Biodegradation of phenanthrene by *Pseudomonas* sp. BZ-3, isolated from crude oil contaminated soil

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**Abstract**

A phenanthrene (PHE) degrading bacterium strain BZ-3 was isolated from the crude oil contaminated soil in Binzhou, China. The isolate was identified as *Pseudomonas* sp. BZ-3 on the basis of 16S rRNA gene sequence. Various experiments were conducted to investigate the effect of pH, salinity and PHE concentration on the degradation efficiency of PHE. The degradation efficiency and degradation metabolites of PHE were detected by using GC–MS and HPLC–MS analyses. The strain BZ-3 could degrade 75% of PHE at an initial concentration of 50 mg/L under 20 g/L salinity in 7 days. PHE degradation kinetics was estimated in a first-order degradation rate model and the rate coefficient was calculated as 0.108 d<sup>−1</sup>. On the basis of the identified degradation metabolites, the strain BZ-3 could degrade PHE in the salicylate metabolic pathway. In a mixture system consisting of PHE and other PAHs including naphthalene (NA), anthracene (ANTH), and pyrene (PYR), the strain BZ-3 showed an efficiently degradation capability. Further study showed that the strain BZ-3 could also use NA, ANTH, PYR, xylene, 1-hydroxy-2-naphthoic acid, and hexane as the sole carbon and energy source, but did not grow on nitrobenzene-containing medium.

**Keywords:** Biodegradation, Phenanthrene, Halophilic microorganism, Metabolic pathway, *Pseudomonas*

**1. Introduction**

Many marine and coastal sites near urbanized centers are frequently polluted by polycyclic aromatic hydrocarbon (PAH) compounds from occasional accidents, such as using in industrial processes, oil spills, and transport (Christensen and Rorrer, 2009). PAHs are aromatic hydrocarbons with two or more fused benzene rings from natural and human activities (Feng et al., 2012). In the past decade, PAHs as a class of organic compounds which having prolonged persistence, recalcitrance, mutagenic and carcinogenic properties have attracted significant attention (Pinyakong et al., 2000). The U.S. Environmental Protection Agency has categorized 16 PAHs as priority pollutants because of their abundance, toxicity and intrinsic chemical stability (Keith and Telliard, 1979).

Bioremediation is a technique utilizing biological organisms to aid in removal of hazardous substances from polluted area (Lin et al., 2014). Microbial degradation is proved an efficient, cost effective and environmental-friendly technology to detoxify PAHs contaminants (Chauhan et al., 2008). Due to their adaptability, rapid population growth and highly efficient metabolism, microorganisms are accepted as a significant role in treating those PAHs contaminated sites (Roy et al., 2012). Phenanthrene (PHE), a tricyclic aromatic hydrocarbon with three-fused rings in an angular fashion, is commonly used as a model compound for PAH biodegradation studies (Roy et al., 2012; Gao et al., 2013). Halophilic or halotolerant PHE-degrading bacteria have the capability of using PHE as a source of carbon and energy (Bogan et al., 2003). A variety of halophilic microorganisms have been isolated from marine or saline water and sediment for the degradation of PHE (Li and Bai, 2005). Compared with non-halophilic degraders, halophilic degraders could treat the contaminants, as well as maintaining an osmotic balance with their external environment (Zhuang et al., 2010). To the best of our knowledge, even though many studies were conducted on halophilic microorganisms for biodegradation of PAHs (Melcher et al., 2002), less attention has been paid to the environmental operation factors and metabolic products during the biodegradation process (Feng et al., 2012).

In this study, a halophilic PHE-degrading bacterium was isolated from a crude oil contaminated site. The isolated strain was found to be affiliated with the genus of *Pseudomonas* and designated as BZ-3. We investigated the biodegradation efficiency in various parameters, including pH, salinity, PHE concentration and different carbon sources. Using HPLC–MS and GC–MS analyses, the degradation...
efficiency by the halophilic strain Pseudomonas sp. BZ-3 and its metabolites were detected. It emerged that 75% of PHE could be degraded with a pH of 7.0 and a salinity of 20 g/L. According to the results of mass spectrometry, a possible metabolic pathway for the biodegradation of PHE by Pseudomonas sp. BZ-3 was elucidated.

2. Materials and methods

2.1. Chemicals and materials

Naphthalene (NA), phenanthrene (PHE), anthracene (ANTH), pyrene (PYR), xylene, catechol, 1-hydroxy-2-naphthoic acid, salicylic acid, coumarin, 1-naphthol and diphenic acid were purchased from Aladdin Chemistry Co. Ltd. (Shanghai, China) in analytical grade. All organic solvents used in this study were of high-performance liquid chromatographic (HPLC) grade.

2.2. Isolation and identification of the PHE-degrading strain

The crude oil contaminated soil collected from Binzhou, China, was incubated in the mineral salt medium (MSM) with addition of PHE. The MSM solution consisted of the following nutrients per liter: (NaH2PO4 3 g; KH2PO4 0.6 g; Na2HPO4 12H2O 1.50 g; NaCl 20 g; MgSO4·7H2O 0.60 g; FeCl3·6H2O 0.50 mg; FeSO4·7H2O 2.0 mg; MnCl·4H2O 0.30 mg; ZnSO4·7H2O 0.25 mg; CoCl·6H2O 0.30 mg and Na2MoO4·2H2O 0.30 mg. The pH of the medium was adjusted to 7.2–7.4 by NaOH and/or HCl (Tao et al., 2007). An enrichment technique was used to obtain the desired microbial consortia according to the previous literature (Ling et al., 2011; Masakorala et al., 2013). First, 2 g of soil was inoculated into the 250 mL volume Erlenmeyer flask containing 100 mL of sterilized MSM medium with 100 mg of PHE, the flask was incubated at 30 °C with shaking at 180 rpm. After incubation for two weeks, 5 mL of the upper aqueous phase was transferred to another fresh flask with 100 mL of MSM containing different amount of PHE (500 mg, 1000 mg and 4000 mg) as the sole carbon and energy source, respectively. Following one week incubation period, dilutions of final enrich culture in water phase were spread on the PHE coated MSM agar plates through the conventional spread plate technique. Colonies were harvested from dilution plates based on distinct colony morphology, and transferred to fresh PHE coated MSM agar plates several times to ensure culture purity. Single colony of each isolate was transferred to 10 mL of MSM solution, aliquots (1.8 mL) of liquid culture were used for DNA extraction using Ultra-clean microbial DNA isolation Kit (MoBio Laboratories, Carlsbad, CA) while 1 mL residual was cryopreserved at −80 °C with 1 mL of 50% glycerol (Tao et al., 2007).

Further identification was carried out using the 16S rRNA gene sequencing. The 16S rRNA gene of each isolate was PCR amplified and sequenced using universal primers 27F (5′-AGAGTTGATCMTGGCTCAG-3′) and 1492R (5′-CGGYTACCTTGTTACGACTT-3′) as described previously (Lin et al., 2014). Sequences were analyzed using the BLASTn tool at the National Center for Biotechnology Information (NCBI) website. Isolates were presumptively identified according to the identity of closest cultured relative in the top BLAST hits.

2.3. Biodegradation of phenanthrene

The experiments were conducted to investigate the effects of several factors (pH, salinity and PHE concentration) on PHE degradation. Strain Pseudomonas sp. BZ-3 (5.6 × 10^9 cfu) was cultivated in 100 mL Erlenmeyer flasks containing 50 mL of MSM medium supplemented with PHE as a sole source of carbon and energy at 30 °C in a rotatory shaker (180 rpm) (Seo et al., 2006). Hence, the effect of pH on the biodegradation rate was assessed by modifying pH values in medium to the value 4, 5, 6, 7, 8, 9 and 10 individually. pH was adjusted using either NaOH and/or HCl solutions. The influence of salinity on PHE biodegradation was investigated by using different NaCl concentrations set at 20, 30, 50, 70, 90, 120 g/L, respectively. The stock solution (1 mg/mL) of PHE in acetone was injected respective volume into 100 mL Erlenmeyer flasks and allowed to evaporate acetone in the laminar hood under the airflow. After forming a thin film of PHE in the bottom of the flask, 50 mL of sterilized MSM medium was added into each flask to achieve the final concentration of PHE at 25, 50, 100, 200 and 400 mg/L, respectively (Masakorala et al., 2013). Under 7 days’ co-inoculation with PHE at 30 °C and 180 rpm, the biodegradation efficiency was measured. A control containing all of the materials without an inoculum was used to detect any abiotic loss of PHE. All experiments were carried out in triplicate (Feng et al., 2012).

2.4. Analytical method

Biodegradation of PHE experiments were conducted in MSM medium (50 mL, pH 7) which supplemented with 50 mg/L PHE as sole carbon and energy source. The flasks were inoculated with 5.6 × 10^10 cfu bacterial culture of the strain BZ-3 and incubation for 7 days with shaking at 180 rpm, 30 °C. Quantitative analysis of PHE was carried out by extracting 50 mL of the cultures three times with 30 mL cyclohexane. The extracts were combined and dried with anhydrous Na2SO4. The solvents were evaporated under reduced pressure and the residues were dissolved in hexane. PHE was analyzed by gas chromatography (GC, Agilent 7890A, USA) with a capillary column (DB-5 model, 30 m long × 0.32 mm diameter × 0.25 mm thick). The GC condition were as follows: injector temperature was 280 °C; column temperature was 60 °C for 5 min, with a ramp to 180 °C at a rate of 3 °C/min, then programmed to 290 °C at a rate of 10 °C/min which was kept for 10 min; detector temperature was 300 °C. The carrier gas (nitrogen) flow rate was 2 mL/min. In order to assess PHE degradation efficiency, control experiments with varied concentrations of PHE were tested, and the remaining PHE concentrations in the cultures were calculated from the standard curve. All experiments were replicated for three times.

PHE metabolites were analyzed by using LC–MS and GC–MS. The cultures were adjusted to pH 2–3 with HCl, and extracted three times using 30 mL ethyl acetate. The organic extracts were combined, dried with anhydrous Na2SO4, and evaporated at 35 °C by a rotary evaporator (IKA, RV10, Germany). The extraction residues were dissolved in methanol (Tao et al., 2007). The metabolites of PHE degradation were analyzed by GC–MS (Agilent 7890A-5975C) and the separation column and column temperatures were as the same as for GC analysis. Trimethylsilyl (TMS) derivatives of the metabolites were prepared for mass spectrometry. The extracted samples were incubated at 60 °C for 40 min with a derivatization reagent including 99% BSTFA (bis(trimethylsilyl) trifluoroacetamide) and 1% TMCS (trimethylchlorosilane) (Aladdin Chemistry Co. Ltd. Shanghai, China) (Stingley et al., 2004). LC–MS analysis of the metabolites was performed in the ESI mode on an LCQ Fleet LC/MS (Thermo Fisher Scientific, USA). Chromatographic analyses used a Hypersil GOLD column (150 × 4.6 mm, 3 μm). Samples were detected at 230–260 nm with a UV–vis diode array detection. The flow rate was 0.6 mL/min and the mobile phase was 45–95% by volume of methanol in water (liner gradient with 45 min).

2.5. Degradation of other PAHs

The degradation ability of Pseudomonas sp. BZ-3 on mixed PAHs (10 mg/L for NA, PHE, ANTH and PYR, respectively) was investigated. Degradation of other PAHs including NA, PHE, ANTH and PYR
were assessed in liquid culture for 7 days as described in Section 2.3 and 2.4. The treatments were in triplicate.

2.6. Utilizing of other organic compounds

Growth of BZ-3 on other carbon substrates including NA, ANTH, PYR, xylene, nitrobenzene, catechol, 1-hydroxy-2-naphthoic acid and hexane in addition to PHE was tested. The strain *Pseudomonas* sp. BZ-3 was inoculated into MSM medium (50 mL, pH 7) with 50 mg/L given compounds to find out the potential to utilize them as sole carbon and energy sources as described in Section 2.3. All the flasks with an appropriate carbon source without PHE were inoculated with 5.6 \times 10^{10} cfu bacterial culture of the strain BZ-3. Following 7 days incubation at 30 °C on a shaker with the speed of 180 rpm, cell growth was recorded by measuring OD_{600}\text{nm} in the medium (Masakorala et al., 2013).

3. Results and discussion

3.1. Isolation and identification

An enrichment culture was performed in MSM containing PHE as a sole carbon and energy source for the isolation of PHE-degrading bacteria from crude oil contaminated sediment collected from Binzhou, China. A total of 10 bacterial strains were isolated. Each isolate was then tested for its ability to grow in MSM medium with the addition of 50 mg/L PHE. Among them, the strain, designated as BZ-3, showed dramatic degrading efficiency of PHE. The strain BZ-3 formed smooth, transparent and wet colonies, which was slight yellow and circular with diameter 1–1.5 mm within 5 days. The bacterium was Gram-negative, rod-like and grew aerobically. Strain BZ-3 was identified as *Pseudomonas* sp. through the comparison of 16S rRNA gene sequence. The closest reference strain (99% identity) was *Pseudomonas* sp. CEB1G with a GenBank accession no. JF439302.

3.2. Factors affecting the biodegradation of phenanthrene

Fig. 1 showed the degradation of PHE by the bacterium strain *Pseudomonas* sp. BZ-3 under different incubation conditions. The biodegradation of PHE at different values of salinity were investigated in medium with addition of different concentrations of NaCl (20–120 g/L). As illustrated in Fig. 1a, the PHE degradation rates decreased, when the concentration of NaCl increased. In the case of low (20 g/L) and high (120 g/L) salinity, biodegradation could still be detected, this demonstrated that the halophilic degrader, strain BZ-3 displayed a very broad salinity profile. Fig. 1b showed that strain BZ-3 exhibited a well biodegradation efficiency (> 60%) under pH values ranging from 6 to 9. The highest degradation rate (79%) was detected at pH 7. Many previous literature (Zhao et al., 2008; Masakorala et al., 2013) have reported the similar behavior which attributes to the negative impact on enzyme activity at acidic pH, highly basic pH. Five initial concentrations of PHE (25, 50, 100, 200, 400 mg/L) were conducted to investigate the effect of PHE concentration on degradation efficiency by the functional bacterium BZ-3. Fig. 1c showed that addition of PHE concentrations from 25 mg/L to 400 mg/L inhibited the biodegradation activity. At a high concentration of PHE (200 mg/L), the biodegradation efficiency was

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**Fig. 1.** Biodegradation efficiency of PHE by *Pseudomonas* sp. BZ-3 under different salinity (a), pH (b) and PHE (c) concentrations. Error bars represent the standard deviation of three independent measurements.
only 17.7%, this might be due to the imposed toxic effect on the growth of the strain BZ-3 by high concentration of PHE. The above results suggested that pH 7 and 20 g/L salinity are the optimum conditions for the PHE biodegradation by the strain BZ-3.

3.3. Degradation of phenanthrene

Biodegradation of PHE by strain BZ-3 was studied with an initial concentration of PHE (50 mg/L) under optimized conditions (salinity 20 g/L, pH 7). As shown in Fig. 2, during the beginning 7 days, an increase of biodegradation efficiency was observed and the results of GC analysis showed that nearly 75% PHE out of the total amount, which could attribute to the increase of the cell density (Feng et al., 2012). However, there was a gradual platform from 14 to 28 days. PHE degradation kinetics was estimated in a first-order degradation rate model. The first-order rate coefficient K1 (day⁻¹) can be calculated by applying non-linear regression to the experimental data according to the equation:

\[ D = A_0 \left( 1 - e^{-k_1 t} \right) \]

where \( D \) is the PHE biodegradation rate at time \( t \), and \( A_0 \) is the initial PHE concentration. The \( K_1 \) value for PHE degradation by strain BZ-3 under the optimized conditions was equal to 0.108 per day. Kim et al. (2009) reported a synergic degradation of PHE by a consortium of isolated bacterial strains which containing the strains Acinetobacter baumannii, Klebsiella oxytoca, and Stenotrophomonas maltophilia, the \( K_1 \) value was calculated as 0.026 per day. Compared to the above microbial flora, the strain Pseudomonas sp. BZ-3 showed a high degradation activity.

The degradation pathway of PHE by the strain BZ-3 is proposed in Fig. 3. The GC–MS analysis of the acid extract of PHE metabolites gave a metabolite (50.03 min) which was tentatively identified as TMS-derivatized 4-[1-hydroxy(2-naphthyl)]-2-oxobut-3-enoic acid (Fig. 4a). The major ions were 386 (M⁺), 371 (M⁺−15, -CH₃ loss), 269 (M⁺−117, -COOSi(CH₃)₃ loss), 254 (M⁺−132, loss of -CH₃ from 269 ion), 211, 178, 147 and 73. 4-[1-hydroxy(2-naphthyl)]-2-oxobut-3-enoic acid which was considered as an intermediate product of the PHE biodegradation. Those indicated that the strain BZ-3 initiates its attack on PHE by dioxygenating at C-3 and C-4 positions to produce cis-3,4-dihydrodiol (Zeinali et al., 2008). A major metabolite that eluted at 32.93 min in the GC–MS analysis was detected. The major ions were contained a molecular ion at m/z = 282 and fragmentation ions at m/z = 267 (M⁺−15, -CH₃ loss), 193 (M⁺−89, -OSi(CH₃)₃ loss), 178 (M⁺−104, loss of -CH₃ from 193 ion), 149, 135, 91 and 73. This metabolite was tentatively identified as TMS-derivatized salicylic acid (Fig. 4b). LC-MS in the negative electrospray mode showed the compounds at 3.29 min, m/z = 187 [M-H–] and 2.93 min, m/z = 159 [M-H–] were identified as the metabolites of PHE degradation, 1-hydroxy-2-naphthoic acid and 1,2-dihydroxynaphthalene, respectively. Masakorala et al.
Degradation capability. PAHs with two rings was degraded more than 26% in the case of PHE, and ANTH was recaltrance to degradation by bacteria, only 18% degraded. PAHs with four rings was degraded more than 26% in the case of PYR. These results, which are similar to those reported by Ma et al. (2013) and Yuan et al. (2000), indicate that BZ-3 might have an effective enzyme system for the degradation of PAHs.

3.5. Growth on other organic compounds

Utilization of different organic compounds as a sole source of carbon and energy by strain Pseudomonas sp. BZ-3 was evidenced by significant increases of OD

7 days incubation. As shown in Table 1, strain BZ-3 could use NA, ANTH, PHE, xylene, 1-hydroxy-2-naphthoic acid, and hexane as the sole carbon and energy source, but not nitrobenzene. It is worth noting that salicylic acid and catechol could be utilized by the strain BZ-3, which were considered as the intermediates in the PHE metabolism via salicylate route (Tao et al., 2007).

4. Conclusion

A halophilic PAHs degrading Pseudomonas sp. BZ-3 was isolated from the soil at the crude oil contaminated site. The strain BZ-3 could efficiently degrade PHE with a broad range of salinities (20–120 g/L) and pHs (6–9). Moreover, the results suggested that the strain Pseudomonas sp. BZ-3 had the ability to degrade PHE (>50%) at a very high salinity (120 g/L). Biodegradation of PHE by the strain BZ-3 linked well to the first-order rate kinetic model with a rate constant of 0.108 d

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Table 1

Utilization of different carbon sources by BZ-3.

| Substrate                  | BZ-3 |
|----------------------------|------|
| Naphthalene                | ++   |
| Anthracene                 | ++   |
| Pyrene                     | +    |
| Xylene                     | +    |
| Salicylic acid             | +    |
| Nitrobenzene               | -    |
| Catechol                   | +    |
| 1-Hydroxy-2-naphthoic acid| +    |
| Hexane                     | +    |

Growth was followed by measuring the increased of OD

7 days. (++) Good growth: OD

> 0.1; (+) growth: OD

> 0.05; (−) no growth OD

< 0.03.
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