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Laccase Immobilized on Mesoporous SiO_2 and Its Use for Degradation of Chlorophenol Pesticides[1]

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Abstract—In this paper, mesoporous silica with large specific surface area was used to immobilize laccase by the glutaraldehyde cross-linking method, and after screening and optimization experiments, the best enzyme immobilization process conditions were found (25°C, pH 5.4, 4% glutaraldehyde and 0.2 g/L laccase, treatment time 6 h). After that, the removal and degradation ratio of 2,4-dichlorophenol (abbreviated as DCP) under different conditions were also studied. After the degradation process was performed for 6 h at 30°C, pH 5.4, and DCP initial concentration of 50 mg/L in the presence of 0.1 g of immobilized laccase, the removal ratio and the degradation ratio were 42.28 and 15.93%, respectively. Compared with free laccase, the reusability of immobilized laccase is significantly improved.

Keywords: mesoporous silica, modification, immobilized laccase, catalyst degradation, 2,4-dichlorophenol

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INTRODUCTION

Widely used in wood preservation and in the production of anti-rust agents, bactericides, pesticides and herbicides for long periods, chlorophenols exist universally in the environment. Chlorophenol pesticides can alter the properties of biological tissue. They are corrosive and extremely irritating to the skin and the mucous membranes. The toxicity of chlorphenols increases with increase of the degree of chlorination. Difficult to be degraded, chlorphenols can persist and accumulate in the environment for a long term. Effective the degradation of chlorphenol is a research focus all over the world.

The present degradation methods include wet oxidation, photooxidation and ozone oxidation [1]. As an oxidase, laccase has a strong ability of decomposing organic matter and degrading chlorinated pesticides. Gaitan et al. [2] studied the biodegradation of DCP using the laccase from *Trametes pubescens*. The results showed that biodegradation ratio of DCP is 99% after 4 h of reaction. Yin et al. [3] found that photocatalysis prior to laccase treatment can remove chlorophenols more completely. Because immobilized laccase is more stable and has a higher efficiency than free, immobilized laccase has a good application prospect in pesticide degradation.

Enzyme immobilization is a new technology originating from the 1950's. By chemical or physical means, carrier is used to link the enzyme to a certain area, so that its molecules can perform unique and active catalytic action in the bound state. With this technology, the enzyme can be recovered and re-used for a long time. The methods for enzyme immobilization can be classified into three kinds: carrier-bonding, cross-linking and entrapment. Liu et al. [4] prepared the macroporous silica-immobilized laccase and studied its performance in degradation of 2-chlorophenol, and found that as much as 96.4% of 2-chlorophenol could be removed after 5 h. Magnetic composite particles and organic materials can also significantly enhance the thermal, pH, operational and storage stabilities of immobilized laccase [5–7].

In the present work, laccase was immobilized on the mesoporous silica via carrier-bonding using glutaraldehyde as cross-linking agent. The DCP degradation by the prepared material, the conditions of enzyme immobilization and the properties of the immobilized enzyme were studied, respectively. At the same time, the factors affecting degradation process were also studied.

[1] The article is published in the original.

EXPERIMENTAL

Reagents

Cetyltrimethylammonium bromide (CTAB), tetraethyl orthosilicate (TEOS), nitric acid, guaiacol, glutaraldehyde, DCP, 4-aminoantipyrine, potassium ferricyanide, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, (3-aminopropyl)triethoxysilane (APTES), ammonia solution were all of analytical grade and were used as received. Laccase (from *Aspergillus sp.*, activity ≥ 0.1 U/mg, reagent grade) was purchased from Sigma.

Synthesis and Modification of Mesoporous Silica (M–SiO_2)

CTAB (1.01 g) was dissolved in 88 mL of deionized water and then nitric acid (12.5 mL, 2 mol/L) was added to the solution, the mixture was stirred until it become transparent. Following this, 3.48 mL of TEOS was added dropwise slowly, and the mixture was stirred for 24 h at 30°C. The formed white precipitate was collected via filtration and repeated washing with de-ionized water, and dried in air at room temperature, then calcined for 6 h at 550°C in air in the muffle furnace. The obtained white mesoporous silica powder was named M–SiO_2.

Preparation of Amino-functionalized Silica (M–SiO_2–NH_2)

1.5 g of M–SiO_2 was refluxed for 4 h in 50 mL of methylsulfonic acid aqueous solution (30 wt %). Then the material was filtered and washed to neutral pH with deionized water, and then dried for two hours in vacuum.

The obtained product (1.0 g) was dispersed in 50 mL of toluene, and 0.5 mL of APTES was dropped slowly. The mixture was refluxed for 24 h filtered. The solid powder obtained was washed with anhydrous ethanol for three times, and then dried for 2 h at 60°C in vacuum. The resulting product obtained was named M–SiO_2–NH_2.

Immobilization of Laccase

In a typical immobilization procedure, 0.1 g of M–NH_2–SiO_2 and 10 mL of 4% glutaraldehyde as cross-linker were mixed for 8 h. Following this, the mixture was centrifugated to wash away unreacted gutaraldehyde. After that, the cross-linked carriers and 10 mL of 0.2 g/L laccase solution were shaken in 5 mL of acetate buffer solution (pH 5.4) for 6 h. The obtained material was separated by centrifugation and washed with deionized water. Its activity was calculated according to Eq. (1).

To screen out the optimum conditions of laccase immobilization, the experiments were carried out by adjusting one parameter while the other four parameters were fixed. These five parameters are the concentrations of cross-linker and laccase, the time of coupling and immobilization, and the pH of reaction solution.

Determination of the Activity of Immobilized Laccase

Degradation of DCP by immobilized laccase. The determination of laccase activity was based on the method reported by Bains [8]. The activity can be calculated by the Eq. (1).

$$\text{The activity of enzyme} = \frac{1000X}{T}, \quad (1)$$

where X is the increase in absorbency and T is the reaction time (min).

The specific activity of the immobilized laccase (U/g) is determined as the total activity of immobilized enzyme divided by the mass of the dry immobilized laccase.

Analytic determination of DCP concentration. The concentration of DCP was determined by a colorimetric method [9]. In the range of DCP concentration determined, the absorbance and the DCP concentration presented a good linear relationship.

Calculation method of degradation ratio. In the process of DCP degradation by immobilized laccase, mesoporous silica carrier can absorb DCP because of its large specific surface area. The influence of adsorption of DCP by the carrier should be taken into account in degradation efficiency calculations. In the absorption experiment, we used cross-linked carrier under the same conditions to exclude this effect.

The removal ratio, the adsorption ratio and degradability of DCP are calculated by the following equations:

$$\text{Removal ratio} = \frac{C_0 - C_d}{C_0} \times 100\%, \quad (2)$$

$$\text{Adsorption ratio} = \frac{C_0 - C_a}{C_0} \times 100\%, \quad (3)$$

$$\text{Degradation ratio} = \text{Removal ratio} - \text{Adsorption ratio}. \quad (4)$$

where C_0 is the initial DCP concentration, C_a is the DCP concentration after adsorption, and C_d is the DCP concentration after degradation.

The factors influencing DCP degradation with immobilized laccase. In a typical experiment on DCP degradation, 10 mL of 50 mg/L DCP solution was placed into a flask, and then 10 mL of acetate buffer and 0.2 g of immobilized laccase was added. Next, the flask was placed in a shaking incubator at the desired temperature for 6 h. Finally, the reaction mixture was

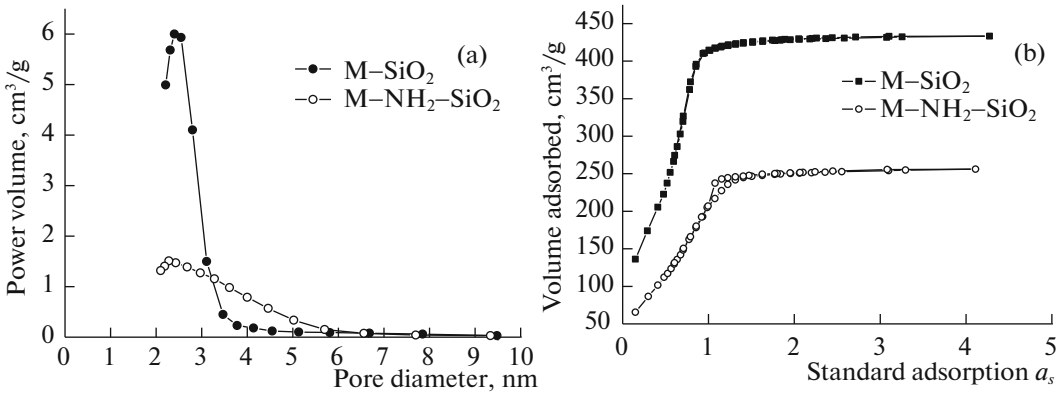


Fig. 1. Pore size distribution (a) and a_s-curves (b) of unmodified and modified mesoporous silica.

filtered, and the concentration of unreacted DCP in it was measured.

In order to study the effects of DCP concentration, pH and temperature, a set of experiments was carried out by adjusting one selected parameter while the other two were kept constant.

Determination of repetition use of immobilized laccase. Immobilized enzyme (0.2 g) was added to the mixture of 10 mL of DCP solution (100 mg/L) with 10 mL of acetate buffer solution (pH 5.5). The resulting solution was reacted for 6 h at 30°C, and then filtered. The cross-linked carrier was used for absorption experiment under the same conditions. The immobilized laccase and cross-linked carrier were separated and washed with deionized water at the end of each experiment. They were refrigerated to be reused in the next day (four times continually), finally degradation ratio was calculated.

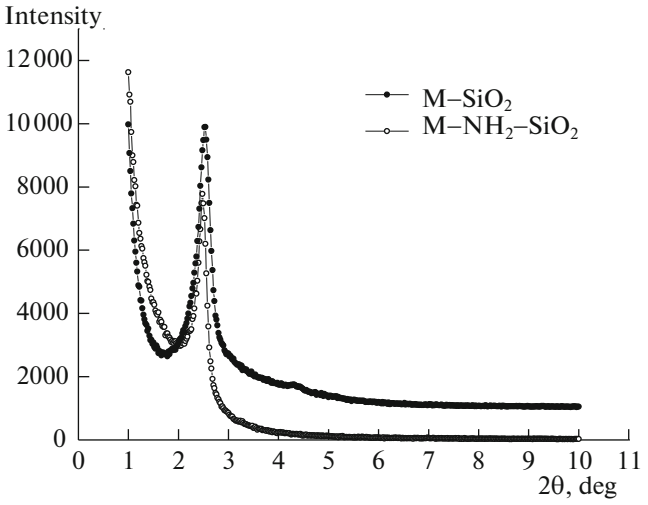


Fig. 2. The XRD patterns of unmodified and modified mesoporous silica.

RESULTS AND DISCUSSION

Characterization of Modified Mesoporous Silica

Mesoporous silica was modified using post-grafting method to obtain amino groups on its surface. The nitrogen content of sample was measured by elemental analysis and found to be 2.96%, corresponding to 2.19 mmol/g SiO_2 grafting density. Using N_2 adsorption and X-ray powder diffraction analysis, it can be found that the specific surface area of the amino-modified sample decreased from 1259 to 566 m^2/g, pore diameter changed from 2.64 to 2.30 nm, indicating that the modified product was still mesoporous material, as shown in Figs. 1a and 2.

In order to demonstrate mesoporous structure of $M-SiO_2$ and $M-SiO_2-NH_2$, the samples were analyzed by a_s-curve method using LiChrospher Si-1000 ($S_{BET} = 25\ m^2/g$) as absorbent medium for reference [10].

Note that the standard reduced adsorption is defined as $a_s = v_{ref}(p/p_0)/v_{ref}(0.4)$, in which $v_{ref}(p/p_0)$ is the amount of reference adsorption at different relative pressures, $v_{ref}(0.4)$ is the amount of reference adsorption at relative pressure of 0.4. a_s-Curve of $M-SiO_2-NH_2$ is plotted with a_s as abscissa and the amount of sample adsorption as ordinate, as shown in Fig. 1b.

The adsorbed volume rapidly increases when a_s value is between 0.14–1.5; the amount of $M-SiO_2$ and $M-SiO_2-NH_2$ adsorption becomes stable with the increase of a_s value above 1.5. Although the amounts adsorbed onto $M-SiO_2$ and $M-SiO_2-NH_2$ samples continue to increase little as the a_s value increases. Such behavior most likely arises from the presence of large amounts of mesopores in the samples together with small amounts of micropores, it reveals that the obtained products $M-SiO_2$ and $M-SiO_2-NH_2$ are mesoporous material [11].

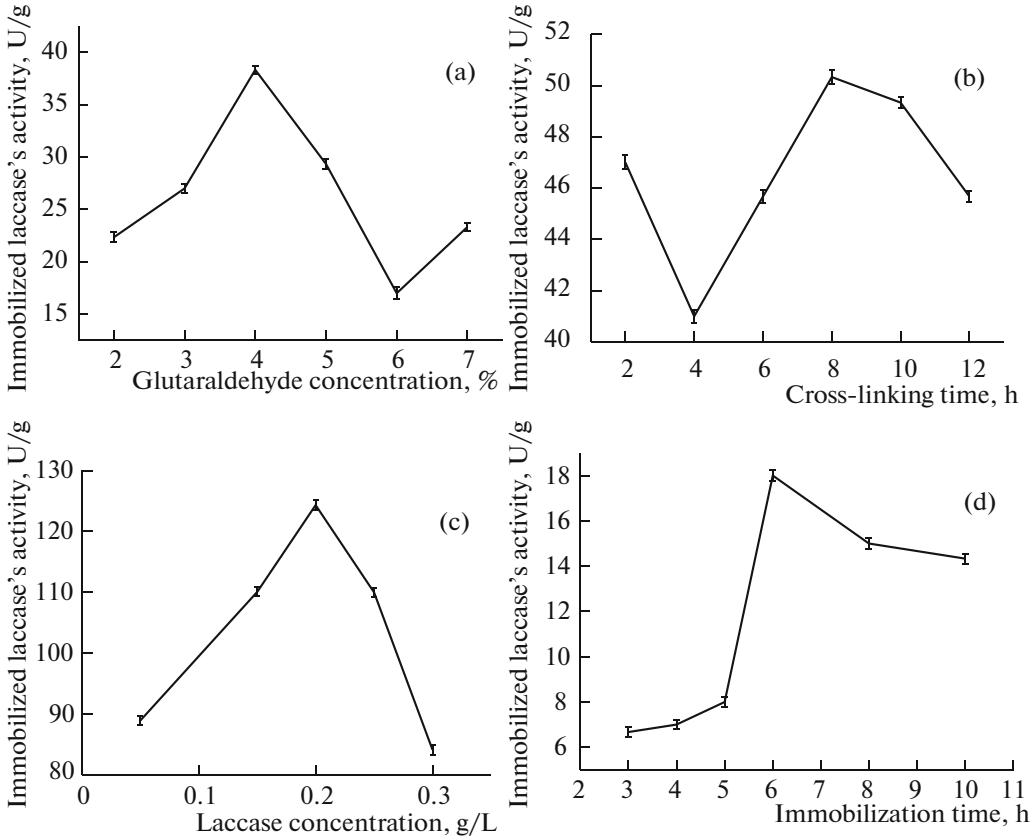


Fig. 3. Effect of immobilization conditions on specific activity of immobilized laccase.

Immobilization of Laccase and Its Properties

Optimal conditions of laccase immobilization. Effect of glutaraldehyde concentration. Specific activity of immobilized laccase increases with increase of glutaraldehyde concentration from 2 to 4% (Fig. 3a), reaching maximum 4%, and decreases at higher glutaraldehyde concentrations.

Low glutaraldehyde concentrations led to fewer suspended aldehyde groups on the mesoporous silica surface and binding less laccase molecules. When the concentration of glutaraldehyde was larger than 4%, more aldehyde groups were grafted on the surface of mesoporous silica, and the immobilization reaction was too rigorous, and multiple binding sites were produced between the aldehyde groups and laccase molecules. The conformation of the laccase was therefore changed and activity of part of the laccase was liable to be lost. The excessive glutaraldehyde also underwent aldol condensation reaction at the same time. The formed polymeric products block some part of the surface of mesoporous silica. This result affected the pore structure of the microspheres and thus prevented covalent immobilization of the laccase. These results demonstrated that glutaraldehyde is not only a cross-linking reagent for immobilization reaction but also a denaturant for laccase. So, in the process of cross-linking, it is essential to guarantee that the obtained laccase has certain activity while avoiding excessive concentration of glutaraldehyde which can cause loss of enzyme activity due to deactivation. Glutaraldehyde concentration of 4% was found to be optimal and therefore is adopted for the cross linking reactions in further experiments.

Effect of cross-linking time. From Fig. 3b, specific activity of immobilized laccase was found to increase with cross-linking time and reached maximum value at 8 h. When the cross-linking time was more than 8 h, specific activity of the immobilized laccase decreased. Because glutaraldehyde has two functional groups, free aldehyde group appeared on the surface of carrier after aldehyde group at one side was coupled with amino group on the surface of the mesoporous silica. When the cross-linking time was relatively short, the glutaraldehyde could not react sufficiently with amino groups on the mesoporous silica, leading to less free aldehyde groups on the carrier surface. The resulting amount of the immobilized laccase molecules and specific activity of the immobilized laccase decreased accordingly.

The content of the aldehyde groups on the surface of the carrier apparently increased with increase of cross-linking time. However, since the amount of

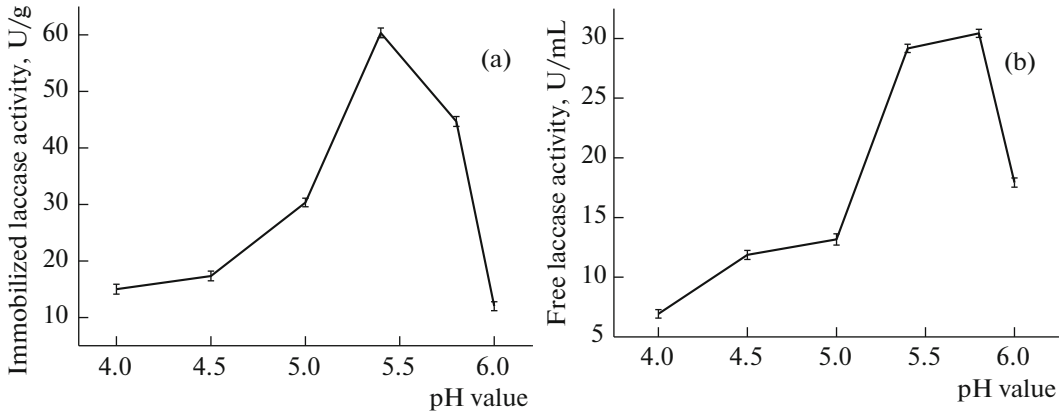


Fig. 4. Effect of pH value on specific activity of laccase (the laccase concentration was 0.2 g/L). Immobilized laccase (a) and free laccase (b).

amino groups on the carrier was limited, when the amount of the linked fixed aldehyde groups reached certain level, equilibrium could be reached and the amount of immobilized laccase would also arrive at the saturation, not increasing further. On the other hand, the excessive aldehyde groups had a denaturizing effect on the laccase and so the optimum cross-linking was found to be 8 h.

Effect of laccase concentration. As shown in Fig. 3c, specific activity of immobilized laccase increased with an increase of laccase concentration, reaching maximum value at 0.2 g/L, and decreased at higher laccase concentrations. This is because the amount of active groups on the carrier surface was limited and specific activity of the immobilized laccase increased with increasing laccase concentration before the binding sites were saturated. Some of the molecular laccase blocked deep pore channels of the carrier after saturation, leading to more difficult substrate accessing the active site. On the other hand, when the amount of laccase absorbed onto the carrier reached a certain amount when the amount of laccase continued to increase, the laccase molecules began to closely gather and overlap, leading to orientation changes of the laccase molecules. The changed spatial orientation of the active centers of the laccase makes it difficult for them to be combined with substrate and the specific activity of the obtained immobilized laccase does not increase but decrease [12]. All the above indicated that absorption and cross-linking of the laccase protein by the mesoporous silica carrier was limited. The optimum concentration of the laccase was 0.2 g/L according to the test results selected for later tests.

Effect of immobilization time. As shown in Fig. 3d, the specific activity of the immobilized laccase increases with increase of immobilization time and reached maximum at 6 h. Further increase of immobilization time had no influence on combination of laccase with glutaraldehyde, possibly due to large amounts of laccase concentrated on the carrier. The increased spatial hindrance affected the effective contact between the laccase and substrate and diffusion of product in the solution, decreasing the specific activity of immobilized laccase [13]. Also, the proportion of specific activity of immobilized laccase increased when immobilization time was increased in this immobilizing environment. So, the specific activity did not increase any more after immobilization time was increased to 6 h.

Effect of pH value on enzyme immobilization. In the process of immobilization, the pH of the solution determines ionization state of carrier and laccase. Due to this reason, the resulting carrier cross-linking with laccase at different degree may result in different specific activity of immobilized laccase.

This is because laccase and mesoporous silica carrier have different isoelectric point (IEP) values. The laccase used in present experiments has an IEP value within the range 3–6 [14]. In solutions with pH values of 3–6, the amino groups of laccase were ionized, leading to polar positively charged $^{+}NH_3$–R' groups. The mesoporous silica, with IEP value less than 3, is ionized at pH within 3–10 range so its surface is negatively charged. This negative charge increases with increase of pH. So when pH of solution was 5.4, the mesoporous silica and laccase have maximum electrostatic interaction between them, leading to maximum amount of immobilized laccase on carrier. As shown in Fig. 4a, specific activity of immobilized laccase attained a peak value at pH of 5.4.

Meanwhile, because of laccase as a protein, changes of its microscopic structure lead to laccase's inactivity when the solution's pH is beyond certain range. According to Bollag [15], laccase's activity is lost at pH ≥ 7.

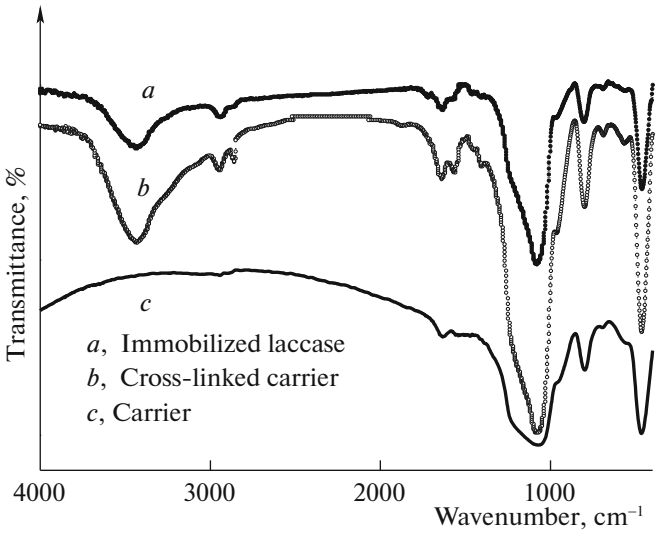


Fig. 5. The IR spectra of carrier and immobilized laccase.

The Properties of Immobilized Laccase

The IR spectra analysis of immobilized laccase. The IR spectra of the carrier, the glutaraldehyde cross-linked carrier, and the carrier with immobilized laccase are shown in Fig. 5. The spectrum of the glutaraldehyde cross-linked carrier displays a distinct strong and wide peak of the corresponding to the amino groups and hydroxyl groups at 3400 cm^{-1}, and the stretching vibration of $-CH_2$ groups at 2940 cm^{-1}. Compared with spectrum of the carrier, the characteristic absorption peak at 1650 cm^{-1} can be assigned to Schiff's base, and the distinct peak at 2870 cm^{-1} can be assigned to the stretching vibration of free aldehyde groups in glutaraldehyde cross-linked carrier. The simultaneous presence of the free aldehyde and imine groups on the surface of the material demonstrates that at the optimal conditions the glutaraldehyde molecules are linked to the carrier surface leaving one of aldehyde groups free for the further enzyme immobilization.

After free aldehyde groups had reacted with amino groups of laccase, Schiff's base was formed again. Therefore, in the IR spectra of immobilized laccase, the characteristic absorption peak of Schiff's base at 1650 cm^{-1} can be also observed, further proving the reaction between free aldehyde groups and the amino groups of the enzyme, indicating successful covalent immobilization.

HRSEM image of immobilized laccase and its carrier. As shown in Fig. 6, $M-SiO_2-NH_2$ sample exhibited an irregular platy shape, with the average length of 4.25 μm and average width of 3.41 μm. There were obvious large amount of gaps between platy shaped particles, probably deducing $M-SiO_2-NH_2$ sample with high porosity. The surface of glutaraldehyde cross-linked carrier without immobilized laccase presented irregular flocculent shape and had small amount of gaps between the particles. This may be due to carrier becoming flocculent shape after being cross-linked. After immobilization of laccase, the mesoporous silica surface was covered forming large blocks and no any other gaps were observed.

The optimal working pH value of immobilized laccase. As can be seen from Figs. 4a, 4b, the optimum working pH of free laccase was 5.8, and that of immobilized laccase was 5.4, indicating that the optimum pH of immobilized laccase had slightly shifted in the acidic direction. This is due to free amino groups on the surface of mesoporous silica, which make microenvironment of immobilized enzyme around mesoporous silica carrier weakly alkaline, and therefore the solution must have lower pH value than optimum pH of free laccase to achieve the best working pH of immobilized laccase. At the same time, the spatial structure of the laccase after immobilization is affected by the carrier, reducing the sensitivity of immobilized enzyme to pH value changes.

The stability of immobilized laccase. In order to compare the stability of free laccase with that of immobilized laccase, the free laccase solution and wet immobilized laccase were stored at 4°C for 7 days. Figure 7 shows that the specific activity of the free enzyme solution decreased significantly, and it lost 66.61% of the initial activity after 7 days, while immobilized enzyme was relatively stable and its specific activity is almost retained, demonstrating that stability of the laccase immobilized by the $M-SiO_2-NH_2$ was markedly improved. The immobilization could overcome the drawbacks of instability and inactivation of free laccase, in order that immobilized laccase can be reused repeatedly in the actual operation.

Degradation of DCP with Immobilized Laccase

Effect of DCP concentration on the DCP removal by immobilized laccase. As can be seen from Fig. 8, when concentration of the DCP was low, degradation ratio of DCP by immobilized laccase was relatively low, but the adsorption ratio was relatively large, leading to removal ratio getting higher. With increase of DCP concentration, the degradation ratio increased gradually. When DCP concentration reached 50 mg/L, the degradation ratio reached the maximum value, while the adsorption ratio decreased to the minimum value. When DCP concentration was further increased to 100 mg/L, the removal ratio and degradation ratio both decreased. The adsorption ratio changed a little.

As the carrier used in the present study is the mesoporous silica and have large specific surface and is a good adsorbent, this absorption factor must be taken into account in the experiments. When the DCP concentration is low, the adsorption played a major role because of low content of DCP, so most of the DCP was adsorbed on the carrier, and small amount of

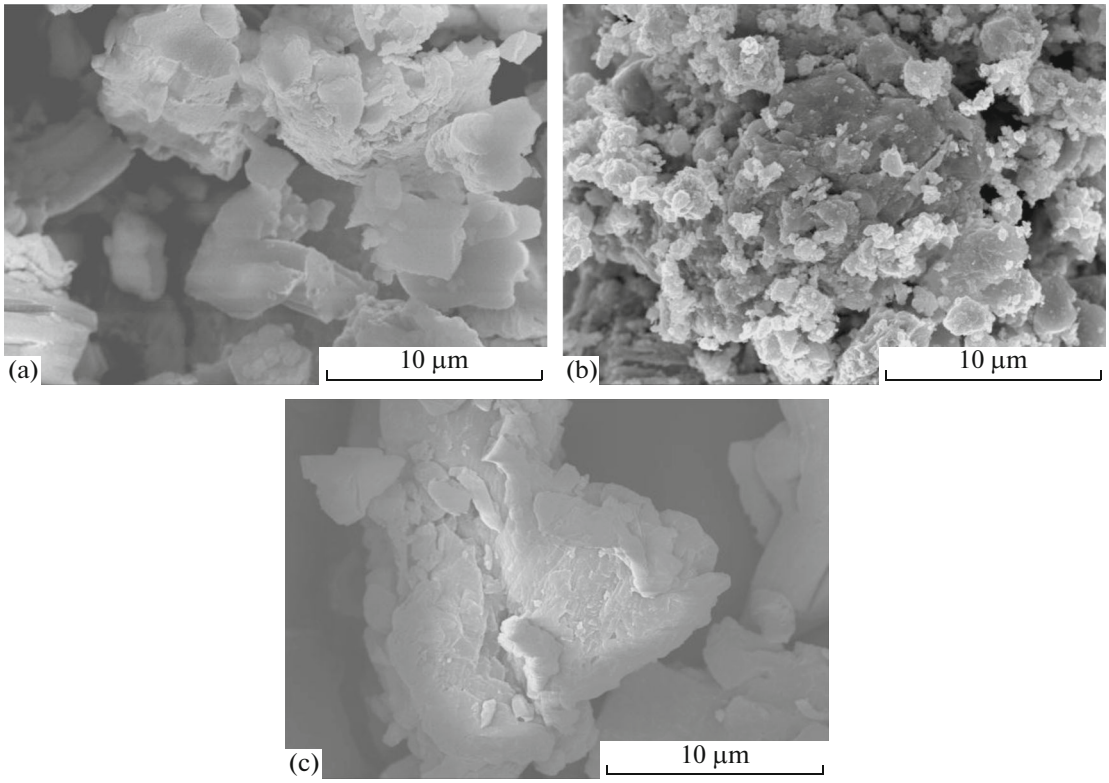


Fig. 6. HRSEM images of three sample (a) HRSEM image of M–SiO_2–NH_2, (b) HRSEM image of M–SiO_2–NH_2 cross-linked by glutaraldehyde, (c) HRSEM image of immobilized laccase.

DCP can get access to enzyme active sites, leading to decrease of degradation ratio. When concentration of the DCP increased, more DCP could get access to enzyme active sites, leading to an increase of degradation ratio, but adsorption ratio decreased significantly, because when certain amount of DCP was adsorbed on the surface of mesoporous silica to form first saturated adsorption layer, the surplus DCP could only be adsorbed on the first layer to form second layer of adsorbed DCP, leading to a decrease of adsorbed DCP amount.

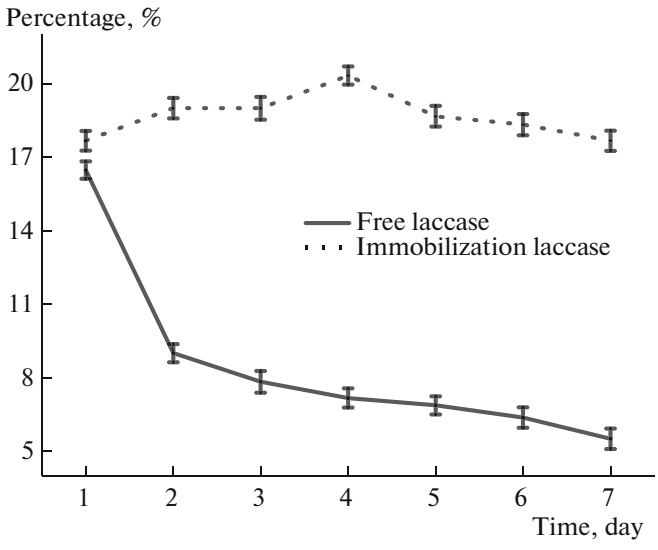


Fig. 7. The stability of free laccase and immobilized laccase, the standard deviations less than 1%.

When the DCP concentration exceeded 50 mg/L, the degradation ratio decreased, because the number of enzyme molecules immobilized on carrier was limited, and only a certain amount of DCP could be degraded. On the other hand, too many DCP molecules gathered around immobilized laccase were prone to generate spatial hindrance, preventing the other DCP molecules from being in contact with the enzyme molecules. These led to the decline of degradation ratio. Therefore, taking all aspects of a comprehensive selection into consideration, concentration of 50 mg/L of initial solution is most appropriate.

Effect of pH value on the degradation process. Figure 9 shows that the degradation ratio of DCP was low when the pH value was near 4.0. The degradation ratio and removal ratio of DCP reached maximum at pH 5.5. At pH 6.5, some part of laccase was inactivated and the degradation of DCP reached minimum value. The adsorption ratio was observed to be relatively stable.

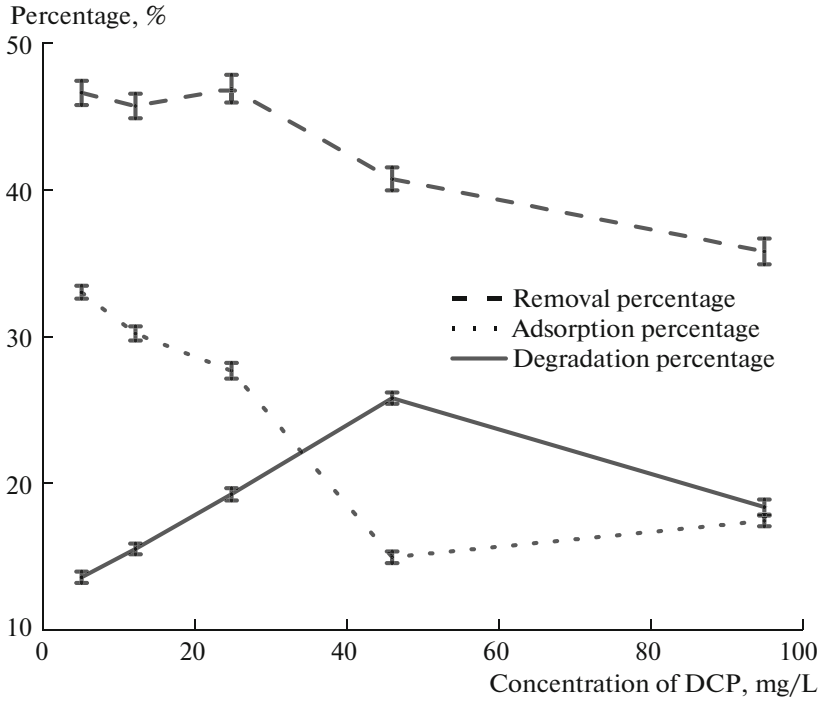


Fig. 8. Effect of DCP concentration on the DCP removal by immobilized laccase, the standard deviations less than 1%.

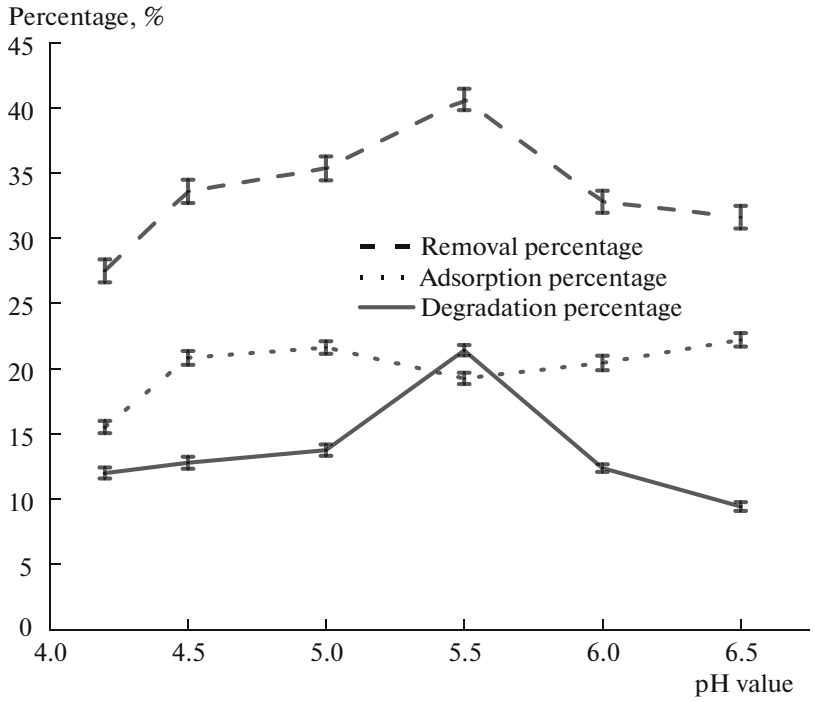


Fig. 9. Effect of pH value on the degradation of DCP by immobilized laccase, the standard deviations less than 1%.

The experiment shows that the optimum pH for degradation of DCP by immobilized laccase is different from the pH at which immobilized laccase's activity reaches maximum, which indicated that the optimum pH for degradation of DCP is related to chemical structure of substrate. For example, the optimal pH of the peroxidase falls with increase of the number of chlorine atoms on phenol aromatic ring from

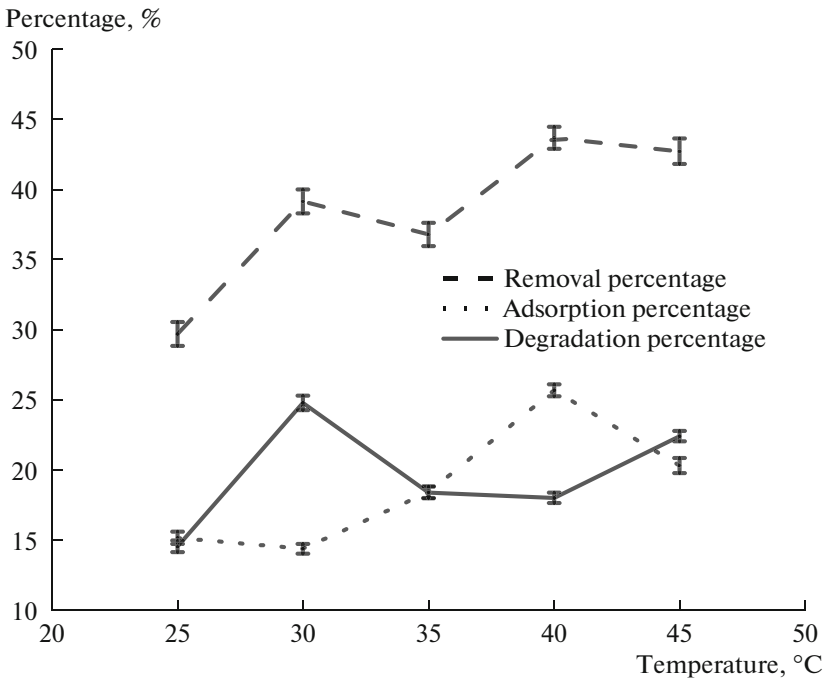


Fig. 10. Effect of temperature on the DCP degradation by immobilized laccase, the standard deviations less than 1%.

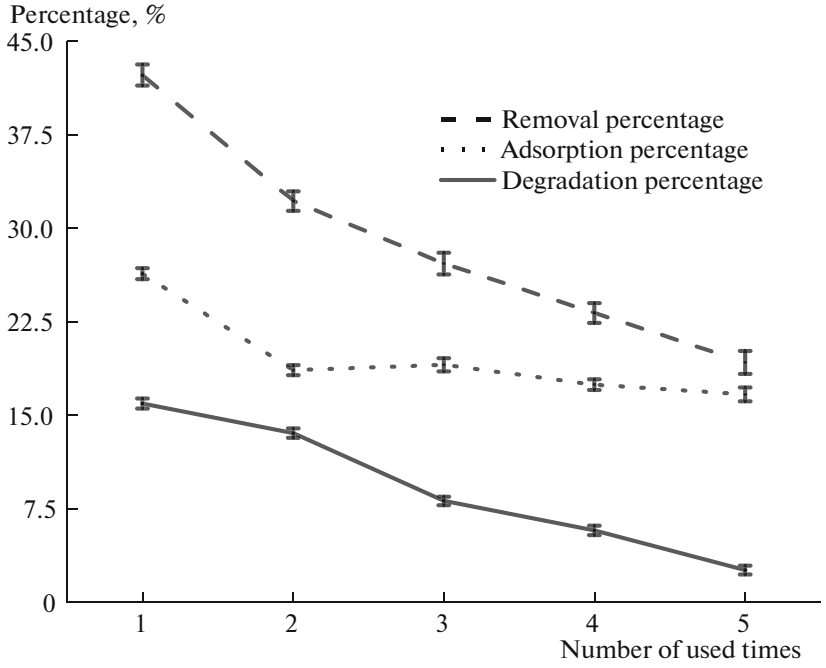


Fig. 11. The use of immobilized laccase for the continuous elimination of DCP, the standard deviations less than 1%.

pH 8.3 for 4-chlorophenol to pH 6.5 for dichlorphenol, and then from pH 6.1 for 3-chlorophenol to pH 5.4 for pentachlorophenol [16].

The optimum pH 5.4 of immobilized laccase moves towards acidity end as compared with the optimum pH 5.8 of free laccase (Fig. 4b). The change of the optimum pH is determined by the electrostatic charge of the carrier. Because isoelectric point of mesoporous SiO_2 is in the range of 2–3 [17] and the optimum pH value in the experiment is 5.4, the surface of the mesoporous SiO_2 carrier is negatively charged. When the laccase is combined with the carrier with

negative charge, only surface positive charged laccase protein can absorb this carrier surface strongly, leading to increased positive charge of zymoprotein on the immobilized enzyme, and the pH of immobilization reaction area is more alkali than that of the external solution. Actually, enzymatic reaction occurs in the alkali part of the solution, so the optimum pH for immobilized enzyme shifts to the acidity end [18]. These results are analogous to the observed for positively charged chitosan carrier [19].

Effect of temperature on the degradation process. The experimental results in Fig. 10 show that the degradation ratio of DCP arrived at maximum and the removal ratio had a general trend of increase at 30°C. The adsorption ratio arrived at maximum at 40°C and the removal of DCP by immobilized laccase was mainly based on adsorption at this time. This is because when the temperature increased, the molecular thermal motion accelerated, and the specific activity of laccase increased accordingly but also lost its initial activity when temperature was over 40°C, due to dissolution of oxygen in the system, unfavorable for enzyme catalytic reaction.

The experimental results show that the removal ratio and degradation ratio of DCP by immobilized laccase were comparatively high within the temperature range 30–45°C, demonstrating that immobilized laccase can degrade DCP in a relatively wide temperature range. This is because after the laccase is immobilized, its spatial structure is influenced by the carrier and the effect of temperature changes on laccase spatial structure becomes weaker, so the immobilized laccase becomes less sensitive to the change of temperature. These experimental results also show that the immobilized laccase has potential application value to treat pesticide in polluted water. Due to temperature sensitiveness, free laccase is denatured or inactivated very easily; the immobilized laccase can be applied in a more wide temperature range however, so it can make the actual operation possible.

Repetitive use of immobilized laccase. It can be seen from Fig. 11 that under optimal conditions the removal ratio and the degradation ratio were 42.28 and 15.93%, respectively. When the regenerated immobilized laccase was re-used for a second time, the degradation ratio decreased slightly, but the removal ratio decreased obviously with a marked decrease of adsorption ratio.

With the number of cycles of the degradation, removal ratio and degradation ratio of DCP all decreased significantly, the adsorption ratio was almost kept at the value of 17.5% however. When the number of cycles reached 5, the degradation and removal ratios for DCP were still retained over 19.23 and 2.57%, respectively. In contrast to the free enzyme, the reusability of immobilized laccase has been improved significantly.

Durán et al. [20] pointed out that the efficiency of use of immobilized laccase to degrade phenols in waste water is affected by two factors. First, the colored products generated during reaction are absorbed on carrier surface; second, insoluble substance is produced by catalytic oxidation of phenol by immobilized laccase. The colored products adsorbed on the carrier surface prevent the carrier and the enzyme molecules from further contacting with the chlorphenol molecule. Therefore, the degradation products and the adsorption of them both reduce degradation ratio with the repeated use of immobilized laccase. Accordingly, in order to reduce these effects on specific activity of the immobilized laccase, the immobilized laccase can be repeatedly washed with buffer solutions.

It has been found that undissolved substance generated during catalytic oxidation of phenols by laccase, might be from the product of non-enzymatic reaction. When immobilized laccase is repeatedly used, the accumulation of precipitate will lead to slowdown and even blocking of flow in the pore channel of carrier, obstructing the catalytic degradation of DCP. In addition, a small part of laccase will be lost in the repetitive use due to centrifugation and washing. Besides, oscillating process is also one factor inducing loss of part of the specific activity of laccase. It thus leads to conclusion that above unbeneficial factors are all able to lower the DCP degradation ratio.

Discussion of Degradation Mechanism

The mechanism for the glutaraldehyde cross-linking method. The mechanism for the glutaraldehyde cross-linking method can be described as following:

First, amino modified mesoporous silica was cross-linked with glutaraldehyde to form cross-linking carrier with free aldehyde groups, as can be seen in the Eq. (5). Second, the cross-linking carrier continued to be coupled with amino group in laccase protein, and as a result, covalent bond of cross-linked laccase onto mesoporous silica was produced, as can be seen in the Eq. (6).

$$\begin{aligned}&\text{Meso\_Silica}-NH_2 + OHC-(CH_2)_3-CHO\\&\rightarrow \text{Meso\_Silica}-N{=}CH-(CH_2)_3-CHO,\end{aligned} \tag{5}$$

$$\begin{aligned}&\text{Meso\_Silica}-N{=}CH-(CH_2)_3-CHO + NH_2-\text{Laccase}\\&\rightarrow \text{Meso\_Silica}-N{=}CH-(CH_2)_3-CH{=}N-\text{Laccase}.\end{aligned} \tag{6}$$

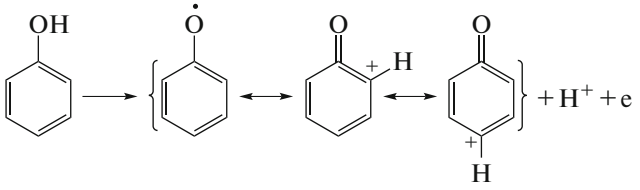
Degradation mechanism of DCP by immobilized laccase. Laccase is a single electron redox enzyme, having different types of reaction mechanism in two aspects. In the presence of oxygen, chlorophenol was oxidized to quinones and other free radicals by laccase (scheme 1); these substances undergo coupling reactions forming polymer, which reduced their solubility, leading to reduced toxicity. The reaction mechanism includes the transfer of electron and proton on phenol to form free radicals. Laccase active site contains Cu(II), which was reduced to Cu(I) in the catalytic cycle, and then reoxidized to Cu(II). The mechanisms of phenols oxidation catalyzed with laccase are shown in Eqs. (7) and (8), in which the mesoporous silica can act as a stabilizer.

$$\begin{aligned} &2\text{Meso\_Silica}-\text{N}-(\text{Cu}^{2+})\text{Laccase} + \text{Phenol} \\ &\quad \rightarrow 2\text{Meso\_Silica}-\text{N}-(\text{Cu}^{+})\text{Laccase} \\ &\qquad + \text{Radical} + 2\text{H}^{+}, \end{aligned} \tag{7}$$

$$\begin{aligned} &2\text{Meso\_Silica}-\text{N}-(\text{Cu}^{+})\text{Laccase} + 1/2\text{O}_2 + 2\text{H}^{+} \\ &\quad \rightarrow 2\text{Meso\_Silica}-\text{N}-(\text{Cu}^{2+})\text{Laccase} + \text{H}_2\text{O}, \end{aligned} \tag{8}$$

where, Meso_Silica–N=CH–$(CH_2)_3$–CH=N–Laccase was abbreviated in Scheme 1 as:

Meso_Silica–N–(Cu^{2+})Laccase



Scheme 1. The generation of the substrate free radical intermediate.

In the presence of oxygen, laccases use oxygen as the electron acceptor to remove protons from the phenolic hydroxyl groups. Chlorophenol stripped an electron and a proton to form three free radical intermediates, then two of them randomly form stable aromatic ring product, but at the same time, oxygen may be reduced into water. There are three resonance forms of the radical (Scheme 1), which can further generate oligomeric and polymeric products [19], or undergo further transformations to the organic acids, i.e. maleic acid through an open-loop process [21].

CONCLUSION

The optimum conditions for the immobilization of laccase on modified mesoporous silica are as follows: room temperature, 4% of glutaraldehyde, 8 h crosslinking time, 0.2 g/L concentration of laccase, immobilization time of 6 h, and immobilization pH 5.4. The optimum working pH of immobilized laccase is 5.4, which is slightly lower than for free laccase. Immobilized laccase is more stable than the free enzyme. The optimum conditions for immobilized laccase in DCP degradation are as follows: DCP concentration 50 mg/L, pH 5.5, temperature range is 30–45°C, and degradation time is 6 h. The removal ratio of DCP was 19.86% after immobilized laccase was used continuously to degrade DCP four times under the optimal conditions. Compared with the free laccase, the immobilized laccase is much more reusable.

REFERENCES

1. A. Zaleska, J. Hupka, M. Wiergowski, and M. Biziuk, J. Photochem. Photobiol. A **135**, 213 (2000).
2. I. J. Gaitan, S. C. Medina, J. C. González, et al., Bioresour. Technol. **102**, 3632 (2011).
3. L. Yin, Zh. Shen, J. Niu, et al., Environ. Sci. Technol. **44**, 9117 (2010).
4. X. Liu, Q. Zhang, Y. Liang, and R. Zhang, Adv. Mater. Res. **1010–1012**, 830 (2014).
5. X. Zheng, Q. Wang, Y. Jiang, and G. Jing, Ind. Eng. Chem. Res. **51**, 10140 (2012).
6. D. Li, L. Luo, Z. Pang, et al., ACS Appl. Mater. Interfaces **6**, 5144 (2014).
7. Y. Liu, Zh. Zeng, G. Zeng, et al., Bioresour. Technol. **115**, 21 (2012).
8. J. Bains, N. Capalash, and P. Sharma, Biotechnol. Lett. **25**, 1155 (2003).
9. Amer. Publ. Health Assoc., Amer. Water Works Assoc., and Water Pollution Control Federation, *Standard Methods for the Examination of Water and Wastewater,* 13th ed. (Amer. Public Health Assoc., 1971).
10. M. Kruk, V. Antochshuk, and M. Jaroniec, J. Phys. Chem. B **103**, 10670 (1999).
11. R. Xu and W. Pang, *Chemistry–Zeolites and Porous Materials* (Science Press, Beijing, 2004) [in Chinese].
12. D. Jiang, S. Long, J. Hong, et al., Acta Microbiol. Sin. **45**, 630 (2005).
13. F. He, L. Liu, and R. Zhuo, Ion Exchange Adsorp. **13**, 290 (1997).
14. G. J. Davies and V. Ducros, in *Handbook of Metalloproteins,* Ed. by A. Messerschmidt, R. Huber, K. Wieghardt, and T. Poulos (Wiley, New York, 2001), Vol. 2, p. 1359.
15. J. M. Bollag, K. L. Shuttleworth, and D. H. Anderson, Appl. Environ. Microbiol. **54**, 3086 (1988).
16. W. Qin, H. Yu, and S. Fu, J. Biotechnol. **5**, 58 (2001).
17. J. Shao, W. Deng, Y. Yang, et al., Adsorpt. Sci. Technol. **27**, 147 (2009).
18. W. Zhang and X. Yang, Chin. J. Nature **22**, 282 (2000).
19. J. Zhang, X. Liu, Zh. Xu, et al., Int. Biodeterior. Biodegrad. **61**, 351 (2008).
20. N. Durán, M. A. Rosa, A. D. Annibale, et al., Enzyme Microb. Technol. **31**, 907 (2002).
21. Y. Huang, Y. Xi, Y. Yang, et al., Chin. Sci. Bull. **59**, 509 (2014).