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Degradation of phenanthrene by bacterial strain isolated from soil in oil refinery fields in Shanghai China

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ABSTRACT

A bacterial strain *Pseudomonas stutzeri* ZP2 was identified with phenanthrene-degrading ability based on Gram staining, oxydase reaction, biochemical tests, FAME analysis, G + C content and 16S rDNA gene sequence analysis. It is the first time that *P. stutzeri* is reported to process the capability for phenanthrene degradation. The strain was isolated from soil samples contaminated with polycyclic aromatic hydrocarbon (PAH)-containing waste from an oil refinery field in Shanghai, China. Strain *P* sp. ZP2 can utilize naphthalene, phenanthrene and Tween 80 as its sole carbon source and can degrade phenanthrene very fast, 6 days for 96% phenanthrene at 250 ppm concentration. The optimal growth conditions of strain ZP2 was determined to be at pH 8.0, 37 °C, respectively. The results also indicate that strain ZP2 can remove more than 90% of phenanthrene at any concentrations ranged from 250 to 1000 ppm in 6 days. It suggests that strain ZP2 can endure high concentrations of phenanthrene. Besides, the effects of non-ionic surfactants such as Brij 30, Triton X100 and Tween 80, on the phenanthrene degradation were examined. Therefore, this strain may find great application in bioremediation practices.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are common environmental pollutants produced by industrial operations using fossil fuels as well as by natural events such as forest fires [1,2]. PAHs are ubiquitous pollutants found in soil at wood preservation plants, gas works, oil refineries, runoff from asphalt pavement, and combustion process. PAHs are often highly toxic, mutagenic, and carcinogenic [3], so they represent considerable environmental concerns [4–6]. The US Environmental Protection Agency has monitored PAHs as priority pollutants in ecosystems since the 1970s [7]. PAHs released into the environment could be removed though many processes, including volatilization, photo-oxidation, chemical oxidation, bioaccumulation, and adsorption. However, the principal process for successful removal and elimination of PAHs from the environment is the microbial transformation and degradation [8,9]. Nowadays, in order to study the fate of these compounds in natural environments, considerable efforts have been focused on the isolation of microorganisms able to degrade them. Bioremediation, based on certain species of microorganisms, is a cheap and effective way to decontaminate PAHs-contaminated soils. During the last few decades, a variety of bacteria capable of degrading PAHs, particularly low-molecular weight compounds, were discovered [10,11]. Most of these bacteria belong to the genera *Agmenellum, Aeromonas, Alcaligenes, Acinetobacter, Bacillus, Berjerinckia, Burkholderia, Corynebacterium, Cyclotrophicus, Flavobacterium, Micrococcus, Moraxella, Mycobacterium, Nocardioides, Pseudomonas, Lutibacterium, Rhodococcus, Streptomyces, Sphingomonas, Stenotrophomonas, Vibrio, Paenibacillus* and others [11–15,31]. Moreover, some studies have shown that bacteria such as *Mycobacterium*, *Rhodococcus*, *Alcaligenes*, *Pseudomonas* and *Sphingomonas* are able to grow on the four-ring PAHs [16–19]. As to the practical application of the research, it is important whether microbial isolates could be cultivated or not by using reported approaches in soil and sediment environments bioremediation. Grosser [32] established enrichment cultures in which solid organic phases were used to reduce phenanthrene bioavailability to different degrees. Their results are consistent with the hypothesis that different phenanthrene-utilizing bacteria inhabiting the same soils may be adapted to different phenanthrene bioavailabilities. While, Yael [33] found that phenanthrene mineralization was substantially enhanced upon sorption to mineral–HA complexes and the degree of enhancement was positively correlated with the fraction of sorbed phenanthrene. The stimulation is thought to be related to sorption of both the microorganisms and phenanthrene to the colloidal surfaces. Their study suggests that when sorbed

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Table 1
Utilization of carbon substrates by phenanthrene-degrading strain ZP2.

Substrate	ZP2[a]
Naphthalene	+[b]
Phenanthrenex	++
Anthracene	–
Fluorene	–
Benzene	–
Toluene	–
Xylene	–
Phenol	–
DMSO	–
Methanol	–
Ethanol	–
Salicylic acid	–
Tween80	+
Cyclohexane	–

[a] Growth was followed by measuring the increase of OD600 nm of the culture for 10 days.
[b] ++ Good growth: OD600 nm > 0.2, + growth: OD600 nm > 0.1, – no growth: OD600 nm < 0.02.

contaminants are still bioavailable, the presence of surfaces may stimulate mineralization.

Recently, attention has been turned toward diverse PAHs-metabolizing bacteria, degradation mechanisms, assimilation of PAHs into bacterial strains and related catabolic genes [20–22]. In order to achieve an efficient bioremediation process, it is interesting to find new bacteria who can degrade PAHs in a new pathway without accumulating potentially toxic metabolites [23]. If this is not the case, it is important to know which metabolites are expected to accumulate so other strains able to degrade those metabolites can be added if necessary. Strains that can degrade PAHs completely and rapidly with good adjustment will be more favored although many bacteria capable of degrading PAHs have been isolated. In this work, the isolation and characterization of high performance PAH-degrading bacteria from the polluted soils in Shanghai has been taken, strain ZP2 is the first representative of *Pseudomonas stutzeri* sp., able to degrade phenanthrene very fast at high experimental concentration. Its phenanthrene degradation pathway also has been studied (Table 1).

2. Materials and methods

2.1. Media

The minimal medium was composed of (l^{-1}): $Na_2HPO_4{\cdot}2H_2O$ 8.5 g, KH_2PO_4 3.0 g, NaCl 0.5 g, NH_4Cl 1.0 g, $MgSO_4{\cdot}7H_2O$ 0.5 g, $CaCl_2$ 14.7 mg. MM also contained trace elements as follows (l^{-1}): $CuSO_4$ 0.4 mg, KI 1.0 mg, $MnSO_4{\cdot}H_2O$ 4.0 mg, $ZnSO_4{\cdot}7H_2O$ 4.0 mg, H_3BO_3 5.0 mg, $H_2MoO_4{\cdot}2H_2O$ 1.6 mg, $FeCl_3{\cdot}6H_2O$ 2.0 mg. Solid MM plate was composed of (l^{-1} MM): 15 g agar. Solid LB plate was composed of (l^{-1}): 10 g NaCl, 10 g peptone, 5 g yeast extract, 15 g agar.

Ten times SSC (saline-sodium citrate buffer) was composed of (l^{-1}): NaCl 87.66 g, $C_6H_7NaO_7H_2O$ (sodium citrate monobasic) 44.12 g. 0.1× SSC: dilute 10× SSC to 100 times. (All these reagents and chemicals were bought from Shanghai Reagent Factory.)

2.2. Isolation of phenanthrene-degrading bacteria

Soil samples were collected from two sites in an oil refinery field in Shanghai, where PAH-containing pollutants had been continually released into environment without any control for more than 30 years. Two grams of soil sample was suspended in 200 ml of MM. Into the suspension 0.025% phenanthrene was added as enrichment substrate and the suspension was incubated with shaking at 180 rpm at 30 °C in the dark. Eight days later, 10-ml aliquot was transferred to 200 ml of a fresh MM containing the same amount of phenanthrene as above and incubated under the same conditions. This process was repeated for four times. Pure cultures were obtained by diluting 1 ml culture to 10^4 times and spreading each 100 μl of aliquots on the solid MM plates, before use, 0.025% phenanthrene dissolved in MM with 1% DMSO (dimethyl sulfoxide), was sprayed on the surface of the medium as the sole carbon source [10,11]. After incubation at 30 °C in the dark for 2 days, colonies, especially those forming clear zones on the sprayed-coated plates, were selected as the candidate phenanthrene-degrading strains [24]. All isolates were stored at −20 °C as the liquid cultures containing 20% glycerol (v/v).

2.3. Phenanthrene degradation assay

The ZP2 liquid culture (300 ml of MM with 0.025% phenanthrene, w/v) was inoculated by transferring 3 ml of pre-culture (MM liquid medium, OD_{600nm} = 0.3, about 3×10^7 cells/ml) of ZP2 (1%, v/v). Control experiment was performed to inoculate the boiled cells of ZP2. The flasks were incubated in darkness at 180 rpm at 37 °C, and the initial pH was adjusted to 8.0. All biodegradation experiments were performed with 250 ppm phenanthrene as the sole carbon and energy source. Some factors were tested including other carbon sources, e.g. yeast extract, peptone, glucose at 1000 mg l^{-1}, and non-ionic surfactants such as Brij 30, Triton X-100 and Tween 80 at 1, 20 and 100 critical micelle concentrations (CMC) respectively. Effects of various pH (6.0, 7.0, 8.0) and different temperature (20 °C, 30 °C, 37 °C) have been investigated too.

Phenanthrene biotransformation by the bacteria under different concentrations was determined in parallel by using Erlenmeyer flasks containing phenanthrene range from 250, 500 to 1000 ppm, respectively. Control experiment was conducted to inoculate the boiled cells of ZP2.

After desired period of incubation, 5 ml of aliquot was sampled every day and the culture was extracted twice thoroughly with 5 ml of chloroform. Every aliquot has three duplicates. The organic phase extractions were combined and dried over with anhydrous sodium sulfate. 1.0 μl of the organic phase was analyzed by gas chromatography (GC) [34] (Thermo FINNIGAN, TRACE GC Ultra) under FID mode, using a fused silica capillary column (30-m length × 0.25-mm ID, 0.25-μm film thickness) and nitrogen as the carrier gas. The temperature program was set as follows: 80 °C for 1 min, then increasing by 15 °C/min up to 240 °C, then lasting for 1 min. Degradation rate was estimated by calculating the GC profile of substrate PAH. Cultures inoculated with boiled dead cells were used in parallel as the abiotic negative controls. Strain cell growth was evaluated by measuring the increase of OD_{600nm} of the culture. Each value represents the mean of three repeats with a standard deviation less then 5%.

2.4. Carbon-source utilization

In addition to phenanthrene, the purified strain was also tested for growth on one of the following compounds at 0.01%: naphthalene, phenanthrene, anthracene, fluorene, benzene, toluene, xylene and other related substrates which were added as sole carbon sources to liquid MM. Sterilized MM medium containing appropriate amounts of PAH or other carbon sources were inoculated with the tested strains and incubated in an orbital shaker as described above. Growth test was followed by measuring the increase of OD_{600nm} of the culture.

All chemicals were of analytical grade and obtained from Sigma or Fluka.

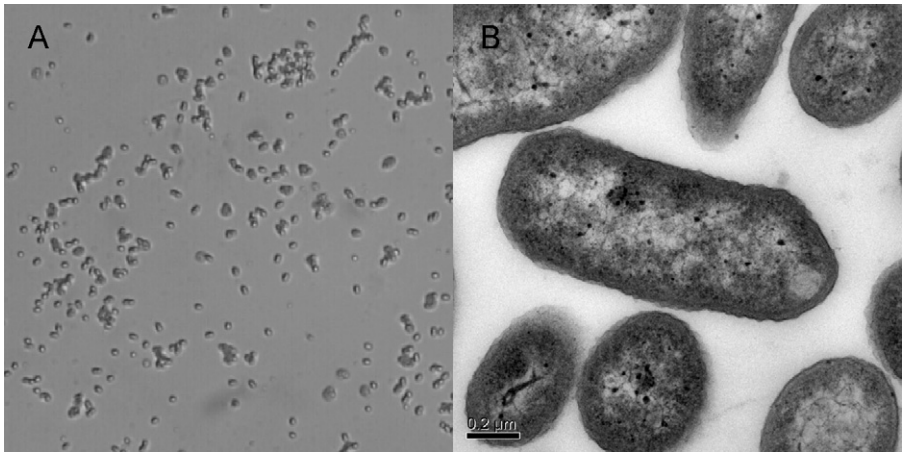

Fig. 1. Photographs of cell of *Pseudomonas stutzeri sp. ZP2*. A: Gram stain photo of ZP2 (10 × 100), B: 2 TEM photo of ZP2 (×80k).

2.5. Biochemical test

The morphology of bacterial cells were examined by transmission electron microscopy (TEM), and included the shape of cell, Gram-stain, the presence of spores, and colony morphology on solid LB plate. Biochemical reactions, catalase reaction, oxidase reaction, acid or gas production from carbohydrates and oxidation or fermentation from carbohydrates were determined by using the API system according to the manufacturer's instructions (Biotech, China).

Whole cell fatty acids analyses were performed by harvesting the cells after culturing at 30 °C for 72 h in MM culture with 0.025% phenanthrene. The cell pellets obtained were first washed with 1 ml of *N, N*-dimethylformamide (DMF) to remove the undegraded phenanthrene and then were washed twice with 0.85% NaCl to remove residual culture medium. Subsequent fatty acids isolation and identification was conducted by the MIDI-MIS method proposed by M. Sakai [25].

2.6. Determination of G + C content

G + C content of genomic DNA from the isolate was determined by making sure the degeneration temperature of DNA (Tm), respectively. DNA has its unique Tm because of the different G + C% content. The DNA of isolates were dissolved in 0.1 × SSC solvent, and the G + C content can be calculated from the empirical equation: 0.1 × SSC G + C% = 2.44 (Tm − 53.9) [35].

2.7. Analyses of 16S rDNA sequences

The chromosomal DNA was isolated by using a method described by Yoon et al. [26]. 16S rDNA was amplified using two primers according to Stackebrandt and Liesack [27]: the forward primer BSF8/20: 5′-AGAGTTTGATCCTGGCTCAG-3′ (primering site corresponding to 8–27 of 16S rDNA of *Eschericha coli*) and the reverse primer BSR1521/20: 5′-AAGGAGGTGATCCAGCCGCA-3′ (primering site corresponding to 1541-1522 of 16S rDNA of *E. coli*). The PCR mixtures were preheated at 94 °C for 2 min prior to running the following cycles: 94 °C for 1 min, 56 °C for 1 min, 72 °C for 2 min. A PCR was run for 30 cycles in a DNA thermal cycle (BIOER TC25/H; BIOER TEC, Hangzhou, China), employing the thermal profile [26]. At the end of the final cycle, a chain-elongation step at 72 °C for 10 min was programmed. The 16S rDNA sequences of the stains were aligned with the BLAST program on the NCBI website (http://www.ncbi.nlm.nih.gov). Phylogenetic trees were constructed using the MegAlign program (DNASTAR).

2.8. Sequence access numbers

The sequence obtained in this study were deposited in the GenBank database. The accession number for the two short fragments is: DQ659594 (strain ZP2).

3. Results and discussion

3.1. Isolation of PAH-degrading bacteria

Strain ZP2 was isolated from PAHs contaminated soil samples in an oil refinery fields in Shanghai, essentially based on the formation of clear zones on solid LB plates with sprayed phenanthrene as the sole carbon source. In contrast to other strains isolated in this experiment which lost their degradation abilities after more than 3 weeks preservation, strain ZP2 showed high phenanthrene degradation ability. As far as our knowledge, *P. stutzeri* sp. ZP2 was the first time to be reported that can use phenanthrene as the sole carbon and energy source. Based on Gram staining, oxydase reaction, biochemical tests, TEM analysis, FAME analysis, G + C content and 16S rDNA gene sequence analysis, Bacteria strain ZP2 was char-

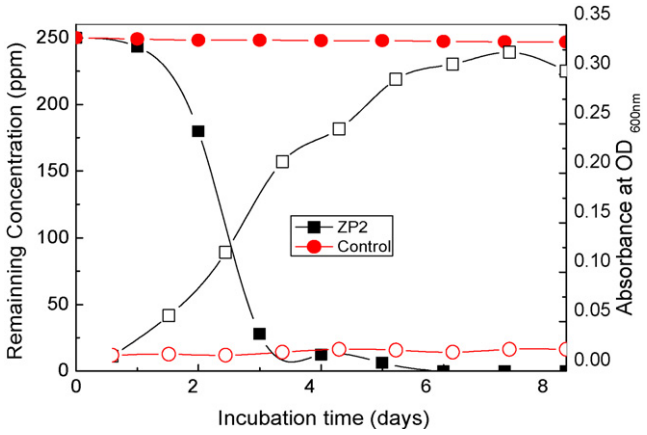

Fig. 2. Growth of ZP2 in phenanthrene at 250 ppm and phenanthrene biodegradation by ZP2. Control was performed by inoculating with dead cells. (Solid symbol is about degradation and hollow symbol is about the absorbance value at OD_{600nm} which indicate the growth of bacterium.)

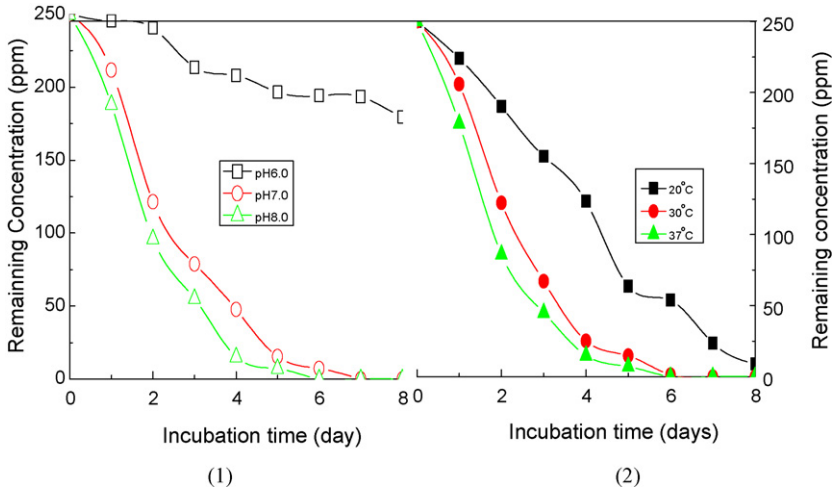

Fig. 3. Effect of different pH value (1) and change in temperature (2) on biodegradation of phenanthrene by strain ZP2 (250 ppm).

acterized belonging to genus *Pseudomonas* (Fig. 1). Many strains from genus *Sphingomonas* and *Pseudomonas* which belong to *α-Proteobacteria* and *β-Proteobacteria* have been reported with the phenanthrene-degradation ability.

3.2. Carbon-source utilization

More than ten carbon sources were tested as carbon substrates for these two isolates, including various low and high molecular weight PAHs and other *n*-alkanes. These chemicals are main components of crude oil and ubiquitous in contaminated soil. Strain ZP2 was found that could not use other PAHs as its sole carbon and energy source except naphthalene, phenanthrene and Tween 80. The carbon-source utilization experiment conveys some useful information about its phenanthrene degradation pathway. In this part, the limited carbon-source utilization of strain ZP2 and its transformation products [36] suggest its potentially different and interesting phenanthrene-degrading pathway.

3.3. Degradation of PAHs

The efficiency of the selected isolates on phenanthrene degradation was individually evaluated by gas chromatography [6]. The results of GC analyses demonstrate that strain ZP2 almost completely degraded phenanthrene within 4 days. As shown in Fig. 2, for one substrate for ZP2, the biomass increases as the remaining concentration of the substrate decreases. This is a very attractive result in that strain ZP2 was first found with the phenanthrene-degrading ability as species *P. stutzeri* [37–39]. It can consume phenanthrene quickly at experimental concentrations. Strains that can degrade PAHs completely and rapidly with good adaptability will be more favored although many bacteria capable of degrading PAHs have been isolated. The genes that encode the large and small subunits of a dioxygenase, *phn* ZP2 and *pha* ZP2 have been identified later. The activity of the dioxygenase indicates the phenanthrene degradation pathway by ZP2.

3.4. Effect of environmental condition on degradation of phenanthrene

A series of phenanthrene degradation tests were carried out at various pH from 6.0 to 8.0 and temperatures from 20 to 37 °C. As shown in Fig. 3(1)–(2), the optimal conditions of ZP2 was determined to be at pH 8.0, 37 °C, respectively. This result is similar to those reported by Kim and Lyudmila [40]. It is also found that strain ZP2 can remove nearly 90% of phenanthrene at any concentration ranged from 250 to 1000 ppm within 6 day (Fig. 4), which is slightly different from the report of Yuan et al. [28]. The result suggests that

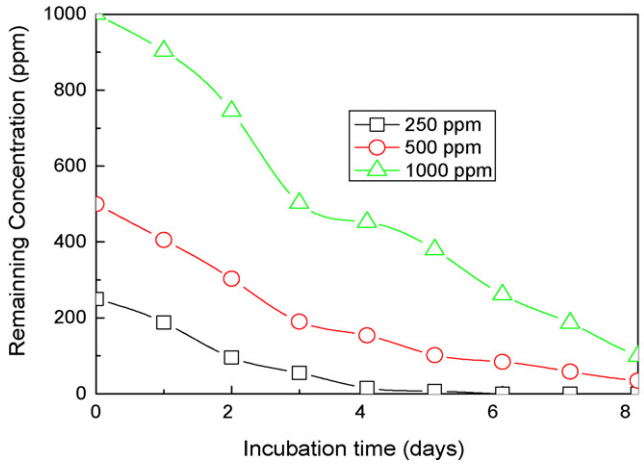

Fig. 4. Effects of changes in phenanthrene concentration on biodegradation of phenanthrene by strain ZP2.

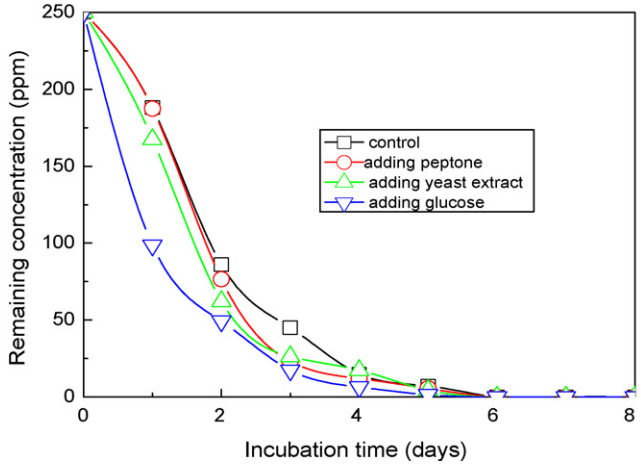

Fig. 5. Effect of various organic nutritions on biodegradation of phenanthrene by strain ZP2 (250 ppm).

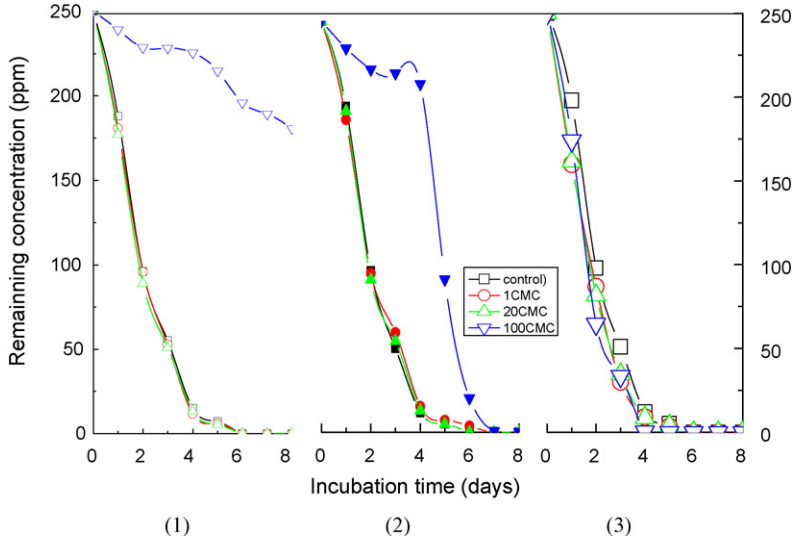

Fig. 6. Phenanthrene degradation by strain ZP2 in the presence of surfactant Brij30 (1) and Trition X-100 (2) and Tween 80 (3).

strain ZP2 can endure high concentrations of phenanthrene and have great potential in bioremediation application. The following study of biodegradation of phenanthrene by strain ZP2 will focus on the microbial transformation and metabolism. Fig. 5 shows the effects of different organic nutritions on the phenanthrene degradation of ZP2. All these tested organic nutritions can improve the efficiency of phenanthrene-degradation of ZP2. This result is consistent with earlier reports with respect to organic carbon source supplementation, which elevated the transformation of PAHs. Zaidi et al. [29] also point out that the supplementation of glucose did not improve degradation of phenanthrene, as carbon was not a limiting factor. In the present study, when these organic nutritions were added, the phenanthrene can be removed nearly two days earlier than without them.

3.5. *Effect of surfactants*

The effects of non-ionic surfactants on phenanthrene degradation were also tested by adding Brij 30, Triton X-100 and Tween 80 at 1 CMC, 20 CMC and 100 CMC, respectively Fig. 6(1–3). To strain ZP2,

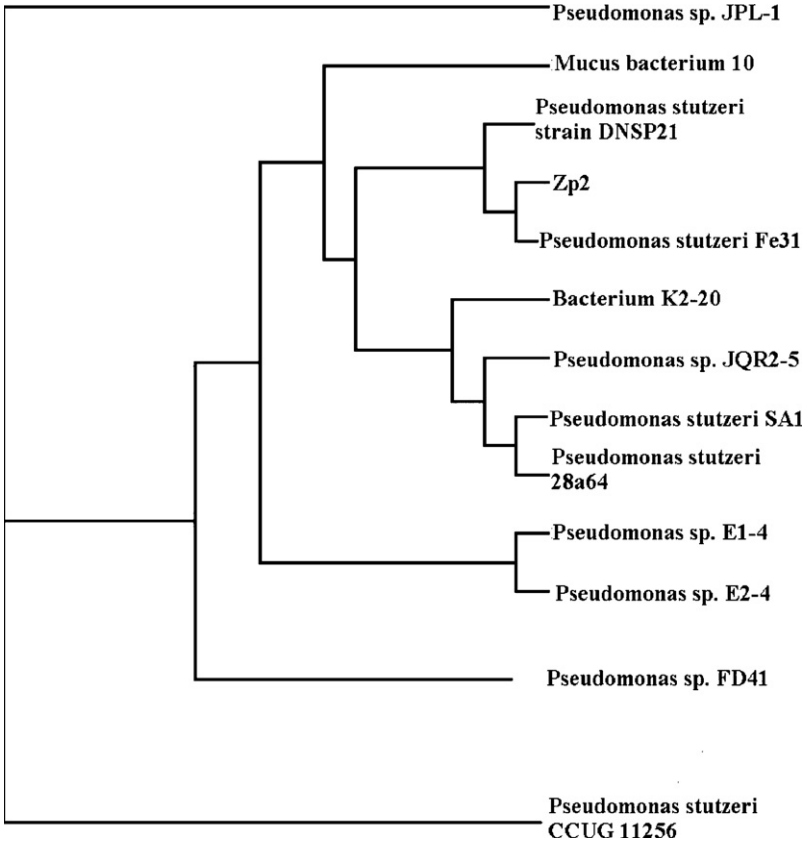

Fig. 7. Phylogenetic trees for the taxonomic location of strain ZP2. This dendrogram was produced by the MegAlign software program of DNASTAR.

both Triton X-100 and Brij 30 can remarkably increase the phenanthrene degradation speed at 1 CMC and 20 CMC, but no significant effect was observed at 100 CMC; as for Tween 80, it can enhance the efficiency of phenanthrene degradation to a large extent at any concentrations. Correspondingly, Kotterman et al. [41] also reported an improvement in PAH bioavailability by adding Tween 80 to fungal cultures. Such phenomenon may be attributed to the higher molecular weight of Tween 80. In carbon-source utilization section, Tween 80 was found could be used by ZP2. Tween 80 was known as good dispersant agent, it can improve the dispersion and solubility of phenanthrene, which in such condition is much easier to be utilized by bacteria. In general, the higher the CMC of surfactants added, the greater the inhibition of phenanthrene biodegradation. Surfactants are known to produce toxicity and therefore decrease the activity of microorganisms [30].

3.6. Taxonomic identification of the isolates

Strain ZP2 was found to be a Gram-negative, oxidase-positive, rod and resistant to cephalexin and ampicillin sodium at concentration of 20 μg/ml. Its optimum growth temperature is 37 °C. Fatty acids mainly contained 9-*cis*-octadecenoic (18:1) (40.3%), hexadecanoic (16:0) (22.9%) and 9-*cis*-hexadecenoic ($16{:}1^{9}$) (26.5%). The G + C content of ZP2 genomic DNA was 63.6%. Analysis of 16S rDNA gene sequence indicates that strain ZP2 belongs to the genus *Pseudomonas* (99% identity) with nearest type strain *P. stutzeri* Fe31 (Fig. 7). Rossello [42] has reported that *P. stutzeri* isolated from ocean can degrade naphthalene. This species has received great attention because of its unique metabolic properties: it has been proposed as a model organism for denitrification studies; many strains have natural transformation properties, making it relevant for study of the transfer of genes in the environment; in addition, several strains are able to fix dinitrogen, and others participate in the degradation of pollutants or interact with toxic metals. To our knowledge, this is the first time that *P. stutzeri* is reported to process the capability for phenanthrene degradation. This ability makes these two strains attractive for phenanthrene bioremediation.

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