

Kinetics Model for Growth of *Pseudomonas chlororaphis* DJ-4 during *m*-xylene Biodegradation under Nitrate Reducing Conditions

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A pure bacterium *Pseudomonas chlororaphis* DJ-4 with the capability of anaerobic growth using *m*-xylene as carbon sources was isolated from gasoline contaminated soil. The substrate of *m*-xylene could be completely degraded by *Pseudomonas chlororaphis* DJ-4 within the incubation time of 12 days and without a significant lag period when the initial concentration was below 150 mg/l, and the degradation rates of *m*-xylene increased with increasing the initial concentration within the range between 11.6 mg/L and 146.9 mg/L. The cell specific growth rates on *m*-xylene obeyed the Haldane-Andrew model when the initial concentration was in the range of 10–150 mg l⁻¹. The Haldane-Andrew model parameters were determined by non-linear regression on specific growth rates and various initial substrate concentrations, and the value of maximum specific growth rate, half saturation constant and inhibition constant was 2.56 d⁻¹, 233.9 mg l⁻¹ and 18.1 mg l⁻¹, respectively. The model provided an excellent prediction of the growth kinetics and the interactions between the substrate.

Key words: *m*-xylene; *Pseudomonas chlororaphis*; anaerobic growth; Haldane-Andrew model.

Benzene, toluene, ethylbenzene and xylenes (BTEX) are important contaminants present in soil and groundwater, which usually originate from the accidental leakage of underground storage tanks containing gasoline and jet fuel or spillage during the transportation. Due to their relatively higher water solubility, BTEX compounds always migrate far away from polluted sources and contaminate drinking water supplies. Xylene isomers are toxic to the liver, kidneys, and the central nervous system when they enter the body by skin contact or breathing (Yoon *et al.*,

2002), and they are classified as pollutants by the US Environmental Protection Agency (Yaws, 1999). Sites contamination by xylene isomers is a very serious problem as these compounds are toxic to humans (An, 2004; Paixão *et al.*, 2007), which is often rendered anaerobic by ¹ oxygen-consuming microorganisms. Aerobic bioremediation of BTEX compounds generally exhibits faster BTEX degradation rates than anaerobic systems (Corseuil *et al.*, 1998). However, aerobic strategies are not universally applicable, and anaerobic bioremediation might be more appropriate to clean up some BTEX contaminated sites². Therefore, in these sites anaerobic degradation of aromatic hydrocarbons may be the determining mechanisms and depend on the activity of bacteria capable of metabolizing hydrocarbons (Lovley, 1997; Cunningham *et al.*, 2001).

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Many pure cultures of anaerobic organisms that can degrade *m*-xylene have been described. Harms et al. (Harms *et al.*, 1999) have successfully isolated the sulfate-reducing strains mXyS1 that could oxidize *m*-xylene, which is the member of the *Desulfobacteriaceae* family in the delta subclass of the *Proteobacteria*. The 16S rDNA sequence analysis revealed that strain mXyS1 was 86.9% similar to *Desulfococcus multivorans*. Morasch et al. (Morasch *et al.*, 2004) isolated the anaerobic *m*-xylene degrading bacterium of sulfate-reducing *Desulfotomaculum* species strain OX39. Chakraborty et al. (Chakraborty *et al.*, 2005) found that *Dechloromonas* strain RCB could degrade *m*-xylene with the rate of two times higher than that for sulfate-reducing strain OX39. Hess et al. (Hess *et al.*, 1997) observed that *Azoarcus toluolyticus* Td-15 could well using *m*-xylene as carbon sources with the simultaneous reduction of nitrate. Achong et al. (Achong *et al.*, 2001) found that *Azoarcus* sp. strain T could degrade mineralize *m*-xylene under nitrate reducing conditions.

In the present study, a new strain of *P. chlororaphis* that could be efficiently degraded *m*-xylene with the reduction of nitrate was isolated. Knowledge on the growth characteristics of the isolated bacterium is necessary for improvements in the process control and removal efficiency. Therefore, a series of batch experiments were designed, where microbial growth characteristic was monitored under various initial substrate concentrations.

MATERIALS AND METHODS

Bacteria growth medium

Mineral salts medium (MSM) of the following composition NH_4Cl (1.0 g l⁻¹), KH_2PO_4 (1.0 g l⁻¹), MgCl_2 (0.1 g l⁻¹), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.05 mg l⁻¹), NaNO_3 (1.5 g l⁻¹) was used in this study. The medium was supplemented with 0.1% of $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$, vitamin solution (1% [vol/vol]) and trace elements solution (1% [vol/vol]). Each liter of trace elements solution contained 30 mg of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.15 mg of CuCl_2 , 5.7 mg of H_3BO_3 , 20 mg of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 2.5 mg of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 1.5 mg of $\text{NiCl}_2 \cdot 2\text{H}_2\text{O}$, and 2.1 mg of ZnCl_2 (Hu *et al.*, 2007). Each liter of the vitamin solution contained 20 mg of biotin, 20 mg of folic acid, 50 mg of riboflavin, 50 mg of thiamine, 50 mg of

nicotinic acid, 50 mg of pantothenic acid, 1 mg of cyanocobalamin, 50 mg of *p*-aminobenzoic acid, and 50 mg of thiotic acid (Zhang *et al.*, 1997). The final pH of the medium was adjusted between 6.8 and 7.2.

Isolation and identification of the isolate

A pure microbial culture utilizing *m*-xylene at strict anaerobic nitrate reducing conditions was isolated from the mixed bacteria that enriched from the gasoline contaminated soil, and the screening procedure of the mixed bacteria was described in our previous paper (Dou *et al.*, 2008). The pure culture was isolated by serial dilution using mineral salts medium mixed with molten agar and *m*-xylene. Each of the colonies produced was removed with an inoculating loop scraped over the agar surface and transferred to liquid medium to check for the ability to grow on *m*-xylene. Controls consisted of uninoculated medium. The plates were inoculated and incubated at 20°C in an anaerobic glove box with a headspace consisting of pure nitrogen gas.

The isolate was characterized and identified by 16S rDNA sequence analysis (Dou *et al.*, 2010), standard morphological, physiological and biochemical plate and tube tests using the criteria and procedures described in Bergey's Manual of Systematic Bacteriology (Krieg *et al.*, 1984). Test kits were used according to the manufacturer's procedures.

The 16S rDNA was amplified from genomic DNA by PCR. Total DNA of the isolated strain was extracted using the DP302 DNA extraction kit (Tiangen Biotech, China), then was purified with DNA purification kit (Dingguo Co. Ltd., Beijing, China). PCR amplification was performed using the universal primers 8F (5' - AGAGTTTGATCCTGGCTCAG-3') and 1492R (5' - TACCTTGTTACGACTT-3') in a 50µl system, consisted of 25µl PCR Master Mix, 2µl 8F, 2µl 1492R, 2µl lysate and 19µl ddH₂O. Amplifications were carried out for 28 cycles (94 °C for 30 s, 55 °C for 40 s, and 72 °C for 90 s) in a 1000-series thermal cycler (Bio-Rad, Hercules, CA, USA) with an initial denaturation at 94 °C for 6min. The PCR products were sequenced using ABI3730 genetic analyzers by Sunbiotech Co. Ltd., Beijing, China. The 16S rDNA sequences were aligned with published sequences from the GenBank database using National Centre for Biotechnology Information (NCBI) BLAST comparison software.

Batch growth tests

To study the biomass growth using *m*-xylene as carbon sources, batch growth tests with different initial substrate concentration in a range of 10-150 mg l⁻¹ were performed using 150-ml glass serum bottles, and the bottles were sealed with Teflon-coated rubber stoppers (approximately 3 mm in thickness, Alltech Associates, Inc.) secured by a crimped aluminum caps. In order to account for abiotic degradation, bottles containing no microorganisms were used as controls. The sterile samples were established by autoclaving at 121 °C for 3 h before amendment of *m*-xylene. Effects of temperature and pH on anaerobic substrate degradation and biomass growth were also examined.

A loopfull from an agar slant of this culture was inoculated to 5-l flask containing 4.0-l mineral salts medium amended with *m*-xylene and nitrate. After the isolated culture was grown anaerobically to late exponential growth phase, cells were harvested anaerobically by centrifugation, washed twice with the anoxic mineral salts medium, and resuspended in the same medium. The substrate of *m*-xylene was added to each mineral salts medium to a final concentration as the experimental design. The experiments were started by inoculating with the washed cells to the serum bottles contained 100 ml mineral salts medium. The final concentration of the inoculated isolates was approximately 10⁷ cells ml⁻¹.

All the experiments were performed triplicate in an anaerobic glove box which was filled with pure nitrogen gas. The maintenance of anaerobic conditions was examined by a preliminary experiment. The microcosms were continuously shaken on a rotary shaker at 20°C in darkness.

Chemical and microbiological analysis

The concentration of *m*-xylene was analyzed by a model GC-14B gas chromatograph equipped with a capillary column (ULBON HR-1 0.25 mm×30 m), with a flame ionization detector (FID) (Shimadzu Corp., Japan). Injector, detector and column temperature was hold at 150°C, 150°C and 100°C, respectively. Nitrogen gas served as carrier gas, and oxygen and hydrogen served as fuel gas for the FID.

The total cell counts were analyzed by DNA intercalating dye 42 , 6-Diamidine-22 -

phenylindol-dihydrochloride (DAPI, Sigma) staining (Porter *et al.*, 1980). For that, suspensions of the samples were concentrated onto 0.2 μm pore-size black polycarbonate membrane filters (Nucleopore, Whatman, USA). Filters were washed with sterilized deionized water and air-dried in the dark. In order to fix the cells, the filters were dipped into a 4% formaldehyde solution for 2~3 h. Then filters were stained with DAPI dye (1 μg/ml) for 15 min in the dark. DAPI-stained cells were identified and enumerated under an epifluorescent microscope equipped with ultraviolet (UV) excitation filter set (Nikon UV-2A, UV excitation at 330~500 nm, dichroic mirror 400 nm, longpass>420 nm, Kawasaki, Japan). The procedure was performed by DAPI staining in at least three independent experiments.

RESULTS AND DISCUSSION

Microbiological characteristics and identifications of the isolated pure culture

The isolated *m*-xylene biodegradative bacterium was anaerobic, gram negative, rod shaped, motile, non-pigmented and varying in size ((0.7—0.8) μm × (2.0—2.8) μm) (Figure 1). The biochemical and physiological characteristics and identifications of the strain were listed in Table 1. Comparison of the above microbiological properties with the relevant criteria and descriptions in Bergy's Manual of Systematic Bacteriology (Krieg and Holt, 1984), the strain was identified as a bacterium belonging to the genus *Pseudomonas* and was named *P. chlororaphis*. The 16S rDNA sequence was most closely related to *Pseudomonas chlororaphis* at a similarity of 99.4 % retrieved from the NCBI nucleotide sequence databases. According to both gene sequence analysis and cell characteristic information above, the isolated strain was classified into the genus *Pseudomonas chlororaphis* and named as *P. chlororaphis* DJ-4.

The effects of temperature and pH on the growth of *P. chlororaphis* DJ-4 were examined. The bacterial growth was maximal at 30°C; temperatures of 10, 20, 25, 30, 35 and 40°C were tested. The pH dependence of bacterial growth was tested in a range between pH 5.0 and 8.5 in 0.5-pH-unit intervals, results showed that the bacterial growth was maximal at pH 7.0 (data not shown).

Table 1. Biochemical and physiological characteristic of the strain

Tested item	Result	Tested item	Result
Oxidase	Positive	Reduction of nitrate	Positive
Catalase	Positive	Citric acid utilization	Positive
Aminopeptidase	Positive	D-Glucose utilization	Positive
Indole production	Negative	L-Arabinose utilization	Positive
Starch hydrolysis	Negative	D-Xylose utilization	Positive
Denitrification	Positive	D-Mannitol utilization	Positive
Gelatin hydrolysis	Positive	Malonic acid utilization	Positive

Biodegradation characteristics of *m*-xylene with various substrate concentrations

The effect of the initial concentration on the biodegradation of *m*-xylene was shown in Figure 2. As can be seen from Figure 2 that *m*-xylene could be completely degraded by the isolated bacteria within the incubation time of 12 days and without a significant lag period when the initial concentration was below 150 mg/l.

Based on the data in Figure 2, the biodegradation rate at each initial concentration could be calculated, and the results were shown in Figure 3. From Figure 3 it could be observed that the degradation rates of *m*-xylene increased with increasing the initial concentration within the range between 11.6 mg/L and 146.9 mg/L, and there were no toxic effects on the isolated bacterium even if the concentration of *m*-xylene was up to 150 mg/l.

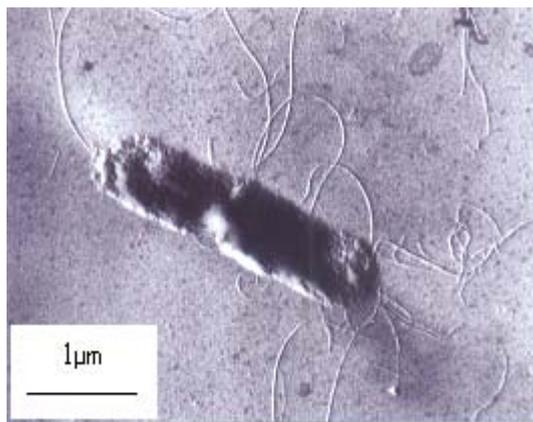


Fig. 1. Photograph of *P. aureofaciens* DJ-4 by the scanning electron microscope ($\times 25000$)

Growth dynamics of the *P. chlororaphis* DJ-4 for *m*-xylene biodegradation

Figure 4 illustrated the results of biomass growth in batch reactors at eight selected initial substrate concentrations (11.6 mg/L, 21.7 mg/L, 39.4 mg/L, 59.3 mg/L, 81.6 mg/L, 99.7 mg/L, 117.5 mg/L, and 146.9 mg/L). From Figure 4 it could be found that the pure culture of *P. chlororaphis* DJ-4 could grow well without a detectable lag phase in mineral salts medium amended with lower concentrations of *m*-xylene. However, there was a lag phase about 3 days when the initial concentration of *m*-xylene was equal to or higher than 117.5 mg/L. It can also be observed that the lag phase generally increased with increase in initial concentration of *m*-xylene.

Growth Modeling of the *P. chlororaphis* DJ-4

The total weight of bacterial biomass

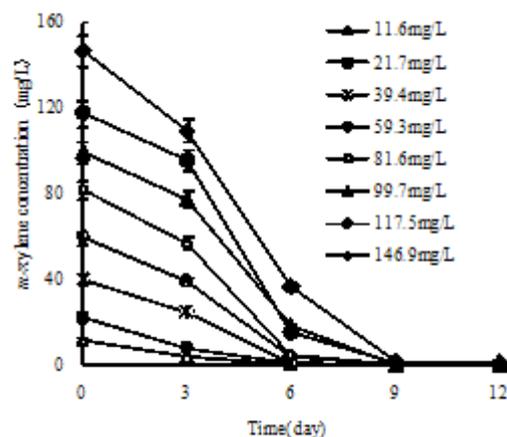


Fig. 2. Time profile of *m*-xylene degradation under different initial concentrations

could be calculated from the total cell numbers using a factor of 1.72×10^{-10} mg/bacterial cell (Balkwill *et al.*, 1988). Thus, the mass of bacterial growth can be calculated subtracting the initial bacterial mass from the final value. According to the experimental data shown in Figure 4, the specific growth rate (μ) of strain *P. chlororaphis* DJ-4 can be calculated for each initial *m*-xylene concentration using the following relationship given in Eq. (1) (Kim *et al.*, 2005):

$$\mu = \frac{\ln(X_2/X_1)}{t_2 - t_1} \quad \dots(1)$$

Where X_2 , X_1 are the cell concentrations at maximum and initial growth time t_2 , t_1 during exponential growth. The specific growth rate of strain *P. chlororaphis* DJ-4 in batch experiments with various initial *m*-xylene concentrations was shown in Fig. 5.

From Fig. 5 it could be found that the specific growth rate increased with an increase in substrate concentration at the beginning, and then the specific growth rate reached its maximum value of 0.33 d^{-1} when the substrate concentration was 81.6 mg l^{-1} . However, the specific growth rate started to decrease when the concentration of *m*-xylene was greater than 81.6 mg l^{-1} . This phenomenon shows that cell growth follows substrate inhibition kinetics, which may be due to cell damage or disruption of membrane integrity at higher *m*-xylene concentrations. The Haldane-Andrew equation (Eq.(2)) was frequently used to quantify the effects of this substrate inhibition (Haldane, 1930; Andrews, 1968).

$$\mu = \frac{\mu_{\max} S}{K_s + S + S^2/K_i} \quad \dots(2)$$

Where μ is the specific growth rate, μ_{\max} is the maximum specific growth rate, S is the concentration of *m*-xylene, K_s is the substrate affinity, and K_i is the inhibition constant. The model equations were solved to fit the experimental data on specific growth rate at different *m*-xylene concentrations by nonlinear regression method using Datafit 9, and the values of the biokinetic constants of the model were estimated with the 99% confidence level. From the result of data fitting,

the predicted value of kinetic parameters μ_{\max} , K_s and K_i were 2.56 d^{-1} , 233.9 mg l^{-1} and 18.1 mg l^{-1} , respectively. A good model fit was obtained with the root mean square (RMS) error of 0.003 and the correlation coefficient (R^2) value of 0.94. Fig. 5

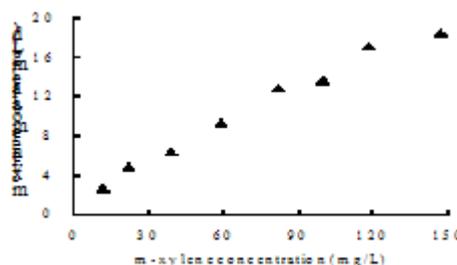


Fig. 3. Relationship between biodegradation rates and *m*-xylene initial concentrations

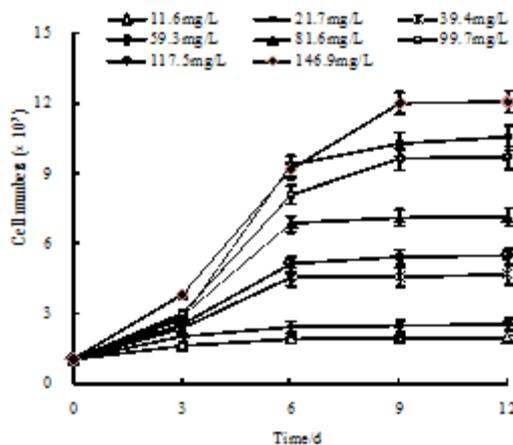


Fig. 4. Increase in cell numbers with time for various initial *m*-xylene concentrations

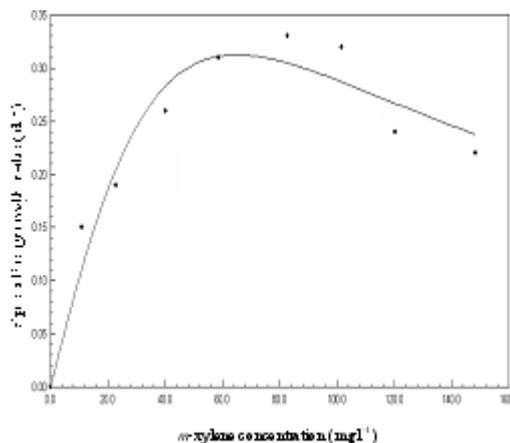


Fig. 5. Experimental and model predicted specific growth rate at different *m*-xylene concentrations

depicts the variation of specific growth rates predicted by the Haldane–Andrew model for μ vs. S using the kinetic parameters described above. From Fig. 5 it could be concluded that the experimental data for specific growth rate over substrate concentration had a good fit to the predicted values (Fig. 5).

CONCLUSIONS

A pure strain was isolated from gasoline contaminated soil and identified as *P. chlororaphis* DJ-4, which could grow well without a detectable lag phase with lower concentrations of *m*-xylene as carbon source. The specific growth rate increased with an increase in substrate concentration at the beginning, and then the specific growth rate reached its maximum value, which obeyed the Haldane–Andrew model when the initial concentration was in the range of 10–150 mg l⁻¹. However, there is a need for further study on the biochemical and genetic aspects of *m*-xylene degradation by the isolated pure culture.

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