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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,