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Influence of pH on the Growth, Laccase Activity and RBBR Decolorization by Tropical Basidiomycetes

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ABSTRACT

The basidiomycete fungi Lentinus crinitus *and* Psilocybe castanella *are being evaluated in a bioremediation process of soils contaminated with organochlorine industrial residues in the Baixada Santista, São Paulo. The aim of the present study was to determine the influence of pH on the fungal growth,* in vitro *decolorization of anthraquinonic dye Remazol Brilliant Blue R (RBBR) and laccase activity. The pH of the culture medium influenced the growth of L. crinitus and P. castanella, which presented less growth at pH 5.9 and pH 2.7, respectively. The fungi were able to modify the pH of the culture medium, adjusting it to the optimum pH for growth which was close to 4.5. Decolorization of the RBBR was maximal at a pH of 2.5 to 3.5. Higher laccase activity was observed at pH 3.5 and pH 4.5 for* L. crinitus *and* P. castanella, *respectively. pH was found to be an important parameter for both the growth of these fungi and the enzymatic system involved in RBBR decolorization.*

Key words: White rot fungi, RBBR, laccase, peroxidases

INTRODUCTION

A series of studies have been conducted with the objective to reduce the level of pollution of organochlorine contaminated soils by the application of Brazillian basidiomycete fungi (Matheus et al., 2000, 2001; Machado et al., 2005). The metabolism of polluting compounds by basidiomycetes seems to be the consequence of the mechanism used by these organisms to degrade the lignin. The degradation of lignin by these fungi is an oxidative, extracellular and nonspecific process which is the result of the coordinated action of a series of intra- and extracellular enzymes (peroxidases, laccases and hydrogen peroxide-producing oxidases) and low molecular mass metabolites (Tortella et al., 2005; Baldrian, 2006; Husain, 2006).

The ability of *Lentinus crinitus* CCB274 and *Psilocybe castanella* CCB444 to degrade hexachlorobenzene has been studied in bioreactors with a capacity of 400 Kg soil (Fig. 1). The growth of these fungi in the bioreactor has been accompanied for 70 to 100 days and different parameters such as temperature, pH, humidity, organic carbon, biomass and ligninolytic enzymes have been monitored. Over time, a reduction in soil pH was observed during the growth of the fungi. In the bioreactor containing *L. crinitus*, the initial pH of 6.67 decreased to 2.87 after 21 days of incubation, stabilizing at 3.84 at the end of 73 days. In the bioreactor inoculated with *P. castanella*, the pH ranged from 4.60 to 3.60 within the same period of time (Matheus et al., 2003).
In general, fungi grow over a wide pH range, with the optimum pH being situated in the acid range

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for most of them. In these organisms, the pH does not only affect the growth but also the production and activity of metabolites due to the effect of pH on cell permeability and the availability of certain metals such as magnesium, phosphorus, iron, calcium, and zinc. The influence of pH on the growth, production and activity of ligninolytic enzymes, and the degradation of aromatic compounds such as lignin and environmental pollutants by basidiomycetes have been the subject of several studies (Galhaup et al., 2002; Lechner and Pappinutti, 2006; Zouari-Mechichi et al., 2006).

The objective of the present investigation was to determine the influence of pH on the growth of *P. castanella* and *L. crinitus* and the effect of reaction pH on ligninolytic activities and decolorization of RBBR by enzymatic extract in order to optimize their application in the bioremediation process of soils.

Figure 1 - Bioreactors with a capacity of 400 kg soil used for fungal culture, São Vicente, SP (Photo: Ricardo R. da Silva, 2004).

MATERIAL AND METHODS

Fungi

Lentinus crinitus CCB274 and *Psilocybe castanella* CCB444 were provided by the Basidiomycete Culture Collection (CCB) of the Institute of Botany, SMA, São Paulo, and were maintained by successive replating on 2% malt extract agar (MEA) (1% glucose, 0.1% peptone, 1% malt extract and 1.8% agar) at 4ºC.

Culture conditions

The fungi were grown in 2% malt extract broth (MEB) whose composition was the same as that of MEA without the addition of agar. The pH of the medium was adjusted with 0.1 M NaOH and 0.1 M H_2SO_4 before sterilization (121°C, 15 min) to 2.5, 4.5, 5.5 and 6.0. Two 5-mm fungal MEA discs (5 to 7 days) were inoculated into 250-mL flasks containing 30 mL MEB. The cultures were incubated at room temperature in a stationary manner. At given time intervals, the content of the flasks was filtered through filter paper and the biomass was determined as dry weight (80ºC for 24 h). The filtrate was used for the determination of pH and decolorization of the anthraquinone dye Remazol Brilliant Blue R (RBBR). The experiments were carried out in duplicate.

In vitro decolorization of RBBR

Decolorization of RBBR was assayed by the decrease in absorbance at 585 nm as described by Machado and Matheus (2006). The reaction mixture contained 5.0 mL of the enzymatic extract, 100 µL distilled water and 50 µL 2% RBBR solution. After 1 h, absorbance was read in samples diluted 1:10 in distilled water. One unit of decolorization was defined as the amount able to catalyze a reduction of 0.01 in absorbance compared to a control prepared with previously heat-inactivated enzymatic extract (10 min).

Effect of reaction pH on ligninolytic activities

P. castanella and *L. crinitus* were grown stationary in MEB at an initial pH of 4.5 for 14 days at room temperature. The content of the flasks was filtered

and the pH of the filtrate was adjusted to 2.5, 3.5 and 4.5. These filtrates were used for the determination of the peroxidase and laccase activities involved in the decolorization of RBBR and in the oxidation of ABTS.

***In vitro* decolorization of RBBR by peroxidases**

The peroxidase activity involved in the decolorization of RBBR was determined using the same method as employed for the *in vitro* decolorization of RBBR replacing distilled water with 2 mM hydrogen peroxide.

***In vitro* decolorization of RBBR by laccase**

The laccase activity involved in the decolorization of RBBR was determined by the same method as employed for the *in vitro* decolorization of RBBR using enzymatic extract previously incubated with 100 µL catalase (Sigma, 1000 mU/L) for 10 min.

Peroxidase and laccase activities

Oxidation of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) by peroxidases was monitored at 420 nm (ε = 36,000 $M^{-1}cm^{-1}$). The reaction mixture contained (in 1 mL) 800 µL of the enzymatic extract, 100 µL 2 mM hydrogen peroxide and 100 µL 1 mM ABTS (Bourbonnais and Paice, 1988). Oxidation of ABTS by laccase was assayed using an enzymatic extract previously incubated with 100 µL catalase for 10 min. One enzymatic unit corresponded to the amount of enzyme able to oxidize 1 µmol of the substrate per minute.

RESULTS AND DISCUSSION

The pH of the culture medium influenced the growth of the basidiomycetes *L. crinitus* and *P. castanella*, which presented less growth at pH 5.9 (Fig. 2) and pH 2.7 (Fig. 3), respectively. The fungi altered the initial pH of the medium. During the growth of *L. crinitus* in medium with an initial pH of 2.5, the pH evolution during the cultivation tended to increase, reaching a value close to pH 4.0 at 14 days. In the other medium, the pH stabilized at about 4.5 by day 7 of incubation (Fig. 2). A similar behavior was observed during the growth of *P. castanella*; however, in medium with an initial pH higher than 4.0, the pH evolution did not stabilize during the incubation period (Fig. 3) Most biochemical studies on the degradation of polluting compounds have used the white rot fungus *Phanerochaete chrysosporium* as a model system. This fungus possesses an optimum pH for growth close to 4.5 (Kirk et al., 1978). The optimum pH for growth of other basidiomycete fungi such as *Polyporus sanguineus* and *Trametes pubescens* is also found in this pH range (Sandhu and Arora, 1985; Galhaup et al., 2002). The present results demonstrated that the optimum pH for the growth of *L. crinitus* and *P. castanella* was also close to 4.5. Acidification of the culture medium during the fungal growth has been related to the production of organic acids (Makela et al., 2002; Aguiar et al., 2006). Tekere and co-workers (2001) observed alterations in the initial pH values of the culture medium during the growth of various basidiomycete species: the pH tended to increase when the initial pH was less than 4.0 and the opposite was observed when the initial pH was higher than 4.5. A reduction of the initial pH of the culture medium from 5.0 to 3.6 was also observed during the growth phase (first 12 days) of *Trametes pubescens* (Galhaup et al., 2002). Rigas and Dritsa (2006) observed redution of the pH from 6.7 to 4-5 during the growth of *Ganoderma australe, Polyporus brumalis and P. ciliatus*.

The use of dyes as model compounds in pollutant biodegradation studies offers a series of advantages compared to conventional substrates: dyes are stable, soluble, possess high molar extinction coefficients and low toxicity, and can be applied in simple, rapid and quantitative spectrophotometric assays. RBBR has been widely used since, as an anthracene derivative, this compound represents an important group of organopollutants (Novotny et al., 2004; Deveci et al., 2004). In the present study, the initial pH of the culture medium influenced the *in vitro* decolorization of RBBR by the fungi studied. Maximum decolorization of RBBR by the enzymatic extract of *L. crinitus* was observed at 14 days in medium with an initial pH of 4.5 (Fig. 2). Maximum decolorization of RBBR by the enzymatic extract of *P. castanella* was also observed after 14 days, but at an initial pH of the medium of 5.9 (Fig. 3). The optimum pH for the decolorization of the dye Poly R478 ranged from 3 to 5 for *Trametes pocas*, *T. cingulata*, *Pycnoporus sanguineus* and *Datronia concentrica* (Tekere et al., 2001).

The production of organic acid by *L. crinitus* and *P. castanella*, which reduces the pH of the culture

medium, might have stimulated the decolorization of RBBR. The role of organic acids in the degradation of aromatic compounds catalyzed by ligninolytic enzymes (peroxidases and laccase) has been well documented in the literature. The organic acid- Mn^{3+} complex acts as a strong redox mediator which is able to attack lignin structures, forming unstable free radicals that tend to disintegrate spontaneously (Hofrichter et al., 1999, Tekere et al., 2001; Hofrichter, 2002; Makela et al., 2002).

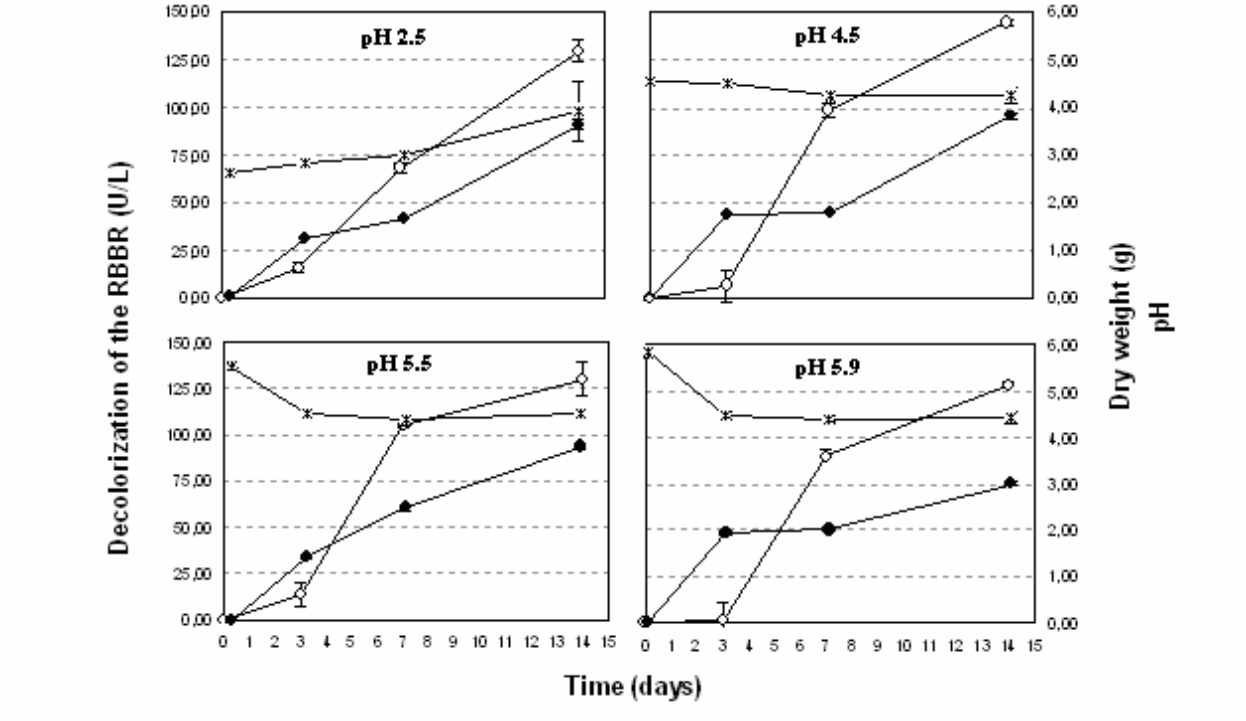

Figure 2 - Decolorization of the dye Remazol R Brilliant Blue (RBBR) by the enzymes extracted from *Lentinus crinitus* CCB274 grown at room temperature at different pH values. (○) Decolorization of RBBR; (●) dry weight; (Ж) pH evolution of the culture medium. The bars indicate the standard deviation between duplicate measurements.

The pH of the reaction influenced the activity of ligninolytic enzymes produced by the basidiomycetes and involved in the *in vitro* decolorization of RBBR. For *L. crinitus*, the optimum pH for RBBR decolorization ranged from 2.5 to 3.5 (Fig. 4). In contrast, for *P. castanella*, the optimum pH for *in vitro* RBBR decolorization was 3.5. The same pH profile was observed for the peroxidase and laccase activities produced by *L. crinitus* and *P. castanella* involved in the decolorization of RBBR. This finding demonstrated the capacity of the enzymatic extracts of these fungi to decolorize RBBR without the addition of hydrogen peroxide, indicating the action of laccase-type oxidases in this process. The identity of the enzymes involved in the degradation of RBBR has not been completely established but degradation is believed to result from the coordinated action of peroxidases and hydrogen peroxide-producing oxidases (Soares et al., 2001; Shin 2004). Recent studies also indicated the involvement of laccases alone or in the presence of specific mediators (Baldrian, 2004; Champagne and Ramsay, 2005; Lu et al., 2007; Murugesan et al., 2007).

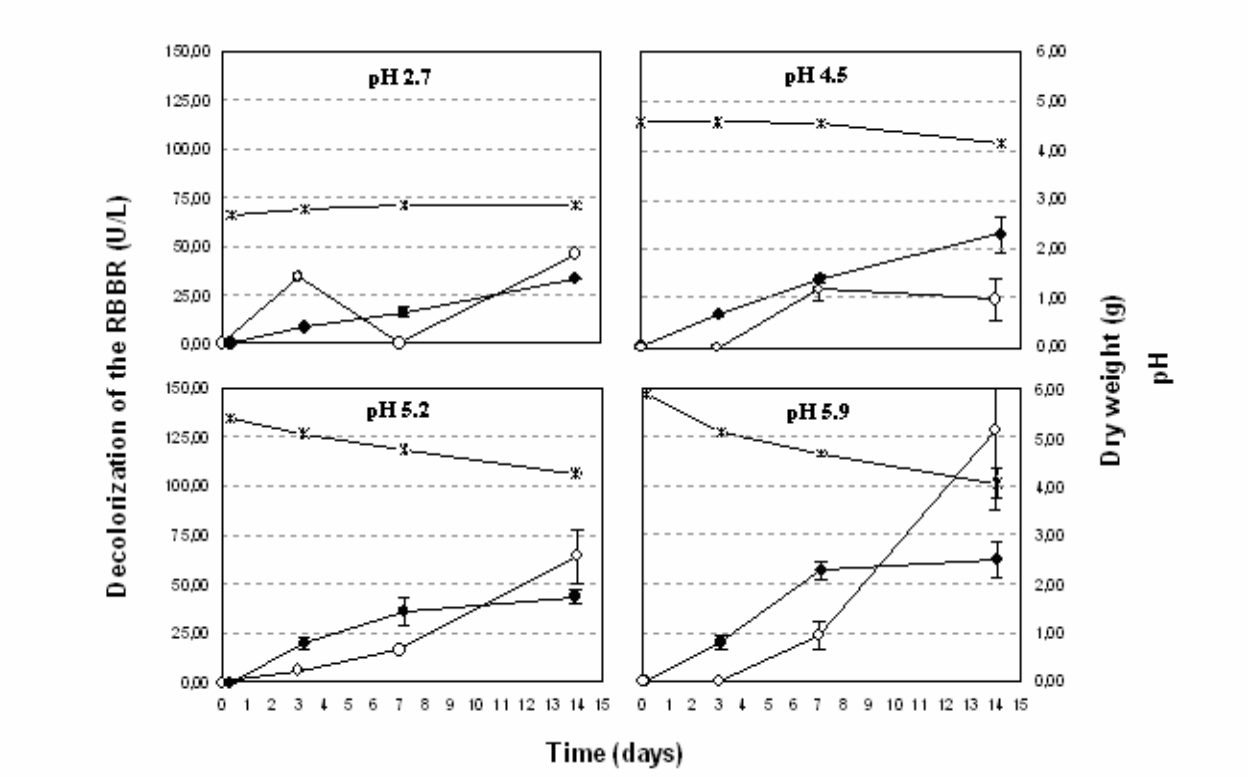

Figure 3 - Decolorization of the dye Remazol R Brilliant Blue (RBBR) by the enzymes extracted from *Psilocybe castanella* CCB444 grown at room temperature at different pH values. (○) Decolorization of RBBR; (●) dry weight; (Ж) pH evolution of the culture medium. The bars indicate the standard deviation between duplicate measurements.

The peroxidase and laccase activities produced by *L. crinitus* and *P. castanella*, which oxidized ABTS, presented the same pH profile, with a predominance of laccase being observed in the enzymatic extracts of these fungi (Fig. 5). For *L. crinitus*, the highest laccase activity was obtained at pH 3.5 (35.0 U/L), whereas for *P. castanella* maximum activity was observed at pH 4.5 (19.5 U/L). These results demonstrated differences in the pH optimum between the laccases produced by these fungi. The optimum pH for laccase activity vary for each fungal strain, such as observed in *P. castanella* and *L. crinitus* laccases from other basidiomycetes presented optimum pH 3.0 using ABTS as substrate (Saparrat et al., 2002; Ryan et al., 2003; Zouari-Mechichi et al., 2006).

The present results demonstrated that i) the pH of the culture medium influenced the growth and *in vitro* decolorization of RBBR by *L. crinitus* CCB274 and *P. castanella* CCB444; ii) the fungi were able to alter the initial pH of the medium so that it would reach the optimum pH for growth; iii) RBBR decolorization by these fungi seemed to be the result of the action of laccase-type oxidases.

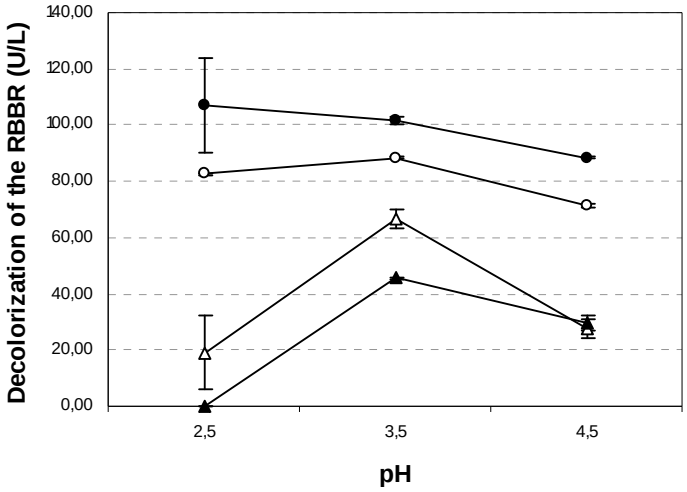

Figure 4 - Effect of pH of the reaction on the ligninolytic enzymatic activity involved in the decolorization *in vitro* of the dye Remazol R Brilliant Blue (RBBR) produced by *Lentinus crinitus* CCB274 (○, peroxidases; ●, laccase) and *Psilocybe castanella* CCB444 (Δ, peroxidases; ▲, laccase) during growth in malt extract broth at room temperature. The bars indicate the standard deviation between duplicate measurements.

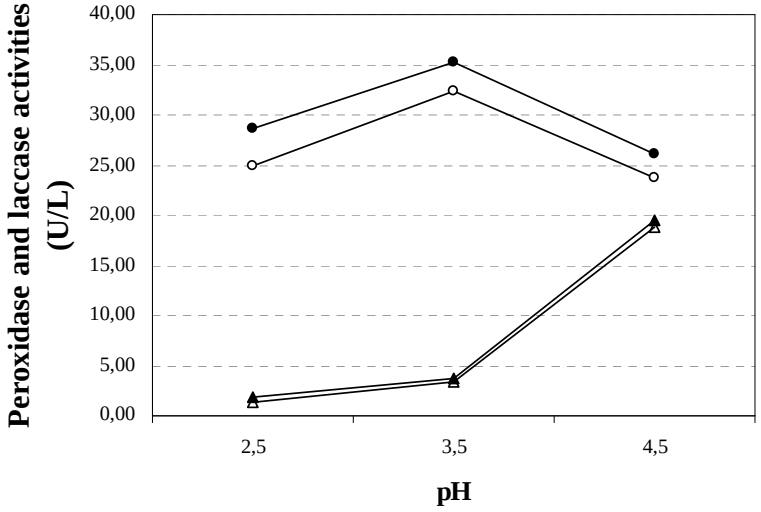

Figure 5 - Effect of pH of the reaction on the ligninolytic enzymatic activity involved in the oxidation of ABTS produced by *Lentinus crinitus* CCB274 (○, peroxidases; ●, laccase) and *Psilocybe castanella* CCB444 (Δ, peroxidases; ▲, laccase) during growth in malt extract broth at room temperature.

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RESUMO

Os fungos basidiomicetos *Lentinus crinitus* e *Psilocybe castanella* estão sendo avaliados em processo de biorremediação de solos contaminados com resíduos industriais organoclorados, na Baixada Santista, SP. O presente estudo avaliou a influência do pH no crescimento, na descoloração *in vitro* do corante Azul Brilhante de Remazol R (RBBR) e na atividade de lacase durante cultivo destes fungos, de forma a subsidiar a otimização do processo. O pH do meio influenciou o crescimento de *L. crinitus* e de *P. castanella*, com menor biomassa em pH 5,9 e pH 2,7, respectivamente. Os fungos foram capazes de modificar o pH inicial do meio de cultura, de modo a ajustá-lo ao valor ótimo de crescimento, próximo a 4,5. Descoloração *in vitro* do RBBR foi máxima em pH 2,5 e 3,5. Maiores atividades de lacase foram obtidas em pH 3,5 e em pH 4,5 para *L. crinitus* e *P. castanella*, respectivamente. Evidenciou-se que o pH é um parâmetro importante para o crescimento destes fungos, atividade de lacase e descoloração *in vitro* do RBBR.

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