



ENGINEERING SCIENCES

Production of *Pleurotus sajor-caju* crude enzyme broth and its applicability for the removal of bisphenol A

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Abstract: Bisphenol A is an endocrine interfering compound, produced and used on a large scale worldwide. Chemical and biologic methods can be used to remove it from the environment. Biological methods are considered less costly, safer and, according to green chemistry definitions, an environmentally correct method. Considering the use of a crude enzyme broth, without any downstream process, the costs could be mostly reduced. Thus, the removal of bisphenol A by *Pleurotus sajor-caju* crude enzyme broth was investigated. Initially, the agro-industrial wastes were characterized and, the composition of the culture medium and the bioreactor culture conditions were defined. The enzyme produced in the highest concentration was characterized and the crude broth used in the bisphenol A removal assays. The OXI45 culture medium presented the highest laccase activity (1,850.7 U L⁻¹, 350 rpm). Greater laccase stability was observed at 20 - 40 °C and pHs 5 - 7. Vanillin and ferulic acid (considered mediator compounds) were identified in the crude broth, probably helping on the obtention of the high value of removal effectiveness (0.052 mg U⁻¹ h⁻¹). The results indicate the potential use of the *Pleurotus sajor-caju* crude enzyme broth to obtain an enzymatic formulation for application in the environmental area.

Key words: Banana wastes, Basidiomycetes, endocrine disruptor, enzymatic activity Submerged culture.

INTRODUCTION

Fungi of the *Pleurotus* genus are considered to be good producers of enzymes such as cellulases, hemicellulases, laccases, manganese peroxidases and lignin peroxidases (Libardi-Junior et al. 2011, 2012, Pandey et al. 2017). They grow in a wide variety of agro-industrial wastes and are easily cultivated in both liquid and solid media (Bonatti et al. 2004, Elisashvili et al. 2008, Quevedo-Hidalgo et al. 2015). The northeastern region of Santa Catarina, Brazil, is a large banana producer and, consequently, a large generator of agro-industrial wastes which can be used in

biotechnological routes, such as fungal enzyme production, reducing production costs and helping in solving environmental problems arising from their accumulation in nature (Bonatti et al. 2004, Osma et al. 2011, Pandey et al. 2017). These wastes are defined as carbon sources in the composition of the culture medium, but, at the same time, also as inductors for the production of lignocellulolytic enzymes by fungi (Elisashvili et al. 2008, Rashad et al. 2010, Libardi-Junior et al. 2012, Tinoco-Valência et al. 2014, Quevedo-Hidalgo et al. 2015).

The cultivation in liquid medium has some advantages in relation to the cultivation in

solid medium such as better process control, less cultivation time and greater enzymatic activity. In this type of cultivation, several factors deserve attention in order to maximize enzyme production, such as the composition of the culture medium, temperature, airflow, agitation rate and the carrying out of the production process (Hess et al. 2002, Fenice et al. 2003, Bettin et al. 2011, Tinoco-Valência et al. 2014). In spite of the substantial literature on the production of lignocellulolytic enzymes by fungi of the *Pleurotus* genus, few studies have been found regarding the influence of culture conditions, mainly in bioreactor, on the production of enzymes by the *Pleurotus sajor-caju* species. Furthermore, information such as optimum pH and temperature for enzyme activity as well as enzyme stability with pH and temperature, are often divergent, which is extremely important for the viability of production on a larger scale.

Oxidative enzymes, such as laccases, are widely used in an extensive variety of industrial segments, such as the chemical, textile, pulp and paper, food segments, etc. (Libardi-Junior et al. 2011, Pandey et al. 2017). More recently, their application has extended to the environmental area, as in the removal of endocrine-interfering compounds such as the bisphenol A (Chang & Chang 2016, de Freitas et al. 2017, Barrios-Estrada et al. 2018). It is considered to be one of the most widely used synthetic compounds on the planet (Jalal et al. 2018). These compounds have the ability to interfere in the endocrine system of organisms causing adverse implications for development and reproduction as well as neurological and immune system changes (Michalowicz 2014, Sifakis et al. 2017, Jalal et al. 2018).

Both chemical and biological methods are proposed in literature for the removal of bisphenol A from the environment. However, chemical methods present some disadvantages

in relation to biological methods, such as high cost and the formation of toxic compounds. Thus, it is believed that biological degradation would be a less costly, safer method and, according to Green Chemistry definitions, environmentally correct (de Freitas et al. 2017, Pandey et al. 2017). Most studies reported in literature use oxidative enzymes, generally in their purified or enzyme extract form, free or immobilized, synthesized by different microorganisms for the removal bisphenol A. However, no study is reported using the *Pleurotus* genus crude enzyme broth, without any downstream processing.

Therefore, considering the biotechnological production of oxidative enzymes by fungi and the environmental aspects, the objectives of this work were to characterize the banana wastes (banana peel powder and immersion water of banana straw) used in the culture medium composition; to study the production of oxidative enzymes by *Pleurotus sajor-caju* in bioreactor and to validate the efficacy of the crude enzyme broth, without any downstream processing, in the degradation of the endocrine interfering bisphenol A.

MATERIALS AND METHODS

Microorganism and maintenance

Pleurotus sajor-caju CCB 019 was obtained from the Center for Basidiomycete Cultivation of the University of São Paulo. The strain was grown in WDA (Wheat Dextrose Agar) (Furlan et al. 1997) medium and stored under refrigeration (4°C).

Chemical characterization of banana wastes

The immersion water of banana straw and the banana peel powder were analyzed in terms of organic matter, organic carbon, total carbohydrate, monosaccharide composition, crude fiber, hemicellulose, cellulose, lignin, total nitrogen, protein, C/N ratio, ash and pH (AOAC

1984, Breene 1990). The analyses were carried out in triplicate.

Enzyme production

A. Influence of medium composition

Three culture media were evaluated: Manachini Solution (2 g L⁻¹ KH₂PO₄; 1 g L⁻¹ (NH₄)₂SO₄; 0,1 g L⁻¹ MgSO₄; 0,9 g L⁻¹ Na₂HPO₄·2H₂O; 1 g L⁻¹ yeast extract, dissolved in distilled water (Manachini et al. 1987)); Manachini Modified Solution (same mineral salts as Manachini Solution, in the same concentrations, but dissolved in immersion water of banana straw. The immersion water of banana straw was obtained by immersing 150 g of dry banana straw in 3 L of water, for approximately 12 hours) (Bonatti et al. 2004) and OXI medium (immersion water of banana straw and 5.4 mM ammonium tartrate (Libardi-Junior et al. 2011)). In these experiments (shake flasks culture) the OXI medium was added of 60 g L⁻¹ banana peel powder, named OXI60 culture medium. All culture media were supplemented with 10 g L⁻¹ glucose. Depending on the enzyme to be produced, laccase (Lac), manganese peroxidase (MnP) or lignin peroxidase (LiP), the following were added to the culture medium: CuSO₄ anhydride 150 μM, Mn²⁺ 0.05 mM or veratryl alcohol 4 mM, respectively. The initial pH of the culture media was adjusted to 6.0 using H₃PO₄ 12 M and NaOH 6 M solutions when necessary (Libardi-Junior et al. 2011, 2012).

The experiments were carried out in 500 mL Erlenmeyer flasks containing 100 mL of culture medium. The flasks were sterilized, inoculated with two 12 mm diameter agar discs containing mycelium and incubated at 30°C and 105 rpm (reciprocal agitation) for 12 days (Libardi-Junior et al. 2012). Samples were periodically collected, frozen and later analyzed for enzyme activities. All experiments were carried out in triplicate.

B. Influence of agitation rate

The agitation rates evaluated were 100, 350 and 500 rpm. The experiments were carried out in batch culture in a stirred tank reactor (B. Braun, Biostat® B model) of 4 L of working volume (0.4 L of inoculum and 3.6 L of culture medium) (Supplementary Material - Figure S5). The cultures were incubated at 100, 350 and 500 rpm, 30°C, and 0.37 L min⁻¹ air flow. The initial pH value was adjusted at 6,0 by the addition of NaOH 6N and H₃PO₄ 12N (Bonatti-Chaves et al. 2014). In these experiments the OXI medium was added of 10 g L⁻¹ glucose, 150 μM CuSO₄ anhydride and 45 g L⁻¹ instead of 60 g L⁻¹ banana peel powder (as described in item A). Therefore, for the experiments in the bioreactor, the medium was named OXI45 culture medium. The concentration of banana peel powder had to be altered due to insufficient homogenization promoted by the high concentration of banana peel powder (60 g L⁻¹) into the bioreactor. The experiments were carried out until total glucose consumption and samples were periodically collected, centrifuged (3000 rpm, 10 minutes) and the supernatant used for enzymatic determinations. All experiments were carried out in duplicate.

The inoculums were prepared in 2 L Duran flasks containing 0.4 L mL of OXI medium added of 10 g L⁻¹ glucose and 150 μM CuSO₄ anhydride. The Duran flasks were sterilized, inoculated with all the mycelium obtained from a Petri dish, after 7 days growth, and incubated at 30°C and 105 rpm (reciprocal agitation) for 6 days (Furlan et al. 2008).

Enzyme characterization

For determining optimum pH, the enzymatic reaction was conducted in McIlvain buffer with pH values ranging from 2.5 to 7.5. For determining optimum temperature, the enzymatic reaction

was conducted with temperatures ranging from 20 to 50 °C. In both experiments, the other conditions foreseen in the enzymatic activity test were maintained (Libardi-Junior et al. 2012). The crude enzyme broth was incubated at different time intervals (2 to 72 hours), and pH values 3.0, 5.0 and 7.0 for determining pH stability, as well as temperatures varying between 20 and 50 °C for determining temperature stability (Libardi-Junior et al. 2012). For determining the Michaelis-Mentes constant (K_m) and the maximum reaction rate ($V_{m\acute{a}x}$), ABTS (2,2'-azino-bis-[3-ethylthiazoline-6-sulfonate]) was used and the assays carried out in optimum temperature and pH. The Origin 8.0 PRO® software was used to calculate K_m and $V_{m\acute{a}x}$ by linearization of Michaelis-Menten equation by the Lineweaver-Burk method (Libardi-Junior et al. 2012). All experiments were carried out in triplicate.

Bisphenol A degradation

Mixtures containing 1.5 mL of crude enzyme broth (200 U L⁻¹ of laccase activity) and 0.5 mL of bisphenol A (500 mg L⁻¹ - 95% purity, Sigma-Aldrich) were incubated at room temperature and samples were taken at 24, 48 and 72 hours. All experiments were carried out in triplicate.

Analyses

Glucose concentration was evaluated using the enzymatic method of glucose oxidase-peroxidase (Glucose-E, CELM, Brasil). The laccase, manganese peroxidase and lignin peroxidase activity were measured as described by Buswell et al. (1996), Glenn & Gold (1985) and Tien & Kirk (1984), respectively.

Bisphenol A concentration was evaluated as follows: prior to the extraction process, a drop of concentrated acetic acid was added to the samples, which were then shaken. The bisphenol A was then extracted using ethyl ether. The supernatant was analyzed for the

bisphenol A concentration using Agilent 6890 gas chromatograph with FID detector. The HP-5 column (5% phenylmethylsiloxane), held at 290°C during injection and then programmed at 140°C min⁻¹ to 310°C, for 10 minutes, was used for quantitative analysis. The bisphenol A was identified by its typical retention times. The concentrations were calculated based on calibration curve established with external standard of bisphenol A.

Gallic acid, ferulic acid and vanillin were identified in the *P. sajor-caju* crude enzyme broth as follows: 1 mL of the sample was diluted in equal volume of ethanol:ultrapure water (1:4) solution and filtered by a membrane filter (0.20 µm) directly in a vial for chromatography analyses. The standard solution was obtained dissolving the standard compounds in equal solution (ethanol:ultrapure water, 1:4). The analyze was performed using high performance liquid chromatography-tandem mass spectrometry (LC-MS/MS method). The spectrometer was operated on the positive ionization mode using multiple-reaction monitoring (MRM). The chromatographic separation was carried out on an Agilent Zorbax SB-C18 (RRHT) column (2.10 x 50 mm, 1.8 µm) maintained at 40°C. The mobile phase consisted of formic acid 0.1% (A), acetonitrile (B) and MeOH (D). The mobile phases A and D were maintained constant at 5%. The gradient started with mobile phase B rising from 5% to 65% in 5 minutes and reaching 80% in 12 minutes. The concentration was then kept in isocratic mode for 8 minutes. Subsequently, the concentration of mobile phase decreased to 5% in 1 minute, and then the column was equilibrated at this condition for 4 minutes. An injection volume of 2 µL was used.

Statistical analysis

All data were submitted to the Dixon Q test (Rorabacher 1991). The results were expressed

as means and submitted to analysis of variance using the Tukey Test, significance level 5% (Origin 8.0 PRO®).

RESULTS AND DISCUSSION

Chemical characterization

A very important factor for enzymatic production by lignocellulolytic fungi is the chemical nature of the compounds used in the culture medium composition (Elisashvili et al. 2008, Tinoco-Valência et al. 2014, Carvalho et al. 2012). According to Table I, the immersion water of banana straw and the banana peel powder presented organic material and organic carbon contents equal to 70.4 and 39.1%, and 76.2 and 42.3%, respectively. The total carbohydrate content for the banana peel powder was 11%. The main carbohydrates in the immersion water were glucose (31.6%)

and galactose (31.5%). The nitrogen and protein contents in immersion water and in banana peel powder were 2.8 and 12.4%, and 2.5 and 11.8%, respectively. According to Carvalho et al. (2012), the excess of mineral or organic N in the culture media, in addition to exercising a negative effect on mycelial growth, may inhibit synthesis of the enzymes that degrade the lignin. In this work, the C/N ratio was 13.8 and 16.9 for immersion water and banana peel powder, respectively.

The crude fiber content found in the banana peel powder was 7%, and the hemicellulose, lignin and cellulose contents were 9.5, 7.2 and 6.6%, respectively (Table I). Crude fiber, lignin, hemicellulose and cellulose are defined as carbon sources as well as inductors for the production of lignocellulolytic enzymes by fungus (Elisashvili et al. 2008, Libardi-Junior et al. 2012, Quevedo-Hidalgo et al. 2015). The floriculture wastes were characterized by Quevedo-Hidalgo

Table I. Chemical composition of banana wastes (mean value \pm standard error).

	Immersion water of banana straw	Banana peel powder
Organic matter	70.4 \pm 1.55 %	76.2 \pm 0.89 %
Organic carbon	39.1 \pm 0.2 %	42.3 \pm 0.5 %
Glucose	31.6 \pm 0.0 %	-
Galactose	31.5 \pm 0.0 %	-
Xylose	25.1 \pm 0.0 %	-
Mannose	11.8 \pm 0.0 %	-
Total carbohydrate	-	11.1 \pm 0.43 %
Crude fiber	-	7.1 \pm 0.21 %
Hemicellulose	-	9.5 \pm 0.33 %
Lignin	-	7.2 \pm 0.05 %
Cellulose	-	6.6 \pm 0.04 %
Total nitrogen	2.8 \pm 0.13 %	2.5 \pm 0.27 %
Protein	12.4 \pm 0.84 %	11.8 \pm 0.0 %
C/N ratio	13.8 \pm 0.51	16.9 \pm 0.64
Ash	28.6 \pm 0.14 %	12.8 \pm 0.02 %
pH	6.05 \pm 0.35	5.18 \pm 0.03

et al. (2015) for enzyme production by *Pleurotus ostreatus* in submerged culture. The wastes presented cellulose content varying from 38.4 to 49.6%, hemicellulose from 7.5 to 8.8%, lignin from 9.5 to 17.5% and N from 1.94 to 2%. The substrate including chrysanthemum waste and the inducers (Cu^{+2} and Mn^{2+}) increased 4.8 times the laccase production. According to the authors, the lignin content in this kind of waste exercises a positive influence on the production of laccase by *Pleurotus ostreatus*.

The addition of lignin in the culture medium by Tinoco-Valência et al. (2014) for enzyme production by *Pleurotus ostreatus* also exercised a strong positive influence on the laccase production. Moreover, when Cu^{+2} was added simultaneously to the lignin to the culture medium, the laccase activity increases more than when both are added separately, showing the synergistic effect between the inducers and on enzyme production.

The ash content in the immersion water and banana peel powder (Table I) were 28.6 and 12.8% respectively. Ash content provides an indication of sample richness in mineral elements (Quevedo-Hidalgo et al. 2015). Some metals are considered enzymatic inducers, such as Cu^{+2} and Mn^{2+} . The pH values found in this study are close to neutrality (around 6.0). Studies showed a pH adjustment close to neutrality for the production of laccases by basidiomycete fungi (Bettin et al. 2011, Pandey et al. 2017).

Influence of medium composition – Assays in shake flasks

According to Figure 1, the highest laccase activity was observed when using the OXI60 culture medium, with the highest activity observed on the 12th day of cultivation ($2,416.5 \pm 35.5 \text{ U L}^{-1}$). For the assays using Mananchini Solution and Manachini Modified Solution it was observed laccase activity equal to each other and

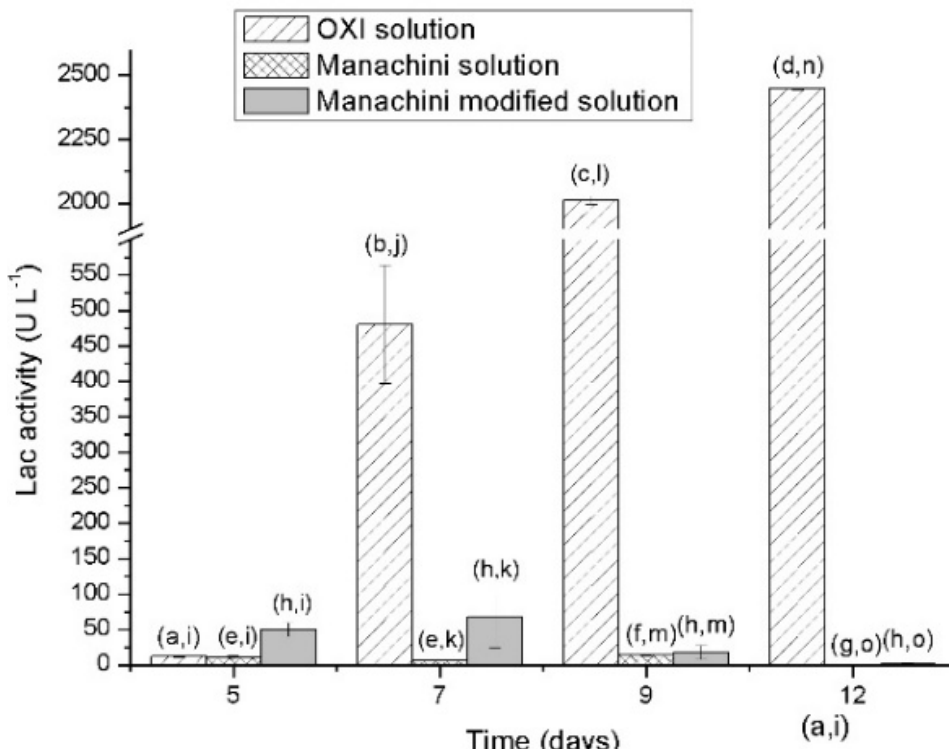


Figure 1. Laccase activity (Lac) mean values obtained in *P. sajor-caju* cultivation using OXI60, Manachini Solution and Manachini Modified Solution culture media. Indication of the existence or not of significant difference among the cultivation times for the OXI60 culture medium (a, b, c, d), for the Manachini Solution culture medium (e, f, g), for the Manachini Modified Solution culture medium (h); among the cultivation media for the time of 5 days (i), 7 days (j, k), 9 days (l, m), 12 days (n, o).

significantly lower than those obtained using the OXI60 culture medium.

According to Figure 2, the greatest manganese peroxidase activity was also observed when using the OXI60 culture medium, with highest activity observed on the 12th day of cultivation ($7.62 \pm 0.36 \text{ U L}^{-1}$). The manganese peroxidase productivity ($0.64 \text{ U L}^{-1} \text{ day}^{-1}$) was lower than that found in literature. However, the laccase productivity ($201.4 \text{ U L}^{-1} \text{ day}^{-1}$) was higher than that found in literature, suggesting the OXI60 culture medium to be efficient on the laccase production.

Libardi-Junior et al. (2012) obtained laccase activity by *Pleurotus ostreatus* equal to $1,575 \pm 93.02 \text{ U L}^{-1}$ on the 9th day (productivity equal to $175 \text{ U L}^{-1} \text{ day}^{-1}$) using culture medium composed of water, 40 g L^{-1} banana peel powder and CuSO_4 $150 \mu\text{M}$. Elisashvili et al. (2008), using mandarin, apple and banana peels in the culture medium composition, obtained manganese peroxidase activity of 58 U L^{-1} on the 8th day (productivity

$7.25 \text{ U L}^{-1} \text{ day}^{-1}$). Quevedo-Hidalgo et al. (2015) evaluated the effect of floriculture wastes in enzyme synthesis by basidiomycetes obtaining as a result 429 and 32 U L^{-1} , on the 7th and 10th days, for laccase (productivity $61.3 \text{ U L}^{-1} \text{ day}^{-1}$) and manganese peroxidase (productivity $3.2 \text{ U L}^{-1} \text{ day}^{-1}$), respectively.

The *Pleurotus sajor-caju* cultivation, regardless of the culture medium, showed no activity for the lignin peroxidase enzyme.

Influence of agitation rate – Assays in bioreactor

According to Figure 3, the highest laccase activity ($1,850.7 \pm 674.8 \text{ U L}^{-1}$) was observed at the assay using agitation rate of 350 rpm, which is statistically different from the other assays (Tukey test $p < 0.05$) (Table II). The maximum laccase activities for cultivation at 100 and 500 rpm were 9.25 ± 2.18 and $207.2 \pm 18.6 \text{ U L}^{-1}$, respectively. The maximum laccase activities for cultivation at 100 and 500 rpm were 9.25 ± 2.18 and 207.2 ± 18.6

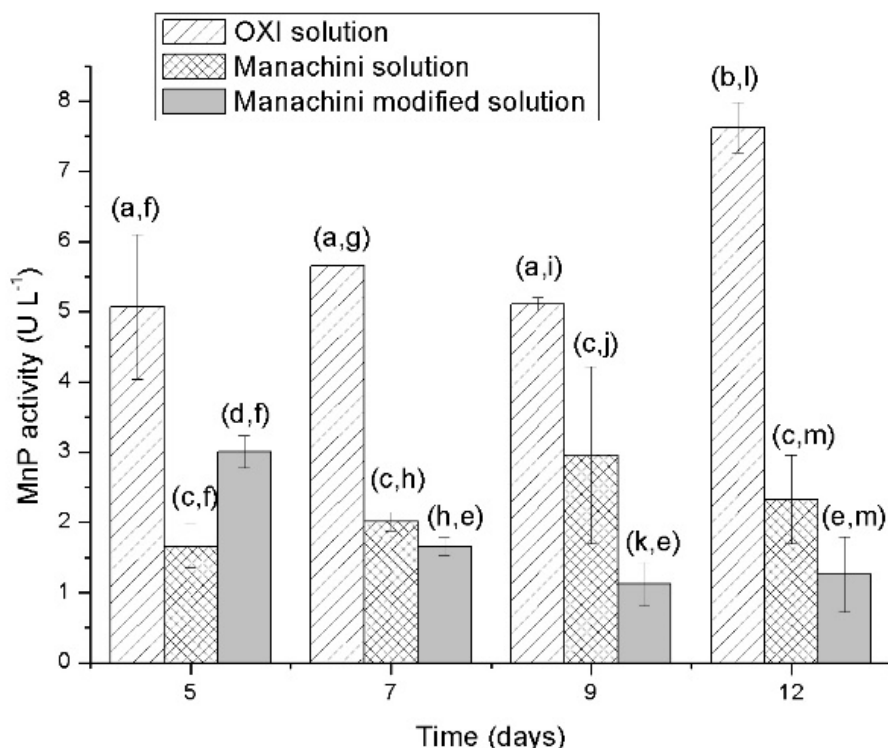


Figure 2. Manganese peroxidase (MnP) mean values obtained in *P. sajor-caju* cultivation using OXI60, Manachini Solution and Manachini Modified Solution culture media. Indication of the existence or not of significant difference among the cultivation times for the OXI60 culture medium (a, b), for the Manachini Solution culture medium (c), for the Manachini Modified Solution culture medium (d, e); among the cultivation media for the time of 5 days (f), 7 days (g, h), 9 days (i, j, k), 12 days (l, m).

U L⁻¹, respectively. By decreasing agitation rate it was hoped that the effect of shear stress on the mycelium would be reduced and, through

this, maybe an increase in the laccase activity by *Pleurotus sajor-caju* would be observed. According to described by Fenice et al. (2003)

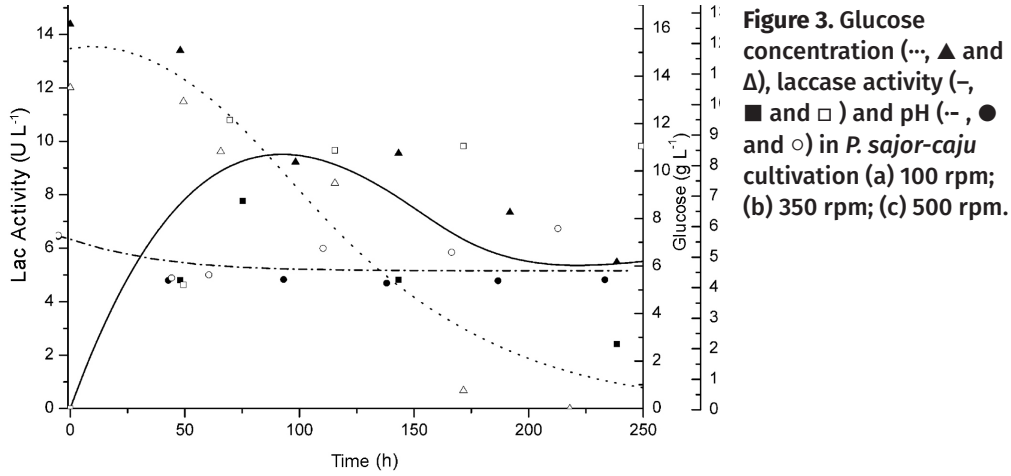
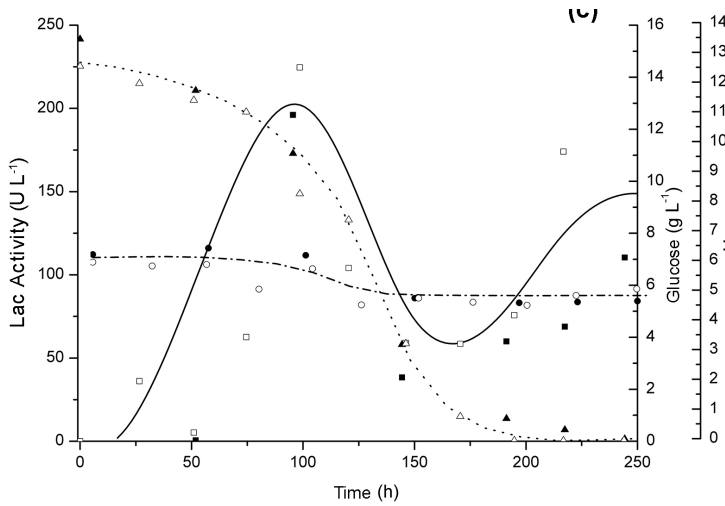
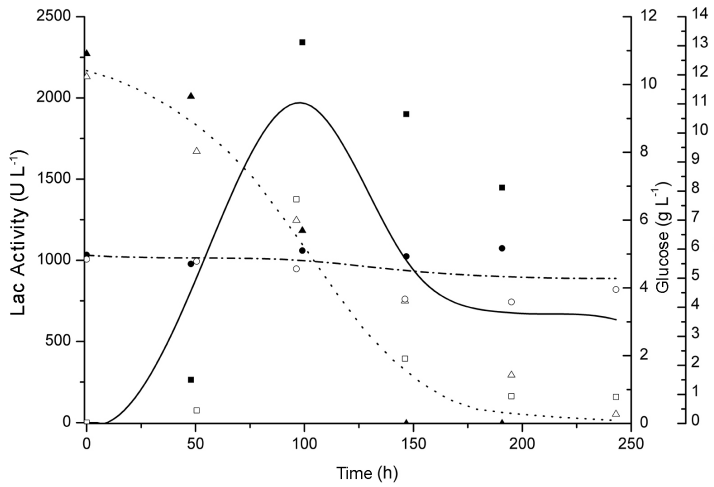


Figure 3. Glucose concentration (---, ▲ and Δ), laccase activity (—, ■ and □) and pH (---, ● and ○) in *P. sajor-caju* cultivation (a) 100 rpm; (b) 350 rpm; (c) 500 rpm.



and Tinoco-Valência et al. (2014), higher agitation rates normally have a negative influence on the production of enzymes by fungus. However, Fernández-Alejandre et al. (2016) observed the opposite effect, that is, the increase in agitation rate encouraged the formation of smaller pellets and higher laccase activity values. The authors justify these results as being due to the non-existence of possible diffusion restriction in the pellets, and consequently, no type of nutritional restriction. In this study, when the agitation rate was increased to 500 rpm, the laccase activity was approximately 22 times greater than in the assay using 100 rpm.

The laccase productivity and the laccase conversion factor were also the highest in the assay using 350 rpm (Table II). The laccase productivity was equal to $18.21 \text{ U L}^{-1} \text{ h}^{-1}$ in the assay using 350 rpm, more specifically, this value is 130 and 9 times greater than in the assays using 100 and 500 rpm, respectively. The productivity value obtained in this study, was superior to productivity obtained by Tinoco-Valência et al. (2014) ($14 \text{ U L}^{-1} \text{ h}^{-1}$) and Aggelis et al. (2003) ($0.38 \text{ U L}^{-1} \text{ h}^{-1}$) using *P. ostreatus* species. Productivity values superior to those obtained in this work were also found (considering only

assays in bioreactor), however none with the *Pleurotus sajor-caju* species. Hess et al. (2002) obtained laccase production by *T. multicolor*, in bioreactor assays, equal to $29 \text{ U L}^{-1} \text{ h}^{-1}$. Liu et al. (2016) obtained laccase production by *Pycnoporus* sp. equal to $0.35 \text{ U mL}^{-1} \text{ h}^{-1}$ ($350 \text{ U L}^{-1} \text{ h}^{-1}$).

Enzyme characterization

Effect of pH and temperature on laccases activity

It can be seen in Figure 4a that the optimum pH for the reaction was 3.0, being pH values above 5.0 not favorable to the reaction (around 50% loss of laccase activity). According to Figure 4b, is observed that laccase activity is stable for all temperatures evaluated.

The optimum pH and temperature values observed in this study can be compared with optimum pH and temperature values found by various authors (Table III), using the same substrate (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) - ABTS) for different *Pleurotus* laccase isoforms. Bettin et al. (2011) observed three optimum pH peaks (2.4, 3.2 and 4.4), the authors justified the three activity peaks as

Table II. Kinetic parameters of the assays using agitations rate 100, 350 and 500 rpm (mean value \pm standard error).

	Agitation rate		
	100 rpm	350 rpm	500 rpm
Maximum laccase activity (U L^{-1})	9.25 ± 2.18^a	$1,850.73 \pm 674.82^b$	207.20 ± 18.61^c
Laccase conversion factor ($\text{U L}^{-1} \text{ g}^{-1}$)	2.40 ± 0.32^a	359.60 ± 13.80^b	38.26 ± 3.02^c
Laccase productivity ($\text{U L}^{-1} \text{ h}^{-1}$)	0.14 ± 0.07^a	18.21 ± 4.06^b	2.06 ± 0.13^c

Different letters in the same line mean that exists a statistical difference between the values according to Tukey test ($p < 0.05$).

being due to the different laccase isoforms produced by fungus. In general, laccases are more stable at acidic pH, with the optimum pH for ABTS substrate oxidation being less than 4.0 (Baldrian 2006). According to Galhaup et al. (2002), the decrease in the reaction rate with increasing pH is characteristic of reactions with non-phenolic substrates, such as ABTS, in which the oxidation of the substrate occurs in a single phase, resulting directly in the ABTS radical cation (Johannes & Majcherczyk 2000).

Stability with the pH and temperature

Greater laccase stability is observed in pH 5 and 7 (Figure 5a). At pH 7 the enzymatic activity remains stable throughout the incubation time and at pH 5 the activity decreases by only 23.7% in 72 hours. At pH 3.0, (optimum pH, as shown in Figure 4a) the enzyme loses 100% of its activity in 8 hours of incubation. Behavior similar was observed by Kumar et al. (2012), the pH 4.0 was observed as the optimum pH and the pH 6.0 was the most favorable with activity around 97%, up to 50 hours of incubation. Libardi-Junior et al. (2012) found greater stability, for laccases from *Pleurotus ostreatus*, in pH 6.0, and pH 4.0 as

the optimum pH. Extreme pH values negatively influenced enzyme stability, with the pH 3.0 and 8.0, in 4 hours of incubation, showing less than 5% activity.

According to Figure 5b, the laccase activity is stable for the range of temperatures 20 to 40 °C (around 100% activity). A loss of 40% activity is observed after 8 hours at 50 °C. Kumar et al. (2012) observed the highest stability (100%) at 25 °C (18 h of incubation). At the temperature of 37 and 45 °C, the decay of 50% of the activity occurs in 14 and 8 hours, respectively. Higher temperatures, such as 50 and 60 °C, the decay of 100% of the activity occurs in 4 h and 2 hours, respectively. Zucca et al. (2011) observed as the optimum reaction temperature, 30 °C (8 hours of incubation, 97% of its initial activity). At temperatures of 40 and 50 °C, after 8 hours, 60% of the initial activity remained present, while only 40% remained active at 60 °C. At 70 °C, after 1 hour, the enzyme was completely inactivated. Libardi-Junior et al. (2012) observed, for laccase from *Pleurotus ostreatus*, that at room temperature, in 72 hours, activity less than 20% and at 50 °C the enzyme had been almost completely deactivated. Bettin et al.

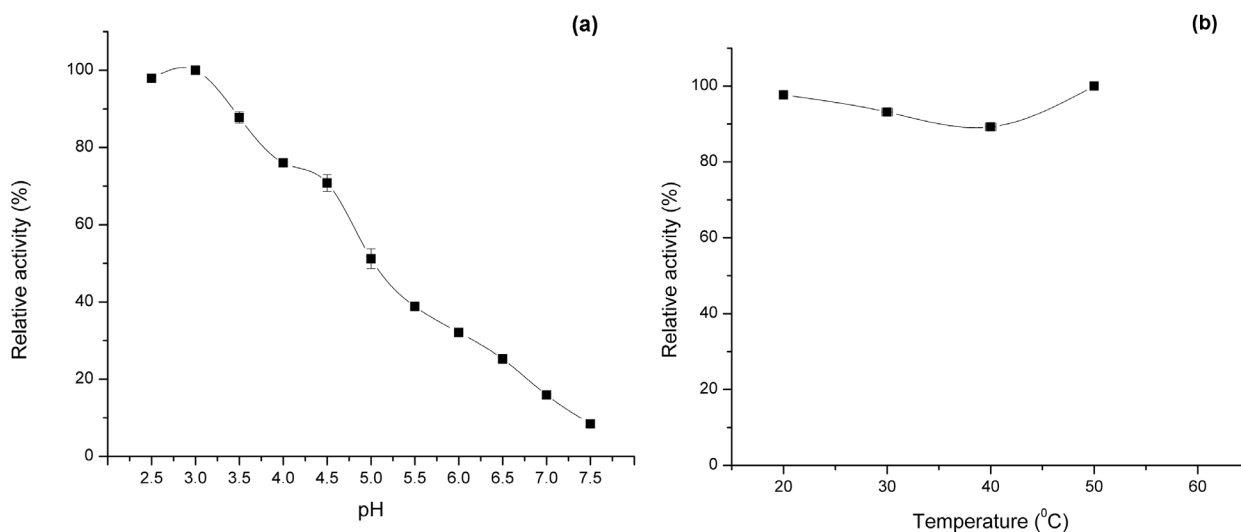


Figure 4. Stability of the *P. sajor-caju* laccase at (a) different pH values and (b) different temperatures.

Table III. Optimum pH and temperature values of the laccase isoforms originating from different *Pleurotus* species.

Fungus	Optimum pH	Optimum temperature (°C)	Reference
<i>Pleurotus ostreatus</i>	4.5	-	Libardi-Junior et al. (2012)
	3.0	50-55	Pakhadnia et al. (2009)
	3.5	30	Rashad et al. (2010)
<i>Pleurotus sajor-caju</i>	3.2	50	Bettin et al. (2011)
	4.0	37	Sahay et al. (2008)
	3.0	50	Results obtained in this study

(2011) observed, for *Pleurotus sajor-caju* laccase, greater stability at temperatures of 20 and 30°C. At 40°C a drop of 50% of activity was observed in 48 hours and, at 50 and 60°C the enzyme was inactivated in less than 5 hours.

Determination of Michaelis-Menten constant and maximum reaction rate

The laccase kinetic K_m and V_{max} parameters for different *Pleurotus* species are presented in Table IV. It is possible to observe values of K_m varying from 0.014 to 0.607 mM and V_{max} values varying from 0.0003 to 1.25 mM min⁻¹. These variations can be explained by the differences in culture media compositions used by the different authors, thus different isoenzymes are produced by each species, as well as laccase activity inhibiting compounds. It also can be explained by the type of substrate used for the enzymatic quantification (ABTS, DPM, syringaldazine, guayacol, etc.). In this study, the K_m and V_{max} values show the affinity of the enzyme for the substrate (ABTS), as well as high catalytic efficiency.

Bisphenol A degradation

A reduction of 40 and 100% in bisphenol A (500 mg L⁻¹) concentration was observed after 24

and 48 hours, respectively, of incubation with *Pleurotus sajor-caju* crude enzyme broth (200 U L⁻¹). The term effectiveness (defined as the amount of bisphenol A removed/(initial laccase activity x time to removal)) was used to discuss the results. Table V presents the values of effectiveness obtained in this study and those obtained by the other authors (0.000015 to 0.052 mg U⁻¹ h⁻¹).

The highest value was obtained in this study (Table V). A possible suggestion for this result can be the presence of the natural small-molecular weight compounds in the *Pleurotus sajor-caju* crude enzyme broth that can act as redox mediators and improve the performance of laccases on the bisphenol A removal. In this study vanillin (3-methoxy-4-hydroxybenzaldehyde) and ferulic acid (3-(4-hydroxy-3-methoxyphenyl) prop-2E-enoic acid) were identified in the *Pleurotus sajor-caju* crude enzyme broth (Figures S1, S2, S3 and S4). According to Piscitelli et al. (2011), phenolic and aromatic compounds with a similar structure to that of lignin or lignin derivatives are usually added to the culture media in order to increase laccase production. Parenti et al. (2013) also found vanillin and ferulic acid, as well as gallic acid (3, 4, 5-trihydroxybenzoic acid), in the

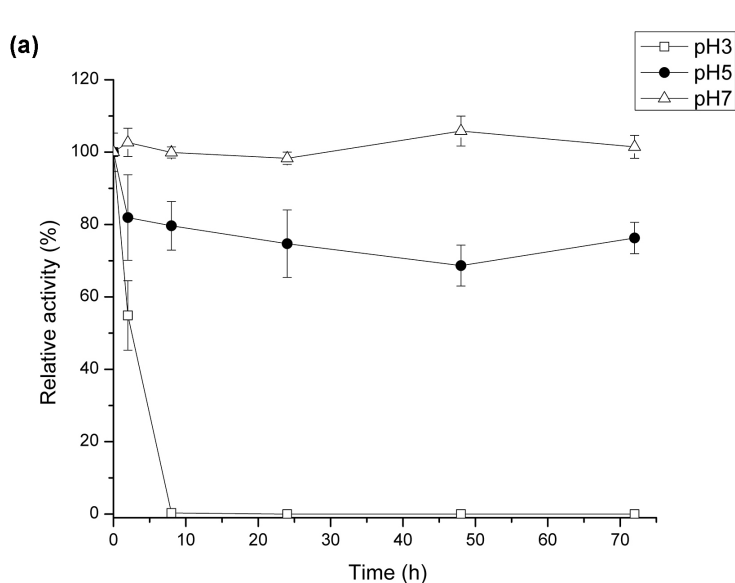
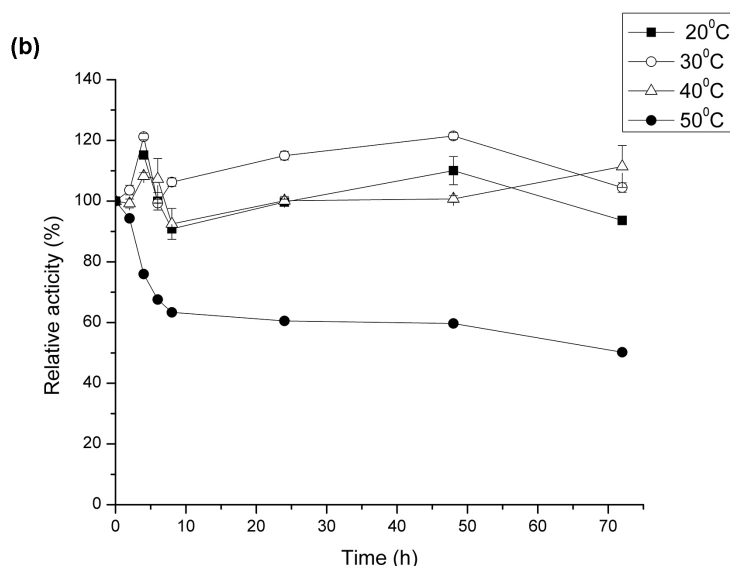


Figure 5. Stability of the *P. sajor-caju* laccase during incubation in (a) pH 3.0, 5.0 and 7.0; and (b) 20, 30, 40 and 50°C.



immersion water of wheat straw used for the laccases production by *Pleurotus ostreatus*. The absence and/or low concentration of the mediators in the extracts obtained from submerged and solid-state culture of *Pleurotus ostreatus*, *Pleurotus pulmonarius* and *Pleurotus eryngii*, as well as in the purified enzyme, can justify the lower effectiveness obtained by some authors cited in Table V.

Erkurt (2015), in assays related to bisphenol A degradation by fungal laccases, observed greater removal percentage (100% in 90 minutes)

as well as higher enzyme stability (60%) using *Funalia trogii* crude enzyme broth and using the inactivated crude broth added from the commercial enzyme purified from *Trametes versicolor*, than when using only the commercial purified enzyme in the assays. The author suggests that the enhanced laccase stability was probably due to an interaction between the polymer products present in the crude enzyme broth which gave protection to the laccase enzyme. De Freitas et al. (2017) observed higher bisphenol A removal using nondialysed

Table IV. Kinetic parameters for laccase enzymes from different *Pleurotus* species.

Species	K_m (mM)	V_{max} (mM min ⁻¹)	Reference
Pleurotus ostreatus	0.014	-	Pakhadnia et al. (2009)
	0.216	-	Libardi-Junior et al. (2012)
<i>Pleurotus</i> sp.	0.25	0.00033	More et al. (2011)
Pleurotus sajor-caju	0.035	-	Sahay et al. (2008)
	0.607	0.018	Bettin et al. (2011)
	0.1	1.25	Results obtained in this study

Table V. Effectiveness values of different studies on BPA removal by *Pleurotus* laccases.

	Effectiveness (mg U ⁻¹ h ⁻¹)	Reference
<i>P. ostreatus</i> crude enzyme broth, concentrated 10 times.	0.005	Libardi-Junior et al. (2012)
<i>P. ostreatus</i> laccase purified.	0.010	Macellaro et al. (2014)
<i>P. eryngii</i> crude enzymatic extracts obtained from submerged culture, solid-state culture and spent mushroom compost.	0.000015	Chang & Chang (2016)
<i>P. ostreatus</i> and <i>P. pulmonarius</i> crude enzymatic extracts obtained from the solid-state culture.	0.008 - 0.032	de Freitas et al. (2017)
<i>P. sajor-caju</i> crude enzyme broth, without downstream processing.	0.052	Results obtained in this study

Pleurotus ostreatus and *Pleurotus pulmonarius* crude laccase extracts than when using dialyzed extracts, showing the positive influence of the natural mediators present in the extracts on the enzyme reaction.

Another possible explanation for the high effectiveness obtained in this study can be due to the polarity of the culture medium (in this study OXI45 culture medium was used, described in item A). Solvents with high polarity tend to increase the solubility of the polar substrates in the culture medium, providing faster and more selective reactions (Baldrian 2006). Additionally,

the water linked to the protein would increase the mobility and flexibility of the active sites of the enzyme and the polarization of the protein structure, enhancing the enzymatic activity (Asadgol et al. 2014). The results presented in this work suggest the potential use of *Pleurotus sajor-caju* crude enzyme broth, without necessity of downstream processing, for obtaining an enzymatic formulation for application in the environmental area in the future.

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SUPPLEMENTARY MATERIAL

Figure S1. Chromatograms by LC-MS/MS method; (a) sample and (b) standard solution reagents; peak 1 = gallic acid, peak 2 = vanillin and peak 3 = ferulic acid.

Figure S2. Chromatograms by LC-MS/MS method; (a) sample and (b) standards reagents; peak area 1 = gallic acid.

Figure S3. Chromatograms by LC-MS/MS method; (a) sample and (b) standards reagents; peak area 2 = vanillin.

Figure S4. Chromatograms by LC-MS/MS method; (a) sample and (b) standards reagents; peak area 3 = ferulic acid.

Figure S5. Schematic representation of bioreactor.

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