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Laccase production by free and immobilized mycelia of *Peniophora cinerea* and *Trametes versicolor*: a comparative study

Sara C. Silvério · Sérgio Moreira · Adriane M. F. Milagres · Eugénia A. Macedo · José A. Teixeira · Solange I. Mussatto

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Abstract The production of laccase by immobilized mycelia of *Peniophora cinerea* and *Trametes versicolor* was studied. In an initial stage, experimental assays were performed in Erlenmeyer flasks using free and immobilized mycelium, and the performance of the fungal strains to produce the enzyme was compared. Both fungi adhered into the support material (a synthetic fiber), growing not only on the surface but also in the interspaces of the fibers. Immobilization of *P. cinerea* provided a 35-fold increase in laccase production when compared to the production obtained by using free mycelium. On the other hand, immobilization of *T. versicolor* caused a decrease in laccase activity. A comparison between the strains revealed that immobilized *P. cinerea* (3,500 U/L) surpassed the enzyme production by free *T. versicolor* (800 U/L). When the conditions that gave the best laccase production to each fungus were employed in a stirred tank bioreactor, very low laccase production was observed for both the cases, suggesting that shear stress and mycelia damage caused by the agitation impellers negatively affected the enzyme production.

Keywords Laccase · Fermentation · Immobilisation · *Peniophora cinerea* · *Trametes versicolor* · Bioreactor

S. C. Silvério · J. A. Teixeira · S. I. Mussatto (✉)
Institute for Biotechnology and Bioengineering (IBB), Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal
e-mail: solange@deb.uminho.pt; solangemussatto@hotmail.com

S. C. Silvério · E. A. Macedo
Laboratory of Separation and Reaction Engineering (LSRE), Associate Laboratory LSRE/LCM, Department of Chemical Engineering, Faculty of Engineering, University of Porto, Rua Dr Roberto Frias s/n, 4200-465 Porto, Portugal

S. Moreira · A. M. F. Milagres
Department of Biotechnology, Engineering College of Lorena, University of São Paulo, Estrada Municipal do Campinho s/n, 12602-810 Lorena, SP, Brazil

Introduction

Laccases are oxidoreductases commonly produced by white-rot fungi, but that can also be found in plants, bacteria and insects [1]. These enzymes contain four catalytic copper atoms distributed in three redox sites and are involved in the degradation of lignin in their natural environment. Besides degrading lignin, laccases are able to catalyze the oxidation of several organic compounds (*o*- and *p*-diphenols, aminophenols, polyphenols, methoxy phenols, aryl diamines, polyamines) and some inorganic ions [2, 3], with simultaneous reduction of molecular oxygen to water (without intermediate production of hydrogen peroxide). Due to these characteristics, laccases may be used in different areas such as delignification of paper pulp, textile dye bleaching, effluent detoxification, modification of biopolymers, biofuel cells, biosensors, soil bioremediation, and drug synthesis. However, significant amounts of laccase must be produced to be used on industrial scale in order to efficiently compete with the traditional oxidizing agents or other chemical intervenient. Fermentation is the most attractive and feasible method for laccase production and during the last decade several efforts have been done in order to find suitable conditions that intensify enzyme production.

Laccases normally occur as extracellular glycoproteins, being produced in the cells and then secreted and accumulated outside the hyphal filaments [4]. Many ligninolytic

fungi producers of laccase secrete multiple isoforms with different physicochemical and catalytic properties [5]. The isoforms can be present in different fractions, and the proportion of enzymes produced depends on the culture composition and operational conditions employed [6]. White-rot fungi usually produce low concentrations of various laccases when cultivated in submerged culture or on wood. Higher concentrations can be obtained by adding aromatic compounds (2,5-xylidine, ferulic acid, veratryl alcohol, guaiacol), lignin preparations, or copper to the fermentation medium [7]. Laccases are usually produced during the secondary metabolism of white-rot fungi growing on natural substrate or in submerged culture. Various cultivation parameters may influence the laccase production and activity, including carbon limitation, nitrogen source and concentration, content of vitamins and microelements, temperature, pH, aeration and agitation [8].

The laccase production in stirred tank bioreactors is sometimes limited by the lack of an efficient production system. The different morphological growth forms of the filamentous fungi have a significant effect on the rheology of fermentation broth and, consequently, in the performance of the bioreactor [9]. Cultures with filamentous fungi generally exhibit viscosity and non-newtonian rheology that may affect oxygen and mass transfer, compromising the overall productivity. For this reason, the control of hyphal extension is a crucial issue to operate successfully in bioreactor mode [10]. Submerged fermentation usually results in uncontrolled growth of mycelium. Fungus immobilization in a support material can be an interesting alternative to control the growth rate. In addition, immobilized fungi are easily separated from the reaction mixture, simplifying the downstream processing [11].

Trametes versicolor and *Peniophora cinerea* are laccase producers' fugal strains. *T. versicolor* (sometimes listed as *Coriolus* or *Polyporus*) is frequently found in trunks in several parts of the world, and is one of the most studied species for the production of laccases. Several studies report the use of laccase from *T. versicolor*, for example for dye degradation [12], kraft pulp [13], or biosensors [14]. *P. cinerea* is a ligninolytic fungus recently isolated from rainforest on coastal ecosystem under marine influence (Forest of Restinga, São Vicente, São Paulo, Brazil). This strain was selected from over one hundred strains for the degradation of xenobiotics such as organochlorine and synthetic dyes [15]. *P. cinerea* produces up to eight laccases isozymes, some of them being induced by copper (unpublished results). These laccases exhibit stability and tolerance to NaCl, attractive features for biotechnological applications such as decolorization of textile wastewater [16], bleaching of paper pulp, or delignification of lignocellulosic substrates.

Several methodologies for using fungal laccase are reported in literature, depending on the area of application or the enzymatic activity achieved after fermentation. When an increase on the enzymatic activity is claimed, some purification and concentration steps have to be performed prior to laccase application [17]. In other cases, process integration can be an efficient alternative: laccase is produced and simultaneously is operating as oxidizing agent [12]. However, the most common procedure is to use laccase as obtained from the fermentation broth [18] without any special treatment, only filtration to separate the extract from the mycelium. This methodology has some advantages over the others because the process becomes more economic and less time-consuming, but it requires final extracts to be more enriched in laccase and with much more enzymatic activity. For this reason, the demand for new and efficient conditions for laccase production has gained much attention during the last decade.

The present study evaluated the production of laccase by free and immobilized mycelia of *P. cinerea* and *T. versicolor*. Experimental assays were performed in Erlenmeyer flasks and in a stirred tank bioreactor, and the performance of the strains in both systems was evaluated and compared. Synthetic fiber was used as immobilization support, since this material demonstrated to be of great potential for fungi immobilization [19, 20]. Additionally, this is a cheap and strong material, easy to obtain and it does not require any complex or time-consuming pre-treatment [11].

Materials and methods

Fungal strains

T. versicolor MUM 04.100 was obtained from Micoteca of Universidade do Minho (MUM), Portugal. *P. cinerea* CCIBT2541was obtained from the Institute of Botany, São Paulo, Brazil. The cultures were maintained at 4 °C on Petri plates containing Tien and Kirk medium (TaK), composed of (g/L): glucose (10), malt extract (10), peptone (2), yeast extract (2), asparagine (1), KH_2PO_4 (2), $MgSO_4$ (1), thiamin-HCl (1), and agar (20).

Support material

The material used as support for immobilization consisted of cubes (0.3 cm^3) of a commercial synthetic fiber (Green Heavy Duty Scour Pad-Scotch Brite™, 3 M Company, Spain). Prior to use in the fermentation experiments, the fiber cubes were pre-treated by boiling during 10 min, and washing thoroughly three times with distilled water. After that, the cubes were dried overnight at 60 °C and autoclaved, at 121 °C, for 20 min [11].

Culture medium

Experiments were carried out using a *Trametes* defined medium (TDM) [21] composed of: glucose (83 mM), glutamine (5 mM), NaCl (5 mM), KH_2PO_4 (5 mM), $MgSO_4{\cdot}7H_2O$ (1 mM), $CaCl_2$ (0.1 mM), dimethyl succinate (10 mM), thiamine-HCl (2.4 μM), and 1 mL per each liter of medium of a trace metals solution containing (μM): $FeSO_4{\cdot}7H_2O$ (20), $MnSO_4{\cdot}H_2O$ (20), $CoCl_2{\cdot}6H_2O$ (6), $ZnCl_2$ (5), $CuSO_4{\cdot}5H_2O$ (2), $(NH_4)_6MO_7O_{24}{\cdot}4H_2O$ (0.5), and $NiCl_2{\cdot}6H_2O$ (0.1). Sterilization of the medium was performed in autoclave at 121 °C for 20 min. Tween-80 (0.5 % w/v) and 2,5-xylidine (30 μM) were also added to the culture medium to stimulate and induce the secretion of extracellular enzyme [9].

Fermentation assays in Erlenmeyer flasks

Three plugs (7 mm diameter) from the culture grown in Petri dishes for 7 days at 28 °C (TaK medium) were inoculated into 250-mL Erlenmeyer flasks containing 50 mL sterile TDM medium, with and without the support for immobilization (1 g/100 mL of medium). The inoculated flasks were incubated during 30 days, at 28 °C, in a rotary shaker at 180 rpm. Samples (500 μL) were taken each 5 days of fermentation and analyzed for laccase activity determination. Fermentation assays were performed in triplicate for each studied condition (free and immobilized *T. versicolor* and *P. cinerea*).

Fermentation assays in stirred tank bioreactor

Initially, 15 plugs (7 mm diameter) from the culture grown in Petri dishes for 7 days at 28 °C (TaK medium) were transferred to each 250-mL Erlenmeyer flask containing 125 mL of sterile TaK liquid medium (same composition described above, but without agar). The flasks were incubated in a rotary shaker, at 28 °C and 180 rpm, for 3 days, to obtain a concentrated suspension of cells (inoculum). The biomass concentration in the inoculum was determined by dry weight, and the volume of this suspension necessary to obtain an initial cell concentration of 70 mg/L was added to the bioreactor containing sterile TDM medium. A 200 μL volume of an antifoam emulsion (Antifoam Y-30 Emulsion, Sigma A6457, Sigma-Aldrich) was also added to the fermentation medium.

A 1.6-L bioreactor (Autoclavable Benchtop Fermenter Type R'ALF, Bioengineering AG, Switzerland) equipped with automatic controls for pH, agitation, and temperature, and operating in a batch mode for 10 days was used in the experiments. Two six-bladed Rushton turbines impeller with a diameter of Di = Dt/2 was placed in the tank. The support material for cells immobilization was added at 10 g/L of medium, and the fermentations were performed using 1 L working volume, at 28 °C, 180 rpm, and pH 5. Compressed air was continuously supplied to the bioreactor to maintain saturation of oxygen in the cultivation medium. The pH was controlled at 5.0 ± 0.1 by addition of NaOH (1 M) or H_3PO_4 (1 M). Samples (3 mL) of the fermentation medium was collected from the reactor once a day, and analyzed for laccase activity determination and glucose quantification.

Fermentation assays were performed in duplicate for each studied condition (free mycelium of *T. versicolor* and immobilized mycelium of *P. cinerea*).

Analytical methods

Laccase activity was determined spectrophotometrically using 1 mM 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonate (ABTS) as substrate in 50 mM phosphate–citrate pH 4.0, at 25 °C. The absorbance was measured at 420 nm ($\varepsilon = 36.000\ M^{-1}\ cm^{-1}$) [22]. One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol of ABTS/min. Laccase activity was expressed in units per liter.

Glucose was quantified by the 3,5-dinitrosalicylic acid method, and confirmed by high-performance liquid chromatography (HPLC) using a Jasco chromatograph equipped with a refractive index detector and a Bio-Rad Aminex HPX-87H column (300 × 7.8 mm). Operating conditions included: temperature of 60 °C, 0.005 M sulfuric acid as eluent at a flow rate of 0.7 mL/min and sample volume of 20 μL. Before injection, the samples were filtered through 0.2 μm filters and diluted with Milli-Q water when needed.

To determine biomass concentration, samples of the culture medium were vacuum filtered through 0.45 μm membranes, and the biomass (with or without the immobilization support) retained in the filters was washed with distilled water and dried at 70 °C until constant weight. For the experiments using the immobilization support, the amount of synthetic fiber (1 g/100 mL of medium) was subtracted from the final weight obtained. All the determinations were performed in triplicate.

Scanning electron microscopy

Micrographs of the synthetic fiber, before and after immobilization with the fungal strains, were obtained by scanning electron microscopy using a Leica Cambridge S360 microscope. The samples to be examined were fixed on a specimen holder with aluminum tape and sputtered with gold in a sputter coater under high vacuum condition. Each sample was examined at 200 and 1,000-fold magnification.

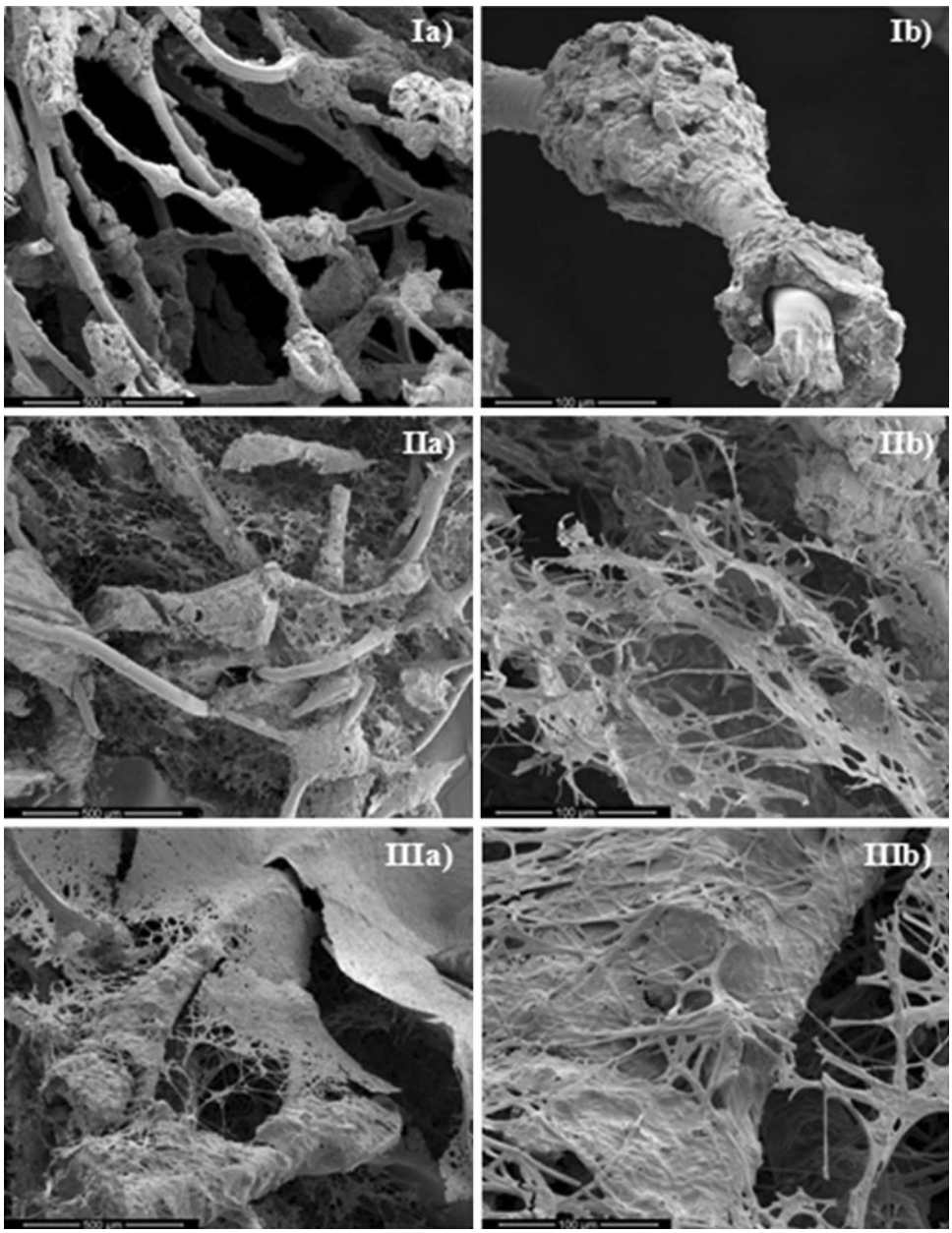

Fig. 1 Micrographs obtained by scanning electron microscopy: synthetic fiber before immobilization (original material) 200-fold (**Ia**) and 1000-fold magnification (**Ib**); synthetic fiber with immobilized *Trametes versicolor* after 30 days of fermentation-200-fold (**IIa**) and 1000-fold magnification (**IIb**); and synthetic fiber with immobilized *Peniophora cinerea* after 30 days of fermentation-200-fold (**IIIa**) and 1000-fold magnification (**IIIb**)

Results and discussion

Fungal immobilization into the support material

A variety of synthetic materials including polyurethane foam, stainless steel sponge, synthetic fibers, pumice stones, zeolites, and foam glass among others, have been tested as support for cells immobilization during fermentation. In the present study, a commercial synthetic fiber was used as support for immobilization of *T. versicolor* and *P. cinerea,* since this material was demonstrated to have great potential for fungal immobilization [19, 20]. According to the manufacturer, the green commercial synthetic fiber, commonly designed as "Scotch-Brite fiber", is constituted by a nylon web covered with a binder polymer resin (phenol/formaldehyde). Figure 1 Ia, Ib shows the characteristics of this material, which is composed by thin and resistant fibers that form an irregular surface and provide a large area available for fungi adhesion. Immobilization of both fungal strains in the synthetic fiber occurred by self-adhesion, and the fungi were able to grow not only on the surface but also in the interspaces of this material (Fig. 1 II and III). However, growth morphology was different to each strain. While *P. cinerea* grew as a compact and uniform film (Fig. 1 IIIa, IIIb), *T. versicolor* produced a more porous frame (Fig. 1 IIa, IIb). Guimarães et al. [23] used the same synthetic fiber for immobilization of *Phanerochaete chrysosporium* and

reported that this synthetic support is a hydrophilic and porous material, and its morphology was more important for the fungus immobilization than the physicochemical properties of its surface. The high surface area, the rough surface and internal pore space may provide the suitable hydrodynamically quiescent environment for fungal immobilization [23].

Laccase production by free and immobilized mycelia in Erlenmeyer flasks

Fermentation experiments were carried out in a medium previously established for *Trametes* strains [21], with slight modifications (addition of Tween-80 and 2,5-xylidine). Nevertheless, the laccase activity obtained for *P. cinerea* was considerably higher than the maximum value obtained for *T. versicolor* (Fig. 2). For both fungal strains, immobilization provided significant changes in laccase production. While the assays using free mycelium of *P. cinerea* presented very low laccase activity (about 100 U/L) during all the fermentation time, the immobilization of this strain clearly enhanced the production of this enzyme, which attained an activity of 3,500 U/L after 15 days of cultivation (Fig. 2a), corresponding to a laccase productivity of 233.3 U/L day (Table 1). An opposite behavior was observed for the fermentations with *T. versicolor* (Fig. 2b), with the highest laccase activity (800 U/L) being also achieved after 15 days of fermentation but using free mycelium.

Besides the laccase activity, the biomass produced during the fermentations (both with free and immobilized mycelium) was also higher in the experiments using *P. cinerea.* In the experiments using free mycelium, the biomass produced by *P. cinerea* was an average 18 % higher than the biomass produced by *T. versicolor*; and for the immobilized mycelium assays, this difference increased to 54 % (Table 1). Such differences in the biomass production reflected also in important differences in terms of biomass productivity, as shown in Table 1. This higher biomass production could be easily visualized during the fermentation time. However, according to Xavier et al. [24], the cell growth of *T. versicolor* is not associated with laccase production, indicating that laccase must be produced by a secondary metabolism.

The color of the culture media at the end of fermentation was also different for both fungal strains. Media cultivated with *T. versicolor* (free or immobilized) presented only slight changes in the color, which became rather brown. On the other hand, media cultivated with *P. cinerea* presented a significant color modification, becoming visibly brown. Such difference can be attributed to the pigments produced by *Peniophora* strains, as previously reported in the literature [25, 26].

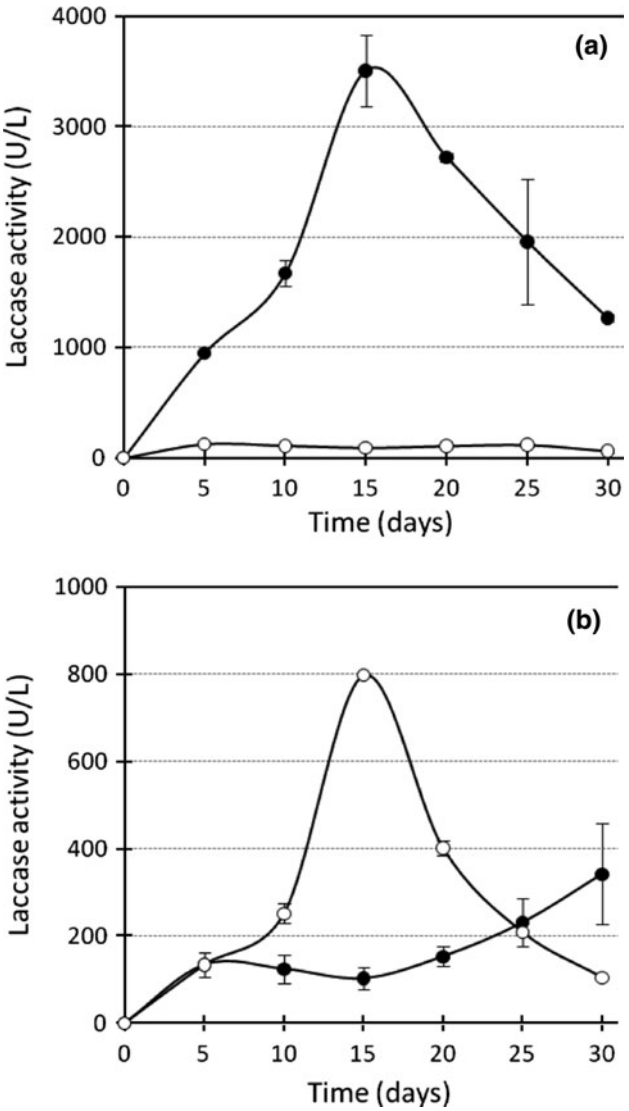

Fig. 2 Laccase production in Erlenmeyer flasks by free (*open circles*) and immobilized (*closed circles*) mycelia of **a** *Peniophora cinerea,* and **b** *Trametes versicolor*

Although some recent studies have described *P. cinerea* as being a laccase producer fungal strain [15, 16], as far as we know, there is not any published study about cultivation conditions for the production of laccase by this fungal strain. However, the 35-fold increase obtained in laccase production by this fungal strain in the present study, corresponding to an enzymatic activity of 3,500 U/L can be well compared with studies using other laccase producer fungi and fermentation systems. For example, the maximum laccase activity obtained for immobilized *P. cinerea* is approximately fivefold higher than the maximum value (650 U/L) achieved for free *T. versicolor* under submerged fermentation and using an analogous culture medium but without glucose [27]. A 30-fold lower laccase activity (114 U/L) was achieved when using *Phanerochaete*

Table 1 Fermentation parameters obtained during the cultivation of free and immobilized *Trametes versicolor* and *Peniophora cinerea* in Erlenmeyer flasks

Fungal strain	Biomass concentration (g/L)		Biomass productivity (g/L day)		Laccase productivity (U/L day)	
	Free	Immobilized	Free	Immobilized	Free	Immobilized
T. versicolor	1.92	2.64	0.06	0.09	53.3	10.7
P. cinerea	2.26	4.07	0.08	0.14	20.0	233.3

Productivity values were calculated per day of fermentation

The volume of the support was excluded when calculating the parameters per liter

chrysosporium immobilized on synthetic fiber in a semi-solid-state culture operated in a static-bed bioreactor [28]. For *Trametes hirsuta* immobilized on synthetic fiber, under submerged fermentation conditions and operating in a fixed-bed bioreactor, the maximum laccase activity was 500 U/L [29]. Maceiras et al. [30] studied the laccase production by *T. versicolor* immobilized on synthetic fiber, under semi-solid-state fermentation conditions and using veratryl alcohol-supplemented cultures, and achieved the maximum laccase activity of 238 U/L. Therefore, it is evident that the increase in laccase production obtained in the present study for *P. cinerea* represents a significant improvement. Furthermore, this maximum laccase activity (3,500 U/L) is in agreement with the enzymatic activity values required for the successful application of laccase in denim washing [18] or synthetic dye decoloration [31].

On the contrary of *P. cinerea*, there are a lot of studies on the production of laccase by *T. versicolor,* and the results obtained in the present work for free *T. versicolor* can be well compared with these literature data. For example, Tavares et al. [27] studied the laccase production by *T. versicolor* using a culture medium analogous to that used in the present work but without glucose, and obtained the highest laccase production (650 U/L) on the seventh day of fermentation. Despite being achieved in a shorter fermentation time, this value was lower than the maximum enzyme activity obtained for *T. versicolor* in the present study (800 U/L). Similar laccase production (690 U/L) was reported by Revankar and Lele [32] using a new isolate of white rot fungus *WR-1* and a culture medium with starch, yeast extract, phenylalanine, and salts. Pazarlıoğlu et al. [18] obtained higher laccase activity (2,575 U/L) with *T. versicolor* using phenol as inductor in a culture medium with glucose, yeast extract, and salts.

Although the immobilization of *T. versicolor* did not contribute to an improvement in laccase production, there are several studies that describe an enhancement in the production of this enzyme when using fermentation systems with immobilized fungal strains. For example, immobilization of *Agaricus* sp. on polyurethane foam promoted a twofold increase in laccase production when compared to the production by free fungus [33]. A fivefold increase in laccase production was obtained when using *Panus tigrinus* immobilized on polycaproamide fibers [34]; and a tenfold increase in the laccase production by *Funalia trogii* was obtained when using this fungal strain immobilized by entrapment in Na-alginate beads [35]. Such improvement was also observed in the present study for immobilized *P. cinerea*, which provided a 35-fold increase in enzyme production when compared to the free fungus.

Laccase production by free and immobilized mycelia in stirred tank bioreactor (STB)

The best conditions for laccase production in Erlenmeyer flasks (free mycelium of *T. versicolor* and immobilized mycelium of *P. cinerea*) were evaluated in a larger scale, using a 1.6-L STB. In this scale, *T. versicolor* grew faster than in Erlenmeyer flasks, and after 8 days of fermentation, this culture medium was visibly more viscous. *P. cinerea* was also able to efficiently grow in this system. In this case, a complete adhesion of the inoculated mycelia to the support fibers was observed after only 2 days of fermentation. At the end of fermentation, *P. cinerea* looked like "cotton" on the surface of the fibers. As observed in the flasks assays, the culture medium in STB also became more brownish after the fifth day of fermentation with this fungal strain, probably due to the production of pigments, as mentioned before.

Regarding the laccase production, the maximum production of this enzyme in bioreactor was obtained on the fifth day of fermentation using free *T. versicolor* (Fig. 3). However, this value (100 U/L) was eightfold lower than the value obtained in Erlenmeyer flasks. Such decrease in the enzyme production was even more significant for *P. cinerea*. When this immobilized fungus was cultivated in the STB, the maximum laccase production (75 U/L) was reached on the eighth day of fermentation, and corresponded to only 2 % of the production achieved in Erlenmeyer flasks. A decrease in the laccase production when the fungal strains are cultivated in bioreactors has also been reported by other authors [36–38]. It has been suggested that the agitation causes shear stress and mechanical forces that represses the laccase production [37, 38]. According to

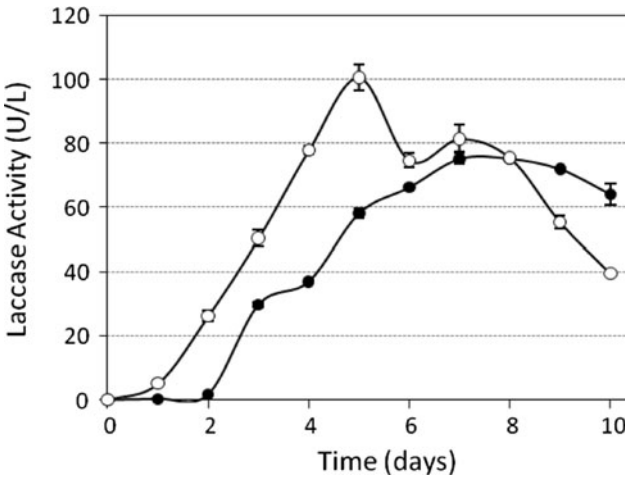

Fig. 3 Laccase production in stirred tank bioreactor by free *Trametes versicolor* (*open circles*) and immobilized *Peniophora cinerea* (*closed circles*)

Hess and co-workers [38], a possible damage in mycelia caused by high shear stress, mainly near the agitation impellers, would have been responsible for the considerable decrease in the laccase activity observed when the fungus was grown in a stirred tank reactor. In fact, Rushton turbines as used in the present study, particularly when used at high speeds, may have a detrimental effect on product yield when using shear-sensitive cells. In this case, such turbines may lead to shear forces that damage mycelia agglomerates and their peripheral hyphal growth zones, as the number of cut filaments (ratio of the number of cut hyphae to the total number of hyphae) can be increased drastically [39]. This fact could be a possible explanation for the decrease in laccase production observed in the present study when the fungal strains were cultivated in the STB. Pictures at the beginning and at the end of the cultivation of free *T. versicolor* in STB (Fig. 4) clearly show mycelia damage (presence of numerous cut mycelia) at the end of the fermentation. This fact can be easily visualized in the area within the circle in Fig. 4b. Similar mycelia damage was observed during the cultivation of *P. cinerea* in STB.

Another important aspect to be considered is glucose consumption during the fermentation time (Fig. 5). For immobilized *P. cinerea*, total glucose consumption occurred after the fifth day of fermentation, and maximum laccase production was achieved 3 days later, suggesting that the enzyme production was connected with the glucose concentration in the culture medium. Nevertheless, total glucose consumption was not achieved by free *T. versicolor* during all the fermentation time, revealing that this fungal strain has a slower metabolism when compared to *P. cinerea*. The connection between glucose concentration and laccase production has also been reported by other authors. According to Galhaup et al. [40], laccase production by *T. pubescens* took place only when glucose was depleted from the culture medium. Tavares et al. [27] also verified that high glucose concentrations affected the laccase production by *T. versicolor*, and conditions of carbon

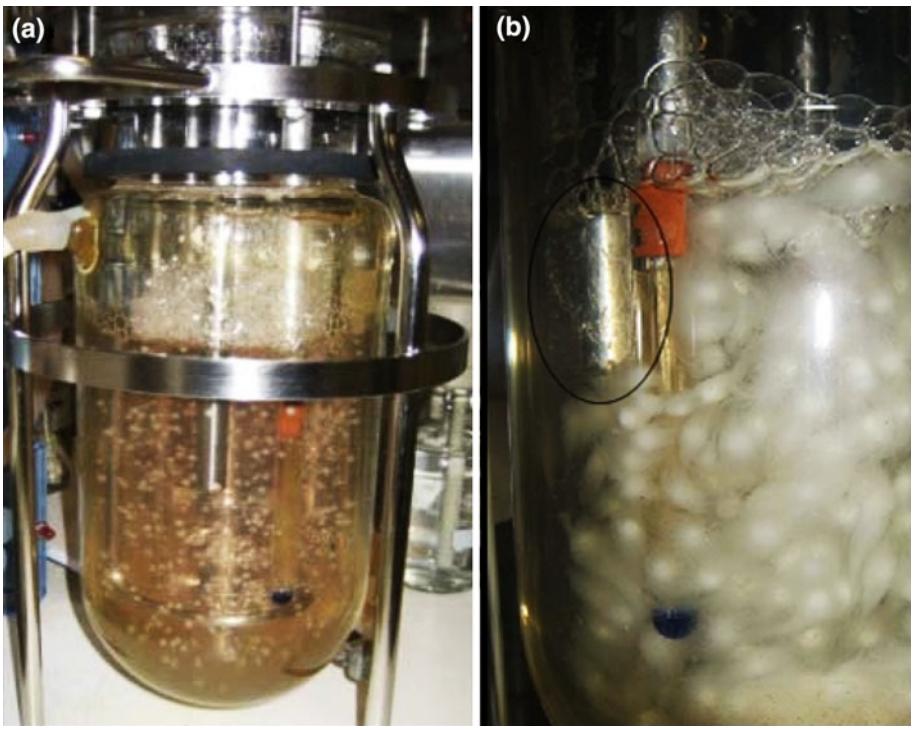

Fig. 4 Cultivation of free *Trametes versicolor* in stirred tank bioreactor. Appearance of the mycelia at the beginning (**a**) and at the end (**b**) of fermentation

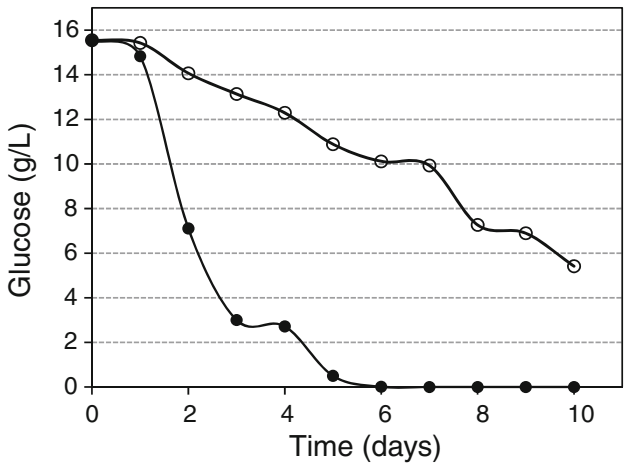

Fig. 5 Glucose consumption by free *Trametes versicolor* (*open circles*) and immobilized *Peniophora cinerea* (*closed circles*) in stirred tank bioreactor

limitation induced a secondary metabolism providing a considerable increase in the production of this enzyme. Based on these facts, it can be concluded that, besides a possible negative effect of the agitation when using STB, the low laccase production obtained in this system using free *T. versicolor* may also have been a consequence of the glucose presence in the fermentation medium during the considered fermentation time.

Conclusions

Synthetic fiber was proved to be a suitable material for use as support for immobilization of *P. cinerea* and *T. versicolor*, since both fungal strains were able to adhere its surface, growing not only on the surface but also in the interspaces. Immobilization of *P. cinerea* was a highly advantageous strategy to improve the laccase production, which was increased 35-fold attaining 3,500 U/L after 15 days of fermentation. Similar improvement on the enzyme production was not observed for *T. versicolor*, but on the contrary, the enzyme production by this fungus decreased approximately fivefold when using immobilized mycelium. In terms of laccase production, immobilized *P. cinerea* produced 4.4-fold more enzyme than free *T. versicolor* (3,500 U/L against 800 U/L). *P. cinerea* provided also higher biomass growth, both in free and immobilized forms (18 % and 54 % higher, respectively) than *T. versicolor*. These results revealed that *P. cinerea,* which is a recently isolated fungal strain, has great potential for use on the production of laccase. Finally, the production of this enzyme was also demonstrated to be greatly affected by the reactor system used for fermentation. The use of stirred flask was better than stirred tank bioreactor, probably due to the shear stress caused by the agitation impellers that damaged the mycelia.

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