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Degradation of polycyclic aromatic hydrocarbons by *Pseudomonas* sp. JM2 isolated from active sewage sludge of chemical plant

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Abstract

It is important to screen strains that can decompose polycyclic aromatic hydrocarbons (PAHs) completely and rapidly with good adaptability for bioremediation in a local area. A bacterial strain JM2, which uses phenanthrene as its sole carbon source, was isolated from the active sewage sludge from a chemical plant in Jilin, China and identified as *Pseudomonas* based on 16S rDNA gene sequence analysis. Although the optimal growth conditions were determined to be pH 6.0 and 37°C, JM2 showed a broad pH and temperature profile. At pH 4.5 and 9.3, JM2 could degrade more than 40% of fluorene and phenanthrene (50 mg/L each) within 4 days. In addition, when the temperature was as low as 4°C, JM2 could degrade up to 24% fluorene and 12% phenanthrene. This showed the potential for JM2 to be applied in bioremediation over winter or in cold regions. Moreover, a nutrient augmentation study showed that adding formate into media could promote PAH degradation, while the supplement of salicylate had an inhibitive effect. Furthermore, in a metabolic pathway study, salicylate, phthalic acid, and 9-fluorenone were detected during the degradation of fluorene or phenanthrene. In conclusion, *Pseudomonas* sp. JM2 is a high performance strain in the degradation of fluorene and phenanthrene under extreme pH and temperature conditions. It might be useful in the bioremediation of PAHs.

Key words: polycyclic aromatic hydrocarbons; *Pseudomonas* sp.; biodegradation; formate; salicylate

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Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous xenobiotics in the environment which consist of two or more fused benzene rings and/or pentacyclic molecules that are arranged in various structural configurations (Kanaly and Harayama, 2000; Zhan et al., 2010). PAHs are introduced into the environment through natural events, such as forest fires, as well as anthropogenic activities, such as incomplete fossil fuel combustion and oil refining. PAHs are persistent because of their low aqueous solubility, low volatility, resistance to biological degradation and strong absorbance on soil and sediments. In particular, PAHs are among the most frequent contaminants of groundwater. The 2- and 3-ring PAHs are of major concern as they are water soluble in the 1–200 µg/L range and are transported with the groundwater over significant distances (Nelkenbaum et al., 2007). Due to their toxic, carcinogenic and mutagenic properties, PAHs are of environmental and human concern and 16 PAHs have been listed by the US Environmental Protection Agency as priority contaminants in ecosystems.

The northeast area of China is an important old industrial base in China. Vehicle exhaust as well as coal and biomass combustion for domestic heating are two

major PAH sources in this area, particularly in winter and spring. It has been shown that sediment samples containing the highest PAH concentrations were found in the upstream area of the Songhuajing River located near the Jilin petrochemical industrial company, in both the flood and icebound seasons. The total PAH concentrations in sediments samples ranged from 84.44 to 14938.73 µg/kg, with average concentration of 2430.37 µg/kg (Guo et al., 2007). The concentration of petroleum hydrocarbon in the surficial sediments from the Songhuajiang River varied from 22.64 to 91.45 mg/kg (Guo et al., 2011). Furthermore, it has been reported that the sum of 15 PAHs ranges from 190 to 8595 µg/kg with phenanthrene accounting for 10.2% in surface soils collected from Dalian, which is a relatively less polluted city in Northeast China (Wang et al., 2007). It is necessary to resolve the PAH pollution problem in Northeast China.

Microbial degradation is the most dominant and significant process for removing and eliminating PAHs from the environment. Since the 1960s, considerable effort has been put toward selecting microorganisms with the ability to degrade PAHs. Many microorganisms capable of metabolizing PAHs were discovered including bacteria, fungi and algae. Most of the bacteria isolated belong to genera *Pseudomonas*, *Sphingomonas*, *Burkholderia*, *Mycobacteria*, *Rhodococcus*, *Alcaligenes*, *Ralstonia*, *Ne-*

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cardioides and others (Cerniglia, 1992; Juhasz and Naidu, 2000; Maeda et al., 2009). Furthermore, some studies have shown that bacteria of the *Rhodococcus*, *Sphingomonas* and *Mycobacteria* genera are able to use the four ring PAHs as sole carbon and energy sources (Mueller et al., 1990; Schneider et al., 1996; Walter et al., 1991). Usually, contaminated sites are polluted by a mixture of PAHs. Thus for an efficient remediation process it is important that the bacteria involved have a complete degradation pathway so that no potentially toxic degradation products accumulate.

Although many bacteria capable of degrading PAHs have already been isolated, it is still important to screen strains that can be acclimatized to Northeast China and can completely and rapidly decompose PAHs. In this work, the isolation and characterization of high performance PAH-degrading bacteria isolated from wastewater sludge in Jilin China was studied.

1 Material and methods

1.1 Chemicals and growth medium

Fluorene, phenanthrene, anthracene and *m*-terphenyl were purchased from Alfa Aesar China (purity > 98%). Mineral salts medium (MSM) was used in enrichment culture and degradation studies. The defined MSM was prepared based on the medium described by Xu et al. (2011). Luria-Bertani agar plate was composed of: 10 g/L NaCl, 10 g/L peptone, 5 g/L yeast extract, and 20 g/L agar (solid MSM plate). All organic solvents used were of high-performance liquid chromatographic (HPLC) grade (SK Chemicals, Ulsan, Korea). Other chemicals used for media were reagent grade (Shanghai Chemical Agent Company, China).

1.2 Isolation and identification of the phenanthrene-degrading strain

A sludge sample was collected from a wastewater treatment facility in the northeastern region of China (Jilin, China). The contaminated sludge (6 mL) was placed in MSM (100 mL), supplied with 4 g phenanthrene and incubated at 28°C for 6 days in a rotary shaker at 170 r/min. After several sub-cultures were performed, the final enriched bacteria culture was spread on MSM agar plates that had been overlaid with 50 mg/L phenanthrene and allowed to dry prior to inoculation with the bacteria. Colonies with different morphologies were individually selected and incubated a second time in MSM containing 4 g phenanthrene. All isolates were tested for their phenanthrene degrading capacity as discussed. One pure strain of bacteria with a high PAH degradation rate was named JM2 and selected for further study. The 16S rDNA of strain JM2 was amplified using a TaKaRa 16S rDNA Bacterial identification PCR kit (Takara Bio, Japan). The 16S rDNA sequences were aligned using the program Clustal X. A phylogenetic tree was constructed according to the neighbor joining method with the program MEGA. The 16S rDNA sequence was deposited in GenBank under accession No. FJ472854.

1.3 Biodegradation of three mixed PAHs

To conduct the biodegradation experiments, bacteria were cultured in MSM supplemented with PAHs to an optical density (OD₆₀₀) of approximately 1.5–2.0. A 150-mL aliquot of the mixed PAH was placed in a sterilized 12-mL amber glass vial and the solvent was evaporated under a fume hood. To investigate the effect of inoculum size on the rate of PAH degradation, a single bacterial clone was cultured in fresh MSM and diluted to an OD₆₀₀ of either 0.1 or 1.0 and added to the glass vial containing the PAH mixture. The inoculum quantity used in the following experiments is OD₆₀₀ = 0.1.

To study the effect of temperature on the degradation of PAHs, fluorene, phenanthrene and anthracene were combined together at a concentration ratio of 1:1:1 in dichloromethane with 50 mg/L each. The acclimated cells were inoculated in vials containing MSM with pH 7.0. The vials were then incubated on a shaker at 4, 10, 18, 28 and 37°C, respectively.

To study the effect of pH on degradation of PAHs, the cells were inoculated in vials containing MSM with pH values between 4.5 and 9.3. The vials were then incubated at 28°C on a shaker.

To determine the influence of additional carbon sources on the bacterial metabolism of PAHs, the medium was supplemented with 1 g/L peptone or 1 g/L yeast extracts. Effects of formate and salicylate addition were also tested by adding sodium formate (20 mmol/L) or sodium salicylate (20 mmol/L) to the system. Then the vials were incubated at 28°C, 170 r/min in the dark.

During degradation, parts of the medium were removed at regular intervals to determine bacterial growth (OD_{600 nm}) and residual PAH concentrations. Cultures inoculated with boiled dead cells were used as controls. All experiments were carried out in triplicate and the data presented are the average of three values.

1.4 Analytical methods

Residual PAHs were extracted ultrasonically for 15 min using 5 mL ethyl acetate, and then a 1 mL aliquot of the organic layer was removed from the vials and transferred to fresh glass vials sealed with a Teflon-lined septum for GC/FID analysis. To each sample, 0.1 mg/L *m*-terphenyl was added as the internal standard. The PAHs in the extracts were separated and quantified with a GC 2010 (Shimadzu, Japan) equipped with a flame ionization detector (FID) and a HP-5 column (length, 60 m; inner diameter, 0.25 mm; film thickness, 0.25 μm; J&W Scientific, Folsom, USA). The samples (1 μL) were injected while the temperature of the splitless injector was maintained at 250°C and the detector temperature was set at 310°C. The oven temperature was initially at 140°C for 1 min, programmed to 240°C at a rate of 10°C/min, and then held at 240°C for 2 min. The PAHs were identified by comparison of the retention times with those of PAH standards.

1.5 Identification of PAH metabolites

Strain JM2 was grown at 28°C and 170 r/min in MSM supplemented with fluorene or phenanthrene or anthracene (2 mg/mL) as the sole source of carbon. After incubation for 1 or 4 days, the cultures were filtered through 0.45 µm water film followed by centrifugation (6000 r/min, 5 min) to remove the undissolved PAHs and bacterial cells. Then the supernatant was acidified with hydrochloric acid to obtain a pH of 1.8, and extracted three times with 50 mL of ethyl acetate. Anhydrous sodium sulfate was then added to the combined ethyl acetate extracts to remove any water, and the extracts were concentrated by rotary evaporation under vacuum at 38°C. Then, the remaining solution was dried under a gentle stream of nitrogen. This was followed by dissolving the residual solution in 100 µL of hexane, derived by adding 100 µL of bis(trimethylsilyl)trifluoroacetamide (BSTFA+TMCS, volume ratio = 99:1), and incubated at 60°C for 60 min. Finally, hexane was added to 1 mL of the samples for metabolite analyses using GC/MS (HP 6890N GC with HP 5975 Inert MSD, Agilent, USA), equipped with a HP-5 column (30 m×0.25 mm×0.25 µm). The mass spectrometer was used in the 70-eV electron impact mode, and a quadrupole mass filter (150°C) was used to scan from m/z 50 to m/z 550 per second. The injector and ion source temperatures were 250 and 230°C, respectively. The carrier gas was helium at a flow rate of 1 mL/min. The column temperature program started at 120°C for 2 min, was raised to 280°C at a rate of 2°C/min, and was held at 280°C for 10 min. The active hydrogen atom(s) of the PAH metabolite was replaced by trimethylsilyl (TMS) group (Si(CH₃)₃), m/z 73) due to the derivatization by BSTFA.

2 Results and discussion

2.1 Identification and characteristics of the phenanthrene-degrading strain

It is well known that the mean annual temperature of Northeast China is 6°C. Although many bacteria that are capable of degrading PAHs have been isolated, strains that can degrade PAHs completely and rapidly with good

adaptability to the environmental conditions will be favored. To obtain strains which could live and degrade PAHs under low temperature conditions in Northeast China, bacteria strains were isolated from the sludge of an active wastewater-treatment plant located in Jilin, China. From these isolations, a strain named JM2 was chosen based on its utilization of phenanthrene as was observed through the formation of clear zones on solid selective-MSM plates where phenanthrene was the sole carbon source. After three weeks preservation, strain JM2 showed high PAHs degradation ability. Strain JM2 degraded more than 90% of fluorene and phenanthrene, and around 40% of anthracene within 4 days. The 16S rDNA gene sequence of JM2 was aligned with the sequences deposited in the GenBank database using a BLAST search and was revealed to be 99% identical to *Pseudomonas pseudoalcaligenes* strain B50 (GenBank Accession No. DQ837704). The results of the phylogenetic analysis also showed that strain JM2 belonged to the genus *Pseudomonas* (Fig. 1). This genus is one of the most widely identified and studied degraders of PAHs as well as of other organic recalcitrant pollutants (Chen and Aitken, 1999; Zhao et al., 2009).

2.2 Effect of environmental conditions on degradation of PAHs

In general, as temperature increases the microbial activity, enzymatic reactions and PAH solubility also increase, thereby increasing biodegradation (Boopathy, 2000; Sartoros et al., 2005; Zhang et al., 2009). Our study showed that both the degradation rate of PAHs and the growth rate of JM2 increased as temperature increases (Fig. 2). The optimum temperature for PAH degradation by JM2 was 37°C, while only a lag time occurred at 18 and 28°C. More than 90% of fluorene and phenanthrene and around 40% of anthracene were degraded at 18, 28 and 37°C during 96 hr (Fig. 2). The result at 18°C is consistent with an early report of Eriksson et al. (1999), who showed a complete biodegradation of naphthalene at 20°C. Interestingly, JM2 still degraded both fluorene and phenanthrene at 4 and 10°C by 50% and 30%, respectively. Anthracene was degraded by 16% at 10°C (Fig. 2) while Eriksson et al. (1999) demonstrated that there was no biodegradation of

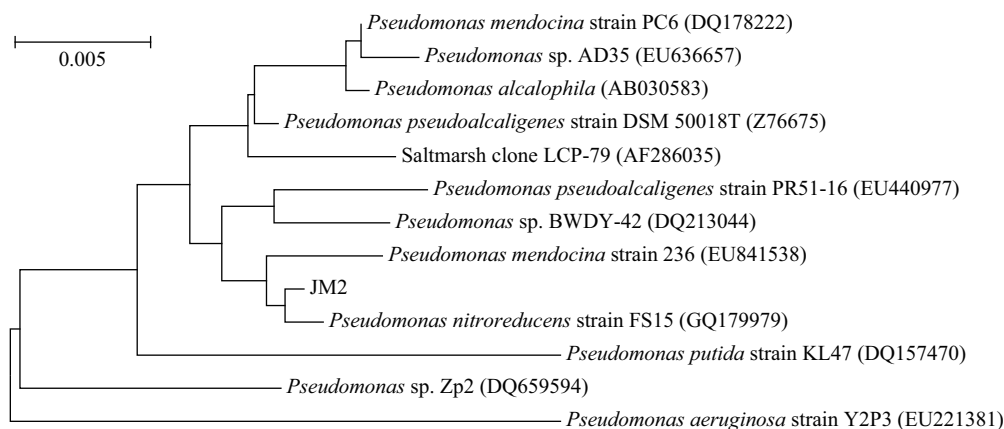


Fig. 1 Phylogenetic neighbor-joining tree with bootstrap values based on 16S rDNA sequences, showing the position of strain JM2 (FJ472854) relative to related strains.

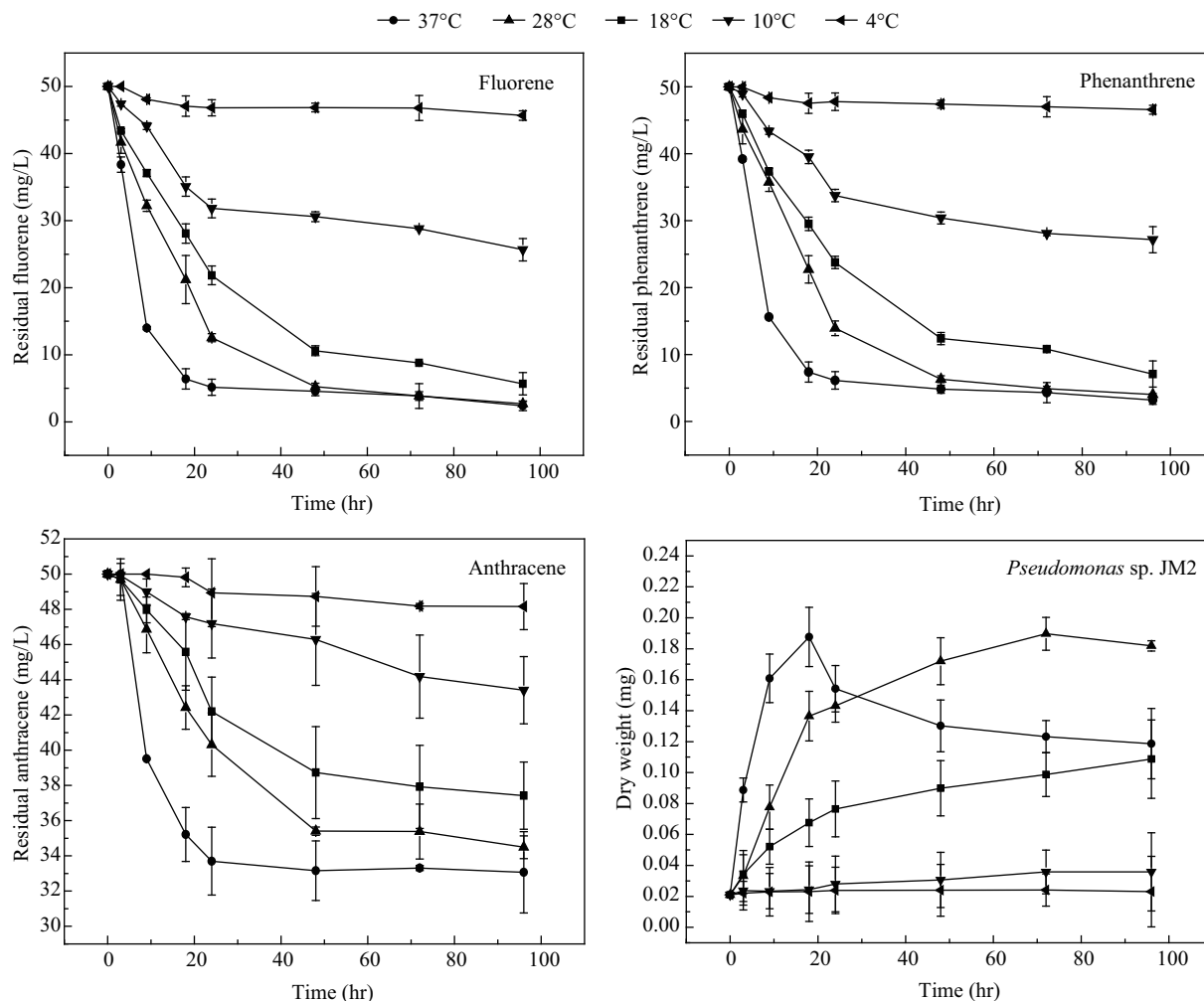


Fig. 2 Effect of temperature on biodegradation of mixture of fluorene, phenanthrene and anthracene (each 50 mg/L) and growth of *Pseudomonas* sp. JM2.

naphthalene at 6°C. A negative effect of decreasing temperature on PAH mineralization was also reported during the biotransformation of anthracene and pyrene by an enrichment culture (Sartoris et al., 2005). The degradation of PAHs by JM2 at low temperatures would be useful in the bioremediation of PAHs in Northeast China where the temperatures are lower in winter.

The pH could affect the physiological and biochemical properties of specific microorganisms in the biotransformation of PAHs. Also, pH is a selective environmental factor affecting microbial diversity and activity, controlling enzyme activity, transport process and nutrient solubility; its effect on the biodegradation and growth of JM2 was assessed (Fig. 3). The isolate showed no significant change in growth and PAH degradation from pH 6.0 to 8.0 (Fig. 3). This is similar to the report by Wong et al. (2002), who observed that pH 5.5 affected phenanthrene degradation, but the growth of the isolates over the pH range 5.5–7.5 was not significantly different. After 96 hr, JM2 degraded more than 94% of fluorene and 92% of phenanthrene between pH 6.0 and 8.0. These results revealed that the bacterium was tolerant to a wide pH range, which may be an advantage when using JM2 for large-scale field-based remediation. However, the pH value had no significant effect on the anthracene degradation. The degradation

percentage of anthracene was between 20% and 40% at all pH levels. Comparatively, lower bacterial growth or PAH degradation was observed at either end of the pH spectrum tested (pH 4.5 or 9.3). At a high pH (9.3), the bacterial growth rate was the lowest and fluorene and phenanthrene were degraded approximately 40%. At a pH of 9.3, floc formation was also observed during the experiment, suggesting that a basic environment was probably inhibitory to bacterial growth. Acidification of the medium to pH 4.5 degraded fluorene and phenanthrene content by more than 60%. A pH of 4.5 might increase the cell membrane permeability and result in the accumulation of PAH substrates and their toxic metabolites in the cytosol (Wong et al., 2002; Yuan et al., 2000). All the results showed that JM2 grew and metabolized PAHs within a broad pH range which is advantageous in bioremediation, particularly for application in Northeast China where the black soil typically has a low pH.

2.3 Effect of additional nutrition, formate and salicylate

Biostimulation agents are commonly used to overcome limitations in microbial activity. The addition of nutrients has proven effective in enhancing PAH degradation, which was attributed to increased growth of microorganisms, thus enhancing degradation (Lee et al., 2003). The effects of

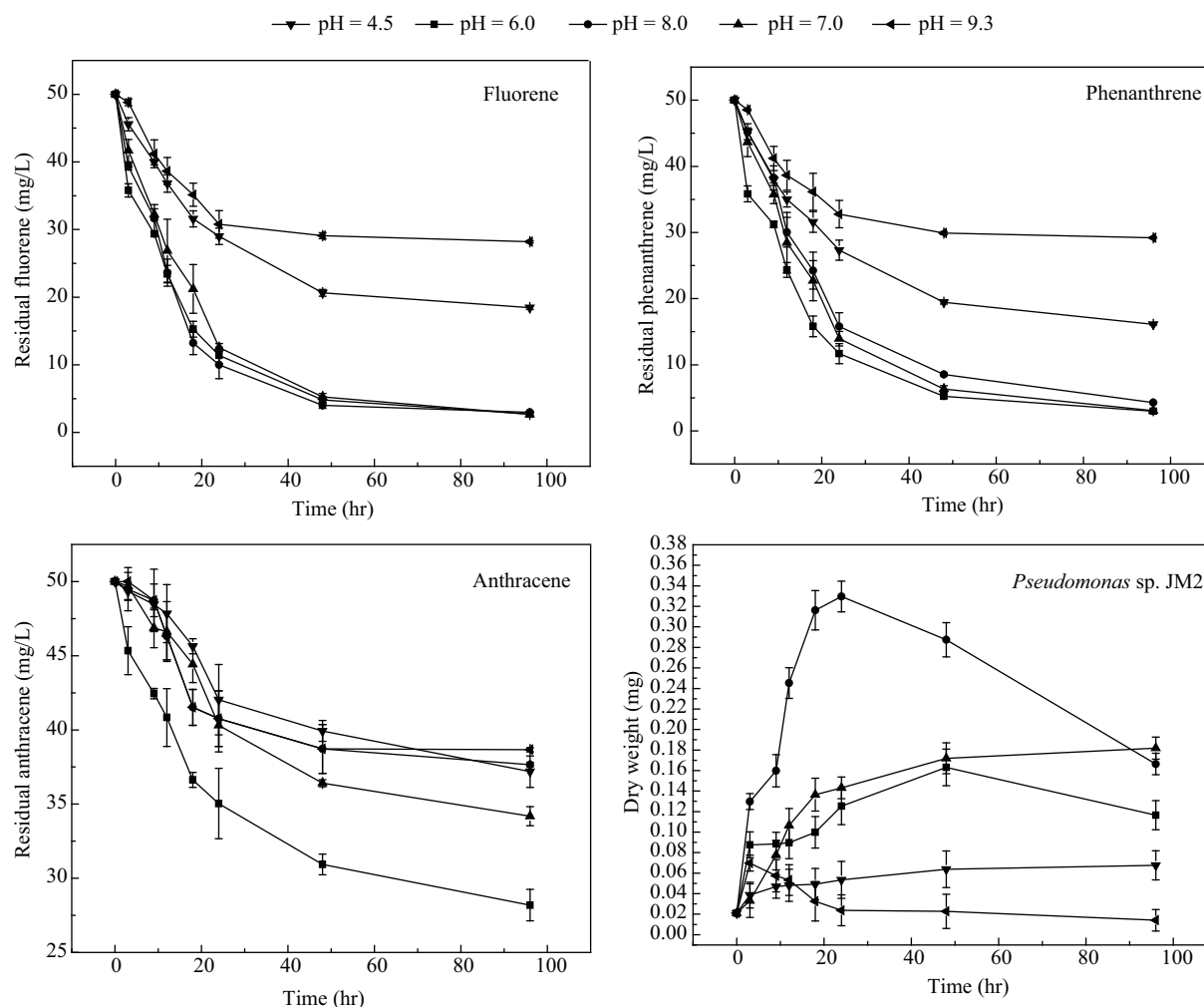


Fig. 3 Effect of different pH on the biodegradation of mixture of fluorene, phenanthrene and anthracene (each 50 mg/L) and growth of *Pseudomonas* sp. JM2.

different organic nutrients on the degradation of PAHs by JM2 were assessed (Fig. 4). All the nutrients tested were able to accelerate the growth rate of JM2 (Fig. 4d). In the first 48 hours, peptone, yeast extract and salicylate increased the biomass of JM2 by 10, 10 and 20 times, respectively, while the effect of formate was limited (Fig. 4d).

The efficacy of nutrient additions on PAH degradation was also investigated (Fig. 4a–c). The addition of peptone and yeast extract to the growth media had no significant effect on the degradation of fluorene and phenanthrene, while they increased the biodegradation of anthracene from 19.49% to 40%–52%. It has been reported that organic carbon source supplementation can prove effective in enhancing PAH degradation, which was attributed to the increased growth of microorganisms (Kim et al., 2003; Zhong et al., 2007). This finding agreed with the report that addition of nutrition increased the degradation of PAH to a greater extent than phenanthrene for *Burkholderia cepacia* 2A-12 (Kim et al., 2003).

The observation that peptone and yeast extract did not promote the biodegradation of fluorene and phenanthrene in this study may be related to the high degradation ability of JM2, which degraded all of them in a short time in MSM medium. Fluorene and phenanthrene had

disappeared before the promotion effects of peptone and yeast extract occurred. Conversely, because the biodegradation of anthracene by JM2 was slow, the degradation of anthracene by JM2 increased as a result of the enhanced biomass caused by the addition of peptone and yeast extract.

Some alternate energy substrates that are not oxidized by the oxygenase enzymes, such as formate, acetate and ethanol, may be used to increase the PAH degradation rate in aerobic co-metabolism by providing reductant NAD(P)H, which did not result in competitive inhibition (Chu and Cohen-Alvarez, 1998). This is the first study to examine the addition of formate on the degradation process of PAHs by a *Pseudomonas* sp. strain. The results showed that the addition of formate increased the microbial biomass up to 3 times (Fig. 4). The degradation rate of fluorene and phenanthrene by JM2 also increased within the first 48 hours. Moreover, the addition of formate increased both the rate and amounts of anthracene degradation. It is known that oxygenase enzymes consume molecular oxygen and reductants such as NAD(P)H or ubiquinone during the oxidation of both primary and co-metabolic substrates under aerobic conditions. However, when an alternate energy substrate is present, such as formate was in this study, it could be utilized by a microorganism to

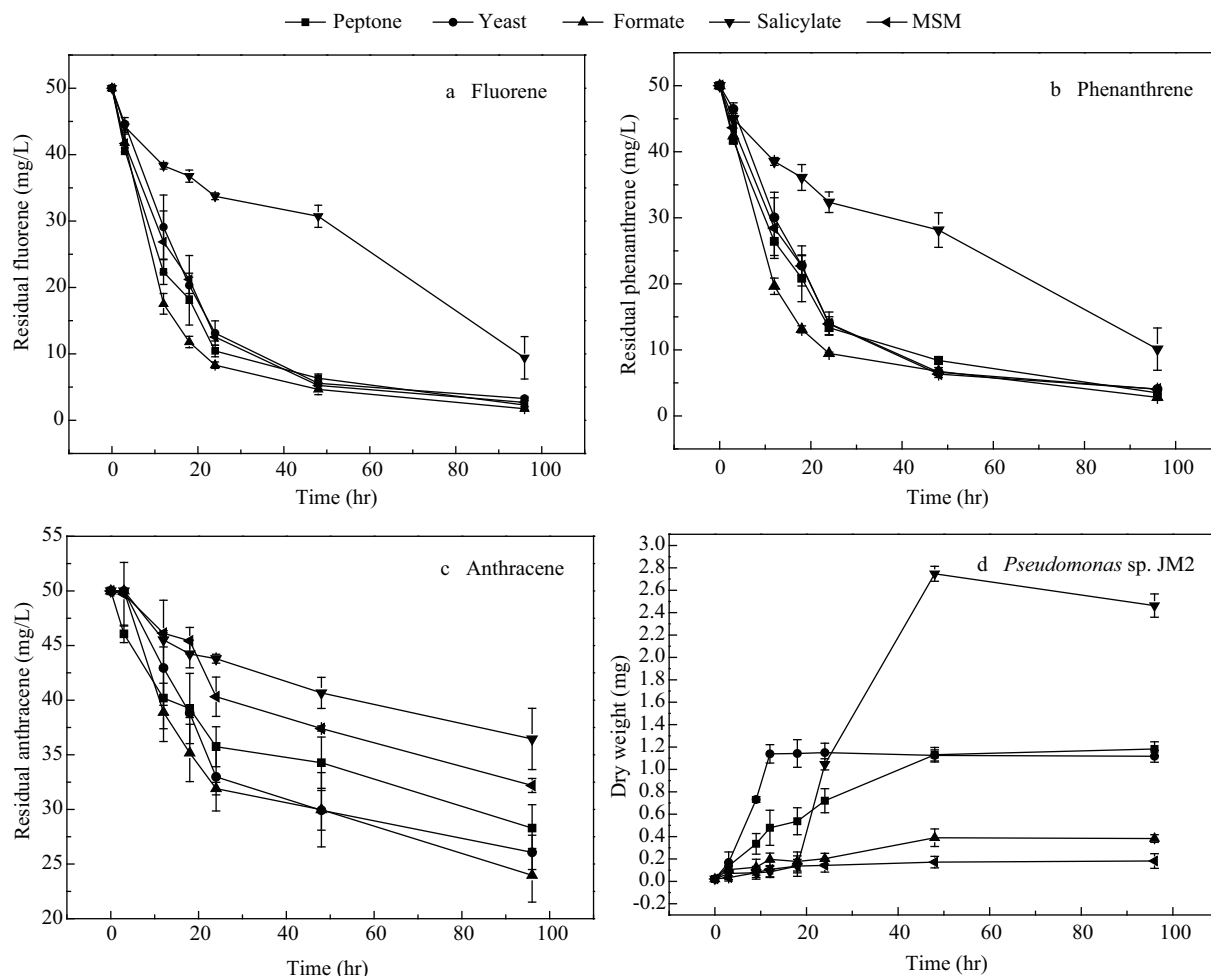


Fig. 4 Effect of adding 1 g/L peptone, 1 g/L yeast extract, 20 mmol/L formate and 20 mmol/L salicylate on biodegradation of mixture of fluorene (a), phenanthrene (b) and anthracene (c) (each 50 mg/L) and the growth of *Pseudomonas* sp. JM2 (d). MSM: mineral salts medium.

regenerate the reductant, allowing co-metabolic oxidations to be carried out without limitations due to either reducing energy depletion or competitive inhibition (Alvarez-cohen and Speitel, 2002).

It has been hypothesized that the addition of relevant pathway intermediates might affect the number of bacteria capable of degrading a range of PAHs (Chen and Aitken, 1999; Rentz et al., 2004). Salicylate, an intermediate in the metabolism of some PAHs, has been shown to induce PAH degradation in some bacteria and has also been used as a biostimulant to increase the abundance of naphthalene-degrading bacteria in soils (Herwijnen et al., 2006; Tian et al., 2003). However, our results showed that the biodegradation rate of JM2 was inhibited by salicylate even though the biomass of JM2 increased up to 20 times. We postulated that salicylate was more feasible as a carbon source for JM2 compared with PAHs. Also, before salicylate was used by JM2, only limited PAHs were consumed.

2.4 Identification of fluorene and phenanthrene metabolites

The metabolism of PAHs by bacteria has been measured for many years. Most bacteria utilize the *nah* pathway to degrade PAHs in aerobic conditions. In the *nah* pathway, phenanthrene is metabolized from an initial

dioxygenation, and after several additional biochemical reactions, 1-hydroxy-2-naphthoic acid is formed. Then it is further degraded either through salicylic acid and catechol or through phthalic acid and protocatechuic acid (Seo et al., 2007; Stingley et al., 2004; Tony and Sanro, 2010). GC/MS analysis of intermediate metabolites showed fluorene was biotransformed to 9-fluorenone (m/z , 180) detected at 17.721 min (Fig. 5a1). We postulated that JM2 initiated fluorene degradation at the C-9 position through mono-oxygenation to 9-hydroxyfluorene, which was then dehydrogenated to 9-fluorenone in aerobic conditions (Luan et al., 2006; Selifonov et al., 1993). Moreover, phthalic acid (m/z , 310) was detected at 18.938 min during degradation of fluorene (Fig. 5a2) and phenanthrene (Fig. 5b1), and salicylate (m/z , 280) was detected at 20.585 min when phenanthrene was transformed by JM2 (Fig. 5b2). These indicated two pathways, including a salicylic acid pathway and phthalic acid pathway, involved in further degradation after one of the PAHs rings was broken.

3 Conclusions

In summary, JM2, a strain of *Pseudomonas* sp., is capable of transforming polycyclic aromatic compounds, and can utilize phenanthrene as its sole carbon source. Optimal degradation conditions for strain JM2 were determined to

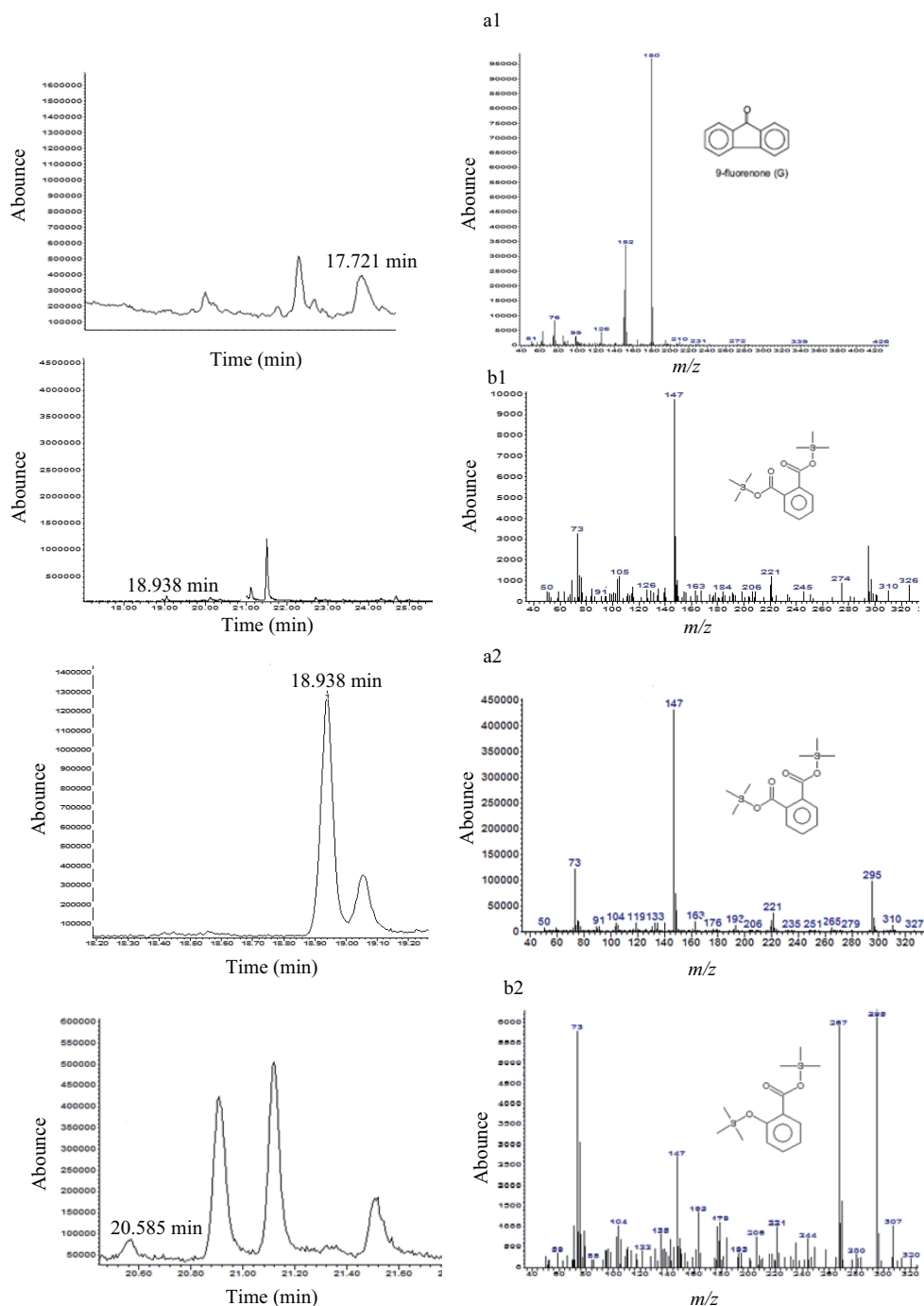


Fig. 5 GC/MS analysis of metabolites after fluorene (a1, a2) and phenanthrene (b1, b2) was biotransformed by *Pseudomonas* sp. JM2.

be at pH 6.0 and 37°C. JM2 was also shown to have a tolerance to lower temperature down to 4°C and pH to 4.5. Moreover, the addition of nutrients showed different effects on the degradation of PAHs by JM2. The investigation of intermediate metabolites indicated that the *nah* pathway was involved in PAH degradation. Our results indicated *Pseudomonas* sp. JM2 possesses the capability for PAH degradation under aerobic conditions and might be useful in bioremediation, particularly in northeastern China.

Acknowledgments

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