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Decolorization of reactive dyes by laccase immobilized in alginate/gelatin blent with PEG

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Abstract

To achieve effective decolorization of reactive dyes, laccase immobilization was investigated. Laccase 0.2% (*m/V*) (Denilite IIS) was trapped in beads of alginate/gelatin blent with polyethylene glycol (PEG), and then the supporters were activated by cross-linking with glutaraldehyde. The results of repeated batch decolorization showed that gelatin and appropriate concentration of glutaraldehyde accelerated the decolorization of Reactive Red B-3BF (RRB); PEG had a positive effect on enzyme stability and led to an increase of color removal. While the beads contained 0.2%, 2.0%, 2.0%, and 0.5% (*m/V*) of laccase, alginate, gelatin, and PEG, respectively. The dye of 50 mg/L initial concentration of RRB was decolorized down to 50% during the tenth repeated batch. As far as the decolorization mechanism was concerned, the thermal and pH stabilities of the immobilized laccase were also investigated and were both appreciably improved. The study indicates that the immobilized laccase can be potential candidate for utilization in biodecolorization processes.

Key words: decolorization; reactive dyes; laccase; immobilization; alginate; gelatin

Introduction

Residual dyes released from textile dyeing plants are visible even in low concentration; they are highly recalcitrant to physical or chemical wastewater treatment systems. The conventional treatment technologies such as coagulation, oxidation, and filtration are usually inefficient in the removal of color; moreover, they are costly and less adaptable to a wide range of dye wastewater. There is a great need to develop an economic and effective way of dealing with the textile dyeing waste in the face of the ever increasing production activities (Ibrahim *et al*., 1996).

As a multi-copper oxidase, laccase (oxygen oxidoreductase) is able to catalyze the oxidation of various phenols, substituted polyphenols, aromaticamines, benzenethiols, and a series of other easily oxidizable compounds (Elias *et al*., 2000). It was evaluated that laccase played an important role in the decolorization of wide spectrum dyes having diverse chemical structure, which suggested its implications in treating textile effluents as a low-cost and environmentally friendly technology (Ivana *et al*., 2006; Marco *et al*., 2006). Nevertheless, laccase is often easily inactivated in waste treatment for the wide variety of treating conditions and is also difficult to be separated from the residual reaction system for reuse, which limits the further industrial applications of laccase. Enzyme immobilization technology is an effective means to make laccase reusable and to improve its stability (Fágáin, 2003), which is considered as a promising method for the effective decolorization of textile effluents. According to the known reports, several different types of supporters were applied to immobilize enzyme, including activated carbon, controlled porosity glass, chitosan microspheres (Jiang *et al*., 2005).

The aim of this study was to achieve effective decolorization of reactive dyes by laccase immobilized in alginate/gelatin. After the free laccase was mixed in sodium alginate, the formed beads were hardened in calcium ions. Additives as gelatin, polyethylene glycol (PEG), and glutaraldehyde were used to optimize the immobilization condition. The influence of additive concentration on the decolorization and stability of immobilized laccase was also studied.

1 Materials and methods

1.1 Materials

Laccase (Denilite IIS) was supplied by Novozymes A/S, China. Na-alginate, guaiacol, gelatin and PEG (molecular weight 4000), glutaraldehyde (25%, *V/V*, aqueous solution), and succinic anhydride were purchased from Sinopharm Chemical Reagent Co. (Shanghai, China). Reactive Red B-3BF (RRB) was obtained from Shanghai Wande Dyestuff Co., Ltd. (Shanghai, China). All other chemicals were of analytical grade and no further purification was required.

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1.2 Immobilization of laccase

Different quantities of gelatin (0, 1.0, 2.0, or 4.0 g) and PEG (0, 0.5, 1.0 or 2.0 g) were previously added into 100 ml of solution containing 0.5%–4.0% (*m/V*) concentration of Na-alginate; 0.2 g of laccase was subsequently added and further mixed thoroughly for 10 min at 25°C. Then, the mixture was withdrawn using a sterile 5 ml syringe and extruded through an 18 gauge needle into $CaCl_2$ solution (2.0%), almost instantly forming beads of 2.0–3.0 mm diameter. The beads were allowed to harden in the $CaCl_2$ solution at 4°C for about 2 h, after which the $CaCl_2$ solution was removed and the beads were washed twice with distilled water. Then the beads were subsequently incubated in 100 ml of glutaraldehyde solution (0%, 0.3%, 0.6%, or 0.9%) and stirred at 4°C for 2 h. After the beads were washed several times with phosphate buffer (0.1 mol/L, pH 7.0), the immobilized laccase was filtered and stored at 4°C.

1.3 Decolorization of reactive dyes

The decolorization capability of the immobilized laccase was accessed with the repeated batch experiment using the dye RRB. The beads with immobilized laccase were moved into 100 ml of buffer solution (pH 5.0) containing 5.0 mg of reactive dyes. The reaction mixture was incubated in an electric-heated thermostatic bath (Rapid Co., Ltd., China) at 60°C for 60 min, and then, the decolorization efficiency was measured by monitoring the decrease in absorbance maximum (540 nm) and expressed in terms of percentage, using a UV-2100 spectrophotometer (Unicon, China). The beads were washed three times at the end of each decolorization cycle, and then consecutive operating cycles were repeated with a fresh aliquot of the substrate. The decolorization efficiency (R, %) was calculated as follows:

$$R = \frac{A_{\text{initial}} - A_{\text{observed}}}{A_{\text{initial}}} \times 100\%$$

where, A_{initial} is the initial absorbance, and A_{observed} is observed absorbance.

1.4 Analysis of laccase activity

Activity of the free or immobilized laccase was assayed using guaiacol as substrate at pH 5.0 at 25°C. After 10 mg of immobilized laccase (or 10 ml of free laccase solution at concentration of 120 mg/L) was added into 40 ml of succinic anhydride buffer containing 20 μmol substrate, the reaction was initiated immediately. Thirty minutes later, the increase in the absorbance at 465 nm was measured using a UV-2100 spectrophotometer. The molar extinction coefficient of guaiacol is 12,000 L/(mol·cm), and one activity unit of laccase was defined as the amount of enzyme required to catalyze 1 μmol of substrate per minute.

1.5 Determination of laccase stability

Thermal stability was determined by incubating the free and immobilized laccase in the phosphate buffers at 60°C and pH 7.0 for variable periods. The free laccase was at a concentration of 120 mg/L, and the immobilized laccase was at a liquor ratio of 10:1. Laccase samples were withdrawn every 30 min, and immediately assayed for residual activity. The pH stability of the free or immobilized laccase was studied by incubating at 25°C in phosphate buffers of varying pH from 3.0 to 8.0 for 1 h, and then the residual activity was determined.

2 Results and discussion

2.1 Effect of alginate concentration on enzyme immobilization and decolorization

Different alginate concentrations of 0.5%–4.0% (*m/V*) were used in enzyme immobilization without any other additives or following cross-linking. The longevity of the decolorization with the immobilized laccase was measured with repeated batch experiment, which was performed to investigate the reusability of the immobilized laccase. The results from repeated batches are shown in Fig.1; the beads containing 0.5% or 1.0% alginate show high efficiency during the first batch, however, both decline rapidly from the second batch, which is because the beads are very soft and the immobilized laccase easily escapes into the dye bath during decolorization. While alginate was at the concentration of 2.0% or 4.0%, it was observed that reusability of the immobilized laccase was improved in some sort, but was still far from an acceptable condition for enzyme immobilization. Moreover, when the alginate concentration was above 4.0%, the beads were hard and not formulation of regular spherical morphology, relevant efficiency of decolorization was also less improved.

2.2 Effect of gelatin blent with alginate on enzyme immobilization and decolorization

To achieve preferable decolorization, gelatin as an additive was blent in 2.0% alginate during the following enzyme immobilizations. As a denatured type collagen, gelatin molecules contain repeating sequences of glycine-*X*-*Y* triplets, where, *X* and *Y* are frequently proline and hydroxyproline (Choi *et al*., 1999). These sequences are responsible for the triple helical structure of gelatin and its ability to form gels where helical regions form in the gelatin protein chains. Owing to its low intensity and high brittleness, gelatin is rarely used alone, and is often used after cross-linking, grafting, or blending (Wang

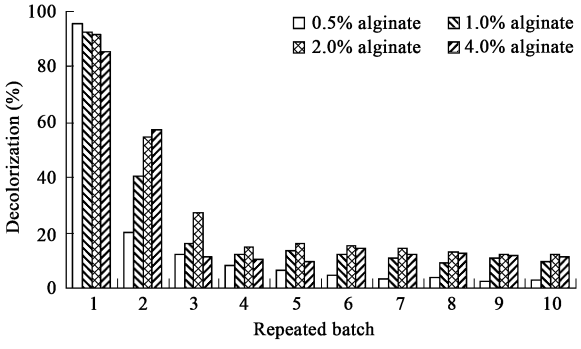

Fig. 1 Effects of the different concentrations of alginate on the decolorization.

et al., 2003; Dong *et al*., 2006). It is well known that blending is an effective and convenient method to improve its performance and easily trap enzyme in beads during immobilization. Different concentrations of 0–4.0% (*m/V*) of gelatin blended with 2.0% alginate were used in laccase immobilization, and the results of decolorization are shown in Fig.2.

As can be seen in Fig.2, the decoloring capability of the immobilized laccase increases with more gelatin used; decolorization of 25% was observed during the last batch when the beads contained 2.0% or 4.0% gelatin. Comparing the results with Fig.1, the advantages of the combination of alginate and gelatin in enzyme immobilization were noticeable; gelatin in blend narrowed the large pore sizes of calcium alginate beads and reduced enzyme leakage out of such beads, which potentially led to visible improvement of the decolorization.

2.3 Effect of glutaraldehyde for cross-linking on laccase immobilization and decolorization

Gelatin has abundant free amino, hydroxyl, and carboxyl functions in molecule chains (Emel *et al*., 2006), and glutaraldehyde can interact with free amino in gelatin or enzyme, which makes water easily exude from the beads during the cross-linking and prevents the leakage of immobilized enzyme from beads (Naganagouda and Mulimani, 2006). To obtain insight into the effect of glutaraldehyde concentration on enzyme immobilization, the alginate/gelatin beads with immobilized laccase were hardened and cross-linked with different concentrations of glutaraldehyde ranging from 0.3% to 0.9% at 4°C for 2 h. The results of the repeated batch decolorization are shown in Fig.3.

Figure 3 shows the effects of glutaraldehyde on the decolorizations by the immobilized laccase; the decolorizations increase first and then decrease, obtaining maximal efficiency with accumulated decolorization of 500% after ten cycles at the concentration of 0.6%. This can be explained as follows: the beads cross-linked with low concentrations of glutaraldehyde had poor mechanism strength; higher concentration of glutaraldehyde accelerated the interactions with free amino functions in gelatin or enzyme, and accordingly increased the intensity of the blent beads and the decolorization efficiency. Furthermore, when the concentration of glutaraldehyde was at

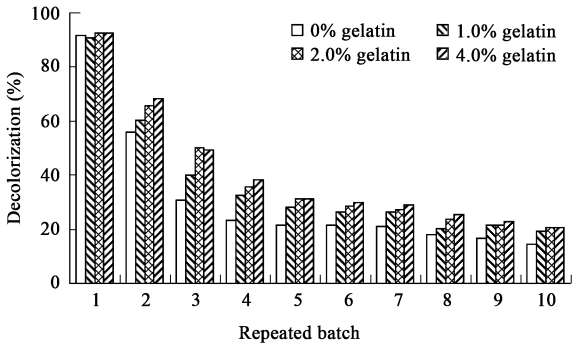

Fig. 2 Effects of the different concentrations of gelatin blent with alginate on the decolorization (beads contained 2.0% (*m/V*) alginate).

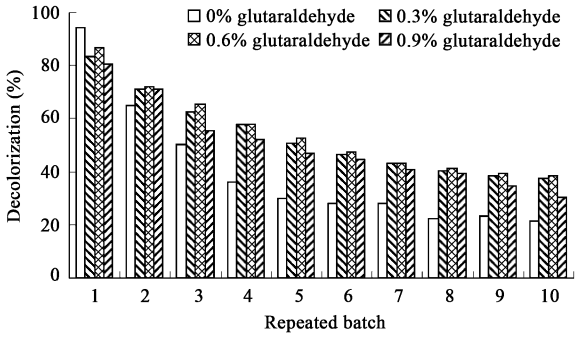

Fig. 3 Effects of different concentrations of glutaraldehyde on the decolorization. Conditions: beads contained 2.0% (*m/V*) alginate and 2.0% (*m/V*) gelatin.

nearly 0.9%, the decolorization decreased in some sort, which was probably owing to the partial inactivation of the laccase by more glutaraldehyde during cross-linking (Jegan and Emilia, 2006). Thus, 0.6% was as an optimum concentration of glutaraldehyde for cross-linking.

2.4 Effect of PEG as additive on laccase immobilization and decolorization

The decolorization by immobilized laccase processed at 60°C, and the enzyme activity decreased after several repeated batch experiments, which will lower the decolorization efficiency. PEG as an additive has been demonstrated to be particularly effective at suppressing the inactivation in horseradish peroxidase (Wu *et al*., 1998) and soybean peroxidase (Caza *et al*., 1999) was added into the alginate/gelatin blend beads to protect the enzyme from inactivation in the laccase immobilization. The effects of different PEG concentrations of 0–2.0% (*m/V*) on the decolorization were studied.

As seen in Fig.4, the beads with different concentrations of PEG achieved high decolorization compared to the control (no PEG blent during immobilization), especially obvious during the later batches. This was probably because the polyol of PEG provided more hydroxyl groups and these polyhydroxylic additives imparted to the enzyme a stronger resistance from inactivation, or had a positive effect on the enzyme stability, even though the mechanism of protection was not fully understood. According to Fig.4, the optimum concentration of PEG should be the minimum

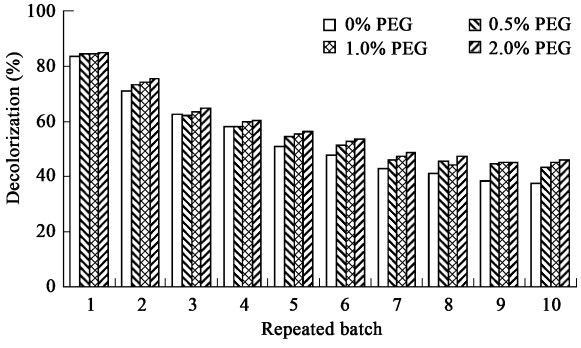

Fig. 4 Effects of the different concentrations of PEG on the decolorization. Conditions: beads contained 2.0% (*m/V*) alginate; 2.0% (*m/V*) gelatin; 0–2.0% (*m/V*) PEG; cross-linked with 0.6% glutaraldehyde.

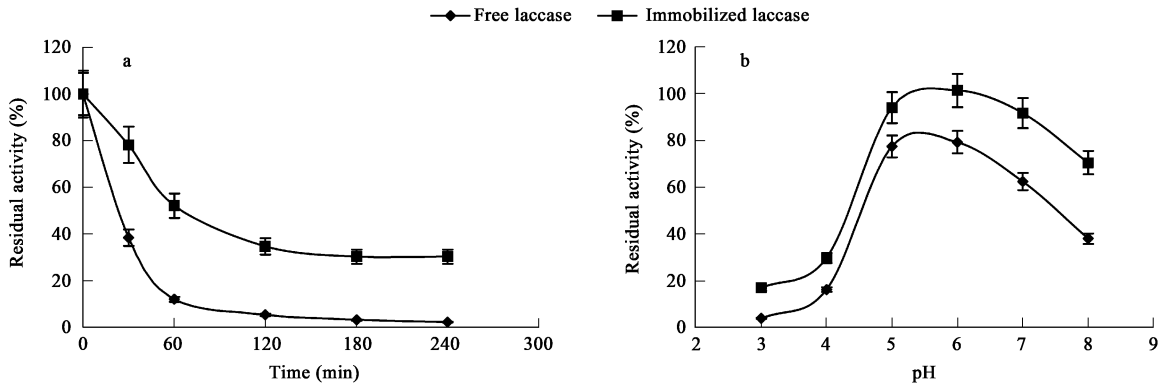

Fig. 5 Thermal (a) and pH (b) stabilities of free and immobilized laccase. Conditions: beads contained 2.0% (*m/V*) alginate; 2.0% (*m/V*) gelatin; 0.5% (*m/V*) PEG; cross-linked with 0.6% glutaraldehyde.

amount necessary to provide maximum protection to the enzyme, and thus, 0.5% was considered acceptable.

2.5 Thermal and pH stabilities of immobilized laccase

Enzyme immobilization often limits its freedom to undergo drastic conformational changes, and increases the probability of enzyme inactivation. The free and immobilized laccase were incubated at 60°C for 30–240 min; the thermal stabilities of the free and immobilized laccase in terms of the residual activities are compared in Fig.5a. The activity of free enzyme decreased significantly after 60 min incubation. After 240 min, the residual activities of the free and immobilized enzymes were 2.4% and 30.4% of the initial activity, respectively. Laccase immobilization in alginate/gelatin beads with PEG as additive led to a significant stabilizing effect towards heat denaturation.

The variation of residual activity of the free and immobilized laccase with pH is shown in Fig.5b. The immobilized laccase was stable in the pH range 5–7 with over 80% residual activity after incubating for 60 min at 25°C, while the free laccase was stable in the pH range 5–6. This indicated that the immobilization appreciably improved the stability of laccase.

3 Conclusions

The laccase (Denilite IIS) immobilized in alginate/gelatin beads showed reusability during repeated batch experiment; appropriate concentration of glutaraldehyde could increase the decolorization efficiency by cross-linking. PEG as an additive had a positive effect on the stability of the immobilized laccase, which enhanced the longevity of the decolorization. While Na-alginate, gelatin, and PEG were at the concentrations of 2.0%, 2.0%, and 0.5% (*m/V*), respectively, the beads with immobilized laccase had preferable decoloring capability after cross-linking with 0.6% glutaraldehyde, and the removal percentage of RRB exhibited about 50% even during the tenth decolorization cycles. The thermal and pH stabilities of the immobilized laccase were also appreciably improved compared to those of the free laccase. Further investigations should focus on effective utilization of the immobilized laccase on different types of dyes, which is expected to widen its application in bio-decolorization processes.

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