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Decolorization of synthetic dyes by laccase immobilized on epoxy-activated carriers

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Abstract

The laccase from the ascomycete *Myceliophthora thermophila* was covalently immobilized on polymethacrylate-based polymers (Sepabeads EC-EP3 and Dilbeads NK) activated with epoxy groups. The enzyme immobilized on Sepabeads EC-EP3 exhibited notable activity (203 U/g) along with remarkably improved stability towards pH, temperature and storage time, but no increased resistance to organic solvents. In addition, the biocatalyst also showed good operational stability, maintaining 84% of its initial activity after 17 cycles of oxidation of ABTS. The immobilized laccase was applied to the decolorization of six synthetic dyes (Reactive Black 5, Acid Blue 25, Methyl Orange, Remazol Brilliant Blue B, Methyl Green and Acid Green 27) with or without the redox mediator 1-hydroxybenzotriazole. The laccase immobilized in Sepabeads EC-EP3 retained 41% activity in the decolorization of Methyl Green in a fixed-bed reactor after five cycles. The features of these biocatalysts are very attractive for their application on the decolorization of dyes in the textile industry in batch and continuous fixed-bed bioreactors.

Keywords: Covalent immobilization; Epoxy carriers; Acrylic polymers; Laccase; Decolorization; Synthetic dyes

1. Introduction

Synthetic dyes are extensively used in several industries including textile, paper, printing, cosmetics and pharmaceuticals [1]. There are many structural varieties of dyes respecting the type of chromophore (part of the molecule responsible for its color), such as azo, anthraquinone, acridine, arylmethane, cyanine, phthalocyanine, nitro, nitroso, quinone-imine, thiazole or xanthene dyes. On the basis of dyeing process, textile dyes are classified as reactive dyes, direct dyes, disperse dyes, acid dyes, basic dyes and vat dyes. It is estimated that 10–15% of the dyes are lost in the effluent during dyeing process [2,3]. Many synthetic dyes are difficult to decolorize due to their complex structure. Decolorization of textile dye effluent does not occur when treated aerobically by municipal sewage systems [4]. Brightly colored, water-soluble reactive and acid dyes are the most problematic, as they tend to pass through conventional treatment systems unaffected [4]. Color can be removed from effluent by chemical and physical methods including adsorption, coagulation–flocculation, ion-exchange, oxidation and electrochemical methods [5,6]. The above ways for clean-up are expensive, which limit their application [7]. Dye decolorization is also achieved by routine anaerobic treatment of the effluents. However, reduction of azo dyes (up to 50% of the total amount of dyes used in the textile industry) by the bacterial reductases produce uncolored highly toxic aromatic amines. Alternatively, enzymatic oxidation of the dye using fungal oxido-reductases such as laccases has received great attention in recent years due to the efficient decolorization of the effluent [8–12].

Laccases (EC 1.10.3.2) are blue multi-copper-containing enzymes that catalyze the oxidation of a variety of organic substances coupled to the reduction of molecular oxygen to water [13–15]. Because of their broad specificity for the reducing substrates, laccases from white-rot fungi are receiving increasing attention as potential industrial enzymes in various applications, such as pulp delignification, wood fiber modification, dye or stain bleaching, chemical or medicinal synthesis, and contaminated water or soil remediation [16]. Further, the presence of small molecular

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weight redox mediators enhance the range and the rates of compounds to be oxidized (including recalcitrant dyes) by the so-called laccase mediator system (LMS) [17]. Recently Camarero et al. [18] reported that some lignin-derived compounds served as eco-friendly laccase mediators that showed higher decolorization abilities against recalcitrant dyes than some synthetic mediators. Regardless of the nature of the mediator, several limitations prevent the use of free LMS for these applications. The stability and catalytic ability of free enzymes are dramatically decreased by highly polluted wastewaters; besides, mediator by-products can inactivate the laccase. The use of immobilized enzymes can overcome some of these limitations and provide stable catalysts with long life times. Taking into account that LMS works through the specific oxidation of the redox mediator, the immobilized laccase can be readily recovered for reuse. In particular, immobilization by covalent coupling retains very high enzyme activity and is effective in removing color and phenolic compounds over wide ranges of pH and temperature [19,20].

The purpose of the present study was to develop an effective method for the immobilization of laccase to decolorize an array of synthetic dyes in the presence of mediators. The specific objectives of the study were to (i) immobilize laccase on epoxy-activated acrylic polymers; (ii) determine the optimum pH and temperature, stability towards storage and organic solvents and re-usability of the immobilized laccase as compared to that of soluble enzyme; and (iii) evaluate the performance of the immobilized laccase in decolorization of synthetic dyes.

2. Materials and methods

2.1. Materials

Sepabeads EC-EP3 and Dilbeads NK were kindly donated by Resindion S.R.L (Mitsubishi Chemical Corporation, Milan, Italy) and DilComplex (Fermenta Biotech Ltd., India), respectively. *N*-Hydroxybenzotriazole (HBT), 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), Reactive Black 5 (RB-5), Acid Blue 25, Methyl Orange, Remazol Brilliant Blue B (RBBR), Methyl Green and Acid Green 27 were purchased from Sigma–Aldrich. All other reagents were used of analytical grade.

A DeniLite II S sample was kindly provided by Novozymes A/S. This formulation is commonly used in the textile industry in the finishing process for indigo-stained cloths. The laccase (EC 1.10.3.2) is produced by submerged fermentation of a genetically modified – using recombinant DNA techniques—*Aspergillus* microorganism containing the laccase gene derived from *Myceliophthora thermophila* [21].

2.2. Laccase immobilization

DeniLite II S was dissolved in 0.1 M sodium acetate buffer (pH 4.5), centrifuged and filtered to a final protein concentration of 5 mg/ml. The DeniLite II S working solution (20 ml) was adjusted to pH 9.0 with 1 M sodium carbonate buffer, and mixed with Sepabeads EC-EP3 or Dilbeads NK (1 g). Approximately 100 mg protein was added per gram of carrier. The suspension was incubated at room temperature with roller shaking for 24 h. The immobilized biocatalyst was then filtered through glass filter, washed (3 × 15 ml) with 0.1 M sodium acetate buffer (pH 4.5), dried under vacuum, and stored at 4 °C.

2.3. Textural characterization of immobilized biocatalysts

Mercury intrusion porosimetry analyses of the biocatalysts were performed using a Fisons Instruments Pascal 140/240 porosimeter; samples were dried at 100 °C overnight, prior to measurement. The specific surface area (S_{BET}) of the supports was determined from analysis of nitrogen adsorption isotherms at −196 °C using a Micromeritics ASAP 2010 device; the samples were previously degassed at 100 °C for 12 h to a residual vacuum of 5×10^{-3} Torr, to remove any loosely held adsorbed species. Water content of the supports was assayed using a DL31 Karl–Fisher titrator (Mettler). Scanning electron microscopy (SEM) was performed using an XL3 microscope (Philips) on samples previously metallized with gold.

2.4. Enzyme assay and protein estimation

Standard laccase activity was determined by oxidation of ABTS at room temperature. The reaction solution was composed of 0.5 mM ABTS in 0.1 M sodium acetate buffer (pH 4.5). A suitable amount of enzyme was added, and the oxidation of ABTS was followed by measuring the absorbance increase at 418 nm ($\varepsilon_{ABTS^{\cdot+}} = 36{,}000\ M^{-1}\ cm^{-1}$). One unit of laccase activity corresponds to the oxidation of 1 μmol ABTS per min under these conditions. For the determination of immobilized enzyme activity, 20 mg of biocatalyst was incubated in 1 ml of 0.5 mM ABTS solution with stirring. Aliquots (20 μl) were withdrawn at fixed time intervals, diluted with water and the absorbance at 418 nm measured.

Protein estimation of laccase solution was performed using the method of Bradford (Bio-Rad protein assay). The quantity of protein bound to the support was calculated by subtracting the protein recovered in the combined washing of the support–enzyme complex from the protein used for immobilization.

2.5. Characterization of free and immobilized laccase

2.5.1. Optimum pH and pH stability

Laccase activity (free or immobilized) was assayed using 0.5 mM ABTS as substrate in 0.1 M citrate-phosphate and Tris–HCl buffers (pH 2.0–9.0) at 30 °C. The effect of the pH on the enzyme stability was studied by incubating enzymes in 0.1 M citrate-phosphate buffers (pH 2.0–6.0) for 24 h at 30 °C. Samples were transferred to standard reaction mixtures to determine the residual laccase activity with ABTS.

2.5.2. Optimum temperature and thermostability

The study was carried out at various temperatures (30–80 °C) and laccase activity was then assayed in standard conditions. Thermal stability was determined by incubating free or immobilized laccases at the selected temperatures (60 and 70 °C) for different time periods. After cooling, laccase activity was assayed at 30 °C in standard conditions with ABTS.

2.5.3. Stability in organic solvents

The stability of the enzymes in organic solvents (methanol, ethanol, dimethylsulfoxide (DMSO) and acetonitrile) at different concentrations (10, 20, 30 and 50% v/v) was measured by incubating free or immobilized enzyme in 1 ml of the solutions at 30 °C for 24 h. Initial and final enzyme activity was measured at 30 °C.

2.5.4. Storage stability

For testing the storage stability of enzymes, free and immobilized laccases in 0.1 M sodium acetate buffer (pH 4.5) were stored in a refrigerator at 4 °C for several days. Then the remaining activity of the enzyme was measured at 30 °C in standard conditions.

2.5.5. Reusability

Several consecutive oxidative cycles were performed using 0.5 mM ABTS in order to assess the operational stability of the immobilized laccase. At the end of each oxidation cycle, the immobilized laccase (20 mg or 5 U) was washed three times with 0.1 M sodium acetate buffer (pH 4.5) and the procedure repeated with a fresh aliquot of substrate, as described by Davis and Burns [19].