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Production of laccase and manganese peroxidase by *Pleurotus pulmonarius* in solid-state cultures and application in dye decolorization

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Abstract The production of ligninolytic enzymes (laccase and Mn-dependent peroxidase) by the white-rot fungus *Pleurotus pulmonarius* (FR.) Quélet was studied in solid-state cultures using agricultural and food wastes as substrate. The highest activities of laccase were found in wheat bran (2,860±250 U/L), pineapple peel (2,450±230 U/L), and orange bagasse (2,100±270 U/L) cultures, all of them at an initial moisture level of 85 %. The highest activities of Mn peroxidase were obtained in pineapple peel cultures (2,200±205 U/L) at an initial moisture level of 75 %. In general, the condition of high initial moisture level (80–90 %) was the best condition for laccase activity, while the best condition for Mn peroxidase activity was cultivation at low initial moisture (50–70 %). Cultures containing high Mn peroxidase activities were more efficient in the decolorization of the industrial dyes remazol brilliant blue R (RBBR), Congo red, methylene blue, and ethyl violet than those containing high laccase activity. Also, crude enzymatic extracts with high Mn peroxidase activity were more efficient in the in vitro decolorization of methylene blue, ethyl violet, and Congo red. The dye RBBR was efficiently decolorized by both crude extracts, rich in Mn peroxidase activity or rich in laccase activity.

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Introduction

White-rot fungi are known for their ability to degrade or modify lignin by enzymatic processes. The major enzymes associated with the lignin-degrading ability of white-rot fungi are lignin peroxidase (LiP, EC 1.11.1.14), manganese-dependent peroxidase (MnP, EC 1.11.1.13), versatile peroxidase (VP, EC 1.11.1.16), and laccase (EC 1.10.3.2) (Maciel et al. 2012). All these enzymes act via the generation of free radicals which represents an efficient way to gain access to their substrates. White-rot fungi usually secrete one or more of the ligninolytic enzymes in different combinations. Generically, white-rot fungi can be distributed into four groups, according to their ability to produce laccases and peroxidases (LiP, MnP, and VP) (Kuhar et al. 2007: (1) laccase and both peroxidases, MnP and LiP (*Trametes versicolor*, *Bjerkandera adusta*); (2) laccase and at least one of the peroxidases (*Lentinula edodes*, *Pleurotus eryngii*, *Ceriporiopsis subvermispora*); (3) only laccase (*Schizophyllum commune*); and (4) only peroxidases (*Phanerochaete chrysosporium*). The most frequently observed ligninolytic enzymes among the white-rot fungi species are laccases and MnP and the least are LiP and VP (Maciel et al. 2012).

The demand for application of ligninolytic enzymes in industry and biotechnology is ever increasing due to their use in a variety of processes. Ligninolytic enzymes have potential applications in a large number of fields, including the chemical, fuel, food, agricultural, paper, textile, and cosmetic industrial sectors in addition to others (Karigar and Rao 2011). These enzymes are also directly involved in the degradation of various xenobiotic compounds. Due to

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the fact that ligninolytic enzymes are basically nonspecific, they are able to attack a series of molecules chemically similar to lignin including pesticides, polycyclic aromatic hydrocarbons, synthetic polymers, and synthetic dyes (Maciel et al. 2012).

Mushrooms of the genus *Pleurotus* are very easy to cultivate (Cohen et al. 2002). The two most important species cultivated in large scale are *Pleurotus ostreatus* and *Pleurotus pulmonarius* (formerly *Pleurotus sajor-caju*). In nature, they grow on wood, usually on dead standing trees or on fallen logs. Various substrates that contain lignin and cellulose can be used for *Pleurotus* cultivation such as wood chips, corn cob, rice straw, cotton stalks, waste hulls, and other agricultural wastes, some of which can be recycled and upgraded for use as animal feed or for preparation of other products (Cohen et al. 2002). These substrates are also frequently used to study the production of MnP and laccase by *Pleurotus* spp in both submerged and solid-state cultures. *P. pulmonarius*, when cultured under submerged and solid-state conditions using wheat bran as a substrate, produces laccase as the main extracellular enzyme (Souza et al. 2002). The capability of *P. pulmonarius* to decolorize textile dyes in both types of cultures has already been described. This capability of the fungus was mainly due to its laccase activity if one considers that the Mn peroxidase production was very low in those cultures (Tychanowicz et al. 2004; Zilly et al. 2002). More recently, however, it has been found that when *P. pulmonarius* was cultured in wheat bran solid-state medium with a low initial moisture level, it produced elevated amounts of both enzymes Mn peroxidase and laccase (Souza et al. 2006). The objective of this study was to compare the production of laccase and Mn peroxidase by *P. pulmonarius* in solid-state cultures using different agricultural residues at several initial moisture levels. An attempt was also done to evaluate the capability of *P. pulmonarius* and its ligninolytic enzymes to decolorize some synthetic dyes.

Material and methods

Waste material

Seven agricultural residues were used in this work, and they were obtained from local producers: yellow passion fruit waste, orange bagasse, banana stalk, pineapple peel, corn cob, rice hull, sugar cane bagasse, and wheat straw. All residues were washed and dried in an air-circulating oven at 50 °C until their weight became constant. The dried materials were then milled, and the resulting products were used as substrates. Wheat bran was obtained from the local market and used without modification.

Microorganism

P. pulmonarius CCB-19 was obtained from the Culture Collection of the Botany Institute of São Paulo. It was cultured on potato dextrose agar (PDA) medium for 2 weeks at 28 °C. When the plates were fully covered with the mycelia, mycelial plugs measuring 10 mm in diameter were made and used as inocula.

Culture conditions

The cultures were performed in cotton-plugged Erlenmeyer flasks (250 mL) containing 5 g of one of the following nine substrates: yellow passion fruit waste, orange bagasse, banana stalk, pineapple peel, corn cob, rice hull, sugar cane bagasse, wheat bran, or wheat straw. The following salts were added to give a final salt concentration of (in milligram per gram): K_2HPO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.2; and $CaCl_2 \cdot 2H_2O$, 0.1. Water was added to obtain initial moisture levels varying from 50 to 90 %. A total of nine different moistures were tested. The pH of the media was 6.0±0.1. Prior to use, the mixtures were sterilized by autoclaving at 121 °C for 15 min. Each flask received three mycelial plugs and was incubated statically under an air atmosphere at 28 °C and in complete darkness. After 10 days of cultivation, fungal growth was measured by glucosamine estimation of the fungal cell wall (Scotti et al. 2001).

Extraction of enzymes

After 10 days of cultivation, a volume of 50 mL of cold water was added to the contents of each flask. The mixtures were stirred for 1 h at 4 °C and centrifuged at 5,000 rpm for 10 min. The supernatants obtained were stored at 4 °C and used as crude enzyme extracts.

Enzyme assays

The laccase activity was determined by measuring the oxidation of 1 mmol/L 2,2′-azino bis (3-ethylbenzthiazoline-6-sulphonic acid (ABTS) in 50 mmol/L sodium acetate buffer (pH 4.5). Formation of the cation radical of ABTS was monitored at 420 nm (ε=36 L $mmol^{-1}$ cm^{-1}) (Hou et al. 2004). The Mn peroxidase activity was assayed spectrophotometrically by following the oxidation of 1 mmol/L $MnSO_4$ in 50 mmol/L sodium malonate, pH 4.5, in the presence of 0.1 mmol/L H_2O_2. The reaction was initiated by adding H_2O_2, and the rate of Mn^{3+}–malonate complex formation was monitored by measuring the increase in

absorbance at 270 nm (ε=11.59 L $mmol^{-1}$ cm^{-1}) (Wariishi et al. 1992).

In vivo dye decolorization experiments

To test the ability of *P. pulmonarius* cultures to decolorize synthetic dyes, each dye was membrane filtered through a 0.45-μm cellulose nitrate filter and mixed with the cultures previously autoclaved, to a final concentration of 200 ppm. After 10 days, the residual dyes in the cultures were extracted firstly with 50 mL of water followed by 50 mL of a mixture of methanol/acetone/water (1:1:1). Dye disappearance was determined spectrophotometrically by monitoring the absorbance at the wavelength of maximum absorbance for each dye: remazol brilliant blue (RBBR), 595 nm; ethyl violet, 596 nm; methylene blue, 665 nm; poly R-478, 530 nm, and Congo red, 497 nm. In control cultures, either the dye or the fungus (abiotic control) was omitted. To calculate the residual dye in the cultures, the total dye extracted with water and organic mixture in the abiotic control was considered as 100 %.

Dye decolorization by crude enzyme extracts

A volume of 0.5 mL of each dye to give a final concentration of 100 ppm and 0.5 mL of crude enzyme extract were added to 4.0 mL of 50 mmol/L malonate buffer, pH 4.5, containing 1 mmol/L $MnSO_4$ and 0.1 mmol/L H_2O_2. The mixtures were incubated in a rotary shaker at 40 °C for 2 h. Dye disappearance was determined spectrophotometrically by monitoring the absorbance at the wavelength of maximum absorbance for each dye. Boiled crude enzyme extracts were used as negative controls. For determination of dye decolorization by crude enzyme extracts at pH 6.5, malonate buffer was replaced by phosphate buffer.

Statistical analysis

The data were analyzed by Student's *t* test and one-way ANOVA with Tukey's multiple comparison test ($p<0.05$) using the statistical program pack GraphPad Prism® (Graph Pad Software, San Diego, USA). All data are presented as mean ± SD of three independent experiments.

Chemicals

The enzymatic substrates were obtained from Sigma Chemical Corp., St Louis, MO. PDA was obtained from DIFCO Laboratories, Detroit, MI. All other reagents were of analytical grade.

Results

Influence of the substrates and the initial moisture levels on the production of *P. pulmonarius* ligninolytic enzymes

The influence of the substrates on the growth of *P. pulmonarius* and on the activity of ligninolytic enzymes was evaluated using eight agricultural residues (banana stalk, corn cob, orange bagasse, rice hull, sugar cane bagasse, wheat straw, pineapple peel, and yellow passion fruit waste) and wheat bran. The initial moisture level of these cultures was 75 % (Table 1). After 10 days, all media were completely colonized by the mycelial biomass. Fungal chitin hydrolysis into *N*-acetylglucosamine was used to determine the fungal biomass. This analysis showed that *P. pulmonarius* grew well in all solid systems, but that orange bagasse, yellow passion fruit waste, pineapple peel, and wheat bran were the best substrates for growth. Under the conditions used in this experiment, laccase was the main ligninolytic enzyme produced by the fungus. The highest laccase activities were obtained by using the following substrates: wheat bran (830±90.0 U/L), pineapple peel (1,400±100 U/L), orange bagasse (1,800± 160 U/L), and yellow passion fruit waste (1,600± 180 U/L). Mn peroxidase activities were low, with the exception of the pineapple peel cultures, where a high activity of Mn peroxidase (2,200±205 U/L) was present.

The effect of the initial moisture level on the production of ligninolytic enzymes was tested using four substrates: wheat bran, pineapple peel, corn cob, and orange bagasse (Fig. 1). In pineapple peel cultures (Fig. 1a), both enzymes were produced at high amounts, with the initial moisture level of 80–90 % being the best condition for the production of laccase (around 2,400 U/L) and an initial moisture content of 70–75 % as the best condition for the production of Mn peroxidase (around 2,000 U/L). The substrate for which the initial moisture level had the strongest effect on the production of enzymes was corn cob (Fig. 1b). The initial moisture level of 85–90 % was the best condition for the production of laccase (900–1,000 U/L) whereas very low Mn peroxidase activities were detected in the filtrates. The best initial moisture level for the production of Mn peroxidase was 50–65 % (500–600 U/L), a condition for which the production of laccase was very low. Laccase was the main ligninolytic enzyme produced by the fungus in wheat bran cultures, and its production was positively affected by increases in the initial moisture level. The highest laccase activities were obtained with an initial moisture level of 85 % (2,860±

Table 1 Effect of substrate on the growth and laccase and Mn peroxidase activities by *P. pulmonarius* in solid-state cultures. The cultures were developed for 10 days at 28 °C

Substrate	Enzyme activity (U/L)		Fungal biomass[a]
	Laccase	Mn peroxidase	
Wheat bran	830.0±90.0	110.0±14.0	24.0±6.0
Wheat straw	670.0±54.0	315.0±30.0	20.0±7.0
Corn cob	450.0±32.0	250.0±21.0	21.0±7.0
Sugar cane bagasse	640.0±75.0	150.0±10.0	19.0±6.0
Rice hull	600.0±50.0	180.0±15.0	17.0±4.0
Pineapple peel	1,400.0±100.0	2,200.0±205.0	25.0±6.0
Banana stalk	510.0±30.0	150.0±10.0	15.0±4.0
Orange bagasse	1,800.0±160.0	90.0±15.0	26.0±8.0
Yellow passion fruit waste	1,600.0±180.0	180.0±20.0	25.0±7.0

[a]As glucosamine content (in milligram per flask)

250 U/L) (Fig. 1c). It is interesting to point out that in orange bagasse cultures, the production of laccase was enhanced (up to 2,000 U/L) by elevating the initial moisture level, and a low Mn peroxidase activity was found in all cultures, less than 100 U/L even in cultures developed with low initial moisture level (Fig. 1d).

Effect of the initial moisture level on the capability to decolorize industrial dyes by solid-state cultures of *P. pulmonarius*

The synthetic dyes used in this work were selected on the basis of their stability over a wide range of pH (3–11), thermostability,

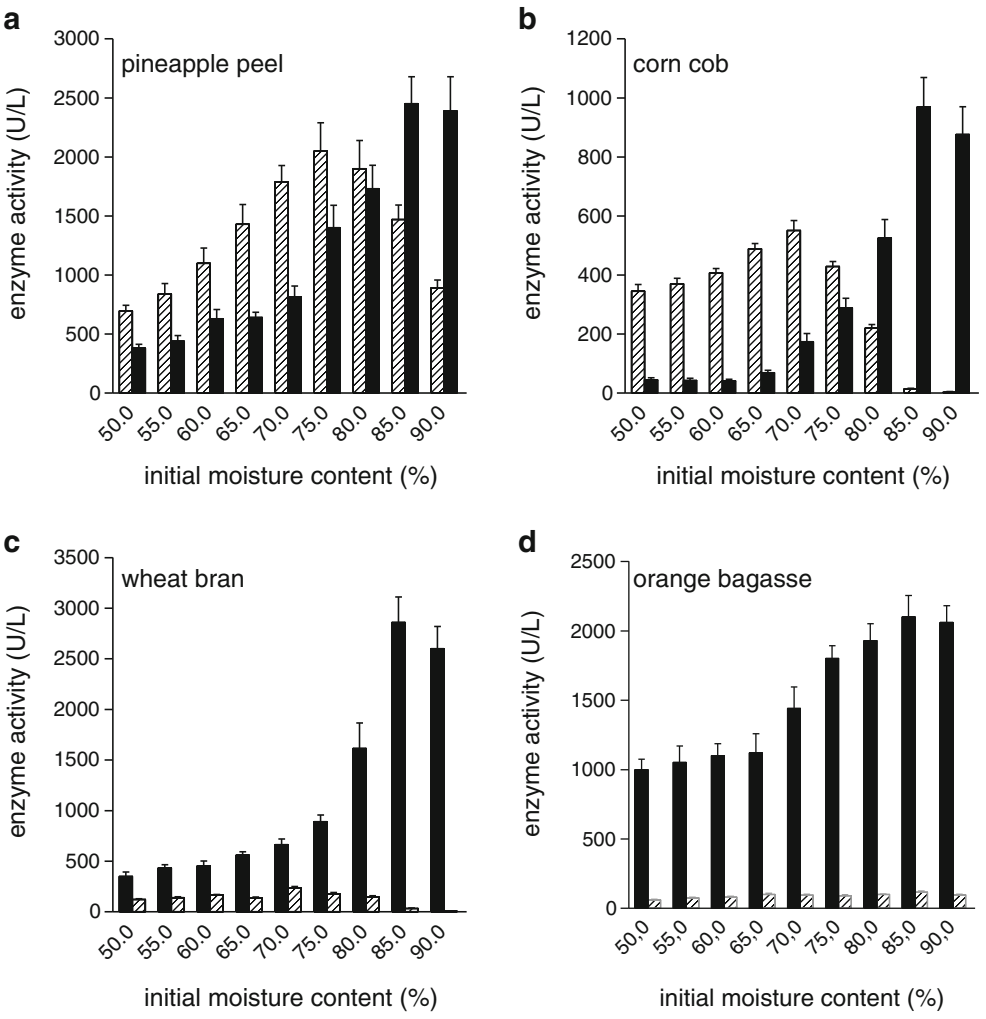

Fig. 1 Effect of the initial moisture level on the laccase and Mn peroxidase activities by *P. pulmonarius* in solid-state cultures. The cultures were developed for 10 days at 28 °C. Laccase, *full columns*; Mn peroxidase, *dashed columns*

and stability under culture conditions in noninoculated flasks, and they were representative for each chemical category (anthracene derivative, azo, heterocyclic, polymeric, and triphenylmethane dyes)

The fungal cultures developed with initial moisture levels of 60 and 85 % were able to decolorize RBBR completely. The other dyes were partially decolorized in the following order: Congo red > ethyl violet > methylene blue > polyR 478 (Table 2). Alcoholic extracts from mycelia and residual substrates showed that less than 8 % of the dyes were adsorbed by the mixture of fungi plus substrate.

Capability of crude extracts from *P. pulmonarius* corn cob cultures to decolorize synthetic dyes with high and low Mn peroxidase/laccase ratios

The selection of corn cob culture extracts for the in vitro decolorization experiments was based on two main points: first, in corn cob cultures, it was possible to obtain the lowest and highest Mn peroxidase/laccase ratio, at 60 and 85 % of initial moisture levels, respectively (Fig. 1); second, the corn cob crude extracts were clear due their low amount of natural colored pigments, when compared to other crude extracts. Corn cob culture extracts were tested for their ability to decolorize the synthetic dyes under two different conditions: (a) to obtain the best condition for Mn peroxidase activity (50 mmol/L malonate buffer, pH 4.5, with 1 mmol/L $MnSO_4$ and 0.1 mmol/L H_2O_2) and (b) to obtain the best condition for laccase activity (50 mmol/L phosphate buffer, pH 6.5). The results are shown in Fig. 2. Both crude extracts (from 60 and 85 % initial moisture content cultures) efficiently decolorized RBBR. The crude enzyme extracts obtained from cultures developed with an initial moisture level of 60 % were more efficient to decolorize the dyes methylene blue, ethyl violet, Congo red, and poly R-478.

Table 2 Decolorization of synthetic dyes by solid-state cultures of *P. pulmonarius* at two initial moisture levels

Synthetic dye	Residual dye (%)	
	Initial moisture content	
	60 %	85 %
Anthracene derivative dye: remazol brilliant blue R		
Corn cob cultures	3.7±2.8a	4.4±2.2a
Orange peel cultures	2.1±1.4a	1.9±1.3a
Wheat bran cultures	3.3±0.6a	3.0±1.0a
Pineapple peel cultures	1.4±1.0a	2.0±0.7a
Azo dye: Congo red		
Corn cob cultures	22.0±5.0a	47.3±5.6b
Orange peel cultures	50.4±7.6a	45.6±5.6a
Wheat bran cultures	25.4±3.7a	49.1±4.8b
Pineapple peel cultures	10.8±3.3a	14.7±3.6a
Heterocyclic dye: methylene blue		
Corn cob cultures	52.1±5.7a	67.9±7.3b
Orange peel cultures	60.0±4.7a	63.0±4.2a
Wheat bran cultures	51.0±4.9a	68.0±8.1b
Pineapple peel cultures	35.0±7.2a	38.0±6.0a
Triphenylmethane dye: ethyl violet		
Corn cob cultures	39.5±6.2a	62.5±2.3b
Orange peel cultures	58.4±7.1a	61.3±5.8a
Wheat bran cultures	43.1±4.9a	56.1±5.4a
Pineapple peel cultures	21.7±2.4a	23.1±1.9a
Polymeric dye: poly R478		
Corn cob cultures	67.8±6.8a	81.8±7.2a
Orange peel cultures	83.0±7.9a	80.5±8.3a
Wheat bran cultures	78.1±6.3a	81.1±4.9a
Pineapple peel cultures	66.7±6.5a	63.0±9.1a

Values labeled with different letters in each line are significantly different ($p<0.05$)

Discussion

The potential application of ligninolytic enzymes in biotechnology has stimulated investigations for selecting promising enzyme producers and for finding convenient substrates to obtain large amounts of low-cost enzymes. Wheat bran is the most commonly used substrate for the cultivation of white-rot fungi in solid-state cultures. However, the list of possibilities is very large and includes several lignocellulolytic wastes such as cane bagasse, corn cob, wheat straw, oat straw, rice straw, and food processing wastes such as banana, kiwi fruit, and orange wastes (Alexandrino et al. 2007; Couto 2008). Even so, it is worth to search for new substrates, especially if they are available in large amounts, allow the growth of white-rot fungi without further supplementations, and facilitate the obtainment of valuable products. Recently, yellow passion fruit

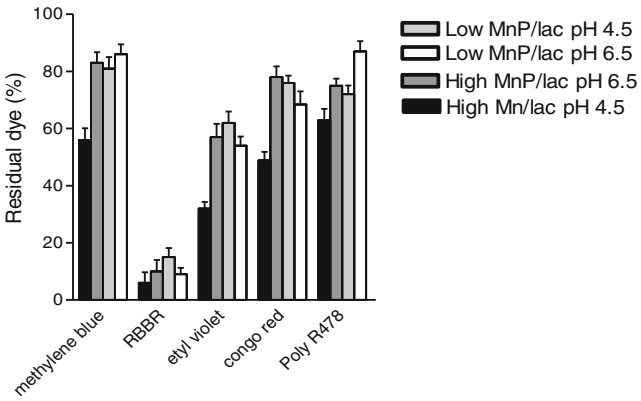

Fig. 2 In vitro decolorization of industrial dyes by *P. pulmonarius* corn cob crude extracts

waste was tested by our group as a substrate for growth and production of ligninolytic enzymes by several white-rot fungi with results comparable to those obtained with wheat bran (Zilly et al. 2012). In the present work, the main oxidative enzyme produced by *P. pulmonarius* was laccase. This result is in agreement with the general observation that laccase is the main ligninolytic enzyme for the genus *Pleurotus* (Arora and Sharma 2010). However, corn cob cultures developed under low moisture levels produced elevated levels of Mn peroxidase. By varying only the initial moisture level, the use of corn cob as a substrate allowed the obtainment of crude extracts rich in either laccase or Mn peroxidase. Another point worthy of being emphasized is the elevated activity of Mn peroxidase obtained in pineapple peel cultures. To our knowledge, this is the first report of the use of pineapple peel to produce ligninolytic enzymes.

Textile industries consume large volumes of water and chemicals for wet processing of textiles. The presence of very low concentrations of dyes in effluents is highly visible and undesirable (Nigam et al. 2000). Due to their chemical structure, dyes are resistant to fading on exposure to light, water, and many chemicals (Poots and McKay 1976), and decolorization of textile dye effluents does not occur when they are treated aerobically by sewerage systems (Willmott et al. 1998). Many white-rot fungi have been intensively studied in connection with their ligninolytic enzyme production and their decolorization ability (Boer et al. 2004; Chagas and Durrant 2001; Jarosz-Wilkołazka et al. 2002; Kasinath et al. 2003; Pointing and Vrjmoed 2000). However, most studies on dye decolorization have been carried out using liquid or solid cultures on agar plates, which do not reflect the natural living conditions (i.e., in wood and other lignocellulosic substrates) of the white-rot fungi. Our results show that solid-state cultures of *P. pulmonarius* were able to decolorize, at least partially, all dyes tested. In addition to this, our results demonstrate the dye decolorization capability of corn cob culture extracts rich in laccase or Mn peroxidase activities. The employment of ligninolytic enzyme preparations can bring considerable benefits over the direct use of white-rot fungi, considering that preparations can be more easily standardized, facilitating accurate dosage. Enzyme application is simpler than the use of microorganisms, and it can be rapidly modified according to the characteristics of the dye or dyes to be removed. Furthermore, analysis of metabolite compounds produced by enzyme preparations is easier than analysis of metabolites produced when the whole white-rot fungus is used.

An additional advantage for using the corn cob culture extracts instead of those obtained with wheat bran or pineapple peel is the low amount of natural colored pigments in corn cob. This allows the use of corn cob crude extracts in experiments of decolorization without any additional treatments, because the color of the extracts does not interfere with the determination of residual dyes. From the results obtained in this work, it is possible to suggest that Mn peroxidase was the main enzyme responsible for the decolorization of polyR 478, Congo red, ethyl violet, and methylene blue. Our data also suggest that laccase and Mn peroxidase cooperated in the RBBR decolorization process. To elucidate the mechanisms involved in the dye decolorization by the ligninolytic enzymes of *P. pulmonarius* as well as to evaluate more properly the toxicity of the decolorized products, it is necessary to conduct experiments using purified preparations.

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