Study on the Preparation of the AOPAN/MMT Composite Nanofibers and Their Application for Laccase Immobilization

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ABSTRACT

In this paper, a novel nano-material was prepared, and then was applied for enzyme immobilization. Amidoxime ployarcylonitrile /montmorillonite (AOPAN/MMT) composite nanofibrous membranes were generated by electrospinning and amidoxime modification, then the nanofiber membranes were modified by glutaraldehyde; the modified AOPAN/MMT composite nanofibers were applied in laccase immobilization by adsorption and crosslinking method. Scanning electron microscopy was used to visualize the morphology of the nanofibers, and Fourier transform infrared spectroscopy was used to provide information on the surface chemistry of original and modified nanofibers. At the same time, the optimization of immobilization conditions and the relative properties of the immobilized laccase were also studied in this thesis. According to the experimental results the optimum conditions of laccase immobilization were obtained: glutaraldehyde concentration, crosslinking time, enzyme immobilization time were determined as 5%, 10 h and 12 h, respectively. The immobilized laccase showed a better stability resistance to temperature and pH change. Comparison of storage stabilities showed that the immobilized laccase retained more than 62.6% of its initial activity when stored at 4°C for 20 days. However, the free laccase lost most of its activity under the same conditions. The immobilized laccase retained 64.5% of its initial activity after 10 repeated batches of reaction.

INTRODUCTION

Enzyme is protein-based biocatalyst with high efficiency and superior selectivity, and the conditions for enzyme-catalyzed reactions are generally mild with low energy consumption [1-3]. Laccase is a multicopper oxidase that can catalyze various phenolic compounds in the environment (e.g., polyphenols, methyl substitution phenols, aromatic amine, benzene thiol, poly methoxy benzene), and it has been widely applied in areas such as paper manufacturing, wood processing, bioremediation, food industry, as well as textile engineering wastewater treatment [4-6]. Nowadays, most kinds of the laccase is mainly produced in fungi, fungal laccase as a biological catalyst has shown a good prospect of application in the treatment of phenolic pollutants [7].

However, some properties of free laccase also limit its further use, such as instability, non-reusability, and high-cost. Immobilization of laccase to water-insoluble supports has become an effective way to solve these problems to some extent. Therefore, great efforts have been made to immobilize laccase in recent years, and it has been reported that immobilized laccase not only had various different kinds of immobilization methods, but also had many types of substrates for immobilization enzymes [8-10], for instance, physical adsorption, chemical cross-linking, covalent binding, entrapment and encapsulation. Among these methods for enzyme immobilization, the adsorption and cross-linking might be the most appealing methods. Because the physical adsorption method was the easiest method of enzyme immobilization, and high catalytic activities could be retained; the method of chemical cross-linking could improve stability and application of free laccase [11,12].

Electrospun nanofibrous membranes have recently been investigated for enzyme immobilization [13,14]. Because these nanofibers possess a large number of interaction sites for enzyme immobilization due to high surface-to-mass ratios. Besides, MMT also gives a better choice for laccase immobilization, for it possesses excellent intercalation and swelling properties, strong adsorption, and a high affinity for several substances including heavy metal ions and biological materials [15-17]. As we know, no studies have been reported using this strategy to create a substrate for laccase immobilization to this day.

At present, the carrier of immobilized laccase is mainly nanoparticles and magnetic microspheres [18]. In this study, the modification of AOPAN/MMT composite nanofibers with glutaraldehyde has been prepared to immobilize laccase though physical adsorption and chemical cross-linking methods. PAN/MMT composite nanofibers were prepared by electro-spinning first. AOPAN/MMT composite nanofibers membranes were generated by amidoxime reaction of electro-spun PAN/MMT nanofibrous membranes in hydroxylamine hydrochloride aqueous solution. And then AOPAN/MMT composite nanofibers were modified by cross-linking reaction with glutaraldehyde. Finally, the membrane was used for laccase immobilization. Meanwhile, the effects of glutaraldehyde concentration and treatment time on the immobilized laccase were studied. The differences in the performance of immobilized laccase and free laccase were compared and analyzed. Besides, the relative properties of the immobilized laccase and free laccase were also studied. Such as, the amount/capacity, activity, stability, and reusability of the immobilized laccase were evaluated. This study suggested that the modified AOPAN/MMT composite nanofibers membrane might be promising as efficient supports for high-density laccase immobilization.

EXPERIMENTAL

Materials

Laccase (EC 1.10.3.2) was a fungal laccase from Ganoderma lucidum, purchased from Sigma. Poyarcylonitrile (PAN, Mw=90000 g/mol), 2, 20-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), N,N-dimethylformamide (DMF), hydroxylamine hydrochloride, CH_3COOH (36 wt% in water), CH_3COONa, H_3PO_4 (85 wt% in water), CH_3CH_2OH (95 wt% in water), Coomassie brilliant blue (G250) were obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China) and used without further purification. Glutaraldehyde (GA, 50 wt% in water), montmorillonite (MMT) were purchased from Aladdin Chemical Reagent Co. Ltd (Shanghai, China).

Preparation of AOPAN/MMT Composite Nanofibrous Membranes

Prior to electrospinning, a solution of 11 wt% PAN/MMT (25:1(wt: wt)) in DMF was obtained by magnetically stirring for 10 h at room temperature. PAN/MMT solutions were placed in syringes (content of 10 mL) with a blunt needle (the nozzle diameter was about 0.7 mm), and the solution flow rate was controlled by a micro-infusion pump (JZB-1800D, Changsha, China). The high-voltage supplier (DW-P503-4AC, Tianjin, China) was used to connect the grounded collector and metal needles for forming electrostatic fields [19]. During electrospinning, a positive high voltage of 18 kV was applied to the needle, solution flow rate was 0.5 mL/h, and collecting distance between the syringe needle tip and the grounded collector was 20 cm. The PAN/MMT composite nanofibers were collected as an overlaid membrane on the electrically grounded aluminum foil that covered the roller. The membrane was dried at 40°C for 3 h after electrospinning.

Then, the PAN/MMT composite nanofiber membranes (0.5 g, dry weight) were accurately weighed. These nanofiber membranes were added to 400 mL 0.15 mol/L hydroxylamine hydrochloride aqueous solutions. The pH of the reaction solution was adjusted to 7 by adding sodium carbonate solution. The reaction was carried out at 65°C for 2 h. After the completion of reaction, the modified PAN/MMT nanofibrous membranes taken from reaction medium were washed with distilled water and dried at 40°C in a vacuum oven. At the same time, the quality of nanofiber membranes also were accurately weighed, and conversion rates of amidoxime were calculated as our former reports [19].

Immobilization of Laccase on AOPAN/MMT Composite Nanofibrous Membranes

Before immobilized laccase, AOPAN/MMT composite nanofiber membranes (0.01 g, dry weight) were immersed in 50 ml glutaraldehyde aqueous solution and shaken gently at 25°C, then nanofiber

membranes have a certain time of crosslinking, and the excess glutaraldehyde on the surface of nanofiber was washed away by distilled water. Then, the cross-linked AOPAN/MMT nanofiber membranes were immersed in 50 mL, 3 mg/mL laccase solution (pH = 4.5), and at 4°C for 12 h. Finally, the composite nanofibrous membranes were removed from solution and rinsed with the same HAc–NaAc solution until no soluble protein was detectable. And the immobilized laccase stored in a HAc–NaAc buffer at 4°C. Schematic of laccase immobilization was shown in *Figure 1*.

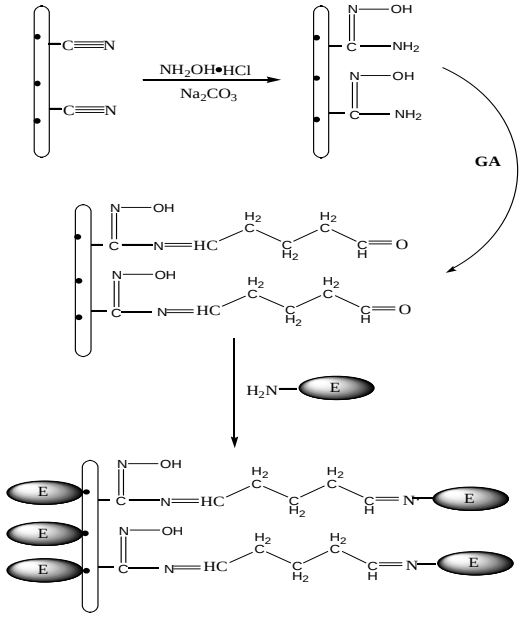


FIGURE 1. Schematic of AOPAN nanofiber immobilized laccase.

In the course of the laccase immobilization, enzyme concentration was determined by the method of Bradford [20]. The amount of bound enzymes was calculated by Eq. (1):

$$G_e = \frac{(C_0 - C_1) \times V_0}{M_d} \quad (1)$$

Here, G_e is the amount of lacase bound onto unit mass of nanofibrous membranes (mg/g), V_0 is the volume of the laccase solution, C_0 and C_1 are the initial and equilibrium laccase concentrations in the solution (mg/mL), and M_d is the mass (dry weight) of the AOPAN/MMT nanofibrous membranes.

Activity Assays of Free Laccase and Immobilized Laccase

To test the respective activities of the free and immobilized lacase, 0.5 mL of laccase solution (3 mg/mL, pH=4.5) or 0.01 g of AOPAN/MMT nanofibrous membranes immobilized laccase were mixed with 14.5 mL of 0.5 mM ABTS solution (pH=4.5). The system was kept at 30°C for 3 min, and 5 replicates were tested for each sample. The activities of the free lacase or immobilized laccase were determined spectrophotometrically by measuring the decrease of absorbance at 420 nm, as a consequence of ABTS consumption [21]. The specific activity of enzyme was then calculated by using the following equation.

$$\nu = \frac{(A_0 - A) \times V}{T \times K \times E_w} \quad (2)$$

where ν is the specific activity of free or immobilized laccase (μmol/mg·min), A_0 and A are the initial and final absorbencies of the solution at 420 nm, V is the volume of ABTS solution (mL), T is the reaction time (min), K is the molar extinction coefficient of ABTS at 420 nm, and Ew is the enzyme amount (mg).

Characterization of Electrospun Nanofibrous Membranes

Field-emission SEM (Hitachi S4800, Japan) was employed to examine morphological structures of different nanofibrous membranes. Prior to SEM examination, the specimens were sputter coated with gold to avoid charge accumulation. FT-IR spectrometer (Nicolet nexus 470, USA) was used to investigate the functional groups on the surface of the nanofibers. Samples of PAN, PAN/MMT, AOPAN/MMT and MMT were sliced into pieces and mixed with KBr. The compound was manually pulverized and pelletized before the analysis which was in the 4000-450 cm^{-1} range.

Effect of Glutaraldehyde Concentration and Crosslinking Time for the Immobilized Laccase

The effect of glutaraldehyde concentration and crosslinking time in the process of laccase immobilization were very important. To determine the optimal conditions for the immobilization of laccase, the AOPAN/MMT composite nanofibrous membranes (0.01 g) were immersed in 30 mL

glutaraldehyde solution. The concentration of glutaraldehyde solution was varied from 1% to 7%. After agitating the mixtures at 40 rpm for 10 h, the nanofibers were withdrawn from the solution, and then each cross-linked AOPAN/OMMT nanofiber membrane was immersed in 50 mL, 3 mg/ml laccase solution (pH=4.5) for 12 h at 4°C. Then, the AOPAN/MMT composite nanofiber membranes (0.01 g) were immersed in 30 mL glutaraldehyde solution (under the optimal GA concentration) reacted for different time (i.e., 4, 6, 8, 10, 12, 15 and 18 h). The relative activity of immobilized enzyme was calculated with the free laccase activity of 100%, and each group was tested three times, the average value was calculated.

Effect of Immobilization Time

The time required for immobilized laccase onto GA-AOPAN/MMT composite nanofibrous membrane to reach equilibrium was examined. The nanofibers (0.05 g) were immersed in sample bottles containing 50 mL 3mg/ml solutions of laccase (pH=4.5) at 4°C. The nanofibers were withdrawn from the solution after pre-determined time intervals and the enzyme concentration in the solution were determined the method of Bradford.

Temperature and pH Dependence of Free and Immobilized Laccase

To determine the temperature dependence, free and immobilized laccase were, respectively, mixed with ABTS solution (pH = 4.5) first. The activities were then measured in the temperature range from 30 to 70°C. To investigate the pH dependence, the activities of free and immobilized laccase were determined at different pH values (from 2.0 to 7.0). The highest enzyme activity as 100%, calculated relative activity under various conditions.

Analysis of Thermal Stability and Storage Stability of the Immobilized Laccase

Thermal stability was investigated by incubating the free and immobilized laccase at different temperatures (i.e., 50, 55, 60, 65, 70 and 75°C) in HAc-NaAc (100 mM, pH 4.5) for 4 h; thereafter, the free and immobilized laccase was studied for enzymatic decomposition of ABTS in the same conditions. Storage stability of the free and immobilized laccase was ensured upon calculating the residual activity of immobilized laccase and free laccase after stored at 4°C in HAc–NaAc (pH=4.5) for 20 days.

Reusability of the Immobilized Laccase

To test the reusability of immobilized laccase, the activity of immobilized laccase was tested 10 times within 24 h. Before each test, the nanofibrous membrane with immobilized laccase was rinsed with HAc–NaAc solution (pH=4.5) to remove any residual substrate, and the activity of immobilized laccase was then tested in a fresh reaction medium.

RESULTS AND DISCUSSION

Morphological Structure of Different Nanofibers

In this study, morphologies of different nanofibrous membranes were observed by SEM, and the results were shown in *Figure 2*. The electrospun PAN nanofibers and PAN/MMT composite nanofibers formed a fibrous membrane with random orientations. Two kinds of nanofibers diameter were very uniform and their average diameter ranged from 300 to 450 nm, as shown in *Figure 2(a)* and *Figure 2(b).* However, compared with PAN nanofibers, the average diameter the electrospun PAN/MMT composite nanofiber was increased. After amidoxime modification (conversion rate of amidoximation 30.8%), the diameter of the AOPAN/MMT composite nanofibers did not change substantially and the fibrous structures was not obviously distorted. But the surface of the composite nanofibers slightly roughened, as revealed in *Figure 2(c).* This indicated that the morphological structure of the nanofibrous membrane could be well-retained in the chemically modification. The SEM images of the nanofibrous membrane with immobilized laccase was displayed in *Figure 2(d).* It was observed that the immobilized laccase on the composite nanofibers and increased the diameter of composite nanofiber.

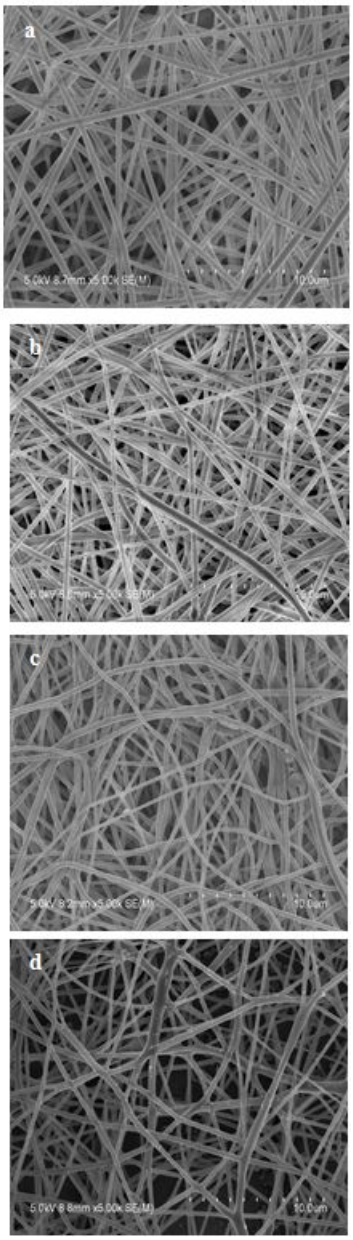


FIGURE 2. SEM images of (a) PAN nanofibrous membrane, (b) PAN/MMT nanofibrous membrane, (c) AOPAN/MMT nanofibrous membrane, and (d) AOPAN/MMT nanofibrous membrane with immobilized laccase.

FT-IR Analysis of Different Nanofibrous Membranes

The FT-IR spectroscopy was an analytical technique generally applied for the qualitative determination of functional groups in a wide range of samples. In this work, the FTIR spectra of PAN, PAN/MMT, AOPAN/MMT nanofibrous membranes and MMT powder were presented in *Figure 3*.

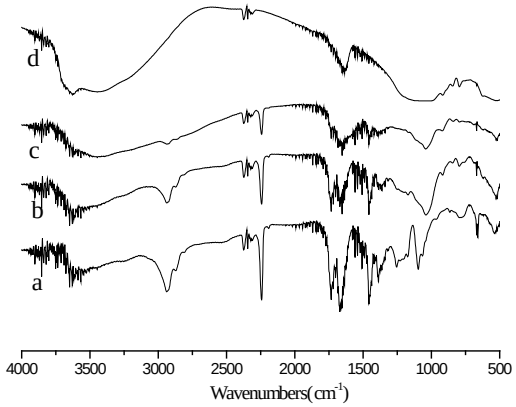


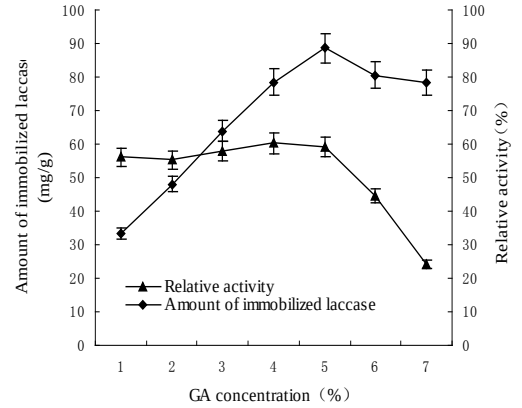
FIGURE 3. FTIR spectras of (a) PAN nanofibrous membrane, (b) PAN/MMT nanofibrous membrane, (c) AOPAN/MMT nanofibrous membrane, and (d) MMT powder.

The characteristic bands of the electrospun PAN nanofibers at 2941 cm^{-1} (CH stretching in CH, CH_2, and CH_3 groups), 2243 cm^{-1} (C≡N stretching), 1739 cm^{-1} (C=O stretching), 1456 cm^{-1} (CH blending) can be observed in the *Figure 3(a).* Compared with the pure PAN nanofibrous membrane, the FTIR spectrum of the PAN/MMT and AOPAN/MMT nanofibrous membrane showed some different peaks. The spectrum *Figure 3(b)* had bands around 3621, 1240-983 cm^{-1}, an indication of Si-O-H, Al-O, and Si-O stretching vibration peak in PAN/MMT and AOPAN/MMT composite nanofiber membranes. After amidoxime modification, the characteristic stretching vibration C=N and N–O absorption bands at 1653 cm^{-1} and 1040 cm^{-1} were also shown in the FTIR spectrum. In addition, a decrease in the absorption band around 2243 cm^{-1} indicated the conversion of the nitrile to the amidoxime group [22].

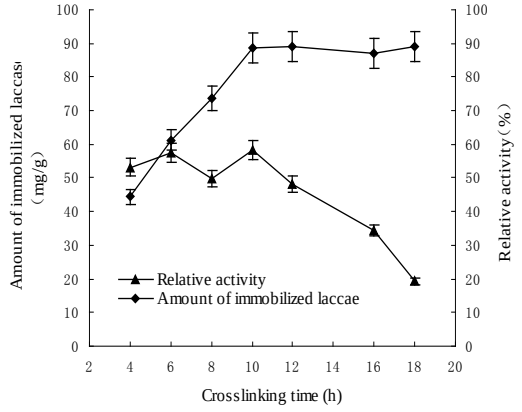
Effect of Glutaraldehyde Concentration and Crosslinking Time for the Immobilized Laccase

Figure 4 showed that the effect of glutaraldehyde concentration and crosslinking time on the immobilized enzyme. As can be seen from *Figure 4(a)*, with the increase of glutaraldehyde concentration, the amount of immobilized laccase was gradually increasing, while the activity of immobilized laccase reflected a faint change to some extent. When the concentration of glutaraldehyde reached 5%, the fixed amount of laccase reached to the maximum (89.12 mg/g, dry weight), when the concentration of GA was further increased, the amount of immobilized laccase began to decline, the activity of immobilized laccase also began to decline. Simultaneously, with the

extension of glutaraldehyde crosslinking time, the amount of immobilized laccase increased, but the activity of immobilized enzyme was relatively little change. When the treatment time was 10 h, the amount of immobilized enzyme reached the maximum and lengthening treatment time, the amount of immobilized laccase and its activity began to decrease. Therefore, when glutaraldehyde concentration was 5%, and the cross-linking time was 10 h, the immobilized enzyme had the best effect in this study.



(a)



(b)

FIGURE 4. The effect of glutaraldehyde concentration (a) and crosslinking time (b) on the immobilized enzyme.

Effect of Immobilization Time

Figure 5 shows that the effect of immobilization time on the immobilized enzyme. Initially, the amount of immobilized laccase generally increased with the extension of immobilization time; after the amount reached equilibrium value, it would be discharged gradually when further prolonging the immobilization time. Upon the basis of the acquired results (*Figure 5*), the optimal immobilization time of AOPAN/MMT composite nanofibers was determined at 12 h, the amount of immobilized laccase reached 89.26 mg/g during this time. The reason was that the composite nanofibers although had a lot of enzyme immobilization sites, with the extension of time, the sites of carrier were gradually reduced due to the immobilized laccase. When all of the active sites were used for the immobilized enzyme, the amount of immobilized laccase reached the maximum equilibrium value.

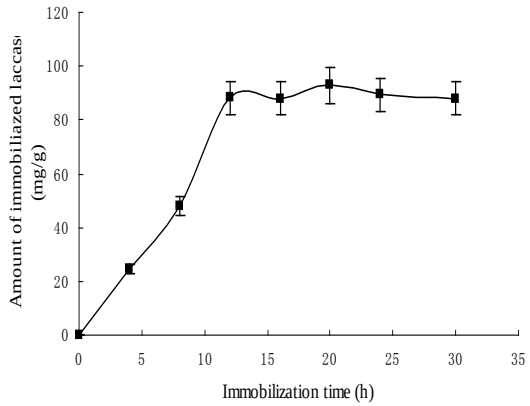
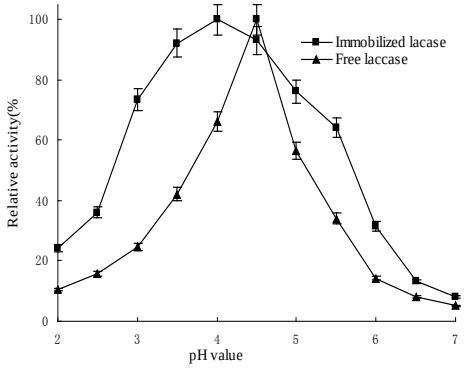


FIGURE 5. The effect of immobilization time on the immobilized laccase.

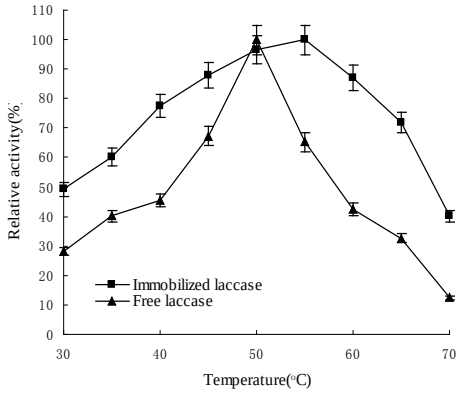
Temperature and pH Dependence of Free and Immobilized Laccase

The effect of temperature and pH value on relative activity of free and immobilized laccase is depicted in *Figure 6*. The relative activity of laccase would be higher with the increase of reaction temperature initially, and it would then become lower when the temperature was further increased. The highest relative activities of immobilized and free laccase were discovered at 55°C and 50°C, respectively; besides, the immobilized laccase generally had higher relative activities than the free laccase in the entire temperature range from 30 to 70°C, especially at 50°C. The higher relative activity would primarily be attributed to the increased structural stability of immobilized laccase molecules, while the multipoint interactions between laccase molecules and functional surface on the supports might provide the further protection against inactivation at a higher temperature. It was obvious that the optimal value of pH for the free laccase and immobilized laccase were identified at

4.5 and 4 respectively, while the optimal pH values for laccase immobilization moved to 4.0. Furthermore, noted that the immobilized laccase exhibited lower degrees of sensitivity to change of pH, and the corresponding residual activities of immobilized laccase were generally higher than that of free laccase.



(a)

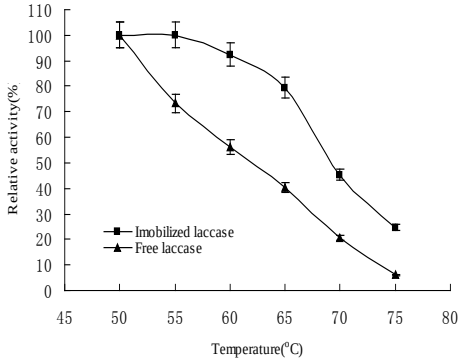


(b)

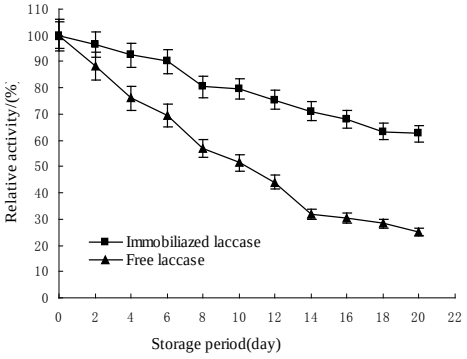
FIGURE 6. Effect of pH (a) and temperature (b) on the activity of free and immobilized laccase.

Thermal Stability and Storage Stability of Laccase

Thermal stability of immobilized laccase was an important parameter in consideration of practical applications. *Figure 7(a)* showed the thermal stability variations of free and immobilized laccase incubated at different temperatures (i.e., 50, 55, 60 , 65, 70 and 75°C) in 100 mM HAc–NaAc (pH=4.5) for 4 h. Laccase activities were observed at the optimum temperature were defined as 100%. Generally, the activities of both free and immobilized laccase were reduced with the increase of incubation temperature. The AOPAN/MMT composite nanofibers membrane immobilized laccase remained 45.28% of the initial activity after incubation at 70°C for 4 h. However, the activity of free laccase was reduced for almost 80% under the same conditions. The higher residual activity and thermal stability of immobilized laccase could be attributed to spatial restrictions on molecular movements, which would limit conformational changes of immobilized laccase molecules, thus resulting in the improved stability against inactivation. The relative activities of free laccase and immobilized laccase were 21.21% and 63.34% after being stored at 4°C in HAc-NaAc (100 mM, pH=4.5) for 20 days, when the corresponding initial activities were set as 100%. The reduction of enzyme activity is a time-dependent natural phenomenon; however, the degree of enzyme activity reduction could be mitigated considerably through immobilization. The immobilized enzyme molecules could better retain their conformational structure; therefore, the inactivation upon long-term storage would be mitigated, thus ameliorating the storage stability of immobilized laccase [23].



(a)



(b)

FIGURE 7. Thermal stability (a) and storage stability (b) of free and immobilized laccase.

Reusability of Immobilized Laccase

The reusability is an important advantage of immobilized enzymes for many practical applications. As shown in *Figure 8*, the residual activities of AOPAN/MMT composite nanofibers immobilized laccase retained 64.5% of their initial activities after was used for 10 times. The results indicated that the AOPAN/MMT composite nanofibers immobilized laccase exhibited the excellent performance on reusability.

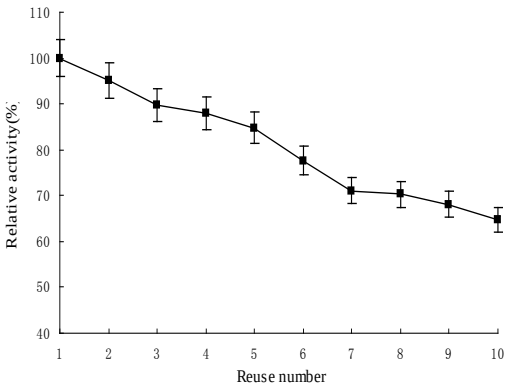


FIGURE 8. Effect of reuse number on the activity of immobilized laccase.

CONCLUSION

To summarize, laccase was very efficiently immobilized on the crosslinked electrospun AOPAN/MMT composite nanofibers. And when the enzyme retains the highest activity, the amount of immobilized laccase up to 89.26 mg / g. Meanwhile, SEM micrograph, FTIR spectra, and Bradford protein assay also confirmed that the enzyme was covalently bonded on nanofiber surface. In addition, the thermostability and storage stability of immobilized laccase were obviously better than free laccase. The composite nanofibrous immobilized laccase also has excellent reusability compare with the free laccase. Moreover, the immobilized laccase exhibited obviously higher resistance to the variation of pH (tested from 2 to 7) and temperature (tested from 30 to 70°C) than free one. These results confirm that the immobilized laccase had a high affinity with the support and gained more stable character compared to free one, which demonstrated the modification of AOPAN/MMT composite nanofibrous membrane could be used as a promising material for immobilizing a wide range of bioactive molecules.

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