518-523.

Tien, M. & Kirk, T.K. (1983). Lignin-degrading enzyme from the hymenomycete *Phanerochaete chrysosporium. Burds. Science*, **221**, 661-663.

Winter, B., Fiechter, A. & Zimmerman, W. (1991). Degradation of organochlorine compounds in spent sulfite bleach plant effluents by actinomycetes. *Appl. Environ. Microbiol.*, **57**, 2858-2863.

Nanocomposites for advanced degradation of industrial residues

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ABSTRACT

In this work, a porous glass with nanometric pore dimensions (Vycor) was used to immobilize traditional photocatalysts (ex. CdS and RuO_2) and metalloporphyrins with mimetic ligninase activity. Both types of nanocomposites were prepared from adequate precursors, completely characterized by spectroscopic methods and used to study the degradation of chemical compounds of environmental relevance. The immobilized semiconductors were used in a conventional photochemical reactor (oxygenated-UV systems) and in a microwave-activated photoreactor (without oxygenation). By using the microwave-activated system almost total decolorization of several reactive dyes using in the dyestuff activities (ex. reactive black B and reactive blue 19) was observed for relatively short reaction times. Unfortunately, the decolorization process is mainly due to homogeneous photochemical reactions, which are favored by the photosensibility of the dyes. The iron (III) porphyrin complex was quickly immobilized into the inorganic glass, showing high oxidation potential toward hexane and o-dianisidine (the typical ligninase substrate). Studies to investigate the potential of this new catalytic nanocomposites toward more recalcitrant chemical species are in progress.

Keywords: nanocomposites, semiconductors, photocatalysis, dyes, effluent treatment

INTRODUCTION

In recent years, the critical character of the environmental pollution has promoted the investigation of new processes oriented to the remediation of industrial residues. Among others, the photochemical (Lisenbigler *et al.*, 1995, Hoffmann *et al.*, 1995, Mills and Le Hunte, 1997) and enzymatic (Khindaria *et al.*, 1995, Hammel, 1996, Dezotti *et al.*, 1995) processes have occupied a prominent place, mainly due to their high degradation capacity toward recalcitrant chemical species. Unfortunately, both processes present important practical limitations mainly related to the difficult separation of the photocatalyst at the end of the photochemical process and the laborious production of ligninolytic enzymes from white-root fungi. To overcome these problems, we are proposing the immobilization of traditional photocatalysts (ex. CdS, Fe_2O_3 and RuO_2) and metalloporphyrins on the nanometric pores of an inorganic matrix (Vycor glass), and the use of this nanocomposites as catalysts in photochemical and mimetic-enzymatic degradation processes.

The Porous Vycor Glass (PVG) is a transparent porous material obtained by acid leaching of a phase-separated alkaline borosilicate glass. The soluble borate phase is dissolved, leaving an open porous structure of essentially pure silica with interconnecting pores of narrow size distribution (between 20 and 200 A), and a pore volume of nearly 28% (Corning Glass, 1979). The pore surface contains slightly acidic silanol groups that can be used as active sites on the incorporation of organometallic and semiconductor compounds (Zarbin *et al.*, 1999, Luang, 1988).

The catalytic oxidation and dechlorination of many chemical compounds of environmental relevance by chemical ligninase models has been reported in recent years. Among others, water-soluble iron or manganese porphyrin complexes were found to be highly efficient for the oxidation of chlorinated compound in the presence of potassium persulfate or hydrogen peroxide (Shukla *et al.*, 1996).

EXPERIMENTAL

Reagents

The dyes were purchased from a textile mill located in Americana (São Paulo, Brazil). Porous Vycor Glass (PVG) was obtained from Corning Glass.

Preparation of the PVG/CdS and PVG/RuO_2 catalysts

The incorporation of CdS in the porous glass was carried out according the procedures previously reported by Luang (1988). Cd (II) was initially incorporated on the PVG matrix by ion exchange with a $Cd(NO_3).4H_2O$ aqueous solution. After this, the cadmium ion-exchanged glass was immersed in an aqueous solution of

thiourea, washed with water and heated at 300°C by 3h. The incorporation of RuO_2 was carried out according the procedures previously reported by Zarbin *et al.* (1999). The precursor ($Ru_3(CO)_{12}$) was incorporated by placing the PVG plate in a n-hexane solution of the cluster for 4h, at room temperature. Finally, the PVG plate was washed several times with n-hexane, dried under vacuum and heated at 300°C by 3h. Both nanocomposites were characterized by UV-VIS spectroscopy.

Preparation of the PVG/$Fe^{III}TPP^+$ catalyst

Initially, the metallation of the meso-tetraphenylporphyrin (TPP) (Aldrich) was carried out by the standard method of Iamamoto *et al.* (1997), using $FeBr_2$/DMF. The metalloporphyrin was purified from the unreacted porphyrin by silica colunm chromatography. The $Fe^{III}TPP^+$ was incorporated in the porous glass by contacting a plate of PVG with a solution of ironporpphyrin in DMF. The solid materials were characterized by UV-VIS spectroscopy.

Catalytic oxidation with PVG/$Fe^{III}TPP^+$ catalyst

Catalytic oxidation reactions were carried out in a 2 mL thermostatic glass reactor equipped with a magnetic stirrer inside of a dark camera. In a standard experiment, within the reactor, vycor-ironporpphyrin and iodosylbenzene were suspended in 0.300 mL of solvent (dichloromethane or acetonitrile) and degassed with argon during 10 min. The substrate (cyclohexane, 0.150 mL) was added and the oxidation reaction was carried out during one hour, under magnetic stirring. To eliminate the excess of iodosylbenzene, sodium sulfite was added and the products of the reaction were separated from the solid by exhaustive washing of the vycor with acetonitrile. The acetonitrile-extracted solution was analyzed by capillary GC and the amount of the products were determined by using n-octanol as the internal standard. No products were detected when the catalyst used was vycor without ironporphyrin.

Photochemical oxidation with PVG/CdS and PVG/RuO_2 catalysts

The photochemical treatment of the substrates was carried out in a conventional reactor equipped with water refrigeration, magnetic stirring and oxygenation (10 $mLmin^{-1}$). Aqueous dye samples of 25 mL were irradiated from the top with a 125 W Philips medium pressure mercury lamp without the glass cover (fluence rate: 15 $Jm^{-2}s^{-1}$ at λ>254 nm) at a distance of 12 cm from the solution surface. The photocatalyst was used as a thin plate (10x10x1 mm), located at the center of the reactor and close of the solution surface. For analytical control, samples were taken at convenient times and analyzed by UV-VIS spectroscopy (Hitachi U-2000 spectrophotometer).

Additional photochemical experiments were carried out in a microwave-activated photoreactor (UMEX). In these experiments samples of 10 mL were treated without oxygenation.

RESULTS AND DISCUSSION

Characterization of the nanocomposites

In view of the transparency of the Vycor matrix in the UV-VIS region, both nanocomposites were preliminarily characterized by UV-VIS spectroscopy. The presence of the characteristic UV-VIS absorption signals (Figure 1) indicated that the proposed experimental procedure leaves to effective incorporation of CdS and $Fe^{III}TPP^+$ into the porous of the Vycor Glass. Additional confirmation of the PVG/$Fe^{III}TPP^+$ nanocomposite was obtained by EPR analysis.

Reactivity of the nanocomposites

The photochemical activity of the PVG/CdS system was tested in relation to the decolorization efficiency toward reactive dyes. The decolorization kinetics for remazol brilliant blue R and remazol black B is presented in Figure 2. It is possible to verify from these results that the decolorization of the dyes was not substantially enhanced by the presence of the immobilized form of the semiconductor. As similar decolorization ratios were obtained by the isolate action of the UV light, we assume that the decolorization process is mainly due to homogeneous photochemical reactions, which are favored by the photosensibility of the dyes. By using the microwave-activated photoreactor very resembling result were obtained. However, in this case the decolorization ratio was higher than 80%. Very similar results were observed for the PVG/RuO_2 system.

As both semiconductors show high decolorization efficiency in the free form, an explanation for the low decolorization ratio observed for the immobilized systems can be the low concentration of semiconductor on

the PVG support (approximately 2% in weight). Studies looking for the immobilization of greater amounts of the semiconductors are in progress at the present moment.

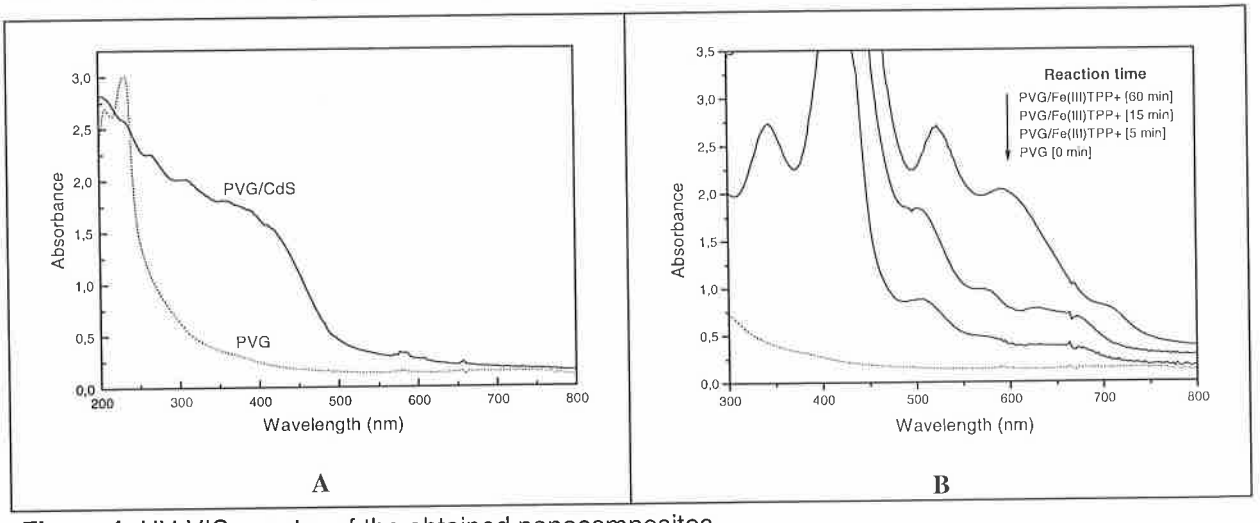

Figure 1. UV-VIS spectra of the obtained nanocomposites.

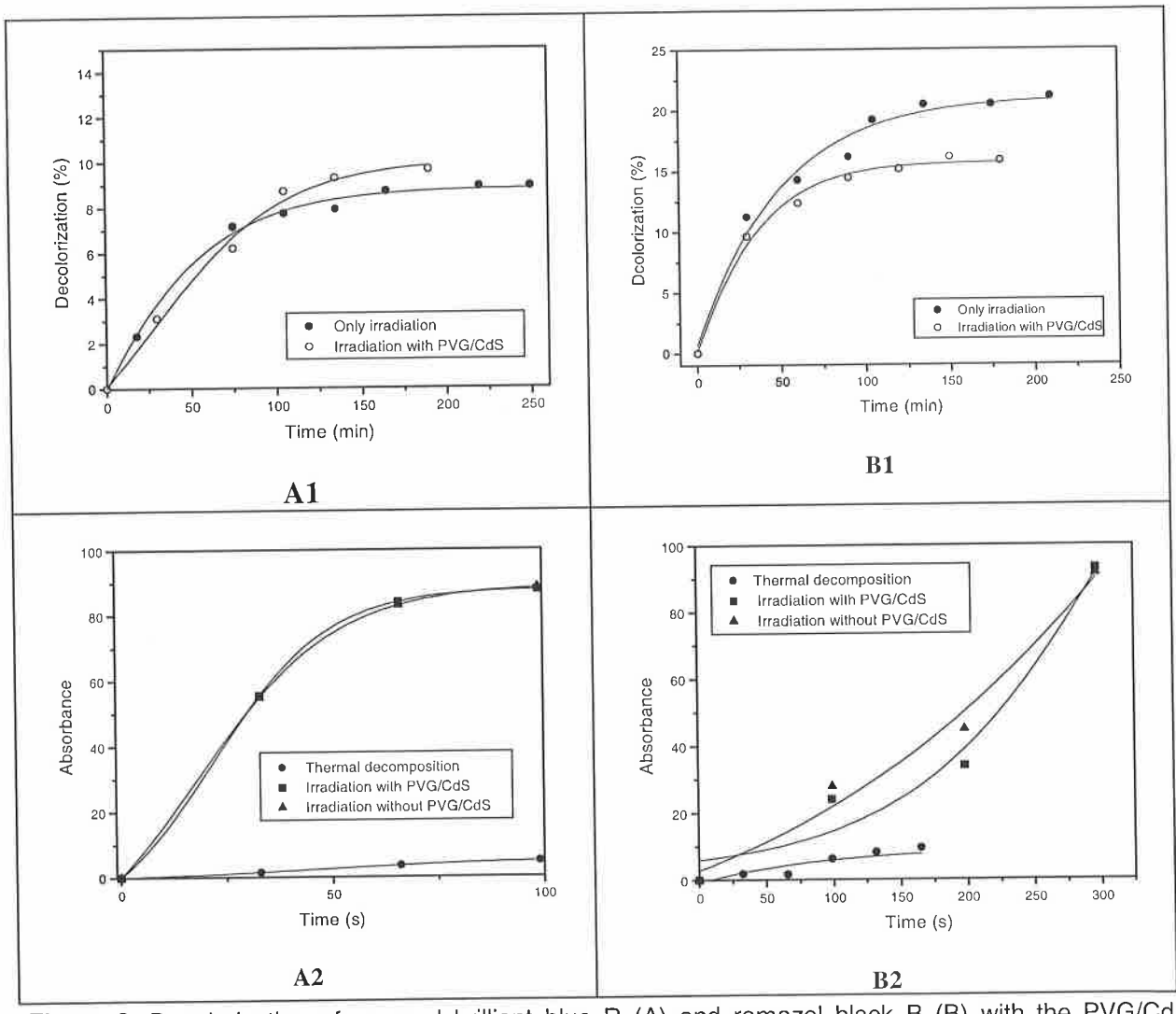

Figure 2. Decolorization of remazol brilliant blue R (A) and remazol black B (B) with the PVG/CdS system A1 and B1: Conventional photoreactor, A2 and B2: Microwave-activated photoreactor.

The catalytic activity of the PVG/$Fe^{III}TPP^{+}$ system was assayed using cyclohexane as substrate. Monitoring the reaction products by hplc, a total conversion ratio of about 11% was observed (5% of cyclohexanol and

6% of cyclohexanone). This is a very promissory result, because in reaction involving metallic porphyrins in a liquid media, the typical conversion ratio is lower than 8%. Using o-dianisidine as substrate, and measuring the formation of the respective dimer at 460 nm, the activity of the proposed system was also confirmed.

FINAL REMARKS

The Vycor glass appears as a very promissory matrix for immobilization of semiconductors and metallic porphyrins. The photochemical activity of the immobilized semiconductors is low, but studies are in progress to enhance their efficiency, mainly toward reactive dyes. The PVG/$Fe^{III}TPP^{+}$ system presents a high oxidizing activity toward classical substrates. For instance, the efficiency of cyclohexane oxidation is higher than those presented by the classical system that operate in liquid phase.

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REFERENCES

Corning Glass Work (1979). A porous glass that resists chemical attack. *Mater. Eng.*, **90**, 92.

Dezotti M., Innocentini-Mei L.H. & Durán N. (1995). Silica immobilized enzyme catalyzed removal of chlorolignins from eucalyptus Kraft effluent. *J. Biotechnol.*, **43**, 161-167.

Hammel K.E.(1996). Extracellular free radical biochemistry of ligninolytic fungi. New J. *Chem.*, **20**, 195-198.

Hoffmann, M., Martin, S.T., Choi, W. & Bahnemann, W. (1995). Environmental Applications of Semiconductor Photocatalysis, *Chem. Rev.*, **95**, 69-96.

Iamamoto, Y., Ciuffi, K. J., Sacco, H. C., Iwamoto, L.S., Nascimento, O.R. & Prado, C.M.C. (1997). Characterization and catalytic activity of 2,6-dichlorophenyl substituted iron (III) porphyrin supported on silica gel and imidazole propyl gel. *J. Mol.Cat. A: Chem.*, **116**, 405-420.

Khindaria A., Grover T.A. & Aust S.D. (1995). Reductive dehalogenation of aliphatic halocarbons by lignin peroxidase of *Phanerochaete chrysosporium. Environ. Sci. Technol.*, **29**, 719-725.

Lisenbigler, A.L., Lu, G. & Yates, J.T. (1995). Photocatalysis on TiO_2 surfaces: principles, mechanism, and selected results, *Chem. Rev.*, **95,** 735-758.

Luang, J. C. (1988). Semiconductor microcrystallites in porous glass and their applications in optic. *Superlatt. Microstruct.*, **4**, 385-390.

Mills, A. & Le Hunte, S. (1997). An overview of semiconductor photocatalysis, *J. Photochem. Photobiol. A: Chem.*, **108,** 1-35.

Shukla, R. S., Robert, A. & Meunier, B. (1996). Kinetic investigation of oxidative degradation of aromatic pollutant 2,4,6-trichlorophenol by an iron-porphyrin complex, a model of ligninase. *J. Molec. Catal. A: Chem.* **113**, 45-49.

Zarbin, A.J.G., Vargas, M. D. & Alves, O.L. (1999). Thermal decomposition of $[M_3(CO)_{12}]$ (M=Ru, Os) physorbed onto porous Vycor glass: a route to a glass/RuO_2 nanocomposite. *J. Mater. Chem.*, **9**, 519-523.

Optimization of D-xylose, L-arabinose and D-glucose production obtained from sugar cane bagasse hydrolysis process

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ABSTRACT

The hydrolysis process of sugar cane bagasse was evaluated for D-xylose, L-arabinose and D-glucose production. An experimental design was used to study the effect of the combinations of temperature and sulfuric acid concentration on monosaccharides production. Three reaction times were used: 18, 30 and 50 min. The sugars were determinate using HPLC. At 18 min, the regression model was significant ($p<0.05$) with an excellent R^2 (0.97) and F_{model} (34.49). The total sugar yield, under the best conditions (time of reaction: 18 min; 126°C and 3.10% (v/v) of sulfuric acid), was 333.41 mg/g of bagasse dry weight. It is composed by 266.73 mg of xylose, 40.09 mg of glucose and 26.59 mg of arabinose per gram of bagasse. For anhydroxylanose, these results represent 96% of theoretical maximum, while for anhydroglucose it was ca. 100% of composition of the bagasse cellulose. Besides, the energy and time saving aspects are significantly relevant using this reaction time.

Keywords: sugar cane bagasse, acid hydrolysis, experimental design

INTRODUCTION

Lignocellulosic materials, containing cellulose, hemicellulose, and lignin as major components, are the most abundant renewable resources found on Earth. It has great potential as an ecologically advantageous feedstock for the value - added products including a number of useful chemicals and liquid fuels (Kern, 1998). Among the several potential biomass sources, sugar cane bagasse has frequently been mentioned in the literature as one of the most promising for the biomass to sugar process (Saska *and* Ozer, 1994). In Brazil, it is a particularly suitable waste for conversion since it is produced in significant quantities (ca. $4.5x10^7$ ton dry mass/year) (FAO, 1995). The advantages of using bagasse as a substrate for bioconversion is that it has high carbohydrate content (cellulose and heteroxylan), is a cheap substrate (waste product), and there is a constant, although seasonal, supply generated within the sugar cane industry. Sugar cane bagasse is composed of 25-35% (dry weight) hemicellulose xylan, of which D-xylose is the major component (Dekker *and* Wallis, 1983; Toit *et al.*, 1984; Zyl *et al.*, 1988).

Otherwise, it is possible to hydrolyse hemicellulose by several processes. Enzymatic, physical, chemical or a combination of these have been explored to produce monomeric sugars in maximum yield and purity (Singh *and* Mishra, 1995). Selective hydrolysis of plant materials, possible due to the hydrolysis of hemicellulose fraction is easily compared to the hydrolysis of cellulose (Ladisch, 1979; Toit *et al.*, 1984; Singh *et al.*, 1984). Diluted acid hydrolysis of biomass wastes to reducing sugars appears to be more feasible commercially than hydrolysis, using concentrated acid or enzymes (Singh *et al.*, 1984).

However, under acid conditions, the decomposition of sugar takes place and xylose decomposes five times faster than glucose. Xylose is dehydrated into furfural, while glucose is chemically transformed to hydroxymethylfurfural (Singh *and* Mishra, 1995). In order to prevent the decomposition of sugars, mainly pentoses, it is very important to use diluted acid solutions, short reaction time, low temperatures, and the rapid removal of hydrolytic agents is required (Gong, 1981).

This study was designed to determine which are the optimum hydrolysis conditions that lead to high D-xylose, L-arabinose and D-glucose yields, using sugar cane bagasse as substrate. The response surface methodology allowed for the verification of the effects of individual factors (temperature, time of reaction and sulfuric acid concentration) and their interactions for the response thus obtained.

EXPERIMENTAL

Pre-treatment of sugar cane bagasse

Sugar cane bagasse obtained from a sugar cane mill (Campinas, São Paulo State, Brazil) was washed, dried in oven at 60°C and milled in a pilot knife-mill to pass through a 0.84 mm sieve (sieve shaker – Produtest) before using.

Bagasse samples (3.0 g dry matter) were placed in Erlenmeyer flasks of 125 mL and soaked in sulfuric acid solution in the proportion of 1:5 (w/v). The samples stayed at room temperature overnight. After this period, the bagasse samples were placed in an autoclave that was heated by direct saturated steam injections and pressurized with compressed air. The temperature, during the hydrolysis, was controlled by electronic system made to keep the temperature constant. The samples of bagasse were submitted to temperature and time according the experimental design (Tables 1 and 2). After this pre-treatment, the resultant hemicellulosic fraction was filtered under vacuum. The hydrolysate was neutralized with $Ca(OH)_2$ solution to pH 5.5 and centrifuged (2.23 x 10^4g; 5°C; 15 min). The precipitate was discarded.

Analytical Methods

Moisture content into the milled bagasse was determinate in oven (105°C until constant weight) and corrections were made to dry basis in all samples.

D-xylose, D-glucose and L-arabinose present in bagasse hemicellulose hydrolysates were determinate using high performance liquid chromatograph equipped with a refractive index detector (Shimadzu Co., LC-10AD). Sugar components were separated on a RT-CH-CA Merck column maintained at 80°C using deionized water as eluent (flow rate = 0,5 mL/min), previously degassed with helium.

Experimental designs

A 2^2-factorial experimental design with three replicates at the centre point (Barros, 1996; Box, 1978) was used to study the effect of the combination of temperature and sulfuric acid concentration on monosaccharides production. The lowest and highest levels of temperature were set from 111.5 to 128.5°C, and acid concentrations were set from 0.2 to 3.6% (v/v). Three reaction times were used: 18, 30 and 50 min.

Response surface methodology (RSM) was adopted to optimize the conditions for hydrolysis of sugar cane bagasse. The temperatures studied and the concentrations of sulfuric acid are presented in the Table 1. The experimental data were analysed by the software Statistica for Windows version 5.0.

Table 1. Variables at different levels during the hydrolysis of sugar cane bagasse.

Variables	Symbol	LEVELS				
		-1.414	-1	0	+1	+1.414
Temperature (°C)	A	111.5	114.0	120.0	126.0	128.5
H_2SO_4 (%v/v)	B	0.2	0.7	1.9	3.1	3.6

RESULTS AND DISCUSSION

The hydrolysis involves solubilization of hemicellulose and partial destruction of reducing sugar produced. As on result, the amount of sugar recovered from the pretreated bagasse is dependent on the hydrolysis conditions, such as time, temperature and acid concentrations (Kling, 1987).

A central composite experimental design was used in this work and its experimental results for the two-factor-two level response surface analysis are shown in Table 2. The results demonstrate that the process conditions had influence on xylose production.

It is observed that hydrolysis time had influenced xylose production at 120°C and 0.2% of sulfuric acid (treatment number 7). The xylose formed increased with increasing time of process of 18min (17.43 mg/g), 30 min (44.75 mg/g) and 50 min (80.52 mg/g), although the results had been the lowest, when they were compared to the other results.

On the other hand, with 18 min of reaction, the best results for xylose were obtained at 126°C and 3.10% of sulfuric acid solution (treatment 4). From 266.73 mg/g (18 min) to 246.36 mg/g (30 min) and 258.42 mg/g (50 min), such results demonstrate the importance of process conditions to optimize xylose formation, because high temperatures and high acid concentrations can degrade pentoses and hexoses to furfural and

hydroxymethyl furfural, respectively (Singh *and* Mishra, 1995). This decreasing of time (from 18 to 50 min) was due to the degradation of pentoses, by this result we can conclude that the best time was 18 min.

Table 2. 2^2 factorial design with coded values and experimental results obtained by hydrolysis of sugar cane bagasse, at 18, 30 and 50 min for xylose, at 18 min for glucose and arabinose.

Treatment number	Variables		Results (mg/g of bagasse, dry wt)				
	A	B	Xylose 18 min	Xylose 30 min	Xylose 50 min	Glucose 18 min	Arabinose 18 min
1	-1	-1	62.75	151.10	188.09	16.75	20.70
2	-1	1	254.63	266.90	264.39	23.76	21.51
3	1	-1	180.03	277.84	264.71	20.68	17.57
4	1	1	266.73	246.36	258.42	40.09	26.59
5	-1.414	0	140.32	211.41	253.44	18.52	21.29
6	1.414	0	260.03	269.21	250.46	36.60	24.90
7	0	-1.414	17.43	44.75	80.52	0.00	18.18
8	0	1.414	247.43	264.54	262.06	34.00	25.04
9	0	0	185.34	258.20	255.66	23.10	21.60
10	0	0	201.83	242.91	257.69	25.10	21.89
11	0	0	203.32	247.21	296.52	24.50	21.42

The analysis of variance presented in Table 3 showed that a polynomial model is adequate for xylose at 18 min and experimental factors. The regression model had highly significant ($p<0.05$) value of R^2 (0.97) and F_{model} = 34.49 for xylose production at 18 min. This result indicates a good agreement between the experimental and predicted values of xylose formed. However, the polynomial model had no satisfactory R^2 value on xylose produced at 30 and 50 min with value of 0.80 and 0.77, respectively. Also, the model had no statistical significance with F_{model} as 4.11 (30 min) and 3.34 (50 min).

Table 3. Analysis of variance for the regression model of xylose production obtained from the response surface experiments.

Source	Xylose 18 min			Xylose 30 min			Xylose 50 min		
	SS	df	MS	SS	df	MS	SS	df	MS
Model	64818.08	5	12963.61	39061.02	5	7812.20	23904.16	5	4780.83
regression	1879.26	5	375.85	9513.15	5	1902.63	7145.60	5	1429.12
Residual	1680.12	3	560.04	9388.80	3	3129.60	7143.24	3	2381.08
Lack of fit	199.14	2	99.57	124.35	2	62.17	2.36	2	1.18
Pure error	66697.34	10		48574.17	10		31049.76	10	
Total									
F model		34.49			4.11			3.34	

$F_{5.5}$ (5%) = 5.05
SS = sum of squares
df = degrees of freedom
MS = mean square

The regression coefficients and t-values are presented in Table 4. In 18 min of hydrolysis, the xylose production was determinated by the linear terms of temperature and sulfuric acid. The quadratic term of sulfuric acid of the model and these two-factors had interaction. Therefore, at 18 min of hydrolysis, temperature and sulfuric acid concentration had positive effect on xylose production.

Table 4. Standardized effects for the variables tested in the 2^2 factorial design with 3 centre points for xylose production at 18 min.

Source of Variation	Regression coefficients	t-value
A	37.33	10.58
B	75.47	21.39
AB	- 26.29	- 5.27
A^2	7.85	1.87
B^2	- 26.01	- 6.19

By applying multiple regression analysis on the experimental data, the second-order polynomial equation obtained for xylose production was:

$$Y = 196.83 + 37.33A + 7.85\,A^2 + 75.47B - 26.01B^2 - 26.29AB$$

The response surface and contour plots of temperature versus sulfuric acid for xylose production are shown in Figures 1 and 2.

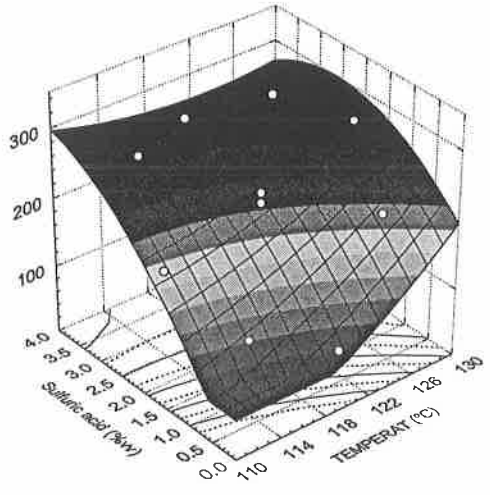

Figure 1. Describes the effects of temperature and acid concentrations on xylose production.

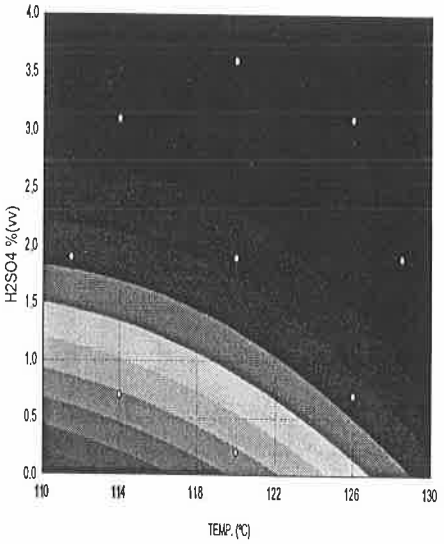

Figure 2. The contour plot of temperature versus sulfuric acid for xylose production.

According to data obtained and statistical analysis, both results were decisive to select time reaction at 18 min as the best condition.

The study showed that bagasse could be hydrolysed for the production of xylose, glucose and arabinose with maximum yield using dilute sulfuric acid. The total sugar yield, under the best conditions (time of reaction: 18 min; 126°C and 3.10% of sulfuric acid), was 333.41 mg/g of bagasse, 266.73 mg of xylose, 40.09 mg of glucose and 26.59 mg of arabinose. For anhydroxylose, these results represent 96% of theoretical maximum, while for glucose it was ca. 100% of bagasse composition in cellulose (Purchase, 1995; Morjanoff and Grey, 1987).

The results indicated that hydrolysis conditions for specific plant materials depend on its composition, time reaction, temperature, and acid concentration. Further researches are being studied in our laboratory.

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REFERENCES

Barros, B.; Scarminio, I.S. & Bruns, R.E. (1996). *Planejamento e otimização de experimentos*. Ed. Unicamp. São Paulo. Brazil. pp.133-165.

Box, G.E.P.; Hunter, W.G. & Hunter, J.S. (1978). *Statistics for experimenters: an introduction to design, data analysis, and model building*. Wiley and Sons, New York, pp. 653.

Dekker, R.F.H. & Wallis, F.A. (1983). Enzymatic saccharification of sugar cane bagasse pretreated by autohydrolysis-steam explosion. *Biotechnology and Bioengineering*, **25**, 3027-3048.

FAO - Food Agriculture Organization of the United Nations. (1995). *Food Production Yearbook*, **49.**

Gong, C.S.; Chen, L.F.; Flickinger, M.C. & Tsao, G.T. (1981). Conversion of hemicellulose carbohydrates. *Advances Biochemical Engineering*, **20**, 93-118.

Kern, M.; Nidetzky, B.; Kulbe, K. D. & Haltrich, D. (1998). Effect of nitrogen sources on the levels of aldose reductase and xylitol dehydrogenase activities in the xylose-fermenting yeast *Candida tenuis*. *Journal of Fermentation and Bioengineering*, **85**, 196-202.

Kling, S.H.; Carvalho Neto, C.; Ferrara, M.A.; Torres, J.C.R.; Magalhaes, D.B. & Ryu, D.D.Y. (1987). Enhancement of enzymatic hydrolysis of sugar cane bagasse by steam explosion pre-treatment. *Biotechnology and Bioengineering*, **29**, 1035-1039.

Ladisch, M.R. (1979). Fermentable sugars from cellulosic residues. *Process Biochemistry*, **14**, 21-25.

Morjanoff, P.J. & Gray, P.P. (1987). Optimization of steam explosion as method for increasing susceptibility of sugar cane bagasse to enzymatic saccharification. *Biotechnology and Bioengineering,* **29**, 733-741.

Purchase, B.S. (1995). Products from sugar cane. *International Sugar Journal*, **97**, 70-81.

Saska, M. & Ozer, E. (1994). Aqueous extraction of sugar cane bagasse hemicellulose and production of xylose syrup. *Biotechnology and Bioengineering*, **45**, 517-523.

Singh, A.; Das, K. & Sharma, D.K. (1984). Production of xylose, furfural, fermentable sugars and ethanol from agricultural residues. *J. Chemistry Technology Biotechnology*, **34A**, 51-61.

Singh, A. & Mishra, P. (1995). Extraction of pentosans from lignocellulosic materials. *Microbial pentose utilization: current applications in biotechnology*, **33**, 71-98.

Toit, P.J.; Olivier, S.P. & van Biljon, P.L. (1983). Sugar cane bagasse as a possible source of fermentable carbohydrates. I. Characterization of bagasse with regard to monosaccharide, hemicellulose, and aminoacid composition. *Biotechnology and Bioengineering*, **26**, 1071-1078.

Tsao, G.T. (1978). Cellulosic material as a renewable resource. *Process Biochemistry*, **13**, 12-14.

Zyl, C.V.; Prior, B.A. & Du Preez, J.C. (1988). Production of ethanol from sugar cane bagasse hemicellulose hydrolysate by *Pichia stipitis*. *Applied Biochemistry and Biotechnology*, **13**, 357-369.

Organosolv lignin-copper as natural preservative

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ABSTRACT

Radiate pine wood *(Pinus radiata* D.Don) must be impregnated with chemical products to improve its durability when is exposed to outdoors, in soil contact. Very good results are obtained with different commercial products as biocides (CCA salts, creosote, sodium pentachlorophenate), however they present environmental drawback due to their toxicity. For this reason, it has been shown an increasing interest to use natural products, with biocide characteristics, at industrial level. This research work deals with the biocide quality of organosolv lignin extracted with formic acid-acetone, 9:1 v/v. The aim of this work is to test some organosolv lignin, with different chemical treatments, and define which lignin is the most useful. Three types of lignin were treated with copper (II) sulfate solution ($1 \cdot 10^{-3}$M): crude lignin (unpurified lignin), purified lignin and carboxymethylated lignin. Wood samples were impregnated by double vacuum process in acetone medium, and the highest retention (1.04 kg/m^3) was reached with purified copper-lignin. The biocide aptitude was tested under ASTM standard (D3273) adapted. The wood samples were exposed, for a month, under simulated environmental conditions (relative humidity of 96±1%, and temperature of 33±1°C) to promote the growth of mould *(Penicillium sp, Aspergillus niger, Aureobasidium pullulans,* etc.). Wood samples without treatment (control samples) were attacked very fast in relation to those treated with the test products. The results were not so successful, because all the samples were contaminated with moulds before completing one-month exposure, however the infection intensity was lower in wood samples impregnated with copper-purified lignin (17 % mould infection). All impregnated wood samples were less infected than control (without treatment). Copper-carboxymethylated lignin impregnated samples reached 27% infection, but retention was only 0.69 kg/m^3. These results encourage us to optimize the treatments to improve the bio-attack resistance.

Keywords: lignin, wood preservation, wood decay, copper compounds

INTRODUCTION

Lignin is a natural barrier to wood cellulose protection, it has been frequently named as "natural wood preservative"(Shimada, 1991), however if wood is not conveniently protected, even the lignin is degraded by environmental oxidant agents, washed by rain and affected by biological agents. Wood is an important building material, with a long life, if it is properly protected and used according to natural durability. A good design help to overcome decay problems when wood is used in exterior parts of a building. Aerobic or anaerobic environmental, pH, moisture, and temperature are the main factors affecting wood biodegradation.

Phenolic substances, as wood dry distillation products, and salts, were used as wood preservatives from remote age. Cooper sulphate was recommended from the early 18th century and it was patented for wood preservation in 1837 (Richardson, 1993). The antiseptic properties of copper sulphate have never been questioned, but its water solubility makes it unsuitable in wet conditions because is partially leachable. Nowadays copper sulphate is the most important component in wood preservatives; usually it is used at 1% aqueous solution. Chromate copper arsenate (CCA) is one of the most important wood preservatives but is considered an environmental unfriendly substance, particularly due to its arsenic content for general uses and soil contamination.

Pinus radiata is the most important commercial specie in Chile; its natural durability (refered to fungal decay resistance, in ground contact) is considered non-durable wood, less than 10 years in use. In this study the initial work to analyze the organosolv lignin effect, previously treated with cooper II salt is presented. The organosolv lignin properties are its water insolubility, important phenolic constitution and human non-toxicity. The lignin-cooper retention in the wood, might improve the wood performance for building and construction, with moderated risk of decay.

EXPERIMENTAL

Lignin preparation

Crude lignin was obtained by precipitation from formic acid/acetone-pulping liquor of *Pinus radiata* sawdust. Its purification was performed in acetic acid solution according to procedure informed before (Urízar *et al.*, 1999). Purified lignin was reticulated and carboxymethylated according to informed method (Gómez *et al.*, 1998).

Lignin-copper preparation

Lignin was treated with $3 \cdot 10^{-3}$ M copper (II) sulphate water solution. The suspension was stirred during 8 h.

Wood samples

50 *Pinus radiata* samples (10x80x100 mm) obtained from sapwood, without defects, were obtained from larger pieces. They were sanded and conditioned properly.

Wood samples impregnation

Wood samples were impregnated with lignin-copper acetone solution by double vacuum treatment. Air-dry samples were wrapped in plastic for 48 h. Absorption and retention were evaluated according to standard methods and expressed as percentage.

Lixiviation

The samples were exposed to artificial rain chamber. The accumulated water was analysed by UV-Vis spectroscopy.

Biodegradation test

Wood samples were exposed to different mould in an environmental chamber. The mould growth was evaluated according to ASTM-3273 - 76 standard. Superficial mould growth on the samples was observed by visual inspection every week.

RESULTS AND DISCUSSION

Organosolv lignin has low solubility in neutral organic solvents, resulting inconvenient to get solutions with high concentration. Acetone was chosen as solvent to prepare lignin solutions to impregnate radiata pine wood samples at 0.25% w/v (L0.25), 0.50%w/v (L0.50) y 1.00%w/v (L1.00) concentrations. Lignin lixiviation, and lignin retention in wood samples were evaluated; results are shown in Table 1.

Table 1. Lixiviation and retention results at different lignin content.

Lignin	% Retention[a]	% Lixiviation[b]
L 0.25	0.098	0.001
L 0.50	0.170	0.0013
L 1.00	0.320	0.0017

[a] lignin grams in 100cc of wood
[b] lignin grams in 100ml solution

The retention increases proportionally to the solution concentration used in this study. The lixiviation also increase but in a lower proportion.

Wood samples absorption of copper-lignin solution and copper lignin retention results are shown in Table 2.

The wood samples lignin retention is directly related to the absorption and independent of the lignin type used. The best retention was obtained with pure lignin-copper and the lower retention with lignin carboxymethylated-copper. All the results are related with fractions solubility:

L-Cu > Lc-Cu> Lcm-Cu

The impregnated samples were exposed to fungi under controlled conditions, during a month, and the results are shown in Table 3.

Table 2. Retention of different formulation of lignins.

Lignins	Absorption %	Retention %
L c – Cu	18.9	0.095
L – Cu	20.7	0.104
L cm –Cu	13.8	0.068

L – Cu: Pure lignin – copper (II)
Lc – Cu: Crude lignin – copper (II)
Lcm – Cu: Carboxymethylated lignin – copper (II)

Table 3. Infection level of the samples impregnated with different lignin solutions.

LIGNINS	1st week	2nd week	3rd week	4th week
Lc – Cu	5 %	14 %	17 %	28 %
L - Cu	3 %	12 %	15 %	17 %
Lcm- Cu	2 %	14 %	19 %	27 %

The best results were obtained with L-Cu that showed the best solubility and consequently best retention. The pure lignin, without copper sulphate treatment, results three times better retention than L-Cu and the wood infection level reached 31 % in a month exposure to fungi, (Villagrán and Mondaca, 1998) and in the same work, wood samples without treatment were 46,6% infected by the same mould in the same condition.

These results let us to conclude that the wood impregnation with copper II ions treated lignin solution is promissory, although the pure lignin is a weak preservative for wood, the copper added to lignin provides additional effect to improve the preservative action over the wood. The best results obtained with L -Cu are in direct correlation with retention increase and this may be increased using other solvents, changing the solution acidity or with more than one impregnation consecutive treatment. Therefore it could be possible to obtain a good performance as wood preservative with this product, and it could replace the traditional human-toxic wood preservatives.

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REFERENCES

Freer, J., Rodríguez, J., Baeza, J., Durán, N. & Urízar, S. (1999). Analysis of Pulp and Lignin extracted with Formic Acid-Acetone mixture from *Pinus radiata* and *Eucalyptus globulus* Woods. *Bol.Soc.Chil.Quim.*, **44 (2)**, 199-207.

Gómez, E., Winkler, A. & Peternale, W. (1997). Absorption of Cd(II) and Pb(II) onto functionalized formic lignin from sugar cane bagasse. *4th Braziliam Symposium on the Chemistry of Lignins and other Wood Component,*. Proceedings, *pp 26-33*

Richardson, B. (1993). *Wood Preservation*. ed. E&FN SPON, 2nd ed. G. Britain, pp. 1-12

Shimada, K. & Higuchi, H. (1991), Microbial, Enzymatic, and Biomimetic Degradation of Lignin, in *Wood and Cellulosic Chemistry*, ed. David N-S.Hon and Nobuo Shiraishi, Marcel Dekker,Inc. New York.

Villagràn, A. & Mondaca, H. (1998). Evaluaciòn de lignina organosolv y taninos de pino insigne (*Pinus radiata* D. Don) como biocidas naturales. *Seminario de Título Ingeniero Ejecución en Maderas*.U.Bìo.Bìo.Chile.

Organossolv delignification of steam exploded wheat straw

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ABSTRACT

It has been increasing the interest in the study of alternative pulping processes that conciliate high performance and protection to the environment. In the same way, the use of agricultural residues has been more and more reminded as promising raw material for production of chemical products. The wheat straw is a very abundant agricultural residue and it has been used in many countries for the production of cellulosic pulps. This work describes the effect of the pretreatment by steam explosion on the acetone-water pulping of wheat straw. The samples of wheat straw were treated by steam explosion at 190 and 210°C for 4 min and later submitted to the treatment with acetone-water (1:1, v:v) at different temperatures (140, 160 and 200°C) and 60 min as reaction times. The pretreatment lead to an increase in the amount of extracted lignin in the organosolv pulping. This effect was more pronounced in the pulping performed at lower temperatures. The use of higher temperatures in the treatment by steam explosion was more significant as shown by lower pulp yield and higher delignification extent.

Keywords: steam explosion, wheat straw, organosolv delignification, cellulose pulp

INTRODUCTION

The utilization of wood as raw material for pulp production is restricted in several countries due to forest resources limitations. In this situation and even when the wood supply is abundant, the utilization of fast growing plants is an attractive alternative. There are a lot of annual plants that can be used as source of lignocellulosic fibers for pulp production. These plants can be processed directly for the pulp industry or after a first treatment carried out by the food industry. In these cases the agricultural wastes produced will be the raw material for pulp and paper production. This alternative is nowadays in use in some developing countries with particular emphasis for the utilization of rice and wheat straw and also sugar cane bagasse. From the last decade, even some traditional wood producers started to stimulate enterprises to use agricultural wastes for cellulose production. Brazil is the eleventh world producer of paper and cardboard and the seventh world producer of cellulosic pulps and it still possesses an incalculable potential for increase of your production. In this regards it should be include not only the wood, but also the annual plants and the agricultural wastes as source of fibers (Gerber *et al.*, 1999).

The wheat culture is one of the most important agriculture activities worldwide and generates an expressive volume of agricultural residues (ca. 750 x 10^6 ton/year) (Rowell and Young, 1997). The development of pulping processes for these fast growing plants has to take into account their specific morphological and chemical characteristics. The pulping processes nowadays in operation at industrial scale were developed considering particularly the utilization of wood as raw material and must also be adapted for the treatment of non-wood fibers. The low density of these non-wood species and their well spread production areas claims to specific pulping process and small or middle size mills. In this new perspective, the utilization of steam explosion followed organosolv delignification can contribute to a valorization of the agricultural residues. (Sciaraffia and Marzetti, 1988).

The steam explosion (SE) treatment consists basically in a short cooking at high temperature and pressure in saturated steam. Following the cooking, the digester is discharged by a fast decompression. This treatment consists essentially in a thermochemimechanical process in which hydrolytic reactions prevail. The mechanical effects are due to the forces acting in the decompression step, which lead to a massive liberation of the fibers (Marchessault, 1988).

The conditions for the effective use of this process to the treatment of straws and wood should ensure an effective saturation of the lignocellulosic material by the steam. The equipment should allow fast heating and

the pressurization of the system and be adapted to produce the fast depressurization of the reactor (Marchessault, 1988).

The main reactions that happen during the process are the cleavage of easily hydrolyzed glycosidic bonds, cleavage of some ether bonds in lignin and cleavage of lignin-carbohydrate linkages (Focher, 1988). The treatment occurs with a predominant hemicellulose extraction and preserved the crystalline structure of the cellulosic moieties. The application of this process to hardwood was already reported as appropriate for the production of pulps, which presented good properties for cardboard production (Capretti, 1988). The production of pulps with low residual lignin contents is only reached by complementary delignification processes. In this case, steam explosion is used as a pretreatment to prepare the lignocellulosic material for the following stages of delignification.

In this work, are presented the results obtained in the acetone-water delignification of steam exploded wheat straw. The steam explosion and organosolv treatment were carried out at different experimental conditions in order to evaluate its influence on the yield and chemical composition of the pulps.

EXPERIMENTAL

The wheat straw was collected from plantations located at Paraná State - Brazil. The straw was cut to 5cm long pieces. The steam explosion was carried out on laboratory scale at the pilot unit of FAENQUIL (Lorena, SP, Brasil). The experiments were performed using 7g of wheat straw in the temperature range of 190-210°C and 4 min as reaction time. After each steam explosion treatment the pretreated material was washed with hot water, dried at room temperature and weighed. The pretreated samples were submitted to acetone-water delignification at different temperatures (140, 160 and 200°C) for 60 min. The delignification was performed in 120 mL stainless steel tube shaped reactors fitted into an oil bath for heating. Each experiment was performed with 2.0g of air dried steam exploded sample and 40.0 mL of acetone-water (1:1/v:v) mixture. The residual straw was filtered, washed and dried (Rosa, 1996).

The residual lignin content was determined gravimetrically (Klason lignin- T230–om82 TAPPI method). The sugars were determined by High Performance Liquid Chromatography (HPLC) using procedure described in the literature (Rocha, 1997).

RESULTS AND DISCUSSION

The association of two different techniques in order to achieve a better performance claims to a complementary behavior between these processes. The production of cellulosic pulps with high cellulose content will be found with high polyoses and lignin removal. The Steam Explosion process is more selective for polyoses removal and should be complemented with an efficient process for lignin dissolution. The results presented in Table 1 show that the processes employed in the wheat straw were not always complementary. The conditions employed in the steam explosion were enough to produce high dissolution yelds with only 4 min reaction times. The similar yields obtained at 200°C and 210°C do not mean a similar chemical composition for the steam exploded wheat straw, as will be showed in the discussion of organosolv delignification results.

Organosolv delignification of wheat straw can also produce high dissolution rates. The pulp yield obtained at 200°C and 60 min reaction time were in the same order of magnitude than that found when steam explosion was performed at higher temperatures (Table 1). The combined use of steam explosion pretreatment and organosolv delignification shows complementary results only for the delignifications performed at low temperature (140°C). The delignification yields obtained at higher temperatures were systematically higher than those obtained from unexploded wheat straw samples. The low performance of the organosolv delignification performed with steam-exploded samples can be explained on the light of chemical composition modifications occurred during steam explosion.

Despite the higher yields obtained in the organosolv delignifications the pretreatment with steam explosion lead to lower accumulated yields (Table 1).

The mechanistic in organosolv delignification involves the action of acid catalysts and nucleophiles in order to produce the cleavage of ether bonds present in the lignin structure. Organosolv delignifications performed with no external catalyst are efficient only at high temperatures due to production of acetic acid from the

acetyl groups present in the polyoses. The removal of polyoses is also an important requisite to obtain higher delignification yields. In the steam explosion treatment, the main reactions involve hydrolysis of polyoses and acetyl groups. Although the removal of polyoses bring some advantages to the following delignification step, the decrease in acetyl content reduces the production of acetic acid and decrease the effectiveness in the lignin removal.

Table 1. Steam explosion and organosolv pulping yields obtained in the treatment of wheat straw. The reaction times in the steam explosion and organosolv treatments were 4 and 60 min, respectively.

Steam Explosion Temperature (°C)	Organosolv Pulping Temperature (°C)	Steam Explosion Yield (°C)	Organosolv Pulping Yield (%)	Accumulated Yield (%)
---*	140	---*	97.4	97.4
	160		87.7	87.7
	200		54.6	54.6
190	140	77.6	92.3	71.6
	160		84.9	65.9
	200		57.0	44.2
200	140	59.1	91.7	54.2
	160		87.7	51.8
	200		68.2	40.3
210	140	58.6	85.3	50.0
	160		82.0	48.1
	200		74.0	43.4

* Organosolv pulping performed without the steam explosion pre-treatment.

The chemical composition of wheat straw and wheat straw pulps showed in Table 2 reveals significant differences according the characteristics of both treatments. The auto-catalyzed organosolv pulping produces high delignification yields only at high temperatures. On the other hand, steam explosion lead to an increase in residual lignin contents as consequence of preferential polyoses removal.

Table 2. Chemical composition of wheat straw and wheat straw pulps obtained in the steam explosion and/or organosolv pulping treatments. The reaction times in the steam explosion and organosolv treatments were 4 and 60 min, respectively.

Temperature (°C)		Cellulose (%)	Polioses (%)	Acetyl G. (%)	Lignin (%)
S. E.*	O. P.*				
---**	---**	41.0	26.0	n. d.	22.0
---	140	n. d.	n. d.	n. d.	18.5
---	160	n. d.	n. d.	n. d.	11.4
---	200	n. d.	n. d.	n. d.	8.6
190	---	46.2	25.5	2.2	24.1
200	---	54.0	21.5	1.4	24.2
210	---	60.6	9.7	1.0	27.8
190	140	56.1	17.1	2.5	23.6
190	160	59.4	13.5	3.8	22.9
190	200	74.7	7.4	0.4	9.6
200	140	63.7	13.2	2.2	20.2
200	160	65.6	9.8	1.3	19.7
200	200	72.0	7.0	0.5	9.5
210	140	70.9	6.5	0.9	18.2
210	160	72.2	5.9	1.2	15.7
210	200	73.8	3.8	0.9	12.1

* S. E. = Steam Explosion; O. P. = Organosolv Pulping.
** Untreated sample.
n. d. = not determined.

The benefits of steam explosion were found until a certain level of acetyl and polyoses removal was reached. Steam explosion performed at higher temperatures produced pulps with higher lignin contents (after the organosolv delignification steps) when compared to results obtained when organosolv pulping was performed on unexploded samples.

The pretreatment lead to an increase in the total amount of extracted lignin in the organosolv pulping as consequence of the low accumulated yields obtained after the combined treatment. This effect was more pronounced when the organosolv pulping was performed at lower temperatures.

CONCLUSIONS

From the set of experiments described in this work, one can conclude that best conditions were found in the steam explosion treatment performed at lower temperatures combined with organosolv delignifications carried out at higher temperatures.

The pretreatment carried out using steam explosion lead to an increase in the amount of extracted lignin after the organosolv pulping. This effect was more pronounced for the pulping performed at lower temperatures at the organosolv step. The use of higher temperatures in the steam explosion treatment was more significant considering the amount of removed lignocellulosic components.

Considering the characteristics of both steam explosion and organosolv delignification the addition of an external catalyst in the organosolv step could produce high delignification extent at low temperatures.

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REFERENCES

Capretti, G. & Marzetti, A. (1988). Steam Explosion Pulping of Wheat Straw. *In: Steam Explosion Techniques - Fundamentals and Industrial Applications*, ed. Focher, B., Marzetti, V. & Crescenci, V., Gordon & Breach Science Publishers, Philadelphia, pp 207-217.

Focher, B.; Marzetti, A.; Beltrame, P.L.; Carniti, P.& Visciglio, A. (1988). Steam Explosion of Wheat Straw. Product Fractionation and Enzymatic Hydrolysis of the Cellulosic Component. *In: Steam Explosion Techniques - Fundamentals and Industrial Applications*, ed. Focher, B., Marzetti, V. & Crescenci, V., Gordon & Breach Science Publishers, pp 331-340.

Gerber.W.; Anthonisen, D. & Gerber, M. D.(1999). Fabricação de Papel de Embalagem de Palha de arroz no sul do Brasil. Parte I – Definição e Estudo das Matérias-primas. *Seminário de Polpação não-Convencional*, Piracicaba-SP.

Marchessault, R. H. (1988). Steam Explosion: A Refining Process For Lignocellulosics. *In:Steam Explosion Techniques - Fundamentals and Industrial Applications*, ed. Focher, B., Marzetti, V. & Crescenci, V., Gordon & Breach Science Publishers, Philadelphia, pp 1-19.

Rocha,G.J.M.; Silva, F. T., Curvelo,A.A.S. & Araújo,G.T. (1997). A Fast and Accurate Metho for Determination of Cellulose and Polyoses by HPLC. *In 5th Brazilian Symposium on The Chemistry of Lignins and Other Wood Components*, Proceedings, **6**, 3-8.

Rosa, M. R. (1996). Estudo Cinético de Deslignificação Organossolve da Palha de Trigo, MSc thesis, Instituto de Química de São Carlos, USP.

Rowell, R.M. & Young, R. A. (1997). Paper and Composites from Agro-Based Resources, CRC Press Inc,.

Sciaraffia, F. & Marzetti, A. (1988). Enhancement of Wheat Straw Digestibility by Steam Explosion Pretreatment. Steam Explosion Techniques. *In: Fundamentals and Industrial Applications*, ed. Focher, B., Marzetti, V. & Crescenci, V., Gordon & Breach Science Publishers, Philadelphia, pp 365-374.

Oxidation of kraft lignin and liquor using oxygen and application in the removal of metals from aqueous solutions

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ABSTRACT

The chelating capacity of lignin can be increased with the insertion of oxygenated groups in its macromolecule. The main objective of this work was to verify the chelating power of both lignin and kraft liquor after direct oxidation. The oxidation was carried out by bubbling O_2 (120 mL.min^{-1}) at 60°C in a basic solution (NaOH 0.5 eq.L^{-1}). This oxidation procedure should be very soft preserving the macromolecular properties of the lignin and inserting oxygenated groups in its structure. The liquors and oxidized lignins were submitted to chelation tests involving the addition of aqueous solutions of metallic ions. After the precipitation of the liquor at pH 1, the mixtures were filtered and the filtrates analyzed by ICP-AES (Inductively Coupled Plasma Atomic Emission Spectrometry). The results demonstrated that the removal of Cd^{+2} and Cr^{+3} from aqueous solutions increased strongly in comparison to the original lignins and liquors, reaching more than 63% removal for the Cd^{+2} ion. The application of the oxidized liquors and lignins to the treatment of a chromium-platting effluent failed due to its composition, essentially CrO_4^{-2} which cannot be chelated. Further work is concerning to the reduction of CrO_4^{-2} to Cr^{+3} before chelating tests.

Keywords: oxidation of lignins; kraft lignin; chelation of metals

INTRODUCTION

Lignin is a macromolecule that can be easily oxidized. The oxidation processes can be classified in three categories, corresponding to the three stages of lignin degradation which are: 1) formation of carbonyls groups conjugated to aromatic rings; 2) direct degradation by the opening of aromatic rings; 3) formation of specific groups in the lignin structure (Chang and Allan, 1971).

The first type of lignin oxidation is more suitable to increase the chelation capacity of the lignin. Polar groups are thereby introduced into the lignin structure, due to the formation of carbonyls linked to the aromatic rings. Besides preserving the macromolecular structure of the lignin (Cotrim *et al.*, 1995), this mild oxidation can be performed by molecular oxygen in alkaline medium. Other reagents, such as nitrobenzene, metallic oxides, nitric acid and nitrogen dioxide, can be used for the same purpose, but they degrade the macromolecular strucutre of the lignin, leading to the formation of small fragments (Dimmel *et al.*, 1997; Chang and Allan, 1971; Villar *et al.*, 1997).

The oxidation of organosolv lignins with molecular oxygen and the chelating properties of the oxidized lignins have been studied by our research group since 1995 (Cotrim *et al.*, 1995)). The chelating capacity of kraft lignins and oxidized kraft liquors still needs to be tested in order to verify if the increased removal of metallic ions from aqueous solutions corresponds to increased oxygenated groups in the lignin structure.

EXPERIMENTAL

Analysis of the Effluent by ICP-AES

After the chelation test with the effluent, the filtrate was analyzed by ICP in an ICP-AES 3410 ARL/Applied equipment. The effluent was diluted in the same proportion and analyzed for comparison. Through software coupled with the equipment, elements expressing significant peaks in certain wavelengths were scanned.

Oxidation of Kraft Lignin

In a round-bottom flask, 5 g of lignin was mixed with about 150 mL of 0.5 mol.L^{-1} NaOH solution. The mixture was homogenized until complete solubilization of the lignin. A condenser, a thermometer and an oxygen inlet were coupled to the flask, and the oxygen flow was adjusted to 120 mL.min^{-1}. The reaction was maintained at 60°C for 1 h and, subsequently, a 3.5 mol.L^{-1} HCl solution was added to the oxidized mixture (100 mL) to adjust the pH to 3-4. After precipitating, the lignin was filtered out, oven-dried at 60°C for 1 h and ground.

Oxidation of Kraft Liquor

Kraft liquor (75 mL) was oxidized using the same system described in the previous item, but with 75 mL of 1 mol.L^{-1} NaOH solution. Temperature, oxygen flow and the other experimental conditions were also the same as before.

RESULTS AND DISCUSSION

Chelation Tests with Liquors and Oxidized Lignins Using Several Metallic Ions

The direct oxidation of kraft pulping liquor generated oxidized liquor (1) and oxidized lignin (1), after precipitation with concentrated H_2SO_4. The oxidation of kraft lignin in alkaline medium also generated oxidized liquor (2) and oxidized lignin (2), after precipitation with concentrated H_2SO_4. Oxidized lignins and liquors were tested to evaluate their chelating capacity, which was compared with the capacity of the original liquor and lignin for metallic ions removal.

Table 1 shows the results of removal of the chelation tests used to evaluate the capacity of lignins and liquors for metallic ions removal.

Table 1. Removal of metallic ions using several chelate agents (100 mL of each type of lignin and 1 mL of each type of liquor) with 10 mL of 0.01 mol.L^{-1} metallic ions solution)

Metallic Ions	Removal of Metallic Ions (%)					
	Original Liquor	Original Lignin	Oxidized Liquor (1)	Oxidized Liquor (2)	Oxidized Lignin (1)	Oxidized Lignin (2)
Pb^{+2}	98.1	18.8	99.7	99.1	22.0	28.5
Cu^{+2}	76.2	20.2	10.5	6.5	20.4	17.2
Cd^{+2}	0.5	0.0	8.8	9.6	24.1	22.7
Cr^{+3}	5.8	7.0	16.8	33.6	27.5	26.5

The number of C_9 units in lignin necessary to chelate the metal can be calculated from the values of ion removal by the original and oxidized lignins. Assuming that the molar mass of the C_9 unit is 207.42 g.mol^{-1} (Souza, 1995), each 0.1 mmol metal has a maximum of 0.482 mmol of C_9 units. The ideal ratio for a larger removal is 1 metal to 6 C_9 units. This is valid only for lignin, because in the case of liquor, not only the complexation, but also the adsorption of the metallic ions should be taken into account. The equation below shows the amount of C_9 units ($^nC_9$) that participated in the linkage with the metal:

$$n_{C9} = \frac{0.482}{0.1 \times \%_{removal}}$$

For original lignin 69 C_9-units are necessary to remove one Cr^{+3} ion and for oxidized lignin (1) this value is just 17.

From analysis of Table 1 it is evident that oxidation is an efficient way to obtain lignins with higher chelation capacity. As can be seen, the removal of Pb^{+2} ions by oxidized lignins (1) and (2) increased by about 14.5% and 34%, respectively, in comparison with the Pb^{+2} removal by the original lignin. These significant increases can, probably, be associated with the higher amount of oxygenated groups incorporated in the lignin macromolecule. In the case of the oxidized liquors, increase was not a significant, because the value was already high (above 98%), as a function of the low solubility of $PbSO_4$ (1.38 x 10^{-4} mol.L^{-1} at 30°C).

With regard to the removal of Cu^{+2} ions the value obtained with the original liquor (76.2%) decreased by 86% when oxidized liquors were used. This value, obtained in duplicate, cannot be attributed to experimental errors or to total precipitation of the liquor at pH 1. Concerning the oxidized lignins, the results did not vary significantly, the average removal being 22%, which means that the maximum chelation capacity was reached or that the insertion of oxygenated groups in the lignin macromolecule was inefficient. Data on the removal of Cd^{+2} and Cr^{+3} ions confirm the first hypothesis, because the removal of Cd^{+2} by all liquors and oxidized lignins was considerably high, the only exception being oxidized liquor (1). The reason this liquor gave a little lower result is probably that the high solubility of $CdSO_4$ makes the removal of Cd^{+2} more difficult.

Analysis of several chelation tests with industrial effluent and lignins and liquors oxidized or not

Table 2 shows the concentrations of the elements found in the effluent before and after the chelation tests. The values are expressed in ppm.

Table 2. Concentration in ppm of the elements in the effluent and in the filtrates after the chelation test (OE: Original Effluent; lig.: lignin; liq.: liquor; ox.: oxidized; or.: original).

Elements	OE #1	ox.lig.(1) + ox.lig.(2) + ox.liq.(1) + ox.liq.(2) + or.lig. OE #2	OE #3	OE #4	OE #5	OE #6	+ or.liq. OE #7	+ ox.liq.(1) +water #8
B	0.1	10.0	9.0	13.0	12.5	15.0	2.0	18.0
Ca	3.0	7.5	10.0	13.5	10.5	21.0	12.0	10.5
Cr	17.0	11.5	12.0	19.5	20.0	12.5	13.0	3.7
Cu	0.2	0.7	0.7	1.1	0.7	0.8	0.3	0.8
Mg	0.2	0.9	1.4	2.5	1.2	1.9	1.5	2.0
Na	180.0	285.5	283.5	30.5	1090.0	212.5	990.0	3115.0
P	6.1	0.0	0.5	1.1	6.6	3.5	0.0	32.9
Zn	1.0	43.0	83.0	118.0	99.0	192.5	28.0	75.0

Test # 1 (original effluent) presents a small amount of B, Ca, Cu, Mg, P and Zn, and high concentrations of chromium and sodium. Original and oxidized lignins removed up to 33% of the total chromium in the effluent. In the case of tests #4, #5 and #7, the amount of chromium increased considerably, being observed in test #8 (liquor oxidized with distilled water) the presence of chromium. The Cr^{+3} removal from the liquor was not high, due to the presence of CrO_4^- and $Cr_2O_7^{-2}$ ions in the effluent, evidenced by the yellow color. High concentrations of B, P, Cu and Cr can be observed in the oxidized liquor, because this liquor is transported in tubes in the pulp industry, and stored in containers that are rich in these elements.

CONCLUSIONS

The amount of lignin is an important variable to enhance the removal of the metallic ions. The percentages of chromium removed from the effluent tested were high (up to 30%), considering that chromium is present mainly in the form of chromate and dichromate. Larger removal values were obtained with lignins and oxidized liquors, which show the advantage of incorporating groups with high electronic density in the lignin structure.

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REFERENCES

Chang, H.M. & Allan, G.G. (1971). Ultraviolet Spectra in Lignins Occurrence. In *Formation, Structure and Reactions*, ed. K.V. Sarkanen & C.H. Ludwig. Wiley-Interscience, New York, pp. 433-475.

Cotrim, A.R., Ferraz, A. & Silva, F.T. (1995). Chelanting Agents through Oxidation of Sugar Cane Bagasse Lignin. *8th International Symposium on Wood and Pulps Chemistry*, Proceedings, pp. 313-318.

Dimmel, D.R., Kuroda, K. & Pan, X. (1997). Pulping Catalysts from Lignin (8). Nitrogen Dioxide Oxidation of Lignins to Benzoquinones. *J. Wood Chem. Technol.*, **17 (3)**, 235-258.

Souza, J.A. (1995). Utilização de Lignina para Obtenção de Formulações de Liberação Controlada do 2,4 D. MSc thesis, Universidade Estadual de Campinas.

Villar, J.C., Caperos, A. & García-Ochoa, F. (1997). Oxidation of Hardwood Kraft-Lignin to Phenolic Derivatives. Nitrobenzene and Copper Oxide as Oxidants. *J. Wood Chem. Technol.*, **17 (3)**, 259-285.

Oxygen-assisted organosolv delignification of sugar cane bagasse

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ABSTRACT

Oxygen-assisted organosolv pulping of sugar cane bagasse was studied in ethanol-water and acetone-water solvent mixtures. The delignification efficiency of both organic solvent mixtures was monitored through pulp yields and chemical composition of the obtained pulps. The processes were carried out at fixed temperature (160°C) and oxygen pressure (80psi) for different reaction times (from 100 to 180 min). The utilization of oxygen produced a significant increase in the delignification extent for both organic solvent mixtures. In the best experimental conditions was reached 77% (ethanol-water) and 91% (acetone-water) of lignin removal. The chemical composition of the obtained pulps revealed higher polyoses removal in the acetone-water medium. This behavior can be attributed to the higher solubility of polyoses and lignin oxidized derivatives.

Keywords: sugar cane bagasse, organosolv delignification, oxygen pulping

INTRODUCTION

Organosolv pulping process shows attractive advantages when compared with traditional chemical pulping technologies (sulfate and sulfite processes). Among these advantages, the low environmental pollution, low installation costs and feasibility in the recovery and utilization of polyoses and lignins are the most important (Sarkanen, 1990; Aziz and Sarkanen, 1989; Jonhansson *et al.*, 1987; Balogh *et al.*, 1992). Organosolv delignifications can be performed using different solvent mixtures and experimental conditions. The utilization of external catalysts and/or acidic solvent mixtures produces high delignification extent even at low pulping temperatures (Parajó *et al.*, 1993; Benar and Schuchardt, 1995; Nimz and Casten, 1986; Nimz *et al.*, 1986; Curvelo *et al.*, 1990). Non-acidic organic solvents can be applied in organosolv delignifications only at high temperatures (170 – 190°C). To overcome this limitation, Russian researchers developed a lower-temperature method of wood delignification using oxygen in low-molecular-weight alcohols and acetone. This oxygen-assisted process delignifies wood in the absence or acidic or basic catalysts (Deineko *et al.*, 1992; Evtugin *et al.*, 1996; Zarubin *et al.* 1989; Evtuguin and Pascoal Neto, 1996; Deineko *et al.*, 1997).

Oxygen-assisted organosolv method can be applied not only in the pulping of wood, but also for the treatment of non-wood plants including agriculture residues. The obtained pulps can be used directly or may be submitted to further treatments aiming to valorize the application of the own biomass as well as the polysaccharides and lignins dissolved during the delignification process (Pascoal Neto *et al.*, 1994).

Aiming to study the performance of this process applied to sugar cane bagasse this work describes the results obtained in oxygen delignification performed in ethanol-water and acetone-water.

The integral use of the chemical compounds present in sugar cane bagasse is a desirable procedure in both economical and environmental aspects. The oxygen delignification performed in aqueous-organic media combines the advantages of organosolv pulping and can produce lignins more suitable for technical applications.

EXPERIMENTAL

Delignification process

The delignification reactions were performed in an 1L shaking autoclave with the mixing been carried out by means of vibratory movements of a vessel in a horizontal plane with amplitude of fluctuations of 20 cm and 60 cycles/min.

The reactions were performed from 10g of integral sugar cane bagasse (supplied by Usina Tamoio). The bagasse was previously sieved and it was employed the fraction with particle size higher than 0.149mm

(retained on the 100 mesh sieve). The reactions were carried out in pressurized autoclave at 160° C using organic solvent-water 60% solution (v/v) and liquor to bagasse ratio = 20:1. The oxygen pressure was fixed in 80 psi (at room temperature) with reaction times in the range of 100 – 180 min. After each experiment the pulps were filtered, washed (until pH ~ 7) and dried in a stove at 100° C. The pressure reached 330 and 280 psi, respectively in the pulpings performed with acetone and ethanol.

The reactions carried out without pressurized oxygen followed the same experimental procedure above described. In the blank experiments the pressure were 190 and 160 psi in acetone and ethanol, respectively.

Pulps characterization

A small pulp fraction collected from each experiment was treated with 72% H_2SO_4 at 45^0C for 7 min and then submitted to post-hydrolyze with 3% H_2SO_4 in a hermetically closed glass tube at 127^0C for 30 min. The residual lignin (insoluble) was determined by gravimetry. The soluble lignin was determined by UV spectroscopy (280nm) (Rocha *et al.*, 1993).

The polysaccharides were determined from the correspondent sugars. An aliquot of 5ml of the hydrolyzed (above described) was eluted through a SEP PAK C_{18} column to separate the soluble lignin and sugar degradation products. The filtrated was then analyzed by HPLC (Shimadzu Chromatograph CR 7A model), using an Aminex column HPX-87H BIO-RAD and refractive index detector (RID 6A) at 45°C. H_2SO_4 0.005 mol/L was the mobile phase at a flow of 0,6 ml/min. The cellobiose and glucose concentrations were converted to cellulose, xylose and arabinose to polyoses and acetic acid to acetyl groups. The conversion factors used to obtain cellulose contents from cellobiose and glucose were 0,90 and 0,95, respectively. To obtain polyoses content the conversion factor was 0.88. Finally, the conversion of acetic acid to acetyl groups and formic acid to cellulose were calculated using the conversion factors of 0,72 and 3,09, respectively. The concentration of individual sugars and carboxylic acids were determined from calibration curves plotted individually for each component (Rocha *et al.*, 1997).

Hydroxymethylfurfural and furfural were determined by HPLC (Shimadzu Chromatograph CR7A model) using a RP-18 Hewlett-PACKARD column, UV detector – 274nm – (Shimadzu SPD-10A model) at 25^0C. The mobile phase was a solution of acetonitrile/water 1:8 with 1% of acetic acid at flow rate of 0,8 mL/min. The conversion factors to obtain cellulose from hydroxymethylfurfural and polyoses from furfural were 1,29 and 1,37, respectively.

The ash contents were determined according to TAPPI Standards.

RESULTS AND DISCUSSION

The utilization of oxygen in combination with organic solvent mixture produced significant effects on the delignification of sugar cane bagasse. At fixed oxygen pressure (80psi) and temperature (160°C) the effect of the reaction time and solvent mixture on the pulping yield and chemical composition of the obtained pulps was studied. The obtained results are shown in Table 1. The delignification performed in ethanol-water mixture lead to pulping yields ranging from 63% (100 min reaction time) to 51% (180 min reaction time). The correspondent values obtained with acetone-water mixtures were 55 and 45%, respectively. The effect of the oxygen-assisted delignification can be verified in the experiments performed with 180 min as reaction time. This effect was more pronounced for acetone-water delignifications. The pulping yield dropped from 70 to 45%, a decrease of 36% approximately. In ethanol-water the correspondent decrease was in the order of 23%. The reason for the best improvement for the acetone-water mixture can be found in the chemical composition of the produced pulps. The lower efficiency of acetone-water delignification (without oxygen) compared with ethanol-water is consequence of the poor polyoses removal. The utilization of oxygen produces polyoses-oxidized derivatives that present high solubility in acetone and thus increases the polyoses (through its oxidized products) dissolution and improves the delignification extent. The removal of lignin is also improved in acetone-water oxygen delignification, but this effect is less pronounced when compared with the previous one.

The meaningful effect of assisted-oxygen process can be verified from the delignification extent reported in Table 1. The reactions performed with 100 min reaction time reached higher delignification extent when compared with the treatments carried out without oxygen and 180 min as reaction time. This effect was also more evident with acetone-water mixture. The comparison between these experiments (100 min with oxygen

versus 180 min without oxygen) revealed an increase in the delignification extent, which is in the order of 47% for ethanol-water mixture and 274% for acetone-water mixture.

Table 1. Influence of reaction times on the delignification of Sugar Cane Bagasse (Temperature = 160ºC; Oxygen Pressure = 80psi).

Solvent[1]	Pulping Time (min)	Pulp yield (%)	Cellulose (%)	Polyoses (%)	Lignin (%)	D. E.[2] (%)
Ethanol	100	63	56.7	23.4	16.5	53.2
Ethanol	120	62	62.3	23.2	14.1	60.7
Ethanol	150	55	62.2	19.2	12.8	68.3
Ethanol	180	51	68.2	17.7	9.9	77.3
Ethanol[3]	180	66	56.3	15.6	21.5	36.2
Acetone	100	55	69.7	16.2	11.1	72.5
Acetone	120	51	71.6	17.5	9.5	78.2
Acetone	150	47	76.5	16.6	4.2	91.1
Acetone	180	45	74.7	14.5	6.9	86.0
Acetone[3]	180	70	51.3	20.0	25.6	19.4

1. Organic solvent-water 60% (v/v).
2. Delignification extent.
3. Reactions performed without oxygen.

The cellulosic portion of the oxygen treated pulps was only slight degraded to soluble fragments (~ 5%). The changes in cellulose contents showed in Table 1 were due to the different amounts of polyoses and lignins removed in the processes.

CONCLUSIONS

Sugar cane bagasse delignifications were strongly affected by the utilization of pressurized oxygen. The oxygen-assisted organosolv process lead to higher delignification extent. The effect of oxygen was more pronounced in acetone-water medium. The overall process showed high efficiency even with short reaction time. Oxygen-assisted organosolv delignification proved to be also very effective in the sugar cane bagasse treatment and will certainly contributes to the valorization of this abundant and renewable raw material.

REFERENCES

Aziz, S. & Sarkanen, K., (1989). Organosolv pulping: - a review. *Tappi J.,* 169-175.

Balogh, D. T. Curvelo, A. A. S. & De Groote, R. A. M. C. (1992). Solvent effects on organosolv lignins from *Pinus caribaea hondurensis*. *Holzforschung*, **46**, 343-348.

Benar, P. & Schuchardt, U. (1995). Polpação Formacell de bagaço de cana e de madeira de eucalipto. *O Papel* **11**, 44-49.

Curvelo, A. A. S., Alaburda, J., Botaro, V. R., Lechat, J. R. & De Groote, R. A. M. C. (1990). Acetosolv pulping of Sugar Cane Bagasse. *Tappi J.*, **73 (11)**, 217-220.

Deineko, I. P., Makarova, O. V. & Zarubin, M. Ya. (1992). Delignification of wood by oxygen in low-molecular-weight alcoholic media. *Tappi J.*, **73 (9)**, 136-140.

Deineko, I. P., Evtuguin, D. V. & Tsarev, G. M. (1997). RU patent N 2076125.

Evtugin, D. V., Robert, A., Zarubin, M. Ya. & Deineko, I. P. (1996). The effect of oxygen-organosolv pulping conditions on pulp properties. *Cellulose Chem. Technol.*, **30**, 105-115.

Evtuguin, D. V. & Pascoal Neto, C. (1996a.). Delignification by Oxygen in Organic Solvent – Water Media: State of the Art & Future Development. *In 29º Congresso Annual de Celulose e Papel – ABTCP*, Anais, pp 81-95.

Jonhansson, A.; Aaltonen, O. & Ylinen, P. (1987). Organosolv pulping: Methods and pulp properties. *Biomass* **13**, 45.

Nimz, H. H. & Casten, R. (1986). Chemical processing of lignocellulosics. *Holz als Roh- und Werkstoff*, **44**, 207-212.

Nimz, H. H., Granzow, C. and Berg, A., (1986). "Acetosolv pulping". *Holz als Roh- und Werkstoff – Kurz-Originalia*, 362.

Parajó, J. C., Alonso, J. L., Vásque, D. Santos, V. (1993). " Optimization of Catalyzed Acetosolv Fractionation of Pine Wood". *Holzforschung* **47**, 188-196.

Pascoal Neto, C., Evtugin, D. V. and Robert, A. (1996). "Chemicals generated during oxygen-organosolv pulping of wood". *J. Wood Chem. Technol.* **14**, 383-402.

Rocha, G. J. M., Silva, F. T., Shuchardt, U., (1993). "Improvement of a rapid UV spectrophotometric method for determination of lignin in alkaline solutions". 3th *Brazilian Symposium on the Chemistry of Lignin and other Wood Components.* pp.73. Programme and Abstract.

Rocha, G. J. M., Silva, F. T., Araujo, G. T., Aprigio, A. A. S., (1997). "A fast Method for Determination of cellulose and Polyoses by HPLC", In: 5th *Brazilian Symposium on the Chemistry of Lignin and other Wood Components.*

Sarkanen, K. V., (1990). "Chemistry of solvent pulping". *Tappi J.* 215-219.

Zarubin, M. Ya., Deineko, I. P. and Evtugine, D. V. (1989). "Delignification by oxygen in acetone-water media". *Tappi J.* **69(9)**, 163-168.

Photochemical bleaching of an organosolv cane-bagasse pulp using the association of tio_2 and different additives

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ABSTRACT

A new process in two stages for bleaching peroxyformic sugar cane bagasse pulp combining photosensitization and photocatalysis is presented. The first stage consists of an irradiation (1h), with mercury lamps, of the pulp under alkaline pH in aqueous suspension at a consistency of 5% with Ti O_2 and methylene blue (MB) or 3,4,9,10 - perylenetetracarboxylic acid (PTCA) or Fe (II) 4,4´,4´´4´´´ - tetrasulfophthalocyanine (TSPC) (concentration $\approx 10^{-5}$ mol L^{-1}) in the presence of bubbling oxygen and hydrogen peroxide (2% pulp basis). The second photobleaching stage performed with hydrogen peroxide (3%) efficiently completed the delignification and the brightness gain, removing completely the sensitizer from the pulp. Under those conditions, efficient bleaching of the pulp was obtained, using uv/vis light and PTCA or MB (unbleached pulp: kappa number:13, brightness:44, viscosity: 900 dm^3 Kg^{-1}; bleached pulp: Kappa number: 1.2-1.3, brightness: 86, viscosity: 510-550 dm^3 Kg^{-1}).

Keywords: cellulosic pulp, bleaching, photocatalysis, titanium dioxide, lignin, photobleaching

INTRODUCTION

The principal goals of chemical pulp bleaching are to remove residual lignin and to decolorize the pulp without adversely damaging the cellulosic matrix. Due to stricter pollution regulations, new non-pollutant bleaching processes are highly desired and the Totally Chlorine Free (TCF) bleaching technology based on the action of oxygen, ozone, hydrogen peroxide and peracids appears to fulfill the conditions for a closed cycle mill (Nelson, 1998). The delignification performed in the TCF bleaching is mainly based on redox reactions (Gierer, 1997). It is known that excitation of molecules in their electronic excited states increases their redox properties compared to the ground state. In that respect, the photochemical bleaching, based on the generation of oxygen active species, appears very promising (Machado *et al.,* 1997; Nascimento *et al.,* 1995). We have recently reported a three stage photochemical bleaching of *Eucalyptus grandis* organosolv pulps (Machado *et al.,* 1997; Ruggiero *et al.,* 1998). The bleaching was attributed to the combined action of superoxide anions, hydroxyl radicals, and singlet oxygen produced by irradiation in alkaline solutions (Ruggiero *et al.,* 1998; Machado *et al.,*1994a; Machado *et al.,*1994b; Ruggiero *et al.,* 1997; Machado *et al.,*1997). Some improvement in the photobleaching was reached by the use of the photocatalytic action of TiO_2 (Da Silva Perez *et al.*, 1998a). The presence of TiO_2 as a photocatalyst showed several advantages such as the reduction of reaction time, preservation of the pulp viscosity, increase of the selectivity during the photobleaching process and decrease of the consumption of the bleaching chemicals (Da Silva Perez *et al.*, 1998a). This article describes the photobleaching of chemical pulp combining the use of a photocatalyst TiO_2, and a photosensitizer. The study was focused on peroxyformic sugar cane bagasse pulp, because it was easy to obtain and the process involves a very abundant raw material in Brazil (Da Silva Perez *et al.*, 1998b). The bleaching sequences were accomplished using oxygen and hydrogen peroxide as the source of oxygen active species in the presence of uv light.

EXPERIMENTAL

Pulp

The unbleached pulp was prepared from sugar cane bagasse using the one stage peroxyformic procedure previously described (Da Silva Perez *et al.*, 1998b). The pulping of the bagasse was carried out at 80°C(3 h) to profit from the action of peroxyformic acid, followed by a formic acid reflux (110°C, 2 h), using the same liquor. The pulping process was completed by a 0.25 N NaOH extraction of the pulp at 60°C in order to dissolve more lignin. Good quality unbleached pulps were obtained. The pulp used in this study has the

following characteristics: kappa number (KN) = 13, intrinsic viscosity [η] = 900 dm^3 Kg^{-1} brightness index (BI) = 44.1.

Photobleaching

During the photobleaching, the aqueous suspesion containing the pulp (5% consistency) was kept at constant temperature (85°C) with constant stirring. The Pyrex cylindrical reactor was placed between two lamps during the irradiation. Two medium pressure mercury lamps (Mazda 400 W) were used for the uv/visible irradiation. The Pyrex glass acts as a filter, it eliminates the uv ligth with wavelengths below 300 nm, which are very detrimental to the cellulosic matrix. The bleaching was done in two stages. During the first stage, the pulp suspension was irradiated for 1 h in the presence of TiO_2 (0.5% relative to oven dried pulp, odp), hydrogen peroxide (2% odp), sodium hydroxide (3% odp) to maintain alkaline the suspension and $MgSO_4$ (0.5% odp) to prevent the degradation of the pulp. A dye, methylene blue (MB), 3,4,9,10 - Perylene tetracarboxylic acid (PTCA) or Fe (II) 4,4´,4´´,4´´´ - tetrasulphophatalocyanine (TSPC) was added to the solution (final concentration ≈ 5 x 10^{-5} mol L^{-1}) in order to absorb the uv/visible light and therefore to increase the photobleaching of the pulp. Oxygen was continuously bubbled through the solution during irradiation. After the irradiation, the pulp was filtered and washed to a neutral pH level with distilled water. For the second stage, the aqueous suspension included H_2O_2 (3% odp), Na OH (3% odp), bubbling oxygen and also sodium metasilicate (3% odp) to limit the thermal degradation of the hydrogen peroxide. The irradiation was maintained for 1 h and after the bleaching, the pulps were washed with water to a neutral pH.

Quantitative and physical measurements

The lignin content of the pulp was estimated using the micro Kappa number method, according to the Tappi Method UM 246. The viscosity measurements were performed using the capillary viscometer method, according to the ISO 5351/1 method. Handsheets of paper (10 cm of diameter) were made from the different bleached pulps (basis weight ≈ 450 g m^{-2}) for measuring, brightness and uv/visible diffuse reflectance spectra at infinite thickness expressed by R_∞. The brightness at 457 nm was obtained with na Elrepho 2000 Datacolor Reflectometer and the uv/visible diffuse reflectance spectra were recorded with a Hitachi U-3300 spectrometer equipped with an integrating sphere.

RESULTS AND DISCUSSION

The photobleaching was performed on sugar cane bagasse, which appears to be most promising raw material pulped with peroxyformic acid (Da Silva Perez *et al.*, 1998b). The bleaching performance was evaluated by measuring the amount of residual lignin (kappa number), pulp viscosity (η) which is indicative of the damage of the cellulosic chain and brightness (BI) of papers made from the pulp after each stage. The bleaching yields were foud to be greater than 90%.

In the first stage of the photochemical bleaching, the pulp was treated with oxygen and hydrogen peroxide in the presence of both a photosensitizer and a photocatalyst (TiO_2) in an alkaline medium. The beneficial effect of TiO_2 to delignify and to bleach the chemical pulps under uv/visible irradiation had already been observed (Da Silva Perez *et al.*, 1998a). The presence of the sensitizer should allow the utilization of light to photobleach the pulp, and may eventually allow the use of sunlight as an irradiation source. The second stage gives high brightness to the pulp as well as eliminating the residual dye over the pulp arising from the first stage. Among different sensitizers which might be used, we have selected methylene blue (MB) for its singlet oxygen sensitizer ability and because it was shown to efficiently photobleach the pulp (Machado *et al.*, 1997; Ruggiero *et al.*, 1998). 3,4,9,10 - Perylenetetracarboxylic acid (PTCA) has been described as a potential visible light transfer agent semiconductors. Finally, Fe (II) 4,4´,4´´,4´´´- tetrasulphophtalocyanine (TSPC) was used to promote the thermal oxidative degradation of catechol and veratry alcohol in the presence of hydrogen peroxide. In an alkaline water solution, MB and TSPC have an intense absorption between 520 and 720 nm, whereas the absorption of PTCA is centered at 450 nm.

The results of the photobleaching of the sugar cane bagasse pulp at 85°C are shown in Table 1.

Examination of Table 1 shows that the bleaching of the peroxyformic bagasse pulp was operating quite well without light in stages at 85°C. Important viscosity loss was observed (37%) .This could be due to the detrimental action of metal ions such as Fe^{2+} , Fe^{3+}, Mn^{2+} on the cellulosic matrix during the peroxide bleaching, as no chelating treatment was done on the unbleached pulp; though the objective of the study was to evaluate the improvement of the bleaching using light in the presence of a dye and a photocatalyst.

As previously observed (Da Silva Perez *et al.*, 1998a) , the presence of TiO_2 alone improves the photodelignification in the first stage and consequently in the second one but this improvement is dependent upon the irradiation light. Also, the photocatalyst limits the viscosity loss and improves the brightness of the pulp with the experiments performed at 85°C being the best. Examination of Table 1 indicates that the compound TSPC does not bring significant enhancement to the delignification process. Thus TSPC; due to its oxidative instability, is not the most useful sensitizer and is unable to augment the activity of TiO_2 in the photochemical bleaching of pulps.

In contrast to TSPC, methylene blue (MB) and the perylenetetracarboxylic acid (PTCA) enhanced the delignification and the brightness gain and limited the viscosity loss under the mercury lamp irradiation.

Table 1. Photobleaching of peroxyformic sugar cane bagasse pulp in two stages at 85ºC

	After the first stage			After the second stage		
System	KN	BI	[η] ($dm^3 Kg^{-1}$)	KN	BI	[η] ($dm^3 Kg^{-1}$)
pulp+ TiO_2	5.0	67.7	732	2.8	76.2	560
pulp+uv/vis	4.9	66.6	575	2.8	78.6	450
pulp+ TiO_2+uv/vis	4.6	71.1	635	1.5	83.1	500
pulp+ TiO_2+PTCA+uv/vis	3.4	74	630	1.2	86.1	510
pulp+ TiO_2+MB+uv/vis	3.5	73.3	670	1.3	85.9	565
pulp+ TiO_2+TSPC+uv/vis	4.1	69.5	690	1.5	83.3	515

Unbleached pulp (15 g, 5% consistency): KN=13; BI=44.1; η = 900 mL/g. *First stage*: TiO_2 0.5% oven dry pulp (odp); H_2O_2 2% odp; $MgSO_4$ 0.5% odp; NaOH 3% odp; O_2 bubbling; photosensitizer ≈ 5 x 10^{-5} mol L^{-1}; irradiation time 1 h. *Second stage*: H_2O_2 3% odp; $MgSO_4$ 0.5% odp; NaOH 3% odp; O_2 bubbling; $NaSi_2O_3$ 3% odp; irradiation time 1 h. uv/vis: two medium pressure Hg lamps (400W) with vessel.

In the first stage, a very similar increasing delignification was observed for MB (26%) and PTCA (24%). After the second stage, both MB and PTCA, in conjunction with TiO_2, were able to cause delignification to a great extent (PTCA: 64%;MB:63%) reaching similar brightness levels (BI=86). Moreover, MB brings some protection to the cellulosic matrix, the viscosity loss being less than with PTCA or without a sensitizer.

CONCLUSION

The present study is an approach chlorine-and sulfur-bleached pulp under mild conditions from a lignocellulosic raw material, sugar cane bagasse. It demonstrates that the combination of peroxyformic acid pulping followed by photochemical bleaching using a photocatalyst TiO_2 , dyes such as 3,4,9,10 - perylenetetracarboxylic acid (PTCA) or methylene blue (MB) can produce almost fully bleached pulp with uv/vis light (KN= 1.2 - 1.3; BI= 86; η = 625 dm^3 Kg^{-1} for MB/TiO_2). Nevertheless, it is necessary to improve the efficiency and the selectivity of the system photocatalyst/sensitizer, to photobleaching to an acceptable level for use. Efforts are being made in this direction in our cooperative program CAPES/COFECUB between the University of Uberlândia and the University of Bordeaux.

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REFERENCES

Da Silva Perez, D.; . Terrones, M. G H., Grelier, S.; Nourmamode, A.; Castellan, A.; Ruggiero, R.; Machado, A.E. H.; (1998b). Peroxyformic acid pulping of eucalyptus grandis Wood chips and Sugar Cane Bagasse in one stage and characterization of the isolated lignins. *Wood Chem. Technol.*, **18**, 333 - 365.

Da Silva Perez, D.; Castellan, A.; Grelier, S.; Terrones, M. G H., Machado, A.E. H.; Ruggiero, R. & Vilarinho, A. L. J. (1998a). Photochemical bleaching of chemical pulps catalyzed by titanium dioxide. *Photochem. Photobiol., A: Chem.*, **115**, 73-80.

Gierer, J. (1997). Formation and Involvement of Superoxide ($O_2^{\cdot}$/$HO_2^{\cdot}$) and Hydroxyl (OH) Radical process: A Review. *Holzforschung*, **51**, 34-46.

Machado, A. E. H.; Gomes, A. J., Campos, C. M. F.; Terrones, M. G H., Da Silva Perez, D.; Ruggiero, R. & Castellan, A. J. (1997). Photoreactivity of lignin model compounds in the photobleaching of Chemical pulps. 2. Study of the degradation of 4-hydroxy - 3-methoxy-benzaldehyde and two lignin fragments induced by singlet oxygen. *Photochem. Photobiol., A: Chem.* **110**, 99-106.

Machado, A. E. H.; Ruggiero, R. & Neumann, M. G. J., (1994b). Fotodegradação de Ligninas Acelerada por Peróxido de Hidrogênio: Evidências da Partição do $^1O_2$ ($^{14}\Delta_g$) nas Reações em Meio Alcalino. *Química Nova*, **17**, 111-118.

Machado, A.E. H.; Ruggiero, R. & Neumann, M. G. J. (1994a). The Photodegradation of Lignins in the presence of Hydrogen Peroxide, *Photochem. Photobiol., A: Chem.*, **81**, 107 - 115.

Machado, A.E. H.; Ruggiero, R. & Terrones, M. G.; (1996). Photodelignification of Eucalyptus grandis Organosolv Chemical Pulp. Nourmamode, A.; Grelier, S.; Castellan, A.J. *Photochem. Photobiol. A: Chem.*, **94**, 253 - 262.

Nascimento, E. A., Machado, A.E. H. ; Morais, S. A. L.; Brasileiro , L. B. & Pilo - Veloso, D. J. (1995). Photochemical Hydrogen Peroxide Bleaching of Eucalyptus Organosolv Pulp; *Braz. Chem. Soc.*, **6**, 365-371.

Nelson, P. J. (1998). *Elemental chlorine free and Totally chlorine free bleaching of pulps*, in Environmentally friendly technologies for the pulp and paper industry, ed. R. A. Young & M. Akhtar. John Wiley & Sons, New York, pp 215-256.

Ruggiero, R.; Machado, A. E. H.; Castellan, A. & Grelier, S. J., (1997). Photoreactivity of Lignin Model Compounds in the Photobleaching of Chemical Pulps. 1. Irradiation of 1-(3,4-dimethoxyphenyl) -2(3´-methoxyphenoxy)- 1,3-dihydropropane in the Presence of singlet oxigen Sensitizer or Hidrogen Peroxide in Basic Methanol Solution. *Photochem. Photobiol., A: Chem.*, **110**, 91-97.

Ruggiero, R.; Machado, A.E.H.; Da Silva Perez, D.; Grelier, S.; Nourmamode; A. & Castellan, A.; (1998). Bleached Chemical Pulp from Eucalyptus grandis Wood Produced by Peroxyformic Acid Pulping and Photochemical Bleaching. *Holzforschung*, **52**, 325 - 332.

Preparation of 4,4-dimethyl-2-(methoxyaryl)-2-oxazolines from *eucalyptus* wood tar constituents

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ABSTRACT

Eucalyptus tar fraction rich in syringyl derivatives was methylated and subsequently oxidized furnishing 3,4,5-trimethoxybenzoic acid **1** as the main product. Bromination (NBS) of this acid gave the 2-bromo derivative **2** and treatment with NaOMe-Cu displaced the bromine furnishing the 2,3,4,5-tetramethoxybenzoic acid **3**. Acids **1** and **3** obtained from *Eucalyptus* tar constituents together with commercially available 3,4-dimethoxybenzoic acid (veratric acid) **4** were transformed into the acid chlorides ($SOCl_2$) which were allowed to react with 2-amino-2-methyl-1-propanol affording 4,4-dimethyl-2-(methoxyaryl)-2-oxazolines derivatives. The reaction of 3,4,5-trimethoxybenzoyl chloride with 2-amino-2-methyl-1-propanol afforded as by-product the 4,4,-dimethyl-2-(4-hydroxy-3,5-dimethoxyphenyl)-2-oxazoline.

Keywords: wood tar, methoxybenzoic acids, 4,4-dimethyl-2-(methoxyaryl)-2-oxazolines

INTRODUCTION

Reforestation in Brazil involves mainly plants of the genus *Eucalyptus*. Paper and cellulose industries, and charcoal steel mills mainly use wood from this reforestation. Some steel mills recover *Eucalytpus* tar and chromatographic analysis of this tar showed that 2,6-dimethoxyphenol (2,6-DMP) and its 4-alkyl derivatives are the main constituents (Stuckenbruck *et al.*, 1989). The difficulty to separate these constituents has induced us to plan its transformation to obtain fine chemicals.

The 2-oxazoline ring system, a simple cyclic imino ester, has demonstrated its vast synthetic potential as a masked carboxylic acid and efficient director for *o*-lithiation of the benzene nucleus. The *o*-lithiooxazoline can then be treated with electrophilic reagents and hydrolyzed to afford the *orto*-substituted benzoic acids. In addition to its ability to direct *o*-lithiation, the oxazoline is a remarkably efficient director for nucleophilic aromatic substitution (Reuman *et al.*, 1985).

Commercially, the main interest has been in 2-oxazolines derived from amino alcohols and low-cost carboxylic acids. The amino alcohols of interest are those having an amino group and a hydroxyl group on adjacent carbon atoms. Those of greatest interest are ones having complete substitution on the carbon atom on which the amino group is attached. The amino alcohols react readily when heated with acid chloride to give high yields in 2-oxazolines (Frump, 1971).

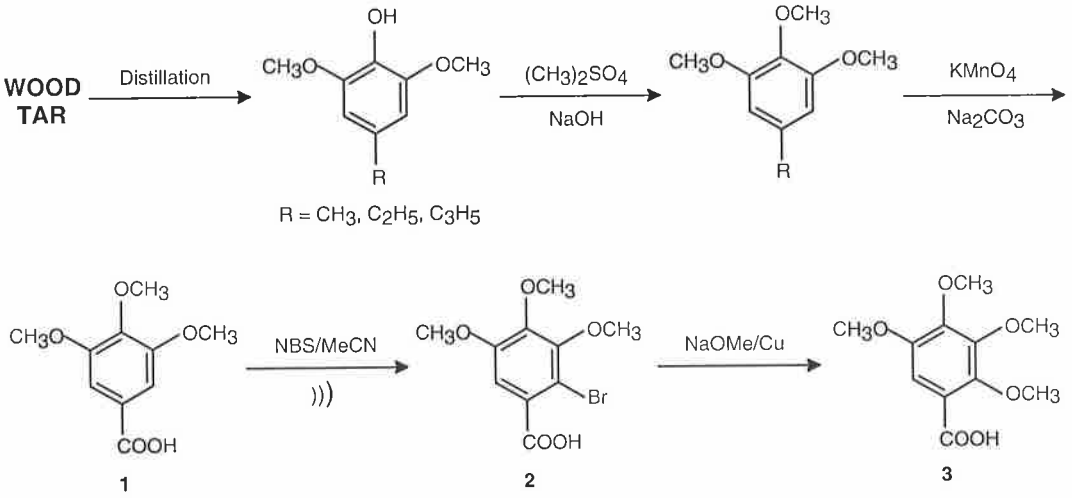
Based on these facts, we decided to prepare 4,4-dimethyl-2-(methoxyaryl)-2-oxazolines from *Eucalytus* tar constituents (Schemes 1 and 2).

One *Eucalyptus* wood tar fraction containing (11%) 2,6-DMP, (45%) 4-methyl-2,6-DMP, (24%) 4-ethyl-2,6-DMP, and (5%) 4-propyl-2,6-DMP could be, after methylated, oxidized furnishing 3,4,5-trimethoxybenzoic acid **1** as the main product (Carazza *et al.*, 1991). Bromination (NBS) of this acid gave the 2-bromo derivative **2** and treatment with NaOMe-Cu displaced the bromine furnishing the 2,3,4,5-tetramethoxybenzoic acid **3** (Meyers *et al.*, 1987) (Scheme 2). Acids **1** and **3** obtained from *Eucalyptus* tar constituents together with commercially available 3,4-dimethoxybenzoic acid (veratric acid) **4** were transformed in its correspondent acid chlorides ($SOCl_2$) which were allowed to react with 2-amino-2-methyl-1-propanol affording 4,4-dimethyl-2-(methoxyaryl)-2-oxazolines derivatives (Scheme 2).

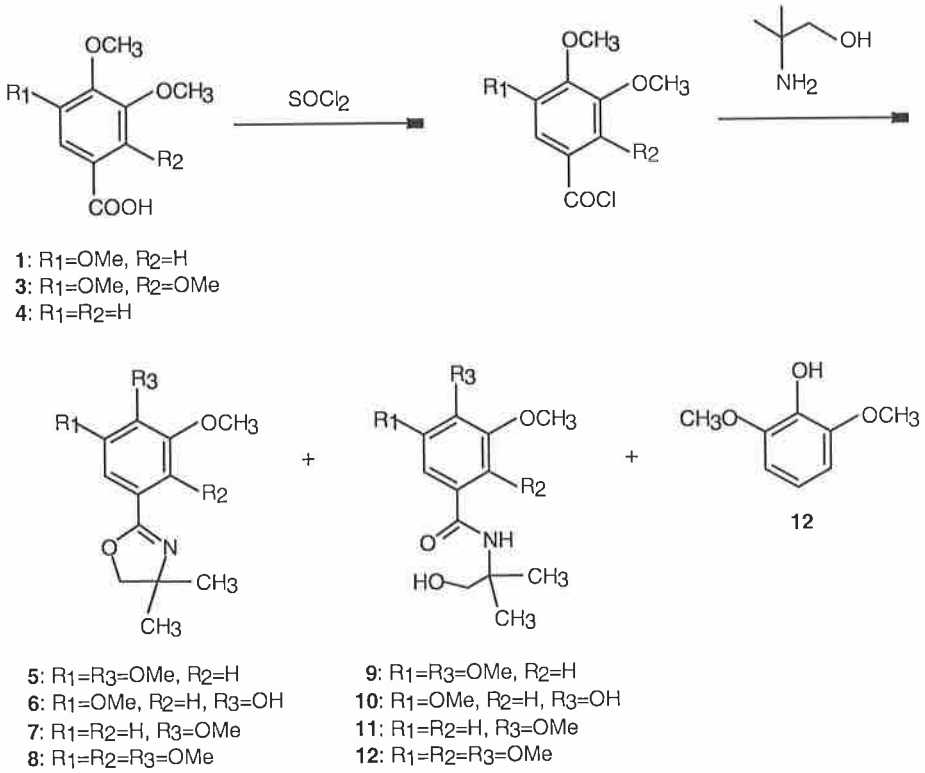
EXPERIMENTAL

N-bromossuccinimide (NBS), thionyl chloride ($SOCl_2$), 2-amino-2-methyl-1-propanol and solvents were purified according to standard procedures before use (Perrin *et al.*, 1980). Other commercially available organic and inorganic compounds were used without further purification. Organic layers from extractive

workups were dried over Na_2SO_4. Rotary evaporation was used to concentrate dried extractants. The reactions were monitored by Thin Layer Cromatography (TLC, silica gel 60G Merck) and High Resolution Gas Cromatography (HRGC).



Scheme 1.



Scheme 2.

Flash column chromatography was carried out with silica gel 60G Merck (40-63 μm).

The melting points reported are uncorrected. Infrared analyses were performed on Shimadzu/IR-408 Spectrophotometer. ^1H and ^{13}C NMR spectra were recorded on Brucker Avance DRX200, using TMS as the internal standard. HRGC-MS/C analyses were carried out with an HP 5890 (column HP-1: 25 m, 0.2 mm i.d., 0.33 μm p.d.; using He as carrier gas) coupled with HP 5989-A (70 eV). HRGC analyses were performed on HP-5890 (column SE-54: 30 m, 0.25 mm i.d., 0.25 μm p.d.; using H_2 as carrier gas). Oven 140-280 ^0C, 10 $^0C.min^{-1}$.

2-Bromo-3,4,5-trimethoxybenzoic Acid (2). A solution of 1.060 g (5 mmol) of 3,4,5-trimethoxybenzoic acid **1** and 1.034 g (5.5 mmol) of N-bromossuccinimide in 100 mL of acetonitrile was immersed in a water-bath

and irradiated with ultrasound using a high frequency processor (Thornton T 50, 40 KHz). The reaction was carried out at room temperature for 7 h, the solvent evaporated, and yellow solid dissolved in methylene chloride. This solution was washed with water (3 x 30 mL), dried, filtered, and concentrated to afford 1.3 g (89%) of **2** as a pale yellow solid which was used without further purification: mp 146-148 ^{0}C; IR (KBr) 3300-2500, 1700, 1590, 1560, 1490, 1420, 1390, 1340, 1280, 1240, 1200, 1170, 1120, 1020, 1000, 920, 850, 810, 770 cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.37 (1H, s), 3.94 (3H, s), 3.89 (3H, s), 3.88 (3H, s); ^{13}C NMR (50 MHz, $CDCl_3$) δ 170.49, 152.22, 151.70, 147.02, 125.43, 111.24, 110.88, 61.19, 61.03, 56.27; EIMS m/z (%) 292 (98) $[M+2]^{+\cdot}$, 290 (100) $M^{+\cdot}$, 275/277 (21/20), 247/249 (9/9), 232/234 (27/21), 211 (23), 196 (31), 181 (37), 153 (21), 124 (48), 109 (23), 93 (85), 77 (81), 69 (61), 65 (88), 59 (33), 53 (100), 45 (33).

2,3,4,5-Tetramethoxybenzoic Acid (3). To a stirred saturated solution of Na metal in anhydrous methanol (7.5 mL) was added 600 mg (2.06 mmol) of 2-bromo-3,4,5-trimethoxybenzoic acid **2**. Once the acid dissolved, 131 mg (2.06 mmol) of Cu powder was added and the mixture refluxed for 48 h. The mixture was cooled, filtered through a bed of silica, and concentrated in vacuum to give a crude white solid. This was dissolved in water and acidified to pH 3 with 10% HCl. The solution was extracted with methylene chloride (3 x 20 mL), dried, filtered, and concentrated to produce a light orange oil. Recrystallization [hexane/ethyl acetate (5:1)] yielded 462 mg (92%) of **3** as white prisms: mp 86-87 ^{0}C; IR (KBr) 3300-2500, 1700, 1680, 1600, 1500, 1470, 1430, 1400, 1340, 1290, 1240, 1200, 1130, 1070, 1040, 1000, 970, 920, 860, 800 cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.41 (1H, s), 4.08 (3H, s), 3.99 (3H, s), 3.93 (3H, s), 3.89 (3H, s); ^{13}C NMR (50 MHz, $CDCl_3$) δ 165.12, 150.08, 148.02, 147.25, 146.05, 115.70, 109.01, 62.71, 61.45, 61.28, 56.29; EIMS m/z (%) 242 (100) $M^{+\cdot}$, 227 (55), 209 (26), 199 (20), 197 (15), 184 (38), 167 (18), 113 (20), 69 (13), 53 (31).

4,4-Dimethyl-2-(methoxyaryl)-2-oxazolines (5, 6). A mixture of 3,4,5-trimethoxybenzoic acid **1** (1.0 g, 4.35 mmol) and thionyl chloride (1 mL, 13.77 mmol) was refluxed for 2 h. The excess of thionyl chloride was removed by heating under reduced pressure to give residue (acid chloride). This was refluxed in the presence of 2-amino-2-methyl-1-propanol (1 mL, 10 mmol) for 6 h. The reaction mixture was cooled to ambient temperature and acidified to pH 6 with 10% acetic acid. This solution was extracted with methylene chloride (3 x 20 mL), dried, filtered, and the solvent distillated under reduced pressure to produce a dark yellow oil. Flash cromatography (hexane/ethyl acetate) afforded the oxazolines **5**, **6**, the benzamides **9**, **10**, and 2,6-DMP **13**.

4,4-Dimethyl-2-(3,4,5-trimethoxyphenyl)-2-oxazoline (5): 184 mg (16%), yellow solid, mp 85-86 ^{0}C; IR (KBr) 3000, 2950, 2900, 2850, 1650, 1600, 1500, 1450, 1425, 1350, 1275, 1225, 1175, 1125, 1100, 850, 725 cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.19 (2H, s), 4.10 (2H, s), 3.91 (6H, s), 3.88 (3H, s), 1.39 (6H, s); ^{13}C NMR (50 MHz, $CDCl_3$) δ 161.75, 152.98, 140.65, 123.36, 105.42, 79.14, 67.69, 60.89, 56.26, 28.38; EIMS m/z (%) 265 (41) $M^{+\cdot}$, 250 (100), 222 (15), 194 (21), 178 (11), 150 (7), 135 (7), 125 (12), 77 (6), 66 (7), 53 (6).

4,4-Dimethyl-2-(4-hydroxy-3,5-dimethoxyphenyl)-2-oxazoline (6): 368 mg (34%), yellow solid, mp 125-127 ^{0}C; IR (KBr) 3600-3200, 2950, 2850, 1650, 1590, 1550, 1500, 1450, 1400, 1325, 1240, 1190, 1125, 1050, 1000, 850, 800 cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.20 (2H, s), 5.30 (1H, s), 4.09 (2H, s), 3.93 (6H, s), 1.38 (6H, s); ^{13}C NMR (50 MHz, $CDCl_3$) δ 161.95, 146.72, 137.61, 119.12, 105.22, 79.08, 67.55, 56.43, 28.40; EIMS m/z (%) 251 (30) $M^{+\cdot}$, 236 (100), 208 (14), 180 (22), 164 (8), 118 (10), 90 (5), 53 (6).

N-(2-Hydroxy-1,1-dimethyl)-3,4,5-trimethoxybenzamide (9): 99 mg (8%), white crystals; EIMS m/z (%) 283 (6) $M^{+\cdot}$, 252 (7), 250 (5), 212 (12), 195 (100).

N-(2-Hydroxy-1,1-dimethyl)-4-hydroxy-3,5-dimethoxybenzamide (10): 33 mg (3%), white solid; EIMS m/z (%) 269 (5) $M^{+\cdot}$, 238 (8), 198 (11), 181 (100), 153 (8), 123 (6), 108 (5), 58 (9), 44 (13).

2,6-Dimethoxyphenol (13): 138 mg (21%), brown solid; EIMS m/z (%) 154 (100) $M^{+\cdot}$, 139 (55), 111 (20), 96 (25), 93 (35), 79 (11), 68 (12), 65 (28), 55 (11), 53 (12), 51 (15).

4,4-Dimethyl-2-(methoxyaryl)-2-oxazolines (5, 7, 8). The following procedure illustrates the general method used for preparation of oxazolines **5**, **7** and **8**. A mixture of methoxybenzoic acid (1 mmol) and thionyl chloride (3 mmol) was refluxed for 2 h. The excess of thionyl chloride was removed by heating under reduced pressure, and the residue (acid chloride) was dissolved in xylene. To this solution was added 2-amino-2-methyl-1-propanol (3 mmol) and the resultant mixture was heated under reflux with azeotropic removal of water (Dean Stark trap) for 6 h. The reaction mixture was cooled to ambient temperature and acidified to pH 6 with 10% acetic acid. This solution was extracted with methylene chloride, dried and

filtered. The solvent was removed by distillation under reduced pressure to afford a crude yellow oil, which was purified by flash chromatography (hexane/ethyl acetate) to give the oxazolines **5**, **7**, **8**, and its correlated benzamides **9**, **10** and **11** as products.

4,4-Dimethyl-2-(3,4-dimethoxyphenyl)-2-oxazoline (7): 420 mg (71%), yellow oil; IR (film) 2980, 2950, 2850, 1720, 1650, 1600, 1580, 1520, 1460, 1425, 1360, 1310, 1270, 1240, 1175, 1140, 1080, 1025, 975, 880, 820, 770, 740, 720 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 7.53 (1H, dd, *J* = 8.4, 1.9 Hz), 7.47 (1H, d, *J* = 1.9 Hz), 6.87 (1H, d, *J* = 8.4 Hz), 4.09 (2H, s), 3.93 (3H, s), 3.92 (3H, s), 1.38 (6H, s); ^{13}C NMR (50 MHz, $CDCl_3$) δ 161.93, 151.54, 148.63, 121.66, 120.68, 110.84, 110.39, 79.08, 67.50, 56.06, 55.94, 28.43; EIMS m/z (%) 235 (34) $M^{+\cdot}$, 220 (100), 205 (5), 192 (23), 164 (26), 148 (8), 120 (5), 92 (7), 77 (8), 65 (5).

4,4-Dimethyl-2-(3,4,5-trimethoxyphenyl)-2-oxazoline (5): 117 mg (67%).

4,4-Dimethyl-2-(2,3,4,5-tetramethoxyphenyl)-2-oxazoline (8): 94 mg (32%), pale yellow solid, mp 136-138 ^0C; IR (KBr) 2980, 2940, 1640, 1600, 1490, 1475, 1410, 1380, 1200, 1120, 1070, 1030, 1010, 980, 840, 780, 700 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 7.04 (1H, s), 4.10 (2H, s), 3.93 (6H), 3.87 (3H, s), 3.83 (3H, s), 1.39 (6H, s); ^{13}C NMR (50 MHz, $CDCl_3$) δ 160.91, 149.15, 147.71, 147.54, 145.50, 117.00, 107.96, 79.04, 67.35, 61.79, 61.42, 61.26, 56.25, 28.34; EIMS m/z (%) 295 (52) $M^{+\cdot}$, 280 (34), 266 (11), 252 (14), 238 (24), 234 (20), 222 (34), 211 (40), 208 (100), 196 (29), 193 (23), 178 (13), 165 (14), 140 (12), 122 (6), 94 (8), 72 (6), 55 (9).

N-(2-Hydroxy-1,1-dimethyl)-3,4-dimethoxybenzamide (11): EIMS m/z (%) 253 (2) $M^{+\cdot}$, 222 (77), 182 (11), 165 (100), 137 (8), 122 (5), 92 (5), 77 (11), 51 (5), 44 (16).

N-(2-Hydroxy-1,1-dimethyl)-3,4,5-trimethoxybenzamide (9): 40 mg (21%).

N-(2-Hydroxy-1,1-dimethyl)-2,3,4,5-tetramethoxybenzamide (12): 127 mg (40%), white crystals; EIMS m/z (%) 313 (6) $M^{+\cdot}$, 282 (9), 225 (100).

RESULTS AND DISCUSSION

The methoxybenzoic acids **1**, **2** and **3** (Scheme 2) were obtained with high yields from *Eucalyptus* wood tar constituents.

Oxazolines are usually prepared by treatment of the acid chloride with the appropriate amino alcohol followed by cyclization of the amide in the presence of thionyl chloride (Reuman *et al.*, 1985). Thus, the 3,4,5-trimethoxybenzoyl chloride was allowed to react with 2-amino-2-methyl-1-propanol in order to prepare the correspondent benzamide. Flash chromatography of the reaction product afforded unexpected results. By this approach 2,6-DMP **13**, benzamides **9** and **10**, and oxazolines **5** and **6** were prepared. The formation of this products could be associated with the harsh conditions used. In addition, the presence of hydrogen chloride (HCl) generated *in situ* promoted the demethylation furnishing the phenolic compounds **6** and **10**. Finally, the hydrolysis of benzamide **10** or oxazoline **6** followed by decarboxylation afforded the 2,6-DMP.

In order to avoid by-products formation, an alternative approach that employs xylene as solvent and azeotropic agent was utilized. By this approach, the di-, tri- and tetramethoxybenzoic acids were converted into the acid chloride and allowed to react with 2-amino-2-methyl-1-propanol in the presence of xylene. This milder method provided the oxazolines **5**, **7**, **8** and its correlated benzamides **9**, **11** and **12**. The drawback of this approach is the incomplete reaction that results in low yields of the oxazolines.

The introduction of methoxyl groups in the aromatic ring decreases the benzamides conversion into oxazolines. The conversion degree was determined during GC analysis. The continuity of the conversion into oxazoline was observed during the elimination of the solvent. In the case of the benzamide **11** the conversion was almost fully obtained.

The purity of the benzamides prepared was not enough for spectrometric analyses and the mass spectra were obtained by GC-MS analyses.

This is the first report on the preparation of the 4,4-dimethyl-2-(4-hydroxy-3,5-dimethoxyphenyl)-2-oxazoline **6** and 4,4-dimethyl-2-(2,3,4,5-tetramethoxyphenyl)-2-oxazoline **8**.

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REFERENCES

Carazza, F., Pereira, M. O. S., Pereira, N. S., Alcântara, M. F. C. & Braga, J. V. (1991). Fine chemicals from syringyl rich fractions of tar obtained from *Eucalyptus* SP wood. *In 1st European Workshop on Lignocellulosic and Pulp*, Proceedings, **Vol I**, pp 207-212.

Frump, J. A. (1971). Oxazolines. Their preparation, reactions, and applications. *Chem. Rev.*, **71**, 483-505.

Meyers, A. I., Flisak, J. R. & Aitken, R. A. (1987). An asymmetric synthesis of (-)-Steganone. Further application of chiral biaryl syntheses. *J. Am. Chem. Soc.*, **109**, 5446-5452.

Perrin, D. D. & Armarego, W. L. F. (1980). *Purification of Laboratory Chemicals*. 2 ed. Pergamon Press, New York.

Reuman, M. & Meyers, A. I. (1985). The synthetic utility of oxazolines in aromatic substitution. *Tetrahedron*, **41**, 837-860.

Stuckenbruck, P., Aquino Neto, F. R. & Carazza, F. (1989). Análise do Óleo de Alcatrão Vegetal de *Eucalyptus* Citriodora por CGAR e CGAR-EM/C. *II Encontro de Carboquímica*, Anais, pp 3-20.

Preparation of sugarcane bagasse hydrolysates for alcoholic fermentation by yeasts

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ABSTRACT

During the last years there has been an increasing interest in using ethanol as a substitute for fossil fuels, since ethanol does not contribute to the greenhouse effect. Industrial production of ethanol in tropical countries is based mainly on fermentation of sugarcane molasses and juice. However, the availability of these substrates is not enough for satisfying the potential demands for fuel ethanol. Bagasse, the solid residue of the cane plant, has a very high carbohydrate content. In addition, it is a cheap and readily available lignocellulosic substrate. Therefore, bagasse should be considered as a feedstock for ethanol production. In the present work, methods for preparing bagasse hydrolysates as substrates for ethanol production by yeasts were investigated. The composition of the bagasse was analysed and the major components were glucans (45.0 %), xylans (25.0 %) and lignin (20.7 %). Bagasse was subjected to different steam pretreatment conditions (205 or 215°C for 5 or 10 min). The pretreated material was subsequently hydrolysed using cellulolytic enzymes. The chemical composition of the hydrolysates was analysed and glucose (21.4 - 23.6 g/l) and xylose (5.4 - 9.0 g/l) were the most abundant monosaccharides. The maximum glucose yield after hydrolysis was achieved in the material pretreated at 205°C and 10 min and it corresponded to 75.5 % of the theoretical yield. Compounds derived from the degradation of carbohydrates and lignin were also found, such as acetic acid, formic acid, furfural, HMF and phenolic compounds. These compounds are potential inhibitors of alcoholic fermentation. The hydrolysates produced by pretreatment at 205°C contained higher levels of all sugars and lower levels of all fermentation inhibitors than the hydrolysate produced by pretreatment at 215°C. After pretreatment and enzymatic hydrolysis, the best ethanol productivity and yield was obtained with the sample subjected to pretreatment at 205°C for 5 min. Enzymatic treatment with the phenoloxidase laccase was performed in order to detoxify the hydrolysates by removing the phenolic compounds. Increased ethanol yield and volumetric productivity were achieved in the detoxified hydrolysates.

Keywords: sugar cane bagasse, cellulases, ethanol production, alcoholic fermentation

INTRODUCTION

Bagasse is the lignocellulosic residue separated after juice extraction from sugar cane (*Saccharum officinarum)* during sugar production. Bagasse represents 12-14 % of the cane dry weight (ICIDCA, 1987). Most bagasse produced in the sugar industry is burned in low efficiency boilers to generate steam and power required by the sugar factories. Since bagasse is a process waste, the thermal efficiency of the combustion unit has not been a concern: the less efficient the boiler is, the more bagasse is consumed, the smaller the disposal problem (Cuzens and Miller, 1996). If the efficiency of the combustion units is improved, 30 to 50 % of the bagasse would become available for alternative use (ICIDCA, 1987). Bagasse thus represents a cheap and abundant source of carbohydrates with a high potential to be hydrolysed and fermented to ethanol (Roberto *et al.*, 1991).

Acids and enzymes can hydrolyse lignocellulosic materials. Acid hydrolysis is easy to perform, but it has the disadvantages of non-selectivity and by-product formation (Palmqvist, 1998), as well as the requirements of expensive construction material for the equipment (Olsson, 1994). Enzymatic hydrolysis is performed with cellulolytic enzymes. The lignocellulosic material must be pretreated in such a way that the cellulosic fraction becomes more accessible for the enzymes. Steam explosion has generally been recognised as one of the most effective methods for pretreating lignocellulosic feedstocks (Gregg and Saddler, 1996). In addition, steaming is relatively inexpensive, non-hazardous to humans, and does not require addition of chemicals (Galbe, 1994). However, under steam pretreatment conditions, fermentation-inhibiting substances, such as aliphatic acids, furan derivatives and phenolic compounds, are released. Chemical, physical and biological methods

can be used in order to remove the inhibitory compounds from the hydrolysate (Palmqvist, 1998). The detoxification with the phenoloxidising enzyme laccase is a method that can selectively remove phenolic compounds from hydrolysates. Laccase treatment has successfully been used for willow (Jönsson *et al.*, 1998) and spruce (Larsson *et al.*, 1999) hydrolysates, but there are still no reports on using this detoxification method for bagasse hydrolysates.

There are some previous reports about using steam pretreatment for sugarcane bagasse (Dekker and Wallis, 1983; Morjanoff and Gray, 1987; Glasser and Wright, 1995; Kaar *et al.*, 1998). However, most of the studies found in the literature on detoxification of bagasse hydrolysates are related to acid hydrolysis (Watson *et al.*, 1984; van Zyl *et al.*, 1988; 1991; Roberto *et al.*, 1991; Gong *et al.*, 1993). In this work different steam pretreatment conditions for preparing enzymatic hydrolysates of sugarcane bagasse were investigated, the chemical composition and fermentability of the hydrolysates were analysed and the effect of detoxification by laccase treatment was studied.

EXPERIMENTAL

Raw material

Fresh bagasse was kindly provided by the sugar mill "J. López" (Matanzas, Cuba) and transported to Lund University, Sweden, by air. Bagasse was fractionated to a particle size between 2.2 and 10 mm. The composition was analysed by the Hägglund method for raw material analysis (Hägglund, 1951). This is a traditional wood analysis procedure. For carrying out the hydrolysis of the polysaccharides, 72% sulphuric acid was used. The results of the analysis are given in Table 1.

Table 1. Composition of raw material.

Component	Content, % of DM (st. dev.)
Glucans	45.0 (0.49)
Xylans	25.0 (1.10)
Lignin	20.7 (0.07)

Pretreatment

Pretreatment of bagasse was performed in a pressurised vessel. The pretreatment vessel had an inner diameter of 0.1 m and a volume of 2.4 dm^3. Steam was introduced into the reactor from a boiler, which produced saturated steam up to 4000 kPa. A computer, ABC 800 (Luxor AB, Motala, Sweden) was used to control valve operations. 100 g of bagasse chips with a dry matter content (DM) of 92 % were loaded into the reactor. Steam was supplied from a boiler and the material was heated up to either 205 or 215°C with a residence time of either 5 or 10 min. Then, the pretreated material was discharged into a cyclone connected to the outlet of the reactor. The pretreated material was collected and samples from the supernatant were analysed to determine the yield of sugars and by-products. The fibrous material was washed thoroughly with water to remove all soluble substances and the fibre yield was determined.

Enzymatic hydrolysis

Saccharification of pretreated bagasse was performed by the cellulase mixture Celluclast 2L and the β-glycosidase preparation Novozym 188 generously donated by Novo Industri A/S (Bagsværd, Denmark). The bagasse slurry was mixed with 2.3 g Celluclast and 0.5 g Novozym. Water was added up to a final weight of 500 g. Hydrolysis was performed with 5% (w/w) dry matter fibre content with stirring for 96 h. The temperature was 40°C and the pH was 4.8. Samples were withdrawn after 0, 2, 4, 24, 48, 72 and 96 h. Finally, the hydrolysates were vacuum filtered and fermented.

Detoxification

Enzymatic detoxification with the phenoloxidase laccase from *Coriolus versicolor* (E 014, Jülich Enzyme Products, Germany) was performed at pH 5.3 (after pH adjustment with 10 % (w/v) NaOH). The laccase preparation (or water instead of the enzyme for the control) was then added to the hydrolysate to 10 % of the final volume. The samples were incubated at 30°C for 12 h in a rotary shaker (90 rpm) following a previously described procedure (Jönsson *et al.*, 1998).

Fermentation

Compressed baker's yeast *Saccharomyces cerevisiae* (Jästbolaget AB, Rotebro, Sweden) was used for fermenting all hydrolysates. The hydrolysates were supplemented to final concentrations of 1 g/l yeast

extract, 0.5 g/l $(NH_4)_2HPO_4$, 0.025 g/l $MgSO_4 \cdot 7H_20$ and 1.38 g/l NaH_2PO_4. The pH was adjusted to 5.5 with 2 M NaOH. Fermentations of 20-22 g/l glucose solutions supplemented with other nutrients as described above were performed as references. The fermentations were carried out in 22-ml flasks containing 20 ml medium, sealed with rubber stoppers and equipped with syringes for sampling and cannulas for CO_2 removal. The flasks were inoculated to an initial cell concentration of 5 g/l dry weight (DW) and incubated at 30°C with stirring. Fermentations were run for 24 h under nonsterile conditions. Samples of 100 µl were withdrawn at the start and after 2, 4, 6, 12 and 24 h. Each fermentation was repeated three times. The glucose consumption rate (g/(lh)), the ethanol yield (g/g) (calculated as produced ethanol after 12 h divided by initial glucose concentration) and the maximum volumetric ethanol productivity (g/(lh)) within the first 6 h of the fermentation were used as criteria of fermentability.

Analyses

All samples were analysed by HPLC (Gilson, Middletown, Wisconsin, USA). The samples were filtered through a 0.20 µm filter and diluted prior to analysis. In all hydrolysis samples, cellobiose, glucose, xylose, galactose and mannose/arabinose were separated on an HPX-87P column (Bio-Rad, Hercules, California, USA) operating at 80°C with degassed ultrapure water as the mobile phase at a flow rate of 0.6 ml min^{-1}. Acetic, formic and levulinic acids, furfural and 5-hydroxy-methyl-furfural (HMF) in the hydrolysates as well as ethanol and glucose in the fermentation samples were separated on an Aminex HPX-87H column (Bio-Rad, Hercules, California, USA) operating at 45°C with 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 ml min^{-1}. Sugars and aliphatic acids were detected with a RI-detector (Shimadzu, Kyoto, Japan). Furfural and HMF were detected with a UV-detector (Shimadzu, Kyoto, Japan). The total content of phenolic compounds was determined colorimetrically by the Folin-Ciocalteau method (Singleton *et al.*, 1999), with vanillin as the calibration standard, using a U-1100 spectrophotometer (Hitachi Ltd., Tokyo, Japan).

RESULTS AND DISCUSSION

Pretreatment

Glucose, xylose and by-product yields, obtained in the pretreatment of the bagasse, as well as the recovery of fibrous material, are shown in Table 2.

Table 2. Yields after pretreatment , g/100g DM

Severity (T,°C; t,min)	Fibre	Glucose	Xylose	Acetic acid	Formic acid	HMF	Furfural
3.8(205; 5)	75.4	3.0	3.2	3.6	0.6	0.4	1.2
4.1(205; 10)	72.0	4.8	6.6	6.0	1.8	0.5	2.6
4.1(215; 5)	71.4	5.2	8.4	7.4	2.2	0.7	3.0
4.4(215; 10)	67.5	7.8	7.0	7.8	2.8	1.2	3.8

The severity factor (Ro) was used as a measure of the reaction conditions during steam pretreatment. It was defined as by (Kaar *et al.*, 1998):

$$\text{Log Ro} = \text{Log}\,[t * e^{(T-100)/14.75}] \qquad t = \text{time (min)};\quad T = \text{temperature (°C)}$$

The concentration of glucose increased during the course of hydrolysis, as a result of the conversion of the cellulose. The glucose content reached 23.6 g/l after 96 h. However, more than half of that concentration was formed during the first four h, when the hydrolysis rate was the highest. The observed gradual increase of xylose and acetic acid are due to an incomplete hydrolysis of hemicellulose during the pretreatment. Cellobiose was found in detectable amounts only in very few samples. The amounts of formic acid, furfural and HMF remained roughly constant (Figure 2).

The final composition of all hydrolysates is shown in Table 3. The chemical composition of all hydrolysates was qualitatively very similar. The main components were the sugars glucose and xylose, derived from the hydrolysis of cellulose and hemicellulose, respectively, acetic acid from the deacetylation of acetyl groups in hemicellulose, phenolic compounds from the degradation of lignin and furfural from the dehydration of the released sugars. Small amounts of arabinose, mannose, formic acid and HMF were also present in the hydrolysates. Mannose and arabinose could not be separated by the HPLC method used. The concentrations of cellobiose, galactose and levulinic acid were too low to be accurately determined. The low concentrations of formic and levulinic acids indicated that the degradation of furfural and HMF during the steam pre-

treatment of bagasse was very limited. The main quantitative differences between the different hydrolysates are a consequence of the different conditions used for pretreating the bagasse prior to enzymatic saccharification. The hydrolysates produced by pretreatment at 205°C contained higher levels of all sugars and lower levels of all fermentation inhibitors than the hydrolysate produced by pretreatment at 215°C.

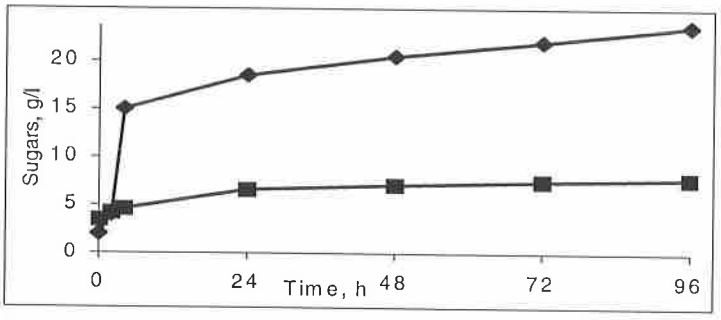

Figure 1. Variation of sugar concentration in steam-pretreated bagasse during enzymatic hydrolysis. Pretreatment conditions: 205°C, 10 min. Glucose (♦), xylose (▪).

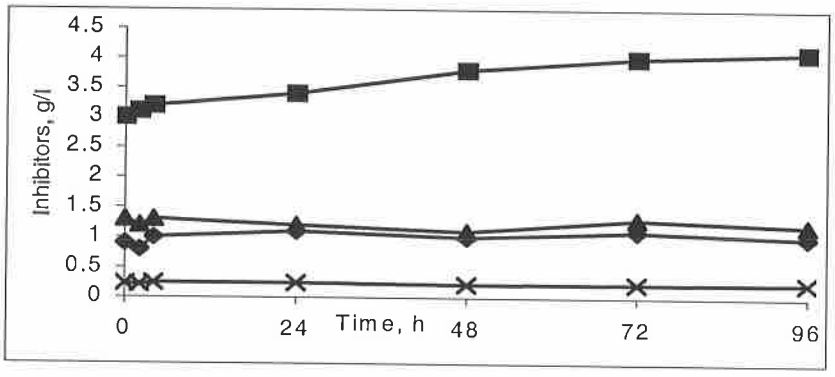

Figure 2. Variation of concentration of inhibitory compounds during enzymatic hydrolysis. Pretreatment conditions: 205°C, 10 min. Acetic acid (▪), formic acid (♦), furfural (▲) and HMF (x).

Table 3. Chemical composition of the hydrolysates, g/l.

Severity (T,°C; t,min)	Glucose	Xylose	Arabinose /Mannose	Acetic acid	Formic acid	HMF	Furfural	Phenolic compounds
3.8(205; 5)	22.3	9.0	2.3	3.7	0.3	0.20	0.6	3.7
4.1(205; 10)	23.6	7.8	2.1	3.9	0.9	0.25	1.3	4.1
4.1(215; 5)	21.4	7.1	1.5	4.3	1.1	0.35	1.5	4.5
4.4(215; 10)	22.3	5.4	1.4	4.4	1.4	0.60	1.9	4.5

The calculated maximum glucose yield after hydrolysis was 34.7 g per 100 g DM. It was achieved at 205°C and 10 min. Based on the raw material analysis, this value corresponds to 75.5 % of the theoretical yield.

The yield of fibre residue after hydrolysis was relatively high and ranged from 45.4 to 62.8 g per 100 g of raw bagasse (data not shown). Since the lignin content in the raw material was 20.7 g per 100 g bagasse, there should be a potential for increasing the yields of sugars.

Detoxification

The detoxification methods for bagasse acid hydrolysates most frequently described in the literature include active charcoal treatment (Roberto *et al.*, 1991 and Gong *et al.*, 1993), ion exchange resin treatment (Watson *et al.*, 1984; van Zyl *et al.*, 1991; Gong *et al.*, 1993) and neutralisation and pH adjustments with different bases (Watson *et al.*, 1984; van Zyl *et al.*, 1988; Roberto *et al.*, 1991; Gong *et al.*, 1993; Katzen and Fowler, 1994). The scope of ion exchange resin and active charcoal treatments is limited by economical reasons. Detoxification procedures including pH adjustments are different in methodology and controversial in results.

Enzymatic treatment with the phenoloxidase laccase is a new detoxification method, which has proven to be efficient in removal of phenolic compounds from wood hydrolysates (Jönsson *et al.*, 1998; Larsson *et al.*, 1999). However, this method has not been tested in bagasse hydrolysates. Since the bagasse hydrolysate obtained after pretreatment at 215°C during 10 min contained very high amounts of phenols, it was chosen to be detoxified by laccase treatment.

Laccase treatment led to the removal of around 76 % of the phenolic compounds from the hydrolysate (Table 4). This high degree of phenol removal is in agreement with the data found in the literature (Jönsson *et al.*, 1998; Larsson *et al.*, 1999). The content of glucose, acetic acid and the furan derivatives furfural and HMF was not significantly affected by the enzymatic treatment. The apparent removal of these compounds, as well as the phenolic compounds in the laccase control can be explained by the dilution effect.

Table 4. Effect of detoxification on the hydrolysate composition, % of the concentration of inhibitory compounds left after detoxification. Pretreatment conditions: 215°C, 10 min.

Method	Glucose	Acetic acid	Furfural	HMF	Phenolic compounds
Laccase	91	87	94	92	24
Laccase control	91	89	94	92	89
None	100	100	100	100	100

Fermentability

The fermentation of all hydrolysates was inhibited compared to the reference fermentation. The strongest inhibition was observed in the hydrolysates obtained from the bagasse pretreated at 215°C, especially when the residence time was 10 min (Fig. 3A). The ethanol concentration in that hydrolysate after 6 and 12 h was only 27 and 46 %, respectively, of that in the reference fermentation. The volumetric productivity was 3.7 times lower than in the reference (Table 5). This strong inhibition of the fermentation was due to the high concentrations of inhibitory compounds in the hydrolysate.

A less strong inhibition was observed in the fermentation of the hydrolysates obtained after pretreatment at 205°C (Fig. 3A). The lag phase was shorter, glucose was consumed faster and ethanol yield and volumetric productivity were higher than in the hydrolysates obtained after more harsh pretreatment conditions (Table 5). Both hydrolysates produced after pretreatment at 205°C had a very similar fermentation pattern (Fig 3A). However, the 5-min hydrolysate fermented slightly faster and gave a slightly lower final ethanol concentration than the 10-min hydrolysate. That was due to the lower concentration of inhibitors in the 5-min hydrolysate and the higher glucose concentration in the 10-min hydrolysate.

Since the ethanol concentration after 24 h in all hydrolysates did not differ very much from the reference fermentation, it can be affirmed that the inhibitory compounds present in bagasse hydrolysates caused a decreased rate of fermentation, but they did not affect the final ethanol concentration.

The effect of detoxification by treatment with laccase on the fermentability of the hydrolysate obtained after pretreatment at 215°C and 10 min, is shown in Fig. 3B. The detoxification led to an evident improvement of the fermentability. The ethanol concentration in the detoxified hydrolysate after 12 h of fermentation was 2.3 times higher than in the undetoxified hydrolysate. The ethanol yield, the volumetric productivity and the glucose consumption were also considerably improved after detoxification (Table 5).

The positive effect of the laccase treatment on the fermentability of bagasse hydrolysates can be attributed to the removal of phenolic compounds (Jönsson *et al.*, 1998; Larson *et al.*, 1999)). Since the concentrations of other toxic compounds, such as furan derivatives and aliphatic acids were not affected by the detoxification method described in this work, there is still potential for increasing the fermentability of bagasse hydrolysates by detoxifying them using methods directed to the removal of also these compounds.

This study has demonstrated that pretreatment of sugarcane bagasse by steam explosion at 205°C followed by enzymatic hydrolysis gives hydrolysates which can be fermented to ethanol more efficiently than hydrolysates obtained after pretreatment at 215°C. It has also shown the feasibility of using laccase treatment as a detoxification method for bagasse hydrolysates.

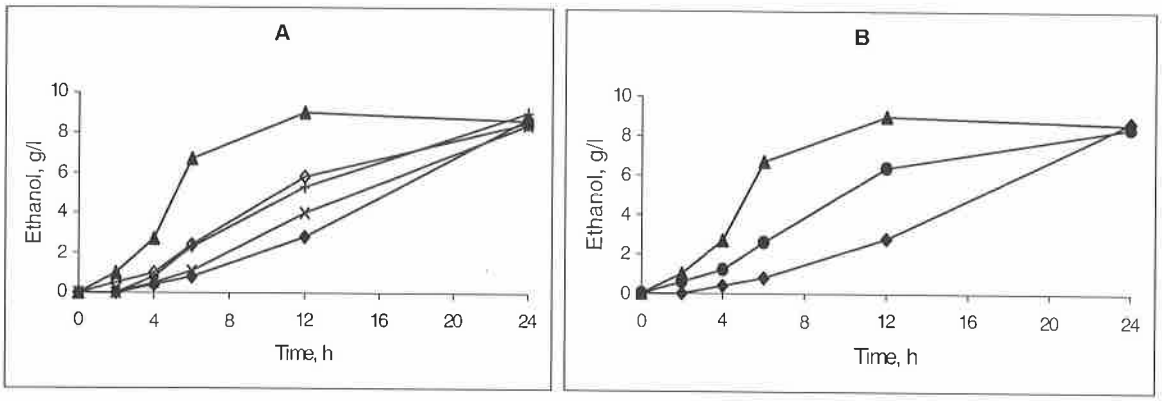

Figure 3. Ethanol production during fermentation of hydrolysates. (A) Undetoxified hydrolysates. Pretreatment conditions: 205°C, 5 min, ◇; 205°C, 10 min, +; 215°C, 5 min, x; 215°C, 10 min, ♦; reference, ▲. (B) Effect of detoxification: undetoxified 215ºC-10 min hydrolysate, ♦; detoxified 215ºC-10min hydrolysate ●; reference ▲.

Table 5. Fermentability of bagasse hydrolysates by yeast.

Hydrolysate	Glucose consumption, g/(lh)	Ethanol yield, g/g	Ethanol productivity, g/(lh)
205-5	0.96	0.26	0.40
205-10	0.88	0.23	0.38
215-5	0.83	0.19	0.28
215-10	0.79	0.13	0.23
Detoxified 215-10	1.07	0.29	0.43
Reference	1.55	0.42	1.05

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REFERENCES

Cuzens, J. C. & Miller, J. R. (1996). Acid hydrolysis of bagasse for ethanol production. *Renewable Energy,* **10**, 285-290.

Dekker R. F. H. & Wallis, A. F. A. (1983). Enzymatic saccharification of sugarcane bagasse pretreated by autohydrolysis steam explosion. *Biotechnol. Bioeng.*, **25**, 3027-3048.

Delgenes, J. P., Moletta, R. & Navarro, J. M. (1996). Effects of lignocellulose degradation products on ethanol fermentation of glucose and xylose by *S. cerevisiae, Z. mobilis, P. stipitis* and *C. shehatae. Enzyme Microb. Technol.*, **19**, 220-225.

Galbe, M. (1994). Ethanol from wood: An experimental study of pretreatment and hydrolysis process simulation. PhD Thesis. Department of Chemical Engineering I, Lund University, Sweden.

Glasser W. G. & Wright, R. S. (1998). Steam-assisted biomass fractionation. II. Fractionation behavior of various biomass resources. *Biomass and Bioenergy*, **14**, 219-235.

Gong, C. S., Chen, C. S. & Chen, L. F. (1993). Pretreatment of sugar cane bagasse hemicellulose hydrolyzate for ethanol production by yeast. *Appl. Biochem. Biotechnol.*, **39/40**, 83-88.

Gregg, D. & Saddler, J. N. (1996) A techno-economic assessment of the pretreatment and fractionation steps of a biomass-to-ethanol process. *Appl. Biochem. Biotechnol.*, **57/58**, 711-727.

Hägglund, E. (1951), in Chemistry of Wood, Academic Press, New York, NY.

ICIDCA. (1987) La industria de los derivados de la caña de azúcar. Editorial Científico-Técnica. La Habana.

Jönsson, L. J., Palmqvist, E., Nilvebrant, N.-O. & Hahn-Hägerdal, B. (1998) Detoxification of wood hydrolysates with laccase and peroxidase from the white-rot fungus *Trametes versicolor. Appl. Microbiol. Biotechnol.*, **49**, 691-697.

Kaar, W. E., Gutierrez, C. V. & Kinoshita, C. M. (1998) Steam explosion of sugarcane bagasse as a pretreatment for conversion to ethanol. *Biomass and Bioenergy,* **14**, 277-287.

Katzen, R. & Fowler, D. E. (1994). Ethanol from lignocellulosic wastes with utilization of recombinant bacteria. *Appl. Biochem. Biotechnol.*, **45/46**, 697-707.

Larsson, S., Palmqvist, E., Hahn-Hägerdal, B., Tengborg, C., Stenberg, K., Zacchi, G. & Nilvebrant, N.-O. (1999) The generation of fermentation inhibitors during dilute acid hydrolysis of softwood. *Enzyme Microb. Technol.,* **24**, 151-159.

Larsson, S., Reimann, A., Nilvebrant, N.-O. and Jönsson, L. J. (1999) Comparison of different methods for the detoxification of lignocellulosic hydrolysates of spruce. *Appl. Biochem. Biotechnol.*, **77/79**, 91-103.

Morjanoff, P. J. & Gray, P. P. (1987) Optimization of steam explosion as a method for increasing susceptibility of sugarcane bagasse to enzymatic saccharification. *Biotechnol. Bioeng.*, **29**, 733-741.

Olsson, L. (1994) Ethanol production from lignocellulosic materials: Fermentation and on-line analysis. PhD. Thesis. Department of Applied Microbiology, Lund University, Sweden.

Palmqvist, E. (1998) Fermentation of lignocellulosic hydrolysates: inhibition and detoxification. PhD Thesis. Department of Applied Microbiology, Lund University, Sweden.

Palmqvist, E., Hahn-Hägerdal, B., Galbe, M. & Zacchi, G. (1996) The effect of water-soluble inhibitors from steam-pretreated willow on enzymatic hydrolysis and ethanol fermentation. *Enzyme Microb. Technol.,* **19**, 470-476.

Roberto, I. C., Lacis, L. S., Barbosa, M. & de Mancilha, I. M. (1991) Utilization of sugar cane bagasse hemicellulosic hydrolysate by *Pichia stipitis* for the production of ethanol. *Process Biochem.* **26**, 15-21.

Singleton, V., Orthofer, R. and Lamuela-Raventós, R. (1999) Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteau Reagent. *Methods Enzymol.* **299**, 152-178.

Stenberg, K.,Tengborg, C., Galbe, M. & Zacchi, G. (1998) Optimisation of steam pretreatment of SO_2-impregnated mixed softwoods for ethanol production. *J. Chem. Technol. Biotechnol.*, **71**, 299-308.

Van Zyl, C., Prior, B. A. & Du Preez, J. C. (1988) Production of ethanol from sugar cane bagasse hemicellulose hydrolysate by *P. stipitis. Appl. Biochem. Biotechnol.*, **17**, 357-369.

Van Zyl, C., Prior, B. A. & Du Preez, J. C. (1991) Acetic acid inhibition of D-xylose fermentation by *P. stipitis. Enzyme Microb. Technol.*, **13**, 82-86.

Watson, N. E., Prior, B. A. & Lategan, P. (1984) Factors in acid treated bagasse inhibiting ethanol production from D-xylose by *P. tannophilus. Enzyme Microb. Technol.*, **6**, 451-455.

Production and characterization of *Bacillus pumilus* alkaline xylanases for the paper industry

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ABSTRACTS

Alkaline active xylanases produced from four different strains of *Bacillus pumilus* were characterized. The optimal pH and temperature for maximum enzyme activity were pH 9.0 and 55°C for strain 5_2, pH 9.0 and 60°C for strains 13_a and 5_{14}, and pH 8.0 and 60°C for strain 4_a. Under these conditions the following activities were found for the crude extracts after 2.5 min in the presence of 1% xylan (birchwood): 111 $U.mL^{-1}$, 482 $U.mL^{-1}$, 105 $U.mL^{-1}$, and 235 $U.mL^{-1}$, respectively, for the four strains. Around 40% of the xylanase activity remaining after 2 hours at 40°C for the enzymes of strain 5_2 and 60% for the other three strains. Stability at 50°C was improved by addition of glycerol. Taking into account the conditions under which kraft pulps are bleached during the manufacture of paper, xylanases from *B. pumilus* exhibit favorable potential for application to bleaching in the paper making process.

Keywords: xylan, xylanase, *B. pumilus*, kraft pulp bleaching

INTRODUCTION

Bleaching in the kraft papermaking process consists in decolorizing and removing the highly colored residual lignin from washed pulp. Most present bleaching sequences are based on elemental chlorine (Cl_2), chlorine dioxide, and alkaline extraction of the pulp (Tremblay and Archibald, 1993). Viikari *et al.* (1986) reported on the ability of xylanase to facilitate subsequent chemical bleaching of kraft pulps, known as xylanase prebleaching. This results in a lower chlorine dosage, a lower chemical cost, and lower chloro-organic concentrations in pulp and effluent. This discovery led to extensive further studies (Clark *et al.*, 1990; Jurasek and Paice, 1988; Viikari *et al.*, 1987; Viikari *et al.*, 1994; Yang and Eriksson, 1992).

Although many bacteria and fungi have been studied for xylanase production (Bailey *et al.*, 1993; Shoham *et al.*, 1992; Raj and Chandra, 1995; Angelo *et al.*, 1997), the several xylanases commercially available are active at a neutral or acidic pH and their optimum temperature for activity is below 45°C. Enzymes, which are active under alkaline conditions, have great potential for industrial applications as a bleaching process without any need for changes in pH or temperature (Shoham *et al.*, 1992).

In our previous investigations (Duarte *et al.*, 1997; Duarte *et al.*, 1998a,b; Bim *et al.*, 1998), four different strains of *B. pumilus* were isolated and were potentially able to produce alkaline xylanases at pH 10.0 and 45°C, which are appropriate conditions for the bleaching process. In this report, the characterization of xylanases produced by *B. pumilus* is described. The enzymatic assays were performed at pH and temperature ranges close to those at which kraft pulps are bleached.

EXPERIMENTAL

Microorganism

B. pumilus strains 5_2, 5_{14}, 13_a, and 4_a were isolated from wood decomposition material and were maintained in a previously described media (Mandels and Stenberg, 1976), with xylan as the carbon source, which contained (g/L): birchwood xylan (Sigma), 10.0; peptone, 1.0; Tween 80, 1.0; $(NH_4)_2SO_4$, 1.4; KH_2PO_4, 2.0; urea, 0.3; $CaCl_2$, 0.3; $MgSO_4.7H_2O$, 0.3; agar-agar, 20.0; and solutions of the following salts (mg/L): $FeSO_4.7H_2O$, 5.0; $MnSO_4.H_2O$, 1.6; $ZnSO_4.7H_2O$, 1.4; and $CoCL_2$, 2.0. The pH was adjusted to 10.0 with 2 N NaOH. The cultures were grown at 45°C for 48 hours.

Inoculum preparation
The cultures were transferred to 250 mL Erlenmeyer flasks, containing 50 mL of the liquid media described above, and incubated at 45°C in a rotary shaker (250 rpm) during 20 hours. Cell concentrations of the different cultures were adjusted to 3% T (transmittance) in relation to distilled water at 600 nm.

Enzyme production
The initial number of cells for fermentation was equivalent to 10^8.mL^{-1}. For xylanase production, the bacteria were grown in the media described, with optimized xylan and peptone concentrations for each strain as shown in Table 1. These concentrations were determined by means of response surface methods in a previous study (Duarte *et al.*, 1998b). Fermentation was carried out in shake flasks under the same inoculum conditions (20 h; 45°C; 250 rpm). The fermented media was centrifuged for 15 min at 12000 x g to the assay activity.

Enzyme activity assay
Xylanase activity was assayed with birchwood xylan 1% solution as the substrate, as described by Bailey *et al.* (1992), and the amount of reducing sugars released was determined by the dinitrosalicylic acid method (Miller, 1959).

Filter paper cellulase (FPase) activity was assayed as an indicator of overall cellulolytic activity and determined according to IUPAC recommendations (Ghose, 1987), using Wathman No. 1 filter paper (50 mg) as a substrate in 100 mM glycine-NaOH buffer, pH 10.0. One unit of enzyme activity was defined as 1μmol of xylose or glucose equivalents produced per minute under the given conditions.

Effect of pH and temperature on xylanase activity
The effect of pH on xylanase activity was studied in the following buffers (100 mM): sodium phosphate, pH 8.0; glycine-NaOH, pH 9.0 and pH 10.0; and carbonate-bicarbonate, pH 11.0. The reaction mixture containing 0.9 mL of xylan solution and 0.1 mL of the crude enzyme preparations was incubated at 35-55°C or until activity declined, and enzyme activity was determined for different times.

Effect of temperature and pH on xylanase stability
The remaining xylanase activity was determined after preincubation of the crude enzyme preparations at 30-60°C in buffers of optimal activity, without substrate, for 1, 2, 4 and 6 h. The effect of pH on xylanase stability was measured over the pH range of 8.0 to 11.0 at a defined optimal temperature. After incubation, residual activity was determined under optimal assay conditions for each strain.

Protein measurement
Protein concentration was measured by the method of Sedmak and Grossberg (1977). Bovin serum albumin was used as a standard. The results were used to calculate specific activity.

Kinetic determinations
K_m and $V_{máx.}$ values were determined from Lineweaver-Burk plots (Lineweaver and Burk, 1934) using xylan concentrations varying from 0.5 to 3.0% (w/v).

RESULTS AND DISCUSSION

Strain characterization and previous results
We had previously isolated four microorganisms, identified as different strains of *B. pumilus*, which were able to produce xylanase under alkaline conditions (Duarte *et al.*, 1997). All microorganisms are capable of growing at 40°C; however, the 13_a strain grows at 55°C. Enzyme production was observed at pH 8.0 to 11.0, but higher levels were observed at pH 10.0.

During the initial studies the strains showed different requirements for xylan or peptone, as shown in Table 1 (Duarte *et al.*, 1998b). We also defined that the cultivation time for enzyme production must be around 16-20h.

Cellulase production from *B. pumilus* strains
FPase activity was assayed as an indicator of overall cellulolytic activity in crude enzyme preparations obtained from cultivations at pH 10.0 and 45°C. The results are shown in Table 2. The cellulolytic activity found was lower than 0.01 FPU.mL^{-1} for all strains.

Xylanase solutions for industrial uses must be cellulase-free. Contaminating cellulase in commercial xylanase preparations can result in a loss of fiber strength (Paice *et al.*, 1992). When cellulase activity is very low, culture filtrate can be used for treating pulp without further purification (Yang *et al.*, 1995). Our results indicate that enzymes produced by *B. pumilus* strains meet this requirements.

Table 1. Ideal concentration of xylan and peptone in the fermentation media, and xylanase activity for different strains of *B. pumilus* obtained in previous experiments.

B. pumilus strains	Optimized Medium			Original Medium* xylanase activity ($U.mL^{-1}$)
	xylan (g/L)	peptone (g/L)	xylanase activity ($U.mL^{-1}$)	
5_2	30.0	6.0	33	3
5_{14}	58.3	6.0	9	2.5
13_a	10.0	10.0	23	1.5
4_a	50.0	10.0	25	4

* xylan = 10 g/L; peptone = 1.0 g/L

Table 2. Cellulase activity from different strains of *B. pumilus* in the presence of filter paper (FP).

	Strain			
	5_2	5_{14}	13_a	4_a
FPase Activity $FPU.mL^{-1}$	0.0076	0.0041	0.0053	0.0059

Effects of temperature and pH on activity and stability

Xylanases from *B. pumilus* strains were tested for the effect of pH and temperature on activity. Initially, the activity was determined after incubation for 5, 10, and 20 min. Maximum xylanase activity was found after 5 min at pH 9.0 and 55°C for strain 5_2 (132 $U.mL^{-1}$); pH 9.0 and 60°C for strains 13_a and 5_{14} (436 $U.mL^{-1}$ and 91 $U.mL^{-1}$, respectively), and pH 8.0 and 60°C for strain 4_a (185 $U.mL^{-1}$), (Table 3). At pH 10.0, enzymatic activities were very low and show little variation in the temperature range studied. At pH 11.0 xylanase activity decreased rapidly.

Measurement of enzymatic activities at intermediary times (2.5 and 7.5 min) was carried out only at the pH and temperature of maximum activity. When incubation time was decreased to 2.5 min, xylanase activity increased for all cases, showing a brief time period for optimal activity. The same was observed for specific activity (Table 4).

Several reports have revealed that the optimum pH for the activity of xylanolytic enzymes produced by other bacteria not usually exceed pH 7.0, as in the case of enzymes from *Bacillus* sp BP-23 (Blanco *et al.*, 1995) *Bacillus* sp (Ynag *et al.*, 1995), *Thermotoga maritima* (Bronnenmeier *et al.*, 1995) and *Thermoanabacterium* sp (Shao *et al.*, 1992). In these cases, maximum enzymatic activity was observed, at pH 5.5 to 7.5, pH 7.0, pH 6.0 to 7.5, and pH 5.4, respectively. These bacteria were able to produce active enzymes at temperatures between 45°C and 60°C, and only *T. maritima* produces xylanases which are highly thermostable at 95°C. Comparable enzymatic levels obtained in this study for *B. pumilus* 5_2 (482 U/mL) and 4_a (393 U/mL) were observed for *T. maritima* (585 U/mL) and *Thermoanabacterium* (393 U/mL). However, the optimum pH range for xylanase activity of *B. pumilus* was higher (pH 8.0 to pH 9.0). The latter are more suitable for application of enzymes at different stages of the bleaching process without the need for changes in pH.

After the enzymes were preincubated at several temperatures and pHs during different periods of time, the xylanase activity tests were carried out under the optimal conditions determined for each *B. pumilus* strain. According to results, the enzymes from *B. pumilus* 5_2 retained around 40% of their original activity after 2 h, while 60% was retained by the others strains (Figure 1). However, activity decreased gradually over time, with 30% of the activity remaining for strain 5_2, 50% for strain 5_{14} and 40% for strains 13_a and 4_a after 6 h (results not shown).

At 50°C and 60°C the enzymes were denatureted very rapidly, which is not appropriate for industrial purposes. However, according to literature, this problem is solved by an addition of a 50% w/w glycerol solution (Angelo *et al.*, 1997). Our results confirmed this statement since the stability of the xylanases from *B. pumilus* strains 5_2, 13_a and 4_a could be improved by glycerol, and there were increases from 8% to 56%,

from 15% to 36% and from 25% to 66%, respectively, after 2 h at 50°C for these strains (Table 5). On the other hand, this protective effect of glycerol wasn't observed in enzymes from *B. pumilus* 5_{14}.

Table 3. Effects of temperature and pH on the xylanolytic activity of ***B. pumilus*** strains after 5 min of incubation.

pH	strain	Enzyme Activity (U.mL^{-1})						
		35°C	40°C	45°C	50°C	55°C	60°C	65°C
8	5_2	46	68	94	101	118		
	5_{14}	41	51	59	83	90		
	13_a	136	202	225	184	196		
	4_a	80	104	128	148	172	**185**	
9	5_2	46	61	88	108	**132**	117	
	5_{14}	32	44	57	71	**91**	93	
	13_a	131	171	219	320	375	**436**	351
	4_a	68	93	100	101	108		
10	5_2	10	14	17	21	16		
	5_{14}	21	18	30	24	39		
	13_a	46	61	71	98	74		
	4_a	25	39	62	44	40		
11	5_2	2	2	1	0.1	0.4		
	5_{14}	6	6	6	6	5		
	13_a	3	4	1	1	1		
	4a	6	6	5	6	69		

Table 4. Effects of incubation time on the xylanase activity of different *B. pumilus* strains.

Incubation time (min)	***B. pumilus*** 5_2 (pH 9; 55°C)*		***B. pumilus*** 13_a (pH 9; 60°C)*		***B. pumilus*** 5_{14} (pH 9; 60°C)*		***B. pumilus*** 4_a (pH8; 60°C)*	
	enzyme activity	specific activity	enzyme activity	specific activity	enzyme activity	specific activity	enzyme activity	specific activity
2.5	**111**	0.42	**482**	2.60	**105**	0.58	**235**	1.00
5	105	0.40	406	2.19	84	0.46	183	0.78
7.5	101	0.38	320	1.73	67	0.37	170	0.72
10	98	0.37	282	1.52	57	0.31	157	0.67

* Optimal conditions for enzyme activity

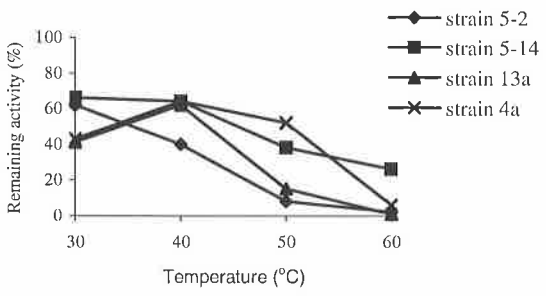

Figure 1. Xylanase activity remaining after incubation for 2 h at different temperatures.

The results obtained after incubation of the enzymes at different pH values did not show variations in xylanase activity after 2 h at pH 8.0 and pH 9.0 for enzymes of the several strains (Figure 2). However, at pH 10.0 the activity began to decrease rapidly and inactivation was observed at pH 11.0.

Table 5. Effect of 50% glycerol on xylanase stability at 50°C on birch xylan of enzymes from different *B. pumilus* strains. Relative activity (%), after 2 h of incubation.

Strain	with glycerol	without glycerol
B. pumilus 5_2	56	8
B. pumilus 5_{14}	25	38
B. pumilus 13_a	36	15
B. pumilus 4_a	66	25

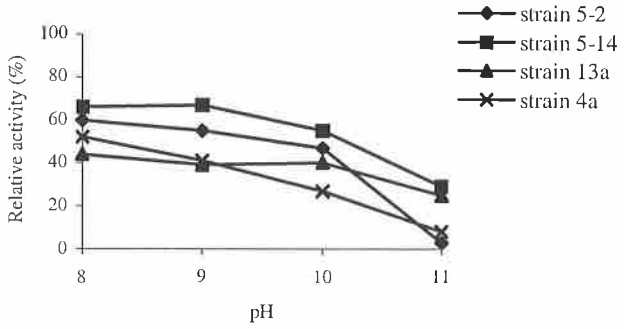

Figure 2. Xylanase activity remaining after incubation for 2 h at different pH values.

Kinetic determinations

Kinetic parameters of the four *B. pumilus* xylanases in birchwood xylan are summarized in Table 6. The crude enzymes produced from different bacterial strains showed quite different K_m and V_{max} values under the conditions studied.

Table 6. Kinetic parameters of *B. pumilus* xylanases in birchwood xylan.

B. pumilus	K_m (mg/mL)	$V_{máx.}$ (μmol/mL/min)
5_2	8.9	178.57
5_{14}	1.1	112.36
13_a	33.3	1666.67
4_a	71.4	1428.57

CONCLUSIONS

The conditions used in our study on the activity and stability of xylanases from *B. pumilus* strains were based on the temperature, pH and time ranges of bleaching in the kraft pulp process. Taking into account industrial conditions such as dosage between 2 and 5 units (U) per gram of dry pulp, alkaline pH, temperature around 50°C and 2 h for reaction (Daneault *et al.*, 1994; Wong *et al.*, 1997), the enzymes from *B. pumilus* exhibit favorable potential for application to the bleaching of kraft pulps.

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REFERENCES

Angelo, R., Aguirre, C., Curotto, E., Esposito, E., Fontana, J. D., Baron, M., Milagres, A. M. F. & Durán, N. C. (1997). Stability and chemical modification of xylanase from *Aspergillus* sp. (2M1 strain). *Biotechnology ppl.Biochem.*, **25**, 19-27.

Angelo, R., Aguirre, C., Curotto, E., Esposito, E., Fontana, J. D., Baron, M., Milagres, A. M. F. & Durán, N. (1997). Stability and chemical modification of xylanase from *Aspergillus* sp (2MI strain). *Biotechnology ppl.Biochem.*, **25**, 19-27.

Bailey, M. J., Biely, P. & Poutanen, K. (1992). Interlaboratory testing of methods for assay of xylanase activity. *J. of Biotechnol.*, **23**, 257-270.

Bailey, M. J., Buchert, J. & Viikari, L. (1993). Effect of pH on production of xylanase by *Trichoderma reesei* on xylan and cellulose-based medium. *Appl. Microbiol. Biotechnol.*, **40**, 224-229.

Bim, M. A., Tagliari, C. V., Duarte, M. C. T., Portugal, E. P., Ponezi, A. N. & Franco, T. T. (1998). Optimization and extraction of an alkaline xylanase produced of *Bacillus pumilus*. *In Fifth European Workshoop on Lignocelullosics and Pulp*, Proceedings, pp. 27-31.

Blanco, A., Vidal, T., Colom, J. F. & Pastor, F. I. J. (1995). Purification and properties of xylanase A from alkali-tolerant *Bacillus* sp strain BP-23. *Appl. Env..Microbiol.*, **61**, 4468-4470.

Bronnenmeier, K., Kern, A., Liebl, W. & Staudenbauer, L. (1995). Purification of *Thermotoga maritima* enzymes for the degradation of cellulosic materials. *Appl. Env. Microbiol.*, **61**, 1399-1407.

Clark, T. A., McDonald, A. G. & Senior, D. J. (1990). Mannanase and xylanase treatment of softwood chemical pulps: effects of pulp properties and bleachability. In: *Biotechnology in Pulp and Paper Manufacture* (T. Kent Kirk and Ho-Min Chang, eds). Ch 14, Butterworth-Heinemann, Raleigh, NC, pp. 153-167.

Daneault, C., Leduc, C. & Valade, J. L. (1994). The use of xylanases in kraft pulp bleaching: a review. *Tappi J.*, **77**, 125-131.

Duarte, M. C. T. , Portugal, E. P., Ponezi, A. N. Franco, T. T. (1998b). Otimização da produção de xilanases alcalinas por diferentes cepas de *Bacillus pumilus*. *In XII SINAFERM – Simpósio Nacional de Fermentações*, UFSCAR, *Anais*, MSC-F3.

Duarte, M. C. T., Portugal, E. P., Ponezi, A. N. & Franco T. T. (1997). Alkalophilic xylanases production from bacteria. *In 5th Brazilian Symposium on the Chemistry of Lignins and Other Wood Components*, Proceedings, pp 340-345.

Duarte, M. C. T., Portugal, E. P., Ponezi, A. N., Bim, M. A., Tagliari, C. V. & Franco, T. T. (1998a) Production and purification of alkaline xylanases by partitioning in aqueous two-phase systems. *Biores. Technol.*, **66**, 49-53.

Ghose, T. K. (1987). Measurement of cellulase activities. *Pure Appl. Chem.*, **59**, 257-268.

Jurasek, L. & Paice, M. G. (1988). Biological treatment of pulps. *Biomass*, **15**, 103-108.

Lineweaver, H. & Burk, D. (1934). The determination of enzyme dissociation constants. *J. Am.Chem. Soc.*, **56**, 658-666.

Mandels, M. & Stenberg, D. (1976). Recent advances in cellulase technology. *J. Ferm. Technol.*, **54**, 267-286.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.*, **31**, 426-428.

Paice, M. G., Gurnagul, N., Page, D. H. & Jurasek, L. (1992). Mechanisms of hemicellulose-directed prebleaching of kraft pulps. *Enz. Microbiol. Technol.*, **14**, 272-276.

Raj, K. C. & Chandra, T. S. (1995). A cellulase-free xylanase from alkali-*tolerant Aspergillus fischeri* Fxn1. *Biotechnol. Lett.*, **17**, 309-314.

Sedmak, J. J. & Grossberg, S. E. (1977). A rapid, sensitive, and versatile assay for protein using Coomasie Brilliant Blue F-250. *Anal. Biochem.*, **79**, 544-552.

Shao, W., Schwartz, Z., Khasin, A., Gat, O., Zosim, Z. & Rosenberg, E. (1992). Delignification of wood pulp by a thermostable xylanase from *Bacillus stearothermophilus* T-6. *Biodegradation*, **3**, 207-218.

Shoham, Y., Schwartz, Z., Khashin, A., Gat, O., Zosim, Z. & Rosenberg, E. (1992). Delignification of wood pulp by a thermostable xylanase from *Bacillus stearothermophilus* T-6. *Biodegradation*, **3**, 207-218.

Tremblay, L. & Archibald, F. (1993)Production of a cloned xylanase in *Bacillus cereus* and its performance in kraft pulp prebleaching. *Can. J. Microbiol.*, **39**, 853-860.

Viikari, L., Kantelinen, A., Sundquistand, J. & Linko, M. (1994). Xylanases in bleaching: from an idea to the industry. *FEMS Microbiol.*, **13**, 335-350.

Viikari, L., Ranua, M., Kantelinen, A., Linko, M. & Sundquistand, J. (1987). Application of enzymes in bleaching. *In 4th International Symposium on Wood and Pulping Chemistry*, EuCePa, Proceedings, **1**, pp 151-154.

Viikari, L., Ranua, M., Kantelinen, A., Sundquistand, J. & Linko, M. (1986). Bleaching with enzymes. *In Third International Conference Biotechnology Pulp Paper Industry*, Proceedings, pp. 67-69.

Wong, K. K., Jong, E., Saddler, J. N. & Allison, R. W. (1997). Mechanisms of xylanase aided bleaching of kraft pulps. Part 1: Process parameters. *Appita*, **50**, 415-422.

Yang, J. L., Lou, G. & Eriksson, K. L. (1992). The impact of xylanase on bleaching of kraft pulp. *Tappi J.*, **75**, 95-101.

Yang, V. W., Zhuang, Z., Elegir, G. & Jeffries, T. W. (1995). Alkaline-active xylanase produced by an alkaliphilic *Bacillus* sp isolated from kraft pulp. *J. of Ind. Microbiol.*, **15**, 434-441.

Protein adsorption on alkali-soluble lignin derived from acid-catalysed steam-exploded *Eucalyptus grandis*

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ABSTRACT

Steam explosion of *Eucalyptus grandis* was carried out under eleven distinct pretreatment conditions (200-210°C, 2-5 min and 0.25-0.5% H_2SO_4) to optimize lignin recovery. The lignin component of each water insoluble fraction was then extracted with mild alkali (NaOH 0.1 M) and recovered after acidification with dilute H_2SO_4. To investigate the adsorption profile of *Trichoderma reesei* cellulases onto steam-exploded lignin, the alkali-soluble lignin (ASL) derived from pretreatment of acid-impregnated chips at 210°C for 5 min was selected. Bovine serum albumin (BSA) was used as an experimental control. Compared to cellulases, BSA adsorbed more strongly onto lignin and this was partly attributed to its relatively higher hydrophobicity and pI (isoelectric point). At 50°C and neutral pH for a loading of 25 μg protein/mg lignin, nearly 38% of the BSA was adsorbed while *T. reesei* cellulases had an adsorption efficiency of only 22%. However, at lower adsorption temperatures of 4 and 20°C, there was no difference between the adsorption profile of both protein samples. When the concentration of cellulases was raised from 5 to 50 μg enzyme/mg lignin, there was a considerable effect on the amount of adsorbed protein, suggesting that this lignin fraction could adsorb protein concentrations beyond the limits tested in this study. We also attempted to follow the adsorption profile over a 210 min contact time at 50°C and the amount of adsorbed protein increased with time, particularly at the highest protein loading of 50 μg enzyme/mg lignin.

Keywords: lignin, cellulases, steam explosion, Eucalyptus

INTRODUCTION

The utilization of lignocellulosic materials as chemical feedstocks for fuels and chemicals is hindered by the low efficiency that is obtained when these materials are hydrolyzed by chemical and enzymatic treatments. Previous results have suggested that the enhanced accessibility of enzymes towards steam-treated substrates is correlated to the relative easiness in the extraction of hemicellulose and lignin after pretreatment (Ramos *et al.*, 1992). The extraction results in a substantial increase in the available surface area of the substrate (substrate porosity) (Wong *et al.*, 1988). This in turn leads to a corresponding increase in hydrolysis efficiency. On the other hand, the effect of other structural factors such as cellulose crystallinity and degree of polymerization may also be important in the increase in hydrolysis yields (Sinitsyn *et al.*, 1991).

Although several substrate-related factors have been suggested to explain the incomplete enzymatic hydrolysis of cellulosic substrates at high substrate concentrations, enzyme-related factors may also contribute significantly to the incomplete hydrolysis. These include the accumulation of sugars during hydrolysis leading to end-product inhibition (Ramos *et al.*, 1992), the inactivation of key components of the cellulase system and the irreversible and/or non-specific adsorption of cellulases onto the hydrolysis residue (Ooshima *et al.*, 1990).

The actual role of lignin during the enzymatic hydrolysis of steam-treated substrates is still unclear. Lignin apparently coats the surface of steam-treated fibers and hinders the accessibility of cellulases to the target substrate (Ramos *et al.*, 1999). Also, the adsorption pattern of both cellulases and β-glucosidases increases with the pretreatment severity in which the steam-treated material is obtained. In the specific case of cellulases, this observation was also attributed to enzyme inactivation and/or nonspecific binding onto lignin fragments, reason for what enzyme recycling proceeds better if lignin is previously removed from the steam-treated substrate (Ramos *et al.*, 1993).

The optimum steam treatment conditions for enhanced enzymatic hydrolysis and recovery yield of *Eucalyptus grandis* have been determined (Emmel, 1999). In general, the more drastic the pretreatment conditions, the lower the recovery yield of the water-soluble fraction and the better enzymatic accessibility

towards the resulting substrate. A subsequent alkaline extraction of steam-exploded substrates was shown to be beneficial because the alkaline process resulted in (a) better separation of the majority of the lignin component for further processing, (b) increase in the relative cellulose content of the steam-treated material, and (c) improvements in further steps of enzyme recycling (Ramos *et al.*, 1993). Although alkaline washing could remove a substantial amount of the original lignin present in steam-treated eucalyptus, the efficiency of enzymatic hydrolysis was only marginally increased after alkaline extraction. The redistribution of the alkali-insoluble lignin was then suggested as being responsible for the relatively lower susceptibility of the alkali-washed, steam-exploded substrates towards the enzymatic hydrolysis (Ramos *et al.*, 1992, 1999).

In this work, the alkali-soluble lignin component of water-washed steam-treated eucalyptus was isolated and tested for its capability to adsorb protein samples such as bovine serum albumin and *Trichoderma reesei* cellulases. Our goal was to show that this alkali-soluble lignin is a chemically modified lignin type (Emmel, 1999) and that its redistribution onto the surface of cellulose fibers may restrict hydrolysis by creating a hydrophobic, physical barrier that limits substrate accessibility and causes an irreversible, non-productive adsorption of the enzymes.

EXPERIMENTAL

Lignin preparation from *E. grandis* wood chips

Steam explosion of *E. grandis* was carried out under eleven distinct pretreatment conditions (200-210°C, 2-5 min and 0.25-0.5% H_2SO_4) to optimize lignin recovery. The lignin component of each water insoluble fraction was then extracted with mild alkali (NaOH 0.1 M) and recovered after acidification with dilute H_2SO_4. Increased alkali concentrations (1M) and extraction temperatures (100°C) had no effect on the yield obtained after alkali extraction. The carbohydrate-free alkali soluble lignin (ASL) was washed thoroughly with water, oven-dried at 45°C and characterized by HPLC (including size exclusion), UV and FTIR spectroscopy, thermal analysis and H^1-NMR (Emmel, 1999). Higher pretreatment temperatures and residence times resulted in a more extensive depolymerization of lignin. Lignin condensation also occurred at higher residence times in the steam gun and this was shown to be greater than the effect derived from increasing the pretreatment temperature.

Experimental design

To investigate the adsorption profile of *Trichoderma reesei* cellulases onto steam-exploded lignin, the ASL derived from pretreatment of acid-impregnated chips at 210°C for 5 min was selected. Bovine serum albumin (BSA, Sigma) was used as an experimental control, whereas a commercial powdered *T. reesei* cellulase preparation (Sigma) was used in the absence of any exogenous β-glucosidase activity.

Adsorption was carried out under the conditions described in Table 1. The variables used were adsorption temperature, pH, contact time, lignin mass and enzyme concentration. Each adsorption experiment was carried out under continuous agitation, using a rotatory shaker incubator. Lignin was recovered after adsorption by centrifugation and the amount of protein adsorbed was calculated by difference after the adsorption supernatant was assayed for protein, using UV spectroscopy at 280 nm and the Bradford colorimetric assay. Experiments were always carried out in triplicates against protein and lignin blanks.

Table 1. Experimental design for protein adsorption onto lignin.

Conditions	Variables	Level -	Level +
1	pH	4.8	6
2	time (h)	2	6
3	temperature (ºC)	2.5	50
4	mass (mg)	50	150
5	[E] (mg/ml)	0.5	2

RESULTS AND DISCUSSION

Compared to *T. reesei* cellulases, BSA adsorbed more strongly onto lignin and this was partly attributed to its relatively higher hydrophobicity and/or isoelectric point (pI) (Figure 1). At 50°C and neutral pH for a loading of 25 µg protein/mg lignin, nearly 38% of the BSA was adsorbed while *T. reesei* cellulases had an adsorption

efficiency of only 22%. However, at lower adsorption temperatures of 4 and 20°C, there was no difference between the adsorption profiles of both protein samples.

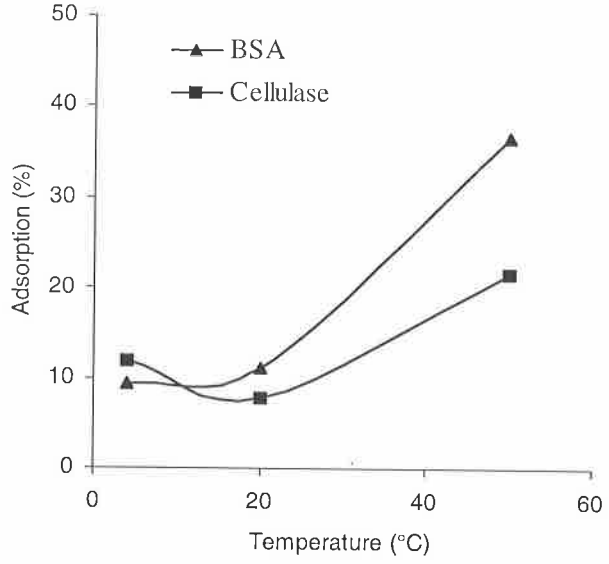

Figure 1. Adsorption of protein as a function of temperature.

The amount of *T. reesei* cellulases that adsorbed onto steam-exploded alkali-soluble lignin increased when the enzyme (protein) concentration was increased from 5 to 50 μg enzyme/mg lignin (or 0.5 to 5 mg enzyme/100 mg lignin) (Figure 2). This was the case for contact times as long as 180 min and could be a result of agitation, since this could produce a gradual increase in the lignin surface area available for adsorption. However, the profile obtained at longer adsorption times of 210 min was distinct from those obtained previously for shorter adsorption times, suggesting severe changes in the mode of interaction between cellulases and lignin after long incubation times such as those usually used for the enzymatic hydrolysis of lignocellulosic substrates.

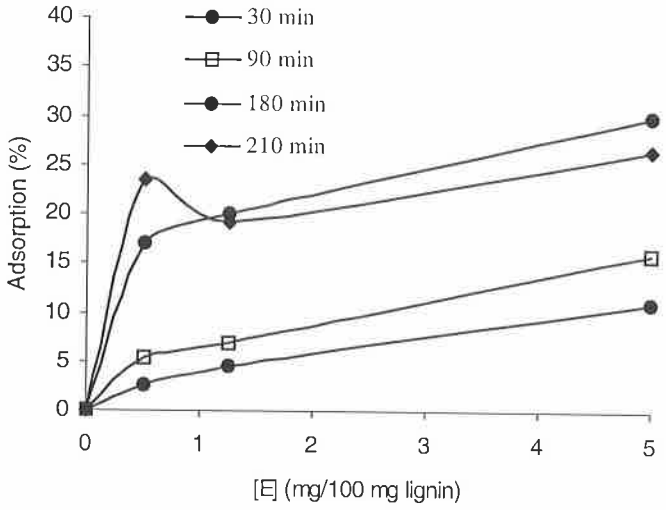

Figure 2. Effect of protein concentration and contact time on the adsorption of *T. reesei* cellulases onto lignin.

The adsorption experiment over a 210 min contact time at 50°C also indicated that the amount of adsorbed protein increased with time, particularly at the highest protein loading of 50 μg enzyme/mg lignin. Therefore, it seems that the ASL sample under investigation is capable of adsorbing a much greater amount of enzyme

and that adsorption is the greatest exactly at the conditions usually used for bioconversion of pretreated cellulosic materials.

Table 2 shows an outline of the experimental design and the results obtained therefore, whereas Figure 3 provides a graphic representation of the trends observed as a result of these experiments, particularly in relation to the most important process parameters for the enzymatic hydrolysis of biomass: temperature, time and pH of the reaction mixture.

Table 2. Adsorption of *T. reesei* cellulases onto steam-exploded alkali-soluble eucalyptus lignin.

	Design					Eads* (%)		Effect
Experiments	*1*	*2*	*3*	*4*	*5*			
1	-	-	-	-	+	4.69	*I*	18.44
2	+	-	-	+	-	10.80	*1*	**7.00**
3	-	+	-	+	-	15.91	*2*	**16.41**
4	+	+	-	-	+	20.63	*3*	**10.86**
5	-	-	+	+	+	11.03	*12*	2.26
6	+	-	+	-	-	14.41	*13*	1.59
7	-	+	+	-	-	28.11	*23*	**5.88**
8	+	+	+	+	+	41.92	*123*	2.96

* **Eads** = amount of enzyme adsorbed onto lignin

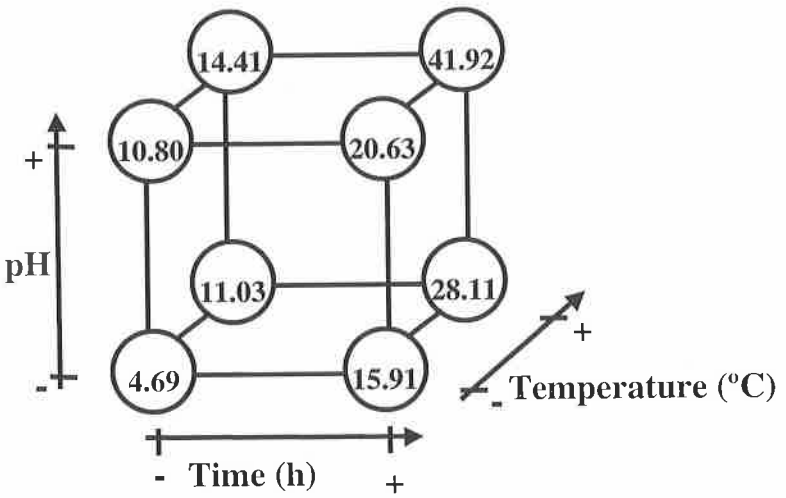

Figure 3. Trends on enzyme adsorption as a function of pH, time and temperature.

As discussed before, the effect of temperature is very important for the adsorption of *T. reesei* cellulases onto steam-exploded alkali-soluble lignin. This observation was confirmed by the strong effect that increased adsorption temperatures had on the experimental design shown above (+10.86 over the average, or a total adsorption yield of 29.30% of the enzyme initially found in solution). However, the greatest effect of an isolated parameter was that obtained for extended contact times between lignin and enzymes found in the cellulase preparation. In this case, a +16.41 over the average could be detected, suggesting that this is indeed the strongest effect that one could expect over enzyme adsorption. On the other hand, the pH had the lowest yet important effect on the adsorption of enzymes onto lignin.

The lignin obtained after acid precipitation of the alkali extract derived from water-washed steam-exploded eucalyptus is a black powder with a large surface area available for adsorption. Many models have been proposed to explain that these lignin particules are tridimensionally arranged from a hard core and that the shearing effects of agitation may brake down these particules to smaller fragments, causing a further increase of surface area for adsorption. Therefore, our results are consistent with this model since the longer the residence time into the shaker incubator, the stronger the effect of shearing over the lignin particles and the greater the availability of surface area for the enzymes to adsorb upon through hydrophobic interactions.

It is also worth noticing that the greatest synergism among the three variables tested in this study (see Figure 3) was that observed for the pair temperature/time (coded *23*), with the pH having little interaction with either one (codes *12* and *13* in Table 2). Once again, since hydrolysis of lignocellulosic materials are usually long and tedious processes, carried out at temperatures usually within 45°C, it is likely that delignification is a desirable operation as it could reduce considerably the amount of non-productive adsorption of the enzymes onto steam-treated substrates.

We are currently finalizing an 2_{III}^{5-2} experimental design to evaluate the adsorption profile of *T. reesei* cellulases onto the above mentioned ASL. Thus, wider ranges of temperature, time, lignin concentration, enzyme loading and pH are being assessed in relation to their effect on the adsorption profile. Once this study is finished, other ASL samples will be tested to investigate which structural properties are more closely involved in these lignin/protein interactions.

CONCLUSIONS

Cellulases adsorb strongly on the alkali-soluble lignin derived from acid-catalyzed steam explosion of *E. grandis*. The highest effect on enzyme adsorption was attributed to long adsorption times. Since hydrolysis of lignocellulosic materials are usually long and tedious processes, it is likely that delignification is a desirable operation as it could improve hydrolysis efficiency and help enzyme recycle by considerably reducing the amount of non-productive and/or irreversible adsorption of enzymes onto the substrate. Finally, it is important to emphasize that the enzyme preparation used in this study is a powdered preparation obtained from Sigma, therefore free of polypeptides and other contaminants usually present in crude extracts. Furthermore, the adsorption levels described in this paper cannot be attributed to β-glucosidases because our commercial enzyme is very poor in this specific activity.

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REFERENCES

Emmel, A. (1999). Caracterização do Efeito da Explosão a Vapor sobre as Propriedades Fisico-químicas da Lignina de *Eucalyptus grandis* Labill. M.Sc thesis, Departamento de Química, Universidade Federal do Paraná.

Ooshima, H., Burns, D. S. & Converse, A. O. (1990). Adsorption of cellulase from *Trichoderma reseei* on cellulose and lignacious residue in wood pretreated by dilute sulfuric acid with explosive decompression. *Biotechnol. Bioeng.*, **36**, 446-452.

Ramos, L. P., Breuil, C. & Saddler, J. N. (1992). Comparison of steam pretreatment of eucalyptus, aspen and spruce wood chips and their enzymatic hydrolysis. *Appl. Biochem. Biotechnol.*, **34/35**, 37-48.

Ramos, L. P., Breuil, C. & Saddler, J. N. (1993). The use of enzyme recycle and the influence of sugar accumulation on cellulose hydrolysis by *Trichoderma* cellulases. *Enzyme Microb. Technol.*, **15**, 19-25.

Ramos, L. P., Cotrim, A. R., Silva, F. T. & Chen, C.-L. (1999). Characterization of residual lignin after SO_2-catalyzed steam explosion and enzymatic hydrolysis of *Eucalyptus viminalis* wood chips. *J. Agric. Food Chem.*, **47**, 2995-2302.

Sinitsyn, A. P., Gusakov, A. V. & Vlasenko, E. Y. (1991). Effect of structural and physico-chemical features of cellulosic substrates on the efficiency of enzymatic hydrolysis. *Appl. Biochem. Biotechnol.*, **30**, 43-59.

Wong, K. K. Y., Deverell, K. F., Mackie, K. L., Clark, T. A. & Donaldson, L. A. (1988). The relationship between fiber porosity and cellulose digestibility in steam-exploded *Pinus radiata*. *Biotechnol. Bioeng.*, **31**, 447-456.

Reactivity of peracids towards lignin model compounds

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ABSTRACT

The reactivity of peracetic acid (Pa), permonosulfuric acid (Px) and their mixture (Pxa) in reactions with some lignin model compounds was studied. Aromatic hydroxylated products were observed in reactions with guaiacol and syringic acid. The reactivity of Pa, Px and Pxa in aromatic electrophilic substitution reactions was low, especially when compared to the reactivity of the subsequent oxidation. The influence of the aromatic substituent groups was confirmed in the reactions of those peracids with syringic acid, 3,4-dimethoxybenzoic acid and 3,4,5-trimethoxybenzoic acid. The experiments with compounds containing α-carbonilic and unsaturated groups bonded to the aromatic nucleus (as in acetovanilone and isoeugenol) highlighted the importance of the oxidation of the aliphatic side chains, compared to the substitution reactions of the aromatic nucleus. The reactions studied confirmed that Pa is a better nucleophile than Px, which on the contrary is a better electrophile. In some cases, a synergistic effect was verified when Pxa was used.

Keywords: lignin, lignin model compounds, bleaching, peracids

INTRODUCTION

The peracids are strong oxidants that can be used efficiently in the bleaching of chemical pulps. The main reactions, described in the literature, between these oxidants and lignin (or lignin model compounds) are the hydroxylation of the aromatic rings by electrophilic substitution leading to the formation of muconic acids and the related lactones (Farrand, 1971; Strumila, 1975; Johnson, 1980; Ni, 1997), as shown in Figure 1. This is a very important reaction since the formation of new phenolic structures, which is probably the determining reaction step (Marshall, 1974), activates the residual lignin for the subsequent bleaching stages, especially those using hydrogen peroxide.

Hydroxylation of the aromatic ring may lead to its demethoxylation forming quinones that are also very reactive towards the peracids. The cleavage of quinone rings results in the formation of muconic acids and lactones. This cleavage occurs generally between adjacent carbonyl groups through the hydrolysis of a cyclic anhydride formed by a Baeyer-Villiger reaction (Marshall, 1974; Strumila, 1975). The opening of the aromatic ring increases the hydrophilic character of lignin, contributing to its solubility in the bleaching liquor (Gellerstedt, 1997; Liukko, 1998). The formation of acidic groups also favors the solubility of lignin fragments, especially in the alkaline stages.

Also very important are the reactions of peracids with lignin side chains, which may compete with the electrophilic substitution reactions depending on the reaction conditions and the substituent groups bonded to the aromatic nucleus (Sakai, 1972; Nimz, 1979). An example is the electrophilic cleavage of the β-aryl ether bonds (Figure 2), which are responsible for the connection of the arylpropanoid lignin units in its macromolecular structure.

Lignin side chain also participates in nucleophilic reactions as the Baeyer-Villiger oxidation of carbonyl compounds (Figure 3). The hydrolysis of the esters formed leads to the formation of hydroquinones. According to Nimz (1979) these reactions compete with the aromatic ring hydroxylation, depending on the pH used, and are not much influenced by phenolic or etherified structures, neither the presence of methoxyl groups bonded to the aromatic nucleus.

Epoxidation of olefins is also a well-known reaction of peracids. Swern (1953) presents a compilation of hundreds of ethylenic compounds, which can be epoxidated by organic peracids. In the oxidation of isoeugenol, Oki (1974, in: Johnson, 1980) suggested that the double bond epoxidation was one of the first reactions steps.

According to Chang (1995), peracetic acid (Pa) and permonosulfuric acid (Px) reaction mechanisms with lignin aromatic structures are identical, but significant differences are found in their reactivities. The Pa reactivity was higher with α-carbonilic aromatic structures, while Px was far more reactive with α-hydroxylated aromatic structures and compounds with no lateral side chain. These results were explained considering that Pa has a better nucleophilic character in the Baeyer-Villiger oxidation and Px has a better electrophilic character in hydroxylation reactions.

In order to understand the behavior of peracids (Pa, Px and their mixture, Pxa) in the bleaching of kraft pulps, Brasileiro (1997, 1999) studied their reactions with lignin model compounds. Besides the identification of some reaction products it was evaluated the general reactivity of those peracids in the reactions considered.

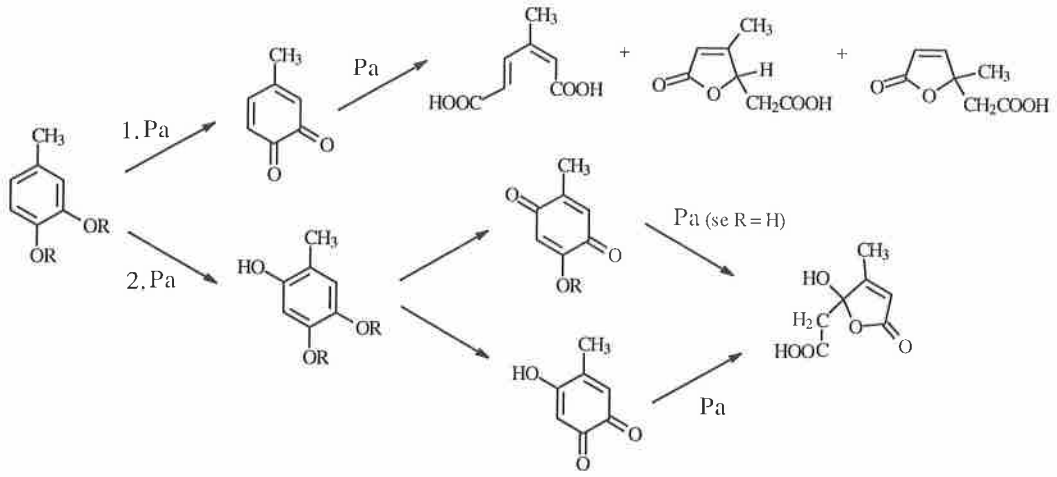


Figure 1. Formation of muconic acids and lactones through the oxidation of aromatic structures by peracetic acid, Pa (Farrand, 1971).

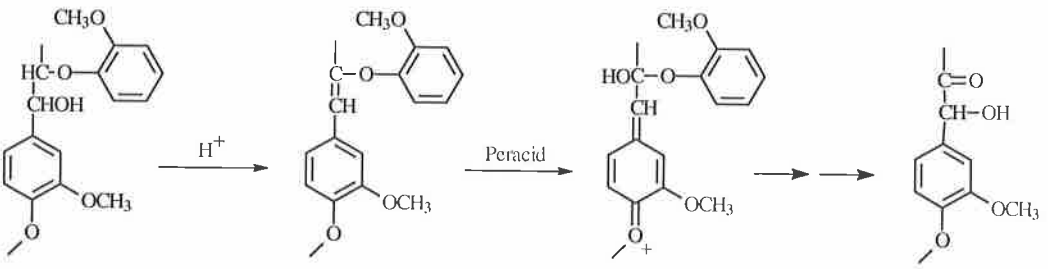


Figure 2. Cleavage of the β-aryl ether bonds by electrophilic addition reaction with peracids (Gierer, 1982).

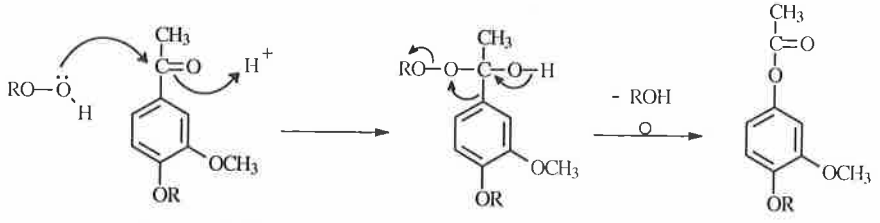


Figure 3. Baeyer-Villiger oxidation of an α-carbonyl compound (Chang, 1995).

EXPERIMENTAL

The peracid solutions were prepared by oxidation of the appropriate acid with hydrogen peroxide 50%. In the case of Pa it was used acetic acid, hydrogen peroxide and sulfuric acid in a molar proportion of 3.5:1:0.03, and in the case of Px it was used hydrogen peroxide and sulfuric acid in a molar proportion of 1:2.75 (Amini, 1995). For the preparation of Pxa it was used acetic acid, hydrogen peroxide and sulfuric acid in a molar proportion of 1:1:1.5. The reaction conversion and the final concentration of hydrogen peroxide and peracid were evaluated according to Greenspan (1948).

The oxidation reactions were done during three hours in aqueous solutions containing 2.5 mmol of the lignin model compound (acetovanilone, isoeugenol, guaiacol, syringic acid, 3,4-dimethoxybenzoic acid and 3,4,5-trimethoxybenzoic acid) and an amount of the peracid solution equivalent to 0.75 g of hydrogen peroxide initially added. The reactions with Pa and Pxa were done at initial pH 5 and 70°C. The reactions with Px were done at initial pH 4 and 50°C. The reactions were interrupted by refrigerating the reaction flask and adding sodium bisulfite.

The reaction mixtures were extracted with methylene chloride at pH 8 and pH 2. The organic fractions were properly derivatized with BSTFA and submitted to the analysis by gas chromatography-mass spectrometry (GC-MS).

RESULTS AND DISCUSSION

Acetovanilone oxidation

The acetovanilone oxidation was already described in the literature, either in neutral or in acidic conditions (Strumila, 1975; Wayman, 1965 and Ishikawa, 1964-1966 in: Strumila, 1975; Nimz, 1979). The α-carbonilic oxidation according to the Baeyer-Villiger mechanism seems to be one of the main steps of this reaction. The use of peracids in such reactions is especially important considering the stability of the carbonyl groups in the oxidation with other reagents.

In the acetovanilone oxidation with Pa, Px and Pxa it was observed that one of the main reaction products was 2-methoxy-4-acethoxyphenol (**1**), resulting from the Baeyer-Villiger oxidation. The hydrolysis product of this compound, methoxy-*p*-hydroquinone (**2**), was also detected. The compounds **1** and **2** (Figure 4) were practically the only observed reaction products, which is in accord to the results of Strumila (1975) and Nimz (1979).

To evaluate the reactivity of the peracids used in this oxidation reaction, the areas of the chromatographic peaks corresponding to acetovanilone and to compound **1** were integrated and compared. The results (Table 1) indicate that Pa was more reactive than Px or Pxa in the Baeyer-Villiger reaction. This is in accordance with the higher nucleophilic character of Pa, as reported by Chang (1995) and Kawamoto (1994 in: Chang, 1995). In pulp bleaching, these higher nucleophilic character may result in higher brightness gain, as demonstrated by Brasileiro (1999).

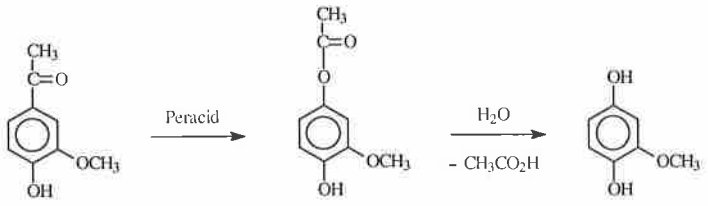


Figure 4. Acetovanilone oxidation products.

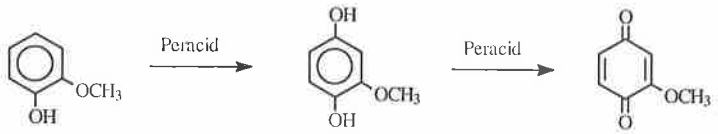
Table 1. Peak area ratio observed in the chromatogram of the oxidation products of acetovanilone extracted in pH 8.

Peracid	1 / acetovanilone
Pa	484.5
Px	0.6
Pxa	1.8

Guaiacol oxidation

In the oxidation of guaiacol by peracids were identified methoxy-*p*-hydroquinone (**2**), formed after the hydroxylation of guaiacol in the *para* position to the hydroxyl group, and methoxy-*p*-benzoquinone (**3**), formed in the oxidation of **2** (Figure 5). Guaiacol was not significantly consumed in these reactions. The importance of the reactions of peracids with the side chains of these lignin model compounds can be demonstrated by comparing this result with those obtained in acetovanilone oxidation, which was almost completely consumed in the reaction with Pa.

The reactivity of Pa, Px and Pxa in the oxidation of guaiacol was estimated by integrating and comparing the areas of the chromatographic peaks corresponding to guaiacol, **2** and **3** (Table 2). The results indicate that the reactivity of peracids in the hydroxylation of the aromatic ring was very low, especially for Pa, which is not strong as an electrophile as Px and Pxa. On the other hand, the reactivity of the peracids in the oxidation of **2** is superior to that obtained in the ring hydroxylation. The easy oxidation of hydroquinones to quinones may be responsible for these results, being more favored by Pa. Probably, the difference in reactivity among the peracids used is related to the oxidizing species involved or to the oxidant concentration, which is different for each peracid because of the different conversion ratios.



guaiacol (2) (3)

Figure 5. Guaiacol oxidation products.

Table 2. Peak area ratio observed in the chromatogram of the oxidation products of guaiacol extracted in pH 8.

Peracids	**2** / guaiacol	**3** / **2**
Pa	0.00005[a]	6.3
Px	0.002	0.97
Pxa	0.003	1.8

[a] saturation observed in the peak corresponding to guaiacol

Syringic acid oxidation

At least three different compounds were present in all the extracted fractions of the syringic acid oxidation (Figure 6). One of them was the substrate, corresponding to the most intense chromatogram peak of the acidic fractions. The others were 2,4-di-hydroxy-3,5-dimethoxybenzoic acid (**4**), the hydroxylation product of syringic acid, and 3-methoxy-5,6-dihydro-2-pirone-5-carboxylic acid (**5**), a δ-lactone resultant of the aromatic ring opening.

The areas of the chromatographic peaks corresponding to syringic acid and the compounds **4** and **5** were integrated and compared to evaluate the reactivity of Pa, Px and Pxa in these oxidation reactions (Table 3). All ratios were below one, what indicates the low reactivity of syringic acid towards peracids. This was already expected, considering the deactivating effect of the carboxylic group in electrophilic aromatic substitutions. Because of this the formation of product **4** was not favored. However, the lowest reactivity of Px observed in the reactions was not expected, since this peracid is a better nucleophile than Pa. Such result may be associated to the sterical hindrance at the *orto* position to the carboxylic group: being smaller than Px, Pa could attack this *orto* position. Through the results shown in Table 3, it can be seen that the formation of the compound **5** is favored, comparing to the formation of **4**. The higher reactivity of Pxa may be associated to a synergism caused by the mixture of peracids and is in accord to the bleaching experiments done (Brasileiro, 1999). Nevertheless, the same effect was not observed in the reactions of peracids with acetovanilone and guaiacol, indicating that the substrate structure, and also the residual lignin structure, is of crucial importance to the reactivity of these oxidants.

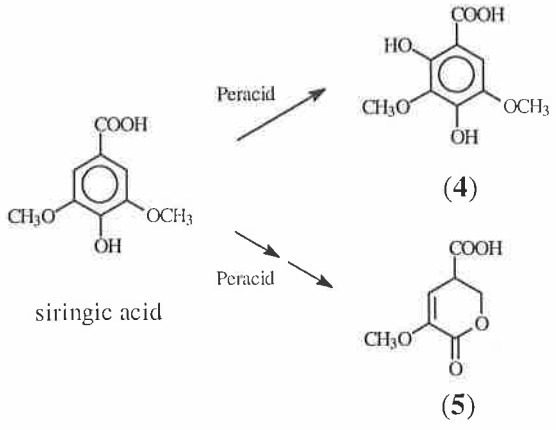


Figure 6. Syringic acid oxidation products.

Table 3. Peak area ratio observed in the chromatogram of the oxidation products of syringic acid extracted in pH 2.

Peracid	**4** / syringic acid	**5** / syringic acid
Pa	0.012	0.037
Px	0.005	0.039
Pxa	0.132	0.199

3,4-dimethoxybenzoic acid and 3,4,5-trimethoxybenzoic acid oxidation

In the peracid oxidation of 3,4-dimethoxybenzoic acid and 3,4,5-trimethoxybenzoic acid only a small amount of the substrate was consumed. Table 4 shows the percentage of substrate recovered (as a white solid) after the oxidation. Although the reactivity of Pa, Px and Pxa with both acids were low, 3,4,5-trimethoxybenzoic acid was more consumed than the other one, since the most oxygenated substrates are the most reactive (Sakai, 1966 in: Johnson, 1980). By comparing the results obtained for 3,4,5-trimethoxybenzoic acid and syringic acid, which was not recovered as a solid, it is reinforced what was proposed by Farrand (1971) that hydroxylated compounds are also more reactive than the etherified ones.

Table 4. Percentage of 3,4-dimethoxybenzoic acid and 3,4,5-trimethoxybenzoic acid recovered after the oxidation reactions with peracids.

Peracid	3,4-dimethoxybenzoic acid, %	3,4,5-trimethoxybenzoic acid, %
Pa	59.3	15.7
Px	57.8	39.2
Pxa	53.5	25.5

Isoeugenol oxidation

After the oxidation of isoeugenol with peracids it was observed that all the organic extracts (those obtained in pH 8 and pH 2) contained isoeugenol. Other compounds were also observed and their mass spectrum suggested the presence of vanillin (**6**), 4-(1,2-epoxypropyl)-guaiacol (**7**), which is the isoeugenol epoxidation product, and 2-(4-hydroxy-3-methoxyphenyl)-propanal (**8**), formed probably from the epoxyde but not observed in the Pa reaction products. It were also observed the presence of isomeric diols, 4-(1,2-dihydroxypropyl)-guaiacol (**9**), formed in the hydrolysis of **7**. These diols showed different retention times but their mass spectrum were identical, constituting probably a diasteroisomeric pair. They were formed in large quantities, being observed in the organic extracts obtained in pH 8 and pH 2. One of them was formed preferentially, which constitutes an indication that the mechanism of the epoxide hydrolysis was not essentially S_N1. The reaction products are shown in Figure 7.

To evaluate the reactivity of peracids in the oxidation of isoeugenol the integrals of the chromatographic peak areas of the substrate and compounds **7** and **9** were compared. For the diols **9** it was considered the sum of the peak area of both diasteroisomers. The results are presented in Table 5. The low ratio calculated between the peak areas of the epoxide **7** and isoeugenol could suggest a small reactivity of the peracids in this reaction. However, large amounts of other products, including **9**, were obtained, making clear that the major part of the epoxidation product (**7**) was rapidly consumed. This is confirmed by the high values calculated for the ratio of **9** and **7**.

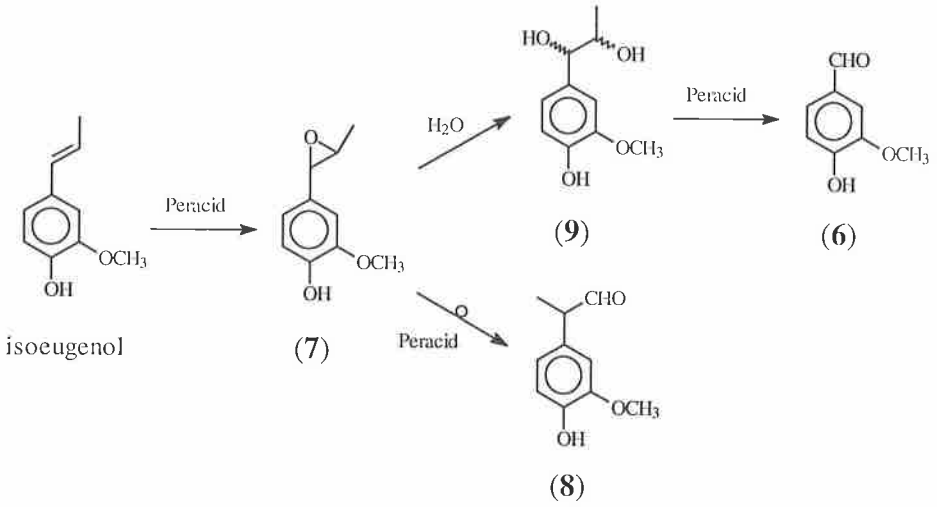


Figure 7. Isoeugenol oxidation products.

Table 5. Peak area ratio observed in the chromatogram of the oxidation products of isoeugenol extracted in pH 8.

Peracid	**7** / isoeugenol	**9** / **7**
Pa	0.05	514.4
Px	0.70	10.0
Pxa	0.77	17.1

The diols **9** were not completely extracted in pH 8, being also obtained in the acidic fraction. Because of this it was not possible to compare directly the peracids used in order to verify which of them was the most reactive with isoeugenol, although Pa seems to be the best one. Anyway, the observation of products like **6**, **7**, **8** and **9** indicates that for this kind of substrate, the main reactions are those that involve the double bond of the side chain. This idea is reinforced by the apparent absence of electrophilic aromatic substitution reaction products.

CONCLUSIONS

The main conclusions of this work are: (1) the reactivity of the peracids towards the aromatic ring hydroxylation was very low, especially when compared to subsequent oxidation reactions; (2) the more oxygenated a lignin model compound, the higher the reactivity of peracids in its oxidation; (3) the reactions of peracids with unsaturated and α-carbonilic side chains demonstrated the importance of the oxidation of these substituent groups compared to the hydroxylation of the aromatic nucleus; (4) Pa is a better nucleophile than Px, which is a better electrophile. A synergism occurred when Pxa was reacted with some of the lignin model compounds studied.

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REFERENCES

Amini, B. (1995). Bleaching with peracids. *Emerging Pulping and Bleaching Technologies Workshop*, Proceedings, non-paginated.

Brasileiro, L.B., Colodette, J.L. & Piló-Veloso, D. (1997). A química do branqueamento de polpa com perácidos. *O Papel*, **11**, 81-89.

Brasileiro, L. B. (1999). A utilização de perácidos no branqueamento e na deslignificação de polpas kraft. PhD thesis, Departamento de Química, Universidade Federal de Minas Gerais.

Chang, H.-M. (1995). Peracids as a bleaching agent: basics. *Emerging Pulping and Bleaching Technologies Workshop*, Proceedings, non-paginated.

Farrand, J.C. & Johnson, D.C. (1971). Peroxyacetic acid oxidation of 4-mehylphenols and their methyl ethers. *J. Org. Chem.*, **36 (23)**, 3606-3612.

Gellerstedt, G. & Heuts, L. (1997). Changes in the lignin structure during a totally chlorine free bleaching sequence. *J. Pulp Pap. Sci.*, **23 (7)**, J335-J340.

Gierer, J. (1982). The chemistry of delignification. A general concept. Part II. *Holzforschung*, **36**, 55-64.

Greenspan, F. P. & MacKellar, D. G. (1948). Analysis of aliphatic per acids. *J. Anal. Chem.*, **20 (11)**, 1061-1063.

Ishikawa, H. & Oki, T. (1964). *Kami-pa Gikioshi*, **18**, 477.

Ishikawa, H.. (1965). *Kami-pa Gikioshi*, **19**, 393.

Ishikawa, H., Oki, T & Okubo, K. (1966). *Kami-pa Gikioshi*, **20**, 435.

Johnson, D.C. (1980). Lignin reactions in delignification with peroxyacetic acid. *International Symposium on Delignification With Oxygen, Ozone And Peracids*, Proceedings, **Vol 1**, pp 217-228.

Kawamoto, Chang & Jameel (1994). *International Symposium on Fiber Science and Technology.*

Liukko, S. & Poppius-Levlin, K. (1998). Analysis of peroxy compound effluents by capillary electrophoresis. *International Pulp Bleaching Conference*, Proceedings, pp.637-642.

Marshall, R. A. G. & Naylor, R. (1974). Investigation of the model enzyme oxidation of phenol by peracetic acid. *J. Chem. Soc., Perkin II*, 1242-1246.

Ni, Y. & D'entremont, M. (1997). Peroxyacetic acid oxidation of lignin model compounds. *International Symposium on Wood and Pulping Chemistry*, Proceedings, **Vol 9**, pp D6-1-D6-5.

Nimz, H.H. & Schwind, H. (1979). Oxidation of monomeric lignin model compounds with peracetic acid. *Cell. Chem. Technol.*, **13**, 35-46.

Oki, t. Okubo, K. & Ishikawa, H. (1974). *J. Japan Wood Res. Soc.*, **20 (2)**, 89.

Sakai, K & Kondo, T. (1966). *J. Japan Wood Res. Soc.*, **12**, 57.

Sakai, K. Kuroda, K. & Kishimoto, S. (1972). Delignification in peracid bleaching. VI. Reaction of the β-aryl ether type model compounds with peracetic acid. *Tappi*, **55 (12)**, 1702-1706.

Strumila, G. & Rapson, H. (1975). Reaction products of neutral peracetic acid oxidation of model lignin phenols. *Pulp Pap. Can.*, **76 (9)**, 74-78.

Swern, D. (1953). Epoxidation and hydroxylation of ethylenic compounds with organic peracids. In *Organic Reactions*. John Wiley & Sons, New York, pp.378-433.

Wayman, M., Rapson, W. H. & Anderson, C. B. (1965). *Tappi*, **48**, 113.

Kraft lignins in the synthesis of modified novolac resins

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ABSTRACT

This work describes the preparation/characterization of phenolic resins (novolac) modified with kraft lignin and the preparation/characterization of phenolic molding powders. The preparation of the resins followed industrial procedure and it was observed that the lignin reacts through the aromatic ring and the lateral chain of its units. The incorporation of the lignin causes differences in the resins's glass transition temperatures, larger reactivity with hexamethylenetetramine and smaller formaldehyde consumption in the polimerization reaction. The lignin based phenolic molding powders presented higher modulus of elasticity and smaller tensile and flexural strength (around 20%) when compared with the standard prepared without lignin.

Keywords: phenolic resins, lignin

INTRODUCTION

The utilization of lignins in the preparation of resol resins applied in adhesives systems is relatively well described in the literature (Nimz, 1983; Danielson and Simonson, 1998; Kazayawoko *et al.*, 1992; Kuo, 1991; Shimatani *et al.*, 1994; Ysbrandy *et al.*, 1992). These adhesives are used to produce plywoods and particleboards. Some works were developed in Brazil (Piccolo, 1993; Benar, 1996; Piccolo *et al*, 1997; Paiva, 1997; Carvalho, 1997). The utilization of lignin as fillers and chain extenders were also described in the literature (Muller *et al.*, 1984a; Muller *et al.*, 1984b; Ysbrandy *et al.*, 1997). The reaction of wood with phenol and formaldehyde may be cited as a reaction between lignin present in wood and these reactants (Lin *et al.*, 1995). The utilization of lignin as simple filler was also described (Matuana, 1983). On the other hand, it exists very few published works concerning the preparations of novolac type resins using lignins as co-reactant (Keslarek, 1995; Santos, 1996; Santos and Curvelo, 1998). These resins are synthesized in acidic media employing molar excess of phenol relatively to formaldehyde. Novolac resins are an interesting alternative to introduce lignins in applications such as injection molding plastics (Santos, 1999; Kharade and Kale, 1998).

Aiming to contribute in the field of lignin valorization, this work describes the preparation and characterization of novolac resins modified with Kraft lignins. Phenolic Moulding Powders (PMF) were prepared using the lignin modified novolacs. The effects of the fiber reinforcement (wood flour or cellulosic pulps) were also evaluated by mechanical and thermal properties of PMF's.

The mechanism of reaction of lignins in novolac preparations is similar to the mechanism observed in the synthesis of phenolic resins. The reaction starts with methyleneglycol (formaldehyde in solution) protonation and its addition to the free positions in the lignin aromatic rings. At low pH may also occur the participation of the aliphatic chains and aromatic positions at *meta* phenolic hydroxyl group of the lignins moieties. The reaction will produce self-condensation lignin derivatives as well as lignin-phenol condensation.

EXPERIMENTAL

The lignins were recovered from industrial Kraft spent liquors and characterized by chemical and spectroscopic analyses. Novolac resins were prepared using different amounts of Kraft lignins following procedure usually used in the industrial resin preparations. The analyses performed include: Free phenol and free formaldehyde contents, Flow, Size exclusion chromatography (SEC), Infrared spectroscopy (FTIR), Nuclear magnetic resonance (^{13}C NMR), Differential scanning calorimetry (DSC) and Thermogravimetric analysis (TGA).

The lignin was recovered from industrial Kraft spent liquor (from ARACRUZ Celulose S. A.). The liquor was acidified until pH 3 with sulfuric acid and the precipitated lignin filtered and washed with distilled water. The resins were prepared in 1 Liter 3-neck flask dotted with mechanical stirrer, thermometer and exit to

perform distillation under reduced pressure. In the kettle were added phenol, lignin (except in RSL standard) and sulfuric acid. When the system reached the reflux temperature it was slowly added the formaldehyde solution (30 min approximately). The system was kept under reflux until obtaining a free formaldehyde content smaller than 1%. The determination of free formaldehyde was performed as follow: Samples of the reaction mixture were added to excess of sodium sulfite solution. The excess of sodium sulfite was then determined by titration with standardized sulfuric acid. When the formaldehyde content was lower than 1%, the reaction medium was neutralized with ammonium hydroxide solution until pH 5. The distillation of water was started, first at atmospheric pressure and in the final stage at reduced pressure. The distillation was monitored by controlling the "flow" of the remaining solution. The flow analysis was performed using an industrial procedure that consists in the reaction of the resin and the cure agent, HMTA (hexamethylenetetramine). The resin and the cure agent were mixed and triturated together in the proportion of 10:1 w/w. A sample of 0.5g of this mixture was molded, under pressure, to form a tablet with 12 mm diameter and 6mm height. This tablet was placed in a glass plate (2 mm of thickness) and transferred to a stove maintained at 125±1°C. After 3 min the glass plate was inclined in 63° angle and kept in this position for 20 min. The distance slip away by the tablet corresponds to the flow distance. The flow analysis of resins indicates the extension of the condensation reactions (that is evaluated by viscosity) and the resin reactivity.

The experimental procedure describe above was employed in the preparations of the lignin-novolac resins with lignin content ranging from 15% to 34%. The resin prepared with 44% lignin content follow a different procedure in the distillation step, which was performed with toluene addition.

The molar ratios of the reagents employed in the resin's preparations are showed in Table 1.

Table 1. Molar ratios of resins modified with lignin.

Resin	Phenol	Lignin (191.7g/mol)	Formaldehyde (37%)	Sulfuric acid (%)	% of lignin (by weight)
RSL*	1.000	-	0.850	0.14	0
R-15	0.926	0.073	0.650	0.20	15
R-26	0.861	0.138	0.606	0.30	26
R-34	0.810	0.190	0.542	0.38	34
R-44	0.738	0.262	0.421	0.50	44
R-44N**	0.738	0.262	0.421	0.50	44

*Resin prepared without lignin/ **water distillated with toluene addition

The free phenol contents were determined by Gas Chromatography in a Hewlett Packard equipment using the following conditions: column: HP-5 - 25m x 0.2mm x 0.33mm; programming temperature: 75°C for 10 min; split: 1:100; carrier gas: Hydrogen; injected amount: 1.0 ml; internal standard: benzyl alcohol. The molar mass distribution was performed by Size Exclusion Chromatography (SEC), using a Shimadzu equipment, with pre-column PL Gel followed by PL Gel 1000, 500 and 100 Å; refraction index detector; room temperature, THF as eluent at 1.0 ml/min flow rate; sample concentration of 1.2 mg/ml; injection volume of 5μl; polystyrene standards molar masses of 450,000; 195,000; 68,000; 50,000; 28,000, 12,500; 8,500; 4,000; 1,800; 1,050; 580 g/mol and ethylbenzene (106g/mol).

The kinetics of the cure reactions was monitored by Differential Scanning Calorimetry (DSC) using a Du Pont and Shimadzu equipments from samples of pure phenolic resin and resins mixed with HMTA (15% w/w). The following experimental conditions were used: mass sample ≈2mg; nitrogen atmosphere (20ml/min); temperature range: ambient to 250°C and heating rate: 10°C/min.

The Phenolic Molding Powders (PMF's) were prepared following industrial procedures (calendering) with two different reinforcement materials, separately, wood flour (80 mesh) or cellulosic pulps (with different residual lignin contents). The composition of the PMF's studied are showed in Table 2.

Table 2. Phenolic Molding Powder (PMF) compositions.

Amount	Phenolic Resin	HMTA	Wood flour or cellulosic pulp	Carnauba wax	Calcium Stearate
Based on 100g of PMF (g)	72.40	10.80	14.50	0.72	1.45
Based on resin's mass (%)	-	15.00	20.00	1.00	2.00

ARACRUZ Celulose SA supplied the cellulosic pulps employed in this work. They were used bleached (PB) and unbleached pulps (PN with lignin content of 6.35% and PI with lignin content of 4.22%). Pinho Pó S.A supplied the wood flour. The components described in Table 2 were mixed and calendered in a laboratory calender with cylinder temperature of 80°C. After 3 min of calendering the material was removed from the cylinder plate, cooled and milled to 80 meshes.

The Phenolic Molding Powders were analyzed in relation to their mechanical properties. Tests of tensile and flexural strength were realized according to ASTM D-638 and D-790 standards, respectively. The tests were performed in a Universal Test Machine Instron 5500R, in the following conditions: for traction, specimen type I (regarding reinforced rigid materials) and head rate of 5.0 mm/min; for flexure test, method I (3 points) and head speed of 2.8mm/min.

RESULTS AND DISCUSSION

The preparation of novolac-Kraft lignin resins can be performed easily up to 34% of phenol replacement. For higher amounts of Kraft lignin the high viscosity difficult the removal of the water liberated in the condensation reactions. In table 1 it was observed that the amount of consumed formaldehyde decreased with the increase of the lignin content in the resins. The participation of lignin in the condensation reactions lead to a smaller formaldehyde consumption and reduces the reaction time. The ^{13}C NMR spectra showed that the lateral chain of the phenylpropanic lignin units also participates in the reaction.

It can be noticed that the lignin is totally incorporated in the resins as confirmed by SEC analysis. This behavior can be attributed to the polymerization reaction involving a "lignin macromonomer". Through the SEC analysis, it could be observed the absence of low molar mass material, except a small amount of free phenol, which is normal for this type of synthesis. In addition, the data presented in Table 3 indicates the control obtained in average molar mass leading to polydispersities in the range of 1.8 to 3.1. This behavior indicates that the participation of the lignin produced resins with higher homogeneity. The lower amount of formaldehyde consumed are indicative that the functional groups present in the lignin molecules acted as important sites for the incorporation of phenol / formaldehyde molecules. The differences observed in Table 3 concerning the average molar mass determined by SEC can be attributed, at least in part, to the different amounts of residual phenol and formaldehyde molecules. Independent of the application, the decrease in formaldehyde amounts configures an advantage, because it can reduce the final resin's cost.

The resin prepared with 15% of phenol replacement (R-15) showed a decrease in the glass transition temperature when compared with the value obtained for the non-substituted resin (RSL). The utilization of higher amounts of lignin promotes an increase in the glass transition temperatures, which is almost proportional to the amount of Kraft lignins incorporated in the resins. Thus, the glass transition temperature can be adjusted in function of the desired industrial application. The T_g values can be also affected the amount of low molar mass compounds present in the resin.

Table 3. Characteristics of the Novolac-Kraft lignin resins.

Resin	Flow (mm)	Free Phenol (%)	T_g(°C)	Mn	Mw	Mw/Mn
RSL	35	3.6	46.6	1,440	21,300	14.8
R-15	55	7.2	24.8	1,030	1,900	1.8
R-26	35	-	46.8	1,450	4,200	3.0
R-34	43	5.3	53.3	1,300	3,500	2.7
R-44	46	-	55.2	1,050	2,300	2.2
R-44N	25	3.5	19.9	1,400	4,400	3.1

The analysis of the cure reactions were studied by DSC and furnished the values reported in Table 4. It is observed that all the resins prepared with lignins started to cure at lower temperatures. The activation energy for the cure process was also smaller than the value found for the resin prepared without lignin (RSL). This means that the Novolac-Kraft lignin resins presented higher reactivity with cure agent.

The modified novolac resins consumed higher amounts of cure agent, as the decomposition peak related to HMTA appears only in the resins with lignins contents up to 26% (R-26). The decrease in the energy involved in the HMTA degradation (for R-15 and R-26 samples) confirms the higher HMTA consumption and it suggests that the resin prepared with higher lignin contents were not completely cured.

The kinetic parameters permitted also the calculation of the reaction order for the cure process. All the resins presented the same reaction order, except the resin R-34. The reaction order obtained indicates that the contribution of reactants to the kinetics is proportional to their concentrations.

Table 4. Kinetics data determined for the Novolac-Kraft lignin resins.

Resin	Cure reaction				HMTA decomposition		Cure kinetics parameters		
	T_i * (°C)	T_{max} * (°C)	T_f * (°C)	Energy (J/g)	T_{max}* (°C)	Energy (J/g)	E_a (KJ/ mol)	n	ln A
RSL	116	143	180	53.50	186	3.25	210	1.0	56.60
R-15	97	131	168	123.70	181	0.77	169.4	1.0	46.02
R-26	101	136	174	59.21	179	0.11	155.3	1.0	41.48
R-34	107	139	176	75.92	-	-	180.9	1.2	48.48
R-44	95	132	168	64.02	-	-	177.7	1.0	48.43

* T_I = Initial temperature; T_{max} = Temperature at maximum; T_f = Final temperature.

Two kinds of Phenolic Moulding Powders (PMF's) were prepared from the novolac resins. In the first series it was used wood flour as reinforcement agent. These samples were tested to evaluate how the mechanical properties are affected by the lignin content in the synthesized resins. In the second set of samples it was used cellulosic pulps (with different residual lignin contents) as reinforcement agent. These samples permitted the analysis of this alternative reinforcement agent as well as the influence of the residual lignin contents (in the cellulosic pulps) in the mechanical performance of the correspondent PMF's. In the last series it was employed the resin prepared with 34% of phenol replacement (R-34). The code SL employed in the notation of PMFSL indicates that this PMF's were prepared using the resin without phenol replacement. The numbers present in the remaining sample notation (as in PMF-15) indicates the lignin content in the resins employed in the PMF's preparations. The experimental results obtained from the mechanical analysis are showed in Table 5.

Table 5. Mechanical properties of PMF's prepared from novolac resins.

PMF	Specific gravity (g/cm^3)	Tensile strength (MPa)	Deformation at rupture (%)	Elasticity modulus (MPa)
PMFSL-FM*	1.22	34.8±1.6	1.02±0.09	4,000±110
PMF-15-FM	1.20	18.7±0.9	0.57±0.05	3,400±140
PMF-26-FM	1.24	27.9±1.8	0.76±0.03	4,200±130
PMF-34-FM	1.20	16.0±1.0	0.50±0.02	4,200±170
PMF-44N-FM	1.24	28.3±1.6	0.81±0.05	4,400±170
PMFSL-PB**	0.93	20.6±0.3	0.97±0.16	3,000±220
PMFSL-PI#	0.84	19.9±3.0	0.68±0.20	3,500±100
PMFSL-PN##	1.01	19.3±0.9	0.61±0.08	3,300±700
PMF-34-PB	1.04	21.6±3.6	0.87±0.16	3,700±350
PMF-34-PI	0.95	23.3±4.7	0.64±0.12	4,000±300
PMF-34-PN	1.15	20.9±4.4	0.65±0.17	3,700±350

*FM: wood flour
**PB: bleached pulp / #PI: unbleached pulp (higher lignin content) / ##PN: unbleached pulp (lower lignin content).

The specific gravity values for the PMF's prepared with wood flour were very similar showing no dependence with the amount of lignin in the correspondent resins. The tensile strength of the lignin containing samples were lower than that obtained for the non-substituted samples. The deformation at rupture showed also lower values for the PMF's prepared with novalac-Kraft lignin resins. The deviations observed in the tensile strength and deformation at rupture values for the lignin containing PMF's are probably due to the low homogeneity of these samples. The effect of the phenol replacement was more significant when the elasticity modulus are considered. The increase in the lignin content produced more rigid (fragile) PMF's. The low value obtained for PMF-15-FM can be associated with the high amount of free phenol present in this sample.

The utilization of cellulosic pulp (instead of wood flour) as reinforcement agent promoted a significant decrease in the specific gravity values. As observed for wood flour based PMF's, the presence of lignin does not modify the specific gravity of the samples. Already for PMF's that used R-34 and cellulosic pulps, the RT

values were similar. The tensile strength for the PMF's prepared with cellulosic pulps presented similar values independently of the phenol replacement and residual lignin contents in the cellulosic pulps. The utilization of bleached cellulosic pulps produced samples with higher deformation at rupture for the samples prepared with and without phenol replacement. The different amounts of residual lignin in unbleached cellulosic pulps did not modify the deformation at rupture values. The elasticity modulus values were higher for the PMF's prepared from novalac-Kraft lignin resins as already showed for the samples prepared with wood flour.

CONCLUSIONS

The results obtained in this work demonstrated the feasibility of the Kraft lignin use in the preparation of novolac resins and phenolic moulding powders. The novolac resins were prepared with up to 34% replacement of phenol employed procedures nowadays in use at industrial scale. The utilization of higher amounts of Kraft lignin lead to an increase of the solution's viscosity and the removal of the water liberated in the condensation reactions became difficult.

The novolac resins modified with lignin presented higher reactivity with HMTA in the cure reactions. The presence of lignin lead also to a higher consume of HMTA. The activation energies for the cure process were smaller in the resin containing lignins.

The glass transition temperatures (T_g) of the resins were affected by the amount of lignin employed in the synthesis. This dependence can be used to adjust the resin's T_g according to desirable applications.

Phenolic Moulding Powders (PMF's) prepared from the modified resins presented higher elasticity modulus than the correspondent PMF prepared without lignin. The utilization of lignin turned the PMF's more fragile (more rigid). The utilization of cellulosic pulps as reinforcement agent seems to be a feasible alternative in the development of new composite materials.

Novolac-Kraft lignin resin consumes lower amounts of phenol (due to the replacement) and formaldehyde (due to the higher reactivity) and thus the final product is also economically attractive. The higher reactivity of the modified resins are also very interesting because lead to shorter moulding (pieces) cycles.

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REFERENCES

Benar, P. (1996). Ligninas Acetosolv e Formacell de Eucalipto e de Bagaço de Cana: Isolamento, Fracionamento, Caracterização e Uso como Componente de Resinas Fenólicas do Tipo Resol. PhD Thesis, Instituto de Química, Universidade Estadual de Campinas.

Carvalho, G. (1997). Ligninas em espumas fenólicas. PhD Thesis, Instituto de Química de São Carlos, Universidade de São Paulo.

Danielson, G. & Simonson, R. (1998). Kraft lignin in phenol formaldehyde resin. Part 1.Partial replacement of phenol by kraft lignin in phenol formaldehyde adhesives for plywood. *J. Adhesion Sci. Technol.*, **12 (9)**, 923-939.

Kazayawoko, J.S.M. (1992). A Lignin-Phenol-Formaldehyde Binder for Particleboard- *Holzforschung*, **46 (3)**, 257-262.

Keslarek, A. (1995). Preparação e Caracterização de Resinas Fenólicas (novolacas) a partir de ligninas Organossolve. MSc thesis, Instituto de Física e Química de São Carlos, Universidade de São Paulo.

Kharade, A. Y.; Kale, D.D. (1998). Effect of lignin on phenolic novolak resins and moulding powder. *Eur. Polym, J.*, **34 (2)**, 201-205.

Kuo, M. (1991). Alkali Treated Kraft Lignin as a Component in Flakeboard Resins. *Holzforschung,* **45 (1)**, 47-54.

Lin, L.Z. et al (1995). Preparation and properties of phenolated wood/phenol/ formaldehyde cocondensed resin. *J. Appl. Polym. Sci.*, **58 (8)**, 1297-1304.

Matuana, L.M. et al (1993). Caracterisation cinetique par analyse enthalpique differentielle des resines phenol-formaldehyde a base de lignosulfonates. *Eur. Polym. J.*, **29 (4)**, 483-490.

Muller, P.C.; Kelley, S.S. & Glasser, W.G. (1984a). Engineering plastics from lignin. VIII.Phenolic resin prepolymer synthesis and analysis. *J. Adhesion*, **17**, 157-174.

Muller, P.C.; Kelley, S.S. & Glasser, W.G. (1984b). Engineering plastics from lignin. IX.Phenolic resin synthesis and characterization. *J. Adhesion*, **17**, 185-206.

Nimz, H.H. (1983). Lignin-Based Wood Adhesives. In: *Wood Adhesives-Chemistry and Technology*, A. Pizzi, Marcel Dekker, New York, pp 247-288.

Paiva, J.M.F. (1997). Compósitos lignocelulósicos: matrizes poliméricas de resinas fenólicas reforçadas com fibras de bagaço de cana-de-açúcar. MSc thesis, Instituto de Química de São Carlos, Universidade de São Paulo.

Piccolo, R.S.J. (1993). Síntese e Caracterização de resinas lignina-fenol formaldeído. MSc thesis, Instituto de Física e Química de São Carlos, Universidade de São Paulo.

Piccolo, R.S.J.; Santos, F. & Frollini, E. (1997). Sugar Cane Bagasse lignin in resol type resin: alternative application for lignin-phenol-formaldehyde resins. *J. Macrom. Sci. - Pure and Applied Chemistry*, **34**, 153-164.

Santos, F. (1996). Utilização de lignossulfonatos na preparação de resinas fenólicas tipo novolaca e pós de moldagem fenólicos. MSc thesis, Instituto de Química de São Carlos, Universidade de São Paulo.

Santos, F. & Curvelo, A.A.S. (1998). Utilização de lignina kraft na preparação de novolacas e pós de moldagem fenólicos. *In Simpósio Interunidades de Ciência e Engenharia de Materiais*, Proceedings, **1**, pp 3-4.

Santos, F. & Curvelo, A.A.S. (1999). Utilização de ligninas em resinas fenólicas. 1. Preparação de novolacas a partir de lignossulfonatos. *Polímeros: Ciência e Tecnologia*, **9 (1)**, 49-58.

Santos, F. (1999). Lignina kraft em novolacas e pós de moldagem fenólicos. PhD Thesis, Instituto de Química de São Carlos, Universidade de São Paulo.

Shimatani, K; Sano, Y & Sasaya, T. (1994). Preparation of moderate-temperature setting adhesives from softwood kraft lignin. *Holzforschung,* **48 (4)**, 337-342.

Ysbrandy, R.E.; Sanderson, R.D. & Gerischer, G.F.R. (1992). Adhesives from autohydrolysis bagasse lignin, a renewable resource. Part I. The physical properties of laminates made with phenolated lignin novolacs. *Holzforschung*, **46 (3)**, 249-252.

Ysbrandy, R.E.; Gerischer, G.F.R. & Sanderson, R.D. (1997). Preparation of moulding resins from inexpensive by-products. II.Properties od mouldings made with various catalyzed phenolated and unmodified pitch/lignin systems. *Cellulose Chem. Tecnol.*, **31**, 213-226.

Reduction of eucalyptus black liquor viscosity by applying new techniques

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ABSTRACT

New technologies have been developed to overcome problems dealing with processing black liquor at high solids contents, such as: the heat treatment of liquor and/or addition of salts to decrease its viscosity. As these technologies have been applied for pine black liquor, their use for reducing eucalyptus black liquor viscosity should be tested previously. The purpose of this work is to carry out experiments concerning the heat treatment and the addition of salts to eucalyptus black liquor in order to analyze its rheologycal behavior and the actual decrease of its viscosity at high solids contents (above 80%).

Keywords: kraft black liquor, Eucalyptus, lignin

INTRODUCTION

In the kraft process of cellulose production, the wood chips are heated in a pressurized vessel with cooking liquor, composed of an aqueous solution of sodium hydroxide (NaOH) and sodium sulfite (Na_2S). During this step, the wood chips change into individual fibers, originating the pulp of cellulose and the black liquor. This liquor goes to the recovery unit to generate energy and to regenerate inorganic compounds for reutilization. The recovery unit of kraft process is composed, basically, by the multiple effect evaporators, responsible for concentrating black liquor until 75% of solids, and by the recovery boiler, where the concentrated liquor is burnt to produce steam. Early studies have shown that a significant increase of the boiler performance is achieved when the solids content of the pine black liquor is raised. However, the industrial operation with concentrated liquor above 75% of solids is limited by the liquor viscosity, which enhances greatly with solids contents. New technologies, such as the heat treatment and the salt addition, are now available for reducing the pine black liquor viscosity at high solids concentrations.

The heat treatment technique (LHT) is based on the studies developed by Soderhjelm (1988) and Kiiskila (1985), which show that the black liquor viscosity is highly reduced by depolimeration or heat cracking of lignin macromolecules. The depolymerization occurs when the liquor is heated in a pressurized reactor during 2 to 4 hours. The operation temperature for this reactor is 180-190°C. Figure 1 shows the lignin molecule structure on liquor before and after the heat treatment.

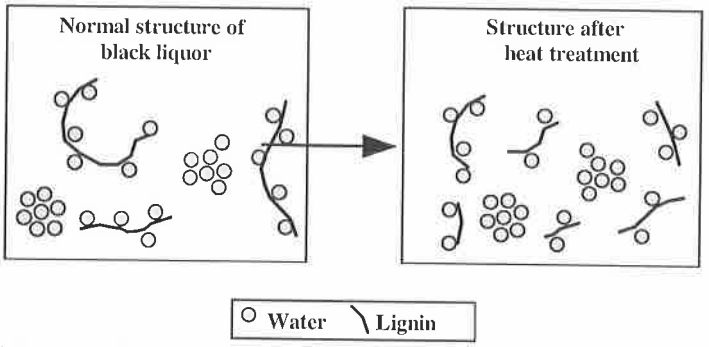

Figure 1. Lignin molecule structure on liquor before and after heat treatment.

The results obtained from Soderhjelm (1988) are the only ones found in literature for the heat treatment of hardwood black liquor. Data about liquor originating from *Eucalyptus grandis*, commonly found in Brazil, do not exist in the literature.

The other new technique involves addition of thyocianate salts to concentrated black liquor for reducing its viscosity. This technique has been tested by Roberts *et al.* (1996). These authors carried out experiments using commercial black liquors composed of 80% (in mass) of softwood liquor and 20% (in mass) of

hardwood liquor at two different temperatures (25 and 60°C) and three levels of solid contents. The salts (sodium thiocyanate NaSCN; potassium thiocyanate KSCN; guanidine thiocyanate GuSCN and ammonium thiocyanate NH_4SCN) were added to liquor samples at concentrations of 0.25, 0.50, 0.75 and 1.00 M. It was noted that the viscosity was reduced by the addition of these salts. Roberts *et al.* (1996) explained this reduction on the "salting-in" mechanism, which should have increased the solubility of dissolved lignin producing a change on the solution rheologycal behaviour. Figure 2 shows the liquor molecule structure before and after the thiocyanate salt addition.

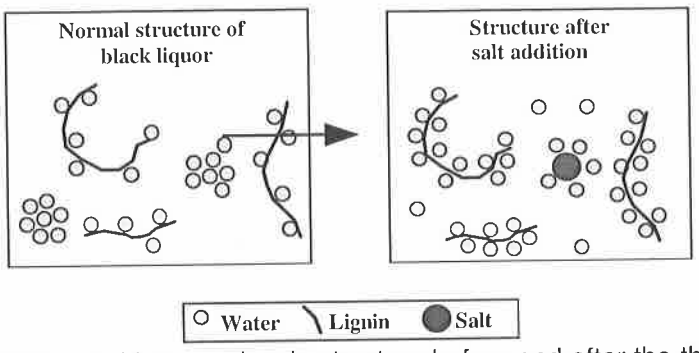

Figure 2. Liquor molecule structure before and after the thiocyanate salt addition, (Roberts *et al.*, 1996).

EXPERIMENTAL

Commercial eucalyptus black liquor obtained form Cenibra in Minas Gerais State was used. Experiments involving the heat treatment of this liquor were carried out using a cilindrical autoclave with 15 cm of height and a 10 cm diameter. The autoclave was heated by a heating plate on its bottom and by a thermal band on its cylindrical vessel. The temperature was registered using a thermometer located inside of an inox steel capsule immersed on the liquor at 1 cm from the bottom of the autoclave. For stabilizing the temperature, the experiments began after 3 to 4 hours of heating the black liquor in the autoclave. The increase in the operation pressure, resulting from the steam and gases produced during lignin macromolecules cracking, was registered using a manometer on the top of the equipment. The amount of solid contents in the samples were 45% and 73% and the experiments were performed at 150°C and 210°C for 2, 6, 8, 24 and 42 h (Soderhjelm, 1988). In order to verify the degree of lignin macromolecules cracking, the liquor viscosity at room temperature (26°C) and the molar mass of lignin were measured before and after the experiments. The lignin was precipitated using the procedure proposed by Kupidlowswki *et al.* (1997). The molecular weight distribution of the lignin was determined by HPLC using Shimadzu gel permeation columns (GPC-810 and GPC-803). The liquor viscosity was measured by a rotational viscosimeter (Cole Parmer, models E-98936-00 and E-98936-20).

Experiments regarding the salt addition were carried out using liquor samples with 73% solid contents. The salts used were the same proposed by Roberts *et al.* (1996): guanidine thiocyanate (GuSCN), potassium thiocyanate (KSCN), sodium thiocyanate (NaSCN) and ammonium thiocyanate (NH_4SCN) at concentrations of 0.25, 0.50, 0.75 and 1.00 M. Two temperatures were chosen for the experiments, 26°C and 80°C, in order to determining the reduction of the liquor viscosity at room temperature and at one closed to the inlet liquor temperature in the industrial boiler (100-120°C). This further temperature had to be kept below 90°C to avoid thermal lignin degradation. The liquor viscosity before and after the experiments was measured by the same viscosimeter equipped with the temperature-controlled unit.

RESULTS AND DISCUSSION

The maximum value of the black liquor viscosity reduction registered after the heat treatment was 43%, corresponding to a normalized viscosity of 0.57 (ratio of liquor viscosity after heat treatment and the one before the treatment). This reduction (80%) is less than the one reported for pine black liquor under the same treatment conditions (Soderhjelm, 1988; Kiiskila, 1985; Kaila and Polwski, 1997). This result was expected because *Eucalyptus grandis* is a hardwood, showing less lignin *in situ* (20 to 25%) then softwood lignin from pine wood (26 to 33%) (Soderhjelm, 1988; Cardoso, 1998). Also, the eucalyptus black liquor has a high

sodium ion concentration (21.4% of total dry solids weight). These ions help the cracking of lignin molecules clusters, decreasing its molecular weight and the polarization of these molecules, increasing its solubility in water (Cardoso, 1998). In this way, the lignin molecules in eucalyptus black liquor were dissociated before the treatment, and this is the reason for a lower viscosity reduction on this type of liquor. The Figure 3 shows one the chromatograms obtained, which proves that the lignin molecules before and after the heat treatment have the same retention time into GPC columns. It can be concluded that the molecular weight distribution of the lignins does not change with the heat treatment; the lignin cracking was not significant and these molecules have been dissociated before the treatment because the high concentration of sodium ions in the eucalyptus black liquor.

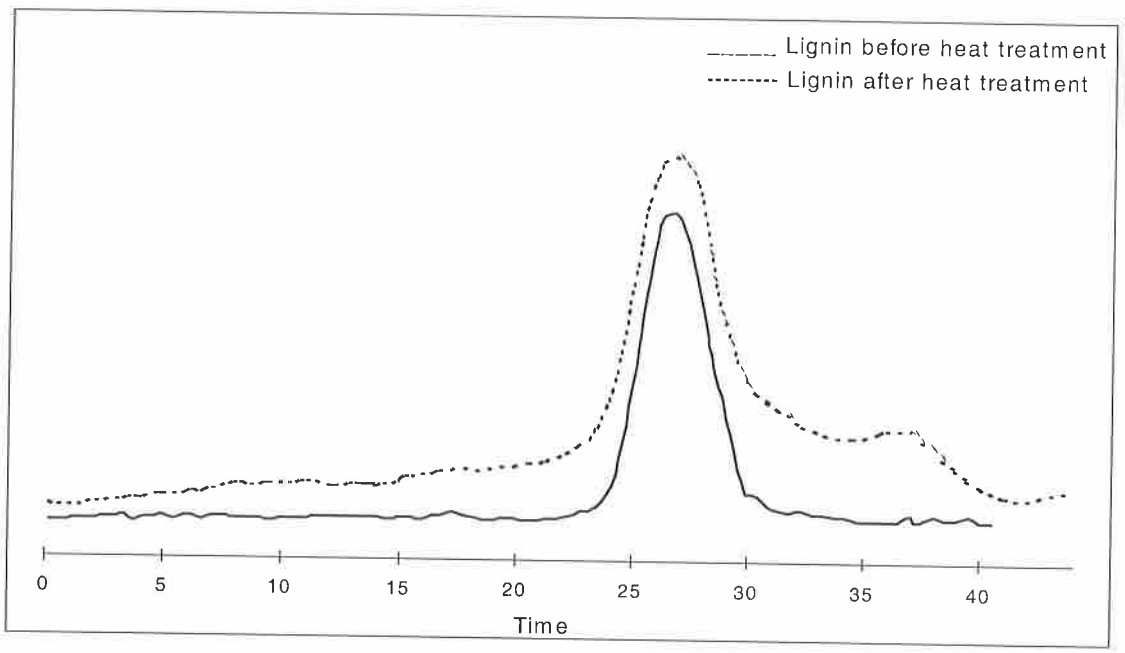

Figure 3. Molecular Weight Lignin Distribution on GPC columns using tetrahydrofuran as the mobile phase.

The Table 1 shows the results obtained for the normalized viscosity as a function of the temperatures used during the experiments, the type of salt and its concentration. The results showed that:

i) there was a liquor viscosity reduction with salt addition that was higher as the salt concentration increased;

ii) the liquor viscosity reduction was highest and lowest when guanidine thiocyanate and sodium thiocyanate were used, respectively. Similar results were also noted for pine liquor, as reported by Roberts *et al.* (1996), showing that the higher is the cation size associated with thiocyanate, the higher is the liquor viscosity reduction;

iii) comparing the normalized viscosity reported by Roberts *et al.* (1996) with those obtained in this work, it can be shown that the eucalyptus black liquor viscosity reduction is lower than the one showed by pine liquor. For example, at temperatures near 25°C, the normalized viscosity for pine liquor is 0.006 when using guanidine thiocyanate at a concentration between 0.5 and 1.0 M. At the same conditions, the eucalyptus liquor showed a normalized viscosity of 0.04 (see Table 1). This fact can be explained by the high sodium ion concentration on eucalyptus liquor. As mentioned, these ions are responsable for the degradation and polarization of lignin molecules in black liquor. As the polarization of lignin molecules increased, its solubility in water also increased and, therefore, the effect of salt addition on viscosity reduction decreased because the molecules were already dissolved in water;

iv) increasing the temperature (from 27 to 80°C) the normalized viscosity also increased and reached values of 0.87 with addition of potassium or sodium thiocyanate, and values of 0.31 with addition of guanidine thiocyanate. Comparing these results with those reported for pine liquor (Roberts *et al.*, 1996), the increase on normalized viscosity with an increase in temperature is higher for eucaliptus black liquor. Higher

temperatures promoted higher desorder on water molecules, decreasing the number and intensity of hydrogen bounds among these molecules. This reduced the effect of thiocyanate ions on the cracking of these water molecules structures and, consequently, the effect of salt addition on liquor viscosity reduction.

Table 1. Eucalyptus black liquor normalized viscosity as a function of the type of salt, its concentrations and the 2 temperatures used for the experiments (liquor's solids concentration equal 75%).

Salt concentration (M)	Normalized Viscosity (viscosity of black liquor after salt addition/viscosity of liquor without salt)					
	Potassium Thiocyanate KSCN		Guanidine Thiocyanate GuSCN		Sodium thiocyanate NaSCN	
	Temperature 27°C	Temperature 80°C	Temperature 27°C	Temperature 80°C	Temperature 27°C	Temperature 80°C
0.00	1.00	1.00	1.00	1.00	1.00	1.00
0.25	0.292	0.992	0.238	0.781	0.409	0.948
0.50	0.150	0.932	0.107	0.619	0.170	0.874
0.75	0.109	0.939	0.073	0.479	0.141	0.909
1.00	0.070	0.868	0.040	0.312	0.103	0.943

CONCLUSION

It can be concluded from the analysis of the results that the viscosity reduction of eucalyptus black liquor using the heat treatment and salt addition was lower, in both cases, than the one reported for pine black liquor. Therefore, these two techniques do not seem to be promising for processing concentrated eucalyptus black liquor. Ragarding the salt addition technique, another fact to take into account is the high price of salts in relation to liquor (30.00 to 100.00 US$/Kg of salt). Therefore, the salt addition technique is unfeasible concerning the economic issue.

Concerning the heat treatment technique, the results in this work indicate that the liquor viscosity reduction is low comparing with the cost for this technique implementation. New experiments should be done using different operation conditions for a better evaluation of its feasibility for a eucalyptus black liquor viscosity reduction.

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REFERENCES

Cardoso, M. (1998). Análise da Unidade de Recuperação do Licor Negro de Eucalipto no Processo "Kraft", Avaliando Alternativas de Processamento. PhD thesis, Instituto de Química, Universidade Estadual de Campinas.

Kaila, J. & Polowski, S. (1997). State of the Art Trends in Black Liquor Evaporator and Concentrator Technology. *In 30° Congresso Anual de Celulose e Papel*, Anais, **1**, pp 675-684.

Kiiskila, E. (1985), Suom. Finnish Patent Hak n° 854732.

Kupidlowski, S. C., Cardoso, M., Passos, M. L. A. & Carazza, F. (1997). Characterization of Eucalyptus Kraft Black Liquor, *In 5th Brazilian Symposium on the Chemistry of Lignins and Other Wood Components*, Proceedings, **1**, pp 76-84 .

Roberts, J. E., Spontak R. J., Jameel, H. & Khan, S. A. (1996). A Novel Approach to Black Liquor Viscosity Reduction Using Salt Additieves, *tappi Journal*, **79**, pp 167-172.

Soderhjelm, L. (1988). Factors Affecting the Viscosity of Strong Black Liquor, *Appita Journal*, **41**, pp 389-392.

Selectivity of sugarcane bagasse degradation by the white-rot fungus *Panus tigrinus*

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ABSTRACT

Biological pretreatments with three selected strains of *Panus tigrinus* were used for delignification of sugarcane bagasse. The fungi with potential for delignification was analysed by determining the chemical composition of the decayed bagasse samples and the selectivity in terms of weight loss of the different components was evaluated. All the strains grow abundantly on bagasse as unique carbon source. After determining chemical composition of degraded bagasse, *P. tigrinus* FTPT-4745 was selected as the most efficient strain on a 6-g scale, since the carbohydrates were preserved. *P. tigrinus* FTPT-4741 and FTPT-4742 were the most efficient strains on a large scale (100 g).

Keywords: *Panus tigrinus*, selectivity of biodelignification, sugarcane bagasse

INTRODUCTION

Biodegradation of lignocellulosics is one of the most important processes in nature. Sugarcane bagasse is a lignocellulosic residue with a complex structure and can be efficiently decayed, specially by white-rot fungi (Zadrazil and Puniya 1995; Esposito *et al.*, 1995, 1996, Gao *et al.*, 1996; Breccia *et al.*, 1997). The introduction of this natural process into the pulp and paper industry has great economic potential and contributes to the environmental preservation. White-rot fungi are able to degrade cellulose, hemicelluloses and lignin at similar rates. Some strains belonging to this group of fungi degrade lignin selectively. An example of selective degradation by white-rot fungi was reported by Akhmedova *et al.* (1994). These authors utilized 34 strains of xylophagous fungi and screened 15 that were able to oxidize gallic and tannic acids, presenting also the high potential of these strains to degrade lignin. *Panus tigrinus* UzBi-013 stood out as a producer of peroxidase, laccase, cellobiase, endoglucanase and xylanase. The same authors also observed an increase in biopolymers degradation along the first 10 days of incubation (Akhmedova *et al.*, 1994).

The production of ligninolytic and hemicellulolytic enzymes has been deeply studied in the last years (Prasad *et al.*, 1996a, 1996b, Pessoa *et al.*, 1997, Abd El-Nasser *et al.*, 1997). Prasad *et al.*, (1996a) investigated the influence of enzymatic preparations from raw extracts on the bleachability of thermomechanical pulps obtained from sugarcane bagasse and found that the brightness of the pulps increased by 2-3 points in relation to a control not treated with enzymes.

Gonçalves *et al.* (1998) evaluated the action of *P. tigrinus* strains on the pretreatment of sugarcane bagasse, using FTIR techniques together with enzymatic activities and pulp properties to screen the strains. In the present work, three strains of the white-rot fungus *P. tigrinus* previously screened (Esposito *et al.*, 1996; Gonçalves *et al.*, 1998) were utilized (FTPT-4741, FTPT-4742, FTPT-4745) for biological delignification of sugarcane bagasse to be employed in the production of pulp. The reason for choosing this kind of treatment is that it gives rise to a most efficient and economic pulping process, without damaging the environment.

EXPERIMENTAL

Fungi

Three strains of *Panus tigrinus* (FTPT-4741, FTPT-4742, FTPT-4745) from a collection belonging to Fundação Tropical de Pesquisa e Tecnologia André Tosello (Campinas, SP, Brazil) were grown from a vegetative inoculum on malt extract agar (4%) at 28°C for five days. The three strains had been previouly screened by Esposito *et al.* (1996) and Gonçalves *et al.* (1998).

Sugarcane bagasse

The sugarcane bagasse used in our experiments contained glucan (51%), polioses (23%), acetyl groups (2%), Klason lignin (21%), H_2SO_4-soluble lignin (1%) and ash (3%), its particles averaging 2.1 cm in length and 0.12 cm in width.

Culture conditions

Three semi-solid state fermentation cultures were carried out at 28°C with no additional carbon source in 250 mL-Erlenmeyer flasks with and without shaking during 10 days. Samples (6 g) of depithed and sterilized sugarcane bagasse were supplemented only with 1 mL salts solution ($CaCl_2$, $(NH_4)_2HPO_4$, FeCl, KH_2PO_4, $MgSO_4$) and 49 mL distilled water. Another set of experiments was carried out for 30 days, using plastic bags containing 100 g sterilized sugarcane bagasse supplemented with 17 mL of the same salts solution and 816 mL distilled water.

Chemical analysis of decayed sugarcane bagasse

The modified Klason method was utilized (ASTM 1956). Samples (1 g) of decayed and undecayed sugarcane bagasse were treated with 5 mL H_2SO_4 72%. After a 7-min stirring at 45°C, 25 mL of water was added to the mixture, which was post-hydrolyzed under 1.05 bar for 30 min. The product was filtered and the insoluble portion (Klason lignin) quantified. The hydrolysate was acidified to pH 1-3, filtered in a Sep-Pak C_{18} cartridge and analyzed by HPLC in a Shimadzu LC10 chromatograph with an Aminex HPX-87H column at 45°C. Mobile phase was H_2SO_4 0.005 $mol.L^{-1}$ at 0.6 $mL.min^{-1}$. The hydrolysis products were determined by refraction index and quantified by using calibration curves (Silva 1995). Soluble lignin was determined as described by Rocha et al. (1993) using absorption at 280 nm of alkaline solutions obtained from the hydrolysate.

Figure 1 shows the scheme of the experimental part.

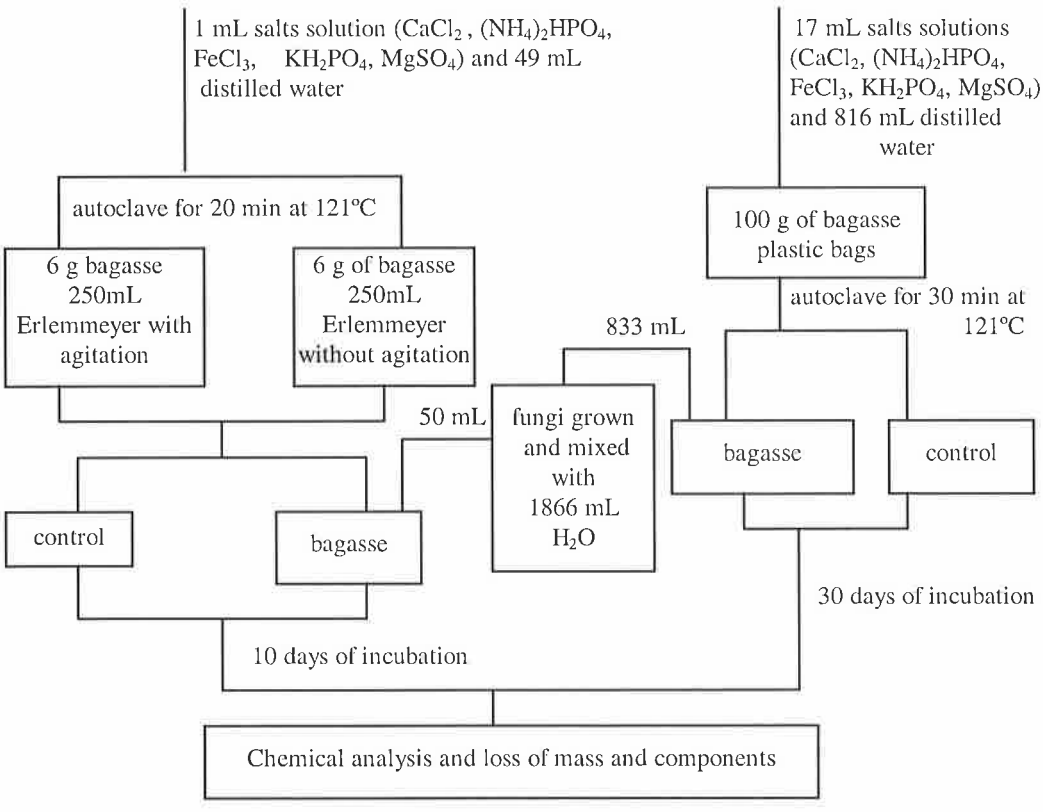

Figure 1. Scheme of the experimental part.

RESULTS AND DISCUSSION

Chemical analysis

Chemical analysis of decayed samples of sugarcane bagasse is useful for the determination of the loss of mass and components after treatment with fungi strains. Selectivity values were calculated from these parameters. Quantification procedures were based on hydrolysis of the samples adopting standard methods: Klason for the quantification of lignin and HPLC for the quantification of sugars and derivatives (Rocha *et al.*, 1997).

Previous results of degradation of sugarcane bagasse by the three strains show that all the samples had about the same chemical composition after degradation (Esposito *et al.*, 1998). FTPT-4741 preserved carbohydrates and lowered the lignin content, but only FTPT-4742 preserved the xylan/glucan ratio. FTPT-4745 showed the highest enzymatic production and this fact was correlated to the highest amount of soluble lignin.

Tables 1 to 3 show the chemical composition of the decayed and undecayed (control) bagasse samples in the three systems utilized. Unlike Esposito *et al.*, (1996), we increased the agitation during hydrolysis to favour the reaction and measured the ash content.

Table 1. Chemical composition of decayed and undecayed (control) sugarcane bagasse after shaking the flasks.

	FTPT-4741	FTPT-4742	FTPT-4745	control
glucan (%)	39.76	37.87	40.58	41.1
acetyl (%)	1.87	1.97	2.21	2.32
xylan (%)	22.55	21.5	23.34	23.57
Arabinan (%)	1.62	1.68	0.24	1.89
total carbohydrates (%)	65.8	63.0	66.37	68.8
Klason lignin (%)	18.82	18.59	19.90	19.66
soluble lignin (%)	5.14	7.50	2.51	3.27
ash (%)	0.51	0.28	0.028	0.28
total lignin (%)	23.96	26.09	22.41	22.93
total ash (%)	0.64	0.32	0.70	0.57
mass balance (%)	90.4	89.41	89.48	92.34
xylan/glucan ratio	0.567	0.567	0.575	0.574
Polioses/glucan ratio	0.655	0.664	0.636	0.677

Table 2. Chemical composition of decayed and undecayed (control) sugarcane bagasse in the stationary flasks.

	FTPT-4741	FTPT-4742	FTPT-4745	Control
glucan (%)	40.54	40.81	43.0	40.48
acetyl (%)	1.56	2.19	2.34	2.27
xylan (%)	24.03	25.02	26.07	23.24
Arabinan (%)	1.29	1.27	1.33	1.84
total carbohydrates (%)	67.42	69.29	72.74	67.83
Klason lignin (%)	19.59	18.26	18.17	19.68
soluble lignin (%)	4.19	5.87	4.79	2.59
ash (%)	0.70	0.28	0.26	0.26
total lignin (%)	23.78	24.13	22.96	22.27
total ash (%)	0.49	0.37	0.29	0.45
mass balance (%)	91.69	93.79	95.99	90.55
xylan/glucan ratio	0.593	0.613	0.606	0.574
Polioses/glucan ratio	0.663	0.698	0.692	0.676

Table 3. Chemical composition of decayed and undecayed (control) sugarcane bagasse in the plastic bags.

	FTPT-4741	FTPT-4742	FTPT-4745	Control
glucan (%)	43.96	41.63	40.63	44.19
acetyl (%)	2.42	2.32	2.22	2.37
xylan (%)	24.27	23.71	23.82	25.41
Arabinan (%)	1.66	1.78	1.67	1.79
total carbohydrates (%)	72.31	69.44	68.35	73.76
Klason lignin (%)	19.80	19.00	19.60	21.90
soluble lignin (%)	2.5	5.0	4.4	3.9
ash (%)	0.4	0.4	0.3	0.2
total lignin (%)	22.3	24.0	24.0	25.8
total ash (%)	0.3	0.3	0.1	0.4
mass balance (%)	94.87	93.71	92.47	99.98
xylan/glucan ratio	0.552	0.570	0.586	0.562
Polioses/glucan ratio	0.646	0.668	0.682	0.610

Although mass balance increased by 4 to 12%, approximately 5% of the mass probably corresponding to extractives could not be determined, as evaluated by Silva (1995).

In some decayed samples the xylan/glucan ratio did not change while in others it increased (in comparison with the control), showing that the fungus does not attack xylan very strongly.

Results from Table 1 (shaken flasks) show that FTPT-4742 produced samples with a small glucan content but with a soluble lignin content (7.5%) 1.3 times higher than that of the control.

In the stationary system (Table 2), the amounts of glucan were very similar in the samples decayed by FTPT-4741 and FTPT-4742 strains, and higher in the samples decayed by FTPT-4745. Acetyl content of *in natura* bagasse was 2% and only sample decayed by FTPT-4741 showed a smaller value, due to its deacetylation. This sample showed also a lower xylan/glucan ratio. The some low xylan/glucan ratio was provided by FTPT-4741 when using the plastic bags system. The percentage of glucan preservation increased 5.6% and 8.1% in comparison with the values provided by FTPT-4742 and FTPT-4745, respectively. Acetyl contents ranged from 2.2 and 2.4% both for the control and for the decayed samples.

Losses of mass and components were calculated for a better evaluation of the action of the strains on the bagasse, since chemical analysis is restricted to the verification of changes in the composition of the samples. These values together with the selectivity values (loss of lignin/loss of glucan ratio) are shown in Tables 4 to 6.

Table 4. Losses of mass and components of the bagasse samples treated with *Panus tigrinus* strains in shaken flasks. (Selectivity is the loss of lignin/loss of glucan; values related to the control).

Losses (%)	FTPT-4741	FTPT-4742	FTPT-4745
mass	13.32	24.10	7.40
glucan	16.58	30.00	8.50
xylan	17.10	30.84	8.32
lignin	9.42	13.61	9.46
selectivity	0.60	0.45	1.11

Table 5. Losses of mass and components of the bagasse samples treated with *Panus tigrinus* strains in stationary flasks. (Selectivity is the loss of lignin/loss of glucan; values related to the control).

Losses (%)	FTPT-4741	FTPT-4742	FTPT-4745
mass	21.10	16.65	7.76
glucan	20.97	16.02	2.03
xylan	18.42	10.13	3.48
lignin	15.79	9.82	4.98
selectivity	0.75	0.62	2.43

Table 6. Losses of mass and components of the bagasse samples treated with *Panus tigrinus* strains in plastic bags. (Selectivity is the loss of lignin/loss of glucan; values related to the control)

Losses (%)	FTPT-4741	FTPT-4742	FTPT-4745
mass	5.10	5.15	9.31
glucan	5.58	10.61	16.60
xylan	9.37	11.53	15.34
lignin	18.10	12.00	15.71
selectivity	3.24	1.13	0.95

Higher values of glucan and xylan losses occured in the shaken and stationary flasks (Tables 4 and 5), corresponding to a rapid fungic colonization in the bagasse. On the other hand, the losses of mass and carbohydrates in the plastic bags were lower than in the flasks, and the lignin was efficiently removed.

Lignin should be removed without damage to the carbohydrates. This behaviour is reflected by the selectivity, which should be greater than 1.0 for a preferential remotion of lignin in respect to the degradation of glucan.

Only the FTPT-4745 strain was selective concerning lignin degradation in flasks, mainly in the stationary flasks. In such system, the aeration is not effective, decreasing the action of the oxidative laccase enzyme, which also decays carbohydrates. With the plastic bags, the situation was the inverse, but FTPT-4745 showed a selectivity value close to 1.0. The mass losses in plastic bags were very small for FTPT-4741 and

FTPT-4742 strains, confirming that the enzymatic activity varies according to the system, as reported in other papers (Esposito *et al.,* 1998; Gonçalves *et al.,* 1999).

CONCLUSIONS

It is possible to evaluate the action of *Panus tigrinus* on the sugarcane bagasse fibers by determining the chemical composition of the bagasse and its losses of mass and components. The results show that this fungus is adequate for the pretreatment of sugarcane bagasse, causing delignification and preserving the fiber properties. Consequently, this process can be utilized in pulp production. FTPT-4745 was seletive for lignin degradation in flask, mainly in stationary flasks. The mass losses in plastic bags were very small for FTPT-4741 and FTPT-4742 strains.

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REFERENCES

Abd El-Nasser, N.H., Helmy, S.M. & El-Gammal, A.A. (1997). Formation of enzymes by biodegradation of agricultural wastes with white rot fungi. *Polym. Degrad.* Stab, **55,** 249-255.

Akhmedova, Z.R., Beletska, O.P., Dalimova, G.N., Khalikova, M.M., Azimkhodzhaeva, M.N., Davranov, K.D. & Sharipova, A. (1994). Selection and cultivation of cellulose-degrading and lignin-degrading fungi. *Microbiology*, **63**, 523-527.

ASTM Methods (1956). D 271-48. Standard test methods for lignin in wood.

Breccia, J.D., Bettucci, L., Piaggio, M. & Siñeriz, F. (1997). Degradation of sugar cane bagasse by several white-rot fungi. *Acta Biotechnol*, **17**, 177-184.

Esposito, E., Speranza, M., Siñeriz, F. & Bettucci, L. (1995). Delignification of lignocellulosic materials with wood-rotting fungi. *In 4th Braz.Symp.Chem.Lignins Other Wood Compon.*, Proceedings, pp 121-125.

Esposito, E., Benar, P., Speranza, M. & Gonçalves, A.R. (1996). Growth of ligninolytic fungi on sugar cane bagasse as a pre-treatment for organosolv delignification. *In 3th. Inter. Nonwood Fibre Pulping Papermaking Conf.*, Proceendigs. pp. 12-16.

Gao, Y., Wang, S., Lin, L. & Chen, J. (1996). Studies on bagasse lignin biodegradation by white-rot fungi. *Ziran Kexueban,* **24**, 37-43.

Gonçalves, A.R., Esposito, E. & Benar, P. (1998). Evaluation of *Panus tigrinus* in the delignification of sugarcane bagasse by FTIR-PCA and pulp properties. *J. Biotechonol*, **66**, 177-185.

Pessoa, A. Jr., De Mancilha, I.M. & Sato, S. (1997). Evaluation of sugar cane hemicellulose hydrolyzate for cultivation of yeasts and filamentous fungi. *J.Ind.Microbiol. Biotechnol*, **18**, 360-363.

Prasad, D.Y., Rajesh, K.S., Praburaj, T., Rao, N.R.M. & Joyce, T.W. (1996a). Enzyme-assisted bleaching of bagasse. *Cellul. Chem. Technol*, **30**, 463-472.

Prasad, D.Y., Rao, N.R.M., Rajesh, K.S., Praburaj. T.T. & Joyce, T.W. (1996b). Enzymes improve the bleachability of bagasse mechanical pulp. *Tappi J*, **79**, 133-138.

Rocha, G.J.M., Silva, F.T, Curvelo, A.. A. S. & Araújo, G. T. (1997) A fast and accurate method for determination of cellulose and polyoses by HPLC. *In: fifth Brazilian Symposium on Chemistry of Lignins and other Wood Components*, Poceedings, **V 6**, pp.3-8.

Rocha, G.J.M.; Silva, F.T. & Schuchardt, U. (1993), Improvement of a rapid UV spectrophotometric method for determination of lignin in alkaline solutions. *In 3th Brazilian Symposium on the Chemistry of Lignins and Other Wood Components*, Proceedings, pp 73.

Silva, F.T. (1995), Obtenção de Insumos Químicos a partir do Aproveitamento Integral do Bagaço de Cana, PhD. Thesis, Instituto de Química, Universidade Estadual de Campinas.

Zadrazil, F. & Puniya, A.K. (1995). Studies on the effect of particle size on solid-state fermentation of sugarcane bagasse into animal feed using white-rot fungi. *Biores. Technol*, **54**, 85-87.

Solvent effect on extraction of polyflavonoids from *Pinus radiata* D. Don bark

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ABSTRACT

This work evaluates the effect of the solvents mixture composition on the extraction of polyflavonoids from bark of *Pinus radiata*. It was selected a non-reactive solvent, specifically methanol, and its mixtures with water. The obtained fractions were characterized by chemical assays, chromatographic (GPC) and spectrophotometric (IR) methods.

Keywords: *Pinus radiata*, wood extractives, polyflavonoids

INTRODUCTION

The condensed tannins, a type of natural poliflavonoid, are present in many vegetable tissues, such as pine bark (*Pinus* sp.), acacia bark (wattle or mimosa), quebracho wood (*Schinopsis* sp.), etc. (Pizzi and Stephanou, 1994; Masson *et al.*, 1997).

Many attempts have been done to optimize the isolation of this type of metabolites from natural sources, for different uses, and to do that it has been utilized different solvents, generally reactive, between them: sodium sulfite solutions (von Leyser and Pizzi, 1990); sodium carbonate and sodium hydroxide solutions (Vázquez *et al.*, 1987).

However, this study is centered in obtaining natural, non-modified tannin from the *Pinus radiata* bark, in order to evaluate its reactivity and its application in adhesives formulation for wood. For this end, methanol was selected as a non-reactive solvent.

In order to evaluate the better solid/liquid extraction conditions, the following extracting solvents were used: water, water:methanol (7:3), water:methanol (1:1), water:methanol (3:7) and methanol 100%, the extraction percentages and some characteristics of the isolated tannin were determined. The other experimental conditions were maintained constant.

EXPERIMENTAL

Bark of 27 years old *Pinus radiata* trees, grown in Concepción (Chile), was dried with a hot air current at an average temperature of 40-50 °C. Once dried, the bark was classified as bulk bark, mean bark (smaller particles), bark with cambium and bark with other particled material.

The bulk bark was grounded to 40-60 meshes in a blade mill, brand Retsch, model SM1 and extracted as described below. The moisture percentage of the grounded bark was determined.

For the extractions, it was used a modified procedure of the suggested by Ayla (1983). Ten samples of grounded bark of 25 g each one, exactly weighed, were extracted in pars, three consecutive times with 200 ml of the following solvents: water 100%, water:MeOH (7:3), water:MeOH (1:1), water:MeOH (3:7) and MeOH 100%. Each extraction was accomplished by a period of two hours, in a water bath at 70°C, provided of a frontal shaker with an agitation frequency of 100 cpm approximately. After the first extraction, the hot mixture was vacuum filtered quickly, obtaining an extract and a solid residue, and the last one was successively extracted by the same procedure, twice. The extracts of the five pars of samples, in each one of the extraction stages (3 for each par), gave rise to 15 different extracts (Table 1), which were concentrated, freeze dried, weighted and analyzed according to is thereinafter described.

The Stiasny number for each freeze-dried extract was determined according to the method described by Suomi (1985).

The samples (1,2)-1 and (9,10)-1, and the (5,6)-1, which represent the extremes and the middle point respect to solvent composition, i.e. with water 100%, MeOH 100% and water:MeOH (1:1) respectively, were selected to study the separation by Sephadex LH-20 and to analyze by GPC and spectrophotometric methods.

Samples of the crude extracts were acetylated, according to method described by García-Vallejo *et al.* (1997), and the acetylated products were analyzed by GPC. Other part of each one of these crude fractions (1 g), were filtered on Sephadex LH-20, following the procedures described by Makkar and Becker (1994), the fractions eluted with acetone:water (1:1) were concentrated, freeze dried, acetylated, and analyzed by GPC. Finally, other part of each one of these fractions (1 g) were previously purified by successive solid/liquid extraction (Soxhlet extraction) with ethyl ether (50 ml) and ethyl acetate (50 ml), during 4 hours with each one, and the resulting products were thereinafter acetylated for their analysis by GPC. All GPC analyses were accomplished in a Merck-Hitachi chromatograph, model L-4250, provided with a Merck-Hitachi pump, model L-6200 A, and a Merck-Hitachi integrator, model D-2520. Three Styragel Waters columns serially connected (HR 0.5, HR 1 and HR 2, for molecular weight ranges of 0-1000, 100-5000 and 500-20000 Da, respectively) were used, at 60°C, with a THF flow of 0.4 ml/min, and with UV detection at 280 nm. The columns were calibrated with polystyrene standards.

The same procedures were followed for drying, milling and extraction of bark samples from different sections of other tree. Samples of the inferior, intermediate and superior sections of a same 25 years old *Pinus radiata* tree, 4 meters long approximately each one, were collected. For these samples, it was realized a unique extraction step, by duplicate, using the same solvents mentioned above. The yields, the chemical and chromatographic analyses of the freeze-dried extracts were obtained.

RESULTS AND DISCUSSION

The obtained results of the three extractions stages with each one of the selected solvents, for the first sampled bark, corresponding to 27 years old trees, are shown in Table 1.

Table 1. Extraction stages results from the bark of *Pinus radiata*, 27 years old, with the following solvents: water 100%, MeOH 100% and water:MeOH (7:3), (1:1) and (3:7).

Sample Code	Extracting Solvent	Extract Code	% w/w Extracted	% w/w Total Extracted	Stiasny Number	% w/w Extracted as Reactive Polyphenol	% w/w Total as Reactive Polyphenol (Stages 1 and 2)	% of Reactive Polyphenol Respect to Total
(1, 2)	Water 100 %	(1,2)-1	14.70	24.90	77.20	11.35	15.81	71.76
		(1,2)-2	6.61		67.56	4.47	(-)	28.24
		(1,2)-3	3.59		50.88	1.83	(-)	-
(3, 4)	Water:MeOH (7:3)	(3,4)-1	25.50	35.95	93.29	23.79	29.89	79.59
		(3,4)-2	6.91		88.29	6.10	(-)	20.41
		(3,4)-3	3.54		(-)	(-)	(-)	(-)
(5, 6)	Water:MeOH (1:1)	(5,6)-1	29.06	48.13	81.85	23.79	30.56	77.82
		(5,6)-2	10.07		67.31	6.78	(-)	22.18
		(5,6)-3	9.00		(-)	(-)	(-)	(-)
(7, 8)	Water:MeOH (3:7)	(7,8)-1	43.61	61.88	70.38	30.69	36.48	84.13
		(7,8)-2	7.94		72.94	5.79	(-)	15.87
		(7,8)-3	10.33		(-)	(-)	(-)	(-)
(9, 10)	MeOH 100 %	(9,10)-1	35.09	65.78	87.15	30.58	35.15	87.00
		(9,10)-2	16.85		27.12	4.57	(-)	13.00
		(9,10)-3	13.84		(-)	(-)	(-)	(-)

(-) Not performed measurement

The greater extractions are achieved in the first stage of each sequence (from 50% to 70% w/w of the total extracted), reducing the yields in the second stage (from 12% to 25% w/w), and consequently in the third (from 9% to 21% w/w). The bulk of extractives and the total percentage extracted, are increased with the methanol proportion in the extracting solvents mixture.

The Stiasny numbers for the first stage, of each one of the series analyzed with each solvent, were generally greater than those obtained for the other stages. It was not observed trend of the Stiasny numbers with the composition of the solvent used in the extraction.

The reactive polyphenol percentage (calculated as the product of the extracted fraction by the Stiasny number) represents the w/w percentage of each extract able to reacts with formaldehyde, in an acid middle, under boiling. The value obtained for this parameter (Table1) is greater for the extracts of the first stage. In the first stage, this percentage increases as it is increased the proportion of methanol in the extracting solvent, being 11% w/w for water 100%, 24% w/w for solvents with 30% and 50% v/v of methanol, and 30% for solvents with 70% v/v and 100% of methanol. The same trend is observed for the total of reactive polyphenol percentage (adding the yields obtained in the first two stages) with each one of the tested solvents, a smaller yield for the water 100% (15.81% w/w), intermediate yields for the water:methanol (7:3) and (1:1) systems, with a 30% w/w approximately, and greater for the water:methanol (7:3) and methanol 100% systems, with a 35-36% w/w approximately.

The quantities extracted with water were lower than those with the mixtures containing methanol. However, it is not observed large variations in the percentages of extraction when varies the methanol proportion in the solvent, even at the lowest proportion used (30 % v/v). The percentages of extraction are smaller in the inferior section of the tree, being almost equal for the other two sections. Nevertheless, in terms of reactive polyphenolic it is observed that this value increased as the methanol proportion in the solvent mixture used in the extraction do. The values for this parameter are greater for the tannins obtained from bark of the intermediate section of the tree with all the used solvents.

The identity of the extracted material was confirmed by the IR spectrum. All the extracts showed the characteristic bands of this type of substances. The IR spectrum of the freeze-dried extract (1,2)-1, as an example, is shown in Figure 1.

In Table 2 are summarized the results obtained from the three bark sections sampled from a same 25 years old tree.

Table 2. Extraction results from bark of the three height sections of *Pinus radiata* tree, 25 years old, with the following solvents: water 100%, MeOH 100% and water:MeOH (7:3), (1:1) and (3:7).

Extracting Solvent	Tree Section	Extract Code	% w/w Average Extracted	Average Stiasny Number	% w/w Average Extracted as Reactive Polyphenol
Water 100 %	Inferior	A1PS-1,2	14.60	48.91	7.14
	Media	A2PS-1,2	21.92	40.60	8.90
	Superior	A3PS-1,2	20.06	28.69	5.76
Water:MeOH (7:3)	Inferior	A1PS-3,4	18.69	67.83	12.68
	Media	A2PS-3,4	25.97	55.86	14.50
	Superior	A3PS-3,4	25.64	43.59	11.18
Water:MeOH (1:1)	Inferior	A1PS-5,6	19.38	68.36	13.25
	Media	A2PS-5,6	24.19	58.87	14.24
	Superior	A3PS-5,6	24.47	48.20	11.79
Water:MeOH (3:7)	Inferior	A1PS-7,8	19.67	73.87	14.53
	Media	A2PS-7,8	25.95	61.68	16.00
	Superior	A3PS-7,8	25.34	50.68	12.84
MeOH 100 %	Inferior	A1PS-9,10	18.51	80.58	14.92
	Media	A2PS-9,10	24.62	69.84	17.19
	Superior	A3PS-9,10	22.06	59.36	13.09

In Table 3 are given the Stiasny numbers and recovery percentages of the fractions obtained by elution from Sephadex LH-20 with the solvents MeOH:water (8:2), acetone:water (1:1) and acetone 100%, of each one of the extracts (1,2)-1, (5,6)-1 and (9,10)-1, in their crude form (non-pretreated), as well in their form previously extracted with ethyl ether and ethyl acetate.

The non-tannic fraction of the extracts was isolated by eluting the extracts through Sephadex LH-20 with methanol-water (8:2), and then the tannic fraction was eluted with acetone-water (1:1). The yields are summarized in Table 3.

It is observed that for crude extracts passed by Sephadex LH-20, the non-tannic fractions represent 75.0% w/w, 70.9% w/w and 67.6% w/w of the extracts (1,2)-1, (5,6)-1 and (9,10)-1, respectively; while the tannic fractions represent 24.1% w/w, 24.8% w/w and 27.1% w/w, respectively. The Stiasny numbers for non-tannic fractions range from 64 to 82, being greater in the samples extracted with the methanol rich solvent. In the tannic fractions, the Stiasny numbers were between 93-95, values considerably greater than those obtained for the non-tannic fractions.

Table 3. Stiasny numbers and recovery percentages of the eluted fractions, from Sephadex LH-20, of the extracts (1,2)-1, (5,6)-1 and (9,10)-1, in their crude form (non-pretreated) as well in their previously extracted form.

Extract Treatment	Extract Code	Extract Stiasny Number before Sephadex LH-20	Elution Solvent from Sephadex LH-20	% w/w of eluted fraction	Stiasny Number of eluted fraction
Crude + Sephadex LH-20	(1,2)-1	77.20	MeOH:water (8:2)	75.04	63.70
			Acetone:water (1:1)	24.11	95.37
			Acetone 100%	0.18	(-)
	(5,6)-1	81.85	MeOH:water (8:2)	70.86	74.01
			Acetone:water (1:1)	24.76	93.76
			Acetone 100%	0.33	(-)
	(9,10)-1	87.15	MeOH:water (8:2)	67.58	82.08
			Acetone:water (1:1)	27.13	92.92
			Acetone 100%	0.39	(-)
Pre-extracted + Sephadex LH-20	(1,2)-1	72.94	MeOH:water (8:2)	60.28	58.38
			Acetone:water (1:1)	34.75	96.21
			Acetone 100%	0.10	(-)
	(5,6)-1	77.59	MeOH:water (8:2)	59.40	68.06
			Acetone:water (1:1)	36.36	95.63
			Acetone 100%	0.63	(-)
	(9,10)-1	85.67	MeOH:water (8:2)	54.22	77.95
			Acetone:water (1:1)	38.65	95.58
			Acetone 100%	1.18	(-)

(-) Not performed measurement

On the other hand, for the previously purified extracts, by successive extractions with ethyl ether and ethyl acetate, the non-tannic fractions represent 60.3% w/w, 59.4% w/w and 54.2% w/w of the extracts

(1,2)-1, (5,6)-1 and (9,10)-1, respectively; while the tannic fractions were 34.8% w/w, 36.4% w/w and 38.6% w/w, respectively. In spite, that the trends were similar than those observed for the crude extract, the amounts of the non-tannic fractions were smaller and those of the tannic fractions greater. These last fractions presented Stiasny numbers between 95-96%.

In this way, it is demonstrated that a previous purification of the extracts by extraction with ethyl ether and ethyl acetate gave better yields from the filtration by Sephadex LH-20. However, the benefit of including a stage most in the purification process is not considerable, therefore would not be necessary.

Tannin is a polymer with a wide range of molecular weight and when removed from bark, the values and distribution depend on the isolation procedure. GPC experiments were carried out to estimate the molecular weight (M) and the molecular weigh distribution (MWD) of the different tannin samples. In order to diminish the adsorption and association caused by hydroxyl groups, the acetylated derivatives were used. In addition of minimize the interactions, the acetylated tannins are soluble in solvents such as THF, which was used as eluent. Columns calibration was carried out by using polystyrene standards.

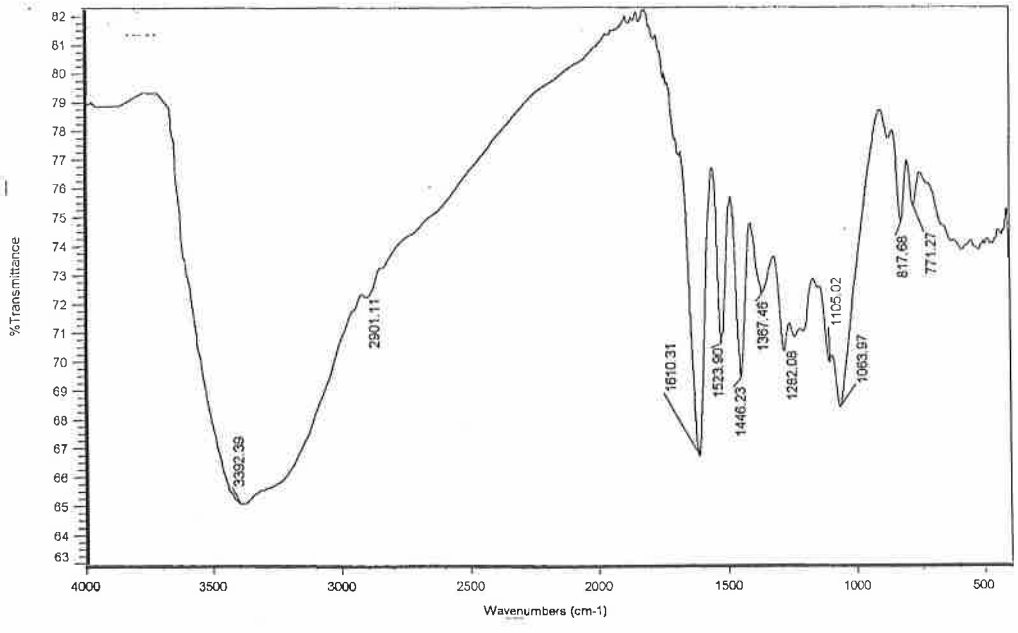

Figure 1. FT-IR spectrum of the freeze-dried extract (1,2)-1, taken at diffuse reflectance mode, with a Perkin–Elmer spectrophotometer, model 2000 FT-IR.

The SEC permeation chromatograms of tannins showed multimodal distributions. In Figure 2 are shown the chromatograms for crude (a), and the purified tannins (crude+Sephadex LH-20 and pre-extracted +Sephadex LH-20), (b, c). The two main signs were not well resolved, but two maxims are clearly observed, one at 36 min. approximately (Mn =27-37*10^3), and the other at 38 min. approximately (Mn = 8-9*10^3). The crude tannins, besides this two peaks, present other one at higher retention times, which is not present in the purified extracts, indicating that in the purification process were eliminated the lower-molecular weight portions. The relative intensity of the two main peaks depend on the nature of the solvent used in the extraction, to greater methanol content greater is the proportion of the high molar weight fraction.

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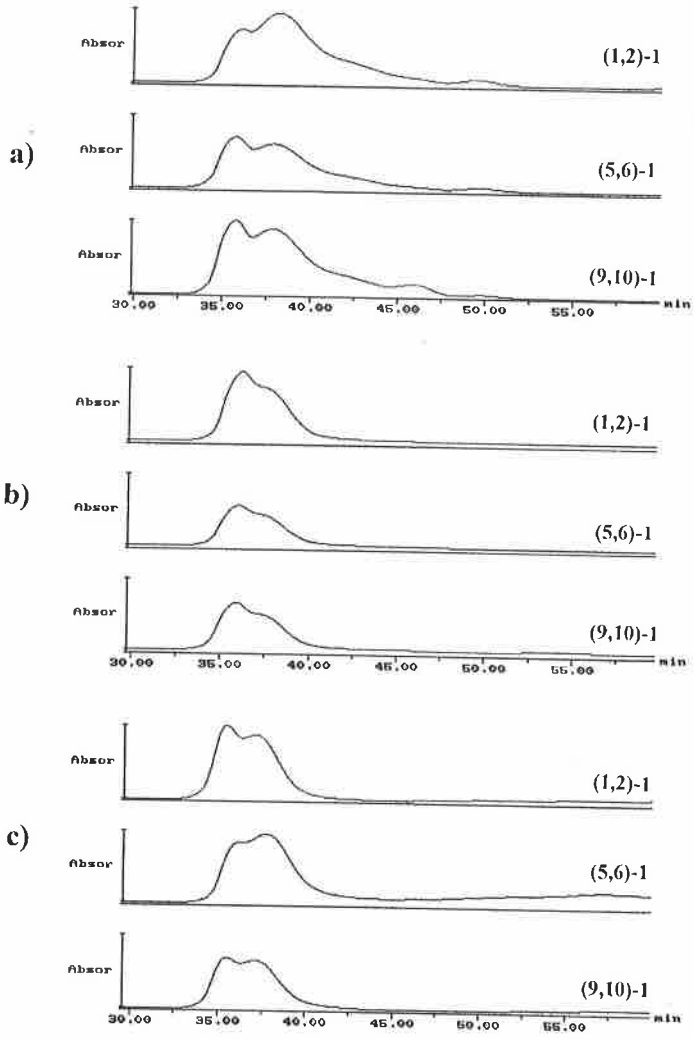

Figure 2. Comparison of GPC cromatogramas of acetylated derivatives of the extracts (1,2)-1, (5,6)-1 and (9,10)-1: a) acetylated derivatives of the crude extracts, b) acetylated derivatives of the extracts filtered through Sephadex LH-20, c) acetylated derivatives of the extracts previously extracted with ethyl ether and ethyl acetate, and then filtered by Sephadex LH-20. Chromatographic conditions: Three serially connected Styragel Waters's columns, HR 0.5, HR 1 and HR 2. Temperature: 60ºC. Eluent THF flow: 0,4 ml/min. UV Detector at 280 nm.

REFERENCES

Ayla, C. (1983). *Wood Adhesives : Present and Future*, John Wiley & Sons, New York, pp. 69.

García-Vallejo, M. C.; Cadahía E. & Conde E. (1997). Hydrolizable and Condensed Tannins in Wood and Bark of Eucalyptus. *Fifth Brazilian Symposium on the Chemistry of Lignins and other Wood Components*, Proceedings, **Vol 6**, pp 176-185.

Makkar, H. P. S. & Becker, K. (1994). Isolation of Tannins from Leaves of Some Trees and Shrubs and Their Properties. *J. Agric. Food Chem.*, **42**, 731.

Masson, E.; Pizzi, A. & Merlin, M. (1997). Comparative Kinetics of the Induced Radical Autocondensation of Poliflavonoid Tannins. II. Flavonoid Units Effects. *J. Appl. Polym. Sci.*, **64**, 243.

Pizzi, A. & Stephanou, A. (1994). Fast vs. Slow-reacting Non-modified Tannin Extracts for Exterior Particleboard Adhesives. *Holz als Roh-und Werkstoff*, **52**, 218.

Suomi, L. (1985). Bark Extracts and Their Use in Plywood Bonding. *Pap Puu*, **2**, 65.

Von Leyser, E. & Pizzi, A. (1990). The Formulation and Commercialization of Glulam Pine Tannin Adhesives in Chile. *Holz als Roh-und Werkstoff*, **48**, 25.

Vázquez, G.; Antorrena, G. & Parrajó, J. C. (1987). Studies on the Utilization of *Pinus pinaster* Bark. Part 2 : Kinetics and Yields of Alkaline Extractions. *Wood Sci. Technol.*, **21**, 155.

Steam explosion of sugar cane bagasse catalysed by H_2SO_4: I. Effect of temperature, time and acid content on sugarcane bagasse solubization

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ABSTRACT

This paper describes the pretreatment of water-free sugar cane bagasse (10g) by steam explosion in a 0.65 L stainless-steel reactor, varying the temperature from 118 to 222°C, time from 4.1 to 59 min and H_2SO_4 contents from 1.0 to 12.9 mmoles. The influence of these variables on the amount of solubilized glucan was investigated by means of multivariate statistical analysis applying factorial design techniques and the response surface methodology. The response surface showed that the amount of solubilized bagasse increased with the severity of the process and occurred in two phases: the first controlled by xylan hydrolysis and the second by cellulose hydrolysis. Under high severity conditions the amount of solulilized bagasse decreased due to condensation reactions of lignin and furan compounds derived from dehydrated-sugar reactions.

Keywords: steam explosion, sugarcane bagasse, factorial design

INTRODUCTION

Sugarcane bagasse is obtained in large quantities in Brazil as a by-product of sugar and alcohol industries. Usually bagasse is used as a fuel to generate energy for industries (Armas *et al.*, 1990). Even so there is an enormous surplus of bagasse that could be used at least in part to produce chemicals of high value such as organic acid, furfural, phenols and other aromatics compounds (Deschamps *et al.*, 1996; Carrasco, 1996; Purchase, 1994; Capek-Ménard *et al.*, 1992).

However, the utilization of lignocellulosic materials for chemical and/or biotechnological purposes requires the splitting of the lignin-cellulose-polyoses complex and the removal of each fraction by pretreatment and delignification (Silva, 1995).

The steam explosion process has been used as a pretreatment to improve the biotechnological conversion of lignocellulosic materials (Stenberg *et al.*, 1998; Grethlein, 1984; Saddler *et al.*, 1983). During the pretreatment, hydrolysis is the main reactions that occur on the components the lignocelullosic materials. On the other hand, the sugars produced from hydrolysis of cellulose and polyoses can be dehydrated to furfural and hydroxymethyfurfural, which condense with lignin to form high molecular weight compounds. The lignin is also partially solubilized to low molecular weight compounds, which are potentially toxic to the microorganism used in enzymatic and fermentative processes (Sun *et al.*, 1996; Browneel *et al.*, 1986). All the biomass components are affected by the steam explosion process, in which the hydrolysis, dehydration and condensation reactions are dependent on pretreatment conditions (Silva, 1995).

This study reports the influences of temperature, time and sulfuric acid on the solubilization of sugarcane bagasse applying multivariate statistical analysis, factorial design techniques and response surface methodology.

EXPERIMENTAL

Steam Treatment

The bagasse samples (10 g) were soaked in 110 mL H_2SO_4 sulfuric acid solutions ranging from 0.1 to 1.29 mmoles, at room temperature for 1h. The excess acid present in the samples was drained prior to their introduction into the reactor. The drained H_2SO_4 was separated from the bagasse fines by filtration and titrated with NaOH 0.1 mol L^{-1}. The total acid absorbed was determined by the difference between the acid concentrations before and after soaking. The bagasse excess retained during the filtration was determined gravimetrically. The drained bagasse was transferred to a 0.65 L stainless steel reactor and pretreated, using the temperature, residence time, and amount of acid absorbed by sugarcane bagasse established in the statistical design shown in Table 1 (Mattos, 2000).

The material was steam-treated and separated by filtration. The amount of the solubilized bagasse was calculated by the difference in dry weight between the bagasse transferred to the reactor and the pretreated bagasse.

Table 1. Levels and factors used in the factorial design.

Levels	Temperature (ºC)	Time (min)	$n_{H_2SO_4}$ (mmol)
-1	118	4.1	1.0
0	170	31.5	6.9
1	222	59	12.9

Experimental Design

The effects of temperature (T), residence time (t), and acid content on the solubilization of sugarcane bagasse were investigated using the response surface methodology described by Barros Neto *et al.* (1985). The design outlined in Table 2 consisted of 42 different experiments. The results were statiscally analyzed by fitting equations 1, 2 and 3 to the experimental response data (Y) by multiple linear, quadratic and cubic regressions. The analysis was performed with STATGRAPHICS statistical software version 6.0 and STATISTICA program version 5.0.

$$Y = b_0 + b_1T + b_2t + b_3\, n_{H_2SO_4} \qquad \textbf{equation 1}$$

$$Y = b_0 + b_1T + b_2t + b_3\, n_{H_2SO_4} + b_{11}T^2 + b_{22}t^2 + b_{33}\, n_{H_2SO_4}^{\ 2} + b_{12}Tt + b_{13}T\, n_{H_2SO_4} + b_{23}\, t\, n_{H_2SO_4} \qquad \textbf{equation 2}$$

$$Y = b_0 + b_1T + b_2t + b_3\, n_{H_2SO_4} + b_{11}T^2 + b_{22}t^2 + b_{33}\, n_{H_2SO_4}^{\ 2} + b_{12}Tt + b_{13}T\, n_{H_2SO_4} + b_{23}\, t\, n_{H_2SO_4} + b_{111}T^3 + b_{222}\, t^3 + b_{333}\, n_{H_2SO_4}^{\ 3} \qquad \textbf{equation 3}$$

As a result, an empirical model for each dependent variable (temperature, residence time and amount of H_2SO_4 absorbed) was obtained. The terms statistically insignificant at 95% confidence level were excluded from equations 1, 2 and 3 to improve the adequacy of fit. The coefficient of regression (R^2) and the significance tests (t and F tests) were used to indicate the adequacy of fit for each empirical model.

RESULTS AND DISCUSSION

The sugarcane used in this study was composed of glucan 39 ± 0.9%, xylan 22 ± 1%, arabinanose linked to xylose oligomers 1.7 ± 0.3%, acetyl groups linked to xylan 3.3 ± 0.1%, total lignin 24 ± 1%, ash 2.5 ± 0.1% and extractives 2.8 ± 0.8%.

The pretreatment conditions and the amount of solubilized sugarcane bagasse are shown in Table 2.

The variance analysis of the solubilized bagasse showed that the cubic model was significant at the 95% confidence level and that the model did not suffer lack of fit (R^2 = 75%). The cubic model, represented by equation 4, can be used to estimate responses at the experimental interval studied.

$$\%\ \text{Bag. Sol.} = (37{,}4 \pm 0{,}8) + (5 \pm 3)T + (8 \pm 2)t - (6 \pm 3)T[n_{H_2SO_4}] + (5 \pm 2)t[n_{H_2SO_4}] - (6 \pm 2)\,[n_{H_2SO_4}]^2 + (9 \pm 3)T^3 - (7 \pm 4)t^3 \qquad \textbf{equation 4}$$

The influences of the temperature and acid content on the amount of solubilized bagasse are shown by the response surfaces in Figure 1, which was built fixing the time of reaction at the coded levels -1, 0 and 1.

The amount of solubilized bagasse increased when the temperature was high, the time of pretreatment was short and the content of acid was low (level –1, Figure 1A), as well as when higher amounts of absorbed acid were used. The response surface also shows that the concentration of acid had some influence on the

sugarcane bagasse solubilization at the temperatures comprised in level –1. The bagasse solubilization increased when the acid concentration changed from level –1 to 0 and decreased when it changed from level 0 to 1, showing that acid contents above level 0 promoted condensation reactions between lignin and degradation products of the carbohydrates. At 222°C (temperature at level 1) the bagasse solubilization decreased when the acid concentration increased as a consequence of condensation reaction.

Table 2. Matrix of the factorial design and experimental response. Amounts expressed as g of solubilized bagasse per 100g of dry sugarcane bagasse.

Pretreatment	Temperature (ºC)	Time (min)	H_2SO_4 (mmoles/g bagasse)	Solubilized bagasse (%)
1	-0.58	-0.6	0.65	24.61
2	-0.58	-0.6	-0.61	23.94
3	0.58	0.5	0.53	42.82
4	0.58	-0.6	0.48	35.34
5	0.58	0.5	-0.58	42.62
6	-0.58	0.5	-0.66	27.05
7	0	-0.05	0.01	35.41
8	0.58	-0.6	-0.61	39.61
9	-0.58	0.5	0.58	31.89
10	0	-0.05	0.09	34.96
11	0	-0.05	-0.01	35.72
12	0.58	0.5	-0.65	41.07
13	0	-0.05	-0.01	35.58
14	0	-0.05	0.98	36.92
15	-1	-0.05	0.08	24.41
16	0	-0.05	0.16	40.72
17	0	-0.05	0.03	41.55
18	0	-0.05	-1	28.69
19	0	-1	0.19	41.72
20	0	0.9	-0.01	38.12
21	1	-0.05	0.04	50.12
22	1	-0.05	0.04	47.88
23	-0.38	0.68	-0.48	35.86
24	-0.38	0.68	0.23	36.86
25	-0.19	0.5	-0.21	43.04
26	-0.38	0.68	0.19	39.45
27	-0.38	0.68	-0.04	42.26
28	-0.38	0.68	0.78	41.16
29	-0.38	1	0.06	41.15
30	-0.58	0.5	1	39.17
31	-0.19	0.86	0.51	43.84
32	-0.38	0.36	0.19	36.61
33	-0.71	0.68	0.11	31.88
34	-0.19	0.5	0.55	40.20
35	-0.38	0.68	0.16	37.81
36	-0.38	0.68	0.14	38.63
37	-0.06	0.68	0.14	43.46
38	-0.19	0.86	-0.24	39.31
39	-0.58	0.5	-0.23	32.32
40	-0.58	0.86	0.60	39.41
41	-0.58	0.86	-0.24	28.95
42	-0.38	0.68	0.16	37.17

The results show that the bagasse solubilization as a function of temperature occurred in two phases. At temperatures from level –1 to –0.5 the solubilization of the bagasse depended on the solubilization of the polyoses and amorphous cellulose; at temperatures above level -0.5 it depended on hydrolysis reactions of the crystalline cellulose (data not shown). From levels –0.5 to 0.5 the hydrolysis reactions occurred more slowly due to the low diffusion of catalyst within the crystalline cellulose structure. The increase in solubilization between 0.5 and 1 occurred by virtue of a higher acid diffusion, which accelerated the hydrolysis rate (Figure 1A). Moreover, the model shows that the maximum of solubilization bagasse was 53% for pretreatment at 222°C (level +1), using bagasse containing from 1 to 4 mmol of H_2SO_4 (levels –1 and –0.5).

The profiles of the surfaces for time projected at levels 0 and +1 (figures 1B and 1C, respectively) are similar to that shown in Figure 1A, but the slopes are quite different. The amount of solubilized bagasse at temperature levels close to 1 did not decrease, due to condensation reactions of sugar degradation products with lignin occurring at the same time as hydrolysis of carbohydrates.

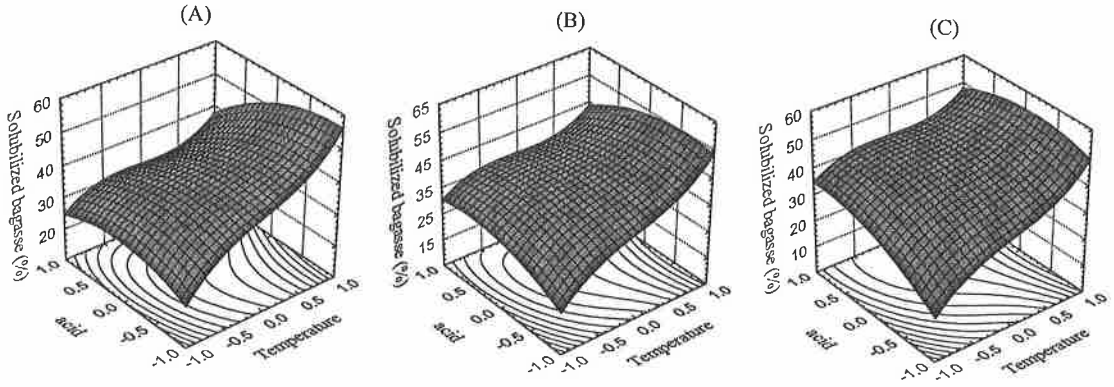

Figure 1. Response surfaces and contour lines of the cubic model representing the amount of solubilized sugarcane bagasse built by fixing the time at the coded levels –1 (A), 0 (B) and 1 (C).

The influences of temperature and time on the amount of solubilized bagasse are shown by the response surfaces in Figure 2, which was built fixing the $n_{H_2SO_4}$ at the coded levels -1, 0 and 1.

The profiles of response surface in Figure 2 are similar. The same figure shows that the amount of solubilized bagasse was more dependent on temperature than on reaction time. Increasing the time from level -1 to -0.5 practically resulted in no solubilization. On the other hand, the effect of time was more pronunced for pretreatments with high acid concentrations (Figure 2C).

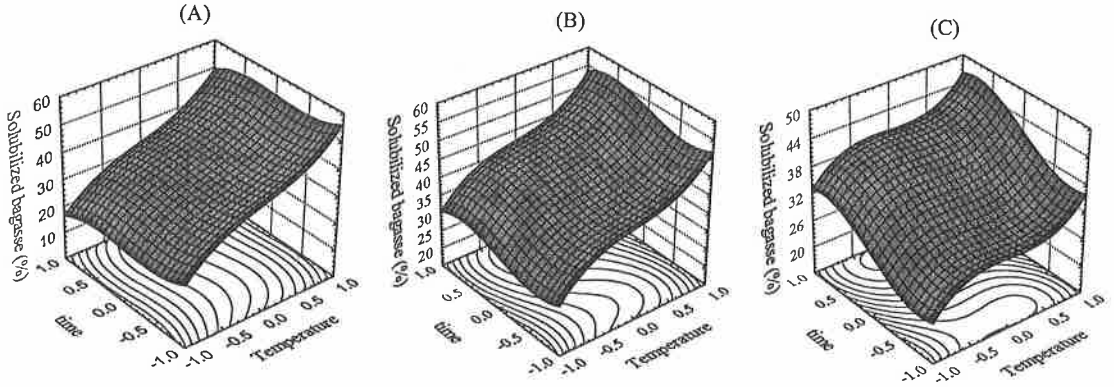

Figure 2. Response surfaces and contour lines of the cubic model representing the amount of solubilized sugarcane bagasse built by fixing the $n_{H_2SO_4}$ at the coded levels –1 (A), 0 (B) and 1 (C).

The influences of time and acid content on the amount of solubilized bagasse are shown by the response surfaces in Figure 3, which was built fixing the temperature at the coded levels -1, 0 and 1.

The solubilization of the sugar cane bagasse increased with the increase in time and in the amount of acid absorbed by bagasse (Figure 3A, temperature at level -1). However, the solubilization was more influenced by acid concentration than by reaction time, principally when times were at level +1.

The response surface also shows that the bagasse solubilization was caused by hydrolysis reactions of the cellulose, hemicelluloses and lignin (data not shown). Under the conditions shown in Figure 3A condensation reactions may have not occurred.

Because of the temperature increase (level 0, Figure 3B) the maximum value projected for the amount of solubilized bagasse was 40% in the region with acid concentration at levels ranging from 0 to 0.5 and time at level 0.5. Besides, the use of high concentration and extended reaction times caused a decrease in the amount

of solubilized bagasse as a result of the condensation reactions. At 222°C (level 1, Figure 3C) the maximum solubilization value (54%) was located between levels –0.5 and 0 of acid concentration and at level 0.5 of time. In these conditions (high temperatures) the increase in acid content favored the condensation reactions.

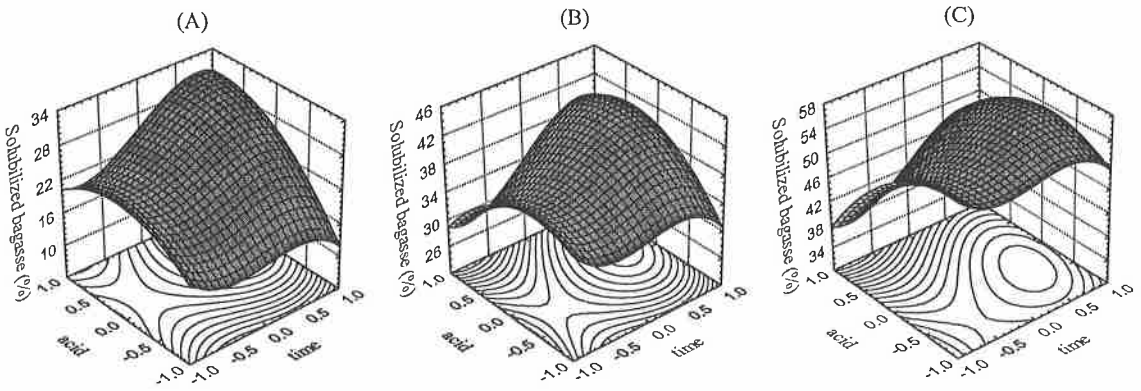

Figure 3. Response surfaces and contour lines of the cubic model representing the amount of solubilized sugarcane bagasse built by fixing the temperature at the coded levels –1 (A), 0 (B) and 1 (C).

CONCLUSION

The sugarcane bagasse solubilization increased with the severity of the process and occurred in two phases: one controlled by xylan and amorphous cellulose and another by crystalline cellulose hydrolysis. Under high severity conditions the amount of solubilized bagasse decreased due condensation reactions of lignin and furan compounds derived from dehydrated-sugar reactions.

The bagasse solubilization caused by cellulose hydrolysis was strongly dependent on reaction time and acid diffusion since it is necessary to disrupt the crystalline region of the cellulose.

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REFERENCES

Armas, C.M. & Bianchi, E. (1990). Aporte Energético da Indústria em Usina Sucro-Alcooleira-Viabilidade Econômica. *Stab*, **8 (5/6)**, 41-45.

Barros N.B., Scarminio, I.S.& Bruns, R.E. (1995). Planejamento e Otimização de Experimentos. ed. UNICAMP, Campinas, pp. 79-86.

Browneel, H.H., Yu, E.K.C. & Saddler, J.N. (1986). Steam-explosion pretreatment of wood: effect of chip size, acid, moisture content and pressure drop. *Biotechnol. and Bioeng.*, **28**, 792-801.

Capek-Ménard, E., Jollez, P., Chornet, E., Wayman, M. & Doan, K. (1992). Pretreatment of waste paper for increased ethanol yields. *Biotechnol. Lett.*, **14**, 985-988.

Carrasco, F. (1996). Yield of furfural obtained by acid hydrolysis of hardwood in a pilot plant. *Inf. Tecnol.*, **7**, 57-69.

Deschamps, F.C., Ramos, L.P. & Fontana, J.D. (1996). Pretreatment of sugar cane bagasse for enhance ruminal digestion. *Appl. Biochem. Biotechnol.*, **57/58**, 171-181.

Grethlein, I.S. (1984). Pre-treatment for enhance hydrolysis of cellulosic biomass. *Biotechnol. Adv.*, **2**, 43-62.

Mattos, L.R. (2000). Separação dos constituintes do bagaço de cana por explosão a vapor na presença de ácido sulfúrico diluído. MsC thesis, Faculdade de Engenharia Química de Lorena.

Saddler, J.N., Mes-Hartree, M., Yu, E.G.C. & Brownell, H.H. (1983). Enzymatic hydrolysis of various pretreated lignocellulosic substrates and the fermentation of the liberated sugars to ethanol and butanediol. *Biotechnol. Bioeng. Symp.*, **13**, 225-238.

Silva, F.T. (1995). Obtenção de insumos químicos a partir do aproveitamento integral do bagaço de cana, PhD thesis, Universidade Estadual de Campinas.

Stenberg, K., Tengborg C., Galbe M. & Zacchi G. (1998). Optimisation of Steam pretreatment of SO2-Impregnated Mixed Softwoods for Ethanol Production *J. Chem. Technol. Biotechnol.*, **71**, 299-308.

Sun, R.C., Lawther, J.M. & Banks, W.B. (1996). The fractional composition of polysaccharides and lignin in alkaline pre-treated and steam pressure treated wheat straw. *Cellul. Chem. Technol.*, **30**, 57-69.

Studies on isolation of polyphenoloxidases from potatoes and their use in the treatment of lignin-containing effluent

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ABSTRACT

A comparative study on techniques for isolation of polyphenoloxidases (PPO) from potatoes aiming their use for lignin effluent treatment is reported. Enzyme inactivation during the extraction procedure is a drawback and is related to the high amounts of phenolic compounds occurring in potato tissues. The addition of L-cysteine as a stabilizer was evaluated to overcome this problem. Furthermore, some simple purification methods as dialysis and phenolic compounds adsorption in polyvinylpyrrolidone (PVP), silica and activated coal were also compared. The disruption of whole potatoes in presence of 1.7 mM of L-cysteine.IU^{-1} of enzyme activity followed by dialysis for 12 h was found to be the best procedure to prepare a crude extract with high PPO activity (0.2 $IU.mg^{-1}$ after freeze-drying). This freeze-dried extract was used to initiate polymerization reactions of low molecular weight lignin fragments contained in a model effluent. The polymerization reaction was evidenced by the formation of a brown precipitate, which was a consequence of the polymerization of lignin fragments, as showed by gel permeation chromatography. The effluent suffered color reduction from 4445 to 2690 APHA color units after the treatment. Also, a significant decrease in phenolic compounds content was observed since around 50% of its initial concentration was removed as a brown precipitate.

Keywords: polyphenoloxidases, lignin-containing effluents, potato enzymes, lignin

INTRODUCTION

The studies on the use of some oxidoreductive enzymes in wastewater treatment raised considerable attention in recent years (Aitken *et al.*, 1994, Rocha, 1995, Xu *et al.*, 1995, Guerra *et al.*, 1997, Guerra *et al.*, 1998). These enzymes can initiate polymerization reactions of phenolic compounds causing their coupling. Considering that the end product of the enzyme reaction is water-insoluble, it is possible to precipitate out some phenolic compounds and generates a less toxic wastewater (Aitken *et al.*, 1994; Miland *et al.*, 1996).

Most studies on Polyphenoloxidase (PPO) (E.C. 1.10.3.1) concerns with the enzymatic browning in fruits and vegetables (Batistuti and Lorenço, 1985). This enzyme catalyzes the conversion of the phenolic compounds present in tissues to o-quinones, which polymerizes given brown pigments (Weemaes *et al.*, 1997). This enzymatic browning reaction is considered to be undesirable because fruits undergo an unpleasant appearance and concomitant development of an off-flavor (Murata *et al.*, 1995). Although several aspects of PPO in plants are well documented, less attention has been given to the PPO use in effluent treatment.

Certainly, fruits and vegetables or its subproducts could be a source of oxidative enzymes. One of the drawbacks of the use of vegetable oxidative enzymes is that their isolation involves disruption of the tissues and concomitant mixing of substances which in living organisms were compartmentalized (Loomis and Battaile, 1966). This may result in a loss of activity decrease or complete inactivation of the enzymes due to interactions of proteins and others protoplasmic constituents, such as nucleic acids and phenolic compounds (Loomis and Battaile, 1966). Then, isolation of PPO from vegetables without altering their activity during the extraction procedure is a key step for their use in effluent treatment. The aim of this work is to obtain a partially purified extract from potatoes with high PPO activity suitable for using in removal of low molecular lignin fragments contained in a model effluent.

EXPERIMENTAL

Enzyme source and extraction

Potato-polyphenoloxidases were prepared by disrupting 50 g of washed potatoes cut in small pieces. Disruption was performed for 1 min in a blender containing 100 mL of 0.2 M KH_2PO_4, pH 7.5 and 0 - 10 μmol of L-cysteine/g of potatoes. The resulting suspension was filtered through cheesecloth and centrifuged at 25°C and 4500 g for 20 min. The supernatant was collected and used as a crude extract of PPO.

Measurement of PPO activity

The polyphenoloxidase activity was measured in a spectrophotometer at 400 nm using chlorogenic acid as substrate. The standard reaction mixture consisted of 1.6 mL of 0.2 M phosphate buffer pH 6.6, 0.2 mL of 1mM chlorogenic acid and 0.2 mL of enzyme solution. The unit of enzyme activity was defined as the amount of enzyme necessary to produce 1 μmol of quinone.min^{-1} (Sánchez-Ferrer *et al.*, 1993).

Phenolic compounds assay

The total amount of phenolic compounds present in the enzymatic extract was determined as described by Kim and Yoo (1996). In a 2 mL quartz cell, 1.5 mL of enzymatic extract, 0.1 mL of 8 mM $K_3Fe(CN)_6$ and 0.1 mL of 0.1 M $FeCl_3$ 6 H_2O dissolved in 0.1 M HCl were added. The resulting absorbance was detected at 700 nm after 5 min. The amount of phenolic compounds was determined using 2-methoxyphenol as standard.

Partial purification of the PPO

To avoid inactivation of PPO by phenolic compounds, the supernatant of the extraction procedure was collected and submitted to three simple phenol adsorption methods and also to dialysis. In the adsorption methods, 10 mL of the crude PPO-extract were shaken overnight with 5–10 g of active coal, polyvinylpyrrolidone (PVP) or silica. The mixture was filtered through Whatman-42 filter paper and the filtrate was submitted to determination of PPO activity and phenolic compounds concentration. Dialysis was conducted with 20 mL of crude extract in a 1000 Da cutoff membrane against distilled water for 12h at 4°C.

Effluent preparation and polymerization reaction

The soluble lignin fraction (effluent) treated was generated from black liquor produced after alkaline delignification of steam-exploded sugar cane bagasse (Silva, 1995). This liquor, which contains a lignin concentration of 22 gL^{-1}, was acidified with concentrated sulfuric acid under vigorous stirring until pH 2.0. Precipitated lignin was filtered-off and the soluble fraction was recovered and stored at 4°C. Before use, the soluble lignin fraction was filtered again through a Whatman-42 filter paper.

The enzyme reaction started by one-way addition of 100 mg of freeze-dried PPO dialyzed extract over 10 mL of effluent, which contains 5 g of soluble ligninL^{-1} previously adjusted to pH 5. The reaction was conducted under stirring at 25-30°C for 0.5 h. At the end of the reaction, the suspension was acidified until pH 2.0 and the insoluble polymer was recovered by filtration through a Whatman-42 filter paper. The insoluble residue was dried at 60°C until constant weight.

Molecular weight distribution of the effluent compounds and of the insoluble polymer was determined by gel permeation chromatography (GPC) on a 46.5 x 2.5 cm Sephadex G-50 column eluted at 0.4 $mLmin^{-1}$ with 0.5 M NaOH solution. A volume of 0.4 mL of sample (corresponding to 1mg of lignin) adjusted to pH 12 was directly injected into the column. The column system was calibrated with proteins of known molecular weight (Forss *et al.*, 1987). The void volume (V_0) and the total permeation volume (V_t) were determined by injection of blue dextran and acetone, respectively. Partition coefficients (Kd) were calculated as described elsewhere (Chum *et al.*, 1987).

RESULTS AND DISCUSSION

One of the limiting aspects for the use of vegetables polyphenoloxidases is related to the extraction procedure. In this work the main difficulty on preparing a crude extract with high activity of this enzyme was inherent to the high concentration of phenolic compounds associated to the potato tissues. During mechanical disruption, these phenolic compounds get in contact with PPO, starting oxidation reactions that can decrease

the PPO-activity (Loomis and Battaile, 1966; Miland *et al.*, 1996). Potato peel is known to contain high concentrations of such phenolic compounds and an experiment was conducted to evaluate the influence of peel on the PPO recovered activity (Table 1). The amount of phenolic compounds quantified in the extract was higher when the disruption occurred in not peeled potatoes. However, the PPO activity decreased from 7.9 to 3.0 IU $g^{-1}$of potato when the potatoes were peeled before the extraction procedure. The activity/phenols (Table 1) ratio suggests that PPO extraction from whole potatoes (with peel) is more attractive since this ratio decreased around 43% when peeled potatoes were used.

Table 1. PPO activity and amounts of phenolic compounds present in crude extract from potatoes as a function of peel presence.

Extract	Activity (UI g^{-1} of potato)	Phenols (mg g^{-1} of potato)	Activity / phenols
Not peeled	7.9 ± 0.3	0.35 ± 0.03	22.6
Peeled	3.0 ± 0.2	0.19 ± 0.02	15.8

L-cysteine has been reported to be an efficient PPO stabilizer when added during the extraction procedure (Kermasha *et al.*, 1993). Results for several extractions performed at different L-cysteine concentrations are shown in Table 2.

Table 2. Characteristics of a crude extract from potatoes (not peeled) in the presence of L-cysteine.

Cysteíne/activity (mM IU^{-1}g of potato)	% of color*	Phenolic compounds (mg g^{-1} of potato)	PPO activity (%)*
Control	100	0,2	100
0.04	104	0,3	100
0.07	110	0,3	74
0.13	65	0.7	70
0.20	47	0.7	70
0.26	31	0.7	62
0.42	25	0.7	63
0.85	27	0.7	63
1.27	29	0.7	63
1.70	27	0.7	63

* The values reported were compared to the control that was considered as 100%

A significant reduction of the extract color was observed at concentrations up to 0.13 mM cysteine. The color reduction of the extract can be related to the concentration of the phenolic compounds. Color development is a consequence of oxidation of phenolic compounds to quinones. As L-cysteine stabilizes the phenolic reverting oxidation reactions (Kermasha *et al.*, 1993), the concentrations of measurable phenolic compounds increase simultaneously with color decrease.

In spite of being efficient to avoid oxidation reactions, the L-cysteine was not effective to avoid PPO inactivation (Table 2). Increasing the cysteine/activity ratio from 0 to 0.26, the PPO recovered activity decreased to 62% of the initial value. Kermasha *et al.* (1993) and Weemaes *et al.* (1997) have attributed this activity loss to direct PPO inhibition by cysteine. However, Table 2 showed also that the inactivation reaches a maximum of 38% with 0.26 mM cysteine IU^{-1}g of potato and this value was constant when higher ratios were used. These results show that cysteine/activity ratios higher than 0.26 presented potential for further investigation since these ratios were effective to avoid phenol oxidation and presented a limiting value for PPO inactivation.

Another advantage for using cysteine is to avoid phenols oxidation during cold storage (4°C) of the extracts or during dialysis procedures. The changes of extract color as a function of the cold storage time are shown in Figure 1. During the first day of storage there was a high increase in the color of the extract prepared without cysteine (control). The extracts obtained with 0.42 and 0.85 mM of cysteine UI^{-1}g of potato showed the same color increase trend which suggests that the amount of cysteine was not enough to avoid oxidation during the first day of storage. On the other hand, the extracts prepared with 1.27 and 1.7 mM cysteine presented little and no significant color increase during the first day of storage, respectively. In all cases cysteine was not efficient to avoid extracts oxidation after one day of storage.

Once cysteine was effective to avoid phenols oxidation during 24 h, some simple and quick methods to remove phenolic compounds from the potato extracts were evaluated (Table 3). The most efficient method to

remove the phenols from the crude extract was the dialysis. After 12 h of dialysis a reduction of 55% in the free phenols was detected with around 20% of activity loss. On the other hand, adsorption on PVP provided the best result on activity recovered since loss in the PPO-activity was minimal. The other methods evaluated presented no potential to be used. As the final activity/ final phenols ratio was maximal on the dialysis this treatment was considered the best one for further preparation of partially purified PPO extracts for application in effluent treatment.

Considering the previously discussed results, a crude extract obtained by disruption of whole potatoes in presence of 1.7 mM of L-cysteine $IU^{-1}.g^{-1}$ of potato was dialyzed for 12 h against water and freeze-dried. The freeze-dried extract (containing 0.2 IU of $PPO.mg^{-1}$) was used to initiate polymerization reactions of lignin fragments contained in a model effluent. The polymerization reaction was evidenced by the formation of a brown precipitate. GPC analysis of the brown precipitate confirmed that insolubilization was a consequence of the increase in the average molecular weight of the lignin fragments present in the effluent (Figure 2).

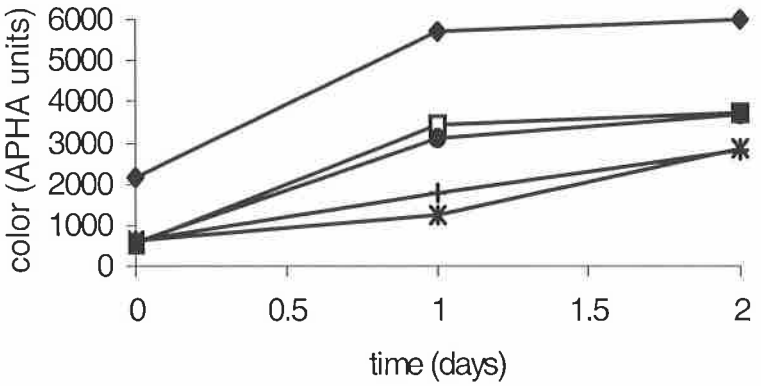

Figure 1. Color variation of a crude PPO extract containing different cysteine/activity ratios as a function of the storage time at 4ºC. (♦) control, ( ) 0.42 mM $IU^{-1}g$(•) 0.85 mM $IU^{-1}g$ (+) 1.27 mM $IU^{-1}g$ (x) 1.7 mM $IU^{-1}g$.

Table 3. Characteristics of the extracts from potatoes after a specific treatment for phenolic compounds removal.

Treatment	Activity ($IU.ml^{-1}$)	Phenols ($mg.l^{-1}$)	Recovered activity(%)	Phenols removed (%)	Activity/phenols
None	2	182	100	0	10.8
PVP	2	104	100	41	18.3
Dialysis	1.6	84	80	55	19.1
Active coal	0.6	129	29	30	4.4
Silica	0	98	0	47	0

The GPC of the brown precipitated presented a high molecular weight fraction which was almost excluded in the G-50 Sephadex column. This fraction afforded to around 30 % of the total chromatogram area. Also, the polymerized material presented an eluted fraction with a molecular weight ranging from 6,500 Da to 58 Da. Additionally, the effluent suffered a color reduction from 4445 to 2690 APHA color units after the treatment. A significant decrease in the soluble lignin content of the effluent should also observed since we previously reported that around 50% of its initial amount was removed as a brown precipitate in the same polymerization system Guerra *et al.* (1998 and 1999).

In summary, the results show that partially purified PPO extracts are suitable to be used to initiate polymerization reactions of soluble lignin fragments present in effluents. Also a simple method to extract and partially purify potatoes-PPO was developed.

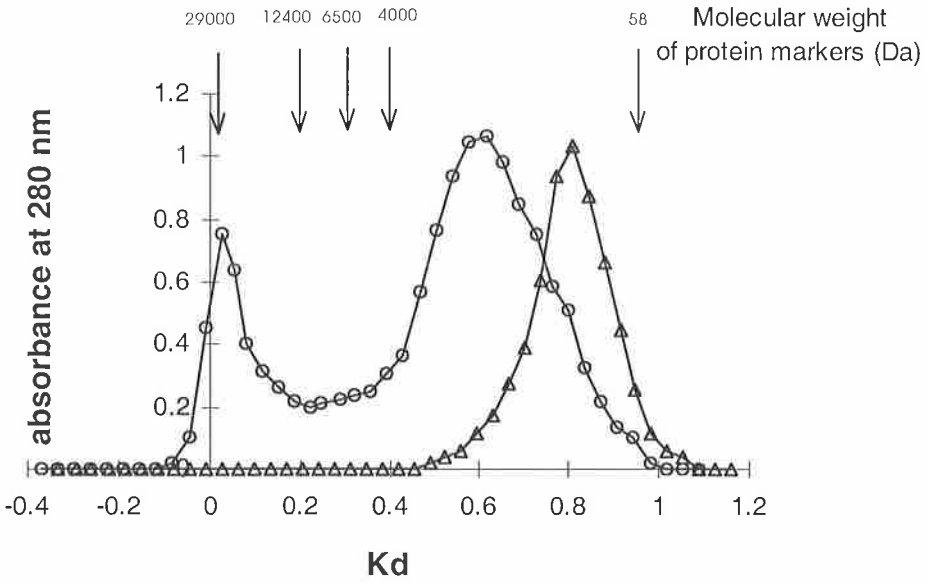

Figure 2. Gel permeation chromatography of the soluble lignin fragments (Δ) and the brown precipitate obtained after polymerization initiated by reaction by PPO (--o--). Arrows show elution Kd values of protein markers.

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REFERENCES

Aitken, M., Massey, I.,Chen, T. & Heck, P. (1994). Characterization of reaction products from the enzyme catalyzed oxidation of phenolic pollutants. *Wat.Res.*, **28**, 1879-1889.

Batistute, J. & Lourenço, E. (1985). Isolation and purification of polyphenoloxidase from a new variety of potato. *Food Chemistry,* **18**, 251-163.

Chum, H. L., Johnson, D. K., Tucker, M. P. & Himmel, E. (1987). Some aspects of lignin characterization by high performance chromatography using styrene divinyl benzene copolymer gels. *Holzforschung*, **41**, 97-108.

Forss, K., Kokkonnen, R. & Sagfors, P. (1988). Determination of the molar mass distribution of lignins by gel permeation chromatography. *ACS Sympsium. Ser.*, **397**, 124-133.

Guerra, A. Ferraz, A., Cotrim, A.,Gonçalves, A. & Silva, F. (1997). The effect of four variables in the polymerization of acid soluble lignin by horseradish/hydrogen peroxide system as showed by statistic experimental design. *In 5th Brazilian Symposium on the Chemistry of Lignins and other Wood Components*, Proceedings, **Vol** 1, pp 293-298.

Guerra, A. (1998). Polimerização de lignina solúvel por polifenoloxidases e peroxidase/H_2O_2. MSc thesis, Departamento de Biotecnologia, Faculdade de Engenharia Química de Lorena.

Guerra A, Ferraz A, Cotrim, A & Silva F. (1999). Polymerization of lignin fragments contained in a model effluent by polyphenoloxidases and horseradish peroxidase/hydrogen peroxide system. *Enzyme and Microbial Technology*, In Press.

Kermasha, S., Goetghebeur, M., Monfette, A., Metche, M. & Rovel,B. (1993). Inhibitory effects of cysteine and aromatic acids on tyrosinase activity. *Phytochemistry*, **34**, 349-353.

Kim, Y. & Yoo, Y. (1996). Peroxidase production from carrot hairy root cell culture. *Enzyme and Microbial Technology*, **18**, 531-535.

Loomis, W., & Battaile, J. (1966). Plant phenolic compounds and the isolation of plant enzymes. *Phytochemistry*, **5**, 423-438.

Miland, E., Smyth, M. & Ófágáin, C. (1996). Phenol Removal by Modified Peroxidases. *Journal Chemical Tech. Biotechnology*, **67,** 227-236.

Murata, M., Tsurutani, M., Tomita, M., Homma, S. & Kaneko, K. (1995). Relationship between apple ripening and browning: changes in polyphenol content and polyphenol oxidase. *Journal of Agricultural and Food Chemistry*, **4**, 115-1121.

Rocha, G. (1995). Polimerização enzimática de lignina solúvel contida em um efluente proveniente da deslignificação alcalina de bagaço de cana. MSc Thesis, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo.

Sánches-Ferrer, A., Laveda, F. & García-Carmona, F. (1993). Partial purification of soluble potato polyphenol oxidase by partitining in an aqueous two-phase system. *Journal of Agricultural and Food Chemistry*, **41**, 1219-1224.

Silva, F.T. (1995). Obtenção de insumos químicos a partir do aproveitamento integral do bagaço de cana. PhD thesis, Instituto de Química, Universidade de Campinas.

Xu, X., John, V., McPherson, G., Grimm, D., Akkara,J. & Kaplan, D. (1995). A combined Chemical-Enzymatic Method to Remove Selected Aromatics from Aqueous Streams. *Applied Biochemistry and Biotechnology*, **51,** 649-660.

Weemaes, C., Cordt, S.,Ludikhuyze, L., Broeck, I., Hendrickx, E., & Tobback, P. (1997). Influence of pH, benzoic acid, EDTA and glutathione on the pressure and/or temperature inactivation kinetics of mushroom polyphenoloxidases. *Biotechnology Progress*, **13**, 25-32.

Studies on the structure of residual and technical lignins by 2D NMR-spectroscopy

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ABSTRACT

Studies were conducted on the structure of pine kraft residual lignins, *Eucalyptus* kraft lignin and organosolv hardwood lignins by heteronuclear 2D NMR-spectroscopy techniques (HMQC and HMBC). The spectra of the technical lignins were also compared with the corresponding spectra of pine milled wood lignin. The results obtained show that β-O-4 structures are still present in all lignins after kraft and organosolv pulping. Stilbene moieties are formed during pulping both in alkaline and acidic media. Vinyl ether structures are present only in kraft lignin, but not in the residual and organosolv lignins. Conjugated carbonyl groups are not involved in β-O-4 structures and mostly present in the form of α-ketone structures in terminal side chain. No structures of coniferyl alcohol or coniferyl aldehyde types have been detected in the lignins after pulping. All the technical lignins, particularly kraft lignins, contain a rather high variety of saturated aliphatic groups. However, the 2D NMR technique does not confirm the presence of biphenylmethane moieties in the lignins, because the corresponding carbon signal (δ_C 29.5 ppm) correlates with a proton signal at δ_H 1.2 ppm, which is rather high for this type of structures.

Keywords: kraft pulps, lignin, NMR, organosolv lignins

INTRODUCTION

The knowledge of the structures of technical lignins is of primary importance for the understanding of the underlying reaction mechanisms in the processes that produce these lignins, and for the exploration to utilize these lignins. The complex and irregular nature of lignin structures creates certain problems in structural studies of the lignins by spectroscopic studies, particularly in NMR-spectroscopy, because of overlapping signals from different lignin moieties. In addition, preparations of residual lignins, particularly those obtained by enzymatic hydrolysis of kraft pulp, contain carbohydrate and protein impurities, which cause problems in analysis of 1D NMR spectra. Advanced 1H-13C correlation 2D NMR spectroscopic techniques, such as HMQC and HMBC, provide resolution of overlapping signals with higher probability, giving a very powerful method to observe qualitative changes in the lignin structure. In spite of a number of studies on the structure of lignin of different botanical origins, there is lack of published data on the 2D NMR-spectroscopy of residual and technical lignins. The aim of this work is to study the structures of technical and residual lignins of different origins by heteronuclear 2D NMR-spectroscopy techniques (HMQC and HMBC).

EXPERIMENTAL

Pine kraft-AQ pulp prepared by extended alkaline cooking (kappa number 21.8) was obtained from Westvaco Corporation Covington Mill, VA. The corresponding residual lignins were isolated from the kraft-AQ pulp by cellulase treatment followed by purification with NaOH (Chang, 1992).

Kraft lignin was isolated from black liquor after kraft pulping of cloned culture of *Eucalyptus globulus* in laboratory conditions (Institute of Forestry and Paper, RAIZ, Portugal). Pulping conditions: 160oC, active alkali of 15%, sulfidity of 25%. Kappa number of the corresponded kraft pulp was 14.1. Organosolv lignin was obtained from Repap Corporation. Both technical lignins were purified in accordance with proceeding described by Chen and Robert (1988).

^{1}H-^{13}C correlation 2D-NMR spectroscopies

The NMR spectra were recorded in a Bruker AVANCE 500 MHz spectrometer (1996 model) with the Oxford narrow bore magnet (1989 model) after dissolving approximately 45-60 mg of each lignin preparation in 0.75 mL DMSO-d_6. The system was controlled by the SGI INDY host workstation and the data was processed using the XWINNMR software. The instrument was equipped with three frequency channels with

waveform memory and amplitude shaping; three channel gradient control units (GRASP III) and one variable temperature unit, as well as one unit for pre-cooling and temperature stabilization. All measurements have been carried out with a 5 mm ID ^{1}H/BB (^{109}Ag-^{31}P) triple-axis gradient probe (ID500-5EB, Nalorac Cryogenic Corp.). The operational frequency for ^{1}H nucleus was 500.128 MHz and conditions for analysis included a pulse width of 10 μsec, PL of 0.00 dB and a 1.5 sec pulse delay (d_1).

RESULTS AND DISCUSSION

The spectra of pine kraft residual lignin and Eucalyptus kraft lignin are presented as examples (Figure1). In general, a lignin HMQC spectrum can be divided into three regions: aliphatic, oxygenated aliphatic and aromatic regions. The HMQC technique shows the correlation between ^{1}H and ^{13}C atoms bonded to each other. The HMBC technique gives information on a long range (3 – 4 bonds) H-C correlation.

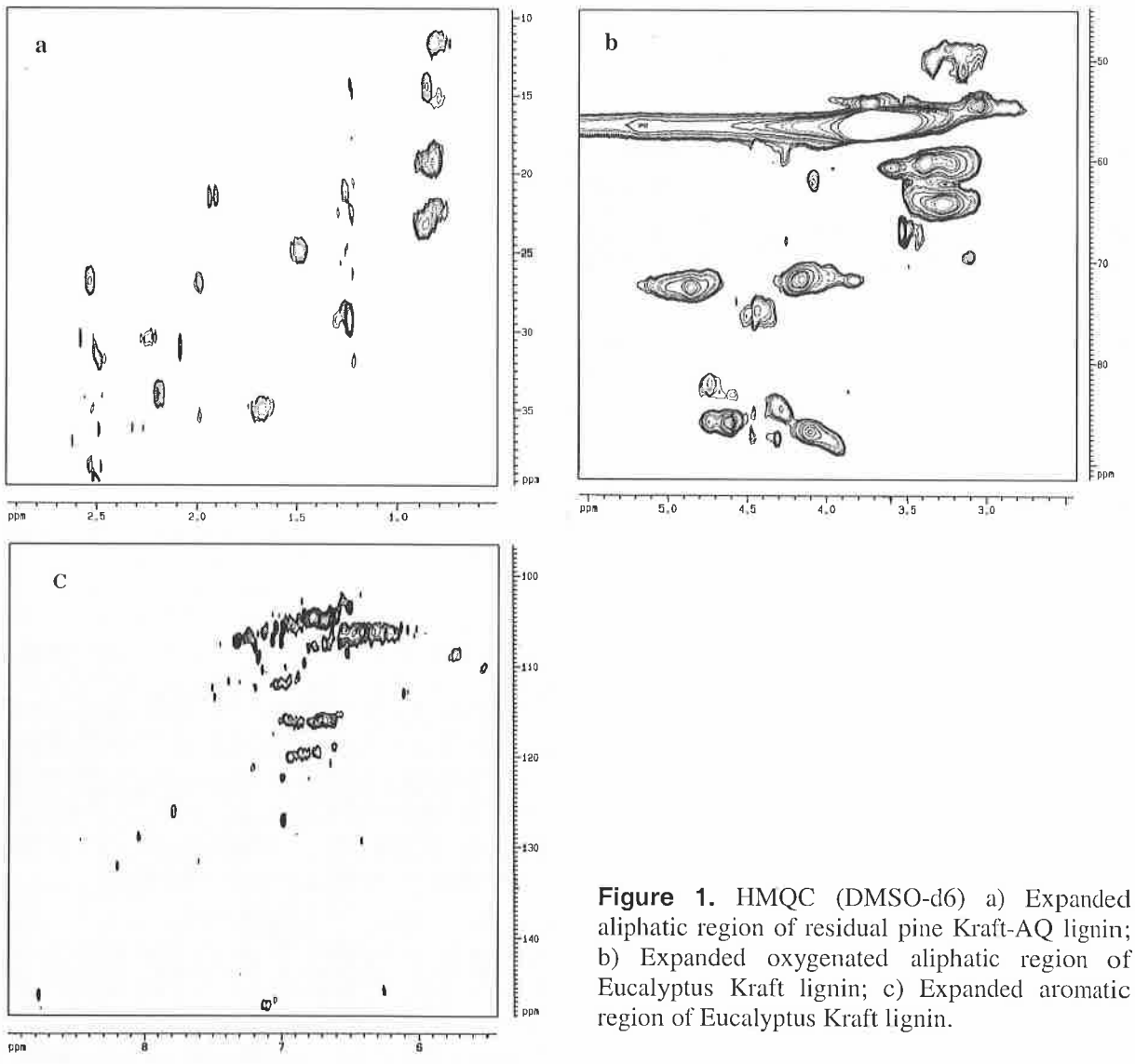

Figure 1. HMQC (DMSO-d6) a) Expanded aliphatic region of residual pine Kraft-AQ lignin; b) Expanded oxygenated aliphatic region of Eucalyptus Kraft lignin; c) Expanded aromatic region of Eucalyptus Kraft lignin.

Signal in the aliphatic region is usually considered as impurities from extractives like fatty acids. Even so, some of these impurities are likely chemically bonded to the lignin structures since exhaustive extraction of wood or pulp before lignin isolation and extraction of lignin preparations after isolation do not eliminate all signals in the aliphatic region. After studying the spin system of aliphatic signals in wheat lignins using HOHAHA technique, Crestini and coworkers (1998) suggested that those signals belong to aliphatic chains bonded to lignin structures by ether or ester bonds. In the technical lignins, the aliphatic region of the spectra contains considerable number of signals with higher intensity than MWL reflecting formation of aliphatic groups during pulping processes. The database of the chemical shift of possible aliphatic moieties in lignins

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