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STUDY OF THE REMOVAL OF BISPHENOL-A BY *Pleurotus sajor-caju* CRUDE ENZYME BROTH

ESTUDIO DE LA REMOCIÓN DE BISFENOL-A POR EL CALDO ENZIMÁTICO CRUDO DE *Pleurotus sajor-caju*

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Abstract

Bisphenol A (BPA) is classified as an endocrine disruptor having adverse effects on health, when in contact with humans and animals. Inadequate disposal of effluents, as well as polycarbonate polymers and epoxy resins in the environment and their use in cooking utensils, increases the risk of human exposure to BPA. An alternative method for removal of BPA is the use of oxidative enzymes produced by lignocellulolytic fungi. Thus, the objectives of this work were to evaluate the capacity of *Pleurotus sajor-caju* crude enzyme broth to degrade BPA and to determine the best reaction conditions (BPA concentration, time reaction, laccase activity, pH and temperature). The experiments to obtain the crude enzyme broth were conducted in bioreactor and BPA quantification was performed using gas chromatography coupled to mass spectrometry (GC-MS). The crude enzyme broth was able to remove 100% BPA (30 ppm) in 10 hours and pH 5.0. The temperature (20, 30 and 40°C) had no influence on the reaction but greater enzymatic stability was observed at 20°C. The *Pleurotus sajor-caju* crude enzyme broth, without any enzyme isolation procedure, has shown to be very promising for use in the environmental field in the future.

Keywords: basidiomycetes, oxidative enzymes, endocrine disruptor, biodegradation, environment.

Resumen

Bisfenol A (BPA) se clasifica como un disruptor endocrino que tiene efectos adversos sobre la salud humana y animal. La eliminación inadecuada de efluentes, así como polímeros de policarbonato y resinas epoxi en el medio ambiente y su uso en utensilios de cocina, aumenta el riesgo de exposición humana al BPA. Un método alternativo para la eliminación de BPA son las enzimas oxidativas producidas por hongos lignocelulolíticos. Así, los objetivos de este trabajo fueron evaluar la capacidad del caldo enzimático crudo de *Pleurotus sajor-caju* para degradar BPA y determinar las mejores condiciones de reacción (concentración de BPA, tiempo de reacción, actividad lacasa, pH y temperatura). Los experimentos para obtener el caldo enzimático se llevaron a cabo en biorreactor y la cuantificación de BPA se realizó usando la técnica de cromatografía de gases acoplada a espectrometría de masas (GC-MS). El caldo enzimático crudo removió 100% del BPA (30ppm) en 10 horas y pH 5,0. La temperatura (20, 30 y 40ºC) no presentó influencia sobre la reacción, pero sí se observó una mayor estabilidad de la enzima a 20ºC. El caldo enzimático crudo de *Pleurotus sajor-caju*, sin ningún proceso de aislamiento, demostró ser prometedor para el uso futuro en el campo ambiental.

Palabras clave: basidiomicetos, enzimas oxidativas, disruptor endocrino, biodegradación, medio ambiente.

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1 Introduction

Bisphenol A (BPA), 2,2-bis(4-hydroxyphenyl)propane (Fig. 1), is classified as an endocrine disrupting compound or an endocrine disruptor (ED). According to the European Union (EU), EDs are exogenous substances that cause endocrine disruption in an organism, affecting their health, growth and reproduction. They can affect the natural action of hormones in humans when they bind to the hormone receptor, generating a false response, or by preventing the binding of the natural hormone to the receptor in the target cell (EFSA, 2010; FAO/WHO, 2010; Fenichel *et al*., 2013; Michalowicz, 2014; Kelley *et al*., 2015; Sifakis *et al*., 2017).

BPA is present in the effluent of several industries, especially in those producing polycarbonate and epoxy resins, and is barely degraded in wastewater treatment plants (Freitas *et al*., 2017). When manufactured products based on polycarbonates or epoxy resin are incorrectly disposed in the environment they can cause contamination due to releasing BPA. Exposure to this type of pollutant can also occur directly when products based on polycarbonates and epoxy resins are used, for example, in food packaging and household utensils (Huang *et al*., 2012; Michalowicz, 2014). Epoxy resins are widely used as varnishes for coating metal products, such as in food cans, bottle caps and water supply pipes. Polycarbonate plastics are used in food and beverage packaging, among others, and can also be found in end products such as adhesives, CDs, thermal paper additives, vehicle coatings, baby bottles, can lids, glass protectors, automotive lenses, electronic component coating, cosmetics, building materials etc. (Sharma, 2009; Bhatnagar and Anastopoulos, 2017).

BPA is considered a micropollutant, i.e., in the environment it is found in small concentrations, to the order of μg L^{-1} and ng L^{-1}, and has increased significantly in recent years (Michalowicz, 2014; Macellaro *et al*., 2014; Wang *et al*., 2016).

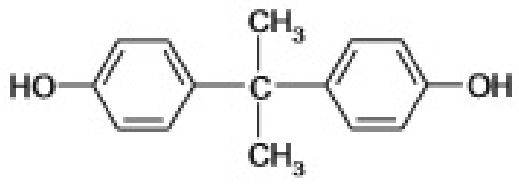

Fig. 1. BPA chemical structure (Michalowicz, 2014).

In natural water it has been found in concentrations between 0.1 and 1 μg L^{-1} (Huang *et al*., 2012). However, it has already been found near industrial zones in concentrations above 1 μg L^{-1} in water samples (Sharma, 2009). Common waste treatments are not able to promote the removal of this class of micropollutants. Thus, other forms of removal/degradation of this substance have been studied, such as membrane separation processes, electrochemical degradation and photo-fenton process (Yu-hong *et al*., 2009; Jiang *et al*., 2014; Pedro-Cedillo *et al*., 2015; Bhatnagar and Anastopoulos, 2017). Another method that has been attracting the attention of researchers is the use of different microorganisms to degrade several types of environmental pollutants (Maciel *et al*., 2010; Majeau *et al*., 2010; Martínez-Trujillo and García-Rivero, 2012). More specifically, fungi producers of oxidative enzymes can be used to degrade BPA. This is considered a very promising and ecologically sustainable alternative method for the BPA removal/degradation process (Furlan *et al*., 2008; Libardi Jr *et al*., 2012; Asadgol *et al*., 2014; Macellaro *et al*., 2014; Arca-Ramos *et al*., 2015; Erkurt, 2015; Chang and Chang, 2016; Daâssi *et al*., 2016; Freitas *et al*., 2017; Brugnari *et al*., 2018).

The *Pleurotus* genus, belonging to the class of basidiomycetes, is recognized for its capacity to produce oxidative enzymes, lignocellulolytics, enabling the fungus, or even the enzymes, to be used in several bioremediation processes for the degradation of several recalcitrant compounds. These fungi adapt and grow in the most diverse types of lignocellulosic wastes, agricultural and agroindustrial byproducts, and can be grown successfully on different substrates, both in solid medium and liquid medium (Bonatti *et al*., 2004; Furlan *et al*., 2008; Álvarez-Cervantes *et al*., 2016; Ramírez-Carmona and Munoz-Blandõn, 2016; García-Reyes *et al*., 2017).

The ligninocellulosic wastes available in the enzyme production region can be used as substrates in culture media formulations for the production of enzymes by fungi, thus reducing production costs and heling in solving environmental problems arising from their accumulation in nature (Bolio-López *et al*., 2011; Bonatti *et al*., 2004; Elisashvili *et al*., 2009; Libardi Jr *et al*., 2012; Ramírez-Carmona and Munoz-Blandõn, 2016). The northeast region of the State of Santa Catarina produces large amounts of residues during banana cultivation and processing, generating large amounts of dried banana leaves and banana peels.

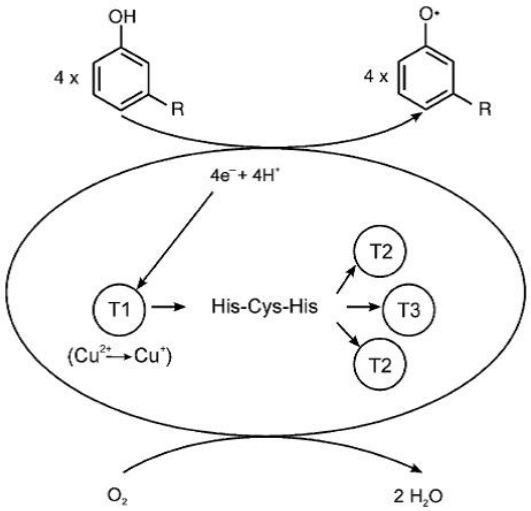

Fig. 2. Catalytic cycle of laccase (Baldrian, 2006).

These residues are defined as carbon sources in the culture medium, but, at the same time, also as inductors for the production of lignocellulolytic enzymes by fungi (Libardi Jr *et al*., 2012; Téllez-Téllez *et al*., 2012; Bertrand *et al*., 2013).

Among the enzymes produced, laccase stands out as it is usually produced in higher concentration (Téllez-Téllez *et al*., 2012; Álvarez-Cervantes *et al*., 2016; Daâssi *et al*., 2016). Named chemically as p-diphenol (dioxygen oxidoreductase, EC 1.10.3.2), it is an oxidative enzyme of potential industrial interest. It is capable of oxidizing phenols and aromatic amines by reducing the molecular oxygen to water through a multi-copper system, resulting in the oxidation of the hydrogen of the substrate and, consequently, in the degradation of the compound. The laccase catalyzes oxidation by extracting an electron from the phenolic substrate, for example, by generating a radical phenoxyl (Mayer and Staples, 2002; Maciel *et al*., 2010; Bertrand *et al*., 2013) (Fig. 2).

Therefore, the objectives of this work were to evaluate the capacity of *Pleurotus sajor-caju* crude enzyme broth to degrade the BPA endocrine disrupting compound and to determine the best reaction conditions, in terms of BPA concentration, reaction time, laccase activity, pH and temperature, with the aim of obtaining an enzymatic formulation for application in the environmental area in the future.

2 Materials and methods

2.1 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 medium (20 g dextrose, 15 g agar dissolved in 1 liter of wheat infusion) (Furlan *et al*., 1997) and stored under refrigeration (4ºC).

2.2 Production of Pleurotus sajor-caju crude enzyme broth

The culture media used was the OXI45 medium: immersion water of banana straw added of 45 g L^{-1} banana peel powder, 5.4 mM ammonium tartrate, 10 g L^{-1} glucose and 150 μM $CuSO_4$. The assays were conducted in batch culture in a B. Braun bioreactor (Biostat® B model) containing 4 L of culture media. The cultures were incubated at 350 rpm, 30ºC, 0.37 L min^{-1} air flow and the initial pH was adjusted to 6.0 using H_3PO_4 12 M and NaOH 6 M solutions when necessary, according previous results obtained by the research group and not yet published. The experiments were carried out in duplicate until maximum laccase activity. The samples were centrifugated at 3000 rpm for 10 minutes to separate the biomass, being the supernatant used for enzymatic determinations.

The inoculum was prepared in 2 L Duran flasks containing 400 mL of OXI45 culture medium, without addition of banana peel powder and $CuSO_4$. The flasks were sterilized, inoculated with all the mycelium obtained from a Petri dish, after 7 days growth, and incubated at 30ºC and 105 min^{-1} (reciprocal shaker) for 6 days.

2.3 BPA degradation assays and definition of the best reaction conditions

2.3.1 Determination of BPA concentration and reaction time

The assays were carried out in test tubes, the total reaction volume being 3 mL, prepared as follows: 1 mL BPA solution (95% purity; Sigma-Aldrichin, concentrations of 30, 15 and 3 ppm), 1 mL citrate/phosphate buffer solution (0.05 M) pH 5.0 and 1 mL of the crude enzyme broth (enzyme activity was measured at the beginning of the experiment, shortly after thawing). The mixtures were incubated at room

temperature and samples were withdrawn at 0, 2, 4, 6, 10, 21 and 24 hours. At these times, in addition to the evaluation of the BPA concentration, the laccase activity was also determined in order to correlate these two parameters. All experiments were performed in triplicate.

2.3.2 Determination of the influence of laccase activity on the BPA degradation

The experiments were carried out in test tubes, as described in section 2.3.1, using the BPA concentration defined in item 2.3.1 and the enzyme broth diluted 1:1 (crude enzyme broth:deionized water). The mixtures were incubated at room temperature for the maximum time defined in item 2.3.1. All experiments were performed in triplicate.

2.3.3 Determination of pH and reaction temperature

The experiments were carried out in test tubes, as described in item 2.3.1, using the BPA concentration defined in item 2.3.1 and the crude enzyme broth (diluted or not) defined in item 2.3.2. To determine the pH value of the reaction (1), McIlvain buffer solution pHs 3.0 and 7.0 were used and the mixtures were incubated at room temperature. To determine the reaction temperature (2), 1 mL of buffer solution defined in the experiment (1) was used, and the mixtures were incubated at temperatures of 20, 30, 40°C. The assay mixtures (1) and (2) were incubated for the time defined in item 2.3.1. All experiments were performed in triplicate.

2.4 Analytical methods

2.4.1 Laccase activity

Laccase activity was evaluated measuring the difference in absorbance (420 nm) produced by the oxidation of 2.2´-azino-bis-[3-ethyltiazoline-6-sulfonate] (ABTS) in sodium citrate buffer 50 mM (pH 5.0) incubated with 100 μL of the crude enzyme broth, at 30ºC (Buswell *et al*., 1995). A unit of enzyme activity was defined as the amount of enzyme that oxidizes 1 μmol of ABTS per minute.

2.4.2 Bisphenol-A concentration

Prior to the extraction process, a drop of concentrated acetic acid was added to the samples (2 mL), which were then shaken. The bisphenol-A was then extracted using ethyl ether (2 mL). The supernatant was analyzed for the bisphenol-A concentration using an Agilent 7869 gas chromatograph with mass detector (5975C) acoupled. An HP-5 column (5% phenylmethylsiloxane), held at 290 ºC during injection and then programmed at 140ºC.min^{-1} to 310ºC, for 10 minutes, was used for quantitative analysis. The bisphenol-A was identified by its typical retention times. Compound concentrations were calculated based on calibration curve (0.5 to 200 ppm) established with external standard of bisphenol-A.

2.5 Statistical analysis

All the replicates were treated by the statistic test for rejection of deviant values (Dixon‘s Q test) with 95% confidence level (Rorabacher, 1991). Mean values were submitted to the variance analysis (ANOVA and Tukey Test) with 95% confidence level to evaluate significant differences between the results using Origin 8.0 PRO® software.

3 Results and discussion

The crude enzyme broth was evaluated to degrade the BPA in three different concentrations (3, 15 and 30 ppm) (Fig. 3). BPA is classified as a micro pollutant, i.e. is found in low concentrations in the environment (Macellaro *et al*., 2014; Wang *et al*., 2016). A control assay was carried out, using the inactive *Pleurotus sajor-caju* crude enzyme broth by heating it at 100ºC for 15 minutes (measured laccase activity equal zero). The BPA removal percentages were constant and equal to zero for all sampling times.

According to fig. 3, it can be observed 100% BPA removal at the concentration of 3 ppm in 2 hours of assay. As in 4 and 10 hours, 100% BPA removal is observed at concentrations of 15 and 30 ppm, respectively. The term effectiveness (defined as the amount of BPA degraded/(initial laccase activity x time to degradation)) was used to discuss the results. Therefore, the effectiveness for the assays using 30, 15 and 3 ppm of BPA concentration were equal to 0.032, 0.040 and 0.016 mg U^{-1} h^{-1}, respectively, considering initial laccase activity equal to 93.33 U L^{-1}. The laccase activity obtained from the submerged cultivation of *Pleurotus sajor-caju* was equal to 370 U L^{-1}.

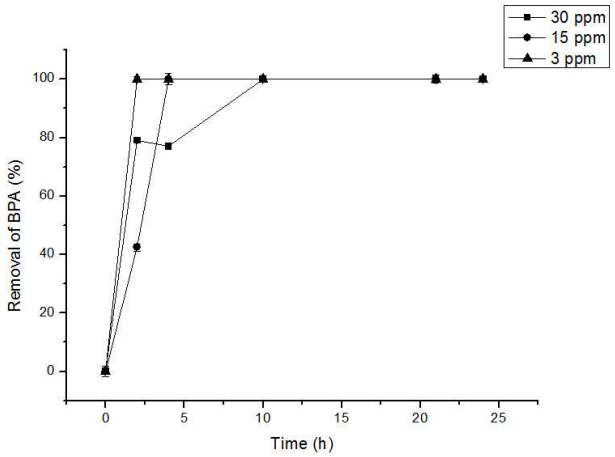

Fig. 3. BPA removal percentage (30, 15 and 3 ppm) promoted by the crude enzymatic broth of *Pleurotus sajor-caju*.

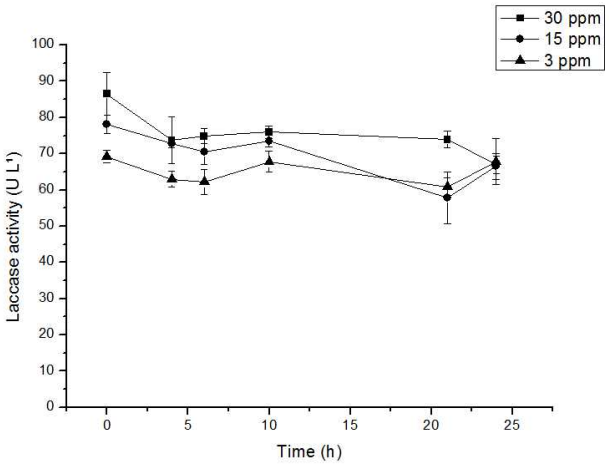

Fig. 4. Laccase activity during the assays of removal BPA by crude enzyme broth.

However, after the thawing process of the enzyme broth the laccase activity decreased from 370 to 280 U L^{-1}. Additionally, a dilution factor equal to 3 must be considered, due to the preparation of the mixtures into test tubs for carrying out the assays (item 2.3.1). Libardi Jr. *et al*. (2012) obtained, from Pleurotus ostreatus culture in submerged culture medium and using banana peel powder in the culture medium, laccase activity equal to 1,117 or L^{-1}. In the present work this waste was also used in the composition of the culture. According to the authors, the addition of banana peel powder in the culture medium induced the production of two isoforms of laccase (58.7 and 21 kDa). The authors observed values of BPA removal (57 ppm) close to zero, on the first day of incubation (the authors used purified laccase in the assays). Macellaro *et al*. (2014), using the mediator ABTS (of 2.2´-azino-bis-[3-ethyltiazoline-6-sulfonate]) in the culture medium to produce laccase from *Pleurotus ostreatus*, observed 95% BPA removal (23 ppm) in 1 hour of incubation. In the BPA removal assays, the authors used the purified and immobilized enzyme (1,500 U L^{-1}), with effectiveness equal to 0.015 mg U^{-1} h^{-1}. According Daâssi *et al*. (2016), the presence of mediators in the culture medium expanded the use of laccases for industrial and environmental purposes since the degradation of non-phenolic compounds also can be observed. In relation of BPA removal, Daâssi *et al*. (2016) observed 100% of removal (concentration of BPA 100 ppm) in 4 hours of incubation (laccase activity in the culture broth 1,000 U L^{-1}; effectiveness equal to 0.025 mg U^{-1} h^{-1}). The authors carried out the cultivation of *Coriolopsis gallica* in semi-solid medium to produce the enzyme laccase and evaluate the efficacy of this enzyme in the degradation of BPA. Chang and Chang (2016) evaluated the BPA degradation (20 ppm) by laccases produced by *Pleurotus eryngii* (crude enzyme broth, laccase activity 53,000 U L^{-1}) and observed in 24 hours of incubation removal of BPA equal to 95.5% (effectiveness 0.0000157 mg U^{-1} h^{-1}).

The variation of laccase enzyme activity with time was monitored during the assays of BPA removal. It can be observed in fig. 4 that the activity remained constant throughout the experiment, that is, the enzyme remained active at the same time that BPA degradation occurred. Erkurt (2015) observed in assays related to BPA degradation by laccases, that greater removal percentage (100% in 90 minutes) as well as higher enzyme stability (60% waste activity in 90 minutes) were obtained using *Funalia trogii* crude enzyme broth and using the inactivated broth added from the commercial enzyme purified from *Trametes versicolor*. Using only the purified enzyme in the assays, the lowest removal percentage was obtained (30% in 240 minutes), as well as lower enzyme stability (totally inactivated in 90 minutes) throughout the assays. The author suggests that an interaction between the polyethylene glycol and the polymer products present in the crude enzyme broth gave protection to the laccase enzyme.

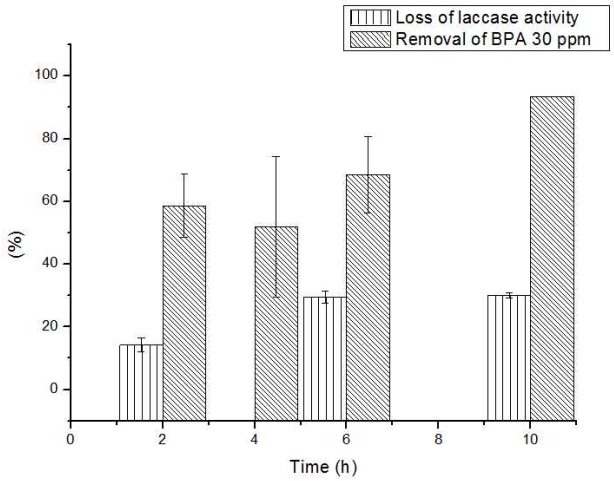

Fig. 5. BPA removal percentage and loss of laccase activity for the assays using crude enzyme broth diluted 1:1.

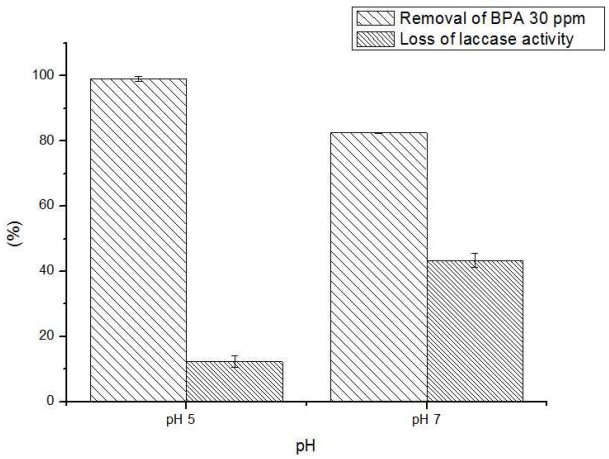

Fig. 6. BPA removal percentages and loss of laccase activity for the assays conducted at pHs 5 and 7.

For the following assays, determination of laccase activity, pH and temperature, which provide maximum performance of the laccase on the BPA removal, was defined: BPA concentration of 30 ppm and reaction time of 10 hours.

Fig. 5 shows the BPA removal percentages, and loss of laccase activity, at 2, 4, 6 and 10 hours using the *Pleurotus sajor-caju* crude enzyme broth diluted in water, 1:1 (initial laccase activity equal to 40.7 U L^{-1}). According to fig. 5, removal percentage around 94% in 10 hours of reaction can be observed (effectiveness equal to 0.069 mg U^{-1} h^{-1}). According to Baldrian *et al*. (2006), the polarity of the culture medium may influence the performance and maintenance of enzyme activity. Solvents with high polarity tend to increase the solubility of the polar substrates in the culture medium, providing faster and more selective reactions. Perhaps this could explain why a higher effectiveness value was obtained in this study by using the diluted enzyme broth rather than the undiluted broth. According to Asadgol *et al*. (2014), the enzymatic activity is related to the amount of water linked to the protein and not to the concentration of water in the organic solvents. Thus, higher enzymatic activity may be related to water activity, regardless of the solvent used. Water would increase the mobility and flexibility of the active sites of the enzyme and the polarization of the protein structure.

Fig. 6 presents the BPA removal percentages and loss of laccase activity in assays conducted at pHs 5 and 7. As observed in fig. 6, removal percent was equal to 100% for the experiment conducted at pH 5. At pH 7 removal percentage was 82.5%, that is, approximately 20% lower. Higher effectiveness value was obtained in pH 5 (0.073 mg U^{-1} h^{-1}) than in pH 7 (0.061 mg U^{-1} h^{-1}). Therefore, for the following assays was determine pH 5. Loss of laccase activity was lower in assays conducted at pH 5 (12%) than in pH 7 (43%), in 10 hours of incubation. In assays conducted at pH 3, in 10 hours of incubation, the enzyme was completely inactivated, and no BPA degradation could be observed. Arca-Ramos *et al*. (2015) evaluated BPA removal (10 ppm) by laccases of *Trametes versicolor* at different pH values (5, 6 and 7). The authors observed a higher percentage of removal (96.3%) in assays conducted at pH 6 and laccase activity equal to 1,000 U L^{-1} (effectiveness equal to 0.00963 mg U^{-1} h^{-1}, assay time equal to 1 hour). Asadgol *et al*. (2014) observed a higher BPA removal percentage (80%) (1,000 ppm) by lacases of *Paraconiothyrium variable* (an isolated soil ascomycete) (5,000 U L^{-1}) at pH 5 (effectiveness equal to 0.32 mg U^{-1} h^{-1}, time assay equal to 30 minutes).

Fig. 7 shows the BPA removal percentages and loss of laccase activity in assays conducted at temperatures of 20, 30 and 40°C. The BPA removal percentages equal to 96.5, 100 and 100% for the assays conducted at 20, 30 and 40°C, respectively, were observed (Fig. 7). According to statistical analysis, there is no statistically significant difference between these

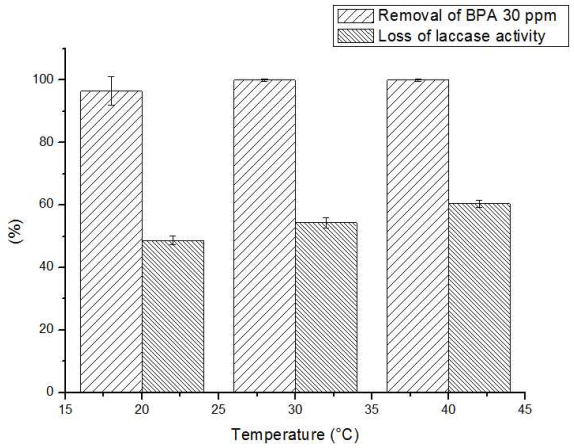

Fig. 7. BPA removal percentages and loss of laccase activity for the assays conducted at temperatures of 20, 30 and 40ºC.

values. The percentage of loss of enzyme activity was 48.8, 54.4 and 60.4% in the assays conducted at 20, 30 and 40°C, respectively. As the temperature increases, denaturation of the enzyme can occur, which could justify the greater percentage of loss of activity in laccase when using a temperature of 40°C. Erkurt (2015) using *Funalia trogii* laccases, taken from the solid culture of this fungus, obtained a removal percentage of nearly 100% using a pH value equal to 5.5 and a temperature of 50°C. The temperatures evaluated by the author were (35, 40, 45, 50, 55 and 60°C).

Freitas *et al.* (2017) observed 40°C as the optimum temperature for BPA removal by *Pleurotus ostreatus* laccase. After 1 hour of incubation 94.42% degradation was observed (initial concentration of BPA equal to 100 ppm and activity in laccase 5,000 U L^{-1}. Therefore, effectiveness equal to 0.019 mg U^{-1} h^{-1}). For *Pleurotus pulmonarius* laccase, the optimum operating temperature was 30°C degrading 88.4% of BPA in 1 hour incubation (effectiveness equal to 0.017 mg U^{-1} h^{-1}). In relation to pH, both enzymes were able to remove the BPA in ranges of 4 to 7 pH, having obtained the best degradation results in 5.0 pH. The authors also investigated the toxicity of the aqueous solution after the enzymatic treatment. Thirteen aromatic and aliphatic BPA metabolites were identified, including *p*-isopropenylphenol, methylpent-3-oic acid, ethyl-3-ethoxy propanoate, and 4-ethyl-2-methoxyphenol. The acute BPA toxicity decreased from 85% to less than 5% using the *Pleurotus ostreatus* laccase. On the other hand, *Pleurotus pulmonarius* laccase, did not cause reduction in toxicity, possibly because at least one BPA metabolite was as toxic as the parent compound itself. The authors suggest the use of crude laccase broth from *Pleurotus ostreatus* as a feasible method for the complete removal of BPA from polluted environments. Brugnari *et al.* (2018) also conclude in their manuscript that the use of laccase can be an economical alternative method for large scale degradation of BPA in aqueous systems. The authors studied the efficiency of *Pleurotus ostreatus* laccase immobilized onto MANAE-agarose (12 U g^{-1}) for degradation of BPA. The authors found effectiveness equal to 0.03 mg U^{-1} h^{-1} and best reaction conditions in pH 5 and temperature 55ºC. The immobilized *Pleurotus ostreatus* laccase retained more than 90% of its initial capability to degrade BPA after 15 cycles of reuse. The results presented in this paper corroborate with the conclusion of these authors and suggest the possibility of obtaining an enzymatic formulation, using *Pleurotus sajor-caju* crude enzyme broth, without necessity of enzyme isolation procedures, for application in the environmental area in the future.

Conclusions

The *Pleurotus sajor-caju* crude enzyme broth was able to degrade the endocrine interfering compound bisphenol A (BPA). 100% of BPA (30 ppm) removal was observed in 10 hours of incubation. The best reaction conditions for BPA removal by *Pleurotus sajor-caju* crude enzyme broth were BPA concentration of 30 ppm, reaction time 10 hours, crude enzyme broth diluted in water (1:1), pH 5.0 and room temperature. Under these conditions, laccase activity was maintained at around 50% and the effectiveness was equal to 0,07 mg U^{-1} h^{-1}. These initial results indicate the great potential of obtaining an enzymatic formulation, without the need for isolation procedures, capable of assisting in the treatment of this class of environmental pollutants, the endocrine interfering compounds.

Acknowledgements

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