EFFICIENT DECOLORIZATION AND DETOXIFICATION OF MALACHITE GREEN DYE BY FREE AND IMMOBILIZED LACCASE OF *Pycnoporus sanguineus*

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ABSTRACT

In this study a laccase of *Pycnoporus sanguineus* was immobilized using cross-linked enzyme aggregates (CLEA) and utilized in the decolorization and detoxification of the dye malachite green (MG). The free and immobilized laccases (1.0 U/mL) decolorized 100 ppm MG with similar efficiencies (94.6% and 97.0%, respectively) after 48 h incubation in distilled water (pH ± 5.0) and 40 ºC under agitation of 110 rpm. The potential of reuse of the immobilized enzyme encompasses 5 decolorization cycles. In addition, the phytotoxicity of MG, evaluated as the capacity of inhibiting the elongation of the seeds of *Lactuca sativa* during germination, was totally abolished after its treatment with both forms of the laccase. Our results indicate the immobilized laccase of *P. sanguineus* as a possible tool for bioremediation, detoxification and decolorization of residual waters from the dyeing industry in the future.

Keywords: Bioremediation. Clean technologies. Synthetic dyes. White-rot fungi.

1 INTRODUCTION

Malachite green is a triarylmethane dye and serves as both dyestuff and, controversially, as antimicrobial agent in aquaculture[1]. Traditionally it is employed for dyeing materials such as silk, leather and paper[2]. Malachite green (MG) is environmentally persistent and acutely toxic to a wide range of aquatic and terrestrial animals[3]. Therefore, it is necessary to remove it from the aquatic systems to protect human health, environment, and aquatic species. White rot fungi exhibit potent lignin-degrading capabilities through the secretion of ligninolytic enzymes such as peroxidases and laccases. Laccases have garnered significant research attention due to their environmental-friendly nature and their ability to oxidize a broad spectrum of phenolic and non-phenolic compounds. Laccases [benzenediol oxygen reductases, EC 1.10.3.2] are multicopper enzymes that catalyze the oxidation of phenols, aromatic amines, and other electron-rich substrates through a concomitant reduction of O_2 to H_2O[4]. They are widely studied for application in technological processes, thanks to their low substrate specificity characteristics, as they use oxygen as an electron acceptor and do not require cofactors or hydrogen peroxide for their action[5]. However, working with enzymes requires some specific care because factors such as temperature and pH can destabilize and reduce their catalytic efficiency in bioremediation processes. An alternative is to immobilize them in order to reduce the risk of denaturation and provide chances of reuse[6]. One of the most studied immobilization techniques is CLEA (cross-linked enzyme aggregates), which consists in the controlled precipitation of enzymes followed by cross-linking with bifunctional reagents such as glutaraldehyde. This methodology provides interesting advantages, such as the obtainment of high enzyme activity, high catalytic stability, and long-term storage stability. Furthermore, these advantages are associated to low production costs due to the elimination of the need for supporting carriers such as encapsulating agents[7]. Considering the above, the objectives of this study were to compare the capability of free and immobilized *P. sanguineus* laccases (CLEA) in the decolorization and detoxification of malachite green.

2 MATERIAL & METHODS

Production of *P. sanguineus* laccase

P. sanguineus belongs to the Laboratory of Biochemistry of Microorganisms, Department of Biochemistry-UEM, and was maintained through successive plating in wheat bran extract agar (WBEA). This isolate is registered in SISGEN (www.sisgen.gov.br) with the code A4E5EC1. For enzyme production, mycelium discs (diameter 17 mm) were transferred to Erlenmeyer flasks (125 mL) containing 5 g of wheat bran and initial humidity of 80%. The inoculum consisted of three mycelium plugs obtained from WBEA cultures. The Erlenmeyer flasks were kept at 28°C and in the absence of light for 7 days. A volume of 30 mL of cold distilled water (4 to 8 ºC) was added to each vial, which was kept for 30 min. in refrigerator. The enzyme extracts were first filtered through gauze and then centrifuged in Falcon tubes for 5 min at 2,000*g*. The supernatants were considered as crude enzyme extracts.

Enzyme assay and immobilization of laccase by cross-linked enzyme aggregates (CLEA)

Laccase activity was measured with ABTS (2,2-azinobis(3-ethylbenzthiazoline-6-sulphonic acid) as substrate in 50 mM citrate buffer, pH 5.0 at a temperature of 40 °C. ABTS oxidation was determined by increasing $A_{420\ nm}$ (ε = 36 mM^{-1} cm^{-1}). One unit of laccase activity was defined as the amount of enzyme required to oxidize 1 µmol of substrate per min. Laccase immobilization was carried out at 4 ºC adapting the previously described methodology with modifications[8]. Ammonium sulfate was slowly added

to the laccase solution to obtain 50% (wt/v). After complete solubilization of ammonium sulfate, glutaraldehyde (25%) was added as a cross-linking agent to obtain a concentration of 100 mmol/L. The suspension was maintained at 4 ºC for 24 h and centrifuged in plastic tubes for 10 minutes at 2,000*g*. The precipitated materials (CLEAs) were washed four times to remove excess ammonium sulfate and glutaraldehyde, and stored at 4 °C in distilled water until use. The two immobilization parameters, immobilization yield (RI) and activity recovery (RA) were evaluated according to the equations below: RI (%) = Total enzyme activity in the supernatant x 100/Total enzyme activity; RA (%) = Activity in the immobilized fraction x 100/Total enzyme activity.

Evaluation of decolorization and detoxification of MG by free and immobilized *P. sanguineus* laccase

The tests of MG dye decolorization were carried out in 125 mL Erlenmeyer flasks containing 10 mL of 50 mM citrate buffer pH 5.0, 100 ppm MG and 1.0 U/mL of free or immobilized laccase. The reaction mixtures were maintained at 40 ºC, in the absence of light, and agitation of 110 rpm. Samples were collected after 6, 12, 24 and 48 hours. The mixtures were introduced into the spectrophotometer cuvette and the spectral wavelength was scanned. The control used to reset the reading (white) used distilled water instead of the dye and the enzymes at the same concentration as in the treatments. The peak absorbance of each spectral curve was used to calculate the percentage of discoloration relative to zero time. The absorbance after 48 h was subtracted in each case.

Toxicity tests were carried out using lettuce seeds (*Lactuca sativa*) according to the methodology described previously with modifications[9]. Twenty lettuce seeds were previously germinated during 2 days in Petri dishes (90 mm diameter) containing filter paper saturated with distilled water. After this time the germinated seeds were transferred aseptically to new Petri dishes containing distilled water, an untreated MG solution, a free laccase treated MG solution or an immobilized laccase treated MG solution. Growth was allowed to continue for additional 3 days when the respective seed elongations were measured. The experiments were conducted in triplicate.

Reusing immobilized laccase in the decolorization and detoxification of MG

A volume of 2 mL of immobilized laccase (20 U) was added to 18 mL of the reaction medium to obtain a final concentration of 100 ppm of MG. The mixture was kept under stirring at 110 rpm at 40 ºC for 48 h. Thereafter it was centrifuged (2,000*g* for 10 min), and after 3 washes, the supernatant was discarded. Finally, a volume of 18 mL of fresh reaction medium was added to start the next reaction cycle.

2 RESULTS & DISCUSSION

Immobilization yield and activity retention values of 100% and 98%, respectively, were obtained using glutaraldehyde at a final concentration of 100 mmol/L after 24 h of reaction. The procedure optimized through CLEA was, therefore, quite efficient. The free and immobilized laccases were efficient in decolorizing the aqueous MG, as the dye solution gradually lost its greenish color depending on the incubation time by 94.57% and 97.02%, respectively (Fig. 1). The MG solution in the presence of denatured enzyme, kept under the same conditions, showed no signs of discoloration after 48 h of incubation. These results demonstrate that both free and immobilized laccases from *P. sanguineus* have good MG color removal activity.

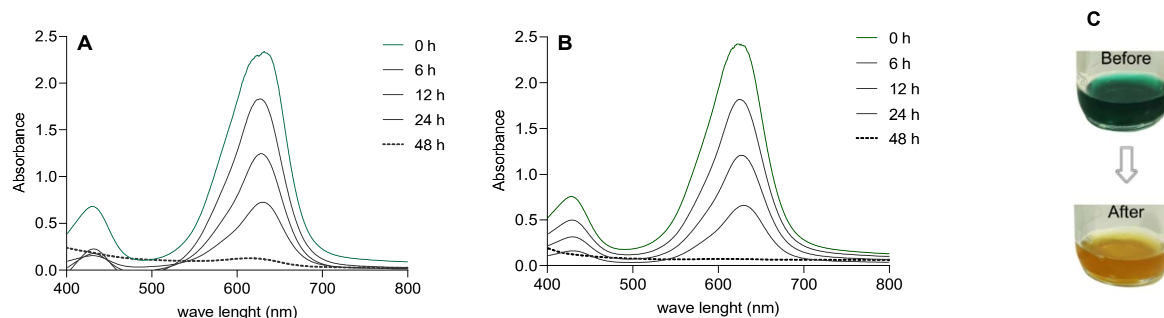

Figure 1 Absorption spectra of MG in the visible range before and after free (A) and immobilized (B) *P. sanguineus* laccase treatments. In C: Dye colour before and after laccase treatment

The results of the toxicity tests are illustrated by Figure 2. Panels A and B illustrate aspects of the various steps of the germination procedure. In the left side of panels A and B roots obtained after the pre-germination step of 2 days are shown. In the right portion of each panel the final appearances of the roots that were allowed to elongate in the presence of free laccase treated MG (A) or immobilized laccase treated MG (B) for additional 3 days are shown. A quantitative evaluation of the action of MG on the root elongation, on the other hand, is shown in panel C. Addition of untreated MG diminished the elongation of the roots by 45%. No such diminution was found when the roots were allowed to elongate in the presence of either free laccase treated MG or immobilized laccase treated MG. Clearly, the transformations catalyzed by the *P. sanguineus* laccase, irrespective of its state, abolish phytotoxicity of MG.

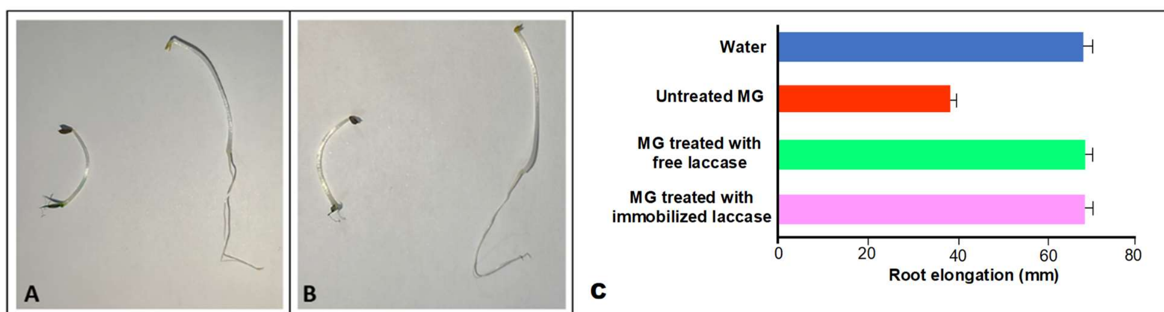

Figure 2 Effects of MG on lettuce seeds (*Lactuca sativa*) germination. Panel A: left, root after 2 days germination in water; right, root after additional 3 days germination in the presence of MG treated with free laccase. Panel B: left, root after 2 days germination in water; right, root after additional 3 days germination in the presence of MG treated with immobilized laccase. Panel C: roots elongations during 3 days following the addition of water, untreated MG, free laccase treated MG and immobilized laccase treated MG. The error indicators are SD.

The reuse of the immobilized laccase was tested in consecutive MG decolorization cycles. In the fifth cycle, approximately 55.4% of the initial activity was found when the substrate was MG (Figure 3). These results suggest that the immobilized laccase was efficient in decolorizing MG for up to 5 cycles. It is important to highlight that each decolorization cycle takes 48 h, which emphasizes the stability of the immobilized enzyme.

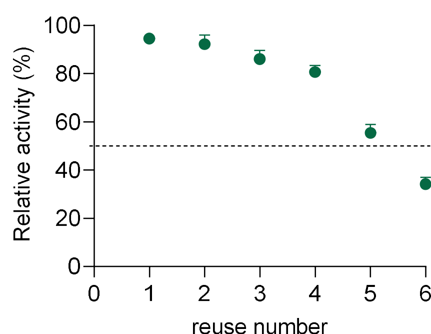

Figure 3 Reuse cycles of immobilized laccase in the decolorization of MG. Data are presented as mean ± SD (n=2).

3 CONCLUSION

The immobilization of the *P. sanguineus* laccase in CLEAs proved to be an efficient alternative for decolorization and detoxification of MG. After 48 hours of treatment, the free and immobilized laccases discolored 94.6% and 97.0%, respectively, and eliminated MG toxicity. Furthermore, the immobilized enzyme showed efficiency in the decolorization of MG along 5 cycles. It must be remembered that although the free and immobilized laccase were equally efficient in decolorizing MG, the immobilized form presents the great advantage of being reusable, a property which is obviously not shared by the free enzyme. Our results indicate the immobilized laccase of *P. sanguineus* as a possible tool for bioremediation, detoxification and decolorization of residual waters from the dyeing industry in the future.

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