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Laccase–Polyacrylonitrile Nanofibrous Membrane: Highly Immobilized, Stable, Reusable, and Efficacious for 2,4,6-Trichlorophenol Removal

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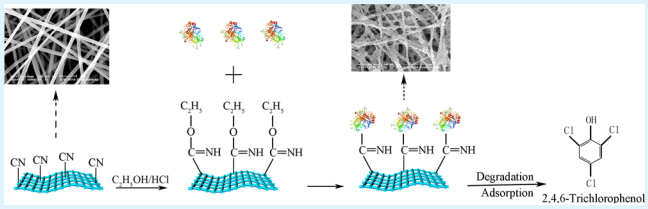
ABSTRACT: Increasing attention has been given to nanobiocatalysis for commercial applications. In this study, laccase was immobilized on polyacrylonitrile (PAN) nanofibrous membranes through ethanol/HCl method of amidination reaction and successfully applied for removal of 2,4,6-trichlorophenol (TCP) from water. PAN membranes with fiber diameters from 200 nm to 300 nm were fabricated via electrospinning and provided a large surface area for enzyme immobilization and catalytic reactions. Images of scanning electron microscope demonstrated the enzyme molecules were aggregated on the nanofiber surface. The immobilized laccase exhibited 72% of the free enzyme activity and kept 60% of its initial activity after 10 operation cycles. Moreover, the storage stability of the immobilized laccase was considered excellent because they maintained more than 92% of the initial activity after 18 days of storage, whereas the free laccase retained only 20%. The laccase–PAN nanofibrous membranes exhibited high removal efficiency of TCP under the combined actions of biodegradation and adsorption. More than 85% of the TCP was removed under optimum conditions. Effects of various factors on TCP removal efficiency of the immobilized laccase were analyzed. Results suggest that laccase–PAN nanofibrous membranes can be used in removing TCP from aqueous sources and have potential for use in other commercial applications.



KEYWORDS: *2,4,6-trichlorophenol, biodegradation, laccase immobilization, nanofibrous membrane, electrospinning*

1. INTRODUCTION

Effluents of industrial wastewater contaminated with chlorophenols are seriously harmful to the environment and human health. Recently, enzyme-based treatments for the removal of chlorophenols receive more and more attention since they offer some advantages over physical and chemical processes, such as mild treatment condition, high efficiency of substrate removal, and ability to handle large volumes of effluents.[1−3]

Laccase is a multicopper enzyme that can oxidize a variety of organic substrates, such as diphenols, polyphenols, diamines, and aromatic amines,[4] and has been widely used in drug analysis, wine clarification, paper manufacturing, and bioremediation of industrial effluents.[5−8] Like all free enzymes, free laccase suffers from the weakness of low stability, recyclability, and reusability in industrial applications. These problems can be solved by enzyme immobilization, which make it applicable in different reaction environments, even under harsh conditions.[9,10]

Nanomaterials including nanoparticles and nanofibrous mats are excellent supports for enzyme immobilization since they have a large surface area leading to high enzyme loading and consequently high volumetric enzyme activity. In comparison with nanoparticles, nanofibrous mats have more advantages, such as high recovery and reusability of the enzymes.[11] Nanofibers can be fabricated by electrospinning, which is the only method that can be used to fabricate fibrous carriers with diameters ranging from several micrometers down to several tens of nanometers.[12] Nanofibers electrospun from poly(acrylonitrile-co-acrylic acids), poly(D,L-lactide), and poly(acrylic acids) have been used for enzyme immobilization.[13−15] But few reports are available on laccase immobilization on polyacrylonitrile (PAN) nanofibers.

PAN is an important polymer which has been widely used in filtration, adsorption and composite materials, with good stability and mechanical properties. In consideration of the inertness and hydrophobicity of an acrylonitrile monomer, a derivative of PAN has been synthesized, in which functional groups are introduced into the polymer backbone for enzyme immobilization.[16] However, this method has the drawbacks of high cost, complicated procedures, and difficult operation.

In this study, we used a method for the direct conjugation of laccase molecules onto the surfaces of PAN electrospun fibrous membrane (EFMs) without using a PAN derivative. Although this method has been reported by Li et al.,[17] to our knowledge, there is few people using this method in the area of enzyme immobilization. Additionally, the degradation and removal of 2,4,6-trichlorophenol (TCP, a chlorophenol) by the immobilized laccase was investigated. Results showed that the performance of immobilized laccase in removing TCP was excellent, thereby providing a novel technique in removing

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chlorophenols in contaminated water, especially industrial wastewater.

2. MATERIALS AND METHODS

Materials. The reagents, namely, PAN, *N*,*N*-dimethylformamide (DMF), Coomassie brilliant blue (G250), hydrogen chloride, ethanol, citrate phosphate buffer solution (CPBS, pH 7.0), 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS), and 2.4.6-trichlorophenol, as well as laccase from *Pleurotus ostreatus* (mushroom), were obtained from Sigma (Shanghai, China). Deionized water was used in all of the experiments.

Preparation of PAN−EFMs Using Electrospinning Method. The electrospinning apparatus used in this study includes a high-voltage power supply, a plastic syringe with a stainless steel needle possessing an inner diameter of 0.8 mm, a syringe pump, and an aluminum foil collector. PAN was dissolved in DMF at 25 °C. The parameters of electrospinning were set as follows: the concentration of the polymer solution was 8% (w/w), the flow rate of the polymer solution was 1.2 mL/h, the applied voltage was 18.0 kV, and the distance between the needle tip and the collector was 18.0 cm. EFMs were collected for 12 h and then dried for 10 h at 50 °C under vacuum to obtain a nonwoven mat. The surface morphologies of the PAN−EFMs were examined using scanning electron microscopy (SEM), which were taken on a field emission XL-30 SEM at 30 kV. The functional groups of nanofibers and enzyme immobilized nanofibers samples were obtained with Fourier transform infrared attenuated total reflectance (FTIR/ATR, Bruker-Vector 22) spectrometer equipped with a germanium crystal. The background spectra were recorded in air.

Immobilization of Laccase. The nitrile groups of the PAN nanofiber was activated through amidination reaction, followed by reaction with the laccase solution. The PAN nanofiber was placed in absolute ethanol, and then hydrogen chloride gas was bubbled through the mixture for 10 min to produce the corresponding imidoester derivatives. After the activation reaction, the PAN nanofiber was removed from the absolute ethanol solution and washed with distilled water several times. A total of 10 mg activated nanofiber was then placed in 15 mL of 1 mg/mL laccase solution for immobilization at 30 °C under gentle shaking (150 r/min). The effects of time (1, 2, 3, 4, 5, and 6 h) and pH (3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, and 6.5) on laccase immobilization were analyzed. After enzyme immobilization, the membranes were taken out and rinsed with CPBS (pH 6.0) until no soluble protein was detected in the washings.

Determination of Immobilization Efficiency. Protein content in the solution was determined using Bradford's method on a UV-1700 spectrophotometer from Shimadzu.[18] Laccase immobilization capacity was defined as the amount of bound protein per gram of PAN membranes. The amount of bound protein was calculated using eq 1:

$$\mathrm{Ae}(\%) = \frac{[(C_0 - C)V - C_w V_w]}{W} \times 100 \quad (1)$$

where C_0 and C are the initial and final concentrations of protein (mg/mL), respectively, V and V_w are the solution and washing volumes (mL), respectively, C_w is the concentration of protein in the washings, and W is the mass of EFM (g).

Activity Assay of Free and Immobilized Laccase. The activity of free and immobilized laccases was determined using ABTS as substrate.[19] The assay reaction contained 1 mM ABTS in 0.1 M CPBS (pH 4.5) and a suitable amount of free or immobilized enzyme. For the free laccase, the reaction time was 3 min, and the absorbance was monitored at 420 nm on a UV-1700 spectrophotometer. For the immobilized laccase, the sample was centrifuged at 4000 r/min for 3 min, and the supernatant was decanted before the assay.

The kinetic parameters V_{max} and K_m of the free and immobilized laccases for ABTS oxidation were calculated based on the Lineweaver−Burk plots.[20] ABTS with concentrations of 30 mM to 240 mM were used.

Stabilities of the Free and Immobilized Laccases. The effects of temperature on the activity of immobilized and free laccases were examined by evaluating the enzyme activity at pH 4.5 from 20 to 70 °C. The effects of pH on the activity of immobilized and free laccases were examined by evaluating the enzyme activity at 30 °C from pH 3.0 to 6.0.

The reusability of the immobilized laccase was determined according to the following procedures. The immobilized laccase was used 10 times within one day at the optimum conditions. After each reaction, the immobilized laccase was washed with CPBS (pH 4.5) to remove any residual substrate.

The storage stabilities of the free and immobilized laccases were determined according to the following procedures. The free and immobilized laccases were stored at 4 °C for 18 days. The residual activity was calculated every three days.

The activity of the laccase was measured as described in the Activity Assay of Free and Immobilized Laccase section.

Removal of TCP. All of the treatments with TCP were performed in 15 mL reaction solution in 20 mL bottles with 0.01 g immobilized laccase. The effects of various pH values (3.0, 3.5, 4.0, 4.5, 5.0, 5.5, and 6.0), temperatures (15, 20, 25, 30, 35, 40, 45, 50, and 55 °C), reaction times (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 h), and concentrations of TCP (5, 25, 50, 75, 100, 125, and 150 mg/L) on the removal of TCP were determined. The TCP solution was treated with PAN membranes, free laccase, and immobilized laccase. All of the reaction bottles were tightly closed with screw caps and placed on a horizontal shaker at 150 r/min. The concentrations of TCP in the reaction mixture were analyzed using high-performance liquid chromatography (HPLC, Agilent 1200), as described in the following section. The amount of TCP degraded by the immobilized laccase was calculated using eq 2:

$$q_D = q_0 - q_S - q_A \quad (2)$$

where q_D is the amount of TCP degraded by laccase-EFMs; q_0 is the initial amount of TCP in the solution; q_S is the amount of TCP retained in the solution; and q_A is the amount of TCP absorbed by the EFMs. All treatments were replicated three times, and the average values were obtained.

Degradation Analysis of TCP Using HPLC. The analyses were conducted using an HPLC instrument that has two pumps, an autosampler, and a photodiode array detector. TCP and its oxidation products were extracted from the bottles using a syringe, and the filtered solution was analyzed using HPLC. Standard solutions of TCP were prepared to obtain a standard curve for analyzing the degradation of TCP and its oxidation products. The elution was performed by pumping methanol and water (90:10 v/v) isocratic ally at a flow rate of 1.0 mL/min. UV light absorption was measured at a wavelength of 280 nm.

Data Analysis. One-way ANOVA was performed to determine statistical significance. Differences between multiple groups of data were determined by multiple comparisons using Tukey's procedure at a family error rate of 5%. Data were considered significantly different from each other if $p < 0.05$. All statistical analyses were performed using Design Expert (version 7.0.0).

Nonlinear regression analysis using first-order hopane-normalized model [eqs 3−5] was used to estimate the first-order rates (k), the time required to obtain 50% of substrate biodegradation ($t_{1/2}$), and the removal efficiency after t(RE_t) of TCP using laccase.

$$C_t = C_0 \exp(-kt) \quad (3)$$

where C_0 and C_t are the substrate concentrations at the beginning of the run and at t time, respectively, whereas k is the first-order reaction constant. From the k values, two efficiency factors, namely, $t_{1/2}$ and RE_t, can be calculated.

$$t_{1/2} = \frac{\ln 2}{k} \quad (4)$$

$$\mathrm{RE}_t = \frac{(C_0 - C_t)}{C_0} \times 100 \quad (5)$$

where C_0 and C_t are the TCP concentrations at 0 h and t, respectively.

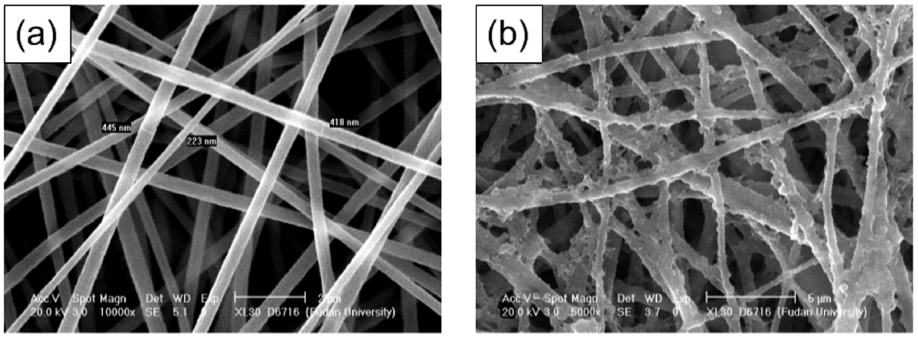

Figure 1. SEM micrographs of PAN–EFMs: (a) original EFMs and (b) immobilized laccase on EFMs.

3. RESULTS AND DISCUSSION

Characterization of Electrospun PAN Fibrous Membranes Before and After Laccase Immobilization. The PAN membranes was activated in ethanol using hydrogen chloride to produce an imidoester derivative, followed by enzyme immobilization through conjugation with the amino groups of laccase molecules. This method is effective in attaching enzyme on the PAN membranes, wherein the immobilized enzyme has good stability during repeated use.

The SEM images of the PAN-EFMs are shown in Figure 1a. The nanofibrous membranes had uniform morphology, and their average diameters ranged from 200 nm to 500 nm. The diameter and structure of the EFMs did not change substantially after activation through amidination reaction. The SEM image in Figure 1b indicates that some laccase molecules form aggregates on the polymer nanofibers after laccase immobilization. This observation could be attributed to the covalent attachment of the enzyme molecules to the surface of the PAN polymer, as well as some enzyme molecules adsorbed on the membrane surface.[21] The immobilized laccase was observed to be attached to the fiber through chemical bond.

To investigate the EFMs further, FTIR spectra for the original and activated PAN–EFMs, as well as the PAN–EFMs with immobilized laccase, were obtained. Compared activated PAN–EFMs (Figure 2b) to original PAN–EFMs (Figure 2a), new peaks at 1647 and 1255 cm^{-1} indicating C═N and C—O—C formation after activation. It can be seen that compared with activated PAN–EFMs (Figure 2b), the laccase–EFMs (Figure 2c) showed new adsorption bands at 1530 and 1635 cm^{-1}, which can be attributed to amide I (the vibration of the C═O bonds) and amide II (a combination of C—N stretching and N—H vibration in protein backbone), respectively. At the same time, a new peak at 1055 cm^{-1} appears, representing C—N bonds formed between the enzyme and PAN after laccase immobilization. These changes suggest that the laccase molecules were successfully immobilized on the PAN–EFMs through chemical bonding.

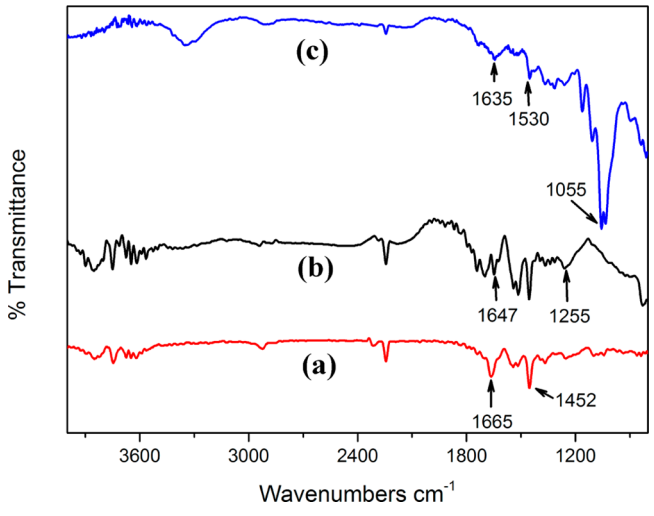

Figure 2. FTIR spectra of PAN–EFMs: (a) original; (b) activated with amidination reaction; (c) immobilized laccase.

Effect of Reaction Time and pH on Laccase Immobilization. Figure 3 shows that the maximum loading of immobilized laccase on PAN membranes was 220 mg/g after 6 h of immobilization, which is higher than that on magnetic particles[22] and magnetic mesoporous silica nanoparticles.[23] The surface area of the PAN membranes was about 20 m^2/g. It was not higher than the mesoporous materials. Therefore, high immobilization efficiency may be attributed to the easier covalent conjugation of amino groups of laccase with the activated membrane surface, not physical adsorption. Figure 3 also shows that pH 4.5 was the optimum pH for enzyme immobilization, wherein the immobilized laccase loading was 223 mg/g. The low and high pH values have obvious effects on enzyme immobilization, which may be attributed to the changes in the diffusion rate of the enzyme and variations in the microenvironment.[24]

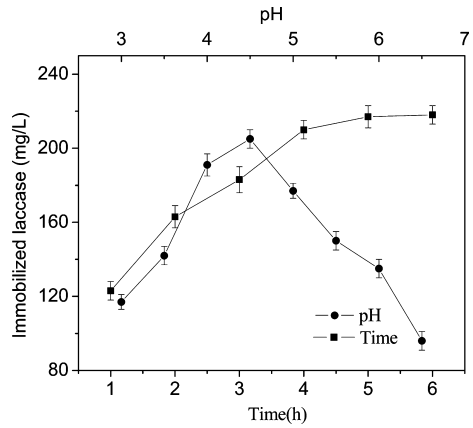

Figure 3. Effects of time and pH on laccase immobilization efficiency: (■) time; (●) pH.

Catalytic Activity of Free and Immobilized Laccases. The laccase loading, specific activity, as well as kinetic parameters K_m and V_{max}, are shown in Table 1.

Table 1. Laccase Loading, Specific Activity, K_m, and V_m of the Free and Immobilized Laccase

laccases	laccase loading (mg/g fibers)	specific activity (U)	K_m (μmol/mL)	V_{max} (μmol/(mg min))
free laccase		88.3	0.9	370.8
immobilized laccase	220	63.7	1.5	289.3

According to the data shown in Table 1, the relative activity of the immobilized laccase was 72% higher than that of the free laccase, which is also higher than the activities of the laccase immobilized on alginate−chitosan microcapsules (59% of its specific activity)[25] and chitosan (52.2% of its specific activity).[26] The difference in the relative activity of laccase may be attributed to the nature of support, method of immobilization, and the laccase source. The loss of catalytic activity of laccase in an immobilized system may be caused by the changes in enzyme diffusion rate and variations in the microenvironment. K_m of immobilized laccase was significantly higher than that of free laccase, whereas V_{max} of immobilized laccase was significantly lower than that of free laccase ($p < 0.05$). The difference in K_m and V_{max} may be due to the lower accessibility between substrate and active points of the immobilized enzyme, which are caused by space barriers of the supports and the increased diffusion limitation, resulting in a lower possibility of forming an enzyme substrate complex.[27−29] In our study, the immobilized laccase has a V_{max} value that is 75% of the V_{max} value of the free laccase; therefore, the immobilization system provides an efficient catalytic ability that is close to that of the free laccase.

Stability of Free and Immobilized Laccases. Stability is very important for various biotechnological applications of immobilized enzymes. Figure 4a shows the effect of temperature on the relative activity of the immobilized and free laccases. Free and immobilized laccases showed maximum relative activity of 98% and 95%, respectively, in this study. The relative activity of the immobilized laccase changed significantly slower than the free laccase with the increase in temperature ($p < 0.05$). This means the immobilized laccase was more stable than the free laccase. This improvement of resistance against temperature may be attributed to the conformational changes caused by the interaction with the matrix and the reduction in molecular mobility.[30] The results also indicated that PAN nanofiber can protect immobilized laccase from environmental factors that can reduce enzymatic activity.

The effects of pH on the activities of free and immobilized laccases are shown in Figure 4b. The immobilized laccase exhibited higher activity than the free laccase in the testing pH range tested. Both laccases achieved maximum activity at pH 4.5. Kouassi et al.[31] found no significant changes were observed in the electrostatic state of the amino acids at the active sites of laccase after immobilization, which may be attributed to the neutral amidine bonds formed after the reaction of the amino groups of the laccase with the imidoester derivatives. The results also indicated that the charge density of the PAN nanofibers was not sufficiently large to change the enzyme microenvironment, consistent with other reports.[32,33] At the same time, we found that the free laccases were more sensitive to pH changes than the immobilized laccases.

Enhanced storage stability and reusability are important advantages of immobilized enzymes. Figure 5 shows the storage stabilities of the immobilized and free laccases. The activity of the free laccase declined significantly faster than that of the immobilized laccase under the same storage condition ($p < 0.05$). The immobilized laccase lost only 8% of its activity within 18 days, whereas the free laccase lost 80% of its activity during the same period ($p < 0.05$). The results show that the storage stability of the immobilized laccase was better than that of the free laccase. The enhancement in storage stability can be attributed to the matrix of the immobilized laccase, which limits the conformational changes of the laccase.

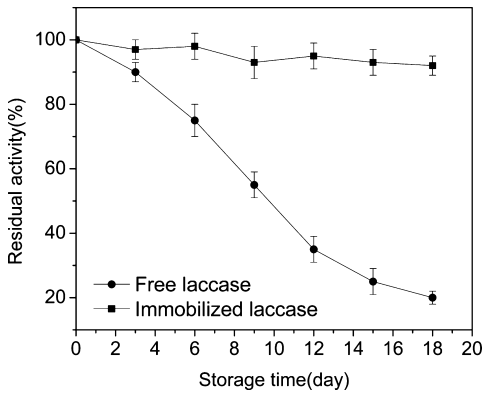

Figure 5. Storage stability of free and immobilized laccase at 4 °C: (■) immobilized laccase; (●) free laccase.

Unlike free enzyme, immobilized enzyme could be easily separated from the reaction solution and reused, which greatly decreases the cost of the enzyme for practical application.[34] Figure 6 shows the operational stability of the immobilized laccase. After five cycles, about 75% of the initial activity of the immobilized laccase was retained. The immobilized laccase also

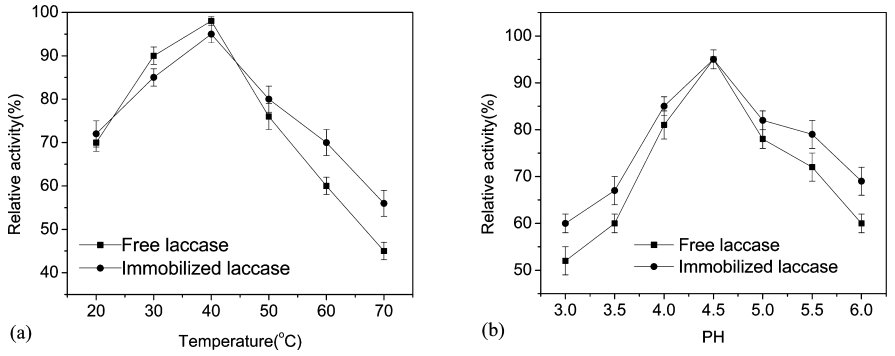

Figure 4. Effects of temperature (a) and pH (b) on laccase activity: (■) free laccase; (■) immobilized laccase.

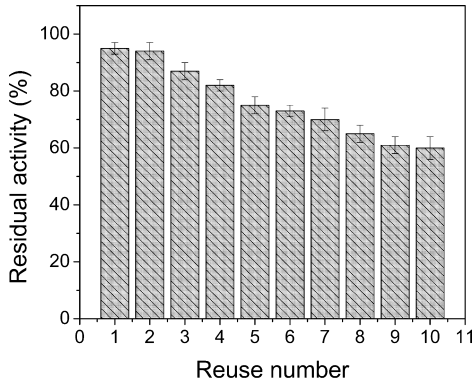

Figure 6. Reusability of immobilized laccase.

retained >60% of their initial activities after 10 cycles, which indicated the reusability of the immobilized laccase. The operational stabilities of the immobilized laccase was better than that of the immobilized laccase on coconut fiber (70% activity retained after five cycles)[35] and poly(D,L-lactide) microfiber (50% activity retained after 10 cycles).[36]

Degradation and Removal of TCP. HPLC analysis of the degradation of TCP revealed the presence of various oxidation products of laccase. Analysis results of mass spectra demonstrated that the peak recorded at about 5.2 min corresponded to TCP. The degradation of TCP in the present study by laccase was higher than that in a previous report by Leontievsky et al.[37] The data indicated that the quantity of TCP decreased by more than 40% in the first hour. More than 90% of the TCP was degraded at the optimum condition in 4 h.

Effect of pH. As shown in Figure 7, the pH values of the solutions varied from pH 3.0 to 6.0. The optimal pH for the

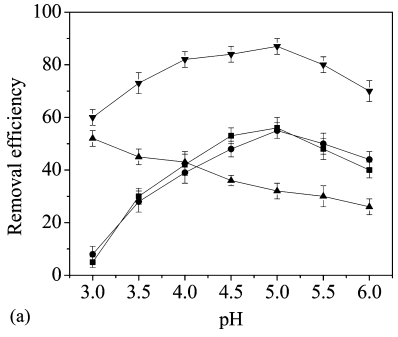

Figure 7. Effect of pH on TCP removal efficiency of free and immobilized laccases (initial substrate concentration is 100 mg/L, temperature is 40 °C, and reaction time is 4 h): (■) free laccase; (●) degradation by laccase−EFMs; (▲) EFMs; (▼) laccase−EFMs.

free laccase was about pH 5.0 with 56% degradation rate of TCP. A higher TCP removal efficiency of laccase−EFMs occurred under a pH range of 3.0−5.0, and the optimal pH was about pH 5.0 with 87% removal rate of TCP. The observed lower TCP removal rate at pH values <4.0 and >6.0 may be due to the suppressed activity of immobilized laccase. Given that the reported pH for effluents from industrial water of paper factories ranges from 5.0 to 9.0,[38] immobilized laccase can be effectively used in removing TCP from industrial wastewater, especially in the paper industry.

Effect of Temperature. Figure 8 shows the TCP removal efficiency in a batch experiment at the temperature range of 15−55 °C. The optimum temperature for the free laccase was

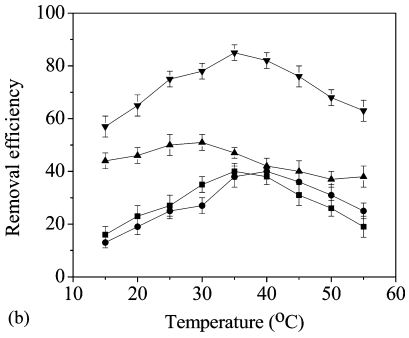

Figure 8. Effect of temperature on TCP removal efficiency of free and immobilized laccases (pH is 4.5, initial substrate concentration is 100 mg/L, and reaction time is 4 h): (■) free laccase; (●) degradation by laccase−EFMs; (▲) EFMs; (▼) laccase−EFMs.

about 35 °C with 40% degradation rate of TCP, whereas the optimum temperature for the immobilized laccase was about 40 °C with 40% degradation rate of TCP. The result of the experiment is similar to the previous study by Morozova et al.,[39] which showed that the removal rate of TCP increases with the increase in temperature. The observed negative effect of higher temperature may be due to the change in enzymatic structure. Given that the effluent temperature of industrial water is lower than 60 °C,[40] the immobilized laccase can be applied in removing TCP from industrial wastewater.

Effect of Initial Substrate Concentration. Figure 9 shows the effect of initial concentration of TCP on its removal

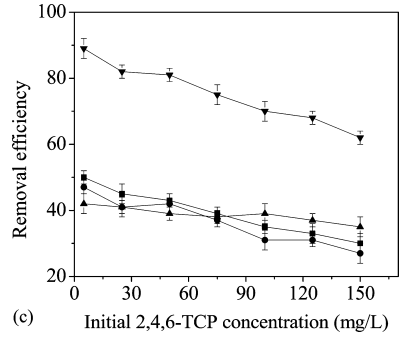

Figure 9. Effect of initial substrate concentration on TCP removal efficiency of free and immobilized laccase (pH is 4.5, temperature is 40 °C, and reaction time is 4 h): (■) free laccase; (●) degradation by laccase−EFMs; (▲) EFMs; (▼) laccase−EFMs.

and degradation efficiencies. As the initial concentration increased from 5 to 150 mg/L, the degradation efficiencies of TCP by both free and immobilized laccase decreased significantly ($p < 0.05$), while removal of TCP by EFMs individually did not significantly change ($p < 0.05$). It can be seen from Figure 9 that the TCP removal efficiency by laccase−EFMs dropped from 89% to 62%. Therefore, the initial concentration of TCP significantly affected its degradation by laccase, not adsorption by the EFMs.

Degradation Kinetics. Figure 10 shows the removal efficiency of TCP in a 4 h batch experiment. The total degradation of TCP by free laccase was about 50% in 4 h. The removal efficiency of laccase−EFMs was much higher than the degradation efficiency. The removal rate of TCP was about 87% in 4 h. The adsorption percentage of TCP by the membrane carrier surface was about 30% after 2 h without

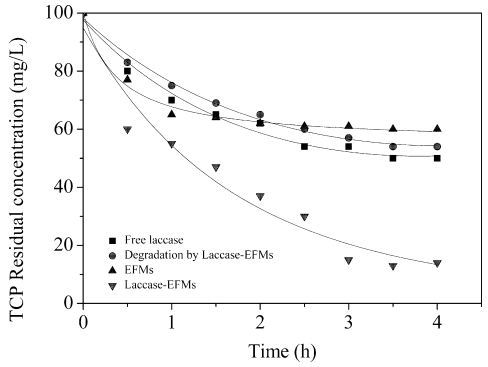

Figure 10. Degradation kinetics of TCP by free and immobilized laccase (temperature is 40 °C, pH is 4.5, initial substrate concentration is 100 mg/L, and reaction time is 4 h): (■) free laccase; (●) degradation by laccase−EFMs; (▲) EFMs; (▼) laccase−EFMs.

significantly increasing in the subsequent 4 h. This finding suggests that physical adsorption may have a great contribution to the TCP removal due to the large specific surface area. The observed higher removal efficiency may be attributed to EFMs adsorption and enzymatic catalysis,[41,42] which is similar to that reported by Xu et al.[43] The kinetic curves of TCP removal followed a first-order reaction.

Table 2 shows that the biodegradation rate of laccase immobilized on EFMs was lower than that of the free laccase.

Table 2. Values of k, $t_{1/2}$, and RE_6 of Free Laccase and Laccase−EFMs toward TCP

sample	k (1/h)	$t_{1/2}$ (h)	RE_6 (%)
free laccase	0.16	4.33	50
laccase−EFMs	0.52	1.33	87
EFMS	0.09	4.95	46
biodegradation by laccase−EFMs	0.14	7.70	40

This observation may be due to the spatial limitations for substrate diffusion and protein flexibility after enzyme immobilization on the carrier, which suggests that the immobilized laccase was partly inactivated during the immobilization process.

4. CONCLUSIONS

PAN nanofibrous membranes with uniform fiber diameter were fabricated via electrospinning and used for laccase immobilization. Laccase was successfully immobilized on the nanofibers using a novel method to directly conjugate laccase molecules on the surfaces of the PAN−EFMs. The laccase loading could reach as high as 220 mg/g fibers. The immobilized laccase retained 72% of the free laccase activity after immobilization. The operational and storage stability of immobilized laccase was significantly improved compared with the free laccase. The method developed in this work offers a simple technique for enzyme immobilization, which could be used in industrial applications. The immobilized laccase was used to remove TCP, resulting in 87% removal efficiency in 4 h. These results indicate that immobilized laccase has potential application in removing chlorophenols, especially TCP, in industrial wastewater.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

PAN = polyacrylonitrile
CPBS = citrate phosphate buffer solution
EFMs = electrospinning fibrous membranes
TCP = 2,4,6-trichlorophenol

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