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Isolation and Identification of Phenanthrene-degrading Bacteria and Increasing the Biodegrading Ability by Synergistic Relationship

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Abstract

Background: Polycyclic aromatic hydrocarbons are a large group of oil contaminants with carcinogenic, mutagenic and teratogenic effects. The release of these compounds in soil destroys animals, plants and microbial diversity and has several negative impacts on physical properties of the soil including the destruction of soil aggregates reduction in pores, and increase in soil bulk density. Many strains of microorganisms isolated have the phenanthrene-degrading ability but this study focused on isolation and identification of a phenanthrene-degrader bacterium for bioremediation of contaminated soils.

Materials and Methods: Enrichment technique was used for isolation and the most effective isolates, were named *pseudomonas aeruginosa* ZF1 and *Serratia marcescens* ZF2. The degradation experiments were conducted in the mineral salt medium (MSM) containing phenanthrene as the sole source of carbon and energy. The selection was based on phenanthrene biodegradation abilities. The isolates were identified using morphological, biochemical tests and 16S rDNA sequencing and after 10 days' incubation at 30 °C and pH = 7, the bacterial growth and Phe-degrading rate were evaluated by protein assay (Bradford) and gas chromatography (GC), respectively.

Results: Biochemical tests and 16s rDNA gene sequence analysis revealed that isolated bacteria are similar to *Pseudomonas aeruginosa* ZF1 and *Serratia marcescens* ZF2 with 99% similarity. The results showed a mixture of ZF1 and ZF2 bacteria could degrade 83% at minimum concentrations of 200 ppm of phenanthrene whereas single strain culture of two bacteria had poor degradation abilities (less than 15%).

Conclusion: Results showed that isolated co-culture bacteria have high potential to degrade phenanthrene with the best results achieved when the enriched consortium was used and this mixture was shown to be an appropriate candidate for bioremediation purposes.

Keywords: Phenanthrene; Co-culture; Biodegradation

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Introduction

Crude oil is composed of a complex mixture of alicyclic, aliphatic and aromatic hydrocarbons (1). The aromatic hydrocarbons have benzene (C6H6) in their structure (2). Polycyclic aromatic hydrocarbons (PAHs) are a large group of organic compounds with two or more fused aromatic rings in linear, angular, or cluster arrangements. These compounds have a relatively low solubility in water, but they are highly lipophilic and very stable in the environment (2, 3).

Some PAHs are toxic, mutagenic and carcinogenic, and represent a potential threat to human health. These compounds are listed as priority pollutants by the US environmental protection agency (4-6). Phenanthrene (Phe) is tri-cyclic aromatic hydrocarbon that is found in high concentrations in polycyclic aromatic hydrocarbon (PAH)-contaminated sediments, surface soils and waste sites (7). Phe is a major constituent of coal derivatives and oil fuels. Phe is

also reported to act as human skin photosensitizer, mild allergen and mutagen in bacterial systems under certain conditions. It has also been proved to induce sister chromatid exchanges and to be a strong inhibitor of gap junction intercellular communication (8, 9).

Bioremediation which is based on microbial transformation and degradation is one of the most promising methods applied in the field of environmental biotechnology to clean up contaminated environments (10, 11). A large number of bacteria with PAH-degrading capabilities have been reported as able to either completely assimilate a defined range of compounds or carry out their transformation to different extents (12). In other words, during the bioremediation process, PAHs that are toxic and hazardous compounds are transformed to non-toxic compounds by microorganisms. However, the success of bioremediation projects has been limited by the scarcity of microorganisms capable of degrading a broad range of PAHs (13).

The potential for degradation of PAH compounds in soil has been a research area of great interest in recent years and many efforts have been made to isolate bacteria that could degrade PAH compounds. This study aims to isolate and identify effective bacteria in Phe degradation available in Arak petrochemical wastewater located in the center of Iran and to determine the capacity of these bacteria for degradation of these compounds.

Materials and methods

Sampling

Samples were collected from wastewater of Arak Petrochemical located at the center of Iran. Sampling site had been naturally exposed to oil pollution because of natural oil seepage over decades. The temperature and pH of the sampling sites in June were 30 °C, and 7 respectively. Samples were aerated every 30 min and transferred to the laboratory in sterile conditions on ice within 24 hours and stored at 4 °C until they were used.

Materials and chemicals

A stock solution of Phe was prepared by dissolving 1 g of Phe in 100 ml acetone (100 mg L-1). Mineral salts medium (MSM) was composed of 1.0 g of NH4Cl, 5.0 g KH2PO4, 0.1 g MgSO4×7H2O, 5 mg of Fe(SO4)2, 1.0 ml of trace elements solution and 2.0 ml of Phe solution as the carbon and energy source. The trace element solution contained (13, 14): 23 mg MnCl2×2H2O, 30 mg MnCl4×H2O, 31 mg H3BO3, 36 mg CoCl2.6H2O, 10 mg CuCl2×2H2O, 20 mg NiCl2×6H2O, 50 mg ZnCl2, and 30 mg Na2MoO4×2H2O per liter. Final pH of MSM was adjusted at 7.0 using 100 mM NaOH (15). In order to produce solid medium, 15 g agar was added to MSM. All the chemicals were purchased from Merck (Darmstadt, Germany), except for the followings: CaCl2, glucose, starch, arabinose (Sigma–Aldrich, Germany); Peptone, SIM and Agar (Difco); MRVP and TSI (Oxid); MgSO4.7H2O and NH4Cl (Riedel-de Haën).

Isolation and identification of Phe-degrading bacteria

In order to isolate of Phe-degrading bacteria, 10 ml of the sample was transferred into the conical flask containing 100 ml MSM supplemented with 200 mg/l Phe (pH 7) and incubated at 30 °C on a rotary shaker at 140 rpm for one week. Phe utilization in the enriched cultures was monitored by the increase in bacterial biomass (absorbance at 600 nm). Then, 1 ml of the enriched culture was transferred to a same fresh medium and subcultures were performed four times. At the end of the enrichment process, a 10-fold serial diluted concentration of inoculated MSM was made, cultured on MSM agar plates and incubated at 30 °C. Colonies with clearing zone (reflects degradation of Phe) were picked and purified by streaking several times on MSM agar. Purified strains were tested for Phe degradation ability as follows. Equal concentration of any of the colonies (an absorbance of 0.3 at 600 nm) was made and cultured in 250 ml flasks containing MSM and incubated at 30 °C for 10 days (140 rpm). MSM with no bacterial inoculums was used as a control. Experiments were conducted in triplicate. At two-day intervals, 1 ml of each medium was taken for measurement of bacterial growth by protein assay with Bradford's method (16, 17). Protein yield was considered as a scale for bacterial growth and Phe degradation. The most produced protein was the most Phe degradation.

In order to identify the isolate, the morphological and biochemical characterization was done referring to Bergey's manual of determinative bacteriology (18). Also, genomic DNA was extracted using phenol/chloroform/isoamyl alcohol according to Sambrook et al., 2001 method (19). 16S rDNA gene fragment was amplified by polymerase chain reaction (PCR) with a set of universal primers including F 27 (5- AGA GTT TGA TCC TGG CTC AG -3) and R 1510 (5- GGT TAC CTT ACG ACT T -3). The PCR amplification mixture of 16 µl, contained the bacterial DNA (of about 200 ng), 1 µl (3 units) of Taq DNA polymerase, 5 µl of Taq buffer (TAPS, pH 8.8, 3mM MgCl2, 50 mM KCl), 5 µl of 2 mM dNTP mix and 5 µl of each primer (10 pM/ µl). Amplification program was set for 30 cycles; each consisted of a denaturation at 96 °C for 10 s, an annealing at 50 °C for 10 s and an extension step at 60 °C for 4 min, followed by a final extension step at 72 °C for 5 min.

Subsequently, PCR products were electrophoresed on 1% agarose gel and visualized using ethidium bromide. DNA fragments were sequenced by a sequencer (SEQLAB, Germany). Finally, all the sequences were compared with reference sequences in NCBI databases (http://WWW .ncbi.nlm.nih.gov/Genbank) using BLAST software.

Evaluation of bacterial growth with Phe

For estimating co-culture (with 1:1 ratio) growth based on total protein production, during 10 days at 48 h intervals, bacteria were harvested by centrifugation of 1 ml of the inoculated MSM at 8000 g for 10 min. Then the pellet was suspended in 1 ml of Ringer's solution (111.2 mM NaCl, 5.7 mM KCl, 2.25 mM CaCl2, and 2.4 mM NaHCO3), vortexed and centrifuged (8000 g for 10 min) again. For cell lysing, the pellet was resuspended in 1 ml of distilled water and 0.5 ml of 0.3 M NaOH and then vortexed. The suspension was incubated at 90 °C for 60 min. This cell lysate was used for protein staining done by Bradford's method (20). Briefly, Bradford's solution was prepared as follows: dissolve 100 mg Coomassie Brilliant Blue G-250 in 50 ml 95% ethanol, add 100 ml 85% (w/v) phosphoric acid was added. Five milliliters of Bradford's solution was added to each glass tube containing cell lysis and a blank glass tube (containing 1ml of distilled water and 0.5 ml of 0.3 M NaOH) while stirring. After incubation in the dark for 20 min, the absorbance of the supernatant was measured at 595 nm (Shimadzu, UV-120-02; Japan). The results were checked against the standard curve of serial dilutions of 0, 10, 50, 150, and 200 mg/l bovine serum albumin (BSA). The degradation rate was evaluated by gas chromatography (GC) after 10 days (14).

Extraction and analysis of Phe degradation

After 10 days, the concentration of Phe in the mixed sample, isolated samples and control flask (containing 100 ml MSM) was determined according to Wu et al (21). 50 ml ethyl acetate was added to the culture medium and shaken for 15 min. After forming two phases, Phe-containing non-polar organic solvent phase was collected and the aqueous phase was re-extracted by another aliquot of 50 ml ethyl acetate. Finally, the two extracts were combined together, dried by anhydrous Na2SO4 and the volume was adjusted to 100 ml. Extract samples were analyzed by GC-FID. GC-FID was equipped with an HP-5MS fused silica capillary column (60 m × 0.25 mm ID × 0.25 l m thickness, Agilent Technologies, USA) with the injector and detector temperature of 280 °C and 300 °C, respectively. Nitrogen was used as the carrier gas. Oven temperature program was 80 °C (for 2 min) to 120 °C at a rate of 10 °C/min and from 120 °C to 300 °C at a rate of 4 °C/min and held at 300 °C for 15 min. The identification and quantification of chemicals were conducted based on matching their retention times of standards (21).

Results

Enrichment of Phe-degrading bacteria and selection of the novel strains

The clear zone around isolated colonies on MSM agar was observed after 4, 5 and 6 days of culture, respectively. Colonies with clear zones were labeled as ZF1 and ZF2. Figure 1 shows the colony of the isolated bacterium on MSM agar.

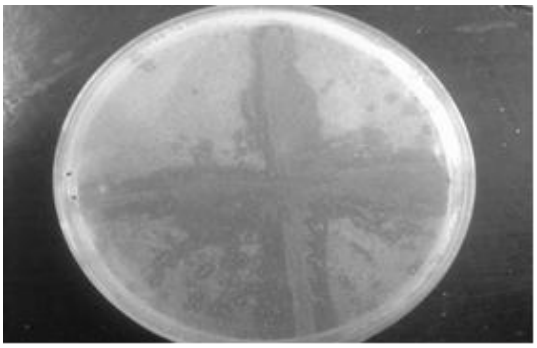

Figure 1. Clear zone on Phe-sprayed MSM agar made by Phe degrading.

Identification of selected strains

A 1516 bp nucleotide DNA fragment of 16S rRNA amplified from the ZF1 isolate and 1436 bp nucleotide DNA fragment of 16S rRNA amplified from the ZF2, were submitted to the BLAST in order to find a homology with other 16S rDNA sequences. Comparing the sequence of the 16S rDNA gene of the isolation with the sequences in GenBank revealed that the ZF1 isolate is similar to *Pseudomonas aeruginosa* and the ZF2 isolate is *Serratia marcescens* with 99% similarity. Morphology and biochemical tests (Table 1) with comparing the sequence of the 16S rDNA gene of the isolation revealed that the isolates were strains of *Pseudomonas aeruginosa* and *Serratia marcescens*.

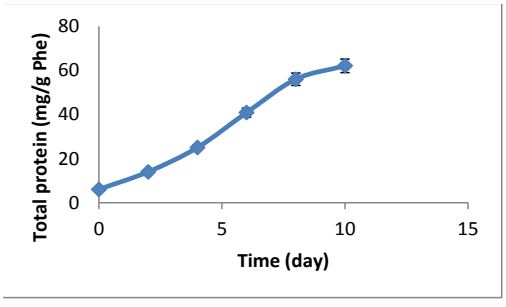

Figure 2. The growth rate for mixed culture with protein assay during 10-day incubation at 30 °C, pH 7 and 120 rpm.

Assessment of mixed bacterial growth

Mixed bacterial growth charts (by means of protein assay) indicated that mixed bacteria had more growth than separated bacterial. Low growth of bacteria in separate cultures was due to producing intermediate materials that are toxic to bacteria.

Table 1. Morphological and biochemical features of isolations ZF1 and ZF2.

Morphological and biochemical features	ZF1	ZF2
Cell morphology	Gram-negative bacilli, non-spore, and non-capsule forming	Gram-negative bacilli, non-spore, and capsule forming
Colony morphology	Circular, smooth-edge, glistening, and yellow–white	Circular, smooth-edge, convex, glistening, and pink
Positive reactions	Oxidase, catalase, mobility, urease, aerobic, nitrate reduction, nitrification, indole production, Utilized d-glucose, Utilized starch	catalase, urease, aerobic, nitrate reduction, Utilized d-glucose, d-fructose, maltose, d-xylose
Negative reactions	Acid-fast, H2S production, and Utilized d-fructose, maltose, d-xylose	Oxidase, Acid-fast, H2S production, and indole production, nitrification, Utilized starch
Tolerance range pH	5-10	6-10
Tolerance range Temperature	20-50 °C	15-45 °C

When bacteria were cultivated in a mixture, intermediate materials reduced by co-metabolism activity. Analysis of total protein charts showed, two mixed cultures of bacteria had low growth rate in the first 24 hours after inoculation and growth rate increased in two eight in two to eight days after inoculation and it reduced again in the last days (Figure 2). Slow growth in the last days was due to the reduction in the carbon and energy source and increase in waste materials.

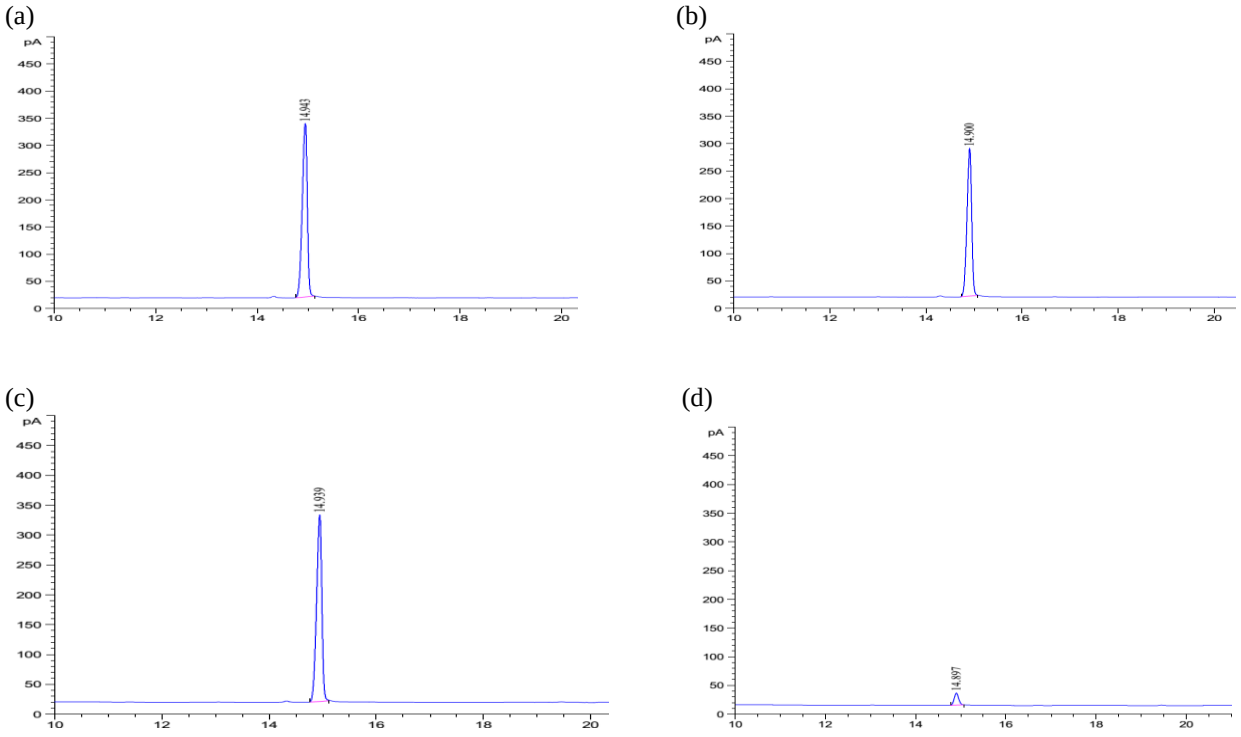

Figure 3. Obtained Chromatograms after 10 days of incubation at 30 °C, pH 7 and 120 rpm.
a) Phe peak at 100 ml MSM containing 200mg/l Phe in bacteria-free medium (control).
b) Remaining Phe peak at 100 ml MSM containing 200mg/l Phe with ZF1 bacteria.
c) Remaining Phe peak at 100 ml MSM containing 200 mg/l Phe with ZF2 bacteria.
d) Remaining Phe peak at 100 ml MSM containing 200 mg/l Phe with a mix of 2 bacteria (ZF1 and ZF2).

Phe degradation analyses

Degradation rate was calculated with the area under the graph of GC to graphic peak. The results of GC analysis showed that the individual culture of bacteria can degrade Phe less than 15%, during10 days. To determine the performance of two mixture bacteria in degradation of Phe, the mixture of two bacteria were added to 100 ml MSM containing 200 mg/l Phe until the OD600 nm reached up to 0.15. Culture media were incubated at pH 7, 30 °C and 120 rpm for 10 days.

Table 2. The amount of pure strain and mix isolated strains biodegradation after 10 days of incubation at 100 ml MSM containing 200 mg/l Phe at 30 °C, pH 7 and 120 rpm.

Bacteria	Phe biodegradation percent
ZF1	13/7 ± 2
ZF2	5 ± 1/2
Mix of 2 bacteria (ZF1 and ZF2)	83/6 ± 4

This mixture could degrade more than 83% (Table 2) of the Phe at this time. GC analysis chromatogram is shown in Figure 3 and Phe remains during 10-day incubation are shown in Figure 4.

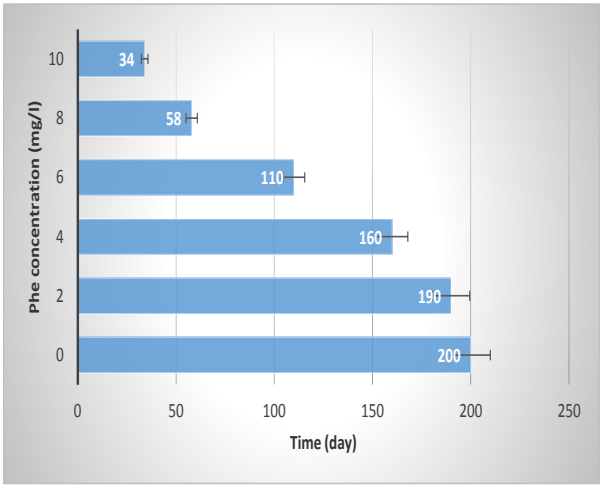

Figure 4. GC chromatograph from Phe remains concentration (initial Phe concentration 200 mg/L, during 10-day incubation by a mixed culture of two bacteria.

Discussion

Considering the dangers of entering PAH compounds (especially, wastewaters as the main source of the environmental contaminants) to the environment and their effects on humans and other organisms, removing these compounds are extremely important. These compounds are considered as a major threat to aquatic and soil ecosystems (22, 23). For this reason, these compounds are considered as a serious threat to humans and other organisms (24). In this study, two bacteria from petrochemical wastewater having high potential to degrade Phe had been isolated. According to the results, this consortium could be valuable in bioremediation of PAHs-contaminated environments. The mixed culture had outstanding performance with a Phe degradation rate of more than 83% when compared with single strains (less than 15%). The present study didn't examine the reasons behind the increase in power to Phe degrading in consortium form. However, further research in the field of PAHs-degradation was showed, ability to break down increased in the mixed culture with synergistic effects. Also, in the mixture growth of bacteria, increasing the enzymatic capacity involved in the degradation of intermediate compounds, bio-surfactant production, production of compounds essential for growth can be the attributed reasons to this increased functionality by synergistic effects (25, 26, 13). When a single strain of the bacteria entered the environment, the began to degrade but after a limited amount of growth, the degradation and growth stopped. Stopping bacterial growth was due to the toxicity of the intermediate compounds. The ability of the single bacterial enzyme was low and therefore it was not able to degrade these intermediate compounds and these compounds increased in the environment and stopped growth. In the mixture growth of bacteria, due to increasing a variety of enzymes involved in degradation of intermediate compounds, these compounds were degraded and their toxicity decreased. The ability of single bacterial enzyme was low and therefore it was not able to break down these intermediate materials and the increase of these materials in the environment would stop growth. Two strains of bacteria isolated in this study with the ability to degradation of Phe and then they were identified using morphological and biochemical tests. According to Han et al (2006) homology with 99% similarity expresses identity in strain level, homology with 97% to less than 99% similarity expresses identity in genus level and homology with 93% to less than 97% similarity expresses identity the new genus and new species (27). According to the above cases ZF1 bacteria with 99% similarity to *Pseudomonas aeruginosa*, and ZF2 bacteria with 99% similarity to *Serratia marcescen*, are probably the strains of *Pseudomonas aeruginosa* and *Serratia marcescen*. Due to the power of the consortium in Phe degradation, further studies on the degradation effect of this consortium on other PAHs such as anthracene, fluorene and pyrene will be useful.

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University of Tehran, Laboratory of Industrial Biotechnology.

Author Contributions

All of the authors performed in this study. EG supervised the experiment, NZ advised and FZ was a researcher. All authors discussed the data and commented on the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest in this work.

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