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Enhancing catalytic performance of laccase via immobilization on chitosan/CeO_2 microspheres

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ABSTRACT

In this study, laccase from *Trametes versicolor* was immobilized onto chitosan/CeO_2 microspheres (CCMS) by adsorption or covalent binding after activating the amine groups of chitosan with glutaraldehyde (GA). The results indicated that the laccase loading on chitosan/CeO_2 microspheres was approximately 73 mg/g under the optimum conditions (pH 5.4, 6 h), and the activity recovery was 66.9%. In comparison with free laccase, the thermal and operational stabilities of the immobilized laccase were significantly improved. The catalytic activity of the immobilized laccase was also demonstrated by the deodorization of two reactive dyes (methyl red and orange II). The laccase immobilized on CCMS was very effective for the removal of textile dyes from an aqueous solution. The removal rates of methyl red and orange II by the immobilized laccase were 83.3% and 92.6%, respectively, which are much higher than that of free laccase (i.e., 49.0% and 67.1%, respectively).

1. Introduction

Q2 Synthetic dyes are widely used in textile, cosmetics, drug, paper, pulp, plastic and printing industries. The effluents of industrial wastewater contaminated with dyes are harmful to the environment and human health [1,2]. Recently, enzyme-based treatments for the removal of reactive dyes have received much attention because they offer some advantages over physical and chemical processes, such as mild treatment condition, high efficiency of substrate removal, and the ability to handle large volumes of effluents [3,4].

Laccase is a type of copper-containing oxidoreductases produced by numerous plants, fungi, and bacteria. In particular, *Trametes versicolor* laccase (Tvl) is a fungal laccase containing four copper ions located in sites denoted T1, T2, and T3 in the active center of the enzyme [5]. This laccase can oxidize a wide range of xenobiotic compounds, such as synthetic dyes, chlorinated phenolics, and polycyclic aromatic hydrocarbons [6]. However, the low stability and poor reusability of this laccase further hamper its industrial application. Immobilization technology is considered to be the most effective method for improving the stability of an enzyme [7,8]. The use of immobilized enzymes normally offers several advantages over free enzymes, such as higher stability, more convenient to handle, easier separation from the product, and more efficient recovery and reuse of costly enzymes [9]. Although these achievements have been realized, some issues including the loss of enzyme reactivity in harsh chemical immobilization conditions, inefficient loading, leakage during usage, and the hindrance of mass transfer caused by encapsulation still limit the applicability of immobilized laccase [10].

The selection of a suitable carrier material is a key factor in enzyme covalent immobilization [11]. Chitosan (CS) is a partially deacetylated polymer of N-acetyl glucosamine that can be obtained via alkaline deacetylation of chitin [12,13]. In addition, CS has been widely applied due to its attractive properties, such as biocompatibility, biodegradability, and nontoxicity. CS consists of a β-(1,4)-linked-D-glucosamine residue with the amine group randomly acetylated [13]. The amine and —OH groups endow chitosan with excellent affinity to a variety of proteins, which makes it applicable in many areas and easily available for immobilization of the enzyme [14]. Chitosan-based materials are used as supports in various forms, such as powder, flake, and gel, with different geometrical configurations for immobilization of several enzymes [15]. In the last few decades, chitosan containing magnetic supports, especially magnetic microspheres, were investigated for enzyme immobilization using physical and chemical immobilization techniques [16–19].

Cerium (IV) dioxide (CeO_2), which is commonly known as ceria, is an important material for solid oxide fuel cells and catalytic applications due to its enhanced capability for absorbing and releasing oxygen via the Ce^{4+}/Ce^{3+} redox cycle [20]. Cerium oxide nanoparticles have been extensively employed in various engineering and

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biological applications [21]. Catalysts based on ceria are promising for low temperature CO oxidation, soot oxidation, and water–gas shift reactions [22]. The oxygen delivery capacity of the ceria nanoparticles embedded in biocompatible chitosan microspheres facilitated enzyme stabilization and operation in oxygen free conditions [23].

In this study, laccase was immobilized onto chitosan/CeO_2 microspheres. To the best of our knowledge, there are few examples where this material is used in the area of enzyme immobilization. Therefore, the removal of reactive dyes by laccase immobilized on chitosan/CeO_2 microspheres (CCMS) was investigated. The results indicated that the performance of immobilized laccase for removing reactive dyes was excellent. Therefore, this method provides a novel technique for removing dyes in contaminated water, especially industrial wastewater.

2. Experimental

2.1. Materials

Laccase (EC1.10.3.2: p-diphenol: dioxygen oxidoreductase; approximately 120 LAMU/g) from *Trametes versicolor*, chitosan (CS) (deacetylation degree, D.D. = 89.2%, Mv = 2.8×10^5) was purchased from AK Biotech Ltd. 2,2′-Azino-bis-(3-ethyl-benzothiazoline-6-sulphonic acid) (ABTS) was purchased from the Sigma Chemical Co. Glutaraldehyde (50%, v/v, aqueous solution) was obtained from the Shanghai Damao Reagent Co. CeO_2, methyl red and orange II were purchased from the China National Medicine Corporation Ltd. The solvents and reagents were of analytical reagent grade and used without further purification.

2.2. Preparation of chitosan/cerium dioxide microspheres

The preparation of chitosan/cerium dioxide microspheres was performed using a suspension crosslinking technique, which has been used in the preparation of chitosan microspheres [24]. In a typical procedure, 100 mg of CS was dissolved in acetic acid solution (1%, 10 mL aqueous phase) containing 50 mg of dry cerium dioxide. Then, this solution was added dropwise to the dispersion medium, which was composed of paraffin wax, petroleum ether (25/35, v/v) and 3 mL of emulsifier (Span-80). During the process, the dispersion medium was stirred with a mechanical stirrer at 1000 rpm at room temperature. One hour later, an additional 3 mL of 5% glutaraldehyde (GA) was added to the dispersion medium to crosslink the microspheres. After the solution of GA had been added, the stirring was continued for an additional hour to allow for curing. The prepared chitosan/CeO_2 microspheres were filtered and rinsed several times with petroleum, ethanol and phosphate buffer (pH 5.0) to remove any potential impurities. For comparison, the pure chitosan microspheres were also prepared under similar conditions, except that the acetic acid solution contained no CeO_2.

2.3. Properties of the chitosan/cerium dioxide microspheres

The morphological characterization of the pure chitosan microspheres (CSMS) and chitosan/cerium dioxide microspheres (CCMS) was evaluated using a scanning electron microscope (DVO-80) operating at 10 kV. The average diameters of the CSMS and CCMS were determined by measuring the diameters of more than 150 microspheres.

The surface area of the CSMS and CCMS was determined using the N_2 sorption isotherm and application of the BET theory.

The FTIR spectra of chitosan, cerium oxide, and the chitosan/cerium dioxide microspheres were recorded using a FTIR spectrophotometer (360 NICOLET INC., USA).

Differential scanning calorimetry (DSC) studies were performed using a Perkin Elmer DSC 7 (USA) at a heating rate of 10 °C/min in a nitrogen atmosphere (20 mL^{-1}) at 30–400 °C.

Crystallographic studies of the pure CeO_2 and CeO_2/chitosan microspheres were performed on an X-ray diffractometer (XRD) (D/Max-IIIC, Japan) using Cu-kα radiation (λ = 1.5406 Å).

2.4. Immobilization of laccase

The immobilization of laccase on CSMS (CSMS-laccase) and CCMS (CCMS-laccase) was performed as follows: first, the microspheres were activated by 5% glutaraldehyde. Second, the activated microspheres with GA were transferred into 20 mL of a 1 mg/mL laccase solution. The immobilization time was varied between 2.0 and 24 h, and the activated microspheres were contained in enzyme solution with a stirring rate of 100 rpm at 4 °C. Finally, the enzyme immobilized microspheres were filtered from the reaction medium. The physically bound enzyme was removed by washing with a phosphate buffer (pH 7.0, 50 mmol/L). The enzyme immobilized beads were stored at 4 °C in the same fresh buffer prior to use.

The amount of immobilized laccase on the glutaraldehyde-activated microspheres was determined by measuring the initial and final concentrations of protein within the immobilization medium using Coomassie Brilliant Blue, as described by Bradford [25]. The laccase immobilization capacity was defined as the amount of bound protein per gram of the microspheres. The amount of bound protein (A_e) was calculated from the following equation:

$$Ae\ (\%) = \frac{[(C0 - C)V - CwVw]}{W} \times 100 \quad (1)$$

where C_0 and C are the initial and final concentrations of protein (mg/mL), respectively, V and V_w are the solution and washing volumes (mL), respectively, C_w is the concentration of protein in the washings, and W is the mass of the CS/CeO_2 microspheres (g).

2.5. Activity assays of free and immobilized laccase

Enzymatic activities for free and immobilized laccase were determined by monitoring the oxidation of 2,2′-azino-bis-(3-ethyl-benzthiazoline-6-sulfonic acid) (ABTS) to its cation radical ($ABTS^+$) at 420 nm ($\varepsilon_{max} = 3.6 \times 10^4$ L/(mol cm)). The reaction mixture consisted of 1 mmol/L ABTS in a phosphate buffer (pH 6.0) and a suitable amount of free or immobilized enzyme, and the reaction time was 5 min. During the process, the increase in the absorbance at 420 nm was measured using a UV-2450 spectrophotometer (Shimadzu, Japan). The molar extinction coefficient of $ABTS^+$ is 36,000 L/(mol cm).

These activity assays were carried out over a pH range of 3.0–8.0 (in phosphate buffer) at 35 °C, and a temperature range of 15–65 °C was employed to determine the pH and temperature profiles for the free enzyme and CCMS-laccase. The results were converted to relative activities (percentage of the maximum activity obtained in that series). Each set of experiments was performed in triplicate, and the arithmetic mean values were calculated.

The activity recovery of the immobilized enzyme was calculated from the following equation:

$$R_a\ (\%) = \left(\frac{A_i}{A_f}\right) \times 100 \quad (2)$$

where R_a is the activity recovery of the immobilized enzyme (%), A_i is the activity of the immobilized enzyme (U) and A_f is the activity of the same amount of free enzyme in solution as that immobilized on the microspheres (U). One activity unit of laccase was defined

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