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Efficient transformation of phenyl urea herbicide chloroxuron by laccase immobilized on zein polyurethane nanofiber

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ABSTRACT

Laccases are widely distributed in plants, bacteria, and fungi, and are used for a wide range of applications, including bioremediation. In the present work laccase was immobilized on an electrospun zein polyurethane nanofiber via crosslinking with glutaraldehyde. FESEM and FTIR analysis clearly demonstrates the binding efficiency of laccase on the nanofiber. The relative activity of immobilized laccase was 85% that of free laccase. Immobilized laccase had a better pH and thermal stability than free laccase. The immobilized laccase completely degraded chloroxuron up to 25 reuse cycles in the presence of 1 mM HoBt. Paddy seeds soaked with solution containing chloroxuron treated with immobilized laccase showed a germination percentage closer to the distilled water control; whereas no damage or fatality of paddy seedlings were noticed in treated chloroxuron solution, which demonstrates the ability of immobilized laccase to detoxify the phenyl urea herbicide chloroxuron.

1. Introduction

Phenylurea herbicides (PUHs), such as chloroxuron: IUPAC name 3-[4-(4-chlorophenoxy)phenyl]-1,1-dimethylurea, are used in agriculture for the control of weed growth in several crops where they act as inhibitors of the photosynthesis and cause growth inhibition, chlorotic, and necrotic effects on foliage. These herbicides are either endocrine disruptors, or have eco-toxic or genotoxic effects. The residues of PUHs and their transformation products were found in aquatic environments causing risk to aquatic flora and fauna [1–5]. Water contaminated with PUHs can be more toxic than the herbicides themselves, as the by-products generated during water disinfection processes based on strong oxidants like chlorine or ozone can be more toxic to human health [3]. Several methods have been proposed for the treatment of the polluted water by herbicides or pesticides, including laccase-based enzymatic remediation for removing phenolic contaminants from the environment [6–9]. Laccases (*p*-diphenol oxidase, EC 1.10.3.2) belong to the family of multicopper oxidases that catalyze the oxidation of aromatic substances, such as diphenols, arylamines, or aminophenols, with the concomitant reduction of O_2 to H_2O. Laccases are widespread in nature and have been found in fungi, in plants, and in some bacteria [10,11]. The laccases have the ability to catalyze a plethora of reactions, including the degradation of polymers, oxidative coupling of phenolic compounds, functionalization of polymers, and ring cleavage; these abilities position laccases as significant industrial enzymes [12–14]. Immobilization technology has been proven to be an effective and most straightforward way to implement efficient and continuous application of enzymatic oxidation [6,15,16]. Similar to the free enzyme, immobilized laccase can be applied in various industrial processes, especially in environmental applications. The search for inexpensive supports and the recovery of activity during the immobilization process is constantly on increase to potentiate the application of laccase in immobilized systems [17]. Biopolymer from renewable resources has gained much attention for economical and environmental reasons. Zein, the major protein of corn and a by-product of the bioethanol industry, is a non-toxic, biocompatible, biodegradable polymer which in recent years had gained much attention because

Abbreviations: FESEM, field emission scanning electron microscopy; SEM, scanning electron microscopy; FTIR, Fourier transform infrared spectroscopy; PUHs, phenyl urea herbicides; PU, poly urethane; HoBT, 1-hydroxybenzotriazole; ABTS, 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonate); DMF, dimethylformamide; THF, tetrahydrofuran; GA, glutaraldehyde; CX, chloroxuron control; ILCX, immobilized laccase treated chloroxuron; IL, immobilized laccase; DW, distilled water.

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of the electrospinning of zein nanofibers [18–21]. High molecular weight substances, which are either natural or artificial with different structures, can be used for enzyme immobilization; one of the best supports for this is polyurethane (PU). The unique properties of PU are its elasticity, toughness, durability, and good resistance to environmental factors [22]. These properties of PU, when combined with the biodegradable polymer zein, can function as efficient and novel support for immobilization of enzymes for use in commercial interests.

In the present study, the novel Zein-based nanofiber was developed by co-spinning with polyurethane followed by immobilization of laccase from *Tinea versicolor* for effective transformation of the PUH chloroxuron.

2. Experimental

2.1. Materials

Laccase (EC 1.10.3.2: p-diphenol:dioxygen oxidoreductase; 20 U mg^{-1}) from *T. versicolor*, 1-hydroxybenzotriazole (HoBT), 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS), ethyl acetate, zein, and glutaraldehyde solution (25%, w/w) were obtained from Sigma–Aldrich Chem. Co., St. Louis, MO, USA. All other chemicals were of analytical grade and were used as received, without further purification.

2.2. Electrospinning of zein–PU nanofibers

Zein (Sigma–Aldrich, USA) and polyurethane (MW = 110,000, Cardio Tech. Intern., Japan) were used in making the solution. PU (10%) solution with 5% and 10% concentration of zein to PU was used to prepare the composite nanofiber mats. A mixed solvent, DMF:THF (1:1) was used to prepare the 5% zein–10% PU and 10% zein–10% PU polymer solution and the composite polymer solution was stirred for 1 h prior to electrospinning. Polymer solution was fed to the 5 mL syringe with a plastic micro-tip. A high voltage power supply (CPS-60 K02V1, Chungpa EMT, South Korea) of 16 kV was supplied to the syringe micro-tip in order to electrospin the nanofibers; whereas, a ground iron drum covered by a polyethylene sheet served as the counter electrode. The solution was kept in the capillary by adjusting the inclination angle. The tip-to-collector distance was kept at 15 cm. Finally, the Zein–PU composite nanofiber mats were vacuum dried in an oven at room temperature for 24 h to remove the residual solvent, and this sample was used for further characterizations.

2.3. Immobilization of laccase on zein–PU nanofiber

All the immobilization steps were carried out by using 5 mg weighed standard pieces of zein–PU nanofiber. Prior to immobilization, the nanofibers were activated with various concentrations of glutaraldehyde (GA) water solution (5, 10, 15, 20, and 25%, w/v), and the mixture was put on a rocker (100 rpm) at 4 °C overnight. The GA activated nanofibers were transferred to new vials, and washed extensively by decanting with 10 mM sodium phosphate buffer, pH 4.5 (buffer A) to remove the excess GA solution. Then, the washed zein–PU was incubated with laccase at 4 °C overnight in a rocker (50 rpm). The excess enzyme from the nanofibers was washed extensively with buffer A until no leaching of enzymes was observed in the washing solution. This washing process took 1 h, with 5 washings. After washing, further cross linking of the immobilized enzymes were achieved with 5% GA solution for 3 h at 20 °C. Again the excess of GA was removed by extensive washing with buffer A. The enzyme coatings on polymer nanofibers were stored in buffer A at 4 °C.

2.4. Physicochemical characterization of laccase–Zein–PU nanofiber

2.4.1. Scanning electron microscopy (SEM) and field emission scanning electron microscopy (FESEM)

The surface topography of the electrospun fibers and laccase immobilized zein–PU nanofiber was analyzed by field emission scanning electron microscopy (FESEM). For FESEM, a thin layer of iridium was coated (2 nm) onto the sample to prevent charging. The image characterization was performed using JSM-6700F (JEOL Ltd., Tokyo, Japan).

2.4.2. Fourier transform infrared measurements (FTIR)

The zein–PU nanofiber and the laccase immobilized zein–PU nanofiber were characterized by Fourier transform infra red spectrometer (FTIR Nicolet 5700). Small amounts of samples were mixed separately with KBr and the prepared pellet was used for FTIR spectra.

2.5. Activity assays of free and immobilized laccase

Laccase activity was measured using ABTS as substrate at 30 °C [23]. Free laccase activity was assessed by initiating the reaction with 50 μL (0.5 U) of enzyme solution and 0.5 mM of ABTS in 100 mM of sodium phosphate buffer (pH 4.5). The activity of immobilized laccase was assayed by incubating five 0.5 cm × 0.5 cm pieces of support containing immobilized laccase with 0.5 mM ABTS in 100 mM of sodium phosphate buffer (pH 4.5) under constant shaking (100 rpm). After 3 min, the membrane was removed using forceps and substrate oxidation by free and immobilized laccase was monitored at 420 nm ($\varepsilon 420 = 36.0\ mM^{-1}\ cm^{-1}$) using a UV–vis spectrophotometer (Shimadzu UV-1800). One unit (U) of activity was defined as the amount of enzyme needed to oxidize 1 μmol of ABTS per minute. Enzyme activity was expressed in U mg^{-1} or mL^{-1}.

2.6. Protein estimation

Protein estimation was performed according to manufacturer's instruction of the Thermo® Scientific Pierce Coomassie Plus (Bradford) Protein Assay Kit (Product No. 23236), which uses bovine serum albumin as a standard. Concentration of the immobilized protein in the ZP nanofiber was estimated inversely by measuring the decrease in dye absorbance at 465 nm, rather than conventional protein estimation by measuring increase in absorbance at 595 nm. The amount of immobilized protein could be directly quantified by this method.

2.7. Optimum pH and temperature

The optimum pH for laccase activity (free and immobilized) was investigated using 50 μM ABTS in a 0.1 M sodium phosphate buffer (pH 2.5–7). The relative activity was calculated as the ratio between the activity at each pH and the maximum attained.

The effect of temperature (20–90 °C) on laccase activity (both free and immobilized) was determined by measuring activity at the corresponding temperature under standard conditions. The relative activity was calculated as the ratio between the activity at each temperature and the maximum attained.

2.8. Determination of kinetic parameters of free and immobilized laccase

The Michaelis–Menten constant (K_m) and maximum reaction rate (V_{max}) for free and immobilized laccase were determined using ABTS as a substrate at 0.025 to 1 mM at 30 °C and pH 4.5.