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ORIGINAL PAPER

Anaerobic biodegradation of phenol by *Candida albicans* PDY-07 in the presence of 4-chlorophenol

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Abstract Biodegradation of phenol and 4-chlorophenol (4-cp) using pure culture of *Candida albicans* PDY-07 under anaerobic condition was studied. The results showed that the strain could completely degrade up to 1,800 mg/l phenol within 68 h. The capacity of the strain to degrade phenol was higher than that to degrade 4-cp. In the dual-substrate system, 4-cp intensely inhibited phenol biodegradation. Comparatively, low-concentration phenol from 25 to 150 mg/l supplied a carbon and energy source for *Candida albicans* PDY-07 in the early phase of biodegradation and accelerated the assimilation of 4-cp, which resulted in that 50 mg/l 4-cp was degraded within less time than that without phenol. While the biodegradation of 50 mg/l 4-cp was inhibited in the presence of 200 mg/l phenol. In addition, the intrinsic kinetics of cell growth and substrate degradation were investigated with phenol and 4-cp as single and dual substrates in batch cultures. The results demonstrated that the models adequately described the dynamic behaviors of biodegradation by *Candida albicans* PDY-07.

Keywords Anaerobic biodegradation · *Candida albicans* · 4-Chlorophenol · Kinetics · Phenol

Introduction

Phenol, a compound regarded as a priority contaminant by the US Environmental Protection Agency, is a characteristic pollutant in many industrial effluents and wastewaters (Alemzadeh et al. 2002; Kavitha and Palanivelu 2004). Its derivative chlorophenol, especially 4-chlorophenol (4-cp) as a typical environmentally significant compound, is detected in wood preservatives, waste incineration, pesticides, fungicides and herbicides (Yuan and Lu 2005; Lee and Lee 2007). Improper treatment of these compounds may lead to contamination of soil and groundwater, and their toxicity seriously affects living organisms even at a low concentration (Kibret et al. 2000). The efficient removal of these compounds is of great importance for environmental protection.

To treat phenolic compounds, biological methods are preferable because of economical advantages and low possibility of the production of byproducts. Some pure microorganisms have been proven to mineralize phenol or/and chlorophenol as the sources of carbon and energy (Wang and Loh 2001; Alexievaa et al. 2004; Santos and Linardi 2004; Bergauer et al. 2005). Among which, *Candida* sp. has been proven to possess high potential to degrade phenol. Fialová et al. (2004) reported the yeast *C. maltosa* with phenol-degrading potential up to 1,700 mg/l under aerobic condition. However, in practice the supply of oxygen is often insufficient, limiting the application of aerobic processes. Anaerobic processes are generally preferred for the treatment of high-strength wastewaters, which also have the advantage in energy saving and low sludge yield. It's necessary to isolate strains with high capacity to anaerobically degrade phenolic compounds. It's noteworthy that Wen et al. (2006) isolated a strain *C. albicans* PDY-07, able to completely degrade 4-cp up to 300 mg/1 under anaerobic condition. However, none has been known about the anaerobic biodegradation of phenol as single substrate and phenol and 4-cp dual-substrate system by *C. albicans* PDY-07.

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Knowledge of the kinetics of biodegradation is important for the evaluation of the persistence of organic pollutant and the biodegradation potential of organisms. The modeling of cell growth on dual substrates was investigated in many studies on biodegradation of phenolic compounds. These kinetic models were proposed based on Monod or Haldane equation. They can describe cell growth behavior at low substrate concentrations (Paller et al. 1995; Kar et al. 1997; Paraskevi et al. 2005). However, these models can't be used in the dual-substrate biodegradation system due to the strong inhibition of high-concentration substrates beyond 1,000 mg/l.

This study investigated biodegradation of phenol and 4-cp as single substrate by *C. albicans* PDY-07, and researched the interaction of phenol and 4-cp in dual-substrate system, and developed the cell growth and substrate degradation kinetics in single and dual substrate biodegradation system, respectively.

Materials and methods

Chemicals

Analytical grade phenol and 4-chlorophenol were purchased from Tianjin Guangfu Fine Chemical Research Institute. Methanol was of HPLC (high performance liquid chromatography) grade. Yeast extract and peptone were products of Beijing Aoboxing Biotech Company. All other chemicals were of analytical grade, commercially available, and used without further purification.

Microorganism and culture conditions

C. albicans PDY-07 was isolated in the lab from activated sludge collected from a refinery plant in China, and was identified based on morphological features and physiological and biochemical tests.

The strain was grown and maintained in yeast extract, peptone and dextrose (YEPD) medium containing 20 g/l peptones, 10 g/l yeast extract and 20 g/l glucose with initial pH 6.0. Biodegradation studies were conducted in the mineral medium, which had the composition (g/l) $(NH_4)_2SO_4$ 1.0, $MgSO_4$ 0.10, $CaCl_2$ 0.086, $FeSO_4$ 0.028, KH_2PO_4 0.10, K_2HPO_4 0.15 and 0.1% (by volume) trace element solution (Olguín-Lora and Razo-Flores 2004), with initial pH 7.0. 1.0 mg of resazurin, as a redox indicator, was added to the medium. The medium was boiled under a stream of N_2 gas and cooled to room temperature, and then autoclaved at 121°C for 20 min. Phenol/4-cp was injected from concentrated anaerobic sterile stock solution to obtain the desired concentrations. The medium was dispensed under anaerobic conditions by flushing the headspace with sterile gas mixture of $N_2/CO_2/H_2$ (80/10/10). The degradation was conducted in 250 ml Erlenmeyer flasks sealed with rubber stoppers at 35°C in a rotary shaker with a speed of 150 rpm.

Phenol and 4-cp biodegradation studies

For each experiment, 20 ml YEPD medium was inoculated with the strain from the slant. After 12 h of incubation, 5 ml of the cell culture was added to 100 ml fresh YEPD medium. Cells in the end phase of exponential stage were harvested as inocula. Ten ml of this subculture was transfused into 100 ml mineral medium containing varying initial phenol/4-cp concentrations. In the process of batch culture, samples were taken periodically for analysis of cell density and substrate concentrations.

Analytical methods

Cell density was determined with a UV spectrophotometer (Model 752, Shanghai Precision Scientific Instrument Co., Ltd) by measuring the absorbance of the microorganism at the wavelength of 600 nm. Then OD_{600} was converted to dry cell weight (mg/l) by a calibration curve, which was obtained by plotting dry cell weight against optical density. Dry cell weight was measured by filtering cell suspension with the filler and drying the filter paper and cells to a constant weight at 105°C. Immediately after analysis of optical density, samples were centrifuged at 7,500 rpm for 10 min, and the cell free supernatants were used to determine the substrate concentration by HPLC (model Series III, LabAlliance, USA), with a C18 column (250 mm × 4.6 mm, LabAlliance, USA) and a UV-detector (Model 500, LabAlliance, USA) at 280 nm. The mobile phase was methanol/water (4/3, v/v) at a flow rate of 1.0 ml/min. The column temperature was 30°C, and the retention time for phenol was 4.89 min and for 4-cp was 8.98 min.

Electrospray ionization mass spectrometry (ESI/MS) analysis were carried out on a HPLC (model Series III, LabAlliance, USA) coupled to a LCQ Advantage MAX instrument of thermo Finnigan (Thermo Electron Corporation, San Jose, CA, USA). The HPLC conditions were the same with the descriptions above. Nitrogen was used as both the sheath gas and the auxiliary gas. Helium was used as the damping and collision gas. The ESI parameters were as follows: heated capillary temperature 300°C; electrospray capillary voltage 5 kV; detection of negative ions 50–500 amu in a full scan mode. The data processing software used was Xcalibur 1.3.

Statistics

All the degradation experiments were repeated three times. The data shown in the corresponding figures in Section "Results and discussion" were the mean values of the experiments.

Kinetics models

It was presumed that the *C. albicans* PDY-07 growth rate and substrate degradation rate were only limited by substrate concentration at fixed initial pH, temperature and shaking rate. For each batch culture with a certain initial substrate concentration, the specific cell growth rate was calculated as:

$$\mu_X = \frac{\gamma_X}{X} = \frac{dX}{dt}\frac{1}{X} \quad (1)$$

where μ_X was the specific cell growth rate (1/h), γ_X the cell growth rate and X the cell concentration (mg/l).

Because of the inhibition of phenol on cell growth, the Haldane's equation was selected for assessing the dynamic behavior of the strain grown on phenol:

$$\mu_X = \frac{\mu_{m1} S_1}{K_{S1} + S_1 + S_1^2/K_{i1}} \quad (2)$$

where μ_{m1} was the maximum specific cell growth rate, S_1 the phenol concentration, K_{S1} the saturation constant (mg/l) and K_{i1} the self-inhibition constant (mg/l).

Because 4-cp imposed a strong inhibition on cells, inhibition constant for cell growth (K_i')was appended in the equation (Wen et al. 2006):

$$\mu_X = \frac{\mu_{m2} S_2}{K_{S2} + S_2 + S_2^2/K_{i2} + S_2^3/K_{i2}'} \quad (3)$$

The growth behaviors of *C. albicans* PDY-07 in the dual-substrate biodegradation system could be described with the proposed equations in this lab using quasi steady state approximation (Bai et al. 2007). For phenol:

$$\mu_{X1} = \frac{\mu_{m1} S_1}{K_{S1} + S_1 + S_1^2/K_{i1} + fS_2 + fS_2^2/K_{i2} + fS_2^3/K_{i2}' + KS_1S_2 + MS_1^2S_2 + QS_1S_2^2} \quad (4)$$

And for 4-cp:

$$\mu_{X2} = \frac{\mu_{m2} S_2}{K_{S2} + S_2 + S_2^2/K_{i2} + S_2^3/K_{i2}' + S_1/f + S_1^2/fK_{i1} + KS_1S_2/f + MS_1^2S_2/f + QS_1S_2^2/f} \quad (5)$$

where (μ_{m1}, K_{S1}, K_{i1}) and (μ_{m2}, K_{S2}, K_{i2},K_{i2}')could be obtained separately from the kinetics of individual cell growth on phenol alone and 4-cp alone, respectively. *f, K, M, Q* were substrate interaction coefficients.

Based on Eqs. 4 and 5 above, the specific growth rate could be obtained as:

$$\mu_X = \mu_{X1} + \mu_{X2} \quad (6)$$

To analyze the utilization of the substrate in cells in more detail, the consumption of substrate for growth, for maintenance and also for product formation, had to be considered (Feitkenhauer et al. 2003). The substrate consumption rates of phenol and 4-cp biodegradation were shown as following (Wen et al. 2006):

$$\mu_S = A\mu_X + B \quad (7)$$

where μ_S was the specific degradation rate, A and B were all kinetic constants and they were regressed using MATLAB software based on the experimental data. Thus, the specific degradation rates of phenol and 4-cp in dual substrates system could be represented as follows:

$$\mu_{S1} = A_1\mu_{X1} + B_1 \quad (8)$$

$$\mu_{S2} = A_2\mu_{X2} + B_2 \quad (9)$$

Results and discussion

Single substrate biodegradation

Single substrate biodegradation behaviors

The biodegradation behavior of 4-cp by *C. albicans* PDY-07 was investigated in previous studies (Wen et al. 2006). Some researchers observed that phenol of high concentration can exhibit inhibitions on cell growth and its own degradation (Allsop et al. 1993; Wang and Loh 1999; Peytona et al. 2002). As shown in Fig. 1, a total of 1,800 mg/l phenol was degraded within 68 h. For a higher phenol concentration, the lag phase of cell growth lasted longer and the specific degradation rate decreased, which demonstrated that high phenol concentration brought about inhibition on cell growth and phenol degradation. Moreover, as the initial phenol concentration increased, cells obtained more carbon source, and the final biomass increased slightly. However, the biomass yield was not in accordance with the stepwise increase of phenol concentration, as from 1,400 to 1,600 mg/l biomass yield increased 40.84 mg/l while from 1,600 to 1,800 mg/l biomass yield increased merely 23.65 mg/l. Cell growth was not in proportion to phenol consumption, which demonstrated that no essential association existed between cell growth and phenol biodegradation as former report, especially at high phenol concentrations (Claußen and Schmidt 1998). The higher the phenol concentration was, the stronger the substrate inhibition exhibited. So the consumption of phenol was not entirely used to synthesize new cells, but was mostly used to counteract strong substrate inhibition at the utmost phenol concentration. In the biodegradation tests, 1,900 mg/l phenol was also investigated. It was found that 1,900 mg/l phenol could not be completely degraded.

The LC-MS results showed the presence of benzoate in the sample solution. When 4-hydroxybenzoate was fed into the culture, its concentration became lower as the biodegradation

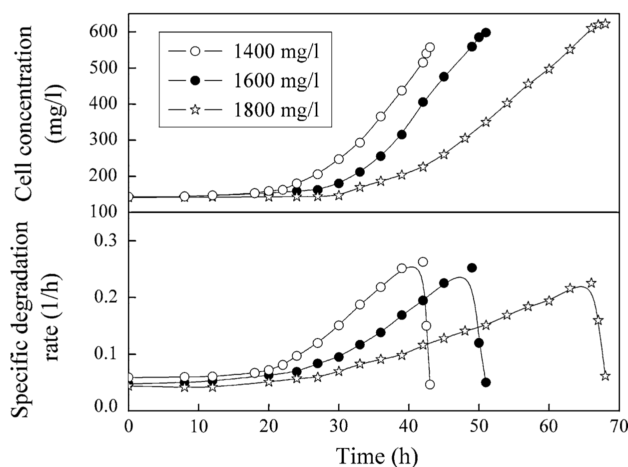

Fig. 1 Cell growth and phenol degradation at initial phenol concentrations of 1,400–1,800 mg/l

got along, which demonstrated that 4-hydroxybenzoate could be assimilated by the strain. Some other researchers reported that phenol was transformed to benzoate via 4-hydroxybenzoate under anaerobic condition (Gallert and Winter 1994; Létourneau et al. 1995; Karlsson et al. 1999). Phenol metabolism in *C. albicans* PDY-07 also followed this pathway.

Figure 2 compared phenol and 4-cp biodegradation behaviors. It was impressive for 200 mg/l phenol to be entirely degraded within 17 h, which was 76 h less than that for 200 mg/l 4-cp. Compared to the long lag phase of cell growth in 4-cp solution, there was no distinct lag phase in phenol solution. It may be attributed to the fact that 4-cp imposed the stronger inhibition on the strain. Additionally, although the same concentration substrate was consumed, a higher final biomass yield was observed in phenol solution. The carbon content in 4-cp solution was 0.009 mol/l, which

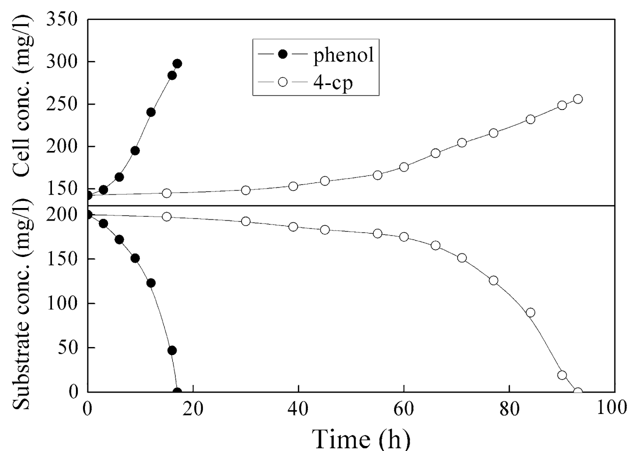

Fig. 2 The biodegradation comparison of *C. albicans* PDY-07 in phenol and 4-cp solution with the concentration of 200 mg/l, respectively

was smaller than that in phenol solution (0.013 mol/l), thus phenol supplied more carbon than 4-cp to synthesize new cells at the same concentration. Another reason for the phenomenon was that the inhibitory effect of 4-cp was stronger than that of phenol, which contributed to the fact that much 4-cp was consumed to overcome substrate inhibition but not to synthesize new cells.

Single substrate intrinsic kinetics

Batch cultures of *C. albicans* PDY-07 were conducted in the mineral medium containing initial phenol concentrations from 0 to 1,800 mg/l and initial 4-cp concentrations from 0 to 300 mg/l, respectively. Model parameters were obtained by a nonlinear least-square regression analysis using MATLAB software based on the experimental data. And the following model equations and their regression coefficients were obtained for phenol alone and 4-cp alone:

For phenol alone:

$$\mu_{X1}^0 = \frac{\mu_{m1} S_1}{K_{S1} + S_1 + S_1^2/K_{i1}} = \frac{0.315 S_1}{19.54 + S_1 + S_1^2/208.57} \quad (10)$$

The value of the squared 2-norm of the residual was 1.24×10^{-3}.

$$\mu_{S1}^0 = A_1^0 \mu_{X1}^0 + B_1^0 = 0.8314 \mu_{X1}^0 + 0.0236 (R^2 = 0.974) \quad (11)$$

For 4-cp alone:

$$\mu_{X2}^0 = \frac{\mu_{m2} S_2}{K_{S2} + S_2 + S_2^2/K_{i2} + S_2^3/K'_{i2}} = \frac{0.04421 S_2}{0.874 + S_2 + S_2^2/456.33 + S_2^3/6397} \quad (12)$$

The value of the squared 2-norm of the residual was 1.18×10^{-3}.

$$\mu_{S2}^0 = A_2^0 \mu_{X2}^0 + B_2^0 = 1.0108 \mu_{X2}^0 + 0.0039 (R^2 = 0.995) \quad (13)$$

As could be seen from Figs. 3 and 4, the simulated values of growth and degradation kinetics agreed well with the experimental data. Both the maximum specific growth and degradation rates occurred at low substrate concentration. With further increase of the initial substrate concentration, the specific growth and degradation rates got lower, which was because of the increased substrate inhibition. The higher specific growth and degradation rates in phenol solution indicated that the strain inclined to utilize more phenol than 4-cp. In addition, sharp drops of curves existed at low substrate concentration in the two figures. It was due to the lack of carbon source in the medium (Banerjee et al 2001).

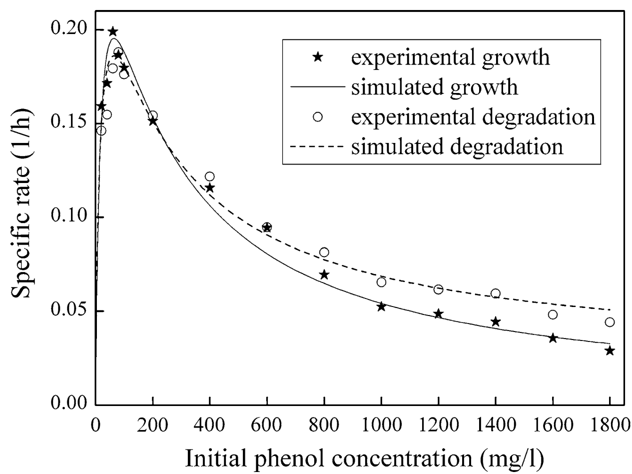

Fig. 3 Kinetic prediction and experimental determined specific growth and degradation rates at initial phenol concentrations of 0–1,800 mg/l

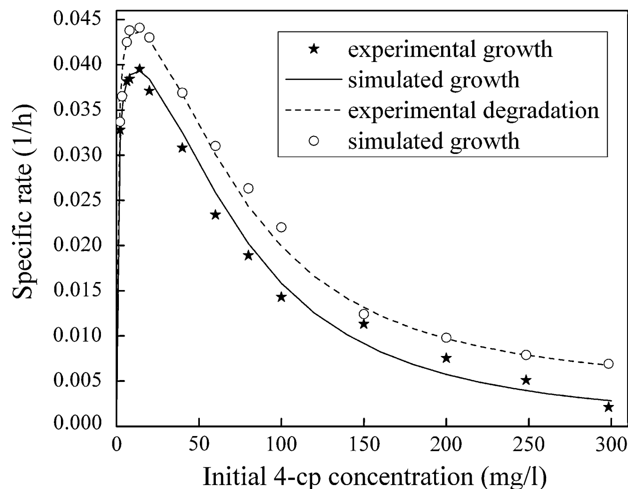

Fig. 4 Kinetic prediction and experimental determined specific growth and degradation rates at initial 4-cp concentrations of 0–300 mg/l

Dual-substrate biodegradation

Effect of phenol on 4-cp biodegradation

From Fig. 5, it was observed that the existence of low-concentration phenol could accelerate 4-cp biodegradation. In the presence of 25 mg/l and 50 mg/l phenol, 50 mg/l 4-cp was degraded within 23.5 h and 20 h, respectively, which were shorter than that without phenol (26 h). In the phenol concentration range of 50–150 mg/l, phenol inhibition exposed, but phenol acceleration still played a key role, and the 4-cp biodegradation rate was higher than that without phenol. When phenol concentration reached 200 mg/l, phenol inhibition property was dominant, and

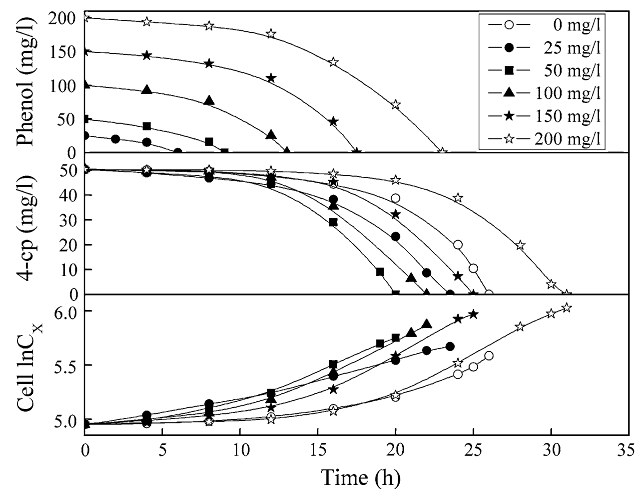

Fig. 5 Effect of phenol on 4-cp biodegradation in dual-substrate system with 50 mg/l 4-cp and initial phenol concentrations of 25–200 mg/l

cell growth was limited, which led to the slow down of 4-cp biodegradation. And the higher the phenol concentration was, the higher the final biomass was. Besides, it was found that in every sample phenol was always beforehand utilized by the strain, and 4-cp was mainly consumed after phenol was largely degraded. Phenol supplied a carbon and energy source for the strain and was easily utilized to synthesize new cells. The biomass accumulation in the early stage of biodegradation ensured the startup of 4-cp biodegradation. Furthermore, there existed two effects of phenol in dual-substrate biodegradation system all along: one was the acceleratory effect on biodegradation, and the other was the inhibitory effect on cell growth. Their competition led to different biodegradation rates. And the competitive balance was optimal in the sample with 50 mg/l phenol, which was the optimal phenol concentration to accelerate 4-cp biodegradation.

Effect of 4-cp on phenol biodegradation

As shown in Fig. 6, the presence of 4-cp inhibited the phenol biodegradation. In the sample of 20 mg/l 4-cp, 8 h more was spent in degrading 1,600 mg/l phenol than phenol biodegradation at the same concentration alone. With the increase of initial 4-cp concentration, the phenol biodegradation rate decreased gradually. When 4-cp concentration reached 80 mg/l, phenol could not be completely degraded. It was clear that although phenol concentration was much higher than 4-cp concentration in the medium, cells still firstly utilized phenol as a carbon and energy source. And rapid assimilation of 4-cp occurred at the late stage of biodegradation.

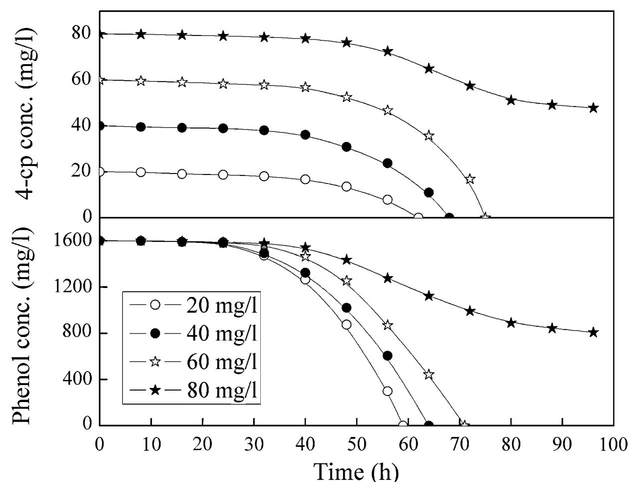

Fig. 6 Effect of 4-cp on phenol biodegradation in dual-substrate system with 1,600 mg/l phenol and initial 4-cp concentrations of 20–80 mg/l

Intrinsic kinetics of dual-substrate biodegradation

Kinetics of overall cell growth on phenol and 4-cp was modeled by Eq. 6. Using the determined parameters for cells grown on phenol (μ_{m1}, K_{S1}, K_{i1}) and 4-cp (μ_{m2}, K_{S2}, K_{i2}, K'_{i2})alone, coupled with experimental data obtained from cells grown on the dual substrates, the parameters f, K, M, Q were determined, and $f = 0.31$, $K = 5.4 \times 10^{-7}$ l/mg, $M = 4.8 \times 10^{-6}$ (l/mg)^2 and $Q = 6.1 \times 10^{-5}$ (l/mg)^2. The value of the squared 2-norm of the residual was 9.86×10^{-3}. The specific degradation rates of phenol and 4-cp in dual-substrate system could be represented based on experimental data:

$$\mu_{S1} = A_1\mu_{X1} + B_1 = 0.968\mu_{X1} + 0.0011(R^2 = 0.971) \quad (14)$$

$$\mu_{S2} = A_2\mu_{X2} + B_2 = 1.226\mu_{X2} + 0.0024(R^2 = 0.963) \quad (15)$$

According to R^2 and the value of the squared 2-norm of the residual, it was concluded that the regression curve was very well consistent with the experimental data. Kinetic equations adequately described the biodegradation behavior of *C. albicans* PDY-07 in dual-substrate system.

Conclusion

Under anaerobic condition, *C. albicans* PDY-07 could completely degrade 1,800 mg/l phenol. The inhibitory effect of 4-cp on the strain was much stronger than that of phenol. The presence of low-concentration phenol enhanced 4-cp biodegradation of *C. albicans* PDY-07. When 25–150 mg/l phenol was introduced into the mineral medium, 50 mg/l 4-cp could be completely degraded within a shorter period than the 4-cp alone, and the maximum 4-cp biodegradation rate was obtained at the existence of 50 mg/l phenol. Comparatively, 4-cp intensely inhibited phenol biodegradation. And the strain always beforehand utilized phenol as a carbon and energy source. In addition, the kinetic models for the specific growth and degradation rates of phenol and 4-cp as single and mixed substrates were obtained, and the simulated values of these models agreed well with the experimental data.

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