

Enzyme production by immobilized *Phanerochaete chrysosporium* using airlift reactor

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Abstract: Enzyme production by immobilized *Phanerochaete chrysosporium* was evaluated in airlift bioreactor and agitated cultures. Free mycelium and immobilized mycelium on alginate beads were tested in the decolourization of 50 and 500 mg/L of Remazol Brilliant Blue R. Dye concentration did not inhibit the fungi development in all tests. In addition, high decolourization percentage of dye was found with free mycelium (99%) in agitated flasks and with immobilized mycelium in airlift (98%). However, decolourization period by immobilized mycelium (120 h) was greater than that by the free mycelium (14 h). Important manganese peroxidase, lignine peroxidase and laccase activities were identified in decolourization process. Manganese peroxidase appeared to be promoted by high dye concentrations during the treatment with immobilized mycelium, but this enzyme was not detected with free mycelium in airlift. Bioreactor prompted also laccase and lignine peroxidase actions in both tests; free mycelium registered a maximum laccase action of 31.569×10^3 U/L in 70 h, whereas immobilized mycelium registered 1.680×10^3 U/L in 170 h, while lignine peroxidase secretion by free *P. chrysosporium* was higher (1.300×10^3 U/L) than immobilized mycelium (1.250×10^3 U/L). Maximum laccase activity coincided with the maximum percentage of decolourization, however, high peroxidase activity was identified from the start of dye treatment.

Key words: manganese peroxidase; lignine peroxidase; laccase; immobilized *Phanerochaete chrysosporium*; alginate beads; airlift bioreactor.

Abbreviations: Lac, laccase; LiP, lignine peroxidase; MnP, manganese peroxidase; RBBR, Remazol Brilliant Blue R.

Introduction

Fungi are recognized for their superior capability to produce a large variety of metabolites that can be adapted for different uses. Actually several fungi have attracted a growing interest for biotreatment of wastewater with different pollutants, such as metals, inorganic nutrients and organic compounds. Important results from fungi action can be seen in recent papers (Wesenberg et al. 2003; Kaushik & Malik 2009; Gao et al. 2010).

Especially *Phanerochaete chrysosporium* has been the subject of intensive research related to degradation of a wide range of recalcitrant xenobiotic compounds and mineralising persistent aromatic pollutants (Chagas & Durrant 2001; Gao et al. 2006; Yu et al. 2006; Zhang et al. 2008). Degradation capacity of *P. chrysosporium* on different synthetic dyes, such as azo, anthraquinone, heterocyclic triphenylmethane and polymeric, has also been showed in several researches (Peralta-Zamora et al. 1999; Faraco et al. 2009; Sharma et al. 2009). Complex molecules are decom-

posed by this fungus due to its ability to transform lignin through unspecific enzymes action, such as laccase (Lac), manganese peroxidase (MnP), lignine peroxidase (LiP), xylanases, cellulases, glucose-1-oxidase and glucose-2-oxidase. In addition, adsorption mechanism of fungal biomass also contributes to the colorants degradation (Rodríguez-Couto 2009).

Ligninolytic processes from *P. chrysosporium* have been applied to industrial effluent detoxification (Pant & Adholeya 2007), mostly coming from textile and petrochemical industries, bleaching and delignification processes in the paper and pulp industries (Wesenberg et al. 2003; Gomathi et al. 2012). The capacities of this fungus to remove xenobiotic substances and produce polymeric products make up a useful tool for bioremediation purposes.

Secretion of lignin-degrading enzymes has been attributed to a nutrient starvation response during primary metabolism of *P. chrysosporium* as well as research related to the influence of lignin activity promoters (veratryl alcohol and Mn^{2+}), specifically on de-

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colourization treatments (Mester et al. 1995; Kaushik & Malik 2009). However, some of the research results have proven to be controversial; the discrepancy is based on enzymes actuation, which is attributed to their selective specificity concerning turn of the substrate (Lee 2007). Several authors agree that nitrogen-limitation increases ligninolytic activities through stimulation of LiP and MnP secretion (Van der Woude et al. 1993; Gao et al. 2008, 2010). On the other hand, other results have shown that strains pre-grown on nitrogen-rich medium also exhibit enhanced decolourization rate (Adosinda et al. 2001, 2002).

Besides nutrients, incubation conditions have also been studied on enzyme actuation. Stationary and agitated cultures of *P. chrysosporium* are reported to be necessary for the expression of the ligninolytic enzymes (Sen et al. 2012). Novotny et al. (2006) and Zahmatkesh et al. (2010) demonstrated that the systems with lower agitation are most likely to have a prolific production of LiP. Apart from agitation, fungal immobilization can also provide a suitable low-shear environment for ligninolytic system of fungi. Immobilize culture of *P. chrysosporium* on supports like polyurethane foam has often been reported for decolourization of various dyes (Minussi et al. 2001; Urek & Pazarlioglu 2004; Park et al. 2006). Shim & Kawamoto (2002) studied the enzymes production from *P. chrysosporium* using a batch reactor system. In this case the secretion of LiP by immobilized mycelium on natural and synthetic carriers was more effective compared to conventional stationary liquid culture.

In this study, we evaluated enzyme production of immobilized microorganisms using *P. chrysosporium* on Ca-alginate beads in agitated cultures and an airlift bioreactor. Remazol Brilliant Blue R (RBBR) was used as a model dye to evaluate the enzymes action. This dye is industrially important because frequently RBBR (anthracene derivative) is used as a starting material in the production of polymeric dyes. For this reason RBBR is found in several industrial wastewaters and it is known as a toxic and recalcitrant dye. Their chemical groups are highly stable to fungal enzymes due to molecules resonance to free biomass. In addition, the competition between the formation of reactive form and the hydrolysis reactions of RBBR has also been revealed as an inhibition factor to their oxidation process by fungal cultures (Carantino et al. 2012). Treatment tests were carried out in agitated flasks and an airlift at two concentrations of RBBR. The experimental results could provide important information on both aspects: (i) the role of mycelium immobilization; and (ii) the role of agitation in airlift reactor. Enzymes secretion and their intervention of RBBR decolourization were analyzed in each treatment.

Material and methods

Microorganism and culture conditions

P. chrysosporium (HEMIM-5) was provided by UAM-CEIB (Biotechnology Research Center) Morelos, México and it

was maintained in potato dextrose agar at 28°C. Strain was stored at 4°C and it was cultured every 2 months.

Treatment tests were performed with fungus *P. chrysosporium* grown in malt extract agar (20% v/v) at 28°C. The mycelium was homogenized and inoculated in a 10% (w/v) proportion in the free mycelium experiments and 13.3% (w/v) proportion in the immobilized fungi experiments (Urrea et al. 2006). The liquid culture medium was based on a wheat straw (20%; v/v), MnSO₄ (40 mg/L), sodium lactate (20 mM), and adjusted to pH 4.5 with a 30% HCl solution. All the experiments were performed with two replicates.

P. chrysosporium immobilization

The immobilization of the fungi was made on alginate beads. The beads were prepared by adding 2.4 mL of homogenized *P. chrysosporium* mycelium to a 30 mL solution of sodium alginate 3% (p/v). The mixture was agitated in a Thermo scientific magnetic stirrer HP130915Q and dropped into a 30% CaCl₂ solution. Nozzle with 3 mm diameter was used to obtain the beads with immobilized mycelium. The beads formed were maintained at 4°C for 24 h before its inoculation.

Enzyme activity of P. chrysosporium on RBBR decolourization

Enzyme activity was evaluated on decolourization experiments of 50 and 500 mg/L RBBR with free and immobilized mycelium of *P. chrysosporium*. Dye concentration was selected in order to test the capacity of fungal decolourization. Flasks of 2 L and Vichi Airlift Bioreactor FAR-4 with volume of 4 L were used in the fermentation processes.

The flasks were incubated at 30°C at 135 rpm in an orbital agitator. Airlift was maintained at 37°C the first 48 h as acclimatization period; after this time the temperature was set at 30°C until the end of the experiment. Air was supplied to the bioreactor in a continuous way at a rate of 2.5 L/min.

The flasks and bioreactor were operated in batch conditions and the dye was added on the third day of growth for both cultures.

Samples were collected every 3 and 24 h during treatment for the free and immobilized mycelium respectively with a 10 mL sterile pipette in a laminar flow hood. The samples were centrifuged at 11350 × g, 10 min in a Thermo scientific Espresso 12 centrifuge 11210800. Percentage dye decolouration, *P. chrysosporium* biomass and enzyme activity were later analysed.

Dye removal was determined by monitoring the absorbance at 593 nm in a Biomate 3 UV-VIS Spectrophotometer and was calculated by means as follows: $D\% = [(AT_0 - AT_t)/AT_0] \times 100$, where $D\%$ represents decolourization percentage, AT_0 the initial absorbance ratio and AT_t absorbance ratio at considered time.

The biomass was determined after the fungi cultivation period. Mycelia were separated from fermented substrates by centrifugation (11350 × g, 15 min) at 4°C and filtered in a fine pore filter paper. The biomass was dried at 60°C for 12 h in a stove to constant weight. Finally, dried biomass at 20°C was placed on silica desiccators and weighed in an Ohaus Pioneer Analytical Balance PA114.

The entire enzyme assays were executed at room temperature. MnP activity was determined by monitoring oxidation of Phenol red by the increase in absorbance at 610 nm ($\epsilon = 4460/\text{Mcm}$). The reaction mixture contained 500 µL of supernatant sample, 100 µL 0.1% Phenol red, 100 µL

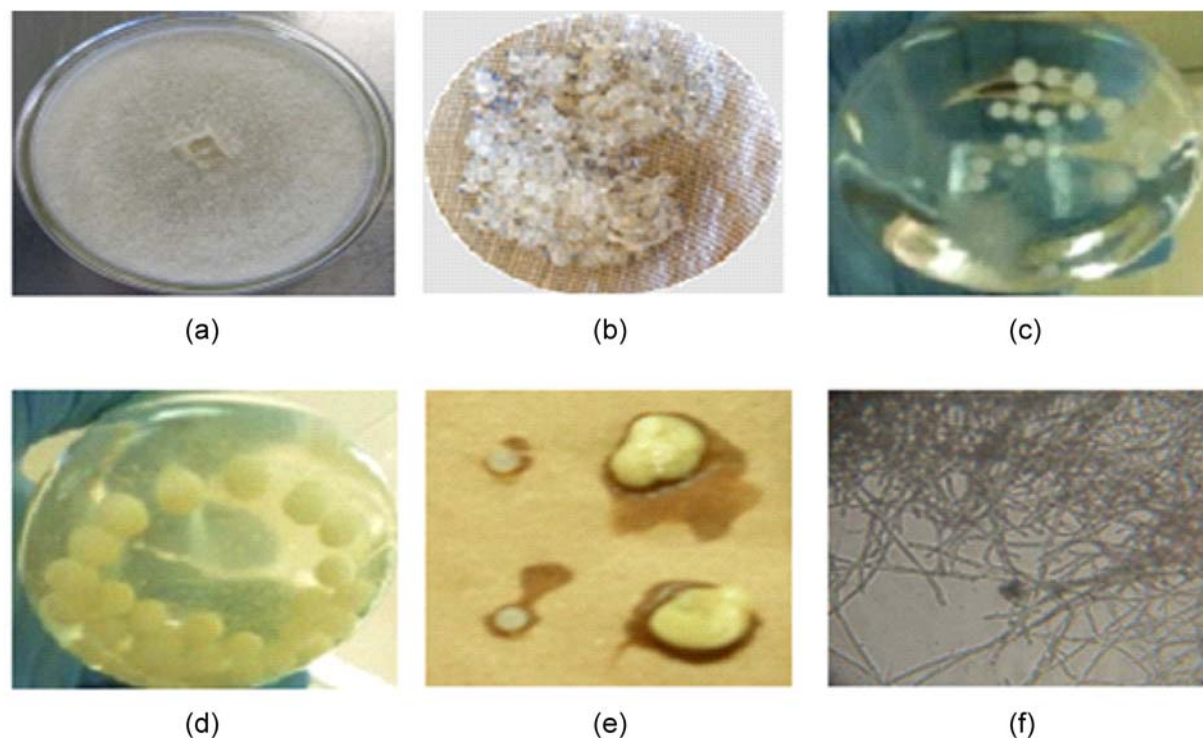


Fig. 1. (a) *P. chrysosporium* mycelium, (b) immobilized *P. chrysosporium* mycelium in Ca-alginate beads, (c) *P. chrysosporium* in Ca-alginate beads after their incubation in liquid medium, (d) *P. chrysosporium* in Ca-alginate beads after three days of incubation in liquid medium, (e) Ca-alginate beads fractured to reveal the internal structure, (f) mycelium structure of *P. chrysosporium* from Ca-alginate beads.

of 250 mM sodium lactate (pH 4.5), 200 μ L bovine albumin, 50 μ L of MnSO_4 and 50 μ L of 2 mM H_2O_2 in sodium phosphate buffer pH 8.0 (Moreira et al. 2000). LiP activity was determined using the veratryl alcohol as substrate. Oxidation of veratryl alcohol was followed by absorbance increase at 310 nm ($\epsilon = 9300/\text{Mcm}$). The reaction mixture contained 500 μ L of supernatant sample, 1000 μ L of 10 mM citrate buffer pH 3.0, 500 μ L of 10 mM veratryl alcohol and 500 μ L of 2 mM H_2O_2 (Shim & Kawamoto et al. 2002). Lac activity was determined using the 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid as substrate by monitoring absorbance intensification at 420 nm ($\epsilon = 36000/\text{Mcm}$). The reaction mixture contained 800 μ L of supernatant sample and 200 μ L of 1.0 mM 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) in 0.1 M sodium acetate pH 5.0 (Pant & Adholeya 2007).

Results

P. chrysosporium immobilization

Immobilization of *P. chrysosporium* biomass was achieved by entrapment into calcium alginate. Spheres of alginate of solid structure with diameters averaging 3 mm were obtained and hyphae were not found on the surface of the beads. In all cases, calcium alginate beads were resistant to high speeds in agitated cultures and the material showed a greater strength and saturated wet density.

Figure 1 shows images of *P. chrysosporium* mycelium produced in agar culture, immobilised mycelium, alginate beads of immobilised mycelium, beads in liquid medium and broken alginate beads showing mycelium

without appreciable biomass changes in its structure. Fractured alginate beads showed that the colour of isolated fungal mycelium is the same as that of mycelium grown on agar. In addition, mycelium into alginate beads maintained its structural form without showing any visible alteration at the end of the experiment. Hyphae appeared to be whole and intact with no evidence of fragmented hyphae.

Alginate calcium showed also low adsorption efficiency (less than 10%). Particles from solution do not exceed the beads saturation; this fact also improves the mass transfer between enzymes and substrate.

Some other inert carriers (stainless steel net, polyamide fibre, fibreglass and polyurethane foam) used to immobilize *P. chrysosporium* have also confirmed that immobilization of their cells can increment the enzyme activity and efficacy on the treatments (Urek & Pazarlioglu 2004; Nurdan et al. 2005; Carletto et al. 2008; Gao et al. 2008). However, one of the most common problems with these types of supports is that fungal hyphae agglomerate into bioreactors (Wang & Hu 2007), whereas biogel alginate protects biomass and provides a homogenous aerobic condition, which helps to avoid impurities into fermentation products. It has been established that other materials as those made of sodium carboxymethyl cellulose or chitosan could have less resistance at pH extremes and high agitated cultures conditions. In addition, structure of mycelium is affected by these materials and enzymes actuation may be also be limited (Urek & Pazarlioglu 2004; Wang & Hu 2007).

Table 1. Data on decolourization process of RBBR.

Process	RBBR ^a (mg/L)	<i>Phanerochaete chrysosporium</i>					
		Free mycelium			Immobilized mycelium		
		Biomass ^b	RBBR ^c (%)	RBBR ^d (h)	Biomass ^b	RBBR ^c (%)	RBBR ^d (h)
Agitated flask	50	2.30 ± 6	84.87 ± 2	47 ± 1	2.55 ± 1	86.55 ± 3	140 ± 5
	500	2.85 ± 3	99.34 ± 1	14 ± 0.8	2.30 ± 2	84.87 ± 2	130 ± 1
Airlift bioreactor	50	14.14 ± 5	88.18 ± 3	65 ± 0.5	11.81 ± 2	90.95 ± 1	100 ± 4
	500	13.97 ± 7	91.57 ± 1	80 ± 0.5	12.25 ± 3	98.42 ± 3	120 ± 4

^a RBBR concentration. ^b Biomass (g) weight dry. ^c RBBR decolourization. ^d RBBR decolourization period.

Enzyme activity of *P. chrysosporium* on RBBR decolourization

Data on decolourization experiments are shown in Table 1. The results are referred to agitated cultures and bioreactor treatment with 50 and 500 mg/L RBBR by immobilized and free *P. chrysosporium*.

High decolourization efficiency was achieved in all tests. Biomass production and decolourization treatment were not inhibited by RBBR concentration. In addition, surprising results were also particularly found in agitated cultures of free *P. chrysosporium*, which achieved 99% of efficiency in the decolourization of 500 mg/L RBBR in 14 h, while immobilized biomass achieved 98% in a period of 130 h.

Airlift cultures with 500 mg/L RBBR registered again a great potential of *P. chrysosporium* to remove high dye concentrations. In this test immobilized mycelium achieved 98.24% of decolourization in 120 h, whereas free mycelium achieved 91.57% in 80 h. Experiments with 50 mg/L RBBR documented a lower efficiency (85–91%) in decolourization period of 47–140 h with free and immobilized mycelium in both treatment types. High efficiencies with large amount of RBBR (500 mg/L) could reveal that this colorant is a suitable substrate for the peroxidases and oxidases produced by the fungus (Balan & Monteiro 2001).

Decolourization of RBBR was observed by the presence of a brown product at the beginning of treatment in all experiments. It has been attributed to polymerization products of oxidative coupling of catechol or aminophenol derivatives (Rodríguez-Couto 2009). Similar decomposition was also reported by treatment of RBBR with pure immobilized Lac (Champagne & Ramsay 2007). The change was described as a pale pink colouration acquired in the medium during decolourization process by the Lac action. Other tests have also shown that Lac causes different changes in the RBBR coloration, such as pink to yellow and finally to colourless (Yyas & Molitoris 1995).

Figure 2 shows in detail decolourization profile of 500 mg/L RBBR by immobilized and free *P. chrysosporium* in airlift bioreactor. A rapid decline of RBBR concentration was observed before 40–70 h of treatment in both tests. Free mycelium cultures achieved 100 mg/L RBBR in this period, whereas immobilized mycelium cultures achieved 200 mg/L in the equivalent

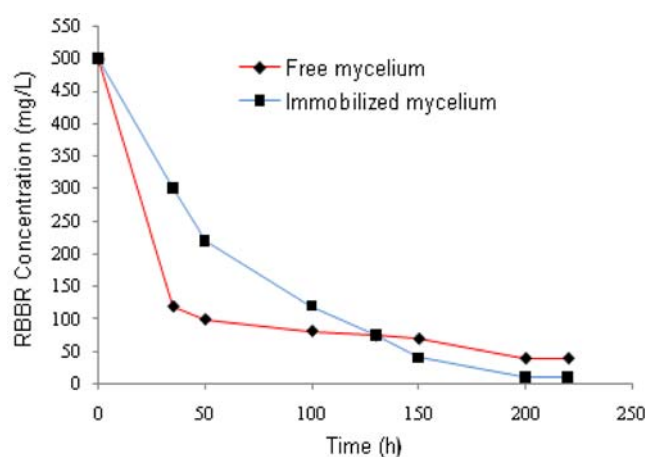


Fig. 2. Decolourization profile of RBBR by *P. chrysosporium* in airlift bioreactor with free mycelium and immobilized mycelium.

range. After 120 h, both tests presented similar decline of RBBR concentration (60 mg/L) and subsequent to 200 h, no changes in decolourization were registered. *P. chrysosporium* achieved in this period 40 and 10 mg/L RBBR for free and immobilized mycelium, respectively.

The behaviour observed in immobilized *P. chrysosporium* during first decolourization period probably suggests three phenomena: (i) diauxic phenomenon, in which microorganisms use a more available nutrient, and afterwards, induced enzymes are synthesized; (ii) transfer mass limitation of enzymes for dye degradation due to microorganism immobilization; and (iii) enzyme inhibition due to some products generated in the oxidation process. However, the last result can be explained by contribution of enzymes (U/L) from immobilized and free *P. chrysosporium* on decolourization percentage of RBBR.

Figure 3 illustrates the enzyme secretion of MnP, LiP and Lac during *P. chrysosporium* culture with 500 mg/L of colorant in agitated culture and airlift bioreactor. Elevated enzyme activity was detected since initiation of culture and 72 h after adding the dye.

Particularly, the free *P. chrysosporium* in agitated flasks showed maximum Lac secretion at 68 h (9.0069×10^3 U/L) after starting the fungus cultivation and later at 100–150 h (among 13×10^3) after adding

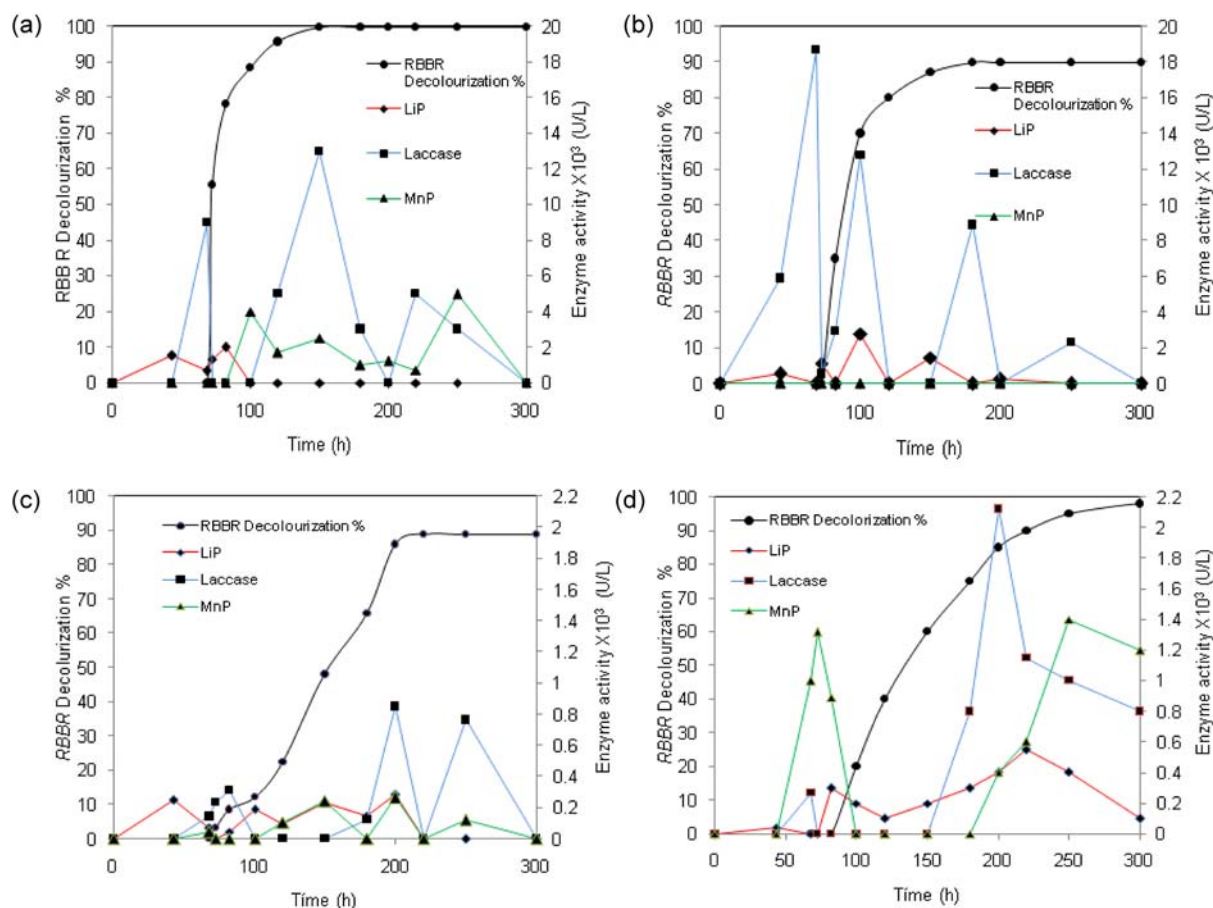


Fig. 3. Decolourization percentage of 500 mg/L of RBBR and enzyme activity (U/L) of *P. chrysosporium*. (a) Free mycelium in agitated cultures, (b) free mycelium in airlift cultures, (c) immobilized mycelium in agitated cultures, (d) immobilized mycelium in airlift cultures.

the RBBR (Fig. 3a), while free *P. chrysosporium* in airlift showed a high Lac activity (Fig. 3b). Enzyme action on this agitation system was higher than in agitated flask. Maximum enzyme activity was achieved at 68 h (19.590×10^3 U/L). However, after adding the RBBR (72 h culture), fewer Lac activity was registered at 100, 180 and 250 h (among $9\text{--}13 \times 10^3$ U/L). These results could indicate that the first decolourization was due to Lac secretion by the fungus and this enzyme production is often enhanced by a suitable agitation (Wesenberg et al. 2003; Dominguez et al., 2005).

Agitated cultures of free *P. chrysosporium* also promoted a high activity of both LiP and MnP enzymes. The greatest enzyme activity of LiP was registered at 43 h (1.129×10^3 U/L) before the RBBR addition, whereas the highest MnP was detected (among $2.5\text{--}5.8 \times 10^3$ U/L) in a period of 40–180 h once the dye was added. However, this enzyme was not detected in airlift and a lesser LiP secretion in this system was also identified.

Immobilized *P. chrysosporium* cultures showed a different behaviour with regard to enzyme secretion compared with free mycelium. Immobilized biomass exhibited always a lower enzyme activity, but airlift bioreactor incremented the enzymes action and in this system the best results of RBBR decolourization were achieved. Maximum activity of Lac (2.1093×10^3 U/L),

LiP (0.5531×10^3 U/L) and MnP (1.457×10^3 U/L) were registered during the process. These results confirmed that production of LiP and MnP is generally optimal at high oxygen tension, but these enzymes can be inhibited in free mycelium by agitation in airlift. However, immobilized mycelium could improve the MnP production due to increase of the contact area between cells and oxygen without sheer stress on mycelia allowing thus an optimal secretion.

Furthermore, conditions of free and immobilized mycelium in these two different agitation systems revealed also that RBBR decolourization is not performed by a single enzyme; however, the registering of certain enzymes during culture experiments coincided in some cases with the coloration change of RBBR. For example, airlift cultures with immobilized biomass exhibited maximum secretion of LiP and MnP enzymes at 120–150 h (5–6 day) after adding the colorant. Decline of enzyme action was observed in a subsequent period. In consequence these enzymes contributed probably on the first decolourization step (80%). Alternatively Lac action may be involved in the finalization of decolourization process since the action of this enzyme achieved 98% of efficiency after 150 h of RBBR treatment.

Concerning the enzyme action of free *P. chrysosporium* (Fig. 3b), it is shown that the absence of MnP in airlift does not affect decolourization process. This

result may be due to high secretion of LiP and Lac in airlift, even after adding the dye. The LiP could have in these tests a greater contribution in decolourization experiment to achieve 90% efficiency due to the absence of MnP, though the record of high Lac activity in all experiments could also indicate that this enzyme is the only one responsible for the RBBR decolourization.

Discussion

Evident limitation by encapsulation material was found in fungi immobilization since the decolourization time of RBBR was higher than that found with free mycelium in the two agitation systems. Mass transfer limitations was seriously observed on alginate beads in agitated flasks, however, this disadvantage was decreased by agitation in airlift; oxygen diffusion on immobilized biomass promoted the enzyme activity. Therefore clogging and mycelium accumulation was avoided by immobilization mycelium in this agitation system. Improving the separation of biomass from solution and reducing biomass dispersion and bioaccumulation of dye was also confirmed with use of pellets (Yesilada et al. 2003; Xin et al. 2012).

Ligninolytic activity of *P. chrysosporium* was principally attributed to LiP, MnP and Lac, although RBBR decolourization was not dependent on one specific enzyme. However, increased activity of MnP and LiP were registered mostly in the first decolourization step, whereas a great Lac activity was also detected in the last decolourization period. Meanly agitated cultures registered a great production of these enzymes by free mycelium, while MnP was inhibited in airlift, whereas immobilized mycelium registered principally MnP and Lac activities with the two agitation systems.

Enzyme action response from LiP and MnP could be explained by high ionization potential of these enzymes to readily oxidized non-phenolics compounds, whereas Lac may be able to oxidize these compounds but with a relatively low ionization potential (Soares et al. 2001; Shim & Kawamoto 2002). Wesenberg et al. (2003) showed that the role of LiP in ligninolysis is additional to action of MnP, since transformation of lignin fragments is initially released by MnP. For this reason LiP is not essential to start the attack on lignin but it is complementary.

Recent results on the enzyme action of free *P. chrysosporium* URM6181 (Miranda et al., 2013) revealed also higher production of ligninolytic enzymes in an aerated bioreactor (Lac – 2020, LiP – 39 and MnP – 392 U/L). The authors attributed these high enzyme activities to the addition of nitrogen in the medium. In addition, aerated bioreactor was found very effective for efficient fungal treatment of textile effluent; 29% of dye was decolourised at the first day and 95% after 10 days of the process.

Anastasi et al. (2012) observed a correlation between Lac/MnP activities and dye degradation of a textile effluent by free *Trametes versicolor*. The strain showed also the highest Lac (820 U/L) production; the

enzyme was produced by the strain, which showed decolourisation efficiency of about 60% after 24 h of process.

Other studies have exposed different values of enzymatic activities, for example, individual addition of purified LiP on olive mill wastewaters exhibited that this enzyme causes more decolourization than the addition of MnP extract does (Sayadi et al. 1996). Lee (2007) found that in batch fermentation, free cells of *P. chrysosporium* can reach to produce 2.8×10^3 U/L of LiP activity, but the MnP was also registered in this fungal culture. In addition, Ollikka et al. (1993) and Yyas & Molitoris (1995) showed that partially purified LiP from *P. chrysosporium* has capacity to decolourise 75% of RBBR, whereas other enzymes are complementary at decolourization process. However, Peralta-Zamora et al. (1999) reported that LiP is capable to decolorize only about 30% RBBR, but MnP was not registered in this test.

Most recent studies have indicated that MnP is the main enzyme activity responsible for the decolourization of a variety of dyes (Moreira et al. 2003; Lopez et al. 2004; Hailei et al. 2009), including also that MnP represents the main oxidative enzyme activity by *P. chrysosporium* with or without LiP cooperation (Faraco et al. 2009). Nevertheless, it has been expressed that mechanically agitated cultures may have an inhibitory effect on this ligninolytic enzyme production (Lopez et al. 2004; Urek & Pazarlioglu 2005). In addition, complex structures of dyes affect enzyme action (Adosinda et al. 2002).

Contribution of MnP and Lac has also been recognized for degradation of selected phthalocyanine dyes, but Lac appears only as a decolourization precursor enzyme (Conneely et al. 2002). However, current decolourization experiments with immobilized Lac showed that this enzyme is able to operate during 15 decolouration cycles losing only 50% of its original activity (Osma et al. 2010). Commercial Lac formulation (0.010×10^3 U/mL) was also tested in the presence of 5.7 mM violuric acid to treat the RBBR and in this test the Lac capacity was confirmed to achieve 90% of decolourization in 20 min (Soares et al. 2001).

Alternatively, it has been proposed that solid or agitated cultures with fixed mycelium on supports may achieve the best results of MnP action (Nurdan et al. 2005; Urek & Pazarlioglu 2005; Park et al. 2006). In some cases the bioreactor has also been used to promote the enzyme secretion from free mycelium (Mielgo et al. 2001; Shim & Kawamoto 2002; Lopez et al. 2004; Enayatzamir et al. 2009; Zahmatkesh et al. 2010; Pakshirajan et al. 2011). Carletto et al. (2008) showed that *P. chrysosporium* immobilized on hazelnut shells and agitated culture exhibited differences in its maximum MnP activity with no dye 0.115×10^3 U/L at 257 h, with 500 mg/L of Congo red 0.091×10^3 U/L at 257 h and with 50 mg/L, 0.075×10^3 U/L at 353 h. Recently Pakshirajan et al. (2011) carried out studies on a rotating biological contactor reactor with polyurethane foam immobilized *P. chrysosporium*. High decolourization ef-

iciency (98.53%) was obtained in 48 h for 50 mg/L of Direct red 80 and Mordant blue 9. Important secretion of MnP (1.033×10^3 U/L in Direct red 80 and 0.737×10^3 U/L in Mordant blue 9) and LiP (1.025×10^3 U/L in Direct red 80 and 1.006×10^3 U/L in Mordant blue 9) was also produced under these conditions.

Recently, moreover, immobilization of fungal biomass by entrapment cell has been studied as a technique to increment the efficiency of metabolic activities (Yesilada et al. 2003; Radha et al. 2005; Park et al. 2006; Zhang et al. 2008). Several tests have demonstrated that mycelium immobilization with fragments of pellets is also an efficient method to improve the performance and economic feasibility of many fermentation processes (Kaushik & Malik 2009). Entrapped microorganisms in different materials, such as agar, alginate, chitosan, cellulose derivatives, and other polymeric matrixes like gelatine, collagen, or polyvinyl alcohol, have been reported for this purpose (Park & Chang 2000; Zhang et al. 2008). Especially decolourization studies in agitated flasks of immobilized fungi on different size of calcium alginate beads were described by Radha et al. (2005). Low decolourization percentage was detected in these tests on some synthetic structures, such as azo, anthraquinone, thiazine and vat dyes. The results were attributed to a little affinity of the hydrophobic substrates and the low rate of dissociation of the dyes probably due to calcium alginate used for the cell entrapment.

Reactor influence on enzymes production of immobilized fungi *P. chrysosporium* has rarely been exposed. In one of the studies (Zahmatkesh et al. 2010) immobilization of cells increased the efficiency of decolourization of azo dye reactive, however, maximum MnP activity of 96 U/L on day 7 and 70% decolourization after 6 h of dye addition were detected. In another study (Xin et al. 2012), the results were attributed only to the intracellular or mycelial-bound enzymes and the type of colorants of this agitation system did not affect decolourization tests.

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