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Kinetics of cometabolic degradation of 2-chlorophenol and phenol by *Pseudomonas putida*

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Abstract: In order to address the complex cometabolic degradation of toxic compounds, batch experiments on the biodegradation of 2-chlorophenol (2-CP) and phenol by *Pseudomonas putida* were carried out. The experimental results show that 2-CP has an inhibitory effect on cell growth and phenol degradation, which demonstrates that the interaction between substrates affects cell growth and substrate degradation. A kinetic model of cell growth and substrate transformation was also developed. The square of the correlation coefficient from the experiment was 0.97, indicating that this model properly simulates the cometabolic degradation of 2-CP and phenol.
Key words: *cometabolism; kinetic model; 2-chlorophenol; inhibition*

1 Introduction

As a group of chemicals with significant economic value, chlorophenols are widely used in herbicides, insecticides, fungicides, and preservatives. Because of their strong toxicity, non-degradability, ready biological aggregation, and strong carcinogenicity, the chlorophenols that universally exist in the soil and water are attracting more and more attention. The U. S. has listed them as priority pollutants (Contreras et al. 2003; Sahinkaya and Dilek 2007; Jiang et al. 2007). The cometabolic biodegradation method of removing these toxic compounds with suitable carbon and energy sources is one of the focuses of environmental pollution control research today. The key ingredients in cometabolic degradation of chlorophenolic organic compounds are proper organic compounds that can be used as the growing substrates (Atuanya and Chakrabarti 2004) and suitable cometabolic degrading bacteria. Generally speaking, it is possible to find degrading bacteria in soil containing chlorophenolic pollutants (Chang and Alvarez-Cohen 1995; Little et al. 1988; Mu and Scow 1994). However, because of their toxicity and non-degradability, chlorophenols, the non-growing substrates, may inhibit the growth of cells during the cometabolic degradation process and hinder degradation

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(Fakhruddin and Quilty 2005; Polymenakou and Stephanou 2005). From the perspective of engineering control, it is necessary to find more suitable conditions to improve the speed and efficiency of cometabolic degradation.

A lot of research has been done on cometabolic degradation kinetics, especially on the cometabolic degradation of trichloroethylene. Different kinetic models have been developed in order to describe different phenomena, such as competitive inhibition (Aziz et al. 1999), product toxicity (Alvarez-Cohen and McCarty 1991; Zhang and Bajpai 2000), the decay and death of microorganisms (Chang and Criddle 1997), the deactivation and repair of enzymes (Ely et al. 1995), and the main factors in cometabolic degradation (Chang and Alvarez-Cohen 1995; Polymenakou and Stephanou 2005). These models play different roles in different cometabolic degradation systems. Whatever the case is, the growth of cells is directly related to the cometabolic degradation. The growing and non-growing substrates coexist in the cometabolic degradation system, which makes the cometabolic degradation process rather complex.

The objectives of this study were to investigate the biodegradation of phenol and 2-chrophenol (2-CP) as a single substrate by *Pseudomonas putida*, to investigate the interaction of phenol and 2-CP in a dual-substrate system, and to research the cell growth and substrate degradation kinetics of *Pseudomonas putida* in a dual-substrate biodegradation system with high substrate concentration.

2 Materials and methods

2.1 Strain sources

The strains were fed from the activated sludge in the aeration tank of the wastewater treatment system of the Sinopec Yangzi Petrochemical Company. Using phenol as the only carbon source, bacterial flora that can degrade 2-CP was obtained through four months of cultivation and domestication. The extracted microorganisms were preserved in the fridge at 4℃ for future use. The strains and culture conditions have been described in previous studies (Liu 2008).

2.2 Culture medium

A 300-mL conical flask with a stopper was filled with 150 mL of culture medium. The culture medium was an inorganic salt solution containing phenol, in which the inorganic components were 3.8 g/L Na_2HPO_4, 1 g/L KH_2PO_4, 1 g/L NaCl, and 0.2 g/L $MgSO_4$. A 1-mL quantity of trace element solution was added to the conical flask. The trace element components were 2 g EDTA-2Na, 0.2 g $FeSO_4 \cdot 7H_2O$, 0.1 g $MnCl_2 \cdot 4H_2O$, 0.1 g H_3BO_3, 0.1 g $CoCl_2 \cdot 6H_2O$, 0.1 g $ZnCl_2$, 0.02 g $Na_2MoO_4 \cdot 2H_2O$, 0.02 mg $NiCl_2 \cdot 6H_2O$, 0.01 g $CuCl_2 \cdot 2H_2O$, and 1 000 mL distilled water.

To decrease pollution as much as possible, instruments such as the suckers and conical flasks were autoclaved before use, and the transferring and sampling of the culture medium

were both undertaken in a bacteria-free environment. Before inoculation, the bacteria grew in the inorganic salt solution, which contained agar gel and a certain amount of phenol (200 mg/L) as the only carbon source.

2.3 Methods

A quantity of 1.5 mL of bacteria solution in the logarithmic growth phase was poured into each conical flask, and then different concentrations of phenol and 2-CP were added. Later, these solutions were cultivated in a constant-temperature shaking table (at 30℃ and 160 r/min). This experiment studied the effects of the different concentrations of phenol on the removal of 2-CP. It also provided relevant control systems in which phenol and 2-CP were removed by the microorganisms separately. The sample flask was placed in the shaking table at a temperature of 30℃, and sampling analyses of the contents of phenol and 2-CP were made over a certain period.

The concentrations of phenol and 2-CP were analyzed using a gas chromatograph (Aglient6890, U. S.), including HP-5 (5% phenyl and 95% polymethyl siloxane), a capillary column (30 m × 0.32 mm × 0.25 μm) and a flame ionization detector (FID). The column flow rate was 3.0 mL/min. The temperatures of the column, vaporizing chamber, and detector were 150℃, 200℃, and 270℃, respectively. The split ratio was 1:5.

Dry cell weight was measured with the constant weight method after centrifugation. The turbidity of the bacteria solution was examined with the turbidimetric method, mainly using a UV-1200V spectrophotometer (Shimazu Company, Japan). A 5-mL sample was taken from each conical flask, and the optical density (OD) value of the growth of the degrading bacteria was measured through spectrophotometric colorimetry (the wave length was 600 nm). Taking the effects of deep color substances on the OD value into consideration, after the transformation of 2-CP, the actual concentration of the microorganisms was determined by testing the OD value of the sample before and after the sample was filtered through the 0.45-μm mesh.

The experimental plan is shown in Table 1.

Table 1 Cometabolic degradation of phenol and 2-CP experimental plan

Serial number	Initial concentration of substrates	
	Phenol (mg/L)	2-CP (mg/L)
A1, A2, A3, A4, A5	150	10, 20, 30, 40, 50
B1, B2, B3, B4, B5	200	10, 20, 30, 40, 50
C1, C2, C3, C4, C5	300	10, 20, 30, 40, 50
D	200	0
E	0	30

3 Model setup

In the system for cometabolic degradation of microorganisms, enzymes can sometimes degrade primary and secondary substrates simultaneously. Thus, competition exists and the

two substrates mutually inhibit each other's degradation. The inhibitory effect should be considered in the cometabolic kinetics. An inhibition constant K_i was introduced into the Monod equation (Eq. (1)) (Tang et al. 2004) and the Andrews equation (Eq. (2)) was obtained (Andrews 1968):

$$-\frac{\mathrm{d}S}{X\mathrm{d}t}=\frac{KS}{K_s+S} \tag{1}$$

$$-\frac{\mathrm{d}S}{X\mathrm{d}t}=\frac{KS}{K_s+S+S^2/K_i} \tag{2}$$

where S is the substrate concentration, K_i is the self-inhibition constant, X is the biomass concentration, K_s is the saturation constant, t is time, and K is the maximum substrate utilization ratio. When K_i goes to infinity, Eq. (2) becomes the Monod equation. Neufeld et al. (1986) proposed the following equation by introducing the inhibition exponent n into Eq. (2):

$$-\frac{\mathrm{d}S}{X\mathrm{d}t}=\frac{KS}{K_s+S+S\left(S/K_i\right)^n} \tag{3}$$

A larger inhibition exponent n indicates stronger inhibition and a slower rate of degradation. When n is equal to 1, this equation becomes the Andrews equation. Therefore, this equation is suitable for describing the inhibition reaction at different inhibition levels. Using the two constants n and K_i to control the inhibition level gives this equation a wider range of application and a more accurate result.

Gupta et al. (1996) proposed to analyze cometabolism more thoroughly. When the primary substrates are directly provided by the outside world, the utilization velocity of secondary substrates is related to the consumption of primary substrates, but when the outside world does not provide any primary substrates directly, the utilization velocity of secondary substrates is related to the self-oxidation of microorganisms during the lag phase. When there are external primary substrates, the utilization velocity of secondary substrates caused by these external primary substrates is much larger than that caused by the self-oxidation of microorganisms during the lag phase. Thus, the utilization velocity caused by the self-oxidation of microorganisms during the lag phase can be neglected. However, when the secondary substrates or their intermediates have a toxic effect on microorganisms, they will, to a large extent, inhibit the degradation of primary substrates and greatly reduce their utilization velocity. At this time, the utilization velocity caused by the self-oxidation of microorganisms during the lag phase cannot be neglected. The total utilization velocity of the secondary substrate can be expressed as

$$-\frac{\mathrm{d}S}{X\mathrm{d}t}=\frac{KS}{K_s+S+S\left(S/K_i\right)^n}+\frac{K'S}{K_s+S} \tag{4}$$

where K' is the maximum substrate utilization ratio of the secondary substrate, $\frac{KS}{K_s+S+S\left(S/K_i\right)^n}$ is the utilization velocity of the secondary substrate caused by the

primary substrate, and $\frac{K'S}{K_s + S}$ is the utilization velocity of the secondary substrate caused by the self-oxidation of microorganisms during the lag phase. The maximum substrate utilization ratios of the two substrates, K and K', are different, but the saturation constants K_s are the same.

Wang and Loh (1999) made use of *Pseudomonas putida* to degrade chlorophenols. They did a lot of research using the models described above, and concluded that the degradation of phenol was in accordance with Eq. (5) and the degradation of 2-CP was in accordance with Eq. (6):

$$-\frac{\mathrm{d}S}{X\mathrm{d}t} = \frac{KS}{K_s + S + \frac{(S_0 - S)^2}{K_i}} \tag{5}$$

$$-\frac{\mathrm{d}S_c}{X\mathrm{d}t} = \frac{K_c S_c}{K_{cs} + S_c + \frac{S_c^2}{K_{ci}}} \tag{6}$$

where S_0 is the original concentration of phenol, S_c is the concentration of 2-CP, K_{ci} is the self-inhibition constant of 2-CP, K_c is the maximum substrate utilization ratio of 2-CP, and K_{cs} is the saturation constant of 2-CP.

By considering the competitive mutual inhibition of phenol and 2-CP in the cometabolic degradation and the introduction of the inhibitor into Eq. (5) according to Eq. (3), we can obtain the degradation kinetics equation of phenol:

$$-\frac{\mathrm{d}S}{X\mathrm{d}t} = \frac{KS}{K_s\left(1 + \frac{S_c}{K_{cp}}\right) + S + \frac{(S_0 - S)^2}{K_i}} \tag{7}$$

where K_{cp} is the coefficient of inhibition of phenol by 2-CP, and $\frac{S_c}{K_{cp}}$ is the inhibitor.

We can also obtain the following degradation speed equation of 2-CP after correction of Eq. (6):

$$-\frac{\mathrm{d}S_c}{X\mathrm{d}t} = \frac{K_c S_c}{K_{cs}\left(1 + \frac{S_c}{K_{pc}} + \frac{S_c}{K_{cc}}\right) + S_c + \frac{S_c^2}{K_{ci}}} \tag{8}$$

where K_{pc} is the coefficient of inhibition of 2-CP by phenol, and K_{cc} is the toxicity coefficient of self-inhibition

In the cometabolic degradation model, Gu and Korus (1995) found that the specific growth rate of cells μ was in accordance with the following equation:

$$\mu = \frac{\mu_m S_0}{S + K_s\left(1 + \frac{S_{c0}}{K_{cs}}\right) + \frac{S_0^2}{K_i}} - K_{d0}\exp\left(\frac{S_{c0}}{K_{dc}}\right) \tag{9}$$

where S_{c0} is the initial concentration of 2-CP, μ_m is the maximum specific growth rate, K_{d0} is the endogenous decay coefficient, and K_{dc} is the toxicity coefficient. In this study, it is assumed that the inhibition coefficient of phenol to 2-CP and of 2-CP to phenol is the same: $K_{pc} = K_{cp}$.

4 Results and discussion

4.1 Cometabolic degradation of 2-CP and phenol

Through the batch experiments on the cometabolic degradation of 2-CP and phenol**,** the cell growth and substrate degradation were studied and the possible modes of degradation were determined. Based on the selection of parameters for the kinetic model and comparison of the experimental data with the model calculation data, an appropriate dual-substrate cometabolic degradation model was developed with consideration of the competition and self-inhibition effect.

Figs. 1(a) and 1(b) are the curves of phenol and 2-CP degradation in experiments B2 and B5, respectively. With the increase of the concentration of 2-CP, the time needed for complete degradation of phenol increased: it was 9.5 hours for experiment D, 13 hours for B2, and about 20 hours for B5. The average degradation rates of phenol were 40 mg/(L·h) for experiment D, 28 mg/(L·h) for B2, and 23 mg/(L·h) for B5. Fig. 1 also indicates that, with the increase of the concentration of 2-CP, the degradation of phenol was inhibited. High substrate concentration brought about strong substrate inhibition, which is quantitatively demonstrated by the semi-log graph of cell growth and the substrate degradation curve. Only due to the small change of 2-CP concentration did the undistinguished changes occur.

Figs. 1(a) and 1(b) show that the degradation rate of 2-CP increased after the phenol was significantly degraded, which indicates that there is a significant mutual inhibitory effect between phenol and 2-CP. When the concentration of 2-CP increased to 50 mg/L, only 35% of 2-CP was degraded, as shown in Fig. 1(b). The reason for the incomplete degradation is that the 2-CP has a toxic effect on the growth of cells, which causes the weakening of the degradation ability of the microorganisms. Phenol still played a role in supplying the necessary carbon for the biodegradation to begin, because of its readier utilization by the microorganisms. The concentration of phenol also influences the degradation of 2-CP by the microorganisms. When the concentrations of 2-CP and phenol were 50 mg/L and 300 mg/L, respectively, the degradation rate of 2-CP was 50%; when the concentrations of 2-CP and phenol were 50 mg/L and 150 mg/L, respectively, the degradation rate of 2-CP was 35%. The reason may be that the increase of the concentration of phenol provides more growing substrates for the degradation of 2-CP. This doesn't mean, however, that the degradation rate of 2-CP can be endlessly increased by increasing the concentration of phenol, because the toxic effect of phenol on the microorganisms also increases with the concentration of phenol, which causes a decrease in the growing speed of the cells and the degradation rate of 2-CP.

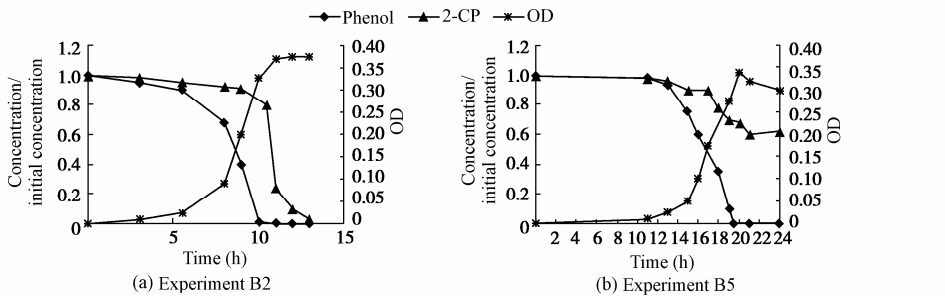

Fig. 1 Degradation curves of phenol and 2-CP

Table 2 presents the μ_m values of the cometabolic degradation experiment of phenol and 2-CP. Fig. 2 shows the effect of 2-CP on phenol degradation when the concentration of phenol was 200 mg/L. As shown in Table 2 and Fig. 2, the maximum specific growth rate of the cells decreases with the increase of the concentration of 2-CP. This is because the toxic effect of 2-CP on the microorganisms and the inhibitory effect of 2-CP on phenol increase.

Table 2 μ_m values of cometabolic degradation of phenol and 2-CP

Serial number	Original substrate content		μ_m (h^{-1})	Serial number	Original substrate content		μ_m (h^{-1})
	Phenol (mg/L)	2-CP (mg/L)			Phenol (mg/L)	2-CP (mg/L)	
A1	150	10	0.562	B5	200	50	0.402
A2	150	20	0.541	C1	300	10	0.416
A3	150	30	0.486	C2	300	20	0.407
A4	150	40	0.454	C3	300	30	0.394
A5	150	50	0.434	C4	300	40	0.384
B1	200	10	0.483	C5	300	50	0.363
B2	200	20	0.462	D	200	0	0.900
B3	200	30	0.444	E	0	30	0.013
B4	200	40	0.422				

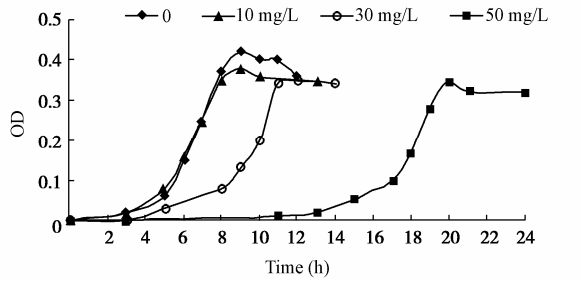

Fig. 2 Effect of concentration of 2-CP on phenol degradation when concentration of phenol is 200 mg/L

4.2 Degradation kinetics of dual-substrate system

4.2.1 Equation of cell growth kinetics

In the dual-substrate system of the cometabolic degradation of phenol and 2-CP, the growth kinetics of cells are simulated with Eq. (9). Related literature (Annadurai et al. 2002; Chung et al. 2003) provided the parameters for this equation, which were $\mu_m = 0.9\ h^{-1}$, $K_s =$

6.93 mg/L, and K_i = 284 mg/L. K_{d0} is equal to 0.002, small enough to be neglected (Haller and Finn 1979). The parameters K_{cs} and K_{dc} are determined by experiments A1 through A5 and C1 through C5, and a fitting curve of the degradation of 2-CP. The values used in this study were K_{cs} = 5.62 mg/L and K_{dc} = 17.7 mg/L. The square of the correlation coefficient R^2 was equal to 0.96. The data from experiments B1 through B5 were used to validate the parameters. Based on the selected parameters, Eq. (9) can be simplified as follows:

$$\mu = \frac{0.9S_0}{S + 6.93\left(1 + \frac{S_{c0}}{5.62}\right) + \frac{{S_0}^2}{284}} - 0.002\exp\left(\frac{S_{c0}}{17.7}\right) \tag{10}$$

Fig. 3 shows a comparison of measured and simulated specific growth rates of cells. It can be seen that the experimental data are consistent with the model calculation results, and the selection of parameters is appropriate. The value of each parameter was determined through the Matlab program and curve fitting of the data from experiments A1 through A5 and C1 to C5. These parameters were validated with data from experiments B1 through B5.

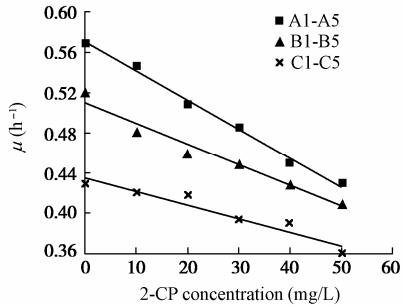

Fig. 3 Comparison of measured and simulated specific growth rates of cells

4.2.2 Degradation kinetics equation of substrate

In the dual-substrate system, the degradation kinetics equation of phenol can be expressed by Eq. (7). Wang and Loh (1999) have conducted systematic research on the degradation of chlorophenols by *Pseudomonas putida*, and the parameters they selected were K_s = 2.19 mg/L, K = 0.819 mg/(mg·h), and K_i = 810 mg/L, so Eq. (7) can be written as

$$-\frac{\mathrm{d}S}{X\mathrm{d}t} = \frac{0.819S}{2.19\left(1 + \frac{S_c}{K_{cp}}\right) + S + \frac{\left(S_0 - S\right)^2}{810}} \tag{11}$$

The degradation kinetics equation of 2-CP in the system can be expressed by Eq. (8). The parameters were determined through the Matlab program and curve fitting of two groups of experimental data (B1-B5 and C1-C5). In this study their values were K_{cc} = 4.40 mg/L (±1.08), K_c = 0.0325 mg/(mg·h) (±0.0075), K_{cs} = 2.83 mg/L (±0.53), $K_{pc} = K_{cp}$ = 3.35 mg/L (±1.06), and K_{ci} = 117 mg/L (±10). Substituting these parameter values into Eq. (8) leads to Eq. (12):

$$-\frac{dS_c}{Xdt}=\frac{0.0325S_c}{2.83\left(1+\frac{S_c}{3.35}+\frac{S_c}{4.40}\right)+S_c+\frac{S_c^2}{117}} \tag{12}$$

Fig. 4 and Fig. 5 show the concentration-time curves of phenol and 2-CP during the degradation process of experiments B1 and B4, respectively.

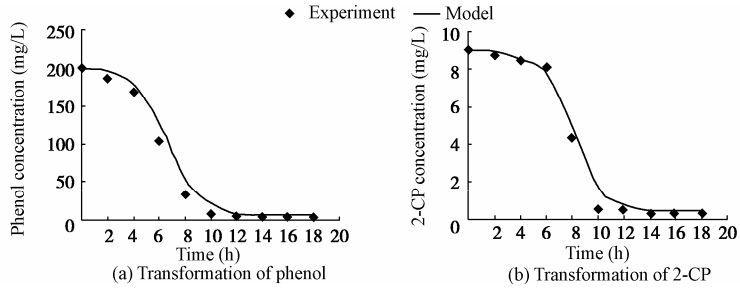

Fig. 4 Comparison of experimental and simulated data in experiment B1

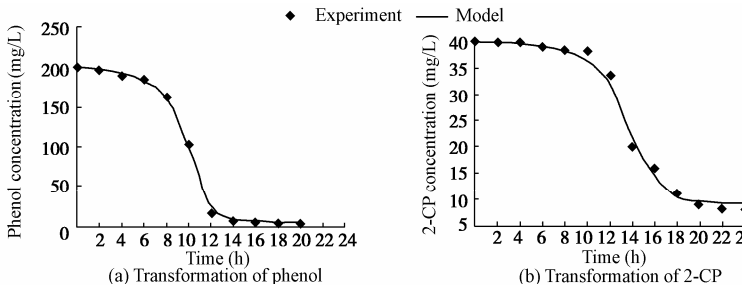

Fig. 5 Comparison of experimental and simulated data in experiment B4

The square of the correlation coefficient R^2 was 0.97. The data show that the model successfully simulates the degradation in the dual-substrate system.

5 Conclusions

The following conclusions can be drawn:

(1) The experiment produced a quantitative description of the mutual inhibitory effect and mutual toxic effect between phenol and 2-CP. The results of the model demonstrate that the main interaction between phenol and 2-CP is competitive inhibition as well as toxic inhibition.

(2) The phenol has a certain inhibitory effect on the degradation of 2-CP in the cometabolic substrates, and this effect grows with the increase of the concentration of phenol. The 2-CP also has a certain inhibitory effect on the degradation of phenol, and this effect grows with the increase of the concentration of 2-CP. Whatever the concentration of phenol is, high or low, its prolonged presence will decrease the removal of the 2-CP in the cometabolic system and will also cause a shift of the main carbon source (that the microorganisms in the dual-substrate system uses) from 2-CP to phenol.

(3) The square of the correlation coefficient from the experiment was 0.97, indicating that

this cometabolic degradation model, with the dual-substrate system, successfully simulated the cometabolic degradation of phenol and 2-CP by *Pseudomonas putida*.

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