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Cross-linked tyrosinase aggregates for elimination of phenolic compounds from wastewater

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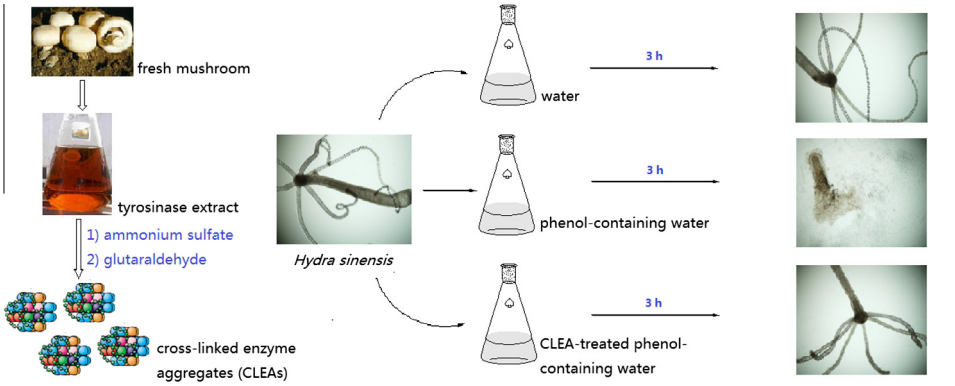
HIGHLIGHTS

- Mushroom tyrosinase was immobilized as cross-linked enzyme aggregates (CLEAs).
- Phenolic compounds in water can be efficiently eliminated by tyrosinase CLEAs.
- Complete conversions were achieved within 0.5–3 h in a batch reactor.
- CLEA/alginate beads are feasible to be used in continuous dephenolization processes.
- After treatment, the toxicity of the phenolic solution was remarkably diminished.

GRAPHICAL ABSTRACT



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ABSTRACT

A novel, practical and inexpensive immobilization method has been developed for mushroom tyrosinase to be used for enzymatic treatment of phenolic wastewater. Catalyzed by the enzyme immobilized in the form of cross-linked enzyme aggregates (CLEAs), phenolic compounds such as phenol, *p*-cresol, *p*-chlorophenol and bisphenol A can be efficiently eliminated, with a complete conversion obtained within 0.5–3 h, superior to other processes catalyzed by the same enzyme which is either free or immobilized with traditional carrier-bound immobilization methods. The effects of reaction time, pH, enzyme dosage and initial concentration of the phenol solution were examined. The sequence of dephenolization rate (*p*-cresol > *p*-chlorophenol > phenol) was in accordance with the substrate selectivity of the enzyme. The reusability of the CLEAs has been tested in a batch reactor for each phenol. In a continuous stirred tank reactor, the CLEAs encapsulated into calcium alginate gels were effective for removing phenol for at least 26 h. The toxicity of the phenol-containing solution was remarkably diminished after treatment with the tyrosinase CLEAs, as demonstrated by the *Hydra sinensis* test.

1. Introduction

Phenolic compounds (i.e., phenol, alkyl-substituted and halogenated phenols, and their derivatives such as bisphenol A) are ubiquitous environmental pollutants generated in the manufacture of many industrial products such as papers, metals, resins, plastics, pesticides, and pharmaceuticals. Because they are frequently discharged into lakes, rivers and oceans in large quantities by industries, these toxic pollutants have adversely affected our environment and ecology. Therefore, removal of these aromatic pollutants from industrial effluents has been a critical issue.

There has been a growing recognition that enzymes can be used in remediation processes that target these phenolic pollutants for treatment. Various oxidoreductive enzymes, such as laccases,

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peroxidases and tyrosinases, have already been utilized for the removal of phenolic compounds from polluted water (Durán and Esposito, 2000; Torres et al., 2003; Ahuja et al., 2004; Ba et al., in press; Kudanga et al., 2012; Mukherjee et al., 2012). The use of tyrosinase for this application was first investigated by Atlow et al. (1984), and the principle behind this is that the enzyme catalyzes the hydroxylation of monophenols to *o*-diphenols and subsequent oxidation of *o*-diphenols to *o*-quinones, the latter then undergoing a non-enzymatic polymerization to form water-insoluble aggregates which can be removed by sedimentation or filtration. A major attraction of tyrosinase-mediated dephenolization over the processes catalyzed by other enzymes is that tyrosinase does not require stoichiometric quantities of other reagents (such as the costly hydrogen peroxide used by peroxidases), apart from molecular oxygen as an oxidant. The potential applications of tyrosinase in the bioremediation of phenolic contaminants have been summarized in a very recent review by Mukherjee et al. (2012).

Given that immobilization can render the enzyme a better stability and applicability for industrial processes, trials of immobilizing tyrosinase with different methods for the purpose of phenol elimination have been reported, including covalent immobilization on magnetite (Wada et al., 1995), on nylon membranes (Burton et al., 1998), on siliceous supports such as silica gel and calcium and sodium aluminosilicates (Seetharam and Saville, 2003), and adsorption on hydrophobic synthetic (polysulphone) capillary membranes (Burton et al., 1998). The use of chitosan as an additive (Sun et al., 1992), a coagulant/adsorbent (Wada et al., 1995), or a support for enzyme immobilization (Abdullah et al., 2006) as well as for "in situ" removal of the *o*-quinones within the immediate environment of the soluble (Yamada et al., 2005) and immobilized (Edwards et al., 1999) enzyme has been successfully introduced. In addition, Girelli et al. (2006) have developed an immobilized enzyme reactor based on tyrosinase immobilized on aminopropyl-controlled pore glass for the removal of a series of phenols.

The major objective of this study was to introduce a new immobilization method to mushroom tyrosinase for efficient removal of phenolic compounds. The enzyme was immobilized in the form of cross-linked enzyme aggregates (CLEAs), with a process of protein precipitation from a crude extract of fresh mushrooms, followed by cross-linking with glutaraldehyde. By combining purification and immobilization into a single operation, this novel carrier-free immobilization method has proved to be superior to other conventional carrier-bound ones in being inexpensive and easy to prepare and handle, yet providing highly stable and recyclable catalysts with remarkable catalytic efficiency (Sheldon, 2007). Due to these attractive features, this new immobilization method has now been successfully applied to a variety of enzymes with widespread applications (Sheldon, 2007). In our recent studies, we have demonstrated that immobilization of tyrosinase via CLEA formation can effectively improve the stability of the enzyme in aqueous solution against various deactivating conditions such as pH, temperature, denaturants, inhibitors, and organic solvents (Xu et al., 2011), and the enzyme in this new immobilized form has been successfully employed for catalyzing with a high productivity the synthesis of L-DOPA (Xu et al., 2012), a drug for treatment of Parkinson's disease. This current study is the first one to demonstrate the use of tyrosinase CLEAs as an efficient catalyst for dephenolization and detoxification of phenol-polluted aqueous solutions. Elimination of four representative phenolic compounds (phenol, *p*-cresol, *p*-chlorophenol and bisphenol A) has been investigated by employing tyrosinase CLEAs, both in a batch reactor and in a continuous stirred tank reactor, and the toxicity of the phenolic solution after such an enzymatic treatment has been examined by following the growth of *Hydra sinensis*, a sensitive test animal for toxicity screening (Beach and Pascoe, 1997). Previously, laccase CLEAs have been tested for their capacity to eliminate bisphenol A in a fluidized bed reactor (Cabana et al., 2007). Bisphenol A is a member of endocrine disrupting chemicals (EDCs), capable of disrupting hormone functions (Husain and Quyyum, in press). In the recent review article by Ba et al. (in press), the potential applications of laccase CLEAs for the biotransformation of micropollutants in wastewater treatment have also been discussed.

2. Materials and methods

2.1. Materials

Fresh mushrooms were obtained from a local supermarket in Shenzhen, China. Glutaraldehyde (50 wt.% in water) was purchased from Sigma–Aldrich China Inc. All the phenolic compounds and other reagents used were of analytical grade from local manufacturers in China. *Hydra sinensis* was collected from Dongjiang River of Huizhou, China.

2.2. Preparation of tyrosinase CLEAs and CLEA/alginate beads

Tyrosinase CLEAs were prepared from fresh mushrooms by precipitating the enzyme with ammonium sulfate and subsequent cross-linking with glutaraldehyde, following the procedures described in (Xu et al., 2011). CLEA/alginate beads were prepared by entrapment of the tyrosinase CLEA powders into the calcium alginate gels, as described in (Xu et al., 2012). The CLEAs had an activity of roughly 113 IU g^{-1}, when assayed in a 20 ml phosphate buffer (pH 6.0) containing 2.5 mM phenol at 30 °C and 250 rpm.

2.3. Dephenolization by tyrosinase CLEAs

For aqueous solution containing a single phenol, a 100 ml phenolic solution with 2.5 mM of each of phenol, *p*-cresol and *p*-chlorophenol or 21.9 μM of bisphenol A in phosphate buffer (50 mM, pH 6.0) was treated with 50 mg of tyrosinase CLEAs. The resulting solution was incubated at 30 °C and agitated at 250 rpm. Periodically, a 1-ml sample was taken for HPLC analysis, as will be described below. To test the feasibility of using CLEAs for removing phenolic mixtures, a solution containing a mixture of three phenols (phenol, *p*-cresol, *p*-chlorophenol, all in 2.5 mM) was prepared in phosphate buffer (50 mM, pH 6.0), and the rest of the procedure was as described above. All tests have been repeated for at least 3 times, with an error within the range of ±5%.

The phenol removal efficiency was assessed by determining the conversion as below:

$$\text{Conversion}(\%) = \frac{[\text{phenol}]_o - [\text{phenol}]_t}{[\text{phenol}]_o} \times 100\%$$

where $[\text{phenol}]_o$ and $[\text{phenol}]_t$ are the initial and residual concentrations of the test phenol in the reaction system, respectively.

2.4. Reusability tests

About 30 mg of CLEAs were reacted with 20 ml of a phenol solution containing 2.5 mM of phenol, *p*-cresol or *p*-chlorophenol in phosphate buffer (pH 6.0, 50 mM) at 30 °C for 1 h before a 1 ml sample was taken for HPLC analysis. The reaction mixture was then centrifuged, the supernatant was decanted, and 20 ml of a fresh phenol solution was added for the next-round reaction. For testing the reusability of tyrosinase CLEAs in converting bisphenol A, the solution containing 21.9 μM of bisphenol A in phosphate buffer (50 mM, pH 6.0) was used as the substrate solution and 45 mg of tyrosinase CLEAs were added to perform the reactions at 30 °C for 30 min each cycle.