Enzyme and Microbial Technology 89 (2016) 31–38

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/emt

Polyamide 6/chitosan nanofibers as support for the immobilization of *Trametes versicolor* laccase for the elimination of endocrine disrupting chemicals

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ARTICLE INFO

Article history:
Received 3 December 2015
Received in revised form 1 March 2016
Accepted 3 March 2016
Available online 8 March 2016

Keywords:
Nanofibers
Laccase immobilization
Bisphenol A
17α
-ethinylestradiol

ABSTRACT

In recent years, there has been an increase in efforts to improve wastewater treatment as the concentration of dangerous pollutants, such as endocrine disrupting chemicals, in wastewater increases. These compounds, which mimic the effect of hormones, have a negative impact on human health and are not easily removed from water. One way to effectively eliminate these pollutants is to use enzymatically activated materials. In this study, we report on the use of laccase from the white rot fungus *Trametes versicolor* immobilized onto polyamide 6/chitosan (PA6/CHIT) nanofibers modified using two different spacers (bovine serum albumin and hexamethylenediamine). We then tested the ability of the PA6/CHIT-laccase biocatalysts to eliminate a mixture containing 50 μM of two endocrine disrupting chemicals: bisphenol A and 17α-ethinylestradiol. The PA6/CHIT nanofiber matrix used in this study not only proved to be a suitable carrier for immobilized and modified laccase but was also efficient in the removal of a mixture of endocrine disrupting chemicals in three treatment cycles.

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1. Introduction

Wastewater treatment plant authorities are increasingly concerned about the concentrations of hazardous water pollutants, including endocrine disrupting chemicals (EDCs) [1], found in wastewater. EDCs like pharmaceuticals, personal care products, polychlorinated biphenyls, dioxins, pesticides and plasticizers can interfere with the human endocrine system (and that of other species), even at low concentrations, by mimicking the effect of hormones [2]. An additional drawback of EDCs is their persistence in the water system and poor degradation into harmless compounds, which leads to their accumulation in the environment.

Bisphenol A (BPA) is used in the manufacture of polymers (e.g. polyvinyl chloride), plastics, polycarbonate plastics, epoxy resins, and flame retardants. There is growing concern over the implication of BPA as a causative agent in some chronic human diseases, including diabetes, obesity, cardiovascular disease, reproductive disorders, birth defects, chronic respiratory and kidney diseases, and breast cancer [3,4]. 17α-ethinylestradiol (EE2) is a synthetic estrogen used in contraceptive pills. Women generally metabolize only 20–48% of the daily EE2 dose; the rest is excreted and enters the wastewater cycle. Exposure to estrogenic compounds has been shown to have a negative impact on several aquatic species and is known to be a risk to human health, being implicated in testicular dysgenesis syndrome, testicular cancer, and breast cancer [5].

The use of enzymes capable of catalyzing the oxidation of these chemicals is regarded as a promising approach to remove EDCs from wastewater. The efficiency of enzyme catalysis is directly dependent on enzyme activity and stability as repeat usage is a necessary feature for its successful industrial application in wastewater treatment [6,7]. However, free enzyme is very sensitive to pH, temperature change, and the presence of inhibitors in the wastewater

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http://dx.doi.org/10.1016/j.enzmictec.2016.03.001
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environment as such factors can cause conformational changes in enzyme molecules, leading to inactivation or direct inhibition.

Enzyme immobilization increases the rigidity of the attached molecule's structure, thereby enhancing its stability and resistance and allowing repeated application [8–13]. Immobilization methods (e.g. enzyme entrapment, enzyme cross-linking, covalent binding, and reversible attachment) rely on different forms of interaction between biocatalyst side-chain functional groups and the immobilization support [14]. The type of interaction and the number and strength of the enzyme-support bonds influence the final activity and stability of the immobilized enzyme. The development of immobilized biocatalysts is especially important in a low added value sector of bioeconomy such as environmental services [15]. Enzymes are the major cost-determining factor of enzyme-assisted bioremediation/biodegradation treatments. Therefore, the increase in enzyme stability and reuse possibility provided by immobilization methods contribute to reduce the overall process cost and to make biocatalysis a feasible and attractive alternative to conventional environmental processes.

Laccase, a multicopper oxidase produced by fungi such as white rot fungus *Trametes versicolor, Pleurotus*, or *Pycnoporus sanguineus* [16], belongs to a group of enzymes that are able to catalyze oxidation of organic and inorganic substrates, including EDCs [17]. Laccase immobilized onto nanoparticles of modified silica, carbon, chitosan, and other biopolymers or metal oxides has resulted in high activity and stability and has opened the possibility to reuse the immobilized enzyme [18–20]. However, there is an increasing concern that such materials may impose health and environmental risks, both during the immobilization process and during the final application [21]. An alternative to the use of nanoparticles for enzyme immobilization are nanofibers, a generally safe and stable material that provides high specific surface area with numerous reactive sites [22]. Furthermore, the use of enzyme-modified nanofiber sheets would be easy to implement during the final step of wastewater treatment by incorporating them into existing filters or other structures. A number of studies have already reported enzyme immobilization on both synthetic and biopolymer nanofiber materials [23–25,40–43], and a number of authors have reported successful covalent immobilization of enzyme onto nylon fibers using glutaraldehyde (GA) activation and hexamethylenediamine (HMD) or polyethylenediamine as spacers [26–29]. Nevertheless, problems still remain concerning maintenance of a reasonable balance between activity and stability in the immobilized enzyme.

In this study, we report on the immobilization of laccase (*T. versicolor*) onto polyamide 6/chitosan (PA6/CHIT) nanofibers modified using two different spacers bovine serum albumin (BSA) and hexamethylenediamine (HMD) and GA as a crosslinker. Activity and stability of the immobilized enzyme were examined under different immobilization process parameters (e.g. pH, temperature, molar strength) and under different operational conditions. The best resulting materials were tested for enzymatic degradation of selected EDCs (bisphenol A and 17α-ethinylestradiol).

2. Materials and methods

2.1. Chemicals

Pelleted polyamide 6 (PA6, B24, Mw 37000 g/mol) was obtained from BASF (Prague, Czech Republic) and chitosan (CHIT, deacetylation rate 80 mol/mol%, viscosity 0–10 mPa·s) was purchased from Wako (Richmond, USA). Glutaraldehyde (GA – 25% solution in water), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), HMD, BPA (≥99% purity) and EE2 (≥98% purity) were purchased from Sigma-Aldrich (St. Louis, USA). All other reagents used were of analytical grade. Laccase from *T. versicolor* (> 10 U/mg, purity and enzyme extracting method were not provided by the supplier) was also purchased from Sigma-Aldrich (St. Louis, USA).

2.2. SDS-PAGE

Protein electrophoresis SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) was used to determine the protein purity of commercial laccase. The upper stacking gel consisted of 2.1 ml of distilled water, 0.5 ml of 30% acryl/bis-acrylamide, 30 μl of 10% SDS, 30 μl of 10% ammonium persulphate, 3 μl of TEMED and 0.38 ml of 1.5 M Tris-HCl buffer (pH 6.8). The bottom running gel contained 3.3 ml of distilled water, 4 ml 30% acryl/bis-acrylamide, 0.1 ml of 10% SDS, 0.1 ml of 10% ammonium persulphate, 4 μl of TEMED and 2.5 ml of 1.5 M Tris-HCl buffer (pH 8.8).

The gel was placed in the running buffer and connected to Enduro 300 V power supply with the voltage of 90 V. The stock laccase with the concentration of 40 mg/ml of distilled water was dissolved in sample buffer consisting of 1.5 M Tris-HCl buffer (pH 6.8), 20% SDS glycerol, 2% 2-mercaptoethanol and 0.04% Coomassie R250. Then it was denatured by heat (100 °C, 30 min) and centrifuged (25 °C, 5 min, 2000 rpm). Subsequently, three different concentrations of the stock laccase were sampled on the top of the gel. When all molecular weight fractions appeared, the gel was removed, transferred to a dye solution (0.1% Coomassie Blue dye in 50% methanol, 10% glacial acetic acid), decoloured by methanol and acetic acid (starting with 50% methanol, 10% acetic acid for 2 h, then using 7% methanol, 10% acetic methanol for 2 h) and finally it was analyzed by software Elfoman version 2.0.

2.3. Preparation of the nanofiber matrix

PA6 was dissolved in a mixture of formic acid and acetic acid (1:2; v/v) at 80 °C to prepare a 12.5% wt. solution. Meanwhile, CHIT was dissolved in the same mixture of formic and acetic acid with a fixed concentration of 8% wt. at room temperature overnight. The final spinning solution consisted of a PA6/CHIT blend at a weight ratio of 4:1 (w/w). PA6/CHIT (90:10; w/w) nanofibers were prepared by electrospinning using Nanospider™ NS 1WS500U equipment (Elmarco, Liberec, Czech Republic). A voltage of −30/60 kV was applied, with the distance between electrodes set at 175 mm. The fiber sheets were stabilized at 105 °C for 15 min after fabrication, whereupon the sheets were immersed in 0.1 M sodium hydroxide for 30 min to remove acidic residues. Finally, the sheets were washed with distilled water twice and dried at room temperature.

2.4. Nanofiber characterization

Prepared nanofiber layers were studied by scanning electron microscopy (SEM) using Carl Zeiss ULTRA Plus microscope (Oberkochen, Germany). Captured images were further analyzed by VEGA TC software used primarily for the measurement of the average fiber diameter obtained as an mean value out of 100 gathered values.

2.5. Determination of enzyme activity

Laccase catalytic activity was measured at 25 °C and 420 nm according to Refs. [30] and [31] using a BioTech Synergy HTX microplate reader (BioTek Instruments inc., Winooski, VT, USA). The reaction was performed in 100 mM citrate-phosphate (pH 3) containing 0.5 mM ABTS. One unit corresponds to the amount of laccase able to oxidize 1 μmole of ABTS per minute under the above

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