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In vitro degradation of polycyclic aromatic hydrocarbons by *Sphingobium xenophagum*, *Bacillus pumilus* and *Pseudomonas plecoglossicida*

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Abstract

Polycyclic aromatic hydrocarbons are a class of organic compounds with carcinogenic and genotoxic properties. Biodegradation of such pollutants using microorganisms, especially bacteria, would be a cheap and environmentally safe clean up method. In the present study, a total of 30 anthracene, phenanthrene and pyrene degrading bacteria were isolated from two petroleum contaminated soils in Isfahan-Iran using enrichment technique. Three isolates, showing the highest growth and the lowest pH in their media, were considered as the best hydrocarbon degraders. These isolates were identified to be *Sphingobium xenophagum* ATAI16, *Bacillus pumilus* ATAI17 and *Pseudomonas plecoglossicida* ATAI18 using 16S rDNA gene sequences analyses, and were submitted to GenBank under accession number of KF040087, KF040088 and KF113842, respectively. They were able to degrade 43.31% of phenanthrene, 56.94% of anthracene and 45.32% of pyrene after 9 days, respectively. Strain ATAI18 produced dioxygenase enzyme as an important metabolite for hydrocarbon degradation. The production of biosurfactant was reported positive for both strains of ATAI17 and ATAI18. It was concluded that these three new strains could be used to improve the bioremediation process in a polluted area.

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Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a class of organic compounds that consist of fused aromatic rings. They are widely distributed in the environment as a result of anthropogenic activities such as incomplete combustion of fossil fuels, petroleum refining, accidental releases of petroleum products and other industrial processes (John *et al*., 2012, Bahrami *et al*., 2004). Anthracene and phenanthrene both with three benzene rings are classified as low molecular weight (LMW) PAHs, while pyrene with four benzene rings is classified as a high molecular weight (HMW) PAH. All the three PAHs are alternate PAHs whose structure is entirely composed of benzenoid rings (Wick *et al*., 2011). They are among the sixteen polycyclic aromatic hydrocarbons (PAHs) which have been classified as priority pollutants by the United States Environmental Protection Agency (USEPA) (Kumar *et al*., 2011). Their presence in the environment is of major concern because of their toxic, mutagenic, and carcinogenic properties (Arulazhagan *et al*., 2010). There are several remediation *methods* for *environmental* pollution.

Physical and chemical techniques used for this purpose are expensive methods that can cause secondary contaminants instead of complete degradation of pollutants. Bioremediation is a cost-effective and safe method which can lead to complete mineralization of organic contaminants into carbon dioxide, water and inorganic compounds (Jain and Bajpai, 2012). Anthracene, phenanthrene and pyrene can be degraded by different bacteria such as species of *Acidovorax*, *Arthrobacter*, *Brevibacterium*, *Burkholderia*, *Comamonas*, *Mycobacterium*, *Pseudomonas*, *Rhodococcus* and *Sphingomonas* (Seo *et al*., 2009). Two bacterial metabolites that play a key role in PAHs degradation are biosurfactants and enzymes (Das and Chandran, 2011). Dioxygenases are multicomponent enzymes which incorporate both atoms of O_2 molecule into the PAHs ring. The dihydroxylated intermediates are further metabolized via either an ortho or a meta-oxygenolytic cleavage pathway and followed by further conversion to tricarboxylic acid cycle intermediates (Chadhain *et al*., 2006). Biosurfactants or biological surface active agents are emulsifier substances produced by microorganisms. These amphiphilic compounds increase water bioavailability of hydrophobic compounds including PAHs (Płociniczak *et al*., 2011). The aims of the present study are isolation and characterization of anthracene, phenanthrene and pyrene degrading bacteria from Isfahan-Iran refinery soils and assessment of their in vitro biodegradation.

Materials and methods

Chemicals

Anthracene (96%), phenanthrene (96%), pyrene (97%), indole, dichloromethane and basal salt medium compositions were purchased from Merck-Germany. Bacteriological culture media were purchased from Quelab-Canada.

Soil characterization

Soil samples were collected from two sites of petroleum refinery in Isfahan-Iran at a depth of 10-20 cm and stored in sterile glass flasks at 4°C prior to analysis. Samples were air-dried and sieved (< 2mm) before the chemical analysis.

The soil pH was determined by preparation of saturated soil paste. The electrical conductivity (EC) of the saturated soil extract was also determined. *Organic matter (OM)* was *determined* by the modified *Walk*ley-*Black method* (Winter and Behan-Pelletier, 2007). Anthracene, phenanthrene and pyrene concentrations in soil samples were measured by gas chromatography (GC Agilent 6890N), equipped with FID detector, HP-5 capillary column (30cm×0.25mm×1 µm) and an autosampler (7683B). *Soxhlet* extraction procedure was performed for 12 h using dichloromethane (DCM) as solvent. The extracts were condensed in a rotary evaporator to approximately 20 ml (IKA RV10) and then transferred to a silica column (1 cm internal diameter × 10 cm length) for clean-up (Rasdy *et al*., 2008). External standards of all the mentioned PAHs were prepared (10-40 mg/L), then internal standard (9,10-

Dihydroanthracene) was added to both extracts and external standards (with the same concentration) before GC analysis. Nitrogen was used as a carrier gas at a constant flow rate of 1.5 ml/min. Splitless injection of 1 µl of the samples was automatically conducted. The GC oven temperature was programmed on 70 to 272 °C with a rate of 5 °C/min. The injector and detector temperatures were 270 °C and the total run time was 40 min.

Screening of indigenous PAH degrading bacteria

PAH degrading bacteria were isolated from contaminated soils using an enrichment culture technique in Basal Salt Medium (BSM) supplemented with anthracene, phenanthrene or pyrene as a sole source of carbon and energy. The BSM contained KH_2PO_4 of 1.0 g, $Na_2HPO_4.12H_2O$ of 1.25 g, $(NH_4)_2SO_4$ of 1.0 g, $MgSO_4.7H_2O$ of 0.5 g, $CaCl_2.6H_2O$ of 0.05 g, $FeSO_4.7H_2O$ of 0.005 g, dH_2O of 1000 ml, and pH of 7.0 (pH meter, Metrohm 827) autoclaved at 121 °C for 20 min (Naama *et al.*, 2010). *Individual stock solutions* of PAHs were sterilized by passing through filter (0.2 µm) and were added to the sterilized flasks at final concentration of 50 mg/L. The solvents were allowed to evaporate prior to inoculation, so that a thin layer of PAH was formed on the flask bottom. Sterilized BSM was added to the flask, and 1g of contaminated soil was suspended in the medium *(this condition was considered for all the PAHs and soils separately)*. All cultures and controls (without PAHs) were incubated for 7 days at 30°C on a dark rotary shaker (Vision 8480 SFN) at an agitation speed of 150 rpm. At the end of each week, 10% of cultured medium was transferred to the fresh medium. After five consecutive enrichments, 0.5 ml of the last enrichment cultures was inoculated on nutrient agar plates. Purification was done by subculturing the nutrient agar through streaking plate method (Bin *et al.*, 2010, Naama *et al.*, 2010).

Selecting the best PAH degrading bacteria

The isolates showed the highest turbidity and the lowest pH in BSM supplemented with 50 mg/L anthracene, phenanthrene or pyrene after 3 days, which were selected as the best degraders for further analyses (Safahiyeh *et al.*, 2011, Nnamchi *et al.*, 2006).

Maximum Tolerable Concentration (MTC) was also assayed in both liquid and solid BSM by increasing PAH concentration from initial concentration (50 mg/L) to concentrations that inhibit the bacterial growth (Bennett *et al.*, 2012).

Characterization of selected isolates

The selected isolates were characterized by colony morphology on nutrient agar and gram staining. Additional biochemical tests were performed according to Bergey's Manual of Systematic Bacteriology (Garrity *et al.*, 2005). Further identification was performed using the 16S rDNA gene sequencing. Genomic DNA from the selected bacteria was extracted using the CTAB method previously described (De Salle *et al.*, 2002). The 16S rDNA genes were amplified by PCR (Thermocycler, Eppendorph 632500) using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The polymerase chain reaction (PCR) program consisted of initial denaturation step at 95 °C for 4 min followed by 30 cycles of 95 °C for 1 min, 60 °C for 30 s, 72 °C for 35 s, and the final extension step was carried out at 72 °C for 5 min (Madueno *et al.*, 2011). Nucleotide sequences were compared to those in the National Center for Biotechnology Information Gene Bank database using BLAST program.

Dioxygenase enzyme test

Dioxygenase activity of the selected bacterial isolates was assayed using indole crystals. The isolates were pre-grown on nutrient agar plates and then indole crystals were placed in the lids of the petri dishes. After 2-3 days incubation at 25°C, colonies producing a blue color were scored positive (Guo *et al.*, 2010).

Biosurfactant production assay

The biosurfactant production of the selected isolates was assayed using drop collapsed test and glass slide

test. For drop collapsed test, isolates were cultured in a liquid BSM with anthracene, phenanthrene or pyrene for 7 days. One drop of supernatant was placed on the surface of sterile liquid paraffin in microtiter wells. After 1 min, collapsed drop was indicated as positive result. In glass slide test, isolates were pre-grown on agar plates. One colony of the selected isolates was mixed with a droplet of sterile normal saline. Flow of droplet over the surface of tilted slide was considered positive (Balogun and Fagade, 2008, Kumar *et al.*, 2006).

Growth rate and biodegradation experiments

The growth and biodegradation of PAHs by the selected isolates were tested in BSM medium containing 400 mg/L phenanthrene, 50 mg/L anthracene or 50 mg/L pyrene as the sole source of carbon and energy. The above concentrations were selected based on the best growth of isolates on them. Standardized bacterial cells suspension (0.5 ml) equivalent to 0.5 McFarland, was inoculated in these media. The control media without inoculation were also considered for determination of abiotic loss of PAH in degradation experiment. All media were incubated at 30°C on a rotary shaker at 150 rpm up to 9 days. The optical density of cultures was measured with 24 h intervals using a spectrophotometer at 600 nm wave length (CE/WPA Biowave II) (Bin *et al.*, 2011). The residual concentration of PAHs was extracted from culture media after 9 days with 3 days intervals using liquid-liquid extraction method. All media were extracted twice by 5 ml dichloromethane (DCM). After dehydration by anhydrous sodium sulfate, the extracts were cold-dried by nitrogen gas flow and diluted in DCM (Shokrollahzadeh *et al.*, 2012). External standards of each PAH were prepared (125-250 mg/L), then internal standard (Acenaphthene) was added to both extracts and external standards (with the same concentration) before GC analysis. Splitless injection of 1 µl of the sample was automatically conducted. Nitrogen flow rate was 1.6 ml/min (for anthracene and phenanthrene) and 1.7 ml/min (for pyrene). The GC oven temperature was programmed on 100 to 250 °C with the rate of 7 °C/min. The injector and detector temperatures were 250 °C and the total run time was 20 min. All experiments were done in triplicate.

Results and discussion

Soil chemical analysis

The pH of soil samples was found to be neutral and their EC was reported to be less than 4 dS/m. According to Richards (1954), they are very slightly saline. The concentration of each analyzed PAH in the soil samples (Table 1) was above the standard level (1-3 mg/kg) reported by Hertel *et al.*, (1998), thus both of the soil samples were considered to be contaminated soils.

Table 1. Chemical properties of soil samples.

Chemical characteristics	Soil 1	Soil 2
pH	7.34	7.30
EC (dS/m)	3.26	3.57
OM (%)	5.04	4.7
Anthracene (mg/kg)	18.48	18.88
Phenanthrene (mg/kg)	39.64	72.50
Pyrene (mg/kg)	36.72	29.88

Table 2. Characteristics of the best PAH degraders.

	Phenanthrene	Anthracene	Pyrene	
Isolate	A	B	C	Control
Turbidity (600 nm) after 72 h	0.139	0.13	0.12	0.00
pH	6.0	6.4	6.5	6.8
MTC (mg/L)	3200	1600	1600	-

Isolation of PAH degrading bacteria

After 5 weeks of enrichment with 50 ppm anthracene, phenanthrene or pyrene as the sole source of carbon and energy, a total of 30 bacteria were isolated from two polluted soils. Among them, 12 isolates showed the ability to degrade anthracene, 13 isolates grew on phenanthrene, and only 5 isolates grew on pyrene. Accordingly, 3 isolates of A, B and C were selected as the best phenanthrene, anthracene and pyrene degrader, respectively. They showed the best growth and the lowest pH in their media (supplemented with 50 mg/L of each PAH) after 3 days of incubation. These isolates could tolerate high concentrations of PAHs and the best growth of isolate A was observed in 400 mg/L phenanthrene with isolates B and C showing the best growth in 50mg/L anthracene and pyrene, respectively (Table 2).

Table 3. Phenotypic characteristics of selected isolates.

Characteristics	Isolate A	Isolate B	Isolate C
Macroscopic character	Opaque, smooth, white colonies with irregular margins	Convex, round, mucoid, yellow pigmented colonies	Convex, round, mucoid, cream colonies
Gram stain	+	-	-
Spore stain	+	ND	ND
KOH test	-	+	+
Catalase	+	+	+
Oxidase	-	+	+
MR-VP	-/-	-/-	-/-
SIM	-/-/+	-/-/-	-/-/+
Urease	±	-	+
Citrate	+	-	+
Nitrate reduction	-	-	+
OF	ND	-/+	+/+
Growth in 5%, 7% and 10% NaCl	+/+/+	ND	ND
Growth at 42°C	ND	-	-
Starch hydrolysis	-	ND	ND
Litmus milk	+	-	-
Acid production from:			
Glucose	+	±	+
Fructose	+	-	+
Mannose	+	±	+
Mannitol	-	-	-
Maltose	-	-	-
Sucrose	+	±	+
Lactose	-	-	-
Suggestive name	*Bacillus sp*	*Sphingomonad*	*Psedomonas sp*

Bacterial identification

The results of morphological and biochemical characteristics of the three selected isolates are given in Table 3. PCR-amplified products of about 1450 bp were obtained from the 16S rDNA genes of the three selected isolates (Fig. 1). Isolates A, B and C were identified by the 16S rDNA gene sequencing as *Sphingobium xenophagum* ATAI16, *Bacillus pumilus* ATAI17 and *Pseudomonas plecoglossicida* ATAI18, respectively. The 16S rDNA gene sequences of the three strains were submitted to GenBank under accession numbers of KF040087, KF040088 and KF113842, respectively. Comparison of 16S rDNA gene sequences revealed that ATAI16 showed about 99% similarity to *Sphingobium xenophagum* strain BN6 and 97% similarity to *Sphingobium Olei* strain IMMIB-HF-1 (Fig. 2A). Comparing the sequence of the 16S rDNA gene of ATAI17 with the sequences in GenBank revealed that this strain exhibited the highest similarity (99 %) to *Bacillus pumilus* strain ATCC7061 and SAFR-032 and also *Bacillus safensis* strain FO-36b (Fig. 2B). *Bacillus pumilus* can be

differentiated from *Bacillus safensis* by casein hydrolysis in litmus milk medium and negative result for acid production from maltose (Satomi *et al*., 2006). Strain ATAI18 also showed 99% similarity to *Pseudomonas plecoglossicida* FPC951 (Fig. 2C). *Sphingomonads* are known to degrade a large spectrum of pollutants including monocyclic and polycyclic hydrocarbons (Schuler *et al*., 2009). Members of the genus *Sphingobium* are common gram-negative, aerobic organisms with yellow pigment that are isolated from a wide variety of environments and degrade a broad range of mono- and polycyclic aromatic compounds (Kertesz and Kawasaki, 2010). In a study by Roy *et al*., (2012), *Sphingobium* sp. strain PNB, capable of degrading phenanthrene as a sole carbon and energy source, was isolated from a municipal waste-contaminated soil sample. Strain PNB was also capable of utilizing anthracene, naphthalene and biphenyl singly as carbon and energy sources. Isolation of *Sphingomonas* sp. strain P2 with the ability to degrade phenanthrene from petroleum-contaminated soil in Thailand was also done by Pinyakong *et al*., (2000). The ability of *Sphingomonas paucimobilis* var. EPA505 to degrade anthracene, phenanthrene, fluoranthene and naphthalene was confirmed by Story *et al*., (2001). *Pseudomonads* are aerobic gram-negative rods with the highest degradative potential (Fritsche and Hofrichter, 2008). *Pseudomonas plecoglossicida* was first isolated from *Plecoglossus altivelis* with bacterial haemorrhagic ascites. This bacterium was similar to *Pseudomonas putida* biovar A in its phenotypic characteristics and on the basis of 16S rDNA gene sequence analysis, but it reduced nitrate to nitrite (Nishimori *et al*., 2000). *Pseudomonas plecoglossicida* with the ability to degrade naphthalene, chrysene, pyrene and fluoranthene was previously isolated from an old industrial site in Bloomington, Indiana (US) inundated with extensive levels of organic pollutants (Nwinyi, 2012). *Bacillus* species are Gram-positive, rod-shaped, obligate aerobes or facultative anaerobes and spore forming bacteria that are ubiquitously present in nature (Vos *et al*., 2009). Eight strains of *Bacillus pumilus* were isolated from solid waste oil samples based on their capacity of growing in the presence of naphthalene, phenanthrene, fluoranthene or pyrene as the sole carbon source (Toledo *et al*., 2006). Also, a strain of *Bacillus subtilis* was previously studied for its ability to utilize naphthalene, anthracene and benzo[a]pyrene as the sole source of carbon and energy (Lily *et al*., 2009).

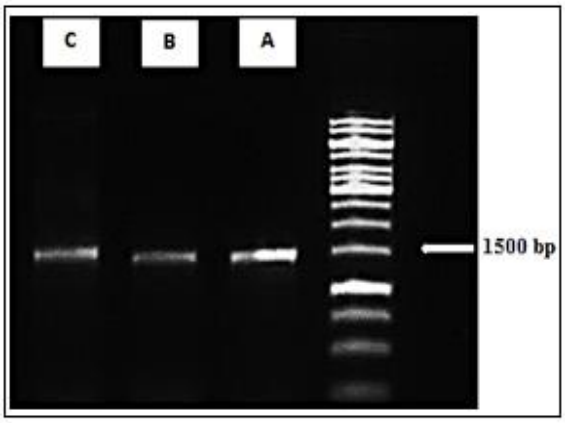

Fig. 1. *PCR-amplified products of 16S* rDNA *genes.*

Metabolite assay

In dioxygenase assay test, only the strain ATAI18 showed dioxygenase activity. The formation of indigo (blue) from indole (colorless) is presumptive for aromatic ring dioxygenases. This strain lost its ability to produce dioxygenase enzyme after several subcultures on nutrient agar plates, showing that the plasmid-encoded genes involved in the production of dioxygenase by this strain. The importance of dioxygenase enzymes in PAH degradation by *Pseudomonas sp*. has been previously confirmed (Kumar *et al*., 2006, Nie *et al*., 2010).

Biosurfactants extracellular amphipathic molecules produced by microorganisms increase the solubility of insoluble compounds and facilitate uptake of hydrophobic molecules (Ward, 2010). Among the three selected strains, ATAI17 and ATAI18 produced biosurfactant. The effects of biosurfactants on biodegradation of PAHs by *Pseudomonas sp*. and *Bacillus sp*. have been previously studied (Das and Mukherjee, 2007, Balogun and Fagade, 2008).

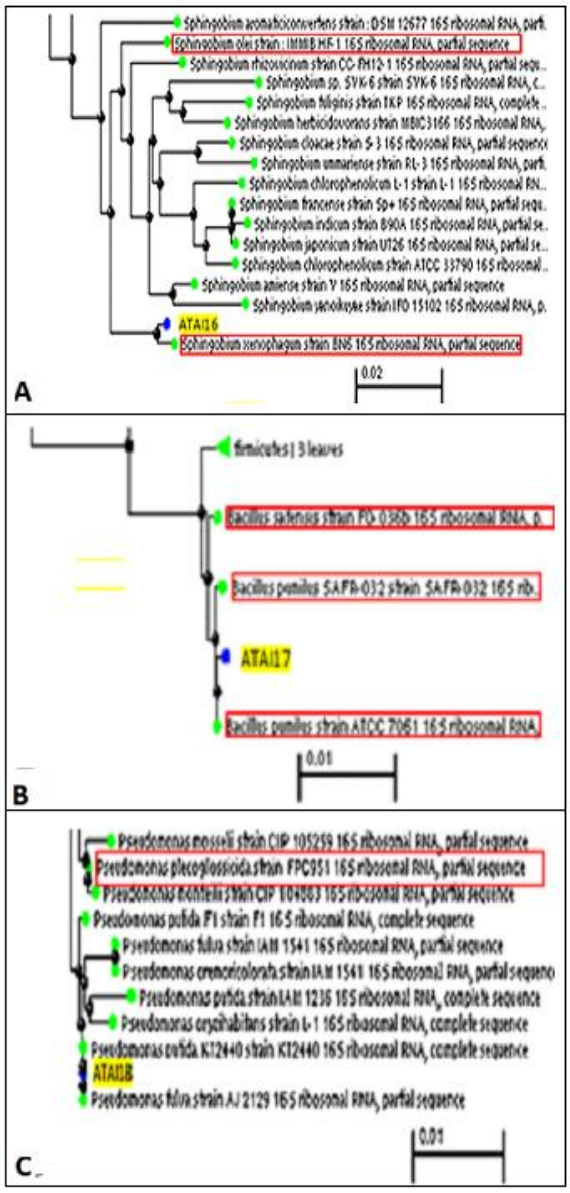

Fig. 2. Phylogenetic trees of *Sphingobium xenophagum*strain ATAI16 (A), *Bacillus pumilus* ATAI17 (B) and *Pseudomonas plecoglossicida* ATAI18 (C).

Growth and biodegradation analyses

In the present study, *Sphingobium xenophagum* ATAI16 was considered as the best phenanthrene degrader. This strain formed clear zone on agar plate sprayed with phenanthrene (Fig. 3). Also, a visible change in the color of growth medium was observed (Fig. 4). After 3 days of incubation, a yellowish brown coloration of the culture was observed, suggesting the production of different metabolites. This could be explained as accumulation of some intermediates such as 1-hydroxy-2-naphthoic acid, showing that phenanthrene degradation by this strain had been performed through salicylate pathway (Coral and Karagoz, 2005, Pinyakong *et al.*, 2000; Tao *et al.*, 2007). Strain ATAI16 lost its ability to grow on solid medium after reaching the stationary growth phase in liquid cultures. This phenomenon has been previously reported for *Sphingomonas xenophaga* strains BN6T and N,N by Stolz *et al.*, (2000). The growth of ATAI16 in BSM contained 400 mg/L phenanthrene and control medium without phenanthrene was shown in Figure 5A. The concentration of phenanthrene in ATAI16 medium and control medium without inoculation was determined during 9 days (Fig. 5B).

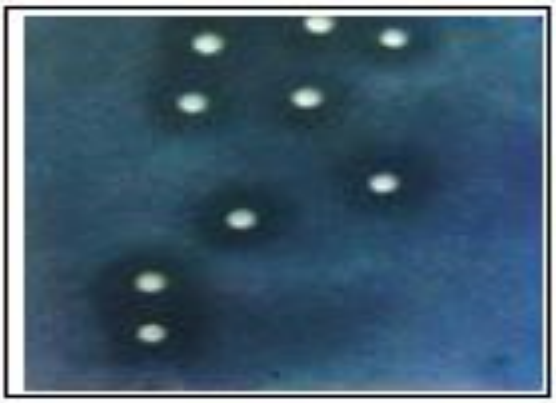

Fig. 3. *Sphingobium xenophagum*strain ATAI16 colonies on the phenanthrene covered agar plate surrounded by clear zone.

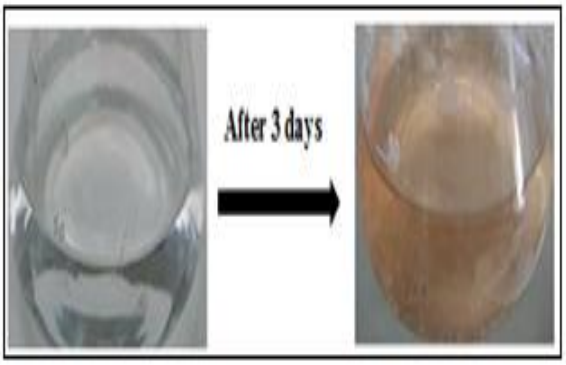

Fig. 4. Color change in ATAI16 growth medium.

This strain degraded 43.31% of phenanthrene with initial concentration of 400 mg/L after 9 days of incubation at 30°C. The maximum growth rate of ATAI16 was observed between the days 5 to 9 and the highest amount of phenanthrene was degraded between the days 3 to 6 (Fig.5C). In a study by Cui *et*

al., (2008), two strains of anthracene-degrading bacteria *Sphingomonas* sp. 12A and *Pseudomonas* sp. 12B were isolated from a long term petroleum-polluted soil. They degraded 35% and 34% of anthracene, respectively, after 18 days. The anthracene, phenanthrene and pyrene degradation ability of *Sphingopyxis* sp. isolated from a petrochemical wastewater in Iran was studied previously. Biodegradation of the selected PAH with initial concentration of 100 mg/L, revealed 98% phenanthrene degradation yield by all *Sphingopyxis* sp., while the maximum 48% and 78% degradations were obtained for anthracene and pyrene after 257 hrs, respectively (Shokrollahzadeh *et al.*, 2012). In a study by Madueno *et al.*, (2011), four bacterial strains were isolated from Patagonian soil in presence of fluorene. All strains belonged to *Sphingomonas* genus and could utilize fluorine, phenanthrene, pyrene and anthracene as a carbon source. The degradation up to 90% of phenanthrene with initial concentration of 26 mg/L was occurred by all strains after 3 days of incubation (Madueno *et al.*, 2011).

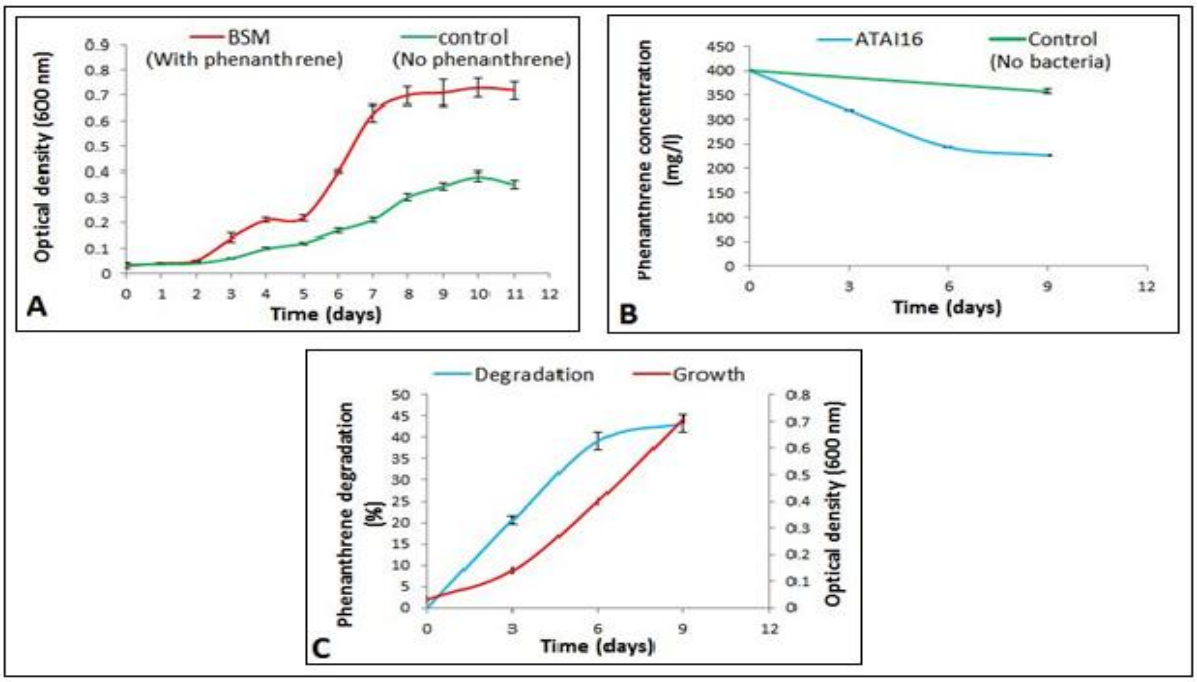

Fig. 5. Strain ATAI16: growth of strain (A) concentration of phenanthrene in medium (B) growth and percentage of phenanthrene degradation (C).

The difference between the phenanthrene biodegradation rate of this study and that of other studies originated from initial concentration of phenanthrene and also bacterial isolates. In the present study, the best anthracene degrader identified as *Bacillus pumilus* ATAI17. Figure 6A shows the growth of ATAI17 in BSM contained 50 mg/L anthracene and control medium without anthracene. Changes in anthracene concentration of ATAI17 medium and control medium without inoculation were determined during 9 days (Fig. 6B).

This strain degraded 56.94% of anthracene with initial concentration of 50 mg/L at 30°C after 9 days of incubation. The highest growth rate of this strain was observed between days 6 to8 and its maximum anthracene biodegradation was determined between the days 6 to 9 (Fig.6C). Similar studies have been done on PAH biodegradation by *Bacillus sp*. In a study by Tulevaa *et al.*, (2005), a naphthalene degrading bacterium *Bacillus cereus* 28BN with biosurfactant activity was isolated from the hydrocarbon-contaminated industrial wastes. This strain degraded up to 72% of naphthalene in a medium contained 2% of this compound. Three bacterial strains *Bacillus* sp. PK-12, *Bacillus* sp. PK-13

and *Bacillus* sp. PK-14 were isolated by Khanna *et al.* (2012) from a crude oil contaminated soil of Patiala, Punjab (India). They were able to cometabolize 64 %, 55 % and 53 % of pyrene (50 mg/L) in the presence of glucose (0.5 %; w/v) at 30 °C, within 35 days, respectively. Cometabolism and temperature optimization seemed to be able to improve the PAHs biodegradation rate. In fact, using the optimal conditions was an important parameter for improvement of PAH degradation by some strains. It was found that number of benzene rings influenced the biodegradation of PAHs. Although the structures of anthracene and phenanthrene were similar, their biodegradation rates were different in the present study. This result proved to be similar to the findings of the previous study by Shokrollahzadeh *et al.*, (2012).

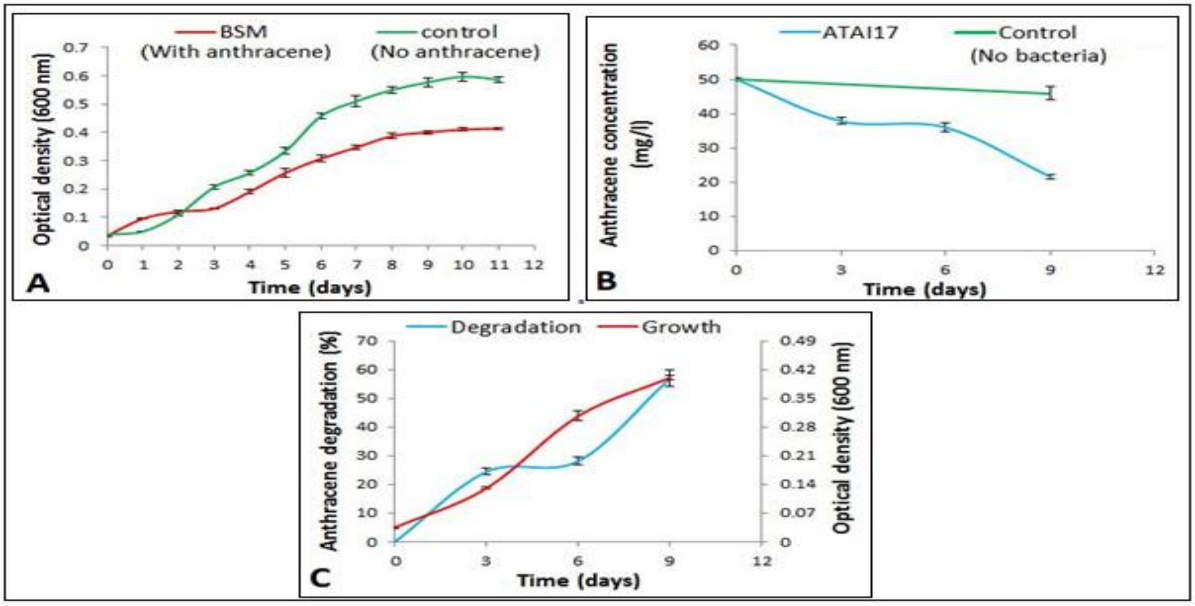

Fig. 6. Strain ATAI17: growth of strain (A) concentration of anthracene in medium (B) growth and percentage of anthracene degradation (C).

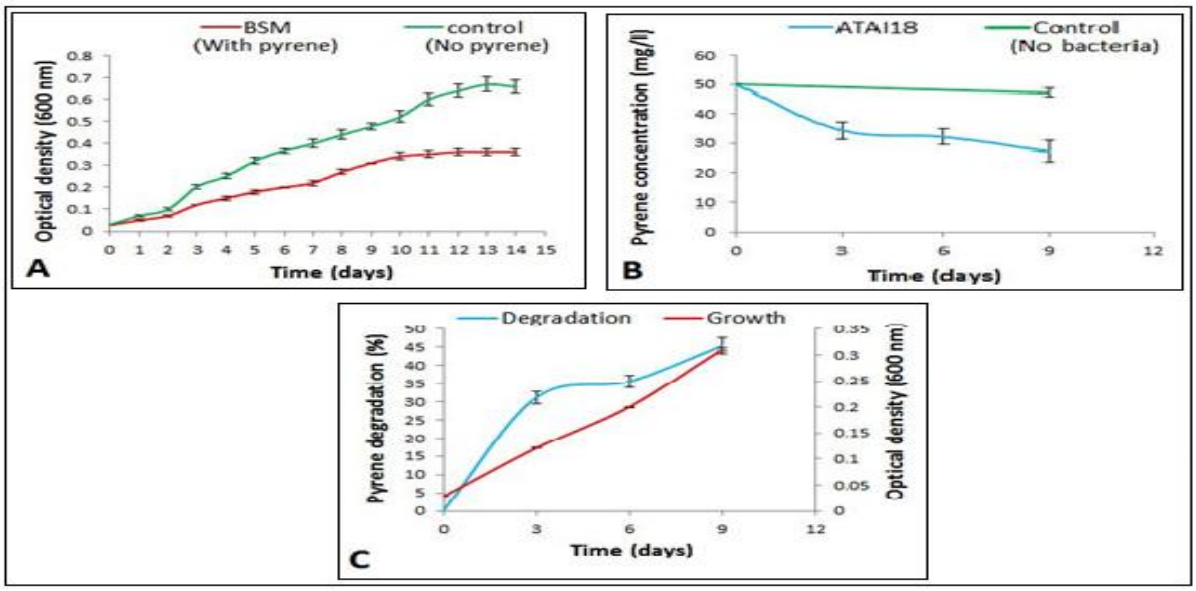

Fig. 7. Strain ATAI18: growth of strain (A) concentration of pyrene in medium (B) growth and percentage of pyrene degradation (C).

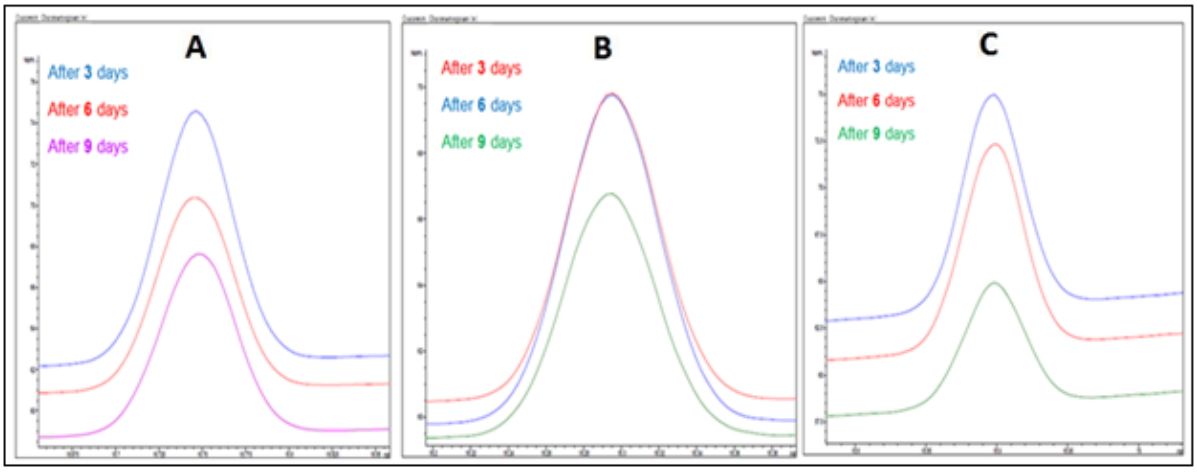

Fig. 8. GC results of phenanthrene (A), anthracene (B) and pyrene (C) biodegradation during 9 days.

In the present study, *Pseudomonas plecoglossicida* ATAI18 was introduced as a pyrene degrading bacterium. The growth of ATAI18 in BSM contained 50 mg/L pyrene and control medium without pyrene was shown in Figure 7A. Pyrene concentrations in ATAI18 medium and control medium without inoculation were determined during 9 days (Fig. 7B). ATAI18 showed a relatively constant growth rate. The highest pyrene degradation was done during the first 3 days of incubation. 45.32% of pyrene with initial concentration of 50 mg/L was degraded by this strain after 9 days of incubation at 30°C (Fig.7C). In a study on oil polluted soils in Lagos, Nigeria, three bacterial strains of *Pseudomonas* sp. LP1, *Pseudomonas aeruginosa* LP5 and LP6 were isolated for their pyrene degradation potentials. Averagely 67.79%, 66.61% and 47.09% of pyrene with the concentration of 50 ppm was degraded after 30 days of incubation, respectively. All the three strains showed catechol 1, 2 dioxygenase activity (Obayori *et al.*, 2008). In a similar study by Ping *et al.* (2011), *Pseudomonas putida* strain PL2, which is capable of growing on pyrene as the sole carbon source, was isolated from hydrocarbons-contaminated soil. This strain was able to degrade 50.0% of the pyrene at 28°C within 6 days in the presence of 50 mg/L pyrene. However, 50.0% of the pyrene was degraded within 2 days in the presence of 50 mg/L pyrene and 50 mg/L phenanthrene by this strain. In fact, phenanthrene as a cosubstrate was shown to increase the PAHs biodegradation efficiency by bacteria. Pyrene is a high molecular weight PAHs with low aqueous solubility that limits its bioavailability and thus the efficiency of a bioremediation process (Shokrollahzadeh *et al.*, 2012). This was in agreement with the result of our study that the biodegradation rate of pyrene was relatively lower than other studied PAHs. Abiotic losses of anthracene, phenanthrene and pyrene in the present study were 8.04%, 10.55% and 5.43%, respectively. The GC results of PAHs biodegradation by the three selected strains are shown in Fig. 8. In conclusion, the present study described three new strains of *Sphingobiumxenophagum*ATAI16, *Bacillus pumilus*ATAI17 and *Pseudomonas plecoglossicida* ATAI18 with the ability to degrade phenanthrene, anthracene and pyrene, respectively. They were isolated from Isfahan- Iran refinery soil for the first time. The importance of some metabolites, such as dioxegenase enzyme and biosurfactant in PAH degradation, was also confirmed. Finally, it was found that the mixed culture of these strains could be useful to improve and accelerate the degradation process of PAHs. So this study can be continued in some contaminated area by these selected strains.

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