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Immobilization of a crude extract of laccase from *T. hirsute* Bm2 on copper alginate for environmental vinasse remediation

Inmovilización de un extracto crudo de lacasas de *T. hirsuta* Bm2 en alginato de cobre para la remediación ambiental de vinaza

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Abstract

Sugarcane vinasses are environmentally aggressive effluents because of their high phenolic content and the presence of other recalcitrant compounds. The aim of this work was to immobilize laccases from *Trametes hirsute* Bm2 in copper alginate spheres in order to oxidize phenols and remove color from vinasses. Respond Surface Methodology (RSM) was used to evaluate enzyme and $CuSO_4$ concentration during immobilization. Laccase activity retained in spheres was the response variable. Lacasse activity in spheres increased by 30 % and the best efficiency in immobilization was 95% with 300 mM of $CuSO_4$ and 150 U/mL of laccase. Vinasse treatment (10%) with free laccase removed 40% of phenols, and the addition of natural mediators increased up to 60%. Immobilized enzymes were able to remove up to 68% of total phenols. Synthetic and natural mediators were used in the immobilization process to improve the removal of phenols by laccases. However, mediators did not significantly improve the process. The biocatalizer was able to remove phenols during four cycles of treatment and the maximum decoloration was 75%. These phenomena were attributed to both laccase activity and adsorption to the support. After treatment, a dark precipitate was observed, suggesting polymerizing activity for laccases. These results reveal that laccases immobilized on copper alginate are a feasible alternative for the treatment of sugarcane effluents.

Keywords: phenols, vinasse, laccase, immobilization, cupper alginate.

Resumen

Las vinazas de azúcar de caña son efluentes agresivos con el medio ambiente por su alto contenido de fenoles tóxicos y otros contaminantes recalcitrantes. El objetivo de este trabajo fue inmovilizar lacasas de *Trametes hirsuta* Bm2 en esferas de alginato de cobre para oxidar fenoles y decolorar vinazas. La Metodología de Superficie de Respuesta (RSM) fue utilizada para evaluar la concentración de enzima y $CuSO_4$ en la inmovilización. La variable de respuesta fue la actividad de lacasa retenida en las esferas. La actividad enzimática en las esferas incrementó 30 % y la mayor eficiencia en la inmovilización fue 95% con 300 mM de $CuSO_4$ y 150 U/mL de lacasas. El tratamiento de vinazas (10%) con lacasas libres redujo 40% de fenoles y con la adición de mediadores naturales aumentó a 60%. Las enzimas inmovilizadas eliminaron hasta 68% de fenoles totales. Un mediador sintético y naturales fueron utilizados en la inmovilización para mejorar la remoción de fenoles por las lacasas, Sin embargo, la adición de mediadores naturales y químico en la inmovilización no mejoró significativamente la remoción de fenoles. El biocatalizador fue capaz de remover fenoles durante cuatro ciclos de tratamiento y la máxima decoloración fue 75%, estos hechos fueron atribuidos a la actividad de lacasas y adsorción al soporte. La presencia de un precipitado obscuro después del tratamiento sugiere una actividad polimerizante de las enzimas. Estos resultados revelan una alta eficiencia en la inmovilización y la actividad que las lacasas inmovilizadas en alginato de cobre podrían ser una alternativa para el tratamiento de efluentes de la caña de azúcar.

Palabras clave: fenoles, vinaza, lacasa, inmovilización, alginato de cobre.

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1 Introduction

The sugarcane industry is an important sector in Mexico, from which alcohol is obtained to be used as an alternative fuel or for the production of alcoholic beverages (Pérez-Fernández and Venegas-Venegas, 2017). In the state of Veracruz, Mexico, large volumes of pollutant effluents called vinasse are generated during alcohol distillation. These are complex mixtures with acid pH, high solids content, high BOD (biological oxygen demand), COD (chemical oxygen demand) and a high concentration of phenols (Noa-Bolaño *et al*., 2020). Phenols are considered priority pollutants by the US Environmental Protection Agency (EPA) due to their toxic effect, even at low concentrations, and their possible carcinogenic properties in living organisms (Chris *et al*., 2017). Color is another characteristic of vinasse, linked to suspended solids and melanoidins that limit the area receiving sunlight, which in turn affects photosynthetic organisms such as algae (Mohana *et al*., 2009). For this reason, the development of processes to improve the quality of wastewater is urgent.

Several technologies have been used for the treatment of sugarcane vinasse, of which anaerobic treatments have proven to be more effective. Farias-Silva and de Souza-Abud, 2016, reported that anaerobic treatment can efficiently remove phenols and other contaminants from sugarcane vinasse in 23 days. Aerobic and combined treatments have also been tested. Reis *et al*., (2019) used ozone, anaerobic and aerobic treatments for the removal of total phenols from sugarcane vinasse. However, these processes have disadvantages such as high energy and reagent requirements, the generation of toxic by-products, and the accumulation of sludge generated after the process (Mathew *et al*., 2018). Ligninolytic fungi, also called white rot fungi, are capable of removing recalcitrant compounds from vinasse by catalysis of the nonspecific enzymes they produce or by bioadsorption to the fungal mycelium (Ahmed *et al*., 2020).

Laccase enzymes (EC 1.10.3.2, benzenediol:oxygen oxidoreductases) are multi-copper enzymes that represent the most versatile group of ligninolytic enzymes due to their broad action on mono-, di- and polyphenols, and aromatic amines. Laccase produced by white rot fungi catalyzes the oxidation of substrates using molecular oxygen (O_2) as the last electron acceptor, producing water (H_2O) as the final molecule of the process (Arregui *et al*., 2019). Laccase production is frequently carried out with lignocellulosic substrates because the fungus can release phenolic monomers that induce enzyme activity (Pérez-Salazar *et al*., 2023). Also, phenols can act as redox mediators (Mani *et al*., 2018). A mediator is a small molecule that acts with the enzyme site and the substrate to oxidize high potential redox-substrates that laccase can not oxidize. Various synthetic mediators such as 2,2'-azino bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), benzotriazole (BTA) and 4-hydroxybenzotriazole (HBT) can increase laccase degradation of other substrates such as pesticides, industrial dyes, lignin, etc. However, these are expensive and toxic to the environment. Natural mediators can be released from lignin breakdown. Previously, Ancona-Escalante *et al*., (2018) reported phenolic extracts produced by *Trametes hirsuta* Bm2 grown on wheat bran, which increased the laccase degradation of industrial dyes.

The ability of laccases to carry out the transformation of phenolic substrates and the detoxification of phenolic contaminants has been demonstrated. However, the application of these enzymes is limited by the high cost of production processes, their low stability in the presence of changes in pH or temperature, and the fact that they are subject to inhibition by various compounds (Couto and Toca, 2006). The immobilization of laccases on a suitable support can provide benefits such as improving pH- and temperature-dependent stability, facilitating product separation, biocatalyst reuse and reducing inhibition by the products present in the medium, all of which would lead to a reduction of process costs (Dajun *et al*., 2020). Alginate is a polymer frequently used for encapsulation of laccases due to its harmlessness, biodegradability, and low cost. Laccases have been immobilized in a matrix combined with divalent ions such as calcium, barium, zinc, and copper, which act as crosslinking agents (Olajuyigbe *et al*., 2019; Sharafi-Badr *et al*., 2023). Although calcium is the most commonly used ion, it has been reported that copper has a high affinity for the D-manuronic and L-guluronic that are part of the alginate. Furthermore, Cu^{2+} can interact with the amino acid residues of the enzymes while maintaining their original structure, providing greater stability (Zang *et al*., 2018). It is important to note that, due to the heterogeneity of phenolic compounds in the effluents, complete removal of these compounds using a single enzyme in the process is difficult. Co-immobilization of two or more enzymes with different properties, or the use of a crude extract with mixtures of oxidative enzymes, could expand the number of target substrates and would also make it possible to improve the efficiency of other industrial processes (Vera *et al*., 2019).

Trametes hirsuta Bm2 is a fungus that produces extracellular laccases (Tapia-Tussell *et al*., 2011). It has previously been shown that *Trametes hirsute* Bm2 is able to decolor sugarcane vinasse in a microbial treatment; and furthermore, that in the presence of phenolic compounds such as guaiacol, ferulic acid, and vanillic acid, there is an overexpression of laccase genes (Tapia-Tussell *et al*., 2015). The immobilization of the crude extract containing mixtures of laccase isoenzymes that have demonstrated synergistic action in the removal of dyes from the textile industry (Zapata-Castillo *et al*., 2015) offers an alternative method of carrying out phenol removal. In this study, the objective was to immobilize the laccases produced by the *Trametes hirsuta* Bm2 fungus in copper alginate spheres for phenol and vinasse color removal and to determine the biocatalyst reuse cycles.

2 Materials and methods

2.1 Vinasse

The sugarcane vinasse was obtained from Ingenio La Gloria (Veracruz, Mexico), an industrial distillery that uses molasses as a raw material for ethanol production and that generates vinasse as wastewater. Samples were collected in containers and kept refrigerated at 4 °C until use.

2.2 Fungal strain

The *Trametes hirsuta* strain Bm2 used in this work was isolated from decaying wood in Yucatan, Mexico (Tapia-Tussell *et al*., 2011). The medium for propagation consisted of 20 g malt extract and 20 g agar per liter (pH 6.0). Plates were incubated for 4 days at 35°C and used to inoculate the liquid medium.

2.3 Laccase production on wheat bran

The liquid medium to obtain inoculum was (g/L): glucose, 10; malt extract, 10; peptone, 2; yeast extract, 2; KH_2PO_4, 2; $MgSO_4{\cdot}7H_2O$, 2; thiamine-HCl, 1 mg/mL.125 mL Erlenmeyer flasks containing 50 mL of medium were inoculated with two 1-cm^2 agar pieces from an actively growing fungus propagated on a malt extract plate. Flasks were incubated at 35°C and shaken at 150 rpm. After 4 days, the culture was homogenized using a sterilized blender. The liquid media for laccase production contained 3% (w/v) wheat bran flakes in 60 mM phosphate buffer (pH 6.0). Two milliliters of mycelia suspension were inoculated to 100 mL of medium and the culture was grown at 35°C for 7 days. Aliquots of the medium were withdrawn each 24 h, filtered and centrifuged to remove the mycelium. To obtain phenolic extracts (natural mediators) from the crude culture fluid, the method described by Ancona-Escalante *et al*. (2018) was used. The crude extract taken each 24 h was filtrated by ultrafiltration tubes (10 kDa, Millipore, US) and the phenol concentration was determined.

2.4 Laccase immobilization in copper alginate

A factorial 2^2 design two factors and two levels was used to determine $CuSO_4$ (300, 700 mM) and laccase concentration in crude extract (50, 150 U/mL), in order to find immobilization parameters. Crude extract of laccases was mixed with sterile sodium alginate (2% w/v) and the solution was stirred gently. The mixture was extruded drop by drop through the sterile syringe into of $CuSO_4$ solution to obtain beads 3 mm in diameter. The spheres were gently stirred for 15 min and then were kept refrigerated for 24 hours. The beads were washed with distilled water to remove non-immobilized enzymes. Laccase activity was measured in spheres of immobilized enzyme under different conditions. With a Fisher test statistical significance was determined through analysis of variance (ANOVA) with 95% confidence. The effects of variables, both independently and in interaction with each other, was also determined. The software used was Centurion statgraphics. Laccases were also immobilized using the conditions selected, with the addition of natural mediators in crude extract (0.14 mg/mL) and reactive mediator 4-hidroxibenzaldehide (20 mM).

2.5 Decolorization and removal of phenolic compounds with free and immobilized laccase

The treatment was carried out in 125 mL Erlenmeyer flasks filled with 10 mL of vinasse diluted by the addition of distilled water to obtain concentrations of 10%, 25%, 50%, and 75% (v/v) in solution. The pH of these solutions was adjusted to 4.5. Treatments with free laccase were carried out with 0.250 mL of crude extract (150 U/mL), also immobilized laccases (150 U/mL) with or without mediators were added into each flask and subsequently incubated for 264 h with orbital rotation (150 rpm) in a New Brunswick shaker at 40°C ± 2 °C. Samples were collected every 24 h. Three replicates were incubated for each vinasse concentration. The concentration of phenols was determined by the Folin-Ciocalteu method (Waterhouse, 2003), which measures the formation of a blue complex spectrophotometrically at 740 nm following the reduction of a phosphomolybic-phosphotungstic reagent by phenols. The phenol content of the samples was expressed as gallic acid equivalents using equation 1 (Singleton *et al*., 1999).

$$Phenols(\%) = \left[\frac{\text{Final phenol concentration in vinasse}}{\text{Initial phenol concentration}}\right] \times 100 \quad (1)$$

2.6 Reusability of immobilized laccase

The reusability of spheres of immobilized enzyme on copper alginate was studied for decolorization and phenol removal from vinasse 10% (v/v). Each catalytic cycle lasted four hours, and the spheres were washed with sterile distilled water and transferred to a new vinasse. Decolorization efficiency was determined according to the equation 2 (Yang *et al*., 2017), by measuring the absorbance before and after treatment.

2.7 Analytical methods

Decolorization was calculated according to the absorbance measurements at 475 nm. The color removal reported was calculated according to equation 2:

$$Decolorization(\%) = \frac{Ao - At}{Ao} \times 100 \quad (2)$$

where Ao = initial absorbance and At = final absorbance for each time.

Laccase activity in cell-free filtrates was measured at 40°C using 2,2'-azino-bis (3- ethylbenzothiazoline-6-sulphonic acid) (ABTS). The assay mixture contained 1M sodium acetate buffer (pH 5.0) and 0.5 mM ABTS in a total volume of 1 mL. The oxidation of ABTS was measured by the increase in absorbance at 420 nm and laccase activity was calculated from the molar extinction coefficient (ε_{max} = 36,000 L/M^{-1}cm^{-1}). One enzyme unit (U) is defined as the amount of enzyme required to oxidize 1 μmol of ABTS per minute under assay conditions. The laccase activity was expressed as U/mL (Niku-Paavola *et al*., 1990).

2.8 Statistical analysis

All the experiments were carried out in triplicate and values were averaged and given with the standard deviation (± SD). Analysis of Variance (ANOVA) was within 95% confidence limits. The statistical significance of the results was tested at $p < 0.05$ level using Tukey's test. Statistical analyses were performed using OriginLab9 (OriginLab, Northampton, MA, EUA).

3 Results and discussion

3.1 Production of laccases and removal of phenols from vinasse

Laccase production by white rot fungi is a process that combines the physiological interaction of the fungus with the components and conditions of the culture, such as pH, temperature, etc (García-Reyes *et al*., 2017). Laccase production can be considerably enhanced by the addition of lignocellulosic substrates or phenolic and aromatic monomers (Bakkiyaraj *et al*., 2013). When *Trametes hirsuta* Bm2 was cultured on wheat bran (Figure 1a), after 72h a linear increase in laccase production was observed, with maximum titers of 9000 U/mL at 192h, which is 60 times higher than the activity previously produced on basal medium (Tapia-Tussell *et al*., 2011). During cultivation, fungi degrade lignin, generating phenolic monomers such as guaiacol, *p*-coumaric acid, syringic acid, sinapylic acid and vanillic acid, which act by promoting transcriptional induction and increasing laccase synthesis. In addition, there are differences in the expression level of laccase genes depending on the phenol structure, causing variation in the transcriptional profiles of laccase isoenzymes (Xiao *et al*., 2004). The increase in laccase synthesis could be linked to a mechanism within the fungi designed to protect them from oxidative stress caused by phenolic compounds.

Figure 1b shows the curve of total phenols, where a slight decrease in phenols is observed during cultivation. In the short term, phenols often increase, decreasing later. However, the behavior observed in this study should be taken with caution, since during cultivation there is a balance between the release and consumption of phenols by the fungus, which also uses them as a carbon source. The composition and concentration of phenols can vary in the extracts obtained at different times and these extracts can also act as redox mediators, favoring the oxidative activity of laccases towards other substrates (Collins *et al*., 1996).

Subsequently, 10% diluted vinasse treatment was carried out with free laccases (crude extract) and laccases supplemented with natural mediators present in the phenolic extract (48h). Figure 2 shows that the free laccases were able to remove 40% of the phenols over 24 h, and that the addition of natural phenols increased the phenol-removing activity of the enzyme by 20%. Colins *et al*., 1996 reported that ultrafiltered phenolic extracts produced in *Pleurotus* cultures with lignocellulosic residues enhanced the biotransformation of benzopyrene and anthracene, and in *Trametes* they increased laccase activity and indigo carmine decolorization when added to the reaction medium (Ancona-Escalante *et al*., 2018). Free laccases failed to reduce the color of vinasse either in the presence or absence of the mediator, suggesting that free laccases from *Trametes hirsuta* Bm2 are not able to act on melanoidins present in vinasse.

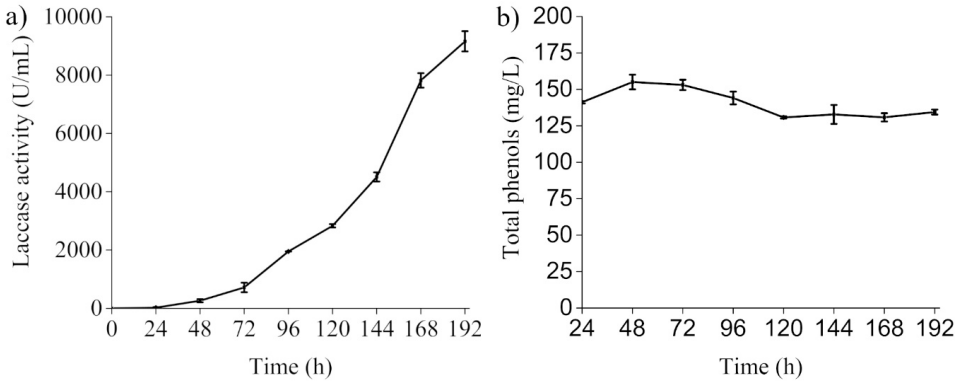

Fig. 1. Laccase production (a) and phenols (b) from *T. hirsuta* Bm2 grown on wheat bran 3%, pH 6, 35°C and 150 rpm.

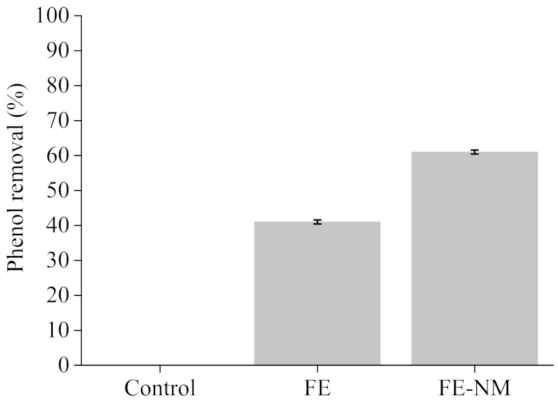

Fig. 2. Vinasse treatment with free enzymes (FE) and free enzymes (FE-NM) with natural mediators 12 h, a 35°C, pH 6 and 130 rpm.

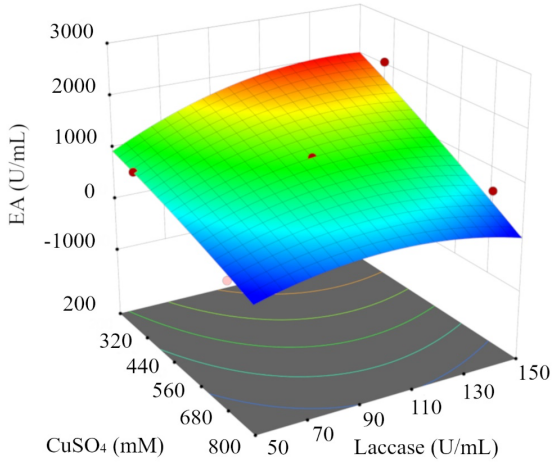

Fig. 3. Response surface to evaluate in laccase immobilization the concentration of $CuSO_4$ and enzyme.

3.2 *Immobilization on copper alginate*

The response surface plot (Figure 3) shows that copper and enzyme concentrations have a significant influence $p < 0.05$ on the activity of immobilized laccases. Activity decreased with increasing copper concentration, probably due to the inhibitory effect that copper ions can have on enzymes. In contrast, activity was higher with increasing enzyme concentration, which can be attributed to the fact that a greater number of enzymes were bound without saturating the support. The spheres with 150 U/mL of laccases and 300 mM copper showed a uniform size of 3 mm, high mechanical stability and an immobilization efficiency of 95%. Similar results were obtained by Sondhi *et al*., (2018) who showed that the optimal copper concentration was 300 mM in the immobilization of laccases from Bacillus sp. Phetson *et al*., (2009) obtained higher stability and better performance in the immobilization of *Lentinus polycrous* Lev. laccases on copper alginate compared to calcium alginate.

3.3 *Removal of phenols by free and immobilized laccases*

The results in Figures 4a-d show that the enzymes ability to remove phenols decreases with increasing effluent concentration. Also, it is clear that the immobilized crude extract was more efficient in removing phenols than the free enzymes. The immobilized enzymes removed 68, 66, 56 and 24.6% of vinasse phenols at 10, 25, 50 and 75% concentration, respectively, and the increase was 30 to 35% higher than the free enzymes in the same conditions. These values differ significantly ($p < 0.05$). The enzymes immobilized with natural mediators and 4-hydroxybenzaldehyde, which is one of the most frequently studied mediators, increased phenol removal by 15% with respect to the free enzymes but, did not significantly improve the efficiency obtained in the absence of the mediator. The higher efficiency of the immobilized laccases compared to the free ones could indicate the protective effect of the alginate against the harsh action of the effluents on the enzymes.

Free and immobilized fungal laccases have shown an ability to remove phenols individually or in mixtures of solutions that simulate an effluent. Degradation of *p*-cholorophenol was obtained using laccases immobilized by 3D bioprinting (Liu *et al*., 2020). Lassouane *et al*. (2022) found that laccases from *Aspergillus oryzae* immobilized on calcium alginate managed to remove 78% of bisphenol. Krastanov, (2000) co-immobilized laccases and tyrosinases on Mikroperl, obtaining a significant reduction (94-97%) of mixtures of phenols such as 4-methoxyphenol, *β*-naphthol, 4-chloro-3-methyl phenol and catechin. *Pycnoporus sanguineus* CS43 laccases immobilized in LentiKats were able to reduce *m*-cresol in synthetic effluent (Gonzalez-Coronel *et al*., 2017). However, there are few application studies of immobilized laccases in real effluents where phenols are present in complex mixtures. Sukan and Sargin, (2013) immobilized laccases from *A. oryzae* (novozyme 51004) on calcium alginate, which removed 50% of phenols present in wastewater from a resin factory.

The treatment of vinasse has been investigated mainly using free fungal cultures, and fungal cultures immobilized on supports. The results have been very satisfactory, as high percentages of phenol and color removal have been obtained, even at high vinasse concentrations (España-Gamboa *et al*., 2018; Junior *et al*., 2019). The process of mycodegradation requires several days of cultivation, raising the costs of the process, while the use of immobilized enzymes considerably reduces the treatment time because laccases can act directly or indirectly on the biodegradation process of recalcitrant molecules through chain reactions.

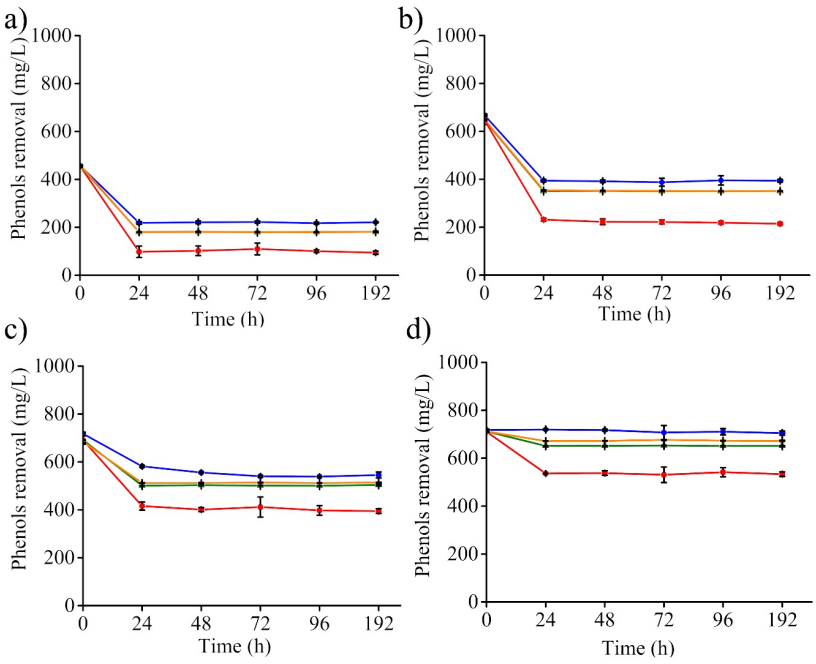

Fig. 4. Comparison of free laccase (FE) and laccase immobilized in copper alginate (IE) for phenol removal from vinasses in different concentrations. a) 10%, b) 25%), c) 50%, d) 75%. FE (blue line),) IE (red line), IE with natural mediators (green line), IE with 4-hidroxibenzaldehido (yellow line). 35 °C, pH 4.5, 150 rpm.

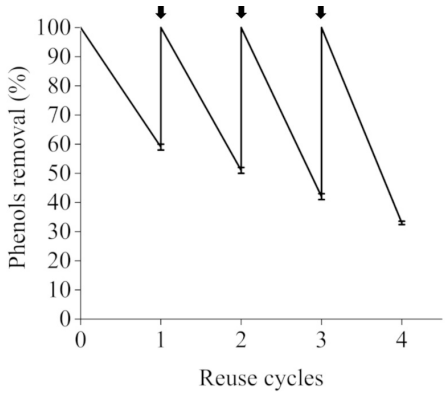

Fig. 5. Reuse cycles of IE. Each cycle lasted 4 hours at 35 ° C and 150 rpm. Black arrows indicate the transfer of spheres to a new vinasse solution.

3.4 Reuse of immobilized laccases

The repeated application of immobilized enzymes is a key factor in industrial processes, since the recovery and reuse of the biocatalyst influences a decrease in the cost of the treatments (Teerapatsakul *et al*., 2008). The crude laccases immobilized on copper alginate were re-used for four consecutive cycles in triplicate and with three replications, giving the significantly different results ($p < 0.05$) shown in Figure 5. The immobilized enzymes showed catalytic activity during the four cycles, although phenol removal efficiency was reduced by up to 30% in the fourth cycle. The persistence of laccase activity can be attributed to the stability provided by the copper alginate, which is a suitable factor for industrial application. During the consecutive treatments, the decolorization of the vinasse was also determined. Figure 6 shows the residual color after each of the treatments with the spheres, with and without immobilized laccases. It can clearly be seen that, especially in the first cycle, the alginate spheres both with and without enzymes adsorb the color of the vinasse. However, this capacity gradually diminishes in the spheres without enzyme until it is lost in the fourth cycle. A dark precipitate was also detected, which could be attributed to the coagulation-sedimentation effects of phenols and melanoidins due to the polymerizing activity of laccases. These results suggest that the decolorization of vinasse occurs by combined effects of adsorption and enzymatic action. Ting-Ting *et al*., (2021) reported that the oxidation of phenol with laccases immobilized on polyethylenimine functionalized with magnetic particles resulted in the formation of quinoid derivatives or homonuclear dimers that initially bound to the support, inhibiting enzyme activity, but were efficiently removed during continuous processing. The polymerizing activity of laccases has been reported by Ameri *et al*., (2021) who observed that, during the treatment of phenols with laccases immobilized on zeolites, a dark precipitate of polymerized compounds was obtained.

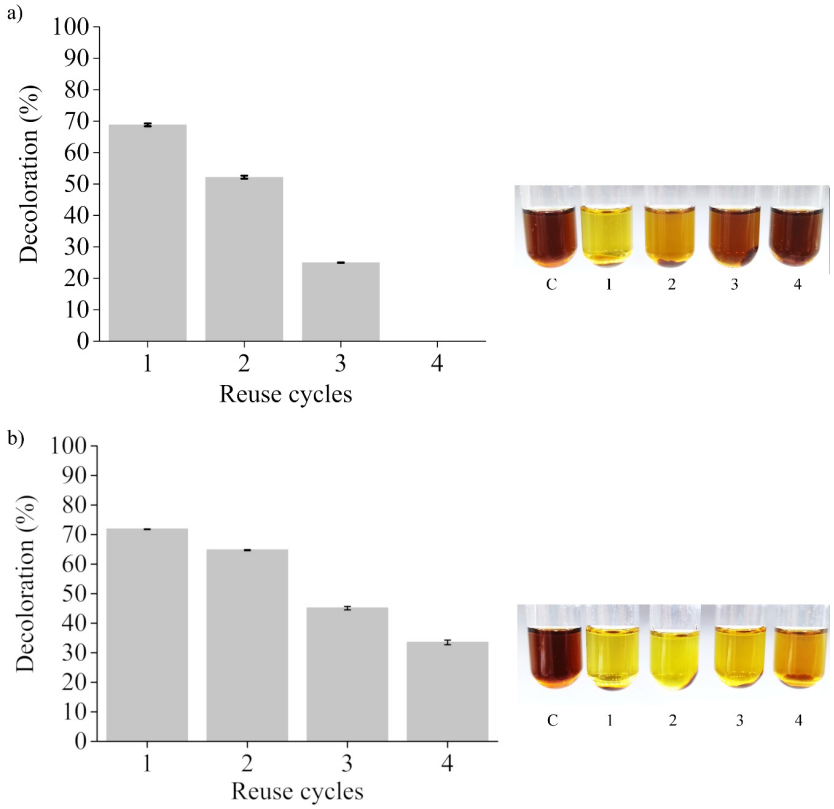

Fig. 6. Reuse cycles of copper alginate spheres (a) and spheres of immobilized enzymes (b) for vinasse decolorization (10 % v/v). Each cycle lasted 4 hours and the washed spheres were transferred to a new vinasse solution. C: control.

Likewise, during the treatment of olive oil mill (OMW) effluents with *Pycnoporus coccineus* laccases, polymerized radicals were produced (Berrio *et al*., 2007). Little is known about the mechanisms of action in melanoidin degradation by laccases. One study performed with purified laccases, degraded 48% of melanoidins extracted from a vinasse from ethanol production; FTIR analysis indicated that laccase can oxidize CH_3, carbonyl groups, haloalkanes, CH and CN bonds present in melanoidins (Toomsan *et al*., 2020).

Conclusions

It was successfully established that laccases immobilized on spheres of copper alginate remove phenol and color from a vinasse. The phenol removal and decolorization of 10% vinasse were 68% and 95 % respectively. Also, immobilized laccase was reused over four cycles. Characteristics of this immobilized system are important for scaling, taking into account that the application of immobilized enzymes may require mechanical strength, uniform size, and the ability to remove color and phenols from vinasse in several continuous cycles. Additionally, immobilization on copper-alginate suggests a protective effect of laccase and shows potential for treatment of other contaminant effluents.

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