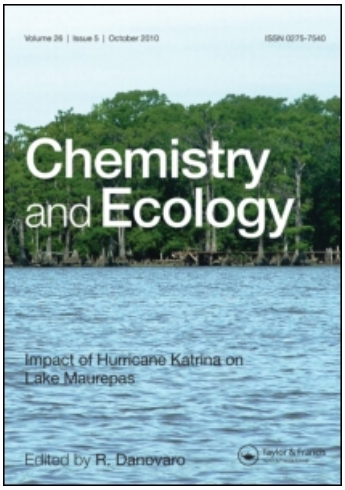

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Characterisation of phenol degradation by *Acinetobacter* sp. XA05 and *Sphingomonas* sp. FG03

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Two new strains named XA05 and FG03 were isolated from activated sludge and phenol-contaminated soils, respectively. Analysis of 16S rRNA gene sequences showed that XA05 belonged to an *Acinetobacter* sp. and FG03 was closely related to *Sphingomonas* sp. Strains XA05 and FG03 were cultivated in minimal medium supplemented with different phenol concentrations as a sole carbon source. Results showed that 99.5% and 78.3% phenol were removed by strain XA05 within 45 h and 60 h, with an initial concentration of 800 mg/l and 1000 mg/l phenol, respectively. In addition, 97.6% and 68.1% phenol were removed by strain FG03 under the same conditions. When two strains were mixed at the ratio of 1:1, 99.8% and 97.2% of phenol were removed within 35 h and 60 h, with an initial concentration of 800 mg/l and 1000 mg/l phenol, respectively. Kinetics of phenol degradation by strain XA05 and FG03 and the influence of other aromatic compounds such as benzophenone, 4-hydroxybenzoic acid, cinnamic acid and resorcin to the growth of two strains were also investigated in this study.

Keywords: phenol; biodegradation; kinetic; *Acinetobacter* sp.; *Sphingomonas* sp.

1. Introduction

Phenols are distributed either as natural or artificial mono-aromatic compounds in various environmental sites as major pollutants. Their existence in wastes from industrial processes such as oil refineries, coking plants wastewater treatment plants, petroleum-based processing, and phenol resin industry manufacturing plants has been well established [1]. Phenol and its derivatives are among the most frequently found pollutants in rivers, industrial effluents, and landfill runoff waters. These compounds are toxic and persistent: they accumulate in the environment and usually affect the performance of industrial as well as urban treatment plants. The development of improved techniques capable of degrading persistent and recalcitrant compounds then become necessary.

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Now, different treatment methods are available for the degradation of phenol, and compared with physico-chemical methods [2,3], the biodegradation methods of phenol reduction are universally preferred, because of lower costs and the possibility of complete mineralisation [4–7]. Applying microorganisms to degrade phenol is the most efficient and prevalent way [8–11], and a large number of phenol-degrading bacteria have been isolated and characterised at the physiological and genetic level [12–15]. However, little information on bacteria with a high phenol tolerance and high metabolising activity is available [16]. Therefore, there is still the need to isolate new phenol-degrading bacteria that can aerobically grow at elevated concentrations of phenol.

In this paper, we studied the phenol degradation ability of two new strains, XA05 and FG03, which were isolated from activated sludge and phenol-contaminated soils, respectively. Kinetics of phenol degradation and the influence of other aromatic compounds such as benzophenone, 4-hydroxybenzoic acid, cinnamic acid and resorcin to the growth of the two strains were also investigated.

2. Materials and methods

2.1. *Materials and growth conditions*

Samples of activated sludge were obtained from Xi'an Beishiqiao Wastewater Treatment Facility, Xi'an China. Samples of phenol-contaminated soils were collected at a site near Fugu Coking Plant, North of Shaanxi province in China. Phenol with greater than 99% purity was purchased from Shanghai Chemical Factory. All other chemicals used were of the highest purity available. Mineral medium (MM) was used for the enrichment and isolation of phenol-degrading strain. The strains were grown aerobically at 30 °C in minimal medium at pH 7.2–7.4 containing (per litre) 0.9 g KH_2PO_4, 6.5 g $Na_2HPO_4{\cdot}12H_2O$, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.4 g $(NH_4)_2SO_4$. and 1 ml of trace element solution [17]. Phenols of different concentrations were added to the sterilised MM as the sole carbon source.

2.2. *Selection and isolation of the phenol-degrading strains*

For selection of the desired microbial consortia, standard batch enrichment culture techniques were performed. A total of 3 g of the soil samples or 5 ml of the activated sludge samples were inoculated into 100 ml flasks containing 40 ml MM in the presence of 1 mg phenol. After 1 week at 150 rpm and 30 °C in an orbital shaker, 1 ml enriched aqueous culture was transferred to another glass flask with 100 ml of MM in the presence of 2 mg phenol for subsequent enrichment. Additionally, 3 mg and 4 mg of phenol in the MM were used for subsequent enrichment. After four consecutive enrichments had been carried out, dilutions of culture were inoculated on LB agar plates. Colonies were harvested from dilution plates based on distinct colony morphology, and transferred to fresh LB agar plates several times to ensure culture purity. Each isolate was then tested for its ability to grow in MM liquid medium with phenol at different concentrations as the sole carbon and energy source.

2.3. *Identification of strains XA05 and FG03 by 16s rRNA gene sequence*

The isolated phenol-degrading strains were identified by colour, Gram staining and morphology. Further identification was performed using 16S rRNA gene sequencing [18]. Genomic DNA from the bacterial strains was extracted according to the procedure given in Pitcher et al. [19].

The fragment of rDNA was amplified using a Gene Amp 2400 PCR System (PE, USA) under the following conditions: 0.1 μg template DNA, 5.0 μl 10 × PCR buffer (100 mM Tris/HCl (pH 8.3 at 20 °C), 15 mM $MgCl_2$, 500 mM KCl, 0.1% (w/v) glutin), 0.5 U Blend-Taq, 0.5 μM upstream primer 27F (AGAGTTTGATCCTGGCTCAG), 0.5 μM downstream primer 1522R (AAGGAGGTGATCCAGCCGCA) [20], 0.1 mM dNTPs, and double distilled water of a total volume of 50 μl. The tubes were incubated at 94 °C for 3 min and then subjected to the following thermal cycling programme: denaturation at 94 °C for 1 min, primer annealing at 54 °C for 1 min, and chain extension at 72 °C for 2 min with an additional extension time of 7 min on the final cycle, for a total of 30 cycles. The BLAST program was used to search the 16S rRNA database for similar sequences. The 16S rRNA genes of strains XA05 and FG03 were deposited in the GenBank data library under accession numbers EU784671 and EU784670, respectively.

2.4. *Kinetics of phenol degradation*

Strains XA05 and FG03 were cultured in LB (5 g of sugar, 3 g of yeast extract, and 5 g of NaCl per litre) liquid medium overnight, from late exponential phase, the cells of bacteria were harvested and rinsed with 50 mM phosphate buffer (pH 6.8) twice, respectively. The cells of two strains were mixed at the ratio of 1:1 (v/v), then 1 ml cells of strains XA05 and FG03, and the mixed cells (10^{10} cells per millilitre) were inoculated into 100 ml MM, which contained 200, 400, 600, 800, 1000 mg/l phenol, respectively, and the beginning value of the OD_{600} was about 0.06. The incubations were grown at 30 °C with shaking at 150 rpm, cell growth biomass and residual phenol concentrations were determined at 5 h intervals for 3 d. All tests were done in triplicate, hence the results are the means of three independent experiments. The initial specific phenol degradation rate ($v_{0,\text{phenol}}$) was determined by Equation (1) [21]:

$$v_{0,\text{phenol}} = \frac{1}{X} \cdot \frac{\mathrm{d}C_{\text{phenol}}}{\mathrm{d}t}\bigg| t \longrightarrow 0, \tag{1}$$

where X denotes the cell concentration (g/l) or in absorbance unit at 600 nm (OD), C_{phenol} denotes the concentration of phenol (M), and t denotes the cultivation time (h). The relationship between $v_{0,\text{phenol}}$ versus C_{phenol} represents the kinetics of phenol degradation by the strains of XA05, FG03 and the mixed cells of two strains (at the ratio of 1:1). To describe the kinetics of phenol-inhibition to the cell growth and phenol degradation, Haldane's substrate-inhibition model was used, because it has a widespread acceptance for phenol degrading and simplicity.

2.5. *Growth on other aromatic compounds*

To determine the inhibition of aromatic compounds to the growth of strains, cells of XA05 and FG03 growing in LB liquid medium overnight to late exponential phase were harvested and twice rinsed with 50 mM phosphate buffer (pH 6.8). The cells of two strains were mixed at the ratio of 1:1 (v/v), then 1ml cells of two strains and the mixed cells (10^{10} cells per millilitre) were inoculated into 100 ml MM, which contained 400 mg/l of phenol, benzophenone, resorcin, cinnamic acid, and 4-hydroxybenzoic acid as the sole carbon source, respectively, and the beginning value of the OD_{600} was about 0.06. Then, the strains were incubated at 30 °C on a shaker preset at 150 rpm. The biomass and aromatic substrates were determined every 10 h for 3 d. Solution without inoculated media was used as a reference.

2.6. *Analysis methods*

Samples (2–3 ml) taken from the culture at different times were used for measurement of the optical density of cells and the phenol concentration. The optical density of cells at 600 nm was measured using a spectrophotometer (UV-1200, Shimazu, Kyoto, Japan). The phenol concentration and other aromatic compounds were determined by HPLC using a Nucleosil 120 C-18 column (250 × 46 mm inner diameter; 5 μm particle size) in combination with a Waters LC-Spectrophotometer. The mobile phase was acetonitrile: water (65:35 v/v) at a flow rate of 0.6 ml/min. Samples (10 μl) were injected into the column and concentrations were measured using a calibration curve by absorbance at 210 nm.

3. Results

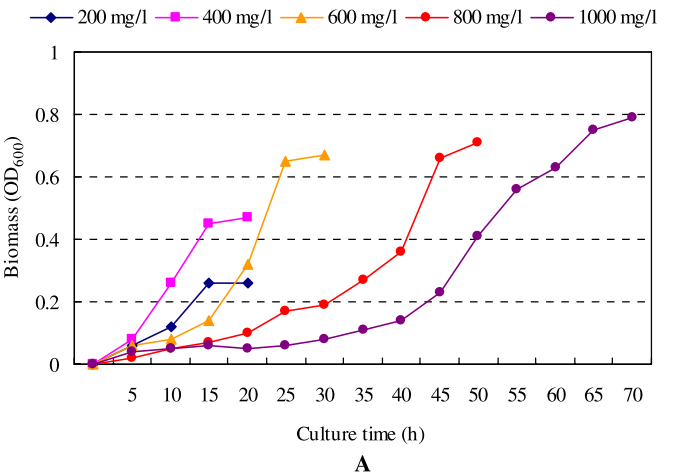
3.1. *Isolation and 16S rRNA gene sequence analysis of phenol-degrading strains*

Strain XA05, a bacterial isolate capable of degrading phenol, was obtained from the activated sludge of a wastewater treatment plant. This strain formed entire, smooth, convex, opaque and wet colonies, which were slightly yellow and circular with a diameter of 0.5–1 mm within 1–2 days. The colony could be easily scraped off from nutrient agar plates incubated at 30 °C for 1–2 days. The bacterium was Gram-negative, long rod-shaped, and grew aerobically. A partial 16S rRNA gene sequence (1389bp) was obtained and sequence alignment revealed that strain XA05 was closely related to the species in the genus *Acinetobacter*. Strain XA05 also exhibited the highest similarity (96.15%) to *Acinetobacter* sp. (AB362299). We thus tentatively classified strain XA05 as *Acinetobacter* sp.

Strain FG03 was isolated by the enrichment culture technique from the phenol-contaminated soil. The strain was a Gram-negative, short rod-shaped bacterium of 0.5–1.0 μm. The strain formed smooth, convex and wet colonies with diameters of 0.8–1.3 mm within 1–2 days. The colony could also be easily scraped off from nutrient agar plates incubated at 30 °C for 1–2 days. By PCR amplification, a partial (1383bp) 16S rRNA gene fragment was obtained and sequenced. We found that the tested strain FG03 was classified in the *Sphingomonas* genera, and the similarities between FG03 and *Sphingomonas* sp. (DQ789172) were 97.36%. Thus, FG03 was identified to be a *Sphingomonas* sp.

3.2. *Kinetics of cell growth and phenol degradation*

Figure 1 shows the cell biomass of strains XA05, FG03 and the mixed cells of two strains in 200–1000 mg/l phenol. Results showed that the lag time of XA05 (Figure 1A), FG03 (Figure 1B) and the mixed cells of two strains (Figure 1C) were prolonged and the biomass of the cells were hardly antiblastic when the phenol concentration increased. The kinetic trends in specific growth rate versus different initial phenol concentration appeared to Andrews's substrate inhibition model (Equation (2)) [22]. Their relevant constant values are listed in Table 1. From the model, the specific growth rate also increased with the phenol concentration increasing. When the phenol concentration exceeded 2830 μM for XA05, 2845 μM for FG03 and 3250 μM for the mixed cells of two strains, respectively, the specific growth rate decreased (Figure 2). According to our data, the strains XA05 and FG03 can both grow in MM with different concentrations of phenol as the sole carbon source, and with almost a same lag time of growth. When the concentration of phenol was increased, the lag time of growth was extended too. However, when two strains were mixed at the ratio of 1:1, the lag time of growth was shorter than that of the strain XA05 and strain FG03 at the same phenol concentration condition. When the initial concentrations of phenol were 800



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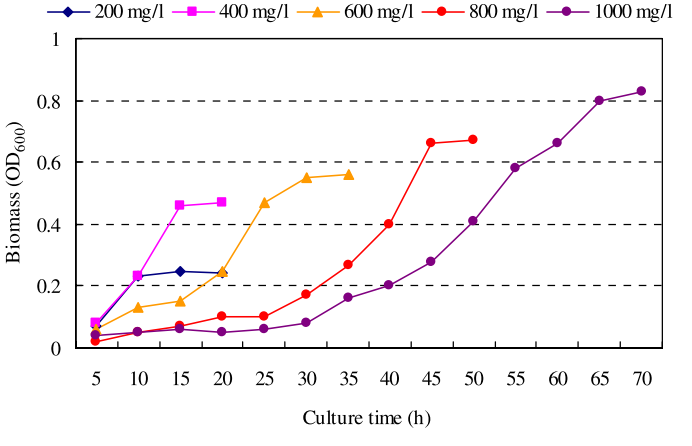

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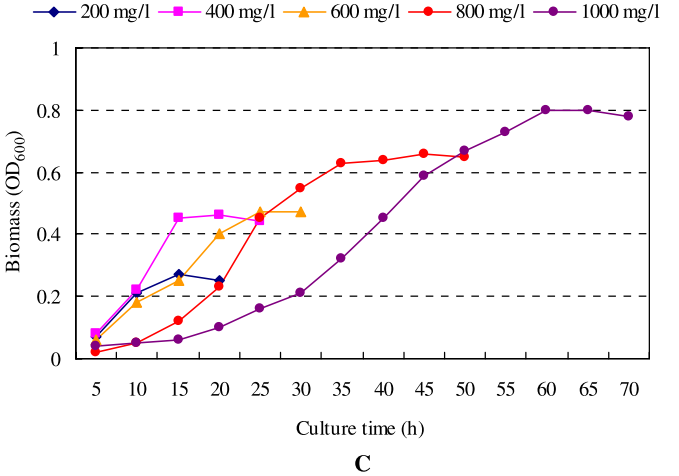

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Figure 1. Biomass growth of strains XA05 and FG03, and the mixed cells of both strains in MM medium containing different initial phenol concentrations. Cells were grown at pH 7.2 for 3 days with shaking at 150 rpm. Values represent the averages of triplicate determinations. A: XA05, B: FG03, C: XA05 + FG03.

Table 1. Kinetic parameters estimated from numerical simulations with Andrews's substrate inhibitory model and Haldane's model.

	Phenol-limited growth kinetics (Andrews's model)				Phenol degradation kinetics (Haldane's model)			
		Strain				Strain		
Parameters index		XA05	FG03	XA05 + FG03		XA05	FG03	XA03 + FG03
Maximum rate	μ_{max} (h^{-1})	0.053	0.056	0.057	$\upsilon_{max,phenol}$ (μM/min/g cell)	45	42	48
Half-saturation constant	$K_{S,G}$ (μM)	820.2	1010.5	780.6	K_S (μM)	760.1	630.5	750.6
Inhibition constant	$K_{SI,G}$ (μM)	6580.3	6720.6	8100.5	K_{SI} (μM)	10100	9500	12000
Coefficient of determination	R^2	0.927	0.935	0.928	R^2	0.931	0.916	0.922

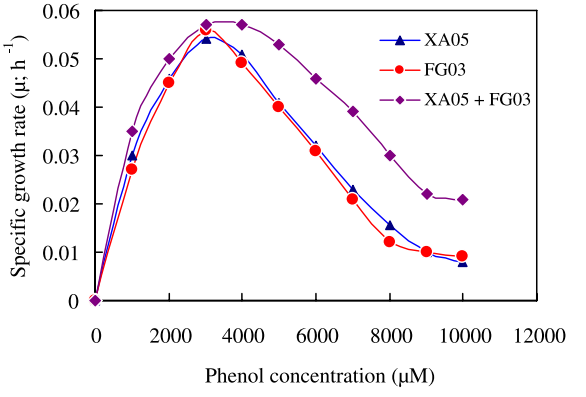


Figure 2. The specific growth rate of strains XA05 and FG03, and the mixed cells of both strains on different initial phenol concentrations. Values represent the averages of triplicate determinations.

and 1000 mg/l, respectively, the lag time of growth was 30 h and 45 h for XA05, 35 h and 40 h for FG03, 20 h and 30 h for the mixed cells of two strains, respectively.

$$\mu = \frac{\mu_{max} C_{phenol}}{K_{S,G} + C_{phenol} + C_{phenol}^2 / K_{SI,G}}, \tag{2}$$

where the μ denotes the specific growth rate of strain on MM medium with phenol as the sole carbon source (h^{-1}), μ_{max} denotes the maximum specific growth rate of strain on MM medium with phenol as the sole carbon source (h^{-1}), C_{phenol} denotes the initial phenol concentration (μM), and $K_{S,G}$ and $K_{SI,G}$ denote the half-saturation and inhibition constant for growth kinetics, respectively.

Under the different initial phenol concentration, the phenol degradation and the specific phenol degradation rate of strain XA05, strain FG03 and the mixed cells of two strains were investigated. Results showed that the high initial phenol concentration had the longest degradation time. Around 99.5% and 78.3% phenol were removed by strain XA05 within 45 h and 60 h with an initial concentration of 800 mg/l and 1000 mg/l phenol, respectively. Under the same conditions, 97.6% and 68.1% phenol were removed by strain FG03. Otherwise, when the two strains were mixed at the ratio of 1:1, 99.8% and 97.2% of phenol were removed within 35 h and 60 h with an initial concentration of 800 mg/l and 1000 mg/l phenol. The specific phenol degradation rate was firstly increased and then reduced until the concentration of phenol was up to 3200 μM for strain XA05, 3225 μM for strain FG03 and 3600 μM for the mixed cells of two strains, respectively (Figure 3).

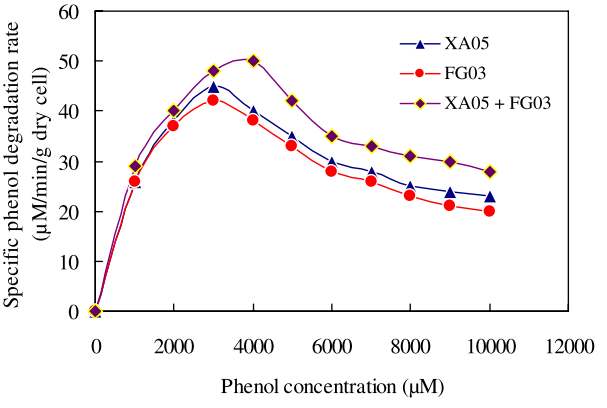


Figure 3. The specific phenol degradation rate of strains XA05 and FG03, and the mixed cells of both strains on different initial phenol concentrations. Values represent the averages of triplicate determinations.

In order to simulate the data of phenol degradation by XA05, FG03 and their mixture, Haldane's mathematical model was used (Equation (3)) [22], and their kinetic parameters are shown in Table 1.

$$\nu_{\text{phenol}} = \frac{\nu_{\text{max,phenol}} C_{\text{phenol}}}{K_{\text{S}} + C_{\text{phenol}} + C_{\text{phenol}}^2 / K_{\text{SI}}}. \tag{3}$$

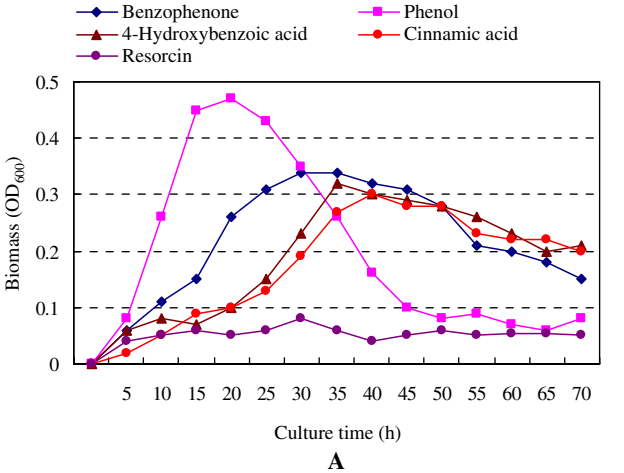
3.3. *Effect of pH and temperature on phenol degradation by strains XA05 and FG03*

The pH optimum experiments for phenol degradation by strain XA05, strain FG03 and the mixed two strains were performed at 30 °C with a phenol concentration of 800 mg/l. The result indicated that, under solution with a pH ranging from 7.0 to 7.5, both strain XA05 and FG03 could grow well and the majority (>95%) of phenol was degraded and utilised as the sole carbon source. Under slightly acidic conditions, the cell growth rate was lowered and phenol degradation was slowed. This indicated that cell growth of two strains and phenol degradation were favoured in slightly alkaline media.

To confirm the influence of the temperature on phenol degradation by strain XA05 and FG03, a series of experiments were performed at a temperature range of 20–45 °C at pH 7.2, with a phenol concentration of 800 mg/l. Results showed that the cell growth of the two strains and phenol degradation were favoured at temperatures of 25–35 °C. It appeared that the optimal growth conditions for strains XA05 and FG03 were in a solution of pH 7.2 and 30 °C, with more than 99.5% of phenol at an initial concentration of 800 mg/l being degraded within 2 d.

3.4. *Aromatic compounds-utilising by XA05 and FG03*

Utilisation of different aromatic compounds as sole substrates by strain XA05, FG03 and their mixture were evidenced by the growth of biomass in the test flasks within 3 days' inoculation. We selected phenol, benzophenone, cinnamic acid, 4-hydroxybenzoic acid and resorcin as the sole carbon source on MM liquid medium. Results showed, besides phenol, XA05 can also utilise benzophenone and 4-hydroxybenzoic acid as the carbon source. The ranking of the aromatic compounds used by XA05 is as follows: phenol > benzophenone > 4-hydroxybenzoicacid > cinnamic acid > resorcin (Figure 4A). Besides phenol, FG03 can utilise 4-hydroxybenzoic acid and cinnamic acid as the carbon source. The ranking of the aromatic compounds used by FG03 is as follows: phenol > 4-hydroxybenzoic acid > cinnamic acid > benzophenone > resorcin



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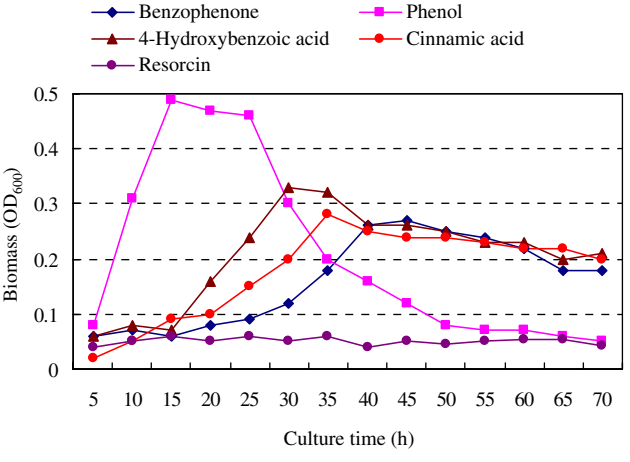

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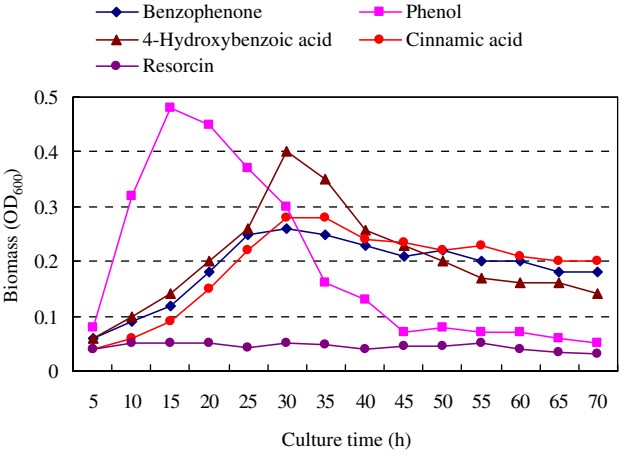

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Figure 4. Biomass growth of strains XA05 and FG03, and the mixed cells of both strains in MM containing 400 mg/l of different aromatic compounds as the sole carbon source. Cells were grown at 30 °C for 3 days with shaking at 150 rpm. Values represent the averages of triplicate determinations. A: XA05, B: FG03, C: XA05 + FG03.

(Figure 4B). Resorcin may not be utilised as the sole carbon and energy source by both of XA05 and FG03. Compared with phenol, the lag time of XA05 and FG03 were all prolonged under the same concentration conditions of other aromatic compounds. The utilising effect of aromatic compounds by the mixed cells of XA05 and FG03 is better than that by strain XA05 or strain FG03 separately. Especially for 4-hydroxybenzoic acid, the utilising effect of the mixed cells is almost as same as for phenol (Figure 4C). However, compared with phenol, the lag time of the mixed cells was also prolonged, and the resorcin can not be utilised as the sole carbon and energy source by the mixed cells of the two strains.

4. Discussion

Bacterial genera capable of degrading phenolic compounds in the environment are soil bacteria which may play an important role in degrading phenolic compounds of toxic organic pollutants. Currently, a number of bacteria were discovered to have excellent capability of phenol degradation. Identification of these bacteria showed the dominance of the genus *Pesudomonas*, especially *Pesudomonas putida*, mainly because of its widespread distribution in soils. Many other genera of bacteria have also been described as degrading strains of phenolic compounds, including *Agrobacterium, Burkholderia, Acinetobacter, Ralstonia, Klebsiella, Bacillus, Rhodococcus,* and *Rhizobium* [12]. In the *Sphingomonas* genera bacteria, phenolic waste degradation was also discovered [23]. Wei et al. [21] reported that the strain *Rhizobium* sp. could remove around 99.5% and 78.3%, with an initial concentration of 900 mg/l and 1000 mg/l phenol in 62 h and 66 h, respectively. Wang et al. [24] reported that the strain *Acinetobacter* sp. was capable of removing 500 mg/l phenol in liquid minimal medium by 99.6% within 9 h. In this study, strains *Acinetobacter* sp. XA05 and *Sphingomonas* sp. FG03 could grow well in 800 mg/l phenol and remove 99.5% and 97.6% within 45 h, respectively. This demonstrated that the strains XA05 and FG03 had excellent phenol degrading ability.

Cell growth on phenol has been observed to display substrate inhibition phenomena at high phenol concentration, and Haldane's equation is often used to describe cell growth on phenol either by pure or mixed cultures [25]. To represent the growth kinetics of phenol inhibition, several kinetic models were fitted to the experimental data for selecting the best models. Out of the models, Haldane's model was used due to its mathematical simplicity and wide acceptance for representing the growth kinetics of inhibitory substrates [26]. Watanabe applied Haldane's model to describe the kinetics of phenol degradation for a number of bacteria and classified the phenol-degrading bacteria into three kinetically different groups, according to their K_S and K_{SI} values [22]. Our research of phenol degradation kinetics showed that XA05, FG03 and the mixed culture of the two strains all possessed a high K_S and K_{SI}, which appeared to belong to none of the three general kinetic groups suggested by Watanabe et al. [22], but were similar to the results of Wei et al. [21], because the lag time of growth for XA05 and FG03 were both prolonged under high phenol concentration conditions. The kinetic characteristics of XA05 and FG03 with high K_S and K_{SI} give better phenol degradation activity. In cell growth kinetic model and degradation kinetic model of XA05 and FG03 in phenol, analysis results of R^2-values also showed that Haldane's model was effective for describing the dynamics of phenol degradation for strains XA05 and FG03.

To our surprise, when the strains of XA05 and FG03 were mixed at the ratio of 1:1, 99.8% of phenol was removed within only 35 h, with an initial concentration of 800 mg/l, compared with the pure culture of strains XA05 and FG03, which take 45 h to remove the same amount of phenol. The efficiency of phenol degradation by the mixed cells is much better than that of their pure culture. At the end of the phenol degradation by mixed cells, we analysed the distribution of the two strains in the mixed culture by microscope, the amount of each type of cell keeping to the ratio of 1:1. It indicated that the two strains did not inhibit each other. Results of aromatic

compounds utilising the pure strains of XA05 and FG03 and the mixed cells showed that they can utilise different aromatic compounds, and the utilising effect of the aromatic compound with mixed cells of XA05 and FG03 is better than that of pure strains. We think that the strain of XA05 and strain FG03 were isolated from different environmental conditions, and they have different enzyme systems and different ways to degradate the phenol and other aromatic compounds. When two strains were mixed, they may remove intermediates that are toxic to the producer strain, and these questions should be further studied.

5. Conclusion

(1) Strain XA05 and strain FG03 were isolated from activated sludge and contaminated soils, respectively. From 16S rRNA sequence analysis, the strains XA05 and FG03 belong to the genera *Acinetobacter* and *Sphingomonas* and are hereby named *Acinetobacter* sp. XA05 and *Sphingomonas* sp. FG03, respectively.
(2) Strain XA05 can remove 99.5% and 78.3% phenol within 45 h and 60 h with an initial concentration of 800 mg/l and 1000 mg/l phenol, respectively. In addition, 97.6% and 68.1% phenol were removed by strain FG03 under the same conditions. When the two strains were mixed at the ratio of 1:1, 99.8% and 97.2% of phenol were removed within 35 h and 60 h, with an initial concentration of 800 mg/l and 1000 mg/l phenol, respectively.
(3) Both strain XA05 and strain FG03 had a high K_S and high K_{SI} in Haldane's model. XA05 and FG03 can also utilise different aromatic compounds as the sole carbon source, and the efficiency of aromatic compounds containing mixed cells of XA05 and FG03 was better than that of pure cultures of strain XA05 or strain FG03.

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