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Pentachlorophenol Removal by *Pleurotus Pulmonarius* in Submerged Cultures

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ABSTRACT

Pentachlorophenol (PCP) removal by Pleurotus pulmonarius grown in submerged cultures in the presence and absence of laccase inducers was studied in this work. When PCP was added to a final concentration of 25 mg·L^{-1} in submerged cultures actively producing laccase, 70% of the PCP was removed after 96 h. The removal of PCP was less than 20% in the cultures with low laccase activity. The results suggested that laccase played an important role in the biodegradation of PCP by P. pulmonarius and that for bioremediation purposes the fungus must be cultured under the conditions of active laccase production.

Key words: laccase, pentachlorophenol, *Pleurotus pulmonarius*, xenobiotics

INTRODUCTION

Pentachlorophenol (PCP) has been widely used as a wood preservative and pesticide. PCP is toxic to all life forms due to its ability to make cell membranes permeable to protons, which leads to dissipation of the transmembrane proton gradients and electrical potentials that are required for energy generation in organisms (McAllistter et al., 1996). Over the last two decades, most developed countries have restricted the use of PCP for domestic and agricultural applications because of health and environmental hazards associated with the compound itself and its impurities (e.g. dioxin, furans and hexachlorobenzene). However, after decades of widespread use as a wood preservative, PCP has become an ubiquitous environmental contaminant. PCP is persistent with a half-life of up to 178 and 200 days in soil and water systems, respectively. In Brazil, PCP was used as a wood preservative until 2006. It is currently one of the most common soil and wastewater pollutants in the country.

Common methods for remediating PCP-contaminated materials include land disposal, incineration, soil washing, and chemical extraction. However, these strategies are expensive and do not eliminate the contaminant from the environment. Alternative strategies, such as transformations mediated by bacteria and fungi (bioremediation) and plants (phytoremediation), are gaining more widespread consideration because they can destroy contaminants or convert them into less bioavailable forms.

In recent years, the capability of white rot fungi (WRF) to biodegrade several xenobiotics and recalcitrant pollutants has generated considerable research interest in the area of industrial/

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environmental microbiology (Baldrian, 2008; Coelho et al., 2010; Moreira Neto et al., 2009; Sene et al., 2010). WRF are the only microorganisms known to be able to degrade the highly recalcitrant natural polymer lignin (a heterogeneous polyphenolic polymer) because they possess a powerful enzymatic system formed mainly by peroxidases (lignin peroxidase, EC 1.11.1.12 and Mn peroxidase, EC 1.11.1.13) and laccases (EC 1.10.3.2). Since these enzymes are non-specific, they can also attack a wide range of recalcitrant compounds, structurally related to lignin, accumulated in soil and water (Asgher et al., 2008). In relation to the capability to degrade PCP, *Phanerochaete chrysosporium* is the best studied WRF (Lamar and Dietrich, 1990; Lamar et al., 1990; Ruttimann-Johnson and Lamar, 1996). In principle at least, other WRF could be useful in the degradation of PCP. For example, *Trametes versicolor* can mineralize PCP, producing pentachloroanisole as intermediate (Ford et al., 2007; Lamar et al., 1992).

Species of the genus *Pleurotus* are among the easiest mushrooms to cultivate. In contrast to *P. chrysosporium*, the genus *Pleurotus* expresses ligninolytic systems during its growth phase. These systems are not inhibited by high nitrogen concentrations. Laccase is one of the most important ligninolytic enzyme of the genus and the induction of laccase by phenolic compounds has been well described in several species, including *Pleurotus sajor caju* (Lo et al., 2001), *Pleurotus eryngii* (Muñoz et al., 1997), and *Pleurotus pulmonarius* (Souza et al., 2004).

For bioremediation purposes, one of the most studied species is *P. ostreatus,* which has a high potential to degrade several organic compounds, including polycyclic aromatic hydrocarbons (PAH) and chlorophenols in liquid and solid-state cultures (Bezalel et al., 1996; Zeddel et al., 1993). In addition, *Pleurotus pulmonarius* is able to decolourize textile industrial dyes under submerged conditions (Zilly et al., 2002) and to degrade atrazine in solid-state cultures (Masaphy et al., 1996). It was also shown that a spent compost of *P. pulmonarius* was able to remove PCP predominantly by biodegradation (Law et al., 2003). However, this PCP biodegradation was actually the result of the combined action of *P. pulmonarius* cells and several kinds of bacteria present in the system. It remains to be shown that *P. pulmonarius* alone is able to remove PCP. This was precisely the main objective of the present study, in which PCP removal by *P. pulmonarius* grown in submerged cultures in the presence and absence of laccase inducers was compared.

MATERIALS AND METHODS

Organism and culture conditions

Submerged cultures of *Pleurotus pulmonarius* (CCB19) obtained from the Culture Collection of the São Paulo Botany Institute were obtained by transferring three disks from the growing edge of the mycelium on PDA plates to a 250 mL Erlenmeyer flask containing 50 mL of potato-dextrose medium. The cultures were incubated at 28°C in a rotary shaker at 120 rpm for five days. Homogenized and standardized pellets from 5-day-old shaken cultures were obtained, and the pellets were used to evaluate the effect of PCP on *P. pulmonarius* growth, enzyme activity, and PCP removal under submerged conditions. Pellets from potato-dextrose medium were transferred to two types of cultures, non-induced and induced laccase media. In the first, the cultures were carried on 25 mL of mineral solution (Vogel, 1956) using glucose (10 $g \cdot L^{-1}$) and ammonium tartrate (0.86 $g \cdot L^{-1}$) as carbon and nitrogen sources (basal medium). In the second condition, corn cob extract rich in phenolic compounds was used as laccase inducer. The corn cob extract was prepared by autoclaving 50 g of corn cob powder plus 500 mL of mineral medium for 15 min. The mixture was then filtered and the soluble material was enriched with glucose (10 $g \cdot L^{-1}$) and ammonium tartrate (0.86 $g \cdot L^{-1}$) (corn cob medium). After five days of cultivation at 120 rpm at 28° C, PCP was added to a final concentration of 25 $mg \cdot L^{-1}$ (85 μM). The *P. pulmonarius* biomass was periodically separated by filtration, and used to estimate the fungal growth in comparison to control cultures without PCP. The effects of PCP on Mn peroxidase and laccase activities were also evaluated by comparing the results with those where no PCP was added.

Determination of PCP remaining in the culture filtrates and PCP adsorbed in the fungal mycelia

The remaining PCP was evaluated in the culture filtrates and in the fungal mycelia. To evaluate the

amount of PCP sorbed on the fungal mycelia, after filtration, the mycelia were washed with distilled water and immediately frozen at -20° C. The material was ground to small particle-size and extracted with ethanol in a 125 mL flask that was shaken at 120 rpm in an orbital shaker for 2 h. The fungal mycelial extract was obtained by centrifugation at 5000 g for 15 min. PCP in the samples was analysed by high performance liquid chromatography (HPLC), using a reversed phase R-Sil C18 column (10 µM, 4.6 x 250 mm), acetonitrile:water:acetic acid (75:25:0.125) as the mobile phase with a flow rate of 0.7 $mL \cdot min^{-1}$, and UV detection at 238 nm. The identification of PCP in the samples was based on the retention time and by spiking the samples with PCP standard. PCP concentrations were determined using calibration curves with peak areas of authentic PCP standards.

Enzyme assays

The laccase activity was determined using syringaldazine as substrate (Leonowicz and Grzywnowicz, 1981). The Mn peroxidase activity was assayed by following the oxidation of $MnSO_4$ in sodium malonate buffer in the presence of H_2O_2 (Wariishi et al., 1992). The lignin peroxidase activity was determined by spectrophotometric measurement at 310 nm of the H_2O_2-dependent veratraldehyde formation from veratryl alcohol (Tien and Kirk, 1984). The enzymatic activity was expressed in international unit (U), defined as the amount of enzyme required to produce 1 µmol product per min.

Determination of phenolic contents

Total phenol content was determined using the Folin-Ciocalteau reagent and expressed as ferulic acid equivalents (Singleton and Rossi, 1965).

Statistical analysis

The data from the different treatments were compared using paired t-test with a significance level of $p \leq 0.05$. The experiments were conducted in triplicate. The data are presented as mean ± standard error. The analyses were conducted using the GraphPad Prism® statistical program pack (Graph Pad Software, San Diego, USA).

Chemicals

The enzymatic substrates and PCP were obtained from Sigma Chemical Corp. (St Louis, MO). PDA was obtained from Difco Laboratories (Detroit, MI). The solvents used in HPLC analysis were of chromatographic grade and all other reagents were of analytical grade.

RESULTS

Previous studies have shown that media supplemented with soluble phenolic compounds from corn cob powder increased the capability of *P. pulmonarius* to produce laccase and to decolourize the industrial dyes (Tychanowicz et al., 2004). For this reason, the same strategy was used in this work. As expected, soluble corn cob extract increased the phenolic compound content from 0 $mmoles \cdot L^{-1}$ (basal medium) to 1.43 $mmoles \cdot L^{-1}$ (corn cob medium). The addition of soluble corn cob extract slightly increased biomass production, however, without statistical significance ($p > 0.05$) (Fig. 1A). The addition of corn cob extract significantly improved laccase activity by more than four times (from 26.6±3.00 $U \cdot g$ dry $biomass^{-1}$ to 116.10±10.00 $U \cdot g$ dry $biomass^{-1}$ (Fig 1B), but did not significantly affect the Mn peroxidase activity (Fig. 1C).

No lignin peroxidase activity was observed in any culture filtrates. The addition of PCP inhibited considerably the growth of the fungus in the basal condition (Fig. 1A). On the other hand, PCP caused only a slight inhibition of the fungal growth in the corn cob medium. The presence of PCP increased the laccase activity five fold in basal medium and two fold in corn cob medium (Fig. 1B), but did not affect the Mn peroxidase activity (Fig. 1C). After 96 h of addition of PCP, about 70 and 20% of the initial PCP were removed in basal and corn cob media, respectively (Fig. 1D). In both types of cultures, less than 8% of PCP was found to be sorbed to the fungal mycelium.

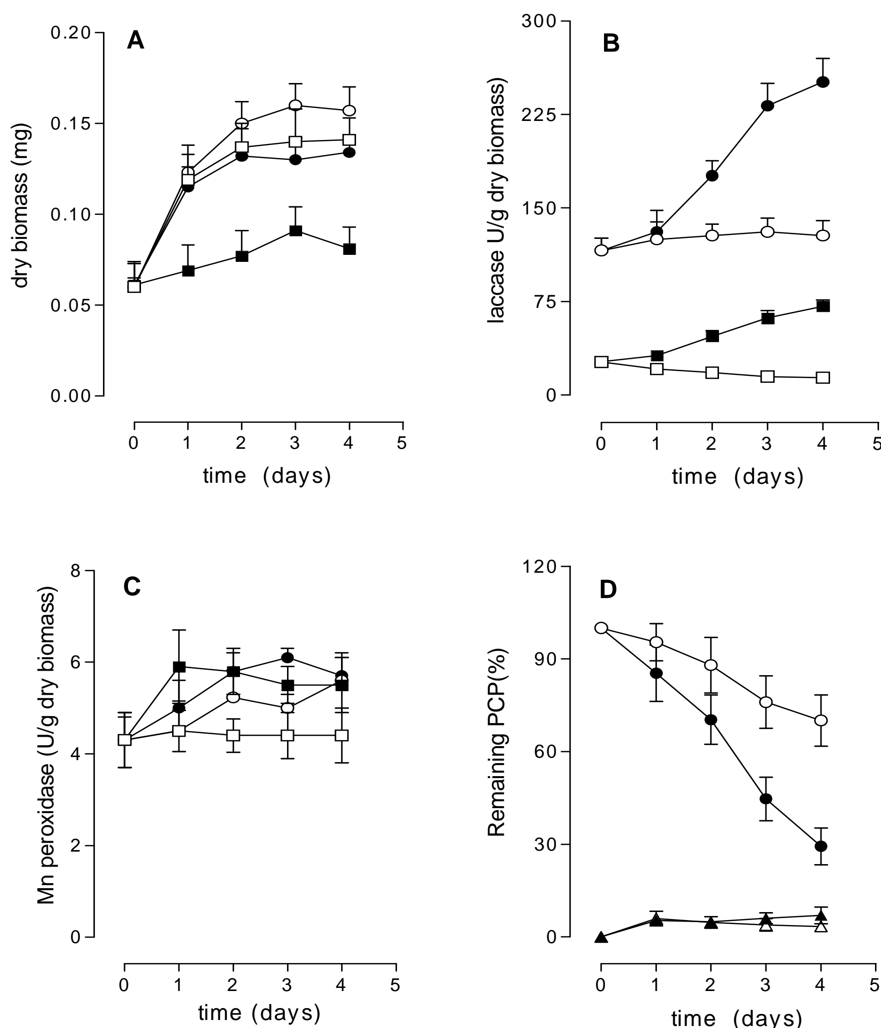

Figure 1 - Effects of PCP in the growth and production of laccase and Mn peroxidase by *P. pulmonarius.* After 5 days of cultivation into basal or corn cob medium, PCP was introduced at the final concentration of 25 mg·L^{-1}. Periodically the cultures were interrupted by filtration. Laccase and Mn peroxidase activities and remaining PCP were determined in the culture filtrates. Dry biomasses were used to estimate the fungal growth and to determine the PCP adsorbed to the mycelia. Legends: (●) induced culture with PCP; (○) induced culture without PCP; (■) non-induced culture with PCP; (□) non-induced culture without PCP. In D: (▲) PCP adsorbed to the mycelial mass obtained in corn cob medium (with laccase inducers); (Δ) PCP adsorbed to the mycelial mass in basal cultures (without laccase inducers). Each marker and error bar represents the mean and standard deviation of three replicates.

DISCUSSION

An increasing range of white rot fungi is being investigated for their bioremediation potential. Xenobiotic removal by some white-rot species such as *Pleurotus, Ganoderma* and *Coriolus* is believed to be catalyzed mostly by laccases. On the other hand, the oxidation of xenobiotics by other white-rot fungi such as *Nematoloma frowardii* (Hofrichter et al., 1998), *P. chrysosporium* (Moen and Hammel, 1994), *Irpex lacteus* (Baboravá et al., 2006) and *Bjerkandera* sp (Eibes et al., 2007) is mainly due to the action of Mn peroxidases. The data obtained in this work showed that laccase was the main ligninolytic enzyme produced by *P. pulmonarius* in submerged cultures, considering that low Mn peroxidase activities and no lignin peroxidase were detected

in the culture filtrates. In the genus *Pleurotus*, laccases are constitutively produced in small amounts; however, their production can be considerably enhanced by a wide variety of substances, mainly aromatic or phenolic compounds related to lignin or lignin derivatives, such as ferulic acid, 2,5 xylidine, *p*-anisidine or veratryl alcohol (Leonowicz et al., 2001; Souza et al., 2004; Tychanowicz et al., 2006)

In this work, the supplementation of submerged cultures with corn cob extract had a very pronounced effect on the laccase activity in *P. pulmonarius*. The laccase inducer effect of the soluble plant extract is considered to be related to its phenolic content. Cotton stalk extract, for example, acted as an inducer of laccase in *Pleurotus ostreatus* submerged cultures (Ardon et al., 1996; 1998). However, this effect was not a general one, considering that the same extract did not stimulate the production of laccase by *G. applanatum, T. versicolor* and *R. solani* (Ardon et al., 1996).

Another important observation of the present work was that *P. pulmonarius* was resistant to PCP, especially when a high laccase activity was present. Results of this study also showed that PCP increased laccase activity in submerged cultures with *P. pulmonarius*, but had no effect on Mn peroxidase activity. It has been suggested that laccases may be important aromatic compound detoxification enzymes for many fungal species by catalyzing nonspecific oxidation and polymerization reactions. For example, laccase production was stimulated by the addition of 0.5 mM PCP to liquid cultures of *Trametes versicolor* (Mougin et al., 2002) and by the addition of 25 µM paraquat to *T. versicolor* and 20 µM paraquat to *A. biennis* cultures (Jaszek et al., 2006).

CONCLUSIONS

Environmental contamination with PCP and other aromatic pollutants are a serious concern world-wide. Many studies have shown that these persistent compounds can be degraded by the ligninolytic system of white-rot fungi. In this study, it was discovered that PCP could be rapidly removed in submerged cultures of *P. pulmonarius*, particularly under culture conditions with high laccase activity. Further studies are necessary to elucidate the types and toxicity of reaction products produced under these conditions. Such studies are important for developing effective bioremediation programs based on the ligninolytic system of *P. pulmonarius.*

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