Degradation of 2,4-dichlorophenol by melamine amine cellulose-immobilized lacasses

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ABSTRACT: Epoxidized dialdehyde cellulose (EDC) was prepared and grafted with melamine to obtain melamine grafted epoxidized dialdehyde cellulose (EDC-melamine); the products were characterized by various methods and were used as carriers to immobilize laccase. Results show EDC-melamine can immobilize laccase effectively and have higher enzymatic activity compared with EDC. Furthermore, the enzymatic activity of EDC-melamine was found to be as high as 865 U•mg^{-1}, compared with 140U•mg^{-1} for EDC. The removal efficiency of 2,4-dichlorophenol (2,4-DCP) for EDC-melamine immobilized laccase was about 71.5% at 40°C for 4 h at 10.0 mg•L^{-1} and dosage of laccase = 0.2 g/L. The removal efficiency can remain greater than 63%, even after six cycles.

Application: This topic provides a new method to immobilize laccases. The product can be used to treat wastewater polluted by phenol and shows excellent performance.

Laccases are among a class of extensively studied oxidoreductase enzymes [1,2]. Due to their prominent ability to oxidize a range of substrates, they have been broadly employed in several industrial sectors, such as decolorization of dyes [3], pulp delignification [4], oxidation of organic pollutants [5], development of biosensors [6], and construction of biofuel cells [7]. Among all the applications, laccase shows excellent performance in organic pollutants degradation, especially for phenolic compounds [8]. However, there were some factors that greatly limited its application in the field of phenolic pollutants removal, such as short service life, low stability, and low reusability. Surface covalent immobilization was considered as a promising method to overcome these problems. At present, the main traditional methods of enzyme immobilization include physical adsorption [9], embedding [10], cross-linking [11], and covalent binding [12].

However, each of these methods has disadvantages, so immobilization is rarely used as a standalone technology. At present, application of several methods in combination became the main method to immobilize laccase to reduce activity loss [13]. It is extremely important to select a suitable substrate to act as carrier materials. Some natural polymers, such as cellulose [14,15] and chitosan [16], have become the main substrates for enzyme immobilization because of low cost and reusability.

In this research, epoxidized dialdehyde cellulose (EDC) and melamine grafted epoxidized dialdehyde cellulose (EDC-melamine) were synthesized and were used as carriers to immobilize laccase to treat 2,4-dichlorophenol (2,4-DCP) in aqueous solution. The products may have great potential for the practical application of cellulose immobilized enzymes in the field of environmental protection.

MATERIALS AND METHODS

Materials

Microcrystalline cellulose was obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Laccase Novozyme 51003 was provided by Novozymes A/S Co. (Tianjin, China; enzymatic activity was 1060 U/g) and 2,4-DCP was purchased from Aladdin Ltd. Co. (Shanghai, China). All other reagents were of analytical grade and used without pretreatment.

Preparation of epoxidized dialdehyde cellulose

A 6.0 g of microcrystalline cellulose was dispersed in 100 ml of 2.0 M sodium hydroxide (NaOH) under stirring, and 30 ml of epichlorohydrin was added while continuing stirring for 20 h at 25°C. The product was filtered and washed with distilled water and acetone until the pH reached approximately 7.0, and then dried at 90°C and stored.

The epoxidized cellulose suspension (50 ml with a concentration of 8%) was mixed with 50 ml of an aqueous solution of 7.9 g of sodium periodate ($NaIO_4$). The pH of the mixture was adjusted to 2.0 with 0.1 M H_2SO_4 in the dark to prevent the photo-induced decomposition of periodate. The reaction mixture was stirred at 35°C for 5 h, and then 4 ml of glycerol was added and continued to react for 1h. Finally, the product was filtered and washed several times with deionized water to remove iodine containing compounds, dried at 60°C, and then stored.

Preparation of EDC-melamine

A 0.5 g of oxidized cellulose was dissolved in 30 ml water while shaking at 80°C and pH was adjusted to 9.0 by 0.1 M NaOH. The 0.5 g of oxidized cellulose was added and allowed

to react for 12 h. Finally, 0.46 mmol sodium borohydride ($NaBH_4$) dissolved in 10 ml water was added and reacted for 4 h. The product was washed repeatedly to neutral pH and dried at 60°C.

Characterization

The Fourier transform infrared spectroscopy (FTIR) analyses of microcrystalline cellulose, epoxidized dialdehyde cellulose (EDC), and EDC-melamine were conducted using an FTIR spectrophotometer (Interspec 2020, Spectrolab Analytical; Newbury, UK) in the range of 4000–400 cm^{-1}. The surface morphologies of these samples were obtained by a scanning electron microscope (SEM) apparatus (Quanta 200, FEI; Hillsboro, OR, USA).

Laccase immobilization

Forty mg of EDC (or EDC-melamine) was dispersed in 10 ml acetic acid-sodium acetate buffer solution (pH=4.5), respectively, and then 20 µL 50% glutaraldehyde solution was added; the mixture was ultrasonicated for 10 min followed by the addition of a certain amount of laccase solution (2 mg/mL) under stirring for 3 h at 25°C. The laccase-immobilized carriers were repeatedly washed with the buffer solution until no laccase was found in the washing liquid. The laccase loading capacity was calculated using the following equation [17]:

$$\frac{M_0 - M_f - M_w}{M} \quad (1)$$

where M_0, M_f, and M_w are the amounts of laccase initially added, recovered in the supernatants, and recovered in the washing buffer, respectively; and M is the amount of carrier.

Enzyme assay

Laccase activity was measured at 420 nm by generation of ABTS radicals from the enzymatic oxidation of ABTS at 25°C using a CECIL 8600 spectrophotometer (Cecil Instruments Ltd.; Cambridge, UK). To determine the immobilized laccase activity, the assay mixture contained 0.2 mM ABTS, 100 mM sodium acetate buffer (pH=5.0) under shaking (100 rpm) at 30°C for 5 min [18]. After the incubation, the substrate oxidation by free and immobilized laccases was monitored at 420 nm (ε_{420}= 36.0 mM^{-1} cm^{-1}) using a UV-vis spectrophotometer [19]. One unit of laccase activity (U) was defined as the amount of enzyme needed to oxidize 1 µmol of ABTS per minute. The unit activity was defined by the following Eq. 2:

$$\frac{A \cdot V}{M_0 (t \cdot \varepsilon)} \times 10^6 \quad (2)$$

where A was the absorbance at 420 nm, V was the reaction volume, t was the reaction time, ε was the molar extinction coefficient for the ABTS oxidation, and M_0 was the mass of the immobilized laccase.

The total activity recovery R (percent) of the immobilized laccase was calculated according to the following Eq. 3 [20].

$$R(\%) = \frac{A_i}{A_f} \times 100 \quad (3)$$

where A_i and A_f were the activity of laccase immobilized on carriers and the starting activity of free laccase, respectively.

Removal of 2,4-DCP by immobilized and free laccases

The removal of 2,4-DCP by laccase was performed in a reaction mixture containing 50 ml of 10 mg/L 2,4-DCP in sodium acetate buffer solution (or NaOH). A suitable amount of carriers for immobilized laccase (5mg–30mg) was added and the mixture was shaken slowly for 4 h. Then, the upper solution was filtered, and the residual concentration of 2,4-DCP was exclusively measured by high performance liquid chromatography (HPLC) using a reversed-phase column (ODS-18, 150 mm × 4.6 mm) and methanol (chromatographic grade) as mobile phase at a flow rate of 0.8 ml•min^{-1}.

The effect of pH on the removal of 2,4-DCP was investigated within the pH range of 3.0–8.0 at 40°C, and the effect of temperature was investigated at pH 4.0 and 25°C–60°C. Blank test was also carried out to assess the effect of EDC, EDC-melamine, and free laccase on the removal efficiency of 2,4-DCP. Relative activity was used for laccase activity assay in this work, where laccase activities observed at the optimum conditions were defined as 100%.

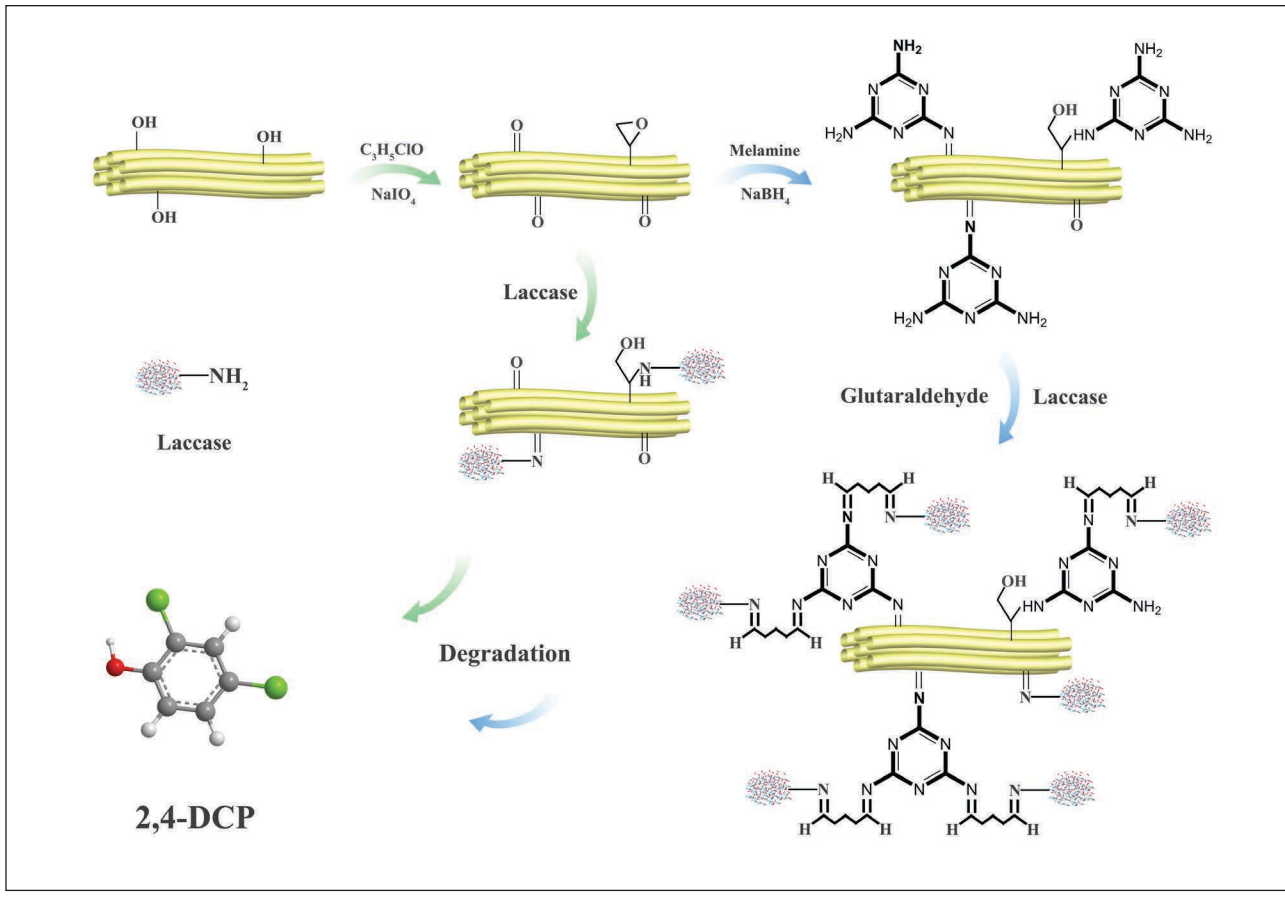
Reusability of immobilized laccase

The reusability of immobilized laccase was accessed using 2,4-DCP as a substrate. After each reaction run, the laccase-immobilized carriers were washed with the previously described buffer solution until no 2,4-DCP was detected in the wash solution.

RESULTS AND DISCUSSION

Synthesis mechanism

Figure 1 illustrates the synthesis process of EDC-melamine and the immobilization of laccase. The hydroxyl groups of cellulose reacted with microcrystalline cellulose to produce oxidized cellulose, since the steric hindrance of the hydroxyl groups at C_2 and C_3 was relatively large and microcrystalline cellulose mainly reacts with the hydroxyl group of C_6 under alkaline conditions. The aldehyde was introduced in epoxidized cellulose by partial oxidation with periodate, which promoted cleavage of the C_2-C_3 bond, thus forming two aldehyde groups per anhydroglucopyranose unit, and EDC was produced. Thereafter, amino groups reacted with epoxy groups through a nucleophilic reaction. Meanwhile, a Schiff base reaction occurred between melamine and the dialdehydes, and the EDC-melamine was synthesized. The products were stabilized by reduction with sodium borohydride. Fi-



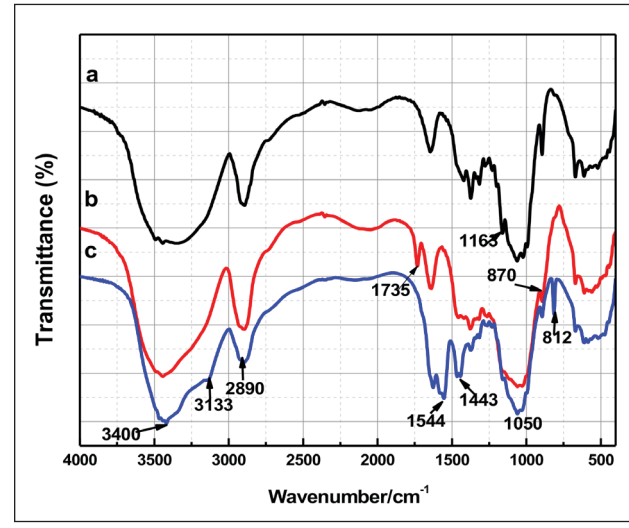
1. Reaction mechanism for the preparation of carriers immobilized laccase

nally, laccase covalently immobilized onto EDC-melamine (Fig. 1).

Properties of immobilized laccase

FTIR characterization

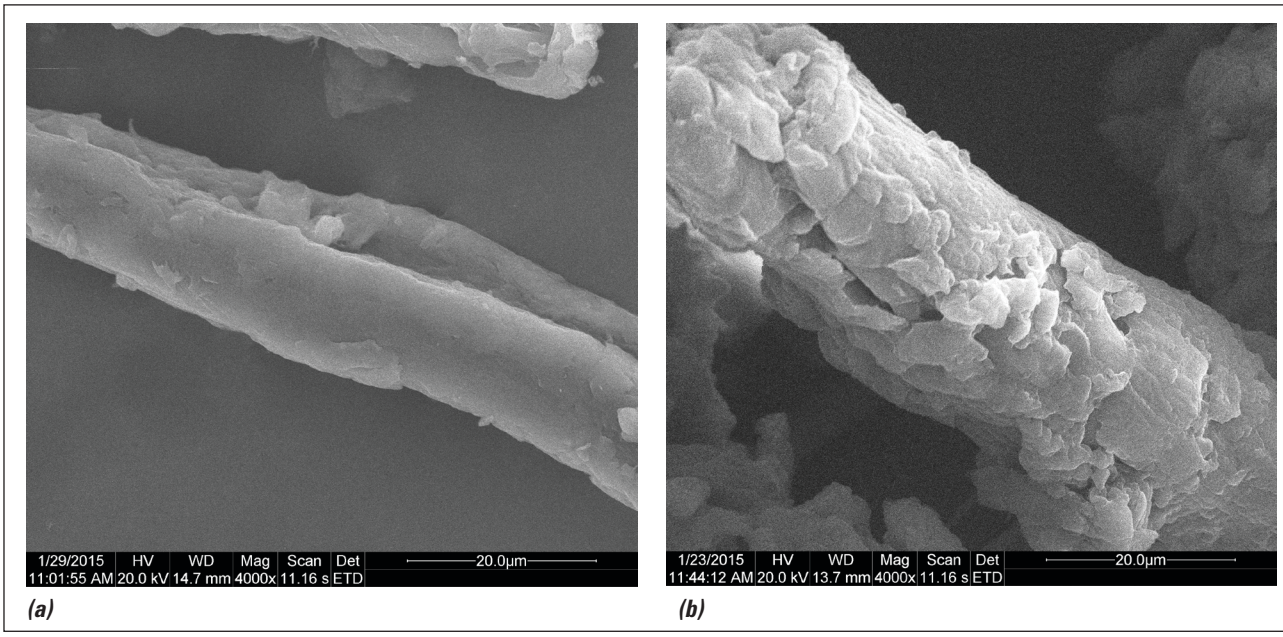
The FT-IR spectra of (a) microcrystalline cellulose, (b) oxidized cellulose, and (c) EDC-melamine are presented in **Fig. 2**. Compared with microcrystalline cellulose (Fig. 2, black line), the main changes in the FT-IR spectrum of oxidized cellulose (red line) is the new peak at 1735 cm^{-1}, a carbonyl stretching peak following oxidation [21]. The peak at 870 cm^{-1} in oxidized cellulose was due to the epoxy groups. Meanwhile, comparing EDC-melamine (blue line) and microcrystalline cellulose, the newly appeared peak at 3133 cm^{-1} in EDC-melamine may be attributed to the bending vibration of amine (N-H), while the stretching vibration peaks of C-N at 812 cm^{-1},1443 cm^{-1} and 1554 cm^{-1} indicate success of amine grafting onto the cellulose [22].



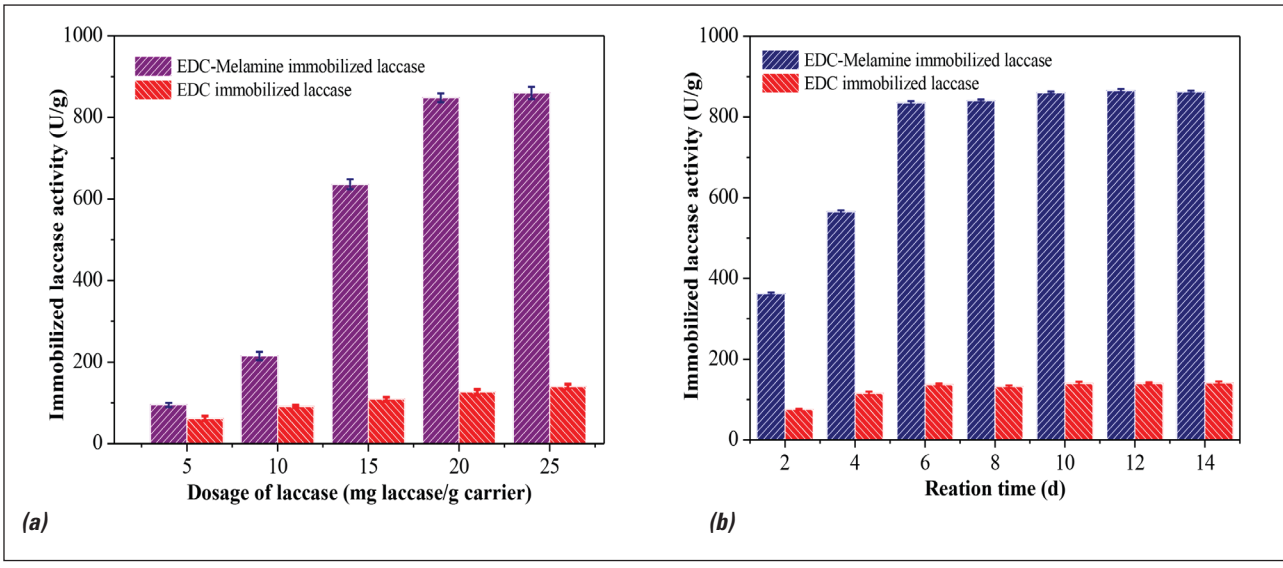
2. Fourier transform infrared spectroscopy (FTIR) analyses for (a) microcrystalline cellulose, (b) epoxidized dialdehyde cellulose (EDC), and (c) EDC-melamine.

SEM characterization

The SEM images of microcrystalline cellulose and EDC-melamine are shown in **Fig. 3**. It is obvious that the surface of cellulose changed significantly before and after modification. The surface of microcrystalline cellulose appeared smooth and simple, but the surface becomes rougher and displays a scaly appearance after modification (Fig. 3b). This increased the specific surface area of the product with much more functional groups exposed to the interface to enhance the reaction probability between laccase and the carrier.



3. Scanning electron microscope (SEM) images of (a) microcrystalline cellulose and (b) EDC-melamine.



4. Effect of (a) enzyme dosage and (b) reaction time on the activity of immobilized laccase.

Effect of enzyme dosage and reaction time on laccase immobilization

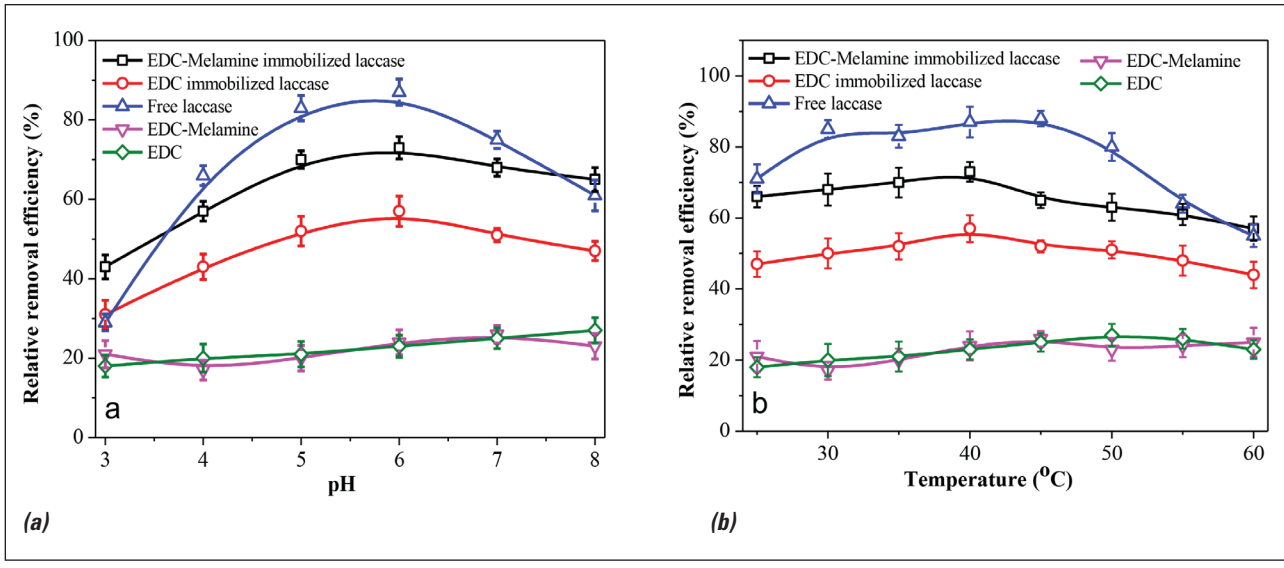
The relationship between laccase dosage and loading capacity of the carriers can be seen in **Fig. 4**. The activity of immobilized laccase increased with the increase of laccase dosage for both carriers (Fig. 4a), but the amount of increase reduced at high dosages. This may be due to the saturation of binding sites. Research found the optimal ratio were 20 mg•g^{-1} and 15 mg•g^{-1} for EDC-melamine and EDC, respectively. The optimal loading capacity of the carriers can be achieved under optimal ratio of 865 U•mg^{-1} for EDC-melamine and 140 U•mg^{-1} for EDC, respectively. Thus, EDC-melamine displays stronger binding ability for immobilized laccase than EDC.

The effect of reaction time was examined under the optimal laccase dosage. The immobilization time was set in a range of 2–14 h, and the interval time between each test was 2 h. As can be seen from Fig. 4b, the activity of immobilized laccase initially increased when time increased from 2 h to 6 h, but showed no significant change thereafter, due primary to steric constraints [23].

Removal of 2,4-DCP

Effect of pH on the degradation of 2,4-DCP

As can be seen from **Fig. 5a**, the highest removal efficiency was achieved at pH = 5.0–6.0 for the laccase immobilized laccase and free laccase. The highest removal efficiency for free



5. Effect of (a) pH and (b) temperature on relative removal efficiency of 2,4-dichlorophenol (2,4-DCP).

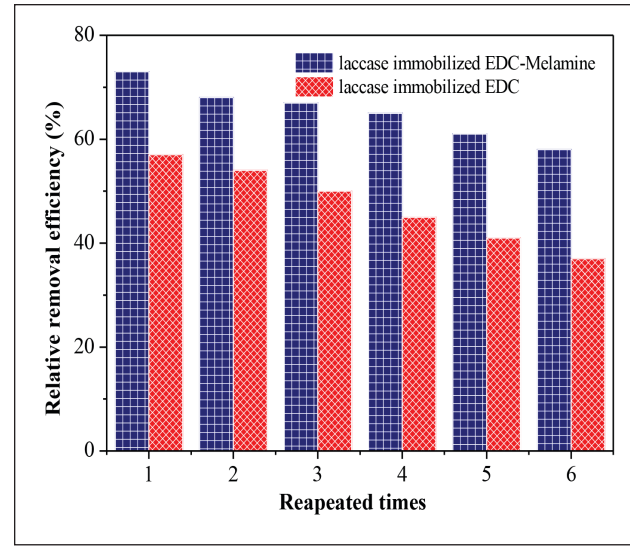
laccase was about 87%, while it was 71.5% and 57% for EDC-melamine immobilized laccase and EDC immobilized laccase. Compared with the free laccase, the immobilized laccase showed better stabilities to environmental change. The suitable pH range for removal of 2,4-DCP was 5.0–8.0 for carriers immobilized laccase. To the carriers, the total removal efficiency was attributed to both adsorption caused by the blank carrier and the degradation caused by the immobilized laccase. Both carriers have adsorption effect on laccase, and there was no obvious difference between them. The adsorption effect was perhaps mainly from physical adsorption. The specific surface of the carriers plays a key role during the physical adsorption. The adsorption removal efficiency by the two carriers was minimum because of the small variations in the specific surfaces between these two carriers.

Effect of temperature on the degradation of 2,4-DCP by laccase

The effect of temperature on removal of 2,4-DCP by immobilized laccase is shown in Fig. 5b. As can be seen from the figure, the optimum temperature was about 40°C for EDC-melamine and EDC immobilized laccase, and 45°C for free laccase. For the immobilized laccase, the impact of temperature was not obvious; for example, the range was about 12% when temperature increased from 30°C to 55°C for EDC-melamine immobilized laccase, while it was 21% for the free laccase. The suitable range of temperatures for oxidation of 2,4-DCP by the immobilized laccase was wider than that for the free laccase.

Reusability of immobilized laccase

Reusability was a significant feature for the immobilized enzyme. As shown in **Fig. 6**, the removal efficiency of 2,4-DCP by EDC-melamine immobilized laccase remained 63% even after six runs. The decrease in removal efficiency may come from two reasons: 1) the decrease or destroy of the grafted functional groups, and 2) the decrease of adsorption ability due to the wreck of the surface structure.



6. The reusability of laccase immobilized EDC and EDC-melamine for the removal of 2,4-DCP.

CONCLUSIONS

EDC and EDC-melamine were synthesized and were used as laccase carriers. Results show EDC-melamine has a strong binding ability for laccase and the immobilized laccase has high enzymatic activity. The enzymatic activity was 865 U•mg^{-1}, while it was about 140 U•mg^{-1} for EDC. EDC-melamine immobilized laccase showed a high removal efficiency for 2,4-dichlorophenol of 73% at 40°C for 4 h at 10.0 mg•L^{-1}, while it was 57% for EDC immobilized laccase. Both carriers showed better stability for pH and temperature compared with free laccase. Furthermore, the EDC-melamine

immobilized laccase also showed excellent reusability. The products have great application potential in the field of immobilized laccase. **TJ**

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ABOUT THE AUTHORS

My (Wang's) research field is related to the modification of biomaterials and their application in wastewater treatment. The research here differs from previous research in this area in that the amount of the immobilized laccase was much lower.

The most difficult aspect of this research was how to tightly keep the laccase on the modified cellulose. To overcome this, we modified the cellulose with different groups and finally found the optimal way. One personally interesting discovery was that the modified cellulose has excellent performance on binding laccase and can maintain its activity. A surprising aspect of the research was that the immobilized laccase can provide higher enzymatic activity and can effectively treat phenol-polluted wastewater.

This research can provide a new way for mills to approach the treatment of phenol-polluted water. Our next step is to find a more effective way to realize the modification process.

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