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REMOVAL OF BISPHENOL A BY A FREE AND IMMOBILIZED LACCASE FROM *Trametes versicolor*

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ABSTRACT

Bisphenol A (BPA) is an endocrine disruptor compound, that is continuously released into the environment and is barely degraded in wastewater treatment plants. In this work, a free and immobilized laccase from *Trametes versicolor* was used to degrade BPA in aqueous solution. The residual BPA was quantified by HPLC. Both free and immobilized laccases were able to remove 80% of BPA after 1 h and 6 h of reaction, respectively, when equivalent amounts of enzyme were used. The reason for this is that the immobilized enzyme has higher K_M and lower V_{max}, phenomena that are likely to result from conformational changes and microenvironmental alterations within the catalytic site induced by the immobilization process. Although the free form was faster in removing BPA than the CLEA-immobilized enzyme, the use of the latter may eventually prove to be advantageous for practical reasons (e.g., possible increased stability and reuse in successive cycles).

Keywords: Bioremediation. Laccase. Endocrine disruptors.

1 INTRODUCTION

Bisphenol A (BPA), also known as 2,2-bis(4-hydroxyphenyl) propane, stands as one of the most extensively manufactured chemicals, primarily utilized in the production of polycarbonate plastics and epoxy resins for packaging food items. Notably, both the US Environmental Protection Agency (EPA) and the World Wide Fund for Nature (WWF) have categorized BPA as an Endocrine Disrupting Chemical (EDC), marking it as a significant social, environmental, and global concern. EDCs, such as BPA, are artificial compounds capable of disrupting the endocrine system, leading to adverse effects on development, reproduction, neurology, mutagenesis, carcinogenesis, and immunity in both human populations and wildlife.[1,2] Various chemical methods, including the Fenton reaction, ozonation, photocatalytic oxidation, and ultrasonic oxidation, have been suggested for BPA degradation. Despite their potential, these methods come with significant drawbacks such as high costs and the generation of toxic byproducts. Alternatively, BPA can undergo metabolism or biodegradation by microorganisms, including microalgae, bacteria, and fungi. Utilizing microorganisms for BPA degradation is generally perceived as a safer and more cost-effective alternative to conventional chemical processes. The oxidative enzyme laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) has attracted attention in the treatment of different pollutants including BPA due to its high reactivity and selectivity in both free and immobilized forms.[3-6] The use of laccase as a catalyst in the enzymatic degradation of BPA is a promising approach, as this enzyme uses molecular oxygen as the final electron acceptor, meeting green chemistry standards, with minimal environmental impacts.[6] However, when working with laccases, it is essential to act with caution, as variables such as temperature and pH can disrupt and diminish their catalytic effectiveness in bioremediation procedures. An alternative approach involves immobilizing them to mitigate the risk of denaturation and facilitate their reusability. One of the most extensively researched methods for immobilization is CLEA (cross-linked enzyme aggregates), which involves precipitating enzymes under controlled conditions and subsequently cross-linking them with bifunctional reagents like glutaraldehyde. This technique offers notable benefits, including enhanced enzyme activity, improved catalytic stability, and extended storage longevity. Moreover, these advantages are coupled with reduced production expenses attributable to the elimination of supporting carriers such as encapsulating agents.[7] In this context, this work aimed to apply free and immobilized laccase from *T. versicolor* to remove BPA in aqueous solutions..

2 MATERIAL & METHODS

Production of laccase

T. versicolor was kept in the laboratory through successive plating in potato dextrose agar (PDA) and is registered in the SISGEN (www.sisgen.gov.br) under the number A4E5EC1 (November 01, 2018). The laccase production was performed in solid state cultures of 7 days using wheat bran as substrate with an initial moisture content of 83%. To extract the enzymes, a volume of 15 mL of cold water was added to the cultures. After extraction, the mixtures were filtered through cheesecloth and centrifuged for 10 min at 2000*g*. Clear supernatants were considered as crude enzyme extracts..

Immobilization of laccase

Briefly, laccase immobilization was performed at 4 °C following the methodology previously described[7] where the enzyme was precipitated with ammonium sulfate and cross-linked using glutaraldehyde as a cross-linking agent. Ammonium sulfate was slowly added to a laccase solution to obtain 55% (w/v) saturation. After complete solubilization of ammonium sulfate, 150 mM of glutaraldehyde was used as a crosslinking agent. The suspension was maintained at 4°C for 24 h and then centrifuged for 15 min at 2000*g*, resuspended in 50 mM acetate buffer pH 5.0, and stored at 4°C until use.

Evaluation of BPA degradation by free and immobilized laccase using high performance liquid chromatography (HPLC)

To evaluate the percentage of BPA degradation by free and immobilized laccase by chromatographic assay, the reaction mixture was prepared with 50 mM sodium acetate buffer (pH 5.0), 200 µM BPA and 0.2 U/mL of laccase The reaction mixture was kept at 40°C for up to 1 h in the assays with free laccase, and for up to 6 h with the immobilized laccase. The trace amounts of BPA in the samples were analyzed using a high-performance liquid chromatography system (Shimadzu, Japan) equipped with a quaternary pump and diode array detector (DAD) and an Ascentis ® C18 column (4.6 mm × 250 mm, 5 µm) thermostated at 40 °C. The mobile phase was water:methanol (30:70, v/v) at a flow rate of 0.8 mL min^{-1} for 10 minutes. Spectrophotometric detection was performed at 290 nm. The residual BPA concentration was calculated using a calibration curve.[8]

Determination of kinetic constants of free and immobilized laccases

For determining the kinetic constants V_{max} and K_M, initial reaction rate measurements were carried out at 40 °C in 50 mmol/L sodium acetate buffer, pH 5.0, using 2,2-azinobis(3-ethylbenzthiazoline-6-sulphonic acid, ABTS as substrate (0.01–2.00 mmol/L). The oxidation of ABTS was monitored for 5 min at 420 nm. The GraphPad Prism version 8.0 (GraphPad Software, Inc., USA) was used to estimate the kinetic parameters K_M and V_{max}. This program allows direct fitting of the Michaelis-Menten equation, $v = V_{max}[S]/(K_M + [S])$, to the experimental data.

2 RESULTS & DISCUSSION

The highest immobilization yield and activity retention values were 100% and 98%, respectively, achieved after 24 hours of reaction with a final concentration of 150 mM of glutaraldehyde. The HPLC quantification was performed immediately after collection of aliquots, which were placed in an ice bath to slow down the enzymatic reaction. Removal of BPA at the initial concentration of 200 µM was 92.9% in an assay of up to 1 h containing 0.2 U/mL of free laccase (Figure 1). However, an equivalent amount of the CLEA-immobilized laccase could remove 94.1% of BPA only after 6 h of reaction (Figure 2). The longer time required for the degradation of BPA by the immobilized enzyme can be explained by probable alterations in the tertiary structure of the active site or by conformational changes in the enzyme molecule. After the immobilization step, the microenvironment around the catalytic site may have been modified leading to changes in the electrostatic interactions between the catalytic groups and the substrate.[9]

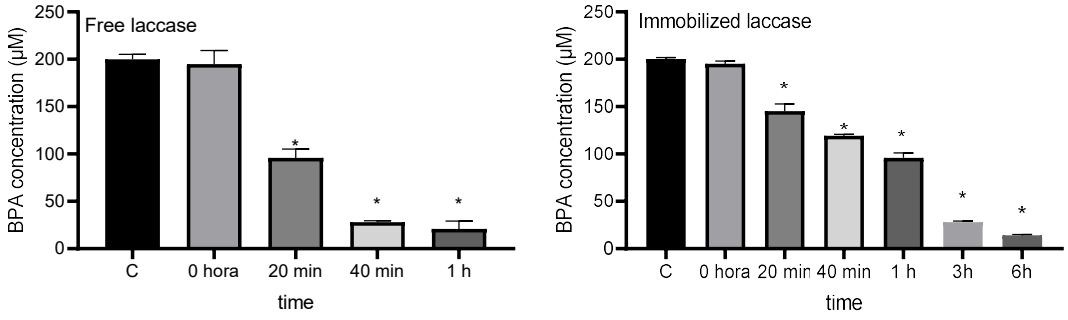

Figure 1 Removal of BPA from aqueous solution by free and immobilized T. *versicolor* laccases. Conditions: the reaction was carried out in 50 mM acetate buffer, pH 5.0, contained 200 µM BPA, 0.2 U/mL laccase at 40°C.

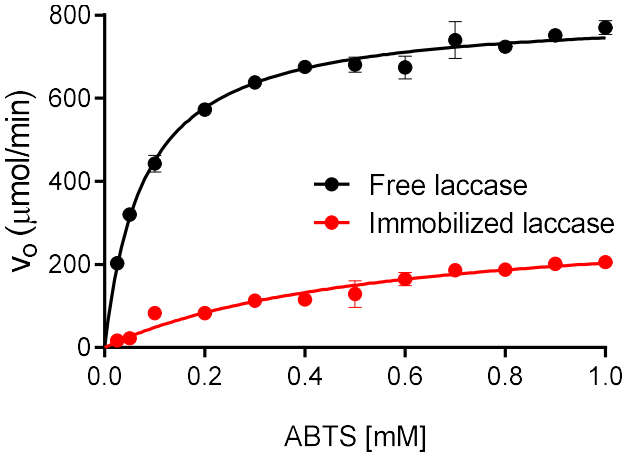

Figure 2 Effect of ABTS concentration on the activities of the free and immobilized laccase from *T. versicolor*. To determine the kinetic parameters (maximal reaction rate, V_{max}, and the Michaelis–Menten constant, K_M), the initial reaction rates of ABTS oxidation were monitored. General conditions: time of reaction, 5 min; pH, 5.0 (50 mM acetate buffer); temperature, 40 °C. Symbols represent the experimental rates. The continuous lines were calculated using the Michaelis-Menten equation, $v = V_{max}[S]/(K_M + [S])$, into which the optimized parameters V_{max} and K_M were introduced. The optimized parameters were: free laccase, V_{max} = 804.7±11.0 µmol/min, K_M = 0.079±0.005 mM; immobilized laccase: 315.7±40.0 µmol/min, K_M = 0.552±0.145 mM. The correlation coefficients for the free laccase and immobilized laccase curves (r) were 0.99 and 0.96, respectively

The interpretation given in the former paragraph is corroborated by kinetic data. Figure 2 shows the substrate (ABTS) concentration dependences of both the free and immobilized enzymes, using equivalent amounts of enzyme in both cases. For

all substrate concentrations, the activity of the immobilized enzyme was smaller than that of the free enzyme. The continuous lines in Figure 2 represent the theoretical curves that were calculated after fitting the Michaelis-Menten equation to the experimental data. Agreement between theory and experiment is fairly good, as can be deduced by visual inspection and from the high correlation coefficients given in the legend of the figure. The immobilization caused a very pronounced increase in the K_M value and a highly significant decrease in the V_{max} (see values in the legend to the Figure 2), phenomena that usually reflect conformational changes as well as microenvironmental alterations within the catalytic site. Despite this, the CLEA-immobilized enzyme still proved to be efficient in degrading BPA. Our results are similar to findings reported in the literature showing that the laccase from *Ganoderma lucidum* was able to remove 80.9% of BPA in 1 h[10] whereas the *Trametes hirsuta* laccase immobilized on Fe_3O_4 nanoparticles achieved a removal of 87.3% in 11 h[11].

3 CONCLUSION

Our results allow to conclude that the laccase from *T. versicolor* is able to degrade BPA by nearly 100%. The free form is more efficient on this respect than the CLEA-immobilized enzyme. Even so, for practical reasons (e.g., possible increased stability, reuse in successive cycles, etc.) the use of the immobilized enzyme may eventually prove to be advantageous, a possibility worth of being examined by future work.

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