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Research Paper

Secretion of laccase and manganese peroxidase by *Pleurotus* strains cultivate in solid-state using *Pinus* spp. sawdust

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Abstract

Pleurotus species secrete phenol oxidase enzymes: laccase (Lcc) and manganese peroxidase (MnP). New genotypes of these species show potential to be used in processes aiming at the degradation of phenolic compounds, polycyclic aromatic hydrocarbons and dyes. Hence, a screening of some strains of *Pleurotus* towards Lcc and MnP production was performed in this work. Ten strains were grown through solid-state fermentation on a medium based on *Pinus* spp. sawdust, wheat bran and calcium carbonate. High Lcc and MnP activities were found with these strains. Highest Lcc activity, 741 ± 245 U gdm^{-1} of solid state-cultivation medium, was detected on strain IB11 after 32 days, while the highest MnP activity occurred with strains IB05, IB09, and IB11 ($5,333 \pm 357$; $4,701 \pm 652$; $5,999 \pm 1,078$ U gdm^{-1}, respectively). The results obtained here highlight the importance of further experiments with lignocellulolytic enzymes present in different strains of *Pleurotus* species. Such results also indicate the possibility of selecting more valuable strains for future biotechnological applications, in soil bioremediation and biological biomass pre-treatment in biofuels production, for instance, as well as obtaining value-added products from mushrooms, like phenol oxidase enzymes.

Key words: *Pleurotus,* laccase, manganese peroxidase, mushroom production, biotechnological applications.

Introduction

Fungi of the genus *Pleurotus* belong to the Basidiomycetes class, order Agaricales, family Tricholomataceae. This genus is distinguished by presenting fruiting bodies with concentric stems in the form of oyster shells, with joined blades. In the blades, there are basidia where the resultant basidiospores are formed by meiosis (Alexopoulos and Mims 1985).

The genus *Pleurotus* is a cosmopolitan group with mushrooms of a high nutritional value, therapeutic properties, and several environmental and biotechnological applications (Cohen *et al.*, 2002). *Pleurotus* fruiting bodies are edible, have a pleasant odour, and contain carbohydrates, proteins, vitamins, mineral salts and lipids, but not cholesterol. Furthermore, they have medicinal properties (Chang *et al.*, 1981). These fungi are also able to secrete enzymes and metabolites that depolymerize hemicellulose and cellulose, besides promoting lignin fragmentation (Kirk and Cullen 1998). After cellulose, lignin is the most abundant organic material on earth, making up 20-30% of the dry weight of wood (Abdel-Raheem and Shearer 2002).

Pleurotus species have been recognized to produce enzymes such as manganese peroxidase (MnP) and laccase (Lcc) (Kamitsuji *et al.*, 2004). Laccase is an oxidoreductase able to catalyse the oxidation of various aromatic compounds (particularly phenol) with the concomitant reduction of oxygen to water (Valeriano *et al.*, 2009).

The interest in the study of ligninolytic enzymes - such as Lcc and MnP - has grown due to their potential use in biotechnological processes. These phenol oxidases may be employed to treat effluents in the textile, paper and pulp industries, to biobleaching cellulose pulp and during the *in vivo* biodelignification of wood chips, to clarify wines and juices, as well as in polymerization reactions. What is more, they can also promote the blanching of fabrics (jeans), with reduced loss of resistance (Camassola and Dillon 2009, Gomes *et al.*, 2009, Machado and Matheus 2006, Munari *et*

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al., 2008), but the most promising applications are in the bioremediation of soils that contain toxic substances (Wu *et al.*, 2008) and in the biological pre-treatment of biomass to produce biofuels (Camassola and Dillon 2009).

Since there may have been variability in phenol oxidase secretion by *Pleurotus* spp., this work evaluated different genotypes of *Pleurotus* isolated from basidiocarpi for Lcc and MnP secretion, using a sawdust-based substrate (*Pinus* spp.) in solid-state fermentation. These experiments target the use of Lcc and MnP in future processes of soil bioremediation and biological pre-treatment of biomass.

Materials and Methods

Macrofungi

Ten fungal strains of *Pleurotus* spp. belonging to the collection of microorganisms of the Enzymes and Biomass Laboratory, Institute of Biotechnology, University of Caxias do Sul, Rio Grande do Sul, Brazil, were used. The strains used are listed in Table 1. These strains were initially isolated from commercial strains.

Table 1 - *Pleurotus* strains used in screening.

Strain	Species
IB01	*P. sajor-caju*
IB02	*P. cytrinopileatus*
IB03	*P. cytrinopileatus*
IB04	*P. ostreatus*
IB05	*P. salmoneo-stramineus*
IB06	*P. sajor-caju*
IB07	*P. cytrinopileatus*
IB09	*P. sajor-caju*
IB11	*P. salmoneo-stramineus*
IB17	*P. ostreatus*

Cultivation media isolation medium, maintenance and inoculum

The medium used for isolation, maintenance and preparation of inoculum contained 2% (w/v) *Pinus* sp. sawdust, ground and swollen, 2% (w/v) ground wheat bran, 0.2% (w/v) calcium carbonate, and 2% (w/v) agar. This medium was autoclaved at 120 °C for 1 min

The medium used for cultivation was prepared using 94% (w/w) *Pinus* spp. sawdust, 5% (w/w) wheat bran and 1% (w/w) calcium carbonate. This medium was supplemented with 100 mg.L^{-1} $(NH_4)_2SO_4$, 0.1 mg.L^{-1} $MnSO_4.H_2O$, and 0.1 mg.L^{-1} $CuSO_4.H_2O$. Water was added to the medium until 66% humidity was achieved. The medium was subsequently homogenized. Solid-state fermentation was carried out in 17.8 x 13-cm polypropylene bags containing 35 g of humid medium, which were sterilized at 120 °C for 2 h. These recipients were called microfermentors.

Cultivation

In order to inoculate the solid-state fermentation, 1-cm-radius disks were taken from cultures grown in Petri dishes with the inoculum medium for 7 days at 25 ± 1 °C. For mycelial development, the cultures were kept in a dark environment at 25 ± 1.5 °C up to 32 days.

Sampling and preparation of the total extracted proteins

The full content of the microfermentors was used to extract the enzymatic broth. After homogenization, the content was mixed with 7 mLof distilled water at 4 °C, in 250-mL Erlenmeyer flasks. The flasks were shaken at 200 rpm for 3 min The solids were removed by filtration and the filtrates were centrifuged at 4000 *g*, at 4 °C, for 3 min The supernatant was used for the enzymatic dosages; four samples (microfermentors containing 35 g of humid medium) were collected on days 4, 8, 12, 16, 20, 24, 28 and 32.

Enzymatic dosages

The enzymatic activities were expressed as international units per gram of dry culture (mass) (U gdm^{-1}). One unit was considered as the quantity (μmol) of product released per minute (IU = μmol min^{-1}). Appropriate dilutions of the samples were done when necessary. The dry mass was obtained by carrying out the drying at 105 °C for 24 h an aliquot of the culture medium.

The stock solutions of H_2O_2 were standardized under UV using the molar extinction coefficient $\varepsilon_{230} = 81$ M^{-1} cm^{-1} against distilled water blank treated with catalase. This solution was used for determination of MnP.

The determination of MnP activity was performed following the method proposed by Kuwahara *et al.* (1984).

The determination of Lcc activity was performed following the method proposed by Wolfenden and Wilson (1982), using ABTS as substrate.

The enzymatic activities are expressed as units per gram of dry mass (U gdm^{-1}).

Protein determination

Protein concentration was determined using the method of Bradford (1976). A minimum of four replicates was carried out for each of these protein assays.

Zymograms

SDS-polyacrylamide gel electrophoresis (PAGE) was performed as described by Laemmli (1970). After electrophoresis, the gel was stained with Coomassie Blue R dye in ethanol-acetic acid-water solution (5:1:4, by volume) for 2 minand destained in the same solution without dye. The sample buffer was prepared without addition of β-mercaptoethanol and the sample was not heated before running.

For the staining of laccase activity, SDS was removed by washing the gel at room temperature in Solution A (sodium acetate buffer, pH 5.0, containing isopropanol 25%) for 1 minand in Solution B (sodium acetate buffer, pH 5.0) for 1 min respectively. The gel was then transferred onto a glass plate and a layer of ABTS-agar (0.02 g of ABTS, 0.4 g of agar, 40 mL of water; heated to dissolve agar) was placed on the gel. These layers were incubated for about 1 min at 25 °C, until the green bands' appearance.

Results

The results showed Lcc and MnP activities in all *Pleurotus* spp. strains studied. Variations between them were also observed (Figures 1 and 2). The greatest of Lcc activity, using ABTS as substrate, was found in a *P. salmoneo-stramineus* strain (IB11) (Figure 1D). As seen in Figure 1, strain IB11 showed an activity of 741 ± 245 U gdm^{-1} of culture on the 32nd day of cultivation, while the others had mean activities ranging from 324 to 459 U gdm^{-1} of culture. However, strain IB09 (*P. sajor-caju*) showed an activity of 347 U gdm^{-1} of culture already on the eighth day, and also a higher productivity, 43 ± 6 U gdm^{-1} day^{-1}, than strain IB11. Although IB11 presented a higher activity peak, it occurred after 32 days, and the productivity was 23 ± 7 U gdm^{-1} day^{-1}.

The evaluations of MnP (Figure 2) showed greater activity on the 12th day for strains IB11 (*P. salmoneo-stramineus* - Figure 2D), 5999 ± 1078 U gdm^{-1}; IB05 (*P. salmoneo-stramineus* - Figure 2D), 5332 ± 357 U gdm^{-1}; IB09 (*P. sajor-caju* - Figure 2A), 5163 ± 739 U gdm^{-1}; and IB01 (*P. sajor-caju* - Figure 2A), 4701 ± 652 U gdm^{-1}. For strain IB06 (*P. sajor-caju*- Figure 2A), the activity peak occurred on the 8th day of cultivation, reaching values of 2817 ± 685 U gdm^{-1}. Strains IB02 and IB07, both of *P. cytrinopileatus*, presented their greatest activity between the 8th and 12th days, keeping stability afterwards.

Besides the observed discrepancies in the enzymatic activities of different strains, distinct band patterns in SDS-PAGE of total proteins and laccases activities were also noted (Figures 3 and 4). Although some strains have been classified as same species, it can be observed in the total proteins SDS-PAGE (Figure 3) that they presented diverse band patterns, as it could be detected in *P. sajor-caju* IB01, IB06 and IB09 strains. The band pattern observed in

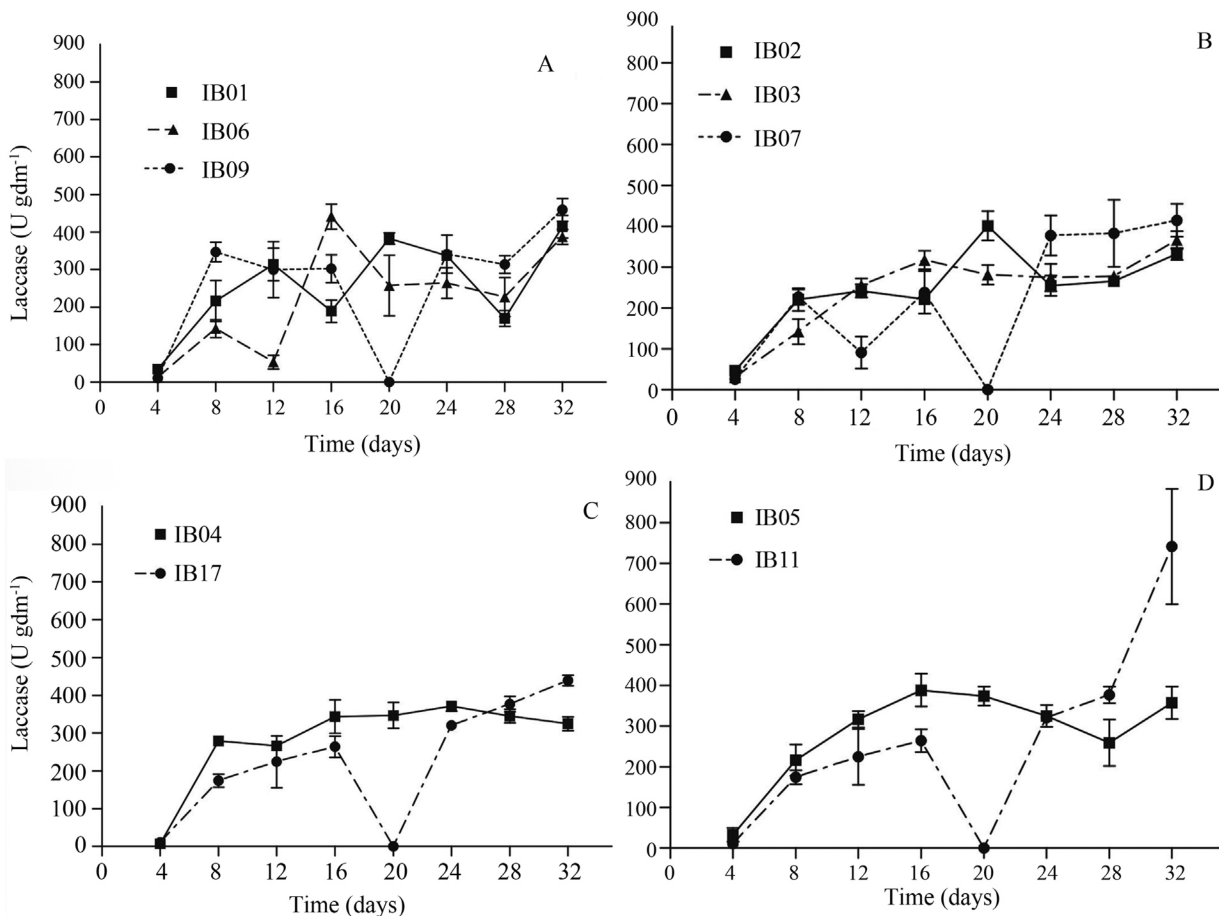

Figure 1 - Laccase activity during solid-state cultivation of strains of *Pleurotus sajor-caju* (1A), *Pleurotus cytrinopileatus* (1B), *Pleurotus ostreatus* (1C) and *Pleurotus salmoneo-stramineus* (1D).

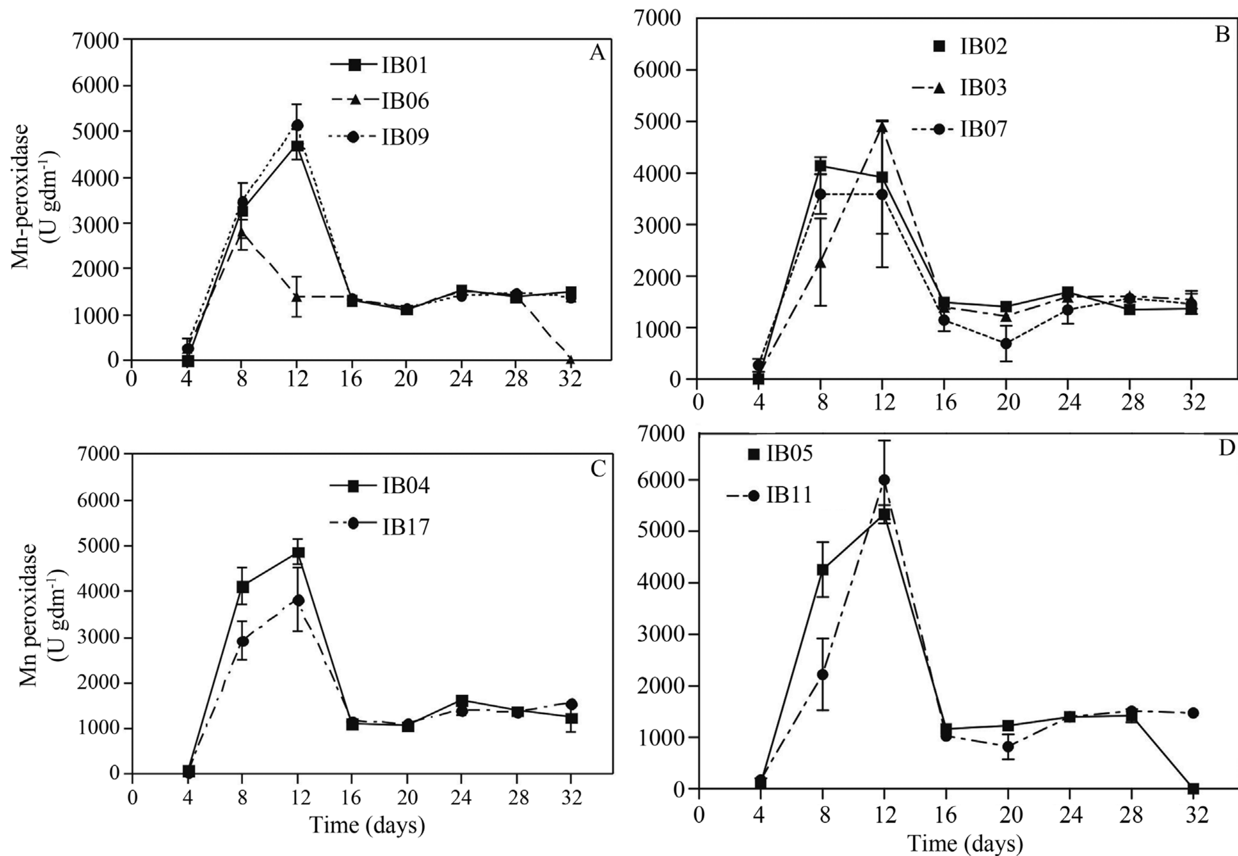

Figure 2 - Manganese-peroxidase activity during solid-state cultivation of strains of *Pleurotus sajor-caju* (2A), *Pleurotus cytrinopileatus* (2B), *Pleurotus ostreatus* (2C) and *Pleurotus salmoneo-stramineus* (2D).

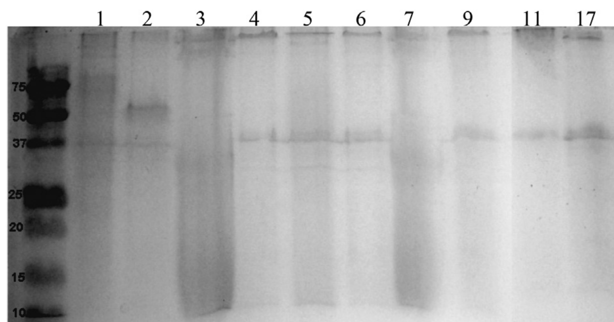

Figure 3 - SDS-PAGE of total extracted proteins stained with Coomassie Blue R dye found in different species of *Pleurotus*. Were used samples obtained at peak of each strain. M: Standard protein molecular weight in kilodaltons. Numbers specify the strains. IB01 - *P. sajor-caju,* IB02 - *P. cytrinopileatus,* IB03 - *P. cytrinopileatus,* IB04 - *P. ostreatus,* IB05 - *P. salmoneo-stramineus,* IB06 - *P. sajor-caju,* IB07 - *P. cytrinopileatus,* IB09 - *P. sajor-caju,* IB11 - *P. salmoneo-stramineus* and IB17 - *P. ostreatus*.

the IB01 strain differs completely from the IB06 and IB09 strains. The presence of glycosylated proteins - diffuse band patterns- and proteins weighing more than 37 kDa can be noted in the result for IB01strain. The strain IB06 presented a band about 34 kDa, yet this outcome was not observed in IB09.

Differences in band patterns were also observed among *P. cytrinopileatus* strains, where IB03 and IB07 presented similar patterns and diffused bands, which indicates the presence of glycosylated proteins. Yet IB02 showed distinctive bands, in comparison to other *P. cytrinopileatus* strains and *Pleurotus* species here analyzed.

Similar band patterns were observed when comparing the results for the morphologically distinctive *P. ostreatus* IB04 and IB17 strains, which have brown and grey colours, respectively. Two bands with similar molecular mass could be noticed (250 kDa e 37 kDa) amongst *P. salmoneo-stramineus* strains. However, other parallel bands (180 kDa e 33 kDa) were only observed in IB05 strains. Moreover, when observing the zymogram for total proteins, a higher similarity can often be noticed among strains from different species than among those that are part of same species (Figure 4).

Laccase activity and the corroboration of their glycoproteic nature can be seen in Figure 4. A greater pattern of diffused bands can be observed in IB01, whereas other *P. sajor-caju* strains here analysed presented bands with equal molecular mass of 57.5 kDa. Yet, *P. cytrinopileatus* IB03 and IB07 strains presented two bands for laccases (37.5 e 42 kDa), as well as equal band pattern in the total proteins gel, which suggests isoenzyme presence.

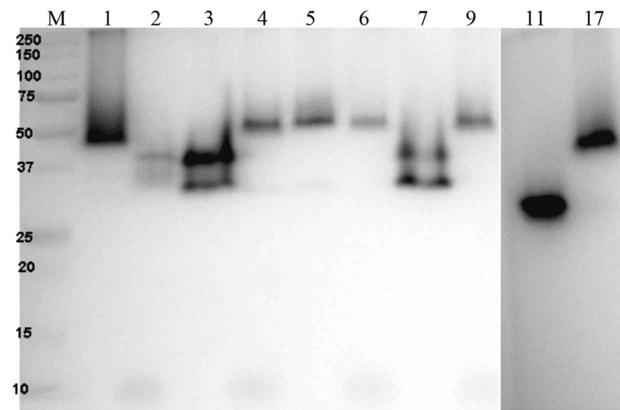

Figure 4 - Laccase identification in different species of *Pleurotus*. Proteins were separated on a 12% polyacrylamide gel electrophoresis. Were used samples obtained at peak of each strain. M: Standard protein molecular weight in kilodaltons. Numbers specify the strains. IB01 - *P. sajor-caju,* IB02 - *P. cytrinopileatus,* IB03 - *P. cytrinopileatus,* IB04 - *P. ostreatus,* IB05 - *P. salmoneo-stramineus,* IB06 - *P. sajor-caju,* IB07 - *P. cytrinopileatus,* IB09 - *P. sajor-caju,* IB11 - *P. salmoneo-stramineus* and IB17 - *P. ostreatus*.

The strain IB02 presented diffused bands with similar molecular masses obtained for *P. cytrinopileatus* IB03 and IB07, but different total proteins pattern. Distinctive molecular masses were observed in laccase band activity, regarding to *P. salmoneo-stramineus* strains. In the case of strain IB05, laccase presented average molecular mass of 60kDa, while IB11had an average result of 30 kDa. *P. ostreatus* IB04 and IB17 strains presented molecular masses of 53.7 kDa and 51.4 kDa, respectively.

Enzymatic specific activities obtained at peak of each sample are presented in Table 2. Laccases specific activities had values below 3U per mg of protein present in the extract. MnP presented the greater specific activity (31 U mg^{-1}) in strain IB04.

Discussion

As observed by Stajic *et al.* (2004), the results showed variations in the activity of extracellular enzymes between the different species of *Pleurotus,* and also between strains of same species. As seen through this work, there is a potential to produce Lcc and MnP from strains IB11 and IB09. Although the substrates and methodologies of enzymatic activity analysis are not the same, it can be seen in Table 3 that the values for this study are higher than those shown before.

Lcc activities using ABTS as a substrate were similar to those observed by Silva (2004) (19) for *P. sajor-caju,* in which activities of 334 ± 148 U gdm^{-1} and 949 ± 164 U gdm^{-1} were obtained after 15 and 30 days of cultivation, respectively, in a medium supplemented with the same salts used in this study.

High activities obtained may be related to the type of medium used in the growth of *Pleurotus* strains. Apart from being rich in salts, the medium also contained lignocellulose, which is essential in the studied enzyme induction.

Table 2 - Enzymatic specific activities obtained at peak of each sample in solid-state cultivation of various *Pleurotus* species and strains.

Strain	MnP (U mg^{-1})	Laccase (U mg^{-1})
IB01	25.17	1.16
IB02	27.03	2.62
IB03	18.33	1.37
IB04	31.32	2.39
IB05	27.69	2.02
IB06	12.72	2.65
IB07	12.79	1.48
IB09	24.29	2.16
IB11	24.71	1.81
IB17	15.61	1.79

Elisashvili *et al.* (2006) proved that the presence of a lignocellulosic substrate is mandatory for MnP production by *P. dryinus* IBB 903, since there was no enzyme production when the fungus was cultivated in a synthetic medium with different carbon sources.

Besides the differences observed in the enzymatic production, it is important to note that different band patterns were obtained in same species strains, while similar patterns were found in diverse species strains, for the SDS-PAGE of total extracted proteins as well as for the laccase activity gel assay.

The observation of two activity bands in some strains suggests the presence of isoenzymes. Such occurrence has already been observed in laccases of other *Pleurotus* species. Munõz *et al.* (1997) verified that *Pleurotus eryngii* produces two laccase isoenzymes, with molecular masses of 65 kDa and 61 kDa, respectively. Both isoenzymes are stable at high pH, retaining 60 to 70% activity after 24 h from pH 8 to 12. Mansur *et al.* (2003) verified that *P. ostreatus* strain V-184 synthesize four laccase isozymes (LCC1, LCC2, LCC3 and LCC4). LCC1 and LCC2 have average molecular masses of 60 and 65 kDa, and exhibited the same pI value (3.0). Their N termini were sequenced, revealing the same amino acid sequence and homology with laccases from other microorganisms. Laccases LCC3 and LCC4 were characterized by SDS-PAGE, estimating their molecular masses in 80 and 82 kDa, respectively. By native isoelectrofocusing, their pI values were 4.7 and 4.5, respectively.

The activity gel assay indicated that most laccases are glycosylated. Glycosylation is important for solubility of enzymes (Barbaric *et al.*, 1984), their catalytic activity, thickening properties and thermostability (Wang *et al.*, 1996) Strains IB03 and IB07 have two clearly defined bands in the activity gel, but the lower specific activities,

Table 3 - Comparison of enzymes production from different *Pleurotus* species grown on lignocellulosic materials.

Organism	Substrate	Enzyme activities (U gdm^{-1}) Substrate used to enzymatic assay		Reference
		Laccase	MnP	
P. salmoneo-stramineus IB11	*Pinus* spp. sawdust, wheat bran, calcium carbonate	741.16[(1)]	5,999.25[(3)]	This work
P. sajor-caju IB09	*Pinus* spp. sawdust, wheat bran, calcium carbonate	346.87[(1)]	5,163.12[(3)]	This work
P. salmoneo-stramineus IB05	*Pinus* spp. sawdust, wheat bran, calcium carbonate	388.3[(1)] 0	5,332.36[(3)]	This work
P. dryinus IBB 903	Tree leaves (*Fagus sylvatica*)	4.00 [(1)]	4.00[(3)]	(Elisashvili *et al*., 2008)
P. ostreatus IBB	Tree leaves (*Fagus sylvatica*)	1.75[(1)]	1.75[(3)]	(Elisashvili *et al*., 2008)
P. ostreatus IBB	Tree leaves (*Fagus sylvatica*)	3.50[(1)]	3.50[(3)]	(Elisashvili *et al*., 2008)
P. ostreatus 2175	Tree leaves (*Fagus sylvatica*)	3.75[(1)]	3.75[(3)]	(Elisashvili *et al*., 2008)
P. ostreatus 2191	Tree leaves (*Fagus sylvatica*)	3.50[(1)]	3.50[(3)]	(Elisashvili *et al*., 2008)
P. tuberregium IBB 624	Wheat straw	5.00 [(1)]	0.53[(3)]	(Elisashvili *et al*., 2008)
P. dryinus IBB 903	Wheat straw	3.25[(1)]	1.43[(3)]	(Elisashvili *et al*., 2008)
P. ostreatus IBB	Wheat straw	1.75[(1)]	1.48[(3)]	(Elisashvili *et al*., 2008)
P. ostreatus IBB	Wheat straw	2.50[(1)]	0.28[(3)]	(Elisashvili *et al*., 2008)
P. ostreatus 2175	Wheat straw	3.00[(1)]	1.88[(3)]	(Elisashvili *et al*., 2008)
P. ostreatus 2191	Wheat straw	4.25[(1)]	0.03[(3)]	(Elisashvili *et al*., 2008)
P. tuberregium IBB 624	Wheat straw	2.50[(1)]	0,55[(3)]	(Elisashvili *et al*., 2008)
P. cornicopiae 32	Grapevine sawdust	5.58[(2)]	-	(Stajic *et al*., 2004)
P. cystidiosus 95	Grapevine sawdust	3.61[(2)]	-	(Stajic *et al*., 2004)
P. cytrinopileatus 602	Grapevine sawdust	0.91[(2)]	-	(Stajic *et al*., 2004)
P. djamor 485	Grapevine sawdust	2.19[(2)]	-	(Stajic *et al*., 2004)
P. eryngii 193	Grapevine sawdust	1.09[(2)]	-	(Stajic *et al*., 2004)
P. eryngii 555	Grapevine sawdust	0.17[(2)]	-	(Stajic *et al*., 2004)
P. ostreatus 207	Grapevine sawdust	3.49[(2)]	-	(Stajic *et al*., 2004)
P. ostreatus var. florida 393	Grapevine sawdust	3.14[(2)]	-	(Stajic *et al*., 2004)
P. pulmonarius 509	Grapevine sawdust	0.77[(2)]	-	(Stajic *et al*., 2004)
P. salignus 328	Grapevine sawdust	0.26[(2)]	-	(Stajic *et al*., 2004)
P. salmoneostramineus 77	Grapevine sawdust	1.13[(2)]	-	(Stajic *et al*., 2004)
P. smithii 138	Grapevine sawdust	1.38[(2)]	-	(Stajic *et al*., 2004)

[(1)]ABTS. [(2)]Syringaldazine. [(3)]Phenol red.

while IB02 and IB06 strains presented the higher specific activities, but only discreet activity bands.

Laccases produced by IB02 e IB06 may have lost activity during electrophoresis, where a separating gel buffer (pH 8.8) was used. Yet, laccases secreted by IB03and IB07, which presented more diffused bands and subsequent indication of glycosylation, showed higher intensity bands. Moreover, the laccase isozymes secreted by IB01 with the lower specific activity presented the more intense band activity, suggesting that the glycosylations may have protected these enzymes during the electrophoretic run, or for any other reason, this enzyme can keep its activity, even in adverse conditions, like during the electrophoresis.

It is important to observe that laccases bands were not detected in the total proteins gel, which indicates their small proportion in relation to the total protein amount produced by the *Pleurotus* strains.

Conclusion

The results obtained in the present study show the importance of further research on the lignocellulolytic enzymes present in different species and strains of *Pleurotus*. Thus, it will be possible to select more promising strains for future biotechnological applications, and obtain value-added products from mushrooms, such as phenol oxidase enzymes. Further studies on the role of laccases in humi-

cation processes and in the transformation of xenobiotics, such as pesticides, should be investigated.

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