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ORIGINAL PAPER

Biodegradation of phenol and *m*-cresol by *Candida albicans* PDY-07 under anaerobic condition

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Abstract Strain *Candida albicans* PDY-07 was used to study the anaerobic biodegradation of phenol and *m*-cresol as single and dual substrates in batch cultures. The strain had a higher potential to degrade phenol than *m*-cresol. The cell growth kinetics of batch cultures with various initial *m*-cresol concentrations was investigated, and the Haldane kinetic model adequately described the dynamic behavior of cell growth on *m*-cresol. When cells grew on the mixture of phenol and *m*-cresol, substrate interactions were observed. Phenol inhibited the utilization of *m*-cresol; on the other hand, *m*-cresol also inhibited the degradation of phenol. However, the presence of low-concentration phenol enhanced *m*-cresol biodegradation; 100 mg/l *m*-cresol could be completely degraded within a shorter period of time than *m*-cresol alone in the presence of 150–300 mg/l phenol. The maximum *m*-cresol biodegradation rate was obtained at the existence of 200 mg/l phenol. Phenol was preferably utilized by the strain as a carbon and energy source. In addition, a sum kinetics model was used to describe the cell growth behavior in binary mixture of phenol and *m*-cresol, and the interaction parameters were determined. The model adequately predicted the growth kinetics and the interaction between the substrates.

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Introduction

Phenol is widely distributed as a characteristic pollutant due to its common presence in effluents of many industrial processes, including oil refineries, petrochemicals, dying, textiles, and coal conversion [5, 18]. Its methylated derivative *o*-, *m*- and *p*-cresol has been detected not only in leachate from creosote sites, but also has been found in a huge range of industrial effluents [10, 14]. 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 [9]. The efficient removal of these compounds is necessary and significant for environmental protection.

Compared with physical and chemical methods, biological techniques are preferable because of economical advantages and low possibility of byproducts production. In spite of phenolic toxic properties, some members of yeast genera *Candida, Trichosporon* and others have been demonstrated to mineralize phenol and/or *m*-cresol as the sources of carbon and energy [2, 4, 8]. These studies were carried out under aerobic conditions. However, in practical application the oxygen supply is often insufficient, which limits the microorganism's potential to degrade phenols. 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 is of great practical importance to isolate strains with high capacity to anaerobically degrade phenolic compounds. Recently, Wang and Wen isolated a strain of yeast *Candida albicans* PDY-07 able to degrade phenol up to 1,800 mg/l

within 68 h under anaerobic conditions [16]. However, nothing has been known about the biodegradation of phenol and the *m*-cresol dual-substrate system by *C. albicans* PDY-07.

Knowledge of the kinetics of biodegradation is important for the evaluation of the persistence of organic pollutants and the design of biodegradation facilities [12]. Therefore, further detailed research is needed to quantify these substrate interactions in the degradation of phenol and *m*-cresol mixtures. Objectives of this study are to investigate biodegradation of *m*-cresol as a single substrate by *C. albicans* PDY-07, to investigate the interaction of phenol and *m*-cresol in the dual-substrate system, and to research the cell growth kinetics of *C. albicans* PDY-07 in single- and dual-substrate biodegradation systems, respectively.

Materials and methods

Microorganism and culture conditions

The strain *C. albicans* PDY-07 was isolated from activated sludge collected from a refinery plant in China and was identified based on morphological features and physiological and biochemical tests [7, 13]. It was a facultative aerobe and could utilize phenol, 4-chlorophenol, *o*-cresol, *m*-cresol, benzoate, and toluene under anaerobic conditions as the sole organic carbon source.

The strain was grown and maintained in yeast extract, peptone, and dextrose (YEPD) medium containing (g/l) peptones 20, yeast extract 10, and glucose 20 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 [11] with initial pH 7.0. Then 1.0 mg resazurin was added to the medium. The medium was boiled under N_2 gas and cooled to room temperature, and then autoclaved at 121°C for 20 min. Phenol/*m*-cresol was injected from concentrated anaerobic sterile stock solution. The medium was dispensed under anaerobic conditions by flushing the headspace with a 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 *m*-cresol biodegradation studies

Twenty milliliters of 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 YEPD medium. Cells in the end phase of the exponential stage were harvested as inocula; 10 ml of this subculture was transfused into 100 ml mineral medium containing varying initial phenol/*m*-cresol concentrations.

Analytical methods

Cell density was monitored spectrophotometrically by measuring the optical density at wavelength 600 nm. Then OD_{600} was converted to dry cell weight (mg/l) by a calibration curve. Samples were centrifuged at 7,500 rpm for 10 min, and the cell-free supernatants were analyzed by HPLC (model Series III, LabAlliance, USA). Phenol, *m*-cresol, and intermediate analyses were performed with a C18 column (250 × 4.6 mm, LabAlliance, USA) and a UV-detector (Model 500, LabAlliance, USA) at 280 nm. The mobile phase was methanol/water (4/3) at 1.0 ml/min. The retention times for phenol and *m*-cresol were 4.89 and 6.12 min, respectively. Aliphatic acids were determined by HPLC (Model 1100, Agilent, USA) with Venusil MP C18 column (250 × 4.6 mm, Agela, USA). The mobile phase was 10 mM dipotassium hydrogen phosphate, pH 2.55 adjusted by phosphoric acid at 0.5 ml/min, and the detection was realized with a UV detector at 210 nm.

Statistics

All experiments were repeated in triplicate. The data shown in the corresponding figures of the "Results and discussion" section were the mean values of the experiments.

Results and discussion

m-Cresol biodegradation

Phenol biodegradation was investigated in previous studies [16]. Figure 1 shows *m*-cresol biodegradation behavior of *C. albicans* PDY-07. The slope of semilog graph represents the specific growth rate. With the increase of initial *m*-cresol concentration, the cell growth underwent a longer lag phase, and the specific growth rate decreased. It demonstrated the presence of inhibitory effects, and higher substrate concentration brought about stronger inhibition on cell growth. Although higher *m*-cresol concentration provided more carbon sources, leading to a higher final biomass, the cell growth was out of proportion to *m*-cresol degradation, as from 100 to 200 mg/l *m*-cresol final biomass increased 67.35 mg/l, while from 200 to 300 mg/l final biomass increased only 44.96 mg/l. The substrate *m*-cresol was consumed mainly for assimilation into biomass and energy for cell growth and maintenance [15].

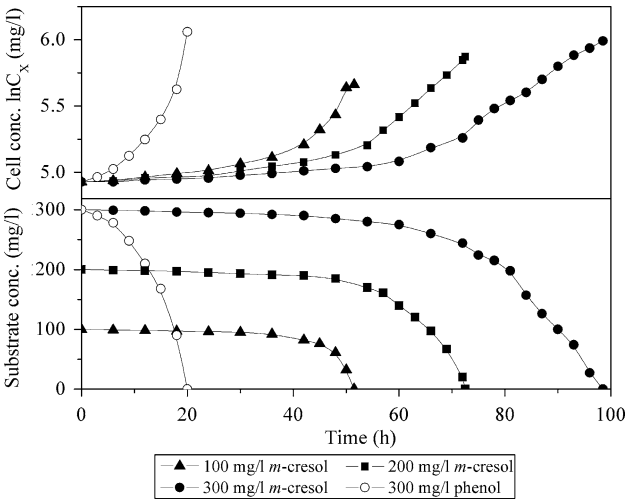

Fig. 1 Single substrate degradation and cell growth at initial phenol concentration of 300 mg/l and initial *m*-cresol concentration of 100–300 mg/l

However, at high substrate concentration, the inhibitory effects on cell growth were stronger and more energy was required to maintain the cell activity, but not to synthesize new cells. Another possible reason for the decreased cell mass was the production and accumulation of various intermediates [3].

Compared to the biodegradation of 300 mg/l phenol, 78 h more was required for complete degradation of 300 mg/l *m*-cresol. And a much higher specific growth rate and higher ultimate cell concentration were observed in phenol solution, indicating that the inhibitory effect on cell growth of *m*-cresol was stronger than that of phenol. In comparison, 300 mg/l 4-chlorophenol was degraded within 243 h [17], indicating that *m*-cresol was more easily utilized by the strain than 4-chlorophenol. Both *m*-cresol and 4-chlorophenol were more toxic than phenol, and 4-chlorophenol was more toxic than *m*-cresol. It could be seen that the position and the type of substituent on the phenol nucleus played an important role in the toxicity properties of phenolic compounds.

Kinetics of *m*-cresol biodegradation

Batch cultures of *C. albicans* PDY-07 were conducted in the mineral medium containing initial *m*-cresol concentrations from 0 to 300 mg/l. For each batch culture with a certain initial substrate concentration, the specific cell growth rate was calculated as:

$$\mu_X = \frac{\gamma_X}{C_X} = \frac{dC_X}{dt}\frac{1}{C_X} \quad (1)$$

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

Because of the inhibition of *m*-cresol on cell growth, Haldane's equation was selected to assess the dynamic behavior of *C. albicans* PDY-07 grown on *m*-cresol:

$$\mu_X = \frac{\mu_{max} S}{K_S + S + S^2/K_i} \quad (2)$$

where μ_{max} was the maximum specific cell growth rate, S the substrate concentration, K_S the saturation constant (mg/l), and K_i the inhibition constant (mg/l). Based on the experimental data, model parameters $\mu_{max} = 0.0311$ l/h, $K_S = 21.26$ mg/l, and $K_i = 102.13$ mg/l were derived using the nonlinear least-square regression program. Among these three parameters, μ_{max} represented the cell growth for the given substrate concentration. In other words, it was the potential of the strain to degrade the substrate effectively. The higher value of μ_{max} for phenol (0.315 l/h) [16] showed that the strain had a greater potential in degrading phenol over *m*-cresol. The low value of K_i indicated that the inhibition could be observed in a low concentration range. The smaller value of K_i for *m*-cresol than that for phenol (208.57 mg/l) [16] demonstrated stronger inhibition on cell growth by *m*-cresol.

From Fig. 2, it was observed that the maximum specific growth rate occurred at a low *m*-cresol concentration of 40 mg/l. In general, a low specific growth rate indicated a very intense inhibitory effect on cell growth. But with the decrease of initial *m*-cresol concentration in the mineral medium, the specific growth rates gradually increased from 0.00787 to 0.01588 l/h until a sharp drop of the curve because of the lack of carbon source in the mineral medium [6]. The higher the *m*-cresol concentration was, the greater the inhibition by substrate was. Thus, both specific growth rate and biomass yield (g/g) were low at the initial phase of biodegradation, and with the consumption of *m*-cresol they increased as the result of the declining inhibitory effect.

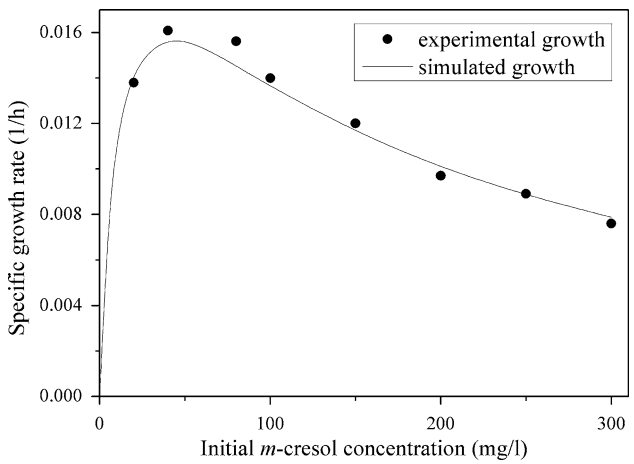

Fig. 2 Kinetic prediction and experimental determined specific growth rate at initial *m*-cresol concentrations of 0–300 mg/l

Dual substrates biodegradation

As shown in Fig. 3, the biodegradation behavior of *C. albicans* PDY-07 in binary mixture of phenol and *m*-cresol differed greatly from that of single substrate. This was due to the availability of more carbon source and the interaction between the two substrates. It was observed that *m*-cresol imposed a significant delaying effect on phenol degradation. In the sample of 25 mg/l *m*-cresol, it took over 6 h to degrade 600 mg/l phenol than without *m*-cresol. With the increase of initial *m*-cresol concentration, the phenol biodegradation rate decreased. On the other hand, the presence of phenol retarded the utilization of *m*-cresol. About 26 h more was spent in consumption of 100 mg/l *m*-cresol with the addition of 600 mg/l phenol. Furthermore, although phenol concentration was much higher than *m*-cresol, phenol still took precedence over *m*-cresol to be utilized by cells as a carbon and energy source, and rapid biodegradation of *m*-cresol occurred at the end of the whole biodegradation.

Although phenol delayed *m*-cresol biodegradation, phenol with low concentration still accelerated *m*-cresol biodegradation. From Fig. 4, it can be seen that in the samples with 150–300 mg/l phenol, 100 mg/l *m*-cresol was degraded within shorter periods than *m*-cresol degradation alone, and 200 mg/l phenol was the optimal phenol concentration to accelerate *m*-cresol biodegradation. When phenol reached 350 mg/l, the inhibition on cell growth by phenol played a key role, and cell growth was limited, resulting in the slow down of *m*-cresol biodegradation, while the strain failed to degrade the substrates with phenol concentration above 900 mg/l, which may be attributed to the sum of concentration effects. Besides, the higher the phenol concentration was, the longer the lag phase of cell growth lasted and the higher the final biomass was (Fig. 5).

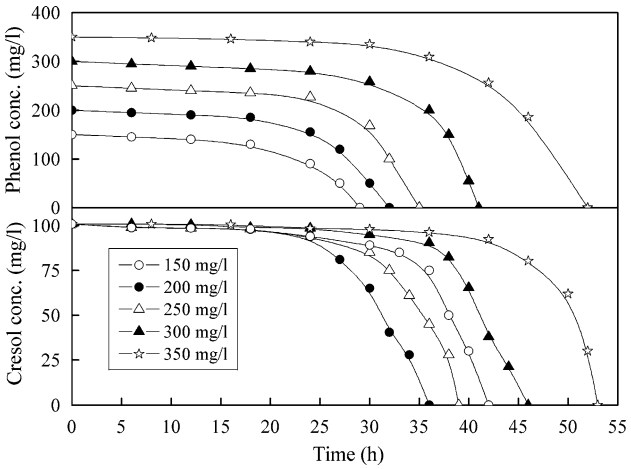

Fig. 4 Phenol and *m*-cresol degradation with fixed initial *m*-cresol concentration of 100 mg/l and initial phenol concentrations of 150–350 mg/l

Cells pregrown on 200 mg/l phenol as a single substrate in the mineral medium were inoculated into the mineral medium containing 100 mg/l *m*-cresol as a single substrate with the same initial cell concentration as that of the above studies. No acceleration of *m*-cresol degradation was observed. The reason might be attributed to the fact that the degradation pathways of phenol and *m*-cresol were different, and the capacity to degrade *m*-cresol had to be induced. When the initial cell concentration was increased by 20%, the degradation rate of *m*-cresol enhanced (data not shown). The accelerated *m*-cresol degradation was mainly attributed to the increased cell concentration. From Fig. 5, it was observed that the cells got rid of the lag phase

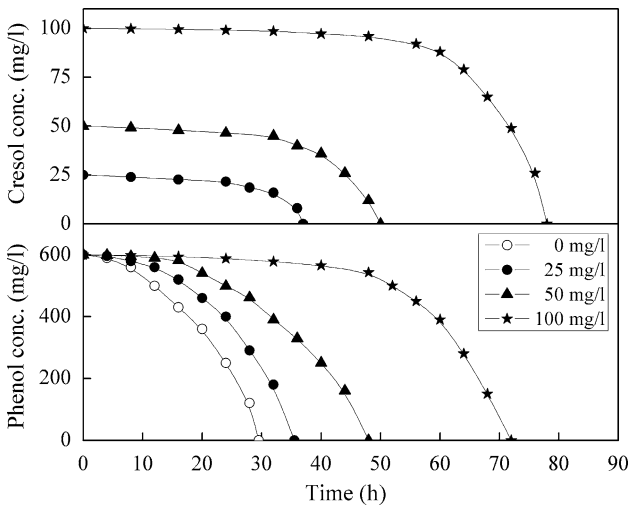

Fig. 3 Phenol and *m*-cresol degradation with fixed initial phenol concentration of 600 mg/l and initial *m*-cresol concentrations from 25 to 100 mg/l

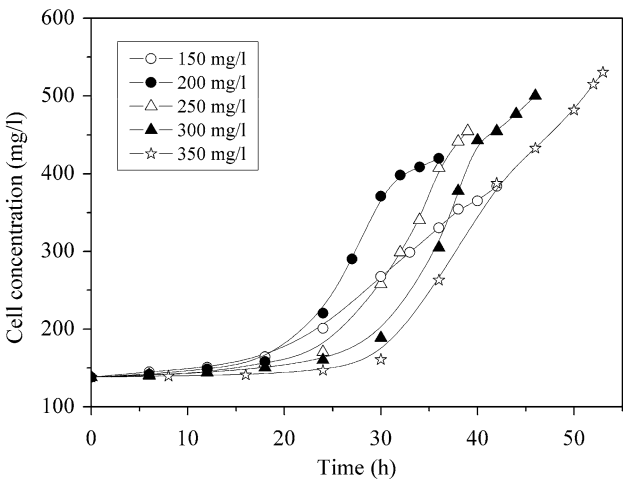

Fig. 5 Cell growth of dual-substrate degradation with fixed initial *m*-cresol concentration of 100 mg/l and initial phenol concentrations of 150–350 mg/l

rapidly in the binary mixture of 150–300 mg/l phenol and 100 mg/l *m*-cresol. Phenol was easily utilized to synthesize new cells, and the biomass accumulation in the initial stage of biodegradation led to increasingly higher *m*-cresol assimilation by the strain. On the other hand, phenol inhibited cell growth as a toxic compound. The accelerator effect and inhibitory effect existed in dual-substrate biodegradation all along, and the competition between them resulted in different biodegradation rates; the competitive balance was optimal in the presence of 200 mg/l phenol.

Kinetics of dual-substrate biodegradation

The kinetics of cell growth on dual substrates in biodegradation has been widely studied. The most widely used model was the cross-inhibition equation proposed by Yoon et al. [19], and Abuhamed et al. [1] modified this model to determine the interactions between benzene, toluene, and phenol. Based on the experimental results showing that both substrates exerted inhibition on cell growth and cross-inhibition occurred between phenol and *m*-cresol, the model equation used by Abuhamed et al. was also adopted to simulate the overall cell growth of our research:

$$\mu_X = \frac{\mu_{max1} S_1}{K_{S1} + S_1 + S_1^2/K_{i1} + I_{2,1} S_2} + \frac{\mu_{max2} S_2}{K_{S2} + S_2 + S_2^2/K_{i2} + I_{1,2} S_1} \quad (3)$$

where the subscript 1 denoted phenol and 2 denoted *m*-cresol (μ_{max}, K_S, K_i), which could be obtained from the kinetics of cell growth on the single substrate. The interaction parameter $I_{i,j}$ indicated the degree to which substrate i affected the biodegradation of substrate j, and a large value corresponded to a strong inhibition. Based on the experimental data, the model equation was solved using nonlinear least-square regression program, and $I_{2,1} = 2.91$, $I_{1,2} = 1.79$ were obtained with $R^2 = 0.92$, while $I_{2,1}$ represented the effect of *m*-cresol on phenol biodegradation and $I_{1,2}$ represented the opposite. A larger value of $I_{2,1}$ indicated that *m*-cresol inhibited phenol utilization more than phenol inhibited *m*-cresol utilization, which was in accordance with the experimental results.

Intermediates study

All samples in biodegradation experiments were analyzed by HPLC for intermediates. The intermediates benzoate, 4-hydroxybenzoate, 3-hydroxybenzoate, succinate and acetate were identified by comparison of the UV spectrum with that of the reference compound. These intermediates formed in a low concentration of less than 10 mg/l. At the end of each experiment, no intermediates were found, which demonstrated the complete degradation of phenol and/or *m*-cresol.

Conclusions

The present studies showed that the capacity of *C. albicans* PDY-07 to degrade phenol was higher than that to degrade *m*-cresol. In the dual-substrate biodegradation system, phenol retarded *m*-cresol degradation, and *m*-cresol imposed a stronger delaying effect on phenol degradation. In spite of that, low-concentration phenol from 150 to 300 mg/l supplied a carbon and energy source for the strain in the early phase of biodegradation and accelerated the assimilation of *m*-cresol, resulting in 100 mg/l *m*-cresol being consumed within less time than *m*-cresol alone. The cell growth kinetics were investigated with phenol and *m*-cresol as dual substrates. The interaction coefficients indicating the degree to which phenol affected the biodegradation of *m*-cresol and vice versa were estimated. These would be useful for modeling and designing the units treating phenol- and *m*-cresol-contaminated wastewaters.

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