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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.

An Eadie–Hofstee plot was constructed to determine the kinetic parameters.

### 2.9. Chloroxuron degradation

Chloroxuron stock was prepared in aqueous methanol (50%, v/v), and appropriate dilutions of stock solution were used for the experiments. Drug transformation experiments were carried out using a 1-mL reaction volume containing chloroxuron (0.175 mM), sodium acetate buffer (100 mM; pH 4.5) and immobilized laccase (5 U) with or without HoBt (1 mM). The final methanol content of the reaction was less than 2.0%, at which no laccase inhibition was observed. Reaction vials were incubated in the dark for various time periods at 30 °C while being agitating at 100 rpm. Appropriate control samples with free laccase were included. Experiments were performed in duplicate and each sample was analyzed twice.

### 2.10. Analytical methods

#### 2.10.1. UV–vis spectral scanning analysis

The biodegradation of chloroxuron by immobilized laccases was monitored by measuring the UV–vis spectra in 1 cm optical path length quartz cuvettes with a Shimadzu UV1800 UV–vis spectrophotometer at a resolution of 1 nm between 200 nm and 1100 nm.

#### 2.10.2. HPLC analysis

Chloroxuron degradation by laccase was analyzed by HPLC (Shimadzu, Japan), which consisted of the following components; pump LC-20A, auto-sampler model SIL-20A, column ACE C18 (250 mm × 4.6 mm, ACE) in column oven CTO-20A at 40 °C, detector model SPD-M20A, and software LC solution. Elution was done with a gradient of water and acetonitrile as follows: 0–2 min, isocratic 40% acetonitrile; 2–12 min, linear gradient to 95% acetonitrile; 12–20 min, isocratic 95% acetonitrile; 22–29 min return to isocratic start conditions; 29–29.5 min, re-equilibration time. The flow rate was 1.0 mL/min. The retention time of chloroxuron was determined by monitoring the eluent at 280 nm.

#### 2.10.3. LC–ESI-MS analysis

After degradation, the reaction mix contains buffer and chloroxuron metabolities were subjected to ethyl acetate extraction to remove impurities. Then it was vacuum dried and the chloroxuron metabolites were dissolved in acetronitrile for liquid chromatography-mass spectrometry analysis for the determination of non-volatile metabolites. For LC–MS analysis, 5 μL aliquots were injected into an HPLC system (Acquity Ultra Performance LC, Waters), comprising a Waters ACQUITY UPLC®BEH C18 column (2.1 mm × 100 mm, 1.7 μm). LC–MS was controlled by MassLynx software (version 4.1). The mobile phase consisted of 0.1% formic acid in distilled water (DW) and 0.1% formic acid in acetronitrile. The flow rate was 0.5 mL/min and employed a gradient from 10% to 100% buffer B for 10 min. The mass spectrometer was equipped with an ESI interface, operating at an ionization voltage of +3000 V and a source temperature of 380 °C. The capillary voltage, con voltage, and source offset were set at 3 kV, 30 kV and 30 V, respectively. Tandem MS analysis was performed using the multi-reaction-monitoring (MRM) mode. Collision energy, the gas flow of desolvation, and cone spray nebulizer were set at 650 L/h, 150 L/h and 7 bar, respectively.

### 2.11. Reusability

The reusability of the immobilized laccase was investigated in repeated batch experiments using ABTS as the substrate and by chloroxuron biodegradation.

### 2.12. Toxicity test

Paddy seeds (plant strain used: Sindongjin, a japonica rice cultivator) were treated with 2-fold dilution of non-degraded chloroxuron solution (CX: chloroxuron control) and degraded chloroxuron solution (ILCX: immobilized laccase treated chloroxuron) by immobilized laccase. Distilled water was used as a control. The seeds (30 seeds for each test) were disinfected or fumigated with 5 mL of these solutions in 15 mL test tube and incubated in plant growth chamber at 25 °C for 14 days. Germination index was calculated from the treated seeds.

Cormophytes of these seeds were selected after treatment with the plant pesticides. Paddy seeds were soaked (imbibition) in CX solution and ILCX without any dilution for 3 days. Then seeds were sowed in nursery soil at a depth of 1 cm. The nursery soils sowed with seeds were irrigated from the bottom of the pot (bottom watering). The seedlings were kept in plant growth chamber at 25 °C for 15 days. The plants grown above 10 cm height and having leaf age of 2.5 days were selected. The selected plants with leaves of age 2.5 days were treated with distilled water, CX, and ILCX solution, by using a painting brush. The laccase treated chloroxuron solution was further checked for plant growth damage and fatality rate index.

## 3. Results and discussion

### 3.1. SEM and FESEM image of zein–PU nanofiber

Fig. 1 shows the SEM and FESEM micrographs of the 10% zein–PU (ZP), 25% GA activated ZP and laccase immobilized on ZP nanofiber cross linked with 5% GA. Among the various percentage of zein used for electrospinning with polyurethane, 10% zein–PU was employed for further studies involving immobilization, as it was tough and flexible. It is evident from the micrograph that the surface modification of ZP nanofiber was achieved with GA (Fig. 1b) and with the subsequent laccase immobilization, a uniform and nicely adhered enzyme layers was produced on the nanofiber (Fig. 1c). The FESEM image of ZP nanofiber after GA activation shows thick coatings on the polymer nanofiber (Fig. 1b). This could be the result of GA molecules attached covalently to the surface of the polymer that had reacted with the $\alpha NH_2$ group of N-terminal, the imidazole ring of histidine and the phenolic group of tyrosine, as zein lacks the ε-amino group of lysine [24]. When GA activated ZP nanofiber was treated with the laccase, a uniform attachment of enzyme to the nanofiber is evident via covalent attachment to GA (Fig. 1c). Further, crosslinking with 5% GA solution of the other unbound enzyme molecules adsorbed onto the bound protein causes the formation of aggregates, wherein GA reacts with ε-amino group of lysine present in the enzyme to form covalent linkages between the enzyme molecules via a conjugated Schiffs base [25]. This is evident from the fully-packed inter-fiber pores of ZP by crosslinked enzyme aggregates (Fig. 1c). Enzyme activity was inversely proportional to the concentration of glutaraldehyde, because extensive cross linking may result in deformation of the enzyme structure. Thus it may lead to the less accessibility of the substrate to the active site of the enzyme, so reducing its biological activity [26]. To avoid this zein polyurethane nanofiber membrane was first treated with glutaraldehyde and washed extensively with distilled $H_2O$. This forms a cross linking of glutaraldehyde molecules with free overhangs.

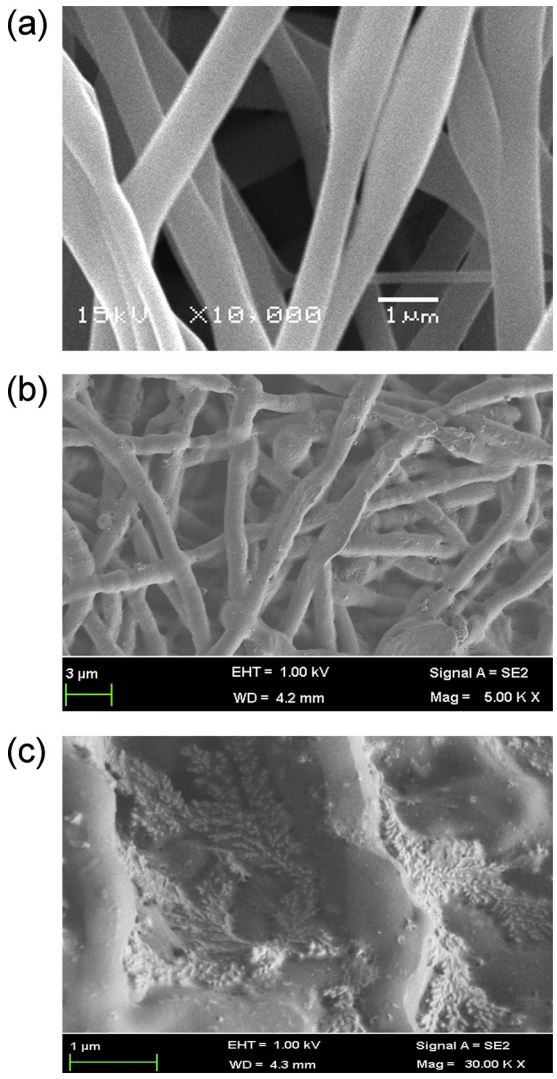


**Fig. 1.** (a) SEM image of 10% zein–PU nano fiber, (b) FE-SEM image of the electrospun zein–PU nanofiber surface topography activated by 25% glutaraldehyde, and (c) FE-SEM image of laccase immobilized on 25% glutaraldehyde activated 10% zein–PU fiber followed by crosslinking with 5% glutaraldehyde.

Then this pretreated membrane was incubated laccase solution. This constitutes a complete cross linking of laccase with all glutaraldehyde molecules bonded with nanofiber membrane. Lower concentration of glutaraldehyde was added to this solution for a complete cross linking of laccase with zein polyurethane membrane. Minimal amount of glutaraldehyde was used to avert the loss of enzyme activity [27].

### 3.2. FTIR spectrum of immobilized laccase

The FTIR spectrum of ZP nanofiber (Fig. 2a) exhibited a broad, but not so intense band around 3300 $cm^{-1}$, which shows the stretching of protons. Absorptions in the region 3650–3250 $cm^{-1}$ correspond to hydroxyl or amino groups, which mainly dominate this region, both giving rise to very characteristic band profiles. The presence or absence of hydrogen bonding is well-delineated in this region [28]. The main absorption band in the region 3600–3000 $cm^{-1}$ was broad and hence the compound probably possesses a hydroxyl or amino group. Since zein contains prolamin and polyurethane have a hydroxyl and an amide linkage, the presence of amino group and hydroxyl groups overlapping in the spectrum cannot be ruled out. According to the FTIR spectrum, urethane linkage

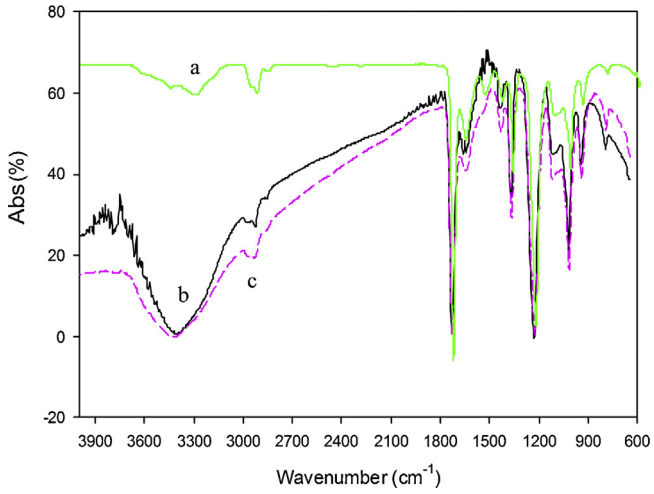


**Fig. 2.** FTIR spectrum of laccase immobilized on 10% zein–PU nano fiber activated with 25% glutaraldehyde followed by 5% crosslinking. (a) Zein–PU nanofiber, (b) laccase immobilized on zein–PU nanofiber after 25% glutaraldehyde activation and (c) laccase crosslinked with 5% glutaraldehyde.

was shown by spectrum peak range between 3329–3290 $cm^{-1}$ (H—O—H stretch) and 1109–1107 $cm^{-1}$ (C—O stretch). Spectrum peak at 2951–2926 $cm^{-1}$ and spectrum peak at 1423 $cm^{-1}$ shows C—H aliphatic stretch and CH bending respectively, and indicates the existence of polyol soft-segment in the chain of polyurethane. In the ZP nanofiber spectra, bands observed in the region correspond to C=O stretching (1750 $cm^{-1}$) and C—H stretching (2820 $cm^{-1}$) vibrations. This can be attributed to the characteristic absorption of aldehyde groups, which act as a binding site for the primary amino groups of enzyme molecules [29,30]. Though the ZP nanofiber is having aldehyde groups the treatment of ZP nanofiber with 25% of glutaraldehyde vastly improves the binding of the laccase to the membrane which is evident from the enzyme activity assay, protein content. The spectrum of laccase-immobilized ZP nanofiber (Fig. 2b) has pronounced bands corresponding to N—H bending (1560 $cm^{-1}$), which may be due to primary amino groups of the immobilized enzyme and N—H bending (1650–1580 $cm^{-1}$). This N—H stretching and bending becomes more pronounced in the FTIR spectrum of ZP nanofiber (Fig. 2c), where laccase is crosslinked with 5% GA which indicates that ZP nanofiber binds laccase effectively and efficiently via GA activation.

### 3.3. Properties of immobilized laccase

The amount of laccase attached to the ZP nanofiber was 0.25 mg (5 U) to 5 mg of ZP nanofiber was estimated according to manufacturer's instructions of Thermo® Scientific Pierce Coomassie Plus (Bradford) Protein Assay Kit. The relative activity of immobilized laccase was 85 ± 4% that of free laccase. After immobilization, a minor decrease in relative activity was observed. The loss in the immobilized system may be due to changes in the enzyme diffusion rate, non-biospecific interactions, and variations in the microenvironment [31].

The pH and temperature profiles of the free and immobilized enzyme are shown in Fig. S1a and b. Both free and immobilized laccase showed optimum activity at pH 4.5 and at 60 °C. These results suggest that there were no significant changes in the electrostatic state of the amino acids at the active sites of the laccase after immobilization [29]. The charge density of the nanofibrous membrane surface does not change the enzyme microenvironment as is the case with other polycations [30]. Compared to the free enzyme, temperature stability was pronounced in the case of immobilized laccase (Fig. S1c). The presence of proline of zein

polymer may influence this observed effect. Proline substitution enhancing enzyme stability is reported elsewhere [32,33].

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2013.10.022.

A kinetic study was performed to obtain $K_m$ and $V_{max}$, for the free and ZP immobilized laccase. These kinetic parameters were determined by analysis of the Eddie–Hofstee plot (Fig. S2). The $K_m$ for the immobilized laccase was 0.67 mM, which varies significantly from the free laccase (0.087 mM), and the $V_{max}$ of the immobilized laccase decreased to 1.9 from 2.1 U $mg^{-1}$ for the free laccase. The decrease in $K_m$ and $V_{max}$ values indicates shift in affinity and catalytic ability of the immobilized enzyme compared to the free enzyme toward the substrate. The increased limitations to diffusion of the substrate to the active site of the immobilized enzymes might be the cause of decrease in affinity of immobilized laccase when compared to the free enzyme [34].

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2013.10.022.

### 3.4. Removal of phenyl urea herbicide chloroxuron

UV–vis spectral analysis of chloroxuron showed a decrease in absorbance at 240 nm indicating the effective degradation of chloroxuron by using the immobilized laccase (Fig. 3). The reduction is more pronounced with increase in time (data not shown). The action of redox mediators on degradation of chloroxuron

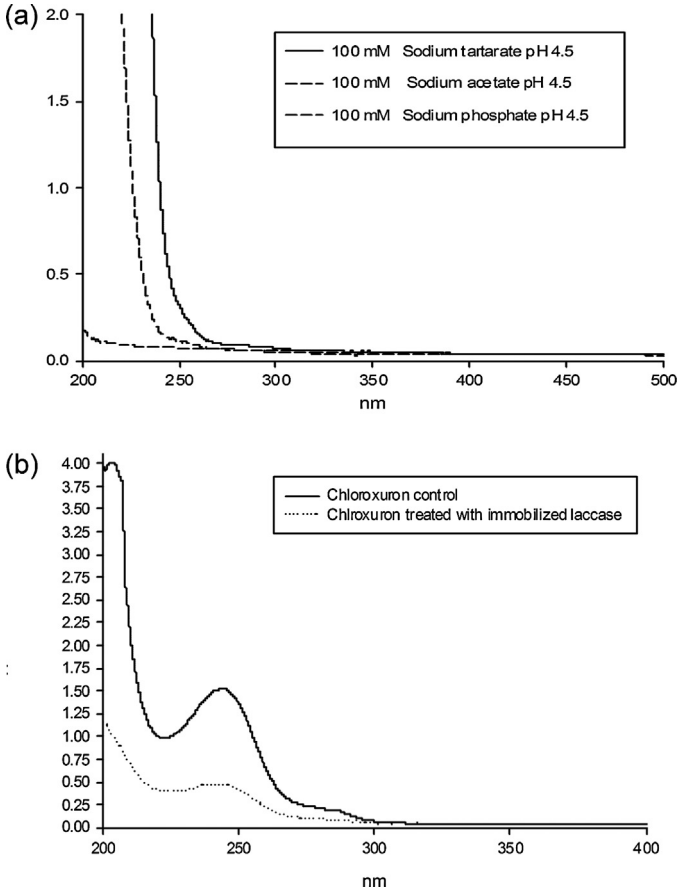


**Fig. 3.** UV–vis spectral scanning. (a) Selection of buffer with less interference for identifying the chloroxuron degradation by UV–vis scanning and (b) UV–vis spectral scanning of 0.175 mM of chloroxuron degradation by immobilized laccase using sodium acetate buffer pH 4.5.

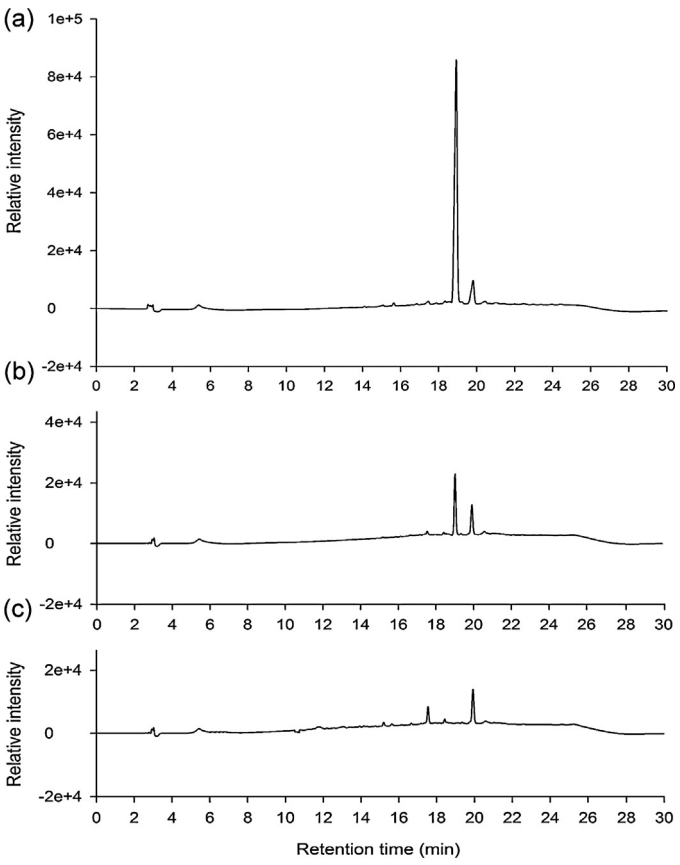


**Fig. 4.** HPLC chromatogram of 50 mg/L chloroxuron degradation. (a) Chloroxuron control, (b) chloroxuron treated by immobilized laccase and (c) chloroxuron treated by immobilized laccase presence of 1 mM HoBt.

cannot be verified, as these mediators have absorption maximum in the UV region, which makes it difficult to interpret the results. The interference of various buffers on chloroxuron detection in UV range was checked (Fig. 3a). It was found that 0.1 M sodium phosphate buffer does not show any interference when compared to citrate or sodium acetate buffers; hence for detection of chloroxuron under UV range 0.1 M sodium phosphate buffer pH4.5 was employed. The degradation of chloroxuron by immobilized laccase was further corroborated with HPLC studies.

HPLC chromatogram of chloroxuron showed a sharp peak with retention time (RT) at 19 min, and a minor peak at RT 20 min (Fig. 4a). Treatment of chloroxuron with immobilized laccase drastically reduces the peak at RT 19 min within 30 min of reaction time (Fig. 4b). Addition of redox mediator HoBt to chloroxuron in presence of immobilized laccase (5 U) resulted in the complete removal of peak at RT 19 min with the formation of new peak with RT 17 min (Fig. 4c). Other redox mediators via syringaldehyde and 3-hydroxy anthranilic acid do not show any effect on chloroxuron removal (data not shown). HPLC results clearly showed that laccase has the ability to decompose chloroxuron to other species, leading to its degradation. This result was further corroborated with LC–ESI-MS analysis.

Fig. 5a shows the decrease in the peak of chloroxuron in LC spectrum at RT 6.30 min when treated with immobilized laccase in presence of 1 mM HoBt. This again clearly demonstrates the ability of laccase to effectively degrade chloroxuron. Fig. 5b (i and ii) shows the ESI-MS analysis of chloroxuron control and chloroxuron treated with immobilized laccase. Analysis of chloroxuron control chromatogram (Fig. 5b (i)) reveals that the peak at $m/z$ 293 as chloroxuron, whereas minor peak at $m/z$ 278

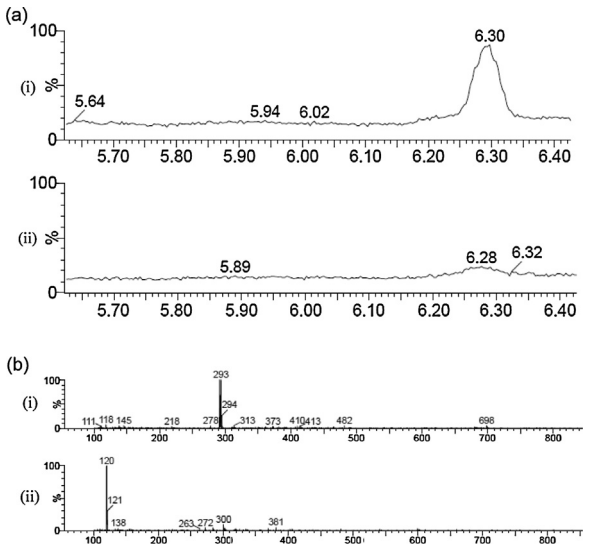


**Fig. 5.** LC–ESI-MS analysis. (a) TIC of LC–MS analysis of ethyl acetate extract of samples. (i) Chloroxuron control, peak corresponds to retention time (RT) 6.30 min; (ii) chloroxuron treated with immobilized laccase in presence of 1 mM HoBt, peak at RT 6.30 min disappears. (b) ESI-MS of chloroxuron degradation. (i) Chloroxuron control; (ii) chloroxuron degraded by immobilized laccase in presence of 1 mM HoBt.

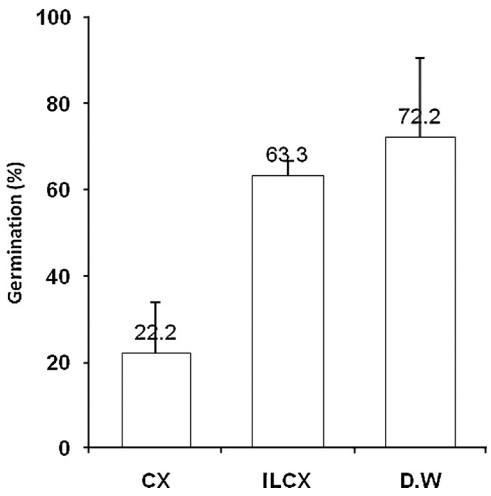


**Fig. 6.** Toxicity assessment of solution containing 0.175 mM chloroxuron treated with and without immobilized laccase in presence of 1 mM HoBt. (CX: chloroxuron control; ILCX: immobilized laccase treated chloroxuron and DW: distilled water control).

may be N-(4-chlorophenoxy)-phenyl-N-methylurea (contains one methyl group). Fig. 5b (ii) shows the ESI-MS spectrum of the eluent at RT 6.30 of chloroxuron treated with immobilized laccase. The major peak at *m*/*z* 121 denotes N-phenylformamide which is the breakdown product of chloroxuron. The minor peaks at *m*/*z* 272 might be N-(4-hydroxyphenoxy)-phenyl-N-methylurea (chloroxuron reacts with $H_2O$ and the $Cl^-$ dissociates to form HCl along with N-(4-hydroxyphenoxy)-phenyl-N-methylurea which further hydroxylated at different phenyl rings to form two different compounds with M.W. 289). The *m*/*z* 263, and 138 denotes a demethylated product of chloroxuron, 1-[4-(4-chlorophenoxy) phenyl] urea and N-phenyl urea or phenyl carbamate respectively. ESI-MS studies reveal the degradation products of chloroxuron by immobilized laccase.

The advantage of immobilized biocatalysts over the free enzyme is their ability of recycling which can be used in a continuous reaction processes. Hence, the operational stability and reusability of the immobilized enzymes are necessary parameters for major industrial applications. Fig. S3 shows the operational stability of immobilized laccase during repeated use for chloroxuron transformation. Results show that the enzyme activity can be retained up to 25 cycles without losing much of its activity and then it gradually decreased with number of recycles. This decrease in enzyme activity could be due to inactivation or loss of enzyme during each cycle. Similar results have been reported for other nanobiocatalysts [35–37].

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2013.10.022.

### 3.5. Toxicity assessment of the laccase treated chloroxuron solution

Effect of chloroxuron (control and treated with laccase immobilized on ZP nanofiber) was checked for germination index of paddy seeds (plant strain used: Sindongjin, a japonica rice cultivar). Fig. 6 shows the pre-mergence application of herbicide had negative bearing on rice germination (22.2%), whereas the control (distilled water) had a germination percentage of 72.2. Chloroxuron treated with immobilized laccase had a germination percentage of 63.3 which is 3-fold higher than the rice seeds treated with chloroxuron alone but not closer to the control. This may be due to the residues or the transformation products of chloroxuron which still remains in the treated solution. However the suppressive influence on paddy seedling by the herbicide chloroxuron is relieved to a greater extent by treating with laccase which is also evident from the effect of chloroxuron and laccase treated chloroxuron on the damaged and fatality on rice plant. Untreated chloroxuron showed 100% damage and 50% fatality to the rice plants whereas the laccase treated chloroxuron did not show any damage or dead plants.

This is the first report on effective degradation and detoxification of chloroxuron by laccase.

## 4. Conclusion

A biocompatible, biodegradable zein polymer-based electrospun nanofiber with polyurethane was prepared to adsorb laccase. The immobilized laccase showed better pH and temperature stability than the free laccase. Kinetic parameters indicated affinity shift between immobilized enzyme and the substrate. By employing the immobilization system along with the redox mediator HoBt, the PHU chloroxuron was completely degraded within 30 min. The immobilized system was able to retain 80% of its activity up to 25 recycles. The immobilized laccase treated chloroxuron solution improved the germination percentage of the paddy seeds and did not cause any damage or mortality to the paddy seedlings.

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