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Immobilization of laccase on magnetic bimodal mesoporous carbon and the application in the removal of phenolic compounds

Yuanyuan Liu [a,b], Zhuotong Zeng [e], Guangming Zeng [a,b,*], Lin Tang [a,b,*], Ya Pang [a,b], Zhen Li [a,b], Can Liu [a,b], Xiaoxia Lei [a,b], Mengshi Wu [a,b], Pinyun Ren [c,d], Zhifeng Liu [a,b], Ming Chen [a,b], Gengxin Xie [a,b]

[a] College of Environmental Science and Engineering, Hunan University, Changsha 410082, Hunan, PR China
[b] Key Laboratory of Environmental Biology and Pollution Control, Hunan University, Ministry of Education, Changsha 410082, Hunan, PR China
[c] College of Physics and Microelectronics Science, Hunan University, Changsha 410082, PR China
[d] Key Laboratory for Micro-Nano Physics and Technology of Hunan Province, Hunan University, Changsha 410082, PR China
[e] State Key Laboratory of Medical Genetics, Central South University, Changsha 410078, PR China

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ABSTRACT

A novel magnetically separable laccase immobilized system was constructed by adsorbing laccase into bimodal carbon-based mesoporous magnetic composites (CMMC). A large adsorption capacity (491.7 mg g^{-1}), excellent activity recovery (91.0%) and broader pH and temperature profiles than free laccase have been exhibited by the immobilized laccase. Thermal stability was enhanced to a great extent and operational stability was increased to a certain extent. The shift of kinetic parameters indicated affinity change between enzyme and substrate. Application of the immobilized system in phenol and *p*-chlorophenol removal was investigated in a batch system. Adsorption effects of the support were responsible for the quick removal rate in the first hour, and up to 78% and 84% of phenol and *p*-chlorophenol were removed in the end of the reaction, respectively, indicating that the magnetic bimodal mesoporous carbon is a promising carrier for both immobilization of laccase and further application in phenol removal.

1. Introduction

In recent years, there have been increasing environmental and toxicological interests about phenolic pollutants due to their widespread occurrence and relative frequency in the aquatic environment (McKinney et al., 2006; Zeng et al., 2008). Most of phenolic compounds, especially phenolic organohalogens, are poorly degradable, and could impose toxic, carcinogenic, mutagenic, and teratogenic effects upon animals and humans even at low concentrations (Meijer et al., 2008; Zeng et al., 2011), thus were listed as priority pollutants by the US Environmental Protection Agency.

The use of oxidative enzymes such as laccase for the treatment of phenol contaminant wastewater has received great attention because of the efficient and low-cost degradation of the pollutants (Tang et al., 2008; Liu et al., 2010). Laccase (benzenediol oxygen oxidoreductase, EC 1.10.3.2) is a copper-containing polyphenol oxidase produced by numerous funguses, several plant species and some bacteria. In comparison with other oxidative enzymes, laccase is reported to be relatively unspecific and be capable of catalyzing the oxidation of a wide range of phenolic compounds and aromatic amines with simultaneous reduction of its co-substrate of oxygen to water (Liebminger et al., 2009; Majeau et al., 2010). Its wide substrate specificity has resulted in its extensive application in biofuel cell, biosensor, bioremediation and wastewater disposal (Couto and Herrera, 2006; Tang et al., 2006). However, low stability and poor reusability of laccase have limited its further industrial application.

Immobilization technology has been proved to be an effective and most straightforward way to implement efficient and continuous application of enzymatic oxidation (Sheldon, 2007; Pang et al., 2011). Mesoporous carbon materials, with their properties such as a large specific surface area, a high pore volume, a porosity made up of uniformed mesopores with tunable sizes and higher hydrothermal resistance compared with mesoporous silica materials and other materials, have been considered as highly suitable candidates for enzyme and molecule immobilization (Lee et al., 2005; Vinu et al., 2007). Magnetic bio-separation technology is a promising technology in the support systems for enzyme immobilization, since on the basis of magnetic properties, compared with conventional filtering separation, rapid separation and easy recovery could be reached in external magnetic field, and the capital and operation costs could also be reduced (Zhang et al., 2007). There have been some reports about the adsorption of lysozyme or hemoglobin in magnetic mesoporous carbon material (Fuertes et al., 2008; Sevilla et al., 2009). Some studies have also reported laccase

* Corresponding authors at: College of Environmental Science and Engineering, Hunan University, Changsha 410082, Hunan, PR China. Tel.: +86 731 88822754; fax: +86 731 88823701.
E-mail addresses: zgming@hnu.edu.cn (G. Zeng), tanglin@hnu.edu.cn (L. Tang).

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immobilization on magnetic mesoporous silica nanoparticles (Wang et al., 2010a; Zhu et al., 2007). However, to the best of our knowledge, there have not been any reports concerned with the immobilization of laccase on the magnetic mesoporous carbon material, nor have any studies researched the application of immobilized enzyme on the carrier.

In view of these problems, in this study, we developed a magnetically separable laccase immobilization system on the basis of carbon-based mesoporous magnetic composites (CMMC) as supports. Effects of laccase concentration on the relative activity, activity recovery and adsorption capacity were investigated. The pH-activity and temperature-activity profiles were evaluated. Affinity changes, exhibited by kinetic parameters, thermo stability and operational stability were also tested. Finally, the immobilized laccase was operated in a rotary shaker to remove phenol and *p*-chlorophenol, with investigation on the adsorption effects of the support.

2. Methods

2.1. Materials

Laccase (EC 1.10.3.2, 23.3 U mg^{-1}) from *Trametes versicolor* was obtained commercially from Fluka. Pluronic copolymer P123 ($EO_{20}PO_{70}EO_{20}$) and 2,2′-azinobis(3-ethylbenzthiazolin-6-sulfonate) (ABTS) were purchased from Sigma–Aldrich (USA). All other chemicals were of analytical grade and were used as received without further purification.

2.2. Preparation of carbon-based mesoporous magnetic composites

The mesostructured SBA-15 silica template was synthesized following the typical method as reported (Zhao et al., 1998). CMMC was synthesized following a co-impregnation method with slight alterations (Wang et al., 2010b). A typical procedure was carried out as follows: 0.75 mmol $Fe(NO_3)_3{\cdot}9H_2O$ and 0.75 mmol $Ni(NO_3)_2{\cdot}6H_2O$ were dissolved in 0.5 ml ethanol, and then 1 ml furfuryl alcohol was added into the mixture under stirring. And then a multi-component solution could be prepared. Afterwards, 1.4 ml of the solution was extracted and infiltrated into 1.2 g SBA-15 template, and then the impregnated sample was cured at 80 °C in air for 10 h to polymerize the furfuryl alcohol. Carbonization and in situ reduction of the metal oxides was performed under a 5% H_2–95% Ar atmosphere at 900 °C for 2 h with a heating rate of 2 °C min^{-1}. The product was washed with heated 2 M NaOH solution to remove silica template, filtrated, washed with distilled water, dried at 70 °C, and stored for further experiments.

2.3. Laccase immobilization

The immobilization experiment was carried out by suspending 4 mg of CMMC into 10 ml of sodium citrate buffer (0.1 M, pH 4.0) containing 1 mg ml^{-1} of laccase. The resulting mixture of the supports and laccase solution was then incubated at 25 °C with shaking at 100 rpm for 3 h to reach adsorption equilibrium. The laccase immobilized on CMMC particles was subsequently separated magnetically, washed with the same buffer, dried at −40 °C under vacuum using lyophilizer and stored at −4 °C for the further use. To investigate the effects of initial laccase concentration on the adsorption capacity of the support, it was changed between 0.1 and 4.0 mg ml^{-1}. Each set of experiments was carried out in triplicate, and the arithmetic mean values were calculated, with the standard deviations less than 5%. The amount of laccase immobilized on CMMC particles was calculated by determining the initial and final concentration of laccase in liquid solution using Lowry's method (Lowry et al., 1951).

2.4. Assay of laccase activity

The activity of free and immobilized laccase was determined spectrophotometrically at 420 nm with 0.5 mM ABTS as substrate in 0.1 M sodium citrate buffer (pH 4.0) at 25 °C (Bourbonnais and Paice, 1990). The oxidation of substrate to $ABTS^+$ was measured for 5 min using a UV–vis spectrophotometer (UV-2250, SHIMADZU Corporation, Japan), with the molar extinction coefficient of 36×10^{-3} M^{-1} cm^{-1}. One unit (U) of laccase activity was defined as the amount of enzyme needed to oxidize 1 μmol of ABTS per minute. The activity recovery of the immobilized enzyme is calculated from the following formula:

$$R\ (\%) = (A_i/A_f) \times 100$$

where R is the activity recovery of the immobilized laccase (%), A_i is the activity of laccase immobilized on CMMC particles (U), and A_f is the activity of the same amount of free laccase in solution as that adsorbed on particles (U).

To determine the pH-activity profiles of the free and immobilized laccase, activities were measured in sodium citrate buffer (0.1 M, pH 2.0–4.0) and phosphate buffer (0.1 M, pH 5.0–8.0) at 25 °C. Effects of temperature on the activities of the free and immobilized enzymes were conducted over the temperature range of 20–80 °C at pH 4.0. Here the activity was expressed as relative forms (%), with the maximal value of enzyme activity at a certain pH or temperature set as 100%.

2.5. Thermal and operating stability

The thermal stability studies on the free and immobilized laccase were conducted by treating laccase in sodium citrate buffer solution (pH 4.0) for variable incubation time at 60 °C. Samples were withdrawn every 30 min and were then taken for the activity measurement immediately. The operating stability of the immobilized laccase was assessed by performing several consecutive operating cycles using 0.5 mM ABTS at 25 °C. At the end of each cycle, the immobilized laccase was separated with a magnet, washed three times with sodium citrate buffer (pH 4.0), and repeated with a fresh aliquot of substrate, as described by Davis and Burns (1992).

2.6. Determination of kinetic parameters

Kinetic parameters of Michaelis–Menten equation (V_{max} and K_m) for the free and immobilized laccase were tested by measuring the initial rates of ABTS oxidation over the concentration range of 0.2–1 mM at 25 °C in sodium citrate buffer at pH 4.0. Lineweaver–Burk plot was used to estimate kinetic parameters, and V_{max} and K_m were determined from the intercepts at *X*- and *Y*-axis of the plot, respectively.

2.7. Removal of phenol compounds

Studies in removal of phenol and *p*-chlorophenol were performed at 25 °C with laccase-immobilized particles in phosphate buffer (0.1 M, pH 6.0) with shaking at 100 rpm. In each set of the experiment, 3.0 mg of the immobilized laccase was added into 10 ml reaction medium with 2 mM phenol and *p*-chlorophenol, with incubation time lasting for 12 h. Before reaction initiation, the solution of buffer and phenols was saturated with oxygen by vigorously shaking for 15 min. The percentage of phenol and *p*-chlorophenol removal was determined at different intervals by separating the upper solution with magnetism, and then the residual concentration of phenol and *p*-chlorophenol was determined using a UV–vis spectrophotometer at 270 nm and 279 nm, respectively (Wu and Yu, 2006). To determine how carrier adsorption

affected removal efficiency of phenol and *p*-chlorophenol, the heat-denatured immobilized enzyme was used in place of the intact immobilized laccase under the same experimental condition.

2.8. Characterization of carbon-based mesoporous magnetic composites

N_2 adsorption–desorption isotherms were obtained on a Micromeritics 2020 analyzer at 77 K. BET analyses were used to determine the surface area. The desorption branches of the isotherms based on BJH model were used to derive pore size distribution, and BJH analyses were also used to evaluate the total pore volume (Wang et al., 2010b). Surface morphology of CMMC was observed using Field Emission scanning electron microscope (FE-SEM, JEOL JSM-6700F). Transmission electron microscopy (TEM) images were performed on a JEOL-1230 electron microscope operated at 100 kV. The magnetic properties of the samples were studied by a vibrating sample magnetometer (VSM) at room temperature.

3. Results and discussion

3.1. Properties of carbon-based mesoporous magnetic composites

From the SEM image of Supplementary data – Fig. 1a, a platelet-like shape with an average size of approximately 0.5 μm could be observed for the composite. Large domains of ordered stripe-like structures could be clearly noticed from the TEM images of Supplementary data – Fig. 1b, and magnetic nanoparticles (denoted with arrows as O and P) with average size about 10 nm were found embedded in the carbon rods over the entire CMMC particles. Large voids indicated with arrows as T and S could also be observed, corresponding to the secondary mesopores of the composite (Sevilla et al., 2009), which would be further convinced by BET and BJH analyses. The isotherm curve (Fig. 1) showed two obvious hysteresis loops, indicating the existence of two pore systems; and the corresponding pore size distributions calculated from the desorption branches (inset of Fig. 1), showing one pore system centered at 4.0 nm, the other 18 nm, could further illustrate the existence of bimodal systems. BET surface area was 1058.6 $m^2 g^{-1}$. The pore volume was 1.8 $cm^3 g^{-1}$, according to BJH analyses. The magnetization saturation value is 4.1 emu g^{-1}, thus it could be inferred that the separation of the material in aqua media can be controlled by magnetic fields.

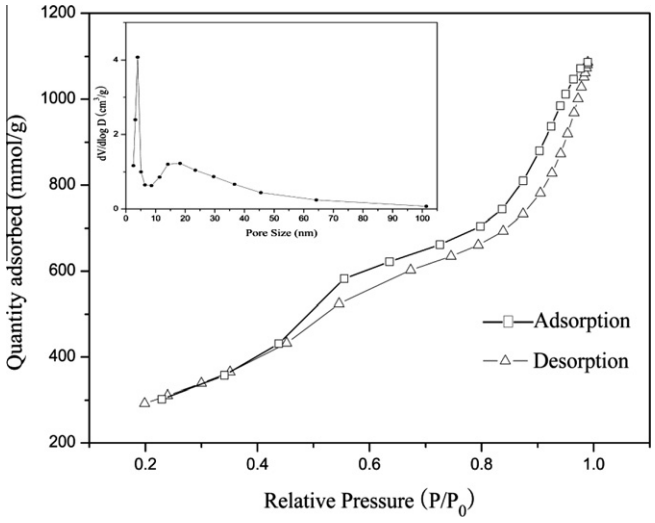

Fig. 1. Nitrogen adsorption–desorption isotherms of CMMC and the corresponding pore size distributions calculated from the desorption branches.

3.2. Immobilization of laccase

As shown in Fig. 2, the maximal activity recovery (91.0%) of the immobilized laccase was obtained when the initial laccase concentration was 0.4 mg ml^{-1}, and the total activity reached its peak value when the laccase concentration was 1.0 mg ml^{-1}. Although the adsorbed amounts of laccase on CMMC increased with the augment of the laccase concentration, the trends of activity recovery and total activity decreased when the laccase concentration was above 0.4 mg ml^{-1} and 1.0 mg ml^{-1}, respectively. Similar phenomenon was also reported in the study of laccase immobilization on magnetic mesoporous silica nanoparticles as a function of laccase concentration (Wang et al., 2010a). Excessive laccase adsorption would easily result in the crowding or agglomeration of enzyme molecules inside the mesopore channels and at the other active sites of the carrier. Thus the resulting steric constraints could subsequently restrain the dispersion and transmission of the substrate and product, exhibited as the reduction of activity.

Compared with the other reported supports, mesoporous materials have exhibited obvious advantages in adsorption capacity (Bautista et al., 2010). In this study, the maximal activity recovery was up to 91.0%, when the adsorbed amount of laccase was 233.3 mg g^{-1}, higher than many reported carriers (shown in Table 1), inferring that the immobilized laccase was not denatured. The higher activity recovery could be due to the physical adsorption, which could avoid the adverse impacts on enzyme activity of covalent attachment (Zhu et al., 2007), lower steric hindrance and the hierarchical bimodal mesoporous structures. As shown in Table 1, in comparison with other supports, the much larger adsorption capacity of 491.7 mg g^{-1} when the highest relative activity reached could be illustrated by the presence of larger secondary mesopores, which greatly overcome the dispersion limits within the mesoporous channels and enhances the loading of large biomolecules (Wang et al., 2010b). In addition, the large surface area and hierarchical mesostructure also played a crucial role in the high adsorption capacity (Wang et al., 2010a).

3.3. Properties of immobilized enzyme

Effect of pH on the activity of the free and immobilized laccase on CMMC was investigated at different pH values varying from 2.2 to 8.0 (Fig. 3). The free and immobilized laccase exhibit the maximal activity at pH 3.0 and pH 4.0, respectively. The shift of optimum pH was also observed in an immobilized laccase on the poly(GMA-MMA-g-4-VP)-Cu(II) beads (Bayramoglu et al., 2010), and was attributed to the electrostatic interaction influenced by carrier microenvironment. The immobilized enzyme also showed broader pH-activity profile than the native enzyme, and higher activity values of the immobilized laccase were obtained from pH 4.0 to pH 8.0, indicating that immobilization preserved the enzyme activity in a broader pH profile (Bayramoglu and Arica, 2009).

Relative activities of both the free and immobilized laccase as a function of temperature were compared in Fig. 4. It could be observed that activities of the two enzymes were strongly dependent on temperature, and both exhibited the maximal activity in 45 °C. However, in comparison with the free laccase, the adsorbed enzyme exhibited a significantly broader temperature profile. In particular, the enhanced activity over 80% for the immobilized enzyme could be observed within the temperature range of 25–55 °C. The improvement in resistance against temperature was probably due to a reduction in molecular mobility and comformational changes by adsorption into mesopores of the support (Wang et al., 2008).

According to the activity comparison of the free and immobilized laccase as a function of incubation time at 60 °C (as shown in Supplementary data – Fig. 2), the adsorbed laccase exhibited a much slower

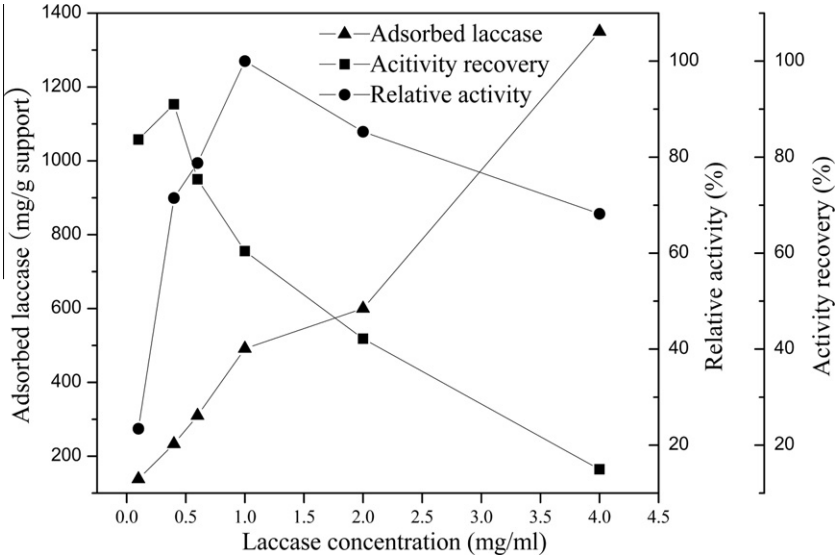

Fig. 2. Amounts of adsorbed laccase, relative activity and activity recovery of laccase immobilized on CMMC as a function of laccase concentration (0.1–4.0 mg ml^{-1}).

Table 1
Performance of immobilized laccase using various supports.

Immobilization support	Adsorption capacity (mg/g)	Activity recovery (%)	Residual activity[a] (%)	Immobilization technique	References
CMMC	491.7	91.0	50.5	Physical adsorption	This work
Green coconut fiber	–	45.7	Nearly 50	Physical adsorption	Cristovao et al. (2011)
Poly(D,L-lactide)/F108 electrospun microfibers	–	67	53.0	Encapsulation	Dai et al. (2010)
Mesostructured siliceous cellular foams	–	69.0	Above 65	Covalent attachment	Rekuc et al. (2009)
Magnetic polymer microspheres	94.1	68.0	87.8	Metal affinity adsorption	Wang et al. (2008)
Magnetic mesoporous silica nanoparticles (MMSNPs)	72.6	81.5	–	Physical adsorption	Wang et al. (2010a)
MMSNPs-CPTS-IDA-Cu^{2+}	98.1	92.5	86.6	Metal affinity adsorption	Wang et al. (2010a)
Magnetic mesoporous silica spheres (MSS)	82	79.4	–	Physical adsorption	Zhu et al. (2007)
MSS-NH2	59	56.9	Above 70	Covalent attachment	Zhu et al. (2007)

[a] Residual activity, residual activity of the immobilized laccase after 10 cycles of oxidizing ABTS oxidation, with the initial activity of the immobilized enzyme set as 100%.

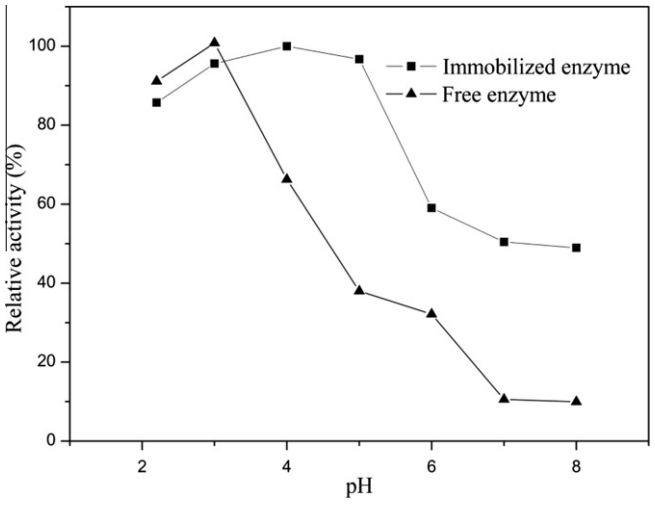

Fig. 3. Effect of pH on the activity of the free and immobilized laccase.

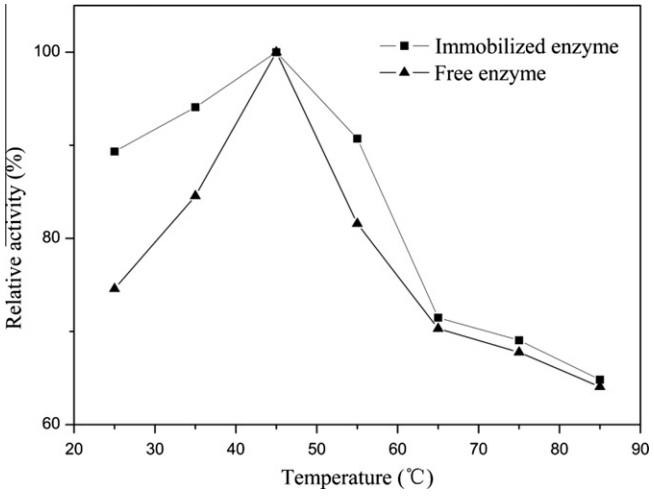

Fig. 4. Effect of temperature on the activity of the free and immobilized laccase.

inactivated rate than the free enzyme, with respective residual activity of 90.3% and 60.8% after 30 min of incubation. Four hours later, the immobilized laccase retained 66.7% of its initial activity, while the free laccase almost completely lost its activity. The more stable form of the immobilized laccase suggested the suitable environment provided by the carrier to maintain enzymatic activity

against heat, which could be attributed to the mesoporous channels of supports and the binding interaction between laccase and carrier, keeping laccase from injuring of direct exposure to environment changes (Zhu et al., 2007).

The operating stability of the immobilized laccase on CMMC was also studied because of its importance of application potential for reducing processing costs. The immobilized laccase could retain above 70% of its initial activity after 5 cycles of oxidizing ABTS and over 50% after 10 cycles (as shown in Supplementary data – Fig. 3), indicating a certain reusability of the laccase immobilized on CMMC. Some reports have presented better operational stabilities than the previous study because of the immobilization method used (Table 1). The physical adsorption is known for the weaker binding interaction, resulting in lower operational stability in this study and the research by Cristovao et al. (2011). However, as shown in Table 1, higher reusability because of covalent attachment was often accompanied with the decrease of adsorption capacity and activity recovery (Zhu et al., 2007). Interestingly, metal affinity adsorption on mesoporous materials could retain the higher reusability without negative effects on activity recovery (Wang et al., 2010a). Thus further studies might adopt metal affinity adsorption on CMMC to improve operational stability without decreasing activity recovery.

Kinetic parameter was estimated from Lineweaver–Burk plot using ABTS as substrate. The apparent K_m value for the immobilized laccase was 0.550 mM, which was 2.4 times higher than that for the free enzyme (0.232 mM). In comparison with the increase of K_m, V_{max} of the adsorbed laccase decreased to 18.75 U mg^{-1} from 20.98 U mg^{-1} for the free enzyme. This shift of K_m and V_{max} value indicated less affinity for the substrate of the immobilized enzyme than that of the free enzyme, which might be caused by the steric hindrance and diffusion limits, resulting in lower accessibility to the active sites of the immobilized laccase due to the comformational changes of the laccase from adsorption into the mesoporous channel of the support (Sari et al., 2006).

3.4. Removal of phenolic compounds

To test the removal rates of phenolic compounds by the prepared immobilized laccase, removal of phenol and *p*-chlorophenol were chosen as model reactions in a rotary shaker. Removal by denatured immobilized laccase was also studied to investigate effects of adsorption by the support, since the adsorption capacity was not fully taken up by the enzyme. As described in Fig. 5, adsorption of phenolic compounds into the inactivated immobilized enzyme was revealed, which was in accordance with many studies, where adsorptions of dyes and phenol by mesoporous carbon and denatured immobilized enzyme on mesostructured cellular foams were reported (Kennedy et al., 2007; Rekuc et al., 2009). Thus the removal of phenolic compounds could be attributed to the combined effects of degradation by the immobilized laccase and the adsorption by the mesoporous support, with nearly 20% of the contribution by the latter one, indicating the advantage of employing the mesoporous support based immobilization system in the removal application. And the extraordinarily rapid removal rate in the first hour of both phenol and *p*-chlorophenol could be deduced as the combined contribution of adsorption and degradation, especially adsorption, since adsorption of phenols reached equilibrium in an hour after the reaction initiated (Fig. 5).

As shown in Fig. 5, about 78% and 84% of phenol and *p*-chlorophenol are removed in 12 h by immobilized enzyme, respectively. The more significant contribution of the catalytic process could be revealed since adsorption only contributed to about 20% of removal. The relative activity order for the removal of phenols by the immobilized laccase, estimated as a function of time was *p*-chlorophenol > phenol, which was in agreement with previous

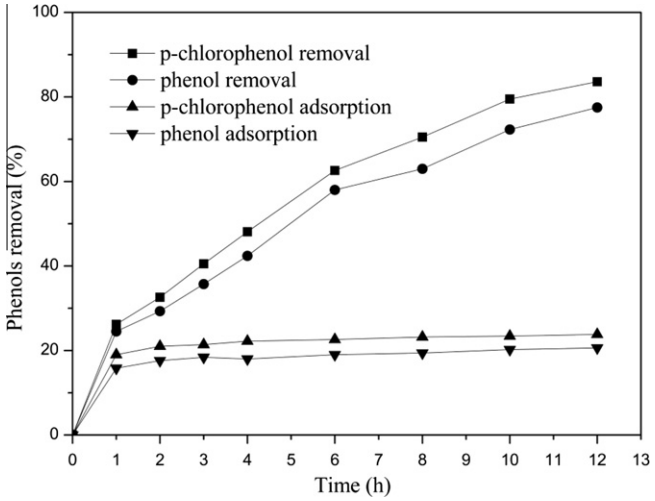

Fig. 5. Removal and adsorption rates of *p*-chlorophenol and phenol by the immobilized laccase and the denatured immobilized laccase.

reports (Bayramoglu and Arica, 2009). Decreased removal rates of phenols after 6 h could be attributed to the decline of degradation of phenols, which were probably due to the accumulation of the catalytic products, causing inhibition of the catalytic process, as reported by previous studies (Bayramoglu et al., 2010). And the reduction of the reactant due to the consumption of phenols could also contribute to the decrease of phenol degradation.

4. Conclusions

A carbon-based mesoporous magnetic composite was prepared to adsorb laccase. Large adsorption capacity of 491.7 mg g^{-1}, favorable activity recovery of 91.0% and broader pH and temperature profiles than the free laccase have been exhibited by the immobilized enzymes. Stability against heat denaturation increased significantly. Operational stability could be maintained to a certain way. Kinetic parameters indicated affinity shift between enzyme and substrate. By employing the immobilization system, 78% and 84% of phenol and *p*-chlorophenol removal reached at the end of the reaction, respectively, with the contribution of removal (20%) in the first hour by the adsorption of mesoporous support.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2011.11.015.

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