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Removal of 2,4-dichlorophenol by chitosan-immobilized laccase from *Coriolus versicolor*

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ABSTRACT

Laccase from *Coriolus versicolor* was immobilized on chitosan using glutaraldehyde as a cross-linking agent. After immobilization, laccase retained 52.2% of its original activity and was used to study 2,4-dichlorophenol (2,4-DCP) removal from aqueous solutions. The optimum pH for 2,4-DCP removal by the immobilized laccase was ~5.5, which was lower than the optimal pH of 6.0 for free laccase catalysis. Immobilized laccase also had an atypically wide optimum temperature range for catalysis of 35–45 °C. Immobilized laccase could be used repeatedly, and its removal efficiency for 2,4-DCP remained above ~50% for up to six usages.

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1. Introduction

Chlorophenols comprise a group of organic pollutants that are resistant to degradation [1] and have been used historically as wood preservatives, antirusting agents, fungicides, and pesticides. For instance, 2,4-dichlorophenol (2,4-DCP) exists in wastewater from pesticide, fuel, and plasticizer production and has been listed as a priority control pollutant in both the U.S. and China. Accordingly, much research is focused on the development of biotechnological methods to reduce chlorophenol pollution.

Laccase is an oxidoreductase that contains Cu^{2+} and is capable of catalyzing the degradation of many phenolic compounds [2]. Its application in environmental fields has been studied extensively [3]. Degradation of chlorophenols by laccase is dependent on oxygen. However, oxidation of chlorophenols results in the formation of radicals and reactive quinoids, which can polymerize and decrease their solubility and toxicity. The Cu^{2+} contained in laccase is reduced during catalytic phenol oxidation, and then being oxidized:

$$4(Cu^{2+})\text{laccase} + \text{phenol} \rightarrow 4(Cu^{+})\text{laccase} + \text{radical} + 4H^{+}$$

$$4(Cu^{+})\text{laccase} + O_2 + 4H^{+} \rightarrow 4(Cu^{2+})\text{laccase} + 2H_2O$$

Electrons are transferred from substrates to oxygen by laccase. In the process of this oxidation, two free radicals randomly combine to form a dimer and then undergo isomerization to form stable aryl chemicals. Furthermore, dimers, trimers, and multimers can be formed by similar mechanisms. Dec and Bollag claimed that the release of the chlorine ion from a chlorophenol substrate can be directly attributed to the oxidization of coupled radicals [4].

However, it easily loses its activity in aqueous solutions [5], which limits its use. Immobilization of laccase could prevent this loss of activity and make the enzyme more effective for chlorophenol removal. Carriers including silica gel, sodium alginate, chitosan, active carbon, calcium alginate, and cellulose could be used to immobilize laccase. Although many experiments have been conducted with several kinds of enzymes immobilized on chitosan, i.e., tyrosinase [6,7], lipase [7,8], phosphor lipase [9], cellobiase [10] and β-glucosidase [11], the effectiveness of laccase immobilized on a chitosan carrier has not been reported yet.

Methods for enzyme immobilization include embedment, adsorption, and covalent linkage or cross-linking [12]; however, each of these methods has disadvantages. For instance, inorganic sorbents may promote denaturation of immobilized laccase and decrease laccase activity. Moreover, covalent or cross-linking immobilization strategies generally lead to severe losses in laccase activity [13]. Hence, the maintenance of high enzyme activity after immobilization is a major concern. The active site of laccase is essential for proper enzyme structure and catalysis [14], and application of several methods in combination might properly immobilize the enzyme and deter its loss of activity.

In this work, laccase was immobilized on chitosan by glutaraldehyde (as cross-linking agent), and the optimum conditions for laccase immobilization and 2,4-DCP removal efficiency

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Table 1
Effect of glutaraldehyde concentration on the activity of immobilized laccase.

Glutaraldehyde concentration (v/v, %)	Immobilized laccase activity (U mg^{-1})
1	102
2	118
3	142
4	166
5	180
6	148
8	104

were assessed, which provide some guidance on further using immobilized laccase to removal 2,4-DCP in sewage in view of degradation efficiency and economical reasonability.

2. Experimental procedures

2.1. Materials and equipment

Laccase from *Coriolus versicolor* was provided by Fluca Co. (Switzerland); 2,4-DCP was purchased from Aldrich Ltd. Co. (United States); potassium ferricyanide ($K_3[Fe(CN)_6]$, pure analytical grade) was purchased from Wenzhou Chemical Product Factory (China); glutaraldehyde (50% analytical grade) was obtained from Beijing Jinli Fine Chemicals Ltd. Co. (China); O-tolidinein was purchased from Beijing Chemical Factory (China); 4-amido antipyrin was purchased from Beijing Xizhong Chemical Engineering Factory (China); chitosan (90.3% deacetylation, pure chemical) was purchased from Qingdao Haolizhong Bio-tech. Co. (China); all other chemicals used were analytically pure.

Sample absorbances were measured using a UV757 spectrophotometer (Shanghai Accurate Scientific Equipment Ltd. Inc., China); pH values were measured with a pHS-3A pH-meter (Hangzhou Wanda Instrument Factory, China); a 79-3 Magnetic Thermostat Mixer was purchased from Shanghai Caoxing Wireless Components Factory (China); immobilized laccase was characterized by Fourier transform infrared spectroscopy (FTIR) with a Magna-IR 750 model instrument (Nicolet, USA).

2.2. Preparation of immobilized laccase

The optimal conditions to immobilize laccase were as follows: 1.0 g chitosan was dissolved in 2% acetic acid solution and diluted with distilled water to 200 mL; a 5.0 g L^{-1} solution of chitosan was prepared. The chitosan solution was added to 2 mol L^{-1} NaOH, resulting in the formation of white flocculent deposit, which was then washed with water to neutrality and filtered through a wet chitosan carrier. Twenty mL of a 5% glutaraldehyde aqueous solution were added to the carrier, mixed for 8 h, allowed to settle overnight, filtered, and washed five times to remove unassociated glutaraldehyde. The product obtained was a wet yellow powdery carrier of chitosan cross-linked with glutaraldehyde. The carrier was added to 50 mL of 0.4 g L^{-1} laccase solution; 5 mL of HAc-NaAc buffer solution (pH 4.37) were added and mixed at room temperature for 16 h and incubated at 4 °C overnight. After filtration, the immobilized laccase was obtained.

2.3. Determination of laccase activity

A unit of laccase activity (U) is defined as the mass of laccase required to increase the absorbance by 0.001/μmol (substrate)/min at 25 °C [15]. O-tolidinein (1 mmol L^{-1}) was added as substrate into the laccase solution, and HAc-NaAc buffer solution (pH 4.0) was used to dilute the mixture. Laccase activity was measured at 600 nm with a scan time of 5 min. Similarly, immobilized laccase was mixed with the substrate and buffer solution for 10 min at 25 °C, and then the upper mixture was filtered and its absorbance was measured each minute at 600 nm. The activity of immobilized laccase was defined as U g^{-1}, i.e., unit activity per gram of immobilized laccase.

Activity was calculated as follows:

$$\text{activity of immobilized laccase (U/mg)} = \frac{\text{total activity of immobilized laccase}}{\text{dry weight of immobilized laccase}}$$

$$\text{recovery of laccase activity (\%)} = \frac{\text{activity of immobilized laccase}}{\text{activity of free laccase in solution}} \times 100$$

2.4. Effect of pH and temperature on the degradation of 2,4-DCP by laccase

Briefly, 25 mL of 50 mg L^{-1} 2,4-DCP and 0.8 g immobilized laccase were added to HAc-NaAc buffer solution (or NaOH) at a pH of 3, 4, 5, 5.5, 6, 7, 8, and 9 and mixed slowly at room temperature for 6 h. The upper solution was filtered, and the residual concentration of 2,4-DCP was exclusively measured by UV757 Ultraviolet-visible Spectrophotometer based on Standard methods for the examination of water and wastewater The procedures for measuring 2,4-DPC are as follows in brief: to add 0, 2, 4, 6, 8 and 10 mL 2,4-DCP solution (50 mg L^{-1}) to each beaker, respectively, and dilute to 50 mL with deionized water, to add $NH_3{\cdot}H_2O$ 1.25 mL 0.5 mol L^{-1} into each beaker, pH is adjusted by H_3PO_4 and Na_2HPO_4 buffer to 7.9 ± 0.02. And then, 0.5 mL, 2% 4-aminoantipyrine and 0.5 mL 80 g L^{-1} $K_3Fe(CN)_6$ are added into each mixturing, well mixing and stewing for 15 min, and then measure their adsorbance with UV757 spectrophotometer. The scanning wavelength ranges 200–800 nm and 510 nm is 2,4-DCP's characterizing wavelength, which has been proved there are no significant adsorption by laccase, 4-aminoantipyrine and $K_3Fe(CN)_6$, etc. [16].

To determine how temperature affected laccase activity, 25 mL of 50 mg L^{-1} 2,4-DCP and 0.8 g immobilized laccase were added to HAc-NaAc buffer solution (pH 5.5) at 20 °C for 6 h, and then the residual concentration of 2,4-DCP was measured. This procedure was also conducted at water bath temperatures of 30, 35, 40, 45, 50, and 60 °C.

To determine how carrier adsorption affected removal efficiency of 2,4-DCP, blank carrier was used in place of immobilized laccase.

3. Results and discussion

3.1. Optimum conditions for laccase immobilization

3.1.1. Effect of glutaraldehyde concentration and cross-linking on laccase immobilization

The immobilization of laccase was conducted as described in Section 2.2; glutaraldehyde concentrations ranged from 1% to 8%. The results are shown in Table 1. The activity of immobilized laccase increased with the concentration of glutaraldehyde; peak activity was reached using 5% glutaraldehyde.

In addition to cross-linking, glutaraldehyde also denatured laccase. Although high concentrations of glutaraldehyde promoted its reaction with the amino radical of chitosan and formed active radicals, concentrations that were too high led to decreases in the ability of chitosan to effectively link with laccase, since amido on chitosan couples within or between the glutaraldehyde molecule in the case of high concentration, and the ability of carrier combing with to enzyme would decrease significantly [14].

The activity of immobilized laccase increased with cross-linking time and reached a maximum at 10 h (Table 2). Glutaraldehyde

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