Fungal Biology 122 (2018) 302–309

Contents lists available at ScienceDirect

Fungal Biology

journal homepage: www.elsevier.com/locate/funbio

Laccase production in bioreactor scale under saline condition by the marine-derived basidiomycete *Peniophora* sp. CBMAI 1063

Pedro H. Mainardi [a], Valker A. Feitosa [b], Livia B. Brenelli de Paiva [c], Rafaella C. Bonugli-Santos [d], Fabio M. Squina [e], Adalberto Pessoa Jr. [b], Lara D. Sette [a, *]

[a] Universidade Estadual Paulista Júlio de Mesquita Filho (UNESP), Departamento de Bioquímica e Microbiologia, Rio Claro, SP, Brazil
[b] Universidade de São Paulo (USP), Faculdade de Ciências Farmacêuticas (FCF), São Paulo, SP, Brazil
[c] Centro Nacional de Pesquisa em Energia e Materiais (CNPEM), Laboratório Nacional de Ciência e Tecnologia do Bioetanol (CTBE), Campinas, SP, Brazil
[d] Universidade Federal da Integração Latino-Americana (UNILA), Instituto Latino Americano de Ciências da Vida, Paraná, PR, Brazil
[e] Programa de Processos Tecnológicos e Ambientais, Universidade de Sorocaba, Sorocaba, Brazil

ARTICLE INFO

Article history:
Received 22 November 2017
Received in revised form
25 January 2018
Accepted 31 January 2018
Available online 9 February 2018

Corresponding Editor: Simon Avery

Keywords:
Aerobic fermentation
Filamentous fungi
Green chemistry
Laccases
Multi-cooper oxidases
Salinity

ABSTRACT

Laccase production in saline conditions is still poorly studied. The aim of the present study was to investigate the production of laccase in two different types of bioreactors by the marine-derived basidiomycete *Peniophora* sp. CBMAI 1063. The highest laccase activity and productivity were obtained in the Stirred Tank (ST) bioreactor, while the highest biomass concentration in Air-lift (AL) bioreactor. The main laccase produced was purified by ion exchange and size exclusion chromatography and appeared to be monomeric with molecular weight of approximately 55 kDa. The optimum oxidation activity was obtained at pH 5.0. The thermal stability of the enzyme ranged from 30 to 50 °C (120 min). The Far-UV Circular Dichroism revealed the presence of high β-sheet and low α-helical conformation in the protein structure. Additional experiments carried out in flask scale showed that the marine-derived fungus was able to produce laccase only in the presence of artificial seawater and copper sulfate. Results from the present study confirmed the fungal adaptation to marine conditions and its potential for being used in saline environments and/or processes.

1. Introduction

Laccases (EC 1.10.3.2) are enzymes that belong to the group of polyphenol oxidases and catalyze the oxidation of various organic substances, typically phenolic compounds, with the simultaneous reduction of oxygen to water through a radical-catalyzed reaction mechanism (Baldrian, 2006). Laccases can be found in many different species of organisms, like plants, fungi, prokaryotes, and insects (Yoshida, 1883; Claus, 2004; Dwivedi et al., 2011). They are referred as "moonlight" enzymes due to their multiple physiologic functions, which includes vegetal lignification and delignification, wound healing, pigment synthesis, anti-stress regulation, and fungal morphogenesis (Sharma and Kuhad, 2008).

The use of chemical mediators in the enzymatic reaction of the laccases greatly expanded their oxidation capacities (Bourbonnais and Paice, 1990). The mediators act as electron "shuttle" between the enzyme and the substrate, enabling the oxidization of complex substrates that could not be oxidized by the enzyme itself (Kunamneni et al., 2008; Cañas and Camarero, 2010). Laccases and their mediators (Laccase-Mediator System) are used to degrade and remove a series of recalcitrant pollutants, such as industrial dyes, pesticides, insecticides, endocrine disrupters, and polycyclic aromatic hydrocarbons (Strong and Claus, 2011; Viswanath et al., 2014). Moreover, laccases are also used in the formulation of biofuels and biosensors, medical analyses, and synthesis of new hybrid molecules (Mikolasch and Schauer, 2009; Senthivelan et al., 2016).

The successful application of laccase requires the production of high amounts of enzyme at low costs (Brijwani et al., 2010). Many strategies were already reported in the literature, such as the isolation of new strains for production, development of optimized culture medium, use of agro-industrial residues as carbon source, and heterologous expression of laccase genes (Osma et al., 2011).

* Corresponding author. Departamento de Bioquímica e Microbiologia-IB, Universidade Estadual Paulista-UNESP, Av. 24A, 1515–Bela Vista, Rio Claro, SP, 13506-900, Brazil. Fax: +55 19 3534 0009.
E-mail address: larasette@rc.unesp.br (L.D. Sette).

https://doi.org/10.1016/j.funbio.2018.01.009
1878-6146/

For industrial purposes, it is also necessary the development of an efficient production system at bioreactor scale (Songulashvili et al., 2015). The strategies include new bioreactors designs and the use of mathematical models to control the fungal fermentation (Znad et al., 2004; Bailey et al., 2007; Couto and Toca-Herrera, 2007).

Basidiomycetes fungi are known to be unique in their ability to degrade lignocellulose components and to produce several lignocellulolytic enzymes, including the laccases (Elisashvili and Kachlishvili, 2009). Fungal laccases are generally produced extracellularly and often exhibits higher reduction potential in comparison to laccases from other organisms (Rodgers et al., 2009). Many terrestrial fungal strains have been reported as good laccases producers at bioreactor scale, including *Trametes pubescens*, *Pycnoporus sanguineus*, and *Pleurotus* sp. (Galhaup et al., 2002; Bettin et al., 2011; Saat et al., 2014). However, studies related to the production of these enzymes by marine-derived fungi are still scarce.

Marine-derived fungal strains, which are strains obtained from the marine environments, are naturally adapted to the ocean conditions and may have unique properties, such as high tolerance against salt stress, oligotrophic conditions, and extreme of pH and temperatures (Bugni and Ireland, 2004; Trincone, 2010; Bonugli-Santos et al., 2015). The ability to growth and reproduce in ocean conditions can be considered as a biological advantage for the use of these microbial resources in saline and alkaline environments and processes (*e.g.* textile dye effluents) (Raghukumar 2008; Bonugli-Santos et al., 2010). Additionally, according to María (2013) the use of seawater in the reaction medium could also reduce the use of potable water on large bioprocesses, a great advantage, since the potable water has become scarcer every day in many world locations.

In earlier studies, the basidiomycete *Peniophora* sp. CBMAI 1063 showed great ability to produce laccases in an optimized media at flask scale (Bonugli-Santos et al., 2010, 2016). The fungus was also capable to decolorize and degrade the textile dye Reactive Black 5 in saline conditions (Bonugli-Santos et al., 2016). In this study, we evaluated the fungus capacities to produce laccases in a Stirred Tank (ST) and Airlift (AL) bioreactors. The main laccase secreted was purified and characterized regarding its optimum pH of activity, thermostability, and secondary structure.

2. Materials and methods

2.1. Microorganisms, nutrient media and cultivation procedures

Peniophora sp. CBMAI 1063 was isolated from the marine sponge *Amphimedon viridis* collected in the region of São Sebastião, São Paulo State, Brazil (Menezes et al., 2009) and taxonomically identified in a previously report by Bonugli-Santos et al. (2010). The fungus is deposited at the Brazilian Collection of Environmental and Industrial Microorganisms–CBMAI (CPQBA, Campina State University- NICAMP, Campinas, SP, Brazil) and Central of Microbial Resource-CRM-UNESP (Biosciences Institute, São Paulo State University-UNESP, Rio Claro, SP, Brazil) under the accession numbers CBMAI 1063 and CRM 205, respectively.

The laccases production medium was prepared according to the patent INPI/BR: 10 2014 0085 and contained 2.00 g L^{-1} of yeast extract, 2.74 g L^{-1} of bacteriological peptone, 1.36 g L^{-1} of malt extract powder, and 2.74 g L^{-1} of glucose, added in 65 % (v/v) of artificial seawater (ASW) (Kester et al., 1967). The medium was supplemented with an aqueous solution of 1.00 M of $CuSO_4$ to a final concentration of 2.0 mM. The initial pH of the medium was established at 4.5 and all the experiments were proceeded at 28 °C and 140 rpm.

The fungus was firstly cultivated in standard Petri plates containing 2 % of Malt Extract Agar and incubated at 28 °C. After seven d of growth, six agar-plugs (0.9 cm in diameter) from the Petri plates were inoculated into 125 mL Erlenmeyer flasks containing 50 mL of the nutrient medium. The flasks were kept at 28 °C under constant agitation (140 rpm) for 7 d. The inoculated flasks were then used as bioreactors inoculum at an equivalent proportion of 8.5 % (v/v) of the stipulated bioreactor working volume.

2.2. Flask experiments

The experiments in flask scale were performed in order to evaluate the influence of the saline condition and $CuSO_4$ on the laccases production. The fungus *Peniophora* sp. CBMAI 1063 was cultured in the optimized medium (see above) formulated with 65 % (v/v) of ASW (artificial seawater) and supplemented with $CuSO_4$ (2.00 mM). The experiments were performed, considering the presence (+) and absence (−) of ASW and $CuSO_4$: ASW(+)/$CuSO_4$(−), ASW(−)/$CuSO_4$(−), ASW(+)/$CuSO_4$(+), and ASW(−)/$CuSO_4$(+). The experiments were carried out in duplicates during 7 d at 28 °C and under a constant agitation rate of 140 rpm.

2.3. Bioreactors

The stirred tank (ST) bioreactors used in the experiments were a 1.20 L working volume BioFlo 115 (New Brunswick Scientific, US) and a 3.50 L working volume vessel Fermac 320 (Electrolab Limited, UK). The BioFlo 115 vessel measured 29.00 cm in height and 24.00 cm in width. The Fermac 320 vessel measured 24.60 cm in diameter and 47.50 cm in height. The 1.20 L ST bioreactor was agitated using one Rushton-type impeller and the 3.50 L bioreactor with two Rushton-type impellers. The compressed air was filtered through a 0.20 μm filter and injected into both bioreactors through a central sparger placed at the bottom of the vessels.

The Air-lift (AL) bioreactor was a modified version of a Microferm MF-441 (New Brunswick Scientific, US), with a vessel measuring 46.00 cm in height and 14.50 cm in diameter and working volume of 3.50 L. Following some modifications proposed by Cui et al. (2007), the impellers were removed and a stainless steel cylinder that measured 14.50 cm in diameter and 45.00 cm in height was added to the vessel. The compressed air, bubbling from the cylinder base, provided an internal-loop recirculating flow, archiving both homogenization and oxygen transfer into the broth, with lower shear stress for the mycelia.

During all the experiments, the temperature was automatically adjusted to 28 °C by a thermal blanket and an internal heat exchanger on Fermac 320 and Microferm MF-441 and by a water-jacketed glass vessel on BioFlo bioreactor. Sterile deionized water was daily added to the broth to reduce the loss of bulk liquid by evaporation, as proceeded by Cui et al. (2007). The concentration of the dissolved oxygen (DO) and the pH of the medium were measured by electronic probes. The biomass concentration and the enzymatic activities were measured by the analytical methods described below.

2.4. Initial k_La determination

The initial volumetric oxygen transfer coefficient (k_La) value were determined in distilled water using the static gassing-out method, as described by Pirt (1975). The method estimates the k_La value as a function of the agitation and aeration conditions, based on the oxygen dissolution rate. In keeping with this method, nitrogen gas was injected through the air sparger at the bottom of the bioreactor up to a deoxygenated condition. Then, air from a pressurized gas cylinder was passed through, replacing the nitrogen and increasing the rate of dissolved oxygen concentration (Marques et al., 2009). The mass balance for the dissolved oxygen in

a well-mixed liquid phase was described through the conventional Pirt mathematical model showed in Eq. (1):

$$dC/dT = k_La\ (Cs-C) \quad (1)$$

where dC/dt is the rate of O_2 accumulation in the liquid phase; k_La is the volumetric mass transfer coefficient (h^{-1}); (Cs−C) is the driving force causing the mass transfer and refer to the liquid phase oxygen concentration and its saturation at any time, respectively.

2.5. Laccase purification

For laccase purification, the crude fungal extract from the ST fermentation was filtered using Whatman® paper filter n.1. The supernatant was concentrated through an Amicon® membrane (30 kDa cut-off–Millipore) and exchanged into a 50.00 mM Tris HCl buffer pH 7.0. The enzyme concentrate was submitted to an ion exchange chromatography (DEAE-Sepharose Fast Flow GE Healthcare 20.00 mL) in 1.00 M NaCl gradient (0–100 %) followed by size exclusion chromatography (Superdex200® HiLoad GE Healthcare 124.00 mL) using Tris HCl 50.00 mM/0:15.00 M NaCl pH 7.0 buffer as the eluent. Both purification steps were carried out using an automated AKTA® system equipped with UV detector (280 nm) at a flow rate of 1.00 mL min^{-1}. To determine the purity of the protein preparation and its molecular weight, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a 12 % polyacrylamide gel containing 0.1 % SDS. Laccase was treated before it was loaded onto the gel with β-mercaptoethanol and boiled at 100 °C for 10 min. The protein was visualized by staining the gel with Coomassie blue G-250 (Bio-Rad, US) and compared with molecular weight markers (PageRuler Unstained Protein Ladder Thermo Scientific®). The protein concentration was determined by the Bradford method (Bradford, 1976).

2.6. Structural analyses

The Far-UV circular dichroism (CD) spectra were recorded on a Jasco J-810 spectropolarimeter (Jasco International Co.). Protein measurements were carried out in a 1.00 mm quartz cuvette, using the wavelength range of 190–250 nm and this represents the accumulation of ten runs. Besides the evaluation of secondary structures, the circular dichroism (CD) technique was also used to verify the thermal stability of the enzyme.

2.7. Analytical methods

For biomass quantification, aliquots of 20.00 mL were taken every 12 h and vacuum-filtered using Whatman® paper filter n. 44. The retained biomass was washed with deionized water and dried at 103 °C until constant weight. The biomass was cooled to room temperature and weighed using an analytical balance (Bailey et al., 2007).

The glucose concentration was spectrophotometrically determined in triplicates by the enzymatically and commercial-available Kit de Glicose-PP® (Gold Analisa, BR), based on Barham and Trinder (1972) description. The samples were denatured at 100 °C for 20 min before the sugar measurement.

The laccase activity was determined using 2.2′-azino-bis-ethylbenthiazoline (ABTS) and Syringaldazine (SGD) as substrates. The ABTS oxidation was measured by monitoring the absorbance increase at 420 nm ($\varepsilon = 36{,}000\ M^{-1}\ cm^{-1}$) in sodium acetate buffer (pH 5.0) at 37 °C for 6 min. One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1.00 μmol of ABTS per minute (Buswell et al., 1995). The SGD oxidation was monitored at 525 nm for 5 min (readings every 20 s) at room temperature in a spectrophotometer Tecan® Infinite. One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1.00 μmol of SGD per minute (Felby et al., 1997). To determine the optimum pH and temperature profiles, the enzymatic reactions were carried out on a different pH buffer system and at different temperatures using SGD as substrate, since it is more stable to chemical oxidation than ABTS and suitable for low pH values reactions. For thermostability evaluation, laccase was incubated at 30, 40, 50, 60, and 70 °C, and aliquots were taken at intervals of 5–120 min and their residual activity was measured in the laccase optimum pH using 0.05 M phosphate-citrate buffer. All assays were performed in triplicates.

3. Results and discussion

3.1. Flask scale experiments

Flask scale experiments indicated that fungus *Peniophora* sp. CBMAI 1063 could produce 2.900 U mL^{-1} of laccase and 3.60 g L^{-1} of biomass in 7 d of cultivation (168 h). The maximum enzymatic and biomass productivity were 0.41 U mL^{-1} d^{-1} and 0.50 g L^{-1} d^{-1}, respectively. In our experiments, we concluded that the fungus *Peniophora* sp. CBMAI 1063 was able to produce laccase only in the presence of both ASW and $CuSO_4$. The lack of copper in the reaction medium inhibited the laccase production by the fungus, and the lack of ASW strongly decreased both laccase activity and the final fungal biomass concentration (Fig. 1).

The highest enzymatic activity was similar to that one obtained in ST bioreactor experiments after seven d of incubation using the same optimized culture conditions. However, maximum enzymatic activity in the bioreactor was achieved in a higher volume and in a shorter period of time (5 d of incubation).

3.2. Laccase production in ST bioreactor

Agitation and aeration rates are considered as important parameters for the production of laccases in ST bioreactors (Saat et al., 2014). According to McNeil and Harvey (2008), both aeration and agitation rates in ST bioreactors supply the fermentation system with oxygen and provides the mixture of the moist. Those

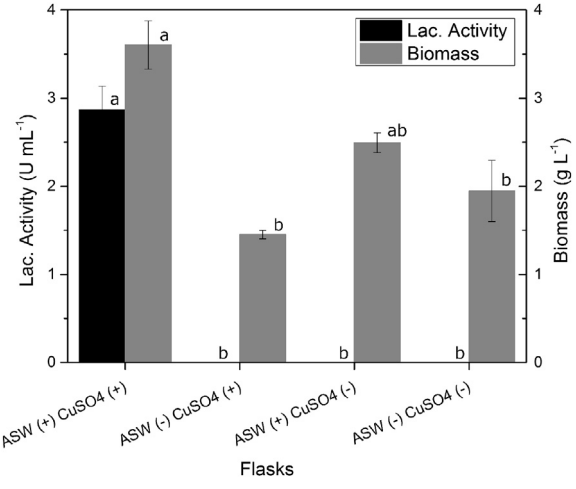

Fig. 1. Laccase activity and biomass concentration obtained after 7 d cultivation of the fungus *Peniophora* sp. CBMAI 1063 in flask scale conditions using ABTS as substrate. The positive sign (+) indicates the presence and the negative sign (−) indicates the absence of the respective constituent: ASW (Artificial Sea Water) and $CuSO_4$ (copper sulfate). The experiments were done in duplicates under a constant agitation rate of 140 rpm at 28 °C. Different letters indicate statistical differences ($P < 0.05$) by the Tukey's test.

parameters also promote deep changes in the fungus morphology and physiology, and direct affect the medium rheology, thus, the solubility of oxygen in the medium (Cui et al., 1997; Fazenda et al., 2008; Posch et al., 2013).

In the present study, the best agitation rate for laccase production was determined in smaller ST bioreactors of 1.20 L working volume. Results revealed that highest laccase activity and productivity was obtained at 150 rpm of agitation rate, with values of 1.40 U mL^{-1} and 0.28 U mL^{-1} d^{-1} (Table 1). In agitation rates of 200 and 250 rpm the enzymatic activities were lower. The effect of excess of agitation in the production of laccases in ST bioreactor were also reported by Hess et al. (2002) and Fenice et al. (2003), using the fungus *Panus tigrinus* and *Trametes multicolor*, respectively. According to the authors, the decrease of the enzyme production was related to the hydrodynamic stress caused by the shearing. Tinoco-Valencia et al. (2014), in the other hand, attributed the reduction of laccase production by the fungus *Pleurotus ostreatus* mainly by the synthesis of proteases than by the hydrodynamic stress itself. Production of proteolytic enzymes by the fungus *P. ostreatus* was also reported by Palmieri et al. (2001) and Mazumder et al. (2009).

The effect of shearing stress on immobilized fungal cells in the production of laccase was investigated by Silvério et al. (2013), using the basidiomycetes *Peniophora cinerea* and *Trametes versicolor*. According to the authors, the immobilization of *P. cinerea* provided an increasing of 35-fold on the laccase production, while the immobilization of *T. versicolor* caused a decreased on the laccase activity. Indeed, despite its great importance, there is still no simple correlation between the fungal morphology and fermentation productivity (Grimm et al., 2005). As suggested by Schürgel (1990), the behavior of each microorganism should be individually studied for an efficient fermentation performance.

Both aeration rates tested (1.00 and 0.50 vvm) showed, indeed, similar results of laccase activity and biomass concentration, with values around 2.80 U mL^{-1}, and 4.50 g L^{-1}, respectively (Table 2). However, there was a 24 h delay to reach the maximum value of laccase activity and biomass concentration in lower aeration rate (0.5 vvm), which culminated in a reduction of nearly 17 % of the enzymatic and 23 % of the biomass productivity (Fig. 2). The low aeration rate also resulted in a decrease of 20 % of the laccase synthesis rate (Table 2).

Laccases are generally produced during the secondary metabolism of the fungus (Viswanath et al., 2014). According to Galhaup et al. (2002), their synthesis starts when the glucose is nearly absent from the medium, below a certain critical level. In the exponential growth of the microorganism, during the consumption of glucose, the metabolism of the fungus is intensified and coincides with a decrease of the dissolved oxygen (DO) concentration in the medium (Bettin et al., 2011; Saat et al., 2014). In our experiments, the DO concentration sharply decreased during the initial day, while the glucose was being consumed (Fig. 2). Then, when the glucose was almost depleted from the medium, the enzyme started to be synthetized.

The cultivation in the 3.50 L ST bioreactor at 150 rpm of agitation rate and 1.00 vvm of aeration rate (k_La of 17.20 h^{-1}) resulted in a maximum laccase activity of 2.80 U mL^{-1} (120 h) and biomass concentration of 4.50 g L^{-1} (84 h) (Table 2). This experiment resulted in a maximum enzymatic and biomass productivity of 0.56 U mL^{-1} d^{-1} and 1.30 g L^{-1} d^{-1}, respectively. The highest value of laccase synthesis rate was 1.09 U mL^{-1} d^{-1}. The glucose took 48 h to be consumed from the medium, which meant a degradation rate of 1.13 g L^{-1} d^{-1} (Fig. 2A). When the experiment was carried out under the same agitation rate (150 rpm) but lower aeration rate (0.50 vvm) the enzymatic and biomass productivity were 0.46 U mL^{-1} d^{-1} and 1.00 g L^{-1} d^{-1}, respectively. The laccase was synthesized at a rate of 0.87 U mL^{-1} d^{-1} and the sugar took around 84 h to be consumed from the medium, with a degradation rate of 1.04 g L^{-1} d^{-1} (Fig. 2B). The reduction of 50 % of aeration rate (0.50 vvm) did not significantly affected the peak of laccase activity (2.80 U mL^{-1}) nor the maximum biomass concentration (4.50 g L^{-1}).

Noteworthy, even though the DO concentration reach the concentration of 5 %, around hour 70, no significantly change was observed in the highest values of enzymatic activity (Fig. 2B). According to Liu et al. (2013) an efficient production of laccase (72.00 U mL^{-1}) by the fungus *Pycnoporus* sp. SYBC-L3 was achieved even with a sharp decrease of the DO concentration to the value of 3 % on the third d of cultivation. Tinoco-Valencia et al. (2014) also reported that the laccase production (3.80 U mL^{-1}) by the fungus *P. ostreatus* CP-50 in a 10.00 L ST bioreactor was enhanced in low oxygen transfer rates.

Other studies related to the laccase production in bioreactor scale have been reported in the literature, however all of them were carried out in non saline conditions. Galhaup et al. (2002) reported the production of 330.00 U mL^{-1} of laccase after 405 h in a 15.00 L working volume ST bioreactor by the white rot fungi *T. pubescens* MB 89. Bettin et al. (2011) described the production of 40.00 U mL^{-1} of laccase in six d by the fungus *Pleurotus sajor-caju* PS-2001 in a 5.00 L ST bioreactor. Fenice et al. (2003) reported the production of 4.60 U mL^{-1} of laccase activity after 13 d by the fungus *P. tigrinus* CBS 577.79 in a 3.00 L ST bioreactor.

The pH of the medium is another crucial aspect in fungal fermentation, influencing the function of the cell membranes, cell morphology and structure, solubility of salts and nutrients, ionic state of the substrates, the biomass, and the metabolite formation (Fazenda et al., 2008). In our experiments, when the initial pH was set to 4.5, the medium suffered a smoothly acidification to 4.3 during the two initial day, meanwhile the sugar was being consumed from the medium (Fig. 2A). Then, the pH of the medium increased until the end of the experiment, reaching the value of 7.5 at 144 h. According to Fazenda et al. (2008) and Eggert (1997), the pH of the medium drifts due to the elimination of waste products and the formation of certain organic acids (*e.g.* cinnabarinic acid) that follows the laccase synthesis. When the initial pH of the medium was set to 8.0, the highest laccase activity and biomass concentration had a reduction around 75 % (0.70 U mL^{-1}) and 50 %

Table 1
Laccase activity, synthesis rate, and productivity obtained after *Peniophora* sp. CBMAI 1063 cultivation in a 1.20 L ST bioreactor under fixed aeration rate of 1.00 vvm and different agitation rates. The laccase activity was measured in triplicates using ABTS as substrate.

Maximum Values*	Agitation Rate (rpm)				
	50	100	150	200	250
Laccase Activity (U mL^{-1})	1.23 ± 0.08 (120 h) b	1.05 ± 0.01 (120 h) c	1.40 ± 0.01 (120 h) a	1.07 ± 0.02 (96 h) c	1.06 ± 0.01 (96 h) c
Enzyme Productivity (U $mL^{-1}d^{-1}$)	0.24 ± 0.01 b	0.21 ± 0.00 c	0.28 ± 0.00 a	0.27 ± 0.00 a	0.27 ± 0.00 a
Synthesis Rate (U $mL^{-1}d^{-1}$)	0.46 ± 0.00 ab	0.35 ± 0.05 c	0.43 ± 0.00 b	0.42 ± 0.01 b	0.50 ± 0.00 a

Different letters on the columns indicate statistical differences ($P < 0.05$) by the Tukey's test.

Table 2
Maximum enzyme activity, biomass concentration, synthesis rate, and productivity obtained after *Peniophora* sp. CBMAI 1063 cultivation in flask and bioreactor (3.50 L ST and AL) scales. The laccase activity was measured in triplicates using ABTS as substrate.

Scale	Cultivation Parameters			Laccase			Biomass		Glucose
	Agitation rate (rpm)	Aeration rate (vvm)	Initial pH	Lac Activity (U mL^{-1}) (Achieved hour)	Enzyme Productivity (U mL^{-1} d^{-1})	Enzyme Synthesis Rate (U mL^{-1} d^{-1})	Biomass Concentration (g L^{-1}) (Achieved hour)	Biomass Productivity (g L^{-1} d^{-1})	Consumption rate (g L^{-1} d^{-1}) (Exhaustion hour)
Flask	140	–	4.5	2.90 ± 0.38 (168) a	0.41 ± 0.05 b	–	3.60 ± 0.38 (168) b	0.50 ± 0.05 c	–
ST1	150	0.50	4.5	2.80 ± 0.09 (144) a	0.46 ± 0.01 b	0.87 ± 0.00 ab	4.50 ± 0.04 (108) b	1.00 ± 0.00 b	1.04 ± 0.04 (84) a
ST2	150	1.00	4.5	2.80 ± 0.05 (120) a	0.56 ± 0.00 a	1.09 ± 0.13 a	4.50 ± 0.43 (84) b	1.30 ± 0.12 a	1.13 ± 0.14 (48) a
ST3	150	1.00	8.0	0.70 ± 0.01 (116) c	0.14 ± 0.00 d	0.59 ± 0.23 bc	2.30 ± 0.26 (104) c	0.50 ± 0.06 c	0.95 ± 0.15 (69) a
AL	–	1.00	4.5	2.30 ± 0.02 (168) b	0.33 ± 0.00 c	0.41 ± 0.06 c	5.80 ± 0.23 (96) a	1.40 ± 0.05 a	0.55 ± 0.14 (120) b

Different letters on the columns indicate statistical differences ($P < 0.05$) by the Tukey's test.

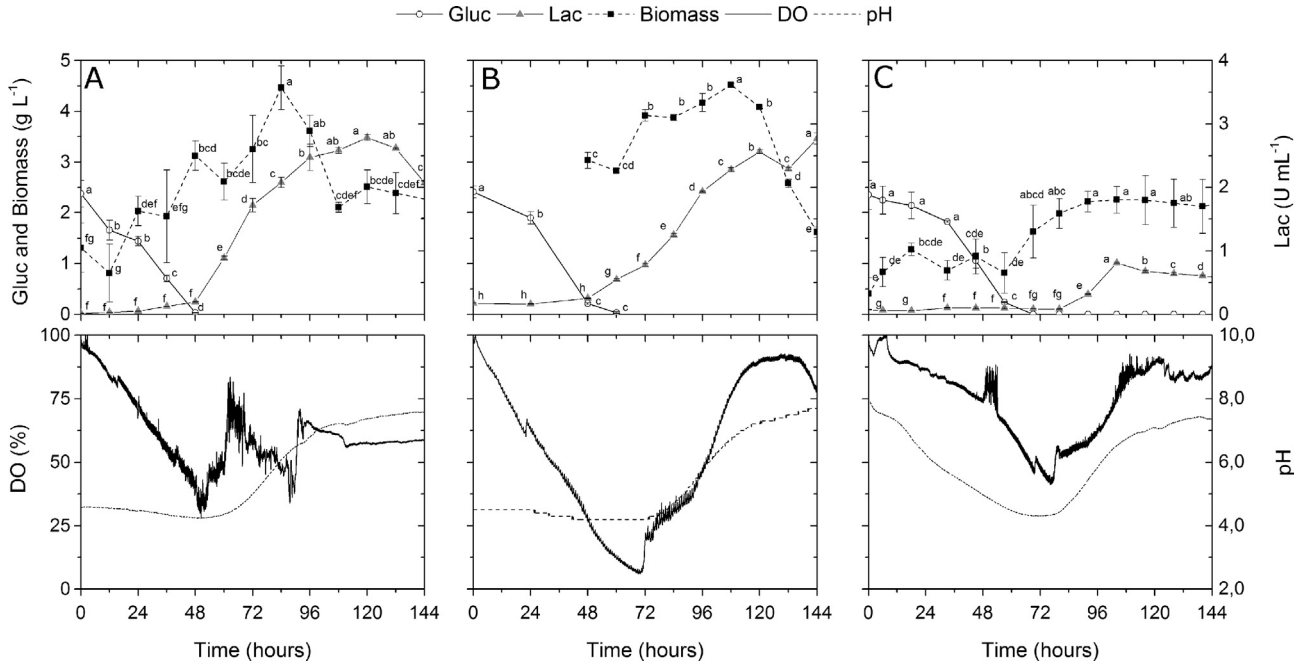

Fig. 2. Time course of laboratory cultivations of the fungus *Peniophora* sp. CBMAI 1063 in a 3.50 L working volume ST bioreactor under constant agitation rate of 150 rpm. The upper graphs indicates the laccase activity (using ABTS as substrate), the glucose and the biomass concentration. The lower graphs indicates the concentration of Dissolved Oxygen (DO) and pH of the medium during the fermentation. (A) Fermentation carried out under 1.00 vvm of aeration rate and initial pH of 4.5. (B) Fermentation carried out under 0.50 vvm of aeration rate and initial pH of 4.5. (C) Fermentation carried out under 1.00 vvm of aeration rate and initial pH of 8.0. Different letters indicate statistical differences ($P < 0.05$) by the Tukey's test.

(2.30 g L^{-1}), respectively (Table 2). There was also a decrease around 55 % on the laccase synthesis rate (0.59 U mL^{-1} d^{-1}), but no significant decrease was observed on the glucose consumption rate (0.95 g L^{-1} d^{-1}) (Fig. 2C).

The DO concentration profile indicated a smoother curve when compared to the previous experiment with initial pH of 4.5 (Fig. 2C). However, the production of laccases in this experiment started when the pH of the medium was around 4.3 (84 h) and the final pH was also the same as the previous cultivation, around 7.5. Díaz et al. (2013) showed highest production of laccases and biomass by the fungus *P. ostreatus* ATCC 32783 also at initial pH of 4.5 (around 70.00 U mL^{-1}) and lowest at pH of 8.5 (16.31 U mL^{-1}). The authors observed that the initial pH of the medium was an important factor for regulating the expression of the laccase isoenzyme genes.

3.3. Laccases production in a modified AL bioreactor

AirLift is a type of bioreactor that do not require mechanical stirrers for mixing and the contents are pneumatically agitated by a stream of air or others gases, generally injected at the bottom of the vessel (Merchuk, 1990). AirLift bioreactors are simple to construct, demands less energy in comparison to ST bioreactors and imposes less hydrodynamic stress to the cells (Znad et al., 2004). On the other hand, the distributions of nutrients and temperature in the AL bioreactors are rather non-uniform due to the low intensity of the medium mixing (Schürgel, 1990). An insufficient mass transfer can limits the availably of oxygen and nutrients to the fungal cells and cause severe impacts in the fermentation performance (Posch et al., 2013).

The successfully production of laccase in AL bioreactors were reported by Couto et al. (2006) in experiments carried out with the fungus *Trametes hirsute* in a 6.00 L vessel, and Liu et al. (2013) in experiments carried out with *Pycnoporus* sp. SYBC-L3 in a large 65.0 L AL bioreactor. In the present study, the cultivation of the fungus in the modified AL bioreactor resulted in a maximum laccase activity of 2.30 U mL^{-1} (168 h) and biomass concentration of 5.80 g L^{-1} (96 h). The highest enzymatic and biomass productivity were 0.33 U mL^{-1} d^{-1} and 1.40 g L^{-1} d^{-1}, respectively. The rate of laccase synthesis observed in the AL cultivation was 0.41 U mL^{-1} d^{-1}. The glucose had a degradation rate of 0.55 g L^{-1} d^{-1} (Table 2).

The fungus *Peniophora* sp. CBMAI 1063 showed 18 % lower maximum enzymatic activity in the AL bioreactor (2.30 U mL^{-1}) in comparison to the ST bioreactor (2.80 U mL^{-1}). Additionally, the highest activity was obtained at 168 h, causing a decrease of nearly 40 % in the enzymatic productivity. The rate of glucose consumption and laccase synthesis in the AL cultivation were 50 % and 60 % lower in comparison to the ST experiments, suggesting that the fungus developed a smoother metabolism.

Schürgel, 1990 reported that a highest productivity of penicillin V by the fungus *Penicillium chrysogenum* could be achieved more in an AL bioreactor than in a ST bioreactor. The author, however, preferred the use of the ST bioreactor due the need of a correct morphological adaptation of the cells for a good production performance in the AL bioreactor. The main issue appointed from the author was the necessity of a sufficient amount of oxygen to achieve a maximum productivity, which requires a low viscosity medium. In our experiments, *Peniophora* sp. CBMAI 1063 showed good ability to adapt to the AL conditions and there was no evidence of a highly viscous medium. The fungal morphology and the medium viscosity, as exemplified by Schürgel, 1990, had a great importance in the fermentation performance.

3.4. Laccase characterization

A typical fungal laccase has an average molecular weight between 50 and 130 kDa (Senthivelan et al., 2016). The crude extract from the ST bioreactor experiment was purified and the main laccase from *Peniophora* sp. CBMAI 1063 appeared to be monomeric with a molecular weight (MW) of approximately 55 kDa (Fig. 3A). Laccases are known to oxidize a wide range of substrates, which includes *ortho* and *para* diphenols, aromatic amines, polyphenols and methoxy phenols (Desai and Nityanand, 2011; Strong and Claus, 2011). After purification, the specific activity of the laccase from *Peniophora* sp. CBMAI 1063 enzyme increased from 1.68 U mg^{-1} to 30.80 U mg^{-1} using SGD as substrate and from 54.50 U mg^{-1} to 986.00 U mg^{-1} using ABTS as substrate.

Laccase activity and thermostability was investigated at different pH values and temperatures using SGD as substrate, a preferable substrate for the enzymatic characterization due do their electron donor dependency to the pH solution (Rodgers et al., 2009). Laccase from the fungus *Peniophora* sp. CBMAI 1063 was fully heat-stable at 30 °C (up to 120 min) and retained 60 % its activity after 20 min when incubated at 40 and 50 °C. The enzyme lost its activity after 20 min when incubated at 60 and 70 °C (Fig. 3B). The optimum pH for laccase oxidation of SGD was 5.0 (Fig. 3C). The enzyme retained more than 50 % of its stability at pH 4.0 and 40 % at pH 6.0.

According to Madhavi and Lele (2009), the optimal temperature of laccases greatly differs from one to another strain. Heinzkill et al. (1998) mentioned that the laccase from the wood rotting fungus *Polyporus pinsitus* remained 27 % active at 60 °C. Schneider et al. (1999) reported a laccase from *Coprinus cinereus* that showed optimum temperature between 60 °C and 70 °C. Patel et al. (2013) reported a laccase from the fungus *P. ostreatus* HP-1 stable at temperatures up to 70 °C. The high stability of fungal laccases is attributed to the presence of covalently linked carbohydrate, from 10 to 45 % of total molecular mass (Claus, 2004). The carbohydrate compounds is formed mainly by mannose, hexamine, glucose, fructose, galactose, and arabinose (Senthivelan et al., 2016). Rodgers et al. (2009) suggests that the glycosylation may stabilize the copper activity centers, directs the protein secretion, prevents proteolysis attacks, and enhances the enzyme thermostability.

The optimum value of pH for laccase activities highly depends on the substrate (Desai and Nityanand, 2011). According to Baldrian (2004), laccases generally forms a bell-shaped profile and exhibits higher activity at acid medium. In our study, the highest activity was obtained at pH 4.5, within the expected when compared to other fungal laccases, whose activity generally shows optimal SGD oxidation at pH between 4.0 and 7.0 (Baldrian, 2004). Laccase from the fungus *Didymocrea* sp. reported by Tetianec et al. (2014) showed optimal oxidation of SGD at pH 8.0. In the experiments carried out by Coll et al. (1993) and Muñoz et al. (1997) fungal laccases ranged from 3.0 to 9.0 and from 3.0 to 10.0, respectively. Additionally, Baldrian (2004) reported a laccase from the white-rot fungus *Daedalea quercina* with half-life of 28 min at pH 5.0 and 6 min at pH 3.0.

The native spectrum of the laccase produced by the marine-derived fungus *Peniophora* sp. CBMAI 1063 obtained from the CD method is shown in Fig. 3D. The CD method allows the evaluation of the denaturation of the protein by monitoring changes in its optical activity as a function of temperature or denaturing agent (Greenfield and Fasman, 1969). The results obtained from the technique revealed that there was little change on the laccase CD spectrum after incubation from 20 to 90 °C (rate of 1 °C min^{-1}), indicating high stability in terms of secondary structure (dark line, Fig. 3D). However, the loss at 215 nm in the CD spectrum indicated a protein unfolding (dark line, Fig. 3D). The CD data was confirmed by the enzymatic assays under the determined conditions.

Fungal laccases often display high number of β-barrel arrangement in their secondary structure, which is considered as a crucial factor for their activity maintenance (Bonomo et al., 2001; Liu et al.,

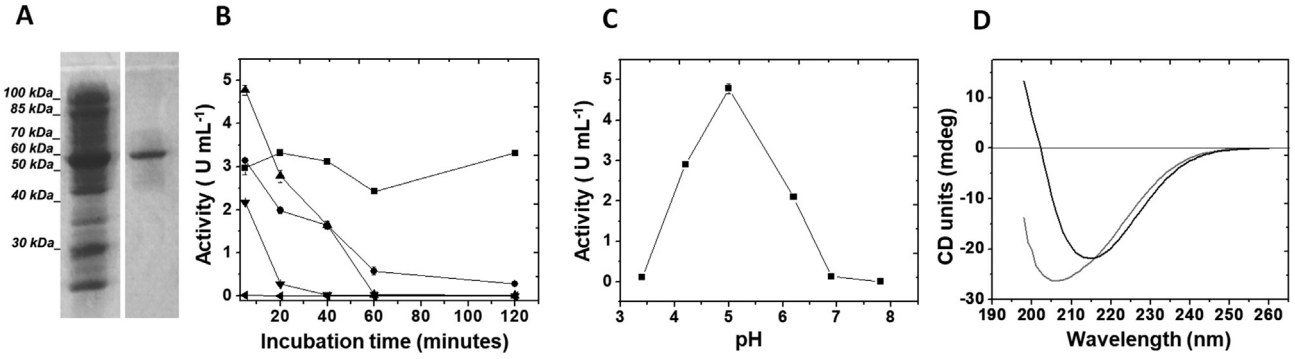

Fig. 3. (A) *Peniophora* sp. CBMAI 1063 extract before gel permeation chromatography (left) and laccase after two purification steps. (B) Thermostability of laccase evaluated at 30 °C (■) 40 °C (●) 50 °C (▲) 60 °C (▼) and 70 °C (◀) using SGD as substrate. (C) Optimum pH value of laccase reaction using SGD as substrate. (D) Circular Dichroism spectra of laccase recorded at 20 °C before (black line) and after incubation from 20 to 90 °C at a rate of 1 °C min^{-1} (dark line). The thermal unfolding was monitored by the change in molar ellipticity at 215 nm wavelength. Error bars represent the standard errors based on the means of triplicate experiments.

2017). According to Schneider et al. (1999) and Liu et al. (2016), laccases from the fungus *C. cinereus* and *Pycnoporus* sp. SYBC-L3 were also mainly composed of β -sheet arrangement. Interestingly, Liu et al. (2017) suggested that laccase from the bacteria *Klebsiella pneumonia* was mainly consisted of α-helix structures. Tang and Coleman (1968) compared the CD spectra of the Blue Copper Protein from *Pseudomonas aeruginosa* and laccase B from *Polyporus versicolar*. The author verified that the Blue Protein had a mixture of approximately 40 % of α-helix, 37 % of β-structures, and 23 % of random coil, while the laccase B indicated that the majority of the peptides bonds were incorporated in a mixture of β-structures. The secondary structure of the proteins and their folding patterns are highly dependent on the pH, ionic strength and presence of salt (Bonomo et al., 2001).

4. Conclusions

The marine-derived basidiomycete *Peniophora* sp. CBMAI 1063 was able to produce significantly amounts of enzyme in both ST and AL bioreactor. The experiments in the ST bioreactor resulted in higher production of laccase, while the AL bioreactor in higher biomass formation. The agitation and aeration rates, as well as the initial pH of the medium, directly influenced the production of laccase and fungal biomass. The purified laccase was mostly composed by β-sheet arrangement and showed good structural stability towards thermal and pH stress. Results from the present study highlight the relevance of the blue biotechnology, since the fungus was isolated from a marine sponge and produced great amounts of laccase only in the presence of salinity, showing to be naturally adapted to the marine environment. *Peniophora* sp. CBMAI 1063 represents a new microbial resource for laccase production and biotechnological applications, mainly under saline conditions and/or processes.

Authors' contribution

P.H.M., L.B.B.P and W.A. performed the experiments and wrote the manuscript. L.D.S., A.P.J. and F.M.S. designed the experiments and revised the manuscript. All authors discussed and commented on the manuscript.

Competing financial interest

The authors declare that they have no conflict of interest.

Acknowledgements

P.H. Mainardi and L.B. Brenelli Paiva were supported by scholarships from the São Paulo Research Foundation - FAPESP (2013/12505-0 and 2013/03061-0). The authors thank FAPESP for its financial support (grants 2013/19486-0, 2014/06923-6, and 2016/07957-7). Lara D. Sette and Fabio M. Squina thank the National Council for Scientific and Technological Development (CNPq) for the Productivity Fellowship (304103/2013-6 and 310186/2014-5).

References

Bailey, M.J., Adamitsch, B., Rautio, J., von Weymarn, N., Saloheimo, M., 2007. Use of a growth-associated control algorithm for efficient production of a heterologous Laccase in *Trichoderma reesei* in fed-batch and continuous cultivation. Enzym. Microb. Technol. 41, 484–491.

Baldrian, P., 2004. Purification and characterization of Laccase from the white-rot fungus *Daedalea quercina* and decolorization of synthetic dyes by the enzyme. Appl. Microbiol. Biotechnol. 63, 560–563.

Baldrian, P., 2006. Fungal laccases - occurrence and properties. FEMS Microbiol. Rev. 30, 215–242.

Barham, D., Trinder, P., 1972. An improved colour reagent for the determination of blood glucose by the oxidase system. Analyst 97, 142–145.

Bettin, F., Rosa, L.O., Montanari, Q., Calloni, R., Gaio, T.A., Malvessi, E., da Silveira, M.M., Dillon, A.J.P., 2011. Growth kinetics, production, and characterization of extracellular Laccases from *Pleurotus sajor*-caju PS-2001. Process Biochem. 46, 758–764.

Bonomo, R.P., Cennamo, G., Purrello, R., Santoro, A.M., Zappala, R., 2001. Comparison of three fungal Laccases from *Rigidoporus lignosus* and *Pleurotus ostreatus*: correlation between conformation changes and catalytic activity. J. Inorg. Biochem. 83, 67–75.

Bonugli-Santos, R.C., Durrant, L.R., Sette, L.D., 2010. The production of ligninolytic enzymes by marine-derived basidiomycetes and their biotechnological potential in the biodegradation of recalcitrant pollutants and the treatment of textile effluents. Water Air Soil Pollut. 223, 2333–2345.

Bonugli-Santos, R.C., Vasconcelos, M.R.S., Passarini, M.R.Z., Vieira, G.A.L., Lopes, V.C.P., Mainardi, P.H., dos Santos, J.A., Duarte, L.A., Otero, I.V.R., Yoshida, A.M.S., Feitosa, V.A., Pessoa, A., Sette, L.D., 2015. Marine-derived fungi: diversity of enzymes and biotechnological applications. Front. Microbiol. 6, 1–15.

Bonugli-Santos, R.C., Vieira, G.A.L., Collins, C., Fernandes, T.C.C., Marin-Morales, M.A., Murray, P., Sette, L.D., 2016. Enhanced textile dye decolorization by marine-derived basidiomycete *Peniophora* sp. CBMAI 1063 using integrated statistical design. Environ. Sci. Pollut. Res. 1, 1–10.

Bourbonnais, R., Paice, M.G., 1990. Oxidation of non-phenolic substrates. An expanded role for laccase in lignin biodegradation. FEBS Lett. 267, 99–102.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Brijwani, K., Rigdon, A., Vadlani, P.V., 2010. Fungal Laccases: production, function, and applications in food processing. Enzym. Res. 1, 1–10.

Bugni, T.S., Ireland, C.M., 2004. Marine-derived fungi: a chemically and biologically diverse group of microorganisms. RSC Adv. 21, 143–163.

Buswell, J.A., Cai, Y., Chang, S., 1995. Effect of nutrient nitrogen and manganese on manganese peroxidase and Laccase production by *Lentinula (Lentinus)*edodes. FEMS Microbiol. Lett. 128, 81–88.

Cañas, A.I., Camarero, S., 2010. Laccases and their natural mediators: biotechnological tools for sustainable eco-friendly processes. Biotechnol. Adv. 28, 694–705.

Claus, H., 2004. Laccases: structure, reactions, distribution. Micron 35, 93–96.

Coll, P.M., Fernández-Abalos, J.M., Villanueva, J.R., Santamaría, R., Pérez, P., 1993. Purification and characterization of a phenoloxidase (laccase) from the lignin-degrading basidiomycete PM1 (CECT 2971), 59, 2607–2613.

Couto, S.R., Rodríguez, A., Paterson, R.R.M., Lima, N., Teixeira, J.A., 2006. Laccase activity from the fungus *Trametes hirsuta* using an air-lift bioreactor. Lett. App. Microbiol. 42, 612–616.

Couto, S.R., Toca-Herrera, J.L., 2007. Laccase production at reactor scale by filamentous fungi. Biotechnol. Adv. 25, 558–569.

Cui, L., Goh, K.K.T., Archer, R., Singh, H., 2007. Characterization and bioactivity of protein-bound polysaccharides from submerged-culture fermentation of *Coriolus versicolor* Wr-74 and ATCC-20545 strains. J. Ind. Microbiol. Biotechnol. 34, 393–402.

Cui, Y.Q., van der Lans, R.G.J.M., Luyben, K.C.A.M., 1997. Effect of agitation intensities on fungal morphology of submerged fermentation. Biotechnol. Bioeng. 55, 715–726.

Desai, S.S., Nityanand, C., 2011. Microbial laccases and their applications: a review. Asian J. Biotechnol 3, 98–124.

Díaz, R., Téllez-Téllez, M., Sánchez, C., Bibbins-Martínez, M.D., Díaz-Godínez, G., Soriano-Santos, J., 2013. Influence of initial pH of the growing médium on the activity, production and genes expression. Profiles of laccase of *Pleurotus ostreatus* in submerged fermentations. Electron. J. Biotechnol. 16, 1–13.

Dwivedi, U.N., Singh, P., Pandey, V.P., Kumar, A., 2011. Structure-function relationship among bacterial, fungal and plant laccases. J. Mol. Catal. B Enzym. 68, 117–128.

Eggert, C., 1997. Laccase-catalyzed formation of cinnabarinic acid is responsible for antibacterial activity of *Pycnoporus cinnabarinus*. Microbiol. Res. 152, 315–318.

Elisashvili, V., Kachlishvili, E., 2009. Physiological regulation of Laccase and manganese peroxidase production by white-rot Basidiomycetes. J. Biotechnol. 144, 37–42.

Fazenda, M.L., Seviour, R., McNeil, B., Harvey, L.M., 2008. Submerged culture fermentation of “higher fungi”: the macrofungi. Adv. Appl. Microbiol. 63, 33–103.

Felby, C., Nielsen, B.R., Olesen, P.O., Skibsted, L.H., 1997. Identification and quantification of radical reaction intermediates by electron spin resonance spectrometry of Laccase-catalyzed oxidation of wood fibers from beech (*Fagus sylvatica*). Appl. Microbiol. Biotechnol. 48, 459–464.

Fenice, M., Sermanni, G.G., Federici, F., D'Annibale, A., 2003. Submerged and solid-state production of Laccase and Mn-peroxidase by *Panus tigrinus* on olive mil wastewater-based media. J. Biotechnol. 100, 77–85.

Galhaup, C., Wagner, H., Hinterstoisser, B., Haltrich, D., 2002. Increased production of Laccase by the wood-degrading basidiomycete *Trametes pubescens*. Enzym. Microb. Technol. 30, 529–536.

Greenfield, N., Fasman, G.D., 1969. Computed circular dichroism spectra for the evaluation of protein conformation. Biochemistry 10, 4108–4116.

Grimm, L.H., Kelly, S., Krull, R., Hempel, D.C., 2005. Morphology and productivity of filamentous fungi. Appl. Microbiol. Biotechnol. 69, 375–384.

Heinzkill, M., Bech, L., Halkier, T., Schneider, P., Anke, T., 1998. Characterization of laccases and peroxidases from wood-rooting fungi (family coprinaceae). Appl. Environ. Microbiol. 64, 1601–1606.
Hess, J., Leitner, C., Galhaup, C., Kulbe, K.D., Hinterstoisser, B., Steinwender, M., Haltrich, D., 2002. Enhanced formation of extracellular laccase activity by the white-rot fungus *Trametes multicolor*. Appl. Biochem. Biotechnol. 98, 229–241.
Kester, D.R., Duedall, I.W., Connors, D.N., Pytkowicz, R.M., 1967. Preparation of artificial seawater. Limnol. Oceanogr. 12, 176–179.
Kunamneni, A., Plou, F.J., Ballesteros, A., Alcalde, M., 2008. Laccases and their applications: a patent review. Recent Pat. Biotechnol. 2, 10–24.
Liu, J., Cai, Y., Liao, X., Huang, Q., Hao, Z., Hu, M., Zhang, D., Li, Z., 2013. Efficiency of Laccase production in a 65-L air-lift reactor for potential green industrial and environmental application. J. Clean. Prod. 39, 154–160.
Liu, J., Yu, Z., Liao, X., Liu, J., Mao, F., Huang, Q., 2016. Scalable production, fast purification, and spray drying of native *Pycnoporus* laccase and circular dichroism characterization. J. Clean. Prod. 127, 600–609.
Liu, Y., Huang, L., Guo, W., Jia, L., Fu, Y., Gui, S., Lu, F., 2017. Cloning expression, and characterization of a thermostable and pH-stable laccase from *Klebsiella pneumoniae* and its application to dye decolorization. Process Biochem. 53, 125–134.
Madhavi, V., Lele, S.S., 2009. Laccase: properties and applications. Bioresources 4, 1694–1717.
María, P.D., 2013. On the use of seawater as reaction media for large-scale applications in biorefineries. Chem. Cat. Chem. 5, 1643–1648.
Marques, D.A.V., Torres, B.R., Porto, A.L.F., Pessoa Jr., A., Converti, A., 2009. Comparison of oxygen mass transfer coefficient in simple and extractive fermentation systems. Biochem. Eng. J. 47, 122–126.
Mazumder, S., Basu, S.K., Mukherjee, M., 2009. Laccase production in solid-state and submerged fermentation by *Pleurotus ostreatus*. Eng. Life Sci. 9, 45–52.
McNeil, B., Harvey, L.M., 2008. Practical Fermentation Technology. John Wiley & Sons.
Menezes, C.B.A., Bonugli-Santos, R.C., Miqueletto, P.B., Passarini, M.R.Z., Silva, C.H.D., Justo, M.R., Leal, R.R., Fantianni-Garboggini, F., Oliveira, V.M., Berlinck, R.G.S., Sette, L.D., 2009. Microbial diversity associated with algae, ascidians and sponges from the north coast of São Paulo state, Brazil. Microbiol. Res. 165, 466–482.
Merchuk, J.C., 1990. Why use air-lift bioreactors. Tibtech 8, 66–71.
Mikolasch, A., Schauer, F., 2009. Fungal Laccases as tools for the synthesis of new hybrid molecules and biomaterials. Appl. Microbiol. Biotechnol. 82, 605–624.
Muñoz, C., Guillén, F., Martínez, A.T., Martínez, M.J., 1997. Laccase isoenzymes of *Pleurotus eryngii*: characterization, catalytic properties and participation in activation of molecular oxygen and Mn^{2+} oxidation. Appl. Environ. Microbiol. 63, 2166–2174.
Osma, J.F., Toca-Herrera, J.L., Rodríguez-Couto, S., 2011. Cost analysis in Laccase production. J. Environ. Manag. 92, 2907–2912.
Palmieri, G., Bianco, C., Cennamo, G., Giardina, P., Marino, G., Monti, M., Sannia, G., 2001. Purification, characterization, and functional role of a novel extracellular protease from *Pleurotus ostreatus*. Appl. Environ. Microbiol. 67, 2754–2759.
Patel, H., Gupte, S., Gahlout, M., Gupte, A., 2013. Purification and characterization of an extracellular Laccase from solid-state culture of *Pleurotus ostreatus* HP-1. 3 Biotech 1, 1–8.
Pirt, S.L., 1975. Principles of Microbe and Cell Cultivation. Halsted Press, New York.
Posch, A.E., Werwig, C., Spadiut, O., 2013. Science-based bioprocess design for filamentous fungi. Trends Biotechnol. 31, 37–44.
Raghukumar, C., 2008. Marine fungal biotechnology: an ecological perspective. Fungal Divers. 31, 19–35.
Rodgers, C.J., Blanford, C.F., Giddens, S.R., Skamnioti, P., Armstrong, F.A., Gurr, S.J., 2009. Designer Laccases: a vogue for high-potential fungal enzymes. Trends Biotechnol. 28, 63–72.
Saat, M.N., Annuar, M.S.M., Alias, Z., Chuan, L.T., Chisti, Y., 2014. Modeling of growth and Laccase production by *Pycnoporus sanguineus*. Bioproc. Biosyst. Eng. 37, 765–775.
Schneider, P., Caspersen, M.B., Mondorf, K., Halkier, T., Skov, L.K., Østergaard, P.R., Brown, K.M., Brown, S.H., Xu, F., 1999. Characterization of a *Coprinus cinereus* laccase. Enzym. Microb. Technol. 25, 502–508.
Schürgel, K., 1990. Comparison of the performances of stirred tank and airlift tower loop reactors. J. Biotechnol. 13, 251–256.
Senthivelan, T., Kanagaraj, J., Panda, R.C., 2016. Recent trends in fungal Laccase for various industrial applications: an eco-friendly approach - a review. Biotechnol. Bioproc. Eng. 21, 19–38.
Sharma, K.K., Kuhad, R.C., 2008. Laccase: enzyme revisited and function redefined. Indian J. Microbiol. 48, 309–316.
Silvério, S.C., Moreira, S., Milagres, A.M., Macedo, E.A., Teixeira, J.A., Mussatto, S., 2013. Laccase production by free and immobilized mycelia of *Peniophora cinerea* and *Trametes versicolor*: a comparative study. Bioproc. Biosyst. Eng. 36, 365–373.
Songulashvili, G., Spindler, D., Jimenéz-Tobón, G.A., Jaspers, C., Kerns, G., Penninckx, M.J., 2015. Production of a high level of laccase by submerged fermentation at 120-L scale of *Cerrena unicolor* C-139 grown on wheat bran. C. R. Biol. 338, 121–125.
Strong, P.J., Claus, H., 2011. Laccase: a review of its past and its future in bioremediation. Crit. Rev. Environ. Sci. Technol. 41, 373–434.
Tang, S., Coleman, J.E., 1968. Conformational studies of copper proteins. J. Biol. Chem. 243, 4286–4297.
Tetianec, L., Chaleckaja, A., Vidziunaite, R., Kulys, J., Bachmatova, I., Marcinkeviciene, L., Meskys, R., 2014. Development of a Laccase/Syringaldazine system for NAD(P)H oxidation. J. Mol. Catal. B Enzym. 101, 28–34.
Tinoco-Valencia, R., Gómez-Cruz, C., Galindo, E., Serrano-Carréon, L., 2014. Toward an understanding of the effects of agitation and aeration on growth and Laccases production by *Pleurotus ostreatus*. J. Biotechnol. 177, 67–73.
Trincone, A., 2010. Potential biocatalysts originating from sea environments. J. Mol. Catal. B Enzym. 66, 241–256.
Viswanath, B., Rajesh, B., Janardhan, A., Kumar, A.P., Narasimha, G., 2014. Fungal Laccases and their applications in bioremediation. Enzym. Res. 1, 1–21.
Yoshida, H., 1883. Chemistry of lacquer (Urushi), Part 1. J. Chem. Soc. 43, 472–486.
Znad, H., Báles, V., Markos, J., Kawase, Y., 2004. Modeling and simulation of airlift bioreactors. Biochem. Eng. J. 21, 73–81.