Desalination and Water Treatment
www.deswater.com
doi: 10.1080/19443994.2014.923337

55 (2015) 1293–1301
July

Taylor & Francis
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Immobilization of laccase on chitosan–halloysite hybrid porous microspheres for phenols removal

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Received 17 January 2014; Accepted 6 May 2014

ABSTRACT

In this paper, we used natural resources of halloysite nanotubes and chitosan to prepare new porous organic/inorganic hybrid microspheres with diameter of about 100–150 μm by reversed-phase suspension cross-linking technique. The SEM image revealed that halloysite nanotubes overlapped together within hybrid microspheres and formed a hybrid porous structure. We demonstrated that the resulted porous microspheres could be used as a new kind of support to immobilize laccase, which showed excellent loading capacity for laccase immobilization as high as 123.1 mg/g. The stability of laccase was also greatly improved after immobilization on the support. While the immobilized laccase was used as biocatalyst to remove phenols from wastewater, the removal efficiency could reach as high as 95.0%.

Keywords: Halloysite nanotube; Chitosan; Hybrid microsphere; Immobilized laccase; Phenol removal

1. Introduction

As excellent green and high-selectivity catalysts, enzymes have attracted significant attention in many fields such as industrial synthesis, pharmacy industry, and environmental protection [1,2]. Immobilization of enzymes on supports is one of the current trends to optimize biocatalytic processes. Compared with the free enzymes, the immobilized enzymes could be more easily separated from the solution for reusing and could retain higher stability in some specific environment involving organic solvent, extreme pH, and extreme temperature [3,4]. The structural characteristics of the support are important since they may influence the catalytic behavior and stability of the immobilized enzyme [5,6], and various natural or synthetic materials have been selected as supports to immobilize enzymes, such as chitosan, kaolinite, montmorillonite and hydrotalcite, polymer beads, and polystyrene microspheres [7–13].

Halloysite is layered aluminosilicate clay mineral and available in abundance in China as well as other locations around the world. Halloysite mineral usually exists in the form of nanotubes, which have inner diameters in the range of 5–20 nm, and are several micrometers in length [14–16]. The pore sizes of

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halloysite nanotubes (HNTs) are comparable to the diameter of biomacromolecules and suitable to entrap enzyme molecules. Moreover, the naturally occurring HNTs are readily obtainable and much cheaper compared with other nanomaterials such as carbon nanotubes (CNTs). For those reasons, HNTs have attracted attention as supports for enzyme immobilization [4].

The polymer of chitosan (CTS) is a natural hydrophilic cationic polysaccharide derived from deacetylation of chitin, the second most abundant natural polymer on earth. It also has been proposed as a potential candidate for the immobilization of biomolecules because of its several outstanding characteristics such as non-toxicity, biodegradability, biocompatibility, antibacterial effect, mucus adhesion, and low cost [17–19]. In the previous studies, we have modified HNTs with chitosan to prepare hybrid chitosan–HNTs for immobilizing enzymes [20,21]. The hybrid nanotubes show a higher loading capacity for enzymes immobilization than the individual HNTs. However, HNTs or HNTs modified with chitosan, like many other nanotubes, are easy to form bundles or agglomerates during the usage, which may result in a reduction of effective specific surface area. As a result, the performance of immobilized enzymes would be severely hindered [22–24]. A potential solution to this problem is to prepare nano/micro hierarchical porous structure with HNTs because this structure can take the advantages of both nanotubes building blocks and microporous assemblies [25]. The 3D hierarchical pores within the microspheres are readily accessible to substrates/products with a small diffusion barrier.

Phenolic compounds usually come from the wastewaters of numerous industries such as pulp and paper, wood, petroleum refining, steel, resins, and plastics. They are widely considered as priority pollutants and their persistence may cause severe environmental problems [26,27]. At present, a number of physical, chemical, and biological methods have been developed for the removal or degradation of phenol [28,29]. Among them, enzymatic wastewater treatment is a promising technology for the degradation of phenol and phenolic compounds [30]. Immobilized laccase has been extensively applied as a biocatalyst to remove phenolic compounds from wastewaters due to its higher thermal stability, broader working pH, and temperature range than the free laccase [31–33].

In present work, we used natural resources of halloysite nanotubes and chitosan to prepare new porous organic/inorganic hybrid microspheres. We further evaluated the enzyme-immobilizing properties on the hybrid microspheres. Compared to the free laccase, the immobilized laccase displayed more excellent properties of thermal stability, storage stability, and reusability. While the immobilized laccase was used as biocatalyst to remove phenols from wastewater, the removal efficiency could reach as high as 95.0%.

2. Experimental and methods

2.1. Materials and instrumentation

Chitosan (92% deacetylated) was purchased from Shanghai Sangon biological engineering technology & services Co., Ltd (Shanghai, China). Halloysite clay from Henan province (China) was milled and sieved to obtain fine powders. Laccase from *Trametes versicolor* (E.C. 1.10.3.2, 13.6 U/mg, product No. 51639), 2,2′-azinobis (3-ethylbenzthiazolin-6-sulfonate) (ABTS), and fluorescein isothiocyante (FITC) were purchased from Sigma-Aldrich (USA). Tween-80 was purchased from Paini Chemical Reagent Co., Ltd (Zhengzhou, China). Phosphate buffer solution (PBS) with desired pH values was prepared by mixing stock standard solutions of disodium hydrogen phosphate and citric acid. All the other chemicals were of analytical grade and were used without further purification. Each solution was prepared with deionized water.

The surface morphology of the microspheres was observed by scanning electron microscopy (SEM, JSM-6701F). The IR spectra of the sample were measured by a Fourier transform infrared (FT-IR, Varian FTS 7000). The thermal decomposition of the microspheres was recorded via TG/DSC technique by a thermogravimetric analyzer (NETZSCH, STA409PC). The activity of free and immobilized laccase was detected by UV–vis spectrophotometer (UV-2450, Shimadzu).

2.2. Preparation of CTS-HNT hybrid microspheres

In a typical synthesis, chitosan (CTS, 1.2 wt %) was dissolved in an aqueous solution of acetic acid (2 wt %). HNTs (3.6 wt %) were dispersed in deionized water under ultrasonic treatment for 2 h. Then the same volume of the HNTs solution and chitosan solution was mixed under constant stirring for 24 h to guarantee full adsorption of chitosan on HNTs. The CTS-HNTs solution (10 mL) was further dispersed in a mixture of liquid paraffin (35 mL), petroleum ether (25 mL), and tween-80 (1.0 mL) in a round-bottomed flask at 25°C. The mixed solution was stirred at 1,000 rpm for 20 min, glutaraldehyde-saturated toluene (10 mL) was introduced into the solution, and then solution was stirred constantly at 25°C for 100 min. The resulting product was filtrated and washed with

petroleum ether for two times, acetone three times, and water three times.

The synthesis mechanism of CTS-HNTs hybrid porous microspheres is illustrated in Fig. 1. First, chitosan (CTS), a polycationic polysaccharide, could spontaneously bond to the negatively charged outer surface of HNTs and form a CTS-HNT assembly. Second, while the aqueous suspension containing CTS-HNTs was introduced into a mixture of oil phase, water-in-oil droplets formed in the microemulsion system under mechanical stirring. Being hydrophilic, CTS-HNTs are encapsulated into the droplets. Third, glutaraldehyde-saturated toluene was added to cross-link with CTS-HNTs in the droplets under stirring, and the stable porous hybrid microspheres were obtained.

2.3. Immobilization of laccase

Laccase was immobilized onto CTS-HNT hybrid microspheres by physical adsorption. For this purpose, CTS-HNT hybrid microspheres (0.1 g) were added in the citrate–phosphate buffer (4.0 mL, 0.1 M, pH 5.0) containing various amounts of laccase. The mixture was stayed in shaking at 25°C for 12 h. After immobilization, the microspheres were washed with the same buffer until no protein was detected in the washing buffer. The immobilized laccase was stored at 4°C prior to use. The amount of laccase immobilized on the support was calculated on the basis of the difference between the total used laccase and the residual laccase in the supernatant after immobilization.

2.4. Assay of laccase activity

The activity of the free and immobilized laccase was determined using ABTS as standard based on the previously described procedure [34]. In the case of free laccase solution, diluted laccase (1.0 mL) was mixed with citrate–phosphate buffer (1.0 mL, pH 5.0), and then ABTS aqueous solution (1.0 mM, 1.0 mL) was added into to initiation reaction system. The immobilized laccase activity was assayed by incubating immobilized laccase in the same citrate–phosphate buffer (2.0 mL) with ABTS aqueous solution (2.0 mL). The increase of absorbance was followed at $\lambda = 420$ nm for 3 min and each sample was detected repeatedly for three times. One unit of activity is defined as the amount of enzyme required to oxidize 1 μmol of substrate per minute.

To determine the effects of pH and temperature on the laccase activity, the activities of the free and immobilized laccase were also evaluated by measuring the activities at different pH values and temperatures. Here, the activity was expressed in relative units [%] where the maximal activity value at a certain pH value and temperature was set at 100%.

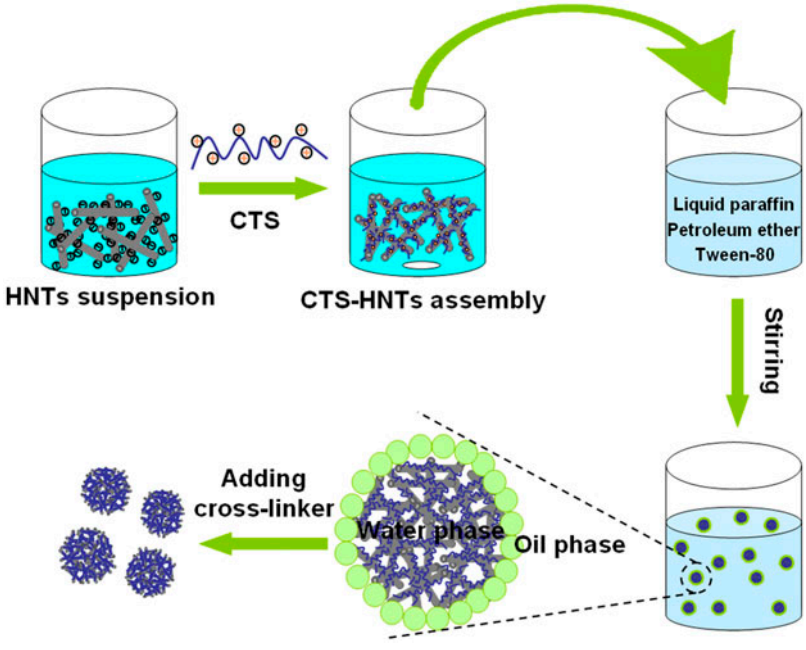

Fig. 1. Schematics of the preparation of the CTS-HNT hybrid microspheres.

2.5. Removal of 2,4-dichlorophen

Phenolic compound of 2,4-dichlorophen was used as target organic contaminants to evaluate the removal efficiency of the immobilized laccase from aqueous solution in the present work. The removal assay was performed at 30°C with a reaction mixture of immobilized laccase and a 2,4-dichlorophen solution. The mixture was incubated for 600 min under continuous vibration. The percentage of 2,4-dichlorophen degradation was determined by measuring the changes of the supernatant absorption using a double-beam UV−vis spectrophotometer, and the more detailed experimental had been present in the previous research [4,33].

3. Results and discussion

3.1. Characterization

The microspheres in wet state were examined by an optical microscope, as shown in Fig. 2(a). The photograph showed that the microspheres have a regular sphere-like morphology with a diameter of approximately 100–150 μm. To examine the fine morphology, the microspheres were also observed by scanning electron microscopy (SEM), and the surface morphology and internal structures were shown in the Fig. 2(b) and (c), respectively. The surface of microsphere was rough and irregular, and numerous halloysite nanotubes grouped together by cross-linked chitosan in Fig. 2(b). The halloysite nanotubes overlapped loosely together and formed a porous structure in the interior of the hybrid microspheres in Fig. 2(c). Furthermore, the specific surface area of the CTS-HNT hybrid microspheres (106.5 m^2/g) was larger than that of halloysite nanotubes (59.6 m^2/g) or chitosan–halloysite hybrid-nanotubes (55.2 m^2/g) [21]. The large surface area and the loose structure were favorable to immobilize enzymes.

The FTIR spectra in the 4,000–400 cm^{-1} wavenumber range of HNTs (a), CTS (b), and CTS-HNT hybrid microspheres (c) have been included in Fig. 3. The spectra of HNTs (Fig. 3(a)) showed characteristic peaks including O–H stretching of water at ~3,462 cm^{-1}, O–H deformation of water at ~1,632 cm^{-1}, and in-plane Si–O stretching at ~1,032 cm^{-1}. Besides, the double peaks at 3,697 and 3,624 cm^{-1} were due to the stretching vibrations of hydroxyl groups on the surface of HNTs. The adsorption peaks at 692 and 537 cm^{-1} could attribute to perpendicular Si–O stretching and deformation of Al–O-Si, respectively. As seen in the FTIR spectrum of CTS (Fig. 3(b)), the peak at ~3,435 cm^{-1} corresponded to stretching vibrations of –OH and –N–H. The –CH stretching vibration of the polymer backbone was manifested through strong peak at 2,925 cm^{-1} and 2,855 cm^{-1}. Also, the peaks at 1,635 cm^{-1} and 1,087 cm^{-1}

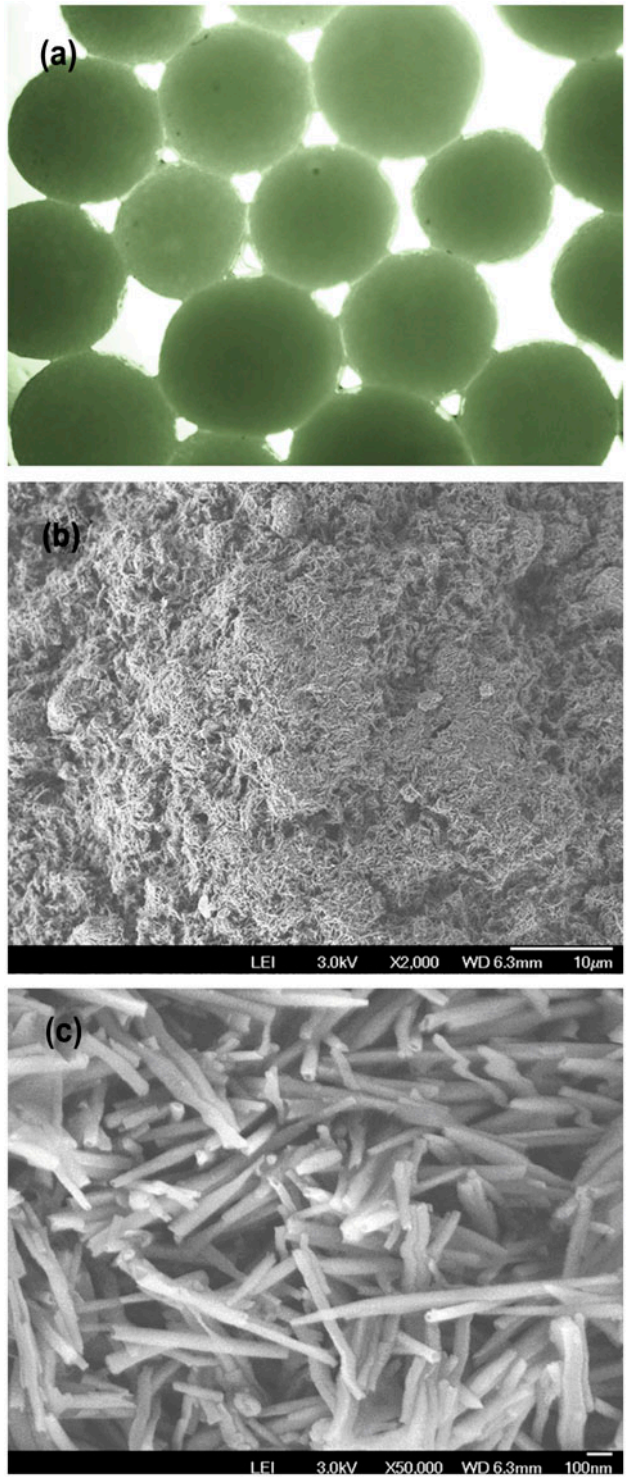

Fig. 2. Optical microscope image of CTS-HNT hybrid microspheres (a), SEM images of the external surface (b) and the internal structure (c).

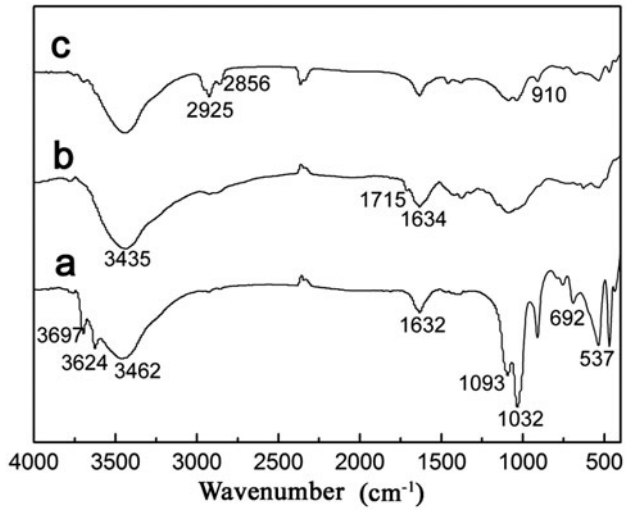

Fig. 3. FTIR spectra of HNTs (a), CTS (b) and CTS-HNT hybrid microspheres (c).

were assigned to C = O (in undeacetylated units) and C–O–C vibrations, respectively. The spectrum of CTS-HNT hybrid microspheres (Fig. 3(c)) existed a peak at ~1,648 cm^{-1}, indicated the presence of the –C=N– vibration which is generated by cross-linking between amine groups of chitosan and aldehyde group of glutaraldehyde.

Thermogravimetric analysis (TGA) of CTS-HNT hybrid microspheres was further carried out in O_2 from room temperature to 800˚C. The thermogravimetric curves that were obtained by measuring the weight loss of microspheres from pyrolysis are shown in Fig. 4. The weight loss of microspheres at temperature below 150˚C is due to the loss of free water. The weight loss in the stage of 150–300˚C is due to chemical dehydration of the CTS. And the weight loss in the stage of 300–800˚C can be attributed to chemical dehydration of the HNTs.

3.2. Laccase immobilization

The loading capacities of the CTS-HNT hybrid microspheres were investigated through incubating the supports in laccase solution with various concentrations. The laccase loading on the CTS-HNT hybrid microspheres increased with its initial concentration which was shown in Fig. 5. The laccase loading on the supports reached 123.1 mg/g when the concentration of laccase increased to 2.0 mg/mL, which was 11 times higher than that of pristine HNTs (11.3 mg/g) [20]. Compared with the previously reported porous supports, such as, CTS (20 mg/g), sepabeads EC-EP3 (32.6 mg/g), magnetic mesoporous silica spheres (82 mg/g), and MMSNPs-CPTSIDA (98.1 mg/g) [35–38], the CTS-HNT hybrid microspheres displayed a higher loading capacity. Such a high-loading capacity could be attributable to the special 3D hierarchical porous structure formed by the overlapped halloysite nanotubes, which could provide larger volume to accommodate enzyme molecules.

The fluorescence microscope was used to detect distribution of the immobilized laccase on the porous microspheres. The fluorescein isothiocyanate (FITC) labeled-laccase was used to obtain fluorescent images. The microsphere displayed in a bright green color on the entire surface (Fig. 6(a)), suggestive of the

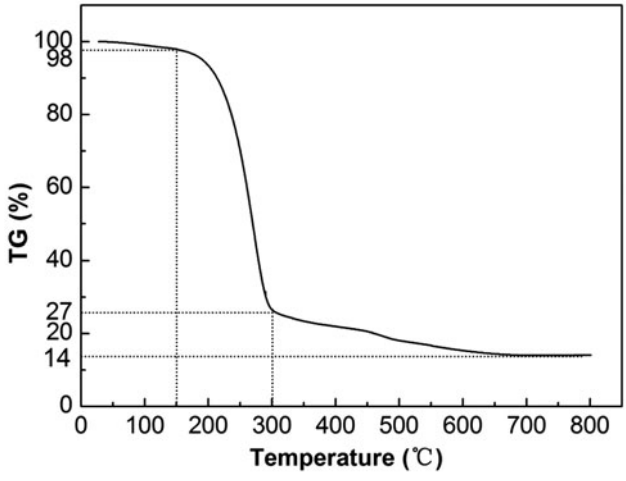

Fig. 4. Thermogravimetric analysis of CTS-HNT hybrid microspheres.

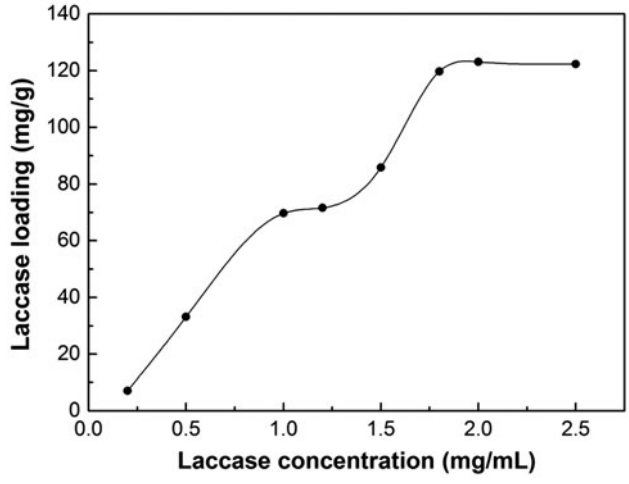

Fig. 5. Effect of the concentration on the laccase loading on CTS-HNT hybrid microspheres.

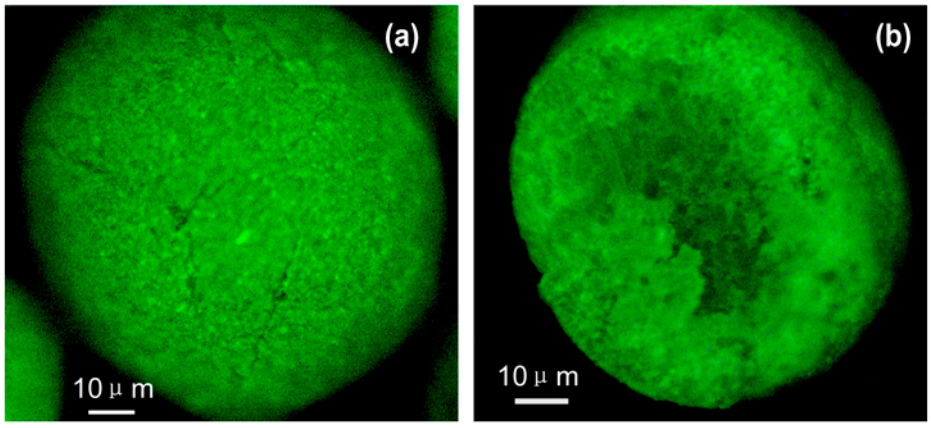

Fig. 6. Fluorescence microscopy image of the surface (a) and the inner microspheres (b) loaded with FITC-laccase.

homogeneous immobilization of laccase on surface of the microspheres. Fig. 6(b) showed the green fluorescence distribution on the cross-section of the microsphere (Fig. 6(b)), which indicated that large amounts of laccase could enter into the hybrid microspheres and disperse uniformly within the entire microspheres.

3.3. The properties comparison of free and immobilized enzymes

The activity of the free and immobilized laccase was evaluated at varied conditions (Fig. 7). Fig. 7(a) showed that the activity of the free and immobilized laccase changed with pH values. Both the activity of the free and immobilized laccase could reach maximal value at the pH of 3.0. However, when pH further increased, the activity decreased and almost lost completely above the pH of 6.0. Compared to the free laccase, the immobilized laccase showed slightly higher resistance to changes in pH value of the medium.

For enzyme immobilization, another important factor needed to be taken into account is the stability at different temperatures. The activity of the free and immobilized laccase at different temperatures was shown in Fig. 7(b). The free laccase had an optimum operation temperature of 35˚C, whereas the optimum operation temperature for the immobilized laccase was shifted to around 60˚C. Hartmeier had proved that the immobilized enzymes were more stable at higher temperatures because enzyme immobilization could enhance its rigidity and prevent conformational changes in the protein's tertiary structure [39]. The maintaining high-enzyme activity at elevated temperature opens a broad range of possible industrial applications.

To further investigate the time-dependent thermal stability of the immobilized laccase, the free and immobilized laccases were handled at a constant temperature of 60˚C in a buffer solution of pH 5.0 as a function of time (Fig. 7(c)). Compared with free laccase, the activity of immobilized laccases decreased more slowly. After an incubation period of 280 min, the immobilized laccase retained 42.1% of its initial activity, while the free laccase retained only 23%. These results suggested that the thermal stability of immobilized laccase was superior at the higher temperature, which could be attributed to the preservation of the structure and active site of the enzyme by immobilization on the support [40].

Unlike the free enzyme, the immobilized enzyme could be easily separated from the solution after reaction, which greatly decreased the cost of the enzyme for practical applications. The reusability of the immobilized laccase was evaluated by cycles of ABTS oxidation. As shown in Fig. 7(d), nearly 50% of initial activity was retained after 10 cycles. The activity loss was attributed to enzyme leakage during washing and enzyme deactivation during repeated uses [41].

3.4. Removal of 2,3-DCP by the immobilized laccase

To test the removal efficiency of phenol compound by the prepared immobilized laccase, 2,4-DCP was chosen as a model. Fig. 8 showed that the 2,4-DCP removal efficiency increased with time. About 76% for 100 mg/L of total 2,4-DCP was degraded in the initial 4 h. When the reaction of 2,4-DCP degradation lasted for 10 h, the removal efficiency of 2,4-DCP could reach 95.0%. Furthermore, immobilized laccase was also utilized to remove other phenol compounds from wastewater, such as 2-cholorphenol and 4-cholorphenol,

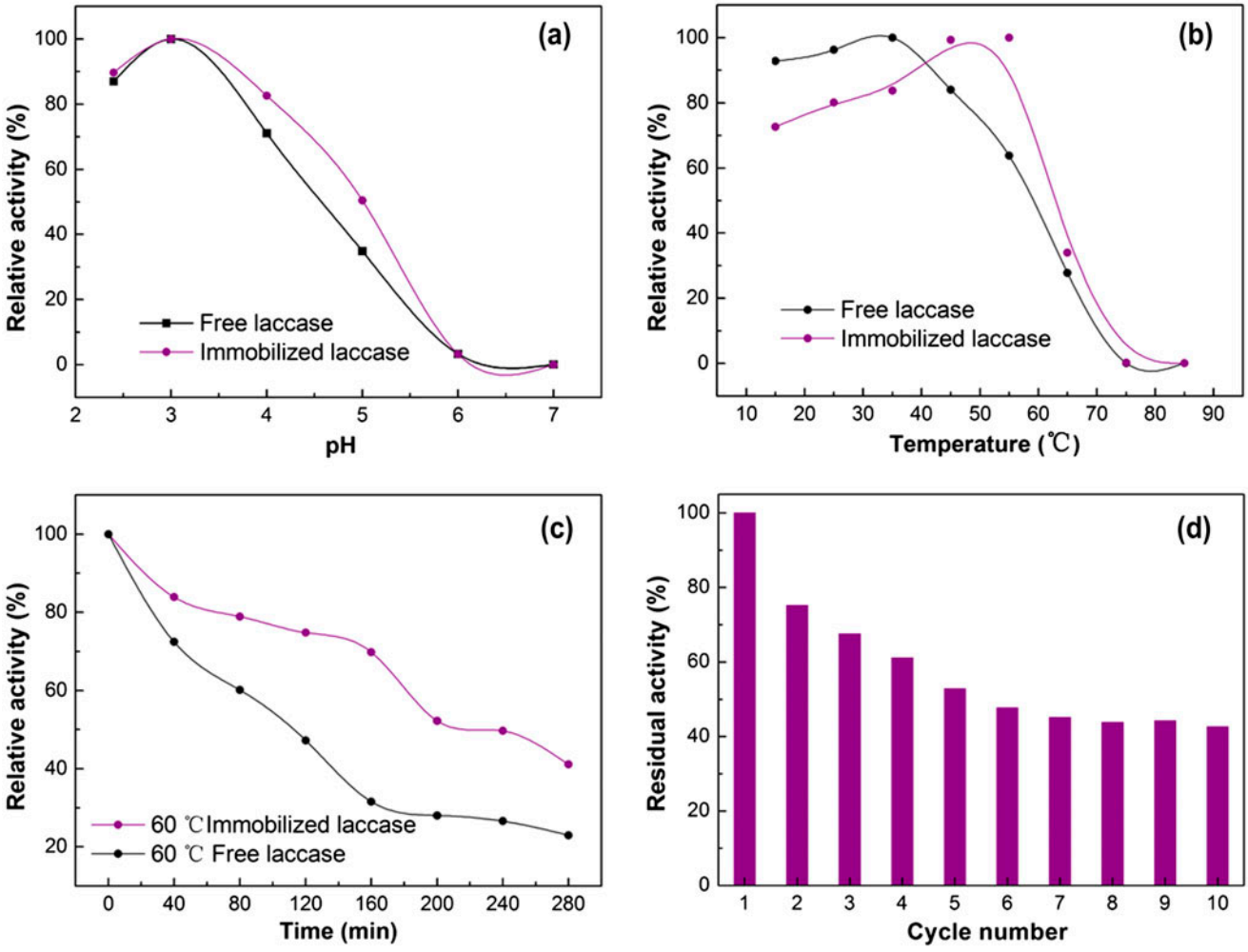

Fig. 7. Properties of free and immobilized laccase: the pH stability (a), effect of temperature on the activity (b), the thermal stability (c), and the reusability of immobilized laccase (d).

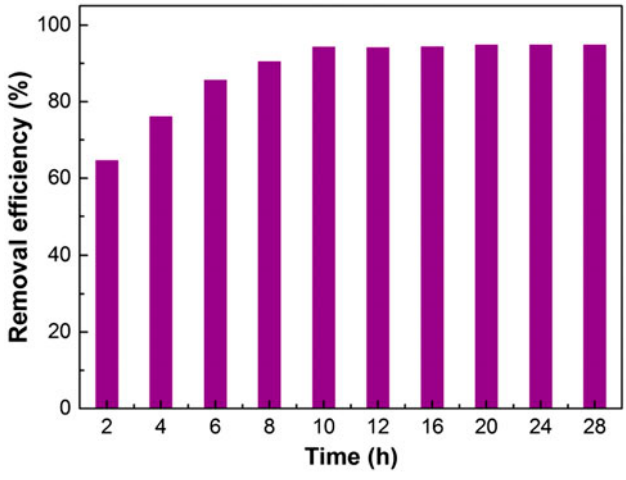

Fig. 8. The removal efficiency of 2,4-DCP by the immobilized laccase.

which achieved high-removal efficiency. On the basis of these results, it was concluded that the CTS-HNT hybrid microspheres could be used as an effective support for biomacromolecule immobilization.

4. Conclusions

We successfully prepared CTS-HNT hybrid microspheres by the suspension cross-linking technique in water-in-oil emulsion using glutaraldhyde as a cross-linker. The halloysite nanotubes overlapped together and formed a hybrid porous structure by cross-linking with chitosan. The porous microspheres exhibited high capacity for laccase immobilization when they were used as enzyme support. The stability of laccase was greatly improved after immobilization on the support. While the immobilized laccase was used to remove 2,4-DCP from wastewater, it exhibited

excellent removal efficiency as high as 95.0%. All the results illustrated that the CTS-HNT hybrid microspheres could be used as an effective support to immobilize enzymes and remove phenolic compounds from wastewater.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 21271158 and 20871105).

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