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Effects of metal ions on the catalytic degradation of dicofol by cellulase

Zihan Zhai[1], *Ting Yang*[1], *Boya Zhang*[2], *Jianbo Zhang*[1,*]

1. *Collaborative Innovation Center for Regional Environmental Quality, State Key Joint Laboratory of Environmental Simulation and Pollution Control, College of Environmental Science and Engineering, Peking University, Beijing 100871, China*
2. *School of Public Health, Peking University, Beijing 100191, China*

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ABSTRACT

A new technique whereby cellulase immobilized on aminated silica was applied to catalyze the degradation of dicofol, an organochlorine pesticide. In order to evaluate the performance of free and immobilized cellulase, experiments were carried out to measure the degradation efficiency. The Michaelis constant, K_m, of the reaction catalyzed by immobilized cellulase was 9.16 mg/L, and the maximum reaction rate, V_{max}, was 0.40 mg/L/min, while that of free cellulase was K_m = 8.18 mg/L, and V_{max} = 0.79 mg/L/min, respectively. The kinetic constants of catalytic degradation were calculated to estimate substrate affinity. Considering that metal ions may affect enzyme activity, the effects of different metal ions on the catalytic degradation efficiency were explored. The results showed that the substrate affinity decreased after immobilization. Monovalent metal ions had no effect on the reaction, while divalent metal ions had either positive or inhibitory effects, including activation by Mn^{2+}, reversible competition with Cd^{2+}, and irreversible inhibition by Pb^{2+}. Ca^{2+} promoted the catalytic degradation of dicofol at low concentrations, but inhibited it at high concentrations. Compared with free cellulase, immobilized cellulase was affected less by metal ions. This work provided a basis for further studies on the co-occurrence of endocrine-disrupting chemicals and heavy metal ions in the environment.

Introduction

Dicofol, or 2,2,2-trichloro-1,1-bis(4-chlorophenyl) ethanol, is an insecticide synthesized from dichlorodiphenyltrichloroethane (DDT). It is used widely in farming to control pest mites on cotton, fruit trees, and vegetables (Qiu et al., 2005). Dicofol is an endocrine-disrupting chemical and difficult to degrade (Hoekstra et al., 2006). It not only interferes with the endocrine systems of fish, birds, and rodents, but also has strong estrogenic effects on humans (El-Din et al., 2011; Guo et al., 2012; Skinner et al., 2011). It can even increase the prevalence of human prostate cancer (Settimi et al., 2003). A risk assessment of manufacturing equipment surfaces contaminated with DDTs and dicofol conducted by Luo et al. (2014) showed that the hazard index of dicofol exceeded the acceptable hazard level. Fujii et al. (2011) confirmed that dicofol was detectable in human breast milk, with a geometric mean concentration in the Chinese breast milk samples of 9.6 ng/g lipid. This indicated the presence of extensive emission sources of dicofol in China. Between 1988 and 2002, 54,000 tons of technical DDT were used to produce about 40,000 tons of dicofol in China (Qiu et al., 2005). Therefore, dicofol is a great threat to the environment and human health.

* *Corresponding author*. E-mail: jbzhang@pku.edu.cn (Jianbo Zhang).

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Biodegradation is regarded as an effective approach to remove dicofol (Osman et al., 2008; Zhao et al., 2010; Zhang et al., 2011). Catalysis with enzymes is one of the biodegradation pathways (Du et al., 2013; Pradub and Wattanachai, 2012). Research has shown that metal ions had impacts on enzyme activity and biological degradation efficiency (Jernejc and Legiša, 2002; Grasso et al., 2012; Madukasi et al., 2010; Gopinath et al., 2011). The co-occurrence of dicofol and heavy metal ions in farmland is common in China (Sun et al., 2012; Zhang and Shan, 2014). However, the effects of metal ions on the degradation of dicofol by cellulase are unknown. Therefore, six metal ions were introduced to test the degradation capability of cellulase.

In this study, an immobilization technique was first applied to cellulase to catalyze the degradation of dicofol. The influence of metal ions on the removal efficiency of dicofol by immobilized and free cellulase was compared. The results will provide support for practical application of this method to treat these environmental contaminants, especially in wastewater and wastewater sludge with high concentrations of toxic metals.

1. Materials and methods

1.1. Materials and equipment

Cellulase (extracted from *Trichoderma longibrachiatum*) was purchased from Ningxia Heshibi Biological Technology Co. (Ningxia, China). Dicofol was purchased from Yangzhou Pesticide Factory (Jiangsu, China). The chemicals glutaraldehyde (50% analytical grade) and 3,5-dinitrosalicylic acid (DNS, pure chemical) were obtained from Beijing Yili Fine Chemical and Sinopharm Chemical Reagent. Carboxymethyl cellulose sodium salt (CMC, pure chemical) were obtained from Beijing Xudong Chemical Works (Beijing, China). Manganous chloride and pyridine were obtained from Shantou Xilong Chemical Works (Guangdong, China). Lead chloride (analytic grade) was purchased from Beijing Hongxing Chemical Works (Beijing, China). Aminated silica was provided by the School of Chemistry and Molecular Engineering of East China University of Science and Technology (Shanghai, China). All other chemicals were purchased from Beijing Chemical Works (Beijing, China).

Instruments included a UV757CRT UV–Vis spectrophotometer (Shanghai Precision Scientific Instrument, Shanghai, China), WMNK-404 temperature controller (Shanghai Medical Instruments, Shanghai, China), and PHS-3A digital pH Meter (Hangzhou Wanda Instruments, Zhejiang, China).

1.2. Preparation of immobilized enzyme

First, 0.15 g of aminated silica carrier was weighed and 5 mL of glutaraldehyde solution was added to it. After the cross-linking reaction and washing away the excess glutaraldehyde, the pale yellow glutaraldehyde cross-linked carrier was obtained. Next, cellulase solution was added to the carrier at the temperature of 40°C, stirred magnetically to mix the cellulase thoroughly, and then washed and filtered to remove excess solution. The product was cellulase immobilized on yellow aminated silica.

1.3. Measurement of cellulase activity

The reaction of the glucose produced by the cellulase hydrolysis of sodium carboxymethyl cellulose (CMC) and 3,5-dinitrosalicylic acid (DNS) generated a brown–red substance with maximum absorption at a wavelength of 540 nm. Then, 0.02 mL cellulase (0.02 mL deionized water as reference) was added to the CMC solution (3.0 mL, 0.51%). Ten minutes later, 1.0 mL of DNS solution was added to 1.0 mL of supernatant, and both were heated in a water bath at the temperature of 100°C for 5 min. After cooling and diluting with water to 10 mL, the absorbance was determined at 540 nm spectrophotometrically. The corresponding cellulase activity was calculated from a standard glucose curve.

The amount of cellulase in 1 mL of enzyme solution completing the hydrolysis of CMC substrate at room temperature generating 1 μmol glucose per min is defined as one enzyme activity unit (U), as calculated by Eq. (1):

$$\text{Free enzyme activity per unit (U/g)} = \frac{\text{Glucose content (mg)} \times 1000}{\text{Enzyme content (mL)} \times 10\ \text{(min)} \times 180}. \quad (1)$$

For the measurement of immobilized cellulose activity, the steps are as follows.

First, 0.15 g of immobilized cellulase was reacted with 3.0 mL of CMC solution for 10 min, and 1.0 mL of supernatant was removed. Then 1.0 mL DNS solution was added. The mixture was heated in a boiling water bath for 5 min and then diluted to 10 mL with water. The absorbance at 540 nm was determined and the activity of immobilized cellulase was calculated from a glucose standard curve.

The unit activity of the immobilized enzyme was defined as the amount of immobilized cellulase completing the hydrolysis of CMC substrate at room temperature generating 1 μmol glucose per min, as calculated by Eq. (2):

$$\text{Immobilized enzyme activity per unit (U/g)} = \frac{\text{Glucose content (mg)} \times 1000}{\text{Enzyme content (g)} \times 10\ \text{(min)} \times 180} \quad (2)$$

1.4. Preparation of dicofol

First, 0.1 g of dicofol powder was extracted and dissolved in 10 mL pyridine. A 5 mL sample containing dicofol was extracted, concentrated with n-hexane, put in a 10 mL centrifuge tube, and evaporated to dryness in a water bath at a constant temperature of 60°C. Subsequently, 0.2 mL of deionized water was added to the centrifuge tube, and then 0.1 mL of 45% NaOH and 2.0 mL pyridine were mixed in and heated in a 100°C water bath for 3 min. After centrifugation for 2 min at 4000 r/min, the absorbance was determined at 530 nm.

1.5. Determination of dicofol concentration

The degradation reaction between 10 mg/L dicofol and 64.98 U/L cellulase was conducted in a 50 mL reaction system at room temperature. Aliquots of the reaction liquid were extracted at specified times and the dicofol concentration was determined to calculate the dicofol removal efficiency. Similarly, 0.2 g of

immobilized cellulase (enzyme activity 62.65 U/L) was added to 10 mg/L dicofol solution in a 50 mL reaction system and the removal efficiency was calculated. Under the same conditions, a carrier adsorption experiment was performed using aminated silica carrier, and the adsorption of dicofol at different reaction times was determined. The catalytic removal efficiency of dicofol by immobilized cellulase was calculated as the total removal efficiency minus the adsorption removal efficiency.

The Michaelis–Menten equation characterizing the relationship between the initial velocity (v) of a one-enzyme catalytic reaction and the substrate concentration (C_0) is shown in Eq. (3):

$$v = \frac{V_{max} \times [C_0]}{K_m + [C_0]} \quad (3)$$

where, v(mg/L/min) is the initial velocity of the reaction, C_0(mg/L) is the initial substrate concentration, and V_{max}(mg/(L·min)) is the velocity at which the enzyme is saturated by substrate. K_m(mg/L) is the Michaelis constant, indicating the affinity between the enzyme and substrate: the larger K_m is, the weaker the affinity. K_m is a property of the enzyme species only. It is independent of the enzyme and substrate concentrations.

In this system, various initial concentrations of dicofol were treated with either free or immobilized cellulase for four hr to determine the dicofol removal efficiency. The values of K_m and V_{max} were determined through Lineweaver–Burk plots.

Various concentrations of metal ions (Na^+, K^+, Mn^{2+}, Cd^{2+}, Ca^{2+}, and Pb^{2+}) were added to the system. The dicofol removal efficiencies of immobilized and free cellulase were determined to study the influences of various metal ions on the degradation reaction. Also, the mechanisms by which Cd^{2+} and Pb^{2+} influence the degradation reaction was explored.

2. Results and discussion

2.1. Effects of cellulase on the degradation of dicofol

The dicofol removal efficiency is shown in Fig. 1. Cellulase could catalyze the degradation of dicofol effectively. The removal efficiency by free cellulase increased continuously and reached equilibrium (72%) after 7 hr. Adsorption by the aminated silica carrier reached saturation after 5 hr, with an adsorption removal efficiency of 38%, while the catalytic removal efficiency of immobilized cellulase was about 50%. The equilibrium was reached after about eight hr with immobilized cellulase, with a total removal efficiency of 90%. Compared with free cellulase, the catalytic removal efficiency of immobilized cellulase was lower. The possible reason was that the immobilization prevented some cellulase inside the carrier particles from contacting the substrate, which decreased the enzyme utilization.

2.2. Kinetic parameters of the catalytic reactions

The initial concentrations of dicofol in the catalytic reaction system were 6, 8, 12, 15, 17, and 25 mg/L. The catalytic removal efficiencies of dicofol by free and immobilized cellulase were determined by the rate, as calculated by Eq. (4)

$$\frac{1}{v} = \frac{K_m}{V_{max}} \times \frac{1}{[C_0]} + \frac{1}{V_{max}} \quad (4)$$

K_m and V_{max} were calculated by plotting $1/v$ *versus* $1/C_0$. For details, see Table 1.

For the catalytic reaction by immobilized cellulase, K_m was 9.16 mg/L *versus* 8.18 mg/L by free cellulase. For immobilized and free cellulase, V_{max} was 0.40 and 0.79 mg/(L·min), respectively. The K_m by immobilized cellulase was higher than that by free cellulase, while V_{max} was the reverse. This reflected weakened substrate affinity after immobilization. The results could be explained by the lack of reaction space, limited diffusion in the immobilized carrier, and molecular conformation change arising from chemical cross-linking during the process of immobilization.

2.3. Influence of metal ions on the catalytic degradation of dicofol by free enzyme

Diverse metal ions at different concentrations were introduced to the catalytic reaction system, and the removal efficiency of dicofol by free enzyme was determined.

2.3.1. Influence of univalent metal ions

The effects of univalent metal ions (Na^+ and K^+) on the degradation of dicofol by free cellulase are shown in Table 2. When the ion concentration gradually increased from 0 to 2 mmol/L, the rate of change remained within 5%, and it stayed almost unchanged when the ion concentration reached 4 mmol/L. Therefore, it could be concluded that Na^+

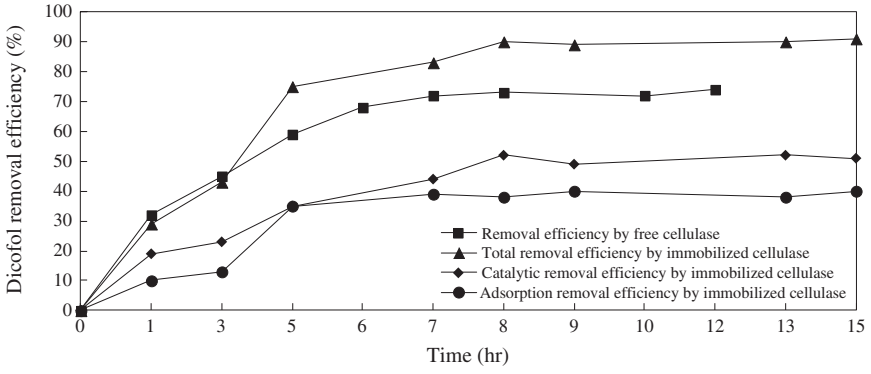

Fig. 1 – Different removal efficiencies of dicofol change with reaction time.

Table 1 – Relationship between the substrate concentration C_0 and reaction rate v.

Substrate concentration		Immobilized cellulase		Free cellulase	
C_0(mg/L)	$1/C_0$	v(mg/L·min)	$1/v$	v	$1/v$
6	0.167	0.16	6.37	0.33	2.98
8	0.125	0.18	5.41	0.39	2.55
12	0.083	0.22	4.44	0.47	2.12
15	0.067	0.25	4.06	0.51	1.95
17	0.059	0.26	3.88	0.53	1.87
25	0.040	0.29	3.45	0.60	1.67

and K^+ had no effect on the catalytic degradation of dicofol by free cellulase.

2.3.2. Influence of divalent metal ions

The effects of the divalent metal ions Mn^{2+}, Cd^{2+}, Ca^{2+}, and Pb^{2+} on the degradation of dicofol by free cellulase were observed (Fig. 2a). When the Ca^{2+} concentration was 0 mmol/L, the removal efficiency was 48.35%. The removal efficiency gradually increased to 58.56% when the Ca^{2+} concentration reached 2 mmol/L, but it decreased to 44.74% when the Ca^{2+} concentration was 4 mmol/L. Therefore, it was concluded that Ca^{2+} could promote the catalytic degradation, but the reaction was inhibited when the Ca^{2+} concentration increased over a certain amount. Cd^{2+} suppressed the catalytic removal efficiency by about 50% when the Cd^{2+} concentration reached 4 mmol/L. When the concentration of Pb^{2+} exceeded 1.2 mmol/L, the removal efficiency decreased to 0.9%. It was much lower than 48.35%, which was the dicofol removal efficiency when the Pb^{2+} concentration was 0 mmol/L. Therefore, Pb^{2+} had a great inhibitory effect on the degradation. Mn^{2+} enhanced the degradation of dicofol by forming a bridge between the enzyme and substrate, which helped to align the substrate with the active center of the enzyme. Cd^{2+} markedly inhibited the degradation, and this effect was strengthened with the increase of ion concentration. A possible reason was that the combination between Cd^{2+} and the non-active center changed the structure of cellulose, leading to the decrease of enzyme activity. Ca^{2+} had both enhancing and inhibiting effects, similar to Mn^{2+} and Cd^{2+}, respectively. It was speculated that it may have acted as a bridge between the substrate and cellulose, which improved the enzyme activity at low concentrations. When the Ca^{2+} concentration increased above 2 mmol/L, the effect of cross-linking was weakened as Ca^{2+} combined with non-active centers to change the structure and properties of the cellulase, reducing the enzyme activity. Pb^{2+} may denature the enzyme, even ultimately eliminating the activity (Hu et al., 2007; Hong et al., 2003).

Table 2 – Effects of Na^+ and K^+ on dicofol removal by free enzyme.

Iron concentration (mmol/L)	Na^+		K^+	
	Removal efficiency (%)	Rate of change (%)	Removal efficiency (%)	Rate of change (%)
0	48.4	–	48.4	–
1	50.4	+4.3	46.3	−4.4
2	46.0	−4.9	49.6	+2.5
4	46.6	−3.7	47.2	−2.5

2.4. Influence of metal ions on the catalytic degradation of dicofol by immobilized cellulase

Neither Na^+ nor K^+ influenced the catalytic degradation of dicofol by free enzyme. Therefore, only the effects of the divalent metal ions Mn^{2+}, Cd^{2+}, Ca^{2+}, and Pb^{2+} on the degradation by immobilized cellulase were explored (Fig. 2b).

At the same ion concentrations, the effects of Mn^{2+}, Cd^{2+}, Ca^{2+}, and Pb^{2+} on the catalytic degradation of dicofol by immobilized enzyme were consistent with those of the free enzyme, but the degree was much weaker than for the free enzyme. In part, the carrier silica adsorbed some metal ions, decreasing the effective metal ion concentration in the microenvironment. In addition, the immobilization of cellulase on the carrier likely altered the molecular conformation of the enzyme, making it difficult for metal ions to access the active centers and to attack cellulase, or simply blocked the active centers. The immobilized enzyme better tolerated the effects of metal ions when compared with free enzyme, making it more useful under complex conditions.

2.5. Mechanisms of the inhibition of the catalytic degradation of dicofol by Cd^{2+} and Pb^{2+}

2.5.1. Mechanism for Cd^{2+}

To determine the degradation efficiency of dicofol at different Cd^{2+} concentrations, the inhibitory efficiency P was calculated as:

$$(R_0–R)/R_0 \times 100\%,$$

where, R_0 is the degradation efficiency when Cd^{2+} is not added. The relationship between the Cd^{2+} concentration and P is shown in Fig. 3.

The relationship between Cd^{2+} concentration and inhibitory efficiency showed the effects of saturation. The reaction velocity at different Cd^{2+} concentrations was used to plot 1/v *vs.* $1/C_0$ to show the inhibition of the catalytic reaction by Cd^{2+}. The results are shown in Fig. 4.

Cd^{2+} did not change the maximum reaction velocity (intercept unchanged) of the catalytic degradation; however, it changed Km. Km increased with increasing Cd^{2+} concentration (the slope of the curve increased), indicating that Cd^{2+} exerts competitive reversible inhibition on the catalytic reaction.

The characteristics of the competitive inhibition by Cd^{2+} showed that the inhibition occurred *via* combination with cellulase molecules, but not at active sites. Because Cd^{2+} was not analogous to the substrate, it would not locate in a site the substrate should occupy. The Cd^{2+} binding location may be outside the active site. The inhibition was speculated to take place by changing the charge distribution or shape of the cellulase.

2.5.2. Mechanism for Pb^{2+}

The effects of the Pb^{2+} concentration on the catalytic degradation reaction were determined. At the same Pb^{2+} concentration, we changed the amount of enzyme added to the aqueous cellulase solution and determined the efficiency

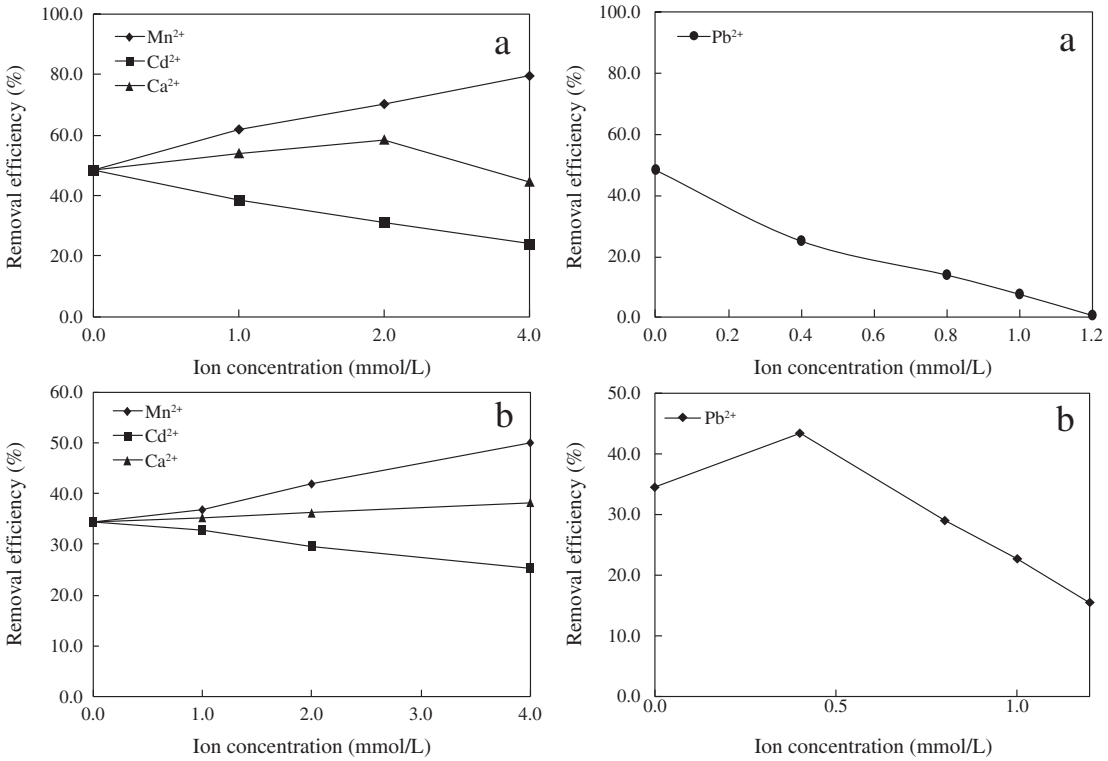

Fig. 2 – (a) Effects of divalent metal ions on dicofol removal by free enzyme, (b) effects of divalent metal ions on dicofol removal by immobilized enzyme.

of dicofol degradation at 4 hr. The results are shown in Fig. 5. The parallel straight lines indicated that the inhibition by Pb^{2+} was an irreversible process. The intercepts on the abscissa became larger and larger with the increase of Pb^{2+} concentration. This suggested that in order to realize the degradation of dicofol at higher Pb^{2+} concentration, increasing the cellulase concentration was an effective strategy.

3. Conclusions

Cellulase immobilized on aminated silica could remove dicofol catalytically. About 8 h was required to reach equilibrium and the removal efficiency was as high as 50%. Compared with the free enzyme, the catalytic removal efficiency decreased with enzyme immobilization because enzyme inside the carrier particles had difficulty contacting the substrate, decreasing enzyme utilization.

The kinetic analysis of the catalytic removal of dicofol determined that K_m = 9.16 mg/L and V_{max} = 0.40 mg/L/min for the immobilized cellulase and K_m = 8.18 mg/L and V_{max} = 0.78 mg/L/min for the free enzyme. The affinity of cellulase for the substrate decreased after immobilization, reducing V_{max}.

Na^+ and K^+ had no effect on the catalytic degradation of dicofol by cellulase. Mn^{2+} promoted the degradation of dicofol by cellulase, while Cd^{2+} caused marked reversible competitive

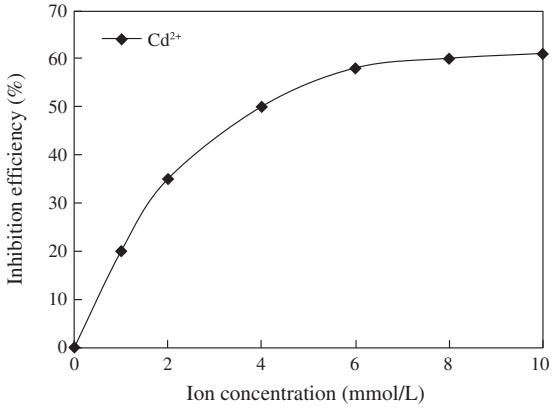

Fig. 3 – Inhibition efficiency changes with Cd^{2+} concentration.

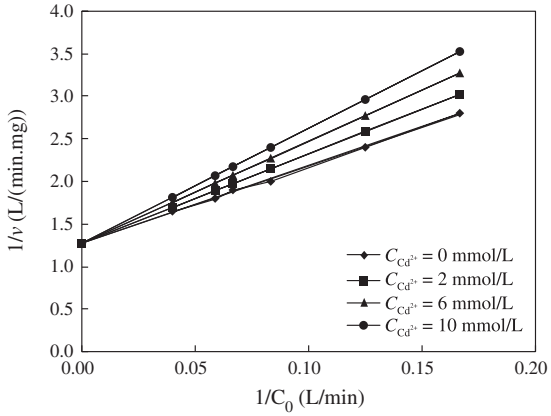

Fig. 4 – Reaction velocity changes with C_0 at different Cd^{2+} concentrations.

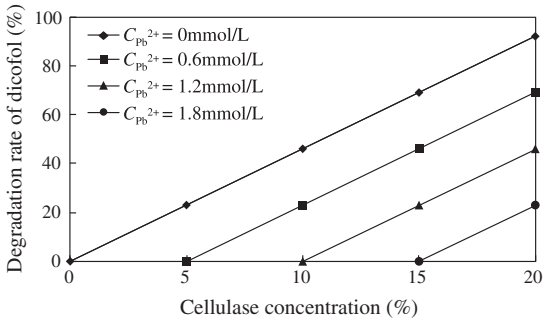

Fig. 5 – Degradation rate changes with cellulase concentration by Pb^{2+}.

inhibition. Ca^{2+} bridged the substrate and cellulase at low Ca^{2+} concentrations, promoting the enzyme activity, but altered the enzyme structure and reduced the activity at high Ca^{2+} concentrations. Pb^{2+} denatured the enzyme, causing irreversible inhibition.

Compared with the free enzyme, the immobilized enzyme better tolerated the metal ions.

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