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Degradation of chlorophenols catalyzed by laccase ☆

Jianbo Zhang[a,*], Xiaopeng Liu[a], Zhenqiang Xu[a], Hui Chen[a], Yuxiang Yang[b]

[a]*Department of Environmental Sciences, College of Environmental Sciences, Peking University, Beijing 100871, China*
[b]*College of Chemistry and Pharmaceutics, East China University of Science and Technology, Shanghai 200237, China*

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Abstract

The degradations of 2,4-dichlorophenol (2,4-DCP), 4-chlorophenol (4-CP) and 2-chlorophenol (2-CP) catalyzed by laccase were carried out. The optimal condition regarding degradation efficiency was also discussed, which included reaction time, pH value, temperature, concentration series of chlorophenols and laccase. Results showed that the capability of laccase was the best, while to oxidize 2,4-DCP among the above-mentioned chlorophenols. Within 10 h, the removal efficiency of 2,4-DCP, 2-CP and 4-CP could reach 94%, 75% and 69%, respectively. The optimal pH for laccase to degrade chlorophenols was around 5.5. The increase of laccase concentration or temperature might result in the degradation promotion. The trends of degradation percentage were various among these three chlorophenols with the concentration increase of chlorophenols. Degradation of 2,4-DCP is a first-order reaction and the reaction activation energy is about 44.8 kJ mol^{-1}. When laccase was immobilized on chitosan, crosslinked with glutaraldehyde, the activity of immobilized laccase was lower than that of free laccase, but the stability improved significantly. The removal efficiency of immobilized laccase to 2,4-DCP still remained over 65% after six cycles of operation.

Keywords: Chlorophenol; Laccase; Catalytic degradation; Immobilized enzyme

1. Introduction

Chlorophenols have been widely used in wood antisepsis, antirust production, fungicide and other pesticides for a long time, such as 2,4-dichlorophenol (2,4-DCP), which was largely used to produce herbicide of 2,4-D (Pandiyan et al., 2002). Chlorophenols have a strong denaturing effect on organisms, cause irritability to skin and mucous membrane, and causticity as well. Chlorophenols may release a special odd smell, while the intoxicating concentrations of these compounds to aquatic organisms are a little higher than the threshold value of osphresis mostly, and several organisms may have toxicity once it is breathed in (Dietz and Traud, 1978). The toxicity of chlorophenols may increase with the increase of its chlorine atom, and what is more, its difficulty to be degraded by microorganisms also contributes to the toxicity (Chen et al., 1999). The removal methods of chlorophenols have attracted much attention. Physical chemistry measures including adsorption, mixing coagulation, extraction and chemical measures such as photo-chemical oxidation, supersonic chemistry process, hydrogenolysis and radiolysis have been applied (Wei et al., 1998). Biological degradation technology has been focused on during recent decades.

Laccase (EC 1.10.3.2) is one of the glucoproteinases containing coppers, including laccase from rhus and fungal laccase, classified by source. Research on chlorophenol degradation by laccase has been reported (Chao and Qian, 2001). In recent years, laccase immobilization was further studied nationally (Yu et al., 1999), and the tested carriers include silica gel, corkwood particulate, sodium alginate, active carbon, calcium alginate, multi-porous glasses, epoxyethane acrylic particulate and micro hydrophilic filter membrane, etc. There are many advantages to immobilize enzyme using chitosan, such as abundant supply, low cost, simple preparation, stable chemical property, excellent immobilization efficiency and without

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*Corresponding author. Tel.: +86 10 62753438.
E-mail address: jbzhang@pku.edu.cn (J. Zhang).

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secondary pollution. The immobilized laccase can be used repeatedly. The stability of laccase was also improved after immobilization (Shuttleworth and Bollag, 1986). Most publications focused on chlorophenol degradation by enzyme; however, studies on laccase were limited, especially on immobilized laccase.

The degradations of 2,4-DCP, 4-chlorophenol (4-CP) and 2-Chlorophenol (2-CP) catalyzed by laccase were studied.

2. Materials and methods

2.1. Materials and equipment

Laccase was from Coriolus versicolor, Fluca, Switzerland; 2,4-DCP was from Aldrich, USA; 4-CP, 2-CP, potassium ferricyanide ($K_3[Fe(CN)_6]$), glutaraldehyde, acetic anhydride, dimethylbenzidine, 4-amidoantipyrine (4-AAP), chitosan, and Petroleum ether were locally purchased.

The UV757 Ultraviolet–visible Spectrophotometer was made in China; GCQ GC-MS in Finnigan, USA; and 79–3 Magnetic Homoiothermal Mixer again in China.

2.2. Principle

Laccase is a single-electron oxidoreductase and able to deoxidize oxygen into water. Without hydrogen peroxide and other secondary catabolite, laccase may catalyze the oxidation of organic pollutants such as chlorophenols (Chao and Qian, 2001). Oxidation of chlorophenols is achieved by four copper ions coordinating in the transferring electron and by variation of the valence state. In the catalytic reaction, chemical constitution, concentration of substrates, pH value of the mixed reaction system, enzyme activity, time, temperature and addition agents may impact the oxidation efficiency of substrates (Qin et al., 2001).

2.3. Determination of chlorophenols concentrations and laccase activities

At pH 7.9 ± 0.1, with the presence of K_3 [Fe (CN)$_6$], chlorophenols may react with 4-AAP, forming chromatic antipyrine dyestuff. The dyestuff has a special absorption peak in the range of visible-light wavelength; hence the concentrations of chlorophenols can be measured by absorbency (American Public Health Association et al., 1985). 2,4-DCP, 4-CP and 2-CP were determined at the wavelengths of 509, 505 and 510 nm, respectively. The standard curves were drawn with the R^2 coefficients higher than 0.9990, the detection limit was 0.1 mg L^{-1} and the recovery efficiency was above 95%.

Due to the strong polarity of chlorophenols, there is strict requirement for a chromatographic column and system to inject samples directly. Chlorophenols should usually be converted into some nonpolar derivants before analysis (Huang and Sun, 2000; Law et al., 2003). Acetic anhydride was used as the derivation agent to convert chlorophenols into corresponding esters in alkaline condition, and then the esters were extracted by petroleum ether and determined by GC–MS.

A unit of laccase activity (U) is defined as the quantity of laccase needed to increase the absorbency of 0.001 per μmol substrate per minute under the specified condition of 25 °C (Wang and Liu, 2000). Dimethylbenzidine aqueous solution (1 mmol L^{-1}) is used as the substrate, and adding HAc–NaAc buffer solution (pH = 4.0), laccase activity is determined at 600 nm.

2.4. Immobilization of laccase by chitosan

The optimal condition to immobilize laccase was determined (Xiao et al., 2003). 1.0 g chitosan was dissolved into 2% acetic acid solution, and diluted to 200 mL; a chitosan solution of 5.0 g L^{-1} was obtained, i.e., a certain amount of chitosan solution was dropped into 2 mol L^{-1} NaOH aqueous solution, resulting in the formation of white flocculent deposition, which was then washed by water to make it neutral and the filtrate was pumped into a wet chitosan carrier. About 20 mL 5% glutaraldehyde aqueous solution was added into the carrier and mixed for 8 h, placed overnight, followed by the pumping of filtrate and washing repeatedly to eliminate the unwanted glutaraldehyde, and then the wet yellow powdery carrier of chitosan crosslinked by glutaraldehyde was obtained. The carrier was added into 50 mL of 0.4 g L^{-1} laccase aqueous solution, and then 5 mL HAc–NaAc buffer solution (pH = 4.37) was added and mixed at room temperature for 16 h, and then placed overnight at 4 °C. After the filtrate was pumped, yellow powdery immobilized laccase by chitosan was prepared.

2.5. Degradation of 2,4-DCP, 4-CP and 2-CP catalyzed by laccase

A certain amount of 2,4-DCP or 4-CP or 2-CP was taken and HAc–NaAc buffer solution with certain pH and laccase aqueous solution were added. The concentration of chlorophenol in the reactor was adjusted by water to 10 mg L^{-1}. The reactor was then placed in an aqueous thermostat, and 10 mL of sample was taken out after a certain time and diluted to 50 mL, 1.25 mL of 0.5 mol L^{-1} $NH_3 \cdot H_2O$ were added and the pH was adjusted to 7.90 ± 0.02 by a phosphate buffer solution (pH = 6.70). About 0.5 mL 2% 4-AAP aqueous solution and 0.5 mL 80 g L^{-1} $K_3[Fe(CN)_6]$ were then added, and then mixed for 30 s and kept for 15 min; the absorbency was measured at the corresponding wavelength by a spectrophotometer.

When the residual concentrations of chlorophenols were lower than the detection limit of the spectrophotometer method, therefore, GC–MS was used to substitute to determine their concentrations.

2.6. Degradation of 2,4-DCP catalyzed by immobilized laccase

About 10 mL 2,4-DCP solution of 100 mg $L^{-1}$was transferred to the reactor, 5 mL HAc–NaAc buffer solution (pH = 5.50) and 0.18 g prepared chitosan-immobilized laccase were added, and then diluted to 50 mL. The reactor was then placed in an aqueous thermostat and the reaction progressed at 30 °C for some time. The solution was filtered to separate the immobilized laccase; the filtrate of 10 mL was collected to determine the concentration of DCP. Then the residual filtrate was mixed with the immobilized laccase again and the reaction continued; this operation was repeated.

3. Results and discussion

3.1. Effect of time on the removal of chlorophenols

With the temperature at 30 °C, pH at 5.5, and, the initial concentrations of chlorophenols and laccase at about 50 and 80 mg L^{-1}, respectively, the removal efficiency (RE) of the three chlorophenols versus time is shown in Fig. 1. For 2,4-DCP, it was much higher than that of 4-CP and 2-CP. 2,4-DCP can be degraded 94% after 10 h; it was 69% and 75% for 4-CP, 2-CP, respectively.

In general, the degradation of o-substituted chlorophenols catalyzed by laccase is faster than that of p-substituted ones. The possible reason is that the −OH on the benzene ring is such an active group that it makes the benzene ring activate and easy to react with an electrophilic substitute (Hirvonen et al., 2000). The electrophilic substitution would firstly react at the o- and p- positions, and then the cyclohexadienyl cation intermediate produced at the

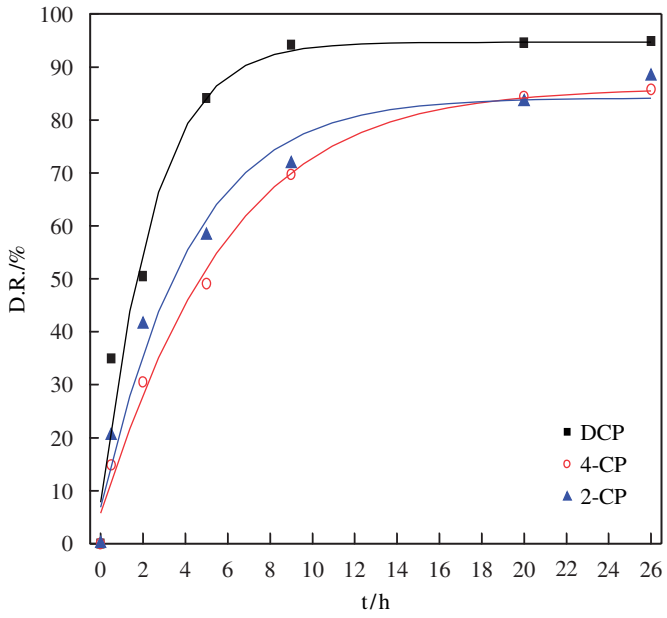

Fig. 1. Removal efficiency of chlorophenols versus time.

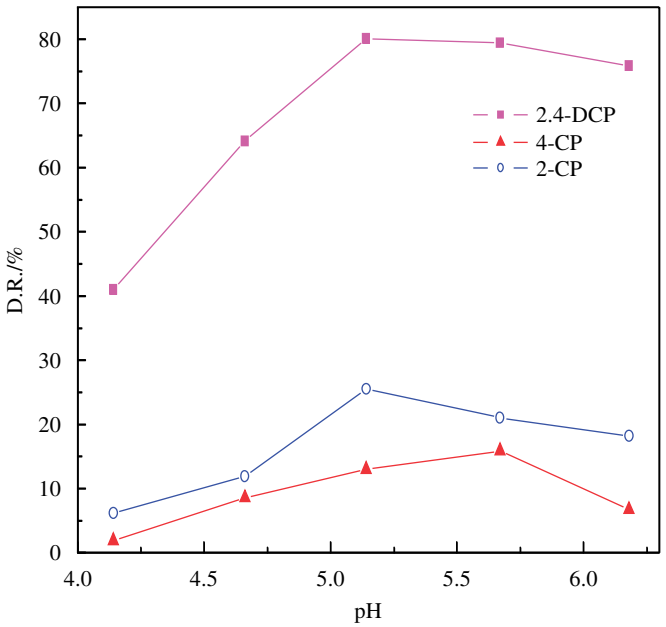

Fig. 2. Effect of pH on chlorophenols degradation.

o- or p- positions in the substitution process, which can be stable with the unpaired electron in the ring. The O- position is relatively easier for electrophilic substitution compared to the p-position, while the cation intermediate produced at the m- position reaction is unstable. With the stability of cation increase, the reaction rate will be improved, and so for the conversion efficiency of phenols.

At the beginning of dechlorination, due to the inhibition of free radical for 4-CP but the promotion for 2-CP, the removal rate for 2-CP is faster than that for 4-CP (Kazuhito et al., 2000). Much later, the effect of intermediate on the catalyzing process of laccase gradually reduced by polymerization, and with further degradation of the intermediate, the RE of two monochlorophenols became close. As 2,4-DCP has two positions available for the chlorine atom to substitute, laccase may catalyze the dechloration on the 2- position firstly, producing free radicals to promote laccase dechlorinating on 2- and 4- positions. This chain promotion made the RE of 2,4-DCP much more than that of the two monochlorophenols; therefore, the degradation is more thorough.

3.2. Effect of pH and temperature on the removal of chlorophenols

At 30 °C, the initial concentrations of the three chlorophenols were about 10 mg L^{-1}, with the laccase being 60 mg L^{-1}. The RE of chlorophenols reacting for 4 h at different pH was determined and the results are described in Fig. 2 degradation rate (DR) refers to the percentage of degraded chlorophenols accounting for the initial system.

It can be seen from Fig. 2, at pH = 4.0, that the RE of the three chlorophenols was relatively low, and the laccase activity is likely to be inactivated in highly acidic condition. In weaker acidic condition, the laccase activity improved. The maximum RE at pH range 5.0–6.0 was more than 80% for 2,4-DCP. For 4-CP and 2-CP, the maximum RE appeared near pH 5.5 and 5.0–5.5, respectively. It was reported (Bollag et al., 1988) that laccase may be gradually inactivated when pH ⩾ 7.

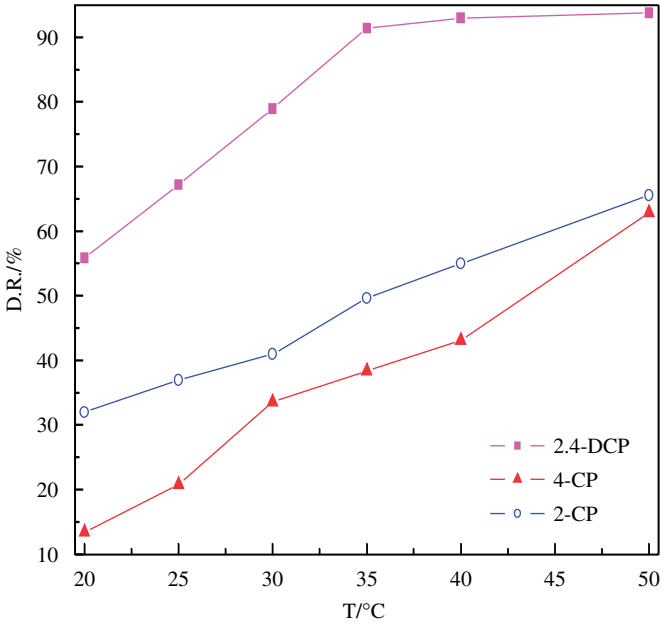

Fig. 3. Effect of temperature on chlorophenols degradation.

The different removal efficiencies of chlorophenols with temperature are shown in Fig. 3. When the initial concentrations of the three chlorophenols were about 10 mg L^{-1}, with the laccase being 60 mg L^{-1} and pH at

5.5, the RE increased rapidly with the rise of temperature at the beginning of the reaction. When the temperature was higher than 35 °C, the increase of temperature had little effect on the RE. In the same condition, the RE of 2-CP was more than that of 4-CP, but with the temperature rise, the increase of the latter was faster than that of the former. Both the removal efficiencies for 4-CP and 2-CP were lower than that of 2,4-DCP.

The results showed that the higher the temperature, the stronger the laccase activity (D'Annibale et al., 1999), whereas the loss of activity will be faster. As the temperature increases, dissolved oxygen in the reaction system will decrease, which is adverse for enzyme catalysis (Aktas and Tanyolac, 2003).

At 50 °C, the parallel experiment was conducted, with no addition of laccase. Results showed that the concentration of chlorophenols was the same, that is, chlorophenols cannot be removed by volatilization, the loss of chlorophenols is the result of degradation catalyzed by added laccase in other experiments.

3.3. Effect of laccase concentration/initial concentrations on the RE of chlorophenols

At pH 5.5, 30 °C and initial concentrations of chlorophenols being 10 mg L^{-1}, reacting for 4 h, the concentrations of laccase changed from 20 to 120 mg L^{-1}, the RE of chlorophenols increased with increasing laccase concentration, and the variation of the degradation quantity (DQ) of chlorophenols/unit mass laccase was different among the three chlorophenols (see Table 1).

It can be seen from Table 1 that the ability of laccase degrading 2,4-DCP is better than its effect on those of the two monochlorophenols. The degradation quantity/unit laccase decreased with the increase of laccase concentration for 4-CP and 2,4-DCP; however, for 2-CP the degradation quantity/unit laccase increased first and then dropped with the increase of laccase concentration. Based on the above results, laccase dosage ranged 20 to 60 mg L^{-1} was appropriate.

At 30 °C, pH 5.5 and concentrations of laccase being 60 mg L^{-1}, the initial concentrations of the three chlorophenols changed from 2.5 to 12.5 mg L^{-1}. The degradation was conducted for 4 h. The RE and degradation quantities of the three chlorophenols are shown in Table 2. It showed that with the increase of 2,4-DCP initial concentrations, the RE decreased gradually; however, the trend was not very obvious. With the increase in the initial concentration of 4-CP, the RE decreased significantly. The RE of 2-CP increased firstly and then dropped with the increase of the initial concentration of 2-CP, dropping from 20% to 35%. The degradation quantities of the three chlorophenols increased as the initial concentration of three chlorophenols increased, especially for 2,4-DCP and 2-CP.

Table 1
Effects of laccase concentrations on the degradation

Concentration of Laccase (mg L^{-1})	20	40	60	80	120
Removal efficiency of 2,4-DCP (%)	46	54	67	78	86
Removal amount of 2,4 -DCP (mg g^{-1})	210	180	148	126	95
Removal efficiency of 4 -CP (%)	5	7	10	12	14
Removal amount of 4 -CP (mg g^{-1})	20	17	15	12	10
Removal efficiency of 2 -CP (%)	5	10	15	20	27
Removal amount of 2 -CP (mg g^{-1})	20	22	22	24	22

Table 2
Effects of initial concentrations of chlorophenols on the degradation

Concentration of chlorophenals (mg L^{-1})	2.5	5.2	7.8	10.0	12.6
Removal efficiency of 2,4-DCP (%)	98	85	85	86	85
Removal amount of 2,4-DCP (mg g^{-1})	36	64	102	130	166
Removal efficiency of 4-CP (%)	18	15	12	8	8
Removal amount of 4-CP (mg g^{-1})	5	8	13	15	18
Removal efficiency of 2-CP (%)	20	24	28	32	32
Removal amount of 2-CP (mg g^{-1})	5	16	30	62	74

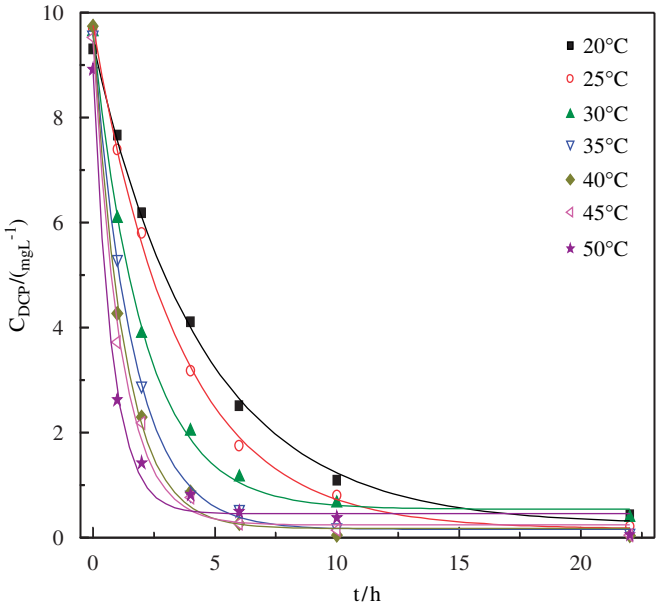

Fig. 4. Degradation curves of 2,4-DCP catalyzed by laccase under different temperature.

3.4. Activation energy of 2,4-DCP degradation catalyzed by laccase

When pH = 5.5, the initial concentration of 2,4-DCP was about 10 mg L^{-1} and the laccase concentration was 60 mg L^{-1}; the kinetical curves of 2,4-DCP degradation at different temperatures are shown in Fig. 4. The kinetical curves followed the first-order reaction: $C = C_0 \exp(-k_1 t)$, in which C_0 is the initial concentration of the reactant, C is the concentration of reactant at time t, and k_1 is the reaction constant of first-order reaction. All the correlation coefficients (R^2) are more than 0.99.

According to the *Arrhenius* Formula $\ln k = -(E_a/RT) + B$, activation energy may be calculated based on the different

Table 3
Calculation of reaction activation energy of 2,4-DCP degraded by laccase

T/K	$C = C_0\exp(-k_1 t)$		$1/T \times 10^3$	$-\ln k$
	$k_1 \times 10^4\ s^{-1}$	R^2		
293	0.621	0.99874	3.413	9.686
298	0.789	0.99894	3.356	9.447
303	1.334	0.99883	3.300	8.922
308	1.713	0.99933	3.247	8.672
313	2.180	0.99731	3.195	8.431
318	2.431	0.99487	3.145	8.322
323	3.532	0.99323	3.096	7.948

reaction constants k (k_1) at different temperatures T (see Table 3).

A schematic may be drawn by ($-\ln k$) and ($1/T \times 10^3$), and then the activation energy of 2,4-DCP degradation catalyzed by laccase at pH 5.5 can be calculated, which should be about 44.8 kJ mol^{-1}.

The activation energy of the typical reaction catalyzed by laccase ranged from 30 to 60 kJ mol^{-}1 (Aktas and Tanyolac, 2001). In general, the lower the activation energy, the faster the reaction. In case it is less than 100 kJ mol^{-1}, the reaction can begin only with little heat. Based on this, the conclusion can be drawn that the reaction of laccase catalyzing 2,4-DCP can begin at a normal temperature and need not be heated.

3.5. Degradation of chlorophenols catalyzed by immobilized laccase

The activity of prepared immobilized laccase (Xiao et al., 2003) was determined for 1550 U mg^{-1}, and the activity of free laccase was about 6250 U mg^{-1}. Both free and immobilized laccase were placed at room temperature, while activities were determined continuously every day (which is shown in Fig. 5). Although the activity of immobilized laccase was lower than that of free laccase, the activity of free laccase was gradually decreasing at room temperature, being close to that of immobilized laccase in 9 days and continued to decrease. The activity of immobilized laccase was constant for quite a long time. That is, the stability of immobilized laccase improved significantly. This property helps in the application of laccase repeatedly and to cut down the cost of wastewater treatment.

In the experiment, 0.18 g immobilized laccase was used repeatedly for several days to degrade 2,4-DCP, and the RE was basically constant (which is shown in Fig. 6). When the reaction was kept for 6 h, the RE of 2,4-DCP ranged from 65% to 90%, which showed that the immobilized laccase can be used repeatedly.

4. Conclusion

The RE at pH 5.5 was higher than that at other pH for the degradation of 2,4-DCP, 4-CP and 2-CP catalyzed by laccase. The RE increased with the rise of temperature. When other conditions were constant, the degradation quantity/unit laccase increased with the debasement of laccase dosage. For a certain range of chlorophenol concentration, the degradation quantity/unit laccase increased with the elevation of initial chlorophenol concentration. It was calculated that the activation energy of degradation of 2,4-DCP catalyzed by laccase was about 44.8 kJ mol^{-1}. In the same condition, the RE of 2,4-DCP was higher than those of 2-CP and 4-CP.

The activities of immobilized laccase were more stable than that of free laccase. At room temperature, the activity

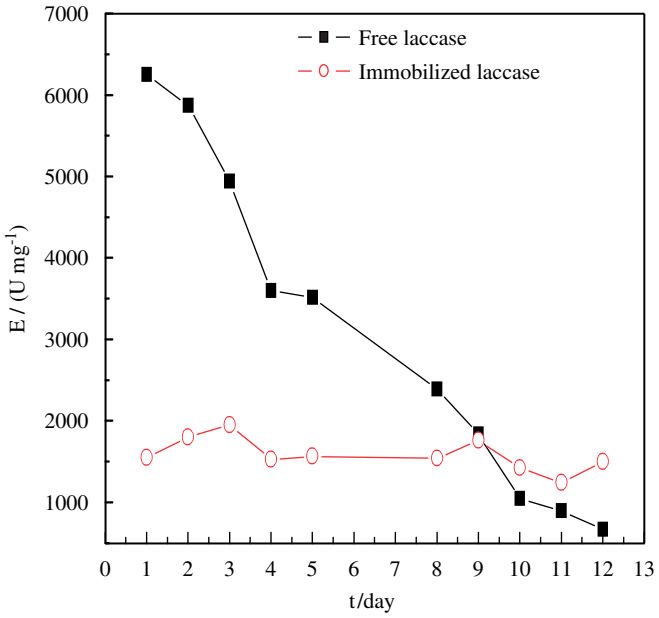

Fig. 5. Variation of laccase activity versus time.

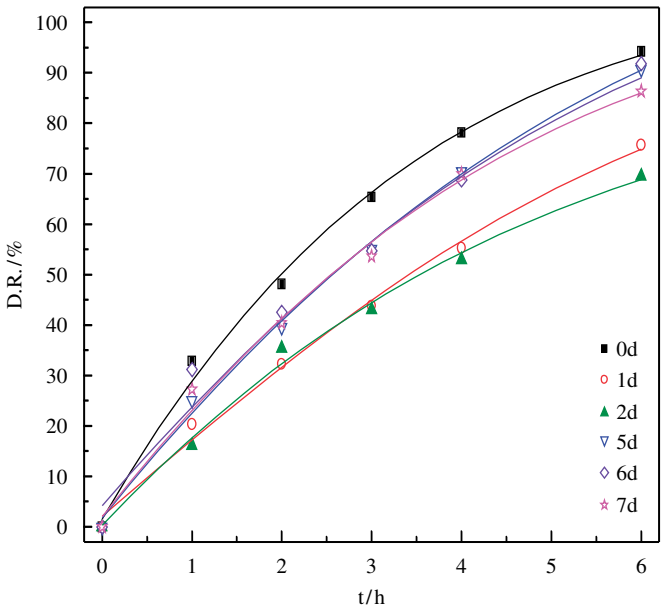

Fig. 6. 2,4-DCP degradation catalyzed by immobilized laccase.

of free laccase decreased gradually, while for immobilized laccase it was almost constant. The prepared immobilized laccase was used repeatedly for 7 days to degrade 2,4-DCP; the RE was more than 60%, which proved that the repeatability of the prepared immobilized laccase was excellent.

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