Applied Biochemistry and Biotechnology (2021) 193:2547–2566
https://doi.org/10.1007/s12010-021-03556-2

ORIGINAL ARTICLE

Preparation of Functionalized Mesoporous Silica as a Novel Carrier and Immobilization of Laccase

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Received: 2 February 2021 / Accepted: 22 March 2021/ Published online: 30 March 2021

Abstract
Amino-modified mesoporous silica (SBA-15-NH_2) was prepared by hydrothermal method, which is a kind of excellent carrier for enzyme immobilization. The structure of SBA-15 was characterized by SEM and FTIR, which proved that amino group was successfully attached to the surface of SBA-15. The carrier had good mesoporous structure proved by nitrogen adsorption and desorption test. Using SBA-15-NH_2 as the carrier, the optimal conditions of laccase immobilization by two different cross-linking methods were explored. At the same time, the properties of the immobilized enzyme and free enzyme were compared. The results showed that the activity of immobilized laccase by two-step method (2977.5 U/g) was much higher than that by one-step method (239.5 U/g). The optimal conditions were as follows: free laccase (35°C, pH=4.5), two-step immobilized laccase (40°C, pH=4.0), one-step immobilized laccase (35°C, pH=4.0). The two-step method was more adaptable to temperature. The pH adaptation range of the immobilized enzyme is wider, and the thermal stability is greatly enhanced. After five cycles of repeated reaction, the residual enzyme activity of two-step and one-step methods was 56% and 43% of the original. The treatment of simulated wastewater containing 2,4-dichlorophenol (2,4-DCP) by immobilized laccase was also studied. Under optimum conditions (40°C, pH=5.0, 20 mg/L), the removal of 2,4-DCP reached 89.06%. The immobilized laccase is really effective for treatment of 2,4-DCP-containing wastewater

Keywords SBA-15-NH_2 · Cross-linking · Immobilization · Laccase · 2,4-DCP · Water treatment

Introduction

In recent years, with the rapid development of industries, the environmental problems are also increasing rapidly. 2,4-Dichlorophenol (2,4-DCP) is a kind of toxic and carcinogenic organic

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pollutant, which seriously endangers human and animal health [1]. Therefore, 2,4-DCP is considered a priority persistent pollutant, which is usually found in agricultural site, pulp, and paper mill wastewater [2]. At present, there are many methods to remove 2,4-DCP from water system, such as chemical reduction or oxidation, extraction, adsorption, biological degradation, RO (reverse osmosis), or NF (nanofiltration) [3]. As a highly efficient biocatalyst with extensive adaptability, enzyme can catalyze reaction under mild reaction conditions, to decompose pollutants. Due to the specificity and selectivity of enzyme to substrates, the degradation process of pollutants can be simplified to a single pathway, reducing the production of by-products.

Laccase (EC 1.10.3.2) is a kind of a copper-containing polyphenol oxidase [4], which was first discovered by the Japanese scholar Yoshida in 1883 from the secretion of Lac tree [5]. It has been found that laccase widely exists in plants, insects, fungi, bacteria, and other organisms [6, 7]. Laccase is a single-electron oxidase, which can catalyze 250 different types of substrates [8]. The various properties of laccase make it have great potential in brewing [9], detection [10], medicine [11–13], and biochemistry [14–16]. Le et al. [17] used different methods to develop bioelectrochemical oxygen reduction using laccase as a biocathode. Wang et al. [18] mechanically treated pulp with laccase mediator system (LMS) and alkaline hydrogen peroxide. Somtimes, enzymatic reaction in the mediator is conducive to the degradation of pollutants by laccase. However, the high cost and non-reusability of laccase limit its application in practice [19]. Immobilization not only maintains the catalytic activity of the enzyme but also increases the stability of the enzyme, which makes the enzyme reusable. Wang et al. [20] immobilized laccase on magnetic mesoporous silica microspheres and used it to treat coking wastewater. The results showed that the treatment efficiency of the immobilized enzyme on coking wastewater was significantly higher than that of free enzyme. After 10 consecutive treatments, the degradation of the immobilized enzyme on coking wastewater was still 71.3%. Niyaz et al. [21] immobilized laccase on zeolite (NZ)-graphene oxide (GO) composite nanoparticles as a carrier to prepare a novel nano-biocatalyst for degradation of Direct Red 23 organic pollutants. The results showed that the immobilized laccase showed good reusability and high storage stability and thermal stability in five cycles. Ahmet et al. [22] synthesized and modified Fe_3O_4 magnetic particles with mercaptan chitosan (TCS) by coprecipitation. The functional magnetic composite can be used to immobilize laccase, and the stability, efficiency, and reusability of the immobilized laccase are greatly improved.

Mesoporous silica nanomaterials are ideal candidates for enzyme immobilization due to their high specific surface area, large pore volume, controllable morphology, and size, and high surface activity of silicon hydroxyl groups can be used to connect multiple functional groups [23, 24]. In recent years, mesoporous silica nanomaterials have been widely used in enzyme immobilization. Li et al. [25] reported that immobilization of lysozyme with SBA-15 nano-material as carrier can improve the enzyme performance. Lei et al. [26] found that organophosphate hydrolase was immobilized on carboxylethyl- or aminopropyl-functionalized mesoporous silica, and its enzyme activity was twice that of free enzyme. Chong et al. [27] showed that the activity of penicillin acylase (PGA) immobilized on vinyl-functionalized mesoporous silica was higher than that of free PGA.

Physical adsorption [28], entrapment [29], covalent binding [30], and cross-linking [31] are all common methods for immobilization of laccase. Physical adsorption method is low cost, but laccase and carrier adsorption binding force is weak, easy to fall off. The reaction conditions of embedding method are mild, but the affinity between laccase and substrate is hindered. Covalent binding method has good stability, but the immobilization operation is

more complex. Compared with the previous methods, the cross-linking method has the advantages of easy preparation of carrier, high degree of immobilization, strong environmental adaptability, recyclability, and wide range of application [32], which is suitable for this study.

Our research group has done some research on immobilization of laccase and the removal of 2,4-DCP [33, 34]. On the basis, as a novel carrier, SBA-15-NH_2 was prepared by hydrothermal method. Two different immobilization methods of laccase were carried out, which were cross-linking followed by immobilization and cross-linking and fixation at the same time. The enzymatic properties of immobilized laccase and free laccase were compared. Its applicaton in the removal of 2,4-DCP was also studied.

Experimental

Materials

Laccase (Ning Xia Xiasheng Industrial Group Co., Ltd., derived from white rot fungi), glutaraldehyde (Tianjin Ruijinte Chemical Co., Ltd.), citric acid (Tianjin Bodi Chemical Co., Ltd.), disodium hydrogen phosphate (Tianjin Bodi Chemical Co., Ltd.), 2,2′-diammonium bis(3-ethylbenzothiazolin-6-sulfonic acid) diammonium salt (ABTS, Budweiser Technology Co., Ltd.), 3-aminopropyl triethoxysilane (APTES, Sigma-Aldrich), 1,3, 5-trimethylbenzene (TMB, Tianjin Guangfu Fine Chemical Research Institute), TEOS (Tianjin Bodi Chemical Co., Ltd.), PEO-PPO-PEO ($EO_{20}EO_{70}EO_{20}$) (P123, Shanghai Saen Chemical Technology Co., Ltd.), 2,4-DCP (Shanghai Kefeng Chemical Reagent Co., Ltd.), potassium ferricyanide (Tianjin BOSF Chemical Co., Ltd.), ammonium chloride (Tianjin Beichen Fangzheng Reagent Factory), 4-aminoantipyrine (Sinopharm Group Chemical Reagent Co., Ltd.), and other reagents used in the experiment are analytically pure.

Other materials used were as follows: SHZ-82 water bath thermostat oscillator (Jiangsu Zhengji Instrument Co., Ltd.), Multiskan GO full-wavelength enzyme reader (Thermo Fisher Scientific Corporation), LD-3 desktop electric centrifuge (Jintan Shenglan Instrument Manufacturing Co., Ltd.), BS124S electronic balance (Beijing Saiduolis Instrument System Co., Ltd.), JB90-D powerful electric mixer (Shanghai Specimen model factory), GZX-9246 MBE digital display blast drying oven (Shanghai Bo News Industrial Co., Ltd. Medical Equipment Factory), Hitachi X650 scanning electron microscope (Karl Zeiss Company of Germany), ASAP 2020 automatic specific surface area and mesoporous/micropore analyzer (Micromeritics, USA), and Nicolet 6700 Fourier infrared spectrometer (Shimadzu Company of Japan).

Preparation of Materials

Preparation of Modified Silica First, non-ionic surfactant PEO-PPO-PEO (4.782 g) was dissolved in HCl (150 mL, 0.75 mol/L), stirred in a 40°C constant temperature water bath to form a homogeneous solution. Then, pore expanding agent TMB (7 mL) was added, stirring for 2 h; TEOS (8.5 mL) was added dropwise, stirring for 1 h; APTES (0.85 mL) was added, stirring for 20 h, still crystallization at 25°C for 2 d, repeatedly washing with distilled water to neutral, filtering. The solid was dried in a drying oven at 80°C to obtain amino-modified mesoporous silica (SBA-15-NH_2).

Characterization

The morphology of the modified silica was analyzed by using a scanning electron microscope (SEM), and characterized by Fourier transform infrared spectroscopy (FTIR). The nitrogen adsorption and desorption curve of the samples was determined by usng an automatic fast specific surface area and mesoporous/micropore analyzer. The specific surface area of the sample was calculated by Bmnauer-Emmett-Teller (BET), and the pore size was calculated by Barrett-Joyner-Halenda (BJH).

Operation Process of Laccase Immobilization

Two methods were used to immobilize laccase.

Cross-linking Followed by Fixation (Two-Step Method) The quantitative carrier SBA-15-NH_2 (0.1 g) was weighed. Then, glutaraldehyde solution (10 mL, 2.0–7.0%) was added. The cross-linking reaction (5–9 h) occurred at 25°C. The excess glutaraldehyde was removed by repeated centrifugation to obtain the activated carrier. Na_2HPO_4-citric acid buffer solution (10 mL, pH=5.0–7.0) and laccase solution (10 mL, 0.4–1.2 g/L) were added into the activated carrier, immobilized (6–10 h) at 25°C. Na_2HPO_4-citric acid buffer solution was used for multiple washing to obtain the immobilized enzyme.

Cross-linking and Fixation Were Carried Out at the Same Time (One-Step Method) The quantitative carrier SBA-15-NH_2 (0.1 g) was weighed. Glutaraldehyde solution (10 mL, 0.1–2%), Na_2HPO_4-citric acid buffer solution (10 mL, pH=4.0-6.5), and laccase solution (10 mL, 0.06–0.6 g/L) were added. The reaction (4–9 h) was carried out in a water bath constant temperature oscillator at 25°C. The immobilized enzyme was obtained by washing with Na_2HPO_4-citric acid buffer solution for many times.

Determination of Enzyme Activity

Free Enzyme Laccase solution (1 g/L, 2 mL) was taken; then, buffer solution (2 mL, pH=6) and ABTS (2 mL, 1 mmol/L) were added, reacting at room temperature for 5 min. Ice bath was used to terminate the reaction. The absorbance at 420 nm was measured by enzyme labeled instrument. The enzyme amount required for catalytic oxidation of 1 μmol ABTS per minute was defined as one enzyme activity unit, and the free laccase activity (U/g) was calculated. At the same time, three parallel samples were made. Laccase solution (1 g/L, 2 mL), buffer solution (2 mL, pH=7), and deionized water (2 mL) were mixed as blank values.

Immobilized Enzyme Immobilized laccase (0.1 g) was weighed; then, buffer solution (4 mL, pH=6) and ABTS (1 mmol/L, 4 mL) were added, reacting at room temperature for 5 min. The supernatant was taken to measure the absorbance at 420 nm, and the enzyme activity (U/g) per gram carrier was calculated. Three parallel samples and one blank sample were also made.

The relative enzyme activity is the ratio of the maximum laccase activity in the same group of experiments as 100%, and the ratio of other points to the value of this point, usually expressed as a percentage.

The recovery rate of the immobilized enzyme was calculated.

$$R = \frac{IU_I}{IU_F} \times 100\% \quad (1)$$

R is the recovery of enzyme activity (%), IU_I is the activity of the immobilized enzyme (U/g), and IU_F is the activity of free enzyme (U/g).

Determination of 2,4-DCP Concentration

After the reaction, the supernatant (5 mL) was taken into a colorimetric tube, which was diluted to 50 mL with distilled water. Then, ammonium chloride ammonia buffer solution (0.5 mL) was added, pluged, and mixed well. 4-Aminoantipyrine solution (1 mL) was added and mixed well. Finally potassium ferricyanide (1 mL) was added and mixed well. After the reaction for 10 min, the absorbance was measured at 510 nm. Three parallel samples were made at the same time. The supernatant was replaced by distilled water as blank, and the determination was carried out simultaneously with the test sample.

The calculation formula of the 2,4-DCP concentration in the supernatant after the reaction was as follows:

$$\rho = \frac{A - A_0 - b}{a \times 5} \times 100 \quad (2)$$

ρ is the mass concentration of 2,4-DCP in the supernatant (mg/L), A is the absorbance value of supernatant, A_0 is the absorbance value measured by blank, b is the intercept of standard curve of 2,4-DCP (0.0195), and a is the slope of standard curve of 2,4-DCP (0.0399).

Calculation of Removal, Adsorption, and Degradation of 2,4-DCP

Immobilized laccase (0.2 g) was weighed; then, buffer solution with certain pH (10 mL) and solution with certain concentration of 2,4-DCP (10 mL) were added to react in a constant temperature oscillation water bath. After the reaction, the absorbance was measured and the concentration of 2,4-DCP in the supernatant was calculated. The immobilized laccase was replaced by immobilized laccase in adsorption test, and the others were the same as above. There were three parallel samples in each group. The calculated result is the concentration of 2,4-DCP left in the solution after the removal of 2,4-DCP by immobilized laccase, so the removal of 2,4-DCP by immobilized laccase can be calculated from the results. In the adsorption experiment, the decrease of 2,4-DCP solubility was only caused by the adsorption of the carrier. Therefore, the adsorption of 2,4-DCP by immobilized laccase was calculated. The degradation of 2,4-DCP by immobilized laccase was the D-value between the removal and adsorption. The removal, adsorption, and degradation of 2,4-DCP by immobilized laccase were calculated as follows:

$$R_r = \frac{C_0 - C_1}{C_0} \times 100\% \quad (3)$$

$$R_a = \frac{C_0 - C_2}{C_0} \times 100\% \quad (4)$$

$$R_d = R_r - R_a \tag{5}$$

R_r is the removal (%), R_a is the adsorption (%), R_d is the degradation (%), C_0 is the initial concentration of 2,4-DCP (mg/L), C_1 is the concentration of 2,4-DCP degraded by immobilized laccase (mg/L), and C_2 is the concentration of 2,4-DCP adsorbed on the immobilized carrier (mg/L).

Results and Discussion

Characterization of Modified Silica

SEM

The SBA-15-NH_2 carrier prepared in this experiment is in powder form and can be directly scanned. The scanning electron microscope results are shown (Fig. 1). It can be seen that the structure of SBA-15-NH_2 is relatively close, and part of it is agglomerated because of the reaction between silicon hydroxyl groups (Si-OH) adjacent to the surface of SBA-15-NH_2, which leads to the formation of agglomeration state of the support.

FTIR

According to the infrared spectrum of the SBA-15-NH_2 carrier, the group information on the carrier can be analyzed, and the access of amino groups can also be judged. The determination results are shown (Fig. 2). According to the analysis chart, the absorption peak near 459 cm^{-1} is the bending vibration peak of skeleton Si-O-Si. The absorption peaks near 797 cm^{-1} and 953 cm^{-1} are the symmetrical stretching peak and bending vibration peak of Si-O-Si tetrahedron, while the absorption peak at 1084 cm^{-1} is the antisymmetric stretching vibration peak of Si-O-Si tetrahedron. The absorption peaks near 1400 cm^{-1} and 2900 cm^{-1} are the bending vibration peak and stretching vibration peak of P123, respectively, indicating that template still exists in the carrier. The symmetry vibration peak of -N-H [35] is near 1491 cm^{-1}, and there is an unobvious peak near 3244 cm^{-1}, which is part of the symmetric and antisymmetric stretching vibration peak of -NH_2. The other part is covered by the stretching vibration peak of Si-OH and adsorbed water O-H at 3466 cm^{-1}. It can be concluded that the amino group has been connected to the surface of SBA-15-NH_2.

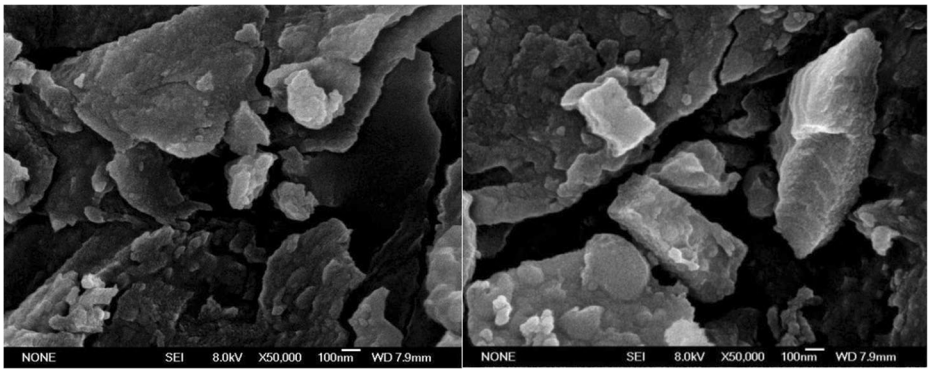

Fig. 1 SEM images of SBA-15-NH_2

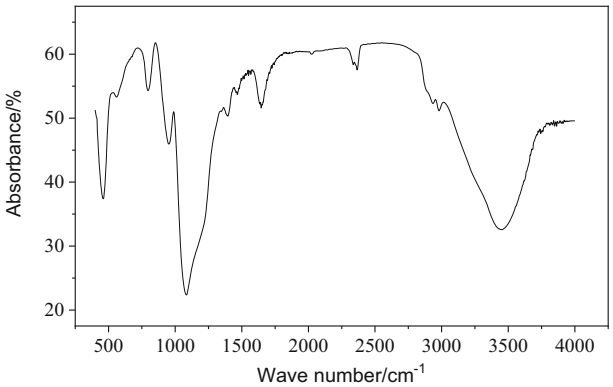

Fig. 2 FTIR spectra of SBA-15-NH_2

Nitrogen Adsorption and Desorption at Low Temperature

The pore size and specific surface area of the materials are mainly measured by nitrogen adsorption and desorption at low temperature. The mesoporous structure of the carrier material can also be seen through the shape of nitrogen adsorption and desorption curve. The nitrogen adsorption and desorption curve of the SBA-15-NH_2 carrier is shown (Fig. 3). It can be seen that the adsorption and desorption curve of SBA-15-NH_2 is consistent with the type IV adsorption and desorption isotherm, which indicates that the support has a mesoporous structure [36]. The reason why the front end of the isotherm cannot be closed is that the pores with small pore size in the carrier material cause irreversible desorption of nitrogen, which makes the hysteresis loop obviously H2 type. The hysteresis ring is produced by porous adsorbate or uniform particle accumulation pore. When the nitrogen in the pore is desorbed, the nitrogen trapped in the bottle suddenly escapes, thus producing the typical curve of H2 type hysteresis ring. The BET method was used to calculate the specific surface area of the carrier which was 33.14 m^2/g, and the pore size of the carrier calculated by the BJH method was 4.13 nm. According to the above data, the effect of TMB on the SBA-15-NH_2 material is very small, far less than that of SBA-15. There are three main reasons. Firstly, the synthesis method of the SBA-15-NH_2 material is the direct synthesis method, which needs to use a relatively mild method to remove the template to avoid the destruction of amino groups, which results in the incomplete removal of the template, resulting in the reduction of pore size. The existence of the template can be verified by the infrared spectrum analysis of the material. Secondly, the internal surface of SBA-15 is also connected with amino groups, resulting in the reduction of pore size. Thirdly, the SBA-15-NH_2 material has lower structure order and different pore sizes. The pore size here is only a relative average value, so there are relatively large pores in the material.

Study on Immobilization of Laccase

Effect of Cross-linking Time

The enzyme was immobilized according to the two-step method described, and the cross-linking reaction time was 5–9 h, the concentration of glutaraldehyde solution was 5%, the pH of buffer solution was 6.0, the concentration of laccase solution was 1.0 g/L, and the reaction

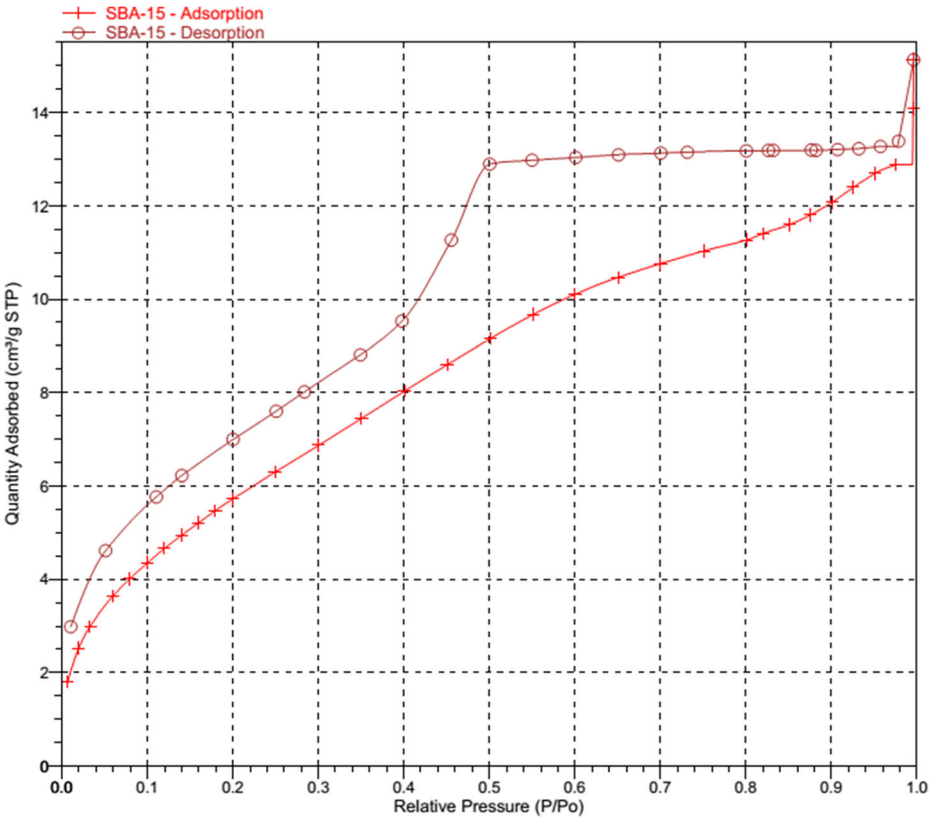

Fig. 3 N_2 adsorption-desorption isotherms of SBA-15-NH_2

time was 7 h. The calculated relative enzyme activity is shown (Fig. 4). It can be seen that the maximum enzyme activity can be obtained when the cross-linking time is 8 h. Because of the short cross-linking time, the reaction between the carrier and glutaraldehyde is not sufficient, resulting in some glutaraldehyde being washed out without combining with the carrier. If the cross-linking time is long, the side reaction may be intensified, and most of the active groups will react with the carrier, affecting the access of enzyme molecules.

Effect of Immobilization Time

In the two-step method, the cross-linking time was 8 h, and the fixation time was 6–10 h. Other conditions were unchanged. The reaction time of the one-step method was 4–9 h. The activity of immobilized laccase was measured. The results of relative enzyme activity are shown (Fig. 5). It can be seen that the best fixed time of the two methods is 6 h and 8 h, respectively. In the reaction system, glutaraldehyde and laccase exist at the same time. When the immobilization time is short, the interaction time of glutaraldehyde and enzyme is short. The main function is cross-linking, and the inhibition effect is not obvious, and the loss of enzyme activity is small. With the prolongation of immobilization time, the amount of cross-linked enzyme increased, and the inhibition of glutaraldehyde on laccase also appeared. The best immobilization time was 6 h. At the same time, due to the long reaction time, the laccase immobilized on the carrier

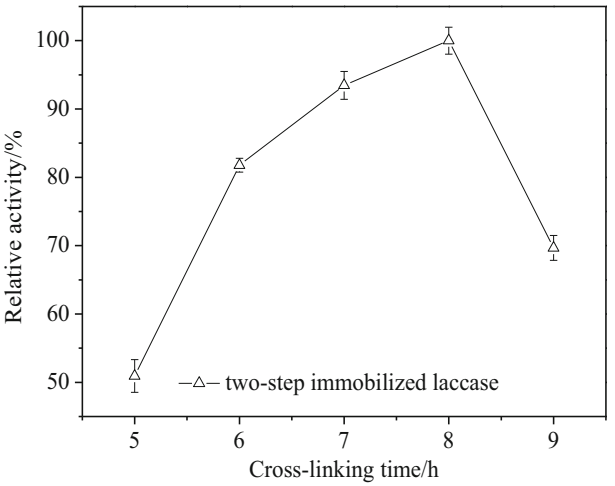

Fig. 4 Effect of cross-linking time on the activity of immobilized enzyme

may react with other active groups, resulting in the loss of activity of groups exposed on the surface, thus reducing the activity of laccase. However, there are only activated carriers and laccase in the reaction system of the two-step method, and the probability of side reaction is smaller than that of the one-step method, so the reaction time can be appropriately prolonged.

Effect of Glutaraldehyde Concentration

The concentration of glutaraldehyde in the two-step and one-step methods was 2.0–7.0% and 0.1–2.0%, respectively. The other conditions were the same as above. The enzyme activity was measured after the immobilized enzyme was obtained. The calculated relative enzyme activity is shown (Fig. 6). It can be seen that the optimal concentration of glutaraldehyde of the two methods is 4% and 0.75% and the enzyme activity by the immobilized enzyme is the largest. With the increase of glutaraldehyde concentration, the amount of laccase cross-linked with glutaraldehyde increased. However, when the concentration exceeded a certain level, the

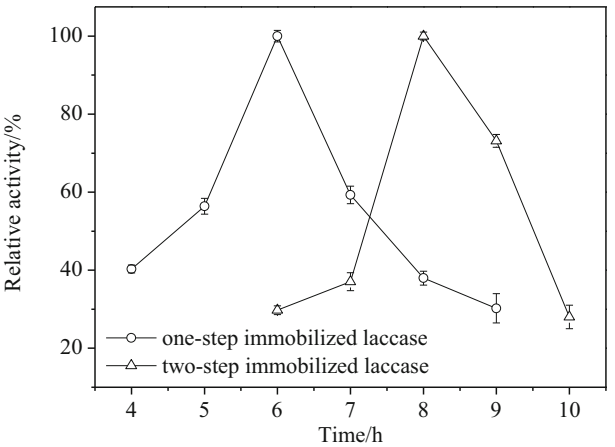

Fig. 5 Effect of immobilization time on the activity of immobilized laccase

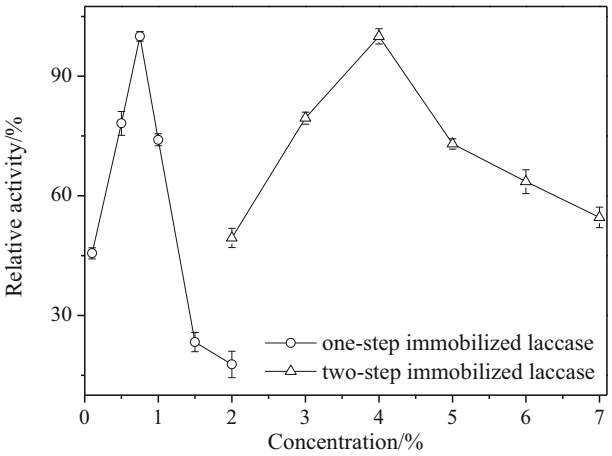

Fig. 6 Effect of glutaraldehyde concentration on activity of immobilized laccase

side effects also increased due to excessive glutaraldehyde. Excessive glutaraldehyde may react with the active groups of laccase, resulting in the decrease of enzyme activity. At the same time, glutaraldehyde can inhibit the laccase activity to a certain extent, and the higher the glutaraldehyde concentration, the more obvious the inhibition effect. Therefore, the activity of laccase decreased. When laccase was added in the two-step method, only glutaraldehyde cross-linked with the carrier existed in the reaction system of the two-step method, and the probability of side reaction was smaller than that of the one-step method. Moreover, there was no free glutaraldehyde in the reaction system, so the inhibition of enzyme was small and the optimal reaction concentration of glutaraldehyde was larger than that of the one-step method.

Effect of pH

The enzyme was immobilized according to the above method. The pH of the two-step and one-step methods was selected as 5.0–7.0 and 4.0–6.5, respectively. The immobilization time was 8 h and 6 h, respectively. The relative enzyme activity results are shown (Fig. 7). It can be seen that the optimum pH of the two-step and one-step methods is 6.0 and 5.0, respectively. The pH of the buffer will change the ionic state of the enzyme molecules and the immobilized carrier. When the pH of the solution is higher than a certain range, the microstructure of the enzyme changes, resulting in the inactivation of the enzyme. The optimum pH of the one-step method is lower than that of the two-step method, because glutaraldehyde and laccase of the one-step method exist in the reaction system at the same time. Under acidic environment, glutaraldehyde exists in free state or in the form of oligomer, and its molecular volume is much smaller than that of polymer under alkaline condition. Therefore, it is easy to enter the pore of mesoporous silica and cross-linked with enzyme molecules, thus increasing the enzyme carrying capacity of the carrier.

Effect of Enzyme Concentration

The concentration of laccase solution in the two-step and one-step methods was 0.4–1.2 g/L and 0.06–0.6 g/L, respectively. The pH was 6.0 and 5.0, respectively. The results of enzyme

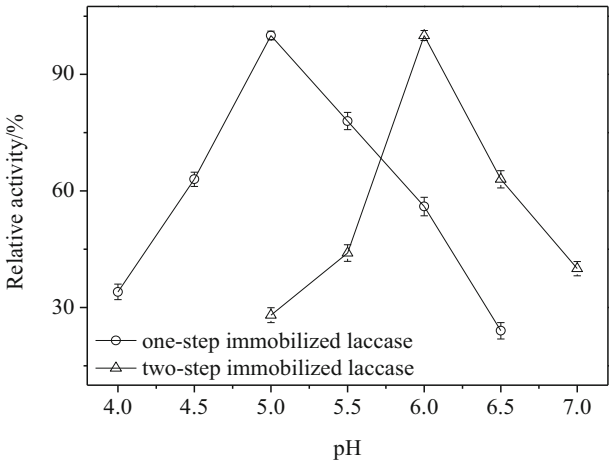

Fig. 7 Effect of pH on the activity of immobilized laccase

activity recovery are shown (Fig. 8). It can be seen that the optimal enzyme concentration of the two-step and one-step methods is 1.0 g/L and 0.2 g/L, and the relative enzyme activity of the immobilized enzyme is the maximum. When the carrier mass and glutaraldehyde concentration were fixed, the molecular weight of glutaraldehyde bound on the carrier was certain, and the free aldehyde group that could bind with the enzyme was also relatively fixed. When the free aldehyde groups which can react with the enzyme are not completely connected with the enzyme molecule, the relative enzyme activity of immobilized laccase will increase with the increase of laccase concentration. However, when the free aldehyde groups that can react with the enzyme have been connected with the enzyme molecule, the increase of enzyme concentration will cause the aggregation of enzyme molecules, and the active sites on the enzyme molecules will be covered, resulting in the decrease of enzyme activity. Moreover, the high concentration of the enzyme will hinder the contact between the substrate and the active site of enzyme molecule, which will indirectly lead to the decrease of relative enzyme activity.

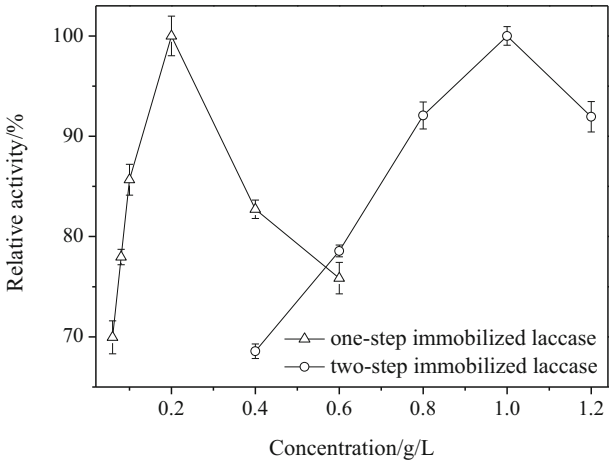

Fig. 8 Effect of enzyme concentration on the activity of immobilized laccase

The calculated enzyme activity of the two-step immobilized laccase was 2977.5 U/g, while that of the one-step immobilized laccase was only 239.47 U/g. The recoveries of enzyme activity were 59.6% and 4.8%, respectively. The difference of enzyme activity between the two methods may be due to the fact that the two-step immobilized laccase is fixed in two steps and laccase does not directly contact with glutaraldehyde molecule. So the glutaraldehyde concentration and enzyme concentration in the fixation process are slightly higher than those in the one-step immobilized laccase. The activity of the immobilized enzyme by the two-step method is larger.

Properties of Free and Immobilized Laccase

Optimum Temperature

Free laccase and two kinds of immobilized laccase were reacted with ABTS at different temperatures for 5 min. The absorbance was measured and the relative enzyme activity was calculated (Fig. 9). It can be seen that the optimum reaction temperature of free laccase is 35°C, and when it exceeds 35°C, the activity of free laccase decreases rapidly, which indicates that free laccase is very unstable at higher temperature. The optimum temperature for the one-step immobilized laccase is 35°C, while that of the two-step immobilized laccase is 40°C, which is slightly higher than that of the free laccase and one-step immobilized laccase. The immobilized laccase can adapt to a wider range of temperature, and both immobilized laccases have higher enzyme activity in the range of 30–45°C. This shows that, after the immobilization of laccase, some active sites of laccase combine with the carrier, the stability of molecular structure is increased, the adaptability to temperature is enhanced, and the applicable temperature range of laccase is enlarged, and the application range of laccase is expanded. At the same time, it can be seen from the figure that the enzyme activity of the immobilized laccase by the two-step immobilized laccase is significantly higher than that by the one-step immobilized laccase after 40°C, so the adaptability of the two-step immobilized laccase to temperature is better.

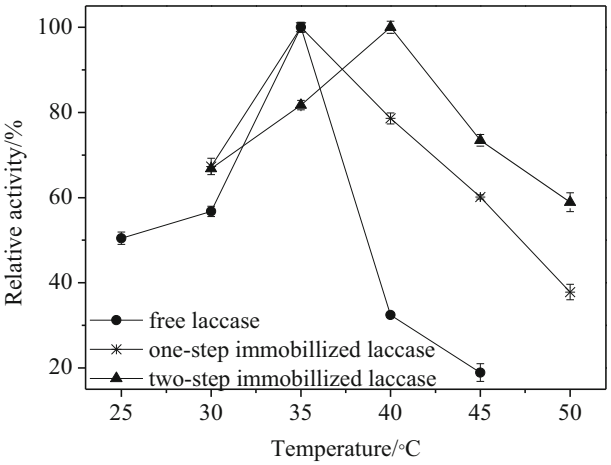

Fig. 9 Effect of temperature on activity of free and immobilized laccases

Optimum pH

In the process of immobilization of laccase, the pH of the reaction system affects the ionization state of the substrate and the immobilized laccase to affect the affinity between the substrate and the enzyme, resulting in the difference in the activity of immobilized laccase. Under different pH conditions, the absorbance of free laccase and two kinds of immobilized laccase was determined, and their relative enzyme activities were calculated (Fig. 10). It can be seen that the optimum pH of both immobilized laccases is 4.0, while that of the free laccase is 4.5, which is slightly higher than that of the immobilized laccase. The charged properties of the immobilized laccase and surrounding microenvironment will be changed. When the pH of the reaction system is too high or too small, the microstructure of laccase molecules will change, resulting in the decrease of enzyme activity. After laccase immobilization, part of its active sites will be fixed on the carrier, and the structural stability will be increased. The effect of pH on the molecular structure of the enzyme was reduced, ensuring that the active group of the enzyme will not be affected in a certain range. So it can adapt to a wider range of pH. It can be seen from the figure that laccase has high enzyme activity between 3.0 and 4.5 after immobilization, which indicates that the adaptability of laccase to pH is enhanced after immobilization.

Thermostability

The free laccase and two kinds of immobilized laccase were respectively placed at different temperatures and kept in a water bath for 1 h, and their absorbance was measured. The calculated relative enzyme activity is shown (Fig. 11). It can be seen that the relative activity of the free laccase increases with the increase of temperature before 35°C, and decreases rapidly when the temperature exceeds 35°C. When the temperature was too high, the conformation of laccase as a protein will change rapidly and irreversibly, which will lead to laccase inactivation. In the temperature range of 25–65°C, the relative activity of the immobilized laccase was high. The reason may be that the interaction between laccase molecule and carrier makes the molecular structure of laccase tend to be fixed, which reduces the change of tertiary structure

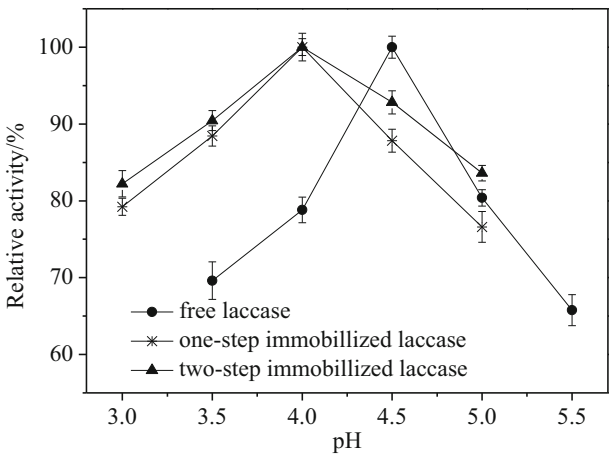

Fig. 10 Effect of pH on activity of free and immobilized laccases

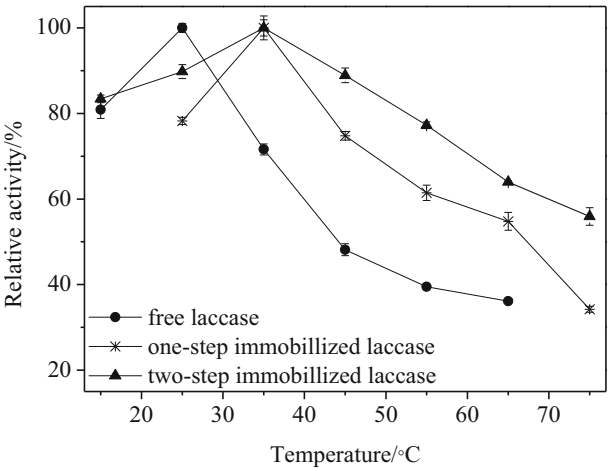

Fig. 11 Thermal stability of free and immobilized laccases

caused by high temperature. Because of the lack of this force, free laccase is prone to folding and denaturation at high temperature. It can be concluded that the thermal stability of laccase was significantly improved after immobilization.

Operational Stability

Free laccase can be dissolved in the solution of the reaction system; it is difficult to separate it. The operation stability of the immobilized laccase was studied. The results of 5 cycles of the immobilized laccase at room temperature are shown (Fig. 12). After five cycles, the residual enzyme activity of the one-step and two-step methods was about 43% and 56% of the original, respectively. The decrease of enzyme activity may be caused by the shedding of laccase molecules which are not tightly bound to the carrier during the reaction. Therefore, the immobilized laccase by two-step methods has good operational stability and can be reused.

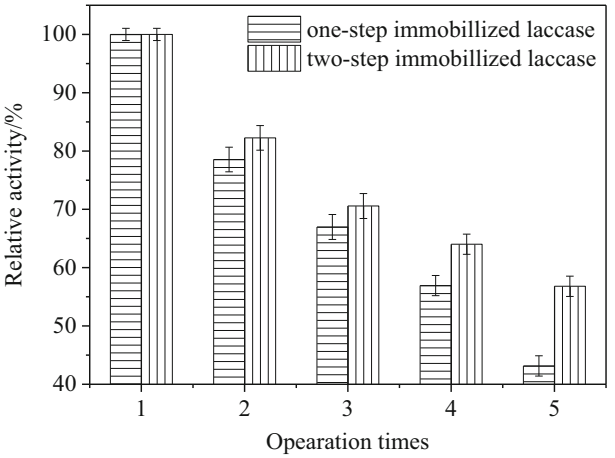

Fig. 12 Operational stability of immobilized laccases

Michaelis-Menten Constants

The free laccase and immobilized laccase were respectively reacted with ABTS solution, and their absorbance was measured at 420 nm. The reciprocal 1/[S] of substrate concentration was taken as the abscissa and the reciprocal 1/V of the initial rate of enzyme reaction was taken as the ordinate. The Lineweaver Burk double reciprocal graph is drawn (Fig. 13). According to the Lineweaver Burk double reciprocal graph, the Michaelis-Menten constant Km was obtained from the intercept (−1/Km) of the straight line on the horizontal axis. The Km of the free laccase and one-step and two-step methods was 3.29×10^{-5}, 6.94×10^{-5}, and 4.73×10^{-5} mol/L, respectively. Km mainly reflects the affinity of the enzyme to the substrate. The smaller the Km, the greater the affinity, and the higher the catalytic efficiency of substrate. According to the data, the affinity of the free laccase to the substrate was greater than that of the immobilized laccase. Therefore, the catalytic efficiency of the immobilized laccase was lower than that of the free laccase. However, the number of laccase molecules in the reaction system of the one-step method was much less than that of the two-step method. Therefore, the affinity of the one-step method is less than that of the two-step method.

From the above analysis, it can be seen that the performance of the immobilized enzyme in the two-step method is much better than that in the one-step method. Therefore, the treatment of 2,4-DCP wastewater with the immobilized enzyme by the two-step method was explored.

The Treatment of Wastewater Containing 2,4-DCP by Immobilized Laccase

Effect of Initial Concentration

Immobilized laccase and inactivated immobilized laccase were used to deal with simulated wastewater with initial concentration of 2,4-DCP of 20–100 mg/L, respectively. The reaction temperature was 40°C and pH was 4.0. After the reaction, the absorbance was measured, and the removal, adsorption, and degradation of 2,4-DCP were calculated (Fig. 14). It can be seen that the removal, adsorption, and degradation of 2,4-DCP gradually decrease with the increase of concentration. The trend of 2,4-DCP removal and degradation was the same. Both of them decreased gently when the concentration was 20–80 mg/L, and decreased rapidly when the concentration was 100 mg/L. When

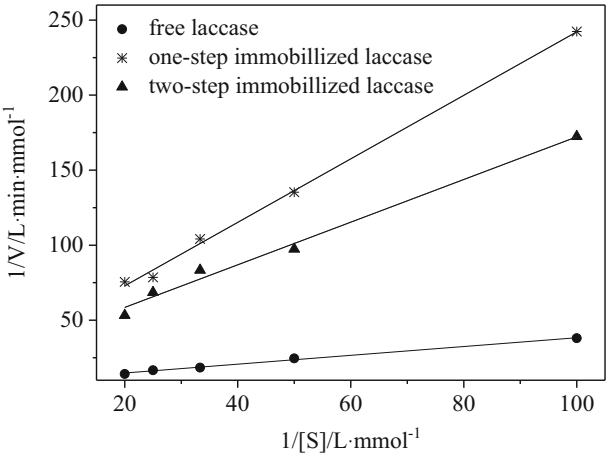

Fig. 13 Michaeil constants of free and immobilized laccases

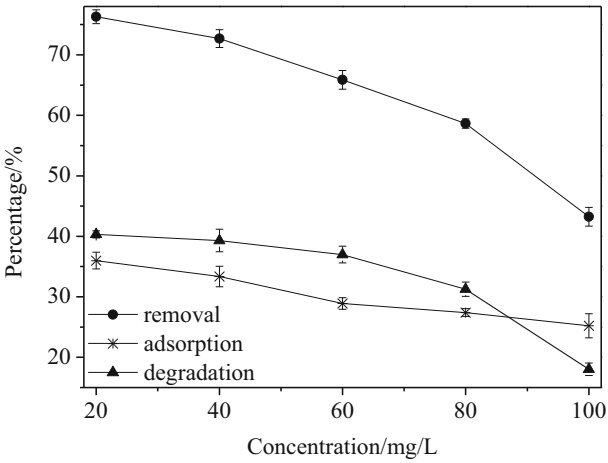

Fig. 14 Effect of initial concentration on 2,4-DCP degradation by immobilized laccase

the concentration was 20 mg/L, the removal of 2,4-DCP (77%) was the highest. When the concentration is increased to 100 mg/L, the removal rapidly drops to below 45%, while the degradation drops to less than 20%. The results showed that the laccase activity was inhibited during the degradation of 2,4-DCP, which led to the rapid decrease of the degradation of 2,4-DCP. The adsorption of 2,4-DCP on the immobilized carrier also decreased gradually, because the active sites that could adsorb 2,4-DCP were certain when the mass of the carrier was constant. When the concentration of 2,4-DCP increased, too many substrate molecules in the solution could not combine with the active sites of the carrier, and there was also a steric hindrance between the crowded 2,4-DCP molecules, which hindered the contact with the carrier, resulting in the decrease of adsorption.

Effect of pH

The initial 2,4-DCP concentration of simulated wastewater was 20 mg/L. The reaction temperature was 40°C and pH was 4.0–6.0. According to the above method, immobilized laccase and

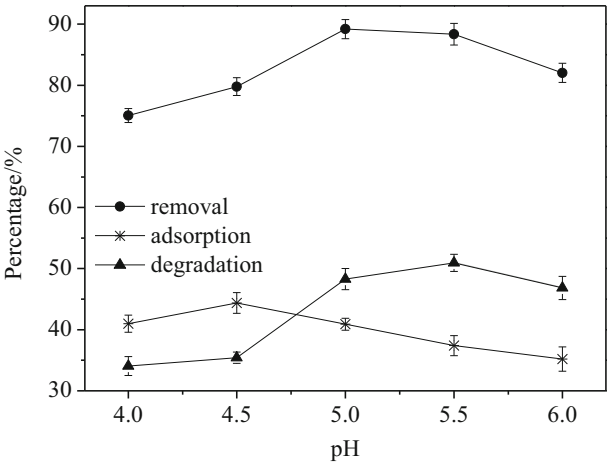

Fig. 15 Effect of pH on 2,4-DCP degradation by immobilized laccase

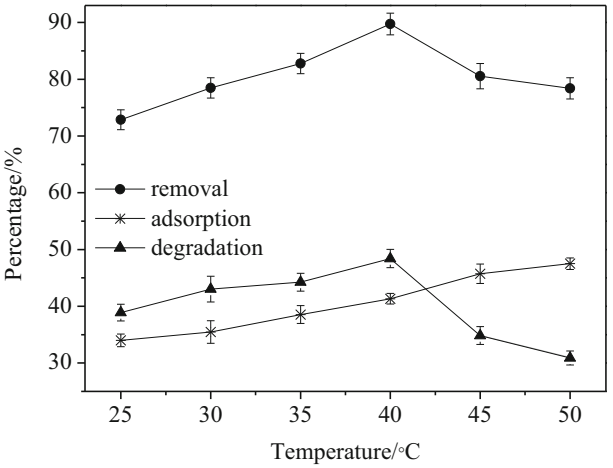

Fig. 16 Effect of temperature on 2,4-DCP degradation by immobilized laccase

inactivated immobilized laccase were used to treat the wastewater. After the reaction, the absorbance of the supernatant was measured. The removal, adsorption, and degradation of 2,4-DCP were calculated (Fig. 15). It can be seen that, when the pH of the solution is 5.0, the removal of 2,4-DCP is the highest; when the pH of the solution is 4.5, the adsorption of 2,4-DCP is the highest; and when the pH of the solution is 5.5, the degradation of 2,4-DCP is the highest. The degradation of 2,4-DCP was more favorable when the pH of the solution was high enough. Because the 2,4-DCP solution system is acidic, which can release the hydrogen ions on the phenolic hydroxyl group to combine with the anions in the solution to form new compounds. In order to get the optimum pH of immobilized laccase catalytic reaction, more anions should be found in the solution to increase the pH of the reaction system. However, when the pH is too high, the space structure of laccase will be changed. It makes the enzyme in the dissociation state which is not conducive to the degradation of 2,4-DCP, resulting in the loss of activity of the immobilized enzyme.

Effect of Temperature

According to the above method, immobilized laccase and inactivated immobilized laccase were used to treat simulated wastewater containing 2,4-DCP at 25–50°C respectively. The initial 2,4-DCP concentration was 20 mg/L and pH was 5.0. The results of removal, adsorption, and degradation of 2,4-DCP are shown (Fig. 16). The results showed that the removal and

Table 1 Performance comparison of different immobilized laccases

Carriers/immobilization		Performance			Enzyme activity/recovery	
Carriers	Methods	pH	T/°C	Stability/%	Activity/U/g	Recovery/%
SBA-15-NH_2	Cross-linking (two-step)	4.0	40	56 (5 cycles)	2978	59.6
	Cross-linking (one-step)	4.0	35	43 (5 cycles)	240	4.8
	Adsorption	5.0	50	69 (20 days)	3005	60.1
	Cross-linking and adsorption	4.5	50	79 (20 days)	3615	72.3
CFLDHs	Cross-linking (two-step)	6.0	55	-	1746	19.2
CS/CFLDHs	Cross-linking (two-step)	6.0	55	-	1271	14.0

Table 2 The removal of 2,4-DCP by immobilized laccase with different carriers

Carriers	Removal/%
SBA-15-NH_2	89.06
CFLDHs	61.78
CS/CFLDHs	81.55

degradation of 2,4-DCP increased gradually from 25 to 40°C. At 40°C, the maximum removal and degradation of 2,4-DCP were 89.06% and 59.06%. When the temperature was higher than 40°C, the removal of 2,4-DCP decreased gradually, but the degradation decreased rapidly. The adsorption of 2,4-DCP increased gradually from 25 to 50°C. The adsorption is divided into physical adsorption and chemical adsorption. The physical adsorption decreases with the increase of temperature, while the chemical adsorption is opposite. It can be concluded that the adsorption of 2,4-DCP by the immobilized laccase is mainly chemical adsorption.

Comparison of Properties of Immobilized Laccase and Its Application

The properties of the immobilized laccase have a great relationship with the carrier and immobilization method. Our group has studied the immobilization of laccase for many years, and the research results are summarized in Tables 1 and 2. It can be seen from Table 1 that, under optimum conditions, the enzymatic properties of the laccase immobilized by the two-step method with SBA-15-NH_2 as the carrier are much better than those by the one-step method. The reusability (5 cycles) and storage stability (20 days) of the immobilized laccase are good, and the thermal stability is also improved. The enzyme activity and recovery of the cross-linking method are slightly lower than those of the absorption method. The performance of the laccase immobilized on SBA-15-NH_2 is much better than that of two hydrotalcite materials (CFLDHs and CS/CFLDHs). In Table 2, compared with the immobilized laccase with SBA-15-NH_2 and two hydrotalcite materials as carriers, the removal of 2,4-DCP (89.06%) to the former is better than that of the latter (61.78%, 81.55%). In general, the two-step immobilized laccase with SBA-15-NH_2 as the carrier has good enzymatic properties, and the removal effect of 2,4-DCP is excellent.

Conclusion

In this work, a novel carrier material was developed by modified mesoporous silica with amino to prepare SBA-15-NH_2 and immobilized laccase was obtained by using glutaraldehyde as cross-linking agent. The successful synthesis of SBA-15-NH_2 was confirmed by SEM and FTIR. The adsorption and desorption tests of cryogenite nitrogen showed that SBA-15-NH_2 had a mesoporous structure. The activity of the immobilized enzyme obtained by the immobilization method of cross-linking first and then immobilization (two-step method) was much more effective than that obtained by cross-linking and immobilization at the same time (one-step method). Compared with the free laccase and one-step immobilized laccase, the SBA-15-NH_2-Lac by the two-step method has a wider range of temperature adaptability and significantly enhanced pH stability, operational stability, and thermal stability. In addition, the SBA-15-NH_2-Lac exhibited excellent catalytic efficiency. And the pH and temperature stability for removing 2,4-DCP has greatly improved. By comparing with the previous studies of our research group, the immobilized enzyme, using two-step SBA-15-NH_2 as the carrier, has good

enzymatic properties and is an excellent material for removing 2,4-DCP. The molecular mechanism of enzyme performance improvement will be further studied in the future.

Funding This study is supported by the National key R & D projects of China (2016YFC0208100).

Availability of Data and Materials Not applicable.

Declarations

Ethics Approval Not applicable.

Consent to Participate All authors consented to participate in the manuscript.

Consent for Publication All authors consented to the publication of the manuscript.

Conflict of Interests The authors declare no competing interests.

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