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Development of strong enzymatic biocatalysts for dye decolorization

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ABSTRACT

Escherichia coli crude laccase was used to develop an immobilized biocatalyst with improved stability. Chelating and entrapment immobilization techniques were studied, and laccase encapsulation in Cu-alginate gels showed the best results. This biocatalyst was active at different conditions of pH, temperature and ionic strength, and was able to decolorize the carcinogenic dyes Trypan Blue, Bromothymol Blue and Coomassie Brilliant blue R with yields close to 90% without mediator addition. The Cu-alginate derivatives retained more than 70% of catalytic activity for at least 430 h of continuous use. The results demonstrated that immobilized laccase has potential applications in dyestuff treatment.

1. Introduction

Laccases (EC 1.10.3.2) are members of the multicopper oxidase family and are potential tools in a wide number of biotechnological processes mainly due to their high and nonspecific oxidation capacity, lack of cofactors, and the use of readily available oxygen as an electron acceptor (Claus, 2003). This enzyme is characterized by having four copper(II) ions per domain and catalyzes the four-electron reduction of O_2 to H_2O coupled with the oxidation of phenolic compounds, diamines or aromatic amines (Thurston, 1994). Additionally, the reactivity of the enzyme can be enhanced by mediators such as 2,2′-azinobis (3-ethylbenzthiazoline-6-sulfonate) (ABTS), which modifies the reactivity towards other substrates that laccase alone cannot oxidize (Galli and Gentili, 2004).

Laccase could be applied in many fields, such as delignification of lignocellulosic biomass, detoxification of recalcitrant pollutants, decolorization of industrial dyes and textile dye effluents, biological bleaching in pulp and paper industries, juice and wine clarification, and biosensors (Rodríguez Couto and Toca Herrera, 2006).

Only a few bacterial laccases have been studied so far, although rapid progress in genome analysis suggests that these enzymes are widespread in bacteria (Sharma et al., 2007).

Escherichia coli CueO is a 53.4-kDa periplasmic laccase involved in the Cu efflux system under aerobic conditions. CueO would be responsible for the oxidation of extremely toxic Cu(I) to less toxic Cu(II) *in vivo* (Rensing and Grass, 2003). Although the native function and *in vivo* substrate of CueO remain unclear, it is able to oxidize catechols (Kataoka et al., 2007), siderophores (Li et al., 2007) and recalcitrant molecules such as polycyclic aromatic hydrocarbons (PHA) (Zeng et al., 2011).

The use of laccases for industrial bio-oxidations has emerged in recent years due to the advantage that these enzymes, in contrast with peroxidases, do not require hydrogen peroxide (Loera Corral Octavio et al., 2006). Furthermore, laccases accept a wide range of phenolic and non-phenolic compounds as substrates and produce water as the only by-product. This makes laccases suitable enzymes for a great number of green oxidation processes.

The great potential and value in industrial and biotechnological applications have aroused a strong interest in obtaining a large amount of laccase for practical use. The stability and catalytic activity of free enzymes are dramatically decreased by process conditions, such as pH, temperature and ionic strength. The use of immobilized enzymes can overcome some of these limitations and provide stable catalysts with longer lifetimes (Singh et al., 2013).

The purpose of the present study was the development of an effective method for the immobilization of *Escherichia coli* crude laccase and their use for bleaching of industrial dyes.

2. Materials & methods

2.1. Materials

Culture media compounds were obtained from Britannia S.A.

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(Argentina). 2,2′-Azinobis (3-ethylbenzthiazoline-6-sulfonate) (ABTS) was acquired from Sigma Chem. Co. (Brazil). Other reagents of analytical grade were purchased from Anedra (Argentina) and solvents, from Sintorgan S.A. (Argentina). Supports (DEAE-Sepharose, IDA-Agarose) were acquired from Sigma Aldrich (Argentina). Sodium alginate was supplied by Saporiti S.A.C.I.F.I.A (Argentina).

2.2. Microorganism and culture conditions

Escherichia coli was cultured at 37 °C and 200 rpm in Luria-Bertani (LB) medium containing 5 g/L meat extract, 10 g/L peptone, 5 g/L NaCl pH 7.0. Biomass was estimated by optical density at 600 nm ($OD_{600\ nm}$) (SHIMADZU, UV-1603). Cell pellets with 1×10^{10} UFC were collected by centrifugation at 11,000 *g* for 10 min, and stored at 4 °C until use.

2.3. Laccase crude extract preparation

E. coli cell pellets were resuspended in distilled water, and crude extracts were prepared by sonication (5 cycles of 5 s at 5 W in an ice bath; Vibra cell, VCX-130PB). Extracts were clarified by centrifugation at 11,000 *g* for 10 min at 4 °C, and supernatants were collected and stored at −20 °C until use. Protein content was determined by the Coomassie blue method (Bradford, 1976).

2.4. Laccase activity test

Laccase activity was measured spectrophotometrically at 420 nm (extinction coefficient $\varepsilon_{420\ nm}$ 36,000 $M^{-1}cm^{-1}$ (Childs and Bardsley, 1975)) using ABTS as substrate. Reaction conditions were 0.1 mg of protein, 5 mM ABTS, 50 mM $CuSO_4$, 100 mM sodium acetate buffer pH 4.6. The initial reaction rates were obtained from the linear portion of the progress curve. One enzyme unit (EU) was defined as the quantity of enzyme that catalyzes the oxidation of 1 μmol of ABTS per minute.

2.5. Laccase production

Laccase production at different stages of microorganism growth was analyzed. *E. coli* cultures were made under the conditions previously described, and fractions with 1×10^{10} UFC were collected at different times. The pellets were used to obtain the protein extract and catalyze ABTS oxidation.

Moreover, the effect of $CuSO_4$ on the expression of laccase was evaluated. *E. coli* cultures were performed using LB medium supplemented with 5 mM $CuSO_4$, and the activity of the resulting protein extracts was analyzed.

2.6. Crude laccase characterization

The stability of crude laccase with temperature and pH was studied. Fractions of crude laccase were incubated at different temperatures (30–80 °C) or pH values (4.0–9.0) for 2 h and were used to catalyze ABTS reaction.

2.7. Crude laccase immobilization

Crude laccase was immobilized on different supports according to the following procedures. Immobilization yields were determined as the difference in protein content of the mixture before and after incubation with the supports. Protein determinations were carried out by the Coomassie blue method. The derivatives obtained were evaluated for laccase activity by ABTS oxidation.

2.7.1. Immobilization by adsorption

One hundred mg of support (DEAE-Sepharose or IDA-Cu-Agarose) was incubated with 0.1 mg of crude laccase in 20 mM Tris-HCl pH 7.0. The immobilization mixture was gently stirred for 16 h at 4 °C, washed with 20 mM Tris-HCl pH 7.0, dried by vacuum filtration and stored at 4 °C until use.

2.7.2. Entrapment in Cu-alginate

Sodium alginate (1% (w/v)) was mixed with 0.1 mg of crude laccase. The mixture was added dropwise to a stirred solution of 0.1 M $CuSO_4$ and incubated in this solution for 5 min at room temperature. The resulting gel beads were washed with distilled water and 0.1 M sodium acetate pH 4.6, and stored at 4 °C until use.

2.7.3. Entrapment in mixed matrix

Sodium alginate (1% (w/v)) and gelatin (4% (w/v)) were mixed with 0.1 mg of crude laccase. The mixture was then added dropwise to a stirred solution of 0.1 M $CuSO_4$ with 4% (w/v) glutaraldehyde. After 5 min incubation, the gel beads were washed and stored until use.

2.7.4. Optimization of immobilization parameters

Different quantities of crude laccase (0.05–0.45 mg) were mixed with sodium alginate (1% w/v) to determine the optimal biocatalyst load. Furthermore, 0.2 mg of crude laccase was mixed with different concentrations of sodium alginate (1, 2 or 2.5% (w/v)). The obtained beads were used to catalyze ABTS reaction.

2.8. Stability and operational profile of immobilized derivatives

The chemical and physical stability of the immobilized biocatalyst were determined. The derivatives were incubated at different temperatures (25, 30 or 50 °C), and pH values (4, 7 or 9) for 2 h, and the activity was compared with the free crude extract.

In addition, the retention of activity in storage conditions was evaluated. Immobilized derivatives were stored at 4 °C and were periodically used to catalyze ABTS reaction.

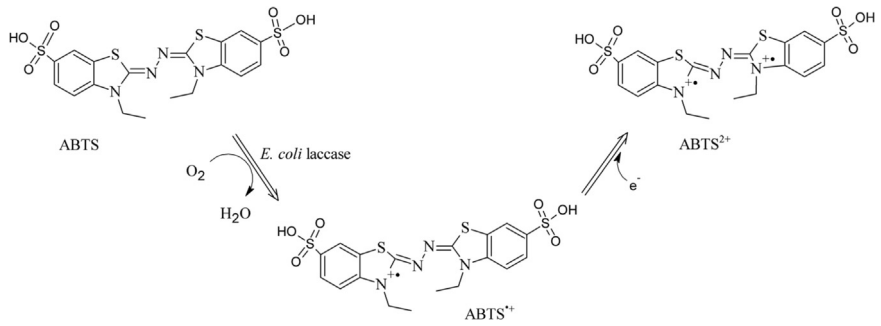
Finally, the deactivation profile of the immobilized biocatalyst was determined. The derivatives were incubated at different temperatures (25, 30 or 50 °C), pH values (4, 7 or 9) and ionic strength (6 g/L NaCl), and the activity was periodically evaluated every 24 h until activity loss.

2.9. Biotransformation of dye by immobilized laccase

Immobilized biocatalysts were used to determinate decolorization rate of four groups of synthetic dyes: anthraquinone, azo, indigoid and triarylmethane. Reactions were performed with 0.012EU of laccase at 30 °C during 3 h. Decolorization yields were calculated as difference in absorbance, at each maximum adsorption wavelength, respect to control without enzyme.

3. Results and discussion

Laccases catalyze hydrogen abstraction reactions from phenolic and related substrates while performing the four-electron reduction of dioxygen to water. The redox process takes place due to the presence of four copper atoms that form the catalytic core of the enzyme, classified in T1 copper ion and a T2/T3 trinuclear cluster (TNC) (Riva, 2006). It has been shown that the T1 site is the primary redox center accepting electrons from the donor substrate. The electrons are quickly transferred, an electron at a time, to the TNC. Molecular oxygen interacts with the fully reduced TNC via a fast 2-electron-transfer process to form a peroxide intermediate,



Scheme 1. Oxidation of ABTS catalyzed by laccase.

and a second 2-electron-transfer to produce the native intermediate. The subsequent release of two molecules of water restores the fully reduced state of the enzyme (Hollmann and Arends, 2012; Jones and Solomon, 2015).

Laccase activity was determined by oxidation of ABTS to its corresponding cation radical $ABTS^{\cdot+}$ (Scheme 1). This radical is highly stable, and could be detected by spectrophotometry at 420 nm (Christopher et al., 2014). It has been suggested that the cation radical $ABTS^{\cdot+}$ may act as a diffusible oxidant of the enzyme. The cation radical can be oxidized further to the dication $ABTS^{2+}$ (Cañas and Camarero, 2010).

3.1. Characterization of *E. coli* crude laccase

Laccase activity at exponential (4 h) and stationary (8 and 16 h) growth phases was studied. *E. coli* cultures had a specific activity of 0.1 U/mg at 8 h, twice the value obtained for the exponential phase. At 16 h, specific activity decreased slightly (0.09 U/mg). Therefore, the stationary phase had the highest laccase expression, which is consistent with previous reports for other laccases (Margot et al., 2013).

It has been reported that laccase expression in *E. coli* can be stimulated by the addition of exogenous copper (Outten et al., 2001). Thus, the addition of $CuSO_4$ to the growth medium was analyzed. Copper added to the growth medium induced laccase expression, with an improvement of 27% in activity. Therefore, up to 40 U/L of laccase may be obtained by adding 0.5 mM $CuSO_4$ to the culture medium. CueO could be involved in the regulation of the response system to exogenous copper, and it has been proposed that the induction of the promoter cueO is positively affected by O_2 and Cu^{2+} (Rensing and Grass, 2003).

To characterize crude preparations, enzymatic stability against temperature and pH was studied. First, crude laccase fractions were incubated for 2 h at 25, 30, 45, 60 and 80 °C, and the activity was determined by ABTS reaction. Laccase crude extracts were fully active up to 45 °C. At 60 °C, the remaining activity was 63%, whereas at 80 °C the enzyme was inactive (Fig. 1A).

The optimal temperature of laccase ranges from 50 to 70 °C, and usually fungal laccases have lower thermal stability than its bacterial counterpart (Baldrian, 2006). Studies of the tertiary structure of CueO and related enzymes indicate that interfacial regions, connecting protein domains, are more densely packed than fungal laccases, and this could be related to the greater thermal resistance observed (Enguita et al., 2003).

Secondly, the stability of crude laccase with pH was analyzed (Fig. 1B). Fractions of crude extract were incubated at different pH (4–9) for 2 h. Then, the pH was adjusted to a standard reaction value, and the extract was used to catalyze ABTS oxidation. In general, bacterial laccases have better stability at neutral-alkaline pH than fungal laccases (Reiss et al., 2011). Consequently, a stimulatory effect was observed at pH near neutrality (7 and 8), obtaining 2-fold more activity in slightly alkaline conditions. Furthermore, although higher pH values did not improve the activity, the enzyme preparation was stable.

Changes in the activity profile at alkaline pH may be the consequence of two effects, (i) the increase in the potential difference between the T1 site and the substrate, and (ii) the reduction in the interaction of OH^- with (T2/T3) the trinuclear cluster. These could increase the oxidation rate (Xu, 1997). These results indicate that the enzyme preparation was not only stable in a wide pH range, but was also overactivated at slightly alkaline pH.

3.2. Stabilization of laccase crude extracts

3.2.1. Support selection

As mentioned above, Cu^{2+} ions interact with specific sites of

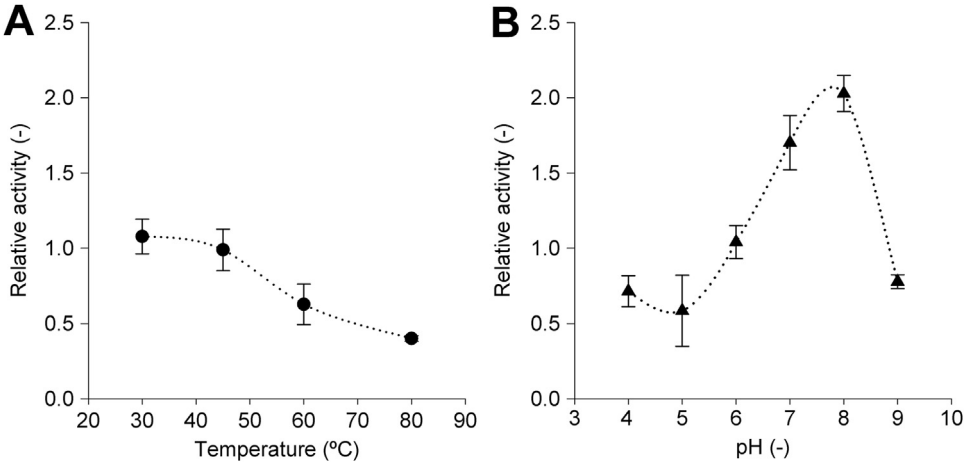


Fig. 1. Characterization of laccase activity in *E. coli* crude extracts. Enzymatic stability with A: temperature, and B: pH.

Table 1
Immobilization of *E. coli* laccase. Crude extract was immobilized in different supports. Activity of derivatives was determinate by ABTS reaction.

Support	Specific activity (U/g h)	Stability (%)[b]
Alginate-Cu	0.55 ± 0.04	79% (60 d)
Gelatin-glutaraldehyde-alginate-Cu	0.45 ± 0.05	82% (60 d)
IDA-Cu-agarose	2.0 ± 0.5	27% (7 d)
DEAE-agarose	ND[a]	ND

[a] Not detected.
[b] Residual activity, in parenthesis storage time in days.

the enzyme, and this property has been exploited to recover laccases from crude extracts by chelating chromatography (Kataoka et al., 2007). Therefore, these matrices could also serve as supports for immobilization.

On the other hand, the immobilization of laccases by entrapment in alginate gels has been reported (Niladevi and Prema, 2008). Although calcium is the most commonly used ion in polymerization, Ca-alginate gels are more porous, less flexible and have lower chemical stability than Cu-alginate (Brandi et al., 2006). In addition, Ca^{2+} ions may inhibit laccase activity (Phetsom et al., 2009).

Additionally, glutaraldehyde treatment can stabilize the alginate beads and is commonly used as a cross-linking agent combined with other composites such as gelatin. Under mild conditions, gelatin can cross-link with glutaraldehyde to form an aldimine bond, a covalent linkage with the lysine residues of protein (Tanriseven and Doğan, 2002). For this reason, two strategies were used to immobilize *E. coli* crude laccase: adsorption and entrapment in alginate (Table 1).

ABTS showed a strong adsorption to the DEAE-agarose support, so it was discarded for future experiments. Moreover, Cu-IDA derivatives showed 4 times more activity than crude laccase immobilized in alginate gels; however, the latter showed better storage stability, retaining more than 70% of activity after 60 days.

Additionally, crude laccase immobilized in alginate-gelatin-glutaraldehyde had comparable activity to the biocatalyst entrapment in alginate gel (0.45 and 0.55 U/gh respectively). In fact, the mechanical stability in operation conditions was evaluated since it has been reported that the addition of gelatin and glutaraldehyde can improve stability (Freitas et al., 2012). Both biocatalysts displayed the same deactivation profile, retaining 50% of initial activity at 300 h of use; therefore alginate without additives was selected as immobilization matrix for the following experiments.

3.2.2. Optimization of immobilization parameters

3.2.2.1. Protein amount. In order to determine the optimal enzyme load, immobilization in 1% (w/v) Cu-alginate with different amounts of protein was performed (Fig. 2A). A mass of protein of 0.2 mg per mL of alginate produced the biocatalyst with the highest specific activity (0.9 U/g h). Derivatives immobilized with more than 0.2 mg of protein did not improve the activity, probably due to mass transfer.

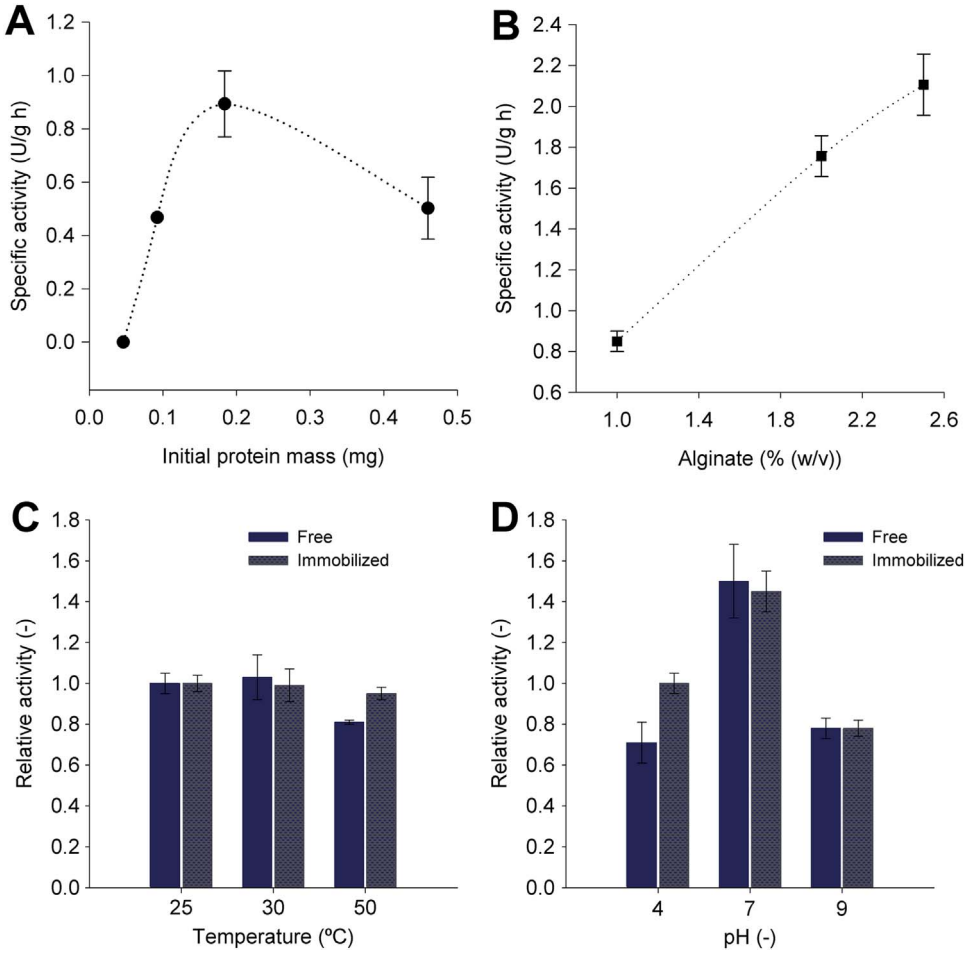


Fig. 2. Optimization of immobilization parameters. Laccase crude extracts were immobilized by entrapment in Cu-alginate, and different parameters were studied. A: Initial protein content, B: Alginate concentration, C: Temperature and D: pH.

3.2.2.2. Alginate concentration. The effect of alginate concentration on the immobilized biocatalyst activity was studied (Fig. 2B). For this, 0.2 mg/mL of crude laccase was immobilized in 1.0%, 2.0% and 2.5% (w/v) alginate, and the derivatives were used to catalyze ABTS reaction. Results showed that the increase in carrier concentration significantly improves the catalytic activity of derivatives. Cu-alginate at 2.5% (w/v) produced a biocatalyst 4.5 times more active than under the reference conditions (1% (w/v)), with a specific activity of 2.1 U/g h.

3.2.2.3. Effects on crude laccase stability. The effect of immobilization was analyzed by comparing the stability of free and immobilized laccase at different pH and temperature conditions (Fig. 2C and D). A stabilizing effect at high temperature and acidic pH was observed when crude laccase was immobilized in alginate. The immobilized biocatalyst was 17% more stable than the free counterpart at 50 °C, while an improvement of 41% in activity retention was observed at pH 4.

3.2.3. Operational stability and reusability of the immobilized biocatalyst

Immobilized crude laccase in Cu-alginate gels was incubated at temperatures (25, 30 and 50 °C), pH (4.5, 7 and 9) and salt concentration (0.1 M) in a rotatory shaker, to study the performance of the biocatalyst under different operation conditions. Periodically, the biocatalysts were washed, and the remaining activity was determined by ABTS reaction. Derivatives were considered inactive when they lost more than 50% of activity.

Fig. 3A shows the stability profile with pH. At alkaline pH the biocatalyst was stable only for 24 h, then the alginate beads began to lose structure, possibly due to electrostatic interactions of H^+/OH^- and the matrix (Phetsom et al., 2009). Conversely, immobilized crude laccase showed high stability at acidic and neutral pH, retaining 70% and 85% of activity at 340 h of operation, respectively.

At 50 °C (Fig. 3B), immobilized crude laccase retained activity for more than 140 h, and then the physical destabilization of the support became evident. When the immobilized biocatalyst was operated at mesophilic temperatures (25–30 °C), it showed to be highly stable, retaining more than 80% of laccase activity before 340 h of use.

Additionally, the immobilized biocatalyst exhibited higher stability at high ionic strength (Fig. 3C). It retained more than 70% of activity before 430 h of operation at a salt concentration comparable with that of industrial and domestic effluents (Lefebvre and Moletta, 2006).

The immobilized biocatalysts were operationally stable for 432 h, which is equivalent to 18 consecutive runs of 24 h each, a value significantly higher than those reported for the same type of immobilization (Niladevi and Prema, 2008). These results indicate that the derivatives of alginate-Cu developed are robust biocatalysts that can be used under different reaction conditions with increased stability and reusability.

3.3. Decolorization assays

Synthetic dyes are one of the major water and soil contaminant released into the environment by different sources. These pollutants are persistent and severely affect ecosystems where they are discharged, causing reduction in photosynthetic activity of natural waters and toxicity on microorganisms involved in soil fertility (Mittal et al., 2010). In addition, the presence of dyes even at low concentrations in water can cause severe health problems and some of them have been classified as carcinogenic and mutagenic for humans (Chequer et al., 2011).

The immobilized biocatalyst was used to decolorize synthetic dyes in batch experiments (Table 2). Generally, color removal by immobilized enzyme is the result of both enzymatic catalysis and support adsorption. In some cases, adsorption contributed significantly to color removal (Lu et al., 2007). For this reason, alginate beads with and without enzyme extracts were made and incubated with dye solution at laccase reaction conditions. In the condition of reaction, alginate beds showed less than 9% of adsorption. Immobilized laccase exhibited a strong decolorization capacity at short reaction times without mediator addition. Results significantly higher than those reported previously for Trypan Blue (Razak and Annuar, 2014), Bromothymol Blue (Ling et al., 2015) and Methyl Orange (Pardo et al., 2013) among others, showing the potential of immobilized biocatalyst developed.

4. Conclusions

Escherichia coli laccase, namely cueO, is involved in the Cu efflux system under aerobic conditions. It was reported that this enzyme could oxidize a large number of xenobiotics of environmental importance. However, their characterization in terms of their thermal and chemical stability, as well as their stabilization by immobilization techniques, has not been reported so far. Laccase crude extract was stable up to 30 °C and alkaline pH, showing its great potential use in effluent treatment.

Laccase crude extract was stabilized by entrapment in Cu-

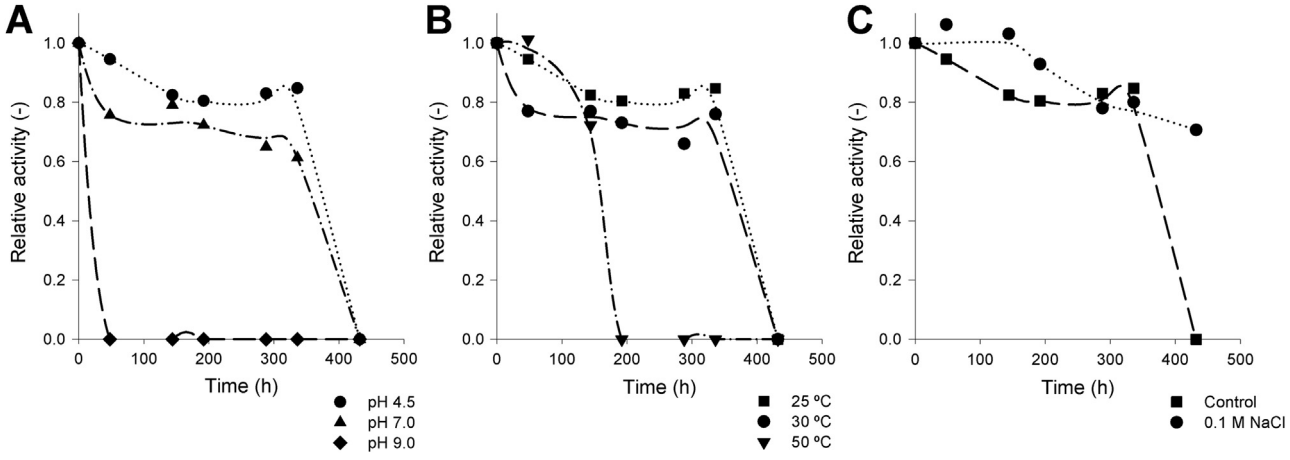


Fig. 3. Operational stability of the immobilized biocatalyst. Laccase crude extracts were immobilized in Cu-alginate at optimized conditions, and derivatives were used in different condition until activity loss. Deactivation profiles at different pH values (A), temperature (B) and ionic strength (C).

Table 2
Decolorization of dyes by immobilized *E. coli* laccase. Reactions were performed by triplicate with 0.012 EU of laccase immobilized in alginate-Cu at 30 °C and 4.5 pH.

Dye $_{(\lambda max)}$[a]	Dye class[b]	Concentration (μmol/L)	Decolorization[c] (%)
Bromothymol blue$_{(425)}$	T	40	85 ± 5
Methyl orange$_{(487)}$	S	40	33 ± 1
Methyl red$_{(550)}$	A	40	17 ± 1
Methyl violet 10B$_{(595)}$	T	40	35 ± 2
Indigo carmine$_{(595)}$	I	10	7 ± 1
Coomassie Brilliant blue R$_{(595)}$	T	40	84 ± 4
Remazol Brilliant blue R$_{(595)}$	AT	40	57 ± 3
Trypan blue$_{(595)}$	DA	20	88 ± 3
Malachite green$_{(660)}$	T	20	16 ± 1
Amido black 10B$_{(660)}$	DA	200	12 ± 1

[a] λ_{max}: maximum wavelength (in nm) determined experimentally.
[b] T: Triarylmethane, S: Sulfonated monoazo, A: Azo, I: Indigoid, AT: Anthraquinone, DA: Diazo.
[c] Reaction yield was calculated at 3 h reaction respect to control without enzyme.

alginate gels. The immobilization process extended the range of stability of the enzyme, which can be used at 50 °C without a significant loss of activity. Furthermore, the immobilized derivatives showed improved stability at different operating conditions, such as pH between 4.5 and 7, temperature from 25 to 30 °C, and a high ionic strength. These derivatives remained active under continuous operation conditions up to 430 h, retaining more than 70% of the initial activity.

The immobilized biocatalyst developed was applied in the decolorization of anthraquinone, azo, indigoid and triarylmethane synthetic dyes. Higher decolorization yields at short reaction times without mediator requirements were obtained. The laccase biocatalyst exhibited improved stability in continuous operation, which confirms its versatility and potential application in industrial processes.

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