Physiological Characterization of Fungal Inoculum for Biotechnological Remediation of Soils

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ABSTRACT

The aim of this work was to study the bioremediating potential of Lentinus crinitus CCIBt2611 according to the physiological condition of the inoculum. Inoculum was prepared using sugarcane ground husk (C:N 90), at several physiological ages and applied in soil contaminated with pentachlorophenol. The inoculum’s potential was assessed by evaluating the mycelium’s vigor at soil’s colonization, determination of peroxidase and phenoloxidase activities, in vitro degradation of Remazol Brilliant Blue R and in vivo degradation of pentachlorophenol. The results showed that the assessed parameters were relevant to identify the quality of the inoculum. For L. crinitus, 10 day old inoculum showed good soil-colonization speed with significant enzymatic activities, indicating the role of Manganese-dependent peroxidase and laccase in degradation, and efficient degradation of pentachlorophenol.

Key words: laccase, manganese-dependent peroxidase, organochlorine degradation, fungal inoculum quality, bioremediation, environmental sanitization

INTRODUCTION

The xenobiotics degradation by basidiomycete is an oxidative, extracellular and non-specific process and can be understood as a multienzymatic process resulting from the coordinated action of peroxidases, laccases and oxidases (Boyle et al. 1992; Leonowicz et al. 1999). The main ligninolytic enzymes produced by the basidiomycetes directly involved in the degradation of natural polymers are manganese-dependent peroxidase (MnP, E.C. 1.11.1.13) and laccases (E.C. 1.10.3.2) (Papinutti et al. 2003; Wesenberg et al. 2003; Rabinovich et al. 2004; Kulikova et al. 2011). Basidiomycetes species from the genus Lentinus have been assessed because of their capacity of degrading recalcitrant compounds such as polyaromatic hydrocarbides (Valentín et al. 2006; Covino et al. 2010), polychlorinated byphenils (Ruiz-Aguilar et al. 2002). L. crinitus CCIBt2611 presented the capacity of mineralizing hexachlorobenzene and pentachlorophenol, as well as reactive textile dyers (Matheus et al. 2000; Matheus et al. 2003; Machado et al. 2005a; Moreira Neto et al. 2009).

Therefore, such fungus has been assessed in bioremediation processes for decontamination of soils containing organochlorine compounds (Matheus et al. 2003).

For bioremediation process, the inoculum must be produced in large scale, with the same technology employed in the cultivation of edible mushrooms...
(Ballaminut and Matheus 2007). The fungal mycelium’s colonization capacity of the substrate is the main quality criterion for the inoculum, which is generally employed at the physiological age of 20 days (Bononi 1997, Lamar and White 2001; Matheus 2003). However, the physiological conditions that knowingly enhance fungal inoculum’s strength vary according to growth time on solid substrate (Ballaminut and Matheus 2007) and may interfere in the efficiency of degradation of the pollutant present in the soil to be treated. A study on L. crinitus cultivated in liquid medium defined both the initial and the exponential growth phase of that fungus; biomass was determined after dry weight, along with ergosterol quantification (Silva et al. 2010). The production of ligninolytic enzymes and their direct or indirect involvement in the xenobiotics-degradation metabolism is well documented in literature (Fragoeiro and Magan 2005; Moreira Neto et al. 2009; Oliveira et al. 2010; Kulikova et al. 2011).

In order to improve the quality parameters used to evaluate the fungal inoculum applied for soil bioremediation, thus optimizing the treatment process, this study aimed to evaluate different times of substrate colonization by Lentinus crinitus CCIBt2611, providing favorable conditions for in vivo degradation of pentachlorophenol in soil, associated with the shorter preparing time of the inoculum.

MATERIALS AND METHODS

Fungus
Lentinus crinitus CCIBt2611 was obtained from the Algae, Cyanobacteria and Fungi Culture Collection of the Botanic Institute of São Paulo (CCIBt) and stored in MEA 2% culture medium at 4°C.

Cultivation in soil
PCP-soil
Two hundred milligram pentachlorophenol P.A. kg⁻¹ from sandy soil were mixed with commercial plaster (2.5%), followed by manual homogenization in propylene bag for 15 min. The soil underwent chemical sterilization with methylbromide in sealed chamber for 48 h (Moreira Neto 2006).

Fungal inoculum
Ten gram solid substrate with 70% humidity (dry mass), with C:N ratio adjusted to 90 (92.3% sugarcane husk, 0.3% soy flour, 7.4% soluble amide) was taken in polypropylene bags and sterilized at 121°C for 90 min. Five mm-diameter mycelium growth disks (MEA 2%) were inoculated in the solid substrate in 1:10 proportion (disk : g humid substrate) and incubated at 28°C for 5, 10, 15 and 20 days. The inoculum age of varying age (T5, T10, T15 and T20) were used.

Cultivation in PCP-soil
Thirty gram sterilized PCP-soil (dry mass) was added into 250 mL capacity flasks containing 5.0% vegetable-oil emulsion and Tween 20 (9:1, p.p.), 10.0% inoculum (dry mass) of different physiological ages and 15.0% sterile water. The control-treatment received solid substrate without fungus. The flasks were incubated at 28°C for 60 days. Humidity correction was made periodically by gravimetric analysis (Matheus 2003).

Soil-colonization analysis
The fungal growth in the PCP-soil was analyzed by visual observation. A value was stipulated for each replication according to the soil’s percent colonization, judged by six independent observers (Ballaminut and Matheus 2007).

Enzymatic assays
The enzymatic extracts were obtained by mixing 10 g soil sample in 30 mL of 50 mm sodium acetate (pH 4.5) (Ballaminut et al. 2009). The contents were homogenized manually for three minutes followed by mechanical stirring (2 hertz) for 1 h (Tecnal®/TE -140). After settling, the supernatant was separated by filtration (0.45 μm Millipore®) and used for the enzymatic assays.

Laccase: Laccase was determined through ABTS oxidation at 420 nm (molar extinction coefficient = 36,000 M⁻¹ cm⁻¹). The reacting solution (1.0 mL) contained 250 μL of 50 mM cytrate-phosphate buffer, pH 4.0; 50 μL ultrapure water; 100 μL of 5 mM ABTS and 600 μL enzymatic extract (Machado and Matheus 2006; Ballaminut et al. 2009). One unit of enzyme corresponded to the enzyme amount to oxide 1 μm ABTS per min.

Total peroxidases
The reaction solution contained the same reagents used for laccases determination, but replacing ultrapure water with 50.0 μL of 2 mM H₂O₂ (Machado and Matheus 2006; Ballaminut et al. 2009).
2009). One unit of enzyme corresponded to the amount of the enzyme required to oxidize 1 µm ABTS per min. Total peroxidases were given by the difference between the value obtained in the reaction and the previously determined laccase activity (Eggert et al. 1996).

**Manganese-dependent peroxidase**

Mn-dependent peroxidase was assayed by phenol-red oxidation determined at 610 nm (Machado and Matheus 2006). The reaction mixture (1.0 mL) contained 300 µL of 0.2 M sodium succinate buffer (pH 4.5), 0.1 M sodium lactate and 0.5 % bovine albumin; 50 µL of 2 mm MnSO₄; 100 µL of 0.1 % phenol-red; 50 µL of 2 mm H₂O₂; and 500 µL enzymatic extract. One enzyme unit corresponded to the amount required to oxidize 1 µm substrate per min.

**RBBR de-coloration:** RBBR de-colorization was determined by the decoloratization of Remazol Brilliant Blue R (RBBR) at 592nm according to Machado and Matheus (2006). The reaction mixture contained the same reagents used for the determination of total peroxidases with RBBR (0.02%) instead of ABTS. One activity unit was defined as the amount necessary to reduce 0.01 in absorbance after 10 min, using enzymatic extract inactivated by wet heat as control.

**Pentachlorophenol in vivo biodegradation**

It was determined through the concentration variation of pentachlorophenol remaining in the soil during cultivation, using as abiotic control the concentration of that residual pollutant in the soil without the fungus.

**Extraction of the remaining pentachlorophenol**

The extraction was carried out in sealed 20 mL flasks containing aliquots of 3.0 g combined soil-sample and fungal inoculum, with 10.0 mL methanol P.A. The flasks were heated in microwave oven for 16 cycles of 20 s at 270 W, alternating ice-baths to avoid boiling and loss by volatilization (Andréa et al. 2001 modified). After cooling, the solvent was recovered and 1.0 mL of each sample was diluted (1:2) in methanol (HPLC grade).

**Pentachlorophenol quantification**

The diluted samples were analyzed by HPLC (Varian ProStar® 215). For pollutant separation, reverse-phase Hipersyl 4.6 x 250 mm column was used with R - SiL C - 18 (10 µm) and acetonitril: water : acetic acid (75 : 25 : 0.125) mobile phase (flow rate 0.9 mL . min⁻¹). The detection was made at 254 nm; pentachlorophenol retention time in the chromatographic column was between 7 and 7.4 min after sample injection (Ullah et al. 2000). The pentachlorophenol amount from the extracts was estimated after a previously settled standard curve, where the amounts found were obtained linear-regression analysis (y = 6000000x + 92782; r² = 0.999).

**Statistical analysis**

Data were assessed using the MiniTab® statistic program version Release 14. The means were compared through Tukey test, following the analysis of variance (ANOVA) (α ≤ 0.1). Percent data were transformed in the following expression, according to Vieira and Hoffmann (1989).

\[ \text{Transformed value} = \text{arcsen} \left( \sqrt{\frac{\% \text{ value}}{100}} \right) \]

Where: arcsen = sine arch and \( \% \) value = pentachlorophenol percent recovery.

**RESULTS**

Figure 1 shows PCP-soil colonization by *L. crinitus*. The treatments T10, T15 and T20 produced superficial colonization, at first faster, reaching up to 40% on the 10th day of cultivation.

![Figure 1 - Colonization of soil contaminated with pentachlorophenol (PCP) by the inoculum of *Lentinus crinitus* CCIBt2611 at different physiological ages: T5 = 5-day inoculum; T10 = 10-day inoculum; T15 = 15-day inoculum; T20 = 20-day inoculum.](image-url)
T20, which reached the same percent colonization after 40 days cultivation. At day 60, the PCP-soil was completely colonized, regardless of the inoculum age.

Figures 2, 3, 4 and 5 show enzymatic activities of laccases, total peroxidases, and RBBR decoloration by *L. crinitus* in PCP-soil after each treatment, T5, T10, T15 and T20, respectively. The enzymatic activities were observed all along incubation (60 days) in PCP-soil independent of the treatment.

Laccase production seemed to be main enzymatic activity in every treatment, presenting a profile similar to RBBR de-coloration’s kinetics. The highest levels of laccase activity were observed 15 to 30 days after T10-incubation. Treatments T10 and T20 results maximum laccase activity in 15 days with 150.32 and 143.84 U L$^{-1}$, respectively. The treatment T10 also presented higher RBBR de-coloration values, 10.93 U L$^{-1}$. Among the peroxidases, Mn dependent peroxidase was highest in T10 (18.44 U L$^{-1}$).
PCP in vivo degradation was maximal on the 5th day of L. crinitus cultivation in the PCP-soil in the first treatments (T5, T10 and T15), with only 1.3% PCP recovery (up to 98.7% depletion) and no significant difference between the treatments. However, treatment T10 presented the highest degradation rate \( (y = 277.14e^{0.871x}) \), as shown in Figure 6. For the 15th day cultivation, no pentachlorophenol recovery was observed in any of the treatments, because the complete degradation occurred on the 5th day (Fig. 7). At the same time, highest activity of Mn-dependent peroxidase was also observed on the 5th day incubation in PCP-soil in the same treatment (Fig. 7). Treatment T10 already presented higher Mn-dependent peroxidase activity after fungal inoculation in the PCP-soil (53.09 U L\(^{-1}\)) as shown in Figure 8.

The data obtained in the degradation of the organic chlorinated was validated through the PCP-soil extraction method efficiency, with 87.26% recovery of the initial pentachlorophenol concentration (277.139 ± 28.739 mg kg\(^{-1}\) soil).

Also, pollutant’s abiotic losses up to 37% were observed, out of which the lowest occurred in the treatment T10 (3%).

Figure 6 - Residual concentration of pentachlorophenol (PCP) in the soils incubated with Lentinus crinitus CCIBt2611 in treatments T5 \((y = 277e^{0.56x})\), T10 \((y = 277e^{0.87x})\), T15 \((y = 277e^{0.61x})\) e T20 \((y = 277e^{0.30x})\). Equations shows degradation rate (differents letters indicate statistical difference – Tukey Test, \(P \leq 0.1\)).

Figure 7 - A) Soil-pentachlorophenol percent decrease (■); B) Mn-dependent peroxidase’s enzymatic activity (□); C) laccase’s activity (│││); D) RBBR de-coloration (≡) in 5th day of cultivation of Lentinus crinitus CCIBt2611, in the different treatments (T5 = 5-day inoculum, T10 = 10-day inoculum, T15 = 15-day inoculum, and T20 = 20-day inoculum). (differents letters indicate statistical difference – Tukey Test, \(P \leq 0.1\)).
Figure 8  - A) Laccase’s enzymatic activities and B) Mn-dependent peroxidase’s, both on solid substrate and in the soil. Enzymatic activities on solid substrate at soil-inoculation in the zero days (□), 5th day (■) and 15th day (///) of soil cultivation, by *Lentinus crinitus* CCIBt2611, in the different treatments (T5 = 5-day inoculum, T10 = 10-day inoculum, T15 = 15-day inoculum, and T20 = 20-day inoculum). Error bar ± average deviation.

DISCUSSION

The analysis of colonization by fungal inoculum can be made either directly through visual analysis, as described in this study, or indirectly through the quantification of the biochemical indicators. In a study employing *L. crinitus* (CCIBt2611) Silva et al. (2010) used as biomass indicator ergosterol, cultivated in the same substrate described in this study, concluding that the exponential growth of that strain occurred after the 7th day of incubation. Corroborating the data from Silva et al. (2010), a slower colonização was observed after employing an under-10-day physiological age inoculum, possibly supporting the use of inocula for a time longer than 7 days incubation, when the growth rate was accelerated. The quick colonization of solid substrates by filamentous fungi, as in the case of PCP-soil, could overestimate fungal colonization when visually observed. That happens because of the extension mode of the hyphae, which initially provide the superficial colonization of the substrate, forming a thin layer of mycelium that only afterwards penetrate the substrate mass (Gowthaman et al. 2001, Ballaminut and Matheus 2007). This was as observed in the inocula assessed (T5, T10, T15 and T20). However, the visual observation of the growth for assessing fungal inocula for application in bioremediation might not be a good evaluation inoculum’s quality, if applied exclusively. Thus, to evaluate the quality of the inoculum, other parameters such as enzymatic activities and their correlation with the degradation rates should also be considered. RBBR de-coloration’s kinetics employed to identify the ligninolytic system’s total action in the de-coloration of the anthracinonic colorant showed positive results. The similarity of laccase and RBBR de-coloration enzymatic profile proved the hypothesis that laccase was the main oxidase involved in pentachlorophenol degradation, rendering it an important biotechnological tool, which was also observed by other authors, including in degradation studies (Jeon et al. 2008; Moreira Neto et al. 2009; Cañas e Camarero 2010; Majeau et al. 2010; Niebisch et al. 2010; Gaitan et al. 2011; Ding et al. 2012; Eichlerová et al. 2012; Moldes et al. 2012; Pardo et al. 2012; Ueda et al. 2012; Janusz et al. 2013; Manavalan et al. 2013). Laccase production, with concomitant Mn-dependent peroxidase production showed that such enzymatic activities might be involved in the in vivo pentachlorophenol degradation under the assessed conditions, since the highest enzymatic activities in the PCP-soil were observed in the treatments with the highest degradation rates. Furthermore, the Mn-dependente peroxidase data in T10, along with the degradation rates, indicated peroxidase as the main involved in the degradation. The results suggested the relevance of the enzymatic levels, especially for the
peroxidases present in the inoculum soon after its inoculation in the soil on the quality of the inoculum to be employed.

The capacity of basidiomycetes to degrade chlorophenols, including pentachlorophenol as already reported in literature (Okeke et al. 1997; Ullah et al. 2000; Seradati et al. 2003; Machado et al. 2005a; Soares et al. 2011) was proved here. There are difficulties to use the concentrations above 200 mg kg\(^{-1}\) pentachlorophenol, given the basidiomycetes’ sensibility to the pollutant (Leontievsky et al. 2001; Ahn et al. 2002; Walter et al. 2004). Hence, the use of \(L. \textit{crinitus} \) CCIBt2611 could be promising as it demonstrated efficacy in the treatment of pentachlorophenol in the same concentration range. Lamar and Dietrich (1990) reported up to 91% pentachlorophenol decrease after using \(\textit{Phanerochaete chrysosporium}\) at concentrations between 250 and 400 mg kg\(^{-1}\) along 6.5 weeks. In a study with \(L. \textit{crinitus} \) CCIBt2611, Machado et al. (2005b) observed the capacity of such basidiomycete in degrading pentachlorophenol at much higher concentrations (around 1,400 mg kg\(^{-1}\) soil), showing the elevated resistance, associated with the rapidity of the fungus to the tested pollutant.

Dzul-Puc et al. (2005), in a study with \(P. \textit{chrysosporium}\) in sugarcane husk and pine seraglio estimated the influence of physiological age and substrate on benzo(\(\alpha\))pyrene degradation, concluding that the removal of the contaminant was most efficient when the inoculum age was 5-days, which was similar for \(L. \textit{crinitus} \) in this work. Some authors have suggested against the use of a too young inoculum in field applications, as the colonization of the contaminated soil would be much slower and hinder the colonization because of the little mycelium biomass present in inoculum (Schmidt et al. 2005; Walter et al. 2005). Similar results were found in this study, which showed 10-days inoculum as the best.

Inocula produced in alginate pellets solid substrate were tested by Lestan and Lamar (1996), who observed that such inocula took up to 8 days for complete colonization, yet, remaining strong enough for their use in the soil, without modifications in their mechanic consistency. In such condition, the fungi removed around 80 to 90% pentachlorophenol in four weeks, confirming their efficiency and supporting the use of younger inocula. Considering this, the young inocula studied here showed degradation above 90% after a much shorter time (5 days cultivation), without pellet possibly favoring degradation in a field scale. The abiotic losses were comparable to those observed in the present study (Lamar and Dietrich 1992; Lamar et al. 1994; Matheus et al. 2000; Machado et al. 2005b).

Table 1 showed 11 criteria assessed in the present study that could help selecting the best condition for the cultivation of \(L. \textit{crinitus} \) for its use in the bioremediation of soils contaminated with pentachlorophenol. Evidently the best treatment was 10-day physiological age inoculum (T10) satisfying at least 80% of the quality criteria proposed. Thus, unlike what is preconized (at least 20 days), the biological treatment can employ younger inocula, optimizing their production for biotechnological application.

| Qualitative criteria | T5 | T10 | T15 | T20 |
|-----------------------|----|-----|-----|-----|
| 1. Higher laccase activity in the inoculum at soil-aplication | x |     |     |     |
| 2. Higher Mn-dependent peroxidase in the inoculum at soil-aplication | x |     |     |     |
| 3. Inoculum with more biomass at soil-cultivation | x | x | x |     |
| 4. Higher laccase activity in the 5\(^{th}\) day of soil cultivation | x |     |     |     |
| 5. Higher Mn-dependent peroxidase in the 5\(^{th}\) day of soil cultivation | x | x |     |     |
| 6. Higher RBBR de-coloration in the 5\(^{th}\) day of soil cultivation | x | x | x |     |
| 7. Higher laccase activity during soil-cultivation | x |     |     |     |
| 8. Higher Mn-dependent peroxidase activity during soil-cultivation | x |     |     |     |
| 9. Higher RBBR de-coloration during soil-cultivation | x |     |     |     |
| 10. Faster soil-colonization | x | x | x |     |
| 11. Higher pentachlorophenol percent degradation | x | x | x |     |
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