Production and characterization of enzymes for use in a biorefinery by Basidiomycetes and plant pathogenic fungi

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Abstract

Biorefineries for production of several products and by-products such as biofuels, heat and/or electricity have been in focus in recent years. In a biorefinery, biomass can be converted to useful biomaterial and/or energy carriers in an integrated manner and thereby it can maximize the economic value of the biomass used while reducing the waste streams produced. Microbial enzymes, mainly hydrolases and oxidases, play a key role in developing of new technologies for processing and utilization of vegetal biomass. In the recent years several researches have been carried out aiming the prospection of new microorganisms and new enzymes which have potential to be applied in industrial bioprocesses. In this work, it was evaluated the effect of carbon source, time of fermentation and cultivation condition (solid state fermentation and submerged fermentation) on production of endoglucanases, β-glucosidases, xylanases and laccases to four Basidiomycetes strains fungi (*Pycnoporus sanguineus*, *Trametes* sp. J-2, *Trametes* sp. J-5 and an unidentified isolated J-129) and two plant pathogenic strains fungi (*Chrysoporthe cubensis* and *Cylindrocladium pteridis*) isolated from eucalyptus plantation. The highest FPase activity (136 U L^{-1}) was reached by *P. sanguineus* cultivated under solid state fermentation using Kraft pulp as a substrate. *P. sanguineus* was also the best laccase producer and a volumetric activity of 11032 U L^{-1} was observed at submerged fermentation with wheat bran. *Chrysoporthe cubensis* secreted the highest endoglucanase (20335 U L^{-1}) and β-glucosidase (1487 U L^{-1}) activities when cultivated on solid state fermentation using wheat bran. *Cylindrocladium* pteridis was the best xylanase producer and a yield of 142519 U L^{-1} was observed in solid state fermentation employing wheat bran. The optimum pH of cellulases and xylanases produced by strains here studied were in a range of 3.5-6.0; however, the optimum pH to laccases has been found in a range of 2.5-3.5. The optimum temperatures to all enzymatic complexes studied were observed in a range of 50-70°C. These results suggest that the fungal strains studied in this work have a great potential to produce enzymes with adequate features for application in biotechnological processes.

Keywords: fungi, enzyme production, biorefinery

1. Introduction

The use of enzymes in transformation industry is rising day by day and nowadays innumerable industrial processes are carried out employing these biological catalysts. The enzymatic catalysis is more advantageous than chemical routes because it provides higher yields of products, diminution at demand for raw materials and reducing emissions and waste resulting in cost savings and reduced generation of pollutants [1]. Lignocellulolytic enzymes such as xylanases, cellulases and laccases are of fundamental importance for the efficient bioconversion of plant residues and they are prospective for the various biotechnological applications *[2, 3]*

Xylanases are utilized in the food, animal feed, fuel and textile industry; however, the most prominent use of xylanases is in the paper and pulp industry where it is employed as a tool

in biobleaching processes *[3]*.

Cellulases are usually applied in industrial processes such as: paper recycling, juice extraction, biostoning, animal feed additives and others *[4]*. However the most relevant application of cellulase is in biorefinery processes where they are employed to degrade lignocellulosic biomass to simple sugars, which are versatile starting materials for further conversion by fermentation, biocatalytic, and chemo catalytic processes to value-added products, including biofuels, biopolymers and chemicals *[5]*

Laccases are versatile oxidases that when act over lignin (or other aromatic compounds) may display ligninolytic and/or polymerizing (cross-linking) abilities, depending on the conditions under which the reaction is conducted *[6]*. Because of such skills, laccases can be applied in several biotechnological processes such as: biobleaching of Kraft pulp *[7]*, bioremediation of phenolic compounds *[8]* and fiber modification to obtaining functionalized papers *[9, 10]*.

Large amounts of enzymes are required for application in biotechnological processes and the cost for production and acquisition of these biocatalysts are still one of the largest limitations for the expansion and consolidation of enzymatic industrial processes. Although various groups of known lignocellulolytic microorganisms have its enzymatic systems well characterized and some of them are already used in industrial scale, worldwide researches have demonstrated that the activities of new isolate have been comparable or superior to the traditional strains. Nature represents an interminable source of lignocellulolytics microorganisms and especially tropical countries as Brazil, which presents a very diversified microbial flora, certainly shelters species of unknown microorganisms of optimum industrial interest *[11]*.

This paper describes the lignocellulolytic enzymes activities produced by four wood-rot Basidiomycetes and two plant pathogenic fungi isolated from eucalyptus plantation. All fungal strains were cultivated under submerged and solid-state fermentation employing different carbon sources and the results to enzymes production were discussed regarding the fungal strain, carbon source and system of cultivation. Furthermore, all enzymatic activities obtained were characterized in relation of pH and temperature optimal to evaluate the feasibility of using these enzymes in industrial processes

2. Material and Methods

2.1. Materials

Substrates including ρ-nitrophenyl-β-D-glucopyranoside (pNPGlc), carboxymethilcellulose (CMC), xylan from birchwood, 2,2'-azino-bis-[3-ethyltiazoline-6-sulfonate] (ABTS), Avicel PH101 (microcrystalline cellulose) and also the chemical reagents monopotassium phosphate, ammonia nitrate, magnesium sulfate, calcium chloride, cuprum sulfate, sodium acetate, sodium carbonate, dinitrosalicylic acid (DNS) and potato dextrose agar (PDA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Yeast extract was obtained from Himedia Laboratories Co. (Mumbai, Maharashtra, India). Potassium sodium tartrate was acquired from Vetec Fine Chemical (Duque de Caxias, RJ, Brazil). Kraft pulp was kindly supplied by the Pulp and Paper Laboratory of the Federal University of Viçosa, Viçosa, MG, Brazil. Milled corn cob and wheat bran were obtained locally. All others reagents used in this study were of analytical grade.

2.2. Microorganism and Inoculum preparation

Four white-rot Basidiomycete and two plant pathogenic fungi collected from eucalyptus plantation in several regions of Brazil were studied in this work. The identification and other pertinent informations about each fungal strain can be observed in Table 1. All fungal strains belong to the mycological collection of the Forest Pathology Laboratory at the Federal University of Viçosa, MG, Brazil.

The fungi were maintained on PDA plates at 28°C and subcultured periodically. The inoculum was prepared by growing the different strains under submerged fermentation in 250 mL erlenmeyer flasks containing 100 mL of medium with the following composition, in g/L: glucose, 10.0; NH_4NO_3, 1.0; KH_2PO_4, 1.0; $MgSO_4$, 0.5 and yeast extract, 2. Each flask was inoculated with 10 agar plugs (cut out of a 5 day-old colony of each strain grown on the PDA

plates) and incubated in a rotary shaker for 5 days at 150 rpm and 28 °C. The obtained culture was aseptically homogenized with a Polytron® device and immediately used to inoculate both, submerged culture media and solid state culture media. This routine for inoculum preparation was employed in all experiment developed in this work.

Table 1: White-rot Basidiomycetes and plant pathogen fungi evaluate during this study. The isolation procedures to each strain were described at respective references

Strain	Classification	Local of isolation	Ref.
Pycnoporus sanguineus PF-2	White-rot basidiomycete	Viçosa, MG, Brazil	*[12]*
Trametes sp. J2	White-rot basidiomycete	Monte Dourado, PA, Brazil	*[12]*
Trametes sp. J5	White-rot basidiomycete	Monte Dourado, PA, Brazil	*[12]*
Isolated J-129	White-rot basidiomycete	Monte Dourado, PA, Brazil	*[12]*
Cylindrocladium pteridis PF-1	Plant pathogen	Lençóis Paulistas, SP, Brazil	*[13]*
Chrysoporthe cubensis LPF-01	Plant pathogen	Belo Oriente, MG, Brazil	*[14]*

2.3. Solid state fermentation (SSF)

SSF was carried out using Kraft pulp, wheat bran and milled corn cob for support and as the main carbon source. The fermentations were carried out in 125-mL Erlenmeyer flasks containing 5 g (dry weight) of substrate moistened with 12 mL of culture media (final moisture of 70%) containing the following composition, in g/L: NH_4NO_3, 1.0; KH_2PO_4, 1.5; $MgSO_4$, 0.5; $CuSO_4$, 0.25 and yeast extract, 2. Furthermore, $MnCl_2$, H_3BO_3, Na_2MoO_4, $FeCl_3$ and $ZnSO_4$ also were added to the medium as trace elements. The flasks were autoclaved at 120 °C for 20 min and then inoculated with 3 mL of inoculum obtained as aforementioned. The flasks were maintained at 28 °C in a controlled temperature chamber and the enzymatic extractions were performed after 4, 8 and 12 days of fermentation. Enzymes secreted during SSF were extracted with sodium acetate buffer, 50 mM, pH 5, at a ratio of 10:1 (buffer/dry substrate) under agitation of 150 rpm for 60 min at room temperature in an orbital rotary shaker. Solids were separated by filtration through a nylon cloth followed by centrifugation at 15000 x g for 10 min. The clarified supernatants were frozen and stored for subsequent enzymatic analysis. All experiments were carried out with three repetitions for each medium composition and for each incubation time.

2.4. Submerged fermentation (SmF)

SmF was carried out using Kraft pulp, wheat bran, milled corn cob and Avicel as carbon sources. The fermentations were carried out in 125-mL Erlenmeyer flasks containing 50 mL of medium with following composition, in g/L: NH_4NO_3, 1.0; KH_2PO_4, 1.5; $MgSO_4$, 0.5; $CuSO_4$, 0.25, yeast extract, 2 and carbon source, 10. Furthermore, $MnCl_2$, H_3BO_3, Na_2MoO_4, $FeCl_3$ and $ZnSO_4$ also were added to the medium as trace elements. The flasks were autoclaved at 120°C for 20 min and then inoculated with three milliliter of inoculum obtained as aforementioned. The flasks were incubated in an orbital shaker at 28 °C and 150 rpm, and enzymatic extractions were performed after 4, 8 and 12 days of fermentation. The liquid extracts obtained were filtered through a cloth filter and immediately centrifuged at 15000 x g for 10 min to separate the solid fraction. The clarified supernatants were frozen and stored for subsequent enzymatic analysis. As was done in the SSF, all SmF experiments were carried out with three repetitions for each medium composition and for each incubation time.

2.5. Enzymatic assays

All enzymatic assays were carried out in sodium acetate buffer, 100 mM, pH 5, at 50° C. All assays were performed in triplicate and the mean values calculated. Relative standard deviations of the measurements were below 5%. FPase and endoglucanase activities were determined using Whatman no. 1 filter paper and carboxymethilcellulose as substrates respectively, according to previously described standard conditions *[15]*. The total reducing

sugar liberated during the enzymatic assays was quantified by the dinitrosalicylic acid (DNS) method *[16]* using glucose as a standard. Xylanase activity was determined using xylan from birchwood (1% w/v at final concentration) as a substrate and the total reducing sugar released was determined by the DNS method using xylose as a standard. β-Glucosidase activity was measured using ρPNGlc as substrate (1 mM at final concentration). Laccase activity was determined employing ABTS (1 mM at final concentration) as substrate. For all activities, one unit of enzymatic activity (U) was defined as the amount of enzyme that liberated 1 μmol of the corresponding product (glucose equivalent, xylose, ρ-nitrophenol and oxidized ABTS) per minute under the assay condition used.

2.6. Determination of optimum pH and temperature

To determine the effects of pH on endoglucanase, β-glucosidase, FPase, xylanase and laccase activities the enzymatic assays were carried out at pH values ranging from 2.0-7.0 using a citric acid/sodium phosphate buffer. The other assay conditions were as previously described. The effect of the temperature was evaluated within a range of 30-80°C at pH 5 according to the standardized methods.

3. Results and Discussion

3.1. Enzyme production as a function of the fungal strains.

The fungi were grown under SmF and SSF employing different carbon sources and all results for xylanase, endoglucanase, FPase, β-glucosidase and laccase production are presented in Tables 2 (SSF) and 3 (SmF). It was noted that the production of a particular enzymatic activity was highly dependent on the fungal specie; however, all strains exhibited a quite diverse response when cultured on different substrates and culture condition.

An outstanding capacity of *C. pteridis* to produce xylanase activity was noted. This fungus was able to produce 142519 U L^{-1} when cultured on wheat bran under SSF (Table 2); nevertheless, considerable xylanase yields also were observed when *C. pteridis* was cultivated under SmF using wheat bran (85111 U L^{-1}) and Kraft pulp (38799 U L^{-1}) as substrates (Table 3). *Chrysoporthe cubensis* showed the second best performance for xylanase production, and yields of 27740 and 25004 U L^{-1} were obtained for cultivation on corn cob-SSF and wheat bran-SSF, respectively (Table 2). The xylanase yields observed for the Basidiomycete strains ranged from 132 to 8529 U L-1 (Tables 2 and 3), indicating that under appropriate cultivations conditions these species also can produce considerable amounts of xylanases.

The greatest endoglucanase activities for SSF were observed in the cultivation of *C. cubensis* (2235 U L^{-1}), *Trametes* J2 (1945 U L^{-1}) and *C. pteridis* (978 U mL^{-1}) using wheat bran as the carbon source (Table 2). On the other hand, considering SmF, the best endoglucanase yields were observed to *P. sanguineus* (890 U L^{-1}) and *C. pteridis* (850 U L^{-1}) cultivated on corn cob and Kraft pulp, respectively (Table 3).

Pycnoporus sanguineus presented the greatest ability to produce FPase activity, regardless of the cultivation system. When *P. sanguineus* was cultured under SmF with corn cob, a production of 55 U L^{-1} was observed (Table 3). Meanwhile, *P. sanguineus* cultivation under SSF resulted in a yield of 136 U L^{-1}; however, in this case, Kraft pulp was used as a substrate (Table 2). The highest FPase concentrations in both SSF and SmF systems were generally obtained for cultures of white-rot Basidiomycetes suggesting that these microorganisms have a great potential to produce an efficient cellulolytic complex. Nevertheless, a high FPase yield was also observed for cultures of *C. cubensis* on wheat bran-SSF (102 U L^{-1}) (Table 2).

A wide variation was observed for β-glucosidase production and titres ranging from 3 to 1487 U L^{-1} were encountered, including SSF and SmF experiments (Tables 2 and 3). *C. cubensis* presented the highest production of β-glucosidase activity, and yields of 501 and 1487 U L^{-1} were observed when the fungus was cultured using wheat bran as a carbon source for SmF and SSF, respectively. Appreciable productions of β-glucosidase were also observed in the enzymatic extracts of *Trametes* sp. J2, *Trametes* sp. J5, *P. sanguineus* and strain J-129 when they were cultured on wheat bran-SSF (Table 2).

Table 2: Maximal enzymatic activity produced by wood-rot Basidiomycetes and plant pathogenic fungi cultured under SSF using corn cob, wheat bran and Kraft pulp as carbon sources.

		*Enzymatic Activity (U L^{-1})				
Strain	Substrate	Xylanase	Endoglucanase	FPase	β-Glucosidase	Laccase
Trametes sp. J-2	Corn cob	^{4}1300 ± 20	^{4}520± 20	^{4}37 ± 4	^{12}69 ± 5	^{4}584 ± 42
	Wheat bran	^{4}5487 ± 51	^{4}1945 ± 162	^{4}75 ± 2	^{8}519 ± 48	^{8}2444 ± 240
	Kraft pulp	^{12}744 ± 23	^{12}89 ± 9	^{8}34 ± 1	^{4}12 ± 1	^{4}4 ±0.5
Trametes sp. J-5	Corn cob	^{4}1626 ± 121	^{4}606 ± 48	^{4}68 ± 7	^{8}91 ± 13	^{4}4318 ± 2
	Wheat bran	^{4}4159 ± 297	^{4}716 ± 55	^{8}92 ± 4	^{8}409 ± 19	^{4}3169 ± 141
	Kraft pulp	^{8}533 ± 43	^{4}406 ± 18	^{4}45 ± 2	^{4}27 ± 1	^{4}12 ± 1
J-129	Corn cob	^{4}3903 ± 155	^{4}392 ± 14	^{4} 30 ± 2	^{12}99 ± 10	^{12}14 ± 3
	Wheat bran	^{4}3472 ± 81	^{8}585 ± 32	^{4}32 ± 3	^{8}373 ± 36	^{8}346 ± 14
	Kraft pulp	^{8}158 ± 12	^{4}68 ± 6	^{8}19 ± 4	^{4}19 ± 1	-
Pycnoporus sanguineus	Corn cob	^{4}865 ± 24	^{8}428 ± 21	^{8}24 ± 1	^{8}83 ± 8	^{4}823 ± 21
	Wheat bran	^{8}6049 ± 393	^{8}654 ± 35	^{4}56 ± 2	^{8}724 ± 49	^{8}11032 ± 391
	Kraft pulp	^{4}1151 ± 98	^{4}818 ± 61	^{12}136± 12	^{4}36 ± 4	^{4}61 ± 2
Chrysoporthe cubensis	Corn cob	^{4}27740 ± 987	^{4}471 ± 27	^{4}42 ± 5	^{4}219 ± 18	-
	Wheat bran	^{4}25004 ± 295	^{8}2335 ± 12	^{4}105 ± 8	^{4}1487 +13	^{4}19 ± 1
	Kraft pulp	^{4}10112 ± 82	^{4}513 ± 35	^{12}32 ± 7	^{4}39 ± 1	-
Cylindrocladium pteridis	Corn cob	^{4}16359 ± 810	^{12}229 ± 2	^{4}24 ± 3	^{12}106 ± 5	-
	Wheat bran	^{8}142519 ± 782	^{4}978 ± 86	^{8}66 ± 5	^{8}373 ± 27	-
	Kraft pulp	^{8}5387 ± 281	^{12}198 ± 8	^{4}44 ± 4	^{4}13 ± 1	-

*The superscripts indicate the fermentation time (in days) that provided the greatest activity.

As expected, high levels of laccase activity were found in enzymatic extracts from white-rot Basidiomycetes, whereas low or no laccase activity was detected for cultures of *C. cubensis*, *C. pteridis* and strain J-129. *P. sanguineus* appeared to be the best laccase producer and titres of 11032 and 2711 U L^{-1} were observed when the fungus was cultured on wheat bran-SSF (Table 2) and Kraft pulp-SmF (Table 3), respectively.

The results reported here showed that the six strains evaluated have an outstanding potential to secrete enzymes of great interest for biotechnological processes. However, each fungal strain showed a specific ability to secrete different enzymatic activity. The white-rot Basidiomycetes *P. sanguineus*, *Trametes* sp. J2 and *Trametes* sp. J5 simultaneously produced considerable amounts of hydrolases and laccase. White-rot Basidiomycetes are the most frequent wood rotting organisms and they are characterized by their ability to degrade lignin, hemicelluloses and cellulose, often giving rise to a cellulose-enriched white material*[17]*. The particular capacity of white-rot Basidiomycetes to produce enzymes which degrade (or transform) all biomass components suggests that these microorganisms may have a central role in the future regarding enzymes production for use in biorefinery processes.

C. cubensis, *C. pteridis* and strain J-129 were not able to produce considerable ligninase activity. However, they showed an outstanding capacity to secrete cellulases and xylanases which suggests that these microorganisms may also be used as enzyme sources for application in several biorefinery processes such as biomass hydrolysis (cellulases and xylanase) and biobleaching of Kraft pulp (xylanases).

Table 3: Maximal enzymatic activity produced by wood-rot Basidiomycetes and plant pathogenic fungi cultured under SmF using corn cob, wheat bran, Kraft pulp and Avicel as carbon sources.

		Enzymatic Activity U L^{-1}				
Fungi	Substrate	Xylanase	Endoglucanase	FPase	β-Glucosidase	Laccase
Trametes sp. J-2	Corn cob	^{12}5611 ± 196	^{12}597 ± 25	^{12}47 ± 4	^{12}118 ± 9	^{4}278 ± 11
	Wheat bran	^{8}1611± 61	^{8}138 ± 3	^{8}21 ± 1	^{8}33 ± 1	^{8}214 ± 25
	Kraft pulp	^{8}830 ± 63	^{12}325 ± 1	^{12}19 ± 1	^{12}30 ± 1	^{4}564 ± 33
	Avicel	^{12}223 ± 17	^{12}63 ± 2	-	-	^{12}320 ± 27
Trametes sp. J-5	Corn cob	^{12}4885 ± 312	^{12}658 ± 42	^{12}44 ± 5	^{12}77 ± 8	^{4}484 ± 39
	Wheat bran	^{8}2262 ± 45	^{8}174 ± 8	^{8}28 ± 2	^{8}59 ± 2	^{8}850 ± 16
	Kraft pulp	^{8}661 ±1	^{12}66 ± 7	^{8}6 ± 1	^{8}3 ± 1	^{8}833 ± 26
	Avicel	^{8}132 ± 4	^{12}42 ± 5	-	-	^{12}852 ± 56
J-129	Corn cob	^{8}8529 ± 385	^{12}669 ± 23	^{8}36 ± 2	^{12}407 ± 10	-
	Wheat bran	^{4}6844 ± 677	^{8}182 ± 17	^{4}21 ± 2	^{12}377 ± 31	^{12}75 ± 12
	Kraft pulp	^{4}1357 ± 58	^{12}369 ± 2	^{8}17 ± 2	^{12}180 ± 9	-
	Avicel	^{12}386 ± 5	^{12}160 ± 7	^{12}16 ± 3	^{12}70 ± 1	-
Pycnoporus sanguineus	Corn cob	^{8}1878 ± 78	^{8}890 ± 9	^{8}55 ± 5	^{8}114 ± 12	^{12}1430±108
	Wheat bran	^{8}931 ± 63	^{4}137 ± 11	^{8}20 ± 1	^{8}56 ± 6	^{8}2177 ± 216
	Kraft pulp	^{12}1217 ± 99	^{12}500 ± 14	^{12}35 ± 3	^{12}103 ± 7	^{12}2711 ± 127
	Avicel	^{12}165 ± 2	^{12}70 ± 8	-	-	-
Chrysoporthe cubensis	Corn cob	^{12}714 ± 49	^{12}120 ± 11	^{12}7 ± 0.8	^{12}82 ± 3	-
	Wheat bran	^{4}7190 ± 530	^{8}521 ± 16	^{4}37 ± 2	^{12}501 ± 36	^{12}54 ± 6
	Kraft pulp	^{12}6283 ± 502	^{8}393 ± 2	^{8}24 ± 2	^{12}70 ± 6	^{4}28 ± 4
	Avicel	^{12}514 ± 31	^{12}259 ± 18	^{12}15 ± 1	^{12}47 ± 2	^{12}15 ± 2
Cylindrocladium pteridis	Corn cob	^{12}7011 ± 685	^{12}239 ± 25	^{12}34 ± 2	^{12}89 ± 6	-
	Wheat bran	^{4}85111 ± 3579	^{12}214 ± 24	^{4}39 ± 3	^{8}185 ± 15	-
	Kraft pulp	^{8}38799 ± 2784	^{8}850 ± 32	^{12}30 ± 2	^{4}8 ± 3	-
	Avicel	^{8}6390 ± 512	^{12}90 ± 3	^{12}14 ± 1	^{12}43 ± 7	-

*The superscripts indicate the fermentation time (at days) that provided the highest activity

3.2. Enzyme production as a function of the carbon source

As it was indicated, the levels of extracellular enzyme activities produced during fermentation of different substrates varied with the fungal strain and fermentation conditions, but some general impressions may be noted concerning the different carbon source. First of all, it was clear that the best enzymatic productions were generally obtained when agricultural residues were used as substrate; however different responses were found depending of cultivation system. Considering SSF, all fungal strains produced greatest enzymatic activities when wheat bran was used as substrate. The only exceptions were laccase production by *Trametes* sp. J5 (highest activity on corn cob), endoglucanase and FPase production by *Pycnoporus sanguineus* (highest activity on Kraft pulp) and xylanase production by *C. cubensis*

and strain J-129 (highest activity on corn cob).

When SmF was considered, it was noted that for the Basidiomycete strains corn cob was the best inducer of cellulase and xylanase production, whereas the highest laccase titres were obtained when they were cultivated on wheat bran or Kraft pulp. Contrarily, the plant pathogenic strains *C. cubensis* and *C. pteridis* produced the highest enzymatic activities under SmF when wheat bran was employed as substrate. The only exception was the maximal endoglucanase production by *C. pteridis* which was found for cultivation on Kraft pulp. Still considering SmF, it was noted for all fungal strains that the lowest enzymatic productions were found for cultivation using Avicel, which it suggests that this substrate was not a good inducer of lignocellulolytic enzyme production.

In this work, wheat bran and corn cob were the best inducers of lignocellulolytic enzymes and this can be considered advantageous because these agricultural residues are inexpensive and their use as substrates would certainly contribute to reduce the costs of enzyme production.

3.3. Enzyme production as a function of the fermentation system

Considering the systems of fermentation some general patterns were detected which may be quite useful in future studies involving the production of enzymes by the fungal strains investigated here. Firstly, the enzymatic extracts obtained via SSF generally showed higher enzymatic activities (higher enzymatic concentrations) that those obtained under SmF. This is quite advantageous because concentrated extracts require fewer steps in downstream processing, thus reducing the operational costs for obtaining enzymatic extracts with adequate characteristics to be applied in biotechnological processes.

Secondly, despite of the higher enzymatic activities found in extracts obtained from SSF, it is worth noting that the enzyme productivity per gram of substrate in SmF was higher (data not shown). This implies that the availability and cost of obtaining of a particular lignocellulosic substrate also should be taken in account at the time of choosing the most appropriate procedure (SSF or SmF) for producing a specific enzymatic activity.

Lastly, it was observed that under SSF the maximal activities were preferentially achieved on the fourth day of cultivation, unlike that observed under SmF, where the maximal activities were generally detected after 8 and 12 days of fermentation. These results suggest that the fungal strains studied in this work could be cultured under SSF to obtain concentrated enzymatic extracts in short fermentation periods which would result in a lower processing cost.

It has been suggested that the SSF is the most appropriated method for aerobic fungi cultivation because these growth conditions are similar to their natural habitats *[4, 18]*. Moreover, other advantages are attributed to the use of SSF for cultivation of microorganisms and obtaining byproducts such as: low water demand, highly concentrated end product, lower catabolite repression, utilization of substrates insoluble in water, higher volume productivity, lower sterility demand and low energy demand for heating *[18]*. However, the major advantages of SSF over SmF are still reported based on laboratorial experiments and there are severe engineering problems such as build-up of gradients - of temperature, pH, moisture, substrate concentration or O_2 pressure - during cultivation, that prevent the establishment of SSF in an industrial scale. Nevertheless, several studies have been developed in recent years aiming to overcome the limitations of SSF at the large scale.

The results observed in this work suggest that the highest enzymatic production could be obtained in the SSF system, however, further studies should be carried out to test new substrates and new culture conditions (humidity, temperature, C/N ratio) so that maximum production is achieved. Moreover, the problems concerning large scale production also should be investigated.

3.4. Partial characterization of enzymatic activities

Xylanase, endoglucanase, FPase, β-glucosidase and laccase activities produced by the six different fungal strains were partially characterized in relation to optimal pH and temperature in order to determine the specific conditions for application of these enzymes in biotechnology processes. The values of pH that ensured maximal activities for xylanase, endoglucanase, FPase, β-glucosidase and laccase produced by the different fungal strains are shown in Table 4.

The optimum pH for xylanase activities ranged from 4.0 (*C. cubensis*) to 6.0 (strains J-129); meanwhile, the maximum cellulolytic and β-glucosidase activities were found in the pH range of 3.0-5.5. Laccase activities were higher at low pH values and the maximal activities were observed in a range of 2.5-3.5.

Table 4: Values of optimal pH found for xylanase, endoglucanase, FPase, β-glucosidase and laccase activities produced by wood-rot Basidiomycetes and plant pathogenic fungi.

	Optimum pH				
Fungal Strain	Xylanase	Endoglucanase	FPase	β-Glucosidase	Laccase
Trametes sp. J2	5.0	3.5	5.0	4.5	3.0
Trametes sp. J5	5.0	4.0	5.0	4.5	2.5
J-129	6.0	4.5	5.5	5.0	3.5
Pycnoporus sanguineus	5.0	3.5	4.5	4.5	2.5
Chrysoporthe cubensis	4.0	4.0	4.5	4.5	2.5
Cylindrocladium pteridis	5.0	3.5	3.5	4.5	-

It was noted that the highest observed values for optimal pH were always associated with the enzymatic activities produced by fungal strain J-129 and these results suggest that enzymes produced by this strain have a particular potential to be applied in biotechnological processes that are carried out at neutral or slightly alkaline pH, such as biobleaching of Kraft pulp *[19]* and biostoning *[20]*.

However, despite the particular characteristics shown by enzymes produced by strain J-129, it was observed that all others fungal strains secreted enzymes with maximal activities at acidic pH values and these results are in accordance with those reported for fungal hydrolases *[4]*. Acidic cellulases are usually more desirable for bioconversion, especially when acidic pretreatment of biomass is employed or when the enzyme makes up part of a cocktail with *T. reesei* (main industrial cellulases producer) enzymes whose optima activity is close to 5 *[4]*.

Table 5 presents the values of optimal temperatures for xylanase, endoglucanase, FPase, β-glucosidase and laccase activities produced by the Basidiomycetes and plant pathogenic fungi investigated in this work. All enzymatic activities showed a maximal hydrolysis/oxidation rate when incubated at temperatures ranging from 50 to 60 °C. The only exceptions were endoglucanase activity produced by the strains *Trametes* sp. J2 and *Trametes* sp. J5 which presented maximal actives at 70 and 65 °C, respectively.

Table 5: Values of optimal temperature found for xylanase, endoglucanase, FPase, β-glucosidase and laccase activities produced by wood-rot Basidiomycetes and phytopathogenic fungi.

	Optimal Temperature (°C)				
Fungal Strain	Xylanase	Endoglucanase	FPase	β-Glucosidase	Laccase
Trametes sp. J2	60.0	70.0	60.0	60.0	55.0
Trametes sp. J5	55.0	65.0	60.0	50.0	55.0
J-129	55.0	60.0	50.0	60.0	60.0
Pycnoporus sanguineus	60.0	60.0	55.0	55.0	60.0
Chrysoporthe cubensis	55.0	55.0	50.0	55.0	55.0
Cylindrocladium pteridis	55.0	55.0	55.0	60.0	-

The incorporation of an enzymatic step in an industrial biotransformation process requires enzymes that showing high stability and high processivity at the conditions in which the process is usually carried out. For instance, alkaline and thermal stable xylanases are indicated for application in biobleaching processes since the conventional pulp bleaching is generally conducted under high values of temperature and pH. The values of optimal temperature found for hydrolytic and oxidizing activities suggest that the enzymatic extract produced by the six fungal strains has potential to be applied in biotransformation processes such as biomass saccharification, biobleaching, biostoning and others since a temperature range of 50-60 °C is

usually employed in these processes.

4. General remarks

In this work, six new fungal strains isolated from eucalyptus plantation were investigated in order to study their potential to produce enzymes of biotechnological interest for application in biorefinery processes. It was observed that the productions of xylanase, endoglucanase, FPase, β-glucosidase and laccase activities were strongly dependent on the fungal strain, carbon source employed and cultivation system, indicating that the exploration of cultivation conditions is a crucial step to evaluate and select new microorganisms with outstanding ability to secrete hydrolases and oxidases.

Among the strains studied, *P. sanguineus* produced the highest FPase and laccase activity, indicating that this microorganism could be a good enzyme supplier.

C. cubensis produced significant amounts of endoglucanase and xylanase activities; however its most prominent characteristic was the ability to produce β-glucosidase activity.

Also deserving attention is the fungus *C. pteridis* which showed an unusual capacity to secrete xylanolytic activity when cultured under SSF using wheat bran as a support and this indicated that this microorganism has high potential to be an efficient producer of inexpensive xylanase.

Finally, the microorganisms investigated here showed to be promising for industrial application since they grow quickly under SSF or SmF using inexpensive agricultural residues and secreting large amounts of important industrial enzymes. Furthermore, the enzymatic activities obtained in this study presented properties that are frequently required for industrial application.

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