PROCEEDINGS OF THE

SECOND BRAZILIAN SYMPOSIUM ON THE CHEMISTRY OF LIGNINS AND OTHERS WOOD COMPONENTS

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SECTION I. WOOD CHEMISTRY

CHEMISTRY AND SURFACE-ACTIVE PROPERTIES OF THE OZONATION PRODUCTS OF TECHNICAL LIGNINS

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ABSTRACT

Kraft lignin was modified by ozonation in alkaline aqueous solution. Kinetic studies were conducted on the effect of pH, ozone consumption based on kraft lignin, formation of carboxylic groups, and changes in molecular weight distribution of the lignin preparations. Characterization of the ozonation products of kraft lignin as well as lignin model compounds indicates that phenols are being polymerized by oxidative coupling. Surface-active properties of ozonated kraft lignins were measured. The synergistic effect of ozonated lignins with a commercial surfactant was also investigated.

1. INTRODUCTION

Lignin is one of the integral components of woody tissues in vascular plants. It comprises approximately one-quarter to one-third of the tissues depending on their particular nature. Thus, lignin is the second most abundant renewable biopolymer next to cellulose in the plant kingdom (1). Recently, interest in better utilization of renewable resources has led to several new applications of technical lignins produced in pulping processes and their chemically modified products (2). The predominance of the kraft pulping process in the United States makes kraft lignin a logical choice as a raw material for the further development of byproducts (2).

The structural moieties and chemical functionalities that render kraft lignin liable to modification are rather well known. However, in addition to the inherent structural complexity of lignin, the structural changes occurring during pulping and isolation of technical lignins are difficult to reproduce. These problems cause difficulty in obtaining uniform technical lignin, and thus uniform modified products. The resulting nonuniformity has handicapped technical lignin products to compete with many similar petrochemical products with homogeneous chemical functionalities, narrow molecular weight distribution and performance characteristics.

The objective of the present study is two-fold: (a) to investigate the modification of pine kraft lignin by ozonation, and (b) to study surface active properties of the ozonated kraft lignins.

2. Pine Kraft Lignin

2.1. Fractionation of Pine Kraft Lignin

Pine kraft lignin (Indulin AT) was provided by Westvaco Corp., Charleston, S.C., USA. The kraft lignin (KL) was fractionated according to the procedure of Lundquist (3) into a high molecular weight fraction (HMWF), an intermediate molecular weight fraction (IMWF), and a low molecular weight fraction (LMWF). The yields of LMWF, IMWF and HMWF were approximately 18, 63 and 15% based on the original crude kraft lignin. The weight average molecular weights (M_w) of LMWF, IMWF and HMWF were approximately 500, 5,500 and 15,000 Dalton, respectively.

2.2. Characteristics of the Kraft Lignin Fractions

Gas chromatographic (GC) and gas chromatography-mass spectrometric (GC-MS) analyses of the LMWF showed that the fraction contains more than thirty compounds with molecular weight of less than 600 Dalton. Most of the compounds identified were degradation products of lignin formed during the kraft pulping of pine. The LMWF was not chemically modified any further.

Elemental composition and hydroxyl content of the IMWF and HMWF were determined. The IMWF has higher methoxyl content than the HMWF. The IMWF also has higher phenolic but lower aliphatic hydroxyl contents than the HMWF. The carboxylic content is nearly the same in both fractions. These findings are consistent with the facts that the increase in the phenolic hydroxyl content results mainly from cleavage of α- and β-aryl ether linkages in lignin during kraft pulping and from O-demethylation of the methoxyl group.

3. Ozonation of The Intermediate Molecular Weight Fraction (IMWF) of Pine Kraft Lignin

3.1. Ozonation of the IMWF

An ozone-air stream with an ozone concentration of 2-2.5% was passed through a solution of 1g of purified IMWF in 100 ml of 0.1N sodium hydroxide solution. The ozone consumed was determined from the concentration of ozone going in and out as a function of reaction time and the flow-rate of the ozone-air stream.

3.2. Kinetics of the Ozonation

A. pH of the Reaction Mixture

The pH of the reaction mixture was measured at ozone consumptions of approximately 0, 10, 20, 26 and 43% based on the weight of IMWF. Figure 1 shows that the pH decreases almost linearly from the initial value of approximately 12.5 with increasing ozone consumption. At an ozone consumption of approximately 44%/IMWF, the pH has dropped to about 7.

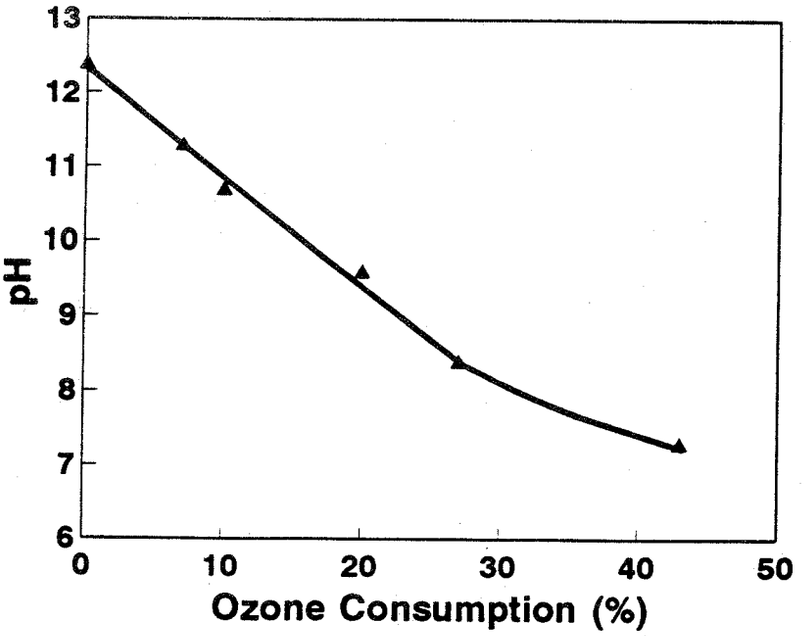


Figure 1. Relationship between pH values of reaction mixture and ozone consumption.

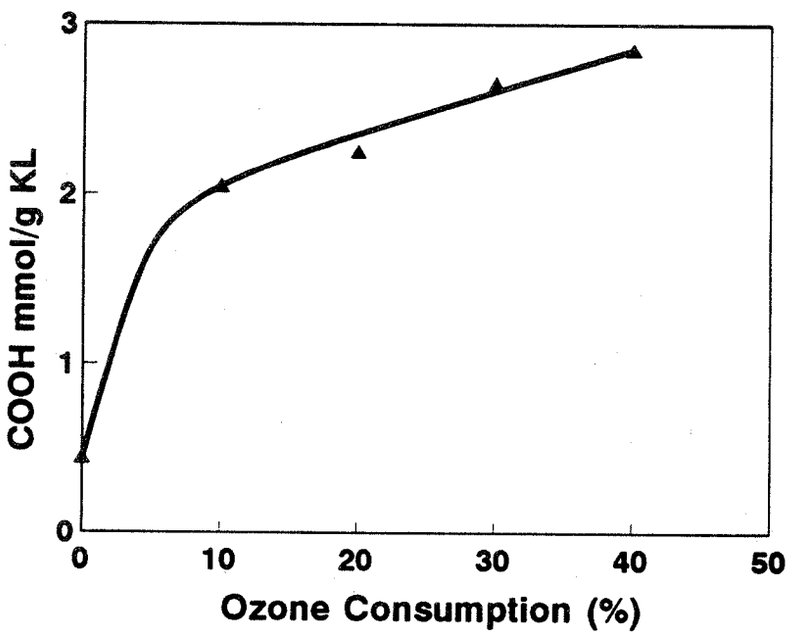


Figure 2. Relationship between carboxylic content and ozone consumption.

B. Carboxylic Acid Content

The carboxylic acid content of the purified IMWF is only approximately 0.4 mmol/IMWF. Figure 2 shows that the carboxylic acid content of the reaction mixture increases rapidly up to an ozone consumption of 10%/IMWF, then more gradually. Moreover, a comparison of Figures 1 and 2 then establishes that the decrease in the pH is caused mainly by the formation of carboxylic acid groups during ozonation of the IMWF.

C. Changes in Molecular Weight Distribution Pattern

Figure 3 shows that the IMWF underwent considerable change in molecular weight distribution during ozonation. With rising ozone consumption, the percentage of the fraction with $M_w \leq 5{,}000$ Dalton suffered a decrease. The fraction with $5{,}000 \leq M_w \leq 10{,}000$ Dalton remained almost unchanged. In contrast, the percentage of the fractions with $M_w \geq 10{,}000$ Dalton showed a marked increase.

The overall M_w of the IMWF steadily increases as ozonation continues. This increase in the overall M_w of the ozonated IMWF is surprising at first glance. It is well established, however, that ozone in alkaline solution leads to the formation of hydroperoxyl and hydroxyl radicals that can induce oxidative coupling of phenol structures in lignin (4). We found, for example, that treatment of isoeugenol with ozone in alkaline solution resulted in the formation of 2,2'-dihydroxy-3,3'-dimethoxy-5,5'-di(1-propenyl)biphenyl and 5-[2-methoxy-4-(1-propenyl)phenoxy]vanillic acid in appreciable amounts.

Thus, the observed increase in the overall M_w (Figure 3) is attributable to oxidative coupling of phenol structures in lignin initiated by hydroperoxyl and hydroxyl radicals. Hydroperoxyl anions prevail in the initial stage of ozonation (5). On the contrary, hydroperoxyl and hydroxyl radicals become predominant over hydroperoxyl anions when the pH of the reaction mixture decreases (4). These radicals account not only for the increase in the overall M_w (Figure 3) but also the formation of substructures giving aromatic acids on potassium permanganate oxidation to be described below.

The kraft lignin also undergoes oxidative cleavage of aromatic rings and double bonds in side chains, leading to the formation of carboxylic acid groups. However, these reactions only lead to scission of side chains in the lignin molecule and thus do not affect the overall M_w of the ozonated KL products.

D. Nitrobenzene-Potassium Permanganate Oxidation

The lignin preparations were subjected to nitrobenzene oxidation (6). The oxidation mixtures were adjusted to pH 14 and extracted continuously with ether to remove unreacted nitrobenzene and its reduction products. The lignin degradation products in the aqueous layer were exhaustively methylated with dimethyl sulfate. The methylated products

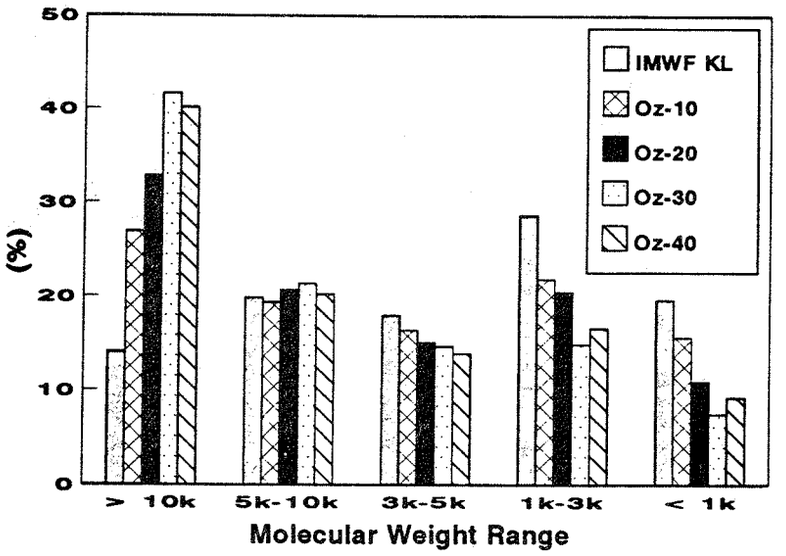


Figure 3. Molecular weight distribution of the IMWF at different stages of ozonation.
Ozone consumptions:
Oz-10, 10%; Oz-20, 20%; Oz-30, 30%;
Oz-40, 40%.
GPC running conditions:
Elution solution: 0.1 N NaOH/0.1 mol. LiCl;
Column: Sephadex G-75;
Flow rate: 17.4 ml/hr.

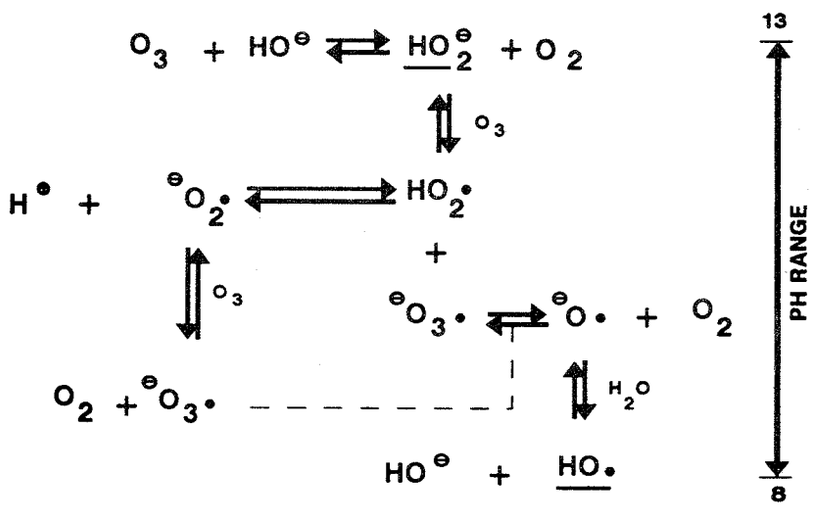


Figure 4. Decomposition of ozone in alkaline aqueous solution.

were then subjected to permanganate oxidation (6).

Table 1 summarizes the results of the nitrobenzene-potassium permanganate oxidation of the original HMWF and IMWF and the ozonated IMWF. Compared to the IMWF, the ozonated IMWF with ozone consumption of 10% gave compounds I-V and VII, in appreciably lower yields (see Figure 5 for structures). The total yield of oxidation products derived from uncondensed units, compounds I-III, decreases from approximately 0.05 mmol/100 mg to approximately 0.035 mmol/100 mg. Similarly, the total yield of oxidation products derived from condensed units, compounds IV-VII, decreases from approximately 0.02 mmol/100 mg to approximately 0.01 mmol/100 mg. Only the yield of compound VI increases from approximately 0.001 mmol/100 mg to approximately 0.002 mmol/100 mg.

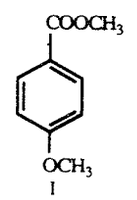
Compared to the IMWF, the ozonated IMWF with an ozone consumption of 30% gave markedly less compound I-III derived from uncondensed units, and compounds V and VII from condensed units, but appreciably more compounds IV and VI from condensed units. The total yield of compounds I-III decreases from approximately 0.05 mmol/100 mg to approximately 0.03 mmol/100 mg. In contrast, the total yield of compounds IV-VII remains unchanged, approximately 0.02 mmol/100 mg. However, the yield of compound VI increases from approximately 0.001 mmol/100 mg to approximately 0.006 mmol/100 mg, and that of compound VII decreases from approximately 0.004 mmol/100 mg to approximately 0.002 mmol/100 mg.

Evidently, the IMWF undergoes extensive oxidative cleavage of double bonds and aromatic rings without decrease in M_w in the initial stage of ozonation. In addition, the IMWF also undergoes, to a smaller extent, oxidative coupling of phenols leading to formation of diphenyl ether structures in the initial stage of ozonation. When the ozone consumption increases, the cleavage of aromatic rings decreases while the oxidative coupling of phenols increases as evidenced by the sudden increase in the yield of compound VI at an ozone consumption of 30%/IMWF. Thus, in the initial stage of ozonation, the cleavage of double bonds and aromatic rings dominate over oxidative coupling of phenols. This is reversed when the consumption of ozone increases.

As discussed previously, the pH of the reaction mixture decreases with increasing ozone consumption. The pH of the reaction mixture is 12.4 at the beginning of ozonation and decreases to approximately 9 at an ozone consumption of 30%/IMWF. Figure 4 summarizes the behavior of ozone in alkaline solution (4,5). At pH 10-13, ozone predominantly reacts with hydroxide anion to give hydroperoxyl anion and molecular oxygen. When the pH decreases to 8-10, ozone starts to react with hydroperoxyl anions to produce hydroperoxyl radicals and its conjugate base, superoxide anions. A reaction between superoxide anions and ozone further leads to the formation of hydroxyl radicals. Thus, the IMWF

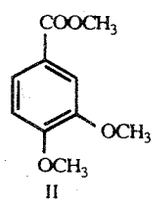
Table 1. Yields of the methyl esters of acids identified from the Nitrobenzene-$KMnO_4$ oxidation products

KL Preps.	Ozone Consumed (%)	Yields of Methylated Products (mmol/100 mg sample. x 10^{-3})						
		I	II	III	IV	V	VI	VII
HMWF	0	3.31	70.15	0.88	16.34	4.49	2.13	9.59
IMWF	0	2.17	47.81	0.88	9.80	2.80	1.01	4.36
Oz-10	10	3.55	31.84	0.27	2.76	2.09	1.91	1.18
Oz-25	25	2.53	40.51	0.75	5.63	1.97	1.54	1.13
Oz-30	30	2.11	24.49	1.06	7.36	3.82	6.49	1.59



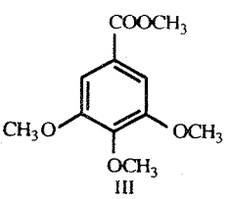
I

p-Anisic acid methyl ester



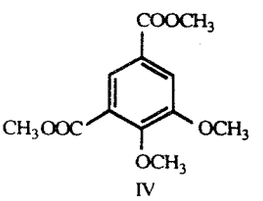
II

Veratric acid methyl ester



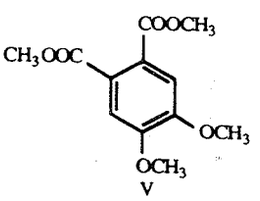
III

3,4,5-Trimethoxybenzoic acid methyl ester



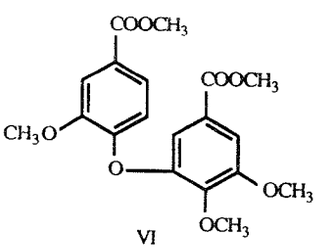
IV

Isohemipinic acid dimethyl ester



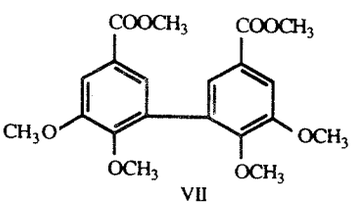
V

m-Hemipinic acid dimethyl ester



VI

2,2',3-Trimethoxydiphenylether -4',5-dicarboxylic acid dimethyl ester



VII

Dehydrodiveratric acid dimethyl ester

Figure 5. Structures of methyl esters of acids identified from the Nitrobenzene-$KMnO_4$ oxidation products.

preferentially undergoes the cleavage of aromatic rings involving hydroperoxyl anions rather than the oxidative coupling of phenols at pH above 10. However, at pH lower than 10, oxidative coupling of phenols initiated by hydroxyl radicals dominates over cleavage of aromatic rings.

4. Ozonated IMWF with Ozone Consumption of 25%/IMWF

The ozonated IMWF at an ozone consumption of 25%/IMWF (Fraction Oz-25) was fractionated into five fractions by ultrafiltration immediately after the ozonation. The reaction mixture (pH approximately 9) was successively passed through hollow fiber (HF) membranes with molecular weight cutoff value of 50,000 (F50k), 30,000 (F30k), 10,000 (F10k) and 2,000 (F2k). The fractions obtained have the average molecular weight of >20,000, 6,000, 2,000, 1,000 and <1,000 (F2kp) Dalton, respectively. The ozonated IMWF Oz-25 comprises approximately 7% of F2Kp, 17% of F2K, 25% of F10K, 24% of F30K and 23% of F50K.

Table 2 summarizes the results of the functional analysis of the original IMWF as well as the ozonated IMWF Oz-25 and its fractions. Compared to the IMWF, the ozonated IMWF Oz-25 and its fractions contain more than four-fold the carboxylic acid groups, but less phenolic and aliphatic hydroxyl groups.

For the fractions of ozonated IMWF Oz-25, the carboxylic acid content decreases with increasing molecular weight. In contrast, the contents of phenolic and aliphatic hydroxyl groups increase with increasing molecular weight. Thus, the low molecular weight ozonated products are predominantly formed by cleavage of aromatic rings during ozonation.

5. Surface-Active Properties of Ozonated IMWF Fractions

5.1. Surface Tension

As discussed previously, the IMWF of kraft lignin was ozonated with different amounts of ozone. Among different ozonated KL products thus obtained, the ozonated IMWF with ozone consumption of 25%/IMWF was fractionated into five fractions by ultrafiltration. The surface-active properties of these lignin preparations were investigated in aqueous solution in the pH range of 6-11.

A. Effects of Concentration and Molecular Weight

The surface tension of aqueous solutions of the Oz-25 fractions F2K, F10K, F30K and F50K at various concentrations were measured at pH 9 by the du Nouy method. In general, the surface tension of these solutions decreases with increasing concentration. Except for fraction F30K, the surface tension of these fractions decreases abruptly in the concentration range of 5-10 mg/ml. This is particularly true for the fraction F2K that has the lowest M_w range among the samples investigated. The surface tension of this fraction drops from

Table 2. Functional groups of ozonated KL fractions

OzKL Fractions	COOH (mmol/g)	Total OH (mmol/g)	Phen. OH (mmol/g)	Aliph. OH (mmol/g)	OCH_3 (mmol/g)
IMWF	0.42	8.34	3.38	4.96	4.36
Oz-25	1.94	5.40	2.33	3.07	2.72
F2K	2.77	3.70	1.44	2.26	1.73
F10K	2.07	5.10	2.11	2.99	2.07
F30K	1.79	5.25	2.23	3.02	2.89
F50K	1.74	5.41	2.46	2.95	3.16

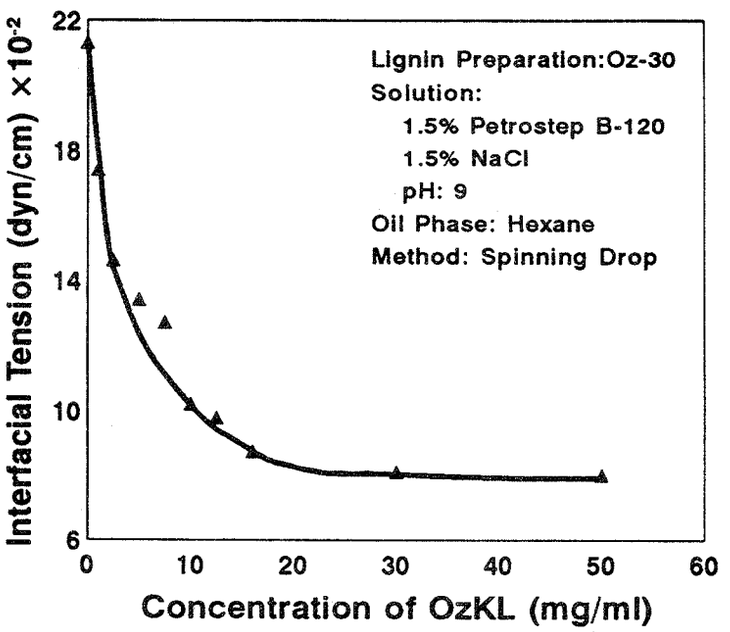


Figure 6. Synergistic effect on oil-water interfacial tension of a surfactant (Petrostep B-120) with ozonated kraft lignin (OzKL) as co-surfactant.

approximately 61 dyn/cm to approximately 53 dyn/cm over the concentration range of 5-10 mg/ml, a drop of 8 dyn/cm, and then levels off. The fraction F30K behaves differently in that its surface tension decreases gradually with increasing concentration.

However, the observed surface tensions of these fractions do not have any meaningful relationship with molecular weight. Thus, the surface tension of fraction F10K is always higher than with other fractions at the same concentration. The surface tension of the fraction F2K is higher than that of fractions F30K and F50K up to a concentration of 5 mg/ml, but lower beyond a concentration of 10 mg/ml.

B. Effect of pH

The surface tension of the fraction F30K at various concentrations and pH were measured. In general, there is no relationship between surface tension and the pH up to a concentration of 8 mg/ml. For example, at a concentration of 2 mg/ml, the surface tension is 66 dyn/cm at pH 6, 62 dyn/cm at pH 9, and 59 dyn/cm at pH 11. At a concentration of 5 mg/ml, the surface tension is 60.5 dyn/cm at pH 6, and 63 dyn/cm at pH 9 and 11. Beyond the concentration of 8 mg/ml, however, it decreases with increasing pH in the concentration range investigated. The surface tension decreases to 52.5 dyn/cm at a concentration of 38 mg/ml and pH 11. At this concentration, the surface tension is approximately 59 dyn/cm at pH 9, and 62 dyn/cm at pH 6.

5.2 The oil-water interfacial tension

The synergistic effects of ozonated KL preparations with commercial surfactants were investigated. An ozonated KL preparation (Oz-30) was added to an aqueous solution containing 1.5% of Petrostep B-120 (Stepan Company) and 1.5% of sodium chloride. When the concentration of the ozonated KL increased from 0 to approximately 20 mg/ml, the oil-water interfacial tension of the solution decreased abruptly from about 10^{-1} dyn/cm to 10^{-2} dyn/cm, and leveled off with further addition of ozonated KL (Figure 6).

6. Conclusions

6.1 Ozonation of Kraft Lignin

A. During ozonation of kraft lignin (KL) fractions, the pH of the reaction mixture decreased markedly and proportional to the ozone consumption. Functional group analysis of the ozonated KL preparations showed that the decrease in pH is caused mainly by the formation of carboxylic acid groups.

B. The KL fractions undergo considerable change in molecular weight distribution during ozonation. The competition between hydroperoxyl anion and hydroxyl radicals strongly affects the molecular weight distribution of ozonated lignin preparations.

The increase in M_w observed on ozonation is attributable to oxidative coupling of lignin phenol structures initiated by hydroxyl radicals.

C. The cleavage of aromatic rings prevails over the oxidative coupling of phenols only during the initial stage of ozonation. At a later stage, kraft lignin predominantly undergoes oxidative coupling.

6.2 The surface-active properties of ozonated KL preparations

A. The surface tension of the aqueous solutions of ozonated KL preparations decreased with increasing concentration over the entire pH range.

B. No relationship between surface tension and molecular weight of the ozonated KL fractions was observed.

C. A synergism was found between ozonated KL preparations and a commercial surfactants (Petrostep B-120). Addition of small amount (less than 20 mg/ml) of ozonated KL preparation (Oz-30) could lead to a significant reduction of oil-water interfacial tension of an aqueous solution containing 1.5% of the commercial surfactant and 1.5% of sodium chloride.

ACKNOWLEDGMENT

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ANALYSIS OF *Eucalyptus grandis* MILLED WOOD LIGNIN

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ABSTRACT

A new method is presented to determine the total contents of lignin and polyphenols in wood of *E. grandis* directly by conventional IR. The Structure of *E. grandis* MWL was studied by functional group analysis, ^{1}H NMR, nitrobenzene oxidation and HPSEC.

I - INTRODUCTION

Eucalyptus wood is one of the most important raw-materials for wood-based industry in Brazil because *Eucalyptus grandis* is extensively cultivated in this country.

The nature of lignins from *Eucalyptus* was studied early [1]. However, only a few works are related to *E. grandis* [2,3]. Therefore, investigation on the chemical characteristics of its lignins was initiated at our University in connection with the University of Uberlândia [4].

Several difficulties were found in the attempt to isolate the pure Milled Wood Lignin (MWL) of *E. grandis* wood.

One of these difficulties is that Eucalyptus wood are very rich in polyphenolic extractives. Therefore, care must be taken to isolate its MWL free of polyphenolic contaminants (PC). For this purpose the Bland and Menshun method was employed [5]. This includes a treatement of wood with alkaline solution which was effective in removal of polyphenols from woods of other Eucalyptus species.

However, the MWL-a thus obtained contains aliphatic contaminants that were detected in the ^{1}H NMR spectrum, having signals at δ 0.9 and 1.1 (Fig. 1A). These signals were still found in the ^{1}H NMR spectrum of the technical lignin of *E. grandis* obtained from acid-catalyzed hydrolysis, as shown in Figure 1C for HL.

Therefore, to obtain a more pure MWL it was necessary to modify the Bland and Menshun method by introducing several chloroform extraction steps [6]. Thus the MWL-b was obtained.

As shown in Figure 1B, ^{1}H NMR spectrum of MWL-b does not exhibit these intense signals corresponding to the aliphatic contaminants. Then, the improved procedure for

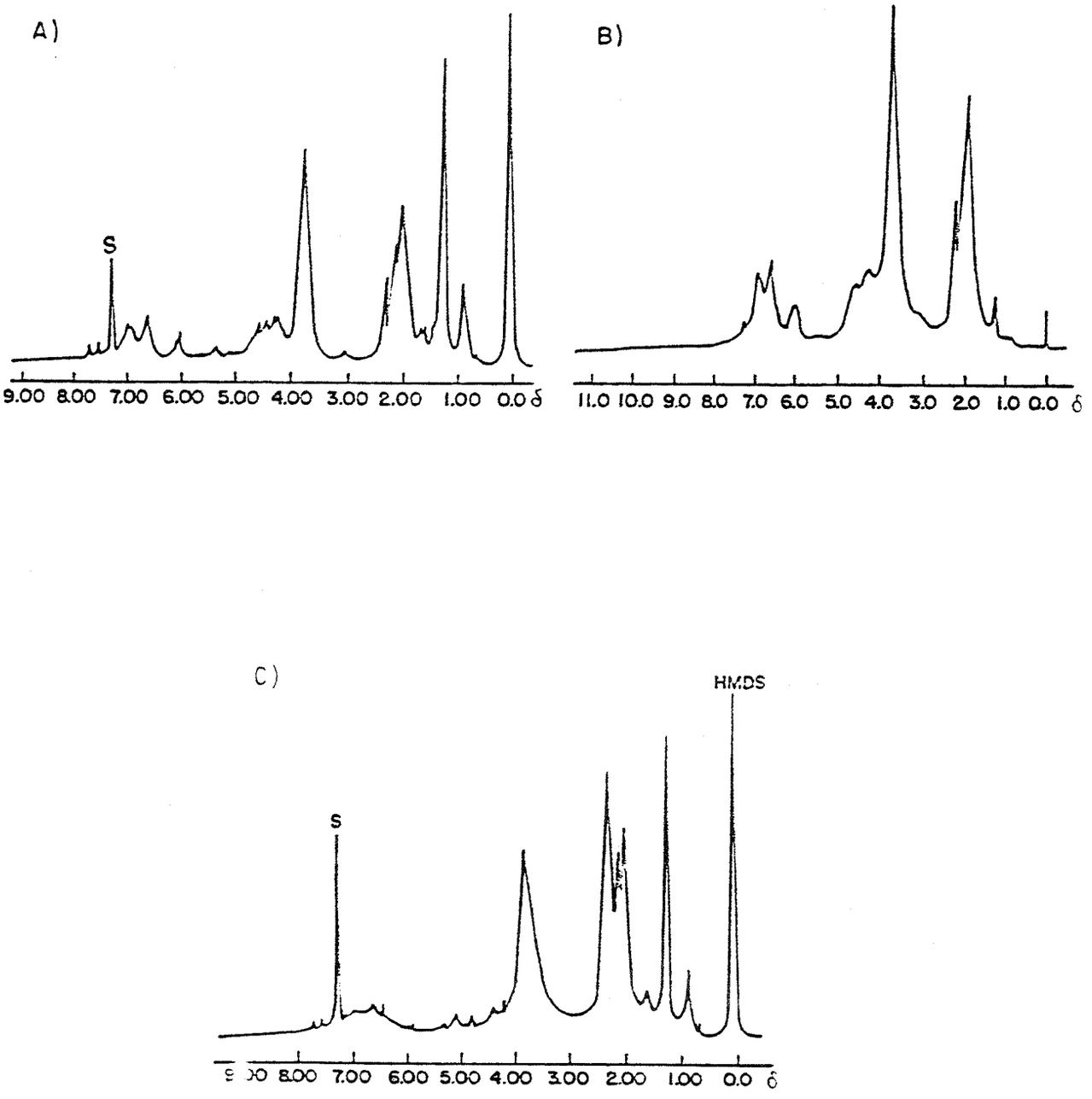


Fig. 1 - ^{1}H NMR spectra $CDCl_3$ of acetylated E. grandis lignins: MWL-a at 400 MHz (A), MWL-b at 80 MHz (B), and AHL at 400 MHz(C).

isolation of lignin discussed above was very important to the structural studies of *E. grandis* MWL.

Table 1 summarizes the results of elemental analysis and C_9- unit formula calculated for both MWL-a and MWL-b. The table also incudes data for the native lignin (NL) isolated by the Brauns method [7].

TABLE 1

	% C	% H	% O	% OMe	C_9- unit formula
MWL-a	60.64	6.86	32.50	21.70	$C_9H_{9.72}O_{2.75}(OMe)_{1.45}$
MWL-b	60.60	6.00	32.00	22.00	$C_9H_{7.90}O_{2.73}(OMe)_{1.50}$
NL	56.50	6.40	37.76	5.86	$C_9H_{11.51}O_{4.23}(OMe)_{0.38}$

It is not surprising that the polyphenolic and aliphatic contaminants contributes differently to the observed characteristics of MWL. Thus, MWL-a that contains only aliphatic extractives as contaminants shows methoxyl groups contents close to that of MWL-b. By contrast, the NL that contains both polyphenolic and aliphatic contaminants has a methoxyl content much smaller than that of MWL-b. This smaller value is also in disagreement with published data for hardwoods, as shown in Table 2 [8].

TABLE 2

MWL	Wood type	% OCH_3
F. silvatica	hardwood	21.4
E. tetraconta	hardwood	20.4
E. diversicolor	hardwood	21.0
P. abis	softwood	15.8

Because Bown's native lignin consists mostly of extractives, it gives a C_9 - unit formula very different from that of MWL-b.

Thus, the MWL-b is evidently the best lignin preparation representing the *E. grandis* MWL.

II - ESTIMATION OF LIGNIN AND POLYPHENOL CONTENTS IN *E. grandis* BY INFRARED SPECTROSCOPY

The MWL-b was then used as a standard to

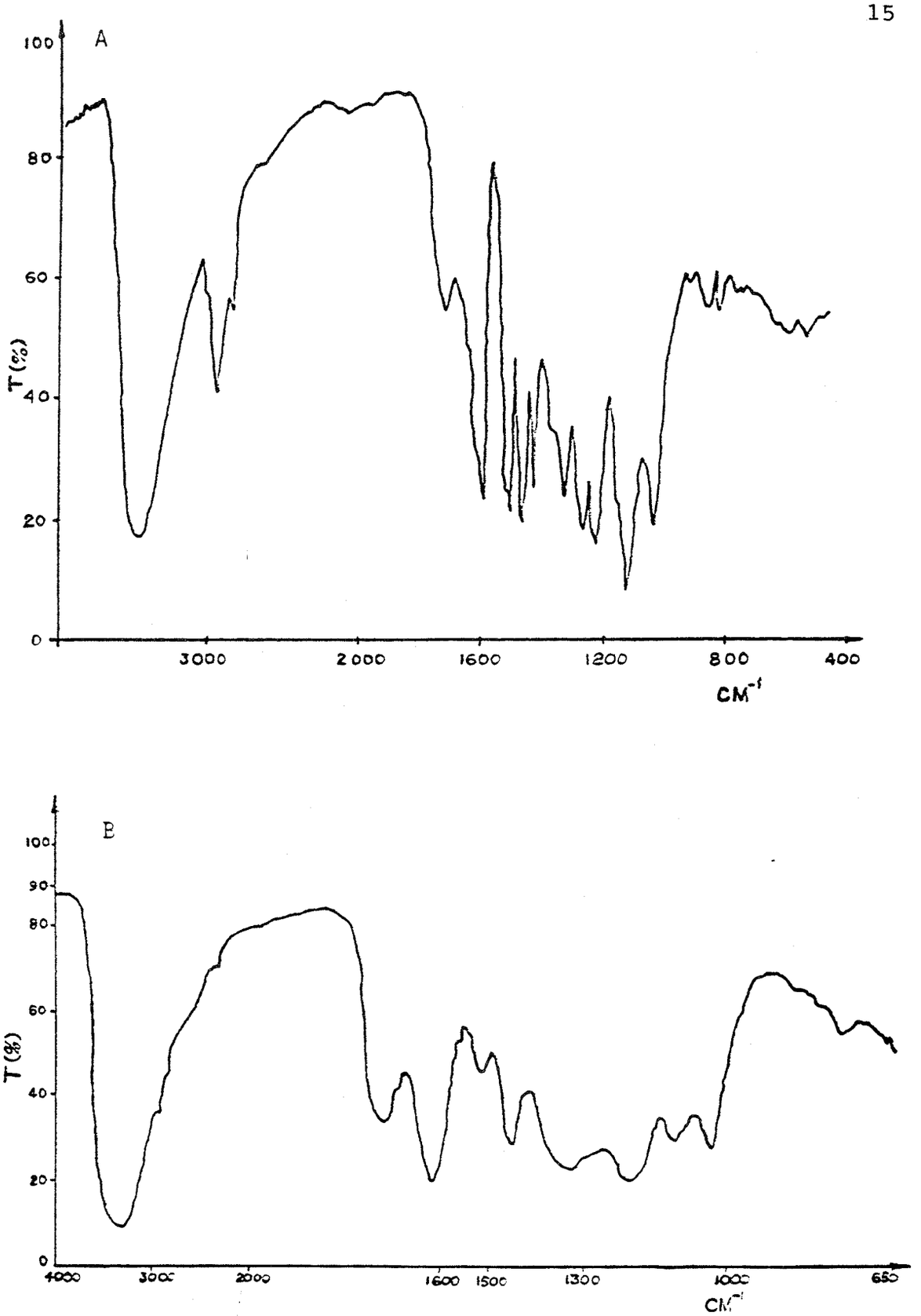


Fig. 2 IR spectra of E.grandis MWL (A) and PC (B)

determine the lignin and polyphenol contents in *E. grandis* wood by infrared (IR) spectroscopy [9]. For this purpose, it was also necessary to isolate the polyphenolic contaminants (PC). They were obtained by exhaustive extraction of *E. grandis* milled wood with water. The milled wood was previously treated with benzene-ethanol 2:1. After removal of water from the water-extractives, residue (PC) was dried in an oven.

The IR spectra of both MWL and PC are presented in Figures 2A and 2B, respectively. In both spectra, it can be observed that the bands around 1600 cm^{-1} of each one are very intense while the bands around 1500 cm^{-1} are less intense. This one in the MWL spectrum is more intense.

Figures 3A, B, and C present only the region of IR spectra containing the 1500 and 1600 cm^{-1} bands, respectively for MWL, PC, and a mixture of PC and MWL extracted from wood with ethanol-water 2:1. In the spectrum 3C, it can be observed that the band at 1500 cm^{-1} is more intense than the corresponding band in the spectrum of PC (spectrum 3B) but less intense than that in the spectrum of MWL (spectrum 3A).

Thus, the intensity ratio of the bands at 1600 and 1500 cm^{-1} could provide a way to determine lignin content in polyphenols of *E. grandis* or even in the wood. To verify this, different amounts of MWL were mixed with PC sample and the IR spectrum was obtained for each mixture sample to be analysed.

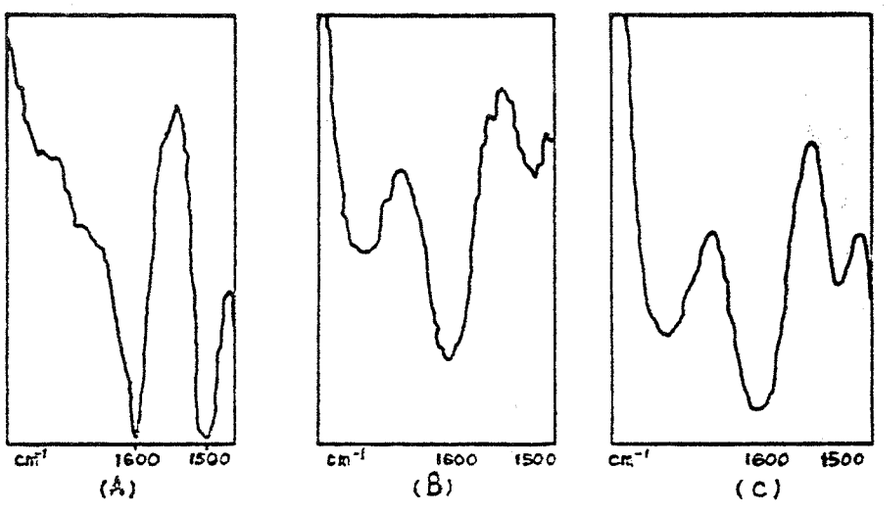
For example, the based lines as shown in Figure 4 were used to determine the band ratios. Six parameters were selected for the procedure. Three of them by comparing the intensity of respective absorption bands related to the base lines 1, 2 and 3.

For instance, in parameter 1 the ratio r is segment AB/ segment CD as illustrated in Figure 4. The other parameters were the absorvances of each maximum related to the baselines 1, 2 or 3. The parameter 4 is the ratio r = Δ absorvance corresponding to segment AB/Δ absorbance corresponding to segment CD.

Table 3 presents the list of ratios obtained by the six different parameters calculated for different mixture samples of MWL and PC.

Figure 5 shows different curves obtained by plotting the increasing percentage of MWL in the mixture samples and the ratio of intensities of 1600 and 1500 cm^{-1} bands calculated for some different parameters explained above. All the curves obtained are exponential and could be used as standard reference. Therefore, curve 1 was selected to be the standard reference bacause it gives the most accurate lignin content when compared with others.

Table 4 presents the lignin content as calculated



Fif.3- IR spectra of MWL(A);MWL+PC(B);and PC(C): 1700 to 1500 cm^{-1} regions.

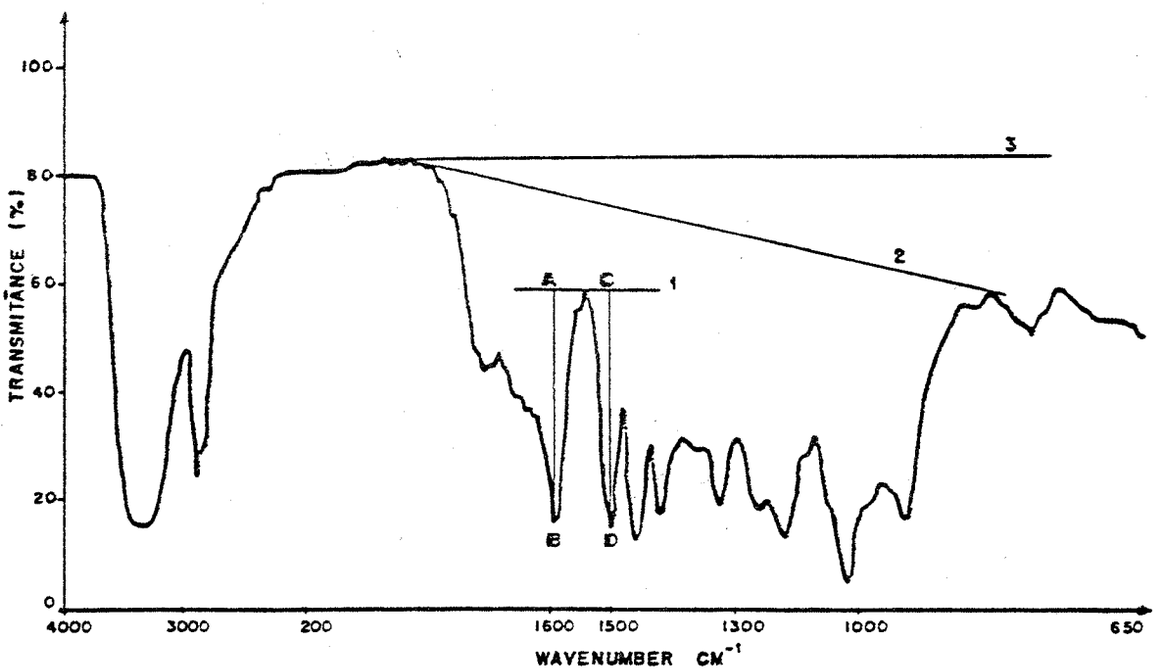


Fig.4
Parameter 1 r = segment AB/ segment CD
Parameter 4 r = Δ absorvance corresponding to segment AB/ Δ absorvance corresponding to segment CD

TABLE 3

Ratios of band intensities at 1600 and 1500 cm^{-1} *versus* lignin content for different procedures

% MWL	Procedures 1	2	3	4	5	6
0	3.28	1.83	1.92	4.93	2.43	2.13
1	2.63	1.67	1.47	4.11	2.31	2.11
5	2.17	1.49	1.37	3.51	2.23	2.08
10	1.95	1.44	1.30	3.10	2.08	1.91
15	1.80	1.38	1.25	2.60	1.98	1.85
20	1.64	1.32	1.22	2.48	1.92	1.79
28	1.47	1.28	1.18	1.87	1.56	1.47
39	1.28	1.20	1.10	1.60	1.43	1.36
50	1.13	1.13	1.06	1.28	1.22	1.18
60	1.04	1.07	1.04	1.13	1.11	1.04
63	1.03	1.05	1.03	1.06	1.07	1.02
70	1.01	1.03	1.02	1.03	1.04	0.98
80	0.95	1.02	0.98	0.97	0.97	0.95
85	0.92	0.98	0.96	0.88	0.88	0.87
100	0.90	0.96	0.93	0.87	0.87	0.87

from several different *E. grandis* extracts using the curve 1. The two first samples obtained have the same MWL content by this procedure.

TABLE 4

Lignin content in some *Eucalyptus grandis* polyphenolic contaminants ("kinos") [5] by IR spectroscopy

Sample	extractive	bands ratio	% MWL
1	ethanol:benzene (2:1)	1.81	15.0
2	ethanol:water (2:1)	1.81	15.0
3	NaOH 0.1N	1.92	11.0
4	dioxane:water (9:1)	1.05	62.0

It can be observed from the data presented in this Table that dioxane-water (9:1) solution is more effective solvent system for extracting lignin from milled wood of *E. grandis* than 0.1 N sodium hydroxyde solution. This result indicates either that the phenolic hydroxyl content in the lignin is not very high or that phenolic hydroxyl groups alone do not contribute significantly to the extraction of lignin by alkaline solution, or both.

This technique may also be applicable to analyse the lignin and polyphenol contents in *E. grandis* wood. However, in this case, first it is necessary to determine

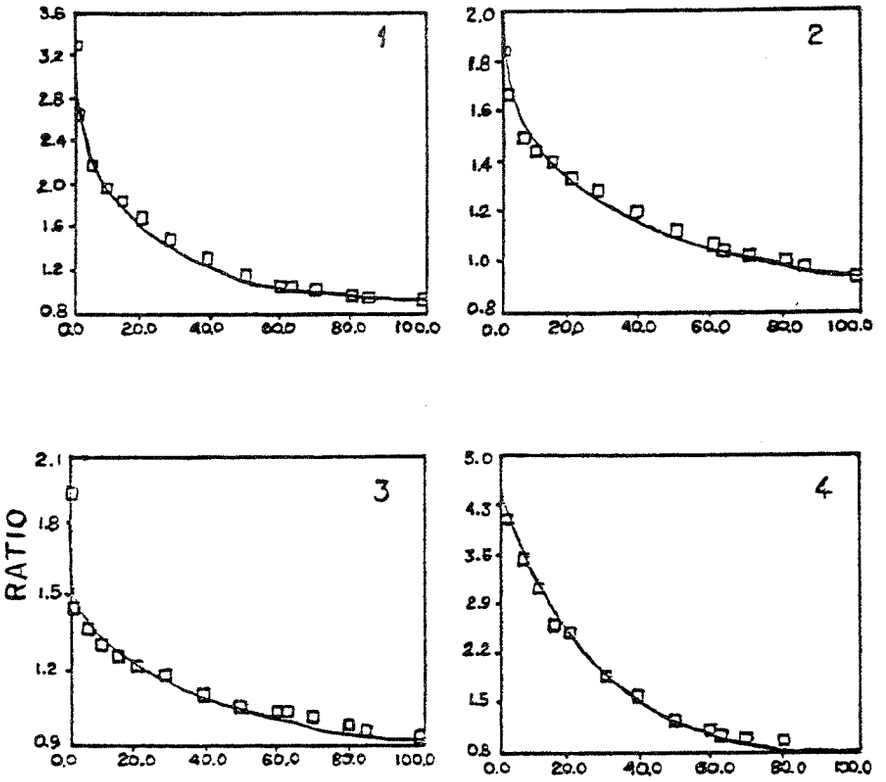


Fig.5 Curves obtained from data of Table 3

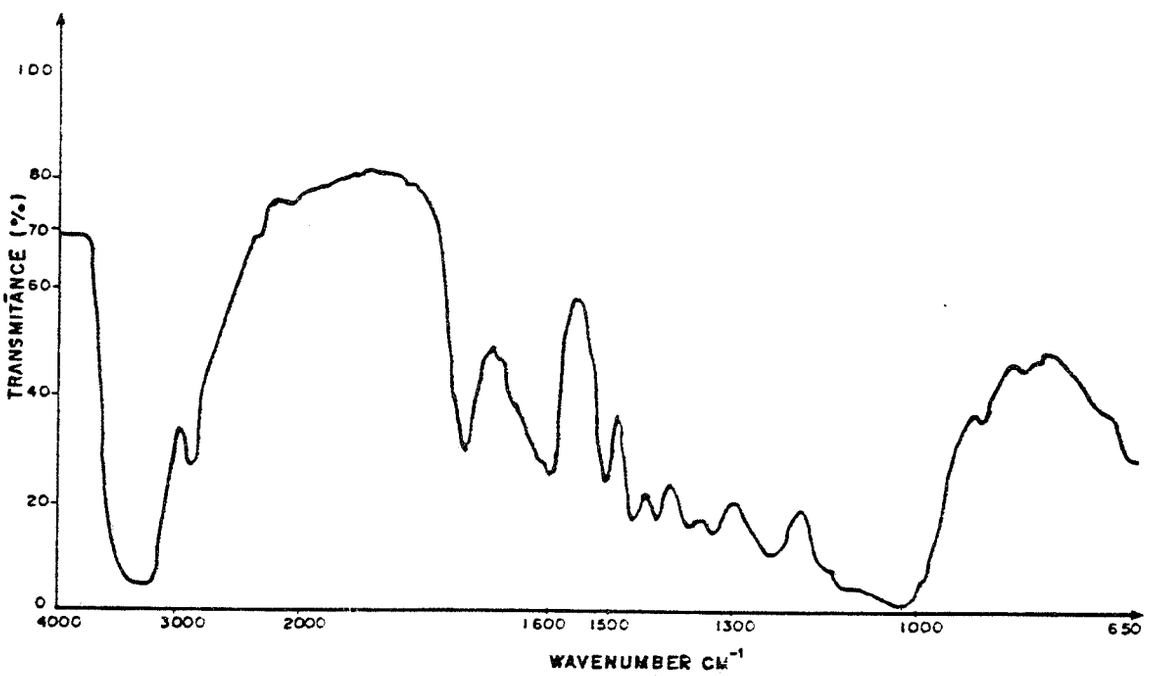


Fig6-IR spectrum of Eucalyptus grandis milled wood

the Klason lignin content of the wood not previously extracted by any solvent. Furthermore, both the soluble lignin and polyphenol contents must also be determined by UV spectroscopy [10]. The addition fo the values of these three determinations will give the total lignin and polyphenol contents of the wood. In the case of *E. grandis* it was calculated 39.8% (Table 5) [11].

TABLE 5
Lignin in *E. grandis* wood [11]

KL	SL	SPC	TOTAL
37.8	2.0	0.005	39.8

KL = Klason Lignin, SL = Soluble Lignin, SPC = Soluble Polyphenol Content.

If this value would be related to the one calculated from the analysis of the IR spectrum of *E. grandis* milled wood by applying the method described above, it will be possible to estimate separately the contents of MWL and PC in this wood..

In Figure 6 can be observed that the 1600 and 1500 cm^{-1} band ratio of the IR spectrum of *E. grandis* milled wood is estimated as 1. Therefore, the MWL content in polyphenol for this wood was calculated as 69%, when the technique proposed above was applied. Thus, the MWL and PC contents of this wood are, respectively, 39.8% x 0.69 = 27.5% and 39.8% - 27.5% = 12.3%.

To compare this result, the total Klason and soluble lignin content of the *E. grandis* milled wood was determined as proposed by Band and Menshum. This procedure includes Klason lignin determination after previous extraction of wood successively with benzene-ethanol (2:1), water, and 0.1 N sodium hydroxide solution. The total lignin content obtained is 29.5%.

The difference between these two values is only 8%.

It is well known that the MWL content of a woody tissue of plant species varies differently in morphological region of the same tree species, as well as geographical location, and ages of trees of the same species [12].

Taking in account all of these possibilities, it is very interesting to apply an analytical method that provides fast result of lignin content in woods of *E. grandis*. The advantage of the method proposed in this work is to provide a direct way to estimate the lignin content of milled wood without the need of tedious chemical treatments.

III - CHARACTERIZATION OF *Eucalyptus grandis* MWL

Besides the studies described above it was also developed MWL analysis aiming for its characterization.

Table 6 presents some of principal characteristics of *E. grandis* MWL.

The methoxyl content of 22% [4,13] reported in Table 2 corresponds to 1.50 methoxyl per C_9 - unit [7]. The methoxyl content was also calculated by infrared spectroscopy (IR), as proposed by Sarkanen for conventional IR [14,15] or as proposed by Faix for FTIR [16], and the value obtained was 21.6%. This corresponds to 1.49 methoxyl per C_9 - unit. Those two values are in good agreement.

The C_9 - unit formula was calculated from the results of the elemental analysis presented in Table 2 and the methoxylcontent.

The syringyl/ vanilin (S/V) molar ratio obtained after GC analysis of the nitrobenzene oxidation product is 1.3 [17]. This value agrees with the values found for other Eucalyptus wood species, S/V molar ratio in the range of 1.2 to 4.7 [18]. Similarly, the methoxyl content in these lignins varies from 20.3 to 22.8% [18]. It has been suggested by Sarkanen and Hegert [17] that S/V values divided by 3 is approximately equal to the S/G molar ratio. However, this rule evidently does not apply to the lignins of Eucalyptus since it gives S/G molar ratio of only approximately 0.4. It is very different from the value of approximately 1.0 which is obtained by both chemical method and IR spectroscopy as described above in this work. As shown in Table 6, it was found that the *E. grandis* MWL consists of approximately 49% of syringylpropane (S) units, 49% of guaiacylpropane (G) units and only 2% of p-hydroxyphenylpropane (H) units [16]. This is a hardwood lignin characteristic .

Finally, ^{1}H NMR spectrum of the MWL (Figure 1B) presented approximate the same hydrogen percentages of syringyl (6.9%) and guaiacyl groups (7.0%). It means that guaiacyl groups are more condensed than syringyl groups.

From the total aromatic hydrogen content in the ^{1}H NMR spectrum of the lignin it was estimated that the degree of condensation/ C_9 - units is approximately 0.50. Thus, approximately half of the phenylpropane units in the lignin is condensed.

The high degree of condensation of lignin is probably one of the factors contributing to its high weight-average molec u lar weight ($\overline{M}_w$) [16] that was determined by high performance size-exclusion chromatography (HPSEC) as 8,400 Dalton [4]. This value was calculated when 8% of chromatogram area corresponding to

TABLE 6

Principal Characteristics of *E. grandis* MWL

- OMe content/	1.50	(Vieböck method) [13]
/C_9- unit	1.49	(IR method) [15]
- C_9- unit Formula	$C_9H_{7.90}O_{2.73}(OMe)_{1.5}$	[7]
S/V	1.3	(by Ø-NO_2 oxidation; GC) [17]
S/G	0.4	
	1.0	(by OMe/ C_9- units; IR, ^1H NMR analysis [4])
- % $C_\alpha C_3$- units	S 49 G 49 H 2	(IR method) [16]
- Aromatic H content	a-2.50/ C_9 unit	(=3x50 + 2x50 from MF) [7]
	b-2.51/ C_9 unit	(=3x49 + 2x40 from IR analysis) [15,16]
	c-2.00/ C_9 unit	(from ^1H NMR analysis)
- Condensation Degree	0.50/ C_9 unit	(a-c)
	0.51/ C_9 unit	(a-b)
- $\overline{M}_w$	8,400 Dalton	(HPSEC, polystyrene standards) [14]

macromolecules larger than 68,000 Dalton were excluded.

IV - CONCLUSIONS

- A method has been developed for the determination of lignin content in polyphenols of *E. grandis* or in *E. grandis* extracts.
- This method shows that the ratio of infrared bands at 1600 and 1500 cm^{-1} provides a way to determine lignin content in *E. grandis* extracts or even in the wood.
- The method requires that Klason lignin content of the wood and both soluble lignin and polyphenol content must be also determined, in the case of its application to analyse directly the lignin content in the *E. grandis* wood.
- This method provides a manner to estimate separately the lignin and polyphenol contents in the *E. grandis* wood.
- The method has the advantages of rapid

and comparative acurate value for lignin content compared with the Klason lignin determination.

- The *E. grandis* MWL contains syringyl- and guaiacylpropane units in approximately 1:1 molar ratio. The guaiacylpropane units are highly condensed.

- The *E. grandis* MWL is a highly condensed lignin. This lignin contains approximately 50 condensed phenylpropane unit/ 100 C_9 units.

- The high weight-average molecular weight ($\bar{M}_w$) of the *E. grandis* MWL may be related to this degree of condensation.

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PRODUCTION AND CHARACTERIZATION OF TECHNICAL LIGNINS : ^{13}C NMR AS A PROCESS CONTROL .

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ABSTRACT.

Lignins obtained from different pulping processes can be characterized precisely and quite rapidly by ^{13}C NMR . Presence of carbohydrates is easily detected and the structural changes in lignins can be correlated to the pulping conditions .

Several technical lignins have been studied :

- kraft lignin from kraft cooks of Pine and Aspen wood in normal batch and flow-through cooks .
- isolated lignins from steam exploded Aspen wood under different conditions with respect to pressure and time .
- organosolv lignin isolated from Spruce TMP (thermo-mechanical pulp) and fractionnated with a flow-through reactor.

Consequences of bleaching procedures can be traced on the structural pattern of lignin, for exemple decreasing or disappearing of some specific chromophores .

From the combination of several NMR sequences were obtained qualitative and quantitative data about functional groups (-OCH3, hydroxy groups, alcoholic and phenolic, β-O-4 structures,) oxygenated side chains, and degree of condensation. These informations are particurlarly useful in the case of heavily and poorly defined lignins .

INTRODUCTION.

It is important to improve our knowledge about the structure of technical lignins, when just by now a double effort is made to both , improve the classical pulping processes and developp new ones, like the organosolv processes.The structure of the technical lignins depend tightly upon the different stages they are going through:

- the dissolution of the lignins (the pulping process itself),
- the recovery of the different lignin fractions,
- eventually the bleaching stage,

The ability of ^{13}C NMR to investigate technical lignins structures has been first demonstrated in the case of lignins from steam exploded wood (I) (in coll. with Dr. M. Bardet CENG Grenoble, France.) It is extended in this study to the case of:

- alkali and kraft lignins from Aspen wood, fractionnated with a flow-through reactor (II) (in coll. with Dr. A. Labidi and Dr. F. Pla, Ecole Française de Papèterie Grenoble, France.)
- kraft lignins from pine wood (III) (in coll. with Dr. G. Gellerstedt KTH Stockolm Sweden.)
- TMP(thermomechanical pulp) lignins from spruce wood organosolv cooks, fractionnated with a flow-through reactor.(IV) (in coll.with Dr. X. Pan and D. Lachenal Ecole Française de Papèterie Grenoble,France.)

The different lignins samples have been characterized qualitatively and quantitatively by ^{13}C NMR, using the appropriate sequences: a variety of chemical groups and structures are observed and quantitated, their ratio are dicussed in relation with the experimental conditions.

RESULTS AND DISCUSSION.

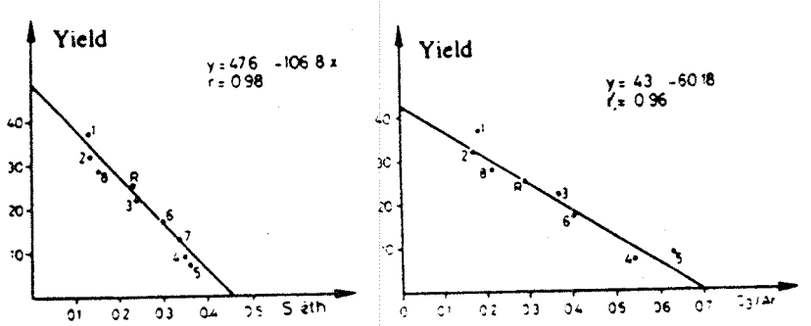
I-Lignins from Steam Exploded Aspen Wood :

- according to the Iotech process wood chips are treated with steam in a reactor at different pressures(10 to55 bars) for different lengths of time (10 to 225 sec.).The lignocellulosic materials are very rapidly extruded from the reactor and the lignins fractions extracted with dioxan-water solvent(9/1).

Characterization (1):

Some of the results are given in Fig.1. A severity parameter appears including pressure and residential time(Fig.1a). It was shown (Fig.1b, and 1c) that a very good correlation, integrating the severity parameter, exists between the yield of lignin material obtained versus the number of syringyl β-O-4 units.One can also plot the yield versus the number of oxygenated propane side chain or the number of free aromatic -OH versus the one of syring.β-O-4, the correlation is still coherent with the severity parameter.It shows the flexibility of NMR as an analytical tool to trace structural changes.

b)



a)

1	45	205°
2	35	240°
3	45	85°
4	25	85°
5	35	10°
6	25	205°
7	20	125°
8	50	125°
9	35	125°

c)

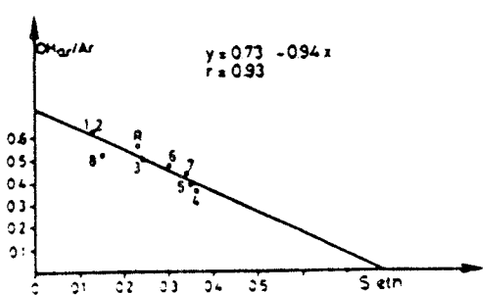


Fig.1 a)-experimental conditions for steam explosion procedure: pressure P,in bars and T residential time, in sec. for 9 samples. 1b), 1c) and 1d) correlation plots integrating the severity parameters corresponding to the samples in 1a).

II- Akali and kraft lignins from aspen

The alkaline phase is continuously replaced in the flow-through reactor by fresh cooking liquor, so that the dissolved lignin can be withdrawn from the reactor shortly after it is formed, it minimize the degration and recondensation reactions which would occur if the dissolved lignins would stay longer in the cooking liquor.

Characterization:(2)

From the results given in Table1 for kraft lignins, we conclude that the real structural changes occur at the earliest stage of the cook:after 9 minutes in the reactor already half of the syring.β-O-4 units have been hydrolysed(sample K1-1) and after 43 minutes 2/3 of them(sample K1-8).If the alkalinity drops from 16.6% to 2% no structural changes occur even after 160 minutes of pulping time, it is a way to test the role played by the alkalinity in the degree of delignification.

Lignin sample OCH_3	Sampling times (min)	s (a) (%)	Δ s (%)	Functional groups in lignin spectra (b) (%) C_{quat}	$S_{\beta O4}$	C_{ali}-O	C_γ	$C_{\beta-\beta}$
MWL	--	--	--	3.50	0.60	0.92	0.83	0.11	1.42
Lignin recovered from series K1 * (Ae = 16.6 %; S = 25 %)									
K1-1	0 - 9	50.9	50.9	3.62	0.36	0.70	0.46	0.05	1.43
K1-2	9 - 14	61.6	10.7	3.46	0.33	0.67	0.37	0.05	1.36
K1-4	18 - 22	73.6	5.6	3.43	0.24	0.48	0.28	0.05	1.19
K1-8	36 - 43	89.2	11.0	3.45	0.19	0.54	0.26	0.06	1.20
Lignin recovered from series K8 (Ae = 2.0 %; S = 25 %)									
K8-1	0 - 15	24.1	24.1	3.57	0.38	0.67	0.43	0.05	1.30
K8-4	25 - 30	35.2	3.1	3.65	0.37	0.62	0.37	0.05	1.30
K8-9	100 - 160	72.8	12.4	3.59	0.37	0.66	0.34	0.06	1.27

MWL = milled wood lignin

(a) : s = mass fraction of soluble lignin which is representative of the delignification degree

Δ s = weight fraction lignin recovered during sampling time

Table 1- Number of functional groups determined by ^{13}C NMR for lignin fractions obtained with a flow-through reactor from aspen kraft cooks. The ratio are expressed per aromatic unit.

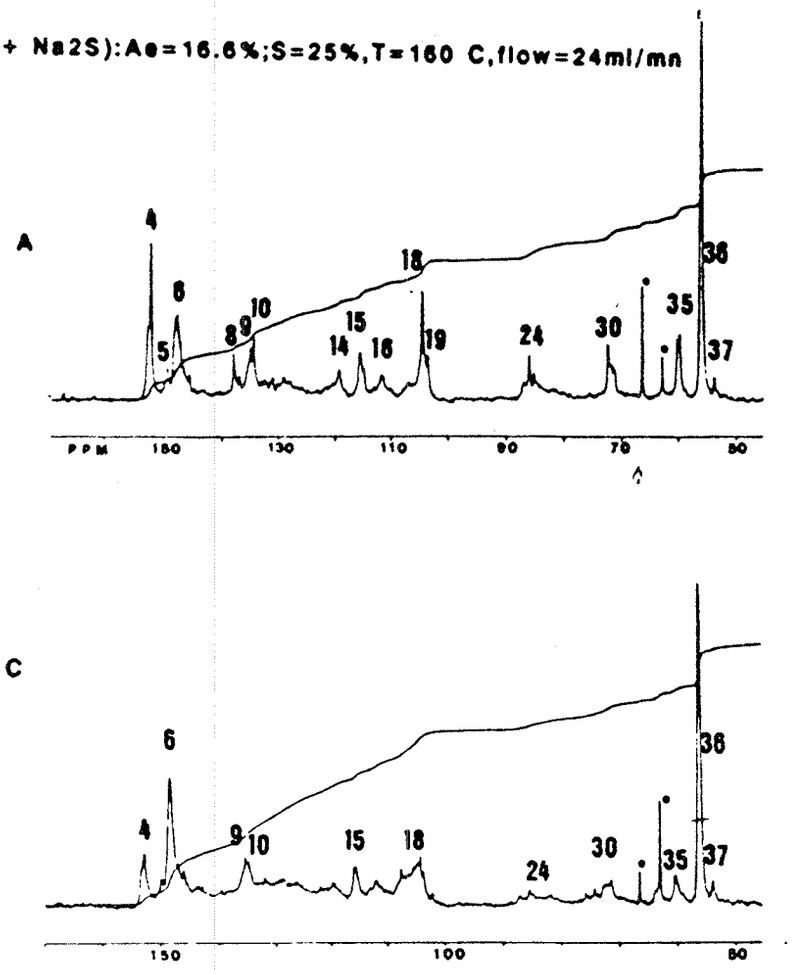


Fig. 2 - ^{13}C NMR spectra of lignins isolated from aspen kraft cooks with a flow-through reactor. Sampling time a) 1-9mn. c) 43-60mn.

On Fig.3a and c) are shown the spectra used for the quantitative analysis of kraft lignins : from the intensity of signals 4 and 6, respectively assigned to C-3/C-5 aromatic carbons in Syr. β-O-4 etherified and non etherified units, one can estimate the degree of splitting of the aryl-alkyl linkages which obviously increases with longer residential times.

The same study was conducted for alkali lignins, for two different NaOH concentrations, the results are summarized in Table 2 : as previously observed, the structural changes occur at the earliest stage of the cook and a lower alkalinity decreases the importance of the hydrolysis. As a side remark : these lignins are far less contaminated by carbohydrates than the kraft lignins.

III- Kraft lignins from Pine(Pinus sylvestris) :

Two series were prepared, one by normal kraft cooks to different pulp yield and another by successive acidification of the black liquors obtained from a flow-through reactor.

Characterization:(3)

The number of functional groups determined by ^{13}C NMR using Inverse Gate Decoupling and DEPT sequences for the different samples are given in Table2 : there is no major changes in the chemical structure of all the dissolved lignins whatever serie it belongs to , it is clear that the major part of the change occurs before or when the lignin is dissolved.

Two other remarks concern the large amount of olefinic double bonds, which are known to belong to stibenes, styrenes or enol ethers structures and of saturated CH_2 and CH_3 aliphatic groups which support the idea of new C-C bonds formed during kraft cooking.

IV- Organosolv lignins from spruce TMP :

The thermomechanical pulps were delignified with ethanol/water(1/1) containing 0.1m acetic acid at 175°C using a flow-through reactor for lignin samples fractionation.

Characterization :(4)

The spectra in Fig.3 show clearly that this lignin has not been seriously degraded and that it is rich in β-5 structures compared to a standard lignin like a Milled Wood Lignin; it is an important difference compared to the classical pulping processes which heavily degrade the

Functional group	Number per aromatic ring			
	Degree of delignification		FTR	
	0(MWL)	0–69 %	29–66 %	69–85 %
Quaternary aromatic C	3.34	3.42	3.43	3.52
Double bonds	0.06	0.18	0.14	0.22
Hydroxy groups, total	1.29	1.25	1.31	1.32
Hydroxy groups, primary	0.78	0.35	0.44	0.43
Hydroxy groups, secondary	0.31	0.25	0.25	0.21
Hydroxy groups, phenolic	0.20	0.62	0.62	0.68
Aliphatic C, 55–90 ppm, CH	1.34	0.49	0.54	0.51
CH_2	0.84	0.35	0.44	0.35
OCH_3	0.97	0.79	0.91	0.88
Aliphatic C, 0–55 ppm, CH	0.25	0.39	0.32	0.38
CH_2	0.18	0.63	0.55	0.59
CH_3		0.18	n.d.	n.d.

Table 2 - Number of functional groups determined by ^{13}C NMR in kraft and in MWL lignins from Pine.

lignin structure; it emphasizes one of the advantages of the organosolv process which offer the possibility to be at the same time a pulping process and a technique for a complete treatement of the biomass.

The TMP has been bleached and then yellowed and the corresponding lignins were analyzed : the cinnamaldhyde group desappears from the bleached TMP lignin and carboxyl groups reappear after the yellowing stage.

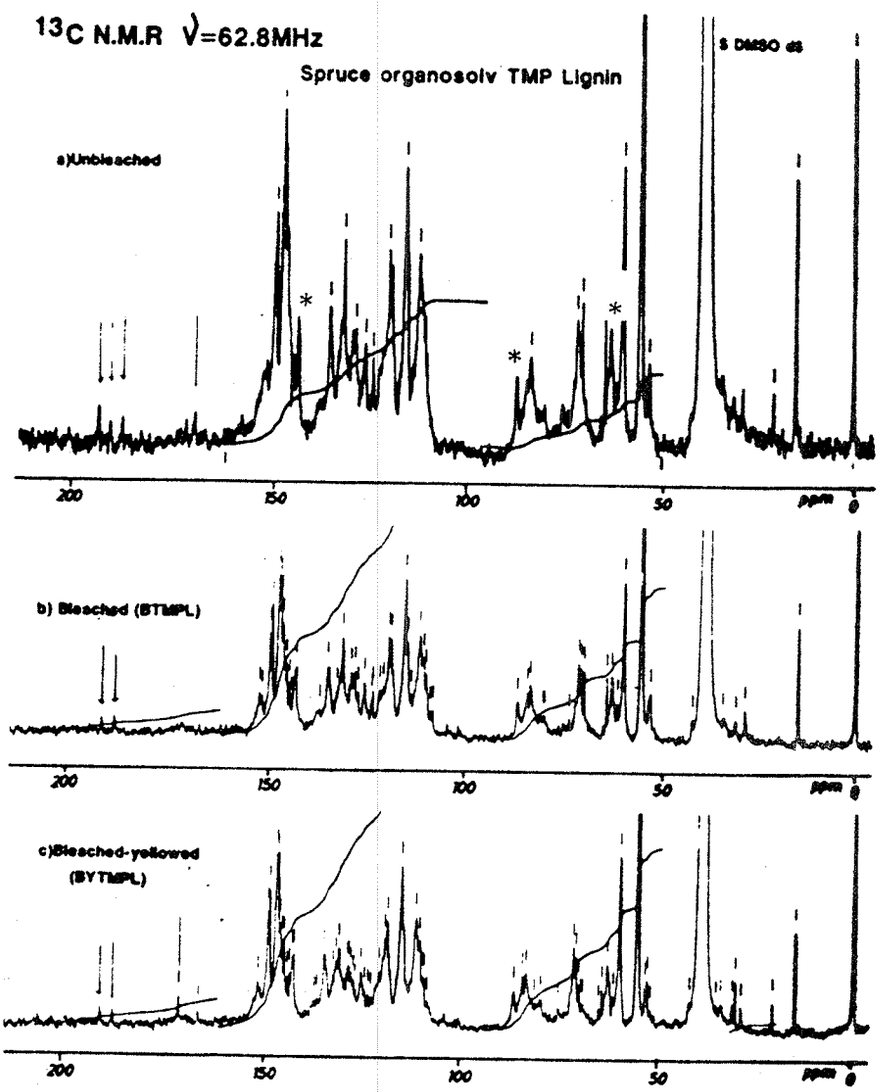


Fig. 3- ^{13}C NMR spectra of thermomechanical pulps lignin from spruce. On 3 a) * signals typical of β-5 units.

The NMR spectra were recorded on a WM250 MHz Bruker spectrometer at 323K and DMSO-d6 as a solvent. Their analysis was made according to established methods (5)(6)

Two types of NMR sequences were used in most of these analysis : the Inverse Gate Decoupling sequence -10 to 12 sec. delay time, 90° pulse, 323K - and the DEPT sequence which gives selectively the sub-spectra for CH, CH_2, and CH_3 groups (and by difference with the total spectrum the quaternary cabons.)

CONCLUSION

If 13C NMR does not yet offer a routine process control for technical lignins , it has nevertheless many real advantages :

- even when the heterogeneity of lignins material is increased by the pulping process, ^{13}C NMR can still give rather reliable and detailed structural informations.

- it gives informations at the same time about details in the structure and about the whole structure

-it is possible to trace qualitatively and quantitatively minor or major structural changes and to correlate them to the experimental parameters, which consequently can be monitored in function of the desired structural characteristic.

Two ways to improve this spectroscopic technique, in the case of technical lignins characterization, would be to developp on line technique analysis of the cooking liquors and ^{13}C solid state NMR to study the pulp itself.

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DETERMINATION OF PHENOLIC HYDROXYL GROUPS IN LIGNINS AND LIGNIN FRACTIONS BY MEANS OF FTIR SPECTROSCOPY

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ABSTRACT

The quantitative determination of phenolic hydroxyl groups is of interest for the characterization of isolated lignins which especially indicates the extent of fragmentation and/or condensation reactions. The paper evaluates the aliphatic and aromatic IR ester bands at about 1745 cm^{-1} and 1765 cm^{-1} of acetylated lignins of different origin and of fractions with different molecular weights. The method was adjusted to the chemical procedure of aminolysis and revealed a high correlation between IR data and chemical analysis.

The IR spectroscopic method described is therefore considered suitable for the quick, easy and reliable quantification of phenolic OH groups in lignins.

INTRODUCTION

The phenolic hydroxyl group is one of the most prominent functional groups of lignins. It plays an important role in degradation reactions involving interunit ether cleavages e.g. in pulping and bleaching processes. The number of phenolic OH groups influences the behaviour and properties of technical lignins with regard to chemical reactions e.g. as co-reactant in resins etc.

The investigations described in this paper aimed at the quantitative IR spectroscopic determination of phenolic OH groups in isolated lignins which is especially suited to routine application in industrial laboratories. This precludes difficult and time-consuming chemical as well as costly and sophisticated spectroscopic methods such as NMR techniques.

On the other hand, the investigations fit into the line of collating reliable chemical lignin data and relating them to IR data for quantitative purposes as suggested and initiated by Faix (1) and Schultz, Glasser (2). Both, chemical and physical methods, are known for phenolic OH determination (Fig. 1) of which we used aminolysis as the chemical reference method.

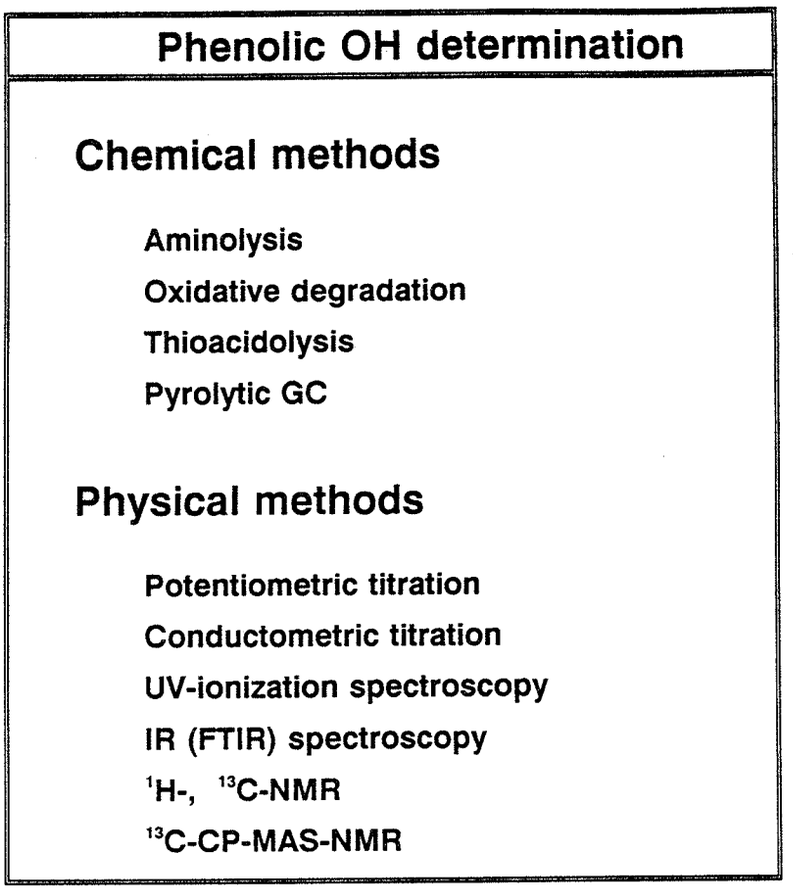

Figure 1: Chemical and physical methods for phenolic OH determination

EXPERIMENTAL PART

Lignins

The lignin samples used should differ in origin and chemical composition and show a wide range of phenolic OH contents. Organocell pilot (P) and demonstration (D) plant lignins from the alkaline stage obtained by acid precipitation (S) or by electrolysis (E) as well as fractions with different molecular weights were therefore used (Fig. 2). A kraft lignin and milled wood lignins from different species were also included in the program. Fractionation according to molecular weight was performed by means of GPC and HPSEC (Fig. 3) (3).

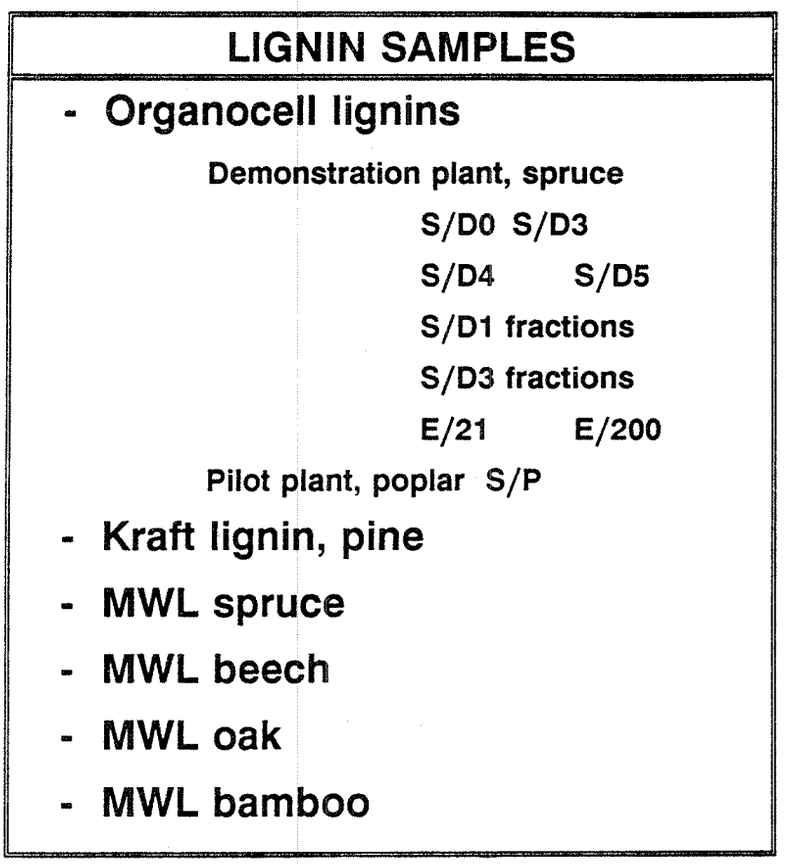

Figure 2: Lignin samples used for chemical and IR spectroscopic analysis

Acetylation

For infrared spectroscopy the lignins were acetylated. Some 100 mg lignin are dissolved in 1 ml pyridine and 1 ml acetic acid anhydride added. The amount of pyridine may be changed to more than 1 ml but the ratio pyridine : anhydride should remain 1 : 1. The mixture was kept for 24 h at 45°C in nitrogen atmosphere. After addition of 1 ml methanol and 8 ml dichloromethane the mixture is shaken out 3 times with 2 n HCl. The organic phase is dried over Na_2SO_4 and evaporated until dry.

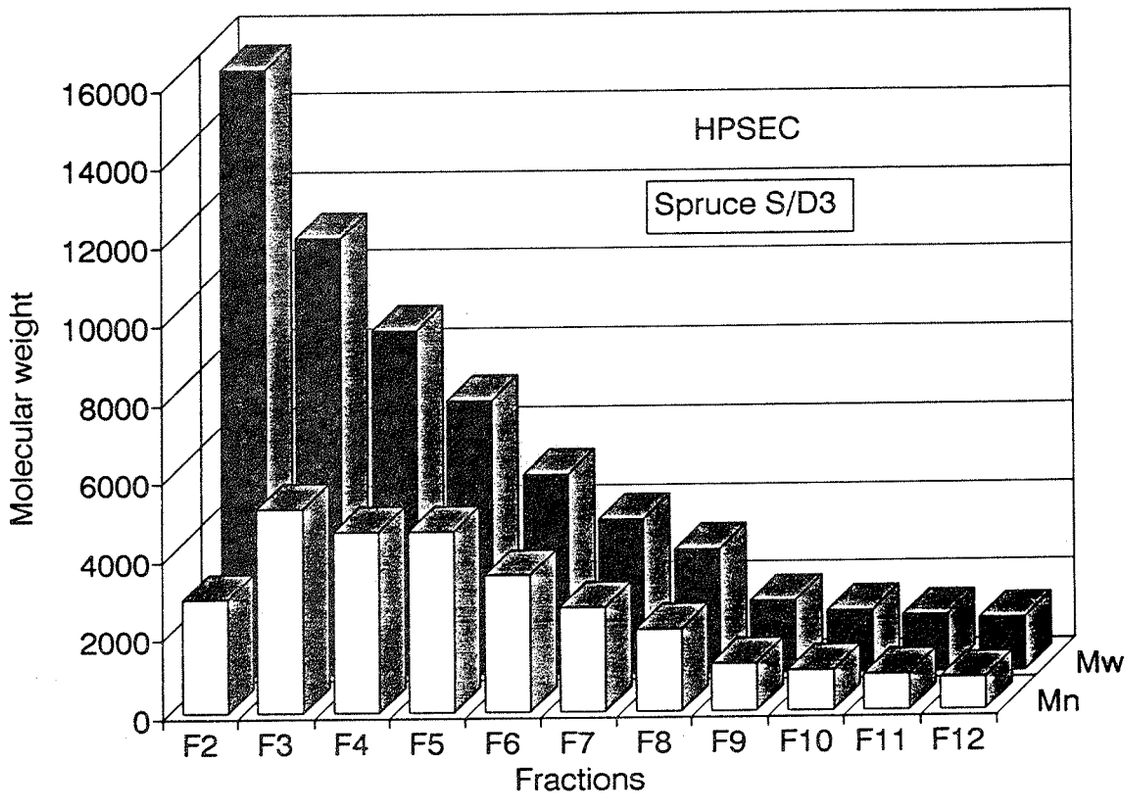

Figure 3: Weight average and number average molecular weights of fractions from S/D3

FTIR measurements

KBr pellets are pressed with about 1 mg of lignin in 300 mg KBr and measured in a FTIR spectrophotometer (FTS-40, Digilab).

The method is based on the evaluation of the phenolic ester band at about 1765 cm^{-1} and the aliphatic ester band at about 1745 cm^{-1}. The measurement of the band intensities is based on the corresponding maxima and not fixed to the mentioned wave numbers. All spectra are normalized by setting the aromatic band at 1510 cm^{-1} to 100% (Figs. 4 - 5).

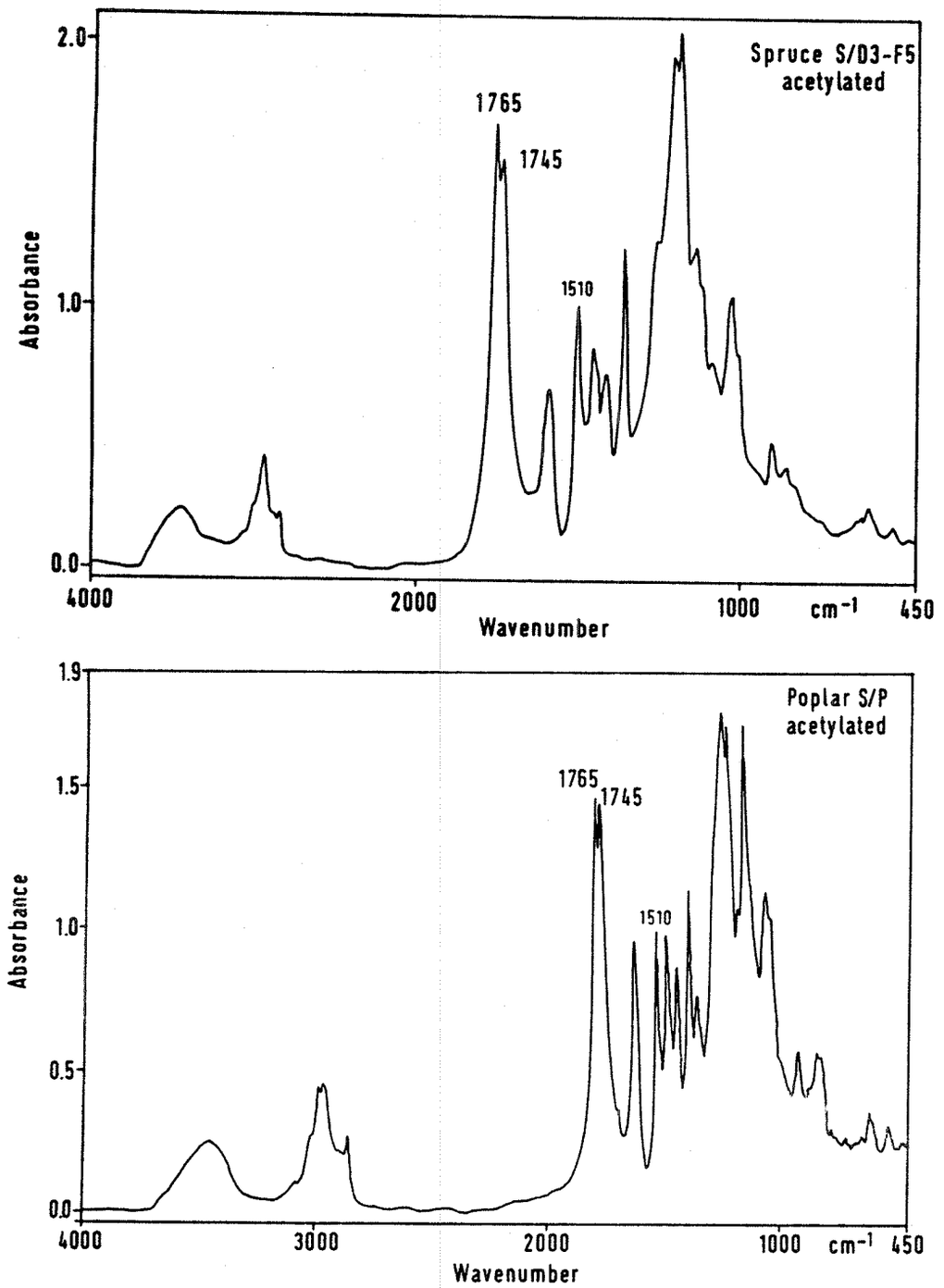

Figure 4: FTIR spectra of acetylated fraction F 5 from spruce lignin S/D3 (above) and of acetylated pilot plant poplar lignin (below)

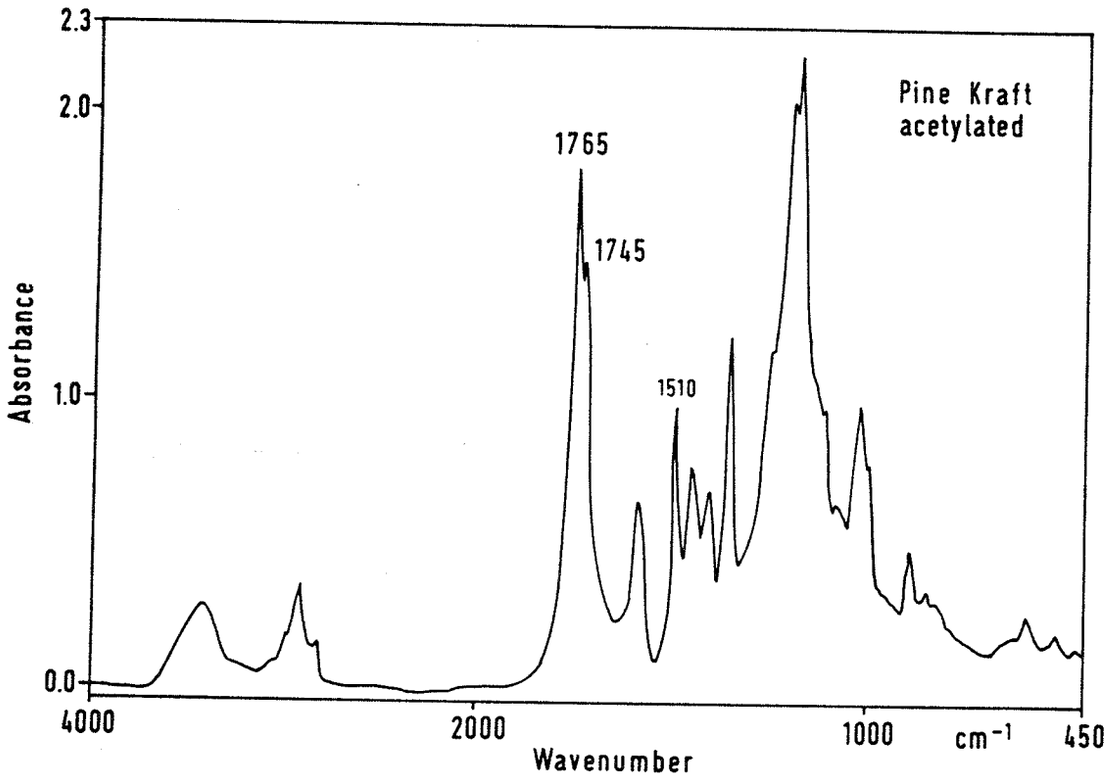

Figure 5: FTIR spectrum of acetylated pine kraft lignin

The quotient of the relative intensities of the ester bands (IR 1765/1745) was found to be the most suitable index for the quantitative evaluation of the phenolic OH groups. In the case of low phenolic OH contents in comparison with aliphatic OH groups, e.g. in milled wood lignins, the 1745 cm^{-1} band supercedes the 1765 cm^{-1} band (Fig. 6).

Resolution of the two maxima is possible by the following techniques:

1. extension of the spectrum in the region between 1700 and 1800 cm^{-1}, yielding a shoulder.
2. measuring the first derivation (Fig. 7).
3. deconvolution of the spectrum (Fig. 8). The deconvolution technique is made possible by an internal program of the FTIR spectrophotometer.

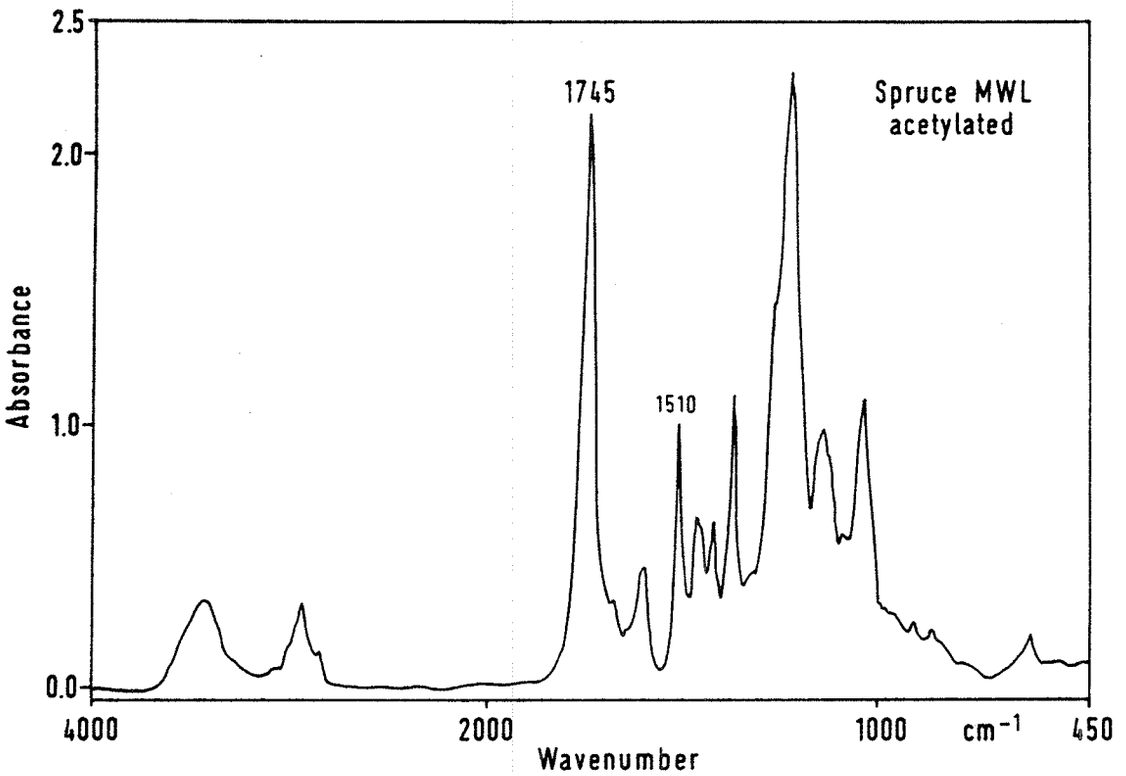

Figure 6: FTIR spectrum of acetylated spruce milled wood lignin

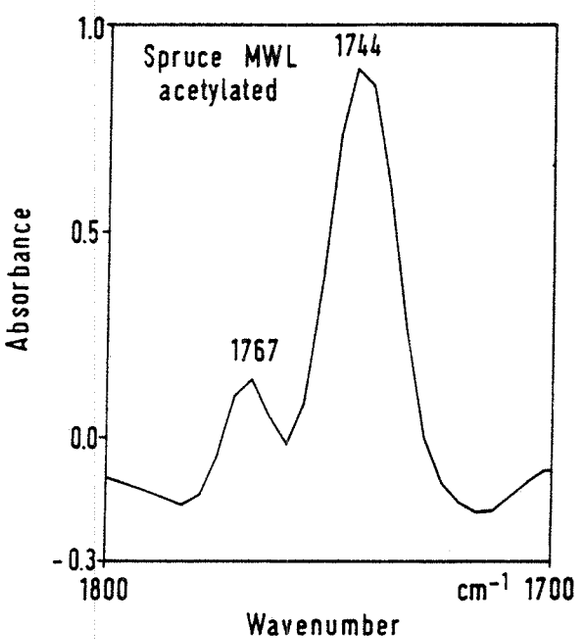

Figure 7: FTIR spectrum of spruce MWL after first derivation

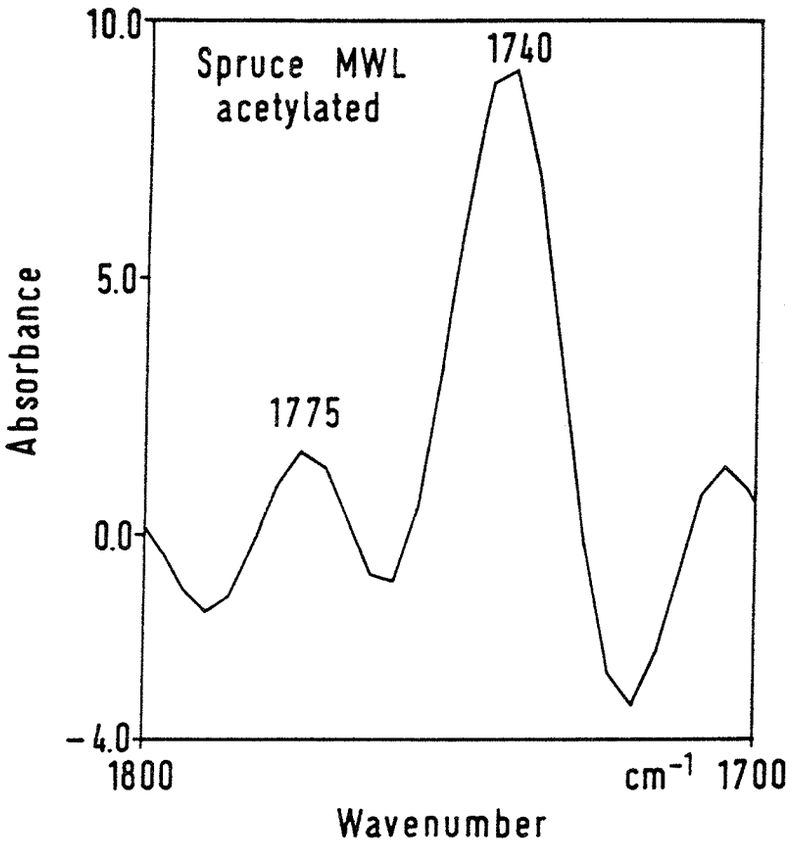

Figure 8: Deconvoluted FTIR spectrum of spruce MWL

Aminolysis

This method involves the selective deacetylation of acetylated lignins by adding pyrrolidine and GC measuring of the acetylpyrrolidine formed within 60 min. The phenolic OH content is quantified by extrapolation to zero time of the right-hand part of the curve representing the slow aminolysis of the aliphatic esters (Fig. 9) (4, 5).

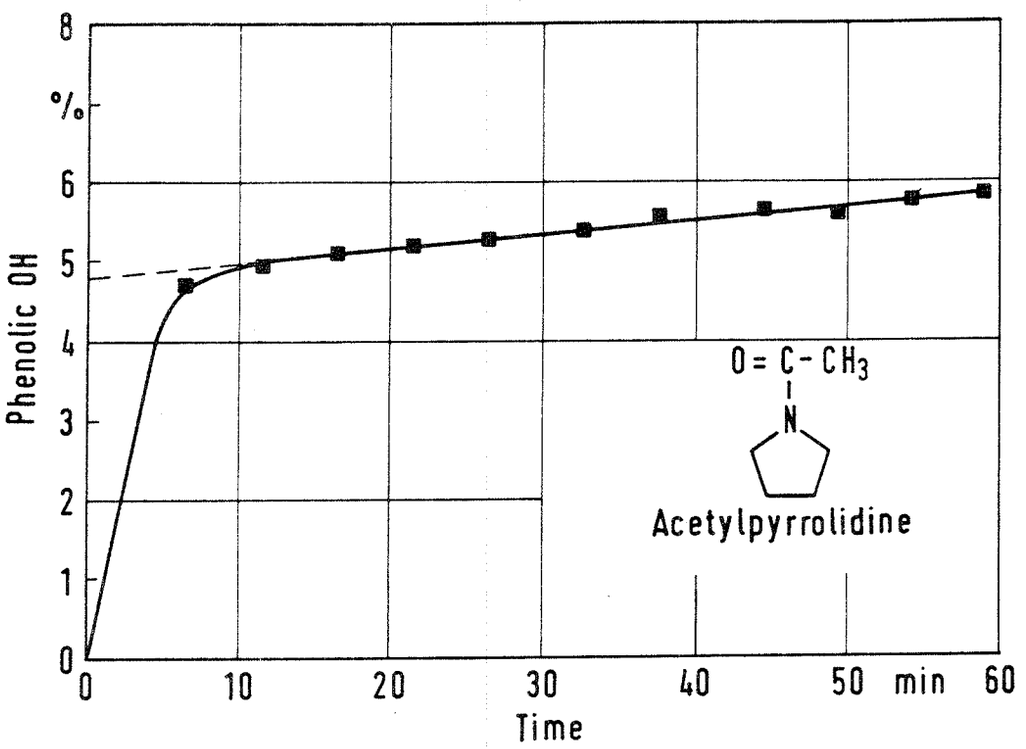

Figure 9: Course of aminolysis of phenolic and aliphatic ester groups

RESULTS AND DISCUSSION

From our experience aminolysis is a very accurate method. Nevertheless it proved to be

- time-consuming
- laborious
- dependent on very stable GC conditions. In routine analysis involving a great number of samples problems occur with column loading
- requires great laboratory skill and experience.

By contrast the spectroscopic method is an easy procedure and much more independent of experience and skill of the person performing the analysis.

The correlation between the results obtained by means of aminolysis and those from IR spectrocopy proved excellent. For a series of lignin fractions with phenolic OH contents between 3.5 and 6 % or 0.37 - 0.64 phenolic OH groups per C_9 the correlation coefficient R^2 was found to be 0.94 and 0.89 (Fig. 10).

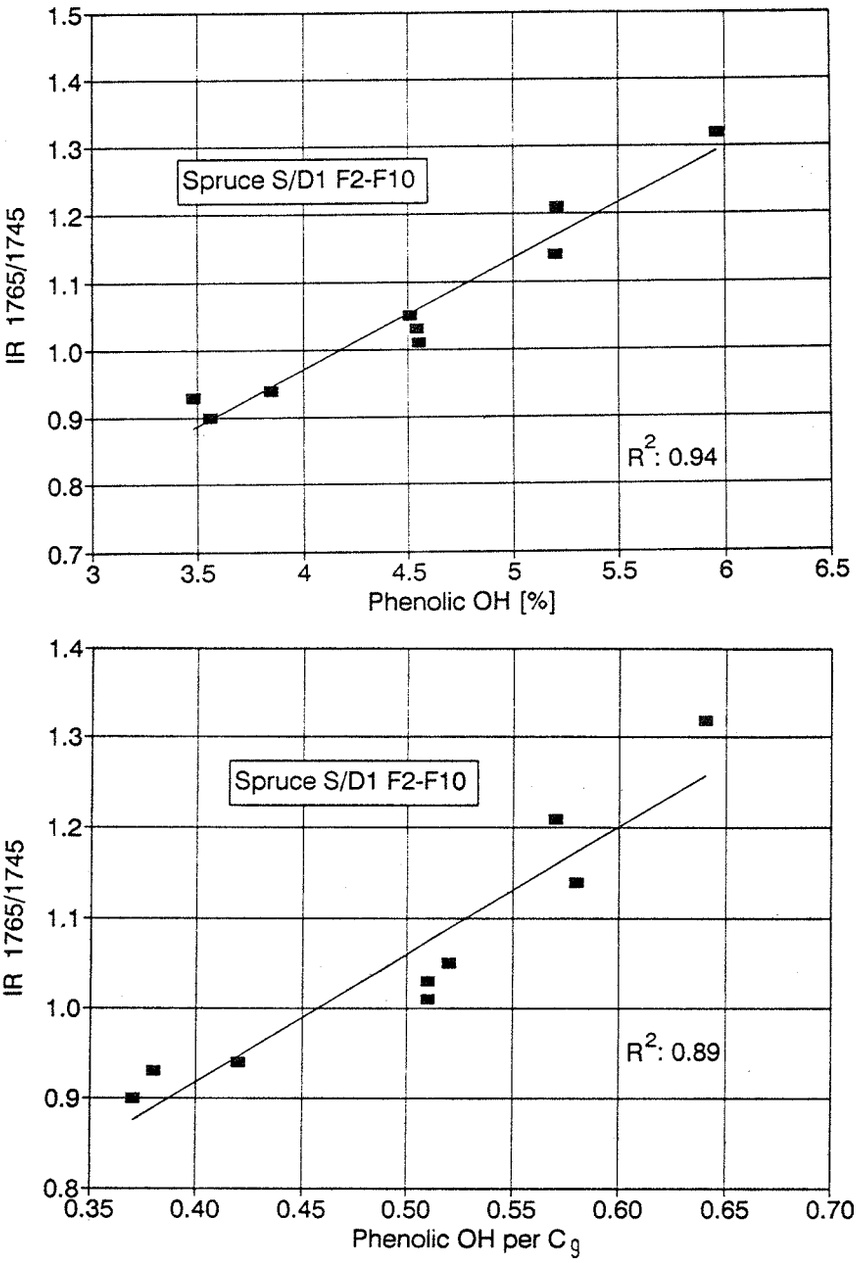

Figure 10: IR index versus phenolic OH content (%) (above) and phenolic OH/C_9 (below) obtained by aminolysis.

For fractions from lignin S/D3 R^2 is 0.96 (Fig. 11).

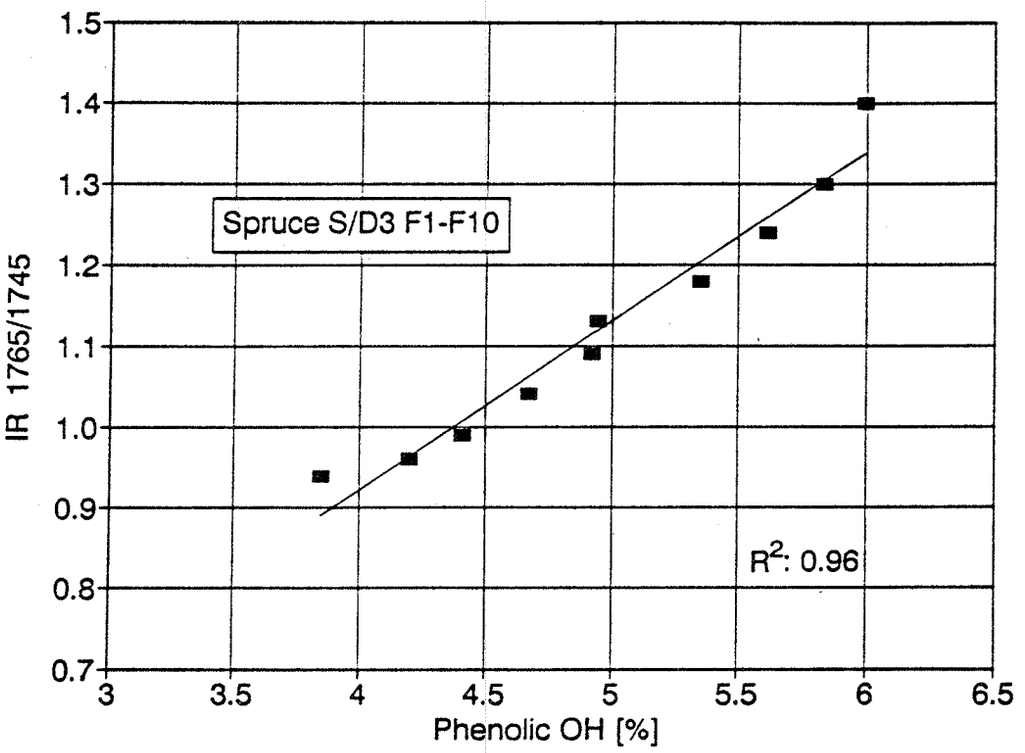

Figure 11: IR index versus phenolic OH content (%) of fractions from lignin S/D3

Plotting the results from 29 different lignins with a phenolic OH range between 1.7 % and 6.6 % a high significance of R^2 = 0.96 was found (Fig. 12).

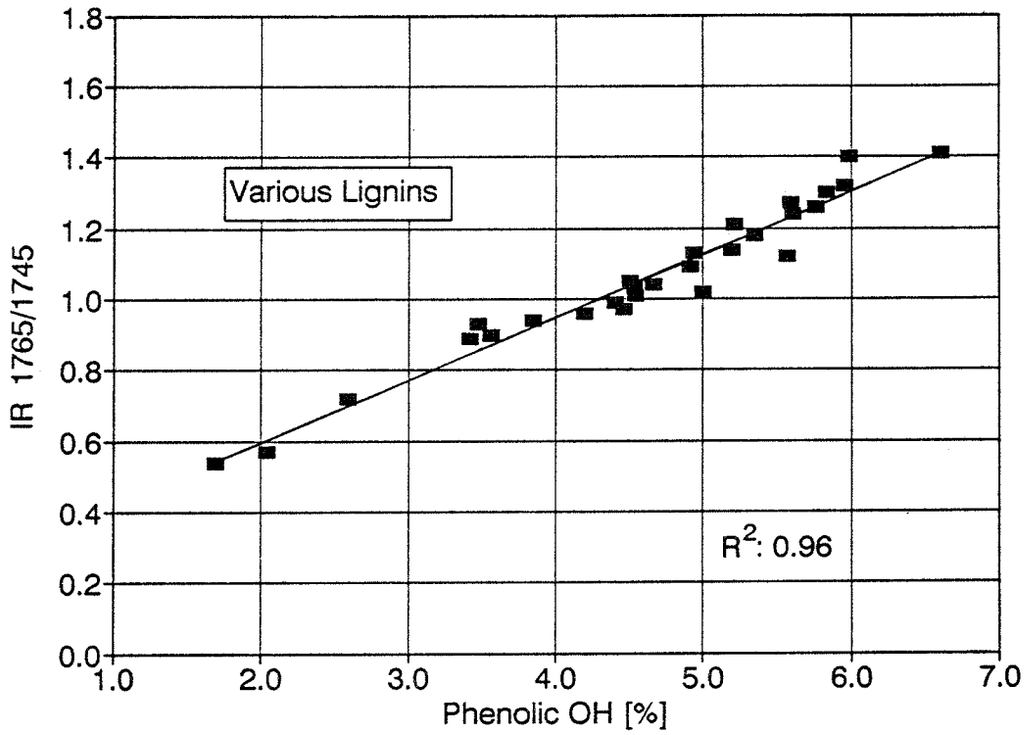

Figure 12: IR index versus phenolic OH content (%) of all investigated lignins

The phenolic OH content of a lignin sample can be calculated from the following equation

Phenolic OH [%] = 5.66 IR index (1765/1745) - 1.37.

The present results demonstrate that FTIR spectroscopy can be used for a quick, easy and reliable quantitative determination of phenolic OH groups in lignins.

Further experiments will include the spectroscopic evaluation of the total hydroxyl groups.

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ORGANOSOLV LIGNINS FROM *PINUS CARIBAEA HONDURENSIS*

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ABSTRACT

The organosolv extractions of lignin from *Pinus caribaea hondurensis* were studied using different organic solvent mixtures, temperatures, extraction times and catalyst concentrations, in order to understand the effect of these parameters on the chemical and macromolecular lignin characteristics. The results indicate the occurrence of topochemical effects in these extractions and that the lignins yields are related to the Hildebrand's solubility parameter of the used solvent mixture.

INTRODUCTION:

The delignification of lignocellulosic materials by chemical pulping processes are based on the solubilization of the lignin fragments generated by depolymerization of the protolignin ("in situ" lignin). The choice of the solvent to solubilize these lignin fragments is related to the chemicals employed in the processes.

The industrial chemical pulping processes nowadays in operation (Kraft and Sulfite) using water as solvent need to produce ionic lignin fragments either by ionization of the phenolic hydroxyl groups by strong bases or introducing sulfonic groups through sulfonation reactions. In both of these processes the reagents are also responsible for the cleavage of specific linkages of

the protolignin by nucleophilic displacements mainly of the ether bonds (1).

In organosolv processes the organic solvents solubilize the lignin fragments without derivatization or ionization due their own capability to solubilize organic molecules. These processes are generally performed in presence of different quantities of water and the depolymerization of the protolignin occurs by hydrolytic and in some cases, also by solvolitic reactions. The use of an acid catalyst is generally necessary in order to activate the reactive centers to compensate the use of weak nucleophiles (water or the organic solvent) (2).

There are also mixed processes based on the use of inorganic reagents and aqueous/organic solvent mixtures to get an improvement of the lignin solubilization (3).

In all these chemical pulping processes, besides the nucleophilicity and the solubility, the accessibility of the reagents through the polysaccharide matrix to the reactive centers of the protolignin plays an important role, due the fact that these processes are performed in heterogeneous media.

The organosolv processes are feasible alternatives to complement worldwide the pulping and paper production. They are very versatile, produce a low pollutant discharge and permit the recovery of the lignins from the liquors in the solvent recycling step (4). Another important advantage is the feasibility to install economically small integrated mills (5).

The most important parameters in the organosolv pulping processes are the temperature, reaction time, catalyst (and its concentration) and the solvent mixture employed.

In this work the influence of these parameters on the yields and characteristics of the *Pinus caribaea*

hondurensis lignins extracted by organosolv processes is discussed. The extractions were performed at 125°C with nine different organic solvents and 0.2 N hydrogen chloride as catalyst. Dioxan lignins were also obtained using dioxan/water 9:1 solutions at reflux temperature with different catalyst concentrations and reaction times. These experiments were proposed for the study of the characteristics of the isolated lignins as function of the experimental conditions of the organosolv process, as well as to find a guide for the choice of the better organic solvent mixture for the delignification of the lignocellulosic materials.

EXPERIMENTAL

Pinus caribaea hondurensis sawdust (30.0% Klason lignin - Tappi T13m - 54) was used as raw material. The lignins were obtained using three different experimental conditions:

1-At 125°C using organic solvent/aqueous 2.0 N HCl (9:1) solution for six hours. The solvents employed in this extractions were the following: chloroform, acetone, 1,4-dioxan, tetrahydrofuran (THF), 2-butanol, 1-butanol, 1-propanol, ethanol and methanol (6).

2-At boiling temperature using dioxan/aqueous 1.0 N HCl (9:1) solution at different extraction times (0.5; 1.0; 2.0; 3.0 and 5.0 hours) (7).

3-At boiling temperature using dioxan/aqueous HCl (9:1) solution for one hour period. The hydrogen chloride solution was prepared in order to obtain final concentrations of 0.1, 0.2, 0.5, 1.0, 1.2 N and also without HCl (8).

The obtained lignins were characterized by chemical analysis (elemental analysis, methoxyl and

phenolic hydroxyl contents) and by High Performance-Size Exclusion Chromatography (6,7,8).

RESULTS AND DISCUSSION

Elemental analysis and the methoxyl contents were used to calculate the C_9 - formula which are shown in tables I, II and III.

All the obtained samples using different solvents at 125°C contain approximately the same methoxyl content, except for the lignin extracted with methanol, with higher methoxyl content indicating the reaction of this solvent with lignin during the extraction process (table I).

Table I - C-9 formula for the lignins obtained using different organic solvents/aqueous 2.0 HCl (9:1) solutions.

SOLVENT	C	H	O	OCH_3
chloroform	9	7.97	2.81	0.70
acetone	9	7.61	2.49	0.69
1,4-dioxan	9	8.09	3.03	0.80
THF	9	8.68	2.83	0.78
2-butanol	9	8.85	2.53	0.78
1-butanol	9	9.32	2.56	0.68
1-propanol	9	8.60	2.77	0.74
ethanol	9	8.24	2.73	0.81
methanol	9	7.74	2.61	1.29

The dioxan lignins extracted with high catalyst concentration (1.0 and 1.2 N HCl) and at 125°C under pressure show a methoxyl content 10 % lower than the lignins obtained under milder conditions. This low methoxyl content can be explained assuming that in these

conditions demethylation took place or that these lignin originated, at least in part, from different regions of the plant tissues (9).

The obtained methoxyl contents using 0.1, 0.2 , 0.5 N (one hour extraction time) and with different extraction times (0.1 N HCl) correspond approximately to the expected values for a softwood lignin (10).

Table II - C-9 formula for the lignins obtained using dioxan/aqueous 1.0 N HCl (9:1) solution at different extraction times.

Extraction Time (hours)	C	H	O	OCH_3
0.5	9	8.67	3.07	0.87
1.0	9	8.57	3.23	0.88
2.0	9	8.46	3.03	0.86
3.0	9	8.07	3.12	0.89
5.0	9	8.06	3.01	0.88

Table III - C-9 formula for the lignins obtained using dioxan/aqueous HCl (9:1) solutions for one hour period and different HCl concentrations.

HCl conc (N)	C	H	O	OCH_3
0.0	9	9.28	3.12	0.68
0.1	9	8.57	3.23	0.88
0.2	9	7.90	3.06	0.89
0.5	9	8.29	3.04	0.89
1.0	9	7.44	3.09	0.80
1.2	9	7.78	3.15	0.80

The phenolic hydroxyl content of the extracted

samples at 125°C are in the range of 3,74% to 6.09%, the lower value corresponding to the lignin extracted in methanol, which confirm the methylation of the phenolic hydroxyl oxygen. The dioxan lignin obtained under these conditions showed a phenolic hydroxyl content of 5.5 %, which is higher than the one obtained at boiling temperature (4.3 - 4.8%), as consequence of α-O-4 and β-O-4 bond cleavage without condensation of the liberated lignin fragments liberated. In both cases these values indicate approximately one phenolic hydroxyl group per two C_9 units.

Molecular weights.

The lignins extracted using different solvents, at 125°C and 0.2 N HCl can be classified in three major groups, considering the most abundant molecular weight fraction and the shoulders present in the chromatograms. This classification can be also obtained from the average molecular weights of the different lignins (11). The dioxan lignin obtained under these conditions belongs to the group which present the higher average molecular weight and a shoulder at molecular weight of 5730 g/mol.

The dioxan lignins obtained using different extraction times (table IV) have approximately the same molecular weight pattern. This result does not agree with those from published works (12, 13, 14) showing an increase in the average molecular weights with the increase of the extraction time.

The obtained chromatograms from the extracted dioxan lignins using different catalyst concentrations show a progressive increase in the high molecular weight region for the samples isolated under more acidic conditions.

Table IV: Average molecular weights of the obtained lignins.

Extraction Time (h)	$\bar{M}n$	$\bar{M}w$	Disp.
0.5	1440	3990	2.77
1.0	1200	3160	2.62
2.0	1260	3400	2.70
3.0	1390	3730	2.68
5.0	1270	3200	2.52

As the high molecular weight fractions (M_i>70,000) correspond at most to 9 %, these fractions do not affect the number-average molecular weight ($M\bar{n}$) in the same extension as they do with the weight-average molecular weight ($M\bar{w}$). The average molecular weights and the corresponding polydispersity were calculated from the chromatograms (not including the high molecular weight fractions) and are shown in table V.

Table V. Average molecular weight ($M\bar{n}$ and $M\bar{w}$) and polydispersity of the lignins extracted.

HCl conc. (N)	$\bar{M}n$	$\bar{M}w$	Disp.
0.0	1110	3500	3.15
0.1	1200	3160	2.62
0.2	1230	3730	3.03
0.5	1140	3650	3.21
1.0	1000	3680	3.68
1.2	920	3690	4.02

The results of the three sets of the performed experiments indicate again that the lignins obtained at higher temperature under pressure (for six hours) are similar to the lignins extracted using higher catalyst concentration and one hour extraction time. As these lignins were extracted in higher yields, the higher average molecular weights observed are consequence of a topochemical effect (the lignins extracted after a certain yield value have higher molecular weights) and/or condensations reactions (more effective with higher temperature, extraction time and concentration catalyst).

Extraction Yields.

The most interesting solvent effect was shown by the yields obtained in the extractions in the different solvents.

The different yields are consequence of three intrinsic solvent factors: (1) the solvent accessibility (and that from the reagents dissolved in it) to the protolignin; (2) the lignin fragments solubility in the extraction solvent; and (3) the efficiency of the reagent to cleave the linkages in the protolignin (reagent nucleophilicity).

Considering that all the systems have the same intrinsic capacity (nucleophilicity) to promote delignification, and since the solubility and accessibility can be expressed by the Hildebrand's solubility parameters, the obtained lignin yields should be directly related to this parameter. The obtained "Bell Shape" curve (plotting the lignin yields versus the Hildebrand's solubility parameters of solvent mixtures) (11) confirms this assumption and shows clearly that this parameter can be used as a guide for the choice of the solvent system employed in the delignification process. The maximum of

the "Bell Shape" curve corresponds to the value of the lignin solubility parameter (23 $MPa^{1/2}$) which is in very good agreement with the value reported by Schuerch (15), as determined from solubility tests of isolated lignins.

The lignin yields obtained in the extractions with dioxan at boiling temperatures are shown in tables VI and VII.

Table VI: Lignin and residual wood yields.

Extraction Time (hours)	Lignin Yield		residual wood yield (%)
	% wood	% Klason	
0.5	3.8	12.7	83.3
1.0	4.7	15.7	80.6
2.0	6.1	20.4	70.6
3.0	7.6	25.4	69.9
5.0	9.4	31.4	65.1

The low lignin yield (31.4% based on Klason lignin) obtained after 5 hours of reaction indicates that the HCl concentration (0,1N) was insufficient to promote pronounced delignification. Pla and Robert (16) obtained similar results in the delignification of Spruce using dioxan/water (10:1) at 70°C. These low yields can be related to topochemical effects as the lignin content in the middle lamella of softwood species (30% based on klason lignin) is in the same order as the yield obtained after 5 hours of extraction. This result is in agreement with the studies of Paszner and Behera (2) using organosolv process, showing that delignification occurs preferentially in the middle lamella during the first stage of the pulping process.

The variation of the catalyst concentration

showed a pronounced effect on the lignin yields (table VII), which grow asymptotically to 80 - 85% with 1.2 N HCl. Other studies (14, 17) also confirm that higher HCl concentration is necessary to increase the delignification.

Table VII. Lignin yields and residual wood obtained for the extractions using different HCl concentrations.

HCl conc. (N)	lignin yield		residual wood (%)
	% wood	% Klason	
0.0	0.4	1.3	99.4
0.1	4.7	15.7	80.6
0.2	7.8	26.1	71.0
0.5	14.7	49.2	57.6
1.0	21.4	71.6	48.7
1.2	23.5	78.6	42.8

The maximum lignin yield for extractions with dioxan/water (9:1) solutions were obtained at 125°C for six hours using 0.2 N HCl and at boiling temperature for one hour using 1.2 N HCl. The analysis of the residual wood yields (table VI and VII) and the molecular weight distributions of the lignins show that the use of higher HCl concentration must be avoided to prevent cellulose losses and lignin condensation.

CONCLUSIONS

The chemical analysis revealed that methoxyl incorporation occurred with the use of methanol as solvent and demethylation using higher HCl concentrations.

The average molecular weights and the molecular weight distributions indicates the occurrence of lignin condensation when the extractions were performed at higher

catalyst concentrations. The different solvents mixtures employed confirm the selectivity of the solvents in the organosolv extractions.

The main solvent effect occurred on the obtained yields. These yields are related to the Hildebrand's solubility parameters of the employed solvents.

The lignin yields show that in the range of temperatures studied the best HCl concentration is about 0.2 N and the increase of the temperature and extraction times must be preferred instead of the increase of the catalyst concentration.

The degree of delignification as well as the analytical results suggest that in the organosolv process the topochemical effect is also operating and that in the first stage of these processes the lignin is removed from the middle lamella region.

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LABORATORY AND PILOT PLANT SCALE ASAM-PULPING OF SOFT- AND HARDWOODS AND CHLORINE FREE BLEACHING OF THE RESULTING PULPS.

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The conventional pulping processes were invented more than hundred years ago and their fundamental parameters remained unchanged until now. In particular, today's environmental requirements are calling for new pulping processes. The drawbacks of the conventional pulping processes can be summarized as follows:

Table 1: Deficiencies of the conventional pulping processes

SULFITE	KRAFT
- limited raw material basis	- high investment cost
- high requirements on raw material quality	- small process flexibility
- low pulping yields for hardwoods	- low pulping yields for softwoods
- inferior pulp quality	- poor bleachability of the pulp and high bleaching chemicals demand
- air pollution by sulfur dioxide emission	- indispensible use of chlorine containing bleaching agents
	- high effluent charge
	- offensive smell of volatile reduced sulfur compounds

A main disadvantage of the sulfite process is the limited raw material basis. The heartwood of pine, Douglas fir and larch cannot be pulped by conventional sulfite processes due to specific phenolic components which react with lignin and surpress lignin dissolutions. So sulfite pulping is limited to few wood species.
The sulfite processes are further very sensitive to raw material quality. Mechanically damaged or biologically decayed wood chips have a dramatic influence on pulp yield and strength properties. In general, the low strength

potential of sulfite pulps compared to kraft pulps limits its utilization for high strength paper products.

The major drawback of kraft pulping is the environmental impact of this process. Today, the odour originated from reduced sulfur compounds can be reduced to an extremely low level, but bleaching of kraft pulps is still highly polluting. The lignin content of the pulps is much higher than that attainable by sulfite pulping and the reactivity of this residual lignin is low. So high chemical charges are required. Moreover, kraft pulps cannot be bleached to high brightness in completely chlorine free bleaching sequences.

It is quite obvious that improved pulping processes are needed. Such a process should meet the following requirements.

Table 2:

Requirements on new pulping processes
• Suitability for a wide range of different raw material, such as softwoods and hardwoods (including certain bark contents), as well as annual plants
• Production of different pulp grades, like paper and dissolving pulps
• Pulps qualities at least as good as kraft
• Good pulp yields, low residual lignin content, and easy bleachability without use of chlorine containing compounds
• No odour problems, low specific water consumption
• High chemical recovery rate
• Availability of reliable, proven technology

The most important prerequisites for the installation of new pulping process are the applicability of all kinds of fibrous raw material suited for pulping, the production of high quality pulp in an environmentally friendly way and the availability of well-known standard technologies. Many developments in this field did not take into consideration that new pulping processes which are based on new technologies and new equipments increase the economical risk of such an installation.

General parameters and results of ASAM pulping

Alkaline sulfite pulping with addition of anthraquinone and methanol (ASAM) is an interesting alternative to the conventional pulping processes. This process uses conventional pulping chemicals such as sodium sulfite, sodium hydroxide and/or sodium carbonate. Catalytically small amounts of anthraquinone have to be added and the liquor contains 10-20 Vol% methanol. The cooking conditions can be regarded as conventional, but due to the presence of methanol in the cooking liquor maximum pressure ranges from 12 - 14 bar. If existing digesters do not allow such a high pressure, ASAM cooking can be modified in such a way that maximum pressure does not exceed 10 bar. As indicated in Table 3, cooking conditions for hardwoods and softwoods are very similar. For hardwoods a somewhat higher methanol charge is beneficial and cooking time can be reduced by half an hour.

Table 4 shows pilot plant pulping results of common European species as average of a brought set of experiments. Compared to kraft pulping, delignification in ASAM pulping can be extended without seriously affecting total yield and strength properties of the pulp. Standard kappa number in ASAM softwood pulping is around 20 whereas hardwood pulping can be extended to kappa number 10. Brightnesses of the resulting pulps are much higher than in kraft pulping. This together with the lower residual lignin content indicates the easy bleachability of these pulps. Total yield in ASAM pulping is superior to kraft, in particular for softwoods. The reject content, however, is fairly high and therefore ASAM pulping requires homogeneous chip size and thorough impregnation.

Table 3: Standard conditions of ASAM cooking of softwoods and hardwoods

	Softwoods Spruce/Pine	Hardwoods Beech/Birch
Total chemicals*	24	24
Na_2SO_3*	17	17
NaOH*	3	3
Na_2CO_3*	4	4
AQ (% on o.d. wood)	0.075-0.1	0.075-0.1
MeOH (% v/v)	10	15
Liquor to wood ratio	4:1	4:1
Steaming (min)	30	30
max. Temperature (°C)	180	180
Heating time (min)	60	60
Cooking time (min)	150-180	120-150

*calculated as NaOH (% on o.d. wood)

Table 4: Pilot plant pulping results

Wood species	Kappa no.	Total yield (%)	Rejects (%)	Bright-ness (% ISO)	Tensile* strength (km)	Tear* index (mNm^2/g)
Spruce	17.2	50.7	1.7	46.1	11.8	16.0
Pine	22.6	49.5	2.0	44.7	11.4	15.2
Birch	9.6	53.8	1.8	56.8	9.8	12.1
Beech	11.6	50.7	2.6	53.7	8.7	13.0

*Strength properties at 20°SR

One of the major advantages of ASAM pulping are the excellent strength properties of the pulps. Figure 1 shows the strength factor of ASAM and kraft pulps from different wood species. In all cases ASAM is superior to kraft. In particular, tensile strength is much higher due to the higher hemicellulose content and viscosity of the pulp. Tear strength is more or less on the same level.

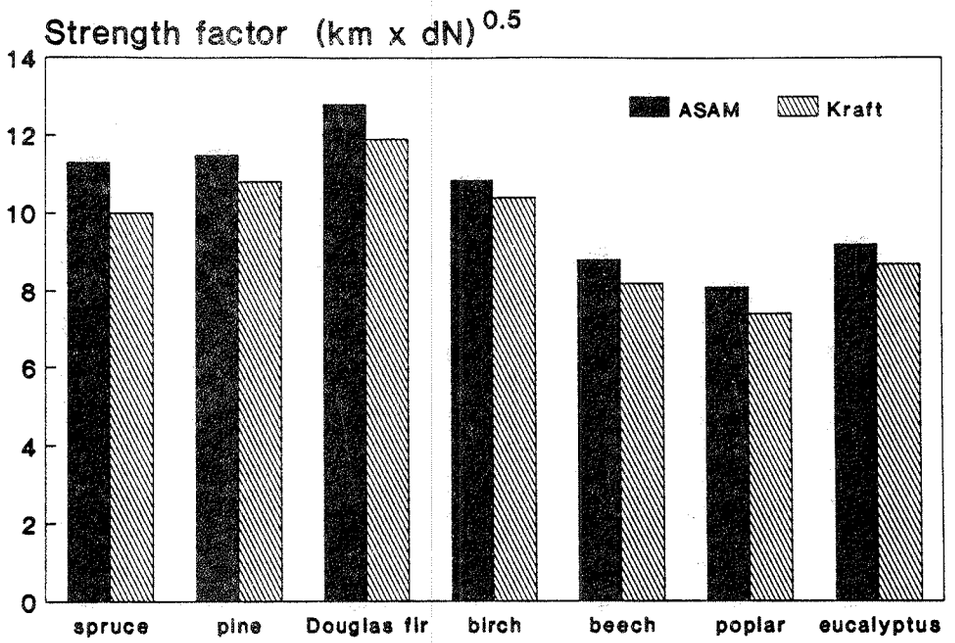

Fig. 1: Strength of ASAM pulps versus kraft pulps of different wood species

ASAM pulping and bleaching of eucalyptus

We conducted laboratory as well as pilot plant scale ASAM pulping and bleaching trials using Eucalyptus from Spain and Brasil. Standard cooking conditions were employed but the ratio of the inorganic cooking chemicals was varied (Table 5). By varying the kind of supporting alkali different cooking results can be attained. Sodium carbonate benefits the pulp yield by protection of the hemicelluloses but delignification velocity is reduced. The resulting pulps are brighter and have improved tensile strength but reduced tear resistence. If sodium hydroxide is applied as additional alkali source more hemicelluloses are removed. So the pulp yield is lowered and the kappa number and pulp brightness are lower as well. The pulps have better tear resistance but the tensile strength is somewhat lower.

Table 5: Effect of alkali ratio on ASAM pulping of eucalyptus

Process	Alkali ratio (Na_2CO_3/total alkali)	Yield (% on wood) total	screened	rejects	Kappa no.	Brightness % /ISO
ASAM I (with NaOH)	0.85	56.7	54.6	2.1	16.6	56.6
	0.8	56.2	52.9	3.3	16.0	47.9
	0.7	56.2	52.9	3.3	14.7	38.6
ASAM II (with Na_2SO_3)	0.85	55.8	53.0	2.8	14.0	48.2
	0.8	54.2	51.7	2.5	11.9	43.3
	0.7	54.0	51.4	2.6	10.9	36.9

Constant cooking conditions: chemical charge: 25 % on o.d. wood, calc. as NaOH, AQ dose: 0.1% on o.d.wood; methanol: 25% by vol. of the total liquor; liquor to wood ratio = 4:1; cooking temperature: 180°C; cooking time at max.temp.: 135 min.

Figure 2 shows the total yield to kappa number plot of different ASAM and kraft cookings. The results clearly show that the ASAM process is able to delignify eucalyptus to low kappa numbers while preserving pulp yield. Depending on the pulping conditions ASAM pulping reduces the kappa number to 10-13, whereas the kappa number of corresponding kraft pulp is 16. In all cases pulp yield is on the same level.

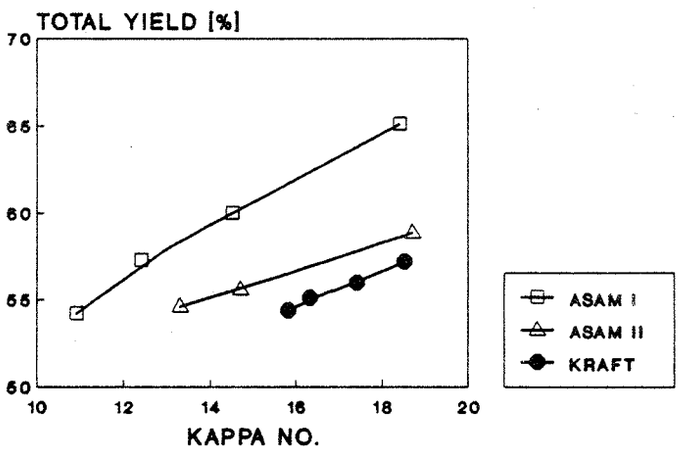

Fig. 2: Yield to kappa number relationship of ASAM and kraft eucalyptus pulps

Bleaching of eucalyptus ASAM and kraft pulps was conducted by using conventional as well as chlorine free sequences. Table 6 shows chemical charges and results of chlorine free ASAM and kraft pulp bleaching.

Table 6: Results of chlorine free bleaching of Eucalyptus pulps

Pulp	Sequence	Chemicals %/o.d.pulp	Kappa no.	Brightness % ISO	Yield %
ASAM kappa 12.7 40.3 % ISO yield 55.6 %	Z	1.0 O_3	3.5	70.0	97.0
	E	1.0 NaOH	2.2	-	95.4
	P	1.0 NaOH 2.0 H_2O_2	-	90.0	95.2
	O	2.0 NaOH	7.8	-	97.6
	Z	0.6 O_3	1.7	79.0	96.6
	P	0.5 NaOH 1.0 H_2O_2	-	87.3	94.5
Kraft kappa 14.6 40.3 % ISO yield 56,2	Z	1.5 O_3	3.7	70.4	96.8
	E	1.0 NaOH	1.4	-	94.9
	P	1.0 NaOH 2.0 H_2O_2	-	90.6	93.8
	O	2.0 NaOH	8.2	-	97.4
	Z	0.7 O_3	1.8	78.6	95.5
	P	0.75 NaOH 1.0 H_2O_2	-	88.4	93.8

For both pulps ZEP and OZP were applied as chlorine free bleaching sequences. In all cases high brightness could be obtained with acceptably low chemical charges. Figures 3 - 7 reveal that ASAM pulps are more suited to chlorine free bleaching. Figure 3 shows that the tensile strength of conventionally and chlorine free bleached ASAM pulp is similar. Maximum tensile strength is about 9 km. The less specific reaction of chlorine free bleaching agents, however, results in a lower tear strength as can be seen in Figure 4. Especially, the application of higher ozone charges in the ZEP sequence reduces the tear resistance, nevertheless the overall strength of both bleached ASAM pulps is very good. The differences between conventionally and chlorine free bleached kraft pulps are more pronounced.

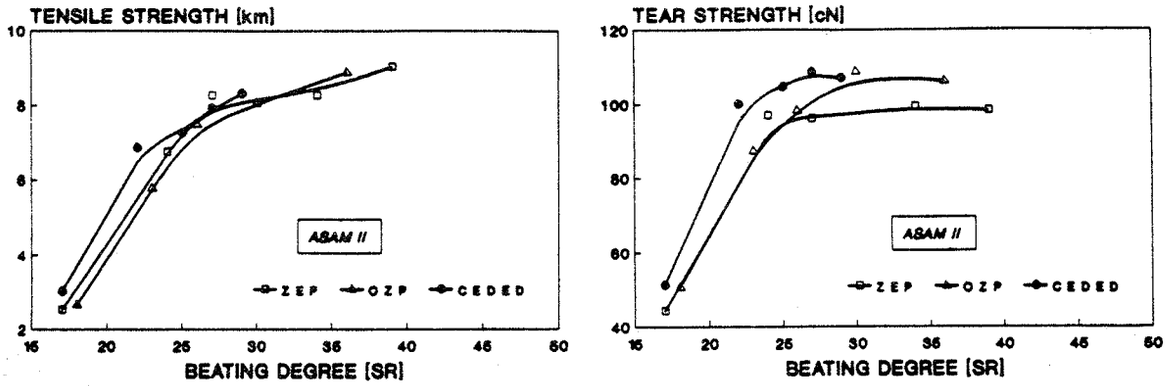

Fig. 3/4: Tensile strength and tear strength of bleached ASAM eucalyptus pulp

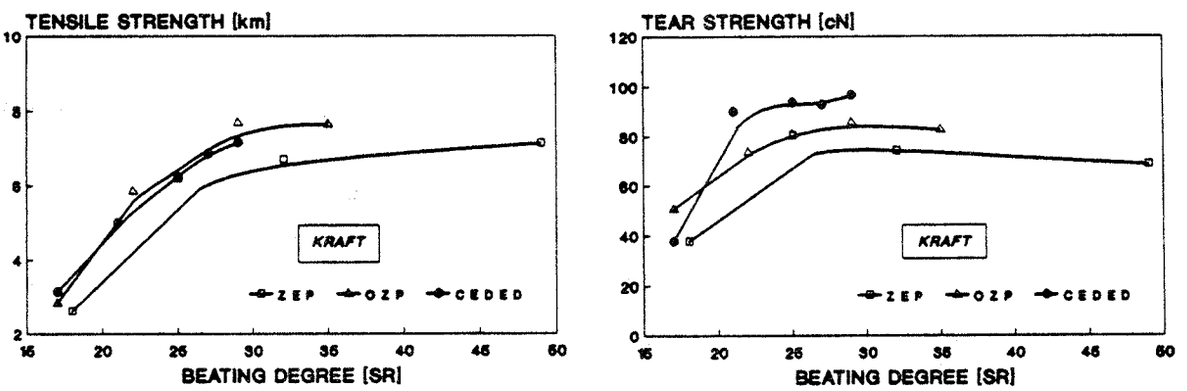

Fig. 5/6: Tensile strength and tear strength of bleached kraft eucalyptus pulp

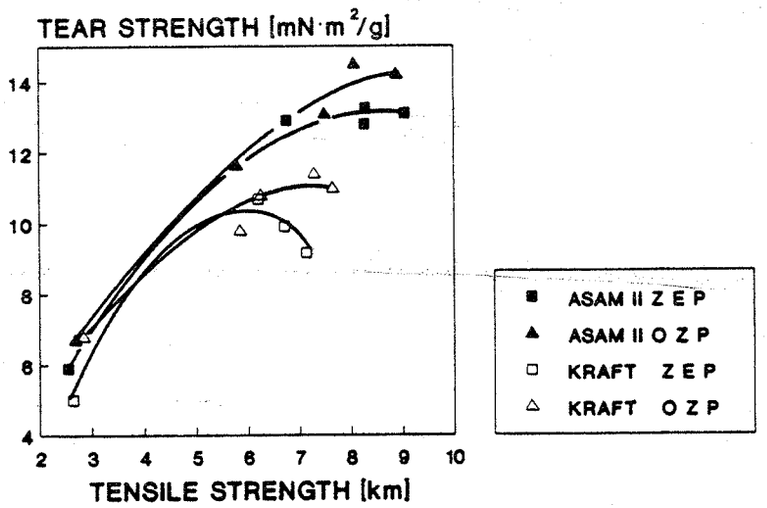

Fig 7: Comparison of chlorine free bleached ASAM and kraft eucalyptus pulps

Figures 5 and 6 indicate that a distinct strength loss has to be accepted in chlorine free bleaching of the kraft pulp. In particular, the high ozone charges in ZEP bleaching reduces the pulp strength. A strength loss between 10 - 20% seems to be inevitable.
Comparing the strength properties of chlorine free bleached ASAM and kraft pulps, it becomes evident that ASAM pulps are superior to kraft pulps (Fig. 7).

ASAM pulping and bleaching of Douglas fir

The potential of ASAM pulping can also be demonstrated by comparing ASAM and kraft pulping of Douglas fir and bleaching of these pulps. Douglas fir is a softwood species which is difficult to pulp. Table 7 summarizes the main conditions and results of ASAM and kraft pulping and bleaching of Douglas fir.
ASAM pulping requires a longer cooking time but the lignin content of the resulting pulp is lower compared to kraft. Even at lower kappa number ASAM pulp yield is higher. Due to the high content of extractives in Douglas fir heartwood the brightness of the unbleached kraft cook is low. In ASAM cooking, the extractives do not influence pulp brightness.
The different response of ASAM and kraft pulps bleaching is reflected by the active chlorine charge required in conventional bleaching of these pulps. Due to the lower residual lignin content and the higher reactivity of this lignin ASAM pulp requires only about 50% of the chlorine charge of a kraft pulp. Also bleaching yield favours ASAM pulping. Tear index of Douglas fir ASAM and kraft pulp at 20° SR is similar, but tensile strength of the ASAM pulp is about 20% over the kraft level. Moreover, completely chlorine free bleaching in a short OZP_N sequence (N = nitrilamine) to high brightness is possible. Compared to conventional bleaching tear index of the bleached pulp is reduced by 20% but the tensile strength is more or less the same. A comparison of a chlorine free bleached ASAM and a conventionally bleached kraft pulp shows that the strength potential of both pulps is adequate. The strength properties of unbleached, conventionally bleached and chlorine free bleached ASAM and kraft Douglas fir pulps are shown in Figures 9 - 17.

Table 7: Results of ASAM and kraft cooking of Douglas fir and chlorine free bleaching

Cooking	**ASAM**	**Kraft**
Cooking time at T_{max} , min	160	85
Kappa No.	22.1	30.2
Screened yield, %	47.0	46.3
Brightness, % ISO	47.8	21.3
Bleaching, C_DEDED		
Active chlorine, %	5.5	10.0
NaOH, %	3.0	3.5
Bleaching Yield, %	94.9	93.7
Yield, % on o.d. wood	44.6	43.3
Brightness, % ISO	90.1	89.4
Tear Index, 25 °SR	21.7	21.7
Tensile Index, 25 °SR	99.8	84.9
Beaching, OZP_N		
Bleaching Yield, %	94.7	-
Yield, % on o.d.wood	44.5	-
Brightness, % ISO	87.3	-
Tear Index, 25 °SR	17.6	-
Tensile Index, 25 °SR	95.7	-

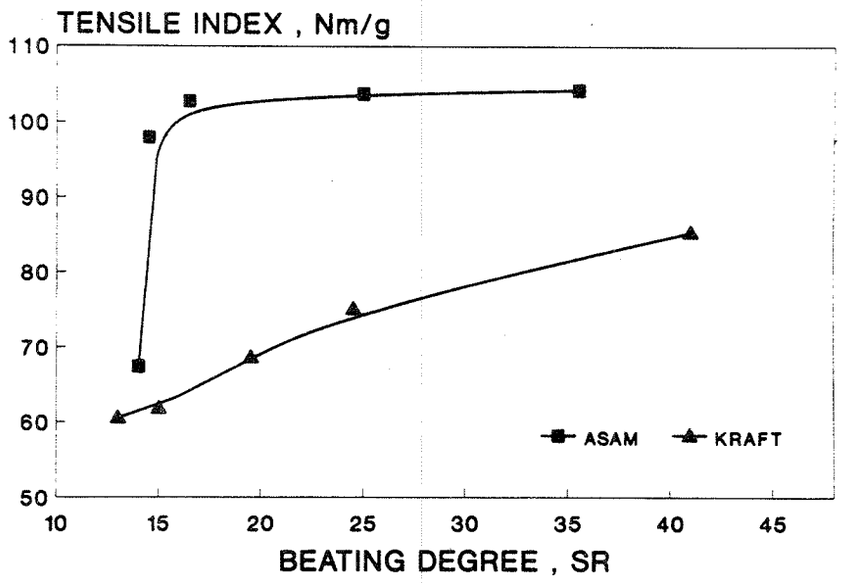

Fig. 8: Tensile strength of unbleached ASAM and kraft Douglas fir pulps

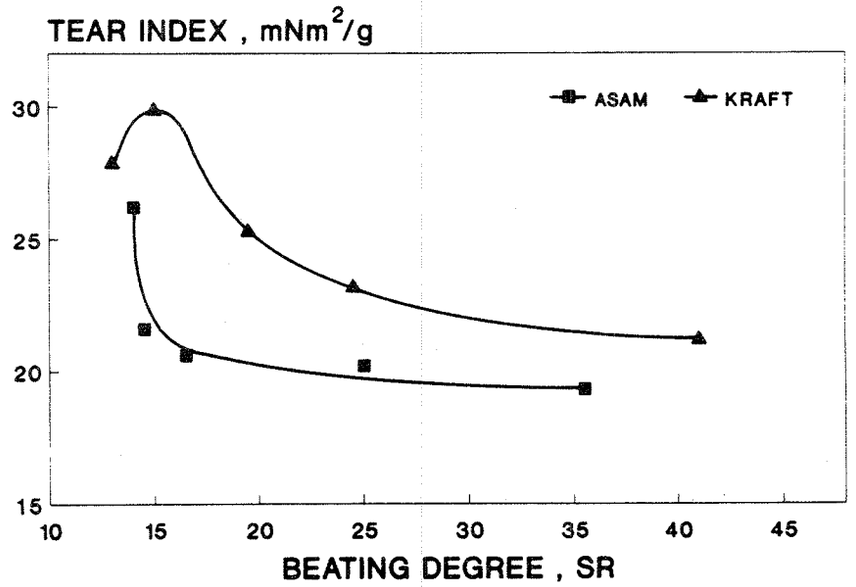

Fig. 9: Tear strength of unbleached ASAM and kraft Douglas fir pulps

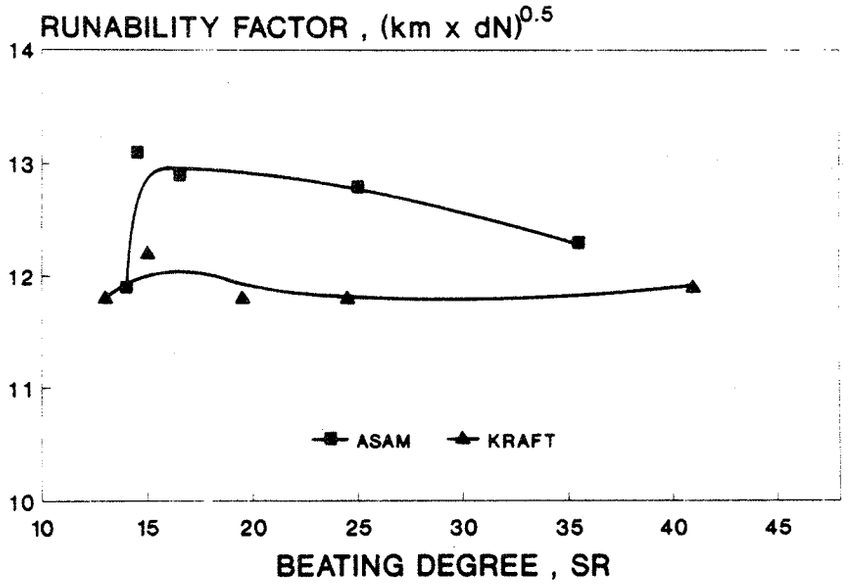

Fig 10: Runability factor of the unbleached Douglas fir pulps

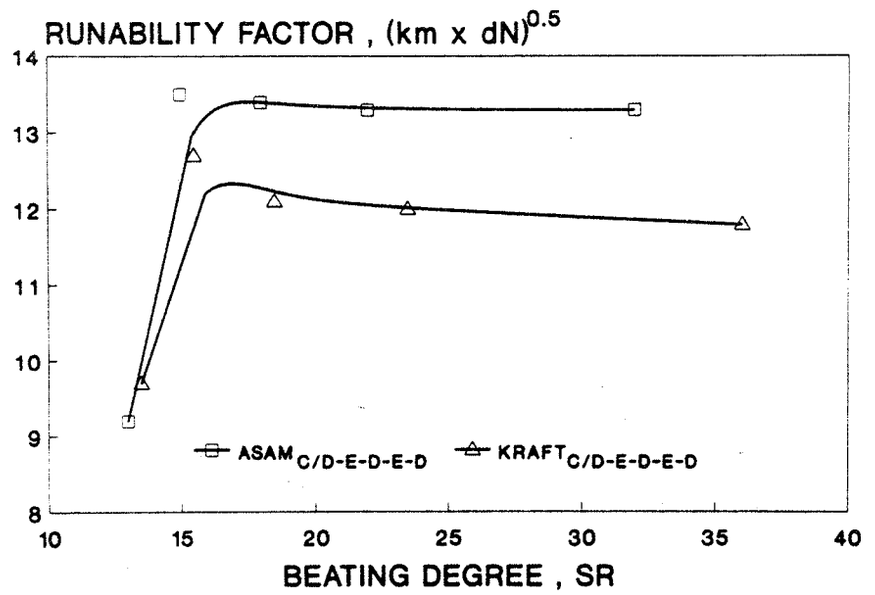

Fig. 11: Tensile strength of conventionally bleached Douglas fir pulps

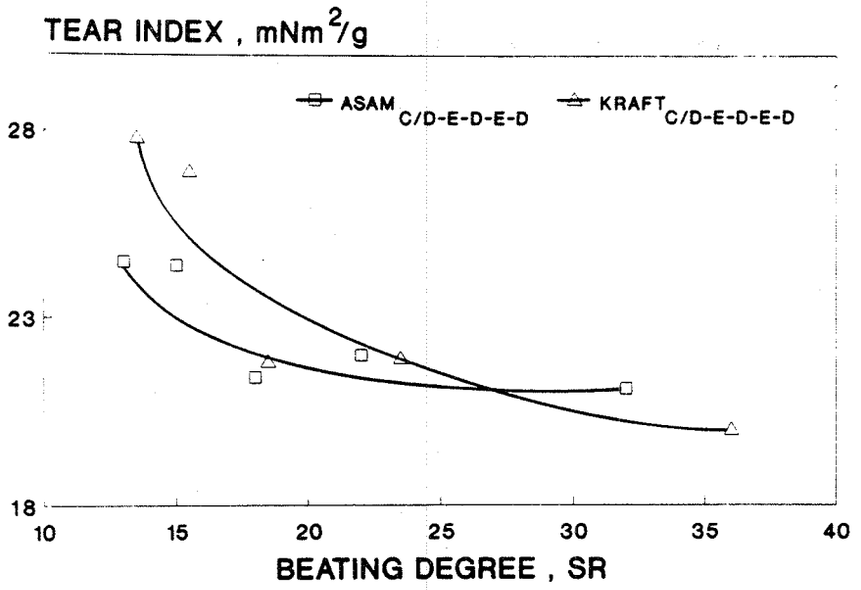

Fig. 12: Tear strength of conventionally bleached Douglas fir pulps

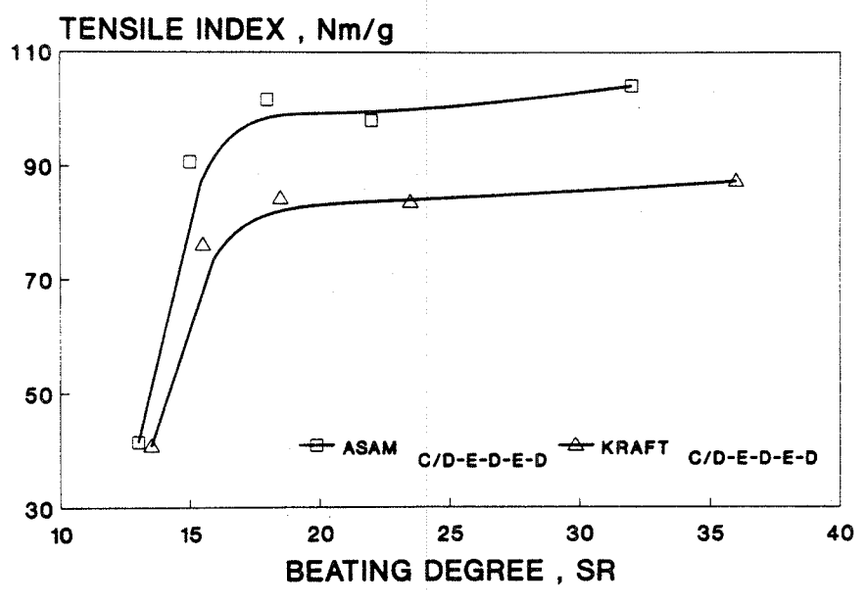

Fig. 13: Runability factor of conventionally bleached Douglad fir pulps

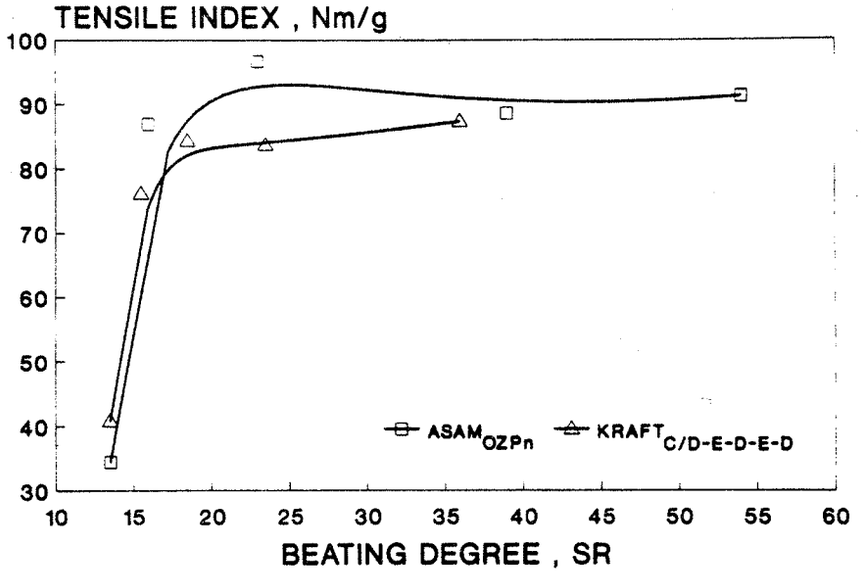

Fig. 14: Tensile strength of chlorine free bleached ASAM pulp in comparison to conventionally bleached kraft pulps

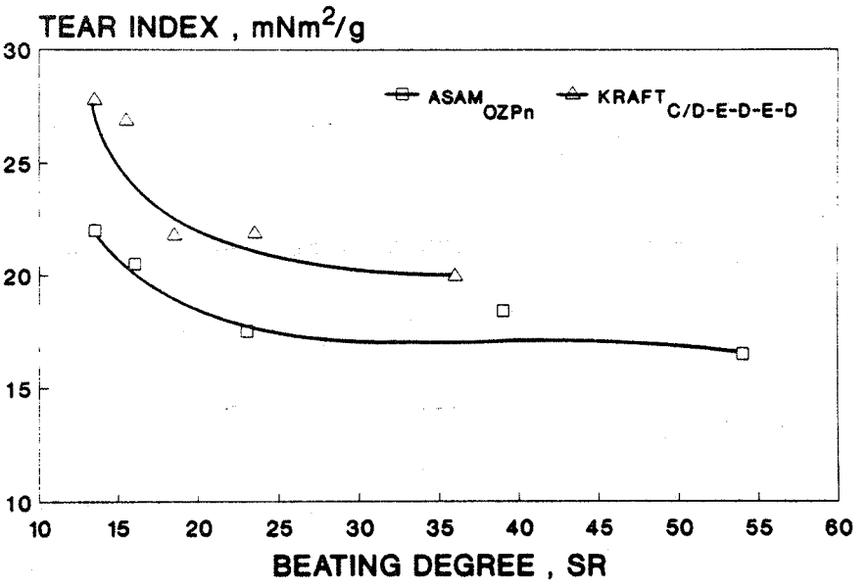

Fig. 15: Tear strength of chlorine free bleached ASAM pulp in comparison to conventionally bleached kraft pulp

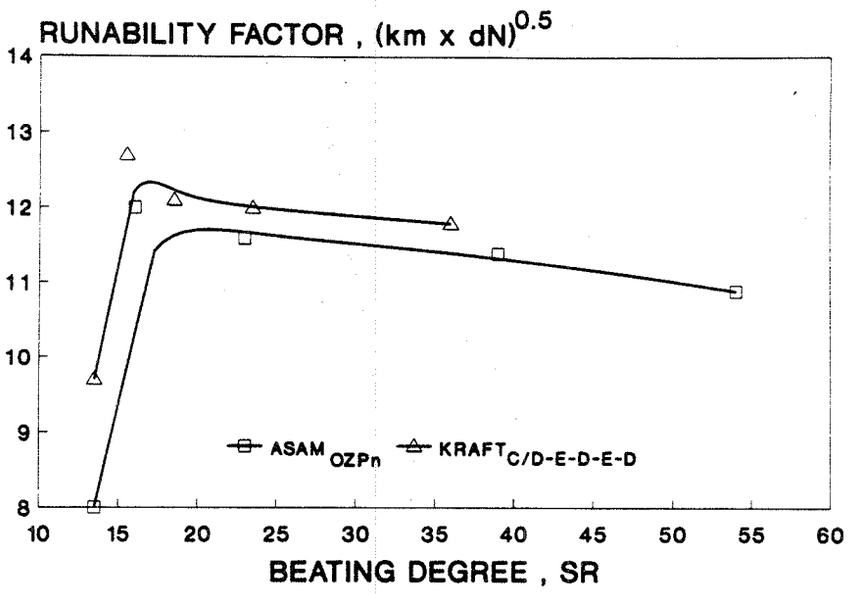

Fig. 16: Runability factor of chlorine free bleached ASAM pulp in comparison to conventionally bleached kraft pulp

ASAM pilot plant trials

Figure 17 shows the flow sheet of the ASAM pilot plant which is in operation since about 2 years. In this time some hundreds of cooks were conducted and it was possible to verify our laboratory results.

The pilot plant consists of a 10 m^3 batch digester and a 4-stage chlorine free bleaching sequence. The oxygen stage is working at medium consistency in an upflow pressurized tower. The ozone stage is working at high consistency of 40-45%. Final bleaching can be conducted in two upflow towers. Washing between the different stages is performed in double sieve presses. The standard bleaching sequence is OZP, one of the two final bleaching towers is used for acidification of the pulp with sulphuric acid before ozone treatment. The pilot plant is equipped with methanol recovery facilities and a 3-stage evaporation unit.

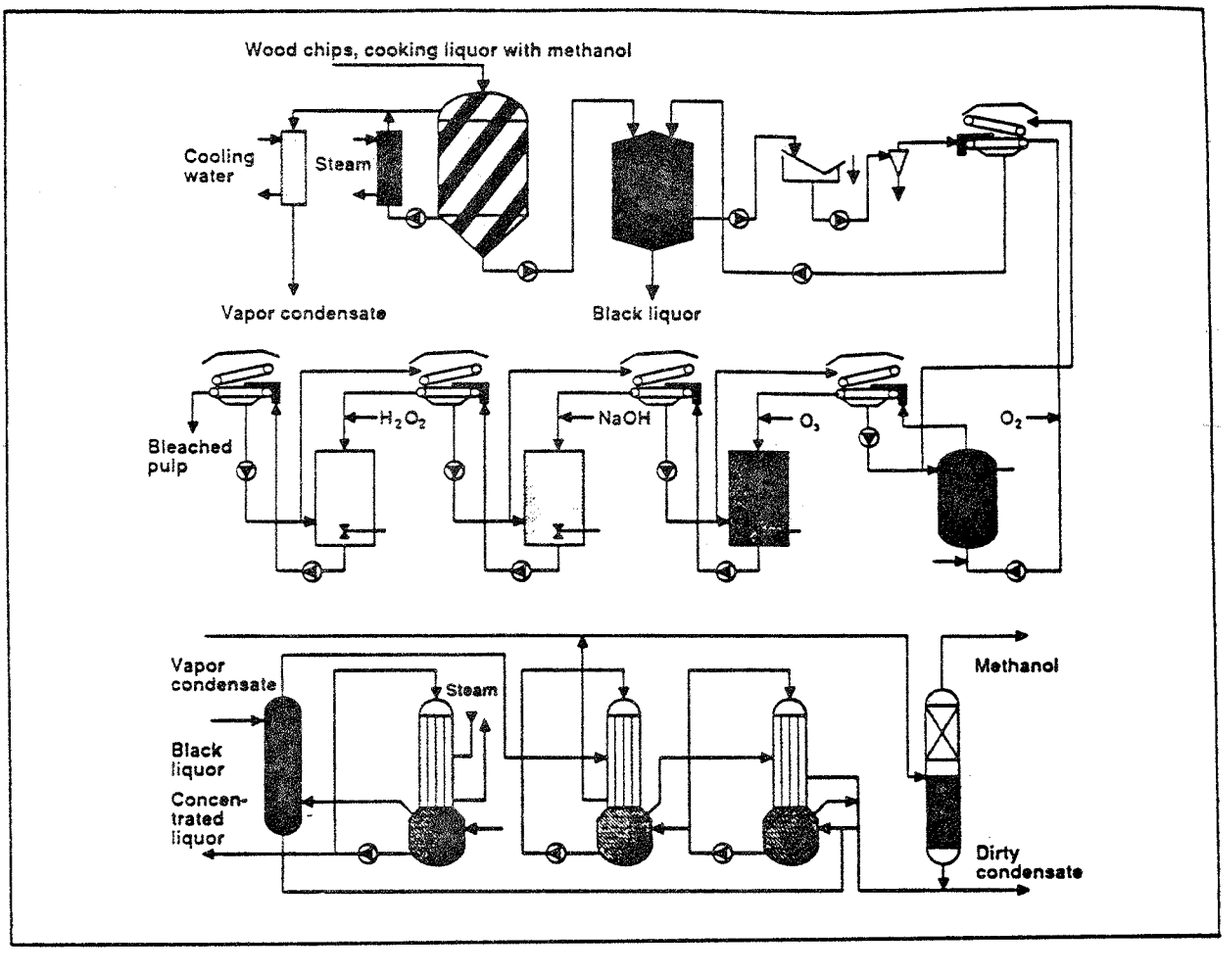

Fig. 17: Flow sheet of the ASAM pilot plant

The pilot plant is operating with countercurrent washing and a closed water cycle. The specific consumption of fresh water in the whole plant is 6-8 m^3/per ton of pulp. The COD load is approximately 8-10 kg/ton of pulp. The organic load of the washing filtrates and its content of transition metal ions impart the effectivity of the different bleaching sequences to a certain extent. Acidification of the pulp before ozone treatment and subsequent removal of transtion metal ions by washing minimize the ozone demand by side reactions. The addition of chelating agents is particularly essential in peroxide bleaching. By this means, fully bleached pulp with excellent strength properties are yielded

DIFFERENT PRODUCTS OBTAINED IN THE THERMOCHEMICAL CONVERSION OF HYDROLYTIC EUCALYPTUS LIGNIN BY DIFFERENT METHODS

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ABSTRACT

Hydrolytic eucalyptus lignin was depolymerized by alkaline hydrolysis, hydrogenolysis and catalytic hydrogenation. Higher reaction temperatures increased the conversion and reduced the molecular weight of the oils obtained. On the other hand, the kind of products depended strongly on the conversion process used. Alkaline hydrolysis at 250°C and hydrogenolysis at 280°C gave mainly guaiacol and 2,6-dimethoxyphenol, hydrogenolysis at 350°C produced mostly catechols while substituted benzenes were obtained in the hydrogenation over a Co-Mo catalyst. Hydrogenation over a palladium catalyst gave the homologous series of 4-substituted phenols, guaiacols and 2,6-dimethoxyphenols. Low voltage high resolution mass spectrometry was found to be an excellent method for the rapid analysis of this kind of oils.

INTRODUCTION

Depolymerization of lignins normally produces a large number of different products in small individual yields. Several different processes may be used for this purpose (Figure 1) but it is not well understood, which kind of bonds are broken under the conditions and which would be the appropriate process to obtain a certain product.

We wish to report here our results on the conversion of hydrolytic eucalyptus lignin by alkaline hydrolysis, hydrogenolysis and catalytic hydrogenation and compare the composition of the oils obtained with that of an eucalyptus wood tar from charcoal production.

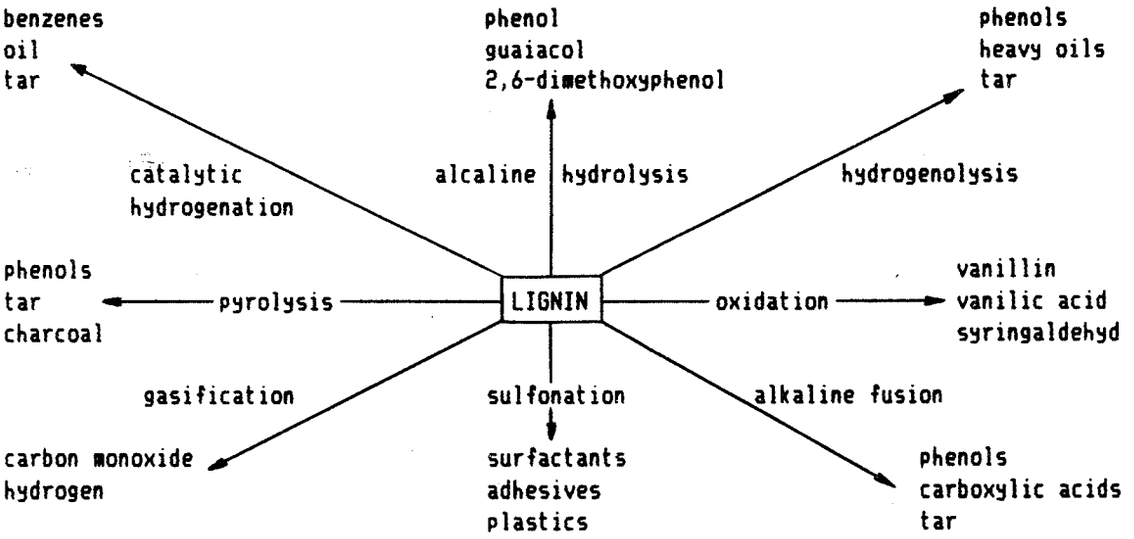


Figure 1. Conversion processes for lignins

EXPERIMENTAL

In the experiments a lignin obtained from the Fundação de Tecnologia Industrial (FTI - Lorena, SP) by hydrolysis of Eucalyptus grandis wood at 170°C with 1% sulfuric acid was used. Lignins from the organosolv processes have been shown in our laboratory to be much more reactive in these depolymerization processes but were not available in sufficient quantities. The immediate and elemental analysis of the washed and ground eucalyptus lignin are shown in Table 1.

Table 1. Immediate and elemental analysis of the washed and ground eucalyptus lignin

Hum. (%)	Ash (%)	Klason[a] Lignin (%)	Soluble[a] Lignin (%)	Polysac-[a] charides (%)	OCH_3/C_9	Elem. Anal. % C	% H	% O[b]
8.6	0.5	74.1	0.6	24.6	1.24	54.0	5.6	40.4

[a] Water and ash free; [b] by difference

The conditions of the lignin conversion are given in Table 2.

Table 2. Conditions for the lignin conversion

LH280:	100 g lignin, 9 g $NaHCO_3$, 400 mL water; pH 9 with 40% NaOH in water; 1 L autoclave, pressurized with argon; 15 min at 280°C; reaction mixture extracted with $CHCl_3/C_2H_5OH$ 3:1; oils dried for 2 h at R.T. in vacuo.
LH350:	idem; 15 min at 350°C.
LC400:	20 g lignin, 60 g xylene, 3 g of catalyst (6% CoO, 13.5% MoO_3 on Al_2O_3), 0.4 g CS_2, 10 MPa H_2; 200 mL autoclave, 30 min at 400°C; distilled at 160°C to remove solvent.
LPd350:	reactor of the Institut für Holzforschung, Hamburg; 110 g lignin, 400 mL acetone, 40 mL water, 10.4 g of Pd (5%) on Al_2O_3 (Degussa), 1 L autoclave; 8 MP H_2, 30 min at 350°C; 1st separator at 230°C, joint with extract (LPd350); 2nd separator at 20°C (LPd350D and acetone); acetone evaporated with rotavapor.
LHA250:	50 g lignin, 200 mL water/dioxane 1:1, 3 g NaOH; 1 L autoclave, 6 MPa argon, 15 min at 250°C; extracted with $CHCl_3/C_2H_5OH$ 3:1; oils dried for 2 h at R.T. in vacuo.
HT:	eucalyptus wood tar from charcoal production (Acesita).

RESULTS AND DISCUSSION

The conversion and oil yields obtained are shown in Table 3.

Table 3. Conversion and oil yields

Sample	Reaction Temp. (T) (°C)	Pressure at T (MPa)	Reaction Time (min)	Conv. (%)	Oil Yield (%)
LHA250	250	15	15	47.6	36.8
LH280	280	22	15	55.2	33.1
LH350	350	27	15	59.9	26.5
LC400	400	32	30	68.0	37.9
LPd350	350	28	30	74.3	59.1

The conversion increases with the reaction temperature but the oil yields are normally low. On the other hand, the real oil yield obtained for LC400 is higher, as the more volatile products were distilled off with xylene. The hydrogenation reaction in acetone using a palladium catalyst clearly gives the best results as the acetone can be easily separated from the products.

The analytical methods used to characterize the oils are given in Table 4.

Table 4. Analytical methods

thermogravimetric analysis:	Varian STA 780 thermogravimetric balance, 25 mL/min argon, 10°C/min until 800°C.
average molecular weight $\overline{M}$:	Knauer 11.00 vapor pressure osmometer, $CHCl_3$ at 37°C.
vacuum distillation:	trap-trap distillation at 1.33 Pa between 30°C and 280°C.
gas chromatography:	Carlo Erba CG 300 gas chromatograph with FID, 30 m x 0.25 mm capillary column with 0.25 µm film of DB-17, cold-on-column injector, 2 min at 35°C, 4°C/min until 280°C.
CG-MS:	Kratos MS 50TC coupled to the GC by a deactivated 0.2 m x 0.25 mm capillary, 70 eV between m/z 50 and 500, resolution 2000.
Low voltage high resolution mass spectrometry (LVHRMS):	Kratos MS 50TC, Data Processor DS 55, 1 mg sample inserted at high temperature inlet (350°C), 12 eV ionization energy, resolution 20,000.

The simulated distillation curves, obtained in the thermogravimetric analysis, are shown in Figure 2.

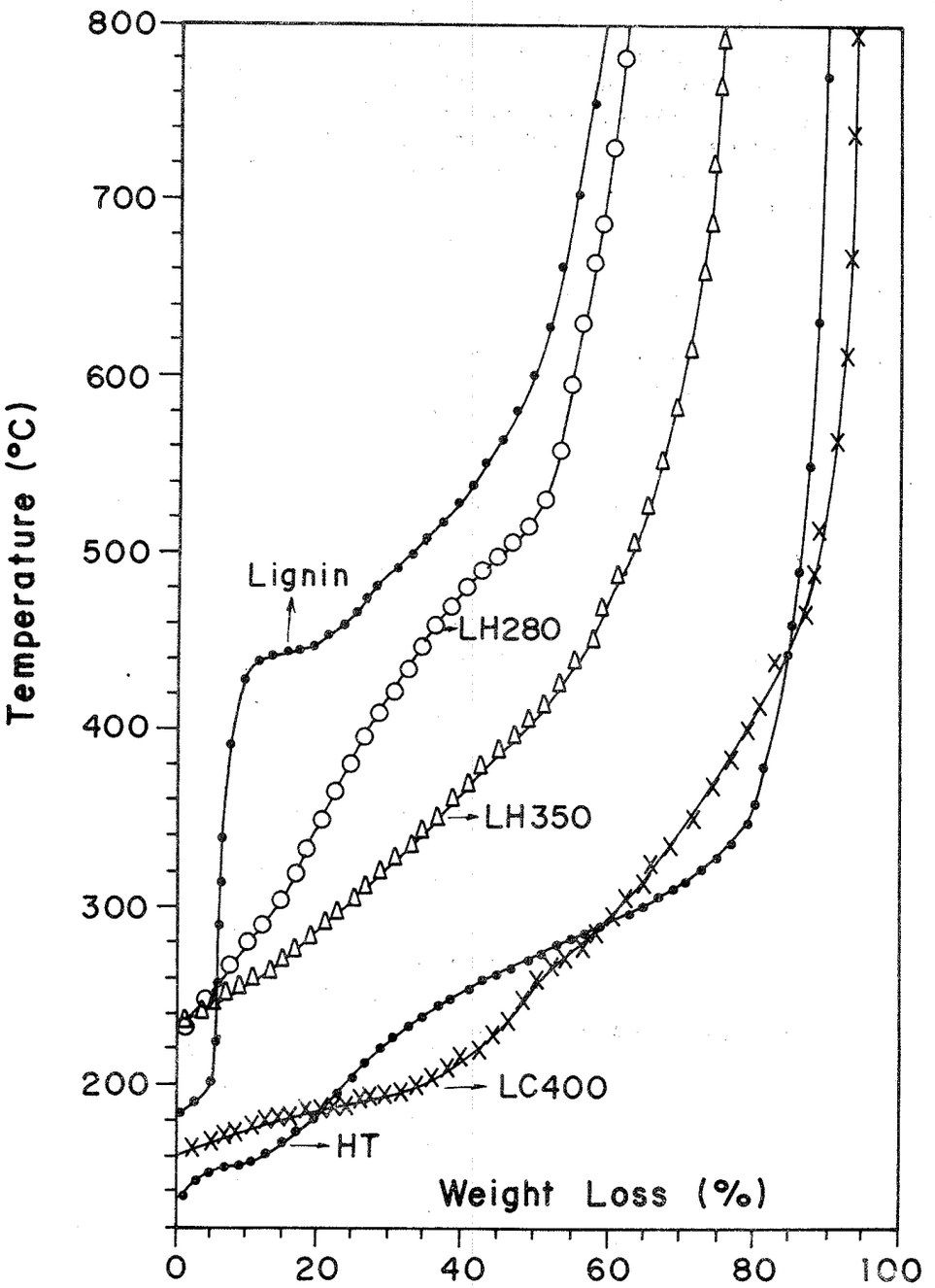


Figure 2. Simulated distillation of the oils

In LH280 only a few bonds of the hydrolysis lignin have been broken, giving a similar distillation curve to that of lignin. With increasing reaction temperature the percentage of volatiles increases, reaching 80% at 400°C in LC400. The wood pyrolysis tar HT has a similar distillation curve to LC400.

The elemental analysis of the oils, their distillates and the residues of the distillations are given in Table 5 together with their average molecular weights.

Table 5. Elemental analysis and average molecular weight of the oils

Sample	Distil. Yield	C (%)	H (%)	$O_{dif.}$ (%)	H/C	O/C	$\overline{M}$ (g/mol)
LH280	-	57.0	6.3	36.7	1.32	0.48	607
LH350	-	72.3	7.6	20.1	1.26	0.21	286
LC400	-	85.3	8.5	6.2	1.20	0.05	217
LPd350	-	71.1	7.6	21.3	1.28	0.22	n.d.
HT	-	63.8	6.7	29.5	1.27	0.35	267
LH280D	40.8	51.6	7.9	40.8	1.83	0.59	223
LH350D	41.2	67.9	8.4	23.6	1.49	0.26	177
LPd350D	28.0	66.3	8.8	24.9	1.59	0.28	n.d.
HTD	73.0	56.1	7.5	36.4	1.60	0.49	198
LH280R	-	73.0	5.9	21.1	0.97	0.22	-
LH350R	-	79.9	6.8	13.4	1.02	0.12	-
HTR	-	73.0	5.8	21.2	0.95	0.22	-

D = distillate, R = residue, n.d. = not determined.

The oxygen content of LH280 as well as the average molecular weight are still high. In LH350 and LPd350 they are reduced to intermediate values, showing the strong influence of the reaction temperature. On the other hand, the low oxygen content of LC400 is not explained only by the higher reaction temperature but is mostly caused by the strong deoxygenating activity of the catalyst used. The oxygen content of HT is still quite high, due to the constant removal of volatile products during the heating-up process. As expected, the oxygen content and H/C ratio of the distillates is higher than of the oils, while their average molecular weight is reduced. LC400 was not distilled but directly analyzed by GC and GC/MS.

The different compounds of the distillates and of LC400 were separated by capillary column GC and identified

by GC/MS. The chromatogram of the distillate of LHA250 showed only two major peaks which were identified as guaiacol (50%) and 2,6-dimethoxyphenol (30%). The compounds identified in the other distillates are given together with their retention times and percentages in Table 6.

As observed for LHA250, guaiacol (11.8%) and 2,6-dimethoxyphenol (23%) are the major components of LH280D. In LH350D aryl-methyl ether bonds have been hydrolyzed and the dimethoxyphenols have been demethoxylated. Dihydroxybenzenes are observed as major components in 6.1%, 3.9%, 2.5% and 3.9% individual yields. In LC400 alkylbenzenes are the principal products. Oxygenated compounds are only observed in very small individual yields. Interestingly LPd350D shows the homologous series of unsubstituted, 4-methyl, 4-ethyl and 4-propylphenol, -guaiacol and -2,6-dimethoxyphenol. The composition of HTD is very similar to LPd350D, but the 2,6-dimethoxylphenols are observed in higher percentages.

Low voltage high resolution mass spectrometry is a rapid and very efficient method to characterize the oils. The relative molar percentages of the different formula classes are given in Table 7 for the oils and their distillates. As already found by GC/MS, guaiacols ($C_nH_{2n+z}O_2$) and 2,6-dimethoxyphenols ($C_nH_{2n+z}O_3$) are the major components of LH280 and LH280D. It is of course possible to further divide the formula classes by their different values of z and n. In agreement with the GC/MS analysis, z has the value of -6 for the most abundant compounds, which corresponds to monoaromatic substances, and n has the value of 7 and 8, which corresponds to unsubstituted guaiacol and 2,6-dimethoxyphenol, respectively. As already found by GC/MS analysis, phenols ($C_nH_{2n+z}O$) and catechols ($C_nH_{2n+z}O_2$) are the most abundant compounds in LH350 and LH350D. More than 50% of LC400 are substituted benzenes (C_nH_{2n+z}), but phenols are also present. Phenols, guaiacols and 2,6-dimethoxyphenols are found in higher percentages in LPd350 and LPd350D. The value of n shows a constant numerical increase in these classes, confirming that all members of the homologous series are formed. In agreement with the GC/MS analysis, HT is very similar to LP350D. In HTD the 2,6-dimethoxyphenols are present at 47%, due to their higher volatility in comparison with the diphenolic compounds found in HT.

Table 6. Individual compounds indentified by GC/MS

Compound	RT (min)	LH280D (%)	LH350D (%)	LC400 (%)	LPd350D (%)	HTD (%)
ethylmethylbenzene	7.55	-	-	1.8	-	-
n-propylbenzene	8.52	-	-	1.2	-	-
2-methyltetrahydrofuran	8.72	1.03	4.33	-	0.08	-
mesitylene	8.88	-	-	3.64	-	-
3-methylcyclopentenone	9.53	0.38	0.37	-	0.15	-
2,5-hexanedione	11.38	-	-	-	1.96	-
phenol	12.20	0.63	1.93	0.24	0.49	0.73
furfural	12.57	0.79	0.64	-	0.78	0.52
2-methylfurfural	14.84	-	1.6	-	-	1.12
o-cresol	15.02	0.52	0.12	0.35	0.15	0.73
m,p-cresol	15.68	0.12	0.96	0.30	0.07	1.05
2-methoxyphenol (guaiacol)	16.64	11.8	1.26	0.35	1.97	1.07
2,5-dimethylphenol	17.83	0.34	0.55	0.58	0.80	0.11
3,5-dimethylphenol	19.10	0.11	0.43	0.21	0.66	0.54
4-ethylphenol	19.43	0.16	0.42	0.49	0.52	0.33
2,3-dimethylphenol	19.78	0.23	0.82	0.20	0.85	0.43
methylnaphthol	19.94	-	-	0.49	-	-
4-methylguaiacol	20.02	0.81	0.71	-	0.65	1.27
2-propylcyclohexanedione	20.54	0.42	-	0.15	0.53	-
1,2-dihydroxybenzene (catechol)	21.50	1.59	1.83	-	0.91	2.43
2,3,6-trimethylphenol	22.06	-	0.11	0.20	0.58	-
4-propylphenol	22.37	-	0.25	0.27	0.32	-
2,3,5-trimethylphenol	22.59	0.42	0.46	0.20	-	-
4-ethylguaiacol	23.16	1.68	0.52	0.16	1.62	0.98
3-methylguaiacol	23.47	-	1.72	-	-	-
4-hydroxyguaiacol	24.05	3.20	-	-	1.57	1.07
4-methylcatechol	24.58	0.57	6.10	-	-	-
4-propylguaiacol	25.63	0.63	0.20	0.34	1.06	0.54
trimethoxybenzene	26.14	0.49	0.81	-	0.30	0.53
dimethylcatechol	26.33	-	3.86	-	-	1.16
3,4-dimethoxyphenol	26.79	0.79	-	-	0.95	-
dimethyldihydroxybenzene	27.80	-	2.52	-	-	-
2,6-dimethoxyphenol (syringol)	27.96	23.0	-	0.24	3.23	8.2
dimethyldihydroxybenzene	28.42	-	3.85	-	-	-
2,6-dimethoxy-4-methylphenol	30.42	2.10	0.92	0.32	2.76	6.1
2,6-dimethoxy-4-ethylphenol	32.61	2.10	-	0.50	1.25	3.8
4-acetonylguaiacol	33.85	0.49	-	-	0.10	0.63
2,6-dimethoxy-4-propylphenol	34.78	0.93	-	0.46	0.83	1.42
4-acetyl-2,6-dimethoxyphenol	40.55	0.48	-	-	-	-
dimethylnaphthol	40.59	-	1.04	-	-	-
Total identified		58.3	43.9	68.2	29.2	42.1

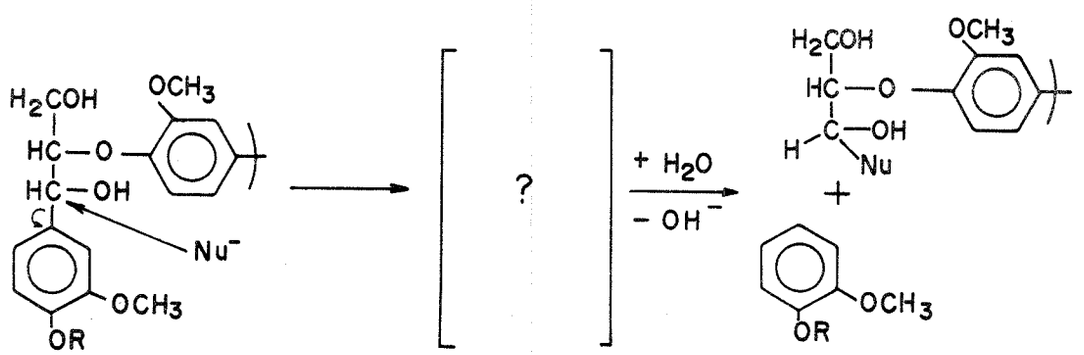
Table 7. Relative molar percentages of different formula classes

General Formula	Sample LH280	LH280D	LH350	LH350D	LC400	LPd350D	LPd350	HT	HTD
C_nH_{2n+z}	2.21	0.76	2.83	3.70	56.74	2.93	2.63	1.46	1.17
$C_nH_{2n+z}O$	8.02	4.65	40.20	31.30	19.47	19.13	13.84	10.19	10.12
$C_nH_{2n+z}O_2$	23.64	24.81	43.61	54.60	5.36	31.20	32.20	26.06	28.82
$C_nH_{2n+z}O_3$	48.36	54.52	1.40	1.10	0.13	27.02	35.66	35.33	46.80
$C_nH_{2n+z}O_4$	5.37	3.86	n.dt.	n.dt.	n.dt.	0.57	8.52	3.17	2.19
$C_nH_{2n+z}O_5$	n.dt.	n.dt.	n.dt.	n.dt.	0.05	n.dt.	n.dt.	0.34	n.dt
$C_nH_{2n+z}S$	n.dt.	0.03	n.dt.	n.dt.	1.39	0.06	n.dt.	n.dt.	0.26
$C_nH_{2n+z}N$	n.dt.	n.dt.	n.dt.	n.dt.	n.dt.	0.70	2.67	n.dt.	n.dt
Other	n.dt.	0.18	3.40	0.15	2.02	0.85	4.23	0.53	0.94
Fragments	12.40	11.20	8.57	9.15	14.85	17.54	0.25	22.93	9.96

n.dt. = not detected

CONCLUSIONS

Hydrogenolysis at 280°C shows a high selectivity for the production of guaiacol and 2,6-dimethoxyphenol, formed by aryl - C_α bond cleavage. We propose the following mechanism for this reaction:



The transition state or intermediate involved is not yet well understood

Alkaline hydrolysis in a dioxane/water mixture at 250 °C gives an even higher yield of these phenols, probably by the same mechanism.

350°C is too high for the hydrogenolysis reaction as aryl-methyl ether bonds are hydrolyzed and substituted catechols formed as the principal products in smaller individual yields.

The $CoO-MoO_3$ catalyst is very effective in deoxygenating the lignin products. The oxygen content of the oil is reduced to 6% and alkylbenzenes are the principal products. Oxygenated products are only found in small individual yields.

The Pd-hydrogen system at 350°C depolymerizes the lignin very effectively but is not very specific in the types of bonds broken. Unsubstituted, 4-methyl, 4-ethyl and 4-propylphenol, -guaiacol and -2,6-dimethoxyphenol are observed, but none of them with more than 3% yield.

The composition of the wood pyrolysis tar is very similar to that found in the oil of LPd350, but 2,6-dimethoxyphenols are more abundant.

ACKNOWLEDGEMENTS

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The 3Rs and 3Es of Wood Chemistry Research for the 21st Century

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ABSTRACT

Contamination of environment and depletion of forest natural resources have forced wood products manufacturers, producers and converters to stop underestimating the environmental, economical and political problems they are facing. These issues also have forced them to take the initiative to promote and develop "complete utilization (whole tree)" strategy. Research, development and commercialization of wood, paper and chemical products have also shifted direction in order to abide by government regulations and guidelines. The classical manufacturing technologies for wood utilization have not been manipulated and operated in an efficient manner and because of this by-products and waste discharge have led to air, water and land pollution. To preserve environment and to salvage raw materials and energy, new strategies have to be established. *Reduction, reuse* and *recycling* (the 3Rs) appear to be the rational approaches to achieve these goals. In addition to raw material control, process improvement is imperative. The *essence* of production, the *economy* and *efficiency* (the 3Es) of process operation have to be considered. Successful studies of using low quality wood, sawdust and newspaper for conversion to thermoplastic materials are discussed. The challenges and opportunities for using wood waste, paperwaste and plastic waste from the municipal solid waste stream to manufacture fiber/plastic composites are also discussed.

INTRODUCTION

Wood chemistry as a science covers a wide spectrum of important applications for an ever growing world population to meet the basic needs of shelter, energy, food, health and clothing (1). It also plays an important role in world economics. The main thrust of this symposium is *Technological*

Development and Environmental Concerns. I would like to take this opportunity to show you some of our concerns in wood and biomass utilization. Although the statistical data is based on the United States of America, I hope the general information is still relevant to Brazil and other countries in Latin and South America.

ENTROPY AND WASTES

Ever since human being started to fabricate articles or to develop technologies, they cautiously planned and reviewed the processes and products. They have realized that while making something useful, they, quite unintentionally, have made something useless simultaneously. They produce WASTE. They produce waste, not because they want to do it, not because they do not have proper planning, and not because they do not have the right tools. They generate WASTE is due to a powerful NATURAL LAW: the second law of thermodynamics, or popularly known as ENTROPY LAW (2). This law states that the entropy (waste) of the universe for an irreversible process is greater than zero. The entropy (waste) of the universe is increasing. Conceptually, entropy can be related to disorder or randomness within a system. In this case, increase in entropy is related to increase waste generation. Thus, generation of waste during fabricating and processing is an inevitable event. Virtually all of our activities generate waste. Civilizations often strangle on their own wastes.

In considering wood and biomass utilization, production of food, energy and material are the objectives. The materials that are generated may be fabricated for structural and chemical uses. Production of pulp and paper from trees is a typical example. However, during this exploitation process, 567 million tons of biomass residues are generated annually in the USA, as shown in Table 1 (3), of which about 99 million tons of biomass

Table 1. Biomass Residues Generated in the U.S.

Source	Amount Generated (Million Tons/Year)
MSW	99
Agricultural residues	300
Forest Residues (Total)	168
Forest residues	145
Wood processing residues	20
Pulp and paper mill wastes	3

residues end up in the municipal solid waste (MSW) stream. Much effort has been expended to utilize forest and agricultural residue, but much biomass waste remains in the municipal solid waste stream.

Recently, public concern for the environmental problems associated with landfills has grown. As a result, we are constructing few new landfills and running out of space in those that already exist. Since the late 1970s, more than two-thirds of the landfills have closed; one-third of those remaining will be full in the next five years (4). The U.S. Environmental Protection Agency estimates that 80% of our landfills will be closed within 20 years (5). The incident of the garbage barge (Mobro 4000) from the New York City area that roamed the seas for several months in a futile search for a landfill that would take it, seems to epitomize the problem and demonstrate its national and international proportions. If no action is taken immediately, we certainly will be buried in our own residential and commercial discards! This is waste of resources, energy, space, and labor. Hence, we have to gravely consider the utilization of recyclable and reusable wastes in the MSW. It is surprising that in the MSW stream, biomass contributes a major portion: paper products, 37.2%; wood products, 3.8%; yard wastes, 17.9% (Figure 1) (6).

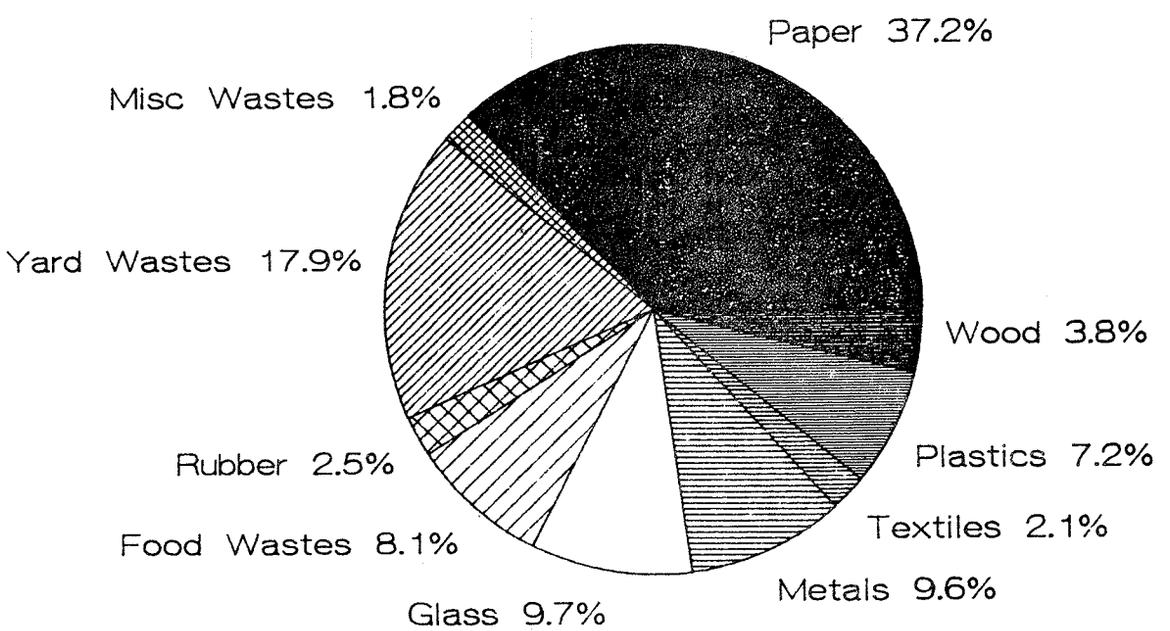

Figure 1. The municipal solid waste stream in the U.S.

In view of this, waste prevention and materials management strategies have to be established. Although the production of useful products provides the driving force for the economy and political society, improper waste management and control cause air-water-land pollution. The ecological and raw material economic requirements are such that the problem of waste can no longer be regarded merely from the aspect of removal, but rather from that of exploitation. Integrating waste into the natural material cycle is becoming more and more a basic of the waste economy. Hence, by reducing waste, we can increase productivity, safeguard the environment, and strengthen the economy.

A 3R AND 3E PROGRAM

With this in mind, we have developed a strategy dealing with waste problems, particularly for wood-based industry. This is the so-called "3R and 3E" program. Literally, 3R is short for REDUCE, REUSE and RECYCLE, and 3E for ESSENTIAL, EFFICIENT and ECONOMICAL. They are briefly described here.

Source Reduction: Source reduction is defined as the design, manufacture, and use of products so as to reduce the quantity and toxicity of waste produced when the products reach the end of their useful lives. Source reduction may occur through the design, manufacture, and packaging of products 1) with minimum toxic content, 2) minimum volume of material, 3) and/or a longer useful life. Source reduction is not a waste management panacea but it can have a positive impact on waste management systems. Although the exact benefits of source reduction are difficult to quantify, the benefits are conceptually clear.

Reuse and Recycling: Reuse and recycling are fundamental parts of any integrated waste management plan. Reuse and recycling alone cannot solve a MSW management problem, but they can divert a significant portion of the waste stream from disposal in landfills or combustion facilities. Recycling is more than the separation and collection of post-consumer materials. Post-consumer materials must also be reprocessed or remanufactured, and only when the materials are reused is the recycling loop complete.

While the "3Rs" are under consideration, we must also simultaneously bear in mind the "3E" factors. With the effort on source reduction, we must consider whether the product is an *essential* product. Without such a product would it make a significant difference in that area of application? While manufacturing such a product, is the process or system operated in an *efficient* manner? Does the process provide highest productivity? Finally is that product *economically* attractive? Halting production of nonessential products also will lead to

source and cost reduction. Optimizing design and consolidating parts are in order, so that we can reduce the number of parts that are required.

Although we cannot win the ENTROPY LAW to eliminate waste generation, we can minimize it. Source reduction, reuse and closed-loop recycling are the recommended methods. Waste generation can potentially be reduced in virtually every aspect of a plant's operation. During the original design and development of a process, raw materials, process conditions and equipment selection are important factors. To maximize productivity and reduce waste, modifications of operation parameters and processes may evolve on the basis of process optimization and yield improvement. To implement a "3R and 3E" program, process design can be divided into three phases, each presenting different opportunities for implementing waste-reduction measures:

Product Conception: What are the raw materials used to manufacture a product? Do we have to use softwood or hardwood? Can we use low quality, inferior wood? Can we use by-products or waste from other products? Are any toxic or hazardous chemicals likely to be used for the process or to be generated during manufacturing? What types of wastes are likely to be generated? Do we currently manage these wastes?

Research & Development: The key process parameters and operating conditions necessary for optimum production have to be determined. How much of the raw materials will end up in the final product, or how much of the raw material will not end up in the final product? How simple is the process? Usually, a simpler process generates less waste than a complex one.

Mechanical Design: With the waste-elimination strategy fully implemented during the product conception and research & development, mechanical design must be appropriately incorporated into the program so that the waste can be control and managed properly.

RESOURCE RECOVERY FACILITY

Based on the simple concepts discussed above, a resource recovery facility may be established (Figure 2). In this facility, forest and agricultural residues as well as biobased materials from MSW are the starting raw materials; and chemicals, energy and composite materials are the final products. Initially, forest and agriculture wastes and municipal solid waste are collected and hauled to the resource recovery facility. There the personnel will determine whether these wastes will be used for generating chemicals, energy or composites, depending on the demand for each end product. These

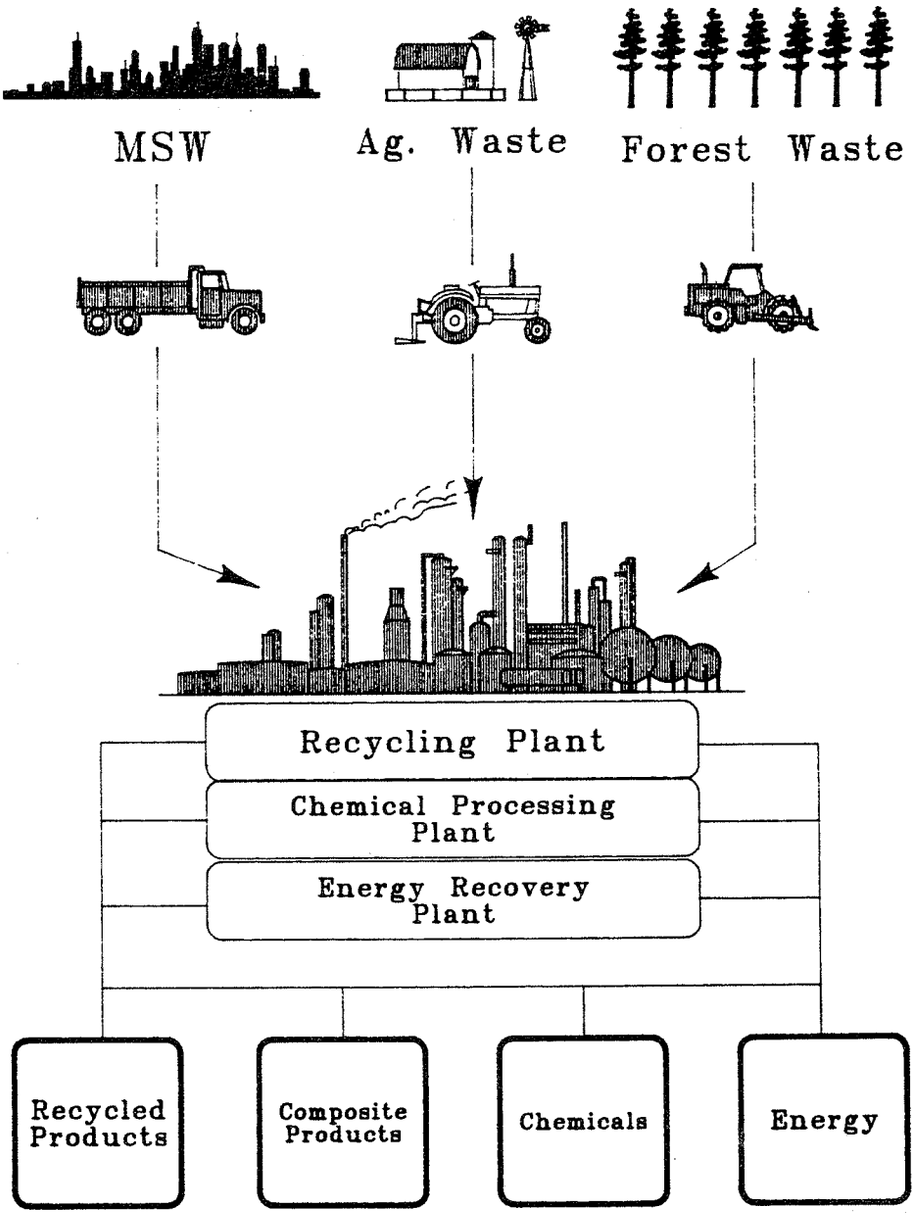

Figure 2. A simplified resource recovery facility diagram.

wastes may be sorted, separated and cleaned for a particular product, manufacture of which requires high purity and uniformity. The paper and plastic wastes in the MSW are first separated from glass and metal so that they can be separated for paper products or plastics for recycling, or, without separation, the paper and plastics may commingle to form composite materials. These composites can contribute to

materials substitution by offering low-cost options in polymer composites. Advantages of polymer composites include light weight with reasonable strength properties and cost, ease of manufacture in continuous processes that achieve parts consolidation and, therefore, lower capital cost. They have been demonstrated at a commercial level and proven to be viable. Low quality wood wastes and paper wastes may be a good source for energy production. Several examples of products generated from the biomass residues and paper and plastic waste from MSW are illustrated here.

New Composites from Wood, Fiber and Plastics

Early work on wood fiber-plastic composites concentrated on laminated structures. New composites have been developed by grafting vinyl monomers to wood pulp to improve mechanical properties of wood fiber. Extensive studies have also been conducted by incorporation of wood fibers into plastic composites. For example, Raj and Kokta (7) studied the mechanical properties of polyvinylchloride-wood fiber composites. In this study, polyvinylchloride was reinforced with chemithermomechanical pulp, wood flour, and steam explosion pulp. Composites from PVC filled with isocyanate-treated stream explosion pulp (30%) exhibited a 45% increase in tensile strength and an 11% increase in modulus compared with composites prepared with unmodified pulp fiber. Comparison of tensile properties of PVC-wood fiber, glass fiber, and mica composites demonstrated the advantage of wood fiber as filler in terms of relative cost and performance. Sean and Hon (8) have produced composites from polystyrene waste and newspaper which have high tensile strength.

New Plastic from Wood and Paper Wastes

Biomass or lignocellulosic residues can be chemically modified to produce plastics. The concept here is to derivative the wood polymers and thus to reduce the crystallinity of the cellulose. The softening temperature of the wood is reduced and the material can be heat formed easily. Shiraishi and coworkers (9) of Japan have studied thermoplasticization of wood for the past ten years. Their approach to rendering wood to thermoplastic materials has been through chemical modification of wood meal. Generally, their work has emphasized esterification of wood. Thermoplasticity of esterified wood was found to depend on the acyl group, the method of preparation, and the degree of substitution. Etherification to rendered wood to thermoplastics have been conducted by Hon and his coworkers (10,11). Benzylation and cyanoethylation can convert wood and paper into thermoplastic materials which can be melted or molded.

CONCLUSIONS

The utilization of biomass residues (forest and agricultural wastes) and the disposal of municipal solid waste (which is comprised of 58.9% biomass) are environmental issues of growing concern. Source reduction, reuse and recycling (3R) appear to be vital approaches to solve this dilemma. Growth of the knowledge and information sector as well as technological change also have accelerated waste management and utilization far beyond its state just a few years ago. Traditionally, materials needs have been met by adapting existing substances. Now, entirely new composite materials can be created by blending various plastics and fiber products from residues and wastes. These developments in material science have led to the advent of remarkable new substances that are challenging standard materials in the traditional markets.
These new materials may be preferred because they offer the opportunity to reduce manufacturing costs sufficiently to offset their higher prices. Hence, to secure long-term viability and competitiveness, forest, wood products and allied industry have to view waste management, especially waste reduction, as an integral component of their strategic business planning. Moreover, to ensure utilization of a new material, the manufacturer will need to employ advanced manufacturing concepts to reduce costs and develop new product designs that utilize his material efficiently. In addition, waste minimization is good business. Besides helping preserve the environment, it enhances the corporate image, can lead to improved customer relations, and often yields cost saving as well.

In any event, *the 3Rs* and the *3Es* are here to stay. We should commit to the belief that *the 3Rs* and the *3Es* play a crucial role in environmental protection and in the long-term growth of business.

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OXIDATION OF HYDROLYTIC EUCALYPTUS LIGNIN WITH OXYGEN IN ACETIC ACID

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ABSTRACT

Hydrolytic Eucalyptus grandis lignin was oxidized with 15 bar of molecular oxygen in glacial acetic acid at 210°C and 240°C, using cobalt(III) acetylacetonate 1 or cobalt(II) acetate 2 as a catalyst and NaBr as a promotor. Catalyst 2 is more efficient than 1 and the best oil yield is obtained at 210°C. NaBr acts as a promotor, increasing both conversion and oil yield to 75.6% and 67.2%, respectively. Analysis of the volatiles by GC-MS was not satisfatory as no molecular ion of the products was obtained and only furfuraldehyde was identified.

INTRODUCTION

Lignin can be converted by several different methods into low molecular weight products. Methods such as hydrogenolysis, catalytic hydrogenation, pyrolysis and alkaline fusion were extensively studied for this purpose (1). Alkaline hydrolysis of lignin under mild conditions can be used to obtain monophenols like guaiacol, 2,6-dimethoxyphenol and phenol in high yields (2). On the other hand, the solvent to lignin ratio must be high in order to get good oil yields.

Oxidation is an efficient method for the depolymerization of lignin and the production of oxygenated compounds. Vanillin and other aromatic aldehydes are the principal products. The industrial yield of vanillin from lignin is between 5-10%. The best yield reported using molecular oxygen is 21% (3).

Until now only studies of the direct oxidation of black liquor, originated from the sulfite pulping process, are described in the literature (4). The objective of this work is the oxidation of lignin from other sources under mild conditions.

EXPERIMENTAL

Hydrolytic eucalyptus lignin was obtained from the Fundação de Tecnologia Industrial (FTI). Its immediate and elemental analysis is shown in table 1.

Table 1. Immediate and elemental analysis of hydrolytic eucalyptus lignin from FTI

water (%)	11.0
ash (%)[a]	1.4
Klason lignin[a]	79.8
soluble lignin[a]	1.0
holocellulose[a,c]	17.8
elemental analysis[b]	
% C	62.0
% H	5.6
% O[c]	32.4

[a] water free; [b] water and ash free; [c] by difference

The experiments were performed as shown in Scheme 1. The oil yield was calculated by equation 1 and the conversion by equation 2.

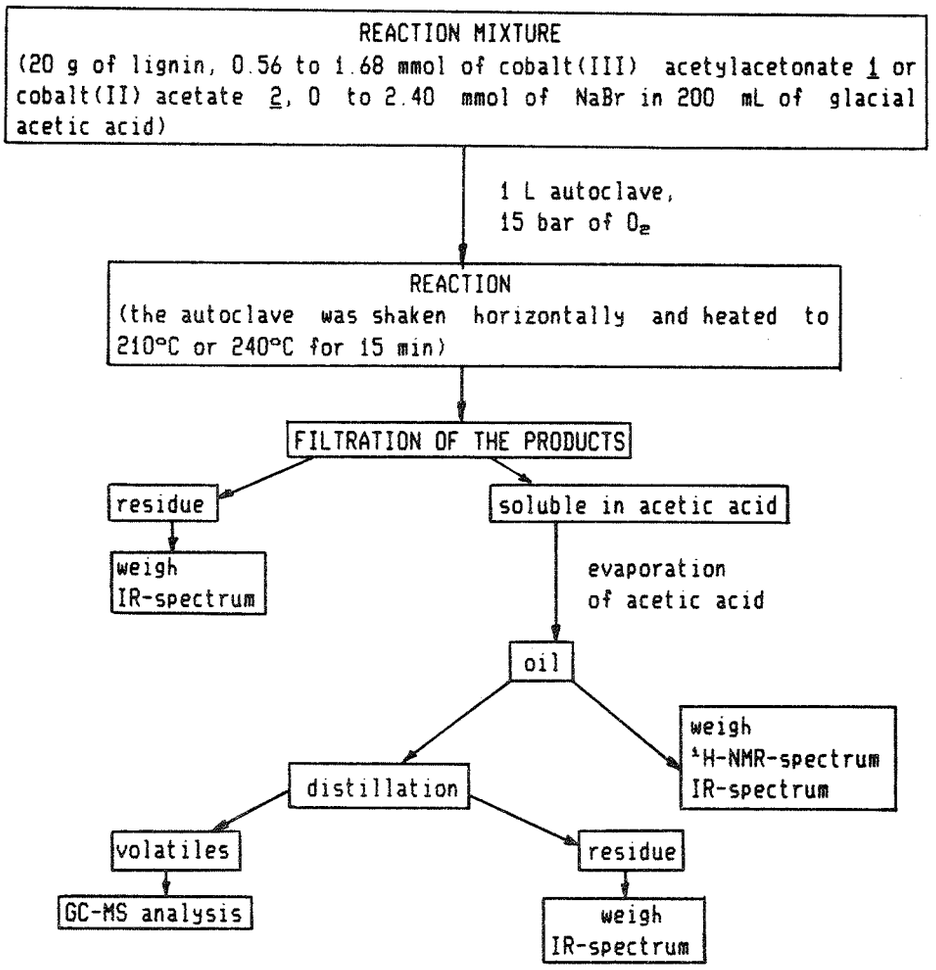
$$\text{oil yield (\%)} = \frac{m_{\text{oil}}}{m_{\text{lignin}}} \times 100 \qquad \text{(eq. 1)}$$

$$\text{conversion (\%)} = \frac{m_{\text{lignin}} - m_{\text{residue}}}{m_{\text{lignin}}} \times 100 \qquad \text{(eq. 2)}$$

m: mass; all masses are water and ash free

The oils were distilled at 165±5°C by 2 h with a heating rate of 2°C min^{-1}, giving approximately 40% of volatiles. The volatiles were analyzed by gas chromatography-mass spectrometry (GC-MS) with a Hewlett-Packard 5890 gas chromatograph using a 25 m x 0.25 mm capillary column (Carbowax 20M on Chromosorb W-HP). The gas chromatograph was connected to a mass detector HP 5970-A which used a 70 eV ionization energy.

Scheme 1. Experimental procedure for the oxidation of lignin



RESULTS AND DISCUSSION

Table 2 shows the conversion and the oil yield obtained in the reactions with 1 or 2 as catalyst.

The lignin is efficiently oxidized at 240°C even without a catalyst. On the other hand, using 2 in larger quantities (1.68 mmol) the conversion increases 11% and the oil yield 28%. As expected, the conversions are lower at 210°C but the oil yields are higher, which shows that at 240°C the products are degradated to non-extractable substances (overoxidation). Experiments performed at even lower temperatures (180°C) showed that the conversions are less than 30% and acetic acid is incorporated into the products.

Table 2. Effect of the catalyst on the oxidation of lignin (20 g of lignin, 200 mL of acetic acid, 15 bar of oxygen).

Exp.	Catalyst	Temp. (°C)	Conversion (%)	Yield (%)
1	-----	240	66.2	32.2
2	0.56 mmol 1	240	63.8	32.8
3	1.12 mmol 1	240	68.9	38.3
4	1.68 mmol 1	240	67.7	36.8
5	0.56 mmol 2	240	66.1	31.2
6	1.12 mmol 2	240	69.0	35.2
7	1.68 mmol 2	240	73.7	41.2
8	0.56 mmol 1	210	56.8	46.8
9	0.56 mmol 2	210	60.5	46.1

Increasing the solvent quantity to 300 mL in exp. 2 the oil yield increases to 37.3%. This shows a better dissolution of the fragments of the lignin due to higher dilution. An experiment carried out with 0.56 mmol of 1 and 27 bar of oxygen at 240°C showed a conversion of 79.3% and an oil yield of 33.5%. This result is better than that obtained with 15 bar of oxygen but under these conditions explosions may occur.

The distillation of the oil of exp. 7 furnished 39.8% of volatiles. A typical chromatogram of the volatiles is shown in Figure 1 and the principal fragments of the eight major peaks are given in Table 3.

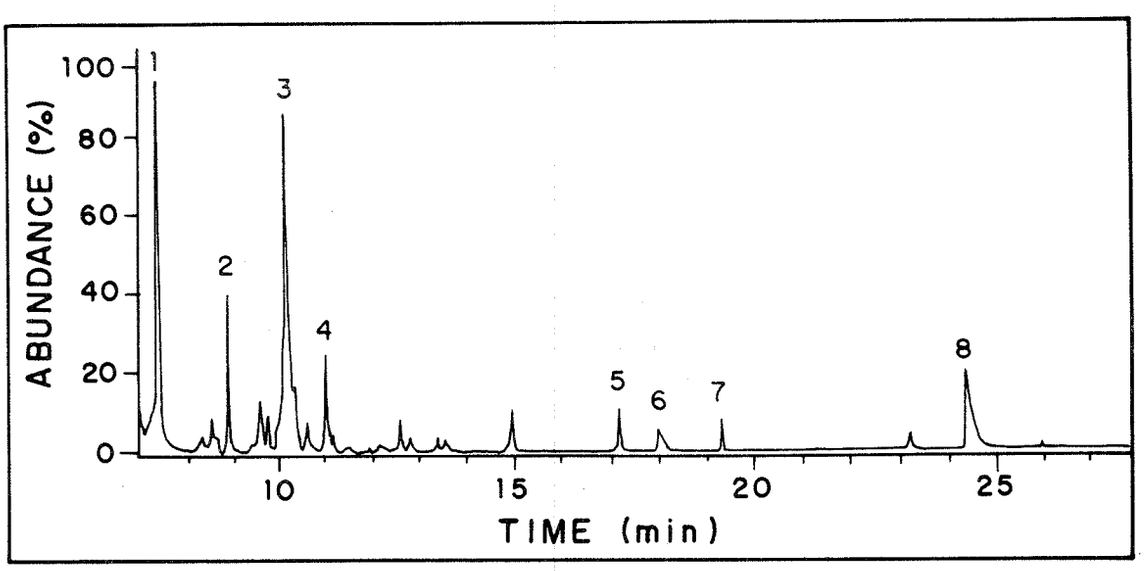


Figure 1: Chromatogram of volatiles of exp. 7

Table 3. Area percentages and principal fragments of peaks of the chromatogram from Figure 1.

peak	retention time (min)	area (%)	principal fragments in the mass spectrum
1	7 367	25.0	96(83%), 95(100%), 67(12%), 50(7%), 43(40%), 39(90%)
2	8 884	3.4	116(34%), 103(23%), 86(100%), 73(54%), 44(48%)
3	10 151	53.4	100(2%), 85(10%), 73(24%), 60(100%), 41(62%)
4	11 048	6.5	100(4%), 61(6%), 43(100%)
5	17 128	1.8	73(31%), 70(13%), 60(100%), 55(17%)
6	17 956	1.8	124(100%), 123(93%), 95(23%), 39(44%)
7	19 276	0.7	101(20%), 84(33%), 73(41%), 80(100%), 55(44%), 41(92%)
8	24 391	7.3	73(18%), 43(100%)

Only furfuraldehyde, formed from holocellulosic residues present in the original lignin, was identified (peak 1). Identification of the other peaks was not possible. Fragments at m/e 43 (peaks 4 and 8) are related to acetates incorporated in the products. We believe that other aromatic adehydes and carboxylic acids are formed but as the ionization energy of the mass detector was too high (70 eV), the molecular ions were not detected.

The results for the reactions using NaBr as a promotor are shown in Table 4. Using three times the quantity of NaBr with respect to the catalyst, there is a large increase in the conversion. With four times the quantity of promotor the oil yield shows a large increase. The quantity of promotor used in exp. 12 is very adequate because 67% of the lignin is converted to oils and the quantity of non-extractable products is very small (8%). The bromide ions react with radicals formed in the reaction, generating bromine radicals which cause the chain propagation.

Table 4. Effect of NaBr on the conversion and oil yield (20 g of lignin, 200 mL of acetic acid, 0.58 mmol of 2, 15 bar of oxygen, 15 min at 210°C)

Exp.	NaBr (mmol)	Conversion (%)	Yield (%)
9	----	60.5	46.1
10	0.58	62.4	51.1
11	1.74	74.2	55.1
12	2.40	75.6	67.2

CONCLUSIONS

Oxidation is a efficient method for the depolimerization of hydrolytic eucalyptus lignin, producing oils in high yields compared to other methods.

The highest conversion of lignin (75.6%) and highest oil yield (67.2%) were obtained with the system acetic acid/cobalt(II) acetate/NaBr/molecular oxygen at 210°C.

Mass spectrometry with an ionization energy of 70 eV is not indicated for the analysis of the oils, as the molecular ion of most products is not observed.

ACKNOWLEDGEMENTS

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EDA/WATER DELIGNIFICATION OF SOYBEAN HARVEST RESIDUES

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ABSTRACT

Samples of soybean harvest residues with and without hemicellulose were used in the delignification process. These samples were treated with ethylenediamine (EDA) aqueous solution.

The soluble product in EDA solutions were precipitated and analyzed by IR spectroscopy. Residual lignin showed characteristics similar to softwood lignins and an increase in the methoxyl content as EDA concentration increases.

INTRODUCTION

Brazil is the second major soybean producer participating with 20% of the total world production. The Paraná State contributed in 1989/90 with 4.650.000 tons, aproximately 23% of the total production in Brazil (1).

On the other hand, the residues of soybean crops are burned or incorpored to the soil for moisture retention. These materials are rich in cellulose, lignin and hemicellulose which have multiple aplications (2).

Considering this facts, this work deals with a delignification process with EDA/H_2O solutions and infrared caracterization of Klason lignins.

EXPERIMENTAL PART.

The soybean residue was obtained from a farm in Maringá. The solid sample was ground to pass trough a 35 mesh screen and retained on a 60 mesh screen.

Hemicellulose extraction: a traditional method were used (3).

Delignification process: Ethylenediamine (EDA) aqueous solution were used, during 30 minutes at 120°C and 10ml solution/1.0000 g of solid.

Lignin Analysis. After the delignification step, supernatant, and solide residue were separated by filtration. The dissolved product was precipitated with HCl, filtered and dried. The residual lignin was analysed as Klason Lignin (4).

Infrared Analysis: KBr pellet technique (1.5 mg/ 100mg KBr) were used and spectra from soluble products and residual lignins were run.

RESULTS AND DISCUSSION

Mass lost in the delignification process with EDA/ H_2O solution were determined. Results in Fig. 1 shows that with concentrations up to 2,5% EDA the residual mass remains almost unchanged.

In the solid residue (obtained after EDA treatment), cellulose and hemicellulose content were determined. These results, given in Fig. 2, showed that the EDA treatment don't affected cellulose content but hemicellulose is extracted until concentration of 5,0%.

The delignification process were also carried out using samples with and without hemicellulose. Comparing these results (Fig. 3) we observed that when hemicellulose

is previosly extracted, the delignification is more efficient. This occurs probably due to a greater contact area between solution and fibers.

Infrared spectra of soluble products and residual lignins showed a considerable variation in peak intensities of 2840, 1700, 1600 and 1500 cm^{-1}.

The absorbance ration (Ax/A_{1500}) x = 2920 (assigned to C-H); 2840 (assigned to CH from $O-CH_3$),1600 and 1500 (assigned to C=C from aromatic rings), are showed in Fig. 4,5 and 6. These absorbance ratios were always greater for the soluble product than for the residual lignins, indicating that soluble residue has different characteristics than residual lignin. Residual lignin has softwood lignin characteristics (5). A decreasing tendency of Ax/A_{1500} for the soluble residue and increasing tendency for residual lignin cohen EDA concentration increasing was observed. For Klason lignin, this tendency shows that in residual lignin the methoxyl contents increases as EDA concentration increase.

CONCLUSIONS.

Soybean harvest residues are difficult to delignify by EDA/H_2O solutions (0-50%). The Klason lignins from treated residues have softwood characteristics. The soluble products show a complex IR spectra, due to a presence of several substances not extracted by EtOH/benzeno 1:1.

The delignification of soybean harvest residues is enhanced by hemicellulose pre-extraction.

ACKNOWLWDGMENTS: This work was supported by CNPq-UEM-CCE, DQI/UEM. M.A. da R. is a CNPq graduate fellow.

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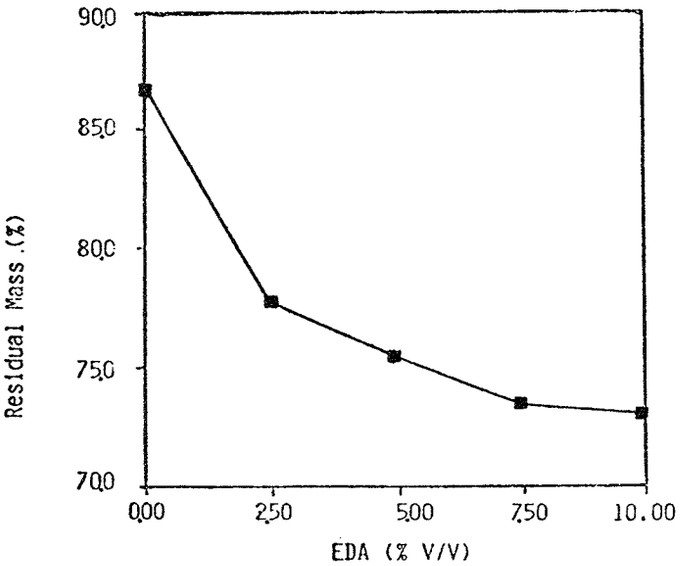


Fig. 1 - Residual mass in the delignification of a soybean residue sample with aqueous EDA solutions.

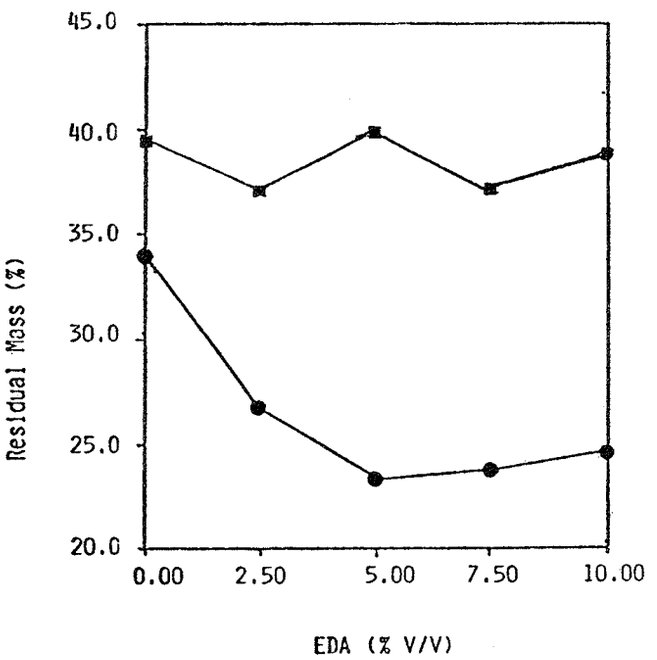


Fig. 2 - Residual mass of cellulose (■) and hemicellulose (●) in soybean residue treated coith. EDA-H_2O solutions of different concentrations.

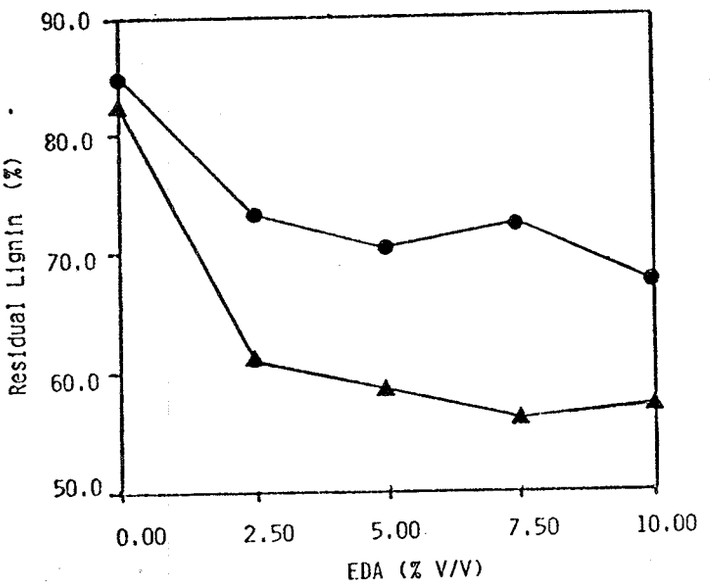
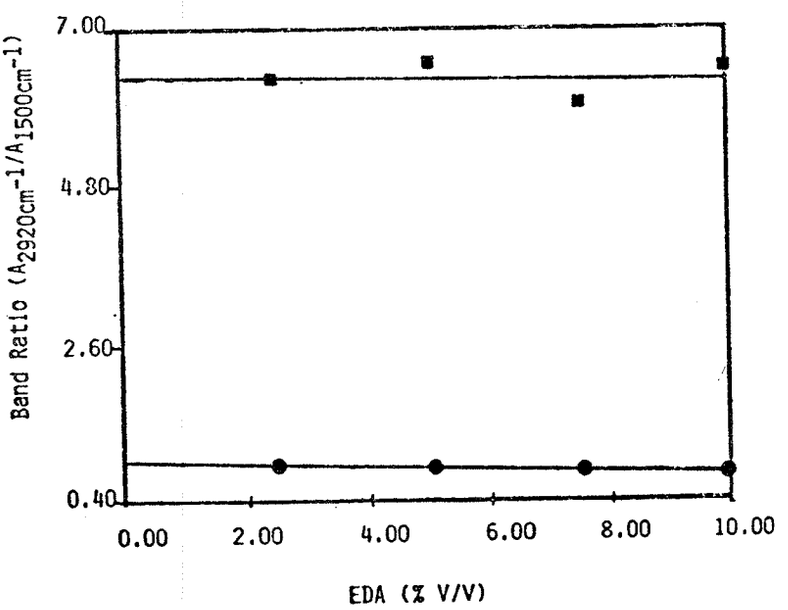


Fig. 3 - Residual lignin in soybean residue treated with EDA solutions. Samples with hemicellulose (●) and without hemicellulose (▲).



Figu. 4 - Absorbance ratio ($\frac{A\ 2920}{A\ 1500}$) for soluble product (■) and residual lignin (●) corresponding to deslignification with different. EDA concentractions.

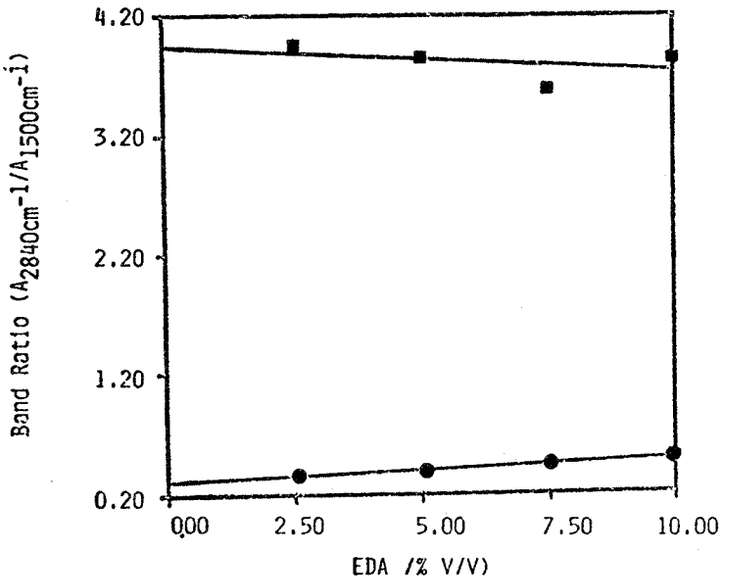


Fig. 5 - Absorbance ($\frac{A\ 2840}{A\ 1500}$) for soluble product (■) and residual lignin (●) corresponding to deslignification with differents EDA concentrations.

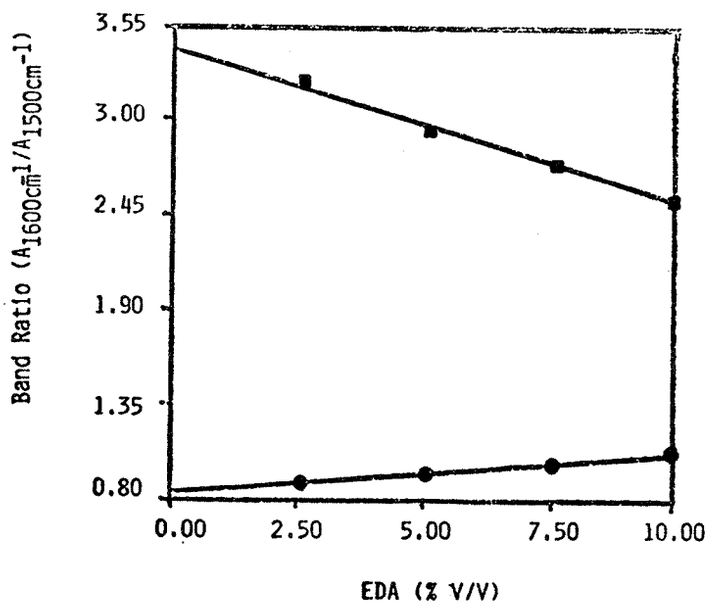


Fig. 6 - Absorbance ratio (A_{1600}/A_{1500}) for soluble product (■) and residual lignin (●) corresponding to deslignification with differents EDA concentration.

Physicochemical Treatment of Corn Harvest Residues

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ABSTRACT

Delignification of corn harvest residues with basic reagents has been studied. The analysis of the samples were performed by chemical methods and infrared spectroscopy. Increasing the base concentration the yield of extracted lignin increases. A complete delignification was attained with NaOH 5%. Linear correlations were obtained ploting absorbance ratios between characteristics peaks of lignin and cellulose, and lignin content.

1) INTRODUCTION

The obtention of raw materials for the chemical industry from lignocellulosic materials is a field which has deserved attention in recent times. The constituents of these materials can be converted in a considerable range of important chemical intermediates which are currently obtained from petroleum (1).

Agriculture residues represent a great potential to be used as alternative in the production of energy and food in the future (2). In Brazil these residues are either burned, causing polution problems, or incorporated in the soil where the process of degradation of these materials is very slow.

In order to use these materials in chemical processes a number of pre-treatments have been suggested, which can be classified in four main groups: mechanical, chemical, physical and biological (3).

Several researchers have used IR spectrometry to examine lignocellulosic materials. Absorbance ratios, such as 1310/900 cm^{-1}, 1500/900 cm^{-1}, 1600/900 cm^{-1}, 1730/900 cm^{-1} and 800/900 cm^{-1}, have been studied to determine lignin content (4-8).Gould et al (9), suggested that the relative lignin content could be determined by the frequency shift of peak maximum in the 2900 cm^{-1} region. Information about cristallinity degree of cellulose can be obtained by infrared spectra too, for example using the absorbance ratio 1370/900 cm^{-1} (10), 1430/900 cm^{-1}(11),etc.

In this work, the influence of treatments with

basic reagents in hemicellulose extraction and delignification of corn harvest residues was studied. The content of the main components (cellulose,hemicellulose and lignin) of these samples was analysed.

2) EXPERIMENTAL

The samples have been collected by hand in the field, washed, air dried, grinded with a rotating knife mill (Manesco & Raniery MR 340) and stored. Humidity was determined by conventional method at 105°C (1)

The chemical treatment was carried out with basic reagents (NaOH, NH_4OH and EDA) in autoclave at 121°C, varying the concentration of each base from 0.1 to 5.0%. The time of treatment was 15, 30 and 60 minutes. The concentration of hemicellulose in the samples before and after treatment was determined by treating 2,0000 g of sample with 20 ml of 2.5% H_2SO_4 at 121°C for 15 minutes (12).

The concentration of cellulose and lignin in the samples previously treated with H_2SO_4, was obtained by treatment with 20 ml of a mixture of HNO_3/AcOH/H_2O (1:8:2) in a water bath at 98°C for 30 minutes. Then the samples were washed with hot ethanol and water and dried at 50°C for 24 h.

The Klason lignin concentration in the samples (untreated and treated) with basic reagent was determined by treatment with 72% H_2SO_4 at 30°C for one hour, in a water bath with shaking, then quickly transferred to a one liter conical flask and 560 ml of distilled water was added. The mixture placed in a autoclave for one hour at 121°C. Then the sample was filtered, washed with hot water dried in an oven at 50°C (13).

The untreated samples and those treated with NaOH for 15 min at 121°C, and those from Klason lignin determination, were analised by infrared spectroscopy. Pellets were prepared with 100 mg of KBr and 1.5 mg of sample.

3) RESULTS AND DISCUSSION

The composition of different parts of the corn plant was firstly determined. The results given in Table (I) show small variations, the major difference was in the stalk with a lower hemicellulose content and higher

cellulose content.

Samples treated with NaOH aqueous solutions (0.1 - 5,0%) suffered mass lost as shown in Fig.1. When the NaOH concentration increases the mass lost shows a clear increasing tendecy and up to 15 min. of treatment time there is no variation.

Table I : Characterization of the samples from corn harvest residues.

	Hemicellulose (%)	Lignin (%)	Cellulose (%)
leaf	47	19	34
corncob	44	17	39
stalk	36	21	43
flower	47	21	32
straw	48	16	36
sample 2	42	19	39
sample 3	46	19	35

The hemicellulose and lignin content in samples treated with the NaOH solutions confirmed that both polymers were extracted during that treatment. This extraction is more efficient as NaOH concentration increases. The cellulose content didn't show variations.

Treatment with ethylenediamine and NH_4OH gave similar results, but showed lower extraction capacities.

Infrared spectra of the original corn harvest residues and of samples after NaOH treatment were run. Major alterations were observed in the 850-750, 1780-1480 and 3000-2800 cm^{-1} regions as showed in Fig.2. Intensity of peaks at 800, 1500 and 1600 cm^{-1} (assigned to aromatic ring) and 1730 cm^{-1} (assigned to C=O) decreases as NaOH concentration in the treatment increases.

In the region of 2900 cm^{-1} (aliphatic C-H)(Fig 2(c)) there is a shift in the band position with the simultaneous disappearance of the shoulder at 2840 cm^{-1} (MeO). This shift is probably due to a decrease in the number of metoxyl groups (lignin) in the sample treated with NaOH.

Figures 3 and 4 show that it is possible to estimate the relative concentration of lignin in the sample in a large concentration range simply correlating the characteristic absorption bands of lignin with the standard band at 900 cm^{-1}, or by the position of the bands in region of 2900 cm^{-1}.

The intensity of the band at 1370 cm^{-1}, relative to

the standard band at 900 cm^{-1}, versus concentration of NaOH (Fig. 5), shows that there is a reduction in the crystallinity of cellulose in the treated sample as NaOH concentration increases.

Analysis of the spectra of the Klason lignins obtained from untreated samples and of the samples treated with NaOH for 15 minutes at 121°C, showed that the lignin present had characteristics of softwood. In Fig. 6 it can be observed that the intensities of the bands at 2840, 1710, 1590 and 1460 cm^{-1}, compared with the standard band at 1500 cm^{-1}, increase with the increase of the concentration of NaOH treatment. This indicates that the residual lignin possesses a greater number of metoxyl groups per unity of aromatic rings, i.e., the lignin has a greater number of siringyl units than the untreated sample. It can be concluded that the treatment with NaOH favours the extraction of lignin with high number of guayacil units.

Acknowledgement
This work was supported by - CNPQ/CAPES/DQI/FUEM.

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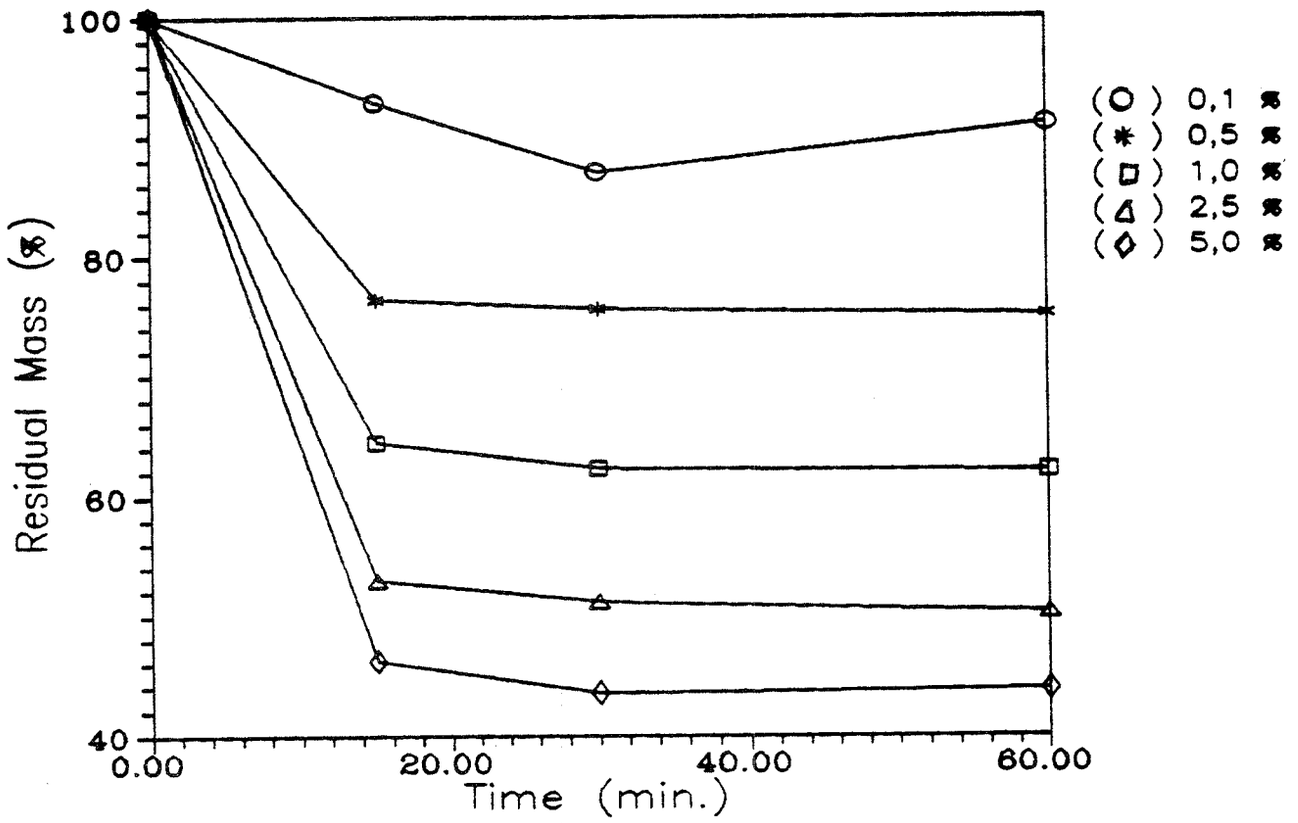

Fig.(1) : Residual mass in function of temperature of treatment with NaOH.

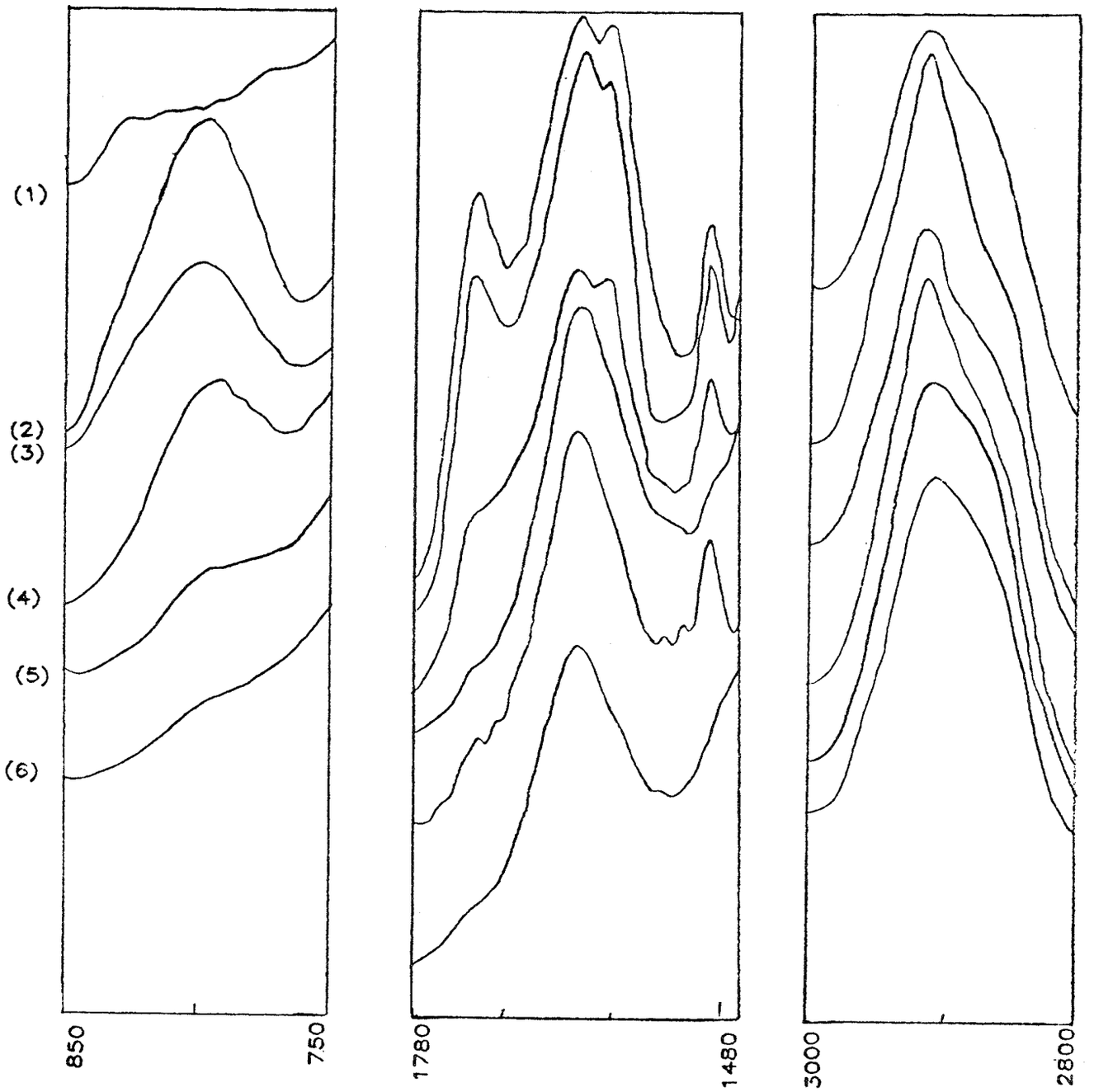

Fig.(2) : Infrared spectra of untreated sample (1) and samples treated with NaOH at 0,1% (2), 0,5% (3), 1.0% (4). 2.5% (5) and 5.0% (6).

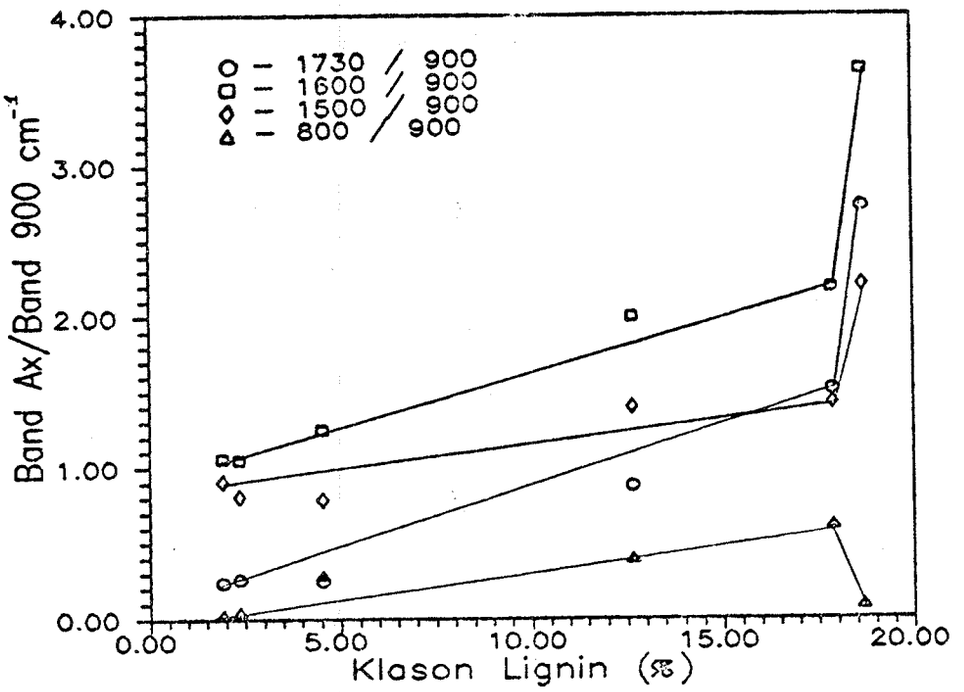

Fig.(3) : Ratio of the intensities of the bands Ax/intensity of standard band at 900 cm^{-1} in the infrared spectra in function of the Klason Lignin concentration (sample treated with NaOH).

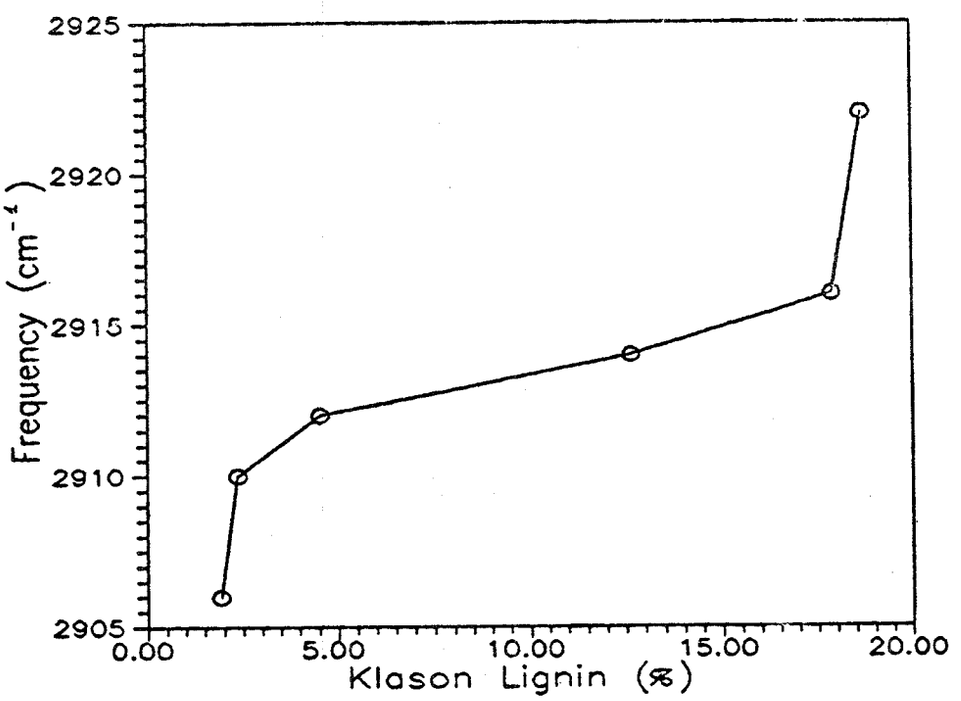

Fig.(4) : Frequency in the region of 2900 cm^{-1} in function of the Klason lignin concentration (sample treated with NaOH)

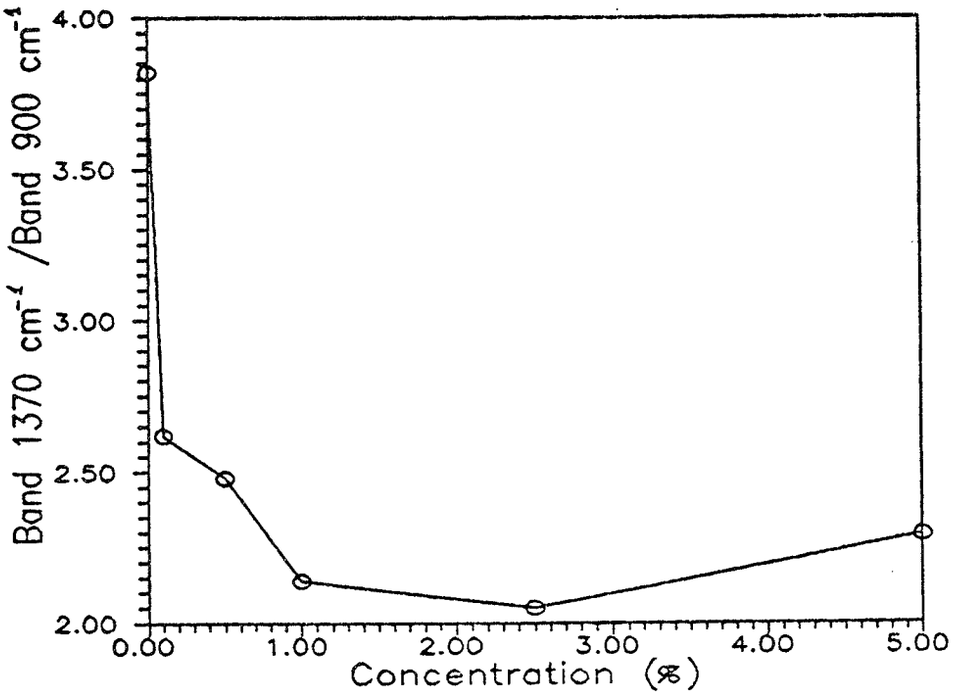

Fig.(5) : Ratio of the intensities of the band at 1370 cm^{-1} / intensity of standard band at 900 cm^{-1} of the infrared spectra in function of NaOH concentration.

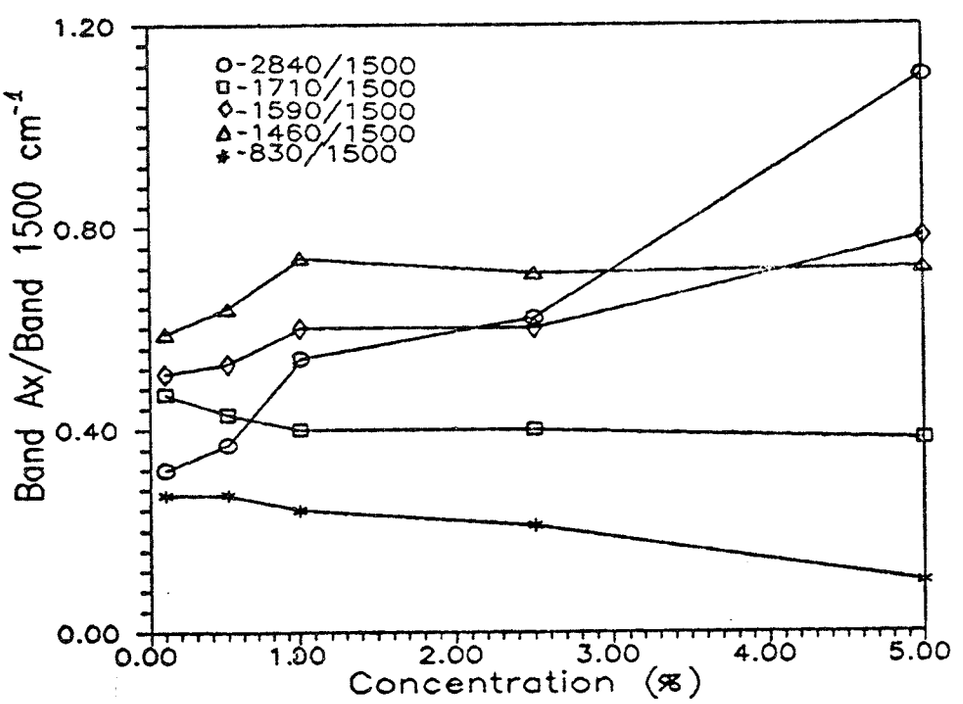

Fig.(6) : Ratio of intensities of the bands Ax/intensity of standard band at 1500 cm^{-1} of the infrared spectra in function of the NaOH concentration (samples of Klason lianin).

FORMIC ACID PULPING: DELIGNIFICATION OF *Eucalyptus grandis*

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ABSTRACT

Eucalyptus grandis wood was delignified with 80% formic acid, 0.1-0.2% (w/v) catalyst (HCl), 90 min of cooking at 90oC, 95oC and reflux temperature (ca. 107oC). The best results were obtained at reflux temperature and 0.2% HCl. For a sample of E.grandis chips furthermore soaked in licor for 3 h and preheated, the pulp yield was 55%, Kappa number 33, Klason lignin 10% and reject content 3%.

INTRODUCTION

As a nonconventional and less polluting pulping process,organosolv pulping methods have attained increasing interest in the last decade. Many organic lignin solvents have been proposed for use in organosolv pulping (1).

Pulping with formic acid offers some advantages over other solvents. Formic acid is a low cost chemical and the pulping process can be carried out at low temperature and atmospheric pressure.

Recently, we have been studying the delignification of *Eucalyptus grandis* with 99% and 86% formic acid. The best pulping results were obtained with 99% formic acid, 0.22% (w/v) HCl, at 95°C and 90min of cooking. Despite the good wood delignification, the pulp fibers were damaged. The critical variable of the fiber breaking was the material of the mechanical stirrer. Substitution of the glass rod and the teflon blade for stainless steel showed a benefical effect on the pulp fiber length and pulp yield (2).

In this work we evaluated the delignification of *E. grandis* sawdust and chips with formic acid: water mixture of 80:20 (v/v).

EXPERIMENTAL

The formic acid cooking of *E. grandis* (7 years old) sawdust and chips (15 x 10 x 2 mm) were done on a small scale. Two grams of wood were treated with 80% (v/v) formic acid, using a charge ratio of wood/solvent of 1/30 (g/ml) and 0.1 - 0.2% (w/v) HCl, at 90°C, 95°C and reflux

temperature (ca. 107°C), with or without mechanical stirring and 90 min of cooking. The rod and blade of the mechanical stirrer were of stainless steel or glass/teflon.

The yield (initial wood basis), the percentage of extracted formic lignin (initial wood basis) and the residual wood composition were considered in the evaluation of the delignification process. In the case of chips, the residual wood was divided into pulp and reject (% of wood basis). The rejects were discarded and the pulp analyzed.

Determination of cellulose (3), Klason lignin (ASTM D-1106-56), Kappa number (4) and fiber length (5) were carried out as described in the literature.

Residual lignin content and percentage of removed lignin were computed as follows:

Total resid. lig. = res. pulp lig. + res. reject lig. [1]

resid. pulp lignin = Klason lig. (%) x pulp yield (%) [2]

resid. reject lig. = Klason lig. in wood x reject (%) [3]

$$\text{Removed lignin} = 100\% - \frac{\text{Total residual lignin}}{\text{Klason lignin (\%) in wood}} \quad [4]$$

where, Klason lignin in wood = 26,7%.

The carbohydrate content of the samples were obtained by:

$$\text{Carbohydrate content (\%)} = 100\% - \text{Klason lignin} \quad [5]$$

The residual carbohydrate content and percent of removed carbohydrate was estimated as follows:

$$\text{Total res. carb.} = \text{res. pulp carb.} + \text{res. reject carb.} \quad [6]$$

$$\text{res. pulp carb.} = \text{carb. content (\%)} \times \text{pulp yield (\%)} \quad [7]$$

$$\text{res. reject carb.} = \text{holocellul. in wood} \times \text{reject (\%)} \quad [8]$$

$$\text{Removed carb.} = 100\% - \frac{\text{Total residual carbohydrate}}{\text{Holocellulose (\%) in wood}} \quad [9]$$

where, Holocellulose (%) in wood = 72,9%.

The selectivity ratio was defined by:

$$\text{Selectivity ratio} = \text{Removed lig./Removed carbohydrate} \quad [10]$$

RESULTS AND DISCUSSION

The results of the delignification process on *E. grandis* sawdust with 80% formic acid are shown on Table I. The selected experimental conditions were close to those optimized for 99% formic acid. The best results were obtained at reflux temperature and 0.22% HCl (assay 6). Increasing the cooking temperature from 90°C to reflux temperature (ca. 107 °C) improved the lignin extraction (Fig. 1.).

Table I. Delignification of *E. grandis* sawdust

ASSAY	1	2	3	4	5	6
CONDITIONS*						
Temperature (°C)	90	90	95	95	reflux	reflux
HCl(%)(w/v)	0.13	0.22	0.22	0.22	0.13	0.22
Stirrer&	none	none	none	S/S	none	none
RESULTS (%)						
Yield	59.2	56.8	52.7	57.4	47.3	46.6
Formic lignin	9.7	11.9	13.9	12.2	15.6	16.5
Klason lignin	16.8	15.4	11.0	16.3	8.1	7.5
Cellulose	81.2	84.1	90.7	84.6	94.4	94.7
Removed lignin	62.8	67.2	78.3	65.0	85.7	86.9
Removed carboh.	32.4	34.1	35.7	34.1	40.4	40.9
Sel. ratio (L/C)	1.94	1.97	2.19	1.91	2.12	2.12

*Unextracted sawdust (40-60 mesh), charge 1/30 (g wood/ml), 90 min of cooking.
&S/S = stainless steel rod and blade, none = without stirring.

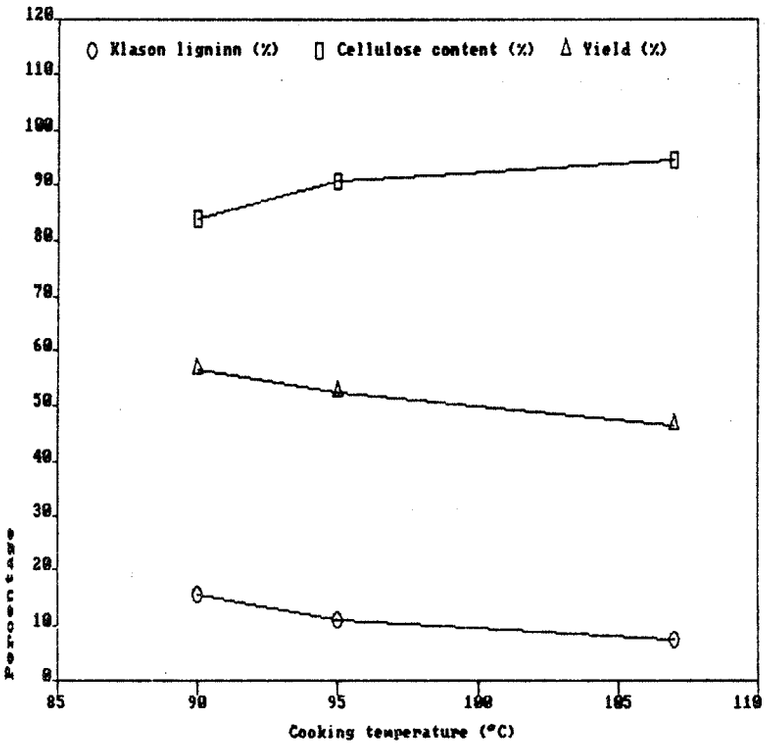

Fig. 1. Effect of the cooking temperature on the yield (Δ), cellulose content (□) and Klason lignin (○) of the formic acid treated sawdust (80% HCOOH, 0.22% HCl, charge 1/30, 90 min, without stirring).

The chips pulping was carried out under the best conditions selected for sawdust. Table II shows the results obtained with chip impregnation and mechanical stirring. Another variable of the process was the material of the mechanical stirrer. The smallest Kappa number and reject content were obtained with a glass rod and teflon blade, instead of stainless steel. The fiber damage

previously observed on the 99% formic acid pulping was absent with the 80% formic acid treatment. The integrity of the pulp fibers, evaluated by the average fiber length, was preserved, regardless of the material of the mechanical stirrer.

Table II. Pulping of *E. grandis* chips with 80% formic acid

ASSAY	1	2	3	4
CONDITIONS*				
Impregnation	--	liquor	liquor	liquor
Impreg. time (h)	--	3	3	3
Rising temp. time (h)	--	--	--	2
Stirrer (rod/blade)&	S/S	S/S	G/T	G/T
RESULTS (%)				
Residual wood	63.8	68.2	50.5	55.2
Reject	40.5	52.3	0.5	3.1
Pulp yield	23.3	15.9	50.0	52.1
Formic lignin	11.5	9.6	17.4	15.4
Klason lignin	14.4	15.1	9.2	9.7
Cellulose	87.3	86.2	93.4	93.7
Kappa number	56.6	61.2	39.1	33.3
Fiber length (um)	711±267	793±231	585±197	700±255
Removed lignin	46.9	38.7	82.3	78.0
Removed carboh.	32.1	29.2	37.2	32.4
Sel. ratio (L/C)	1.46	1.33	2.21	2.41

*0.22% HCl, charge 1/30 (g wood/ml), reflux temperature, 90 min of cooking.
&S/S = stainless steel rod and blade, G/T = glass rod and teflon blade.

Table III summarizes the results of the pulping studies with 99% and 80% formic acid.

Table III. Summary of the best pulping results

ASSAY	1	2	3	4
CONDITIONS*				
HCOOH (%)(v/v)	99	99	80	80
Impregnation	--	--	liquor	liquor
Impreg. time (h)	--	--	3	3
Rising temp. time (h)	--	--	--	2
Maximum temp. (^{o}C)	95	95	reflux	reflux
Charge (g wood/ml)	1/10	1/30	1/30	1/30
Stirrer (rod/blade)&	S/S	S/S	G/T	G/T
RESULTS (%)				
Residual wood	42.9	51.9	50.5	55.2
Reject	0.1	3.5	0.5	3.1
Pulp yield	42.8	48.4	50.0	52.1
Formic lignin	23.4	20.3	17.4	15.4
Klason lignin	8.4	10.7	9.2	9.7
Cellulose	88.3	85.2	93.4	93.7
Kappa number	31.3	39.1	39.1	33.3
Fiber length (um)	286±132	640±201	585±197	700±255
Removed lignin	86.4	77.1	82.3	78.0
Removed carboh.	46.1	37.2	37.2	32.4
Sel. ratio (L/C)	1.87	2.07	2.21	2.41

*0.22% HCl, 90 min of cooking at maximum temperature.
&G/T = glass rod and teflon blade, G/S = glass rod and stainless steel blade.

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PULPING OF **PINUS RADIATA** D. DON WITH ETHYLENEDIAMINE-SODA AND NON TRADITIONAL BLEACHING SEQUENCE

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ABSTRACT

The effect of cooking time and ethylenediamine concentration on the pulping of **Pinus radiata** D. Don by using MeOH-H_2O-NaOH (NaOH 10% o.d.w.) were analyzed. Pulping at 170°C, 670 kPa, 45.5% (v/v) of amine and a charge liquor/wood of 10, yielded 53 -57%, with Kappa number between 18 - 30 depending on the cooking time and no reject was observed. The intrinsic viscosity of the pulp (cooking 120 min, Kappa 30) was 839 ml/mg. The pulps produced possessed higher tear but lower burst and tensile strenghts than kraft pulps. Pulps were bleaching by a sequence free of chlorine, by using oxygen reinforced with hydrogen peroxide.

INTRODUCTION

Addition of certain amines during alkaline pulping increased the rate and selectivity of delignification of different wood species [1]. From kinetic studies, using both hardwoods and softwoods, have been demostrated that the accelerating effect of a series of primary amines is dependent on the molar concentration of amino groups, and almost independent of the alkyl group, suggesting that the influence of the primary amine is achieved by chemical, rather than physical process [2,3].

In recent years, effluents from the bleaching of chemical pulps have become the focus of enviromental concern, and an elimation or reduction in the consumption of chlorine during the bleaching has been suggested as a way to reduce the formation of chlorinated organic compounds resulting from the first-stage chlorination and subsequent alkaline extraction of the traditional sequence (CEDED) [4,5]. The oxygen is one chemical attracting alternative in nonchlorine bleaching delignification. Using hidrogen peroxide to reinforce the oxygen delignification has yielded kraft pulps lower in kappa numbrer and with viscosities of the pulps only slighly lower.

The purpose here is to report the results from a study into the effects of cooking time and ethylenediamine (EDA) concentration on the pulping of **Pinus radiata** D. Don (growing in Chile) by using MeOH-H_2O-NaOH (NaOH 10% o.d.w.). Pulps obtained by alkaline delignification in aqueos methanol

and EDA were bleaching by a sequence free of chlorine, by using oxygen reinforced with hydrogen peroxide (PO) [6]. The PO-PO sequence was compared with the oxygen delignification (O-O sequence).

EXPERIMENTAL PART

Delignification experiments of **Pinus radiata** D. Don (growing in Chile) were carried out in a reactor Parr of 1 liter by a mixture of MeOH-H_2O-EDA-NaOH at 170°C. The amine concentration range between 0 - 45.5% (volume). The conditions used in each experiment are indicated in the Results section. Experiments were also carried out by using a reactor of 15 liters, at the same temperature with a charge of 4.5. The pulps (5-10 g) were bleaching by using oxygen sequences (O-O) and hydrogen-peroxide reinforced sequences (PO-PO) under the following conditions: 85°C, consistence 10%, oxygen pressure 110 psi, H_2O_2 0.5% (on o.d.pulp basis), NaOH 2% (on o.d.pulp basis), time 30 min each stage, $MgSO_4$ 0.5% (on o.d.pulp basis) [6]. Strength properties, kappa number and CED viscosity were evaluated following the TAPPI procedures [7,8].

RESULTS AND DISCUSSION

The effect of the sodium hydroxide concentration on the screened yield, reject and kappa number of pulping of pine chips with methanol-NaOH mixtures is shown in Table I. The cooking was performed at 170°C, 180 psi, for 150 min, with a liquor/wood ratio equal to 7:1. In general, the chips were not sufficiently delignified and the rejects are high, but increasing the concentration of base to 20% the yield was of 54.8% and the reject of 2.5%, the kappa number of the resulting pulp was 60.6 and the length of fibers was high (2.72 mm).

TABLE I

Effect of the NaOH concentration on MeOH-NaOH pulping of pine chips

NaOH % (o.d.w)	Screened yield %	Rejects %	Kappa number
5	7.82	75.7	75.8
10	12.18	71.3	-
15	16.5	61.1	-
20	54.8	2.5	60.6

Another series of cooks was made using MeOH-EDA (withouth NaOH). The cooking was performed with a MeOH/EDA ratio equal to 1:5 gave a 58.2% reject, 19.3% of screenend yield, the chips were not sufficiently delignified to allow fiberization and kappa number determination to be made in the pulp. The addition of EDA to the system MeOH-NaOH cook greatly increases the selectivity of the system with respect to lignin and stabilize hemicelluloses towards alkaline peeling reaction. The results obtained for screened yields, rejects, and kappa number when the chips were pulped with MeOH-NaOH-EDA are shown in Table II. This series of cooks varying concentration of EDA (in the range 1.5 -45.5%) were performed at 170°C, 10% NaOH (o.d.w), with a liquor/wood ratio 10:1 and cooking time 120 min. The rate of delignification increase significatively with increasing the concentration of EDA. A screened yield of 57%, no reject and pulps with kappa number 30 were obtained at 45.5% EDA. It is interesting to note that at lower EDA concentrations the cooked chips were delignified, but the kappa number are higher, for example at 2.8% EDA the yield was 53.5%, rejects 3.5%, but the kappa number was 84.

TABLE II

Effect of EDA concentration on delignifcation of Pinus radiata for MeOH-NaOH-EDA system

EDA (%)	Screened yield (%)	Rejects (%)	Kappa number
45.5	57.0	0	30
22.6	52.4	0.1	42
11.3	54.3	0.1	53
5.0	58.0	0.5	59
2.8	53.5	3.5	84
1.5	35.5	35.5	96

A series of cooks was made at varying cooking times between 120 and 210 min (the conditions are the same that in Table II). The results obtained are resumed in Table III. Much lower kappa number were obtained by increasing the cooking times, and all the screened yields were higher that 50%. These results shown that it is possible to obtain pulps with lower kappa numbers by using lower amine concentration by increasing the cooking time. For example, the kappa

number (27) obtained with 22.6% EDA in 210 min is lower than that obtained with 45.5% EDA in 120 min (30). At this last EDA concentration in 210 min the kappa number of the pulps obtained was 18.

TABLE III

Effect of the cooking time on the pulping of pine with the system MeOH-NaOH-EDA

EDA (%)	Tiempo (min)	Screened yield (%)	Kappa number
45.5	120	57	30
	150	54	23
	180	52	21
	210	53	18
22.6	120	52	42
	150	53	43
	180	56	37
	210	51	27

The physicomechanical properties of the pulp obtained using 45.5% EDA, under the conditions indicated on Table III (120 min) are given on Table IV. The results obtained when the process was scale up to 15 liters, at the same temperature, using a rate liquor/wood 4.5:1 (the other conditions are the same) are included in the same table.

TABLE IV

Physicomechanical properties of the pulp

Properties	PULP	
	1 liter reactor	15 liters reactor
Fiber lenght (mm)	2.39	2.49
Tear factor	159.5	182.1
Burst factor	16.6	27.5
Tensile strenghtt (Km)	3.1	5.2

The pulps produced at the optimun conditions possessed higher tear but lower burst and tensile strenghts than kraft pulps, similar to the results obtained with southern pine by Green et al.[1]. As can be seen in Table IV, the pulp obtained in the reactor of 15 liters had better properties than that obtained in the reactor of 1 liter.

Samples of the screened pulps obtained above were bleached with oxygen sequences (O-O) and with hydrogen peroxide-reinforced oxygen sequences (PO-PO). Delignification were carried out on pulps kappa number 72, 40 and 20. The quality of the pulps were partially evaluated through the viscosities and kappa number determinations. The results obtained in the O-O and PO-PO bleaching sequences (pulp kappa number 72, 85°C, 30 min) are shown in Table V and Table VI, respectively. In a four-stage, hydrogen-peroxide reinforced oxygen delignification , kappa number reduction was of 67.4%, and the pulp viscosity was 18.4 cps. On the other hand, by a four-stage O-O process the delignification achieved was of 54.3%, and the viscosity of the pulp was 24.1 cps. A similar behavior was observed with pulp kappa number 40. Increasing the time of delignification from 30 min to 60 min (kappa number 72) gave pulps with similar viscosity and the kappa number (60 min: 66% reduction of kappa number and 19.2 cps; 30 min: 67.5% reduction of kappa number and 18.4 cps). In a two-stage, hydrogen-peroxide reinforced oxygen delignification at 110°C, 30 min, the reduction of kappa number was of 50.8%, but the viscosity, as consequence of a high degradation of the pulp, was only 13.0 cps.

TABLE V

Four-stage oxygen delignification
(30 min, kappa number 72)

Stage	Kappa number	Total deslignif. (%)	Viscosity (cp)
O	62.1	13.8	*
O	54.1	24.9	*
O	45.0	37.5	25.0
O	32.9	54.3	24.1

* Insoluble in copper(II) ethylenediamine 0.5%.

TABLE VI

Four-stage hydrogen-peroxide reinforced oxygen delignification (30,min, kappa number 72)

Stage	Kappa number	Total deslignif. (%)	Viscosity (cps)
PO	56.6	21.4	*
PO	43.4	39.7	*
PO	30.1	58.2	20.1
PO	23.5	67.4	18.4

* Insoluble in copper(II) ethylenediamine 0.5%

Fig. 1 shown the rates of one stage-delignification achieved with caustic soda alone, which is of 5.3%. The oxygen increases this delignification to 13.8%, but the addition of small amounts of hydrogen peroxide the delignification increase to 21.4%. These results demonstrate that the effects of caustic soda, oxygen and hydrogen peroxide are addittive instead of a synergic effect.

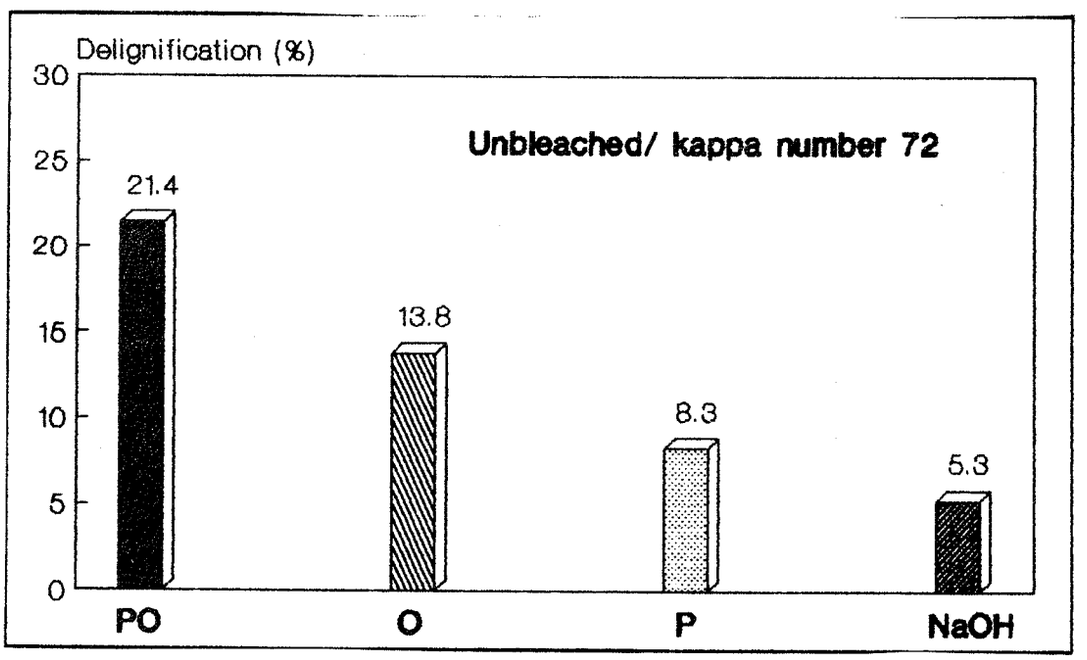

FIG. 1 **Effect of the addition of hydrogen peroxide (0.5% o.d.w) on the delignification of Pinus radiata D. Don pulps with oxygen**

SUMMARY

The concentration of the base in microscopic level would be a crucial factor in determining the averall rate process. The apparent sodium hydroxide adsorbed for the wood depends on the concentration of the amine. At high EDA concentration the effective sodium hydroxide concentration in the chip increases, resulted in an increase of the deliginification rate, in cellulose degradation and the loss of pulp viscosity.

The pulps produced at the optimun conditions possessed higher tear but lower burst and tensile strenghts than kraft pulps. The pulps produced in the process scaled up to 15 L showed better properties.

The addition of hydrogen peroxide in small amounts (0:5%) improved the properties of oxygen bleached. Delignification achieved in the pulps bleached by PO-PO sequence was 13 % higher compared to the oxygen stages without peroxide.

Replacing chlorine by oxygen and hydrogen peroxide bleaching enables a processing of effluents with few problems with regard to enviromental protection, since the TOX-values (organically bound chlorine) are reduced.

The effects of sodium hydroxide, oxygen and hydrogen peroxide in the delignification process are additive.

The hydrogen peroxide added to the oxygen stages improved the selectivity of pulping enhacing delignification, but some viscosity losses are observed. In order to have preservation or lower losses in viscosity of the pulps, a possible option is include a dioxide chloride stage as an intermediate or final stage, depending on the viscosity. The TOX-values can be reduced to a very low value.

Further work is in progress, the PO bleaching will be optimized and especifically for pulps of kappa number 20, in order to get high bleached paper making pulps.

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SUCCESSIVE ORGANOSOLV EXTRACTIONS OF LIGNINS FROM *EUCALYPTUS GRANDIS*

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ABSTRACT

Eucalyptus grandis has been extracted successively with dioxane/aqueous 2N HCl (9:1) solution. The lignins obtained were analyzed by high performance size exclusion chromatography, elemental analysis, methoxyl and phenolic hydroxyl contents. The Klason lignin contents in residual wood has been also determined. The functional group distribution of the extracted lignins show differences depending on the extraction time. The observed increase in molecular weight suggests that higher molecular weight lignin fragments are liberated in the last extractions.

INTRODUCTION

The study of organosolv delignification processes has increased in the last years. These delignification processes can be performed in a batch, a flow or a successive mode.

In a batch process, the condensation reactions between lignin fragments and between lignin fragments and polysaccharides are known to occur (1).

In successive extraction experiments, as well as in flow extraction processes, the lignin concentration is low and the residence time in the solution is short and

thus the condensation reactions are minimized.

As the condensation reactions affect the delignification and also the molecular weight of the extracted lignins, the occurrence of these reactions have to be minimized in order to achieve a great extent of delignification and to study the characteristics of the extracted lignin.

A few papers concerning successive organosolv processes are found in the literature (2,3) and these are related to the analysis of softwoods.

In this work, lignin organosolv extractions from *Eucalyptus grandis* were performed in a successive process and the influence of the extraction time on the characteristics and molecular weights of the obtained lignins were studied.

EXPERIMENTAL PART

Lignin extractions

Eucalyptus grandis chips were pre-extracted with cyclohexane/ethanol (1:1) during 48 hours and with water during 30 hours. The pre-extracted chips were submitted to six successive extractions with dioxane/aqueous 2N HCl (9:1) solution (final concentration = 0.2N HCl). The extractions were performed at reflux temperature (89 - 90°C) during one hour. The liquor to wood ratio used in all extraction was 10:1.

After each extraction, the residual wood was filtered, washed two times with dioxane/water (9:1) and for 6 hours in soxhlet. A residual wood sample was then separated for analysis. The filtrated and washings were

concentrated to ca. 100 ml under reduced pressure and poured into 2000 ml of water with stirring. The precipitated lignin was filtered, washed with water and dried to constant weight in a desiccator containing phosphorus pentoxide. The remaining residual wood was submitted to another extraction and this procedure was repeated 5 times.

Lignin analysis

The obtained lignins were characterized by elemental analysis, methoxyl and phenolic hydroxyl contents and molecular weight determinations by high performance size exclusion chromatography.

Elemental analysis were performed by the Analytical Center of the "Instituto de Química (USP)" in a Perkin Elmer Elemental Analyzer 2400 CHN.

Methoxyl contents were determined by TAPPI standard method T2m-60.

Phenolic hydroxyl contents were determined by a modification of Sarkanen and Schuerch method (4). A lignin sample (100 mg) was dissolved in acetone (3 ml) and ethanol was added (6 ml). Next, water was added dropwise, with constant stirring. The resulting suspension was titrated condutimetrically, at 20°C, with standard sodium hydroxide solution (0.10N). All the solid material dissolved before the titration end point was reached.

The lignin molecular weights were determined in a Waters chromatographic system with a U6K injector, UV detector model 440 (254 nm) and a pump model 6000A. The conditions used were the following:

eluent: THF (1 ml/min)

sample concentration : 2.5 mg/ml

injection volume: 5 µl

columns: PLgel 10^3Å (5µm),500Å (10 µm),100Å (10µm)

standards: polystyrene with nominal molecular weights of 68,000; 28,000; 12,500; 7,600; 3,770; 1,800; 1,050; 580 and ethylbenzene (106).

Lignin content in residual wood

The lignin contents in residual wood were determined by the Klason method (TAPPI standard method T 13m - 54)

RESULTS AND DISCUSSION

Yields and lignin content in residual wood

The lignin and residual wood yields obtained are shown in Table 1 and in Figure 1.

Table 1. - Obtained residual wood and lignin yields

extraction time (h)	residual wood yield (%)	lignin yield (% Klason*)
1	70.8	32.8
2	58.9	47.8
3	55.7	57.1
4	53.8	61.6
5	48.7	64.9
6	47.5	66.4

* Klason lignin content in wood = 26.8%

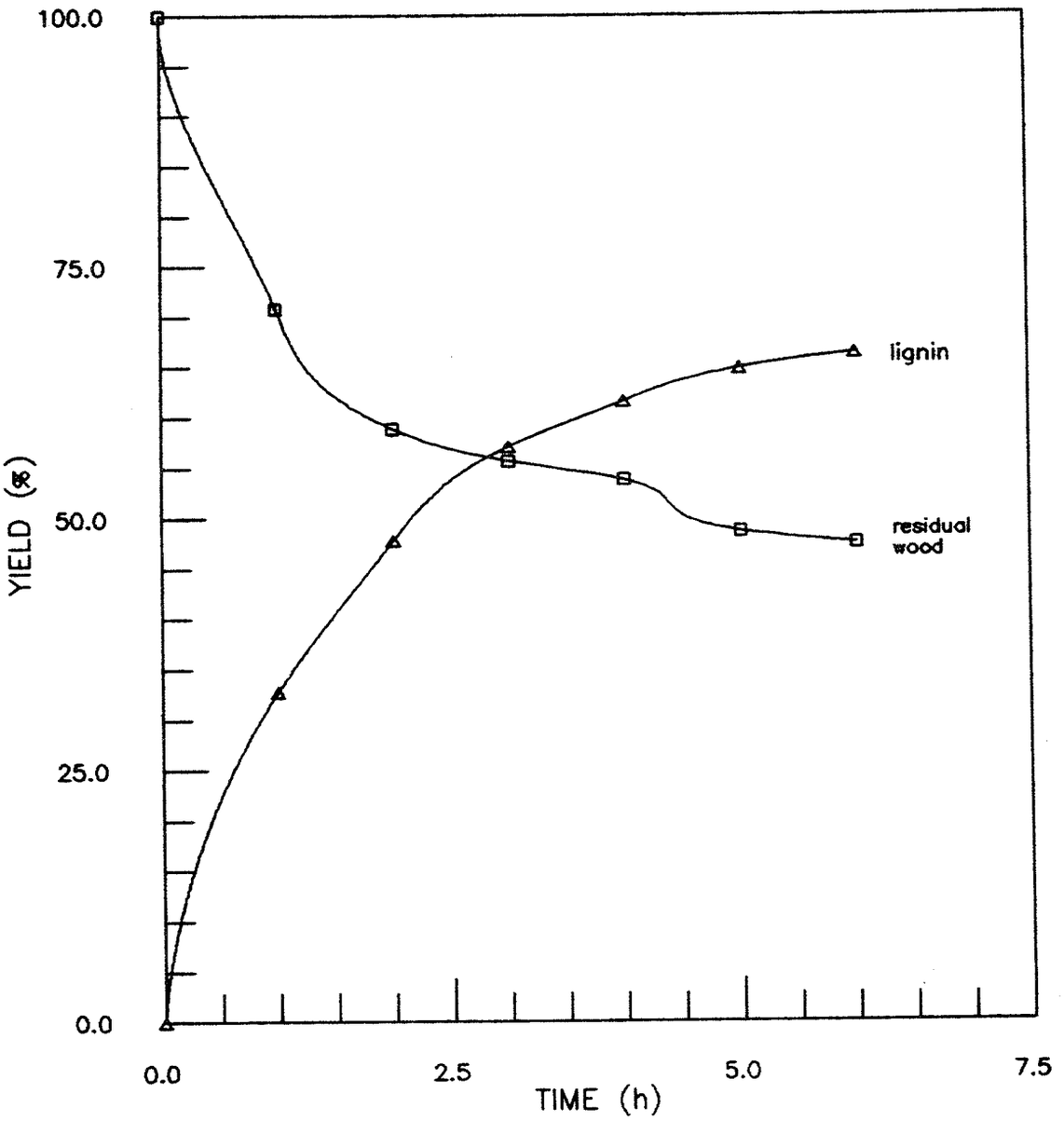

Figure 1 - Residual wood and lignin yields obtained in the successive extractions

The lignin yield increases asymptotically till 70%, indicating that higher delignification would need more drastic conditions.

The residual wood yields decrease homogeneously until the fourth extraction, changing this behavior between the fourth and fifth extractions. This change can be related to cellulose degradation.

Elemental analysis and functional group distribution

Table 2 shows the data obtained for elemental analysis, methoxyl and phenolic hydroxyl contents as C_9 formula. As the carbon content of the obtained sample in the sixth extraction was too low (38%), indicating a high degree of contamination, this sample was discarded.

Table 2 - C_9 formula of the obtained lignins

extraction time (h)	C	H	O	OCH_3	OH_{fen}
1	9	7.22	2.96	1.42	0.47
2	9	7.09	2.97	1.36	0.42
3	9	7.69	2.89	1.33	0.44
4	9	7.25	2.89	1.33	0.48
5	9	7.30	2.89	1.29	0.62

The functional group distribution showed differences with the extraction time as consequence of a decrease in methoxyl content and an increase in the phenolic hydroxyl content. These data can be related to topochemical effects, showing a preferential remotion of lignin from different morphological regions depending on the extraction time

Molecular weights by high performance size exclusion chromatography (HPSEC)

The obtained lignins were partially soluble in THF, solvent which is commonly used in HPSEC experiments, and this solubility decreased with the extraction time. The data obtained from HPSEC measurements for the soluble fraction are shown in Table 3.

Table 3. Number average ($\overline{Mn}$) weight average ($\overline{Mw}$) molecular weights and dispersities ($\overline{Mw}/\overline{Mn}$)

extraction time (h)	$\overline{Mn}$	$\overline{Mw}$	disp.
1	1037	2524	2.43
2	1299	3582	2.76
3	1278	3810	2.98
4	1258	3879	3.08
5	1428	4643	3.25

The smaller molecular weight by number ($\overline{Mn}$) obtained in the first extraction is due mainly to the "dimer" fraction that is more pronounced in the molecular weight distribution of this lignin sample.

Since in successive experiments the condensation reactions are minimized, the increase in molecular weight by weight ($\overline{Mw}$) and consequently the increase in dispersity, with increasing extraction time are due to higher molecular weight lignin fragments liberated in the last extractions.

CONCLUSIONS

The obtained data from lignin and residual wood yields indicate that cellulose loss can start to be significant after the fourth successive extraction and to obtain a higher delignification it is necessary to use more drastic conditions.

Due to topochemical effects,the obtained lignins showed differences in the functional groups distributions (phenolic hydroxyl and methoxyl contents) with the increase of the extraction time.

Considering that in successive experiments, using low acid concentrations, the condensation reactions are minimized, the obtained results from HPSEC measurements suggest that higher molecular weight lignin fragments are liberated in the latter extractions. This conclusion is in agreement with that one reported by Funaoka an Abe (3) for softwood extracted by successive acidolysis.

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DIOXAN LIGNINS FROM PINUS CARIBAEA HONDURENSIS
PART B. EFFECT OF THE EXTRACTION TIME

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ABSTRACT

Lignins were extracted from *Pinus Caribaea Hondurensis* using dioxan/aqueous 1.0 N HCl (9:1) solution at different extraction times (0.5-5.0 hours).

The obtained lignins were characterized by elemental analysis, methoxyl and phenolic hydroxyl contents. The molecular weights and molecular weight distributions were determined by HPSEC.

INTRODUCTION

Lignin is heterogeneously distributed within the cell walls and middle lamella. In the cell corners and middle lamella the lignification is more pronounced than in the secondary wall (1,2).

The lignin concentrations in the middle lamella, cell corners and secondary wall are approximately 50-60%, 80-100% and 22-24% respectively in softwoods (1,2). However the bulk of the lignin is found in the secondary wall, because of its dominant volume. In Black Spruce the secondary wall contain about 72% of the total lignin in the earlywood tracheids, and about 82% of the total lignin in the latewood tracheids (1,2).

Besides the lignin distribution, lignins in different morphological regions differ considerably in terms of chemical characteristics, mainly in hardwood species.

There is a high portion of syringyl units in the secondary walls in hardwood species, while the lignin of the middle lamella is rich in guaiacyl units. In softwood species it was observed that the phenolic hydroxyl content in the secondary wall lignin is higher than in the middle lamella lignin (1,2,3).

There are studies which propose that the macromolecular structure of lignin in the secondary wall is different than the lignin in the middle lamella (4).

The evidences that lignin in the middle lamella is distinct from that in the secondary wall, has suggested that delignification of the middle lamella and the secondary wall occurs in different stages.

Studies of the topochemistry (5,6) of the delignification in the Kraft and acid sulfite pulping, using UV-microscopy, showed that the lignin of the secondary wall was dissolved before any major dissolution of the lignin from the middle lamella. In the neutral sulfite (7) a little preference was found for the secondary wall lignin remotion up to 50% delignification. An absence of the topochemical effect was found on acid chlorite delignification (7).

Saka et al (7) showed that soda/AQ pulping was much more selective in the remotion of the lignin from the middle lamella than soda or Kraft pulping without catalyst.

Paszner and Behera (7) studied the topochemistry of softwood delignification by Alkali Earth Metal Salt Catalyzed Organosolv pulping and showed that, in this process, lignin remotion occurs preferentially from the middle lamella during the first stage.

In this work we studied the organosolv delignification of *Pinus Caribaea Hondurensis*, using a dioxan/water mixture with HCl as catalyst at 5 different

extraction times.

The occurrence of topochemical effect was investigated through the lignin yields, as well as by functional group and molecular weight determinations of the isolated lignins.

EXPERIMENTAL

Lignin Isolation

Pinus Caribaea Hondurensis sawdust (50g, 100 mesh), pre-extracted with cyclohexane/ethanol (1:1) and water, was extracted with dioxan/aqueous 1.0 N HCl (9:1) solution at boiling temperature at different times (0.5; 1.0; 2.0; 3.0 and 5.0 hours). The residue was filtered, concentrated and poured into 1200 ml of water. The precipitated lignin was filtered, washed with water and dried over P_2O_5.

Lignin Analysis

Phenolic Hydroxyl Content: The phenolic hydroxyl content was determined by condutimetric titration (8).

Methoxyl content: The methoxyl content was determined according to TAPPI 2m-60 method (9).

High Performance - Size Exclusion Chromatography (HPSEC): The molecular weight and molecular weight distribution of the lignins were determined by HPSEC, using the following conditions: Detector: UV 254 nm - Waters mod. 440; Eluent: THF; Flow Rate: 1 ml/min; Sample Concentration: 2.5 mg/ml; Injection Volume: 5μl; Column: PL-gel 10^3, 500 and 100 Å; Calibration Curve: polystyrene Standards with molecular weights of 68,000; 28,000; 12,500; 7,600; 3,770; 1,800; 1,050; 580 and 106 (ethyl benzene).

RESULTS AND DISCUSSION

Elemental Analysis and Methoxyl contents: The results are shown as C_9-formula (Table 1).

Table 1: C_9-formula of the obtained lignins.

Extraction Time (hours)	C	H	O	OCH_3
0.5	9	8.67	3.07	0.87
1.0	9	8.57	3.23	0.88
2.0	9	8.46	3.03	0.86
3.0	9	8.07	3.12	0.89
5.0	9	8.06	3.01	0.88

These results are in agreement with the expected values for softwood lignins (16) and show that the obtained lignins have approximately the same chemical composition.

Phenolic Hydroxyl Content: The phenolic hydroxyl content of the obtained lignins were in the range of 4.3-4.8% corresponding to 0.48-0.55 OH/C_9.

Lignin Yields: The lignin yields increase with the extraction time, as showed in Table 2 and Figure 1.

Table 2: Lignin and residual wood yields.

Extraction Time (hours)	Lignin Yield		residual wood yield (%)
	% wood	% Klason	
0.5	3.8	12.7	83.3
1.0	4.7	15.7	80.6
2.0	6.1	20.4	70.6
3.0	7.6	25.4	69.9
5.0	9.4	31.4	65.1

This result agrees with other experimental data (10-13) and with delignification kinetic theories (11,14), showing that the delignification rate decreases with the extraction time.

The Klason content of the isolated lignins showed that all the samples contain approximately the same (7%) quantity of the carbohydrate residues.

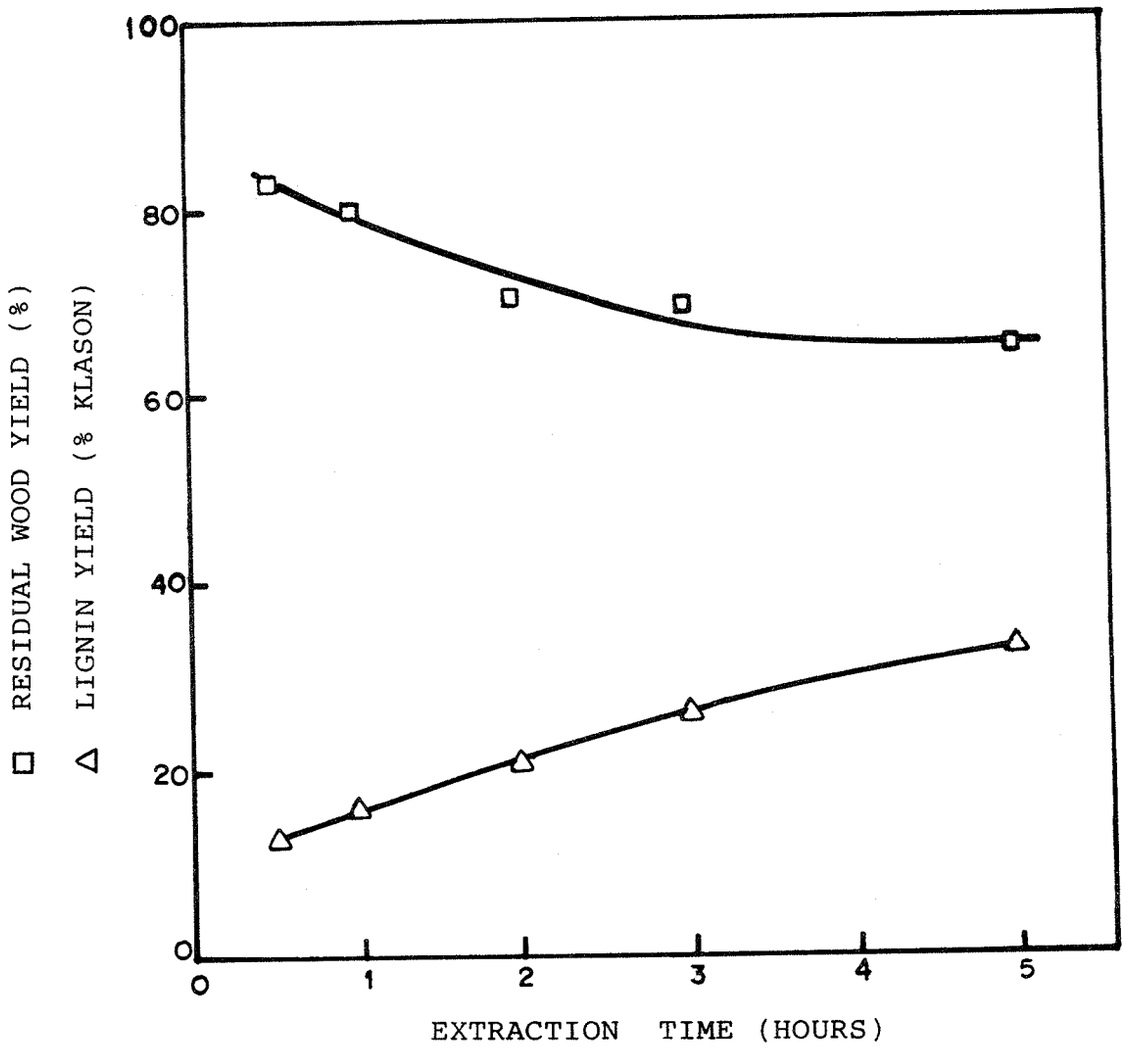

Figure 1: Lignin and Residual Wood Yields

The low value of the lignin yield (31.4% based on Klason lignin) after 5 hours of reaction indicates that the HCl concentration (0,1N) was insufficient to promote pronounced delignification. Pla and Robert (10) obtained similar results using the same HCl concentration (0.1N) in Spruce delignification using dioxan/water (10:1) at 70°C. Other studies (11,12,13,15) showed that higher HCl concentration is necessary to increase the delignification.

The residual wood yields (Table 2 and Figure 1) decrease asymptotically to 65% with the extraction time. After 5 hours the carbohydrate losses reach approximately 25%, indicating the occurrence of polyoses and cellulose degradation.

The limited lignin yield obtained (≈ 30% based on klason lignin) and the similar chemical composition indicate that these lignins came from the same morphological region. The lignin content in the middle lamella of the softwood species is about the same that the maximum lignin yield obtained, indicating that this lignin probably came from the middle lamella region. This result is in agreement with the studies of Paszner and Behera using organosolv process, showing that delignification occurs preferentially in the middle lamella during the first stage of the pulping process.

Average Molecular Weights ($\bar{M}n$ and $\bar{M}w$) and Molecular Weight Distribution: The obtained chromatograms for the lignins are very similar and the $\bar{M}n$ and $\bar{M}w$ calculated from them are shown in Table 3.

In other published works (10,11,13) the average molecular weight reported increases with the extraction time, in agreement with the delignification theories (4). Argyropoulos and Bolker, through lignin recooking experiments,observed that self-lignin condensation reactions

occur during the extraction.

Table 3: Average molecular weights of the obtained lignins.

Extraction Time (hours)	$\bar{M}n$	$\bar{M}w$
0.5	1520	4760
1.0	1430	5390
2.0	1490	4780
3.0	1430	4110
5.0	1470	3290

In this work was observed that the weight average molecular weight decreases, indicating that the solubilized lignins are degraded with increase of the extraction time and that the used conditions are not able to promote condensation reactions.

CONCLUSIONS

The lignin yields show that the delignification rate decreases with the extraction time and that the used conditions (low temperature and catalyst concentration) were insufficient to promote pronounced delignification.

The degree of delignification as well as the analytical results suggest that the extracted lignins came from the same morphological region (middle lamella).

The weight average molecular weight decreases with the extraction time, indicating that the solubilized lignins are further depolymerized in solution.

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PULPING OF EUCALYPTUS WOOD BY THE ACETOSOLV PROCESS

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ABSTRACT

The objective of the present study is the optimization of the acetosolv process for Eucalyptus grandis wood. The variables investigated were solvent/wood ratio (6:1 to 12:1 v/w), chip size (0.1 to 0.9 cm of thickness), cooking time (2 to 3 h), the nature and quantity of the catalyst (0.11 to 0.34% of HCl, 0.14 to 0.28% of HBr or 0.1 to 1.6% of $FeCl_3$). The best results, obtained with 0.4-0.6 cm chips, a solvent/wood ratio of 7:1, 3 h of cooking and 0,14% HBr were 59.9% pulp yield with a Kappa number of 14.7. The Soxhlet extraction of the pulp was found to be an important parameter to reduce the Kappa number.

INTRODUCTION

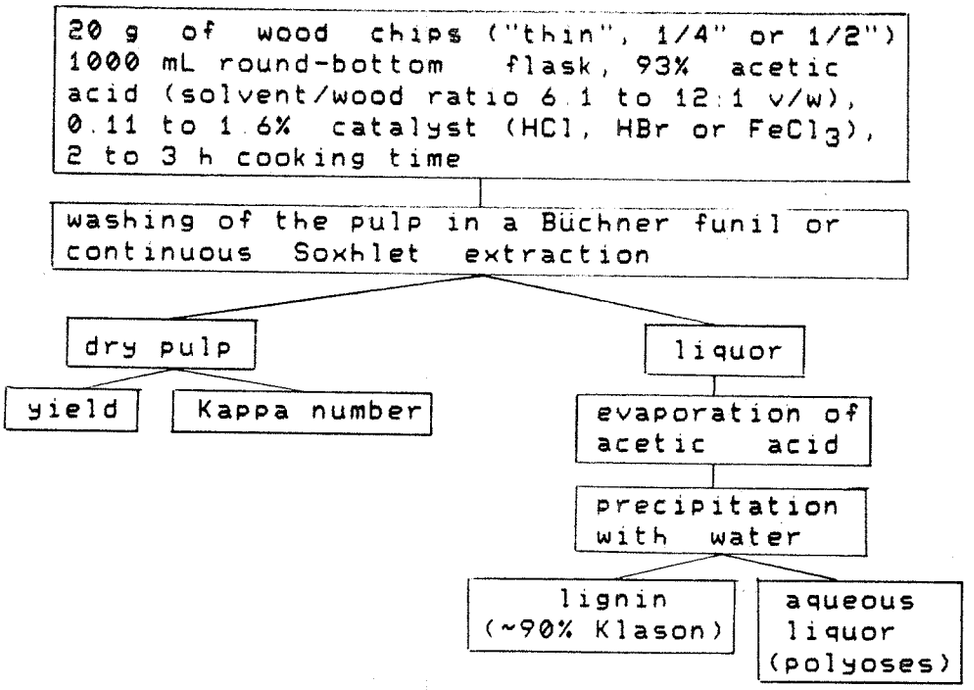
In the last 20 years organosolv processes have been extensively studied as alternative pulping methods. The aim is not to replace the conventional processes but to find auxiliary methods that could satisfy increasing paper consumption. The advantages of organosolv processes are lower investment, economically feasible production in small mills, easy recovery of the solvent (decreasing environmental problems) and byproducts (lignin and polyoses) that are promising chemical feedstocks [1].

The acetosolv process is probably the most promising of the alternative pulping process, as no pressure is required and the pulping solvent is totally recycled. The cooking temperature is lower than in other processes (approximately 110°C), which means less cellulose degradation. The process uses 93% acetic acid under reflux and a small quantity of a strong acid as catalyst. In 1989 Nimz et al. [2] published good results obtained for softwoods and some annual plants. We wish to report here our results on the pulping of eucalyptus wood by the acetosolv process.

EXPERIMENTAL PART

The chips of eucalyptus wood were selected in "thin" (1.5-2.0 cm of length, 0.2-0.4 cm of breadth and 0.1-0.3 cm of thickness), 1/4" (1.5-2.0 cm of length, 1.0-1.5 cm of breadth and 0.4-0.6 cm of thickness) and 1/2" (2.0-2.5 cm of length, 2.0-2.5 cm of breadth and 0.6-0.9 cm of thickness). The procedure for eucalyptus wood pulping by the acetosolv process is shown in the scheme:

SCHEME OF PULPING PROCESS



RESULTS AND DISCUSSION

Table 1 shows the immediate analysis of *Eucalyptus grandis* wood.

TABLE 1: Immediate analysis of *Eucalyptus grandis* wood

water	chips "thin"	14.9%
	chips 1/4"	17.5%
	chips 1/2"	17.5%
ash (water free)		0.53%
extractives (water free)		3.5%
Klason lignin (water free)		26.5%
soluble lignin (water free)		2.5%

The variation of pulp yield and Kappa number as a function of the chip size is showed in table 2.

TABLE 2: Pulp yield and Kappa number as a function of the chip size (solvent/wood ratio 7:1, 0.11% HCl, 3 h)

exp.	chip size	yield(%)	Kappa
1	"thin"	61.6	21
2	1/4"	62.7	24
3	1/2"	63.9	25

Although the pulping of smaller chips is easier because the acessibility of the solvent is increased, the work was continued with chips of 0.4-0.6 cm of thickness (1/4") because this is the size normally employed in the pulping industry.

Table 3 shows the pulp yield and the Kappa number as a function of the solvent/wood ratio. The delignification depends on the solvent difusion into the wood chips and the dissolution and removal of the lignin fragments. This is facilitated at higher dilutions, which are not interesting for the pulping industry. We, therefore, continued to using a solvent/wood ratio of 7:1.

TABLE 3: Pulp yield and Kappa number as a function of the solvent/wood ratio (chips 1/4", 0.11% HCl, 3 h)

exp.	solvent/wood ratio	yield(%)	Kappa
4	6:1	65.3	25
2	7:1	62.7	24
5	10:1	60.6	24
6	12:1	57.9	21

Decreasing the cooking time from 3 to 2 h (chips 1/4", solvent/wood ratio of 7:1, 0.11% HCl) the pulp yield is maintained at approximately 62% but the Kappa number increased from 24 to 27.

The most important parameter to reduce the Kappa number was found to be the Soxhlet extraction of the pulp with glacial acetic acid. Under standard conditions (chips 1/4", solvent/wood ratio of 7:1, 0.11% HCl and 3 h of cooking) and using Soxhlet extraction, the pulp yield is only reduced from 62.7% to 59.9% but the Kappa number suffers a strong reduction to 18, because most of lignin fragments were removed. The work was, therefore, continued using Soxhlet extraction instead of washing in a Büchner funil.

Table 4 shows the variation of the pulp yield and the Kappa number as a function of the nature and quantity of catalyst used.

TABLE 4. Pulp yield and Kappa number as a function of the nature and quantity of catalyst (chips 1/4", solvent/wood ratio of 7:1, 3 h)

exp.	cat.	quantity of catalyst %w/w solvent	%w/w wood	yield (%)	Kappa
7	HCl	0.11	1.0	59.9	18
8	HCl	0.23	2.0	56.9	16
9	HCl	0.34	3.0	55.6	14
10	HBr	0.14	1.3	59.9	15
11	HBr	0.21	1.9	55.7	13
12	HBr	0.28	2.5	56.4	13
13	$FeCl_3$	0.10	0.9	(93.8)*	--
14	$FeCl_3$	0.20	1.8	(83.2)*	--
15	$FeCl_3$	1.6	14	60.7	20

* no pulp obtained

Increasing the quantity of HCl or HBr, the pulp yield and the Kappa number are reduced. The pulp yield obtained with 0.14% of HBr is the same obtained with 0.11% of HCl but the Kappa number is lower (15 and 18, respectively). Increasing the quantity of HBr the Kappa number can be further reduced to 13, but the pulp yield is low (56%) under these conditions. The use of $FeCl_3$ (a Lewis acid) was not efficient as pulp was only obtained with a high quantity of catalyst. Furthermore, the Kappa number of the pulp is higher than that obtained with HBr in a similar yield.

The viscosity of acetosolv pulps from eucalyptus wood were typically in the range of 18 to 24 cP.

CONCLUSIONS

The acetosolv process is more adequate for the pulping of eucalyptus wood than of sugar cane bagasse [3]. The best results are obtained with 0.14% of HBr, allowing a good Kappa number of 15 for a high pulp yield of 59.9%. The pulp yields for conventional pulping of eucalyptus are expected to be less than 50% [4].

ACKNOWLEDGEMENTS

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Removal of Cupric Ion From Solution By Contact With Corn Cobs.

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ABSTRACT

We have studied the Cu(II) ions adsorption by corn cobs in aqueous solution. The adsorption process is pH dependent, with optimum removal of copper occuring in the 5-6 pH range. Hydrogen ions are liberated into solution, showing that the adsorption mechanism is (partly) due to ion exchange.

The same material treated with different concentrations of ethylenediamine (EDA)/formaldehyde (HCOH) showed a higher retention capacity than the untreated material.

1 - INTRODUCTION

The presence of heavy metal ions in waters has generated considerable concern in recent years. Among the toxic heavy metal ions which present a potential danger in industrial waste waters are copper, lead, cadmium chromium and mercury.

Interest has risen recently in removing heavy metal ions from solution with agricultural byproducts (1,2). It has been speculated that the active sites attachment of heavy metal cations to the agricultural substrates are poliphenolic groups.

Corn cobs, which are rich in cellulose, lignins and hemicellulose, could be a good adsorbent for these heavy metal cations. Waste corn are inexpensive and available throughout the country (Brazil) in quantities that would make it an ideal material for the purpose. On the other hand, Parana state is the greater grain producer in Brazil.

We have studied the adsorption of cupric ion by corn cobs and modified corn cobs. The corn cobs composition used is 33% of cellulose,43% of hemicellulose and 23% of lignin.

2 - EXPERIMENTAL

2.1 - Preparation of Adsorbents

Two forms of corn cobs were used. One was unmodified corn cobs, and the other was corn cobs which was

modified with ethylenediamine/formaldehyde, both without hemicellulose.

Fresh corn cob was air dried and crushed in a Manesco & Ranieri Type 340 mill to pass through a 80 mesh screen.

Extraction of hemicellulose from the corn cobs was carried with 2,5% H_2SO_4 out as described in the literature (3).

In a boiling water bath, 2g of corn cobs without hemicellulose were treated with a mixture of 0,0017M of EDA solution and 0,0032M of 35% HCOH solution. The reaction time used was 30 min, then cooled to room temperature and filtered. The residue was washed with cool water and dried at 50°C overnigth. The same treatment was repeated with different concentrations of EDA/HCOH.

2.2 - Adsorption Studies

Solutions containing the metal ion were prepared from stock solutions in 50 cm^3 volumetric flasks and made up with water or buffers. Ten cubic centimeters of the solution were mixed with c.a. 0,2500g of the corn cobs and shaked continuously at 35°C for 2 h. After adsorption, the adsorbent was separated off and the copper concentration in the supernatant was determined by atomic absorption spectroscopy (Varian AA 175). The copper fixed in the adsorbent was calculated (by difference) from the equilibrium concentration in supernatant, and then plotted as a function of the equilibrium concentration to give the adsorption isotherm. The pH values of all supernatants were measured to check changes in the acid-base equilibria established in suspensions of the corn cobs in the presence of the metal ion.

2.3 - Time Course of Copper Adsorption

The samples were analysed for free copper, following the procedure described in 2.2 at different intervals of time between 10 min to 4 h.

2.4 - Infrared Analysis of Materials

The infrared spectra were taken on Jasco IR-700 in KBr pellets (1,5/100mg) (4).

3 - RESULTS AND DISCUSSION

3.1 - Time Course of Copper Adsorption

From Figure 1 it can be seen that the amount of copper adsorbed by the corn cobs increase very rapidly in the first minutes. Thus, for Cu(II) it was assumed that a contact time of 30 min or more was adequate for equilibrium conditions to be reached. In these studies a time of 2 h

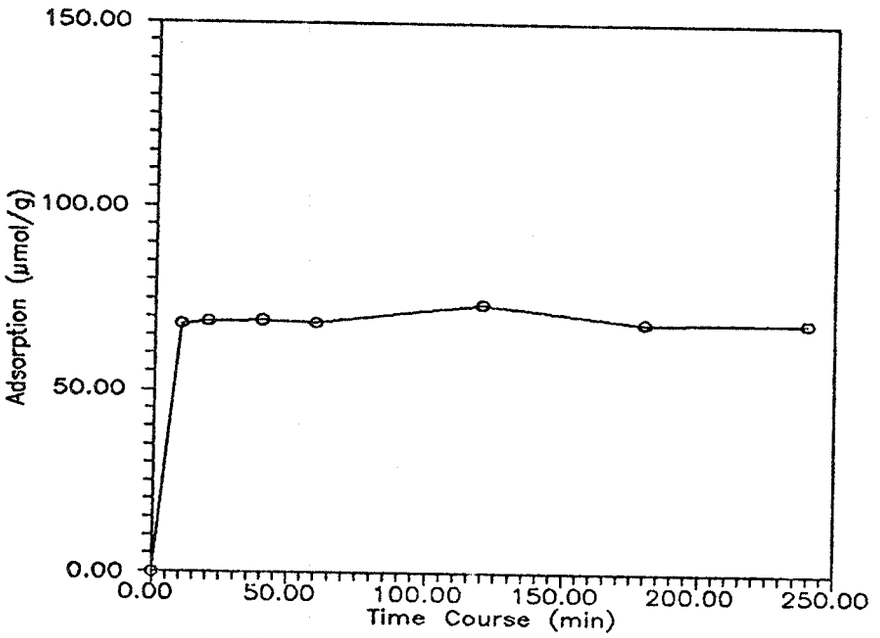

Fig.1. The binding of Cu(II) ions from aqueous solutions at 35 C in corn cobs expressed in umol Cu(II) adsorbed per gram of corn cobs as a function of the time course in minutes.

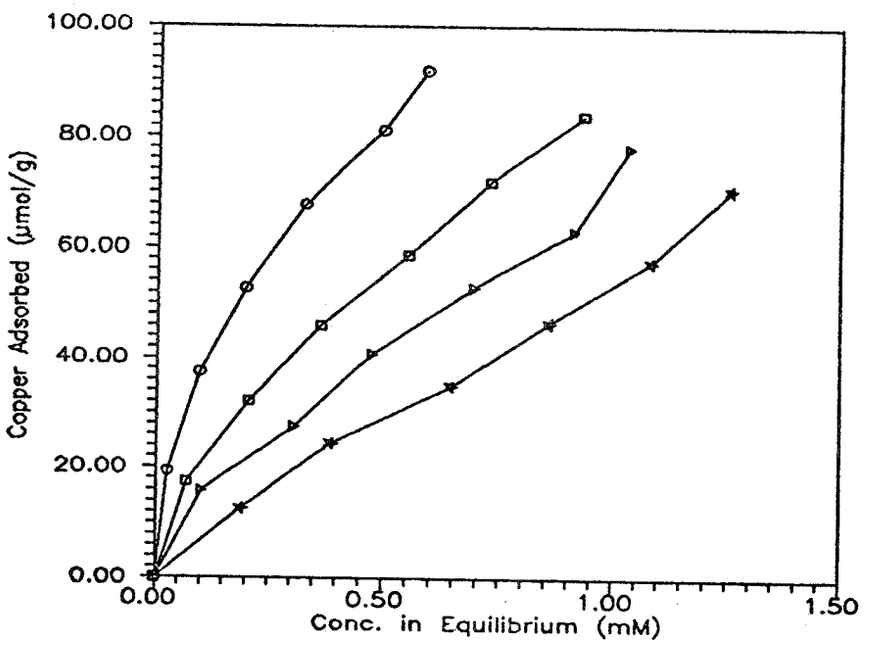

Fig.2. The binding of Cu(II) ions to corn cobs from aqueous acetate buffers at 35 C for several pH values (1–3,39 2–4,56, 3– 5,35, 4– 6,01 respectively).

was utilized.

3.2 - Effect of pH on Copper Adsorption

Experiments were done to determine whether the copper adsorption by the corn cobs is influenced by the pH of the solution. Addition of Cu(II) to aqueous unbuffered suspension of corn cobs results in decrease in the pH of the solution after adsorption. The initial pH of the suspension decreases with Cu(II) concentration increases (table I).

TABLE I - Influence of pH in Removal of Cupric Ion from Solution by Contact with Corn Cobs.

Mass of Corn Cobs	Initial Conc.of $CuCl_2$	pH in Solution	
(g)	(mM)	Before	After
0,2509	0,5012	5.10	3,40
0,2512	1,002	4,66	3,32
0,2511	1,503	4,64	3,03
0,2515	2,005	4,63	3,10
0,2510	2,506	4,47	3,14
0,2510	3,007	4,45	3,27
0,2507	3,508	4,42	3,20
0,2508	4,176	4,38	3,46

Adsorption of copper ions on corn cobs was also followed in acetate buffer as a function of pH (Fig.2). The increase in binding for copper ions with increasing pH, showed that the equilibrium is pH dependent.

It was shown, that copper adsorption by corn cobs, presents a ion exchange mechanism . Nevertheless, considering other sites for attachment of metal, other mechanisms should be considered.

3.3 - Adsorbents

Figure 3 shows the adsorption efficiencies of corn cobs and EDA/HCOH treated corn cobs in taking up and holding cupric ion. The EDA/HCOH modified material showed a higher retention capacity than unmodified corn cobs , c.a. 27% . Higher concentrations of EDA/HCOH showed higher retention capacities.

3.4 - Infrared Analysis

Some information about copper-corn cobs bonds were obtained through IR spectroscopy (4000-400 cm^{-1}).

Diferences were observed in regions between 3500-3100, 3000-2800, 1700-1500 and 1400-1180 cm^{-1} (fig.4 and 5).

These differences observed correspond to some

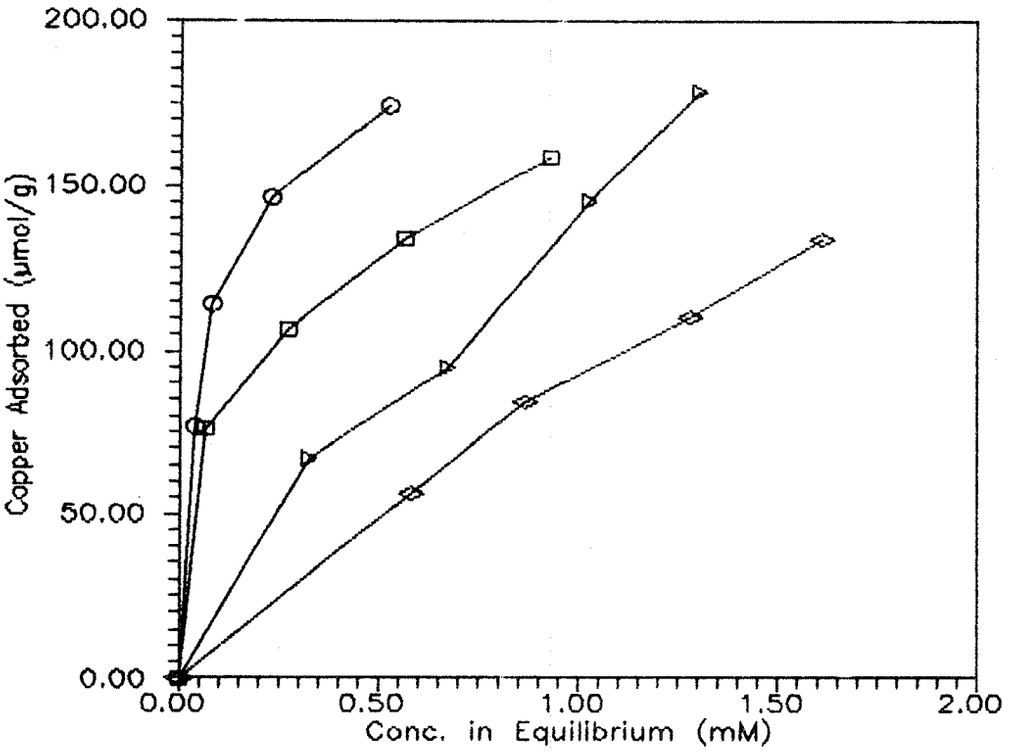

Fig.3. The binding of Cu(II) ions to corn cobs and modified corn cobs with EDA/HCOH (1–unmodified, 2– 0,0033M, 3– 0,0332M, 4–0,332M respectively)

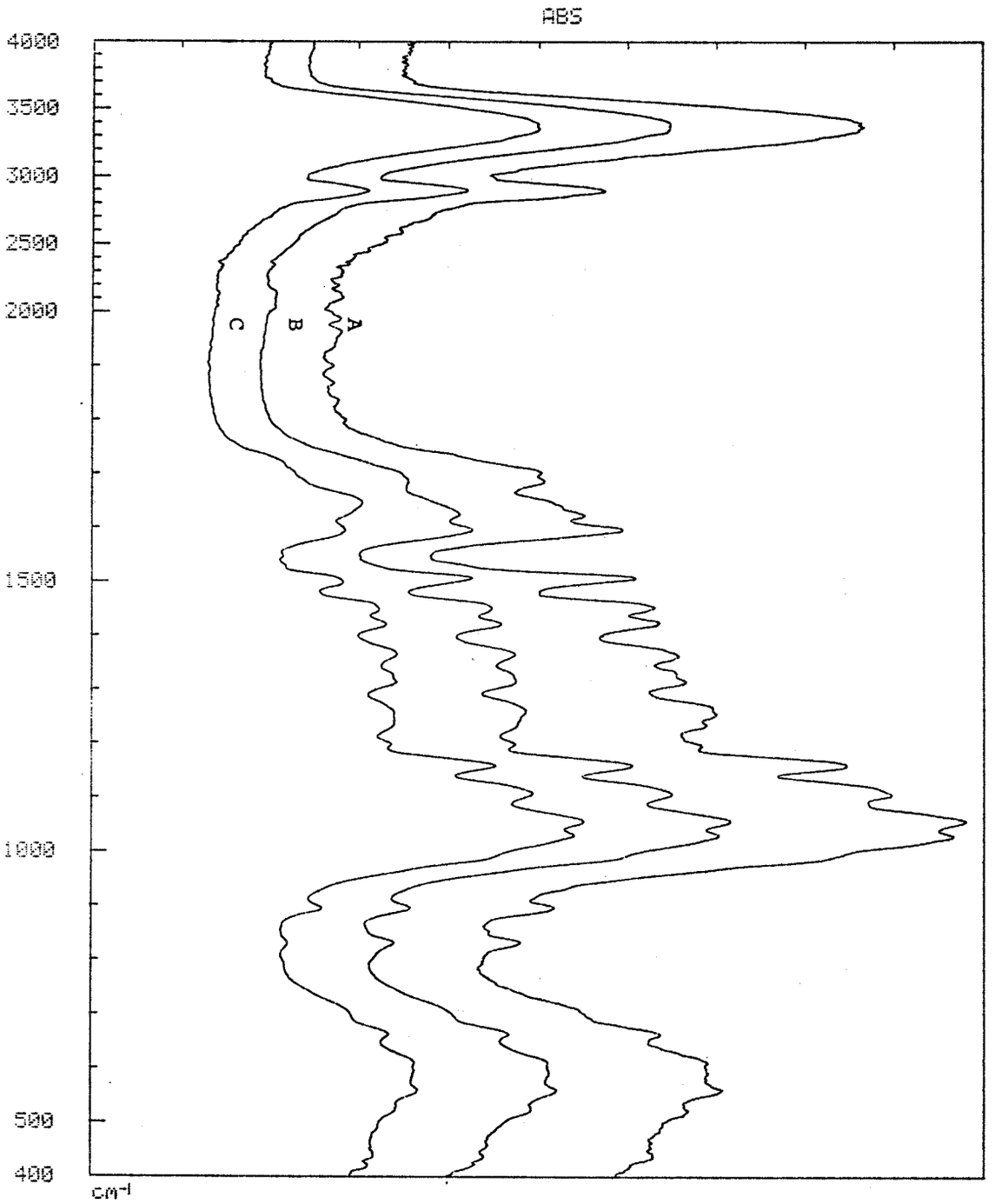

Figure 4. Infrared Spectra.

A - Corn Cobs; B - Corn Cobs-copper; C - Modified Corn Cobs.

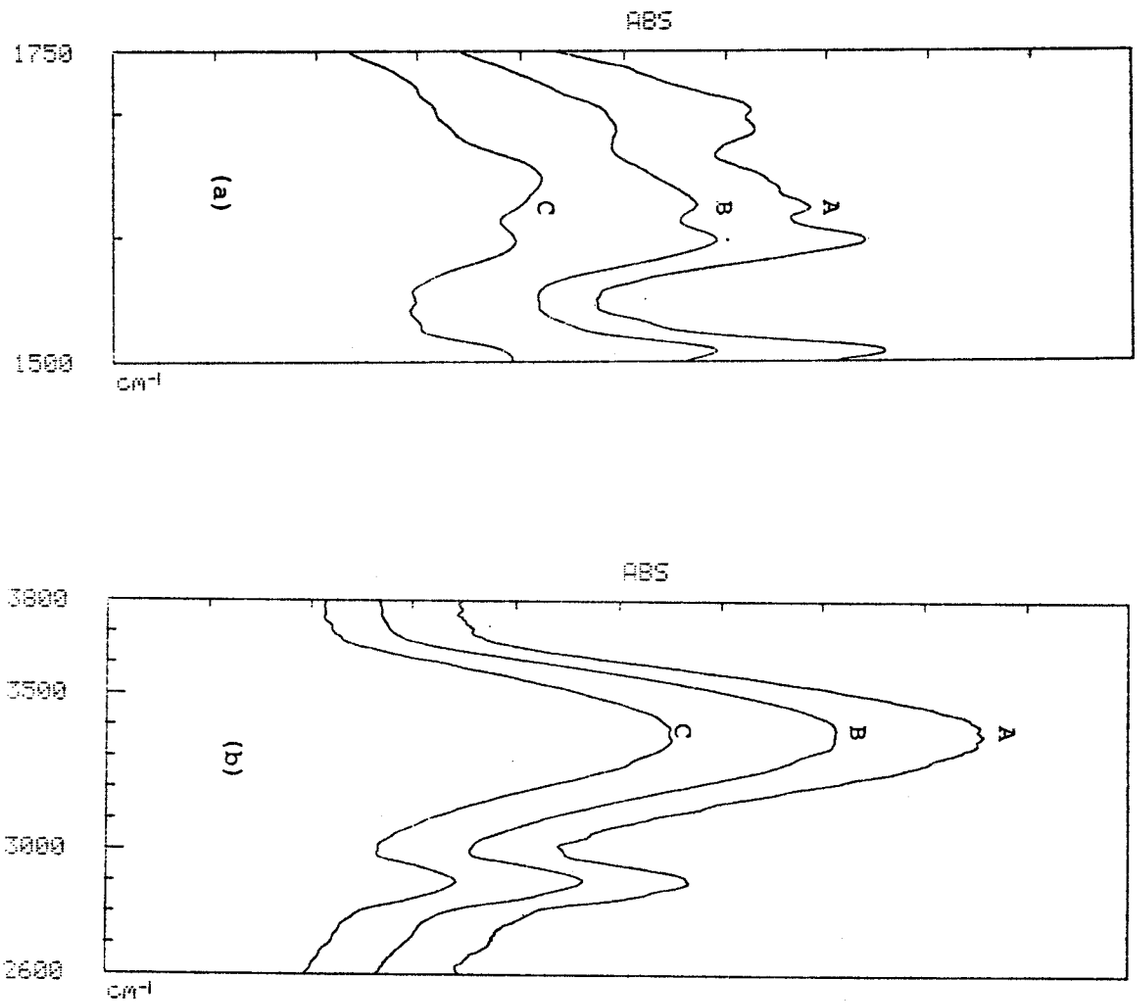

Figure 5 - Expanded Infrared Spectra (from Fig. 4)
a - 1750-1500 cm^{-1}
b - 3800-2600 cm^{-1}

alterations in groups presents in both cellulose (O-H, CH_2) and lignin (O-H, OCH_3, C=O) and incorporations of NH groups.

4-CONCLUSION

Corn cob is a very interesting material as adsorbent for Cu(II) ions. Through simple chemical modifications and pH control the adsorbent capacity can be considerable enhanced.

Finally, the rapid adsorption process, low cost and high disponibility of corn cobs are indicative of its potential industrial application in the purification of waste water containing cupric ion.

Acknowledgement

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EXTRACTION AND INCORPORATION OF METALS IN BARK AND ITS DERIVATES

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ABSTRACT

Studies on the removal and recovery of different metal ions from aqueous solutions were undertaken using modified bark and modified tannins (treated with formaldehyde, to prevent blending of color into the liquid) of **Pinus radiata** as extractants. The formaldehyde treatment, in general, did not disminish the capacity of the bark and tannins to remove the ions from solutions. Experiments of adsorption as function of the pH of the solution, desorption and maximum capacity were carried out for Re(VII), Mo (VI), As(V), Cd(II), Hg(II), Ge(IV), Al(III) and Pb(II). The Ca(II), Mg (II), K(I), Fe(II), Fe(III), Zn (II), Mn(II) adsorbing abilities of unmodified bark and bark free of tannins (bark extracted with water at 70°C) were studied in order to evaluate them as potential matrices for fertilizers.

1. INTRODUCTION

The presence of heavy metal ions in industrial and mining waste waters is a worthwhile subject to investigate, because of the great ecological damage that some of the metal ions such : Hg, Cu, Pb, Cd and Cr represent. In recent years, several studies have been made to establish effective methods of ions removal from aqueous systems, the most likely seems to be the use of agents that have the ability to adsorb them from the aqueous systems (1). Several metal adsorbents have been considered in the removal and recovery of metals (2). Some synthetic compounds and biological substances have been reported to be effective adsorbents (1-12). The low cost of agricultural and forest sludge has an atractive interest. Wood barks, which are high in phenolic compounds, should be ideal adsorbents for metal ions (9,10). Waste bark is inexpensive and disposal of bark is becoming an increasingly difficult problem. Utilization of bark as a substrate to remove heavy metal ions would provide a convenient means of resolving both waste problems.

The bark can also be utilized as matrix to incorporate mineral elements, micronutrients which are necessary for the growth of plants, that can constitute an excellent

fertilizing sistem, with a slow liberation effect of the mineral elements as the bark is degraded by microorganisms present in the soil, enriching it by the formation of "humic substances". The bark has also great capacity of water retention to maintain the humidity of the soil (13-14).

In this paper the ability of bark and tannins to adsorb different metal ions is discussed. The bark and tannins have been contacted with formaldehyde in acid media to prevent blending of color into the liquid. Experiments of adsorption as function of the pH of the solution, desorption and maxima capacity were carried out for Re(VII), Mo (VI), As(V), Cd(II), Hg(II), Al(III) and Pb(II). The Ca(II), Mg (II), K(I), Fe(II), Fe(III), Zn (II), Mn(II) adsorbing abilities of unmodified bark and bark free of tannins (bark extracted with water at 70°C) were studied in order to evaluate the utilization of bark as support matrix to transport micronutrients.

2. EXPERIMENTAL PART

2.1 **Chemicals.**

All chemical were Merck reagent grade and were used without further purification.

2.2 **Preparation of the adsorbents.**

Bark of **Pinus radiata** D. Don were used in all the experiments. The adsorbent used were: bark, modified bark, modified tannins and extracted bark.

Modified bark. Air dried bark was crushed by a Janke S. Kunkel Type 10 mill and particles that pass a 60 mesh screen were gathered for the further treatment. In a water bath at 80°C, ten g of bark were treated for 20 min by a mixture of 150 ml 3% aqueous nitric acid with 0.25 ml of a 35% formaldehyde. The mixture was cooled at room temperature, filtered and the residue washed with cool water and then dried overnight at 50°C.

Modified tannins. Extraction of tannins from bark was carried out with water at 70°C for 45 min, the residue was filtered, and the water evaporated at 70°C and the tannins dried overnight at 70°C. In as water bath at 35°C, five g of tannin were treated by a mixture of 250 ml of 3% aqueous nitric acid solution and 0.40 ml of a 35% formaldehyde. The time of reaction was one hour. The mixture was cooled at room temperature, filtered and the residue washed with cool water and then dried overnight at 50°C.

Extracted Bark. The bark was extracted 4 times with fresh water (ratio 1 bark: 5 water) at 70°C for 45 min each step.

The extracted bark was dried at 50°C. The was crushed and passed through a screen of 240 mesh.

2.3 Adsorption-desorption experiments

Influence of pH on the adsorption of metal ion by the adsorbent. Aqueous solutions containg each 1 g/L of one of the metal were prepared at the corresponding pH. The ability of modified bark and modified tannins to remove Re(VII), Mo (VI), Ge(IV), As(V), Cd(II), Hg(II), Al(III), Pb(II); and the ability of bark itself and extracted bark to adsorbe Mn(II), Zn(II), Ca (II), Mg (II), Cu(II), Fe(II), Fe(III) or K(I)] were analyzed. Ten ml of the solution were mixed with 1.0 g of the dry test adsorbent, and stirred constinuously at room temperature for 1 h. After adsorption, the adsorbent was filtered and the residual metal content in the filtrate was determined by spectrometry (V, Re and Mo) or by atomic absorption spectrometry (Hg, Ge, As, Cd, Mn, Zn, Cu, Mg, Ca, Fe, K). In these experiment, metal uptake by the substrate was calculated from the difference between the initial and final concentrations of metal in the solution.

Desorption of the ion adsorbed on the adsorbent. To obtain basic information on metal recovery, the adsorbed metal were eluated with different eluents, namely H_2SO_4 (1 M - 5 M) solutions and Na_2CO_3 (0.25 - 1 M) for modified bark and modified tannins, and HCl at different pH for bark and extracted bark. In a test tube, 100 mg of the loaded resin were shaken with 10 ml of the eluent for 1 h at room temperature. The adsorbent was separated by filtration and washed with water. The solution was analyzed as before.

Metal adsorption maximun capacity of the adsorbent. In a beaker of 250 ml, 1.0 g of the dry adsorbent was suspended in 50 ml of an aqueous solution containing 1 g/L of the metal ion at the optimun pH and stirring continuously (200 cycles/min in a Elektronische Shuttel Machine THYS 2MLW) for 15 min. After adsorption the adsorbent was separated and washed several times with water, and the residual metal in the liquid phase was determined. These procedure was repeated three times (using the same sample of adsorbent). The metal fixed in the adsorbent was determined by difference.

3. RESULTS AND DISCUSSION

3.1 Metal adsorbing abilities

The utility of a extractant in a particular system requieres the understanding of its behavior starting from the

extraction and elution selectivity when more than one metal is envolved. In this study, therefore, experiments were initially carried out to study the extraction and elution of the different metals separately.

Effect of pH on metal adsorption. Fig. 1 shows the adsorption of different ions by modified bark as a function of the pH. The adsorption by modified tannins follows a similar trend. The adsorption for Re, V, Ge, Mo between pH 0-12 are very different (Fig. 1-A). The loading capacity of the adsorbents was higher for Mo between the pH 8-12 (95%), for Re in the range pH 4-5 ((80%), for V at pH 10-12 (68%) and for Ge the adsorption is of the order of 60% at all the pH. The results indicates that the adsorbents are no specific for any ion but there are different capacities to retain the different ions at this pH range. This selectivity will allow the separation of these ions from other ions and between them. For example, at pH 0 the adsorption for Re and V is almost null but for Ge and Mo are about 60%, therefore, Re and/or V coulb be separated at this pH from Mo and/or Ge The adsorption values by modified bark for Pb, Hg, Cd and As are shown in Fig. 1-B.

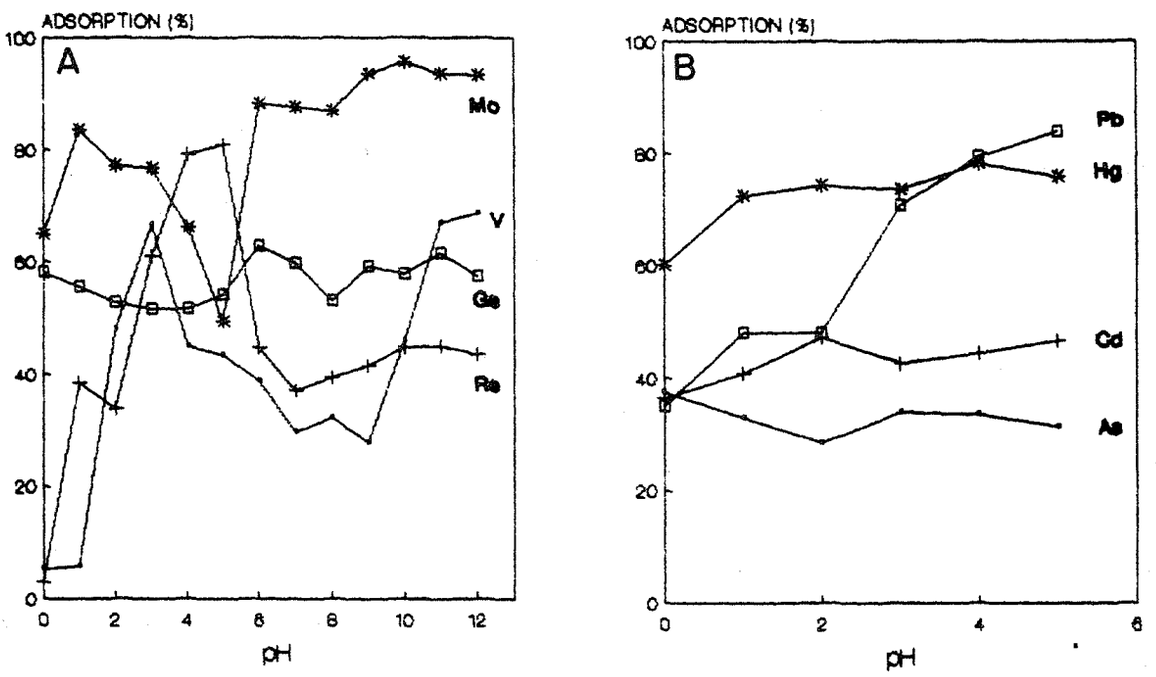

FIG. 1 pH DEPENDENCE OF THE ADOSRPTION
ADSORBENT: MODIFIED BARK

Fig. 2 shows the adsorption of bark itself (A) and extracted bark (B) of the cations Mn^{2+}, Zn^{2+}, Ca^{2+}, Mg^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+} and K^{+}, in the pH range of 0-8. The capacities of adsorption are dependent of the pH.

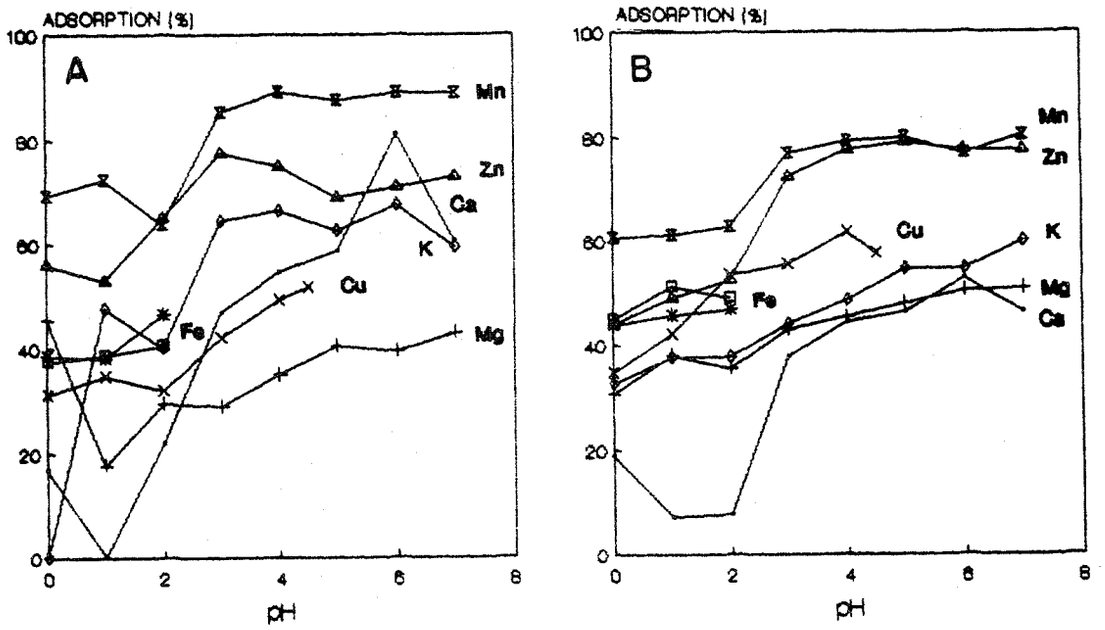

FIG. 2 pH DEPENDENCE OF THE ADSORPTION ADSORBENT: BARK (A), EXTRACTED BARK (B)

Desorption of the ion adsorbed on the adsorbent. The modified bark and the modified tannins adsorbe large amounts of the different ion depending of the pH of the aqueous system. The adsorb ion was eluted with various eluates as shown in Table I. It can be observed that the amount of ion desorbed is highly dependent of its nature and of the eluent. The selection of the conditions of the adsorption and the elution could allow the separation and recovering of different metals.

TABLE I

ELUTION OF THE LOADED ADSORBENT

ELUENT (M)	DESORPTION (%) METAL ION							
	Re	Mo	Ge	As	Cd	Hg	Al	Pb
H_2SO_4	MODIFIED BARK							
1.0	4.3	21.0	12.0	12.7	1.8	36.4	17.7	1.4
2.0	1.1	12.9	12.1	12.8	2.0	-	17.7	1.3
3.0	0.8	11.7	12.2	12.6	1.9	20.0	16.5	1.3
4.0	1.1	14.7	12.1	12.6	2.0	61.8	20.0	1.3
5.0	0.7	16.6	12.2	12.7	1.7	21.8	18.1	0.8
H_2SO_4	MODIFIED TANNINS							
1.0	1.3	29.6	8.6	12.8	0.5	12.0	19.3	0.8
2.0	0.9	34.1	8.7	12.7	0.5	9.7	22.0	0.7
3.0	1.2	37.6	8.6	12.6	0.6	27.1	22.0	0.6
4.0	1.6	32.3	8.8	12.7	0.5	10.9	20.0	0.6
5.0	0.6	27.9	8.7	12.7	0.6	84.5	18.0	0.5
Na_2CO_3	MODIFIED BARK							
0.25	0.8	11.9	1.3	12.7	0.2	14.1	9.6	1.1
0.50	1.0	16.0	1.2	12.6	0.3	7.1	9.7	1.0
0.75	1.2	19.2	1.4	12.8	0.2	12.7	9.6	1.2
1.0	1.1	15.6	1.3	12.7	0.2	9.1	9.8	1.0
Na_2CO_3	MODIFIED TANNINS							
0.25	0.2	21.7	0.9	12.6	0.3	6.2	19.3	0.9
0.50	0.5	21.6	1.0	12.7	0.2	9.0	22.0	0.8
0.75	0.1	22.6	1.4	12.8	0.3	11.4	22.0	1.0
1.0	0.1	13.0	1.3	12.7	0.2	14.6	20.0	0.9

	METAL ION						
	Ca	Mg	Fe(II)	Cu	K	Zn	Mn
pH (HCl)	BARK						
2	3.7	2.4	1.7	12.7	3.6	1.4	1.1
4	2.4	1.8	0.7	5.0	1.3	0.7	0.7
6	0.9	0.7	0.7	3.0	0.7	0.1	0.1
	EXTRACTED BARK						
2	4.8	3.7	1.7	10.7	4.1	1.0	1.9
4	5.9	6.3	2.5	8.4	6.4	1.0	2.5
6	1.2	1.4	0.7	1.5	0.4	1.0	0.3

Determination of maximum capacity. To determine the adsorption capacity of the adsorbents with respect to each ion, succesive experiments using the same sample were carried out in a batch system. On the third run the values obtained in meq of ion per g of dried adsorbent are given in Table II.

TABLE II

MAXIMUM CAPACITY

ION	pH		MAXIMUM CAPACITY meq of metal/g d.a.	
	CM*	TM*	CM	TM
Re	5	5	2.41	2.45
Mo	10	6	0.86	1.53
Ge	6	0	0.51	0.71
As(V)	0	3	0.98	0.63
Cd(II)	2	2	0.22	0.18
Hg(II)	4	5	0.27	0.32
Al(III)	3	3	0.72	0.42
Pb(II)	5	5	0.37	0.41

* CM: MODIFIED BARK, TM: MODIFIED TANNINS

Effect of the formaldehyde treatment of bark
The formaldehyde treated bark and tannins showed zero bleed of color into solutions, and in general the treatment did not decrease the adosrption capacity, and in some cases, a better capacity retention is observed.

CONCLUSIONS.

Our present results show the bark as potential low-cost and readily available substrate for removing and recovery metals from industrial effluents and other water sources. The adsorption and desorption of the metal ions by the adsorbent used was found to be governed by the pH of the medium.The results of incorporation of mineral elements are excellent, as well as maximun capacity and elution, what make possible and convenient the utilization of bark as support matrix to transport micronutrients.

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USE OF LIGNINS AS SUPPORT FOR HERBICIDES IN CONTROLLED RELEASE FORMULATIONS

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ABSTRACT

Pinus kraft lignin, eucalyptus kraft lignin and sugar cane bagasse lignin (2) were formulated separately, under melt conditions, first with trifluralin and then with diuron. The lignins and trifluralin were incompatible while diuron was compatible with the lignins in different proportions. The kinetic study of these formulations showed that the release rates of diuron depend upon the type of lignin.

INTRODUCTION

Most pesticides available in the market are in accordance with conventional formulations. In such cases, the losses of active ingredients either by biological degradation, lixiviation or volatilization, among others, are very accentuated. To avoid or to decrease these problems the controlled release systems have been sought (1).

The requirement for these formulations is that the active ingredient interact physically and or chemically with the matrix and under field conditions be released through the polymer to the surrounding environment by diffusion or another mechanism.

Formulations can be prepared by using synthetic or natural polymers including lignins. The variations of the lignin properties (3, 4) are important when considering the most appropriate utilization (6, 7). Variation in molecular weight, functional groups, degree of condensation, saccharidic character (7) etc., will influence the selection of appropriate formulation types, but the manner of isolation, particularly with regard to cellulose and other materials content will be more limiting (5). Lignins are also available as a by-product of many pulping processes and are appropriate for pesticide formulation as they are insoluble at most soil pHs.

In the present work the preparation, the properties and the controlled release characteristics of herbicide formulations with lignin, that can be applied to the soil are reported.

MATERIALS AND METHODS

Pinus kraft lignin (Indulin AT, Westvaco Inc.), eucalyptus kraft lignin, sugar cane bagasse lignin (2) and the technical grade herbicides

trifluralin and diuron were used to prepare the formulations under melt conditions (1).

Formulations were carried out in an electrically heated open vessel. The active agent was melted in an aluminium dish and the appropriate amount of dried powdered lignin was added under stirring to produce a uniformly plasticised mix. This mix was molded and cooled to make a sheet (1.2 mm thick), which was then cut into pieces containing 15 mg of active agent. The trifluralin and diuron formulations were prepared in the range of 90 to 180°C and 150 to 190°C, respectively. The amount of herbicides used for melting ranged from 35 to 60% for trifluralin and from 5 to 50% for diuron.

The herbicide compatibility with the lignins was determined through a polarized light microscope by observing the presence or absence of herbicide crystals in the formulations.

A sample of formulation was treated by MeOH in a sonicator until overall extraction of the herbicide. The amount of extracted herbicide was determined by HPLC and reported as releasable herbicide.

To determine the possible mechanisms involving the release of herbicide, pieces of formulations sheets with known surface area and weight were prepared. Three pieces of each formulations were weighed and each one was immersed in 100 mℓ distilled water at 23°C. At every sampling time the water phase was removed and replaced by fresh water. The amount of the herbicide released was daily determined by sampling the water phase followed by analysis in a Gilson Isocratic HPLC system with a Spherisorb ODS column (15 cm x 4.6 mm). The eluent was a solution of methanol water at the proportion of 90:10 and 75:25 for trifluralin and diuron analysis, respectively. The detection was made at 275 nm for trifluralin and 254 nm for diuron.

RESULTS AND DISCUSSION

The lignins used presented low content of sugars (Table 1). This

TABLE 1 - CHEMICAL COMPOSITION OF BAGASSE, EUCALYPTUS AND PINE LIGNIS

LIGNIN	KLASON INSOLUBLE (%)	KLASON SOLUBLE (%)	ASH (%)	CARBOHYDRATES GLUCOSE (%)	CARBOHYDRATES XYLOSE (%)	CARBOHYDRATES ARABINOSE (%)	TOTAL (%)
BAGASSE	92.0	3.9	1.92	0.22	0.15	—	98.2
EUCALYPTUS	91.2	5.7	0.59	0.06	0.25	—	97.0
PINE	88.5	4.6	1.56	—	1.0	0.15	95.8

characteristic make the lignins an ideal bioedegradable polymer to formulate by melting the herbicides that are useful as herbicide granules (5, 8, 9).

The ability of the active agent to dissolve or plasticize lignins can be predicted by the comparison of their respective solubility parameters (1). When the values are similar the compatibility may be good. The solubility parameters of isolated lignins range from 10.5 to 12.8 $(can/cm^3)^{1/2}$ while the solubility parameters of trifluralin and diuron are 10.31 $(cal/cm^3)^{1/2}$ (13) and 16.3 $(cal/cm^3)^{1/2}$ (1), respectively. Although trifluralin shows a solubility parameter similar to lignins it was incompatible with them, independently of the proportions of active ingredient and temperatures studied. On the other hand diuron (16.3 $(cal/cm^3)^{1/2}$ showed compatibility with all the lignins tested and readily formed a glassy matrix from 50 down to 20% of active ingredient. The trifluralin incompatibility may be explained by the low melting point of the trifluralin (46 - 47°C), that is insufficient to solubilize the lignins. The diuron compatibility may be explained by the softening temperature of lignins (160 - 180°C) that is close to the melting temperature of diuron (158 - 159°C) and can promote a glassy matrix formulation.

The amount of diuron releasable in the formulations prepared by using 5% to 50% of the herbicide between 160 to 180°C is shown in Table 2.

TABLE 2 - PERCENTAGE OF RELEASABLE AND LOST DIURON AS FUNCTION OF THE INITIAL QUANTITY OF DIURON AND PROCESS TEMPERATURES

LIGNIN	TEMPERATURE (°C)	INITIAL DIURON (%)	RELEASABLE DIURON (%)	LOST DIURON (%)
BAGASSE	174	49.96	42.68	14.57
	180	34.87	24.32	30.26
BAGASSE + 20% OF UREA	168	20.01	2.34	88.31
	164	9.91	1.33	86.60
	162	5.08	0.50	90.16
EUCALYPTUS KRAFT	160	50.00	41.85	16.30
	163	35.23	30.65	13.00
	168	20.15	14.50	18.04
PINE KRAFT (INDULIN AT)	170	49.98	47.10	5.76
	168	35.06	29.56	15.69
	> 170	20.27	11.40	43.76

The minimum amount of initial diuron required to obtain a good formulation with eucalyptus and pine lignins was 20% and with bagasse lignin 35%. When using urea as an additional plasticizer, the minimum amount of diuron in the bagasse lignin formulation decreased from 35% down to 5%. However, the amount of releasable diuron, became lower than 2.5% (Table 2), as an unknown compound was formed by reaction of urea and diuron.

After processing, the formulations prepared with 50% diuron presented 42% of releasable herbicide for bagasse and eucalyptus lignins and 47% for pine lignin. In the formulations using 35% initial diuron, the amount of releasable herbicide was 24% for bagasse and 30% for eucalyptus and pine lignins. With 20% of initial diuron the final formulations using eucalyptus and pine lignins showed 14% and 11% of releasable diuron, respectively.

The amount of diuron decomposed or bounded to the lignin during the process (reported as lost diuron - Table 2) increased as the amount of diuron used decreased, probably because the time required for making these formulations was longer.

The release curves for the bagasse, eucalyptus and pine lignins containing different amounts of releasable diuron are shown in Fig.1, 2 and 3, respectively. After 43 days the bagasse lignin formulations with 43% and 24% of diuron delivered 8% and 10% of the releasable diuron (Fig.1).

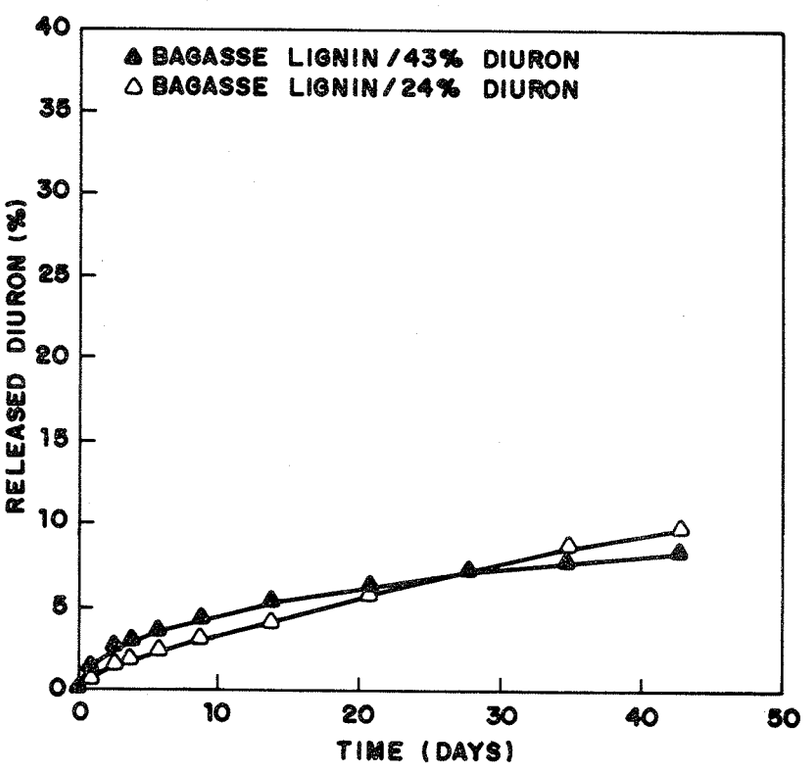

FIGURE 1 - RELEASE OF DIURON FROM BAGASSE LIGNIN FORMULATIONS.

The eucalyptus kraft lignin formulations with 42%, 30% and 14% of diuron delivered 29,36% and 24% (Fig.2) and the pine kraft lignins formulations

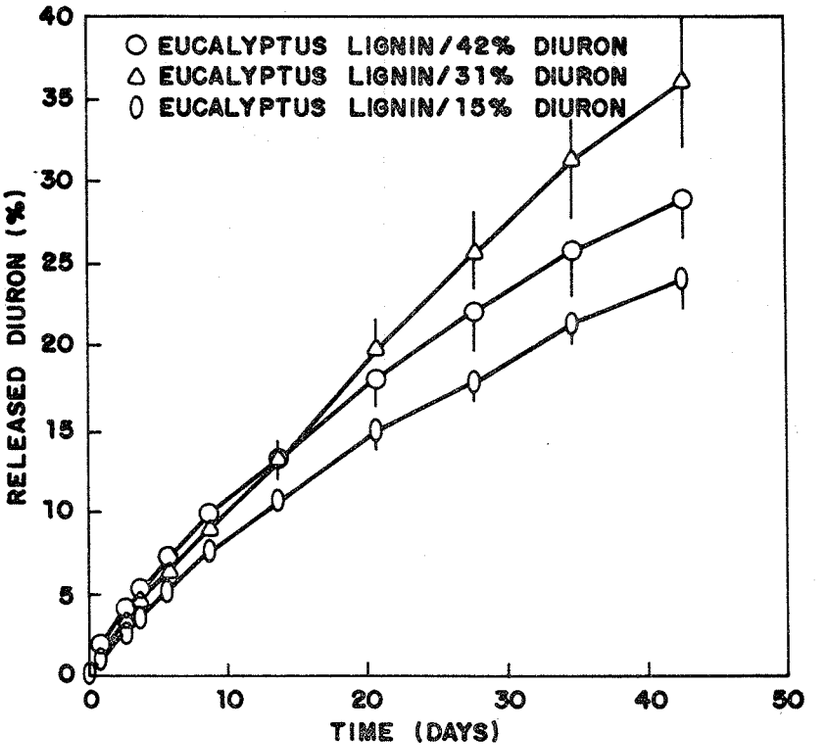

FIGURE 2 - RELEASE OF DIURON FROM EUCALYPTUS KRAFT LIGNIN FORMULATIONS.

with 47%, 30% and 11% of diuron delivered 19%, 29% and 19% (Fig.3) of the releasable diuron.

The release profiles of the formulations suggested that a similar release mechanism was involved. When the amounts of diuron released in bagasse formulations were plotted against $t^{1/2}$ (Fig.4), a linear curve was obtained which characterizes a diffusion dependent release kinetics. Considering the lignin type the order of release rates was: eucalyptus kraft lignin > pine kraft lignin > bagasse lignin.

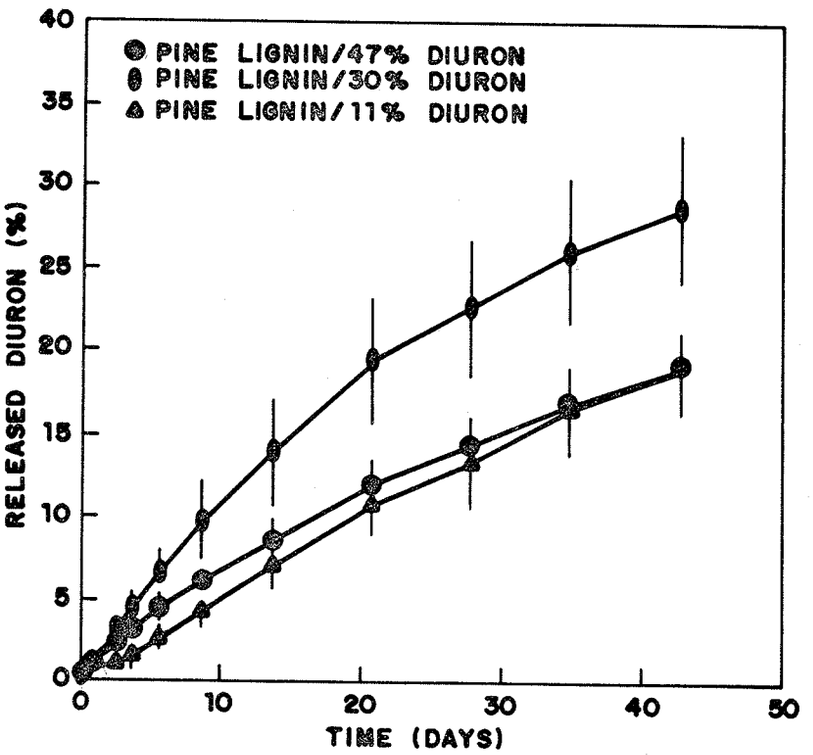

FIGURE 3 - RELEASE OF DIURON FROM PINE KRAFT LIGNIN FORMULATIONS.

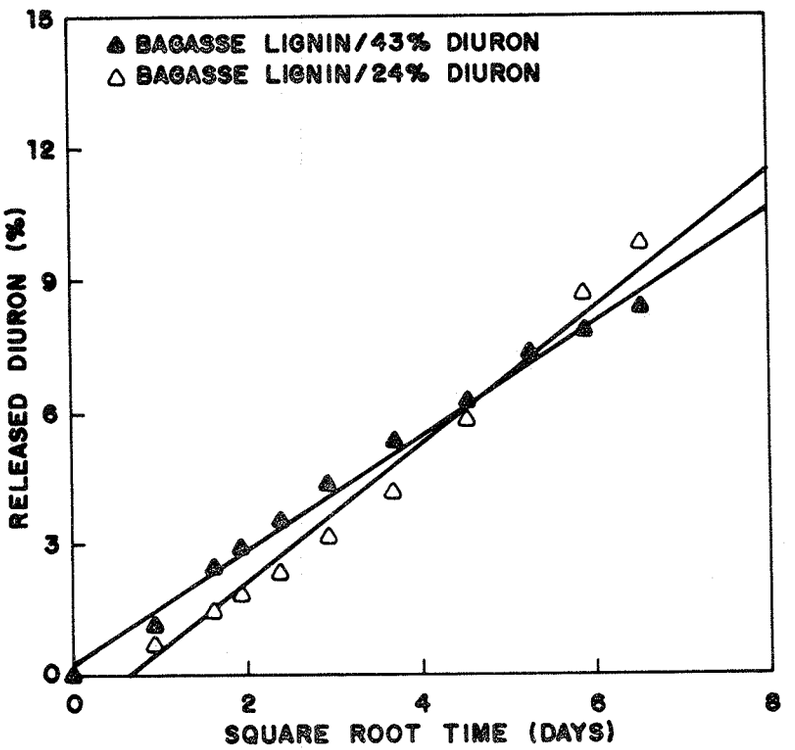

FIGURE 4 - RELEASE OF DIURON FROM BAGASSE LIGNIN FORMULATIONS AS FUNCTION OF THE SQUARE ROOT OF THE TIME.

CONCLUSIONS

Despite the conventional formulations, the lignins act as a not inert support for pesticides. The lignins studied produced formulations which release the active ingredient at different rates. This property can perhaps be used to produce formulations that release pesticides at several rates by mixing them.

As different release rates may be associated with the content of different functional groups and other chemical and physical properties of the lignins, their structural parameters must be better studied.

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CHARCTERIZATION OF LIGNINS USED AS MATRIX IN CONTROLLED RELEASE FORMULATIONS: STEAM EXPLOSION SUGAR CANE BAGASSE AND KRAFT EUCALYPTUS LIGNINS

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ABSTRACT
Steam explosion sugar cane bagasse (SEBL), KRAFT Eucalyptus (KEL) and Indulin AT lignins were characterized. Carbohydrates and insoluble and soluble Klason lignin contents were determined. All samples showed less than 1% carbohydrate content. Total hydroxyl content was 12-13% in SEBL and Indulin AT and 11% in KEL. The phenolic hydroxyl content was 4.5-5.7% in KELs, 6.8% in Indulin AT and 2.9% in SEBL. ^{1}H-NMR and I.R. spectra of lignins showed that KEL and SEBL were siringyl/guaiacyl type.

INTRODUCTION

Several lignin applications have been discribed in the last years. The utilization of this wood component in its polymeric form without further processing to convert it into low molecular weight compounds has been suggested as economically feasible (1). From this point of view lignin can be used as a polymeric matrix for obtention of controlled release formulation of agrochemicals. This was initially proposed by Allan and Neogi in 1975 (2). This type of formulation has several advantages in comparison with conventional formulations. Lignin can act as a protector of the formulated agrochemical and can release it at a known rate unlike conventional formulations (2).
In Brazil there are high amounts of lignocellulosic residues which can be used as a source of different lignins. Also a fraction of the KRAFT efluent from paper industries could be used for obtaining lignin instead of burning to generate heat.
This paper shows the chemical and physical characterization of the lignins steam explosion sugar cane bagasse, KRAFT Eucalyptus and Indulin AT (a commercial KRAFT lignin). All lignins were characterized in order to control their quality and to further understand the different rates of a pesticide or fertilizer released from the formulations.

EXPERIMENTAL

Carbohydrates and insoluble and soluble Klason lignin determination: The crude material was treated with 72% (w/w) H_2SO_4 at 45^{o}C during 7 minutes. Afterward the

material was diluted and hydrolyzed with 3% H_2SO_4 at 120°C during 30 minutes. The insoluble residue was filtered and dried at 105°C until constant weight. The ash content of this residue was determined and the insoluble Klason content was calculated as ash-free insoluble residue. In the soluble fraction, carbohydrates and soluble Klason were determined. Carbohydrates were analysed by HPLC using a BIORAD HPX-87H columm and 0.01 N H_2SO_4 at 0.6 ml/min as eluent. Soluble Klason content was determined by 280 NM absorbance measurement at pH 12 using SEBL as a standard.

Ash determination: The ash content of studied materials was determined after incineration at 800°C during 2 hours.

Density determination: Densities were determined by the ASTM method (3).

Elemental analysis: Carbon, Hydrogen and Nitrogen contents were determined in a Perkin Elmer PE 2400 CHN analyser. Sulfur was determined by a modified ESKA procedure (4).

Hydroxyl groups determination: The total hydroxyl content was determined after acetylation of the lignin followed by titulation of acetic acid produced in the reaction (5). Phenolic hydroxyls were determined by conductometric titulation of lignins using 0.2 M LiOH as titulant (6) and by the differential U.V. method discribed by Wexler (7). Aliphatic hydroxyls were calculated as the difference between total and phenolic hydroxyl content determined by the conductometric method.

Total acids determination: Lignins were agitated for 30 minutes in 0.1 M $NaHCO_3$ solution. The suspension was filtered and the residual $NaHCO_3$ content in the filtrate was determined by potentiometric titulation with standardized 0.1 M HCl. The calculation of the acid content in lignin was based on the $NaHCO_3$ consumed.

^{1}H-NMR, U.V. and I.R. spectra: ^{1}H-NMR spectra were obtained from previously acetylated samples using $CDCl_3$ as solvent in a Bruker AW 80 NMR spectrometer. U.V. spectra were obtained from lignin in dioxane/water solution using a Beckman DU-70 spectrophotometer. I.R. spectra were obtained from 1% lignin/KBr dishes in a Zeiss Specord spectrophotometer.

RESULTS AND DISCUSSION

Table 1 shows the contents of Klason lignin, ash and carbohydrates in lignins. The lignins studied are almost sugar-free materials. The low content of oligomeric sugars is an essential characteristic to obtain formulations by melting technique (2). Glucose and xylose were predominant in SEBLs and KELs while in Indulin AT arabinose was also detected. Low ash amounts and a total lignin content in the range of 93% to 97% were observed.

TAB. 1 - CHEMICAL COMPOSITION OF INDULIN-AT, KRAFT EUCALYPTUS (KEL) AND STEAM EXPLOSION BAGASSE (SEBL) LIGNINS

LIGNIN	KLASON(%)		ASH	CARBOHYDRATES (%)			TOTAL
	INSOLUBLE	SOLUBLE	(%)	GLU	XYL	ARA	(%)
SEBL ppt w/HCl	92.0	3.9	1.92	0.22	0.15	---	98.2
SEBL ppt w/H2SO4	93.4	4.1	1.86	----	0.39	---	99.7
KEL (#) ppt w/ HCl	89.7	4.7	1.71	0.23	0.20	---	96.5
KEL (#) ppt w/H2SO4	87.3	8.2	1.22	0.33	0.76	---	97.8
KEL (##) ppt w/H2SO4	91.2	5.7	0.59	0.06	0.25	---	97.8
INDULIN-AT	88.5	4.6	1.56	----	1.00	0.14	95.8

(#) and (##) = liquor with 37% and 14% of solids, respectively
GLU = glucose, XYL = xylose and ARA = arabinose.

Phenyl propane (C_9) formula, densities, amounts of hydroxyl groups and acids are showed in table 2.

TAB. 2 - ELEMENTAL ANALYSIS, DENSITY AND FUNCTIONAL GROUPS CONTENT OF INDULIN-AT, KRAFT EUCALYPTUS (KEL) AND STEAM EXPLOSION BAGASSE (SEBL) LIGNINS

LIGNIN	C_9 FORMULA	DENSITY (g/cm3)	HYDROXYL GROUPS (%) TOTAL	PHEN.	ALIPH.	TOTAL ACID(%)
SEBL ppt w/HCl	$C_9H_{10}O_{3.1}S_{0.01}$	1.38	12.7	2.9a 2.0b	9.8	0.16
SEBL ppt w/H_2SO_4	$C_9H_{9.9}O_{3.3}S_{0.01}$	n.d.	11.1	2.9a 1.9b	8.2	0.18
KEL ppt w/HCl (#)	$C_9H_{9.5}O_{3.6}S_{0.3}$	1.42	10.5	5.7a 2.7b	4.8	0.12
KEL ppt w/ H_2SO_4 (#)	$C_9H_{9.9}O_{3.5}S_{0.2}$	1.43	11.5	4.6a 3.0b	6.9	0.20
KEL ppt w/H_2SO_4 (##)	$C_9H_{9.8}O_{3.6}S_{0.2}$	1.41	11.0	4.5a 2.7b	6.5	0.21
INDULIN-AT	n.d.	1.38	12.9	6.8a 3.0b	6.1	0.19

n.d. = not determined; (a) = conductometric method (6); (b) = differential U.V. method (7)
(#) and (##) = liquor with 37% and 14% of solids, respectively

Kraft lignins from Eucalyptus showed high sulfur content probally due to sulfur incorporation during KRAFT process. Indulin AT and SEBLs showed similar densities while KELs showed higher values.

KEL from 37% solids Kraft liquor preciptated with HCl showed a higher content of phenolic hydroxyl groups (determined by conductometric method) than the other KELs studied. The content of total hydroxyl groups were similar in Indulin AT and SEBLs (12-13%) while in KELs lower values were observed (11%). The content of phenolic hydroxyl groups were significantly higher in KRAFT lignins than SEBLs. It has indicated that SEBLs shows higher degree of polymerization.

The percentage of ^{1}H obtained from ^{1}H-NMR spectra of SEBL and KEL are showed in table 3. The aromatic and - vinyl region showed lower values for SEBL than for KEL. Also the region of methoxyl groups showed higher values for KEL than for SEBL. These results has indicated that SEBL has lower methoxyl content than KEL. I.R. analysis of these lignins also suggested that KELs have higher syringyl groups content than SEBL.

U.V. spectra showed lower 280/310 NM absortivity ratios for SEBL than for KEL and Indulin AT (table 4). This result showed that bagasse lignin probally has higher amounts of conjugated carbonyl groups and $\alpha-\beta$ unsaturations than KEL and Indulin AT (8).

TAB. 3 - INTEGRATIONS OF NMR-^{1}H SPECTRA OF KRAFT EUCALYPTUS (KEL) AND STEAM EXPLOSION BAGASSE (SEBL) LIGNINS .

CHEMICAL SHIFT (ppm)	TYPE OF PROTON(#)	% ^{1}H SEBL ppt w/HCl	KEL(##) ppt w/HCl
7.15-6.3	AROMATIC AND α -VINYL	11.3	10.1
6.3-5.75	β -VINYL AND α - 1	3.4	3.9
5.75-5.2	α - 3	1.7	1.7
5.2-2.5	α-2 ; β ; γ AND METHOXYL	38.4	42.5
2.5-2.1	AROMATIC ACETOXYL	11.9	15.1
2.1-1.6	ALIPHATIC ACETOXYL	14.1	14.5
1.6-0.0	HIGLY SHIELDED ALIPHATIC	19.2	12.3

(#) α -1 = protons bonded to C-α in β-O-4 structures
α-2 = protons bonded to C-α in pinoresinol structures
α-3 = protons bonded to C-α in phenyl-coumaran structures
(##) = liquors with 37% of solids.

TAB. 4 - U.V. DATA OF INDULIN-AT, KRAFT EUCALYPTUS (KEL) AND STEAM EXPLOSION BAGASSE (SEBL) LIGNINS SPECTRA

LIGNIN	ABSORTIVITY (L/g.cm) 280 NM	310 NM	RATIO 280/310 NM
SEBL ppt w/HCl	24.0	19.5	1.2
SEBL ppt w/H_2SO_4	24.3	19.6	1.2
KEL (#) ppt w/HCl	22.9	15.0	1.5
KEL (#) ppt w/H_2SO_4	23.3	15.5	1.5
KEL (##) ppt w/H_2SO_4	25.3	17.1	1.4
INDULIN-AT	22.1	15.1	1.4

(#) and (##) = liquors with 37% and 14% of solids, respectively.

Overall differences in structure of analysed lignins were observed. These differences may be useful to explain the different release rates of pesticides and fertilizers formulated with these materials. It is a subject of our work in these area.

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AMMOXIDATION OF TECHNICAL LIGNINS IN LIQUID PHASE.

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ABSTRACT.

The main purpose of this study was the incorporation of nitrogen on lignin molecule by covalent bonding, for its possible use as a low-release fertilizer. The technical lignins used in this study consisted of kraft lignins, lignosulfonates and ASAM lignin. the ammoxidation reaction was carried out in a liquid phase in a high pressure-stirred reactor at a bench scale. The effect of temperature and reaction time, as well as the oxygen pressure on the total incorporated nitrogen is discussed.

INTRODUCTION.

The proper characteristics of commercial agriculture development claims for a gradual supply of nutrients. Up to date, the most common way to supply nitrogen to plants has been by means of inorganic salts; however, this practice has some disadvantages. On one hand, the high solubility of such salts causes great economic losses and pollutes underground waters, while on the other hand, due to plants may only take fertilizers in a brief period, it is necessary to apply fertilizer two or three times to annual crops with an additional expense and environment deterioration.

Now there is a tendency for using organic fertilizers as composted residues, which not only supply nutrients but also contribute to improve physico-chemical properties of soil. Another possibility for production of organic fertilizers is by the ammoxidation of lignocellulosic materials.

Lignins are byproducts that can be used as a raw material in the production of organic fertilizers by using an ammoxidation reaction.

The ammoxidation process has been worked out by KIM (1) over pine sawdust, while COCA (2) has applied this process to lignites, and recently MARTINEZ (3) has also applied this process to kraft lignin. These authors have used such process which consist of a solid/gas reaction taking place in a fluidized bed system.

Other studies on lignin ammoxidation have been made by FLAIG (4), MILETZKY (5). These authors have applied the ammoxidation process in a liquid phase-stirred reactor, using only spent sulfite liquor.

The aim of present work was to develop the ammoxidation process in a liquid phase at high temperature and pressure in a stirred reactor, over different technical lignins and one from a pulping process that is at present time at pilot plant scale.

EXPERIMENTAL PROCEDURE.

Raw materials.

The basic raw materials were kraft lignin from the Compañía Industrial de Atenquique, Mexico (LIGNATEN), and kraft lignin from Lignotech Company, Sweden, (KRAFT LIGNOTECH), and additionally it was tried a carbohydrate-free lignosulfonate also from Lignotech Sweden, (LS LIGNOTECH), and finally a black liquor sample from pilot plant of ASAM pulping, supplied by Kraft Anlagen heidelberg (LS ASAM).

Reactor description.

A Büchi stirred reactor of one liter capacity was used for the experiments, fitted with temperature heating control by oil recycling to the body jacket. The reactor has also pressure measurement and control of stirring speed. Figure 1 shows a schematic diagram of the system.

Ammoxidation procedure.

It was taken 45 g of each lignin sample and diluted in 50 ml NH_4OH plus 550 ml deionized water and then this solution was charged to the reactor at room temperature. Inner air was replaced and oxygen was then fed up to the specified initial pressure, after which the reactor was then heated up to the set point temperature in about 20 minutes. As a means of reaching the set point temperature within this period of time, oil was pre-heated up to 15 °C above the set point and once reached, it was again reset to the set point.

it is necessary to comment that the sample identified with time equals to 0 minutes in charts, corresponds to a reaction time of 20 minutes, from room temperature up to getting the maximum temperature, which then was kept during the rest of the process. To achieve a quick cooling of the reactor, it was used an alternate oil bath at room temperature.

Liquor samples were taken by means of a needle valve connected with a cooling coil in the lower section of the reactor as shown in the schematic diagram. Those liquor samples of ammoxidated lignin were taken at different time intervals then dried in a Büchi Mini Spray Dryer model 190.

Fig 1 SCHEMATIC DIAGRAM OF THE REACTION SYSTEM

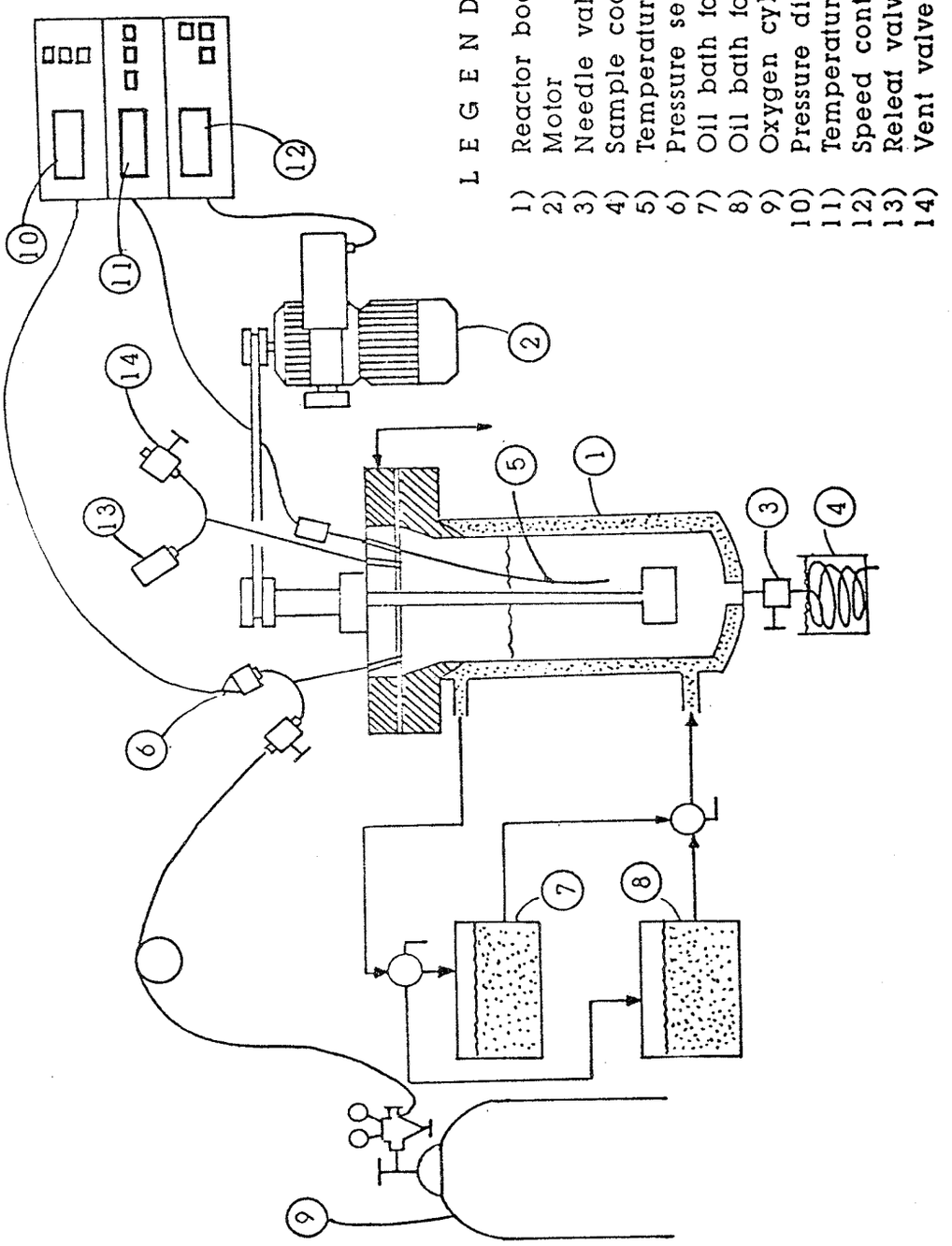
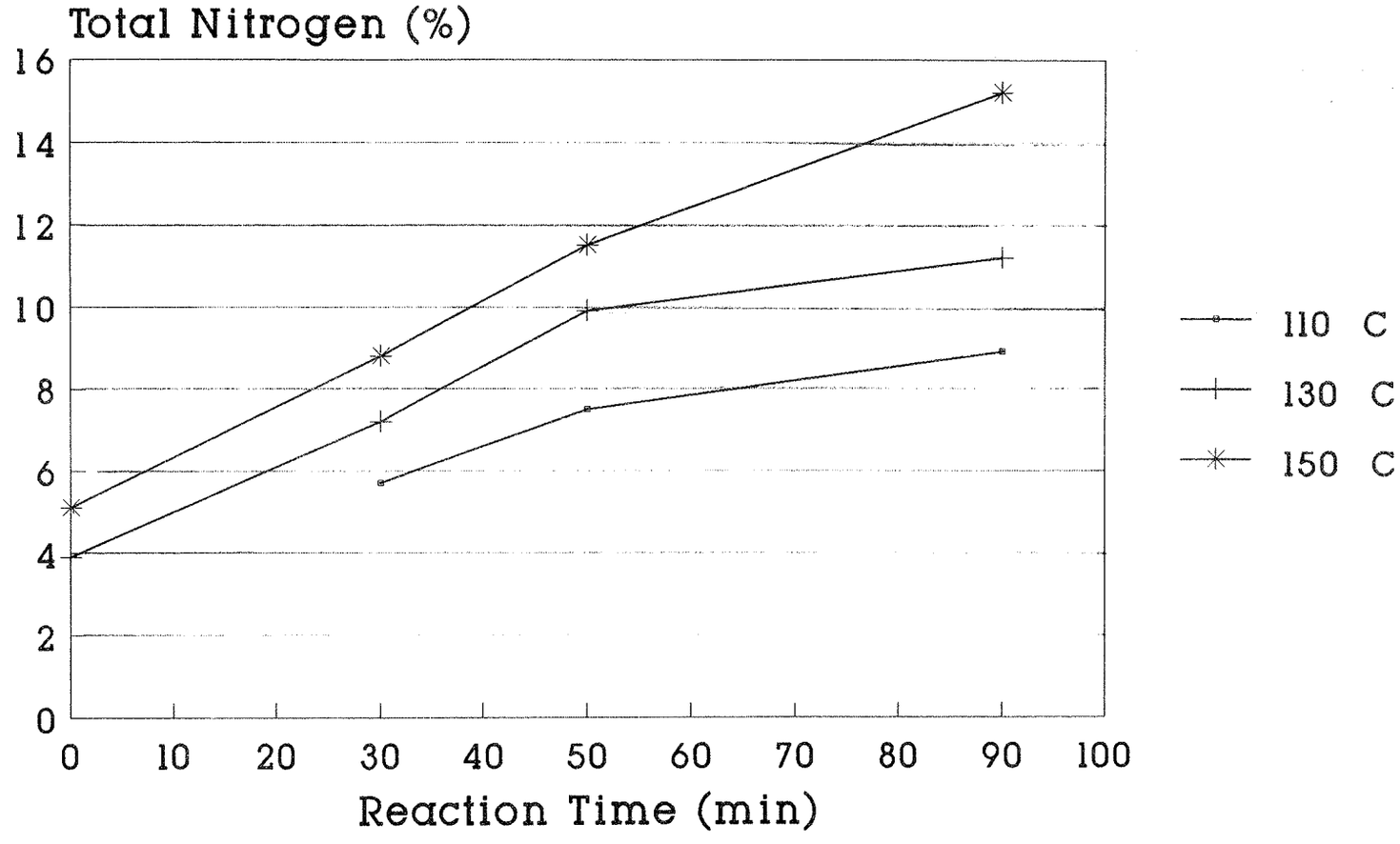
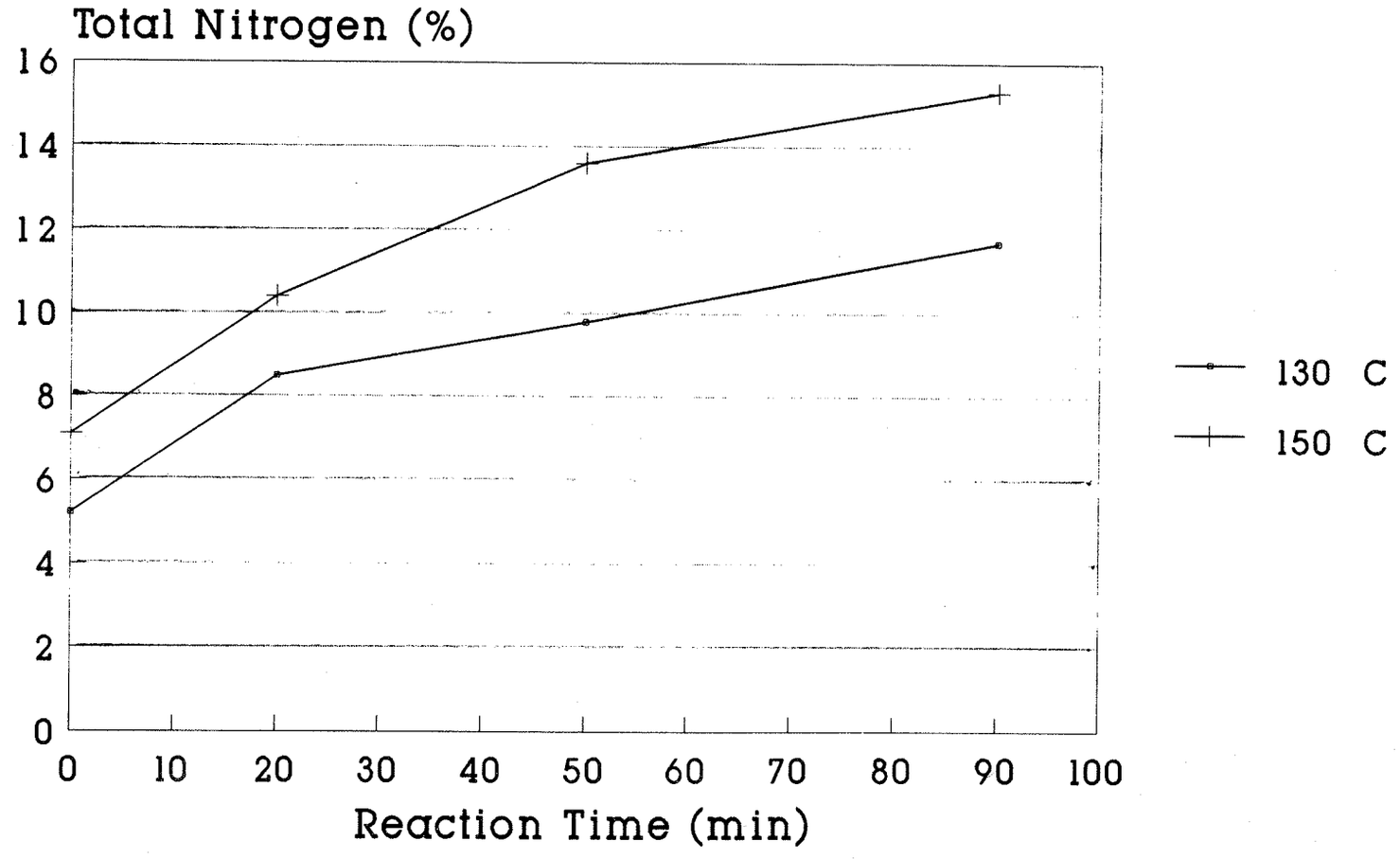


FIGURE 2
NITROGEN INCORPORATION IN LIGNATEN*
- EFFECT OF TIME AND TEMPERATURE -



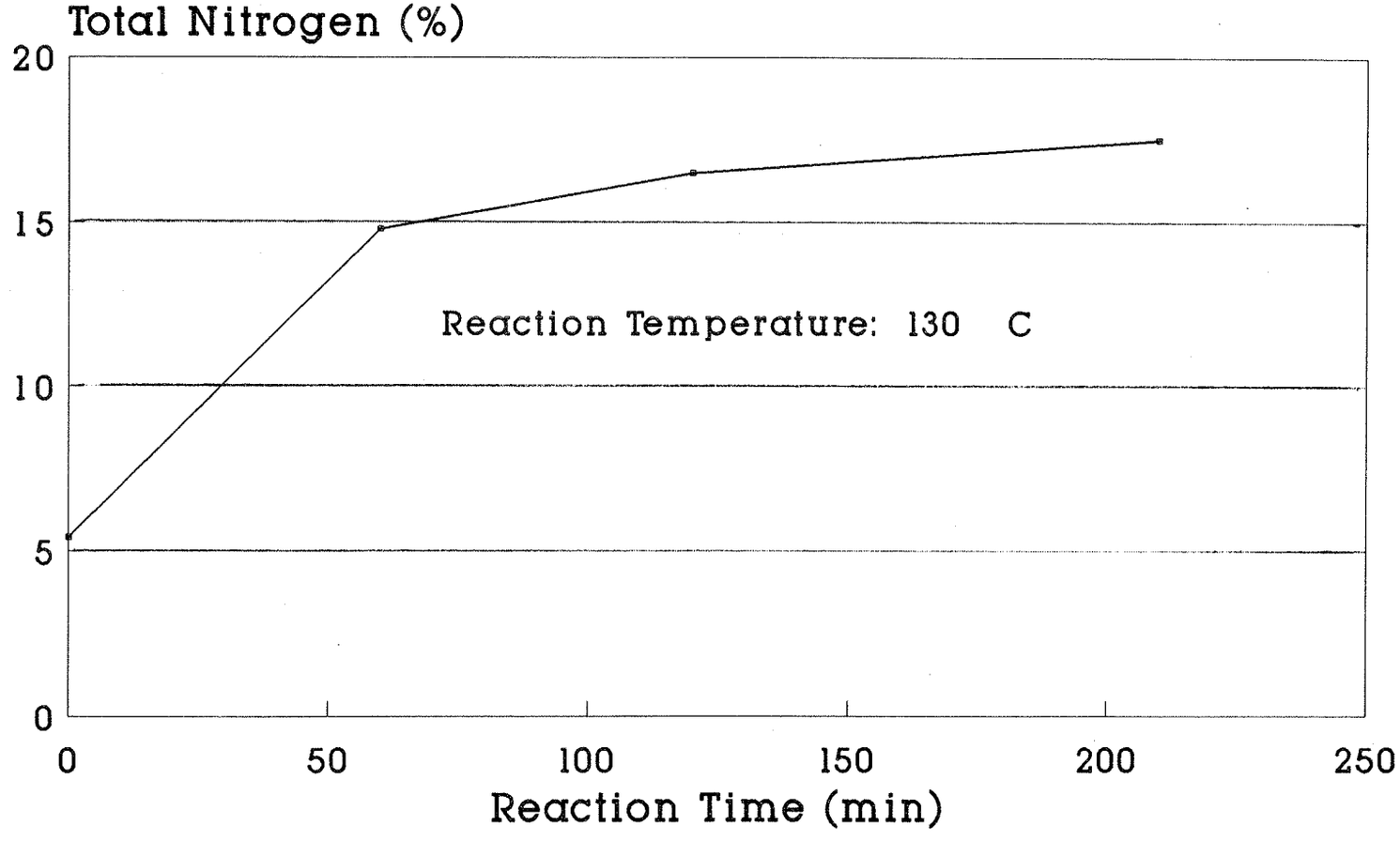
* Lignin from Atenquique, México

FIGURE 3
NITROGEN INCORPORATION IN KRAFT LIGNIN*
- EFFECT OF TIME AND TEMPERATURE -



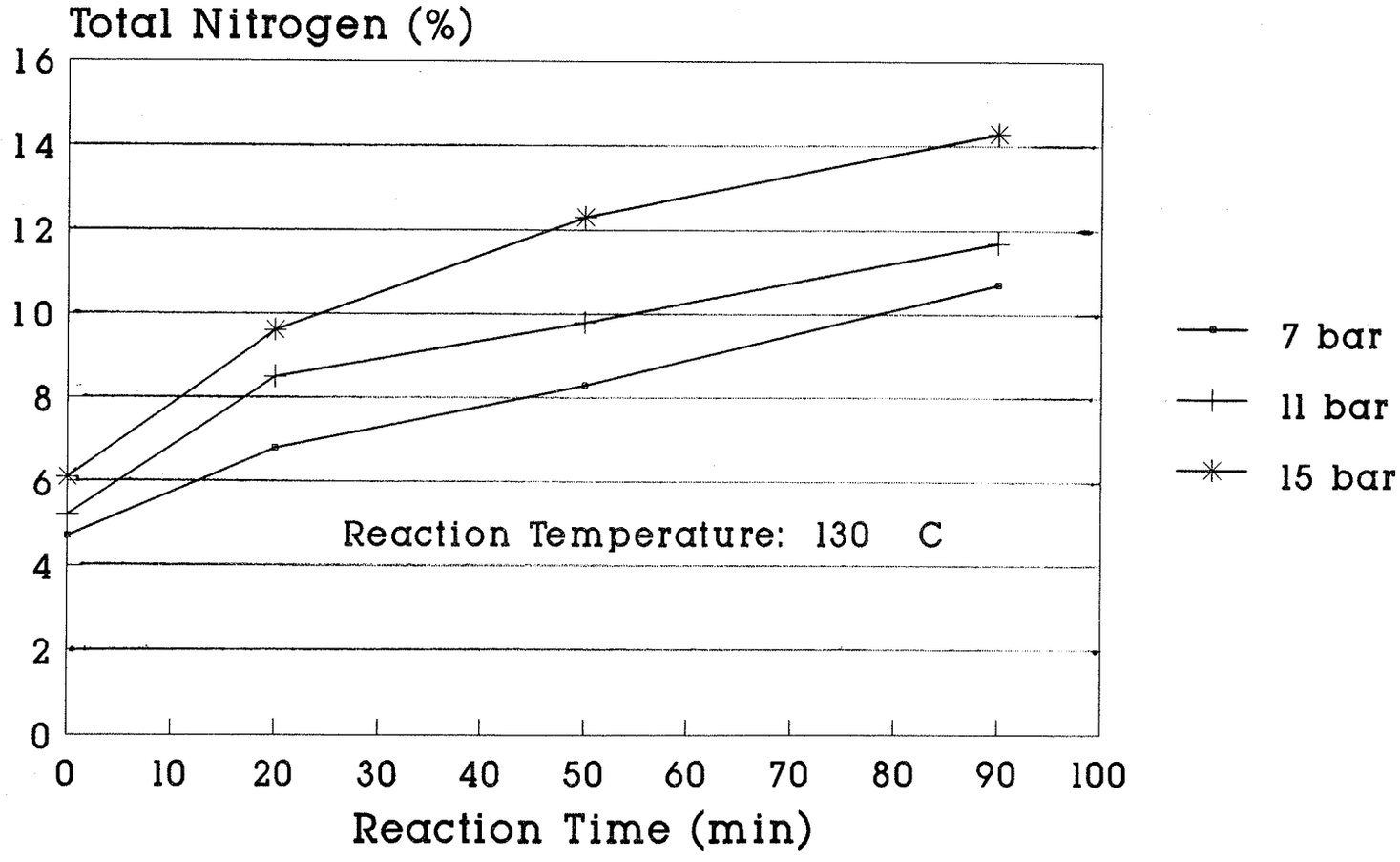
* Lignin from LIGNOTECH, Sweden

FIGURE 4
NITROGEN INCORPORATION IN LIGNATEN*
- EFFECT OF A LONG REACTION TIME -



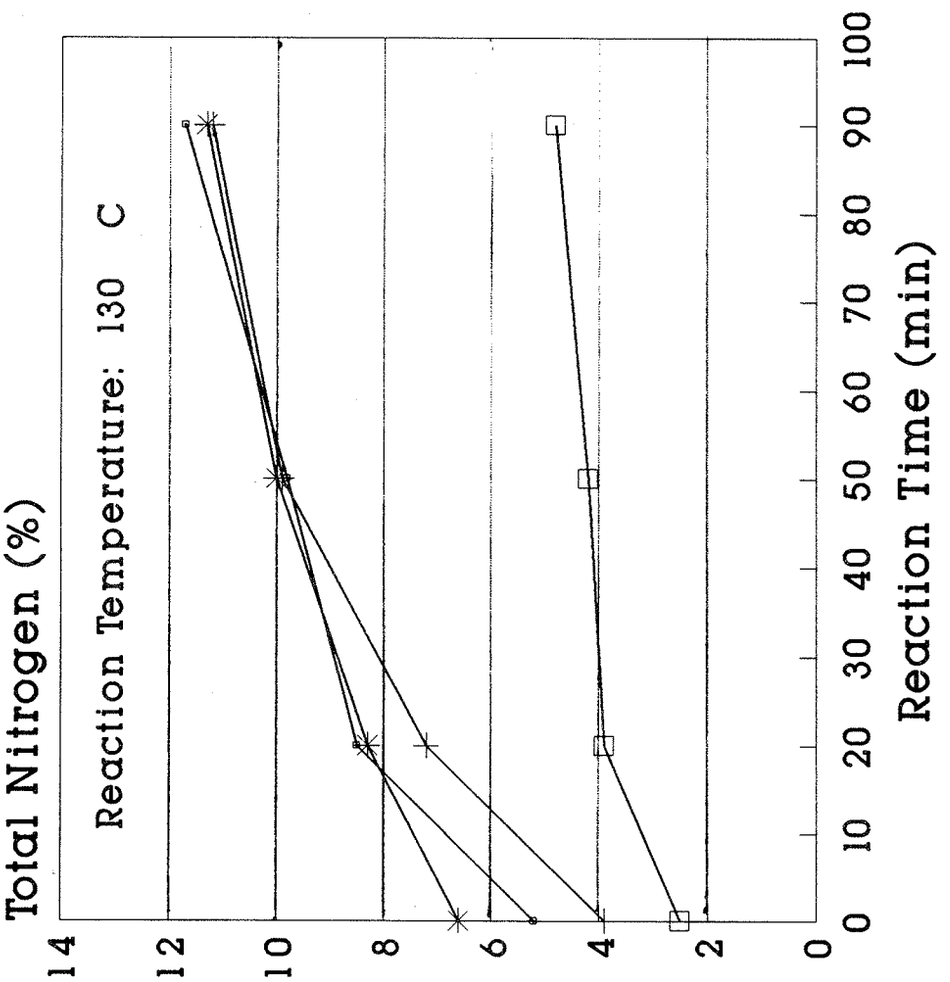
* Lignin from Atenquique, México

FIGURE 5
NITROGEN INCORPORATION IN KRAFT LIGNIN*
- EFFECT OF TIME AND OXYGEN PRESSURE -



* Lignin from LIGNOTECH, Sweden

FIGURE 6
NITROGEN INCORPORATION IN DIFFERENT TECHNICAL LIGNINS



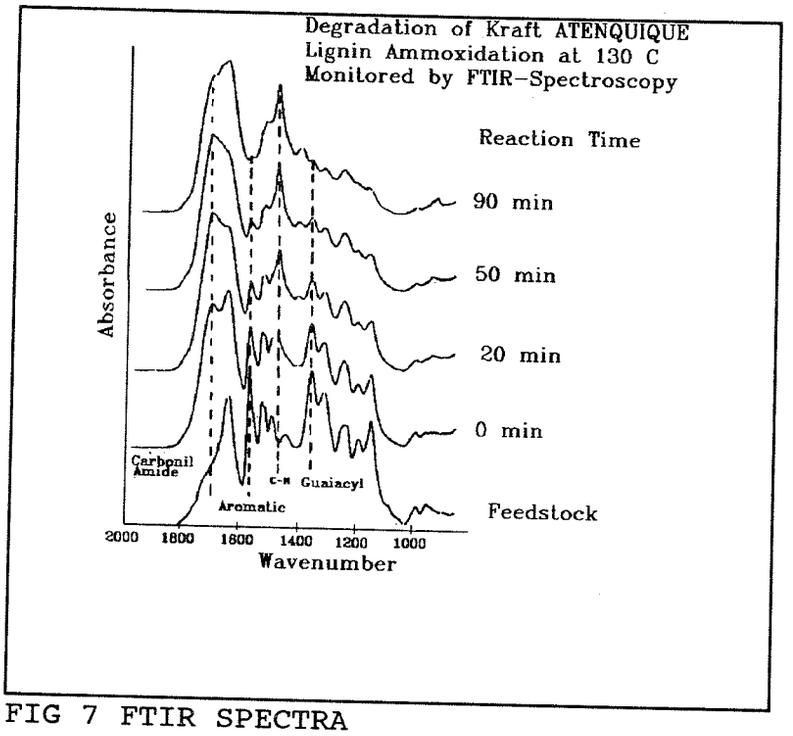


FIG 7 FTIR SPECTRA

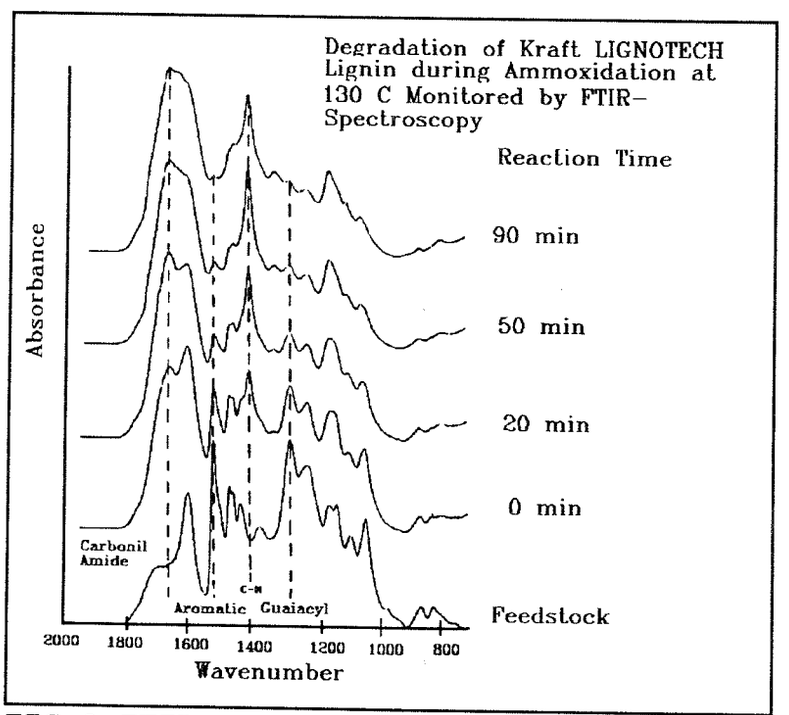


FIG 8 FTIR SPECTRA

RESULTS AND DISCUSSION.

Figures 2 and 3 show time and reaction temperature effects on the nitrogen fixation in kraft lignins (LIGNATEN and LIGNOTECH). In both figures, an increase in nitrogen fixation can be observed when the reaction temperature is rised from 110 °C to 130 °C and 150 °C, respectively. In these three reactions were carried out for 90 minutes and the results showed that nitrogen was fixed at a content of 15%. These data suggest the use of higher reaction temperatures.

The effect of large reaction times on the nitrogen fixation is depicted on Figure 4. It is evident that during the first 80 minutes of reaction, including 20 minutes of temperature rise, the 15% nitrogen fixation is reached. However, from the minute 80 to the end of the reaction, only a 2% gain (17% total) in nitrogen fixation is achieved.

Figure 5 shows the profiles of total nitrogen content with regard to oxygen pressure at different reaction time. It is evident that the limiting reaction of the overall process, is the oxidation stage, which is limited, as well, by oxygen diffusion occurring from the gas to the liquid phase. This was noticed since an increase in oxygen pressure produced an increase in nitrogen fixation.

Figure 6 shows profiles of nitrogen fixation for those technical lignins processed at 130 °C. Kraft and lignosulfonates profiles are very similar, while ASAM lignin shows a lower reactivity maybe due to its high sulfonation degree and ash content.

Feedstock and ammoxidated products FTIR spectra are illustrated in Figures 7 and 8. These spectra show the reduction of aromatic and guaiacyl groups while new groups are formed, such as carbonylamide and C-N.

CONCLUSIONS.

From a practical point of view, 60 minutes at maximum temperature is recommended as the reaction time, since the 90% of total Nitrogen fixation is reached at this time.

Experimental results suggest that higher reaction temperature could increase the amount of Nitrogen fixed.

Results obtained up to date justify field experiments since 17% of Nitrogen content is a good basis.

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ACKNOWLEDGMENT

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AMMOXIDATION OF LIGNACEOUS MATERIALS IN A FLUIDIZED BED REACTOR.

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ABSTRACT

The ammoxidation study of kraft lignin, bark, and sawdust -all from pine- in a fluidized bed reactor at a bench scale, is presented.

By means of the central composite experimental design, the effect of temperature, time and oxygen/ammonia ratio was evaluated on the nitrogen incorporation to such materials.

Temperature had the greatest effect followed by time. With respect to oxygen/ammonia ratio, it was found that oxidation was the controlling reaction in the overall process. Up to now the highest value for nitrogen fixation has been 18% for sawdust.

INTRODUCTION

In order to grow crops effectively it is usually necessary to supply a source of nitrogen. This source is commonty either ammonia, urea, or salts containing ammonia and/or nitrate ions. All of these are very water soluble and even in temperate climes more than half of the applied nitrogen is often lost by volatilization and leaching. In semitropical agricultural areas, there are brief periods of exceptionally heavy rain fall which tend to leach away any soluble fertilizer. Even if there is no rain, the high soil temperature will speed the conversion of the urea into ammonia.

The ammonia (cheapest form of nitrogen) and the great amounts of lignocellulosic residues can be converted into water insoluble form of nitrogen by a low-cost reaction of ammoxidation.

The ammoxidation process has been worked out by KIM (1) over pine sawdust, while COCA (2) has applied this process to lignites. These authors have used such process by a

solid/gas reaction in a fluidized bed system. Other studies on lignin ammoxidation have been made by FLAIG (3), and MILETZKY (4). Those authors have applied the ammoxidation process in a liquid phase, using spent sulfite liquor.

The aim of this study was to determine the effects of reaction time, temperature and the ratio air/ammonia on the extent of ammoxidation attainable in a bench scale fluidized bed reactor for different lignocellulosic residues.

EXPERIMENTAL PROCEDURE

Raw materials.

The raw materials: kraft lignin, bark and saw dust, were collected from a pulp and paper company "Compañía Industrial de Atenquique" in Mexico. This mill use pine as the main source of wood. The lignin was obtained by precipitation of concentrated black liquor with CO_2, and washed with cold water three times in order to eliminate ashes. All the raw materials were dried at room temperature and milled. The fraction between mesh 20-60 were collected for the study.

Equipment.

Fig. 1 shows a schematic diagram of the ammoxidation equipment. The fluidized bed reactor is a glass tube (20 mm i.d.) at the bottom has a distributor mode of sintered glass. The air was heated directly with heaters and the ammonia was heated indirectly as showed in the figure. After heating, both gases, were mixed in a union "Y". The flows of gases were controlled with needle valves and the temperature was controlled with a PID controller. The total flow of gases was 25 l/min for lignin, 30 l/min for bark and 22 l/min for saw dust.

Analysis.

The samples obtained were analyzed for nitrogen content according to AOAC Method (5).

Experimental design.

A central composite experimental design was used (6).

RESULTS AND DISCUSSION.

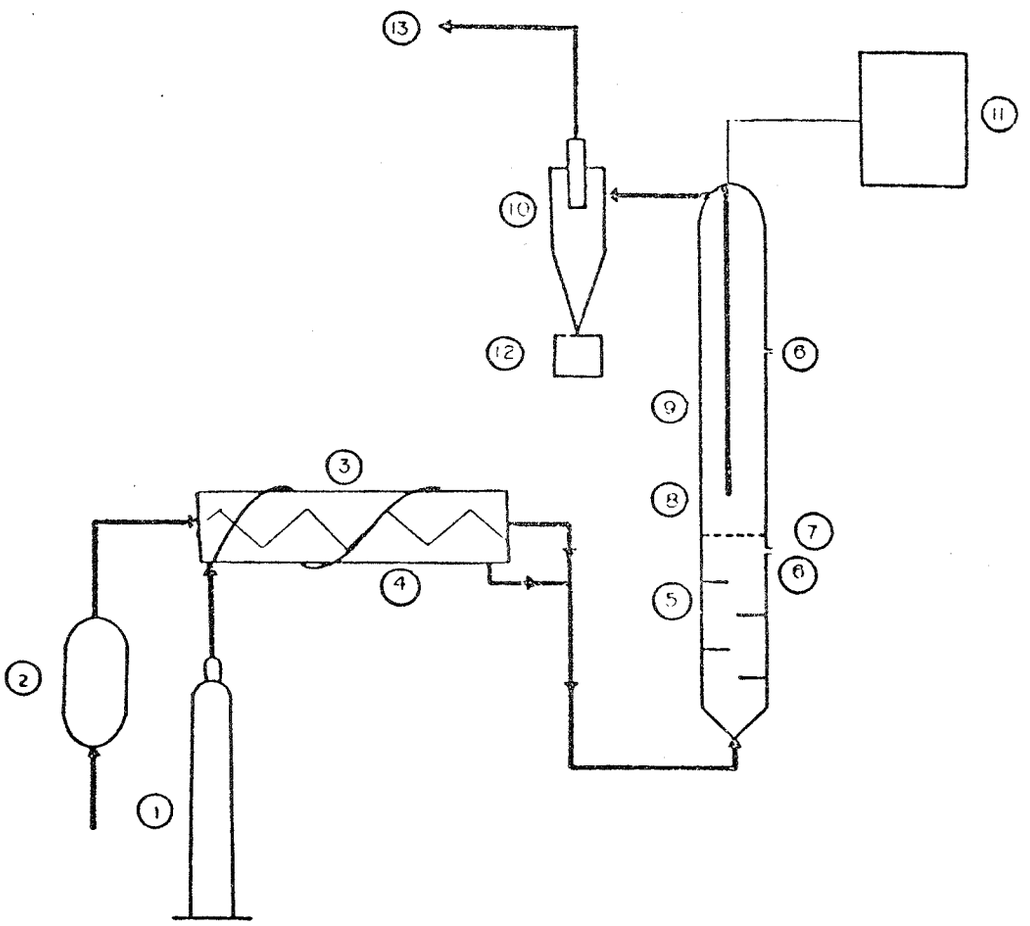
Three independent variables were selected for the study: reaction time, temperature, and ratio air/ammonia maintaining the total flow of gases constant.

The expression which related the variables and their labels is the following:

$$t = X_1 + 3 \qquad \text{or} \qquad X_1 = t - 3$$

Fig. 1 Schematic diagram of the fluidized bed reactor.

1 Ammonia cilinder
2 Air compressor
3 Heaters
4 Heat exchanger
5 Deflectors
6 Manometric outlet
7 Gas distribuitor
8 Reactor
9 Thermocouple
10 Cyclone
11 Temperature controller
12 Fine solid collection
13 Gas outlet



$$T = 25X_2 + 250 \quad \text{or} \quad X_2 = (T - 250)/25$$

$$C_{gases} = 0.75X_3 + 3 \quad \text{or} \quad X_3 = (C_{gases} - 3)/0.75$$

Where t, T and C_{gases} are the independent true variables and X_1, X_2 and X_3 are the independent coded variables.

The model of the experimental design is the quadratic surface between the measured property (in this case nitrogen fixed) and the independent variables coded. The model is as follow:

$$\%N = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + B_{23}X_2X_3$$

Table 1 summarized the values obtained for the coefficients for bark and lignin by the least squares adjustment. The fact that the standard deviation and the pere error are similar for both cases its indicated that the model describes the results with the same precision that the experimental technique

TABLE 1 OBTAINED VALUES FOR THE COEFFICIENTS

Coefficients	Value for Bark	Value for Lignin
b0	8.777956	6.411364
b1	1.230625	1.5
b2	2.285625	1.785
b3	-0.818748	-0.3500001
b4	-0.772732	-0.1068186
b5	-0.610234	0.1306817
b6	-0.172733	3.068161E-02
b7	6.374E-2	0.300001
b8	0.362501	-7.500005E-02
b9	7.875E-2	-7.499993E-02
Correlation coeff.	0.9904	0.99841
Pure error	1.254	1.999996E-02
Standard deviation	0.209	3.333327E-03

The effect of the variables on the nitrogen fixation was analyzed by means of graphics obtained from the models for each raw material.

Figures 2 and 3 show the effect of time and temperature on nitrogen fixation. An increase in reaction time or temperature increased the amount of fixed nitrogen. In the case of bark after 3 hours of reaction time the profiles start to became asintotic. However, for lignin it seen that a longer

FIG 2 Effect of time and temperature on nitrogen fixation in pine bark

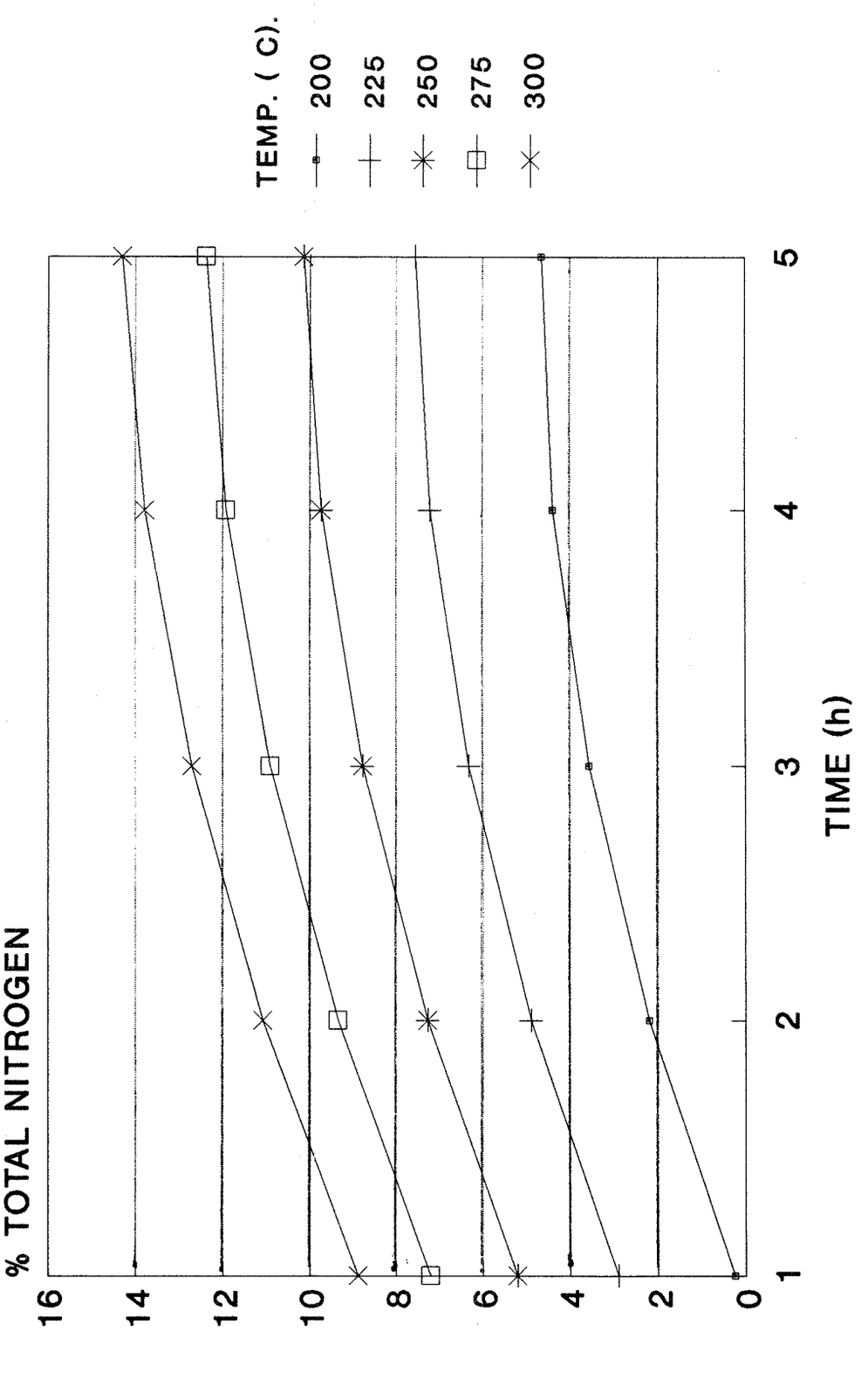
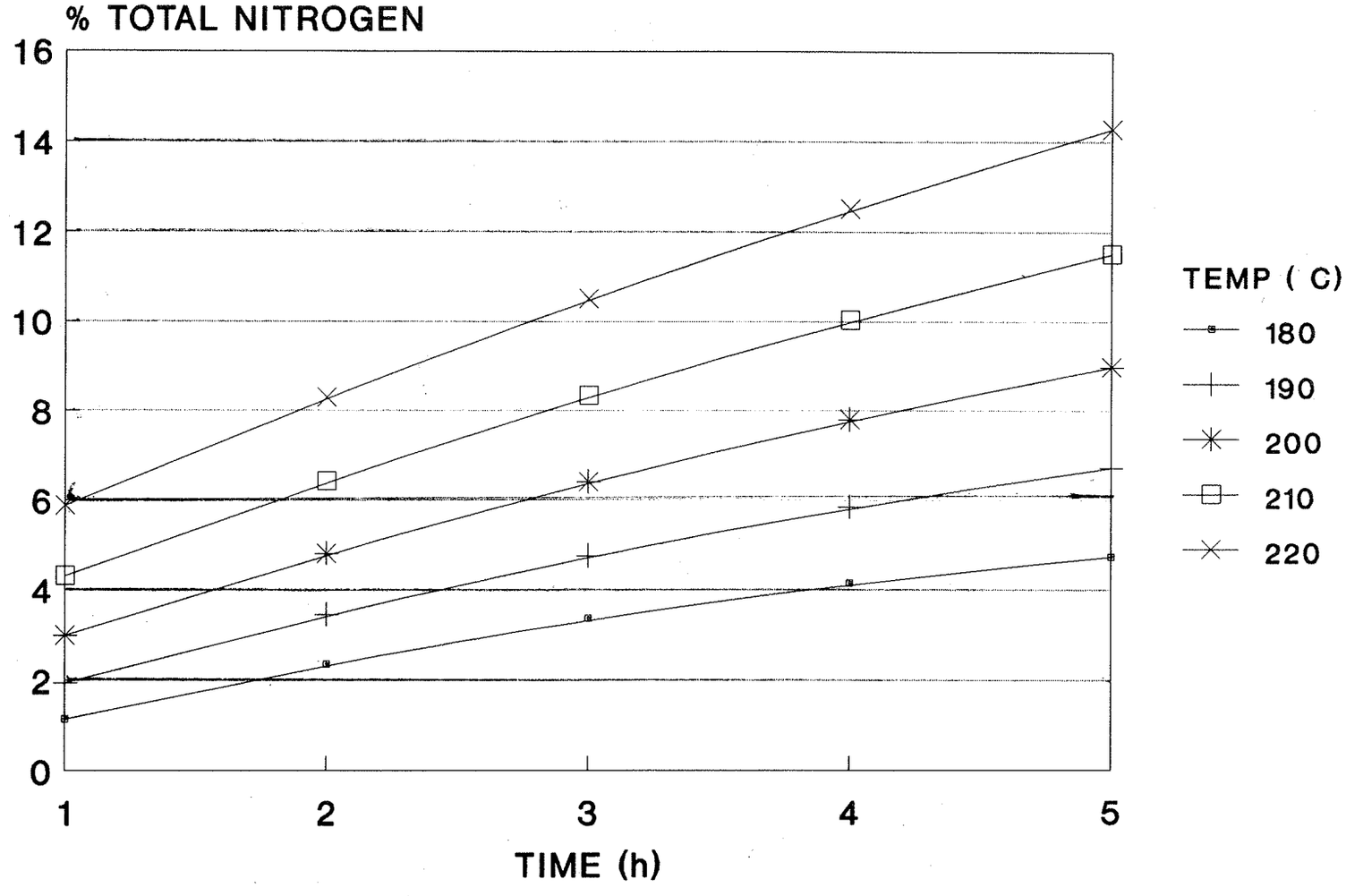


FIG 3 Effect of time and temperature on nitrogen fixation in Kraft lignin



RATIO (Air/Ammonia)= 2.5

FIG 4 Effect of time and ratio of gases on nitrogen fixation in pine bark

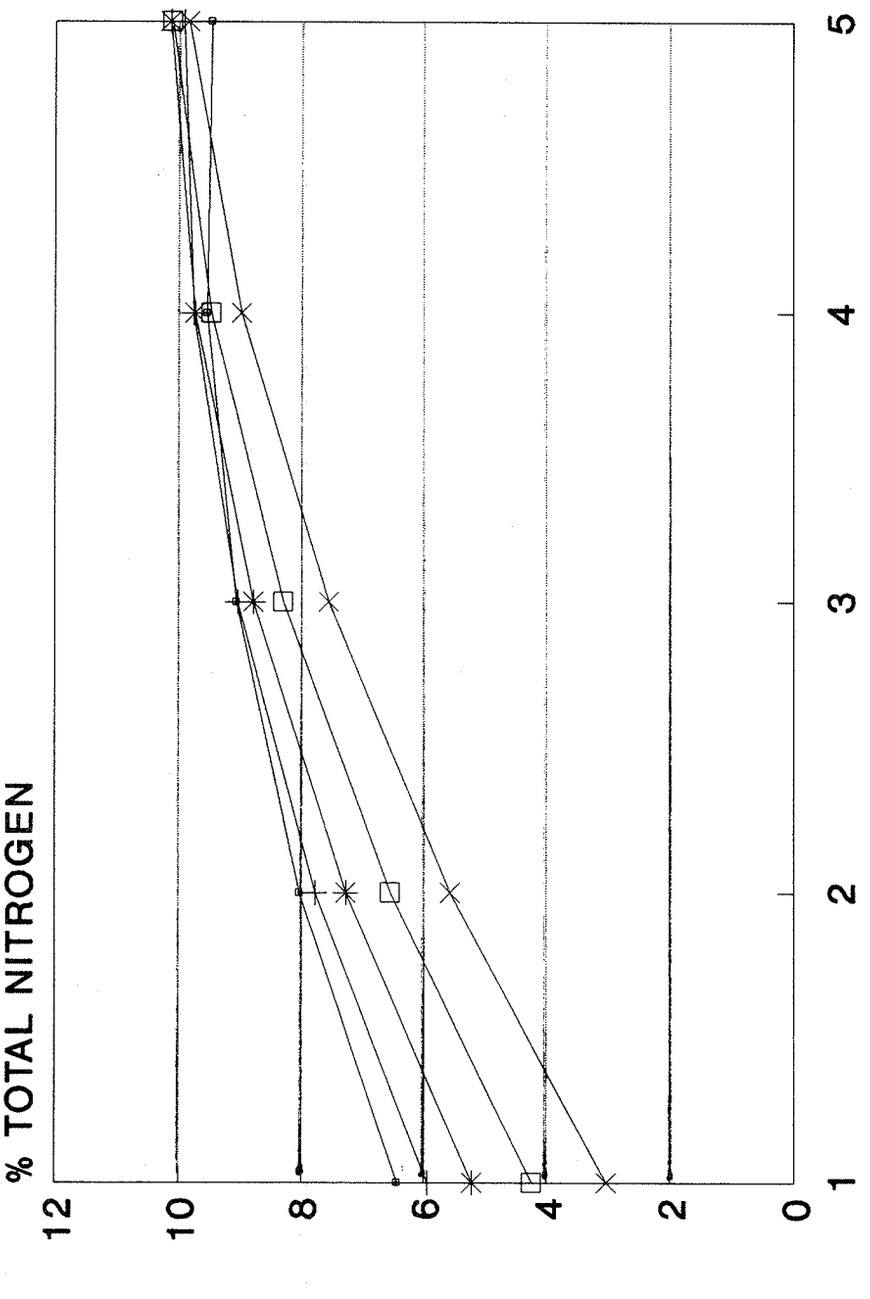
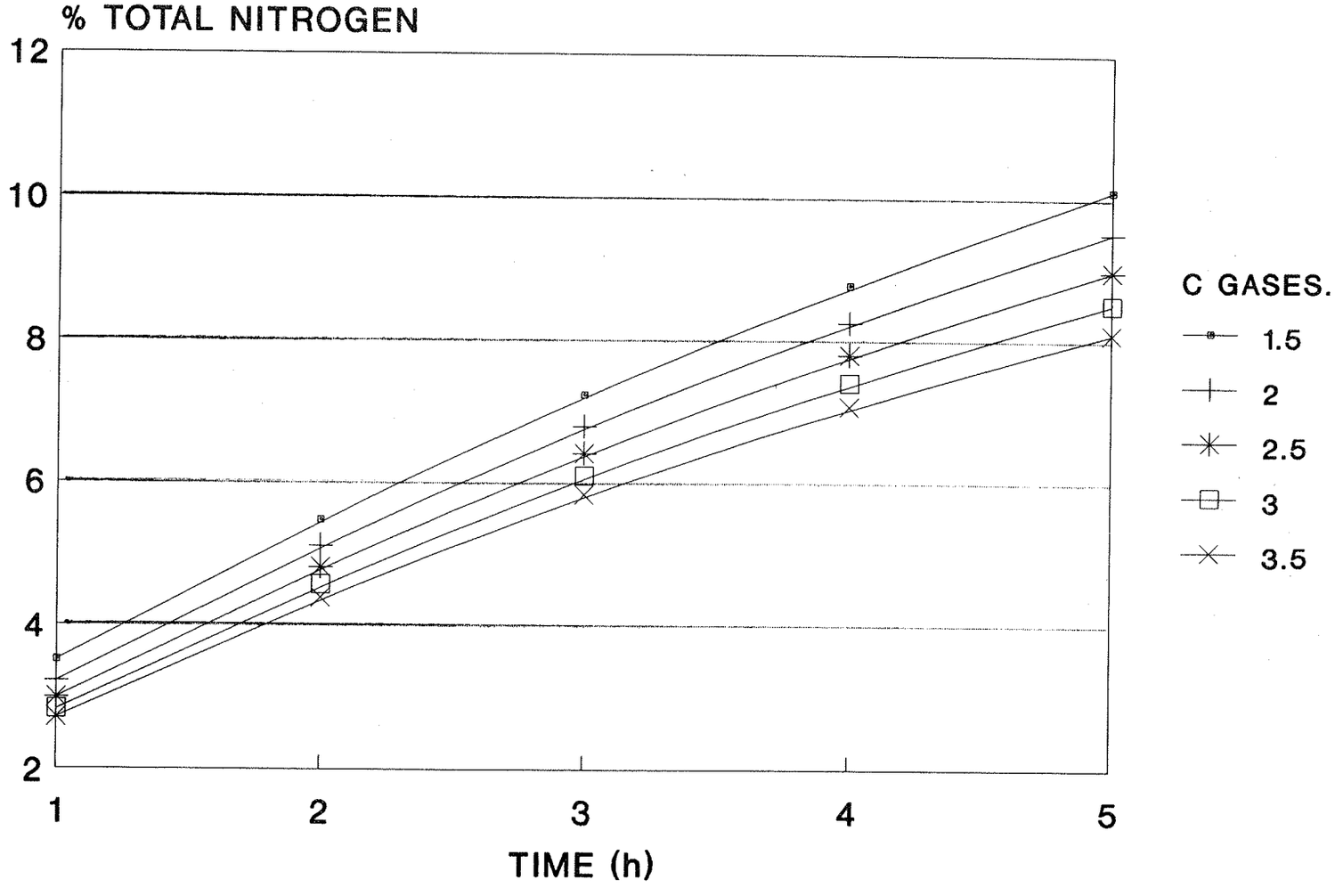
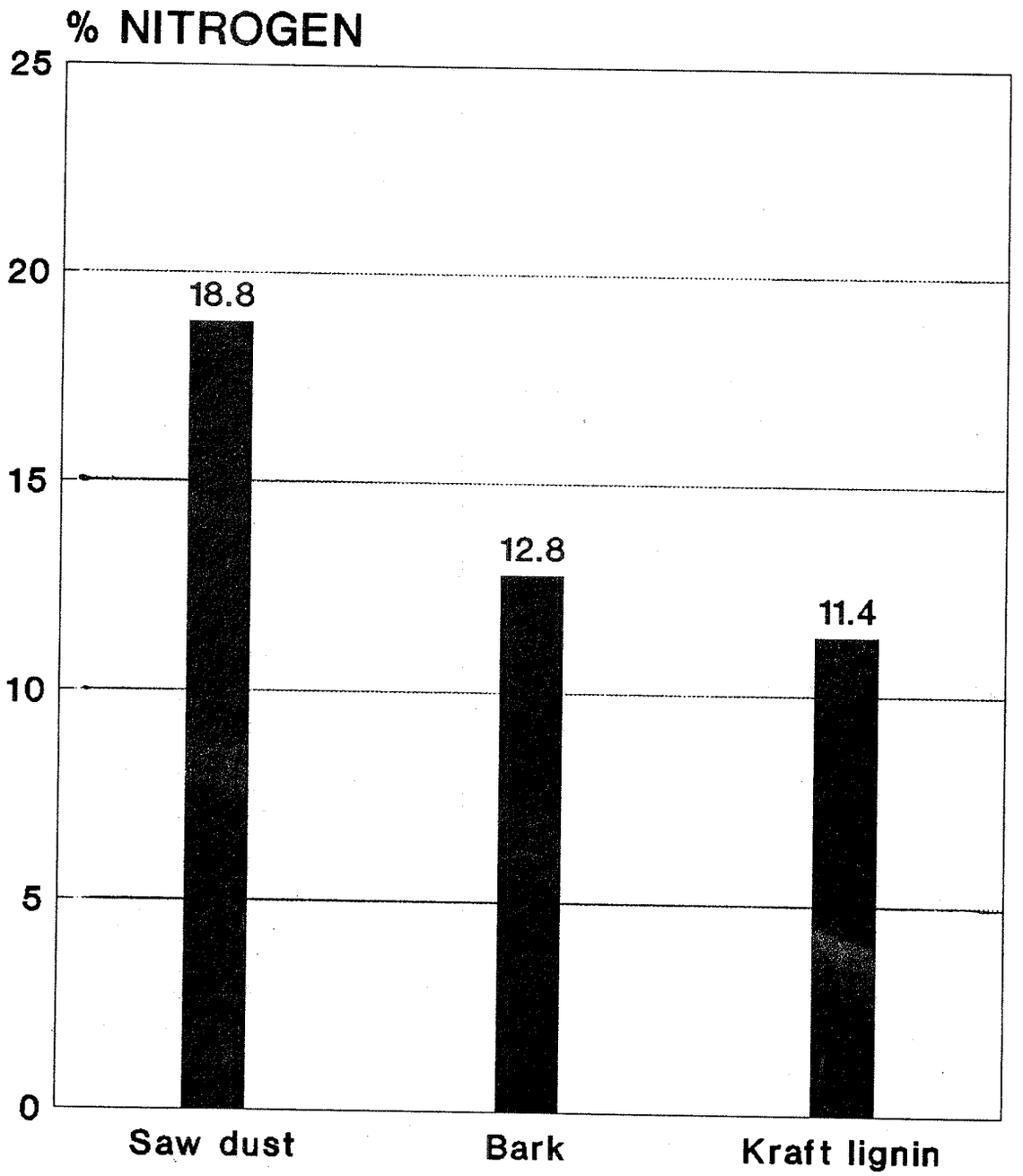


FIG 5 Effect of time and ratio of gases on nitrogen fixation in kraft lignin



TEMPERATURE 200 C

FIG 6 MAXIMUM AMOUNT OF NITROGEN FIXATION OBTAINED EXPERIMENTALLY



time will produce more nitrogen fixation. However in the case of lignin the temperature cannot be increased beyond 220°C because it burns.

Figures 4 and 5 show the nitrogen fixation as function of time and ratio of air/ammonia. In the case of bark at short of reaction time, the effect of the concentration of ammonia is more appreciable, becoming contemptible from 4 to 5 hour of reaction.

The lignin behavior with respect to ammonia concentration is different at the studied condition. Figure 5 shows that at 5 hours of reaction time, the ammonia concentration still is important in the nitrogen fixation, may be it is because the processes with lignin is limited with respect to the temperature, at higher temperature shorter time to reach the saturation of nitrogen.

Figure 6 shows a comparison of maximum fixed nitrogen obtained experimentally for the tested lignocellulosic materials.

Summarizing the analysis of results it is concluded that temperature and time are the major factors which affect the ammoxidation of lignocellulosic materials. The contained of functional groups capable of react with ammonia are in major quantity as follow: saw dust bark lignin. In lignin the functional groups has to be created by oxidation before the reaction with the ammonia take place. For the bark this means that at the beginning of the ammoxidation the step limiting the reaction is the ammoniation and at longer reaction times the oxidation limited the rate of reaction. However for lignin in all the processes the reaction rate is limited by the oxidation.

CONCLUSIONS.

1) The time and temperature are the more important variables in the processes of ammoxidation. The process has to be conducted at high temperature as possible to reduce time and to save ammonia.

2) The quantity of fixed nitrogen achieved up to date for the raw materials used in this study are good to consider the agronomic studies to test the bifunctional property: humic fertilizer as soil conditioner and non-leaching slow release nitrogen source.

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FINE CHEMICALS WITH TRIMETHOXYBENZOYL GROUP OBTAINED FROM WOOD TAR EUCALYPTUS

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ABSTRACT

3,4,5-Trimethoxybenzoic acid was obtained by oxidation of methylated syringyl *Eucalyptus* wood tar fraction. From this compound several fine chemicals like 3,4,5-trimethoxybenzoyl chloride, trimetozine, N-(3,4,5-trimethoxybenzoyl)-imidazole, 3,4,5-trimethoxybenzyl 3,4,5-trimethoxybenzoate, methyl and ethyl 3,4,5-trimethoxybenzoate, and 3,4,5-trimethoxybenzoic anhydride were prepared.

INTRODUCTION

The fractionation of *Eucalyptus* wood tar obtained by distillation furnished several fractions. The 240-270°C fraction (12%, tar) turned out to be rich in syringyl derivatives. This fraction was methylated, oxidized and, after work up, furnished the 3,4,5-trimethoxybenzoic acid[1] as main product. This compound was used as raw material for preparation of several chemicals which contain the 3,4,5-trimethoxyphenyl portion.

The compounds 3,4,5-trimethoxybenzoyl chloride, trimetozine (I), N-(3,4,5-trimethoxybenzoyl)imidazole (II), 3,4,5-trimethoxybenzyl 3,4,5-trimethoxybenzoate (III), methyl (IV) and ethyl (V) 3,4,5-trimethoxybenzoate, and 3,4,5-trimethoxybenzoic anhydride (VI) were prepared. Chart 1 summarizes these preparations.

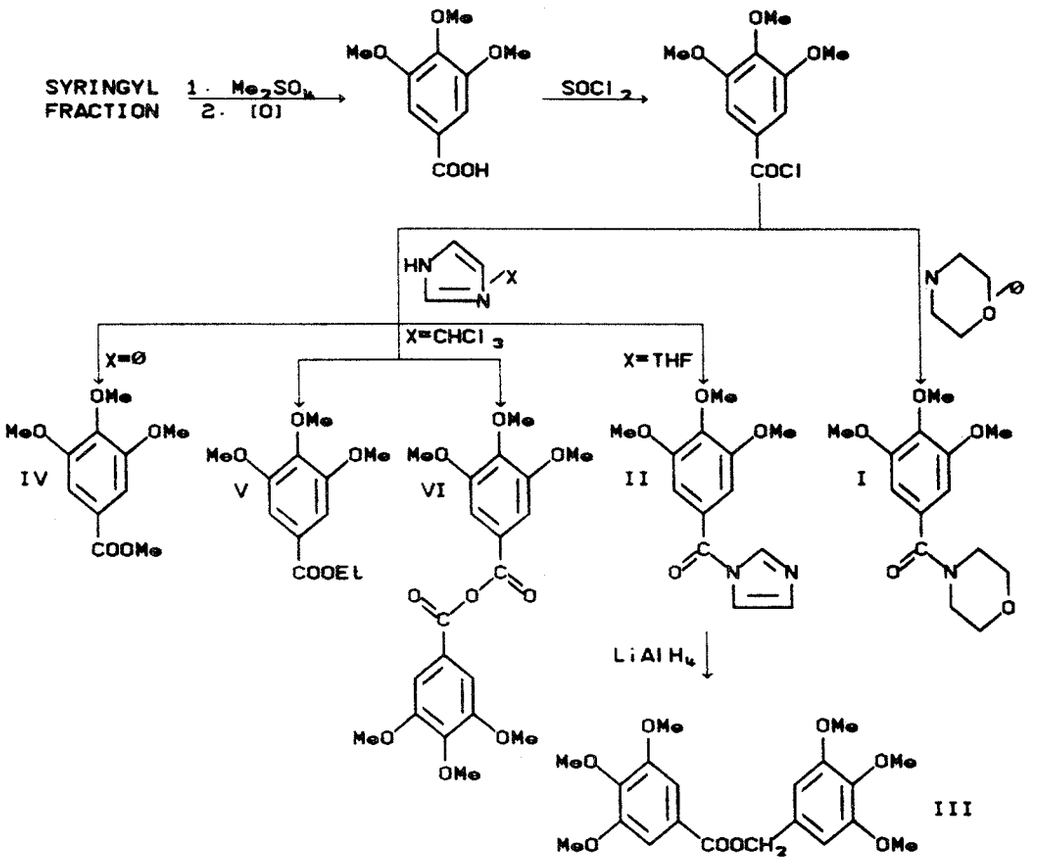


CHART 1 - Fine Chemicals obtained from 3,4,5-trimethoxybenzoic acid

MATERIALS AND METHODS

GENERAL: Melting points were determinated at a 247-Leitz-Wetzlar-Dialux apparatus and are uncorrected. IR spectra were recorded at a IR-408 Shimadzu; ^{1}HNMR (60MHz) at a EM 360 Varian; ^{1}HNMR and ^{13}CNMR (200 and 50 MHz, respectively) at a AC 200 BRUKER.

- 3,4,5-Trimethoxybenzoic acid:

This acid was obtained from *Eucalyptus* wood tar fraction as described by Carazza et al[1].

- 3,4,5-Trimethoxybenzoyl chloride:

212 mg (1 mMole) of the acid and 0.6 mL of the thionyl chloride were refluxed for 3 H. This reaction was monitored by TLC. The excess of thionyl chloride was removed by distillation under reduced pressure furnishing the corresponding acid chloride with 96% yield, used without purification.

-N-(3,4,5-Trimethoxybenzoyl)morpholine (trimetozine)-I:

The acid choride (obtained from 217 mg of the acid) was dissolved in dry benzene (3.5 mL) and morpholine (0.2 mL, 2.29 mMoles) was slowly added with stirring and cooling in ice-water. The stirring was continued for 30 min more, then the reaction mixture was allowed to stand overnight. The solvent was distillated under reduced pressure furnishing trimetozine (253 mg, 88% yield). Mp. 119°C (rec. EtOAc) lit.[2,3] 120-121°C. IR (υ_{max}^{KBr}, cm^{-1}): 2950, 2850, 1640, 1590-1400. ^{1}HNMR ($CDCl_3$, δ): 6.6 (2 H), 3.9 (9 H), 3.7 (8 H). MS (m/z): 281 ($M^{+\cdot}$).

- N-(3,4,5-Trimethoxybenzoyl)imidazole-II:

230 mg (1 mMole) of the acid chloride was dissolved in dry tetrahydrofuran (2 mL) and added dropwise to a stirred solution of the imidazole (2 mMoles) in THF (2 mL) at room temperature and the stirring was continued for 2 h more. After 18 h, the mixture was filtered and the filtrate was concentrated under reduced pressure to give the residual N-acylimidazole[4] with 81% yield. IR (υ_{max}^{KBr}, cm^{-1}): 3100, 2900, 1705, 1600-1400. ^{1}HNMR ($CDCl_3$, δ): 8.2 (1 H), 7.7-7.2 (2 H), 7.1 (2 H), 4.0 (9 H). The substance presented high instability.

- 3,4,5-Trimethoxybenzyl 3,4,5-Trimethoxybenzoate-III:

To a solution containing approximately 1 mMole of the N-acylimidazole in THF (4mL) were added 21.6 mg of $LiAlH_4$ with stirring at room temperature. After 45 min, the mixture was filtered and the filtrate was submitted to chromatography (Si-gel column; eluant: $CHCl_3$/MeOH) furnishing 11.9 mg of the 3,4,5-trimethoxybenzyl 3,4,5-trimethoxybenzoate (3%) and traces of the 3,4,5-trimethoxybenzyl alcohol. This reaction[4,5] was repeated at 0°C and -20°C giving only 3,4,5-trimethoxybenzyl 3,4,5-trimethoxybenzoate. Mp. 101-105°C (rec. $CHCl_3$/MeOH). IR (ν_{max}^{KBr}, cm^{-1}): 2950, 1710, 1600-1400. ^{1}HNMR ($CDCl_3$, δ): 7.4 (2 H), 6.7 (2 H), 5.3 (2 H), 3.9 (18 H).

-Methyl 3,4,5-Trimethoxybenzoate - IV:

This substance was obtained by addition of methanol to a solution of N-acylimidazole (1 mMole) in benzene (1 mL) freshly prepared. Crystallization of the material in benzene/methanol furnished 176.4 mg of IV (78% yield). Mp. 71-5°C. IR (ν_{max}^{KBr}, cm^{-1}): 2900, 1715, 1600-1400. ^{1}HNMR ($CDCl_3$, 200 MHz δ): 7.3 (2 H), 3.9 (12 H). ^{13}CNMR ($CDCl_3$, 50 MHz,δ): 162.2 (COO), 153.2 and 143.8 (PhO), 123.5 and 107.9 (ϕ), 61.0 and 56.3 (OMe). MS (m/z): 226 ($M^{+\cdot}$).

- Ethyl 3,4,5-Trimethoxybenzoate-V:

On the reaction of N-acylimidazole preparation, the material obtained was filtered, concentrated, and submitted to chromatography (Si-gel column; eluant: $CHCl_3$/MeOH) furnishing 21 mg of ethyl 3,4,5-trimethoxybenzoate and the starting materials. The presence of the ethyl esther was atributed to ethanol containing chloroform used on the column. Mp. 54-56°C. IR (ν_{max}^{KBr}, cm^{-1}): 2900, 1710, 1600-1400. ^{1}HNMR ($CDCl_3$, δ): 7.3 (2 H), 4.4 (q, 2 H), 3.9 (6 H), 3.8 (3 H), 1.4 (t, 3 H).

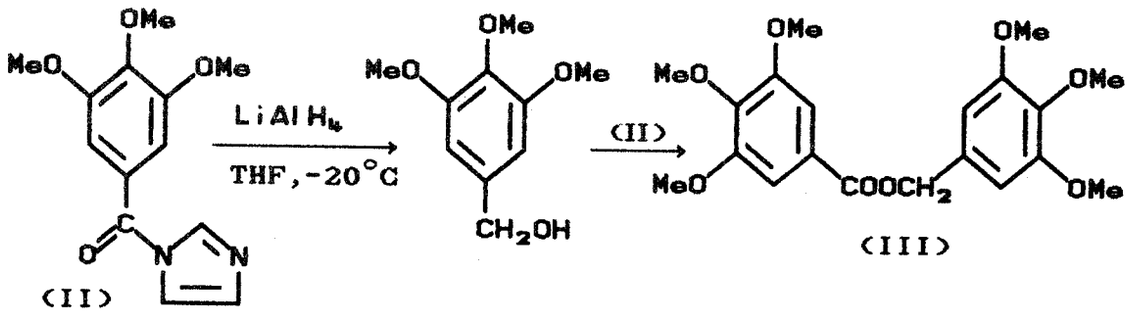
- 3,4,5-Trimethoxybenzoic anhydride-VI:

On the attempt of imidazolide preparation by reaction of 3,4,5-trimethoxybenzoyl chloride and imidazole, the substances were mixtured in $CHCl_3$. The product obtained, after filtration, distillation, and purification by Si-gel column ($CHCl_3$/EtOAc elution) was the anhydride (38 mg). The presence of this substance was atributed to water present in the chloroform used on the reaction. Mp. 159-161°C (rec. EtOAc). IR (ν_{max}^{KBr}, cm^{-1}): 2920, 1790, 1710, 1600-1400. ^{1}HNMR ($CDCl_3$, 200 MHz δ): 7.4 (4 H), 3.9 (18 H). ^{13}CNMR ($CDCl_3$, 50 MHz, δ): 166.7 (COO), 152.9 and 142.1 (PhO), 125.1 and 106.7 (ϕ), 60.9, 56.2 and 52.2 (OMe). MS (m/z): 406 ($M^{+\cdot}$).

RESULTS AND DISCUSION

The 3,4,5-trimethoxybenzoyl chloride, obtained after reaction of 3,4,5-trimethoxybenzoic acid with thionyl chloride, reacts with morpholine or imidazole furnishing trimetozine or 3,4,5-trimethoxybenzoylimidazole. Trimetozine is a sedative[2,3] and the imidazolides are used as intermediates for syntheses of esthers, amides, peptides, hydrazides, anhydrides, aldehydes, ketones, and others[4], due to its high degree of reactivity.

According to H.A. Staab and H. Bräunling[4,5] the imidazolide group can be selectively reduced with $LiAlH_4$ to aldehydes with good yields, at -20°C for 30 to 60 minutes of reaction, in ether or tetrahydrofuran. However, reduction of 3,4,5-trimethoxybenzoyl-imidazole furnished the esther 3,4,5-trimethoxybenzyl 3,4,5-trimethoxybenzoate and traces of the 3,4,5-trimethoxybenzyl alcohol. The aldehyde presence was not detected even at -20°C. This suggests a very high reactivity for the imidazolide, leading to alcohol which reacts with starting material, giving the esther.



During the attempt of imidazolide preparation were obtained methyl 3,4,5-trimethoxybenzoate, ethyl 3,4,5-trimethoxybenzoate, and 3,4,5-trimethoxybenzoic anhydride by reaction of the imidazolide with methanol, ethanol, and water, respectively.

Although most of these preparations have occured unexpectedly they clearly show the potential of the acid chloride and the imidazolide as intermediates for synthesis.

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Preparation of Vanillin from Kraft Lignin from Pinus spp.

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ABSTRACT

An experimental set-up, completely automated, was built in order to study the oxidation of kraft lignin from Pinus spp. for the production of vanillin. Vanillin was not produced by hydrolysis; this was confirmed by HPLC since the MWD did not change. However, when oxygen was fed to the reactor, vanillin was produced with yield of 4 to 6%.

INTRODUCTION

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is mainly used as a flavouring agent in ice-creams, candies, cookies, puddings, cakes, soft drinks and other foods. It is also used in perfumes, deodorants, pharmaceuticals and metal-plating industries. It can be extracted from vanilla beans with alcohol and manufactured from coniferin, eugenol, guaiacol, safrole and lignin. The main routes from lignin are: (a) hydrolysis by alkali or alkaline earth hydroxides at elevated temperature and atmospheric or higher pressure; and, preferentially, (b) oxidation with air, metal oxides, or nitrobenzene in alkaline medium, with or without catalysts (1).

Dry wood is composed by 42-45% cellulose, 27-30% hemicelullose, 20-28% lignin and 1-5% extractive. Lignin is a large by-product of the pulp and paper industry. There are mainly two types of lignin resulting from the sulfite process (lignossulfonate) and from the sulfate process (kraft lignin) (2). Lignin is the most abundant renewable source of aromatic monocyclic compounds on the earth. It is composed of guaiacyl-, syringyl-, and p-hydroxyphenyl-propane units connected to each other by various carbon-carbon or carbon-carbon ether linkages. Gymnosperm lignin is formed by the first monomer, angiosperm includes the second monomer as well, and gramminaea includes all of them. The building blocks of lignin are linked in a random and non-linear fashion forming a heterogeneous network of high molecular weight which is very resistant to hydrolysis (figure 1) (3).

Vanillin can be produced from lignin or lignin containing materials, but, unfortunately, the second route needs an excess of alkaline and oxidant agents. Several variables have been considered for the oxidation of lignin, such as: lignin concentration, lignin type (vegetable source, chemical environment and way of obtaining the lignin), reaction time, temperature, total pressure, oxygen's partial pressure, agitation, presence or absence of catalyst and alkali concentration. If the concentration of lignin is high, the rate of the condensation reaction is very high and the production of vanillin is lower. In a

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quite oxidant medium, vanillin will be oxidized too. In a scarcely oxidant medium, vanillin will not be produced. If the concentration of alkali decreases, the concentration of vanillin decreases as well.

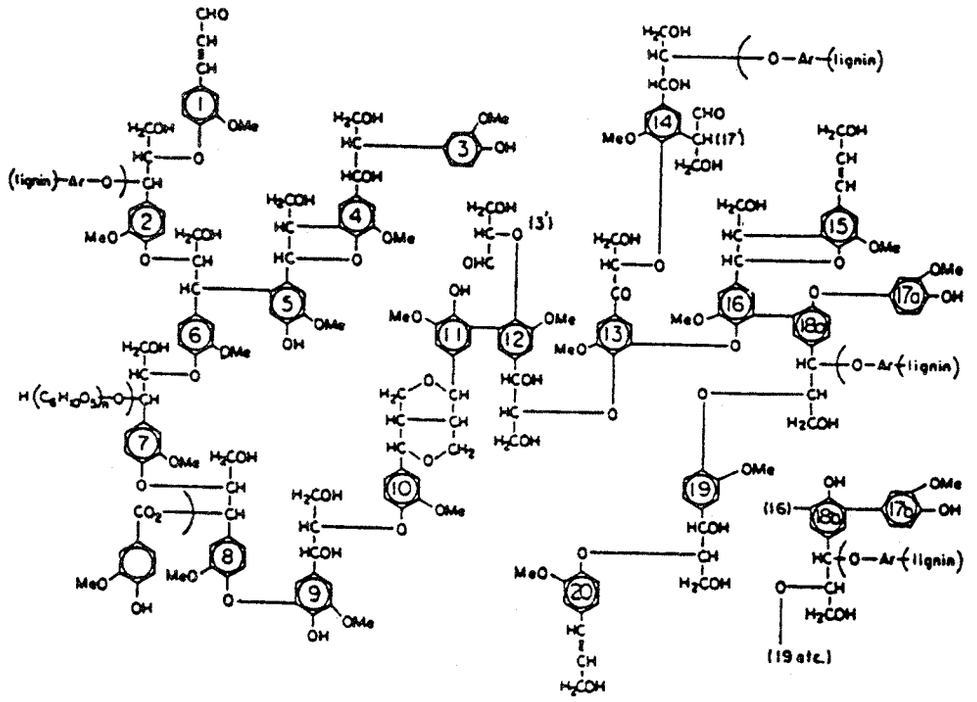

Figure 1. Schematic structure for conifer lignin (3).

EXPERIMENTAL PART

Vanillin, syringaldehyde, p-hydroxybenzaldehyde, acetovanillone, acetosyringone, p-hydroxyacetophenone, vanillic acid, syringic acid, p-hydroxybenzoic acid, ferulic acid, p-coumaric acid and sinapic acid were determined by gas chromatography (GC) as described by Hartley (4). Consumption of oxygen and sodium hydroxide were followed by gas flow mass measurements and acid-base titrimetry, respectively. The degradation of lignin was followed by optical density (280nm) and by high pressure liquid chromatography (HPLC).

We have assembled an automated experimental set-up, with data acquisition, in order to study the lignin oxidation of kraft lignin from pinus for the production of vanillin (figure 2). It includes a glass cylindric autoclave with stirrer, a IBM PS2/55 computer, a data acquisition board, a thermostatic bath, a mass flow meter and controller for gas and several accessories (thermocouple, pressure transducer, pipes, connections, etc.).

A typical example of the use of this apparatus is a follows: valve 15 is directed to admit nitrogen. Valves 6 and 11 are closed, valves 5 and 9b are opened. Nitrogen flows to the autoclave for several minutes (about 5 minutes). This step is mainly important to study the reaction of hydrolysis. Valve 9b is closed and 400ml of liquid phase is added in the autoclave. It contain 10 g/l of lignin and 8 g/l of NaOH. Previously,

nitrogen was bubbled into the solution to take out all oxygen. Valve 5 is closed and nitrogen is admitted in the reactor, with acquisition of the volume of this gas and pressure (pressure transducer 7), until the desired partial pressure of nitrogen is reached. Valve 10 is opened to admit water to cool the upper cover of the autoclave. Motor 2 is turned on to move the stirrer 3 (about 1150 rpm). Thermostatic bath B is turned on and silicone oil is circulated through the jacket 1 of the autoclave, to start heating. The temperature inside the autoclave 4 and of the bath 14 are determined by means of "K" (nickel-chromium/ nickel-aluminium) thermocouples inserted them. The electromotive force of the thermocouples are acquired by the acquisition system.

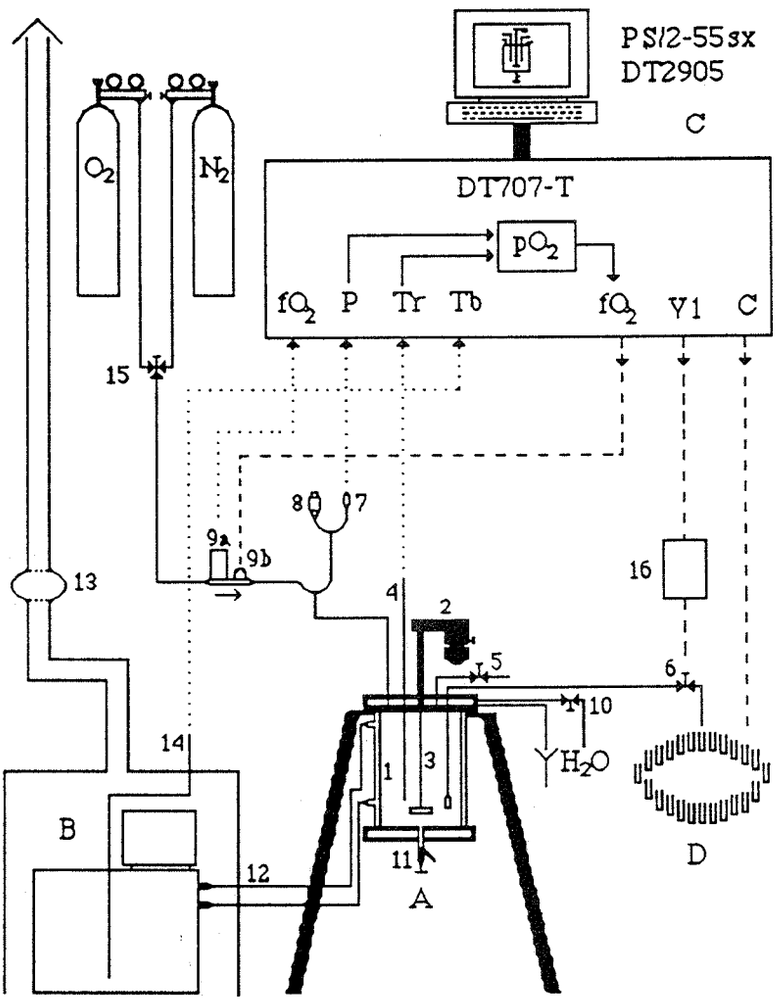

Figure 2. Set-up used to study oxidation of lignin to produce vanillin. Reactor A is heated by circulating the silicone oil from thermostatic bath B. This system is controlled by a computer C and samples are collected with the fraction collector D.

When the temperature inside the autoclave reaches the desired value, valve 15 is directed to admit oxygen and valve 9b is opened. Electromotive forces of the flow meter 9a and pressure transducer 7 are acquired. Oxygen is admitted until the total

pressure reaches the desired value (steam pressure plus nitrogen's partial pressure plus oxygen's partial pressure). As the total pressure is known, oxygen's partial pressure can be determined and this value can be controlled, by controller valve 9b, at the desired value. From time to time, valve 6 is opened for few seconds to clean the sampling line, the computer moves the fraction collector D, valve 6 is opened again to take a sample and the collector is moved again.

RESULTS AND DISCUSSION

TGs (mg) and DTGs (mg/s) of celluloses (from paper or cotton), hemicelluloses (from *Mimosa scabrella* or *Sorghum spp.*) and lignins (from *Pinus spp.*or *Eucaliptus spp.*) were obtained using nitrogen and air (b = 5°C.min-1). Figure 3 show the experimental TG and DTG curves for lignin of pinus.

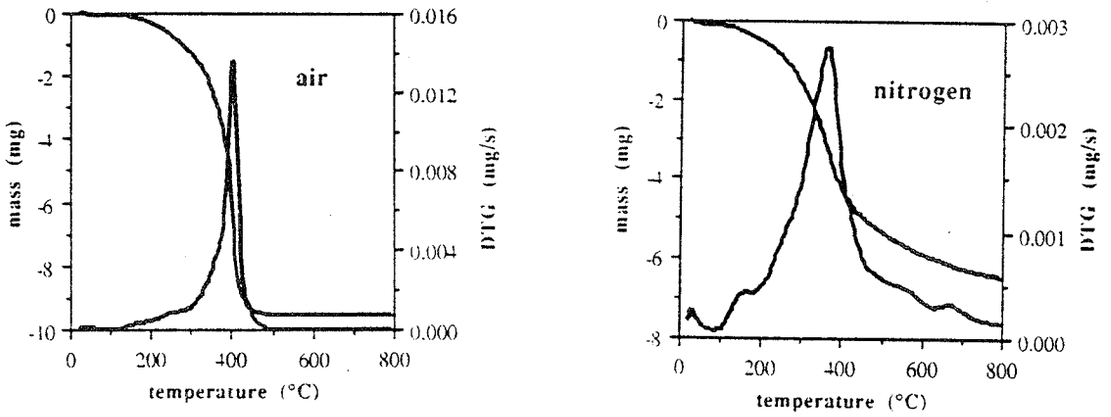

Figure 3. TG and DTG of lignin from pinus using nitrogen and air.

In nitrogen, the major thermal decomposition for cellulose occurs within a step region ranging from 280 and 380°C, where about 89% (paper) and 83% (cotton) weight-loss take place. Weight-loss of hemicelulloses are bigger, between 210 and 420°C, with 65% (mimosa) and 68% (sorghum). Lignins show a main weight-loss region between 220 and 530°C, where 75% weight is lost. The weight-loss peaks occur at 340°C for cellulose, at 275°C for hemicellulose and at 360°C for lignin.

In air, DTGs of cellulose and hemicelullose show two peaks. This is unexpected, mainly for cellulose, which has a simple and defined structure. 78% (paper) and 77% (cotton) are lost first, between 270 and 380°C, and 16% (both), between 380 and 480°C for paper and 380 and 500°C for cotton. Hemicelullose is lost in 49% (mimosa) and 52% (sorghum), between 200 and 365°C, and 32% between 365 and 450°C in the case of mimosa and 29% between 365 and 500°C for sorghum. Lignin shows a single peak with 95% of loss in the region between 210 and 460°C. The loss-peaks of cellulose are 320 and 445°C (paper) or 470°C (cotton), of hemicelullose are 270 and 420°C (mimosa) or 465 (sorghum) and of 405°C to both lignins.

It is not possible to state that lignin is pure due to the wide-range of DTG. Nevertheless similarity between lignins' DTG and difference with the other wood components, at least with one of the gases, lead us to consider lignin with high purity grade (5).

Figure 4 shows the yield of vanillin, at 120-130°C, as a function of time. As the lignin concentration used was 100g/l, vanillin concentration is expressed in g/l too. This experiment shows three steps: a) heating (about 60 minutes), b) hydrolysis (between 60 and 180 minutes) and oxidation (after 180 minutes). Vanillin was not detected in absence of oxygen, but after addition of this oxidant in the reactor, it is quickly produced. After reaching the maximum value, vanillin's concentration decreased quickly.

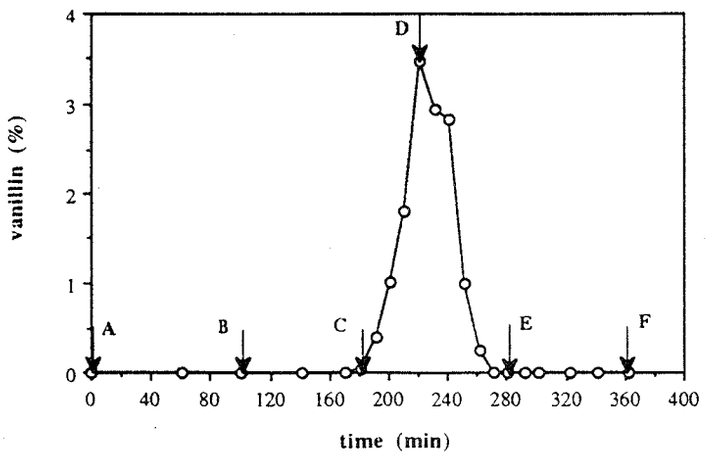

Figure 4. Production of vanillin versus time of the reaction.

During the oxidation of lignin phenoxy radicals are produced , which react with OOH°, and can produce vanillin, diacid and CO_2 (6). This radical could react with vanillin too and therefore it could explain the consumption of vanillin without producing other aromatics compounds. Acetovanillone was detected in some experiments, mainly after a high quantity of vanillin has been produced. Acetovanillone is apparently more resistant to oxidation, because his disappearance is slower than vanillin.

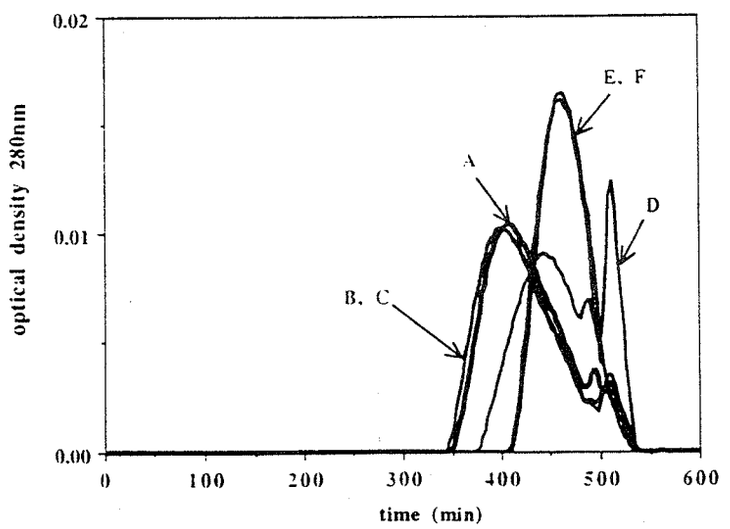

Figure 5. HPLC of lignin at different reaction times.

Lignin HPLC chromatograms at different steps of the reaction are shown in figure 5. Curve A shows the molecular weight distribution (MWD) of the initial lignin. After heating, the lignin was hydrolyzed and oxidized (see also figure 4). Curve B displays lignin after 41 min of hydrolysis, curve C after 121 min of hydrolysis, curve D after 41 min of oxidation, curve E after 101 min of oxidation and curve F after 181 min. In curve D, a narrow peak of vanillin can be seen. This was confirmed with a standard (not shown). The peak of vanillin does not appear in curves E and F, which supports the results of GLC. Curves E and F are very similar, probably due to a limit of oxidation of the lignin molecule in these conditions.

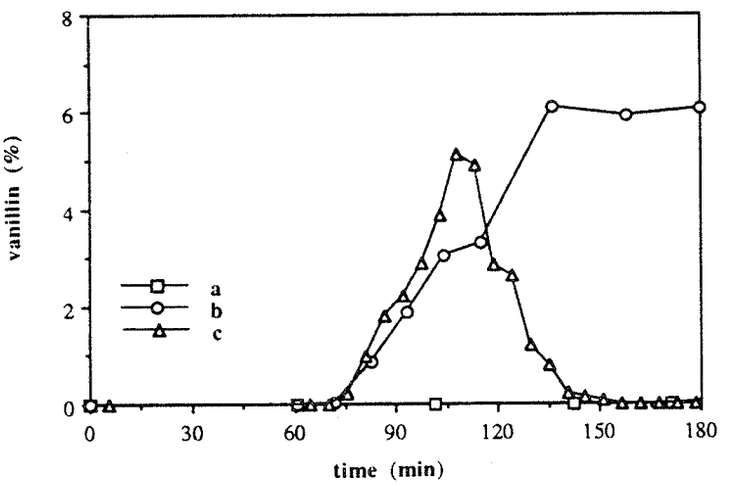

Figure 6. Yield of vanillin in absence of oxygen (a), with 12,5% (b) and 35% of total pressure.

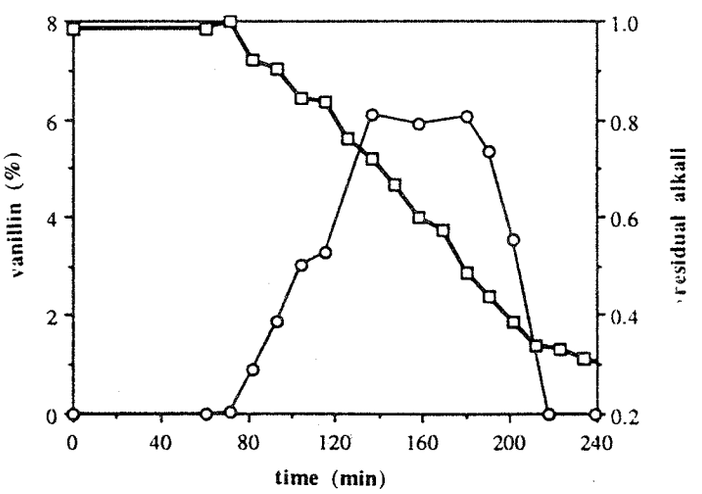

Figure 7. Consumption of alkali during the production of vanillin.

Figure 6 shows the yield of vanillin in the absence of oxygen (a), with 12,5% of oxygen concentration (b) and 35% of oxygen concentration (c) in gaseous phase. If the medium is very oxidant, vanillin is quickly consumed. Figure 7 shows the alkali consumption. Alkali is consumed to neutralize vanillin, diacids and CO_2, and also to reduce oxygen; therefore the consumption is bigger in high oxidant medium. The optical density of the diluted sample was followed at 280 nm and it shows an increase in the range where vanillin is produced (figure 8). Similarly, the reactor temperature increases in this range, because the reaction is exothermic (figure 9). Partial pressure of nitrogen is known and constant. Steam pressure is calculated by reactor's temperature. Total pressure is selected by taking into account the desirable partial pressure of oxygen (figure 10).

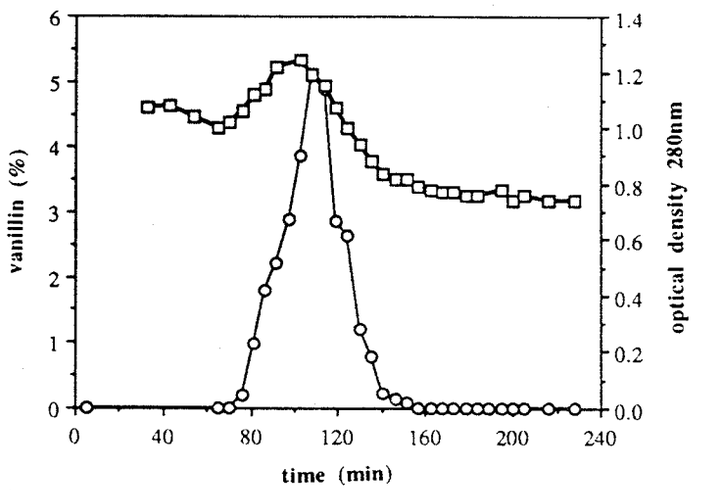

Figure 8. DO_{280} during the production of vanillin.

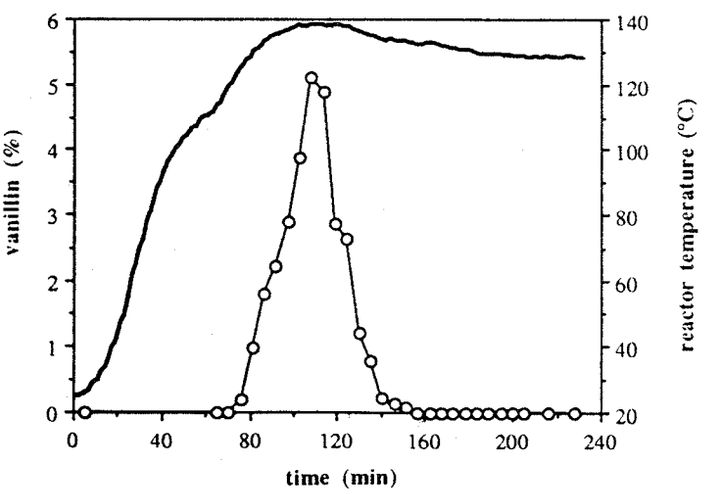

Figure 9. Temperature inside the reactor during the production of vanillin.

In conclusion, vanillin can be easily produced by oxidation of kraft lignin from pinus, but it can be easily consumed too. Pinus is a good source of lignin, producing only vanillin and acetovanillone, as by-product. Probably only part of lignin molecule can be oxidized. Several combinations of these variables are the next step of our study.

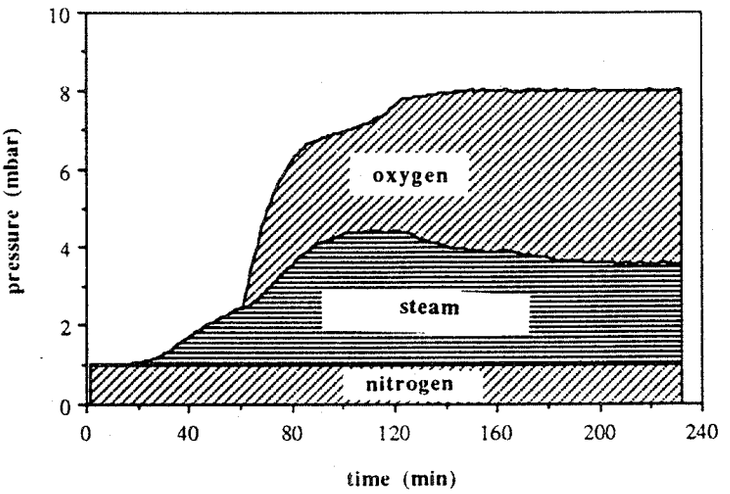

Figure 10. Partial pressures during production of vanillin.

ACKNOWLEDGMENT

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KINETIC STUDY OF THE LIGNIN EXTRACTION OF EUCALYPTUS GRANDIS, BY ETHANOL-SODA PROCESS

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ABSTRACT

The kinetics of the lignin extraction of *Eucalyptus grandis*, with water-ethanol-sodium hydroxide solution, was studied with pulping purposes. Studies were performed in various time intervals, in the optimized conditions of the ethanol-soda method (ALKALC) [1].

INTRODUCTION

The first study about delignification using athanol was performed at 1891 [2]. Kleinert and Tayenthal were the first to use, by 1931, ethanol as pulping agent [3]. In 1936 Aronovsky and Gortner [4] studied the use of different mono and polihydroxilic alcohols as delignifying solvents. It was observed that primary alcohols were more efficient than secondary or tertiary.

Kleinert and other authors [5-9] studied wood delignification by ethanol. The process seems to be more rapid and with a higher yield than the kraft method.

Studies [6,10-16] about wood delignification with ethanol showed that the process occurs in two steps. Initially, in a first order reaction, occurs a rapid extraction of the lignin. In the second step the residual lignin is removed in a lower rate. In this step removing of carbohydrates also occurs.

By 1976, Nakano et alii [17,18] studied the methanol-soda process of pulping. They found a rapid delignification and an yield 5% higher than that obtained by kraft method.

Studies performed by Demuner and Gomide [19] in Brazil, with ethanol-soda pulping of *Eucalyptus grandis* and Eucalyptus urophylla, indicated three phases of delignification.

Tubino and Torres [20], in a study of plant analysis, showed that the 20% v/v ethanol-water solution is an excellent extractor of plant components.

In this article are presented the results obtained in the kinetic study of the extraction of lignin and other components of wood chips of *Eucalyptus grandis*.

EXPERIMENTAL

Materials

Wood - Were utilized chips of *Eucalyptus grandis*, 7 years old, obtained in a industrial Carthage chipper and screened in a sieve system in order to reject oversized (19.0

mm) and undersized (6.4 mm) material. However, bark and fines were used to reproduce the industrial pulping process.

Ethanol - Commercial grade 96% ethanol was diluted to 20% in the digester. Concentration measurements were done in the purchased product using a densimeter.

Sodium hydroxide - Commercial grade 50% product was used, as in the industrial pulping. Concentration was controlled by titration with standard HCl solution.

Pulping Conditions - Ethanol = 20% v/v; Pressure = 9 bar; soda = 20% (as Na_2O); Wood chips weight = 1.0 kg (AD); Liquor to wood rate = 4.26/1.

Digester - Experiments were performed in a 20 liter rotating REGMED digester, with electric heating.

RESULTS AND DISCUSSION

Results are summarized in table 1 and in figures It can be easily observed that lignin was mainly extracted in the first 60 minutes, i. e., in temperatures under 160 °C . About 75% of the lignin was extracted in this period and about 20% was extracted in the last 100 minutes at 160 °C.

The screened yield increases with increases with time, reaching a maximum between 90 and 125 minutes of total time of cooking, with 51.2%.

It is very difficult to propose a mechanism for the extraction of the lignin from wood. In figure 1, it can be observed the plot of the logarithm of the residual lignin versus time of pulping. Kinetically, it can be only considered, rigorously, for mechanistic considerations, only the interval in constant temperature, i.e., above 60 minutes,

when temperature was kept at 160 °C. It seems that from 70 minutes a straight line describes the process. This could indicate a pseudo first order reaction. However, the curve of the reciprocal of the concentration of the residual lignin, versus time of pulping, is a quite good straight line from 60 to 160 minutes indicating a second order reaction through a bimolecular process.

Table 1.
Kinetics of the remotion of lignin from *Eucalyptus grandis*. NaOH 20% (as Na_2O), ethanol 20% v/v, liquor/wood ratio= 4.26/1.0.

Total time of pulping (min)	Pulping Temp. (°C)	Residual Lignin (%)	Kappa Number	Screened Yield (%)
0	31	26.4	-	-
20	74	20.0	-	-
40	117	17.7	-	-
60	160	6.3	-	-
75	160	3.5	49.0	45.8
90	160	2.5	34.2	51.2
105	160	2.1	29.7	51.2
125	160	1.6	24.9	51.3
145	160	1.3	22.2	49.8
160	160	1.1	20.5	47.7

Figure 1.

Logarith of the residual lignin in the wood versus total time of pulping.

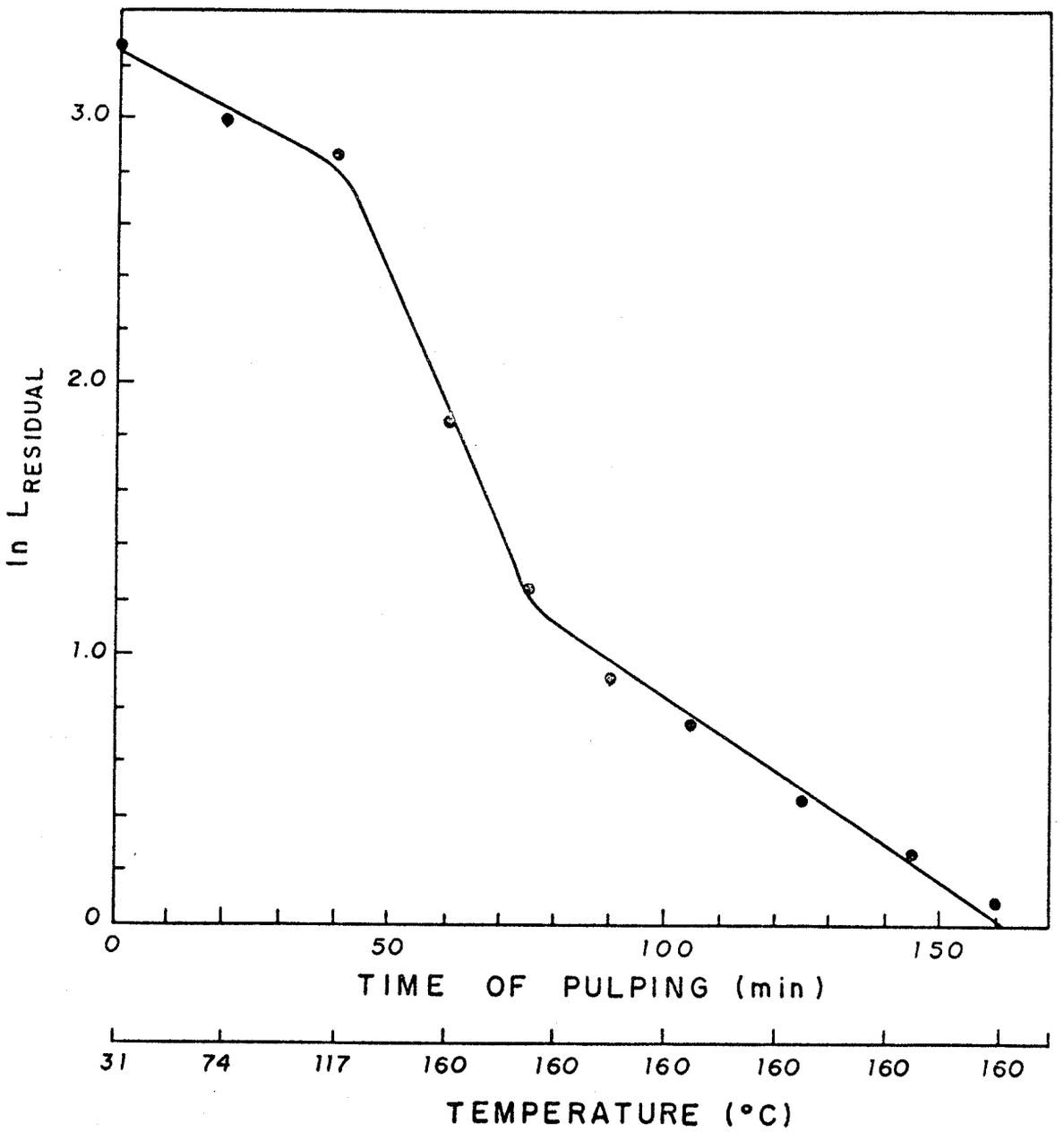


Figure 2.

The reciprocal of the residual lignin concentration versus time of pulping.

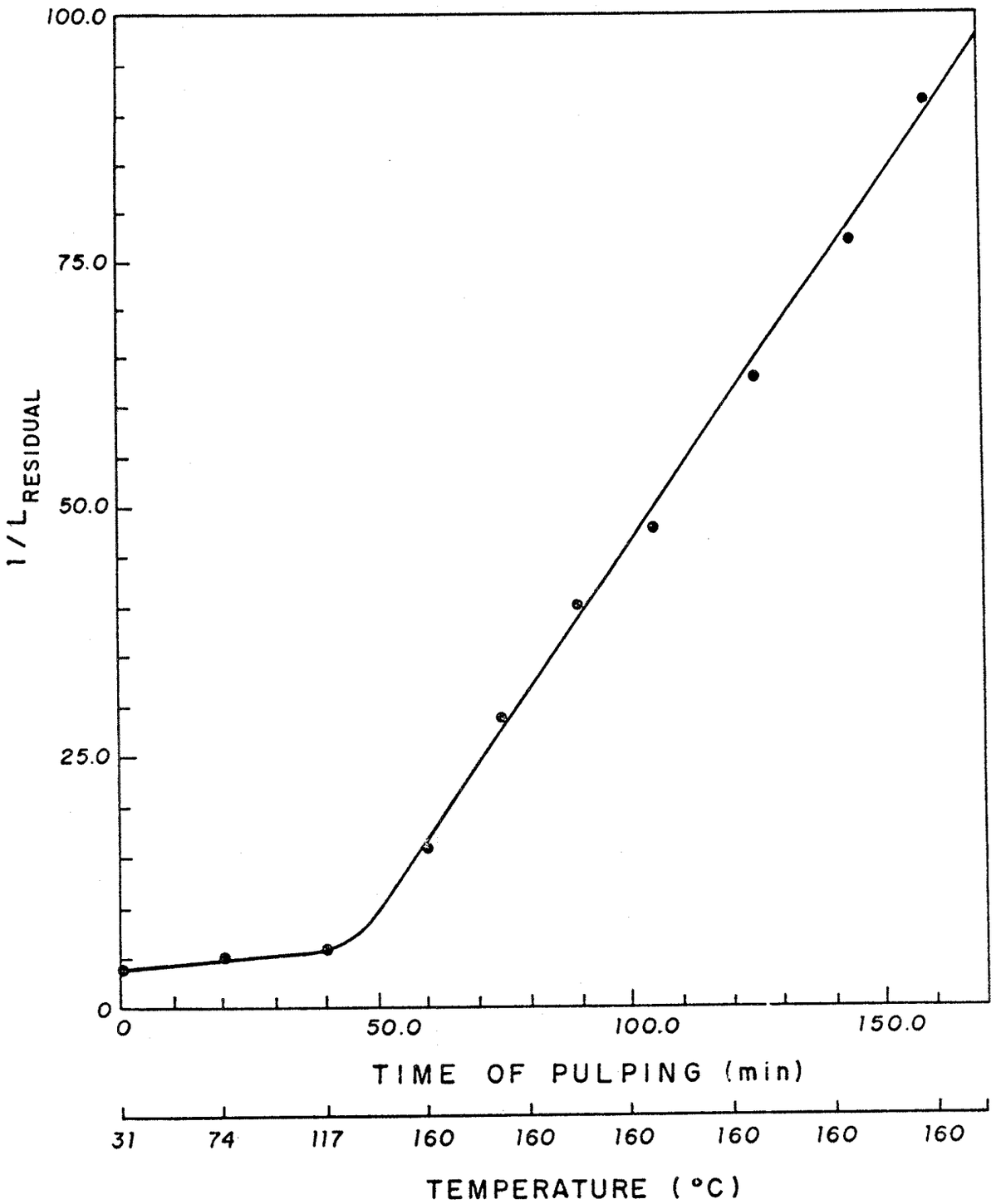
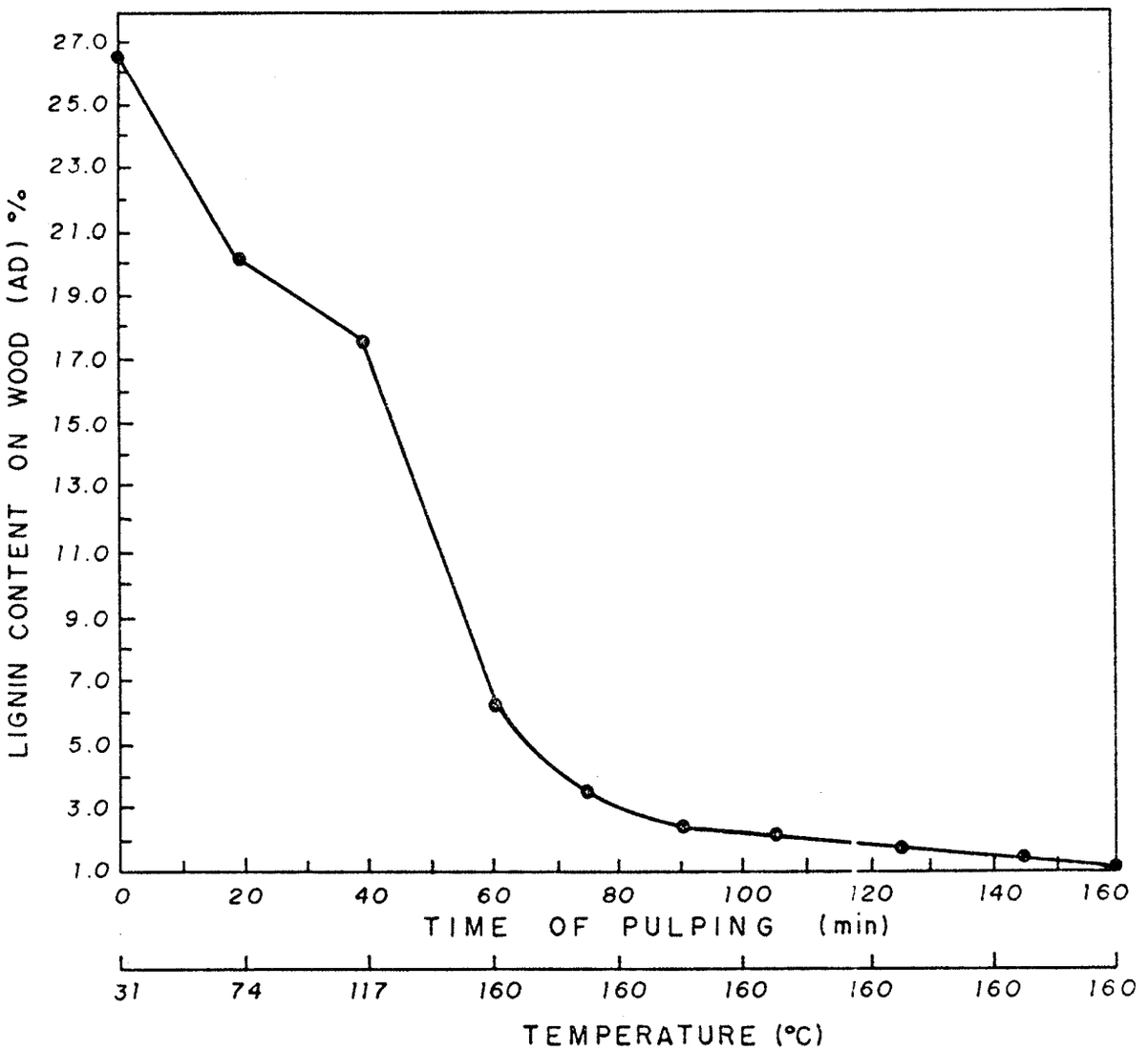


Figure 3.
Lignin content on wood (%) versus total time of pulping.



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EFFECT OF CATALYST AND STEAM-EXPLOSION PRETREATMENT ON SUGAR CANE BAGASSE DELIGNIFICATION

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ABSTRACT

Raw sugar cane bagasse and sugar cane bagasse treated by steam-explosion were delignified in ethanol/water (3:1, v/v) at 152°C for 30 min, in the presence and in the absence of $FeCl_3$. The catalyst highly improved delignification of the raw sugar cane bagasse, but played only a minor role at the delignification of the steam-exploded sugar cane bagasse.

INTRODUCTION

Every year 36.5 million tons of sugar cane bagasse are produced as a by-product of ethanol and sugar production in Brazil[1], of which 80% is burned for the generation of energy and 20% still has no use. This means that approximately 3.6, 1.8 and 1.8 million tons of cellulose, hemicellulose and lignin, respectively, could be transformed into chemicals of high economic value.

Contrary to the traditional commercial pulping methods, the alternative ones, such as steam-explosion, solvolysis with organic solvents and combinations of these with the traditional methods, have been able to produce not only cellulose, but also hemicelluloses and lignins, which can be further utilized.

We have earlier treated sugar cane bagasse by steam-explosion and delignified it with a low concentration NaOH aqueous solution, obtaining cellulose containing only 2.8% of lignin.[2] We now wish to report our results on the delignification of sugar cane bagasse and steam-exploded sugar cane bagasse by solvolysis with ethanol/water ("organosolv process") and the effect of $FeCl_3$ as a catalyst.

EXPERIMENTAL PART

Sugar cane bagasse (SCB) obtained from an ethanol distillery was used as received. Ten kg of SCB were steam-exploded in a 0.2 m^3 reactor at 195°C for 15 min. The reacted material was then transferred to a semicontinuous centrifuge to separate the bagasse, which was washed with water until the filtrate became colorless. The hydrolysate

was separated for further analyses and the bagasse pretreated by steam-explosion (SCBSE) was stored in a refrigerator at 4°C. Chemical analysis of SCB and SCBSE are given in Table 1.

Table 1: Composition of the lignocellulosics used and obtained in the organosolv delignification reactions (10 g sugar cane bagasse, 100 cm^3 ethanol/water (3:1, v/v), 0.3 g $FeCl_3$ in Exp. 02 and 04, 152°C, 30 min).

	Substrates (%)		Cellulosic Residues (CR) (%)			
Components	SCB	SCBSE	Exp. 01	Exp. 02	Exp. 03	Exp. 04
			SCB	SCB+$FeCl_3$	SCBSE	SCBSE+$FeCl_3$
Cellulose	42.5	55.5	48.8	69.6	68.9	75.4
Hemicellulose	25.1	4.1	23.5	15.30	5.43	4.75
Acetyl Groups	2.0	-	2.0	-	-	-
Total Lignin	30.4	37.1	24.8	15.5	21.6	17.5
Ash	2.1	2.5	0.9	1.5	2.3	2.49
Total	102.9	99.2	100.0	101.9	98.2	100.1

SCB: Sugar cane bagasse.

SCBSE: Steam-exploded sugar cane bagasse.

For the delignification reactions approximately 10 g of bagasse (dry basis) and 100 ml of an ethanol/water mixture (3:1, v/v) were put into a stainless steel autoclave[3]. In the reactions with catalyst, 0.3 g of $FeCl_3$ were added to the solvent mixture and thoroughly mixed. The autoclave was shaken horizontally (1.5 Hz) and heated to 152°C, the heating-up time being 15 min. The reaction temperature was maintained for 30 min, when the heater was switched off and the autoclave immersed into cold water. The solubilized lignin was separated from the cellulosic residue (CR) by vacuum filtration. The CR was washed with distilled water (200 ml) for complete removal of the lignin impregnated at the fibers. The solubilized lignin was recovered by evaporating the ethanol of the filtrate in a rotavapor. The precipitated lignin was filtered, washed with distilled water and oven-dried at 60°C for 24 h. Chemical analysis of the CR and the liquor is described elsewhere[2]. The composition of the cellulosic residues is given in Table 1.

RESULTS AND DISCUSSION

Overall hemicellulose solubilization and decomposition, overall cellulose solubilization and overall delignification are shown in Fig. 1, 2 and 3, respectively.

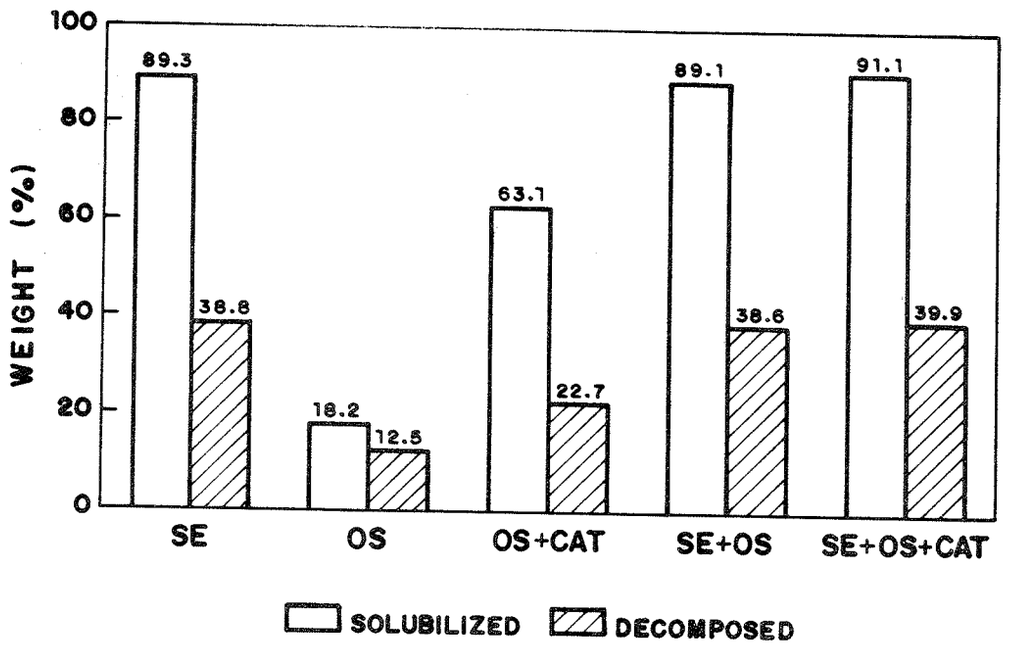

Figure 1: Overall solubilization and decomposition of the hemicelluloses by steam-explosion (SE), organosolv (OS), organosolv in the presence of the catalyst (OS+CAT), steam-explosion and organosolv (SE+OS) and steam-explosion and organosolv in the presence of the catalyst (SE+OS+CAT).

As shown in Fig. 1 the steam-explosion pretreatment was very effective in extracting the hemicellulosic fraction of SCB. The solvolysis in ethanol/water presented solubilization rates of only 18.2% and 63.1% in the presence and absence of the catalyst, respectively. Of the residual amount of hemicellulose in SCBSE (4.1%) less than 0.7% was dissolved in ethanol/water. Thus, the high percentages of hemicellulose solubilization were due to pretreatment of SCB. On the other hand, during the steam-explosion pretreatment approximately 39% of the solubilized hemicellulose was decomposed. The solvolysis of SCB with ethanol/water diminished solubilization of the hemicellulose, and $FeCl_3$ reduced its decomposition.

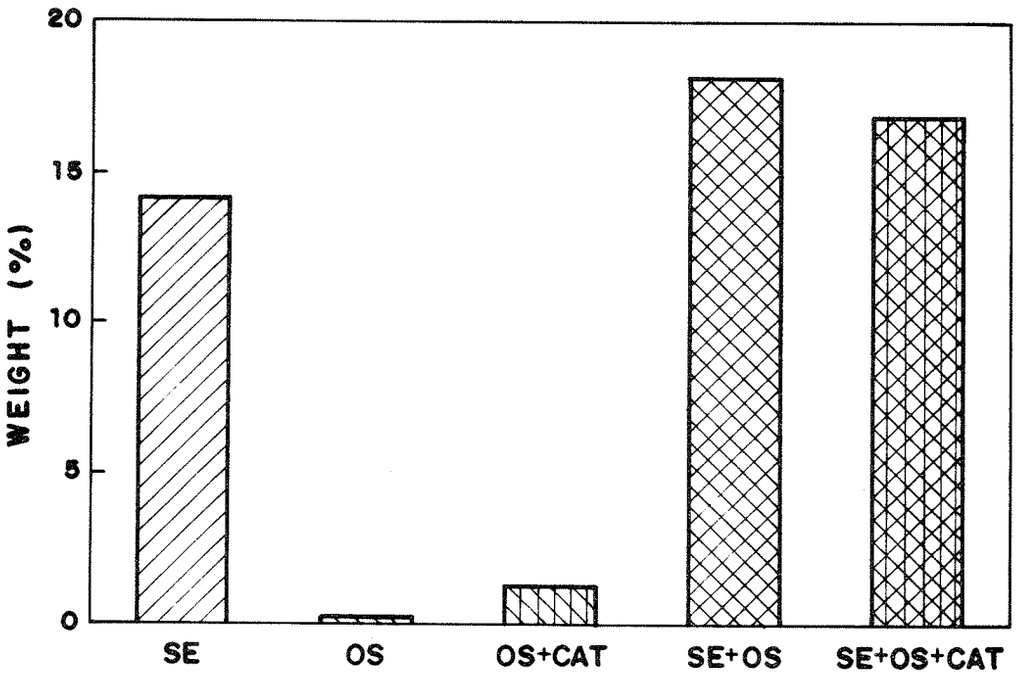

Figure 2: Overall solubilization of the cellulose by steam-explosion (SE), organosolv (OS), organosolv in the presence of the catalyst (OS+CAT), steam-explosion and organosolv (SE+OS) and steam-explosion and organosolv in the presence of the catalyst (SE+OS+CAT).

Cellulose solubilization (Fig. 2) was very high under the conditions of the steam-explosion (14%). During the solvolytic treatment losses of cellulose smaller than 1.3% were achieved. The pretreated bagasse showed high overall solubilization of cellulose, which, within the experimental error, corresponds to the additive effects of steam-explosion and solvolysis in ethanol/water.

The delignification of SCB by the steam-explosion pretreatment was only 20%. Delignification in ethanol/water without $FeCl_3$ was rather higher, as shown in Fig. 3. The presence of $FeCl_3$ improved the solubilization of lignin significantly and a delignification of 64.2% was achieved. Similar results were obtained by treating the SCBSE with ethanol/water, both in the presence and in the absence of the catalyst, suggesting that the steam-explosion is a good pretreatment for the delignification reaction and that the catalyst is not needed under these conditions.

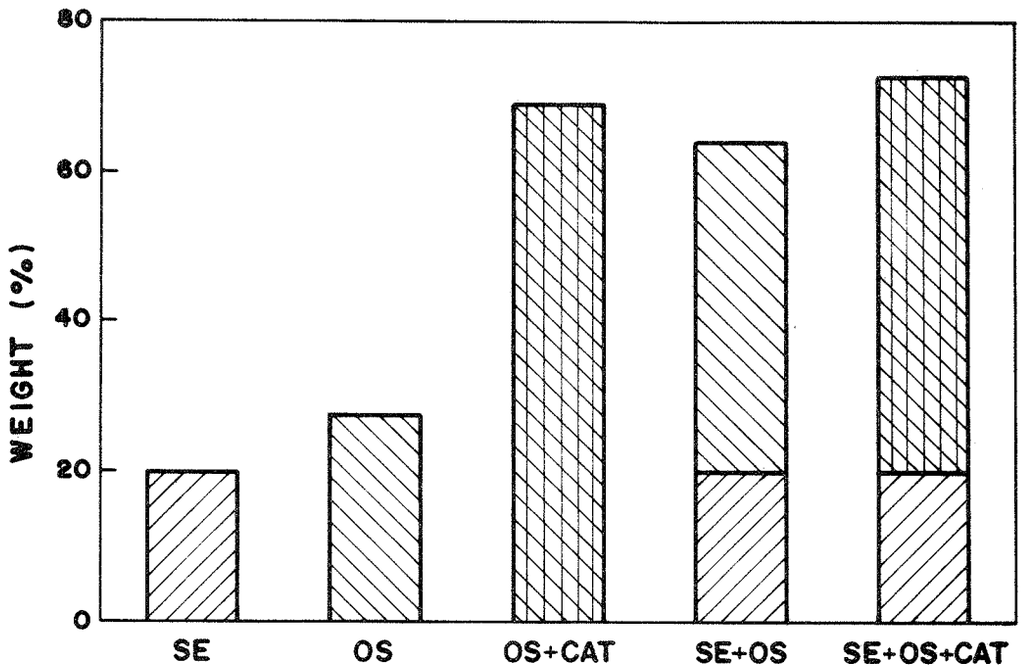

Figure 3: Overall delignification of sugar cane bagasse by steam-explosion (SE), organosolv (OS), organosolv in the presence of the catalyst (OS+CAT), steam-explosion and organosolv (SE+OS) and steam-explosion and organosolv in the presence of the catalyst (SE+OS+CAT).

The composition of the cellulosic residues presented in Table 1 reinforces the effectivity of steam-explosion in the extraction of hemicellulose and the effectivity of the catalyst in the delignification. The combination of both methods (Exp. 04) produced a CR with the highest cellulose content. An evaluation of the suitability of these residues for paper industry and their physical characterization will be carried out in collaboration with another laboratory.

CONCLUSIONS

The data presented show that the best delignification was obtained for SCB by the organosolv process in the presence of $FeCl_3$ (69.2%). The effect of the catalyst on the delignification of the steam-exploded sugar cane bagasse was not significant, as an increment of only 9% was observed. On the other hand, pretreatment by steam-explosion, which allowed the removal of 89.3% of the hemicellulose, was very effective in improving delignification by the organosolv process in the absence of the catalyst.

ACKNOWLEDGEMENTS

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PLANT XYLANS AS POTENTIAL CHEMICAL FEEDSTOCKS

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ABSTRACT.

The availability of xylans as possible source of chemicals is summarized . Xylans are the most promising components of wood and plant hemicelluloses capable of being converted into furfural, xylitol, and other chemicals. The chief sources of xylans are the agricultural, wood, and pulp and paper industries.

Availability is often affected by seasonal variations, and use is frequently complicated by the need to separate them from cellulose and lignin.

At the present time the most viable approach for the efficient utilization of xylans seems to be the direct conversion to furfural. Other alternatives that deserve attention are the hydrolysis of xylans to xylose and its further transformation into xylitol via reduction or into ethanol by means of yeast fermentation.

1. INTRODUCTION.

The need to supplement or supplant fossil resources with renewable resources for the production of fuels and chemicals is now well established and extensively reviewed (1-4).

On a generalized basis, the idea of converting lignocellulosic materials to fuels and chemicals is the fractionation or separation of the feedstock into its three main components, namely cellulose, hemicelluloses and lignin. With further processing, each of these fractions can then be converted into fuels, chemicals, or feed and foodstuffs.

As a source of chemicals, biomass has several intrinsic advantages over fossil mass (5): it is renewable, flexible through crop switching, and adaptable through genetic manipulation. However, biomass lacks of the highly effective technology for production of olefins and aromatics, economies of scale, and equally important the highly developed system of conversion products with large markets that sustain the fossil-based chemical industry.

As far as the carbohydrate system is concerned, most of the research in the past has been concentrated in the

utilization of the cellulosic fraction of biomass. More recently, biological processes for the production of ethanol directly from pentoses (8) have opened new perspectives for the utilization of this type of carbohydrates present in wood and plant materials.

It is the purpose of this review to place the xylans component of hemicelluloses into perspective as a source of chemicals in view of their widespread occurrence, relative ease of extraction from biomass and relative ease with which they may be hydrolyzed and converted to useful products.

2. STRUCTURE AND CHEMICAL COMPOSITION OF XYLANS.

The xylans are the most common hemicellulose and constitute the major non-cellulosic polysaccharide of the widely distributed angiosperms (9). Xylans account for nearly 8 % of the total weight in gymnosperms and 22-28 % in angiosperms being the major hemicellulose in these plants as can be seen in Table 1.

Component	Angiosperm			Gymnosperm
	Bamboo	Wheat Straw	Hardwood	Softwood
Lignin	23	21	24	29
Cellulose	48	50	48	43
Glucomannan	Trace	Trace	3	17
Xylan	28	27	22	8
Miscellaneous polymers, pectin, arabinan,galactan, etc.	3	2	3	3

Table 1 Estimated quantities of high molecular weight components of typical angiosperms and gymnosperms.

Xylans appear in nature as a polymer formed by a basic chain of D-xylopyranosyl units linked mainly in (1 Ü 4)-ß-D fashion. Usually the side chain contains other sugar units as single or multiple unit side chains and sometimes the xylan chain is branched with other sugar units attached (10). The

most common sugar that appears as side chain in the D-xylose backbone is in the form of ß-L-arabinofuranosyl groups although some D-arabinopyranosyl groups may also be present.

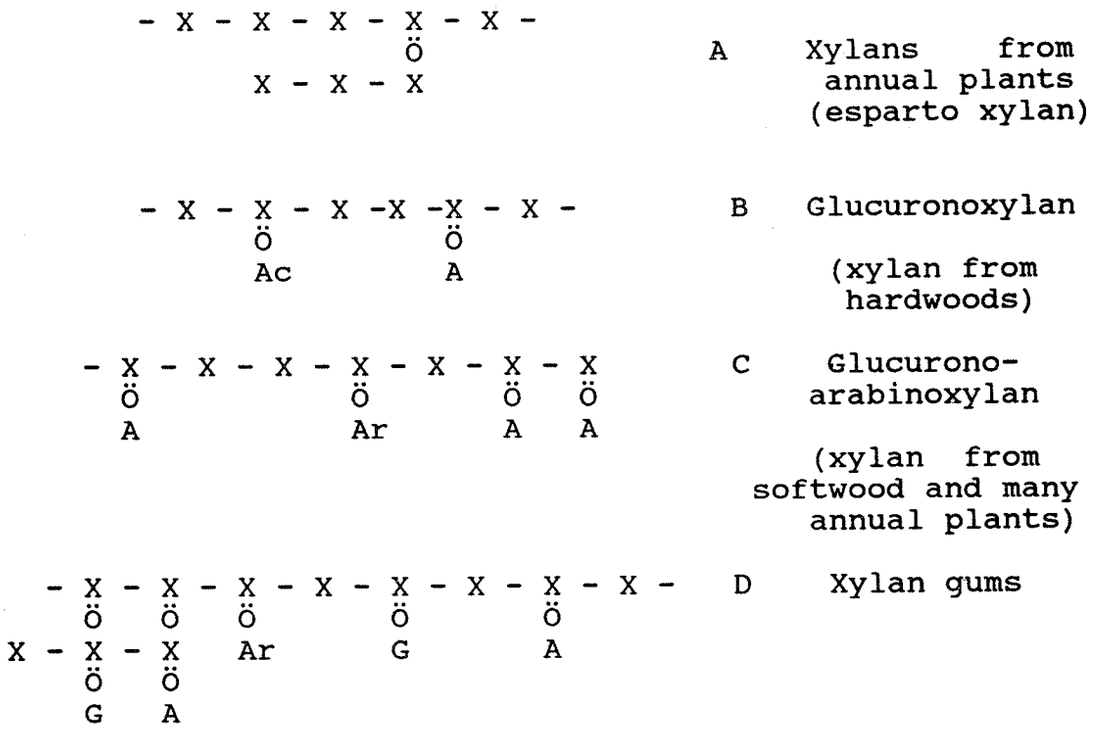
Attachment to the xylan chain is often at C 3 of the side chain units. 4-O-methyl-α-D-Glucuranosyl units occur as end units either on the L-arabinofuranosyl chains or more frequently as side chain linked 1Ü2, or to a less extent 1Ü3 to the main xylan chain. In annual plants D- and L-Galactopyranosyl units are also often attached to the main chain (10).

An important additional feature of many D-xylans, particularly those of hardwoods, is the presence of acetyl ester groups. Approximately, 3.5-7 out of each ten xylose units carry an O-acetyl group corresponding to 8-17 % . All of the ester groups are believed linked to D-xylopyranosyl units of the main chain, predominately at C-2 positions although a small proportion of linkage at C-3 positions has been observed in some D-xylans (10,11,12).

Xylans may be classified into two broad groups according to the complexity of their structure and the absence or presence of uronic groups (13). The simplest member of the first group (Figure 1A) is the typical xylan of annual plants, which has a linear structure with perhaps one or two filiform branches. The main chain is composed entirely of ß(1Ü4) linked xylopyranosyl units and contains no uronic acid (14). One example of this type of xylans is the esparto xylan.

The second group includes three types of xylans carrying side chain uronic groups attached by glycosidic bonds to the xylan chain (Figures 1B, 1C, 1D). In Figure 1B, it is showed the typical structure of hardwoods xylan (glucuronoxylan). The uronic acids are randomly distributed along the chain of many hardwoods xylans and may occur as lactones or esters (13,15,16). Acetyl groups may also be present attached to the 2 and/or 3 position of the main chain (17).

The xylans of monocotyledons and softwoods are more complex examples of the second group of xylans (Figure 1C). They have uronic acids but lack of acetyl groups, being this particularity an important difference between xylans from softwoods and hardwoods. Besides uronic groups, L-arabinofuranosyl units are attached by α(1Ü3) glycosidic bonds to the main chain. The more highly branched nature of these polymers compared with hardwood xylan makes them more soluble after isolation (18).



X = ß (1Ü4) linked-D-xylopyranosyl units
Ac = Acetyl
Ar = L-arabinofuranosyl units
A = D-glucuronopyranosyl or 4-O-methyl-D-glucuronopyranosyl unit.
G = Hexose unit

Figure 1. Possible configurations for xylans.

The most complex structure (highly branched) of xylan carrying uronic groups is shown in (Figure 1D). Side chains in this xylan are formed by L-arabinofuranosyl, (4-O-methyl-)-D-glucuronopyranosyl, hexose and D-xylopyranosyl units. More hexose or uronic units are attached to the side D-xylopyranosyl chain. This type of xylans have been isolated from specialized tissues such as the seed coats of corn, sorghum and sapote (13) and they have unique rheological properties making them ideal candidates as thickeners, stabilizers, and emulsifiers (10).

2. PERSPECTIVES FOR XYLAN UTILIZATION.

This section attempts to revise some of the traditional as well as current alternatives for the industrialization of xylans.

Xylans as polymers

Industrial procedures based on alkaline extraction have been developed to isolate xylan from agricultural wastes (9,19). Some interesting case is the effort to utilize the abundant xylan present in corn fiber (10). The recovery of this hemicellulose is made by extracting corn fiber with lime water to give a water soluble homogenous hydrocolloid with rheological properties similar to other gums used commercially as thickeners, stabilizers, and emulsifiers. However, this utilization has not been successful.

Xylans can also be left as a component of the pulp to obtain higher pulp yields and to improve the mechanical performance of the fibers during subsequent papermaking operations.

It has been known for a long time that well-fibrillated and swollen fibers are needed for the manufacture of high strength paper. Quality of paper is dependent on the amount and quality of hemicellulose attached to the fibers. Hemicellulose with the greatest effect on the strength of the paper may be those with xylan backbones. In general, hemicellulose improve breaking stress, modules of elasticity, yield point stress and work to rupture of fibers and bursting strength, tensile strength and fold endurance of paper (20,21,22,23,24).

Xylo-hemicellulose have been examined in various clinical and biochemical applications (10), specially wheat straw hemicellulose has shown antitumor action.

In one interesting application, O-acetyl derivatives of xylan (25) are able to form cellophane-like films with tensile strengths in the range of 590-885 kg/sq.cm .

A very complete list of other applications for xylo-hemicelluloses ranging from detergent components to flocculents and adhesives has been published somewhere (10).

Direct hydrolysis of easily accessible xylan for xylose production seems to be the preferred route over the isolation of xylan from raw material and application of a subsequent hydrolysis step (42). The principal difficulties associated with the production of pure xylose from acceptable sources are related to the different types of impurities found in the hydrolyzates. These impurities range from other sugars, extractives, lignin, lignin-carbohydrates complexes, unsaponified acetates, and various organic acids. The way these impurities affect the process deals with difficulties in crystallization, contamination of ion exchange resins, alteration of neutralization schemes and poisoning of hydrogenation catalysts.

Furfural

Furfural is the most important chemical derived from xylan or any hemicellulose. The process for furfural production was developed almost simultaneously by La Forge at the National Bureau of Chemistry and by the Quaquer Oats Company at the end of the World War I (42). The Quaquer approach consisted of treating oat hulls with sulfuric acid in rotating digesters while steam was introduced to the desired temperature. Furfural was recovered from a vapor outlet valve as a steam distillate. The distillate is then further purified to give the commercial product.

Nylon was originally produced from furfural starting material and although production from plant material has almost ceased in the United States, a revival of furfural as a source of competitive organic chemicals can be anticipated as oil prices increase (10).

The kinetics of furfural production are complex, and many of the kinetics details have been addresed in the past (25). The initial reaction involves the hydrolysis of xylan followed by the loss of water by the pentose component. The dehydration step proceeds to furfural through intermediate unknown species. In order to avoid secondary reactions leading to resin and colored condensation products, removal of furfural is required as soon as possible after formation.

The acidic catalysts used in most industrial processes are sulfuric acid, hydrochloric acid, calcium phosphate and acetic acid generated by deacetylation of hemicelluloses during the treatment (42).

Xylitol

The most important product derived from xylose is D-xylitol. Xylitol has attracted industrial attention because its sweetness is equivalent to sucrose and because it is not cariogenic (10).

Xylitol gives a initial cool perception when it is used as sweetener in chewing gum due to its high endothermic heat of solution. Unfortunately, xylitol is completely metabolized so that it is not useful as a low caloric sweetener replacement (10).

Xylitol has been tested in a variety of food products but it is still considered as a conditional additive by the Food and Drug Administration (28).

The process for xylitol production is based in the reduction of hydrolyzates containing D-xylose by means of catalysts (42). By far the most used catalyst is nickel on a variety of supports such as active charcoal, silica and kieselguhr.

Bioconversion of xylans

Two modes of biotechnology will have an impact on chemical process development: fermentation and enzyme engineering. The first will primarily change resource entry into the chemical industry and affect the transition from nonrenewable (petroleum) to renewable (biomass) feedstocks. The second will have two functions: to allow the entry of fermentation feedstocks into the chemical intermediate chains, and to compete directly with traditional chemical transformations (29).

As shown in Figure 1, xylose can serve as feedstock for a variety of fermentations from which commodity chemicals such as ethanol, acetic acid, acetone, butanol, 2,3-butanediol, and lactic acid can be produced; these chemicals can then serve as intermediates for the petrochemical, agricultural chemical, plastics, and pharmaceutical industries (31).

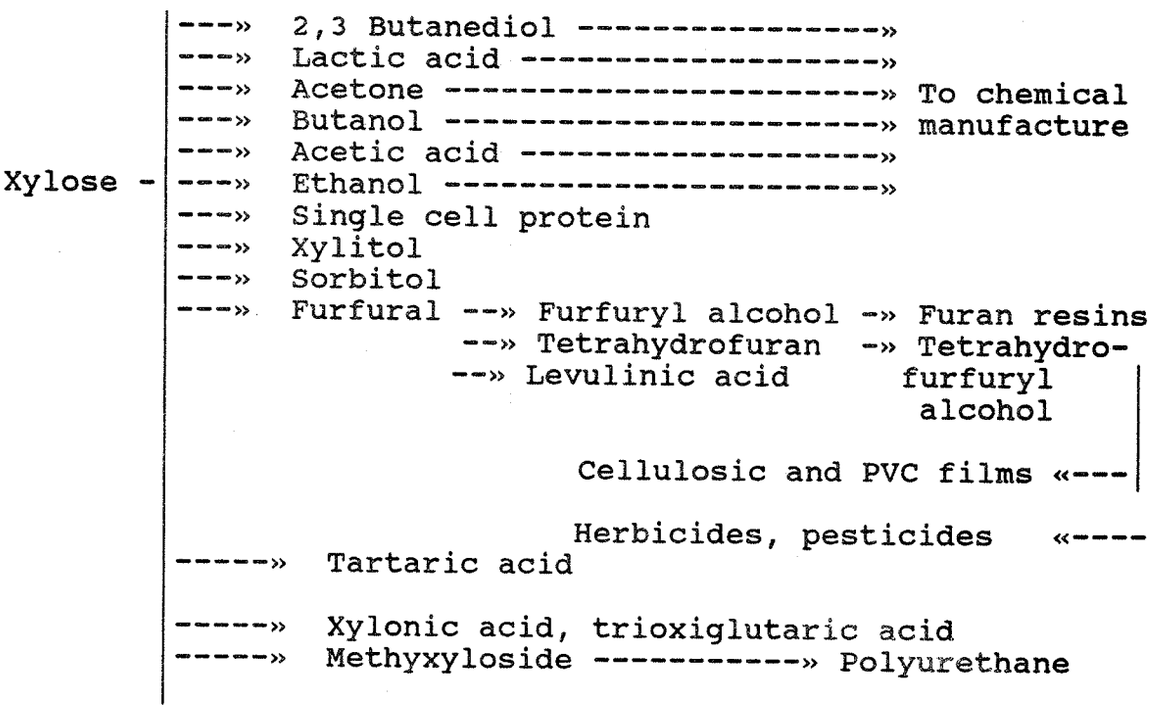


Figure 2 Chemicals and food obtainable from xylose (32).

Xylose can also be fermented to single cell protein, a possible animal feed material. Via enzymes xylose can be hydrogenated to xylitol, a potential attractive but as yet unexploited sweetening agent, or derivatized to compounds such

as xylonic and trioxyglutaric acid and methylxyloside, which can be used in the manufacture of binders, sequestering agents, and polyurethanes (31).

By far the cost of producing chemicals by fermentation routes will depend on the cost associated with the raw material and the efficiency of the conversion steps employed.

Of the many products available from hemicellulose-derived carbohydrates, ethanol has recently received the most attention. This recent interest in ethanol production focuses in his potential use for blending with petroleum to make "gasohol". In addition to its use a fuel or petroleum supplement, ethanol is also a versatile chemical feedstock, and many chemicals products are derived from ethanol such as ethylene, propylene and butylene, which in turn are base chemicals for the production of rubber, fiber, resins and specialty chemicals (30).

Ethanol is produced commercially by both chemical and microbial syntheses. While virtually all industrial alcohol is currently manufactured synthetically from petroleum and natural gas, all beverage alcohol is produced from grain, molasses, and other materials containing starch and sugar. The major sources for industrial ethanol production are carbohydrates in the form of grains, crops residues, cellulosic materials and industrial wastes (30).

A number of biological processes have been investigated for the conversion of hexoses-rich materials to fuels and chemicals; however, little progress has been made toward the conversion of hemicellulose-derived pentoses (30).

Soluble pentoses produced by acid or enzymatic hydrolysis of pretreated lignocellulose are suitable substrates for a variety of homo and hetereo-fermentations by bacteria, yeast and fungi, which have been extensively reviewed (30,32).

Many bacteria are able to assimilate and convert pentoses to a variety of products but this conversion is not satisfactory at the present time due to the production of many products (30).

The inability showed by yeast for converting xylose to ethanol has been mediated by the addition of xylose isomerase , which catalyzes the conversion of xylose to xylulose.

Ethanol yields, ranging from 10 % to 85 % of the theoretical estimation (based on the initial amount of xylose added to the culture) have been achieved in laboratory-scale demonstrations using soluble and immobilized xylose isomerase and xylulose-fermenting yeasts (33-35).

A step further in this context is the identification of yeasts capable of effecting the direct conversion of xylose to ethanol. Yields 52 % of theoretical are obtained in

fermenting periods higher than 4 hours. Alcohol concentrations exceeding 4-5 % inhibits the grow and the fermenting capability of this organism (36).

The economics of the fermenting process of xylose to ethanol has been studied (37-41). From these studies, it is clear that the competitive position of any process for the conversion of lignocellulosic materials is highly dependent on the feedstock costs, the recoverability of any pretreatment chemicals used, the sugar yields obtainable from the hydrolysis, and the product recovery techniques employed.

Xylans as components of dietary fibers

A highly branched hemicellulose existing in corn fiber and constituted by a main bone of xylose has been found responsible, in part, of the notable water absorption that this fiber afford when used in dietary food. Absorption of bile acids and cholesterol are some other interesting properties attributable to this hemicellulose that have been referred in the literature (10).

3. POTENTIAL OF XYLANS AS SOURCE OF CHEMICALS.

This section attempts to present a very brief analysis about the perspectives of the processes currently available for the transformation of xylans.

Tong and Cannel (7) in their well- documented analysis of the economics of organic chemicals from biomass utilized a series of principles that constitute an excellent basis for evaluate the potential of biomass as a source of organic chemicals.

According to this study, the first requirement to fulfill for a biomass-base material in order to qualify as a possible feedstock is the availability of a large, dependable supply of raw material.The second requirement is a competitive price. There are other three requirements that are related to the processing of the raw material. They are: existence of cost-competitive biomass fractionation or refining technologies, availability of efficient conversion and product recovery technologies and compatibility of biomass-derived chemical products with the existing infrastructure of the organic chemical industry.

Other aspects equally important, if not the most important ones, are the existence of a demand for the product, stability of the market and potential markets for a surplus of the chemical, which are not discussed in this paper.

Applying these considerations to the case of xylans, without trying to make an exhaustive analysis, some practical aspects arise that may serve to evaluate its potential as source of valuable chemicals.

With regard to the supply of raw material, forest residues, specially those coming from the processing of hardwoods, can be utilized as acceptable source of xylans. The existence of pulp mills with an infrastructure for collection, storage and transport, as well as the higher density of wood, favors the utilization of forest residues over agricultural residues (for example annual residues). The low price of wood residues and their high carbon content, only comparable to that of coal, also account for the favorable rating of forest residues (7).

Most of the processes studied for xylan transformation face troubles in providing good fractionation of the raw material. Implicit in this matter is the concept of processing all the fraction in the heterogeneous feedstock to the highest overall product and by-product values. Two approaches are commonly utilized for fractionating xylan-rich materials: enzymatic hydrolysis and acid hydrolysis.

Enzymatic hydrolysis requires work to improve the rates of reaction, the recovery of enzymes and the yields. In addition, pretreatment steps to make carbohydrate materials accessible for enzymatic attack result in by-products and soluble lignin present in solution, which inhibit the action of the enzymes.

Acid hydrolysis of biomass requires similar development work to improve yields and reduce operating cost by hydrolyzing the material in high solids concentration. This requires new mechanical feeding devices capable of introducing wood chips to a reactor with short residences times.

Whether or not chemicals should be recovered from the spent liquor instead of be burned is a very difficult question that may be better answered in terms of fuel/chemical value, marketing considerations, economies of scale, recovery technology, capital and energy costs.

Alternative lignocellulose refining schemes that are able to render each of the biomass components in a sufficient pure form have been proposed (7,43).

The carbohydrate components of lignocellulose - cellulose and hemicellulose- are chemically the most compatible components of biomass for producing oxygenated petrochemicals such as organic acids, esters and solvents. These oxygenates typically have atomic oxygen to carbon ratios ranging from 0.5 to 1.0. These oxygen-to-carbon ratio closely match that of xylose (7).

With the development of efficient routes for the xylose conversion to ethanol by yeasts it is expected that biomass and specially xylans become a competitive feedstock for oxygenated petrochemicals.

Still furfural is the most reliable chemical derived from xylans with a high chemical value . However, it has a small market. As a result, additional production would reduce

the market value and cause a surplus supply in the market (7).

CONCLUSIONS.

Xylans are complex groups of related polysaccharides very widely distributed in nature and relatively easily isolated from biomass.

It seems to be two clear tendencies regarding the utilization of xylans: production of furfural and hydrolysis of xylans to produce xylose and xylitol.

Recent innovations in enzymatic and biological hydrolysis have opened new perspectives for the production of organic acids and alcohols.

Conversion efficiencies in this context should be increased in order to be competitive. Yeasts will be the choice for ethanol production from pentose-rich hydrolyzates.

Present processes specially in the wood pulping and food industry may be altered to achieve to provide the required fractionating or refining technologies.

Hardwood residues appear to be the best source for supplying pentose sugars for an industrial approach.

Other interesting uses for xylans are being developed . At present applications such as thickeners, components of dietary food, sweetening agents and additives for papermaking represent only good alternatives that need to be further developed.

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AN EXPERIMENTAL STUDY OF A SIMULTANEOUS HYDROLYSIS AND FERMENTATION OF SUGAR CANE BAGASSE

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ABSTRACT

An experimental study is carried out to evaluate the possibility of production of ethanol from sugar cane bagasse. In this study ethanol is produced in a process on which the hydrolysis of cellulose and the fermentation of the converted sugars take place simultaneously. Based upon the experimental results, the economical advantages and disadvantages of this production method are outlined

INTRODUCTION.

Traditionally, ethanol has been produced from sugar crops. However, in the last decades alternate ethanol production processes have been developed because of the increasing cost of sugar. In countries where sugar is more expensive (due to high labor costs and less advantageous climate) ethanol is produced from other renewable feedstocks, such as starch-containing grains and particularly, lignocellulosic materials.

The production of ethanol from renewable cellulosic materials is attractive and seems promising. Lignocellulose is inexpensive because it cannot be digested and therefore does not compete as a food. However, the production of ethanol from lignocellulose is a difficult process since its inability to be digested makes it difficult to convert to fermentable sugars. Therefore, it is necessary to develop an inexpensive process to convert cellulose to fermentable sugars and then fermentate these to ethanol. One alternative is the simultaneous hydrolysis and fermentation.

This process, proposed by Takagi et al. [1], offers the following advantages:

1) Enzyme requirements are minimized.
2) Glucose and cellobiose accumulation is minimized.
3) Inhibitory effects on cellulases are eliminated during the process
4) Only one reactor is used.

Therefore, the objective in this work is aimed at the development of a simultaneous hydrolysis-fermentation process to produce ethanol from a lignocellulosic material: sugar cane bagasse. In order to increase the accessibility of enzyme activity to carbohydrates, the bagasse is previously treated with high pressure steam. Cellulases from *Trichoderma reesei* and *Saccharomyces sp.* are used in this process.

EXPERIMENTAL SECTION.

Sugar cane bagasse was used as the lignocellulosic material. Cellulases from *Trichoderma reesei QM 9414* and the *Saccharomyces sp.* yeast were used in the simultaneous hydrolysis-fermentation process. The bagasse was treated with saturated steam at 12 kg/cm^2 during 15 minutes [2), the solubilized hemicellulose were later separated by extraction with hot water

Cellulases were produced by using *Trichoderma reesei* with the sugar cane bagasse as inductor and source of carbon and energy. The rest of the nutrients were those used by Mandels [3]. The yeast used in these experiments were taken from a *Saccharomyces sp.* culture which was selected upon its tolerance to cellulases and high ethanol concentrations, as well as its thermotolerance.

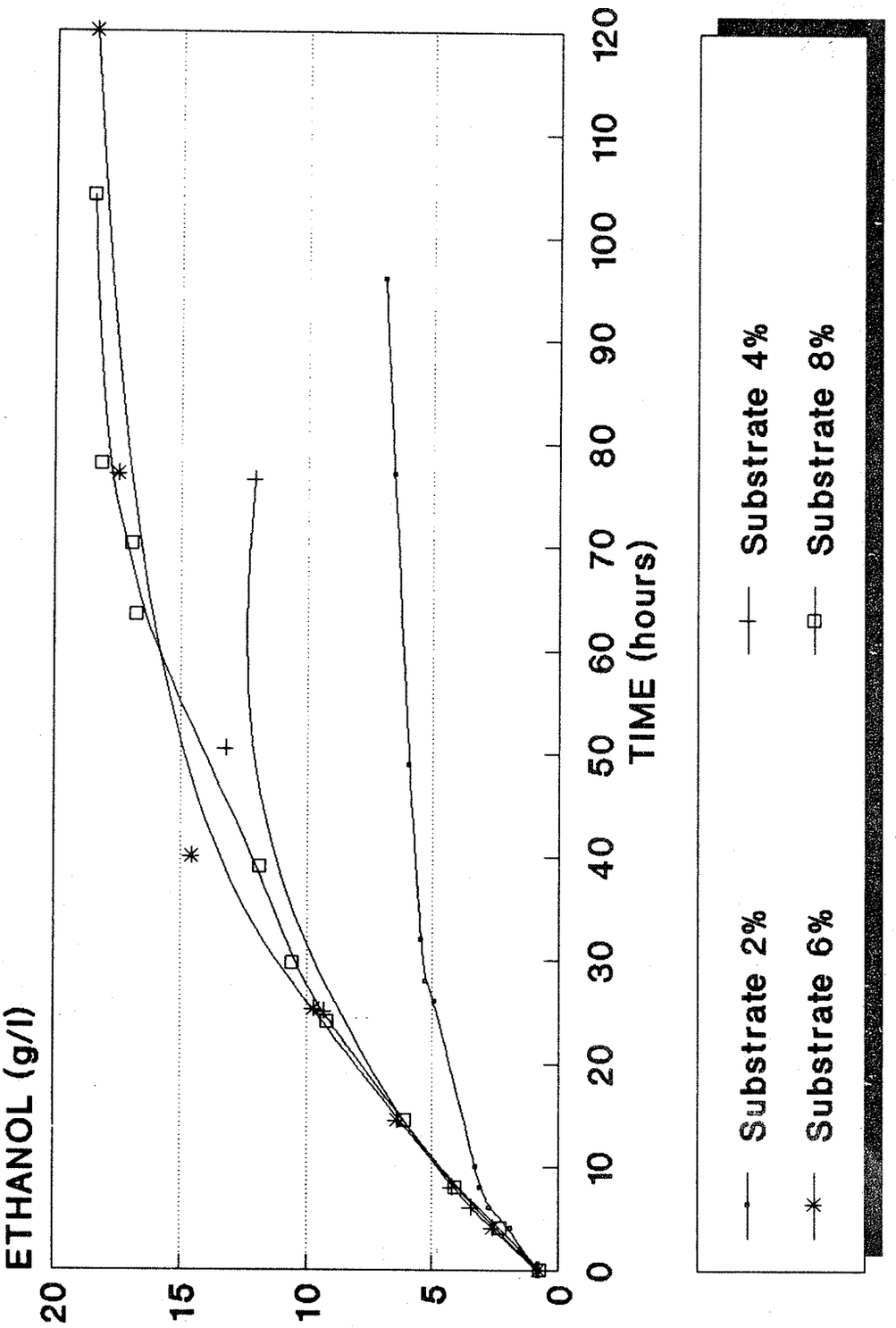
The experiments were carried out at 38°C, pH ranging from 4.5 to 5, in stirred 500 ml flasks with steam traps located at the top. The nutrients were (in gr/l) : yeast extract, 10; monobasic potassium phosphate, 2; magnesium sulfate, 1.7; ammonium sulfates, 3; calcium chloride, 0.2; sodium chloride, 1, and oligoelements in adequate amounts. Substrate and mineral salts were sterilized at 120 C during 30 minutes. Cellulases were sterilized by means of filtration through 0.45 microns "millipore" membrane. The yeast was propagated in stirred flasks, then centrifugated under aseptic conditions in order to inoculate 4.5 gr/l.

Cellulase activity (FPasa) was determined by using Wilkes method [4] with Whatman # 1 filter paper. Cellobiase activity was determined using cellobiose as substrate. The liberated glucose was determined by HPLC. Carbohydrates contents and enzymatic hydrolysis residues were analyzed by liquid chromatography [5] following a quantitative hydrolysis technique reported by Seaman [6]. Ethanol concentration was determined by gas chromatography using a Chromosorb 101 column and propanol as internal standard.

RESULTS AND DISCUSSION.

Figure 1 shows the ethanol concentration profiles for different substrate concentrations. In these experimental runs, enzyme concentration was kept constant at 0.3 Ul/ml.

Figure 1 Effect of substrate concentration on ethanol formation



Temp 37 C, pH 5, Enz 0.3 UI/ml,

These results indicate that up to a 6% substrate concentration, the concentration of ethanol increases with the same rate. However, with a substrate concentration of 8%, ethanol concentration shows the same profile as the one obtained at 6% substrate. This is probably due to the fact that substrate concentration is completely saturated by the enzyme concentration (0.3 Ul/ml).

In order to analyze the effect of the enzymatic activity on the ethanol concentration, two different experiments were carried out. In the first experiment, the enzymatic activity was increased up to 0.6 UI/ml. In the second, the activity was double and 20 mg/l of cellobiase was added. In these runs the substrate concentration was maintained at 8%. A comparison of the activity effect is presented in Figure 2. One can see that there is no difference between the two curves. However, the rate of ethanol produced increased when cellobiase was added. These results show that substrate is saturated with the enzyme concentration of 3 UI/ml, and particularly by the endoglucanase and exoglucanase enzymatic components. Furthermore, at this substrate concentration the inhibition effects of cellobiose are stronger. However, the level of cellobiose is kept at its lowest value when cellobiase is added and therefore the inhibitory effects are minimized.

The effect of substrate concentration was also studied. Another experiment was carried out with 10% substrate and an enzyme concentration of 0.6 UI/ml plus 20 mg/l of cellobiase. In Figure 4, it is possible to observe that the 8% and 10% profiles are remarkably close. This results are mainly due to the enzyme saturation and the reactor mixing difficulties.

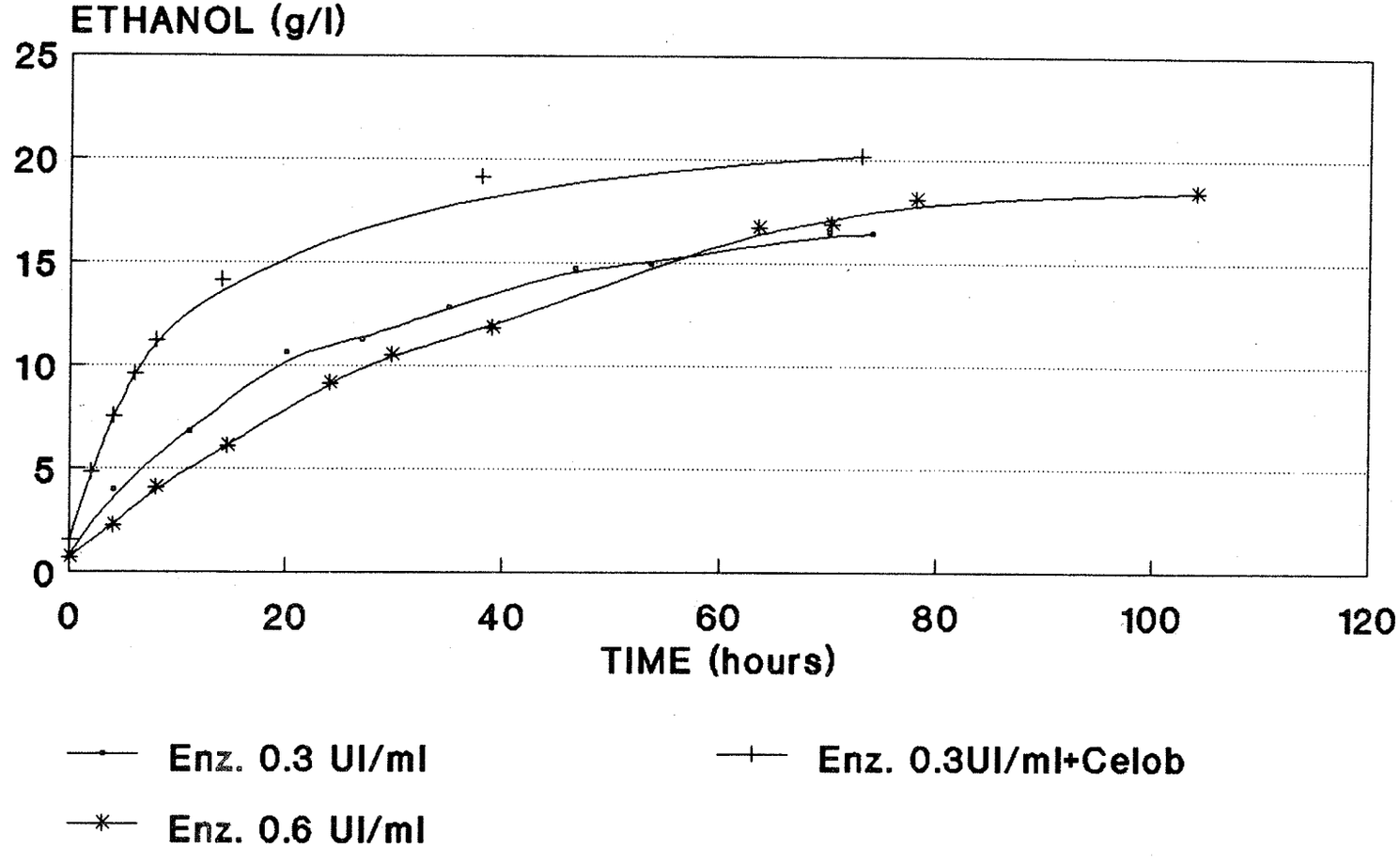
The glucose and cellobiose profiles are depicted in Figures 4 and 5 for both process without and with cellobiase supplementation respectively. At initial hours cellobiose and glucose concentration are increased. When the concentration of cellobiase decrease its faster with cellobiase than without supplementation.

CONCLUSIONS.

1) In the development of the simultaneous hydrolysis - fermentation process of lignocellulosic materials, one must consider the enzyme production as a part of the global process. The enzyme can be used as is produced with no further treatments. In this work, good quality cellulases were obtained from the same substrate which was later used in the hydrolysis-fermentation process.

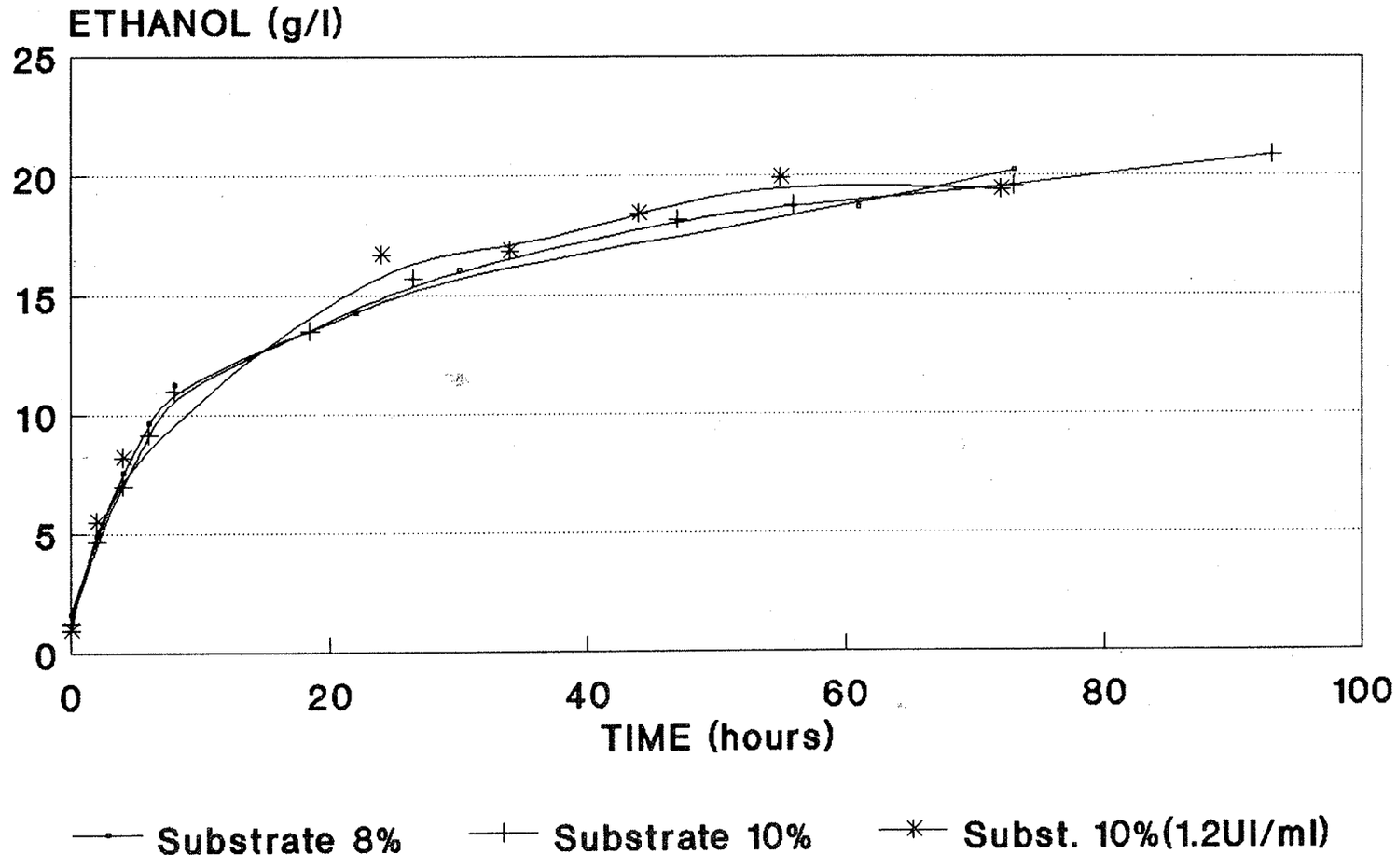
2) It was found that the conversion of cellobiose to glucose is the limiting reaction of this process, particularly when cellulase extract was used and no cellobiase was added.

Figure 2 Effect of enzymatic activity and supplentation with cellobiase (20 mg/l) on ethanol formation.



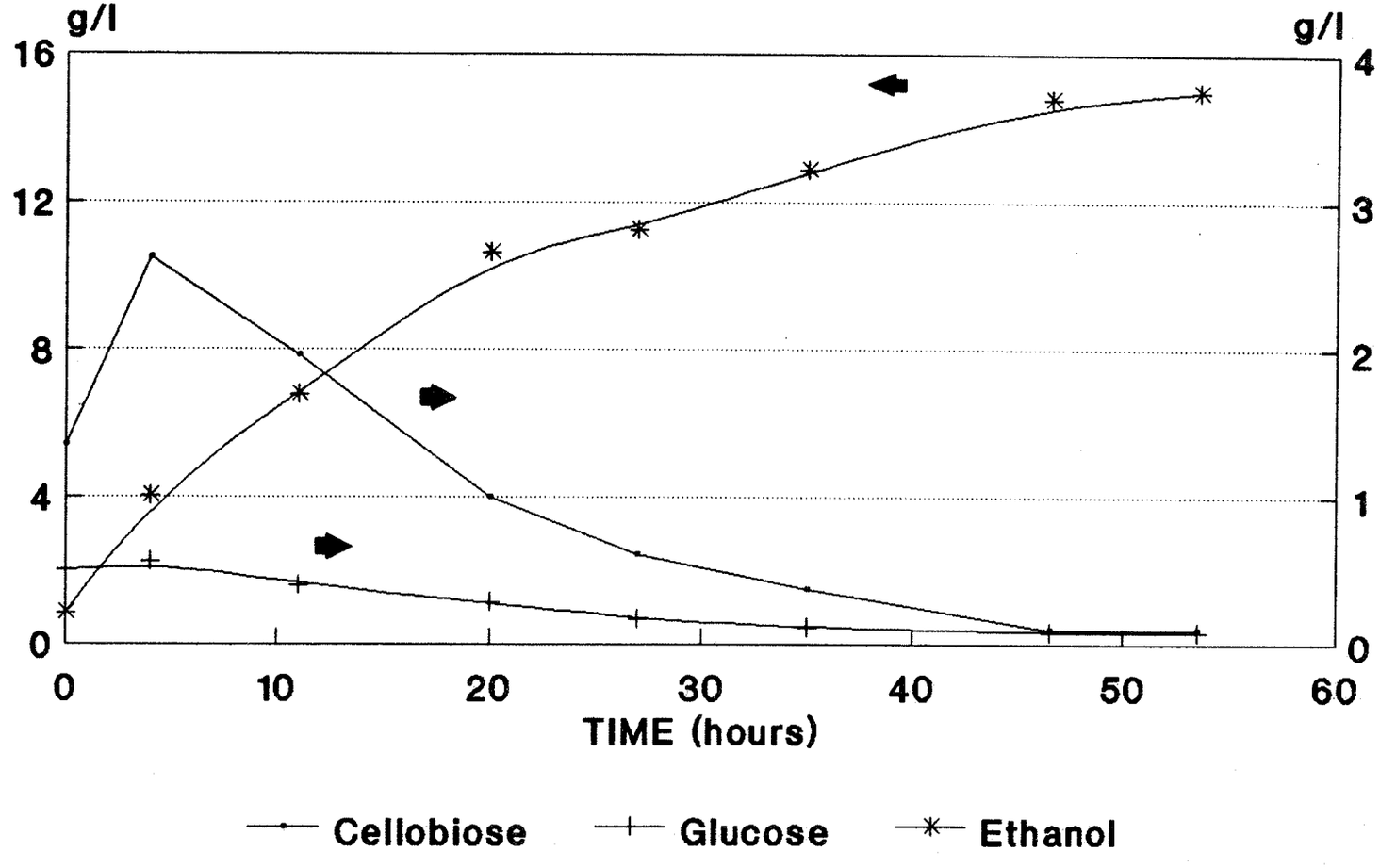
Substrate 8%, Tem. 37 C, pH 5.

Figure 3 Effect of high substrate concentration on ethanol formation.



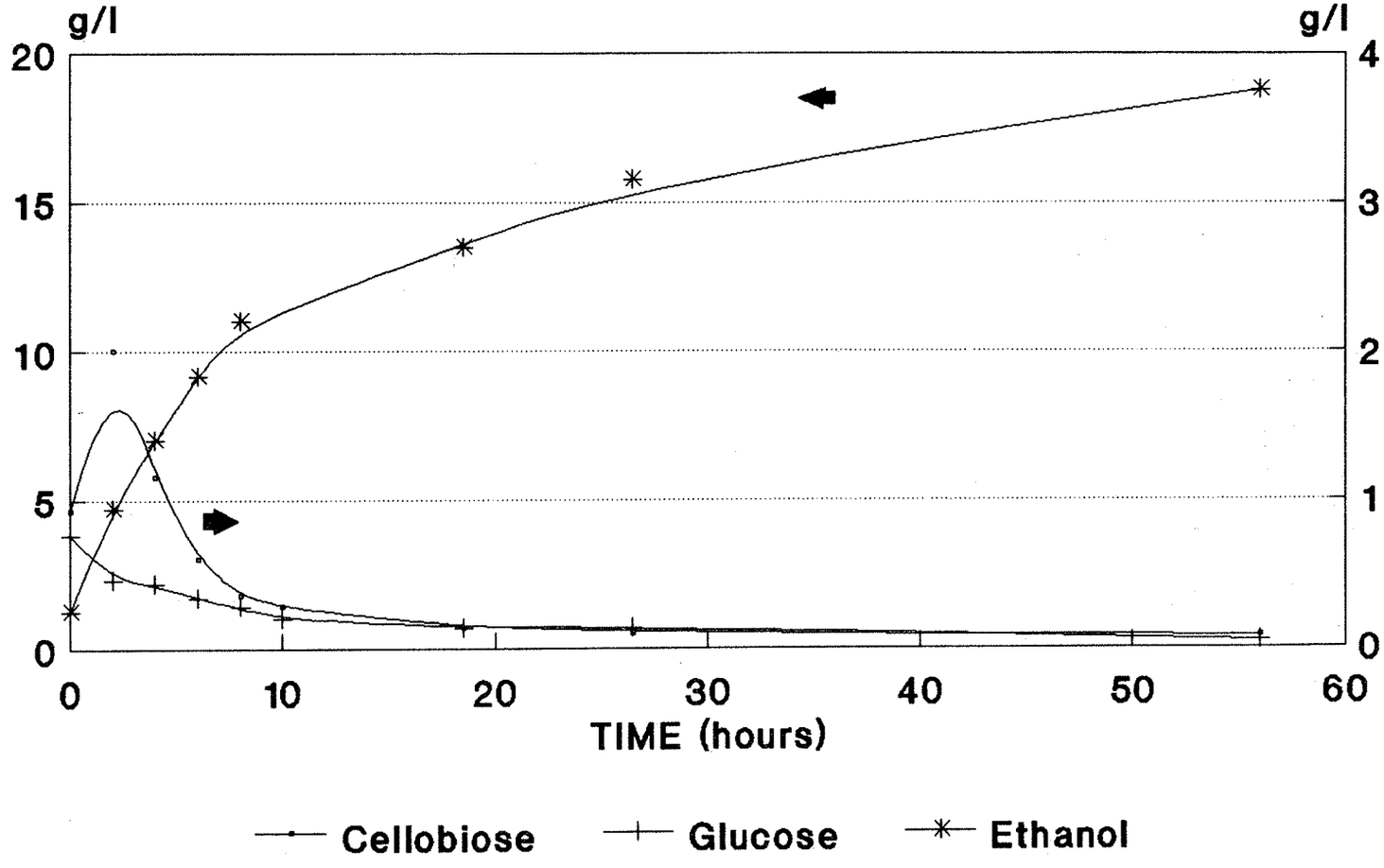
Temp 37 C, pH 5, Enzyme 0.6 UI/ml,
Cellobiase 20 mg/ml

Figure 4 Profiles of glucose and cellobiose during the fermentation, without supplementation of cellobiase.



Sustrato 8%, Temp 37 C. pH 5

Figure 5 profiles of glucose and cellobiose during the fermentation, with supplemented cellobiase (20 mg/l).



Substrate 8%; Enzyme: 0.6 UI/ml

3) The cellobiase addition is important since reduces reaction time and increases hydrolysis yields. This addition should be considered in a commercial process.

4) The results shown here demonstrates the economic potential of the simultaneous hydrolysis-fermentation process. The lignocellulosic materials are readily available and pretreatment of raw materials, such as high pressure steam, are inexpensive.

5) In order to analyze the effect of substrate at high concentration, it is necessary to modify the reactor configuration.

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CHARACTERIZATION OF SPENT SODA COOK LIQUOR AND SULFONATE ALKALILIGNINS

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ABSTRACT

The structures of lignin from spent soda cook liquor and its sulfonate product were examined and compared by IR spectroscopy and gel permeation chromatography.

The evaporation process during spent liquor concentration causes an increment in the number of aromatic rings and a decrease of hydroxyl groups.

The sulfonate alkalilignin has more C-O-C groups vs. OH aliphatic groups than spent liquor.

During the sulfonation process, the sulfonated alkalilignin obtained has two fractions. The insoluble one has higher molecular weight, more C-O-C, OCH_3, S and OH groups than the soluble fraction.

INTRODUCTION

Cellulose used in our paper industry was fundamentally first obtained from wood, but because of felling of trees, it was necessary to look for other raw materials such as bagasse obtained from sugar cane, our principal crop, which contains 30-50% of cellulose.

On bleach pulp process, great amounts of spent soda liquors are spilled in rivers, constituting a source of pollution; therefore one of our mayor objectives is to make this spill useful.

Through the sulfonation process, the alkalilignin

present in the spent liquor could find industrial applications as a surfactant with many potencial uses. We have been working on this project for some years, but first it was necessary to perform an analytical assessment of the material to be processed. The objective of this paper is to present a comparative study between:

-Spent liquor obtained from the Damuji paper industry before and after concentration.

-Concentrated spent liquor and sulfonated spent liquor.

EXPERIMENTAL

IR spectra were obtained on a Philips FT-IR spectrofotometer. GPC analysis were carried out using a MILICROM liquid chromatograph equipped with a variable wave lenght detector set at 280 nm. The column used (10cm x 6mm) was packed with Sephadex G-75, G-100, G-200 (1:1:2) and the elution system (1) was 0,01 mol/l solution of NaCl, NaOH, $NaH_2PO_4.H_2O$ (1:1:1).

The $\overline{Mn}$ was verified by means of a Knauer osmometer with cellulose acetate membrane (2).

Total sulfur content (3) was determined on purified acetylated sulfonated liquors (4). Methoxyl groups were obtained by GLC on a CHROM 5 gas chromatograph with FID detector (5).

RESULTS AND DISCUSSION

Spent liquor was obtained from a soda pulp process on a continuous digester (cooked for 7 min.). It presents 3-6% of solid contents. After a concentration process the % solids increase to 20-30%.

Spectra of spent liquor (SPENTLIQ), concentrated spent liquor (CSPENLIQ) and sulfonated spent liquor (SSPENLIQ),

show differences in the relationships obtained with respect to the absorption band at 1600 cm^{-1}, which corresponds to the aromatic C=C linkages. Results are shown in table I.

Table I: Relationships obtained with respect to absorption at 1600 cm^{-1}.

cm^{-1}	assignation	SPENTLIQ	CSPENLIQ	SSPENLIQ
3330	ν_{O-H}	0.86	0.36	0.19
2920	ν_{C-H}	0.65	0.31	0.15
2850	ν_{C-H}	0.57	0.23	-
1505	$\nu_{C=C}$	0.58	0.34	0.23
1420	$\delta_{C-H(-OCH_3)}$	0.86	0.71	0.65
1120	ν_{C-O-C}	0.63	0.34	0.45
1043	$\delta_{O-H(aliph)}$	0.76	0.37	0.34

The relationships obtained from the concentrated liquor and from the sulfonated spent liquor decrease with respect to the original spent liquor (SPENTLIQ). This finding could reflect the presence of great amounts of aromatic compounds in those products.

IR spectra of concentrated and sulfonated samples show a decrease of the intensity absorption at 3330 cm^{-1} and 1043 cm^{-1} assigned to hydroxyl groups. This last band was designed by Nada et al. (6) to aliphatic hydroxyl groups, which has similar values in concentrated and sulfonated products. This could imply that the sulfonation process does not affect aliphatic hydroxyl groups.

The absorption at 1120 cm^{-1} specific for C-O-C dialkyl

ether groups, decrease in the concentrated spent liquor while it increases in the sulfonated product with respect to the concentrated liquor. If we compare this band with the absorption at 1043 cm^{-1}, specific for aliphatic hydroxyl, we observe a great increment in the relationship C-O-C/OH on the sulfonated product (C-O-C/OH spent liq.=0.83; C-O-C/OH conc. liq.=0.92; C-O-C/OH sulf. liq.=1.32). This indicates that the sulfonated product presents more C-O-C dialkyl ether groups per aliphatic hydroxyl than the other samples. On the other hand there are more aliphatic ether linkages per aromatic C=C in the spent liquor.

To obtain more information about the alkalilignin present in the spent liquor and the sulfonated product, we studied the IR spectra of these products after their acetylation and purification. The relationships of the absorption bands with respect to 1600 cm^{-1} are shown in table II.

Almost all the relationship bands analysed in table II exhibit a decrease in ACETCSL. This confirms the existence of more aromatic C=C linkages in the alkalilignin of concentrated spent liquor. Otherwise acetylated sulfonated alkalilignin does not have the same characteristics. It has an increase in all the relationships found. the most intense are those which correspond to the C-O-C dialkyl ether and C-O-C aryl alkyl ether groups (1130, 1030 cm^{-1}). This suggests that acetylated sulfonated alkalilignin molecules present a great number of aliphatic chains, so its structures should be less aromatic.

The comparison between the sulfonated alkalilignin before and after acetylation (tables I and II) shows that the non acetylated product has more aromatic structures. When acetylation occurs most of these structures are lost during the purification process.

Table II: Relationships of the absorption bands with respect to 1600 cm^{-1} of acetylated spent liquor (ACETSL), acetylated concentrated spent liquor (ACETCSL) and acetylated sulfonated spent liquor (ACETSSL).

cm^{-1}	assignation	ACETSL	ACETCSL	ACETSSL
3330	ν_{O-H}	0.68	0.31	1.93
2920	ν_{C-H}	1.16	0.66	1.94
2850	ν_{C-H}	0.92	0.47	1.86
1740	$\nu_{C=O}$	0.76	1.46	1.40
1505	$\nu_{C=C}$	0.30	0.50	0.57
1460	$\delta_{C-H}(-OCH_3)$	0.86	0.58	1.63
1420	$\delta_{C-H}(-OCH_3)$	0.82	0.66	1.27
1360	δ_{O-H}	0.79	0.85	1.45
1230	ν_{C-O-C}	1.09	1.69	2.53
1130	ν_{C-O-C}	2.04	1.14	6.59
1090	ν_{C-O-C}	1.62	-	4.48
1045	$\delta_{O-H}(aliph)$	1.42	1.19	2.79
1030	ν_{C-O-C}	1.28	1.14	3.06

The sulfonated alkalilignin has one part which is not water-soluble . This fraction was isolated and both parts were acetylated, obtaining the samples SACSLIG (soluble acetylated sulfonated alkalilignin) and IACSLIG (insoluble acetylated sulfonated alkalilignin).

The spectra of these samples were compared with their acetylated raw material (concentrated spent liquor). The results are shown in table III.

Table III: Relationships of the absorption bands with respect to 1730 cm^{-1} of ACETSL, SACSLIG and IACSLIG

cm^{-1}	assignation	ACETSL	SACSLIG	IACSLIG
3330	ν_{O-H}	0.21	0.41	0.71
2920	ν_{C-H}	0.45	1.60	1.72
2850	ν_{C-H}	0.32	1.01	1.12
1600	$\nu_{C=C}$	0.68	0.58	1.05
1505	$\nu_{C=C}$	0.34	0.22	0.55
1460	$\nu_{C-H}(-OCH_3)$	0.40	0.66	1.00
1420	$\delta_{C-H}(-OCH_3)$	0.45	0.58	1.04
1360	δ_{O-H}	0.58	0.57	0.89
1230	ν_{C-O-C}	1.17	0.76	1.51
1120	ν_{C-O-C}	0.78	0.72	1.71
1045	$\delta_{O-H}(aliph.)$	0.81	0.74	2.09

The spectrum of the insoluble fraction shows an increment of absorption at 3330, 1360 and 1045 cm^{-1}. The last band corresponds to aliphatic hydroxyl groups and it is the most intense. This means that the insoluble fraction has more hydroxyl groups than the others, specifically aliphatic hydroxyl groups. Furthermore this insoluble fraction has a great quantity of aromatic groups as reflected by the higher values obtained on the relationship C=C/C=O.

The C-O-C band assigned to aryl alkyl ether groups (1230 cm^{-1}) evidences a high absorption on the three samples that were analysed, but it is predominant in the insoluble sulfonated product. The same occurs with respect to the

absorption band corresponding to the C-O-C dialkyl ether (1120 cm^{-1}). If we calculate the relationship between these two bands, we find a decrease from the acetylated alkali-lignin (C-O-C aryl alkyl/dialkyl = 1.5) to the acetylated sulfonated insoluble alkalilignin (C-O-C aryl alkyl/dialkyl = 0.88). This indicates more dialkyl ether linkages in the insoluble fraction than in the other samples, in spite of the great amount of C=C aromatic rings.

Table IV shows the methoxyl groups content obtained by gas chromatography.

Table IV: Methoxyl groups content.

Sample	% OCH_3
SPENTLIQ	4.57
CSPENLIQ	5.75
SSPENLIQ	5.13
SACSLIG	3.61
IACSLIG	6.93

The concentrated spent liquor and its sulfonated product show a decrease of methoxyl per aromatic linkages (table I). However total content of methoxyl in these products is higher than in the spent liquor (table IV); this may indicate that the concentration process produces ruptures of OCH_3 from aromatic groups, but as these products contain more aromatic structures, the total content of methoxyls is higher than in the spent liquor. Furthermore methoxyl groups could be bonded to the hydrocarbon chains of lignins macromolecules.

These findings,together with results shown in table II, confirm the fact that the acetylated sulfonated alkali-lignin has a higher content of OCH_3 per C=C, suggesting that acetylation preferably occurs in sulfonated

alkalilignin structures which have a great amount of OCH_3 groups. Table IV shows the difference between the methoxyl content of the soluble and insoluble fraction of acetylated sulfonated alkalilignin, which reflects the different chemical structure of these two fractions.

The GPC analysis used to determine the molecular weight of the alkalilignin products are shown in table V.

Table V: Molecular weight of analysed samples.

Sample	$\bar{M}w$	$\bar{M}n$	D
SPENTLIQ	27300	13700	1.99
CSPENLIQ	29600	12260	2.40
SSPENLIQ	21540	17550	1.20
ACETSSL	49220	21330	2.31
IACSLIG	96970	30000	2.55
SACSLIG	31550	17850	1.77

The concentration of the spent liquor produces a great dispersion, that leads to the rupture of lignin macromolecules and the formation of compounds with more aromatic contents and less hydroxyl groups, as confirmed by infrared analysis . On the other hand, it is probable that condensation of some molecules also occurs producing higher molecular weight compounds.

The sulfonated spent liquor has the least dispersion while the insoluble fraction has the highest; the latter also exhibits a high molecular weight.

The insoluble fraction has a higher sulfur content (6.27%) than the soluble one (1.94%). Probably part of the sulfur contained in the insoluble fraction is not present as sulfonate, as reflected by its lack of solubility in water.

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EFFECTS OF O-ACETYLATION OF PHENOLIC HYDROXYL GROUPS ON ^{13}C CHEMICAL SHIFTS OF AROMATIC CARBONS IN BIPHENYL TYPE LIGNIN MODEL COMPOUNDS

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ABSTRACT

Studies have been conducted to elucidate the effects of O-acetylation of phenolic hydroxyl groups at C-4 and C-4' of several biphenyl (5-5') type lignin model compounds. Results show that symmetric biphenyl type lignin model compounds undergo shielding of C-4/C-4' (ipso) and C-6/C-6' (meta) on O-acetylation of phenolic hydroxyl group at C-4 and C-4'. In addition the compounds also undergo deshielding of C-1/C-1' (para), C-2/C-2' (meta), C-3/C-3' (ortho) and C-5/C-5' (ortho) by the O-acetylation. Among them, the ^{13}C chemical shifts of C-1/C-1' and C-4/C-4' are affected significantly by the O-acetylation.

INTRODUCTION

Acetylated lignins are not only used in structural study of lignins by NMR spectroscopy (1-3), but also in functional group analysis of the lignins (4-6). Moreover, acetylated lignins are obtained as the major by-products by acetic acid-based organosolv processes for pulping of woods (7). It is, therefore, important to study effects of O-acetylation of phenolic hydroxyl groups on ^{13}C chemical shifts of aromatic carbons in lignin substructures, such as ß-O-4', cyclic ß-5' and 5-5' (biphenyl) type substructures. In this work, we will present the results observed by comparing ^{13}C NMR spectroscopic data of biphenyl (5-5') type lignin model compounds and their acetates.

EXPERIMENTAL PART

Routine ^{13}C NMR spectra of lignin model compounds were recorded with a Bruker AC-80 spectrometer operating at 21.2 MHz for ^{13}C nuclei frequency with the broad band-noise decoupling technique. Deuterated dimethylsulfoxide (DMSO-$\underline{d}_6$) was used as solvent. The concentration of sample is approximately 100 mg in 0.5 ml of DMSO-$\underline{d}_6$ (ca. 20% $\underline{w}/\underline{v}$). The spectra were run in 5 mm (O.D.) Sample tube with a 90° pulse angle corresponding to 12 μsec pulse width, pulse repetition time of 4 sec, and an average number of scans of approximately 1,000. Tetramethylsilane (TMS) was used as the internal chemical shift (in δ) reference.

RESULTS AND DISCUSSION

^{13}C chemical shifts of aromatic carbons in symmetric biphenyl type lignin model compounds 3-6 have been assigned by

Table 1. Calculated and Observed ^{13}C Chemical Shifts for Aromatic Carbons in 2,2'-Diacetoxy-3,3'-dimethoxy-5,5'-dimethylbiphenyl (3).

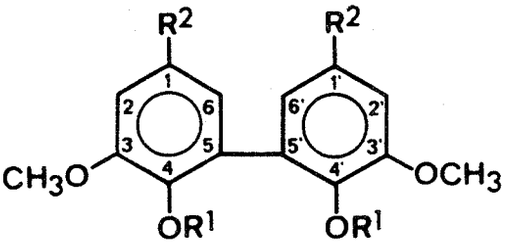
	C-1	C-2	C-3	C-4	C-5	C-6
Compound 1[a]	126.3	112.3	151.2	137.0	130.7	122.0
SCS[b] for $-CH_3$[c]	+8.6	+0.6	-0.4	-2.3	-0.4	+0.6
Calculated Chemical Shift	134.9	112.9	150.8	134.7	130.3	122.6
Observed Chemical Shift	135.6	112.9	150.9	134.8	130.5	122.3
Error[d]	-0.7	0.0	-0.1	-0.1	-0.2	+0.3

[a] Chemical Shift: in δ. Solvent: DMSO-$\underline{d}_6$.

[b] Substituent Chemical Shift (SCS): in $\Delta\delta$, relative to the corresponding carbon of parent compound.

[c] SCS for $-CH_3$ group in biphenyl type lignin model compounds, see Reference Drumond et al. (1989).

[d] Error: in $\Delta\delta$.



1, R^1 = OAc; R^2 = H

2, R^1 = R^2 = H

3, R^1 = OAc; R^2 = CH_3

4, R^1 = H; R^2 = CH_3

5, R^1 = OAc; R^2 = $CH_2CH_2CH_3$

6, R^1 = H; R^2 = $CH_2CH_2CH_3$

Table 2. Calculated and Observed ^{13}C Chemical Shifts for Aromatic Carbons in 2,2'-Dihydoxy-3,3'-dimethoxy-5,5'-dimethylbiphenyl (4).

	C-1	C-2	C-3	C-4	C-5	C-6
Compound 2[a]	118.7	110.8	147.9	143.8	126.2	123.5
SCS[b] for $-CH_3$[c]	+8.6	+0.6	-0.4	-2.3	-0.4	+0.6
Calculated Chemical Shift	127.3	111.4	147.5	141.5	125.8	124.1
Observed Chemical Shift	127.4	111.6	147.7	141.3	126.0	123.5
Error[d]	-0.1	-0.2	-0.2	+0.2	-0.2	+0.6

[a] Chemical Shift: in δ. Solvent: DMSO-d_6.

[b] Substituent Chemical Shift (SCS): in $\Delta\delta$, relative to the corresponding carbon of parent compound.

[c] SCS for $-CH_3$ group in biphenyl type lignin model compounds, see Reference Drumond et al. (1989).

[d] Error: In $\Delta\delta$.

application of the generalized SCS additivity rule developed previously for biphenyl type substructure (8). 2,2'-dihydroxy-3,3'-dimethoxybiphenyl (2) and its O-diacetylated derivative (1) were used as parent compounds for the estimation of ^{13}C chemical shifts.

Tables 1 and 3 show ^{13}C chemical shifts of aromatic carbons in compounds 3 and 5 estimated by the generalized SCS additivity rule. Error between the observed and estimated values for the corresponding carbons is, in general, less than $\Delta\delta$ ±0.7. Moreover, the error is in the same magnitude with the corresponding non-O-acetylated 5-5' type model compounds 4 and 6 as shown in Tables 2 and 4.

Thus, the generalized SCS additivity rule for the biphenyl (5-5') type lignin model compounds is also applicable to O-acetylated phenolic biphenyl type lignin model compounds.

Table 3. Calculated and Observed ^{13}C Chemical Shifts for Aromatic Carbons in 2,2'-Diacetoxy-3,3'-dimethoxy-5,5'-dipropylbiphenyl (5).

	C-1	C-2	C-3	C-4	C-5	C-6
Compound 1[a]	126.3	112.3	151.2	137.0	130.7	122.0
SCS[b] for $-CH_2CH_2CH_3$[c]	+13.6	-0.3	-0.4	-2.2	-0.4	-0.3
Calculated Chemical Shift	139.9	112.0	150.8	134.8	130.3	121.7
Observed Chemical Shift	140.3	112.3	150.9	134.9	130.5	121.5
Error[d]	-0.4	0.3	-0.1	-0.1	-0.2	+0.2

[a] Chemical Shift: in δ. Solvent: DMSO-d_6.

[b] Substituent Chemical Shift (SCS): in $\Delta\delta$, relative to the corresponding carbon of parent compound.

[c] SCS for $-CH_2CH_2CH_3$ group in biphenyl type lignin model compounds, see Reference Drumond et al. (1989).

[d] Error: in $\Delta\delta$.

Table 5 summarizes ^{13}C chemical shifts of aromatic carbons of three O-acetylated symmetric biphenyl type lignin model compounds. These compounds includes 2,2'-diacetoxy-3,3'-dimethoxybiphenyl (1), 2,2'-diacetoxy-3,3'-dimethoxy-5,5'-dimethylbiphenyl (3) and 2,2'-diacetoxy-3,3'-dimethoxy-5,5'-dipropylbiphenyl (5). ^{13}C chemical shifts of aromatic carbons in these compounds are compared with those of the corresponding aromatic carbons in 2,2-dihydroxy-3,3'-dimethoxy-biphenyl (2), 2,2'-dihydroxy-3,3'-dimethoxy-5,5'-dimethylbiphenyl (4) and 2,2'-dihydroxy-3,3'-dimethoxy-5,5'-dipropylbiphenyl (6). The results then give the effects of O-acetylation on ^{13}C chemical shifts on carbons in compounds 2, 4 and 6.

In compounds 2, 4 and 6, O-acetylation results in shielding for C-4/C-4' (ipso) and C-6/C-6' (meta) by

Table 4. Calculated and Observed ^{13}C Chemical Shifts for Aromatic Carbons in 2,2'-Dihydoxy-3,3'-dimethoxy-5,5'-dipropylbiphenyl (6).

	C-1	C-2	C-3	C-4	C-5	C-6
Compound 2[a]	118.7	110.8	147.9	143.8	126.2	123.5
SCS[b] for $-CH_2CH_2CH_3$[c]	+13.6	-0.3	-0.4	-2.2	-0.4	-0.3
Calculated Chemical Shift	132.3	110.5	147.5	141.6	125.8	123.2
Observed Chemical Shift	132.4	110.8	147.7	141.3	126.0	122.9
Error[d]	-0.1	-0.3	-0.2	+0.3	-0.2	+0.3

[a] Chemical Shift: in δ. Solvent: DMSO-d_6.

[b] Substituent Chemical Shift (SCS): in $\Delta\delta$, relative to the corresponding carbon of parent compound.

[c] SCS for $-CH_2CH_2CH_3$ group in biphenyl type lignin model compounds, see Reference Drumond et al. (1989).

[d] Error: in $\Delta\delta$.

approximately Δδ 6.4-6.8 and 1.2-1.5, respectively. In addition, it also results in deshielding for C-1/C-1' (para), C-2/C-2' (meta), C-3/C-3' (ortho) and C-5/C-5' (ortho) by approximately Δδ 7.6-8.2, 1.3-1.5, 3.2-3.3, and 4.5, respectively. Thus, ^{13}C chemical shifts on C-1/C-1', C-3/C-3', C-4/C-4' and C-5/C-5' of these compounds are affected significantly, in particular C-1/C-1' (deshielding) and C-4/C-4' (shielding) on O-acetylation of the phenolic hydroxyl groups.

Table 5. Effects of O-Acetylation of Phenolic Hydroxyl Group in Biphenyl Type Lignin Model Compounds on ^{13}C Chemical Shifts of Aromatic Carbons. Solvent: DMSO-d_6

Compound	Chemical Shift (in δ Scale)					
	C-1	C-2	C-3	C-4	C-5	C-6
1	126.3	112.3	151.2	137.0	130.7	122.0
2	118.7	110.8	147.9	143.8	126.2	123.5
Effect of O-Acetylation[a]	+7.6	+1.5	+3.3	-6.8	+4.5	-1.5
3	135.6	112.9	150.9	134.8	130.5	122.3
4	127.4	111.6	147.7	141.3	126.0	123.5
Effect of O-Acetylation[a]	+8.2	+1.3	+3.2	-6.5	+4.5	-1.2
5	140.3	112.3	150.9	134.9	130.5	121.5
6	132.4	110.8	147.7	141.3	126.0	122.9
Effect of O-Acetylation[a]	+7.9	+1.5	+3.2	-6.4	+4.5	-1.4
Average[a]	+7.9	+1.4	+3.2	-6.6	+4.5	-1.4

[a] In Δδ.

CONCLUSION

O-Acetylation of phenolic hydroxyl groups in biphenyl (5-5') type lignin model compounds significantly affects ^{13}C chemical shifts of aromatic carbons in these compounds. The ^{13}C chemical shifts of C-1/C-1' (para) and C-4/C-4' (ipso) are particularly affected by considerably large deshielding and shielding, respectively. The generalized SCS additivity rule for 5-5' type lignin model compounds is also applicable to their O-acetylated derivatives.

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SECTION II. BIOTECHNOLOGY

Development of New Techniques to Reduce Environmental Impact of Pulp Bleaching

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Introduction

The release of spent liquors from conventional bleaching of chemical pulps into receiving waters represents the most important environmental problem of the pulp and paper industry. Effluents from chlorine bleaching of pulp contain acutely toxic chlorinated compounds measured as total organic chlorines (TOCl) or adsorbable organic halogen (AOX), genotoxic materials and high molecular mass chlorinated materials that slowly transfer into toxic chlorinated catechols and guaiacols [Eriksson, 1990]. In addition, the chlorinated phenolic compounds are generally converted by microorganisms to the corresponding, more persistent and more hydrophobic, chlorinated veratrols. On top of the more than 300 different organic compounds found in waste bleach waters, a small quantity of highly toxic dioxins have also been found. However, the dioxin formation can be reduced to undetectable levels by excluding molecular chlorine from the bleaching sequences.

The primary reason for bleaching chemical pulp is to remove lignin in order to obtain a brighter pulp. The most commonly used brightness index compares the reflectance factor for blue light (457 nm) of a pulp sheet to the reflectance of magnesium oxide (100% brightness). It is essentially impossible with existing techniques to remove enough lignin during the cooking stage to yield pulp of satisfactory brightness. If all the lignins were removed in the cooking process using current techniques, the yield and strength properties of the pulp would be much too low to be acceptable.

In response to the growing concerns for the environment, restrictions on the release of waste bleach waters are growing more stringent. In Sweden, goals have been set to reduce the bleaching effluent problems (Table 1). Other countries will inevitably follow these steps. Given this, it is obvious that there is an urgent need to develop techniques that allow for pulp bleaching without environmental impacts.

The problem caused by bleach plant effluent pollution can be attacked and probably solved in several ways. We have chosen to attack these problems in three different ways: (1) develop a new technique for purification of waste bleach waters; (2) develop a new technique for pulp bleaching; (3) change the amount and structure of lignin in forest trees to make the wood easier to pulp and bleach.

New Technique for Purification of Waste Bleach Waters

The techniques presently in use for treatment of waste bleach waters are aerated lagoons and activated sludge plants. The acute toxicity is normally removed in the aerated lagoons but the reduction of chlorinated compounds is very low. In many countries, treatment with activated sludge is preferred to the aerated lagoon. Such treatments, particularly at a low organic load, have given a better reduction of chlorinated compounds (40%), than the aerated lagoon (25%) [Boman et al. 1988].

A common feature of all biological methods used for purification of waste bleach liquors is that high molecular mass chlorinated materials are not removed. Eriksson and Kolar [1985], using high molecular mass ^{14}C-labelled chlorinated lignins, showed that this material cannot be degraded by bacterial consortia isolated from aerated lagoons. The white-rot fungus *Sporotrichum pulverulentum* (*Phanerochaete chrysosporium*) is, however, able to convert 40-50% of the high molecular mass ^{14}C-labelled chlorinated lignins to $^{14}CO_2$ in 40-50 days. Furthermore, it has been shown [Eriksson et al. 1985] that high molecular mass

Table 1. The Swedish goal to reduce toxic materials in bleaching effluents.

Horizon	AOX-level kg/ton pulp	Indicator Substances % of 1970 level	Introduction of Commercial Substances
Short	1.5	5	1988-1992
Medium	0.5 - 1.0	1	1992-2000
Long	0.1	< 0.4	> 2000

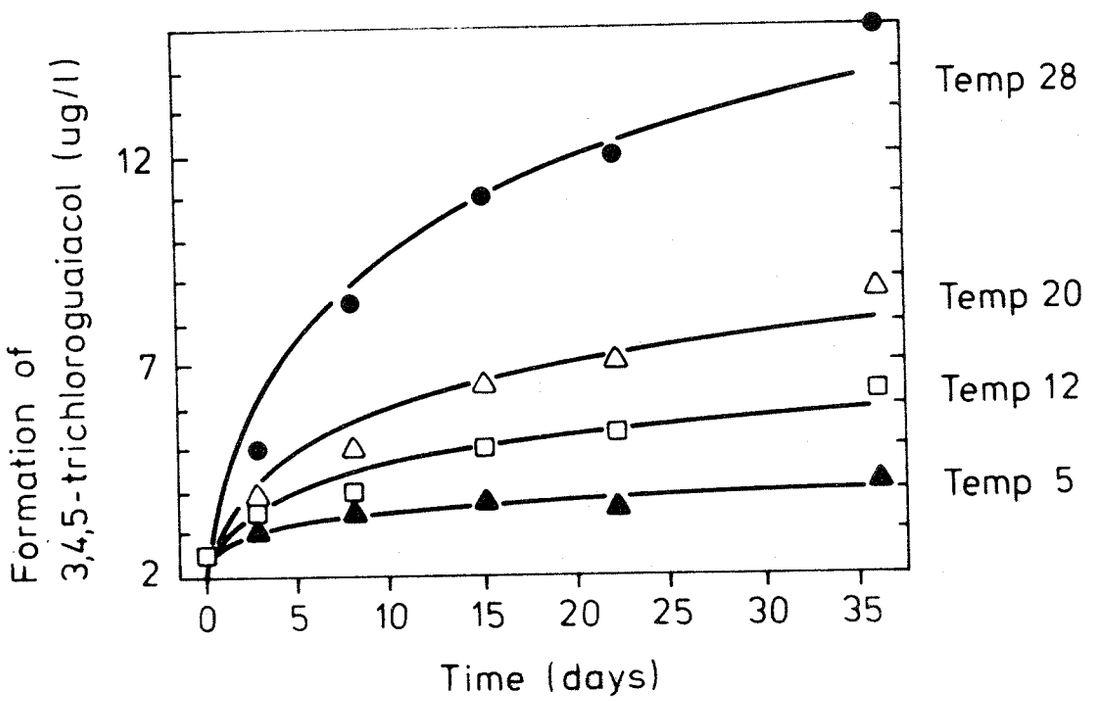

Figure 1. Formation of 3,4,5-dechloroguaiacol under sterile conditions from a solution of high relative molecular mass material from spent alkali extraction liquor from bleaching of softwood kraft pulp, pH 7.2 [Eriksson et al. 1985].

chlorinated materials are not as stable as was earlier thought. These compounds are slowly transformed into, among other substances, chlorinated catechols and guaiacols (Fig. 1).

It is clear that the release of chlorinated organic compounds should be reduced or eliminated in order to avoid harmful effects on the environment. This elimination must involve both low and high molecular mass chlorinated compounds. Regulatory and consumer pressure is now causing pulp and paper companies to do just that.

We thought at an early stage that it would be possible to use white-rot fungi to degrade and eliminate the high molecular mass chlorinated material since these fungi are the only known microorganisms able to completely degrade polymeric lignins. However, there is no known microorganism that can live and grow on lignin alone and lignin degradation by white rot fungi is an energy-requiring process. This energy is derived from easily metabolizable carbon sources, particularly wood polysaccharides and low molecular mass sugars. Such sugars are necessary not only for energy purposes, but also for production of H_2O_2 -- a necessary ingredient in the lignin degrading machinery created by white-rot fungi. However, so far white-rot fungi have not been used commercially for degrading high molecular mass chlorinated lignins in bleach plant effluents. This is because they have very complicated physiological demands for lignin degradation which are difficult to satisfy on a large scale [Eriksson et al. 1990].

Even if anaerobic degradation of lignin does not take place [Zeikus et al. 1982; Colberg and Young, 1985], anaerobic treatment of spent bleach liquors might still be effective, i.e. if anaerobes can cause dechlorination of chlorinated lignins. We have recently demonstrated that this is not the case [Fitzsimons et al. 1990]. Only low molecular mass chlorinated substances seem to be dechlorinated anaerobically.

With this background, it was evident that alternatives to biological processes for elimination of high molecular mass chlorinated materials from waste bleach waters must be found. Ultrafiltration of the alkaline stage effluent is presently the method of choice -- it has a good effect and it can also be accomplished at a reasonable cost [Boman et al. 1988]. Ultrafiltration of the entire bleach plant waste waters is not yet economically realistic because of the large volume of the chlorination stage effluent. Approximately 70% of TOCl is found in the first E-stage effluent from a softwood kraft pulp bleach mill. When ultrafiltration is used on this stream, the total amount of TOCl is reduced by around 35-45%, resulting in a discharge of TOCl of less than 3 kg/ton pulp. However, after the ultrafiltration, the effluent is still acutely toxic and, in addition to ultrafiltration, biological treatment is needed (Fig. 2). As a complement to ultrafiltration, several different biological methods can be used, some of which will now be discussed.

Biological treatment of waste bleach waters normally means that bacteria dominate the biomass. However, by lowering the pH value to about 4, microbial consortia dominated by fungi can be obtained instead. We have investigated the combination of ultrafiltration with fungal treatment of waste bleach waters in an airlift type fermenter. The main reason for selecting fungi instead of bacteria was that filamentous fungi can easily be filtered off and recirculated to the process. High biomass concentration in the purification system is important. Waste bleach waters are meager in terms of utilizable carbon sources and a too high toxicity:microbial biomass ratio will eventually wipe out the microorganisms there to do the cleaning job. Fungi of the white-rot type have proved able to degrade most of the wastes found in bleach plant effluents. Reductions of COD and AOX were also achieved in the fermenter experiments using the white rot fungus *Phanerochaete chrysosporium* --

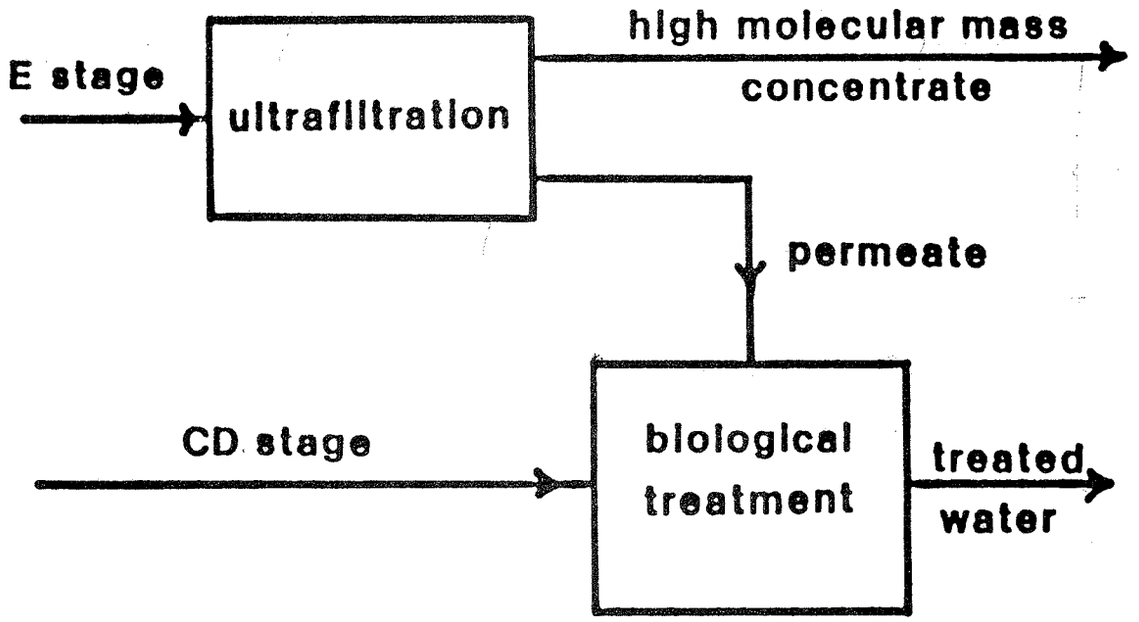

Figure 2. Principle of the suggested combined ultrafiltration-biological process [Boman et al. 1988].

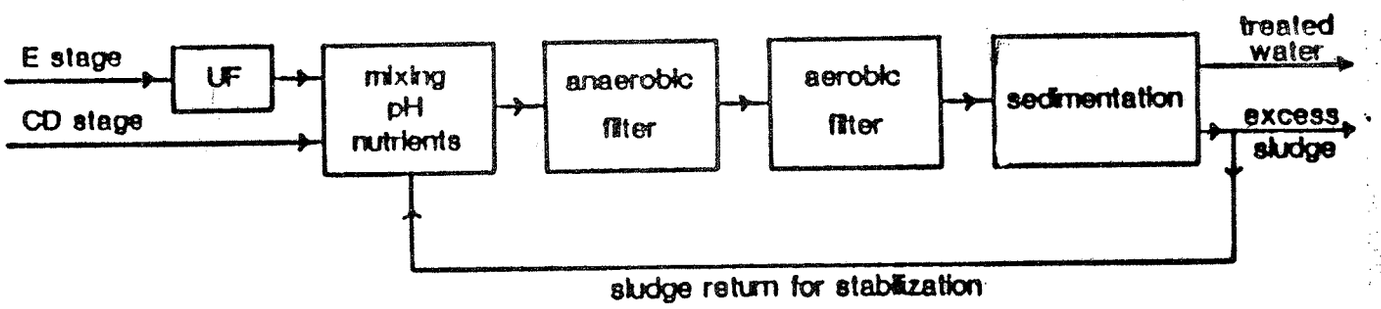

Figure 3. Principle of the bacterial treatment system after ultrafiltration of the alkaline stage effluent [Ek and Eriksson, 1988].

about 70% reduction of COD and chlorinated phenols and 55% of AOX was achieved in the combined process.

Anaerobic treatment is gradually being introduced into the pulp and paper industry. Such treatments are particularly used for warm and concentrated waste waters from recycling paper effluents, semi-chemical pulp effluents and evaporator condensates [Boman et al. 1988]. However, purification of bleach plant effluents is a different story since waste waters from chlorine/chlorine dioxide bleaching are currently more dilute than is desirable for anaerobic treatment. To avoid an excessively long hydraulic retention time in the system, high solids retention must be achieved. This can be accomplished in a system of immobilized organisms, i.e. the use of a filter type equipment to which the microorganisms adhere.

Since anaerobic treatment results in the formation of reduced substances such as hydrogen sulfide and organic acids, there is always risk of creating an odor problem. Therefore, we thought it useful to combine the anaerobic treatment with an aerobic treatment that could be an effective remedy for the potential odor problem. The principle of this system, ultrafiltration/anaerobic, aerobic treatment is given in Figure 3. The results already obtained with this purification technique are about three times as good as those obtained with aerated lagoons or activated sludge techniques. The purification technique outlined in Figure 3 is now being further developed in cooperation with a major company in the United States and it seems as if the developed technique will be used commercially. The process we are aiming at is a completely closed system where the waste bleach waters are recirculated after purification.

New Techniques for Pulp Bleaching

Until the 1970's, oxygen bleaching was not implemented on a large scale because the free radicals generated in the process demonstrated poor selectivity by attacking cellulose as well as lignin. Addition of magnesium salts was found to protect the cellulose and other carbohydrates and oxygen bleaching is now in wider use. However, the extent of delignification in oxygen bleaching is limited [Fossum and Marklund, 1988; Bowen and Shu, 1990].

The partial replacement of chlorine with chlorine dioxide in bleaching is also gaining acceptance. Chlorine dioxide substitution decreases bleach plant effluent color, the amount of chlorinated organic compounds, and particularly the amount of chlorinated dibenzo-*p*-dioxins and chlorinated dibenzofurans [Pryke, 1989]. When the substitution of chlorine dioxide for chlorine goes beyond 50%, the amount of total chlorinated phenolics produced during bleaching decreases dramatically (Fig. 4). With all the advantages of chlorine dioxide substitutions in mind, it must be remembered that chlorate is formed in proportion to the amount of chlorine dioxide used [Boman et al. 1988]. Chlorate is a potent herbicide and certain aquatic plants such as brown algae are particularly sensitive to it. Thus, waste bleach waters from chlorine dioxide bleaching still require biological or chemical treatment before discharge to the recipients.

Several attempts have been made to use biological techniques to degrade and remove lignin. Thus, there are many fungi in nature capable of degrading wood and, among them, white-rot fungi degrade lignin better than other wood-degrading fungi. Some of these white-rot fungi have been used for pulp bleaching but the process is too slow [Reid et al. 1990]. Lignin peroxidase, a haeme-containing enzyme first isolated from the white-rot fungus *Phanerochaete chrysosporium* was, for some time, a candidate for use in pulp bleaching.

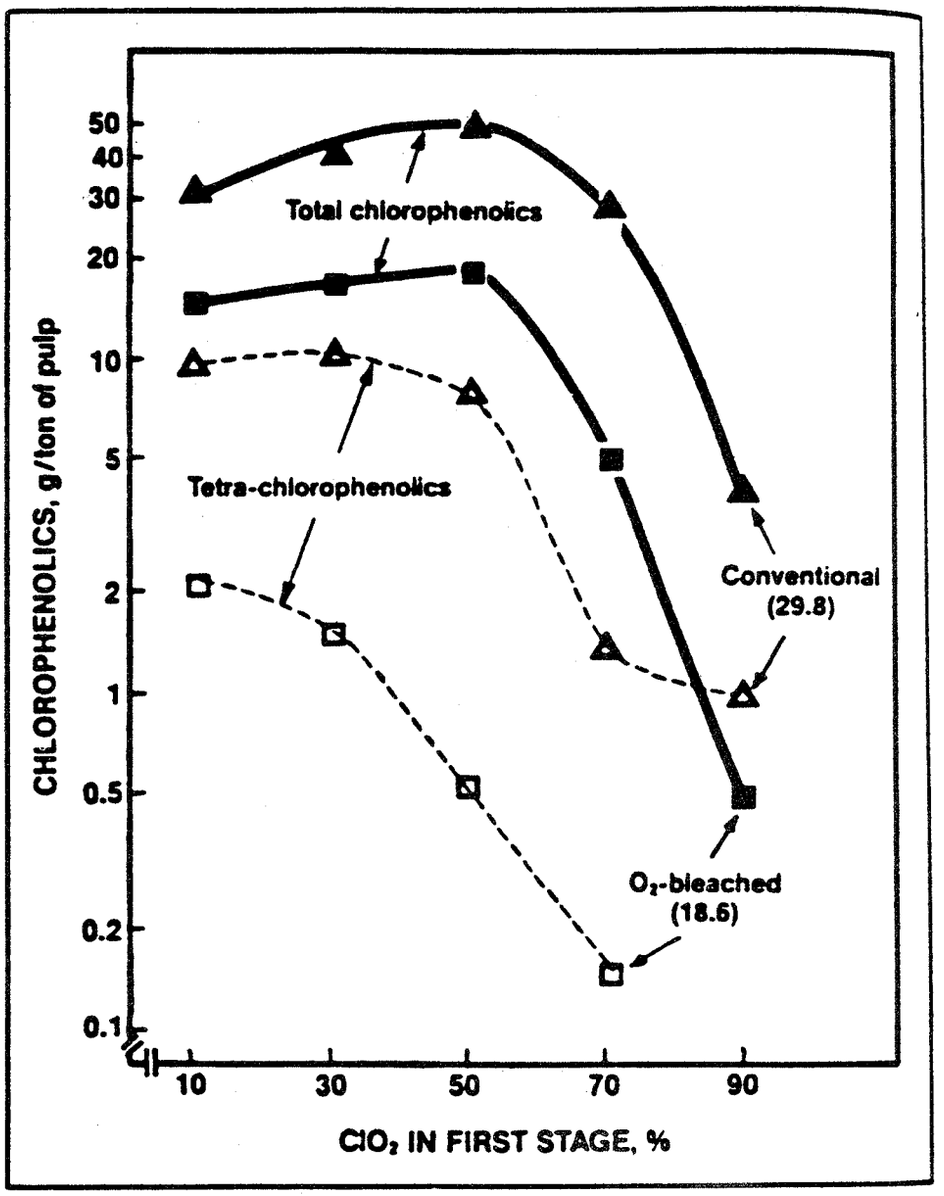

Figure 4. The effect of chlorine dioxide substitution on total and tetra-chlorinted phenolic compounds production [Axegård, 1985].

However, limited lignin degradation was achieved with this enzyme. Biomimetic bleaching, a technique which attempts to simulate the attack of ligninolytic enzymes on lignin, is another approach to reduce the charge of harsh chemicals. Shimada et al. [1989] treated a β-O-4 lignin model compound, as well as unbleached hardwood kraft pulp, with a system containing Mn(II)/Co(II) in 95% acetic acid for 1 h at room temperature. This treatment degraded the model compound, and an increase in pulp brightness from 35% to 80% was accomplished. Palmitoyl chloride-modified hemoglobin was also tested as a bleaching catalyst. Incubation of pine kraft pulp with .75% hemoglobin, based on pulp dry weight, at 60°C for 90 min decreased the Kappa number from 30 to 22.7 with only a minor decrease in pulp viscosity [Pettersson et al. 1988].

A better understanding of the relationship between lignin and other cell wall components is imperative for the development of enzymatic technique as one stage in pulp bleaching. Lignin is not simply deposited between cell wall polysaccharides, but is covalently linked to at least part of them to form a lignin-carbohydrate complex. The bonds between lignin and carbohydrates contribute to the difficulties encountered in removing lignin from wood. In general, hemicelluloses appear to be the primary lignin linked polysaccharide, but bonds between cellulose and lignin created in the cooking process cannot be excluded. Lignin-xylan complexes are found in hardwoods, while both lignin-mannan and lignin-xylan complexes are found in softwoods, maybe also lignin-cellulose complexes in softwood kraft pulps.

Hemicelluloses comprise both linear and branched heteropolymers of D-xylose, L-arabinose, D-mannose, D-glucose, D-galactose and D-glucuronic acid. Most hemicelluloses have 1,4-β-linkages between their backbone sugar units -- an exception are galactose-based hemicelluloses, which are connected by 1,3-β-linkages. The individual sugar units may also

be acetylated or methylated. The sugar composition and structure of hemicelluloses and their content in wood vary from species to species, but xylans, mannans and galactans are the predominant types found in wood. The complex nature of the hemicelluloses implies cooperation between a number of enzymes for their degradation. Figure 5 illustrates the enzymes necessary to degrade a hypothetical xylan. Similar classes of enzymes are required for enzymatic hydrolysis of mannans. However, during alkaline pulping, acetyl groups, arabinose and other branching substituents, as well as part of the xylan-mannan backbones, are degraded. Thus, for the hydrolysis of hemicelluloses remaining in kraft pulps it seems as if enzymes of the endo-type, i.e. endoxylanase and endomannanase, are the only ones necessary. Endoxylanase seems to be the most important enzyme, particularly for treatment of hardwood kraft pulp and a removal of up to 20-30% of xylan from such pulp has been reported [Clark et al. 1990]. The corresponding figure for endomannanase is solubilization of less than 5% of the available glucomannan in kraft or neutral sulfite anthraquinone pulp.

It is generally felt that addition of cellulolytic enzymes to pulp is resulting in a loss of both physical strength yield of the pulp [Linko et al. 1989]. In our search for a microorganism producing essentially a cellulase-free xylanase solution when grown on a xylan-containing substrate, we have settled for the yeast-like fungus, *Aureobasidium pullulans*, which had been reported not to produce cellulases [Leathers et al. 1984; Leathers, 1986]. However, we found in our studies that *A. pullulans*, grown on oat spelt xylan as the sole carbon source, not only produces high amounts of endoxylanases but also low levels of endoglucanase and mannase activities. Using ultrafiltration membranes, we were able to separate the culture solution from *A. pullulans* into a high molecular weight enzyme fraction (10-100), passing through a membrane with cut-off 100 kD but not through a membrane with cut off 10 kD, and a low molecular weight enzyme fraction (5-10), passing through a

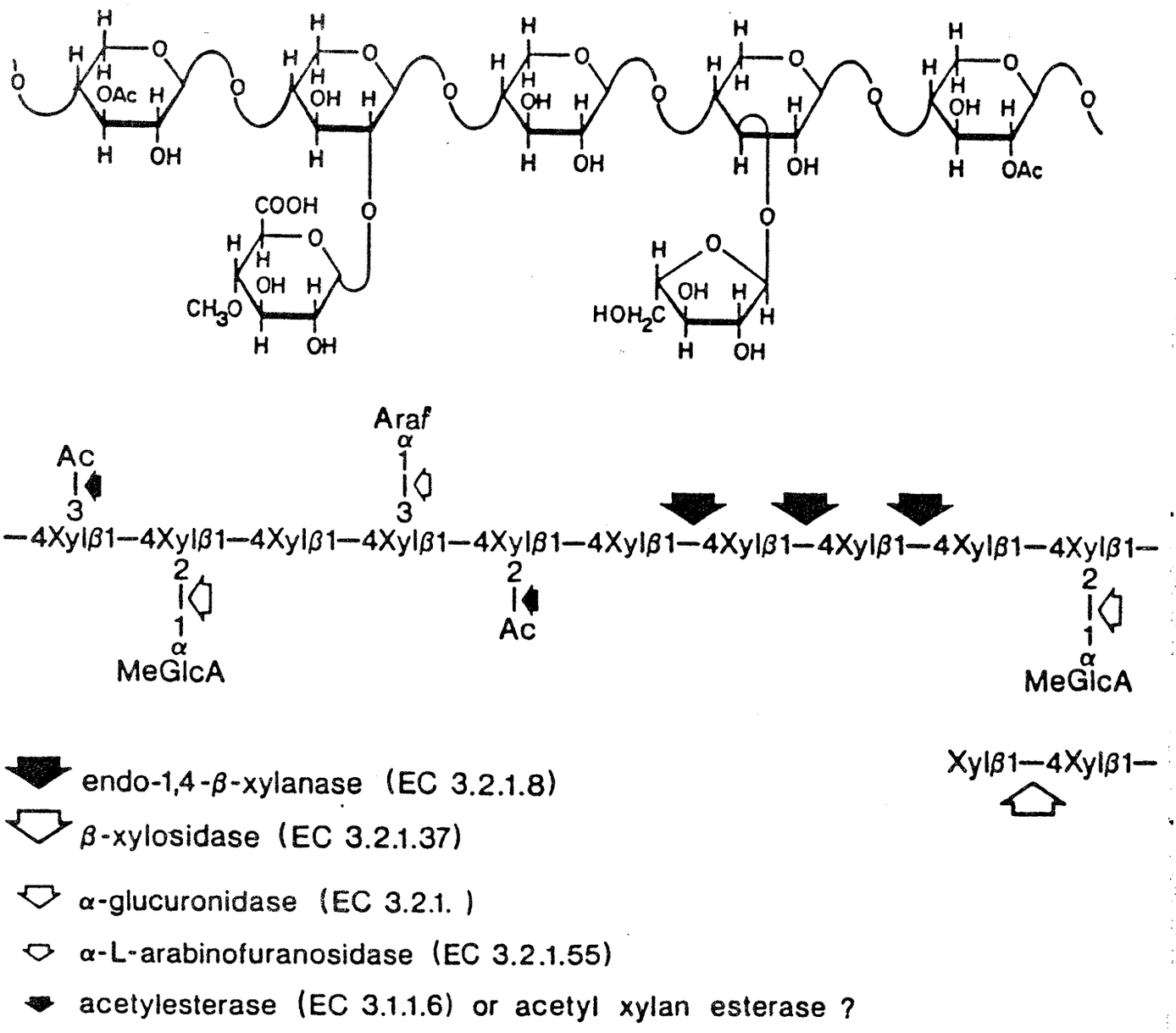

Figure 5. A hypothetical plant xylan and the sites of its attack by microbial, xylanolytic enzymes [Biely, 1985].

membrane with a cut off 10 kD but retained on a membrane with a cut off 5 kD. The low molecular weight fraction contains low levels of endoglucanase and mannase activities in addition to endoxylanase activity. Treatment of both hardwood and softwood kraft pulps, as well as oxygen-bleached kraft pulps, enhance the leaching of lignin-carbohydrate complexes (LCCs) from the pulps with only a moderate decrease of pulp viscosity (Table 2). Leaching of LCCs from various pulps has been reported previously [Favis et al. 1981; Favis and Goring, 1984; Willis and Goring, 1985]. However, in these earlier studies, enhancement of lignin leaching from pulps was only examined at different pH levels, temperatures and different concentrations of electrolytes.

Treatment of both hardwood and softwood kraft pulp, particularly oxygen-bleached pulp, with the low molecular weight enzyme mixture facilitates leaching of lignin-carbohydrate complexes compared to pulps treated with the high molecular weight fraction. The low molecular weight enzyme fraction (which contains endoxylanase, endoglucanase and endomannanase activities) thus seems to be an effective preparation for enhancing lignin removal from hardwood and softwood pulp. Leaching of lignin-carbohydrate complexes from the fibers after enzyme treatment also facilitates bleaching in subsequent stages [Yang et al. 1991].

Our use of enzymes as one stage in pulp bleaching allows for reduced amounts of bleaching chemicals in subsequent stages. Enzyme treatment also has a positive effect on the physical properties of pulp. However, while the enzyme mechanisms involved in degradation of hemicelluloses are relatively well known, the effect of enzyme treatment on pulp fibers is not very clear. This means that we do not yet have a theoretic grounding for the optimization of enzyme treatments for maximal pulp bleaching.

We have in our studies been able to produce in the laboratory a fully bleached pulp

Table 2. Relative LCC content in filtrates after treatment of the pulps with the enzyme fractions, measured as the absorption at 280 nm.

Pulp treatment	HWK	HWKO	SWK	SWKO
Control	0.573	0.436	0.199	0.318
X5-10	1.920	2.290	0.494	1.153
X10-100	1.390	1.530	0.327	0.685

Note:

HWK = hardwood kraft pulp
HWKO = oxygen-bleached hardwood kraft pulp
SWK = softwood kraft pulp
SWKO = oxygen-bleached softwood kraft pulp

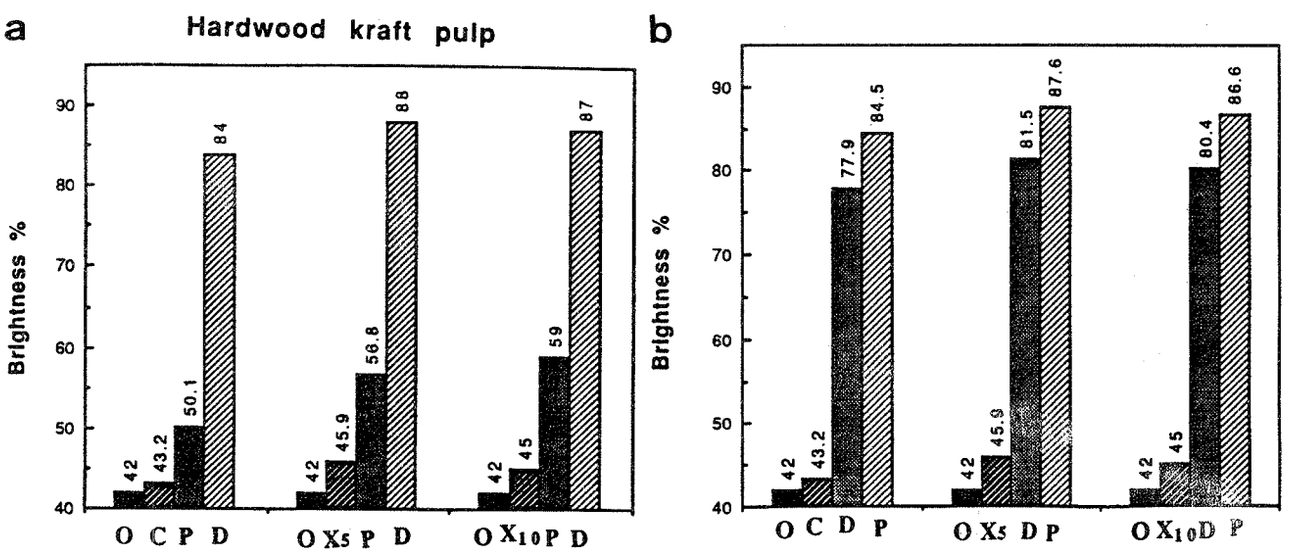

Figure 6. Brightness of hardwood kraft pulp after different bleaching stages in two different bleaching sequences. a) OXDP; b) OXDP. X_0=control without addition of enzymes [Yang et al. 1991].

without the use of molecular chlorine and with a considerably reduced amount of chlorine dioxide. The chemical properties of hardwood kraft pulps, bleached according to the bleaching sequences OXDP and OXPD, are presented in Table 3. A graphic depiction of pulp brightness obtained after the different bleaching stages is shown in Figure 6. The brightness values for the fully bleached pulp recovered in the OXDP sequence were 87.6% (enzyme fraction 5-10) and 86.6% (enzyme fraction 10-100), compared to 84.5% for the control pulp -- the controls being carried out under identical conditions but without addition of enzymes.

The pulp brightness values arising from the OXPD sequence were 88.0% (enzyme fraction 5-10) and 87.0% (enzyme fraction 10-100), compared to 84.0% for the control pulp. However, in the OXPD sequence the hydrogen peroxide dosage was 1% higher than that applied in the OXDP sequence. It should be pointed out that in both sequences only 1.5% total chlorine dioxide was needed to reach a final brightness of approximately 87%.

The viscosities of the fully bleached pulp were generally good. However, the pulps bleached in the OXDP sequence had a better overall viscosity than pulps bleached in the OXPD sequence. From this we conclude that the bleaching sequence OXDP is more suitable for hardwood kraft pulp than is the OXPD sequence.

For bleaching of softwood kraft pulp, it was necessary to add an extra hydrogen peroxide stage in order to obtain a fully bleached pulp. The used sequences have been OXDPD and OXDPP, respectively. The final brightness values for the pulp bleached according to the sequence OXDPP were 85.7% (enzyme fraction 5-10) and 85.3% (enzyme fraction 10-100), compared to 82.7% for the control pulp. However, the final brightness of pulp bleached according to the sequence OXPDP fell short and brightness values of only 83.9% and 82.4%, respectively, were obtained, compared to a brightness of 81.4% for the

Table 3. Chemical properties of hardwood kraft pulp after different bleaching stages.

Bleaching Stages	Kappa Number			Viscosity m·Pa·s			Brightness ISO %		
	C	X5-10	X10-100	C	X5-10	X10-100	C	X5-10	X10-100
O	9.4	9.4	9.4	22.3	22.3	22.3	42.0	42.0	42.0
OX	9.2	6.3	7.1	20.3	18.5	23.1	43.2	45.9	45.0
OXD	---	---	---	20.0	16.1	20.5	77.9	81.5	80.4
OXDP	---	---	---	17.4	14.6	18.8	84.5	87.6	86.6
OXP	7.6	5.8	6.7	18.0	15.7	15.9	50.1	56.8	59.0
OXPD	---	---	---	16.2	14.2	15.5	84.0	88.0	87.0

Note:

O=oxygen bleaching, X=treatment with the enzyme fractions, D=chlorine dioxide, and P=hydrogen peroxide.
C=Control carried out under identical conditions but without addition of enzyme fractions.

control pulp. Since the viscosities of the pulps obtained from either sequence were similar, we assume that the most suitable bleaching sequence for softwood kraft pulp is OXDPP.

We have compared certain properties of our laboratory-produced bleached pulp with the physical properties of mill reference pulps. Our studies show that pulp treatment with hemicellulases reduces the use of other bleaching chemicals and improves pulp quality in what is essentially an environmentally benign process. Studies of large scale production of hemicellulases using agricultural wastes are currently underway. We are also optimizing the enzyme composition to be used as one stage in pulp bleaching. We believe that an enzyme stage in pulp bleaching will prove both economically feasible for the pulp and paper industry and will also relieve a heavy burden on the environment.

Attempts to Make Trees Easier to Pulp and Bleach

The lignin polymer arises from an enzyme initiated dehydrogenative polymerization of three primary precursors: coniferyl alcohol (guaiacyl structure), *p*-coumaryl alcohol (*p*-hydroxyphenol structure), and sinapyl alcohol (syringyl structure). Typically, softwood (gymnosperm) lignin contains primarily guaiacyl with few or no syringyl residues, while hardwood (angiosperm) lignin is composed of approximately equal amounts of guaiacyl and syringyl units [Sarkenen and Ludwig, 1971]. Both types of wood lignins additionally contain low levels of *p*-hydroxyphenol residues, while grass contains significant levels (5-10%) of esterified *p*-coumaric acid in addition to all three phenolic alcohol derivatives [Sarkanen and Ludwig, 1971]. The relative proportion of *p*-hydroxyphenol, guaiacyl and syringyl structure residues in lignin affect the physical properties of the polymer, most likely as a result of the degree of intramolecular cross-linking allowed within the polymer. The level of cross-

linking, commonly referred to as the degree of condensation of the lignin polymer, is thus a direct result of the methoxyl content (syringyl > guaiacyl > *p*-hydroxyphenol) of the lignin.

Although several techniques are available for investigating lignin distribution in plant cell walls [Saka and Goring, 1985], no reliable technique exists for determining composition and spatial differences in lignin at the subcellular level *in situ*. There is sufficient information to illustrate that guaiacyl:syringyl ratio changes from one morphological region to another, but unacceptably high differences are obtained when the different methods are used for estimation. An improved understanding of lignin deposition would greatly advance our knowledge of lignin biosynthesis and help in the development of molecular strategies for genetically altering the production of the key enzymes responsible for lignin structure and abundance.

We currently produce monoclonal antibodies to polymeric lignins emanating from the different alcohols. We will do so also to enzymes important for the lignification process. The probability of successfully using McAbs for lignin determination is high because polyclonal antibodies to spruce mill wood lignin, to unravel the composition of mechanical pulp fiber surfaces, have already been produced, i.e. lignin is immunogenic [Pettersson et al. 1988].

The aim of our work is to develop knowledge and techniques that ultimately allow us to change the biosynthesis of lignin in wood. We particularly have in mind forest trees with lower lignin content, with lignins of modified methoxyl content, or with a reduced association between lignin and the hemicelluloses [Eriksson and Dinus, 1990]. We therefore now purify and characterize key enzymes in lignin biosynthesis, particularly those involved in polymerization. Production of monoclonal antibodies to these enzymes will make it

possible to localize their production in time and place. We will also use antisera produced in mice to the purified enzymes [Dean and Eriksson, in press].

The work to manipulate lignin structures in forest trees to facilitate pulping and bleaching will be carried out in cooperation between the University of Georgia and the Institute of Paper Science and Technology (IPST) in Atlanta. Our colleagues at IPST are developing cell and tissue culture systems that offer significant advantages for research into lignification. The systems permit control and manipulation of genetic and environmental factors and allow for examination of lignification across a continuum from single cells through developing embryos and seedlings, all derived from the same donor tree(s).

We have presently produced extracellular laccase and peroxidases from cell cultures of sycamore cells. The laccase from sycamore has been purified and characterized and it has been demonstrated that laccase can polymerize all three monolignols, particularly coniferyl and sinapyl alcohols [Sterjiades et al., to be published]. The work will now continue by purification and characterization of the peroxidases and the relative importance of laccase and peroxidase for lignification will be estimated.

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BIOLOGICAL TREATMENT OF PULP AND PAPER INDUSTRIAL WASTEWATERS: PROCESSES AND BIOREACTORS.

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ABSTRACT

Pulp and and paper wastewaters are discharged in large amounts in natural receiving waters. These effluents cointain many deleterious substances, mainly chlorinated phenols and high molecular weight chlorinated lignins. Conventional biological treatment processes remove only a fraction of the pulp and paper wastewater pollutants. This article deals with the anaerobic and aerobic bioreactors and processes employed in the treatment of these effluents. The use of fungal reactors for color removal and dechlorination is also focused.

INTRODUCTION

Pulp and paper industry consumes large amounts of water and discharges highly polluted wastewaters. Wastewater discharge volumes range between 80–150 m^3/ton of finished product. Considering the magnitude of the world production of paper and paperboard, estimated at 227 million tons in 1987, the forest industry can be considered as a particularly important source of wastewater [1], discharging daily more than 62.10^6 m^3. This value corresponds to the daily domestic water consumption of about 200 million persons.

Wastewater composition and concentration depend on ligno cellulosic materials used and on the process conditions applied (i.e, chemical pulping, mechanical pulping, bleaching, etc.). The main sources of wastewater in the pulp paper industry are illustrated in Figure 1. In spite of the growing diffusion of the Thermomechanical (TMP) and Chemithermomechanical (CTMP) processes in recent years, chemical pulping processes are still dominant, and kraft pulping process is responsible for more than half of the total pulp production [1].

A comparison of pollution loads and specific water comsuption in mechanical and chemical pulp mills is presented in Table 1. The mechanical pulping process generates less pollution loads, but the TMP, CTMP and kraft pulping processes have similar loads (COD and BOD). The COD/BOD_7 ratios are high, in the range of 2 to 5 , indicating that many compounds which are present in these wastewaters are resistant to biodegradation. Bleaching increases the pollution loads and also the ratio COD/BOD_7, rendering the final effluent less amenable to biological treatment. Bleachinng wastewasters contribute to 50–60% of the total BOD load and 80–90% of the collor pollution of the final industrial effluent. The

quantity of organically bound chlorine formed in the bleaching process is estimated as 2–5 Kg/ton of pulp.

Process modifications like substitution of batch digestion system for continuous digestion may result in significant changes in pollution loads and biodegradability of the final effluent as shown in Figures 2 and 3. Improved design of the water utilisation in pulp and paper industries and process optimization are contributing to a strong reduction of the pollution load of the final effluents as shown in Figure 4.

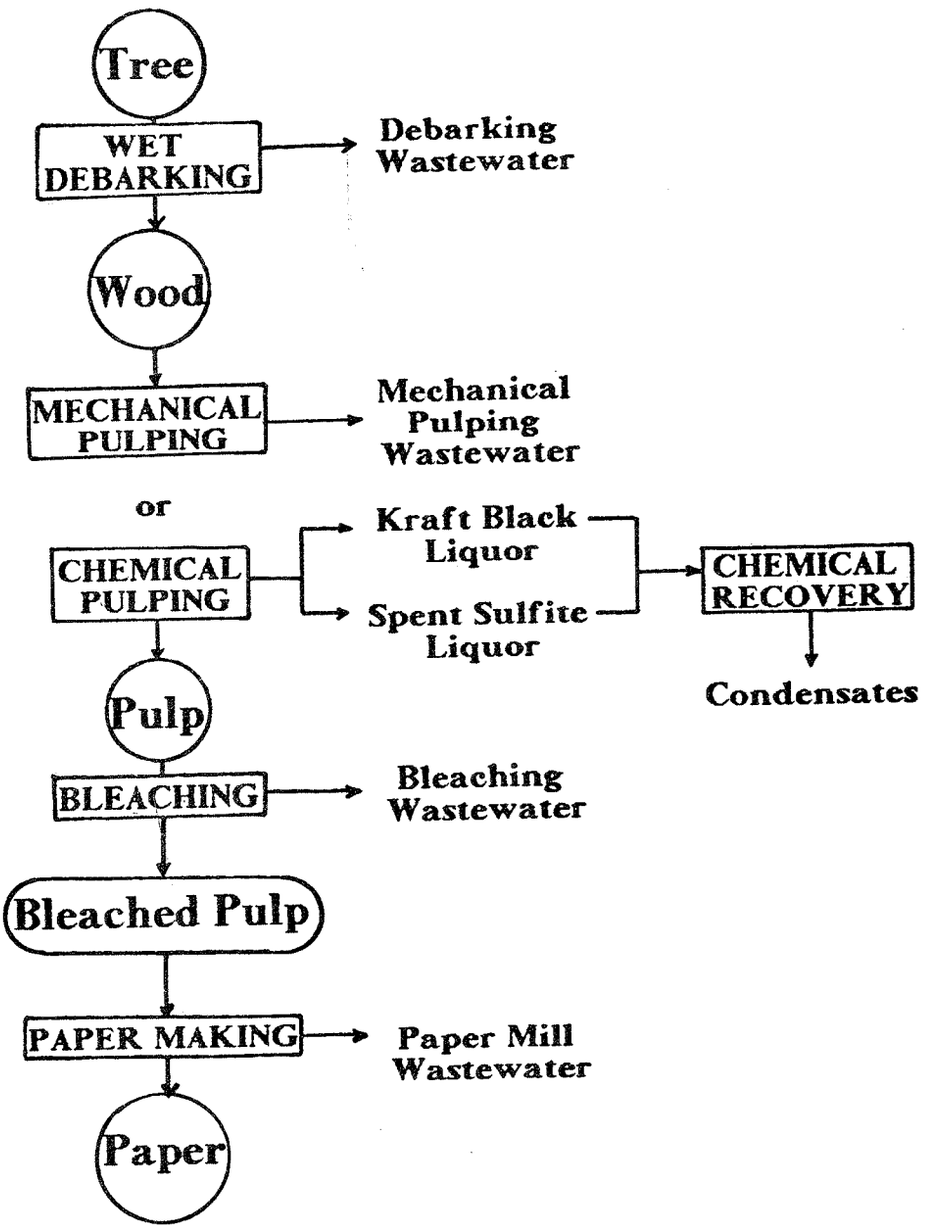

Figure 1. The main sources of wastewater in the pulp and paper industry (R.Sierra – Alvarez [1].

Process	Water Consumption [m^3/ton pulp*]	BOD_7 [kg/ton pulp*]	COD [kg/ton pulp*]	COD/BOD_7
Wet debarking	5 - 25	3 - 14	5 - 20	1.4 - 1.6
GWP	10 - 15	8 - 15	15 - 32	1.9 - 2.1
TMP unbleached bleached	 10 - 30 10 - 30	 15 - 25 20 - 30	 40 - 60 50 - 120	 2.4 - 2.7 2.5 - 4.0
CTMP unbleached bleached	 10 - 15 10 - 15	 30 - 45 40 - 60	 70 - 120 100 - 180	 2.3 - 2.7 2.5 - 3.0
NSSC	20 - 80	15 - 60	30 - 120	2.0
KRAFT unbleached bleached	 40 - 60 60 - 90	 8 - 20 20 - 40	 40 - 60 100 - 140	 3.0 - 5.0 3.5 - 5.0
Ca-SULFITE unbleached bleached	 80 - 100 150 - 180	 30 - 70 45 - 85	 --- 150 - 180	 --- 2.1 - 3.3
Papermaking	10 - 50	1 - 5	---	---

*Pulp = oven dried pulp

Table 1. Pollution loads and water consuption in typical mechanical and chemical pulp mills (adapted from R.Sierra – Alvarez [1].
GWP = ground wood pulping, TMP = thermochemical pulping
CTMP = chemi–thermomechanical pulping
NSSC = neutral sulfite semichemical pulping

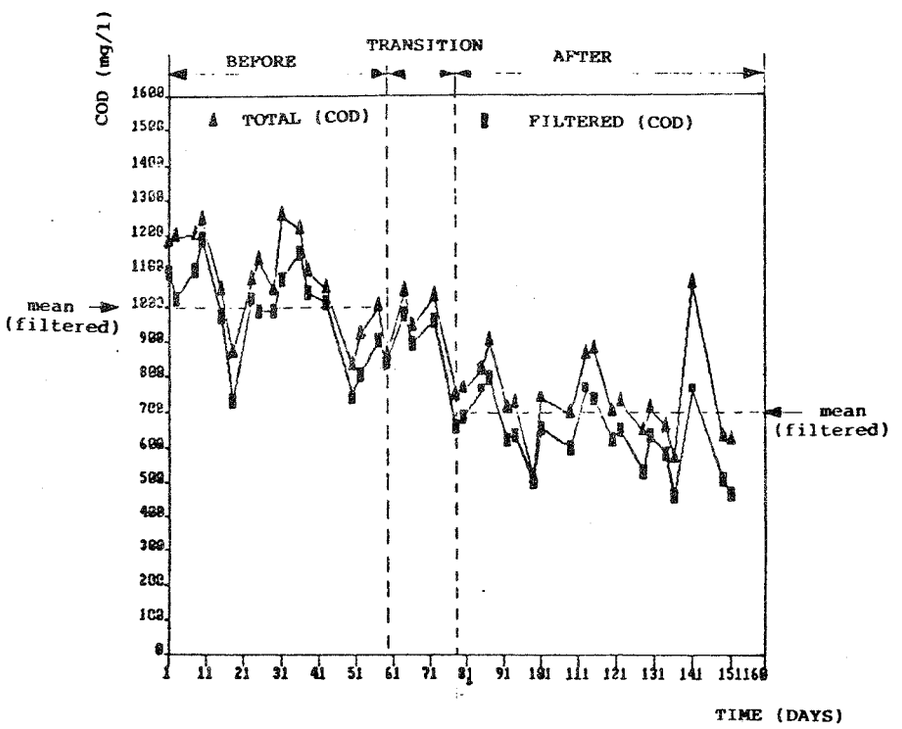

Figure 2. Changes in COD content of a bleached kraft mill effluent due to modification in the industrial process – replacement of the batch digesters by the continuous Kamyr digester (O.Vieira [2]).

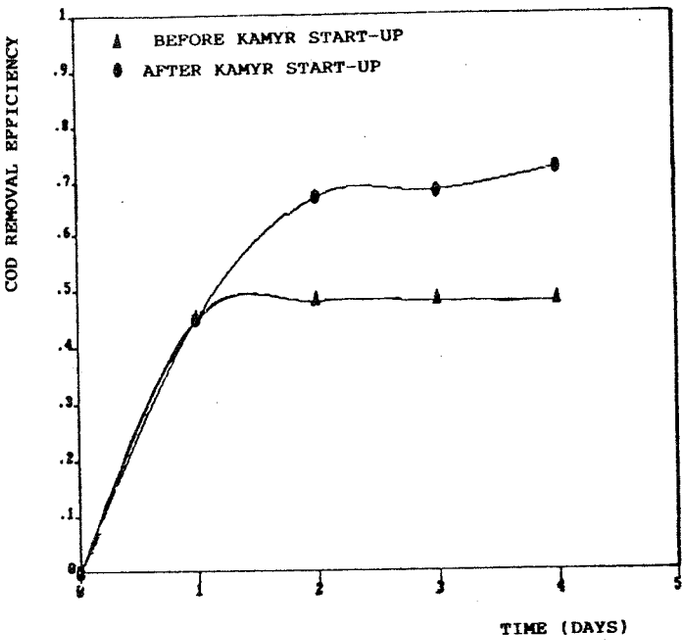

Figure 3. Biodegradability of a kraft mill effluent (O.Vieira [2]).

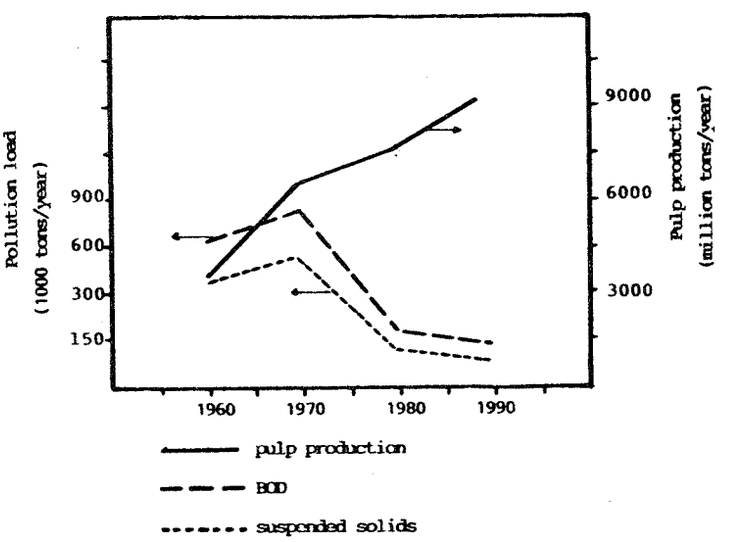

Figure 4. Pulp production and pollution loads in Finnish forest industries (J.Puhakka [3]).

PULP AND PAPER MILL WASTEWATERS – ENVIRONMENTAL EFFECTS

Most of the toxicity in pulp an paper whole mill effluents is attributed to resin and fatty acids, chlorinated phenols and, to a lesser extent; a broad class of neutral compounds. Our comments will be concerned to the kraft process effluent which is the dominant chemical pulping process in many countries. Levels of resin acids which occur in kraft mill effluent are shown in Table 2. Despite the wide range of concentrations observed many of the resin acids are present in toxic concentrations in the untreated kraft effluent. Data from a comprehensive study carried out by the Environmental Protection Agency (USA) concerning level of toxic compounds in treated and untreated effluents from different categories of pulp and paper mills in USA is presented in Table 3 and also confirms the concern with respect to the toxicity of resin acids.

Concentrations of fatty acids in bleached kraft mill effluents are shown in Table 4. Comparing LC_{50} values in Table 4 with those for resin acids it is possible to conclude, as espected, that fatty acids are less toxic to fish than resin acids. They are present in concentrations which usually do not exceed lethal thresholds.

Chlorophenols are produced in bleached kraft mills during the degradation of lignin with chlorine and by chlorination of plenolic residues, produced during pulping, which are present in the unbleached pulp. Table 5 gives the amounts of clorophenols found in bleached mill wastewaters. The concentration of chlorophenols in untreated bleached kraft mill effluents do not exceed lethal thresholds (with the exception of tetrachloroguaiacol).

Bleaching produces high–molecular–weight chlorinated compounds which are probably biologically inactive. These materials however, carry chromophoric structures which are responsible for the intense color of kraft mill effluents. The biological and chemical transformations of these compounds in the receiving waters is still object of research. Figure 5 shows the distribution of organically bound chlorine in the spent chlorination and spent alkali extraction effluents, 70% of the organically bound chlorine is present as high molecular mass material ($M > 1000$) in the chlorination stage wastewater whereas this percentage attains 95% in the alkali extraction effluent.

Screening for the mutagenic properties in forest industry effluents indicates the presence of mutagenic materials in most of the discharges within the industry. Chlorination stage effluents have been identified as the principal source of mutagenicity in paper mills [7]. Chloroacetones, chlorinated derivatives of the chlorofurans and 2–chloropropenal are regarded as major contributors to the mutagenicity of chrorination stage effluents. Table 6 presents some mutagenic compounds that have been identified in bleaching efluents. According to a Canadian report [6] the risk involved in releasing these compounds into rivers and lakes was estimated to be extremely low in view of the low levels these compounds were found in the receiving waters.

	Kraft mill effluent		bleached Kraft mill effluent		96-h LC_{50} [µg/l]
Resin acid	untreated	biotreated	untreated	biotreated	
abietic	30-9970	<20-3630	<20-4800	<10-1780	700-1500
chlorodehydroabietic	--	--	<10-750	<1-260	600-900
dehydroabietic	990-5780	<20-1930	<30-4580	<1-2140	800-1740
dichlorodehydroabietic	--	--	<10-410	<10-152	--
isopimaric	70-4120	<20-1420	<20-4800	<10-930	400-1000
levopimaric	<10-2700	<10-30	<10-2400	<1-1190	700-1000
neoabietic	<50-1200	--	<10-1000	<1-150	610-730
palustric	--	--	90-100	80	500-600
pimaric	100-1830	<20-890	<20-1010	14-540	700-1200

96-h LC_{50}: Median lethal concentration derived for rainbow trout under static bioassay conditions.

Table 2. Resin acids concentrations in kraft mill wastewaters (EPS–Canada [4]).

Resin acid	untreated Kraft mill effluent	treated Kraft mill effluent	bleached untreated Kraft mill effluent	bleached treated Kraft mill effluent
abietic	350 - 12,000	0 - 250	0 - 18,000	0 - 2,500
dehydroabietic	330 - 27,600	6 - 200	10 - 5,200	0 - 1,000
isopimaric	78 - 1,600	0 - 32	0 - 1,300	0 - 590
pimaric	38 - 2,500	0 - 60	0 - 1,900	0 - 790

Table 3. Resin acids concentrations in kraft mill wastewaters (R.W.Dellinger, EPA report [5]).

	Kraft mill effluent		bleached Kraft mill effluent		96-h LC_{50} [µg/l]
Fatty acid	untreated	biotreated	untreated	biotreated	
dichlorostearic	--	--	<40 - 552	<40 - 268	2500
epoxystearic	--	--	<40 -1540	<40	1500-3400
linoleic	<10 -1160	<20 - 60	<20 -9300	<20 - 500	2000-4500
dichlorostearic	<20 - 110	<20	<20 - 260	10 - 30	2000-6000
oleic	40 -2490	<20 - 120	30 -7750	<20 -2340	3500-8200

96-h LC_{50}: Median lethal concentration derived for rainbow trout under static bioassay conditions.

Table 4. Fatty acids concentrations in kraft mill wastewaters (EPS–Canada [4]).

BIOLOGICAL TREATMENT OF PAPER AND PULP MILL EFFLUENTS

Conventional Aerobic Processes

Biological treatment processes are effective in the removal of paper mill effluents biochemical oxigen demand (BOD). BOD removals range from 40 to 90%, COD reductions, however, vary between 40–70% at paper and board mills and range between 25–55% at chemical pulp mills.

	bleached Kraft mill wastewater		96-h LC_{50} [µg/l]
Chlorophenol	untreated	biotreated	
dichlorocatechol	12 - 90	1 - 120	500 - 1000
dichloroguaiacols	22 - 100	12 - 60	2300 [a]
2,4-dichlorophenol	9 - 15	2 - 51	2800
tetrachlorocatechol	22 - 420	2 - 240	400 - 1500
3,4,5-trichlorocatechol	120 - 270	2 - 280	1000 - 1500
trichloroguaiacols	<10 - 340	<1 - 220	700 - 1000 [b]
2,4,6-trichlorophenol	<1 - 51	<1 - 61	450 - 2600

96-h LC_{50}: Median lethal concentration derived for rainbow trout under static bioassay conditions.
a : 4,5 isomer
b : 4,5,6 isomer

Table 5. Range of concentrations [µg/l] of chlorophenols in untreated and biotreated kraft mill effluents (EPS–Canada [4]).

Compound	dose/plate	number of mutants
Trichloroethane	0.1 mg	37
Tetrachloroethane	0.1 mg	31
Monochloroacetone	10 µg	26
1,3-Dichloroacetone	4.5 µg	248
1,1,3,3-Tetrachloroacetone	16 µg	20
Pentachloroacetone	50 µg	14
Hexachloroacetone	1.0 mg	38
Chloroacetaldehyde	45 µg	40
Trichloroacetaldehyde	0.5 mg	19
2-Chloropropenal	0.1 µg	368

Table 6. Mutagenic activity * of some compouds found in spent chlorination liquor.
* Ames test, *Salmonella typhimurium* TA 1535 (K.P.Kringstat et al. [7]).

Aerated lagoons were the first biological purification units constructed for treating paper mill effluents. Large volumes are necessary due to the high residence time required to attain adequate removal efficiencies. Activated Sludge systems were introduced latter in pulp and paper industries, BOD removal efficiencies in the range of 65 to 90% are obtained with loading rates varying from 0.6 to 3.9 Kg BOD/m^3d. Fixed-film aerobic reactors such as trickling filters were also used to treat kraft mill effluents. Table 7 summarizes some published results concerning aerobic treatment processes applied to the treatment of kraft mill effluents.

Process/ Country	BOD load [kg/m^3d]	Treatment/ scale	HRT [h]	Removals[%] BOD	COD	Reference
K/ Finland	0.12	aerated lagoon industrial	144	55/80	—	Luonsi, A. et al. (1988)
K/ Finland	0.11	aerated lagoon industrial	36	49/65	—	Luonsi, A. et al. (1988)
K/ Finland	0.03	aerated lagoon industrial	336	50/70	—	Luonsi, A. et al. (1988)
K/ Finland	0.01	aerated lagoon industrial	624	40/75	—	Luonsi, A. et al. (1988)
KB/ USA	0.07	aerated lagoon pilot	74	75	—	Chen, H.T. et al. (1974)
KB/ Brazil	0.10	aerated lagoons (2) pilot	144	95	57	Galvão, J.B. et al. (1988)
KB/ Brazil	0.05	aerated lagoon pilot	144	88	44	Galvão, J.B. et al. (1988)
K/ Finland	2.4	activated sludge industrial	4	80	—	Luonsi, A. et al. (1988)
K/ Finland	1.2	activated sludge industrial	18	80	—	Luonsi, A. et al. (1988)
K/ Finland	0.6	activated sludge industrial	12	65	—	Luonsi, A. et al. (1988)
KB/ USA	2.3	activated sludge [O_2] pilot	2	90	35	Chen, H.T. et al. (1974)
K/ Canada	1.1[a]	activated sludge pilot	24	—	64	Ganczarczyc, J.et al. (1974)
K/ Canada	3.2	activated sludge [O_2] pilot	2.2	91	60	Grader, R.J.et al. (1973)
KB/ Canada	3.2[b]	activated sludge two stage pilot	2.8	90	—	Jank, B.E. .et al. (1975)
KB/ Canada	0.7	activated sludge pilot	5.5	91	—	Jank, B.E. et al. (1975)

a - COD load
b - first stage load

Table 7. Some reported results on aerobic treatment of kraft mill effluents (adapted from M.C. Cammarota [8]).

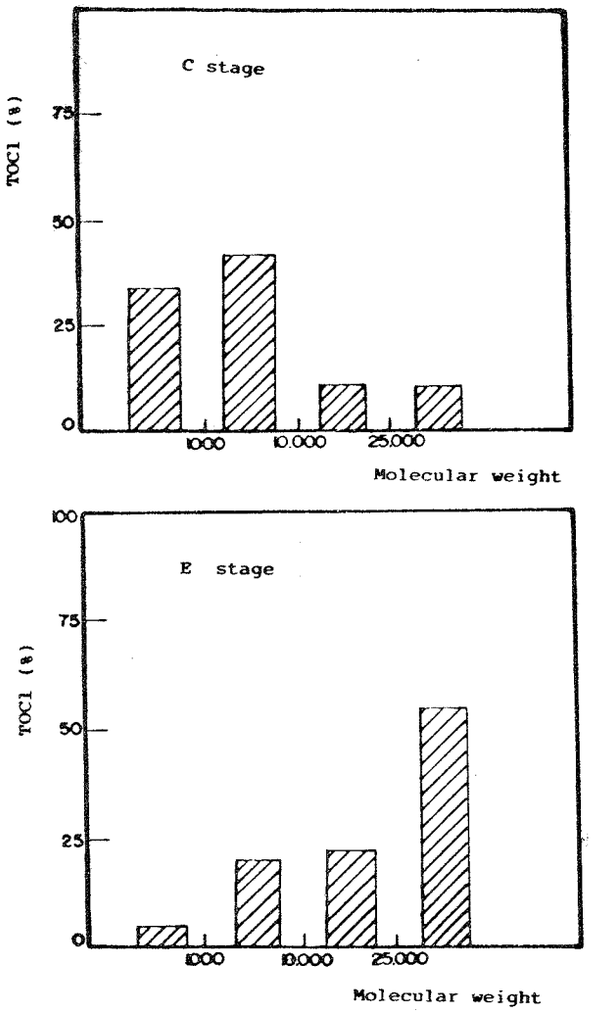

Figure 5. Distribution of total organically bound chlorine (TOCl) in chlorination and alkali extraction effluents (S.A Heimburger et al. [6]).

Resin acids are degraded in biological treatment in appreciable percentages, non–chlorinated resin acids removal in aerobic treatment may attain 90%. Chlorinated resin acids are more resistant to biological treatment. Fatty acids are readily degraded. Low levels of fatty acids are found in biotreated effluents. Chlorophenols are more resistant to biological treatment and conventional aerobic treatment is not very effective in reducing the concentrations found in pulp and paper mill effluents.The rate of removal depends on treatment conditions and can be quite low. Treatment processes which utilize immobilized biofilms such as trickling filters, RBC and others may be of interest, because these systems can retain chlorophenol degrading organisms which grow at slow rates. Such organisms would be washed–out in non–attached biomass reactors.

Anaerobic Treatment

Anaerobic processes applied to the treatment of pulp and paper mill effluents have been studied intensively since 1980. Many experiments were carried out with anaerobic lagoons, anaerobic filters and anaerobic contact process. Like in aerobic treatment moderate COD removals are observed for chemical pulping mill effluents. The development of the up–flow anaerobic sludge blanket reactor (UASB R) in Holland by Prof. Lettinga and co–workers and its successful application to the biological treatment of food–industry wastewater, generated an important interest on the applicability of this type of reactor to the treatment of pulp and paper mill effluents. The UASB reactor is mainly applied to treat the effluents of paper and paper board mills. COD removal efficiencies for TMP, CTMP and kraft mill effluents are about 50%.

Figure 6, shows some results concerning the anaerobic treatment of kraft bleached wastewater (IKPC Paraná) [2]. Appreciable COD and BOD_5 reductions were only attained at high hydraulic retention times (100h).

Anaerobic treatment have a limited capacity to decrease the aquatic toxicity of forest industry wastewaters [9], resin acids and resin compounds such as terpenes are poorly degraded in anaerobic reactors, low tannnis are partially degraded. Monomeric chlorinated phenols may be metabolized during anaerobic treatment. The initial step in the degradation of these compounds seens to be the removal of chlorine atoms from the aromatic ring by a reductive dechlorination reaction [9]. Dechlorination depends on the number and relative position of the chlorine atoms on the aromatic ring and the acclimatation of the microbial consortia. Haggblom and Sakinoja–Salonen [10] report 60–70% of chlorinated phenols removal in an anaerobic fluidized bed reactor. According to these authors theirs results are higher than those obtained in aerobic treatment systems (50–60%).

High molecular weight natural lignins and chlorolignins cannot be taken–up by microorganisms to be attacked intracellularly. The natural extracellular enzymes which are capable of depolymerizing lignin promote a nonspecific oxidative erosion, whereby oxygen derived radicals play a key role [9]. Each type of intermonomeric bond present in lignin can be cleaved by anaerobic microorganisms when they are fed with dimeric lignins, even oligomers of 3 to 7 monomeric units are partly biodegradable in anaerobic environments [9]. Figure 7 shows anaerobic degradation pathways of tannin and lignin monomers and Figure 8 illustrates the role of polymer size on the anaerobic biodegradability of lignin.

A comparison of anaerobic and aerobic processes performances when applied to bleached kraft mill wastewater treatment is presented in Table 8.

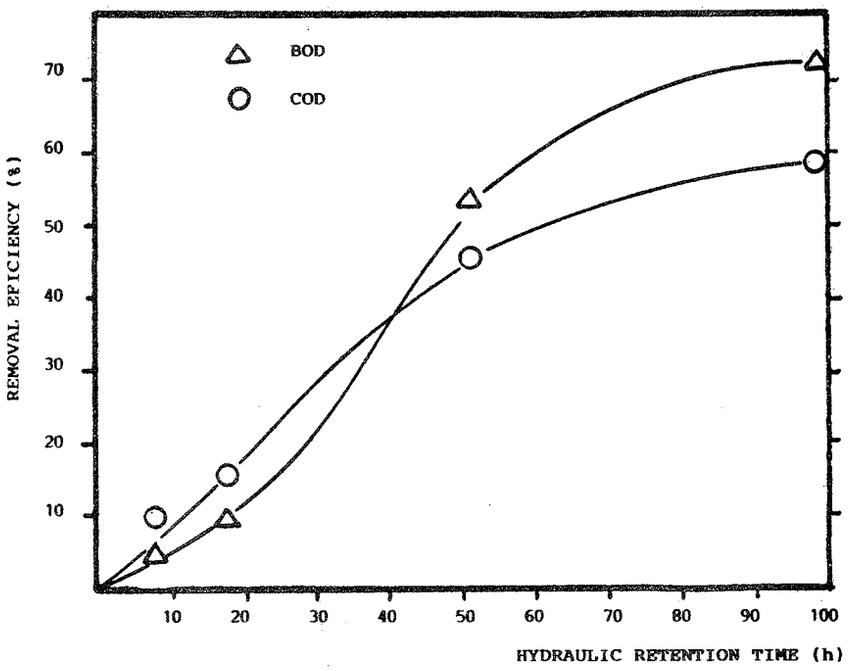

Figure 6. Performance of an UASB reactor treating kraft mill effluents (adapted from O. Vieira [2]).

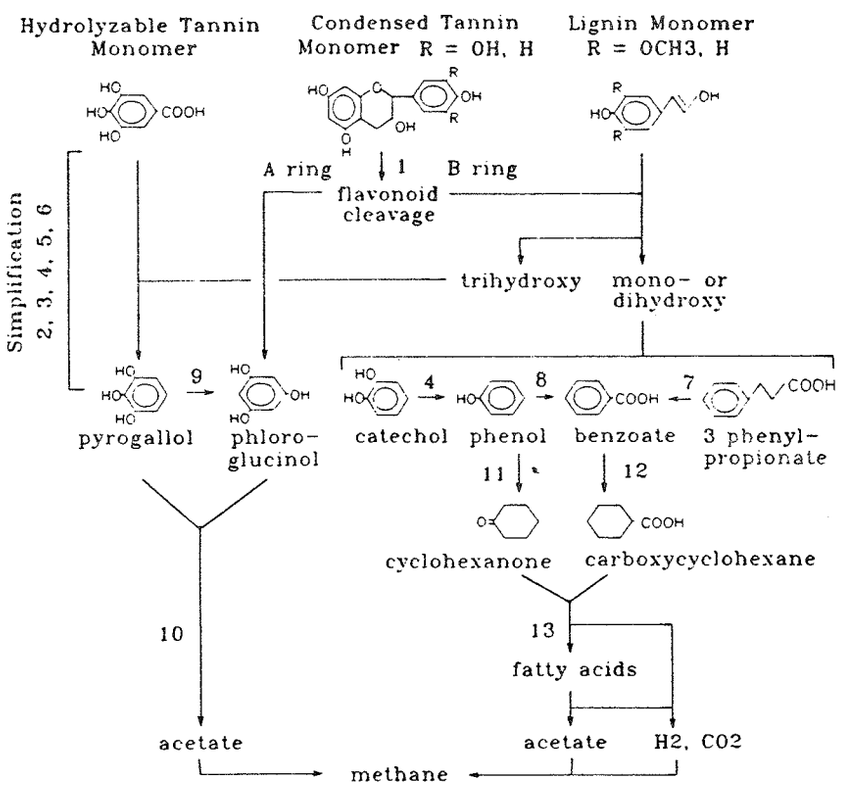

Figure 7. Anaerobic degradation pathways of tannin and lignin monomers (J.A. Field [9]).

Mixed Processes

Some interesting results were obtained with treatment processes which involve an anaerobic stage (normally a fluidized bed–reactor) and an aerobic stage (trickling filter). Hakulinen and Salonen [11] gave some data of the Enso–Fenox process which are reproduced in Table 9. A recent work of Haggblom and Salonen [10] reports removal efficiencies of several chlorinated phenols present in low concentrations in bleaching effluents. An anaerobic fluidized bed reactor and an aerobic trickling filter composed the sequential treatment system. Table 10 summarizes these results.

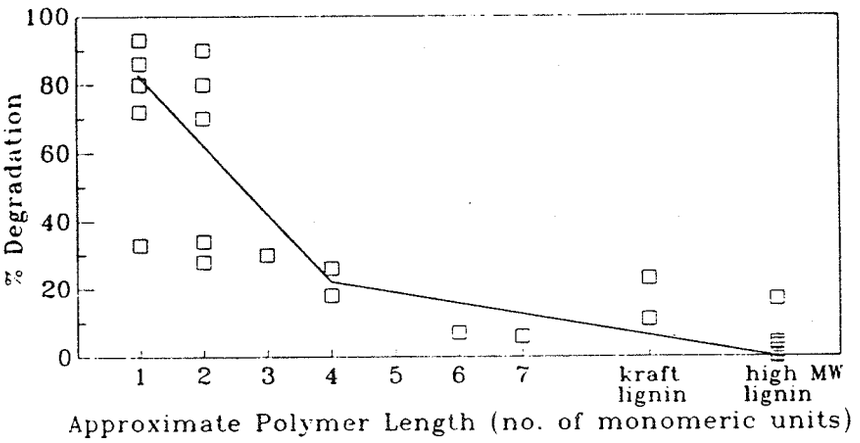

Figure 8. The role of polymer size on the biodegradability of lignin (J.A. Field [9].

Aspect	Anaerobic	Aerobic
Pollutants removal		
BOD	B/C	B/C
COD	C	C
resin acids	C/D	C
fatty acids	B/A	B/A
chlorinated phenols	B/C	C
high-molecular-weigh lignin and chlorolignins	D	D/C
color	C/D	D/C
Operation & design		
Compacticity (reactor volume)	C/D	C/B
Energy saving	B/A	C/D
Stability (operation)	C	B
Sludge disposal costs	B/A	C/D

Table 8. A comparison between anaerobic and aerobic processes applied to the treatment of kraft pulp mill effluents.
A = excellent B = good C = regular D = deficient

Chlorophenols [µg/l]	Influent	After anaerobic	%	After aerobic	%	Overall removal %
2,4,6-TCP	94	28	70	22	21	77
4,5-DCG	300	39	87	44	--	85
3,4-DCC	616	58	91	43	26	93
3,4,5-TCG	30	3	90	2	33	93
TeCG	42	3	93	2	33	95
PCP	2	0.4	80	0.4	0	80
TCC	2	0.3	85	0.3	0	85

TCP: trichlorophenol
PCP: pentachlorophenol
DCC: dichlorocatechol
TCC: trichlorocatechol
DCG: dichloroguaiacol
TCG: trichloroguaiacol
TeCG: tetrachloroguaiacol

Table 9. Removal of chlorophenols from ultrafiltred kraft bleaching effluent by the ENSO–FENOX process (R.Hakulinen et al. [11]).

Parameter	Influent	After anaerobic	%	After aerobic	%	Overall removal %
COD [mg/l]	3570	3010	16	1450	52	59
BOD_7 [mg/l]	580	605	--	130	79	78
AOX [mgCl/l]	175	122	30	56	54	68
Chlorophenols [µg/l]						
2,3,4,6-TeCP	6.1	2.2	64	1.8	18	70
2,4,6-TCP	18.1	11.9	34	0.2	98	99
2,4-DCP	9.6	10.3	--	2.2	79	77
TeCG	48.3	18.8	61	7.7	59	84
3,4,5-TCG	138.1	75.9	45	30.4	60	78
4,5,6-TCG	22.4	13.4	40	4.9	63	78
4,5-DCG	20.6	17.3	16	6.4	63	69
TCS	13.9	8.1	42	5.1	37	63

DCP: dichlorophenol
TCP: trichlorophenol
TeCP: tetrachlorophenol
DCG: dichloroguaiacol
TCG: trichloroguaiacol
TeCG: tetrachloroguaiacol
TCS: trichlorosyringol

Table 10. Removal of COD, BOD, AOX and chlorophenols by anaerobic–aerobic treatment of a bleaching effluent (Haggblom et al. [10]).

Utilization of Fungal Reactors

The ability of white–rot fungi to degrade chlorolignins and to decolorize kraft bleach effluents has been investigated in the last ten years. Bleaching wastewaters are responsible for 85 to 90% of the color of the effluent of a chemical pulping mill. This color is found predominantly in the first alkaline extraction stage (E_1) effluent of the chlorine bleach plant. The main contribution to color cames from polymeric, chlorinated, heavily oxidized degradation fragments of lignin. Figure 9 compares the contributions of the effluents from stages C and E to the final effluent color intensity. The high molecular weight compounds (M > 1000) are responsible for almost all the color intensity of the alkaline extraction stage effluent. Many white–rot fungi were tested for color removal of E_1 effluent (Phanerochaete chrysosporium, Phlebia brevispora, Phlebia subserialis, Poria cinerascens, Funalia gallica, Coriolus versicolor, etc.) One of the interesting results of this research effort was the development of the MyCoR process (Mycelial Color Removal) which is based on the decolorization action of the fungus Phanerochaete chrysosporium immobilized in a Rotating Biological Contactor (RBC) system. Problems observed with the operation of MyCoR process were short fungal lifetime (5–7 days), high oxygen concentration required and biomass wasting problems. Some alternative bioreactors such as packed–bed or fixed–bed reactors were proposed to overcome the drawbacks of the MyCoR process. The reactor shown in Figure 10 is an alternative model which was developed in our laboratory. The reactor is composed of an internal (air–lift) tube and an annular space where cubic particles of polyurethane foam retain and immobilize de fungal biomass (Phanerochaete chrysosporium). Experiments were carried out with the effluent from the first alkali extraction of a kraft bleach plant (IKPC – Paraná, Brazil). The reactor was operated in batch for 5 days to promote fungal growth. A special enriched growth medium was used in the reactor. After that period, the reactor was fed continuously with an induction medium containing predominantly the effluent E_1. Figure 11 shows the results related to color and total phenols removal. The bioreactor operating with a hydraulic retention time of 5.8 days was able to promote 70% of decolorization and removed 64% of the total phenols present at the reactor feed stream. The increase in inorganic chloride content in the exit stream is also showed in Figure 11. The mean removal of COD was 50% and only 7% of the organic bound chlorine was transformed to inorganic chloride. The moderate COD removal and the low dechlorination observed in this work may be attributed to the use of air, instead of pure oxygen in the reactor and to the high chloride content of the reactor influent (14 to 18 mM). The chloride ion may severely inhibite fungal lignin peroxidase as

remarked by Renganatham et al. [13]. Figure 12 indicates that there is a correlation between color and total phenols removals. Tests carried out in parallel showed that aerobic microbial cultures found in aerated lagoons are unable to remove the COD content of the E_1 effluent. These experiments conducted during 240 days showed that effluent E_1 was not biodegraded by the aerobic bacterial consortia. Furthermore conventional aerobic treatment was unable to decolorize E_1 effluent.

Although the bioreactor used in this work could be an alternative to the RBC system, many aspects related to its operating conditions should be further investigated and improved.

The technology of fungal reactors will be more diffused if some of its following negative aspects were overcome: the need of important amounts of organic and inorganic compounds to supplement E_1 effluent in order to ensure high levels of decolorization and dechlorination (mineral salts, vitamins, inducers, etc.) and the short fungal lifetimes still observed in the continuous operation of bioreactors. These aspects should be intensively investigated in future works to enable the utilization of less expensive media and to improve reactor stability otherwise these points may constitute serious drawbacks for fungal process viability.

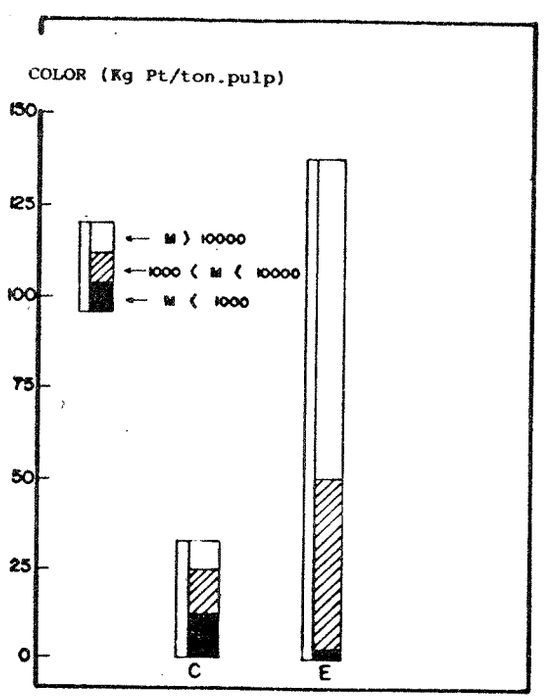

Figure 9. Color distribution in C and E bleaching effluents (K.Pfister et al. [12]).

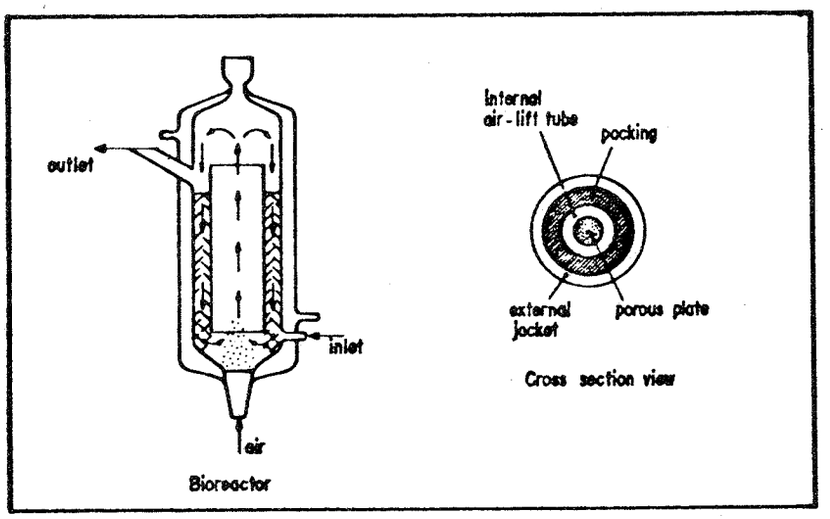

Figure 10. Schematic diagram of a fungal bioreactor (M.C.Cammarota [18]).

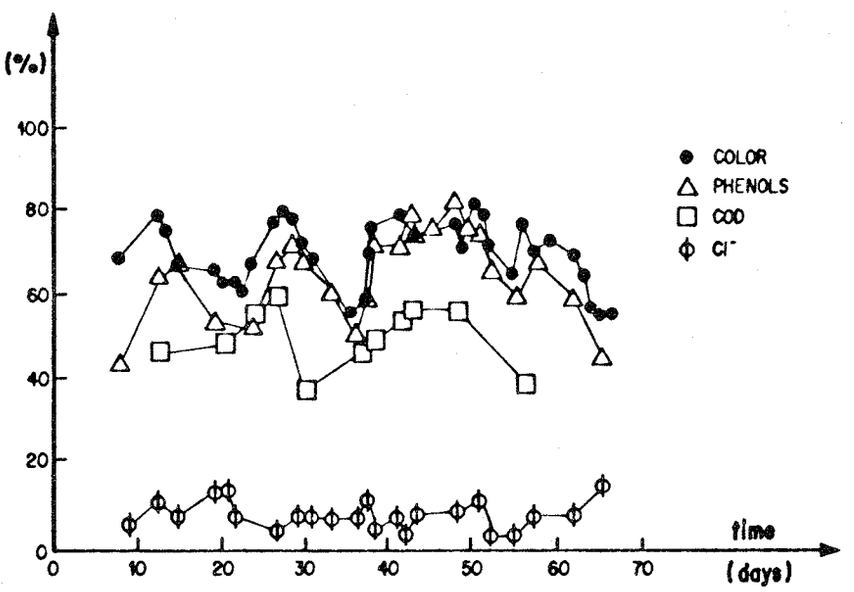

Figure 11. Removal (percentage) of color, phenols and COD and percentage of dechlorination in the fungal bioreactor (M.C.Cammarota [8]).

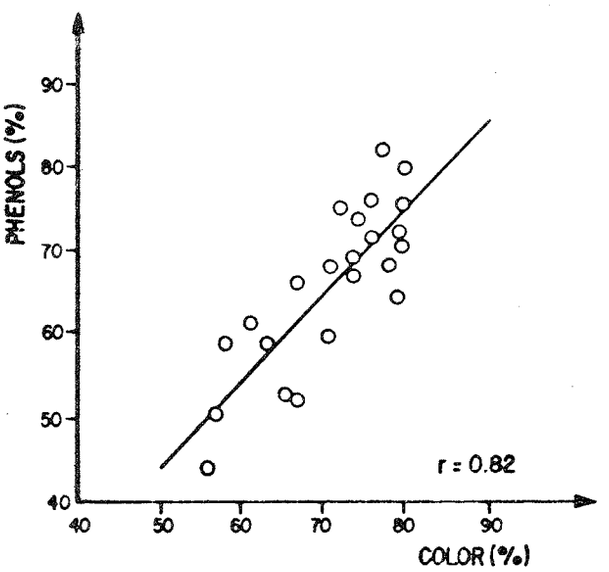

Figure 12. Correlation between phenols removal and color removal (M.C.Cammarota [8]).

TRENDS ON BIOLOGICAL TREATMENT OF PULP AND PAPER MILL EFFLUENTS

Some trends of the biological treatment and some aspects which demand for an intensive research are listed bellow:

- Use of mixed anaerobic–aerobic process employing immobilized microorganisms.
- Search for novel microorganisms and reactors.
- Use of membrane processes associated with biological reactors.
- Combination of physical, chemical and biological processes.

The chemical complexity of pulp and paper mill effluents claims for integrated treatmet systems, which can combine very effective methods to remove specific groups of pollutants.

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LIGNINOLYTIC ENZYMES FOUND IN SUBMERGED CULTURES OF *Phlebia brevispora* AND *Ceriporiopsis subvermispora*.

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ABSTRACT.

Manganese-dependent peroxidase, laccase and the H_2O_2 producing enzymes were found in the extracellular fluid of *P. brevispora* and *C. subvermispora,* two white rot fungi that exhibit good performance in biopulping. Lignin peroxidase, assayed by oxidation of veratryl alcohol in the presence of hydrogen peroxide, was not detected in concentrated media after growing these fungi under several different conditions.

With both species, enzymes could barely be detected unless Tween 20 was added at a concentration of 0.05%. In addition, we found that the levels of MnP in *P. brevispora* are regulated by Mn(II). This phenomenum also applies to the other enzymes of both fungi. In most cases, 11 ppm of Mn(II) was the optimum. For H_2O_2 producing oxidase from *C. subvermispora*, however, the highest levels were attained at 40 ppm Mn(II). Molecular oxygen, flushed either below or above the culture surface, strongly stimulated enzyme production by *P. brevispora*, although it had a slight inhibitory effect on *C. subvermispora*. Veratryl alcohol, a secondary metabolite of some white rot fungi and itself a substrate of lignin peroxidase, showed no effect on enzyme production by both fungi.

INTRODUCTION.

Enzymes thought to play a leading role in lignin depolymerization include lignin peroxidase (LiP) and manganese-dependent peroxidase (MnP). LiPs have the unique ability of abstracting one electron from non-phenolic aromatic residues, whereas MnPs oxidize Mn(II) to Mn(III), which in turn oxidizes phenol rings to phenoxy radicals. Laccase, an extracellular copper-containing oxidase, has also been implicated in lignin degradation. As MnP, it abstracts one electron from phenols, although in the presence of appropiate substrates it is able to act on non-phenolic compounds (1,2). It is striking, however, that this enzyme is not produced by *P. chrysosporium*, one of the most efficient ligninolytic microorganisms known to date.

It was originally found that LiP could partially depolymerize methylated lignin *in vitro* (3), although subsequent work revealed that lignin is instead polymerized by this enzyme (4,5). In addition, LiP seems to be absent in several ligninolytic fungi analyzed (6,7,8). However, two recent findings point to a leading role of LiP in the process. One is the observation by Perez and Jeffries that synthetic lignin mineralization correlates with LiP but not with MnP production (9). The second one is that Hammel and Moen have succeeded in developing an *in vitro* system of lignin fragmentation with a crude preparation of LiPs from *Phanerochaete chrysosporium* (10). On the other hand, MnP appears to have a more widespread distribution than LiP (6) and it is also able to depolymerize synthetic lignin in a cell free system (11).

LiP and MnP both require hydrogen peroxide for activity. Therefore, the enzyme(s) that provides them with this substrate can also be considered as a component of the ligninolytic system. Bearing in mind that production of H_2O_2 must take place outside the cell, two enzymes have been proposed to fulfill this task : a) MnP itself, which can form H_2O_2 from O_2 when either NADH, NADPH or glutathione are present (12) ; b) glyoxal oxidase (Glox), a novel enzyme described to

date only in *P.chrysosporium*, which is produced during secondary metabolism and is activated by LiP plus veratryl alcohol (13,14).

Most studies in this field have focussed on *P. chrysosporium*. Ligninolytic enzymes from other species, such as *Coriolus versicolor* (15,16) and *Phlebia radiata* (17,18), have also been partially characterized. The simultaneous analysis of several strains will help to elucidate the essential features required for ligninolysis. With this purpose, we studied enzyme production by two other white rot fungi, namely *Phlebia brevispora* and *Ceriporiopsis subvermispora* (19).

MATERIALS AND METHODS

Fungal strains and inocula.

C. subvermispora strains L-14807, L-15225, FP-104027, FP-105752, FP-90031-sp and *P. brevispora* strain HHB-7030-sp were obtained from the Center for Forest Mycology Research of the Forest Products Laboratory, Madison, WI. All strains were mantained on agar slants of YMPG media (6). Preparation of the inocula fand fungal growth was as reported by Bonnarme and Jeffries (6).

Determination of enzymatic activities.

Enzyme activities were measured at 30°C using a Shimadzu 160A UV-visible recording spectrophotometer. All experiments were done in duplicate, although most of the data obtained in this work represents an average of several cultures. LiP was assayed as described by Tien and Kirk (3). MnP activity was determined as reported by Paszczynski et al (20). One unit of MnP activity was defined as the amount of enzyme required to oxidize 1.0 µmol of substrate per minute. Laccase activity was measured in 1.0 ml reaction mixtures containing catecol 75 mM as substrate in 50 mM sodium phosphate buffer pH 5.0 and 10-200 µl aliquots of culture fluid (21). The progress of the reaction was monitored at 440 nm

during 10 min. One unit of laccase activity was defined as a change in A_{440} of 1.0 in one min. For determinations of H_2O_2 producing oxidase, reactions (1.0 ml) contained 10 mM methylglyoxal, 50 mM dimethylsuccinate buffer pH 6.0, 0.01% phenol red, 10 µg of horse radish peroxidase (Sigma) and 10-200 µl of enzyme (13). Reactions were halted after 10 min by adding 50 µl of 2 N NaOH and increase in A_{610} was measured. One unit of oxidase activity was defined as the amount of enzyme producing 1.0 nmol of H_2O_2 per minute.

RESULTS.

Enzymes secreted by the two fungal species.

Cultures of *P. brevispora* and of the five strains of *C. subvermispora* were assayed for the presence of LiP, MnP, laccase and oxidase. The last three activities were readily found in all cases. However, oxidation of veratryl alcohol to the corresponding aldehyde in the presence of added hydrogen peroxyde could not be detected. All attempts to detect the latter were unsuccessful. With respect to the specificity of the oxidase measured using methylglyoxal as substrate, the activities from the two fungi are different. *P. brevispora* 's oxidase appears indeed to be a Glox type enzyme, since it does not recognize glucose and galactose as substrates. In contrast, the activity present in *C. subvermispora* 's extracts exhibits a broad specificity, suggesting that more than a single H_2O_2-producing enzyme may be present.

Effect of various parameters on enzyme production.

We confirmed the observation of Bonnarme and Jeffries (6) that the levels of MnP and laccase in *P. brevispora* cultures are regulated by the concentration of manganese in the growth media. We found that this also applies to the H_2O_2-producing activity. Whereas negligible levels of either enzyme were observed in the absence of Mn(II), optimal concentration of Mn(II) for their production was found to be 11 ppm. Higher concentrations were partially inhibitory in each case.

With *C. subvermispora,* MnP production was optimum at 11 pmm Mn(II). Oxidase production, however, was higher at 40 ppm Mn(II), whereas laccase levels were approximately the same at these two Mn(II) concentrations. The same pattern was observed with the five strains of *C. subvermispora.*

Flushing of oxygen into cultures of *C. subvermispora* strains slightly inhibited enzyme production. In contrast, when oxygen was flushed either above or below the surface of cultures of *P. brevispora,* a pronounced stimulation of MnP and H_2O_2-producing oxidase was observed. Oxygen had a somewhat different effect on laccase production. This enzyme seems to be secreted by *P. brevispora* in cycles. Although the levels of laccase are approximately the sameunder air or oxygen, the cycle is altered by oxygen.

The effect of nitrogen concentration on MnP and H_2O_2-producing oxidase production was studied with four strains of *C. subvermispora* using ammonium tartrate at concentrations of 1,0 , 10 and 50 mM. MnP levels were higher at 50 mM ammonium tartrate for strains FP-90031-sp and FP-105752, whereas for strains L-15225 and FP-104027, this ocurred at 10 mM ammonium tartrate. On the other hand, oxidase levels were always higher at 50 mM ammonium tartrate. With the four strains it was observed that regardless the final levels of MnP attained, cultures containing 50 mM nitrogen in the culture media exhibited a short lag period before starting the production of this enzyme.

Addition of detergents to submerged cultures has been shown to facilitate the formation of small fungal pellets, thus optimizing the secretion of ligninolytic enzymes (17,22). When Tween 20 (0.05%) was added to cultures of *P. brevispora,* a two fold increase was observed in the titers of the three enzymes at any time during fungal growth. With *C. subvermispora*, the effect of this detergent was tested with strains FP-104027 and FP-105752. In both cases, MnP and laccase could barely be detected in the absence of Tween 20. In contrast, levels of the H_2O_2-producing oxidase were not affected by Tween 20 at concentrations between 0 and 2,0%.

Finally, in one of the attempts to find LiP in the extracellular fluid of both fungal species, veratryl alcohol was added to the growth media at a concentration of 0.4 mM (22). This compound did not produce any detectable change in the enzyme pattern of either fungi.

DISCUSSION.

LiP seems to play a crucial role on lignin degradation by *P. chrysosporium* (9,10). The fact that this enzyme was not found in the present study suggest that there may be alternative pathways for breaking down the polymer to small fragments. The absence of LiP may also explain the lack of effect of veratryl alcohol on the induction of the ligninolytic system of *P. brevispora* and *C. subvermispora*. This compound may act as an efector only in those strains producing LiP. In support of this hypothesis is the high correlation between LiP production and peroxidase induction by veratryl alcohol found by Waldner et al in several fungi (23).

The higher levels of MnP found in cultures of *P. brevispora* and *C. subvermispora* are similar to those found by Jeffries et al in *P. brevispora* (9) and *P. chrysosporium* (6). These authors measured MnP activity in cultures of *P. brevispora* growing in media containing either 0.35 or 39.8 ppm of Mn(II). The latter is the optimal Mn(II) concentration for MnP production by *P. chrysosporium* (6). In our case, the optimal was 11 ppm for both fungal species tested. For *P. brevispora* , this was also the optimum for laccase and H_2O_2-producing oxidase production. However, with the five strains of *C. subvermispora,* there was not significant difference in laccase production at these two Mn(II) concentrations whereas oxidase levels were higher at 39.8 ppm Mn(II). This may indicate that although the three enzymes are induced by Mn(II), the expression of the corresponding genes in this microorganism, in contrast to those of *P. brevispora* , is not strictly coordinated.

The effect of nitrogen was studied only with *C. subvermispora* strains. These differ in the required ammonium

tartrate concentration to attain the highest levels of MnP. The lag in the appearance of MnP at 50 mM ammonium tartrate suggests that this enzyme is produced during secondary metabolism. However, this lag is not observed with the H_2O_2-producing oxidase(s).

Regardless the precise identity of the H_2O_2-producing oxidases, its levels in cultures of *P. brevispora* are about four fold higher than in those of *C .subvermispora* and *P. chrysosporium* (13). Since as stated above the titers of MnP are similar for these three fungal species, there seems to be no correlation between H_2O_2 requiring peroxidases and oxidase levels. Moreover, *P. chrysosporium* also secretes several isoenzymes of LiP that utilize H_2O_2 as a cosubstrate.

We are presently carrying out *in vivo* synthetic lignin (DHP) mineralization experiments with both fungi, to establish to what extent the enzyme levels observed under various conditions correlate with lignin conversion to CO_2.

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REDUCTION OF CHEMICAL OXYGEN DEMAND IN BLEACH PLANT EFFLUENT BY A COMBINATION OF PHOTOCHEMICAL AND BIOLOGICAL METHODS.

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ABSTRACT.

A screening of 51 ligninolytic strains of fungi to examine their ability to decolorized phenolic industrial effluent was carried out. The selection showed that **Lentinus edodes** (UEC-2019) strain removed 73 % of colour in 5 days, without any additional carbon sources. Under these conditions, L. **edodes** were more efficient than the known **Phanerochaete chrysosporium** (BKM-F-1767) strain (e.g. COD reductions were 60 % and 26 %, respectively). Kraft effluent was also oxidatively decolorized by a semi-conductor photocatalyzed reaction in aqueous solution containing oxygen and a semi-conductor powder. When a pre-irradiated effluent (10 min) in the presence of ZnO was treated with L. **edodes** an efficient enhancement of the decolorization (5.5 folds) at 48 h was obtained. The most active fungi in this pre-treatment and biobleaching were the ones which exhibited higher ligninases and phenoloxidases together with beta-glucosidase activities. The combined photo-biobleaching procedure appeared to be an efficient process of decontamination with a great industrial potential in effluent treatment.

INTRODUCTION.

Bleaching Kraft mills usually discharge large volumes of brown coloured effluents as a result of the different processes applied in wood and pulp. The brown color of the effluents originate mainly from lignin degradation products, are mostly chlorinated and its elimation is of great importance.

Several white-rot fungi besides **Phanerochaete chrysosporium** can readily decolorize Kraft bleach lignins. Attempts have been made to remove the color of effluent from a Kraft mill by **P. chrysosporium**, by **Trictoporia sp.**, by **Aspergillus sp** and by **Coriolus versicolor**. A mycelial color removal process (MyCor) for decolorizing E_1 bleach plant effluent have been published . The potential use of white-rot fungi for biopulping, biobleaching and treatment of pulp mill waste effluents has been reviewed (1,2).

To obtain the best possible species for use in new biotechnological processes, numerous species of wood-rotting fungi have been screened for rapid growth and superior rates of lignin degradation (3-6) were evaluated.

Another aproach of interest in non-classical effluent treatment is based on photochemical degradation. A potential method for effluent treatment is the direct pre-irradiation of lignin or lignin-cellulose complex and subsequent enzyme degradation (7,8). A photocatalyzed reaction using a semi-conductor as photocatalyst may also be a potential method of effluent treatment, since it is known that the photodecomposition of Kraft lignin using TiO_2, ZnO and others is quite efficient (9). In addition TiO_2 produces efficient photochemical degradation of chlorophenols (10, 11).

In the present study, we devised an application of an efficient method for decolorization of Kraft effluent by a combination of a photochemical pre-treatment (12) and a biobleaching with the pre-selected fungi (6), without any extra carbon sources.

MATERIAL AND METHODS

STRAINS. The fungal strains were obtained from UNICAMP Culture Collection and from CCT (Coleção de Culturas Tropical). Stock cultures were maintained in malt agar medium at 5°C. **Chrysonilia sitophila** (TFB-27441)was maintained in a 1,25% malt-agar plates.

CULTURE CONDITIONS. Cultures of strains were grown as previously described (13)

LIGNINASE ACTIVITY. Enzyme activities were measured as published (6,12-14).

PHENOLOXIDASE ACTIVITY. Laccase and peroxidase activities (U/L) were measured as published (15).

GLYOXAL OXIDASE ACTIVITY. This activity was measured by a published method (16)

EFFLUENT. The effluent (RIGESA S.C.) used in this study was the first alkaline extraction stage (pH 9.5 - 13.0) from a bleached kraft pulp (Eucalyptus) and paper mill.

MEASUREMENT OF COLOR IN TREATED KRAFT EFFLUENT. Color removal by fungal strains was measured in duplicate by a standard method (17). The nature of the color before and after decolorization was measured by a published method (18)

SUGAR DETERMINATION. The carbohydrate determination of kraft effluent was carried out by the DNS procedure (19).

COD DETERMINATION. The Chemical Oxygen Demand (COD) was determinated by standard methods (20).

PHOTOCHEMICAL PRE-TREATMENT AND BIOBLEACHING WITH LENTINUS EDODES. The reaction was carried out in a beaker containing 25 ml of effluent, at initial pH value of 5.0,

and adequate ZnO photocatalyst from Aldrich (50 mg). The solution was irradiated from the top using a 250 W Phillips lamp (fluence rate 108 KJ m^{-2} s^{-1} at 254 nm) for 10 min and then a mycelial pellet (2 g of wet mass, 97% humidity) was added to 25 ml of pre-irradiated effluent (initial abs. 0.44 at 465 nm) at pH 5.0. During the ilumination, the solution was magnetically stirred and bubbled with oxygen (12).

DESCOLORIZATION OF E_1 BY FILTRATED CULTURE FROM CHRYSONILIA SITOPHILA(POOL OF CRUDE ENZYMES). After **C. sitophila** growth at standard conditions, the mycelium was filtered and the ligninase and laccase activities were measured in the filtrate. Filtrated broth (20 ml) was added to 20 ml of non-sterilized effluent (when hydrogen peroxide was added the concentration was 0.1 ml of 0.048 M) and shaken at 150 rev. min^{-1} at 30° C and pH 6.5. The mixture was filtered through a 0.47 mm millipore adjusted to pH 7.6 with NaOH and the absorbance at 465 nm was determined. Decolorization was expressed as a percentage of the initial absorbance (absorbance 0.5, 1894 color units).

COMBINED EXPERIMENTS WITH ZnO AND BIOLOGICAL TREATMENT. This experiment was carried out by first pre-irradiating the effluent for a short period of 15 min at $\lambda > 254$ nm as described above. The pre-irradiated effluent (20 ml) (absorbance 0.5 at 465 nm, pH 7.6) was then added to 20 ml of the filtrated culture at 30°C and pH 6.5, which contained the lignin-decomposing enzymes (ligninase 120 U/L, laccase 0.02 U/L) from **C. sitophila**.

RESULTS AND DISCUSSION

EFFLUENT TREATMENT. Fifty one strains of fungi were previously screened (6) and the basidiomycete **P.chrysosporium** BKM-F-1767 and ascomycete **Chrysonilia sitophila** TFB 27441 (13) were used as controls. Among the 25 species which decolorized the effluent efficiently in five days were **Lentinus edodes** (UEC-2019), **L.edodes** (UEC-2021),**Pycnoporus sanguineus** (UEC-2015), **Xylaria sp.** (ICN-155) and **Phaeocoriolellus trabeus**(UEC-2013). No additional carbon source was added to the E_1 effluent which contains 0.06 % carbohydrates. The remaining 25 strains increased the effluent color.

The decolorization capacity did not present any apparent correlation with the lignin peroxidase production capacity. **L. edodes** (UEC-2019 strain) mycellium after 120 h exhibited 11 U/L of laccase, 0.5 U/L of peroxidase and 19.3 U/L of lignin peroxidase,0.003 Abs x min^{-1} x ml^{-1} of Mn-peroxidase, 14 U/L of beta-glucosidase and a 73 % of decolorization with 60 % of COD reduction. Only 13 % of

the decolorization was due to mycelial adsorption.In this case the initial absorbance value was 0.44 at 465 nm.

With **P. chrysosporium** (BKM-1767 strain) under the same conditions, 0.41 U/L of laccase, 0.01 U/L of peroxidase, 7.8 U/L of lignin peroxidase, 0.012 Abs x min^{-1} x ml^{-1} of Mn-peroxidase, 5.5 U/L of beta-glucosidase and a 52 % decolorization and a COD reduction of 26 % were observed.

C. sitophila exhibited no phenoloxidase and 7.8 U/L of lignin peroxidase, 0.008 Abs x min^{-1} x ml^{-1} of Mn-peroxidase, 7.0 U/L of beta-glucosidase and a 41 % of either decolorization or COD reduction.

The enzyme expression in the growth culture medium was different than in the effluent (6)(similar in Archibald et al.(21)). In general, lignin-peroxidase activity decrease while laccase and peroxidase activity increased in both cases.

From our observations it seems that beta-glucosidase exert an important role in the effluent decolorization. The most efficient strain for decolorization, **L. edodes** (UEC-2019), exhibited a higher beta-glucosidase activity than the other, besides those of lignin peroxidase, peroxidase and Mn-peroxidase; meanwhile **P. trabeus** (UEC-2023) although exhibiting high Mn-peroxidase (0.3 Abs x min^{-1} x ml^{-1}) and high lignin peroxidase (273 U/L) was extremely inefficient for decolorization, probably due the complete absence of beta-glucosidase activity in the effluent.

Previous results from others studies, mainly with carbon source variation, emphasize the importance of executing the biological treatment under strictly defined conditions. It was clear that glucose, among the various carbon sources enhances the decolorization process (21). This was generally, attributed to the fungus growth conditions. However, holocellulose or xylan were more effective in lignin-model degradation at low sugar concentrations (22).

Recently, it has benn shown that this degradation depends on the transglucosylation activity of glucosidase secreted by the majority of wood-rot fungi (23). For intance, glucosidated DHP-Lignin can be degraded by peroxidase readily, suggesting that there must exist a system where the formation of glucoside prevents polymerization and accelerates efficient degradation reactions (24). Probably this is one of the reasons for the efficient decolorization by the fungi with the low reducing carbohydrate concentration (0.06 %) present in our effluent and for the presence of a relative high beta-glucosidase activity. These results are in agreement with the observations of Yin et al.(25) that a critical amount (between 0.1 a 0.2 %) of glucose was

needed to mantain the decolorization activity, but only a very low consumption of glucose was observed (around 0.06%).

The majority of the glucose remained unmetabolized in the effluent (25). This was recently confirmed by Prasad and Joyce (22) who found that 0.05 % glucose was the most efficient for decolorization .

Another important aspect which is actually under study in our group is the role of glyoxal oxidase on effluent decolorization. Following the Kersten and Kirk (16) methology we have found this activity in **L. edodes** (UEC-2018) culture. In progress it is the relatioship between this activity and the beta-glucosidase activity with the fungal capacity to decolorize Kraft effluent.

PHOTOCHEMICAL PRE-TREATMENT. At the optimum pH and ZnO concentration, a short pre-irradiation of the effluent (15 min) at pH 6.5, followed by fungal culture filtrate (from **C. sitophila (TFB-27441)**) treatment, results in efficient decolorization, which is enhanced in the presence of hydrogen peroxide which acts as an enzymatic cofactor(Fig.1).

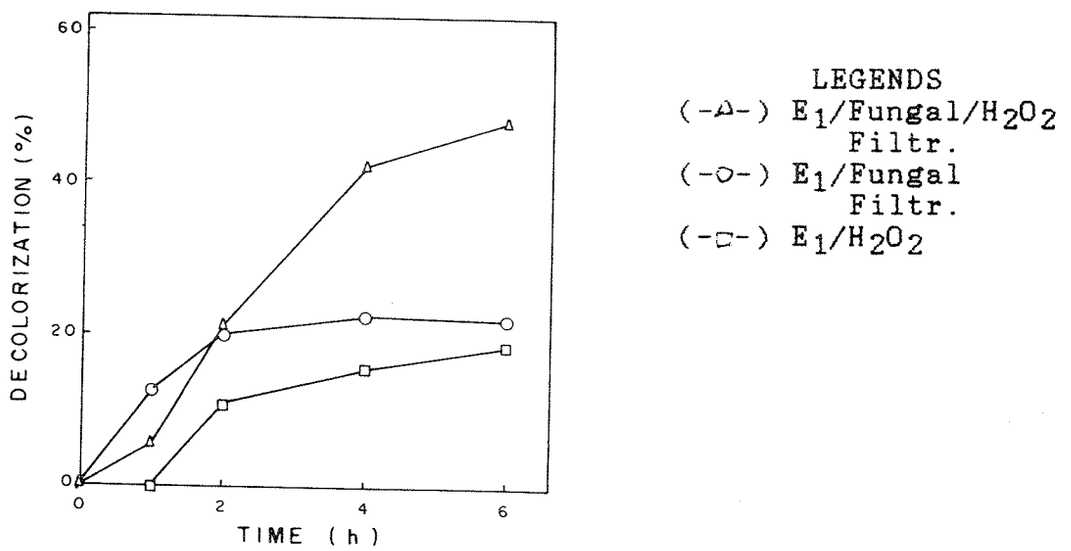

FIG. 1. EFFLUENT DECOLORIZATION BY FUNGAL FILTRATE

As an alternative procedure, when the effluent containing suspended semi-conductor powder is bubbled with oxygen and illuminated, the color fades gradually, being more efficient in the presence of ZnO than TiO_2 (Fig.2). The pH variations in both semiconductors were similar.

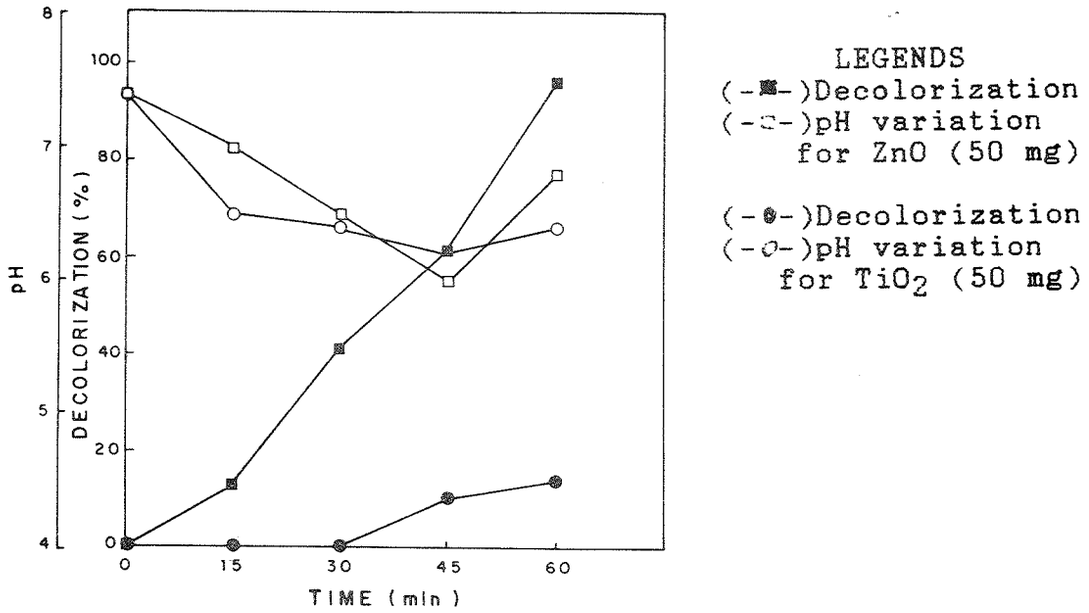

FIG.2. EFFLUENT DECOLORIZATION BY PHOTOCATALYZED REACTION WITH ZnO AND TiO_2.

The molecular weight distribution of the chlorolignins in the effluent at the optimum photochemical conditions indicates that no polymerization occurs (Fig.3). Mineralization is observed, since a pronounced biomass loss occurs with no further pecipitation in the effluent. There are perfect correlations between decolorization, biomass loss and CO_2 formation.

Thus this method appears as an excellent pre-treatment procedure for very short irradiation periods. In order to evaluate this procedure we have tested the pre-irradiation on E_1 effluent.

At the optimum pH and ZnO concentration, a short pre-irradiation of the effluent at pH 6.5, followed by fungal culture filtrate treatment, results in efficient decolorization, which is enhanced in the presence of hydrogen peroxide which acts as enzymatic cofactor (Fig.4)

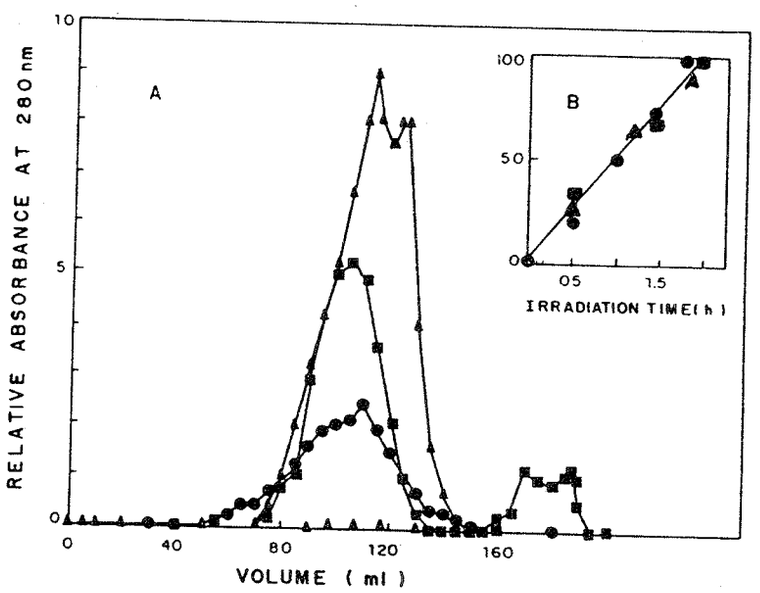

FIG.3. GEL FILTRATION OF EFFLUENT IN SEPHADEX-G-50 AT DIFFERENT TIMES OF IRRADIATION (\ > 254 NM) IN THE PRESENCE OF ZnO (50 mg).

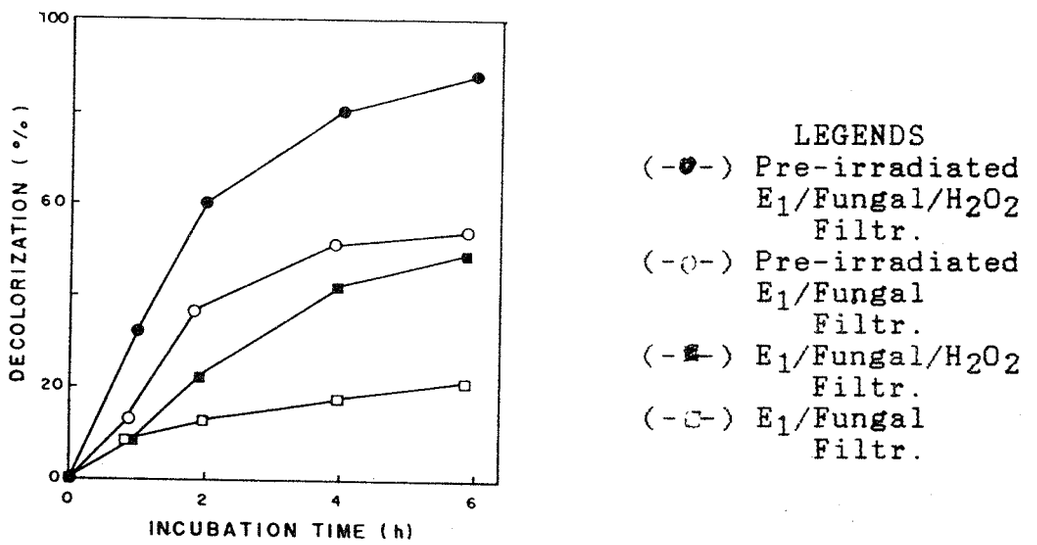

FIG.4. COMBINED PHOTOCHEMICAL AND ENZYMATIC PROCESS.

Since, all the enzymatic content is known in the **C. sitophila** (13) the importance of ligninolytic enzymes was quite evident in the decolorization process.

The next step was then to carry out experiments with the best selected fungi with pre- irradiated Kraft effluent. The fungi selected was **L. edodes** (UEC-2019). The results with the kinetics of decolorization in both systems (biobleaching and pre-irradiated-biobleaching systems) showed that at 24 h and 48 h, the biobleaching exhibited a negative efficiency (-0.4) and a positive efficiency (+0.2), respectively. Meanwhile at 24 h and 48 h the pre-irradiated-biobleaching system exibited a positive efficiency for both periods(Fig.5) (+0.5 and +1.1, respectively). In other words, the pre-irradiated system was 5.5 times faster at 48 h than the biobleaching alone. At 120 h the total decolorization in the biobleaching system and the pre-irradiated-biobleaching system were 73% and 78%, respectively. This is a good indication that the pre-irradiated effluent was kinetically more efficient than the unirradiated one.

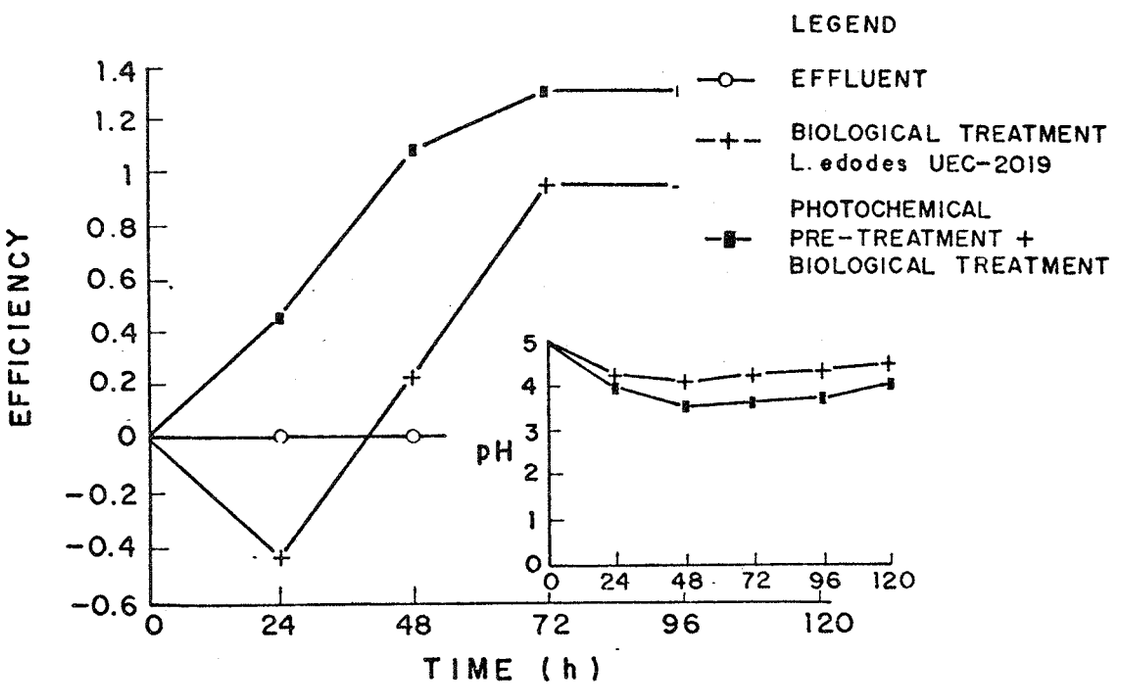

FIG.5. EFFLUENT TREATMENT: KINETIC STUDY OF DECOLORIZATION OF E_1 EFFLUENT AND pH VARIATIONS.

The molecular mass distribution of the treated effluent in both systems at 120 h are presented in Fig. 5. A biomass loss of 70% in the biobleaching system and 80% in the pre-irradiated-biobleaching system were found. The chemical oxygen demand reduction in both systems were 60% and 70%, respectively.

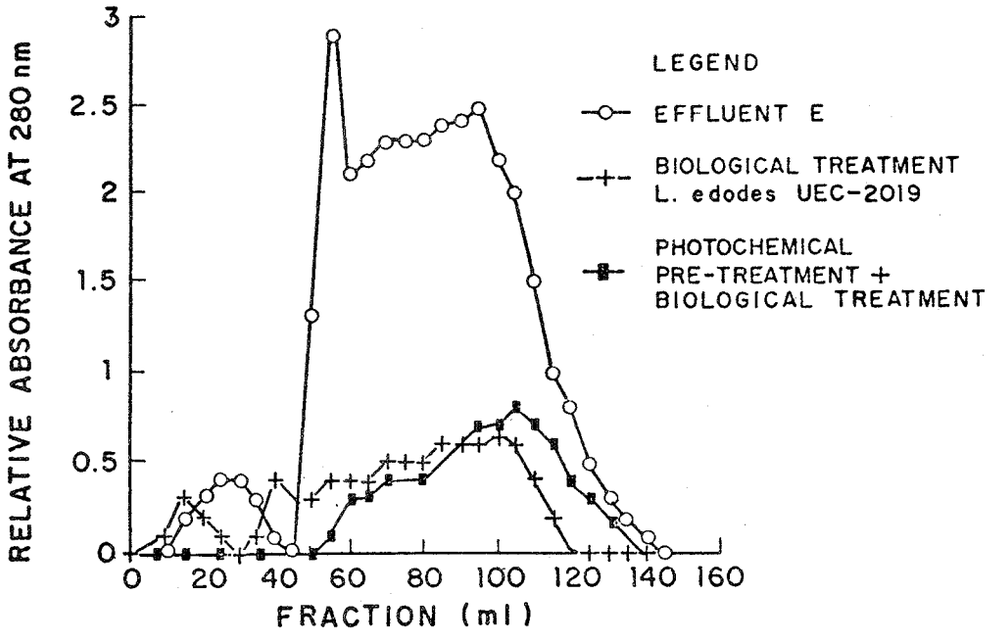

FIG.6. MOLECULAR MASS DISTRIBUTION AFTER 120 HOURS.

DECOLORIZATION MECHANISM OF SPENT BLEACHING LIQUOUR BY **L. edodes** (UEC-2019). In order to understand the possible mechanism in the pre-irradiated effluent, a study on the color distribution was carried out. Due to experimental reasons in this study an absorption value of 0.7 at 465 nm was used.

The color was divided into three types, depending upon their reactivities to dithionite (quinone type color) or to borohydride (total quinone and carbonyl type color) or when unreacted to both reagents (denominated as an other type of color) (18).

The quinone type color from the E_1 effluent from RIGESA occupied 60% of the color. Therefore, the removal of the quinone type color in this effluent is very important for the entire removal. Results of the biobleaching, pre-irradiated, and pre-irradiated-biobleaching systems are in TABLE 1.

Among the three types of colors, the quinone type and carbonyl type colors were removed more rapidly than the other type color in the biobleaching alone. Although no

change in the initial color was observed, for the

TABLE 1. COLOR DISTRIBUTION AFTER 120 H OF TREATMENT WITH **L. edodes** (UEC-2019)(%)(a).

Color Type	Control	Biobleaching Treatment	Pre-irradiated Treatment	Combined Treatment
Quinone	60 (50)(b)	36 (40)	77	27
Carbonyl	15 (10)	8 (20)	9	4
Other	25 (40)	22 (20)	13	8
Color Loss(%)	0	44 (20)	0	61

a) Absorbance value of 0.7 (2700 color units) at 465 nm pH 7.6 was used. b) Values in parenthesis corresponds to removal of color from E_1 effluent by **P. chrysosporium** (18) in the presence of external carbon and nitrogen sources (144 hs of treatment) (8500 color units).

pre-irradiated effluent, the color distribution was largely modified in the sample. In this case the quinone type color increased and the other type color decreased. This pre-treatment led the fungus to act even more unspecifically on the three type of colors, exhibiting a high capacity for effluent decolorization (61%) compared with only the biobleaching process (44%).

In summary, the combined photo-biobleaching procedure appeared to be an efficient process of decontamination with a great potential in effluent treatment. We believe that in combination with other physical methods it should become a comercial feasible procedure.

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DECOLORIZATION OF SUGAR CANE MOLASSES BY P. chrysosporium AND TWO SELECTED WHITE-ROT FUNGI

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ABSTRACT

Sugar cane molasses was decolorized by ligninolytic cultures of P. chrysosporium ATCC 24725. Decolorization appeared to be dependent on the levels of lignin peroxidase present in the culture supernatant. Two white-rot fungi isolates were also able to decolorize molasses but exhibited low levels of lignin peroxidase production. Strain N.1 produced peroxidase as determined by the oxidation of syringaldazine in the presence of hydrogen peroxide. Strain N.2 produced high levels of laccase.

INTRODUCTION

The white-rot fungus Phanerochaete chrysosporium has the ability to degrade a variety of organic compounds such as lignin, lignin model compounds, vanillic acid, syringic acid, chlorinated aromatic compounds and many others (1,2,3). It has been reported that the ability of P. chrysosporium and other white-rot fungi to degrade these compounds is due, at least in part, to the lignin degrading system of these microorganisms (4,5,6,7). Four kinds of enzymes have been described to be envolved in the lignin breakdown: lignin peroxidase, manganese peroxidase, hydrogen peroxidase producing enzymes and laccase (8).

Here, the decolorization of sugar cane molasses by ligninolytic cultures of P. chrysosporium ATCC 24725 and by cultures of two white-rot fungal isolates is described. Molasses is a by-product of either manufacture or refining of raw cane sugar. It is a dark, heavy and viscous liquid from which no further sugar can be crystallized by normal methods. The main non-sugar components from cane molasses have been identified as phenolic and phenylpropanetriolic glucosides coming from the lignin material during juice extraction and processing (9).

METHODS

Lignin peroxidase was produced by P. chrysosporium, under shaking conditions, as described previously (10).

The two fungal strains were isolated after a screening, for white-rot fungi producing enzymes that might be involved in lignin biodegradation, was carried out (11). Lignin peroxidase production by these two strains was as for P. chrysosporium, except that non agitated conditions were used and the temperature for the growth of strain N. 2 was 30 C.

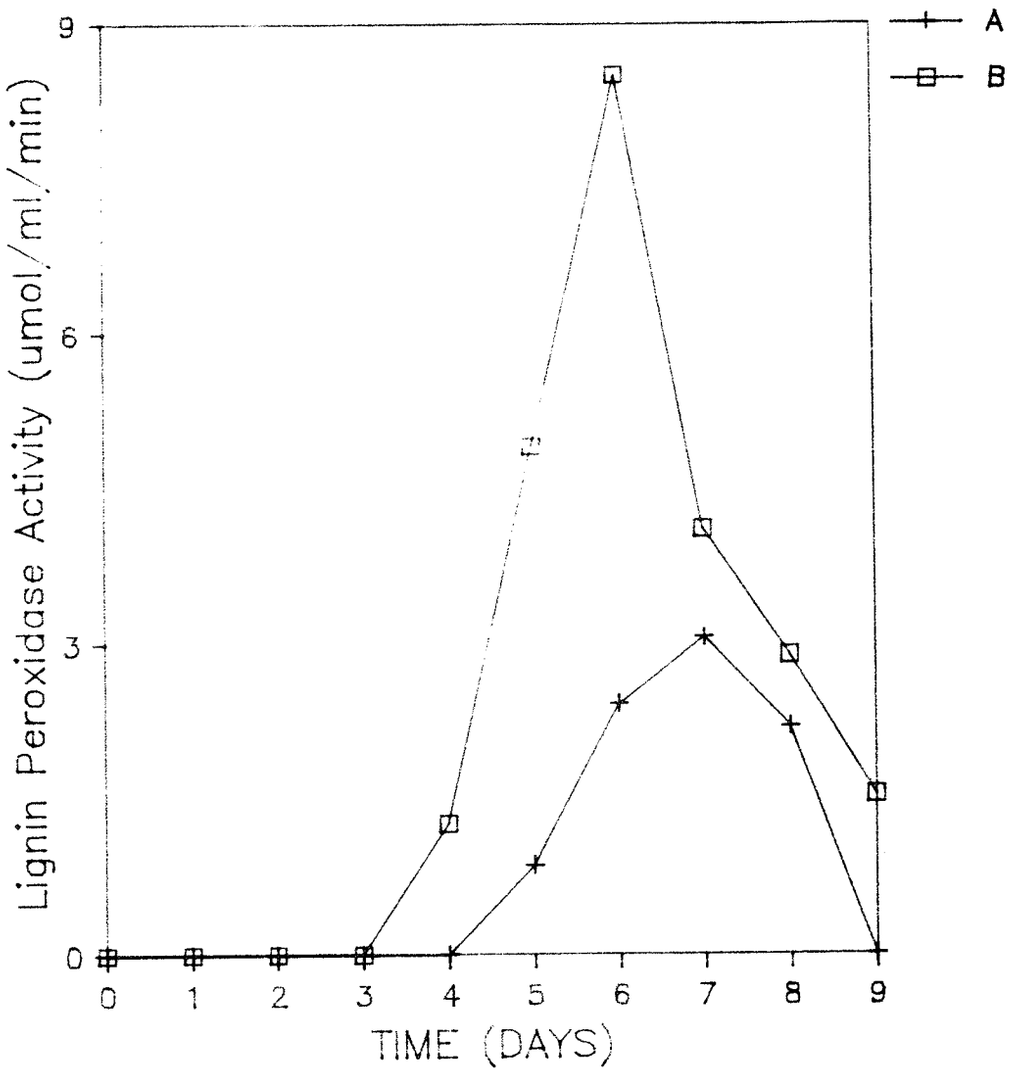
Lignin peroxidase was assayed using veratryl alcohol as described previously (12).

RESULTS AND DISCUSSION

Figure 1 shows the time course for the production of lignin peroxidase by P. chysosporium grown on 1% glucose only and on 1% glucose plus 0.6% molasses as the carbon sources. It is clear that the presence of molasses promoted an enhacement in lignin peroxidase production. It has been reported that the addition of veratryl alcohol or other lignin related compound to P. chrysosporium' cultures, stimulated lignin peroxidase production, but the mechanism through which this stimulation ocurrs is not completely understood (13,14). It might be possible that a similarity exists in the mechanism of "induction" of lignin peroxidase production by veratryl alcohol or lignin model compounds and molasses. The presence in molasses of lignin derived compounds would be the reason for this possible "induction". Decolorization of molasses was observed during lignin peroxidase production by P. chrysosporium.

Figure 2 shows the spectra for decolorized molasses in cultures of P. chysosporium where the levels of lignin peroxidase present on the same day of growth exhibited variation among the flasks. A control, where no oxygenation was carried out on the 3rd day of growth, was used. As can be seen,

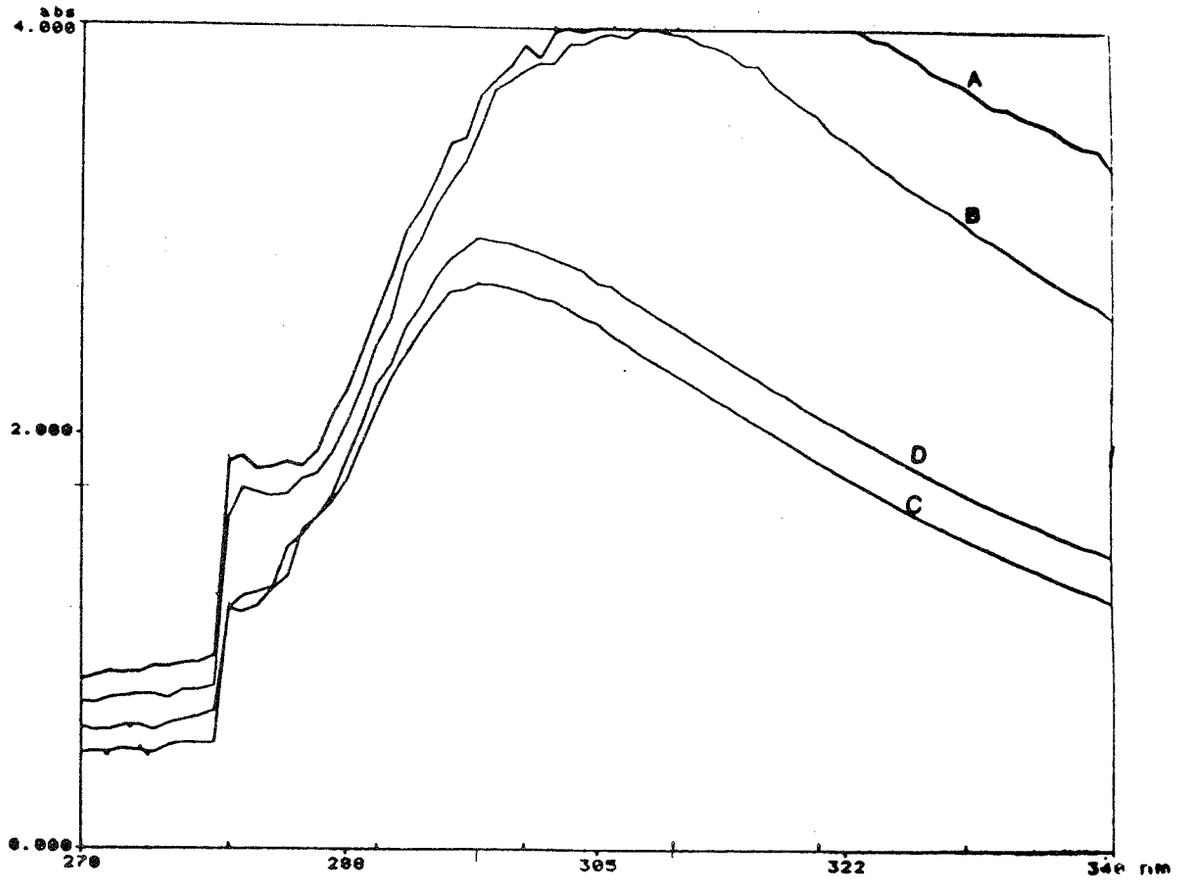
Figure 1 Time Course for the Production of Lignin Peroxidase Under Shaken Conditions



A: Carbon Source - 1% Glucose

B: Carbon Source - 1% Glucose + 0.6% Molasses

Figure 2 The Spectrum of Molasses' Pigments



A: Control

B: Sample showing poor lignin peroxidase activity

C: Lignin peroxidase activity = 100%

D: Lignin peroxidase activity = 70% C

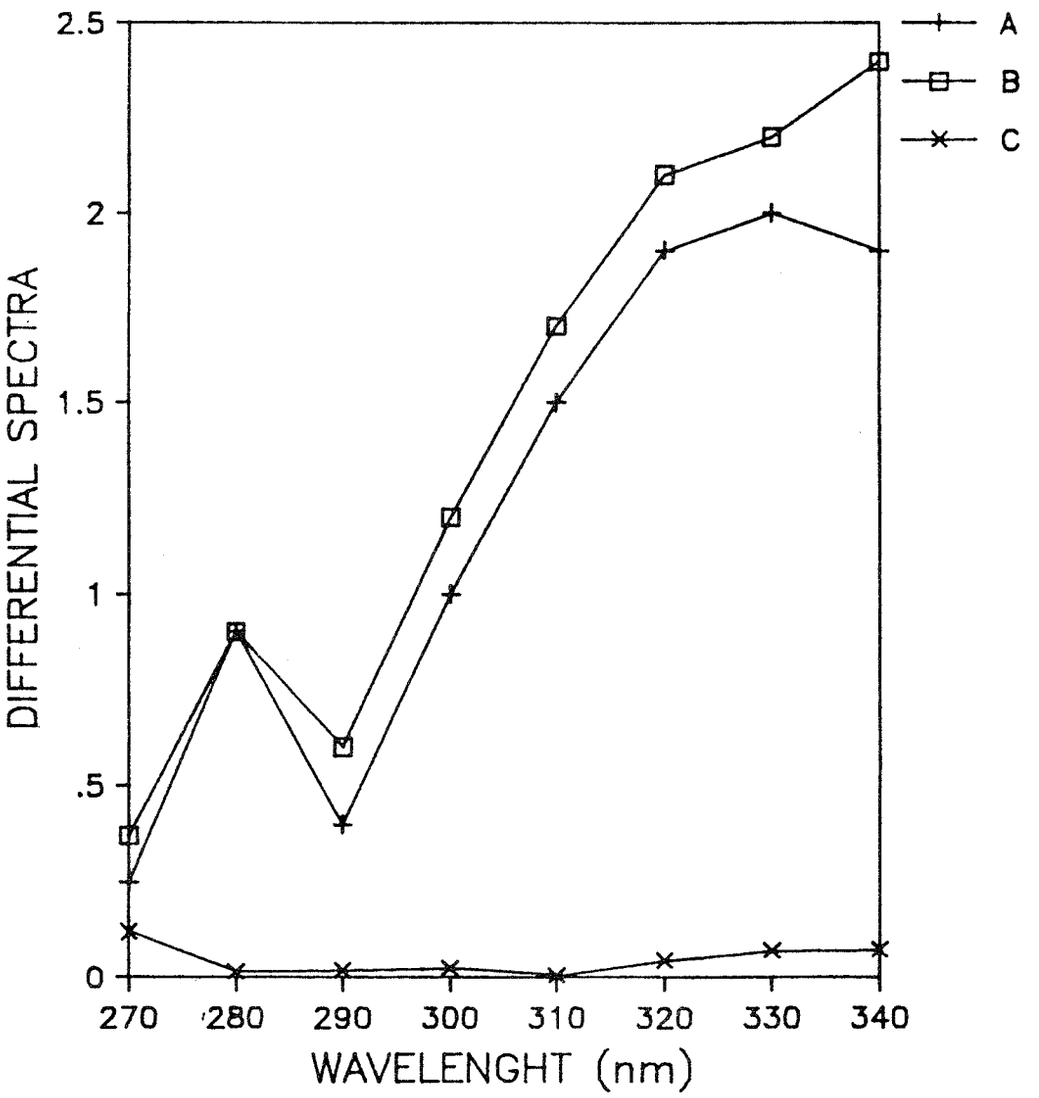
The pigments were decolorized during lignin peroxidase production under shaking conditions.

sample which exhibited high lignin peroxidase activity and were higly decolorized showed a much lower absorbance than the respective control. A shift in the maximum absorbance wavelength can also be observed. It could be said that the decolorization of molasses is directly related to the lignin peroxidase activity in the cultures. However, the ligninolytic system of the fungus, as a whole, might be responsible for the decolorization of molasses.

The differential spectrum for decolorized molasses and the control is shown in figure 3. The differential spectrum was obtained by calculating: (Absorbance of control - Absorbance of the samples) at the various wavelenghts. The various degrees of decolorization, which seem to be dependent on the levels of lignin peroxidase activity in each sample, can be observed.

The two strains selected gave a positive reaction to o-dianisidine (15), indicating the presence of phenol oxidases. Strain N. 2 gave a positive reaction to syringaldazine whereas strain N. 1 needed the presence of hydrogen peroxidase for enzymatic reaction to occur, indicative of a peroxidase (16). Decolorization of molasses was observed, however, low levels of lignin peroxidase

Figure 3 The differential spectra of molasses' pigments



A: Lignin peroxidase activity = 70% B

B: Lignin peroxidase activity = 100%

C: Sample having poor lignin peroxidase activity

activity were produced by the two strains. This might suggest that for these strains, decolorization is not dependent on lignin peroxidase activity. Some improvement in growth conditions for lignin peroxidase production might be required to increase levels of activity.

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BIOTECHNOLOGICAL OBTENTION OF VANILLIN FROM BLACK KRAFT LIQUOR LIGNIN

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ABSTRACT.

Preparation of vanillin by biotechnological treatment of Black Kraft Liquor from Paper Industry with 13% solids was obtained. The process involves a previous physico - chemical treatment of the liquor mainly for separation of inoganic solids, pH adjustement and further biological treatment with a crude enzymatic extract of Acinetobacter anitratus. The vanillin obtained make feasible the preparation of essences from lignin residues.

INTRODUCTION.

Among the different energy sources and chemical products, lignocellulosic residues are the most important because of their renowable characteristics.

In order to obtain molecules of high aggregated value is necessary to develop primary matter transformation processes to separate the components. Lignin extraction and its direct use or its transformation in products of high aggregated value is important.

Studies on kinetic of lignin transformation to low molecular weight molecules are related to microbiological aspects as well as biochemical ones (1 - 5).

Biological methods are mainly related to lignin transformation and recuperation of the products obtained by its polymerization and oxidation.

Previous trials on lignin depolymerization of lignocellulosic substract of Eucalyptus and wheat had showed oxidative and depolymerizant activity in an enzimatic crude extracted from celular membrane of Acinetobacter anitratus. Optimum culture conditions (pH 6 and temperature 25 and 35°C were determined). Kinetical trials done showed a response to a positive cooperative behaviour (6).

EXPERIMENTAL PART.

The aim of the study was the biotechnological treatment of Black Kraft Liquor from a cellulose digestor of a paper industry.

The digestor works with a feeding of 150 ton/day of

Eucalyptus grandis wood chips in the following conditions: temperature 170°C, pressure 9 Kg/cm^2, pH 13 - 14 and residential time 90 minutes.

70 ton/day of cellulose and 4 x 10^2 ton/day of Black Kraft Liquor 13% solids are obtained.

Liquor 13% solids is concentrated to 65% solids in the recuperation plant by multiple effect evaporators so as to be used as fuel with a caloric power of 3300 Kcal/Kg of solids.

The concentrated liquor was used in appropiated conditions as substrate for a biological treatment with an enzymatic extract from Acinetobacter anitratus. to produce depolymerization and oxidation of lignin in order to obtain products of high aggregated value.

Black Kraft Liquor from a cellulose digestor with a 13% and 65% concentration of solids (47% organics of total solids) was used.

Treatment suggests an adequation of liquor conditions to optimize biological treatment. This chemical pretreatment includes the following stages:

A) Chemical pretreatment

1) Separation of inorganic matter from total solids of the black liquor. Mixtures of 0.1 M $BaNO_3$, 0.1 M Barium acetate and 0.1 M $BaCl_2$ were tried.

2) Adjustment of pH 6.2. pH was adjusted to pH 6.2 with HCl. Supernatant solution was used as substrate. Precipitate contained inorganic solids (not separated in the previous treatment) and organic solids with high molecular weight were separated to obtain other subproducts.

B) Biological treatment

This was carried out with the addition of the crude enzymatic extract from Acinetobacter anitratus (0.5 mg protein/mL), 300 µg of enzyme to 200 mL of liquor incubated for 24 hours at 37°C with shaking and the addition of 0.1 mL H_2O_2.

C) Vanillin obtention

The solvent extraction was with benzene and chloroform. After three extractions and separations by decantation in the organic solvents and evaporated partially, the vanillin was crystallized.

The vanillin was identified by:

a) spectrometric UV read at a 307 nM.
b) thin layer chromatography.
c) purification by sephadex (G 100) column 18 cm diameter 1 cm

RESULTS AND DISCUSION

A) Vanillin obtention

The results of vanillin yield, were refered to total

liquor solids (Tables I and II). From these tables we can conclude:

Related to the chemical pretreatment to separate inorganics seemed to be an advantage the use of $BaCl_2$ in the manipulation to separate the precipitate. Barium acetate was discarded because it carries organic compounds, therefore, some low molecular weight substrates are lost.

An enzymatic extract dose of 0.5 mg proteins was adecuated in trials 1 and 3, (substrate concentration ranging 41 and 26g, respectively). This dose was not enough for trial 2 (substrate concentration 54 g), therefore vanillin yield was lower.

Benzene extraction produced solids with 47% aldehides (including vanillin). Chloroform extraction, followed by molecular filtration, produced solids with 10 - 20% pure vanillin; purity was determined by spectrometry and thin layer chromatography.

Trial 3 showed the highest yield and easyest process as it used the not concentrated liquor (13% solids) which came directly from the paper plant. In addition, $BaCl_2$ could be added directly to the liquor during the treatment.

B) Preliminary vanillin costs

Black Kraft Liquor, concentrated to 65% solids, is generally used as fuel with a caloric power of 3300 Kcal/Kg of liquor solids.

This fuel allows us to give it a potential value in function of its energetic petroleum equivalent. So, a ton of black liquor 13% has 130 Kg solids and it is equivalent to 430,000 Kcal. These Kcal can not be considered as a whole because the liquor must be concentrated to 65% solids to be able to be used as fuel. This concentration implies vapour energy in the process (200 Kg vapour per ton of black liquor 13%). Then, 170,000 Kcal are needed to concentrate one ton of black liquor. Then 260,000 Kcal remain from black liquor 13% for energetic use, equivalent to 26 KEP (Kg Equivalent Petrol). Therefore, we can asume an economic value of 2.5 US$/ton of black liquor.

A vanillin yield of 0.1% in solids is equivalent to 130 g vanillin/ton of black liquor, representing 19 US$ in the production of 1 Kg vanillin (market price is 36 US$/Kg).

Operative costs for the process and equipment amortization will be studied later, following pilot scale trials in a feasible project.

TABLE I: CHEMICAL PARAMETERS IN THE TRIALS

TRIAL	Solids in black liquor (%)	Soilds in sample g	Chemical pretreatment	Inorganic precipitates	Decanted volume at pH 6.2 (%)
1	65	325	0.1 M $Ba(NO_3)_2$ dilution 1/4	positive	20
2	65	325	0.1 M $Ba(CH_3COO)_2$ dilution 1/3	positive	20
3	13	130	0.1 M $BaCl_2$ in liquor	positive	22

TABLE II: BIOLOGICAL PARAMETERS IN THE TRIALS

TRIAL	Total solids in substrate (g)	Sample volume (mL)	Solvent extract. (mL x 3)	Cristal solids in solvent (g)	Aldehide yield (%)	Vanillin (% of total solids)
1	41	250	Benzene 40	0.13	47 (vanillin + others)	0.10
2	54	250	Chloroform 40	0.24	10 (pure vanillin)	0.05
3	26	200	Chloroform 40	0.14	20 (pure vanillin)	0.10

Starting with a biological treatment of Black Kraft Liquor from a cellulose digestor and chemical pretreatment to adequate the liquor, it is possible to obtain a low molecular weight product identified as vanillin with a yield of 0.1% expressed in solids of the treated liquor.

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STRATEGY FOR UTILIZATION OF PEROXIDASES ON KRAFT EFFLUENTE TREATMENT

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ABSTRACT

The strategy to enzyme utilization involves production, purification and immobilization processes.

Three Ligninase found in extracellular culture of *Chrysonilia sitophila* were purified by ion exchange chromatography and their kinetics parameters were studied. Colour removal from Kraft effluent by ligninase and HRP was compared. Ligninase and HRP in free and immobilized form and filtered broth removed colour from Kraft effluent. In general in the immobilized form all the studied systems exhibited an average value around 30% of polymer consumption and very little of depolymerization processes.

INTRODUCTION

An interesting microorganism, the ascomycete Chrysonilia sitophila belongs to a class of wood rotting fungi, has being studied in the last five years (1-2). Some of the potential applications for lignin peroxidase of *C. sitophila* include the biobleaching of wood pulp and the degradation of organopollutants(2). Many researchers have suggested the use of these enzymes specially from basidiomycetes as *Phanerochaete chrysosporium*, *Phlebia radiata* and others, for biobleaching pulps as an alternative to conventional chlorine-based bleaching operations that produce hazardous by-products (3-4).

C.sitophila secretes three extracellular lignin peroxidases (5-6). Many practical application with these enzymes were envisioned and it was suggested that a crude rather than a purified enzyme will be used because of the prohibited cost of the enzyme purification(7). Similar limitations were indicated with *P.chrysosporium* enzymes applications (8).

Lignin peroxidase in crude and partially purified culture filtered from *P.chrysosporium* were examined (9).

For a fixed pH and enzyme concentration, lignin peroxidase stability was greatly enhanced in the presence of its substrate veratryl alcohol. Similar protection with manganese in the Mn-peroxidase was observed.

Also studies with lignin peroxidase H2 and L2 from *P.chrysosporium* were carried out.(10-11).

Conventional treatment methods, such as aerated lagoons and activated sludge plants are ineffective in removing this colour or are extremely expensive. Recently the potential uses of peroxidases were extended to removal of toxic wastes and they have been shown in laboratories to efficiently remove carcinogenic aromatic amines from aqueous industrial effluents (12) and low molecular weight colour bodies from mill effluents (13). Despite these uses the industrial application of peroxidases has been limited, mainly because of their relative inestability, high isolation and purification costs, and the difficulty in recovering active enzyme after completion of the catalytic process (14). Laccase and peroxidases oxidize phenolic to aryloxy radicals, which spontaneously polymerize to form insoluble complexes, these can be removed by precipitation, filtration or centrifugation (15).

Lignin peroxidases and Laccase are currently under experiments related to Kraft effluent (16-18).

It is of general acceptance that in order to increase the potential for the use of enzymes as a wastewater treatment method, it is necessary to immobilize the enzyme so that it is biochemically stabilized and reusable. This is the case of laccase (15,19) lignin peroxidase (15,20,21) and horseradish peroxidase (22).

Chloroperoxidase purified from *Caldariomyces fumago* was covalently bound to aminopropyl glass by using a modification of an stablished method(23). In all the cases a more stable enzyme were obtained.

This communication describes the strategy and the methodology for the production and purification of the ligninase. The kinetics and structural properties, the effect of temperature and pH on the ligninase stability are discussed in order to apply them in effluent decoloration processes in a free and immobilized form.

MATERIALS AND METHODS

Organism and culture conditions. *Chrysonilia sitophila* (TFB-27441 strain) from UNICAMP Collection was maintained on Fries-sucrose modified medium at 5°C(1).

Production of lignin peroxidases. The experiments were carried out in a 15 L bioreactor (Superohm F-15 Piracicaba, Brazil) as previously describes (5).

Enzyme preparation. Cultures were filtered through a sintered funnel and to minimize proteolysis, p-methylphenylsulfonyl fluoride (0.2 mM) (SIGMA) was added to the filtrate . Then, it was ultrafiltered in a MINITAN System (Millipore) to 10% of the volume. Purification method published previously was followed (5).

Three enzymes were isolated (See Table I).

Heat denaturation conditons. Five mL enzyme aliquote or crude culture filtrated was placed in test tubes in a water bath at the desired temperature (±0.5°C) and periodically shaken as previously reported (9,10).

pH stability conditions. To study the effect of pH on the stability of the resting enzyme 0.1µM lignin peroxidase, horseradish peroxidase (Type VI, SIGMA), were incubated in 5mM sodim tartrate buffer pH 3.0-6.5 at 28°C as previously described (10).

Immobilization of lignin peroxidase and horseradish peroxidase and filtered culture a published method was followed (24).

Kraft effluent treatment. The Kraft liquor (E_1) was diluted, filtered and the pH adjusted to 5.0 and at an absorbance of 0.5 (1,894 CU). To 10mL of effluent was added an adequated mass of immobilized enzyme complexes and 5mM hydrogen peroxide at 25°C. Colour removal by enzymes was measured in duplicate in 2 mL effluent previously centrifuged for 2 minutes at 15,000 rpm in a Eppendorf Microcentrifuge, and the absorption measured at 465nm.

Molecular weight distribution . 0.5mL of the clean effluent supernatant was applied to a 60 x 1.5 cm Sephadex G-50 column equilibrated with NaOH-LiCl 0.1N. 2.5 mL were collected (flow rate of 2 min 15 s/mL) and the chlorolignins were followed at 280 nm.

RESULTS AND DISCUSSION

Production and purification of Lignin peroxidase. The elution profile of the ligninolytic enzymes from DEAE-Sephadex A-50-120, three fractions were isolated. **Table I** shows the general characteristic of the obtained fractions from the purification process and their kinetics characteristic in Table II.

Table I. General characteristic of the protein fraction from *Chrysonilia sitophila*

Fraction	Enzymatic Activity	Optimum pH	Fe/Hem Ratio	Carbohydr. (%)	Molecular weight	Ip	Optimum temp.°C
I	121 U/L	3	0.8	25.7	68,000	9.05	28
II	210 U/L	5	1.3	14.8	50,200	6.74	28
III	108 U/L	4	1.2	17.4	47,500	4.50	28

Table II. Kinetic constants of lignin peroxidases from C.sitophila (TFB-27441 Strain) culture (a).

Lignin peroxidases	Veratryl aldehyde KM app mM	Veratryl aldehyde V_{max} nmol.mim^{-1}mL^{-1}	Hydrogen peroxide KM app µM	Hydrogen peroxide V_{max} nmol.mim^{-1}mL^{-1}
LIG-I	0.37	9.09	50	6.67
LIG-II	0.30	8.30	37	7.20
LIG-III	0.12	2.50	33	4.80

(a) The kinetic constant were calculated from Lineweaver-Burk plots.

Enzyme properties. In all temperature the heat-denaturation kinetics can be described as first order. (Table III). Culture filtrate from P.chrysosporium(9) from a medium of nitrogen-limited, glucose based medium, gave a half-live of 13.2 h at 50°C meaning that these lignin peroxidases are 3.6 fold more stable at 50°C than lignin peroxidase from C.sitophila. However, we have to keep in mind that both experiments were carried out at different enzyme concentration, meanswhile in C.sitophila was 13.3 U/L in the case of P.chrysosporium was 80 U/L. This point is important, since it is known that the thermal stability of lignin peroxidase in the latter was increased 2.3 fold when a 10 fold of the enzyme concentration was increased. In an experiment with the enzyme concentration on LIG-III, an increase of enzyme concentration from 4.7 U/L to 11.2 U/L a stabilization of 1.6 fold was found (k_D of 0.092 h^{-1} to 0.058 h^{-1}, respectively, at 28°C and pH 4.0). Probably the differences in stability between the enzymes of P.chrysosporium and C. sitophila are exclusively to this fact. The filtered culture from P.chrysosporium in a glycerol-based medium was not a first order kinetics but rather a complex one(9). Then a more complex heat denaturation kinetics were observed with ultrafiltered crude enzyme in P.chrysosporium . The effect of reaction temperature on measured activity of purified lignin peroxidases are on Table III, and all are of first order kinetics (not showed) in a similar profile that the ultrafiltered culture .

Table III. Decay constants ($K_D \times 10^2 [h^{-1}]$) and half-lives ($t_{D0.5}[h]$ for lignin peroxidase and culture filtrate, acting on veratryl alcohol(a).

Temperature	Optimum pH	28°C	35°C	50°C
LIG-I(b)	3.0	9.2 ± 2.3 (7.5)	14.9 ± 3.3 (4.7)	24.6 ± 3.6 (2.8)
LIG-II(b)	5.0	8.8 ± 2.3 (7.9)	15.2 ± 2.5 (4.6)	22.7 ± 0.7 (3.1)
LIG-III(b)	4.0	5.8 ± 1.1 (12.2)	9.5 ± 1.8 (7.3)	11.1 ± 0.4 (6.2)
Culture(c) ultrafiltered	3.0	7.8 ± 1.0 (8.9)	12.8 ± 2.0 (5.4)	18.4 ± 0.5 (3.7)

(a) Parenthesis values are $t_{D0.5}=0.693/k_D$ (b) 0.1 µl (equivalent to 10U/L), (c) 12 U/L.

Under the condition used for these tests, lignin peroxidase begun to deviate from the Arrhenius relationship at approximately 35°C in a similar fashion than in a purified lignin peroxidase L2 from *P.chrysosporium*(10). The activation energy determined from linear portion of Arrhenius plot at 25-35°C are exhibited on **Table IV.**

Table IV. Activation energy values of the thermal deactivation of lignin peroxidases from *C sitophila*. (a).

Enzymes	Optimum pH	E_{act} Kcal/mol	(KJ/mol)
LIG-I	3.0	11.9 ± 0.5	(49.8 ± 2.1)
LIG-II	5.0	13.7 ± 1.1	(57.3 ± 4.6)
LIG-III	4.0	12.6 ± 0.3	(52.7 ± 1.3)
Culture Ultrafiltered	3.0	12.3 ± 0.6	(51.5 ± 2.5)
Lignin Per.L2	3.0	10.5	(43.9)(Ref.10)

(a) At the same conditions on Table I.

Tables V show the decay constants of the lignin peroxidase under different pH's values. All the kinetics were first order at the studied conditions (not showed).

Table V. Decay constant (kD x 10^2 [h^{-1}] at different pH's (a)

pH	3.0	3.5	4.0	4.5	5.0	5.5
LIG-I	9.2±2.3	8.5±1.2	14.6±2.2	-	-	-
LIG-II	-	-	-	-	8.8±2.3	21.7±3.0
LIG-III	-	-	5.8±1.1	11.3±0.5	16.8±2.8	-
Culture ultra-filtered	7.8±1.0	-	13.9±1.7	-	27.4±2.5	-

(a) At 28°C.

Under the condition used for this test, lignin peroxidases deviate from the Arrhenius relationship at approximately 35°C. The same phenomena occurs with purified lignin peroxidase L2 from *P.chrysosporium* (10). Contrary to lignin peroxidase from *P.chrysosporium*, lignin peroxidase from *C.sitophila* did not increase the stability at higher pH. They were more stable at their optimum pH's. The evaluation of these enzymes was compared with that of ultrafiltered culture from the same bioreactor batch. The similarities of the pool of enzymes present in the culture with the isolated lignin peroxidases was evident. All of these results will be important into choose optimal reaction conditions in immobilization processes and to select conditions of pH and temperature to suggest its use

in biological delignification or effluent treatments.

Immobilization Process. The percentage of activity retained on immobilization for each system were the following: HRP-Seph (38%), Lig I-Seph (2%), Lig III-Seph (6%) and liophilized-culture-Seph (1%).

No optimization process to enhance the retention of the activity in the support were attempted. The best activity retention was with HRP and then with LIG-III. Unfortunately the amount of LIG-II in these experiments was not enough for immobilization, but actually the experiment is in progress.

Utilization. **Table VI** shows the percentage of effluent decolorization in the free and in the immobilized forms on Sepharose 4B activated by CNBr.

Table VI . Percentage of decolorization of Kraft effluent (a)

Effluent and enzymes	Time (h)			
	0	24	48	72
HPP (0.06U)	0	14.4	20.2	25.0
HRP-Sepharose (0.06U)	0	26.6	53.5	61.3
LIG-I (0.03U)	0	2.0	2.0	2.0
LIG-I-Sepharose (0.03U)	0	16.9	16.0	2.0
LIG-III (0.10U)	0	10.2	13.3	30.7
LIG-III-Sepharose (0.10U)	0	21.6	38.4	45.7
Lyophilized fungal culture (0.03U)	0	5.4	6.4	2.0
Lyophilized fungal culture-Sepharose (0.03U)	0	27.2	29.3	11.4
Hydrogen peroxide (5mM)	0	2.0	2.0	2.0

(a) 10 mL effluent pH 5, hydrogen peroxide 5mM at 25°C.

LIG-I and in lyophilized culture at 72 h the colour was recovered. In HRP a 2.5 fold increase of decolorization with the immobilized enzyme was obtained. LIG-I did not decolorized the effluent at the concentration studied, but significant decolorization in its immobilized form was observed. In Lig-III a 2.9 fold and 1.5 increase over the free form was found at 48h and 72h respectively,. In the immobilized lyophilized culture a 5 fold at 24 h and then a progressive decrease in the decolorization was found. Then all the immobilized enzymes were more efficient that the free form at the studied conditions. The molecular weight distribution in the Kraft effluent during the incubation with lignin peroxidases, horseradish peroxidases and lyophilized fungal culture free and immobilized in Sepharose 4B shows that in free HRP a repolymerization occurs with 16%

of polymer consumption at 24h. After that chlorolignin was polymerized during the first day of treatment and that the newly formed hig-molecular weight material started then to be degrade (48h). On contrary in HRP Sepharose a 61% and 79% polymer consumption was observed at 24h and 72h, respectively, with no low molecular chlorolignin fragments or repolymerization materials. The molecular weigh in lyophilized fungal culture and in immobilized lyophilized culture show that in the first and second case a 13% and a 24% respectively, of polymer consumption without repolymerization occurred at 24 h.

LIG I free and immobilized show a polymer comsumption of 12% at 24h and 37% and some depolymerization at 24 h respectively occurred. LIG-III free and immobilized show a polymer comsumption of 22% at 72h in both cases but in L-III immobilized was found also a slight depolymerization at 72h.

The repolymerization of HRP was completely eliminated by immobilization. No polymerization of chlorolignin was observed in any lignin peroxidase. A more selective depolymerization in LIG-I and LIG-III was observed.

CONCLUSIONS. In summary the strategy for enzyme utilization on Kraft effluent is the immobilization of purified enzymes or the lyophilized culture, being the latter more convenient in an industrial purpose.

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PHOTOCHEMICAL PRE-TREATMENT OF KRAFT EFFLUENT: BIO-BLEACHING WITH Lentinus edodes.

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ABSTRACT

In a screening of 51 strains which decolorize kraft mill effluent, Lentinus edodes UEC 2019 demonstrated to be the most efficient one. In general, mycelial pellets adsorbed around 13 % of liquor chromophores and over 60 % were oxidized without any external carbon sources. A decrease of Chemical Oxygen Demand (COD) (60 %) was found.

On the other hand, kraft effluent was oxidatively decomposed efficiently by irradiation in the presence of a semi-conductor (ZnO). Under continuing illumination, the solution became colorless and the molecular weight distribution indicated almost total mineralization in 2 h. The effluent pre-irradiation (10 min) followed by treatment with a pellet of fungal culture efficiently enhanced the effluent decolorization in 30 % after 48 h.

INTRODUCTION

The pulp and paper industry produces over 700 billion gallons (3 trillion liters) of colored water annually. These colored effluents are originated by lignin degradation products produced during pulp bleaching (1). The first alkaline extraction stage (E1) bleach plant effluent is the major source of color of the effluents from pulp and paper industry. The main contributors to the colour are polymeric, chlorinated, and heavily oxidized degradation fragments of lignin. Moreover their high chlorine content, 3 kg of lignin bound chorine/ton. of bleached paper (2), is highly toxic. Most microorganisms cannot attack lignin or its polymeric degradation products and, as a result, the colored and toxic materials from bleach plants are largely released in an almost unmodified form (3).

A number of colour removal methods have been developed, such as lime coagulation, rapid land infiltration, membrane processes, and polymeric adsorbents. These

processes have operational difficulties and are very costly (from $ 1.90 to $ 6.50 dollars per metric ton. of pulp produced). Conventional bacterial water treatment processes are relatively ineffective in removing these pollutants (3). However, white-rot fungi, a limited group of basidiomycetes and some ascomycetes, possess an active ligninolytic system, which is able to degrade protolignin as well as heavily modified lignins, such as kraft lignin and chlorinated lignins. Phanerochaete chrysosporium is reportedly capable of at least partially degrading a wide range of organic pollutants, including pentachlorophenol and chlorolignins (4). Previus work in our laboratory with kraft E-stage effluents treated with the Lentinus edodes strain showed a relatively large chemical oxygen demand (COD) decrease. The colour and the molecular mass were also removed. In the same conditions, L. edodes was more efficient than the known P. chrysosporium BKM-F-1767 strain (5). Another alternative treatment method interesting for organic pollutants in water is chemical oxidation catalyzed by the ultraviolet (UV) (6,7,8). Thus, the aim of this research was to develop a method to increase the efficiency in the treatment of wastewater, combining photochemical and microbial technologies.

MATERIAL AND METHODS

STRAIN. Lentinus edodes strain UEC-2019 (UNICAMP, S.P., Brazil) was used. Stock cultures were maintained on 1.25 % malt agar plates at 5°C.

CULTURE CONDITIONS. Culture of strain was grown in agitated conditions for ligninase and phenoloxidase production, using malt extract as carbon source, in 125 Erlenmeyer flasks at 25 °C, in the dark. A culture medium (50 ml) malt liquid at pH 5.0 was used.

ENZYME ASSAYS. Ligninase activities (9) were measured by UV spectroscopy of veratrylaldehyde formed by oxidation of veratryl alcohol (expressed as U/L). Phenoloxidases (Laccase and Peroxidase) were measured using syringaldazine as a substrate in the absence and presence of H_2O_2 respectively (10). Beta-Glucosidase were measured using pNPG as a substrate (11). Mn-Peroxidase was determined by oxidizing phenol red in the presence of H_2O_2 and Mn(II) (12).

EFFLUENT. The effluent (RIGUESA, S.C, Brazil) used in this study was the first alkaline extraction stage (pH 9.5 - 13.0) from a bleached kraft pulp (Eucalyptus) and paper

mill.

MEASUREMENT OF COLOR IN TREATED KRAFT EFFLUENT. Color removal by fungi strain during incubation was measured according to the CPPA method, initially absorbance 0.726 (13). The mycelial pellets (2 g of wet mycelium, 97 % humidity) were inoculated with 25 ml of non-sterile effluent (pH 5.0) at 25°C and 150 rpm in the absence of any additional carbon or nitrogen source. The effluent was diluted and an initial absorbance of 0.44, was used.

DECOLORIZATION WITH DEAD MYCELIUM. The wet mycelium was autoclaved for 30 min at 120 °C, and the experiment was carried out as described for live mycelium.

COD DETERMINATION. The Chemical Oxygen Demand (COD) was determined by standard methods (14).

MOLECULAR MASS DISTRIBUTION. 0.5 ml of the clean effluent supernatant was applied to a 60 x 1.5 cm Sephadex-G-50 column equilibrated with NaOH-LiOH 0.1 N. 2.5 ml were collected (Flow rate of 2 min 15 s/ml) and the chlorolignins were determined at 280 nm (15).
SUGAR DETERMINATION. The carbohydrate determination of kraft effluent was carried out by the DNS procedure (16).

PHOTOCHEMICAL PRE-TREATMENT: The reaction was carried out in a beaker containing 25 ml of effluent, at on initial pH value of 5.0, and adequate ZnO photocatalyst from Aldrich (50 mg). The solution was irradiated from the top using a 250 W Phillips lamp (fluence rate , 108 kJ m^{-2} s^{-1} at 254 nm). During the illumination, the solution was magnetically stirred and bubbled with oxygen (17).

RESULTS AND DISCUSSION

Previous research in our laboratory with Lentinus edodes showed that color removal from phenolic industrial effluents was 73 % in 5 days followed by a significant COD reduction (60 %) (5).

We report here a comparative study between two treatment methods for effluent: first, biological alone and a second one with photochemical pre-treatment followed by biological treatment. In both systems pellets of fungal culture (L. edodes) inoculated in the effluent were utilized, without any additional carbon source and non-sterile conditions.

The molecular mass distribution of treated effluent in two systems shows that degradation to lower molecular

weight and high molecular weight polymer occurs. A biomass loss of 70 % in biological systems and 79.5 % in the pre-treatment systems (measured as peak area and compared with the control sample) were found. In addition, the results of these investigations showed that L. edodes have the necessary enzymatic system (TABLE I) to degrade chlorophenolic compounds from effluent, reducing apparently acute toxicity by depolymerization and dechlorination of the compounds. The mycelial pellets adsorbed around 13 % of liquor chromophores.

Both systems, reach a maximum color reduction in 72 hours. However, the second method is more efficient (30 %) than the first one. A COD reduction after treatment for of 120 hours of 68.5 % and 60.0 % respectively (TABLE II) was also observed.

The pre-treatment enhanced the biodegradation of wastewater. Larger pH decreases in the effluent with photochemical pre-treatment was observed. On the 5^{th} day enzymes activities were measured (TABLE I), and market differences were observed. In the biological system, laccase, peroxidase and beta-glucosidase were higher than in the pre-irradiated effluent which exhibits high levels of ligninase and Mn-Peroxidase activities but little phenoloxidase and beta-glucosidase activities. The pre-treament might change the effluent chemical characteristics with a subsequent susceptibility for ligninolytic attack. More studies are still needed to assess the possibilities refering to the mechanisms of action of this fungi. Recently, Michael et al. (18) showed that extracellular peroxidases (Li-P and Mn-P) from Phanerochaete chrysosporium are important for the decolorization of effluent. Mn-Peroxidase plays a more predominant role in this decolorization process. The change caused by pre-treatment in the effluent can modify the enzymatic system increasing the activity of some

TABLE I. Treated effluent, after 120 hours, with L.edodes: Enzyme Assays.

Effluent	Laccase (U/L)	Peroxidase (U/L)	LiP (U/L)	Mn-P a	β-Glu (U/L)
Biological Treatment	11.0	0.5	19.3	0.003	14
Photochemical Pre-treatment	1.0	0.0	78.3	0.01	6

a) $Abs.min^{-1}mL^{-1}$

TABLE II. Treated effluent , after 120 hours with L. edodes.

Effluent E1	Decol. (%)	E[a]	COD (mg O_2/l)	COD Reduction (%)
Control	-	-	259.0	-
Biological Treatment	73	0.97	105.0	60.0
Photochemical Pre-Treatment	78	1.30	81.5	68.5

a) Efficiency (% decol./mycelial dry mass (mg))

specific enzymes.

In summary: This study shows the potentialities of the combined photo- and biobleaching procedure for industrial effluent treatment.

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LIGNIN BIODEGRADATION PRODUCTS FROM *Pinus radiata* DECAYED BY THE ASCOMYCETE *Chrysonilia sitophila*

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ABSTRACT

Two heavily degraded lignins were extracted from 3 months decayed *Pinus radiata* by *Chrysonilia sitophila* with dioxane (HDLD) and methanol (HDLM). Functional groups, molecular weight distribution and spectral characteristcs of these lignins were determined. $C\alpha$ -$C\beta$ and β-O-aryl cleavages were postulated based on new saturated carbons and carboxylic acids observed in the side chain of HDL's . Ring openning, $C\alpha$ -$C\beta$ reduction and quinone formation were suggested by U.V. and I.R. analysis of HDL's.

INTRODUCTION

Perspectives for technological applications of wood biodegradation have contributed to increase the knowlegement of the chemical, biological and physics aspects of wood decaying. The mechanisms involved in lignin biodegradation during wood decaying by Basidiomycetes have been extensively studied. There are some typical reactions clearly demonstrated to lignin degradation such as oxidative cleavage of side chain of lignins, β-aryl-ether cleavage and openning of aromatic nuclei (1,2).

Now we report the study of lignin degradation during wood decaying by an Ascomycete, *Chrysonilia sitophila*. This Ascomycete degrades rice hull (3), *Pinus radiata* bark products (4), cellulose (5) and also produces ligninolytic and cellulolytic enzymes in liquid cultures containing glucose as carbon source (6). Purified enzymes of this fungus were similar to the ligninases extracted from *Phanerochaete chrysosporium* (7). Recently Nilsson et. al. (8) also reported that some higher Ascomycetes caused significative degradation of birch wood.

This paper shows the characterization of two heavily degraded lignins obtained by extraction of 3 months decayed *P. radiata* with dioxane (HDLD) and methanol (HDLM).

EXPERIMENTAL

Wood biodegradation: Sawdust of *P. radiata* D. Dom 30 years old was degraded during three months in semi-solid cultures by the Ascomycete *C. sitophila*. In order to obtain the heavily degraded lignins the residual decayed wood was treated as reported by Chen et. al. (2). Decayed wood was extrtacted progressively with petroleun ether, chloroform, acetone, methanol and 96% dioxane in a soxhlet apparatus. The three first extracts were not studied and contain fatty acids and resins as reported by Chen et. al.(2). Methanol and dioxane extracts were evaporated and yielded the heavily degraded lignins methanol (HDLM) and dioxane (HDLD), respectively.

Lignin characterization: Lignins studied were characterized by molecular weight distribution (9), elemental analysis and by functional groups such as methoxyls (10), phenolic hydroxyls (11) and carbonyls (12) determinations. Also were obtained U.V.and I.R. spectra of the lignins. Acetylated samples were analysed by ^{1}H-NMR and ^{13}C-NMR spectroscopy (13).

RESULTS AND DISCUSSION

We compare the structural characteristcs of lignins from decayed wood with a control milled wood lignin (MWL) obtained from undecayed *P. radiata*. Phenyl propane formula, molecular weight and functional groups content of the lignins are showed in table 1.

The low molecular weight observed in HDLM and HDLD has indicated that they are fragments produced by decaying process. Phenyl propane formula showed incorporation of oxygen and hydrogen in HDLM and only hydrogen in HDLD.

TAB. 1 - Phenyl propane (C_9) formula, molecular weight and functional groups content of the lignins from decayed *Pinus radiata*

	LIGNIN SAMPLE			
	MWL control	MWL from decayed wood	HDLD	HDLM
C_9 formula	$C_9H_{10.3}O_{4.1}$	$C_9H_{9.9}O_{3.6}$	$C_9H_{12.2}O_{4.0}$	$C_9H_{13.4}O_{4.9}$
M_w	13175	15030	867	753
M_n	5882	7141	693	537
M_w/M_n	2.34	2.10	1.25	1.40
Ph-OCH3 (%)	12.2	11.7	n.d.	n.d.
Ph-OH (%)	2.1	2.1	2.2	1.4
α-C=O/OCH3	0.063	0.075	0.060	0.052
γ-C=O/OCH3#	0.021	0.025	0.026	0.015

(#) = γ-C=O cojugated with α-β unsaturation.

Table 2 shows the U.V. and I.R. data from lignins spectra. A significant decrease in absortivity at 280 nm in HDLD and HDLM was observed. This result probally can be related with openning ring reactions occured in these lignins. As the bands at 1730 and 1660 cm^{-1} increased in HDLM and HDLD spectra(table 2) and carbonyl groups content decreased in these lignins (table 1) we have postulated that substructures with carboxylic acids were produced by wood decaying process. New signals at 171.4 and 170.8 ppm in ^{13}C-NMR spectra of these lignins (table 4) also indicated the formation of carboxylic acids. The decrease of phenolic hydroxyl content and the increase in the 280/405 nm ratio in HDLM (table 1 and 2) probally was related to quinone formation in this lignin.

TAB. 2 - U.V. and I.R. data from spectra of the lignins from decayed ***Pinus radiata***

	LIGNIN SAMPLE			
	MWL control	MWL from decayed wood	HDLD	HDLM
U.V. DATA				
WAVELENGHT (NM)		ABSORTIVITY (L/g.cm)		
280	18.9	17.6	13.8	10.2
310	9.7	8.8	7.4	6.2
405	0.3	0.5	0.7	0.5
		ABSORTIVITY RATIO		
280/310	1.9	2.0	1.9	1.6
280/405	59.1	36.9	20.1	20.9
I.R. DATA				
WAVENUMBER (cm^{-1})		ABSORBANCE RATIO		
1730/1510	0.24	0.24	0.34	0.76
1660/1510	0.37	0.40	0.51	0.78

Table 3 shows the $\%^{1}H$ in lignins calculated from ^{1}H-NMR spectra. Decrease in protons content from aromatic and α -vinyl chemical shift range (7.3-6.3 ppm) was observed in all degraded lignins. This result can be related with ring openning reactions ocurred in wood decaying as also indicated by low absortivity at 280 NM of these lignins. The increase of protons in region of CH_2 bonded to carbons (1.6-0.0 ppm) can be related with reduction reactions ocurred in the side chain of lignins.

TAB. 3 - Integrations of ^{1}H-NMR spectra of the lignins from decayed *Pinus radiata*

Chemical shift range (ppm)	Type of proton (#)	LIGNIN SAMPLE MWL	MWL from control	HDLD decayed wood	HDLM
		SIGNAL INTENSITY (% ^{1}H)			
7.3 - 6.3	aromatic and α-vinyl	15.5	12.5	13.8	8.4
6.3 - 5.75	β-vinyl and α-1	4.5	3.0	2.4	2.3
5.75 - 5.2	α-3	2.6	2.7	2.4	2.8
5.2 - 2.5	α-2, , and methoxyl	37.5	37.6	35.3	28.3
2.5 -2.1	acetoxy aromatic	6.8	6.3	6.0	6.0
2.1 - 1.6	acetoxy aliphatic	23.3	21.5	24.9	22.5
1.6 - 0.0	C-CH2-C-...	9.8	14.5	15.2	29.6

(#) α-1 = protons bonded to C-α in β-O-4 structures
α-2 = protons bonded to C-α in pinoresinol structures
α-3 = protons bonded to C-α in phenyl-coumaran structures

TAB. 4 - Data from ^{13}C-NMR spectra of the lignins from decayed *Pinus radiata*

CHEMICAL SHIFT (PPM)	RELATIVE INTENSITY(#) LIGNIN				ASSIGNMENT(##)
	MWL CONTROL	MWL FROM DECAYED WOOD	HDLD	HDLM	
171.4	-	vw	m	m	C=O/L-O-CH2-COOH or L-CH2-CH2-COOH
170.8	w	w	vw	vw	C=O acetyl aliphatic 1º
169.7	-	vw	vw	vw	C=O acetyl aliph 2º
167.1	-	vw	vw	vw	C=O/COOH aromatic
151.1	w	w	w	w	C-3/G acetylated
140.2	-	vw	vw	vw	C-1/G-CH2-CH2-L
131.5	vw	m	m	w	C-1/G-etherified
129.3	vw	m	m	w	C-5/G and C- and C- in G-CH=CH-CHO
122.5	vw	vw	w	vw	C-5/G-acetylated and C-β/G-CH=CH-CH2OAc
120.3	w	vw	vw	vw	C-6/G
83.2	-	-	vw	-	C-α/α-ethers
80.0	vw	-	vw	vw	C-β/β-aryl-ether
70.1	-	-	-	vw	C-α/L-CH-OAc
68.3	vw	vw	m	vw	C/-CH-OAc and/or CH2/L-O-CH2-COOH
62.3	vw	vw	vw	w	C-γ/β-aryl-ether CH2/-CH2OAc
56.1	S	S	S	S	OCH3-aromatic
50.7	-	vw	-	vw	C-β/phenylcoumaran and/or B1 structures
42.5	vw	vw	vw	vw	C-α/pinoresinol
38.9	-	m	m	vw	C-α/pinoresinol
34.1	-	-	vw	vw	-CH2-
32.2	-	vw	m	m	C-α/G-CH2CH2CH2OAc
29.7	m	VS	VS	VS	-CH2-/G-C5-CH2-C5-G -CH2-/
27.5	-	-	-	W	-CH2-
24.6	w	m	vw	vw	-CH2- ; -CH3 and/or C-β/L-CH2-CH2-CH3
23.1	-	m	m	m	-CH2-
21.1	VS	VS	VS	VS	CH3/acetyl
14.1	W	W	M	M	CH3-γ/G-CH2CH2CH3

(#) vw = very weak; w = weak; m = moderate; S = strong; VS = very strong.

(##) L = lignin; G = guaiacyl;

In general $C\alpha$-$C\beta$ and β-O-aryl cleavages were postulated based on low molecular weight of HDL's and in new saturated carbons and carboxylic acids observed in the side chain of HDL's. Ring openning, $C\alpha$-$C\beta$ reduction and quinone formation were suggested by U.V. and I.R. analysis of HDL's.
Our work with *C. sitophila* has showed that this Ascomycete degrades lignin in the same way of Basidiomycetes. It has indicated that lignin degradation mechanisms probally are similar for these two class of microorganisms.

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ACTIVATED SILICA AS SUPPORT FOR XYLANOLYTIC ENZYMES

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ABCTRACT.
Macroporous silica gel was obtained by hydrothermal treatment, prepared from sodium silicate and hydrochloric acid, at room temperature. The macroporous silica, sieved in differents particle sizes was selected with a pore distribution between 390-600 Angstroms. This silica gel was examined for its suitability as support materials for xylanase immobilization, catalizing the xylan hydrolysis. The enzyme was coupled to the alkylamine derivatives of the supports by glutaraldehyde. The immobilized enzyme did not extended its optimum pH. Although a high protein immobilization was afforded a low percentage of activity was found. Now, we are optimizing the immobilization efficiency and to increase the activities by changing the quality of pore sizes.

INTRODUCTION.

Inorganic carriers employed in biotechnology are usually chemically modified with bifunctional organosilanes, of which the most important are the trialkoxyamino-alkylsilanes (1). Such modified carriers,specially porous glasses having controlled pore dimensions are widely applied in enzyme immobilization. Taking into account the high applicability of ligninases and xylanases in biotechnology of pulp and paper industry, we have previously applied this support into ligninase and peroxidase immobilization (2). Now we have selected the enzyme xylanase from **Penicillium janthinellum** for the immobilization (3,4). The xylanases were immobilized in a synthesized porous silica. The great advanvantage of this support is that it can be prepared in the variety range of particle size to fulfil flow requirement in the reactor.

EXPERIMENTAL PART.

Chemical,if not mentioned otherwise, they are of analytical grade. Substrates used were oat spelts xylan (Sigma Chem. Co.USA),and p-nitrophenyl-beta-xylopyranoside (Sigma. pNPX).The **Penicillium janthinellium CRC 87-M** culture fitrate was produced according to Milagres and Lacis (4).

The enzyme immobilization was carried out using microporous silica gel. This support was obtained by mixing sodium silicate with HCl, washing the gel formed after 6 h standing at room temperature and drying it. The dried gel was covered with water and submitted to a hydrothermal treatment for 5 h at 250°C. The treated silica was washed, dried and sieved before stored. The carrier has the following characteristics: Hg penetration of 0.36 (cm^3/g) Hg retention of 23%, medium pore diameter 460 Angstrons and pore distribution (>70%) between 390-600 Angstrons. The silanization was performed by immersing the dried support in a 2% solution of gama-aminopropyl-trimethoxy-silane, filtering the excess of liquid and drying the wet solids at 50°C for 18 h. The unreacted silane was washed with water until neutral pH and the alkylamine support dried at 100-110°C.
The alkylamine supports were stirred in 25% aqueous glutaraldehyde in citrate-phosphate buffer at pH 7.0 for 2 h (1) and washed thoroughly with distilled water.
P. **janthinellum** xylanase crude extract was immobilized on alkylamino-derivatized controlled pore glass. Different conditions for coupling, as concentration of protein and silica different, were used. The procedure used for xylanase immobilization was described previously for peroxidase (5).The coupling yield was determined from the difference in optical absorbance before and after immobilization. The coupling of endo-xylanase, beta-xylosidase activities and protein content were determined. Endo-xylanase and beta-xylosidase activities were done in 100 mM potassium phtalate buffer pH 5.5 at 40°C and calculated from the increase in reducing end groups as measured by the DNS assay (6) or from the release og p-nitrophenol from p-nitrophenyl-beta-xylopyranoside as measured spectrosphotometrically at 405 nm (7). One unit of activity is defined as the amount of reducing end groups or p-nitrophenol from the substrate per minute at 40°C. Protein determination was measured according to Bradford (8) with bovine serum albumin as standard.

RESULT AND DISCUSSIONS

The P. **janthinellum** CRC 87-M crude culture was immobilized in a porous silica gel and the resulting preparation was able to catalyse the hydrolysis of insoluble D-xylan and pNPx to simple sugars. The percentage of xylanase and xylosidase activities in their free and immobilized form can be seen on TABLE 1. The pH and temperature of immobilization were the same in all the processes.
As observed, the percentage of the immobilized protein was significant, when compared with the not bounded, but the level of attachment for xylanases and beta-xylosidases was

much lower. Probably these low activities of immobilized enzyme reflect an unappropiate bind between the carrier and the xylanases, interfering with the catalytic function of the enzyme.

TABLE 1. IMMOBILIZATION EFFICIENCY OF XYLANOLYTIC ENZYMES FROM PENICILLIUM JANTHINELLUM WITH ACTIVATED SILICA (pH 7, 25°C)

	Protein (%)	Xylanase (%)	Xylosidase(%)
Immobilized	40.4	1.3	5.4
Free	59.6	55.9	72.7

The activity of endo-xylanase was determined also in the presence of different protein concentrations. It can be seen (TABLE 2) that an increase in protein content in up to five times placed a xylanase activity only one and half times higher.

TABLE 2. GLUTARALDEHYDE COUPLING OF XYLANASE ON SILICA (50 mg,34 mesh). EFFECT OF PROTEIN CONCENTRATION ON IMMOBILIZATION.

Protein(mg)	Xylanase Activity (UI/g support)
0.41	1.26
0.83	1.52
1.67	1.82
2.08	1.84

An explanation for this fact is that the protein saturation in the porous carrier prevent the internal diffusion of the substrate or another substance through the pore to the active site of the enzyme. Again this is a good indication that the active site of xylanase underwent a serious interference when associated to the carrier support or other reagents. The question if the quantity of carrier is not enough to an efficient immobilization or if there is an unappropiate bind between enzyme and carrier was studied more into detail with defferent carrier concentration (TABLE 3).

The **P. janthinellum** crude filtrate, having 5 mg of protein content, was incubated with different amounts of silica gel. The immobilized xylanase activity was determined and the conversion of xylan to xylose was not linear with the support concentration.

TABLE 3. GLUTARALDEHYDE COUPLING OF XYLANASE (34 mesh). EFFECT OF SILICA CONCENTRATION ON IMMOBILIZATION.

Silica(mg)	Xylanase Activity (UI/g support).
20	2.1
50	1.2
100	0.8
150	0.2

We were expecting in this experiment the same values for the different support concentration, since the protein content was not the limiting factor, as observed in the previous experiments. However, the results indicated that the number of points of binds in support is very important, to a higher efficiency of immobilization, but the most important aspect is the choise of the carrier having groups of binding that not intefere with the enzyme activity.
In order to improve our results, we are purifying the xylanases at an analytical grade using silica with pore dimameter distribution higher than 600 Angstrons will be done, and changing the immobilization method.
The applicability of this process is large, since with free form of xylanases is possible to decrese the extensive use of chlorination the pulp bleaching. Recent results are indicative that this process is able to decrease at the level of more than 50% chlorination. In the immobilized form the xylanase should led to recover the enzyme and to lower the process cost.

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XYLANASE - LIGNINASE BLEACHING SEQUENCY ON KRAFT AND ORGANOSOLV (FORMIC ACID) PULPS

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ABSTRACT

Ligninases and xylanases were applied to the removal of residual lignin from kraft pulps. In a combined xylanase and ligninase delignification a 16% reduction of the original kappa number. The action of ligninases was studies on kraft and organosolv pulps and both pulps had lowers their lignin content.

INTRODUCTION

Enzymes involved in lignocellulosic degradation in situ continue to be of interest for their potential application to process which utilize lignocellulosic substrates. Xylanase, ligninase and the microorganisms which produce them could potentially be applied to the production of hydrolyzate from agro-industrial wastes, nutritional improvement of lignocellulosic feeds, fiber processing and pulp processing. In the later two cases enzyme preparations with low cellulase activity are desireable. In our group we have studied *Chrysonilia sitophila* (1) from which ligninase wich peroxidase activity were isolated. Recently we have isolated xylanases from *Penicillium janthinellum* with low cellulolytic activities (2).

In the present study the effect of the enzymatic treatment, using ligninases and xylanases, was evaluated as the effectiveness to removal residual lignin from pulps.

MATERIALS AND METHODS

Pulps: Commercial *Eucalyptus grandis* unbleached kraft pulp was obtained from Ripasa S.A. Celulose e Papel, Limeira, S.P. Brazil and rinsed with water before used. Organosolv pulp was obtained from treatment of *E. grandis* with formic acid (99%), using a charge ratio of wood/solvent of 1:30 (g/ml) and 0.22% (W/V) HCl at 95%, with mechanical stirring and reaction time of 90 minutes (3). Kappa numbers (4) were determined before and after enzyme treatment.

Enzymes: The production of ligninase by *C. sitophila* has been studied in our laboratory (1). The culture broth was fries modified (pH 6.0), supplemented with glucose 1%. Xylanase was produced by *P. janthinellum* isolated from decaying wood in Lorena, S.P. (2). Media for fermentors contained 0.1% yeast extrat, 2.0% (v/v) salts solution based on Vogel's medium (5), and either sugar cane bagasse hemicellulosic hydrolyzate.

Enzyme Treatments: Pulp was treated with enzyme at 3% consistency for 48 h at 30°C. The pH was maintained at 3.5 for ligninases and 5.0 for xylanases. Following enzyme treatment, a concentration of 1 M NaOH on pulp extraction was used at 2.0% consistency for 60 min at 65°C. In any cases, it was added H_2O_2 (4.66 mM) associated with ligninase treatment.

RESULTS AND DISCUSSION

Unbleached hardwood kraft pulp was treated with ligninases from *C. sitophila*. The crude enzymatic complex constituting the crude filtrate of this fungus contain three haemproteins with ligninase activity (1). Table 1 shows that ligninase causes a significant reduction in Kappa number. Clearly, alkaline extraction associated at ligninase treatment emerged as the most effective treatment in pulps and 8 until 14% decreases in Kappa number were obtained. The efficiency of this process is apparently based upon the saponification of esters of uronic acids associated with hemicellulose chains (6). The effect of saponification is a breaking of crosslinks. Consequently a marked increase in the swelling cappacity and pore sizes occurs. In the same table the assition of H_2O_2 dind't improve the ligninase treatment.

Two kinds of chemical pulps were compared to investigate the effect of ligninase on decrease of Kappa numbers. Table 2 shows the ligninase treatment on kraft pulp and Organosolv. For both pulps a 8% decrease of Kappa numbers were observed. However, the organosolv pulp was treated with six times more ligninase than the kraft pulp treatment. Others experiments will be necessary to evaluate the ligninase efficiency upon these pulps.

Sequential xylanase - ligninase treatments were also performed (Table 3). The ligninases of *C. sitophila* had a detectable effect on kraft pulp when compared with the reference buffer treatments. Compared with ligninases, the effect of xylanase on the Kappa number was the same. However, when the alkaline extraction was associated with xylanase treatment the decrease in Kappa number enhanced. On the other hand, when applied after the hemicellulase treatment, the ligninolytic enzymes seemed to have a positive effect on Kappa number.

Treatment of unbleached kraft pulp with 50 $U.ml^{-1}$ of xylanase for differents periods of time is shown in Figure 1. In 8 h of xylanase reactions, the Kappa numbers reduced from 14.1 to 11.5. When alkaline extraction was associated to the xylanase treatment the Kappa number deccreased to 10.0. Part of this effect is seen without enzyme, due to alkaline extraction alone. In this case, the Kappa number obtained was 11.0. After 8 h reaction time the Kappa number reached a plateau.

The effect of exposure to different concentrations of xylanase on Kappa number is shown in Figure 2. Again the results are shown before and after alkaline extraction. In 24 h reactions, enzyme charges of 25 $U.ml^{-1}$ decreased Kappa number from 16.0 to 12.8 and from 12.0 to 10.0 using xylanase alone or after on alkaline extraction, respectively. Higher concentrations of xylanase dind't improve the treatment.

TABLE 1: Ligninases (20 U/L) treatment and alkaline extraction bleaching.

TREATMENT	KAPPA NUMBER
Untreated	14.8
Untreated (H_2O_2)	14.8
Untreated + E	12.9
Untreated (H_2O_2) + E	11.8
Ligninases	13.8 (7%)
Ligninases (H_2O_2)	14.1 (5%)
Ligninases + E	11.1 (14%)
Ligninases (H_2O_2) + E	10.9 (8%)

E - Alkaline extraction (NaOH 1M)
(H_2O_2) - 4.66 mM
29°C, 24 h, consistency 5%

TABLE 2: Ligninases treatment followed by alkaline extract of eucalyptus kraft and organosolv pulp.

TREATMENT	KAPPA NUMBER	
	KRAFT PULP[1]	ORGANOSOLV PULP[2]
Untreated + E	-	37.2±0.6
Untreated (H_2O_2) + E	11.8±0.3	-
Ligninases (H_2O_2) + E	10.9 (8%)	34.3 (8%)

E - Alkaline extraction
(H_2O_2) - 4.66 mM
1 - Ligninases (20 U/L), 29°C, pH 3.5, 24 h, consistency 5%;
2 - Ligninases (125 U/L), 29°C, pH 3.5, 24 h, consistency 3.3%.

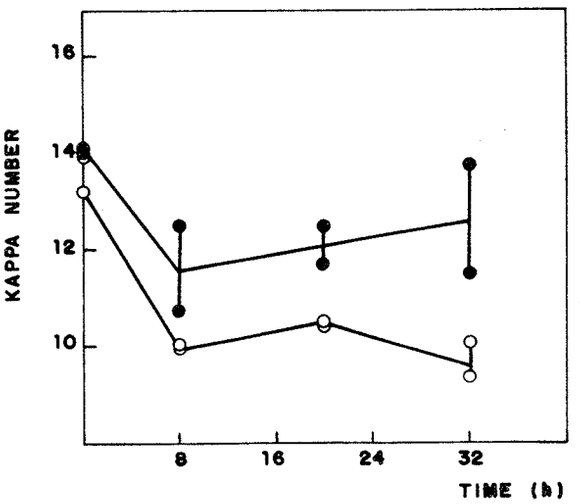

FIGURE 1: The effect of time of expesure to xylanase (50 $U.ml^{-1}$) on lignin removal of kraft pulp. (●–●) xylanase; (○–○) xylanase + NaOH 1.0 M.

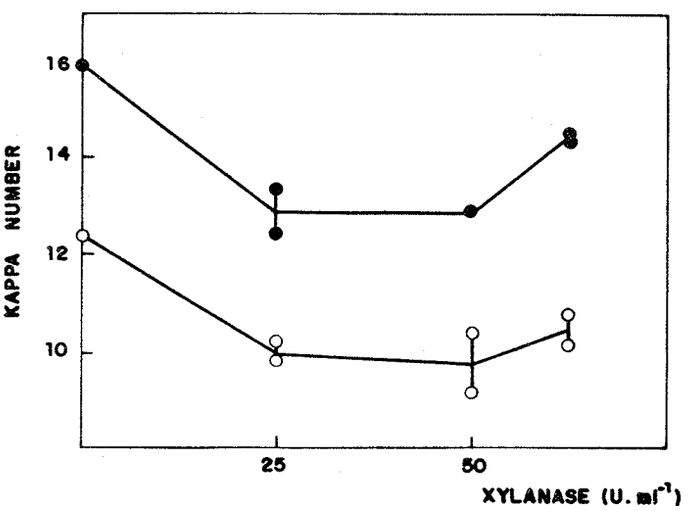

FIGURE 2: The effect of xylanase charge on lignin removal (Kappa number) of kraft pulp.
(●–●) xylanase; (○–○) xylanase + NaOH 1.0 M.

TABLE 3: The effect of xylanolytic and lignolytic enzymes on delignification of eucalyptus kraft pulp.

TREATMENT	YIELD (%)	KAPPA NUMBER
Untreated (H_2O)	97.4	14.1
Untreated + E	90.5	11.0
Ligninase (H_2O_2)	97.1	13.8 (8%)
Lign (H_2O_2) + E	91.1	10.9 (1%)
Xylanase	92.4	12.6(11%)
Xylanase + E	86.5	10.0(9.0%)
Xyl + Lign (H_2O_2)	89.9	11.9(16%)
Xyl + Lign.(H_2O_2) + E	84.3	9.4(15%)

E - Alkaline extraction (NaOH 1M)

30°C, pH 5.0, 48 h, Consistency 3.3%

The values in percentage show the decrease in Kappa numbers upon each control.

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GROWTH OPTIMIZATION OF *Thermoascus aurantiacus*: AN EFFICIENT FUNGUS ACTING ON EXTRACTABLE PHENOLS

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ABSTRACT

The growth conditions of *Thermoascus aurantiacus* were optimized. The horizontal tube method, which relates the advancement of the mycelial frontier culture along the surface of a suitable solid medium was applied. The statistical-mathematical methods of optimization appear as alternative, that permit to examine every possible combination of independent variables at apropriate levels. In this work a two-level factorial designs and simplex search technique were applied.

INTRODUCTION

Utilization of microorganisms in water purification (decolorization of phenolic effluent), development of new bleaching techniques and microbial delignification (biopulping) are processes of increasing importance in the present. Since thermophilic fungi are microorganisms with the ability to carry out the fermentation process at klevated temperatures (Grajek, 1988), the utilization of these fungi in the process above mentioned is of great importance. The thermophilic fungus *Thermoascus aurantiacus*, brazilian strain, has been examined for its ability to produce cellulolytic, hemicellulolytic and ligninolytic enzymes when grown on a variety of substrates (Machuca, 1989). The presence of **Eucalyptus grandis** sawdust seems to induce a phenol oxidase activity in the culture medium by

T. aurantiacus (Machuca, 1991). Analyses of **E. grandis** sawdust treated with this fungus revealed a rapid decrease in extractives and partially removal of lignin. An extractive loss of 64.4 % after 21 days was related to the high phenol oxidase activity (92.7 U/L) (Machuca, 1991).

Improvements in the yield of processes such as enzymes production, microbial metabolites production and wood components degradation could bee achieved through the development of superior strain via mutation. However, other parameters such as the nutritional and physical environment to which a microorganisms is exposed are also known to significantly alter the product yield. That is the principal rason why studing the optimal growth conditions for *Thermoascus aurantiacus*.

Optimization process by the classical method involves changing one independent variable (such as temperature, pH, nutrient, etc.) while fixing the others at a certain level, which frequently does not guarantee determination of optimal conditions. The statistical- mathematical methods of optimization appear as an alternative that permit to examine every possible combination of independent variables at appropiate levels (Greasham and Inamine, 1986).

This communication attemped to define a simple and economically attractive medium that supports optimum growth of *T. aurtantiacus*. Statistical methods of optimization such as factorial designs and simplex search technique will be utilized. Carbon and nitrogen sources concentrations, pH and cultivation temperature will be optimized.

MATERIAL AND METHODS

MICROORGANISM : *Thermoascus aurantiacus*, a brazilian strain, was isolated from **Eucalyptus** sp. chips piles as previously described (Auer et al., 1986). Stock cultures were maintained in Czapek-glucose plates at 5°C.

CULTURE CONDITIONS : The cultures were carried out in Czapek medium containing (g/L): agar-agar, 15.0; K_2HPO_4, 1.0; $MgSO_4$, 0.5; $CaCl_2$, 0.3 and trace elements, 0.1 ml/L. The differents sources of carbon and nitrogen were added to the medium at concentrations of 1.5% (w/v) and 24 mEq N/L, respectively.

GROWTH MEASUREMENTS : The choice of the best carbon and nitrogen sources and the study of the effect of temperature, pH and carbon and nitrogen concentration were evaluated applying the horizontal growth method (Tube method) (Ferraz and Duran, 1989). Standard growth tubes (40 cm of lengths) cointaining Czapek-agar medium were used. After sterilization, inoculations were made by transferring mycelia to the medium at one end of the growth tube. The position of the advancing mycelial frontier was measured at convenient time intervals, and a linear response was obtained.

STATISTICAL TREATMENT : The sequence factorial designs and simplexes procedures were applied.

Factorial designs (Strange, 1990): Two factorial designs requering $2^4 = 16$ runs with each variable at low (-) and high (+) level were used. Four independents variables at two levels were studied: glucose concentration, $NaNO_3$ concentration, temperature and pH. A central or reference point based an the literature was fixed.

Simplex technique(Press et al., 1987): A simplex optimization procedure that consist in a geometric figure defined by experimental points equal to one more than the number of independent variables (N+1, with N= 3). The optimum response is achieved by moving the simplex in the direction of improved response utilizing a computational routine denominated AMOEBA.

RESULTS AND DISCUSSION

1) CHOICE OF CARBON AND NITROGEN SOURCES

The optimal sources of carbon and nitrogen for the growth of *T. aurantiacus* were glucose and $NaNO_3$, respectively. Satisfactory rate of grwoth were obtained when lignin-containing media such as **E. grandis** sawdust, rice hull and bagasse were utilized (Table 1)

TABLE 1: EFFECT OF VARIOUS CARBON AND NITROGEN SOURCES ON THE RATE OF GROWTH OF *Thermoascus aurantiacus*

NUTRIENT	RATE OF GROWTH(mm/h)
CARBON SOURCE (1.5%)	
Glucose	3.98 ± 0.04
Manose	3.40 ± 0.50
Arabinose	3.22 ± 0.40
Xylose	3.17 ± 0.03
Rice hull	2.98 ± 0.20
Eucalyptus grandis sawdust	2.48 ± 0.08
Saccharose	2.29 ± 0.07
Sugar cane bagasse	2.06 ± 0.10
NITROGEN SOURCE (mEq N/L)	
$NaNO_3$	3.22 ± 0.40
NH_4NO_3	2.83 ± 0.30
$NH_4H_2PO_4$	2.52 ± 0.05
$(NH_4)_2SO_4$	2.34 ± 0.07

2) OPTIMIZATION OF RATE OF GROWTH THROUGH STATISTICAL METHODS

FACTORIAL DESIGNS

Identification of an optimum region for *T. aurantiacus* growth was done through successive application of two

factorial designs (2^4).

TABLE 2: DEFINITION OF VARIABLES AND LEVELS FOR 2^4 FACTORIAL DESIGNS

VARIABLE	REFERENCE POINT*	1st F.D. LEVEL (+)	1st F.D. LEVEL (-)	2nd F.D. LEVEL (+)	2nd F.D. LEVEL (-)
Temperature (°C)	50.0	55.0	45.0	53.0	48.0
pH	6.80	7.80	5.80	7.30	6.30
Glucose (%)	1.00	1.50	0.50	1.20	0.70
$NaNO_3$ (mEq N/L)	24.0	44.0	4.00	34.0	14.0

* Reference point defined with basis in the literature (Tansey, 1971; Rosenberg, 1975)

TABLE 3: RESULTS OF FIRST AND SECOND 2^4 FACTORIAL DESIGNS

	T*	pH	C	N	RATE OF GROWTH (mm/h)
Reference Point					3.93 ± 0.2**
1st F.D.	-	-	+	+	3.46 ± 0.04
	-	-	+	-	2.91 ± 0.01
	-	-	-	-	2.95 ± 0.01
	-	-	-	+	3.49 ± 0.01
2nd F.D.	-	+	+	+	3.07 ± 0.04
	-	-	+	+	3.91 ± 0.05
	-	-	+	-	3.87 ± 0.04
	-	-	-	+	4.05 ± 0.06

* T= Temperature (°C), C= Glucose (%), N= $NaNO_3$ (mEq N/L)

** Rate of growth obtained experimentally

TABLE 4: CALCULATED MAIN EFFECTS AND INTERACTION FOR THE FIRST AND SECOND 2^4 FACTORIAL DESIGNS

EFFECT	ESTIMATE ± Standar error 1st F.D.	2nd F.D.
MAIN EFFECTS		
Temperature (°C)	-1.17	-1.25
pH	-0.61	-0.92
Glucose (%)	0.26	0.24
$NaNO_3$	0.31	0.07
TWO-FACTOR INTERACTION		
TxpH	0.55	-0.04
TxC	-0.38	-0.12
TxN	0.24	-0.23
pHxC	0.55	0.14
pHxN	-0.27	-0.21
CxN	0.48	-0.18

The results of Table 4 shows that temperature and pH are the variable with a significative main effect on growth. However, the main effect of a variable should be individually interpreted only if there is no evidence that the variable interacts with other variables, otherwise the interacting variables should be considered jointly. In this study a significative interaction effects between TxpH, pHxC and CxN were observed. These effects were absent after second factorial design.

SIMPLEX OPTIMIZATION

The variables pH, glucose and $NaNO_3$ concentrations were selected for applying the simplex method, while the temperature was kept constant at 48°C.

TABLE 5: MOVEMENT OF THREE DIMENSIONAL SIMPLEX

VERTEX	VARIABLES pH	C	N	RATE OF GROWTH (mm/h)
1*	7.3	1.2	34	3.07 ± 0.04
2*	6.3	1.2	14	3.87 ± 0.04
3*	6.3	1.2	34	3.91 ± 0.05
4*	6.3	0.7	34	4.05 ± 0.06
5	6.8	1.45	34	4.20 ± 0.30
6	5.55	1.08	24	4.55 ± 0.05
7	4.68	1.01	19	4.11 ± 0.10
8	6.18	1.01	49	4.34 ± 0.10
9	6.11	0.92	36.5	4.67 ± 0.10
10	6.0	0.78	37.8	4.74 ± 0.02

* Vertices of original simplex were obtained from the second factorial design (Table 3)

Growth optimization for *T. aurantiacus* was realized through statistical methods, which permited to analyze several factors simultaneously in a short time. The rate of growth in the optimal conditions was 4.74 mm/h (Table 6).

TABLE 6: OPTIMAL CONDITIONS FOR *Thermoascus aurantiacus* GROWTH

VARIABLE	OPTIMUM
TEMPERATURE (°C)	48.0
pH	6.0
GLUCOSE (%)	0.8
$NaNO_3$ (mEq N/L)	37.8

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SCREENING OF WHITE-ROT FUNGI FOR ENZYMES INVOLVED IN LIGNIN BIODEGRADATION

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ABSTRACT

One hundred and three fungal strains were isolated from samples of naturally decayed lignocelluloses collected from various places in Brazil, in media containing either Indulin AT or veratryl alcohol or ferulic acid as the carbon sources. Eleven white-rot fungal type strains were selected. Each gave a positive reaction to o-dianisidine, indicating the presence of phenol oxidases. Six strains gave a positive reaction to syringaldazine whereas five strains needed hydrogen peroxide for reaction to occur.

INTRODUCTION

The potential for the use of lignocellulosic materials in bioconversion processes is well recognized (1). The degradation of the lignin barrier is important for the efficient conversion of these materials to useful products. Biodelignification by microorganisms or isolated enzymes is of interest because it may be cheaper than the processes already in use. Four kinds of enzymes have been implicated in lignin breakdown: ligninase, laccase, manganese peroxide and H_2O_2-producing enzymes. Ligninase (lignin peroxidase) is probably the major enzyme, and the production, properties and mechanisms of Phanerochaete chrysosporium ligninase have been extensively

studied (2).

The purpose of the present work was to isolate white-rot fungi from naturally decayed wood and investigate the presence of enzymes involved in the degradation of lignin. Eleven strains have been selected and the presence of phenol oxidases (laccase and/or peroxidase) has been detected. They are now being checked for the presence of lignin peroxidase.

METHODS

Collection of samples and screening of microorganisms

Samples of decayed wood were collected from various regions of Brazil. A portion containing a reasonable quantity of microorganisms was placed in Erlenmeyer flasks (250 mL), containing 50 mL of the following media: mineral solution; yeast extract 200mg/l; thiamine HCL 3.0 mg/L and carbon source 5.0 g/L. Carbon sources were either veratryl alcohol or ferulic acid or Indulin AT, all obtained from Sigma. The pH of the media was always adjusted to 5.0. The inoculated media were left at room temperature for a period of 30 to 40 days. For the screening, 20 g of agar was added to one liter of each medium, placed in Petri dishes and incubated at 30 C, the colonies formed being transferred to

PDA (potato dextrose agar) slopes.

Selection of microorganisms

A preliminary selection was carried out by inoculating the isolated colonies on media containing the mineral solution, and either sawdust (1.5%) plus 0.2% glucose or ball-milled rice straw (1.5%) plus glucose (0.2%), or glucose (0.2%) only, as the carbon sources. After growth at 30 C the colonies were covered with either 0.001 M o-dianisidine for the detection of phenol oxidases (3) or 0.1% syringaldazine (4) for the detection of laccase, or with 0.1% syringaldazine plus hydrogen peroxide for the detection of peroxidases (4). The colonies which presented a pink colouration, which indicates the presence of phenol oxidases (peroxidases and/or laccase), were selected and the hability of lignin peroxidase production by these strains was determined as follows.

Lignin peroxidase production and activity assay

Growth conditions and oxygenation for the production of lignin peroxidase were as previously described (5). Both oxygenated and non-oxygenated conditions were used. The carbon source was 1% glucose plus 0.6% molasses (14). P. chrysosporium ATCC 24725 was used as a standard microorganism. Lignin peroxidase activity was assayed using

veratryl alcohol, a simple lignin model substrate, which is oxidised to the corresponding aldehyde in the presence of hydrogen peroxide (6). The formation of veratryl aldehyde was followed by an increase in absorbance at 310 nm.

Decolorization of molasses' pigments

The decolorization of molasses' pigments during growth of the microorganisms for the production of lignin peroxidase was followed by changes in the spectra (290nm to 400nm) of the culture supernatant. The controls were run for the same time and under the same conditions, except that the carbon source was 0.6% molasses only.

RESULTS

One hundred and three strains were isolated from the samples of naturally decayed lignocelluloses collected from various places in Brazil. These strains are now being tested for the presence of enzymes that are thought to have roles in lignin biodegradation, such as phenol oxidases (peroxidase and laccase), and lignin peroxidase.

Production of phenol oxidases (laccase and peroxidase)

The best growth was observed with sawdust plus glucose as the carbon source. Many of the microorganisms tested were able to grow on this

medium. However, positive results, i.e the production of a pink coloration with either o-dianisidine or syringaldazine only, or syringaldazine with hydrogen peroxide, were observed with only a few strains (eleven). These strains seem to belong to the white rot fungi type. Each gave a positive reaction to o-dianisidine, indicating the presence of phenol oxidases. Six strains gave a positive reaction to syringaldazine whereas five strains exhibited pink colouration only after the addition of hydrogen peroxide to the colonies containing syringaldazine.

Production of lignin peroxidase and decolorization of molasses' pigments

Four strains were chosen for the determination of lignin peroxidase. Two strains (£1 and £3) exhibited colour with syringaldazine plus hydrogen peroxide whereas strains £2 and £4 did with syringaldazine only. Lignin peroxidase activity was not detected in the culture supernatants of the strains selected, but it was present in the culture supernatant of the standard strain, P. chrysosporium ATCC 24725.

Various degrees of decolorization of the molasses' pigments were observed depending on the strain. The absorbances were also found to vary as a function of the wavelength used. However, the

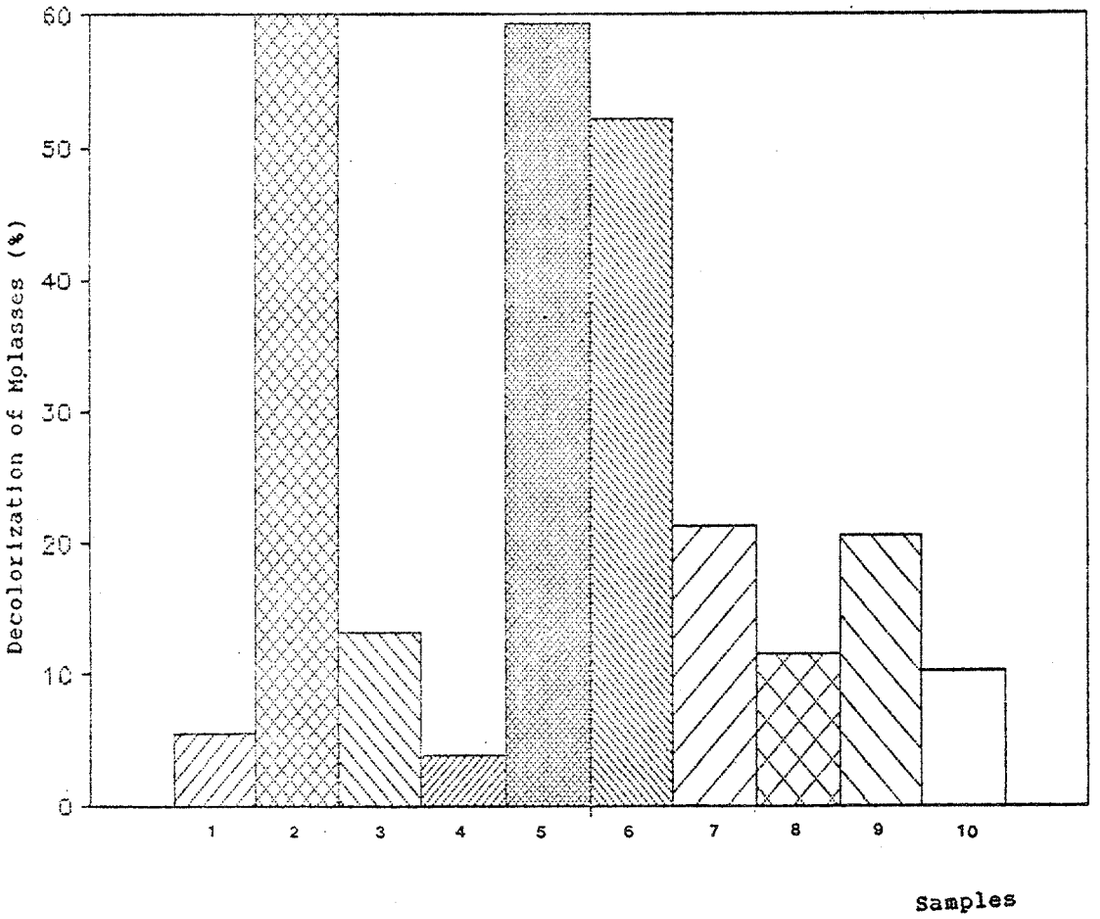
spectra were virtually identical for every control. The absorbance at 400 nm was used to calculate the percentage of decolorization during growth for the production of lignin peroxidase as shown in Fig. 1.

DISCUSSION

Of all microorganisms selected those showing some indication of producing any of the enzymes thought to have roles in lignin biodegradation seem to belong to the white-rot fungi group, which are a kind of wood-rotting fungi, able to degrade the three major components of lignocelluloses, i.e., lignin, cellulose and hemicellulose. The white-rot fungi and related organisms produce a range of lignocellulolytic enzymes when grown on suitable media (7). These enzymes include phenol oxidases, peroxidases, cellulases, hemicellulases and sugar oxidases. Phenol oxidases, either laccase or peroxidase, have been observed in all the strains selected.

No lignin peroxidase activity was detected by the oxidation of the veratryl alcohol assay, in the culture supernatant of the selected strains. However, some decolorization of the molasses' pigments was observed as can be seen in Figure 1. In ligninolytic cultures of P. chrysosporium, the brown molasses' pigments start to disappear at the

Figure 1 Decolorization of Molasses Pigments



Legend to Figure 1

1 P. chrysosporium without oxygenation

2 P. chrysosporium with oxygenation

3 Strain 1 with oxygenation

4 Strain 1 without oxygenation

5 Strain 2 with oxygenation

6 Strain 2 without oxygenation

7 Strain 3 with oxygenation

8 Strain 3 without oxygenation

9 Strain 4 with oxygenation

10 Strain 4 without oxygenation

same time as the appearance of lignin peroxidase (8), the colour removal being proportional to the enzyme activity (8). Thus decolorization is maximum when lignin peroxidase activity is high or the degree of decolorization is dependent on the levels of lignin peroxidase activity present in the culture supernatant (8). The various degrees of decolorization observed in the culture supernatants of the strains selected suggest that lignin peroxidase might be present but was not detectable by the assay used. The role of laccase activity in lignin degradation remains unclear and controversial. However, lignin degradation by a phenol oxidase less mutant lacking laccase was only restored after the addition of laccase (9). Also, it has been reported (10) that a laccase of C. versicolor catalysed not only alkyl-aryl cleavage but also C -C cleavage of lignin models in the same way as lignin peroxidase in lignin biodegradation. It was proposed (19) that degradation may proceed by the cooperation of both lignin peroxidase and laccase.

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SELECTION OF WHITE-ROT FUNGI FOR BIOPULPING

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ABSTRACT

Forty eight white rot fungi were screened for their ability to degrade lignin of *Eucalyptus globulus* and *Eucalyptus grandis*. Phenoloxidase activity, weight and lignin losse was determined. *Poria sp.*, *Stereum sp.* over *E. globulus* ; *Punctularia artropurpurascens* and the strain 5515 for *E. grandis* were the fungi with better lignolytic activity.

INTRODUCTION

Biopolymers constitute a large fraction of the available wood biomass. If gentle and efficient methods were available to degrade the polymer structures into their building blocks, one could start to modify or convert these polymer structures into products of practical interest. White rot fungi are reported for their unique capacity of degrading all wood components (1,2,3). Several researches have demonstrated that some white-rot fungi produce a specific attack on lignin (1,3,4). *Eucalyptus spp.* wood is used in Uruguay for paper pulp production, which is expected to have a certain increase in the coming years and with Brazil are the principal Kraft pulp producers in South America. Nearly 50% of the forested area for industrial purposes corresponds to species of *Eucalyptus*, mainly *E. globulus* and *E. grandis*, with an estimated annual increase of 2,9% during 30 years (5). However, few are the attempts to select fungi with high degrading depolimerization activity in this wood (6,7). Therefore, the screening of other white-rot species, which have the ability to degrade lignin, would be particularly useful in this context, specially those lignin degrading strains for the paper pulp production processes. Moreover several strains of white rot fungi are used in industrial process e.g. biological treatments of wood (8,9,10), treatments of waste effluents generated in pulp and paper production (11,12) and development of animal food sources from lignocellulose (13). The aim of this work is to carry on a primary screening of some white-rot fungi as a survey to achieve microbial delignification of *Eucalyptus spp* wood.

MATERIALS AND METHODS

Fungal strains were isolated from rotting-wood of different plant materials, detached branches, stumps

and standing trees, mainly from *Eucalyptus sp.*, *Pinus sp.*, and some native riparian forests species. Isolates were obtained from fruitbodies and vegetative mycelium associated to decayed wood. They were collected from Uruguay, Rio Grande do Sul (Brazil), and Tucumán (Argentine). Forty eight fungal strains were screened (Table1).

Rate growth

Rate growth of each strain developed on agar-malta was estimated as the diameter of the colony per day.

Enzyme production.

A semi quantitative assay was used to determine the enzymatic activity of the screened strains (14). Strains were

grown on malt-agar (1.25% malt extract, 2% agar) and *Eucalyptus sp.* sawdust-agar (15). The enzymes tested were -laccase, ß-laccase, estearase, phosphatase, peroxidase, tyrosinase and cytochrome-oxidase.

Wood samples and decay.

A total of 1956 wood blocks were cut and polished , 2.5x2x0.5 cm, from *E. grandis* and *E. globulus* and dried at 80 C for 24hs to determine dry weight. Samples composed by 6 wood blocks per plate, with one replicate, were prepared to be decayed on malt-agar and simultaneously inoculated with six inocula alternating with each wood block. Petri dishes were incubated at 27 C and 90 RH, for 30 and 60 days (16). Two noninoculated dishes for each wood were incubated under the same conditions as controls. Degradation of lignin and weight loss were determined in the previously described technique (16,17). Samples for standard deviation (95% confidence interval for the true mean) were calculated for lignin and weight determination. Relative specificity (W/L) of lignin removal was calculated as a rate between the percentage of dry weight loss (W) and percentage of lignin loss (L), in the case with more than 20% of lignin loss.

RESULTS AND DISCUSSION

The tested fungal strains showed a wide range of weight loss in both *E. globulus* and *E. grandis* wood and in different isolates of the same species (5031 and 5054 strains,Table 1). In *E. grandis* wood blocks a weight loss of more than 10% was produced by 20.83% of the strains and in *E. globulus* by 16,66% of the strains, after one month. The number of strains producing a weight loss higher than 10% at two months was greater than at one month, corresponding to 45% of the strains in both woods.

In *E. globulus* ,*Botryobasidium sp.*(5356), showed the lowest weight loss rate 0.26% at 60 days and *Phellinus gilvus* 1.19% at 30 days. The 5534 strain produced the minimun weigth losses 3.10% and

TABLE

STRAIN	DAYS	*Eucalyptus grandis* % Weight loss (a)	*Eucalyptus grandis* % Lignin loss (b)	*Eucalyptus grandis* W/L (c)	*Eucalyptus globulus* % Weight loss	*Eucalyptus globulus* % Lignin loss	*Eucalyptus globulus* W/L	Growth Rate (d)	Enzymatic Reactions (e)
									1 2 3 4 5 6 7
Alleurodiscus mirabilis	30	7.53	20.17	0.37	8.39	31.44	0.26	L	1 2 0 0 1 0 0
MVHC 6376	60	13.14	21.83	0.60	11.86	38.39	0.30		& 4 4 2
Asterostroma sp.	30	2.18	7.37		2.92	8.31		M	4 4 0 0 0 2 3
MVHC 5176	60	3.23	11.07		3.24	29.02	0.11		
Basidiomycete	30	4.27	7.27		3.35	13.43		H	1 1 1 1 2 2 1
MVHC 5370	60	5.61	11.87		6.13	13.8			
Basidiomycete	30	3.86	‡‡		1.93	‡‡		L	4 1 0 0 3 0 1
MVHC 5531	60	5.45	0.30		5.30	0.28			
Bjerkandera adusta	30	1.18	11.18		5.20	13.80		H	0 0 0 0 2 2 2
MVHC 5105	60	4.96	14.88		6.57	22.29	0.29		
Botryobasidium sp.	30	‡	5.19		‡	16.96		M	0 0 0 0 0 0 0
MVHC 5356	60	5.90	10.59		0.26	16.21			
Coriolus sp	30	15.19	12.80		2.14	10.08		H	4 4 3 2 1 0 4
MVHC 5555	60	25.52	17.06		19.52	12.34			
Coriolus pinsitus	30	15.03	15.09		2.10	13.43		H	4 2 1 1 1 0 4
MVHC 5174	60	18.32	40.21	0.45	20.84	36.34	0.57		
Coriolus versicolor	30	6.51	8.17		3.78	8.38		M	4 4 4 4 3 0 0
MVHC 5031	60	20.75	10.14		21.63	11.			
Coriolus versicolor	30	13.51	6.58		9.17	5.70		M	4 4 4 2 2 0 0
MVHC 5054	60	21.35	11.42		20.66	15.92			
Corticiacea	30	6.03	1.63		7.51	‡‡		M	1 1 0 0 0 0 1
MVHC 5503	60	6.92	1.97		7.64	1.52			
Corticiacea	30	6.23	7.16		6.37	11.68		H	1 1 1 1 2 0 4
MVHC 5519	60	8.94	9.34		10.41	15.84			&4 4
Corticiacea	30	2.04	5.36		1.70	9.83		L	3 3 1 1 1 0 3
MVHC 5534	60	3.10	8.55		3.99	11.15			
Corticiacea	30	9.21	12.35		8.81	17.34		H	4 1 1 1 1 2 1
MVHC 5535	60	10.34	13.50		9.76	18.04			
Corticiacea	30	9.81	14.19		13.01	17.75		L	4 4 0 0 3 0 0
MVHC 5544	60	12.71	15.75		16.75	26.00	0.64		
Corticiacea	30	11.74	12.32		10.33	17.17		L	4 4 1 0 3 0 4
MVHC 5515	60	13.04	46.74	0.27	12.10	30.03	0.40		
Corticiacea	30	3.06	4.95		1.68	1.40		L	2 2 1 1 1 0 2
MVHC 5517	60	4.21	14.64		5.53	19.96			
Corticiacea	30	4.34	‡‡		3.04	‡ ‡		M	1 1 1 0 1 2 1
MVHC 5509	60	6.13	‡‡		4.38	‡ ‡			
Corticiacea	30	‡	‡‡		‡	‡ ‡		H	1 2 0 0 3 0 1
MVHC 5510	60	‡	10.02		‡	13.07			

Cont. table 1

STRAIN	DAYS	*Eucalyptus grandis* % Weight loss (a)	*Eucalyptus grandis* % Lignin loss (b)	*Eucalyptus grandis* W/L (c)	*Eucalyptus globulus* % Weight loss	*Eucalyptus globulus* % Lignin loss	*Eucalyptus globulus* W/L	Growth Rate (d)	Enzymatic Reactions (e)
Cyathus pallidus	30	9.40	7.23		10.39	8.85		M	2 2 0 0 4 0 0
MVHC 6396	60	12.37	15.33		11.33	9.27			
Cyathus striatus	30	4.56	8.06		4.45	16.09		M	4 3 1 0 1 0 0
MVHC 6381	60	9.94	18.79		6.52	23.70	0.27		& 2
Dichomytus anectoporus	30	10.94	12.32		12.37	3.86		H	4 4 1 0 3 0 0
MVHC 6377	60	17.31	17.51		17.82	16.75			
Ganoderma applanatum	30	9.70	3.36		7.24	6.10		H	4 4 1 1 2 0 0
MVHC 5347	60	25.04	14.53		22.16	29.11	0.76		
Ganoderma lucidum	30	4.60	17.16		6.42	22.99	0.27	H	4 4 0 0 3 0 1
MVHC 5104	60	4.64	18.90		6.45	27.44	0.23		&4
Ganoderma resinaceum	30	7.62	9.45		7.27	2.65		M	4 4 1 1 4 0 0
MVHC 1009	60	18.38	12.08		14.17	8.43			
Grammothele subargentea	30	2.70	5.43		4.08	9.68			4 4 1 1 2 0 1
MVHC 6401	60	7.36	14.00		10.88	11.35			
Heteroporus biennis	30	4.62	14.05		5.36	14.46		H	4 4 0 0 1 0 1
MVHC 5123	60	7.97	15.23		24.24	15.92			
Hohenbuehelia grisea	30	4.55	11.87		4.87	17.59		M	4 4 0 0 0 0 3
MVHC 5374	60	4.57	14.39		5.03	24.66	0.20		
Panus crinitus	30	5.12	23.04	0.22	3.80	26.32	0.14	H	4 4 1 1 3 0 1
MVHC 5400	60	9.32	25.03	0.37	5.77	34.35	0.16		
Panus tigrinus	30	5.97	8.75		4.37	9.34		H	4 4 1 0 2 0 1
MVHC 5305	60	10.04	19.58		8.92	18.42			
P. chrysosporium	30	8.90	22.35	0.39	3.72	32.69	0.11	H	2 2 2 2 2 1 1
MVHC 5247 BKM 1767	60	14.33	27.89	0.51	9.89	41.84	0.23		
Phellinus gilvus	30	8.55	7.51		1.19	15.26		L	4 4 1 1 4 1 1
MVHC 5142	60	16.91	13.25		5.88	20.62	0.28		
Phellinus punctatus	30	11.20	14.25		11.27	16.20		L	2 2 2 3 1 1 3
MVHC 6388	60	17.46	18.20		14.20	19.79			
Poria sp.	30	8.60	5.58		8.19	4.69		M	4 4 1 0 1 1 1
MVHC 5522	60	9.42	8.20		9.95	14.17			
Poria lignea	30	8.12	13.94		6.04	10.97		M	4 4 4 4 4 1 0
MVHC 5346	60	11.26	15.23		6.92	16.63			& 4
Poria sp	30	2.96	20.03	0.14	2.83	51.04	0.05	L	1 1 0 0 1 0 0
MVHC 5496	60	13.51	25.20	0.53	13.34	53.00	0.25		
Polyporus sp.	30	3.52	‡‡		4.90	‡ ‡		L	2 1 1 0 0 0 0
MVHC 5502	60	9.27	‡‡		6.83	‡ ‡			
Polyporus arcularius	30	3.36	6.47		4.79	7.35		H	4 4 1 1 4 0 1
MVHC 6400	60	4.92	16.61		13.21	9.14			
Punct. artropurpurascens	30	10.56	28.58	0.36	9.02	25.03	0.55	L	4 4 0 2 3 0 1
MVHC 5130	60	15.23	39.31	0.38	15.12	37.68	0.40		

cont. table 1

STRAIN	DAYS	*Eucalyptus grandis* % Weight loss (a)	% Lignin loss (b)	W/L (c)	*Eucalyptus globulus* % Weight loss	% Lignin loss	W/L	Growth Rate (d)	Enzymatic Reactions (e)
Rigidoporus lineatus	30	8.98	5.99		8.07	15.51		H	2 2 2 3 2 1 1
MVHC 5324	60	10.53	6.68		12.82	19.25			
Spongipellis pachyodon	30	5.89	‡‡		5.04	‡‡		M	4 3 1 1 1 1 3
MVHC 5019	60	12.35	10.02		17.10	14.55			
Sp. pulverulentus	30	5.41	12.28		4.21	7.85		H	1 0 3 2 2 1 3
MVHC 5580	60	6.37	13.25		5.48	15.84			
Stereum sp.	30	11.43	16.30		9.63	36.01	0.27	M	4 2 0 1 2 0 4
MVHC 5113	60	13.76	18.34		12.07	54.86	0.22		
Stereum sp.	30	6.28	7.27		4.50	8.18		L	4 1 0 1 2 0 1
MVHC 5532	60	9.71	17.65		7.43	15.00			
Stereum sp.	30	8.88	5.64		8.76	7.52		M	4 4 0 0 4 0 0
MVHC 5524	60	10.96	6.23		12.22	8.60			& 2
Stereum sanguinolentum	30	7.93	13.01		5.69	9.27		M	3 2 0 0 1 0 1
MVHC 5349	60	8.24	13.36		7.61	17.63			
Trametes sp.	30	6.55	2.36		6.85	13.72		M	4 2 1 1 2 0 2
MVHC 5117	60	9.30	6.56		13.40	17.88			& 4 4
Trametes extenuata	30	9.45	10.24		4.15	12.34	0.19	H	4 3 0 0 2 0 1
MVHC 5304	60	6.15	19.03		7.02	21.45			& 4

(a) - Weight losses are based on original and final oven dry weights and expressed as a percentage of the former.
(b) - Lignin losses was determined by the Effland method and expressed a percentage of the former.
(c) - W/L were not calculated for the strains where lignin losses were less than 20%.
(d) - Growth at 27ºC, H (high),>1 cm/day;M (medium),0.5 to 1.0 cm/day;L(low),,0.5cm/day.
‡ , ‡‡ - not determined.
(e) - Enzimatic reactions in malt-agar. Enzymes 1- α- Laccase, 2- β- Laccase, 3- Esterase, 4- Phosphatase, 5- Peroxidase, 6- Tyrosinase and 7- Cytochrome oxidase.
& - Enzimatic reactions in sawdust-agar (only data which presented differences in comparation with the determination in malt-agar are showed)
Untreated wood blocks of E. globulus contain 24% of lignin end E. grandis 28.9%.

Bjerkandera adusta (5105) 1.18% at one and two months respectively in *E. grandis*. The higher weight losses in *E.globulus* ranged from 13.01% produced by the 5544 strain at 30 days and 24.24% for *Heteroporus biennis* (5123) at 60 days.

The maximun weight losses were produced in *E. grandis* for *Coriolus sp.* 5555 at 30 and 60 days corresponding to 15.19% and 25.52%, which were similar for the 5544 and 5123 strains in *E. globulus*. The strain that produced the highest weight loss in *E. grandis* showed lower components remotion activity in *E. globulus*. Moreover approximately 50% of the strains in *E. globulus* , resulted in a low (less than 5%) components remotion at one month.

Variation also existed in the lignin degrading capacity of the same strain over the wood tested (Table 1). In *E. grandis* 25% of the strains produced more than 20% of lignin losses whereas in *E. globulus* were 48%. In *E. grandis* the 5515 strain produced the maximun of lignin losses, 46.74%, at 60 days and *Punctularia artropurpurascens* (5130), 39.31% ,at 30 days. In *E. globulus*, *Stereum sp.*(5113) displayed the highest lignin remotion in boths woods, 54.68% at 60 days. In one month *Poria sp.* (5496) produced in this wood, the highest lignin losses, 51.04%, and showed a low rate of weight loss. The strains that had the most significant lignolytic activity in *E.globulus* presented a reduce activity in *E. grandis* at the same period. *Phanerochaete chrysosporium* (5247,BKM 1767) developed also a good lignolytic activity over *E.globulus* ,32.69% and 41.84%, after one and two months of incubation but an important reduction of this activity in *E. grandis*.

Poria sp. (5496) at 30 days had the highest relative specificity in both woods but the lignin removed in *E.globulus* was 100% more than *E. grandis* at both periods. This double lignolytic activity in *E. globulus* was also achieved by two other strains (5113 and 5176). *Asterostroma sp.*(5176), *Panus crinitus* (5400), *Phanerochaete chrysosporium* (5247), *Poria sp.*(5496), *Stereum sp.*(5113) and *Trametes extenuata* (5304) had also had a good relative specificity in *E.globulus*. In *E.grandis*, *Panus crinitus* (5400), Corticiacea (5515), *Phanerochaete chrysosporium* (5247), *Poria sp.*(5496), *Punctularia artropurpurascens* (5130) and *Alleurodiscus mirabilis* (6376) had a good relative specificity. Generally, the relation of these strains was better in *E.globulus* except for 5130 and 5515 (Table 1).

Enzymatic activity of the strains in malt-agar and sawdust-agar were aproximalety the same. However the same variation appeared in the and β-laccase and citochrome oxidase activities (Table 1). *Poria sp.* (5496), which shown the best lignin remotion, did not present significant enzymatic activity as it is well known for *Phanerochaete chrysosporium*(20). The remainder strains had a high enzymatic activity for both laccase and peroxidase. It has been shown that the presence of these enzymes is

related to the ability of a fungus to effectively degrade lignin (21). The white rot fungi tested removed more lignin from *E. globulus* than from *E. grandis*. This could be due to the chemical and anatomical characteristics of this wood (22,23,24). It was considered that the best suited fungi for selectively lignin remotion in *E. globulus* and *E. grandis* at 30 days were *Poria sp.*(5496), *Panus crinitus*(5400) and *P. chrysosporium* (5247).

CONCLUSIONS

Fungal growth rate, oxidative enzymatic activity, weight and lignin losses seemed to be not correlated with a good relative specificity (18). The strains with weakly positive or negative enzymatic reactions also showed a full range of decay rates and lignin specificity . The inability of certain fungi to oxidize phenols cannot be assumed as an inability to utilize lignin (18,19). However, *Stereum sp.*(5113), *Panus crinitus* (5400) and *Corticiacea* (5515) that had a great relative specificity, also had a high enzymatic activity (21). The results obtained showed that several species of white-rot fungi possesed the ability to decompose lignin in *Eucalyptus sp.*. *Poria sp.* (5496) and *Stereum sp.* (5113) in *E. globulus* , *Punctularia artropurpurascens* and the strain 5515 in *E. grandis* produced the highest lignin remotion. *Poria sp.* (5496), *Phanerochaete chrysosporium* (5247) and *Panus crinitus* (5400) were the fungus that showed a good activity in both woods. *Punctularia artropurpurascens* (5130) that produces asexual spores (14) and had high lignin decomposition ability is an important colonizer of *Eucalyptus spp.* standing trees in Uruguay. This species should be examined more closely and select new strains for lignin degradation and biopulping purpose.

Variation in fungi degradation ability on different substrata has been reported (2,25). A different lignolytic activity of a same strain on the species of *Eucalyptus spp.* tested was also tested. A higher lignin remotion in *E.globulus* than in *E. grandis* was evidenced. It may be due to the fact that *E. grandis* contains a higher ratio of lignin / carbohydrates than *E. globulus* and this could be a restriction to the lignin remotion in *E. grandis* (19,20,21,22). At the same time phenolic compounds from *E.grandis* were reported as inhibitors of mycelial growth (26). Further research, including ecological studies, chemical and micromorphological observations seems necessary when screening basidiomycets for their potential to selectively delignify wood (16,27,28).

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CELLULASES PRODUCTION BY MESOPHILIC AND THERMOPHILIC MICROORGANISMS

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ABSTRACT

Biodegraded cellulosic material collected from various regions of Brazil were cultured on a cellulose-containing medium, and incubated at either 30 C or 45 C. Several colonies with a large clear circle around them were selected and solid state fermentation was carried out. Two mesophilic and three thermophilic strains were selected and exhibited reasonable amounts of cellulose hydrolysing activities after growth on liquid media containing either microcrystalline cellulose or solka-flok 40.

INTRODUCTION

Biomass, carbohydrate containing plant and animal matter, represents an attractive alternate source of energy because it is both renewable and found in various forms, world-wide (1,2). Biomass, in the form of cellulose is the major constituent of plant matter, comprising the most abundant organic resource in the world. Bioconversion, particularly enzymatic hydrolysis, of cellulosic wastes to useful products has great potential and is being actively investigated (3,4,5,6,).

Agricultural and industrial residues, forage and woody crops are significant renewable resources for the generation of fermentable sugars (7).

One disadvantage of enzymatic hydrolysis of

cellulose is the high cost of the enzymes envolved in its degradation and there is now much research effort centered on producing them more cost effectively (8,9). Biotechnological utilization of cellulose requires a very efficient *in vitro* cellulolytic enzyme system active on native cellulose. This system may arise from the combination of enzymes from cellulases hyperproducing microorganisms, obtained by specific selection of truly cellulolytic strains, which are capable of degrading native cellulose.

The present study describes the selection of mesophilic and thermophilic microorganisms which exhibit high yields of cellulolytic enzymes and attempts to maximise the production of the cellulase hydrolysing enzymes.

METHODS

Samples of soil and biodegraded cellulosic materials were collected from various areas in Brazil and placed in plastic bags for transportation. A portion of each sample was placed in a 250 mL Erlenmeyer flask containing 50 mL of sterile distilled water for 24 hours. Inoculation was carried out on a cellulose containing medium (composition given below), and incubated at both 30 C and 45 C.

Cellulose medium: cellulose microcrystalline 5.0 g/L; mineral solution, bengal rose 50.0mg/L; triton X 100 1.0 ml/L and agar 20g/L.

After growth of colonies, the plates incubated at 30 C were transferred to 45C. Colonies exhibiting a clear circle around them were selected and their ability to degrade filter paper was determined by inoculating them in a test tube containing a strip (10x1 cm) of filter paper Whatman N 1 and mineral solution, as described above, sufficient to cover 7-8 cm of the strip (pH 5.5). Incubation was carried out at both 30 C and 45 C for two weeks. The microorganisms able to degrade the filter paper strip in a period of less than 15 days were inoculated in 2 g of wheatbran:water (1:1), for five days at both 30 C and 45 C. Colonies which presented good cellulose hydrolysing activities were chosen for experiments in liquid media.

T. reesei QM 9414 was used as a standard culture.

Extracellular enzyme production in shake flasks

Media composition: carbon source 5.0 g/L; peptone 0.75 g/L; Tween 80 1.0 ml/L; and mineral solution. The pH was adjusted to 5.6. The cultures were incubated at either 30 C or 45 C for a period of 6 days.

Carbon sources used: cellulose microcrystalline (Merck); and solka flok BW 40FCC (James River Corporation).

Enzyme assays

Filter paper degrading activity (FPA) was estimated according to the procedures of Mandels et al. (10). Carboxymethyl cellulose (CMC) was determined by the increase in reducing sugar in 60 minutes from a mixture of 0.5 mL enzyme and 1.0 mL of 1% carboxymethyl cellulose (in acetate buffer), incubated at 50 C. Microcrystalline cellulose degrading activity was carried in a similar way as for CMC activity, using 1% microcrystalline cellulose. One unit of enzyme activity was defined as the number of micromoles of reducing sugars released by one millilitre of enzyme per minute. Reducing sugar expressed as glucose was determined by the DNS (3,5-dinitrosalicylic acid) method (11).

RESULTS

A total of 254 fungal colonies was isolated from the cellulose containing medium. These colonies exhibited a clear circle around them, which varied in intensity from colony to colony. Twenty three strains isolated at 45 C, were able to degrade the filter paper strip in less than two weeks. This was

also observed with seventy mesophilic strains. After solid state fermentation eight thermophilic and nine mesophilic strains exhibited reasonable microcrystalline hydrolyzing activity and were used for growth in liquid media. Table 1 shows cellulase activites in a liquid medium where the carbon source used was micrcrystalline cellulose, whereas in Table 2 the results obtained using Solka Flok BW 40 as the carbon source are demonstrated.

Table 1. Cellulase activities in medium containing microcrystalline cellulose as the carbon source

Microorganisms	Cellulase Activities (umoles glucose/mL/min)		
Classification	CMC	FPA	C1
F 7.14*	0.51	0.65	0.16
F 5.22*	0.50	0.61	0.11
F 5.18*	0.49	0.75	0.18
SG 17.3**	0.37	0.20	0.16
Blu 6.5**	0.42	0.37	0.19
QM 6a**	0.35	0.16	0.09
QM 9414**	0.43	0.35	0.21

* Thermophilic; ** Mesophilic

Table 2. Cellulase activities on Solka Flok BW 40 as the carbon source

Microorganisms	Cellulase Activities (umoles glucose/mL/min)		
Classification	CMC	FPA	C1
F 7.14*	0.50	0.77	0.19
F 5.22*	0.49	0.81	0.23
F 5.18*	0.49	0.75	0.18
SG 17.3**	0.37	0.26	0.15
Blu 6.5**	0.43	0.36	0.19
QM 6a**	0.33	0.26	0.12
QM 9414**	0.40	0.39	0.24

* Thermophilic; ** Mesophilic

DISCUSSION

The three thermophilic microorganisms isolated showed better microcrystalline cellulose hydrolysing (C1) activity when grown on Solka Flok BW 40 as the carbon source. This was particularly true of the strain F5.22, which showed twice as much activity than when it was grown on microcrystalline cellulose as the carbon source. Among the five strains selected F5.22 was the best producer of cellulases, exhibiting cellulase activities (C1, Cx and FP) similar to those of T.

reesei QM 9414. The strain Blu 6.5 also showed good levels of cellulase activity. The strain F 5.22 was able to grow at 45 C and this may offer some advantages for the production of thermostable cellulolytic enzymes and their subsequent use in the saccharification process. The strain Blu 6.5 (mesophile), exhibited faster growth than the strain T. reesei QM 9414 and also produced higher amounts of spores, which might be an advantage for its utilization for classical genetics strain improvement, such as mutation by UV light exposure. The use of other carbon sources, such as sawdust, rice straw and bagasse, and also nitrogen sources and levels are worthy of investigation. Hyperproducing mutants combined with a selected media for high enzyme production may then provide a very efficient cellulolytic enzyme system.

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UTILIZATION OF LIGNOCELLULOSIC MATERIAL: ORANGE WASTE AS CARBON SOURCE FOR SINGLE-CELL PROTEIN

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ABSTRACT

Industrial orange bagasse can be used as a substrate for the production of high quality single-cell protein from the filamentous fungus *Chrysonilia sitophila*. Considerable gain was observed in terms of total protein, methionine and lysine in the product.

INTRODUCTION

With Brazil's leading position as a concentrated orange juice exporter, considerable interest has been given to the more efficient utilization of all by-products from the orange juice industry.

Orange bagasse is the insoluble residue left after the juice, the soluble polysaccharide liquor and essential oils have been extracted. By weight, the solid waste accounts for 40 to 50 % of the fresh fruit. Following neutralization with calcium oxide, the residue is dehydrated and pelletized into an exportable product which is used as cattle feed ingredient. Compositional limitations such as low crude protein and high cellulose content do not permit utilization of this by-product by chickens or other non-ruminants. [1].

It would be desirable therefore to upgrade the nutritional characteristics of the orange bagasse through fungal fermentation. Fungi are known to possess a wide range of hydrolases capable of degrading the complex constituents of most organic wastes [2] and several authors have even proposed the production of single-cell protein using orange waste as a substrate [1,-4].

The choice of fungi for protein enrichment of plant wastes is particularly advantageous because of the existance of cellulolytic species capable of utilizing bulk cellulose, they are obligate aerobes and do not produce the unpleasant odors associated with anaerobiosis, they are good sources of protein bearing low levels of nucleic acids and, in addition, mycelial growth confers them the ability to spread over solid surfaces and penetrate intact tissue without prior treatment.

This paper describes the production and comparative characterization of a protein-enriched orange waste by fermentation with *Chrysolnilia sitophila* (TFB-27441 strain).

MATERIALS AND METHODS

Brown, pelleted orange bagasse, milled to 1 mm particle size, was used as the sole carbohydrate source suspended in Czapek solution (3 % w/v), inoculated with *Chrysonilia sitophila* [5] and incubated in a circular shaker for periods of 10, 20 and 30 days at 30 C. For comparison, a Czapek modified (4 % sucrose) medium was also inoculated and incubated for six days.

The biomass fraction was assayed for total protein by semi-microkjeldahl [6] and amino acids determined in a in sealed tubes with 6N HCl [7].

RESULTS AND DISCUSSION

Fermentation of the orange waste with *C. sitophila* for various periods beyond 10 days resulted in both an increment of the protein content to about two-fold that of the original substrate and a significant improvement of the indispensable amino acid pattern.

The amino acid profiles of the industrial orange bagasse and those of the bagasse plus the biomass developed at 10-day intervals are presented in Table 1. Noteworthy was the improvement of the biological quality due to the increases of the sulfur amino acids methionine/cystine and lysine, nutritionally essential forms of nitrogen, all at the expense of dispensable species such as aspartic and glutamic acids.

Comparing the profiles of the product protein (Table 1) with that of the fungus grown in 4 % sucrose (last column) it can be observed that, in spite of the lysine enrichment in the 30-day fermentation product, the absolute level of this amino acid approached but not reached the content of the mycelium grown in modified Czapek medium. As incubation time increased, the methionine concentration of the biomass approached the characteristically high level of 6.56% found in the last column. Moreover, the slight increase in arginine content from 4.4 to 5.0 (Table 1) represents a significant departure from the characteristic composition of the cell grown in 4 % sucrose, which has a relatively low arginine concentration.

TABLE 1. Amino acid composition of the substrate-fungus (*Chrysonilia sitophila*) protein mixture as a function of fermentation time. Composition of the pure mycelium is shown for comparison.[a]

Amino acid	Orange Waste	10 Days	20 Days	30 Days	Pure[b] Mycelium
Aspartic	10.78	12.04	10.01	8.32	11.66
Threonine	5.20	5.19	5.45	4.96	6.26
Serine	5.55	4.84	5.45	4.63	5.79
Glutamic	14.87	12.53	12.70	11.43	10.63
Proline	5.58	5.51	5.66	5.32	-
Glycine	7.41	6.21	6.00	6.80	6.65
Alanine	4.92	6.75	6.09	6.81	8.64
Cystine	1.05	0.78[c]	2.17	2.46	-
Valine	6.04	6.26	6.02	7.19	8.25
Methionine	0.82	2.56	4.61	5.96	6.56
Isoleucine	8.17	5.36	5.75	5.69	4.57
Leucine	9.81	10.09	9.56	8.78	6.70
Tyrosine	3.39	3.78	3.70	3.90	0.08
Phenylala.	6.89	5.72	5.73	5.44	3.67
Histidine	2.34	1.92	2.00	2.43	2.63
Lysine	2.69	5.03	4.14	4.77	7.08
Arginine	4.48	5.42	4.98	5.00	1.64
Total Prot.	5.30	6.80	-	10.40	- (%)

a. Gram of amino acid per 100g of total amino acid minus tryptophan.
b. Mycelium grown in modified 4% sucrose-Czapek medium.
c. Low recovery probably due to accidental oxidation.

Ammonia levels remained within the range of 3 to 4 % in the mycelium (data not shown). The cell-free filtrate, however exhibited an amino acid profile that resembled the over-all cellular composition except for ammonia which, accounted for 96.4 % of the total nitrogen. Such feature must reflect the microorganism's metabolism in the process of reducing the nitrate ion prior to incorporation and should not jeopardize the nutritional quality of the product for non-ruminants since the soluble nitrogen represents about 1 % of the total.

TABLE 2. Ratios of some amino acids of nutritional significance in conventional protein sources [8] and cultivated *C. sitophila*.

Source	ILE / LEU	TYR / PHE	LYS / HIS	LYS / ARG	THR / SER
Milk	0.65	1.05	2.95	2.12	0.78
Egg (whole)	0.75	0.74	2.66	2.66	0.59
Meat (bovine)	0.64	0.82	2.51	1.35	1.05
Rice	0.54	0.91	2.34	0.68	0.77
Maize	0.35	1.34	1.39	0.82	0.70
Sorghum	0.34	0.55	1.41	0.72	0.71
Wheat flour	0.67	0.46	1.69	0.76	-
Peanut	0.67	0.71	1.45	0.33	0.40
Yeast	0.57	0.95	2.58	1.70	1.04
Orange waste[a]	0.83	0.49	1.15	0.60	0.94
30-Day OW[b]	0.65	0.72	1.96	0.95	1.07
C. sitophila[c]	0.68	0.02	2.69	4.31	1.08

a. Industrial orange bagasse
b. Orange bagasse fermented for 30 days with *C. Sitophila*
c. grown in 4% sucrose-Czapek

Biological quality in a protein can be assessed not only by the absolute amounts of each essential amino acid but also by their internal balance. Isoleucine, for example, should keep a molar ratio not lower than 1:3 with leucine. Threonine is another essential amino acid found in short supply in vegetable proteins and encountered in high quality proteins in almost equimolar ratios. In addition, Table 2 shows that low quality proteins, usually of vegetable origin, are rich in arginine and histidine. in relation to lysine. These data attest to the improvement of the internal amino acid balance throughout fermentation, with the exception of isoleucine/leucine which, nevertheless remains at an acceptable value. The high lysine/arginine ratio of the pure fungus was a result of the unusually low level of arginine found in *C. sitophila*. Likewise, the low content of tyrosine in the fungus was responsible for the extremely

low tyrosine/phenylalanine ratio observed in the 4 % sucrose culture. Low phenylalanine content in novel proteins would be desirable for phenylalanine intolerant individuals.

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