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Short communication

Biodegradation of phenol at high initial concentration by *Alcaligenes faecalis*

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Abstract

Strain *Alcaligenes faecalis* was isolated and identified as a member of the genus *Alcaligenes* by using BIOLOG and 16S rDNA sequence analysis. The phenol biodegradation tests showed that the phenol-degrading potential of *A. faecalis* related greatly to the different physiological phases of inoculum. The maximum phenol degradation occurred at the late phase of the exponential growth stages, where 1600 mg L^{-1} phenol was completely degraded within 76 h. *A. faecalis* secreted and accumulated a vast quantity of phenol hydroxylase in this physiological phase, which ensured that the cells could quickly utilize phenol as a sole carbon and energy source. In addition, the kinetic behavior of *A. faecalis* in batch cultures was also investigated over a wide range of initial phenol concentrations (0–1600 mg L^{-1}) by using Haldane model. It was clear that the Haldane kinetic model adequately described the dynamic behavior of the phenol biodegradation by the strain of *A. faecalis*.

Keywords: Phenol biodegradation; *Alcaligenes faecalis*; Enzyme activity; Kinetics

1. Introduction

Biological methods of phenol removal operate significantly on wastewater treatment. Effluents containing phenol are traditionally treated in continuous activated sludge processes at a relatively lower processing cost and a less possibility of the production of byproducts [1]. However, the application of this technology in practice is greatly limited because of its poor adjustability to fluctuation in the phenolic load [2]. Consequently, many pure bacteria have been confirmed to be able to grow on phenol as a sole carbon and energy source [3]. Annadurai studied the maximum phenol degradation by *Pseudomonas putida* [4]. It showed the bacteria assimilated 85% of 500 mg L^{-1} phenol under optimal control. González also gave the maximum phenol degradation by another strain *P. putida*, and 1000 mg L^{-1} phenol was degraded more than 260 h [5]. Besides, Claußen, Wang, Chung and Alexievaa have also reported some bacteria to degrade phenol with the initial phenol concentration ranging from 0 to 1000 mg L^{-1} [6–8]. Nevertheless, none has been known about phenol biodegradation at initial concentration above 1200 mg L^{-1} by bacteria.

Objectives of the present studies are to isolate and characterize *A. faecalis* from acclimated activated sludge, to test biodegradation potential at the initial phenol concentrations higher than 1200 mg L^{-1}, to determine the character of enzyme activities with different physiological stage, and to investigate the cell growth and phenol degradation intrinsic kinetics of the wild *A. faecalis* [9].

2. Materials and methods

2.1. Acclimation of activated sludge

Activated sludge was collected from a municipal gasworks in China and enriched for a period of 10 weeks using phenol as a sole carbon source in the mineral medium with the phenol concentration varying from 300 to 2000 mg L^{-1}.

2.2. Isolation and culture conditions of organism

Sample of diluted activated sludge was inoculated into shaking flasks with LB medium. After three multiplication cultures,

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the late cultures were plated onto agar plates. Individual isolates on plates were inoculated and stored on slants. Phenol biodegradation was tested for their phenol-degrading potential. A dominant colony type was purified after several transfers to the plates again. Liquid cultures were grown in the mineral medium which has been described in previous studies [10].

2.3. Phenol biodegradation

After the strain was activated twice, the cells in three different phases (OD_{600} = 0.594, 0.917 and 1.226) were harvested as inocula. 2.5 mL of this subculture was transfused into 50 mL the mineral medium containing the varying phenol concentration over the range from 0 to 200 mg L^{-1} at an interval of 50 mg L^{-1}, and 200–1800 mg L^{-1} at an interval of 200 mg L^{-1}. Samples were periodically taken for biomass and phenol concentration.

2.4. Enzyme assay

Enzyme activities were spectrophotometrically determined in cell-free extracts at room temperature using quartz cuvettes of 1 cm path length. Cells grown in different exponential stages in LB medium were harvested and centrifuged at 7500 rpm for 10 min. After being washed twice with 0.1 M phosphate sodium buffer (pH 7.2) and resuspended in the same buffer, the cell pellet was disrupted by sonication for 5 min, and then the cell debris was removed by centrifugation of the homogenized cell suspension at 15,000 rpm for 20 min at 4 °C. The cleared supernatant was immediately used for both assays of enzyme and total protein. Total protein concentration in cell-free extracts was monitored by the method of Lowry [11]. The phenol hydroxylase (EC 1.14.13.7) activity strictly depended on the presence of NADPH, and was assayed spectrophotometrically according to NADPH absorbance at 340 nm [12]. One unit of hydroxylase activity was defined as the amount of enzyme which in the presence of phenol caused the oxidation of 1 μM NADPH/min. Catechol 1,2-dioxygenase (EC 1.13.11.1) and 2,3-dioxygenase (EC 1.13.11.2) activities were spetrophotometrically determined following the formation of *cis*, *cis*-muconic acid and 2-hydroxymuconic semialdehyde at 260 and 375 nm and 25 °C. The former was monitored in the presence of 1 mM EDTA and 33 mM Tris–HCl at pH 8 and 10 μL enzyme extract with the total volume of 2 mL. The reaction was started by adding 0.1 mM catechol. The latter, 2,3-dioxygenase, was evaluated by measuring the formation of 2-hydroxymuconic semialdehyde at 375 nm. The reaction mixture contained 0.1 mM catechol, 50 mM Tris–HCl at pH 7.5 and 10 μL enzyme extract, within a total volume of 2 mL. One unit of catechol 1,2 (or 2,3)-dioxygenase activity was defined as the amount of enzyme producing 1 μmol of *cis*, *cis*-muconate or 2-hydroxymuconic semialdehyde per minute at 25 °C. Specific activities were expressed as units (U) per milligram total cell protein.

2.5. Analysis procedure

The determinations of cell and phenol concentrations were previously described [10].

3. Results and discussion

3.1. Isolation and identification of microbial species

A total of 76 individual isolates were obtained, and based on phenol tolerance, 20 were further screened. Among the 20 isolates, one strain with the maximum degradation velocity was used in the following experiments. The strain was identified by Institute of Microbiology, Chinese Academy of Sciences. The BIOLOG and 16S rDNA gene sequence of the strain was determined. Identifications on the strain clearly revealed the presence of *A. faecalis*.

3.2. Phenol degradation

Fig. 1 showed the results of cell growth and phenol biodegradation with inocula of the different exponential phases. Different inocula greatly affected phenol biodegradation of *A. faecalis*. With the increase of inoculum concentration, biodegradation potential of the strain promoted prominently. It was impressive that *A. faecalis* degraded 1600 mg L^{-1} phenol within 76 h in the sample of OD_{600} = 1.226 (Fig. 1c), which was 36 and 14 h less than the other two samples with low inoculum concentration, respectively (Fig. 1a and b). The similar phenomenon also occurred in other samples with the initial phenol of 1200 and 1400 mg L^{-1}. In Fig. 1a, poor removal efficiency could be observed, even for the sample with 1200 mg L^{-1} phenol, it still took 82 h. It could be confirmed by a little smaller specific degradation rate. Besides, the specific degradation rates decreased for each of the given inoculum with the step increase of phenol concentration, which manifested the strong substrate inhibition. Just because of it, *A. faecalis* failed to degrade 1800 mg L^{-1} phenol, which could be also observed from the semilog plot of cell growth. Before the cells got rid of the lag phase, the cell growth terminated and the cell concentration kept as a constant.

With the augmentation of the inoculum concentration, the phenol-degrading potential of the strain increased gradually, but cell growth was not proportionable to the phenol consumption, which demonstrated there was no essential association between cell growth and phenol degradation as former report, especially at high phenol concentration [6]. The higher the phenol concentration was, the stronger the substrate inhibition was. Hence, the consumption of phenol was not entirely used to synthesize new cells, but was used mostly to counteract strong substrate inhibition at the utmost phenol concentration.

3.3. Enzyme activities

The present studies demonstrate that *A. faecalis* has the property of an efficient phenol-degrading microorganism. The efficiency of a certain catabolic pathway often depends on the properties of the involved key enzyme. In the metabolism of

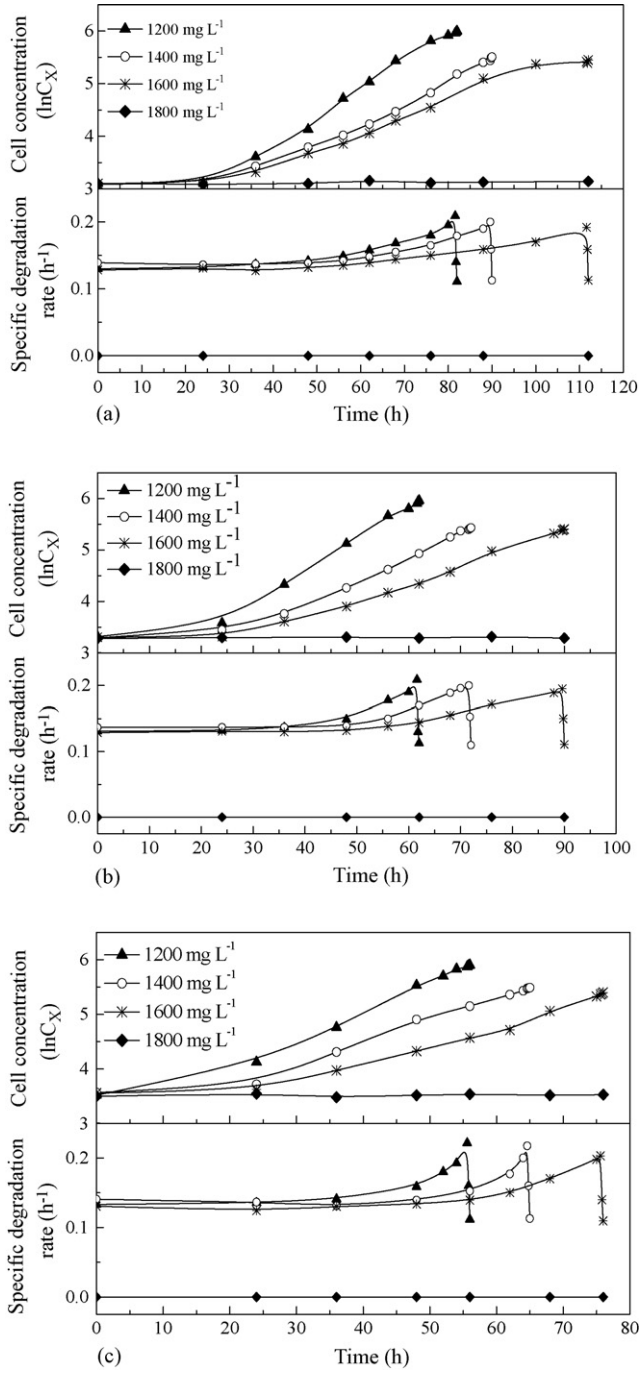

Fig. 1. The cell growth and phenol degradation in the mineral medium containing initial phenol from 1200 to 1800 mg L^{-1} at an interval of 200 mg L^{-1} with optical density of inoculum at (a) 0.594, (b) 0.917 and (c) 1.226.

phenol, the phenol hydroxylase hydroxylates phenol to catechol, then the ring of catechol is cleaved by the catalysis of catechol 1,2-dioxygenase or 2,3-dioxygenase, following ortho or meta fission. In the assay of enzyme activity, the activity of catechol 2,3-dioxygenase was not found in cell-free extracts, which proved that no 2-hydroxymuconic semialdehyde was produced in the ring fission products of catechol. Thus, phenol was assimilated via the 3-oxoadipate pathway by ortho fission of catechol.

Fig. 2 showed that cells began to excrete heavily both phenol hydroxygenase and catechol 1,2-dioxygenase at the middle phase and reached the maximum at the late phase of the exponential stages. It was an important factor that led a higher phenol-degrading potential at the late phase of the exponential stages, especially the change of phenol hydroxylase activity. Between the two enzymes, the phenol hydroxylase, hydroxylating phenol to catechol, was the key enzyme for phenol biodegradation [8,13]. The biodegradation velocity chiefly relied on the reaction which phenol was hydroxylated in phenol metabolism. Accordingly, the phenol hydroxylase activities in different exponential phases played an important role for the phenol biodegradation. Of course, it could not be excluded that the higher cell concentration in the late phase also slightly raised the phenol-degrading velocity of *A. faecalis*.

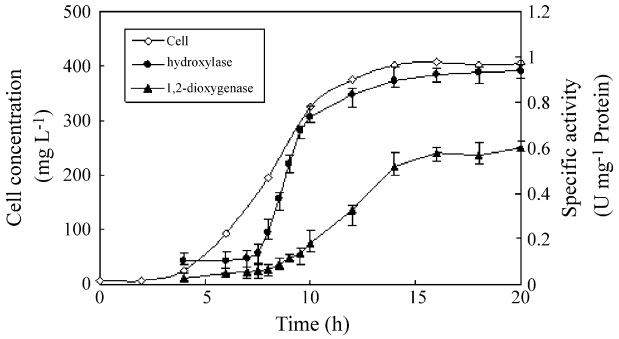

Fig. 2. The cell growth and specific enzyme activities of *Alcaligenes faecalis* grown in the different physiological phases in LB medium with 100 mg L^{-1} initial phenol.

3.4. Intrinsic kinetics

In this work, we assumed that the growth rate of *A. faecalis* was only limited by phenol concentration. Dissolved oxygen value in a shaking flask was determined beyond 5.0 mg L^{-1} by an oxygen electrode (Mettler Toledo 6050, Switzerland) [14]. It was presumed that the aeration provided by shaking the flasks was sufficient to keep the oxygen concentration constant and sufficient.

Batch cultures of *A. faecalis* were conducted in the mineral medium containing initial phenol concentrations ranging from 0 to 1600 mg L^{-1} with inoculum of OD = 1.226. For each batch culture with a certain initial phenol concentration, the specific growth rate of *A. faecalis* 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 growth rate (h^{-1}), γ_X the cell growth rate, and C_X was the cell concentration (mg L^{-1}).

Because of the inhibition of high phenol concentration on cell growth, Haldane's equation was selected for assessing the dynamic behavior of *A. faecalis* grown on phenol,

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

where C_S was the phenol concentration (mg L^{-1}), $\mu_{X,max}$ a model constant, k_S the saturation coefficient, and k_i was the inhibition coefficient. The values of the parameters

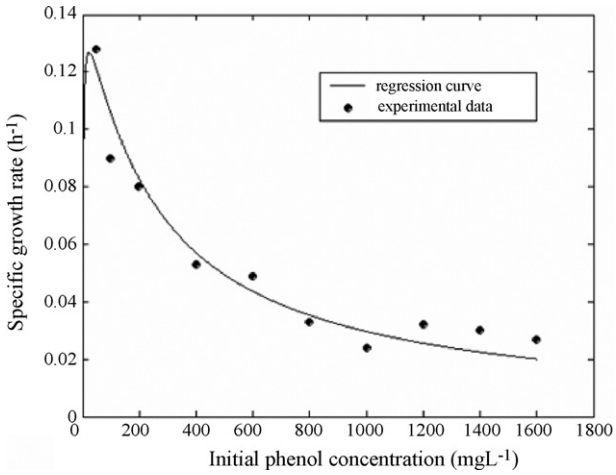

Fig. 3. Comparison between kinetic prediction and experimental determined specific growth rates at different initial phenol concentrations.

$\mu_{X,\max} = 0.15\,h^{-1}$, $K_S = 2.22\,mg\,L^{-1}$ and $K_i = 245.37\,mg\,L^{-1}$ were derived using a nonlinear least-squares regression analysis of MATLAB based on the experimental data obtained in the tests of phenol biodegradation. The value of the squared 2-norm of the residual at these parameters was a very small value (8.34×10^{-3}), which indicated the regression curve agreed with the experimental data very well.

It could be seen in Fig. 3 that the maximum specific growth rate occurred at very low phenol concentration of $38.9\,mg\,L^{-1}$. Generally, a low specific growth rate indicated very intense substrate inhibition to the strain of *A. faecalis*. But with the decrease of initial phenol concentrations in the mineral medium, the specific growth rates gradually increased from 0.027 to $0.128\,h^{-1}$ until a sharp drop of the curve because of the lack of carbon source in the mineral medium [15]. The lower the phenol concentration in the medium was, the weaker the substrate inhibition exhibited. However, in spite of a lower specific growth rate, bacteria kept a high phenol-degrading potential. More energy was required to overcome the effect of substrate inhibition at high phenol concentrations. Thus, both specific growth rate and biomass yield (*g*/*g*) were low at the initial phase of biodegradation, and with the consumption of phenol they increased as the result of the declining inhibit of phenol. Besides, the production and accumulation of various intermediates might also account for the decrease of cell mass yield [16,17]. It was also a reason why there was no essential relation between cell growth and phenol degradation, although phenol was consumed mainly for assimilation into biomass and for the energy for cell growth and maintenance [18].

The Haldane's equation has been used widely to describe the cell growth kinetics on phenol, and this equation has also often been used for prediction of phenol degradation rate directly by assuming a constant cell mass yield [15,17,19]:

$$\gamma_S = \frac{1}{Y_{X/S}}\gamma_X = \frac{\mu_{X,\max} C_S C_X}{(K_S + C_S + C_S^2/K_i)Y_{X/S}} \quad (3)$$

Analyzing the utilization of the substrate in cells in more detail, the consumption of substrate for growth and for maintenance, and also for product formation if possible, has to be considered [20]. The substrate consumption rate of phenol biodegradation was:

$$\gamma_S = \frac{1}{Y_{X/S}}\gamma_X + mC_X + \frac{1}{Y_{P/S}}\gamma_P \quad (4)$$

where $\gamma_p = \alpha\gamma_X + \beta C_X$ was the product formation rate and because $Y_{X/S}$, m, $Y_{P/S}$, α, β, were all constants, Eq. (4) could be reduced to:

$$\gamma_S = A\gamma_X + BC_X \quad (5)$$

or:

$$\mu_S = A\mu_X + B \quad (6)$$

A and B were all kinetic constants and they were regressed using MATLAB based on the experimental data [21], $A = 0.75$, $B = 0.113\,h^{-1}$. R^2 was 0.984, which indicated that degradation kinetics agreed well with the experimental data.

4. Conclusions

Strain *A. faecalis* isolated from acclimated activated sludge has a high phenol-degrading potential. When subculture at late phase of the exponential stages was used as inoculum, $1600\,mg\,L^{-1}$ phenol was degraded within 76 h. After the cell growth reached the middle phase, cells began to heavily excrete phenol hydroxylase and catechol 1,2-dioxygenase until the late phase of the exponential stages. For *A. faecalis*, phenol was assimilated via the 3-oxoadipate pathway by ortho fission of catechol. Haldane's equation was adopted to well describe the growth kinetics of *A. faecalis* at 30 °C and pH 7.2: $\mu_X = 0.15C_S/(2.22 + C_S + C_S^2/245.37)$. The phenol degradation process was associated to the cell growth kinetics: $\mu_S = 0.754\mu_X + 0.1132\,h^{-1}$.

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