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Biodegradation of polycyclic aromatic hydrocarbons by *Pseudomonas* species

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ABSTRACT: Biodegradation of polycyclic aromatic hydrocarbons, toxic compounds widely distributed in the environment by bacteria, is a cheap and safe cleaning up method. The present study attempts to isolate and characterize dioxygenase-producing bacteria which are able to degrade phenanthrene and pyrene from refinery soils. It also aims to assess in vitro biodegradation. To do so, two contaminated soil samples were collected from Isfahan-Iran refinery. The population of phenanthrene and pyrene degrading bacteria were 2.17×10^3 and 1.19×10^3 CFU/g in sample 1 and 21.50×10^3 and 19.40×10^3 CFU/g in sample 2. A sum of 18 phenanthrene and pyrene degrading bacteria were isolated using enrichment culture technique, three of them getting selected which had dioxygenase activity and produced biosurfactant. Identified as *Pseudomonas plecoglossicida* ATAI18, *Pseudomonas aeruginosa* ATAI19, and *Pseudomonas stutzeri* ATAI21, they were submitted to GenBank under the accession number of KF113842, KF113843, and KF113845 respectively. The degradation rate of pyrene (50 mg/L) by strains ATAI18 and ATAI19 was 45.32% and 31.23%, respectively. The strain ATAI21 degraded 39.38% of phenanthrene (50 mg/L) after 9 days. These isolated bacteria can be used to improve microbial population of other hydrocarbon-polluted soils for faster bioremediation of such areas.

Keywords: biosurfactant, dioxygenase, phenanthrene, *Pseudomonas* sp., pyrene.

INTRODUCTION

Polycyclic Aromatic Hydrocarbons (henceforth PAHs) are non-polar organic compounds, widely distributed in the environment. The high hydrophobicity of these pollutants causes their low bioavailability (Haritash and Kaushik, 2009). Petroleum-contaminated soil leads to organic pollution of ground water, environmental problems, and decreased agricultural productivity of the soil (Thapa et al., 2012). Phenanthrene is a Low Molecular Weight (LMW) PAHs with three benzene rings, considered a human skin photosensitizer and mild allergen (Wick et al., 2011). On the contrary, Pyrene is a High Molecular Weight (HMW) PAHs with four benzene rings that is recalcitrant and persists in the environment (Ceyhan, 2012; Kafilzadeh and Hoshyari pour, 2012). They are priority pollutants, listed by United States Environmental

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Protection Agency (USEPA). Although they are not a genotoxic compounds by themselves, their structure is similar to carcinogenic PAHs (Kumar et al., 2011). Remediation of the contaminated soil can be done in several ways which include physical, chemical, and biological methods. Biodegradation is a cost-effective and safe method, leading to complete mineralization of organic contaminants into carbon dioxide, water, and inorganic compounds (Jain and Bajpai, 2012). Among different bacteria *Pseudomonas* sp. plays the most important role in biodegradation of hydrocarbon contaminated soils (Shekhar et al., 2015; Puskarova et al., 2013; Das and Mukherjee, 2007). Several different enzymes and biosurfactants have been identified among PAH-degrading bacteria. Dioxygenases are multicomponent enzymes that catalyze the incorporation of both oxygen atoms into aromatic rings, playing a key role in PAH degradation. The first products of such activities are cis-dihydrodiols. These compounds are further oxidized to aromatic dihydroxy compounds (catechols). Then benzene rings are cleaved either by ortho or meta cleavage. The ortho cleavage produces cis,cis-muconic acid while meta cleavage produces 2-hydroxymuconic semialdehyde. Further oxygenation leads to the production of tricarboxylic acid cycle intermediates (Peng et al., 2008). Biosurfactants are active surface substances produced by microorganisms. Based on their amphiphilic structure, they increase water bioavailability of hydrophobic compounds (Das and Chandran, 2011; Pacwa-Płociniczak, 2011). The of present study aims to isolate and characterize dioxygenase-producing bacteria, capable of degrading phenanthrene and pyrene, from soils contaminated with petroleum. It also tries to assess in vitro biodegradation.

MATERIALS AND METHODS

Chemicals

Phenanthrene (96%), Pyrene (97%), catechol, Indole, dichloromethane (DCM) and basal salt medium compositions have been purchased from Merck-Germany and bacteriological culture media from Quelab-Canada.

Soil Sampling and Chemical Analyses

Two petroleum-contaminated soil samples have been collected from Isfahan-Iran refinery from a depth of 10-20 cm, immediately to be transferred to the laboratory on packs of ice. Samples have been air-dried and sieved (< 2 mm) before chemical analysis, where some of their chemical characteristics, such as pH, Electerical Conductivity (EC) and Organic Matter (OM) have been analysed (Winter and Behan-Pelletier, 2007). Phenanthrene and pyrene concentration have been measured with gas chromatography (GC Agilent 6890N), equipped with FID detector, HP-5 capillary column (30 cm×0.25 mm×1 μm), as well as an autosampler (7683B). PAHs have been extracted by soxhlet procedure for 12 hrs, using DCM as the solvent and the extracts have been condensed in a rotary evaporator to approximately 20 ml (IKA RV10) and passed through silica column for clean-up (Rasdy et al., 2008). External standards of anthracene have been prepared (10-40 mg/L) and the internal one (9,10-Dihydroanthracene) has been added to both extracts and external standards (with the same concentration) before GC analysis. Splitless injection of 1 μl of the sample has been automatically done. Nitrogen has been used as the carrier gas at a constant flow rate of 1.5 ml/min, with the GC oven temperature programmed from 70 to 272°C with a rate of 5°C/min. The injector and detector temperatures have been 270°C and total run time, 40 min.

Bacterial Count

Total Heterotrophic Bacteria (THB) and PAH degrading bacteria are enumerated by means of spread plate technique on nutrient agar and solid Basal Salt Medium (BSM), supplemented with phenanthrene or pyrene, respectively. The BSM was composed of

1.0g of KH_2PO_4, 1.25g of $Na_2HPO_4.12H_2O$, 1.0g of $(NH_4)_2SO_4$, 0.5g of $MgSO_4.7H_2O$, 0.05g of $CaCl_2.6H_2O$, 0.005g of $FeSO_4.7H_2O$, and 1000 ml of dH_2O at pH = 7.0 (pH meter, Metrohm 827) were autoclaved at 121°C for 20 min (Naama et al., 2010). Pyrene and phenanthrene are dissolved in DCM, separately at a final concentration of 0.5 mg/ml, transferred through a 0.22 µm syringe filter. Stock solutions (0.5 ml), sprayed on the BSM agar, allowed the solvent to evaporate before inoculation. The experiments have been carried out in triplicate with the number of bacteria, expressed as CFU/g (Sutton, 2010; Tian et al., 2008).

Isolation and Purification of PAHs Degrading Bacteria

PAH degrading bacteria have been isolated by means of enrichment culture technique in BSM, supplemented with phenanthrene or pyrene as the only source of carbon and energy. To do so, the sterilized stock solution of each mentioned PAHs was added to the sterilized flasks at a final concentration of 50 mg/L and the solvents were allowed to evaporate prior to the inoculation, then sterilized BSM was added to the flask and 1 g of each contaminated soil is suspended in medium. It took 7 days to incubate the inoculated mediums at a temperature of 30°C, for which a dark rotary shaker was employed at 150 rpm (Vision 8480 SFN). At the end of each week, 10% of cultured medium got transferred to the fresh medium. After five such consecutive transfers, 0.5 ml of last enrichment cultures were spread on nutrient agar plates. The isolates were purified by several sub-culturing on nutrient agar through streaking plate method (Bin et al., 2010; Tian et al., 2008).

Dioxygenase and Biosurfactant Production Assay

The dioxygenase activity of isolated bacteria has been verified by two standard methods using indole crystals and catechol solution. In both methods, the bacteria grew on agar plates then the presence of doixygenase enzymes was analysed. In the first method indole crystals were placed on the lid of each plate. After 2 to 3 days of incubation, there appeared blue colored colonies as dioxygenase producers (Guo et al., 2010). Catechol solution (100 mM) spread on colonies, previously grown on agar plates, to verify the presence of catechol 2,3 dioxygenase (Alquati et al., 2005). The biosurfactant production of selected isolates was assayed using glass slide and drop collapsed tests as described previously (Balogun and Fagade, 2008; Kumar et al., 2006).

Characterization of the Selected Isolates

Selected isolates have been identified by morphological and biochemical characteristics according to Bergey's Manual of Systematic Bacteriology (Garrity et al., 2005), for which purpose gram stain, catalase, oxidase, urease, IMVIC test, nitrate reduction, and acid production from some carbohydrates have been carried out. Phylogenetic analysis of isolates was also performed based on 16S rDNA gene sequencing for further identification. Genomic DNA from selected bacteria were extracted via CTAB method, previously described (Nishiguchi 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 taken at 72°C for 5 min (Madueno et al., 2011). Nucleotide sequences have been compared to those in the National Center for Biotechnology Information Gene Bank database by using BLAST program.

Biodegradation Experiment

The ability of the selected isolates to degrade

phenanthrene or pyrene as sole source of carbon and energy has been analysed in BSM, supplemented with 50 mg/L of each mentioned PAHs during 9 days. Standardized bacterial cell suspension (0.5 ml) equivalent to 0.5 Mc Farland, were inoculated to these media. The uninoculated controls have been taken into consideration and experiment has been performed in triplicate. Liquid-liquid extraction of remained PAH in media has been done twice, using 5ml of dicloromethan (DCM). After dehydration by anhydrous sodium sulfate, the extracts were cold-dried by a flow of nitrogen gas, then to be diluted in DCM (Shokrollahzadeh et al., 2012). External standards of each mentioned PAHs were prepared (125-250 mg/L), then internal standard (Acenaphthene) was added to both extracts along with the external standards (with the same concentration) before GC analysis. Splitless injection of 1 µl of the sample has been done conducted automatically, where the Nitrogen flow rate was 1.6 ml/min and 1.7 ml/min for phenanthrene and pyrene respectively. The GC oven temperature was programmed from 100°C to 250°C at a rate of 7°C/min. The injector and detector temperatures were 250°C and the total run time, 20 min.

RESULTS AND DISCUSSION

Soil chemical properties and bacterial populations

Based on chemical analyses (Table 1) the pH of two soil samples have been found to be neutral. Also, they were slightly saline as their EC was reported to be less than 4 dS/m (Richards, 1954). The concentration of phenanthrene and pyrene in two soil samples was more than the standard level (1-3 mg/kg) reported by Hertel et al. (1998), hence both samples were regarded as contaminated soil. According to the previous study, there are positive relationship between PAH concentration in soil and the population of hydrocarbon utilizing bacteria (Saadoun et al., 2008). In this study the concentration of phenanthrene in two soil samples showed significant difference (P value < 0.05; Table 1). The population of total heterotophic, phenanthrene, and pyrene degrading bacteria were 2.50×10^3, 2.17×10^3, and 1.19×10^3 CFU/g in soil sample 1 and 120.40×10^3, 21.50×10^3, and 19.40×10^3 CFU/g in sample 2, respectively (Fig. 1). So as expected, the population of phenanthrene degrading bacteria in sample 2 was more than the one in sample 1.

Identification of PAH Degrading Bacteria and Metabolite Production Assay

After the enrichment period, 10 bacteria (7 phenanthrene degraders and 3 pyrene degraders) were isolated from soil sample 1 and 8 bacteria (6 phenanthrene degraders 2 pyrene degraders) from sample 2. Among all of 18 PAH degrading isolates, only two pyrene degrading bacteria, isolated from soil sample 1 (isolate A and isolate B), showed positive dioxygenase activity in indole test. The conversion of indole (colorless) to indigo (blue), demonstrated the presence of dioxygenase activity (Fig. 2A). Also one phenanthrene-degrading bacteria isolated from soil sample 2 (isolate C) produced catechol 2,3-dioxygenases. This enzyme catalyzes the meta-cleavage of catechol, confirmed by the appearance of a yellow-colored product called 2-hydroxymuconic semialdehyde (2-HMSA) when catechol was sprayed on colonies (Fig. 2B). All three isolates produced biosurfactant (Fig. 3).

Table 1. Chemical characteristics 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
Phenanthrene (mg/kg)	39.64	72.50
Pyrene (mg/kg)	36.72	29.88

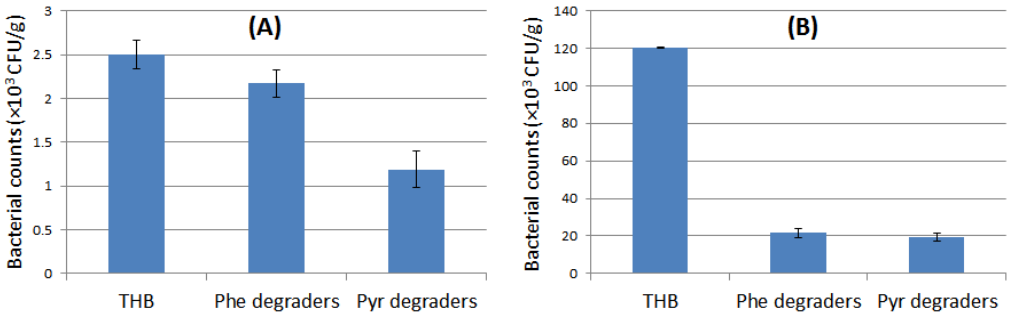

Fig. 1. The bacterial population of soil sample 1 (A) and sample 2 (B)

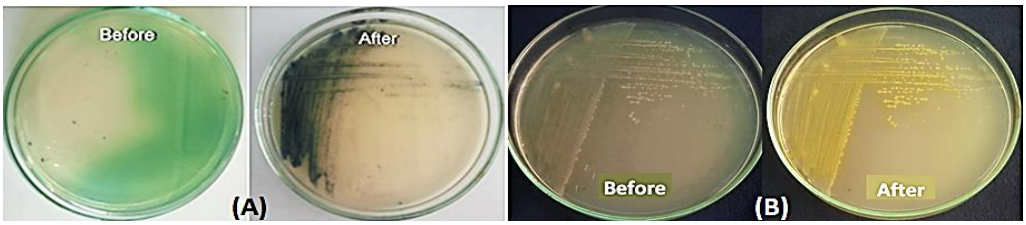

Fig. 2. Dioxygenase assay test by indole crystals (A) and catechol solution (B)

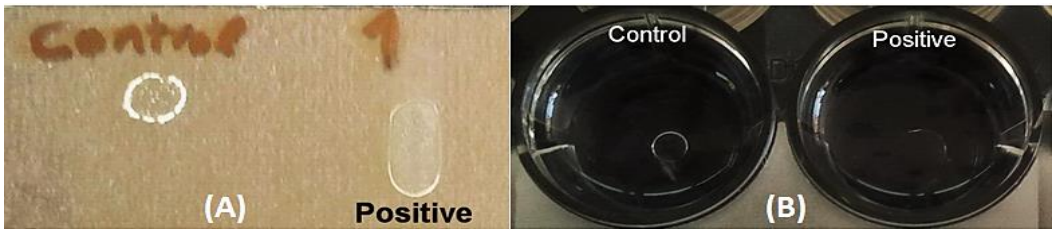

Fig. 3. Biosurfactant production assay by glass slide test (A) and drop collapsed test (B)

Table 2 gives the morphological and biochemical characteristics of the selected isolates. PCR-amplified products of about 1450 bp have been obtained from the 16S rDNA genes of isolates (Fig. 4). Based on the 16S rDNA gene sequencing, isolates A, B, and C are identified as Pseudomonas plecoglossicida ATAI18, Pseudomonas aeruginosa ATAI19, and Pseudomonas stutzeri ATAI21, respectively. The 16S rDNA gene sequences of strains have been submitted to GenBank, their accession numbers being KF113842, KF113843, and KF113845, respectively. Figure 5 (A-C) shows the phylogenetic trees of all strains. Comparing 16S rDNA gene sequences has revealed that *Pseudomonas plecoglossicida* ATAI18 is 99% similar to *Pseudomonas plecoglossicida* strain FPC951 (Fig. 5A); furthermore, *Pseudomonas aeruginosa* ATAI19 is 99% similar to *Pseudomonas aeruginosa* strains PAO1 and 98% to DSM 50071 (Fig. 5B). The 16S rDNA gene sequences of *Pseudomonas stutzeri* ATAI21 also shows a 98% similarity to *Pseudomonas stutzeri* strains A1501 and LMG11199 (Fig. 5C). *Pseudomonads sp.* are aerobic gram-negative rods with a particularly-important role in biodegradation process (Fritsche and Hofrichter, 2008). The importance of dioxygenase enzymes in PAH degradation by *Pseudomonas* sp. has been previously confirmed (Kumar et al., 2006; Nie et al., 2010; Zhou et al., 2013). Biosurfactants are emulsifying agents, produced by microorganisms that enhance PAHs uptake, and play an important role in phenanthrene and pyrene degradation (Khanna et al., 2012; Ward, 2010). The production of biosurfactants by *pseudomonas* sp. during PAH degradation has been previously confirmed (Kumar et al., 2006; Balogun and Fagade, 2008; Nie et al., 2010). *Pseudomonas plecoglossicida* is a gram

negative, rod shaped, non-fluorescent, motile bacterium, placed in the *Pseudomonas putida* group (Boricha and Fulekar, 2009). *Pseudomonas plecoglossicida* (with the ability to degrade naphthalene, chrysene, pyrene, and fluoranthene) has been previously isolated from an old industrial site in Bloomington, Indiana, US inundated with extensive levels of organic pollutants (Nwinyi, 2012). In another study by Mercadal et al. (2010) *Pseudomonas plecoglossicida* J26 has been isolated from intertidal sediments of southern coasts of Argentina by enrichment culture, using naphthalene as sole source of carbon. Similar to *Pseudomonas plecoglossicida* ATAI18, strain J26 shows dioxygenase activity by biotransformation of indole to indigo. Previous studies have demonstrated the ability of *Pseudomonas aeruginosa* to degrade phenanthrene (Roostan et al., 2012), naphthalene (Nnamchi et al., 2006), and some other petroleum hydrocarbons (Das and Mukherjee, 2007; Kuberan et al., 2011). In a similar study two *Pseudomonas aeruginosa* strains have been isolated from a petroleum-oil contaminated soil in North-East India with the ability to utilize pyrene as the sole source of carbon. The biosurfactants, produced by the strains under study, are capable of enhancing the solubility of pyrene in aqueous media (Das and Mukherjee, 2007). The production of biosurfactant by *Pseudomonas aeruginosa* during PAH degradation has also been confirmed by a phenanthrene degradaer strain (Providenti et al., 1995). *Pseudomonas stutzeri* is a ubiquitous bacterium, involved in environmentally-important metabolic activities such as degradation of xenobiotic compounds (e.g. crude oil, oil derivatives, aliphatic and aromatic hydrocarbons, etc.). *Pseudomonas stutzeri* produce catechol 2,3-dioxygenase, responsible for the meta cleavage of catechol (Lalucat et al., 2006). In a study by Stringfellow and Aitken (1995) *Pseudomonas stutzeri* strain P16 has been isolated from a creosote-contaminated soil, capable of using phenanthrene, fluorine, naphthalene, and methylnaphthalenes, and pyrene as the only carbon and energy sources. A naphthalene-degrading bacterium *Pseudomonas stutzeri* DN6 has been reported with catechol 2,3 dioxygenase activity (Jiang et al., 2004) as well.

Table 2. Morphological properties of isolates

Characteristics	Isolate A	Isolate B	Isolate C
Macroscopic characters	Large, round, cream coloured colonies	Large, irregular, opaque, florescent coloured colonies	Wrinkle, reddish brown colonies
Gram stain	Gram negative bacilli	Gram negative bacilli	Gram negative bacilli
KOH	+	+	+
Catalase	+	+	+
Urease	+	-	+
H2S production	-	-	-
Indole	-	-	-
Motility	+	+	+
MR/VP	-/-	-/-	-/-
Citrate	+	-	-
Nitrate reduction	+	+	+
OF	+/+	+/-	+/-
Growth at 42°C	-	+	-
Haemolysis	-	Beta haemolysis	-
Litmus milk	-	Peptonization	-
Glucose fermentation	+	+	+
Fructose fermentation	+	-	+
Sucrose fermentation	+	-	+
Lactose fermentation	+	-	-
Mannose fermentation	+	+	+
Mannitol fermentation	-	-	-
Suggestive name	*Pseudomonas sp*	*Pseudomonas aeruginosa*	*Pseudomonas sp*

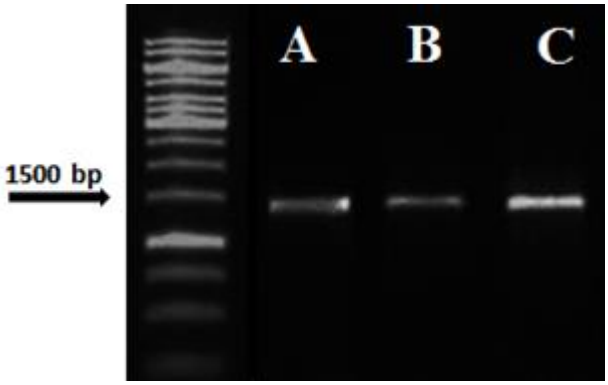

Fig. 4. PCR-amplified products of 16S rDNA genes

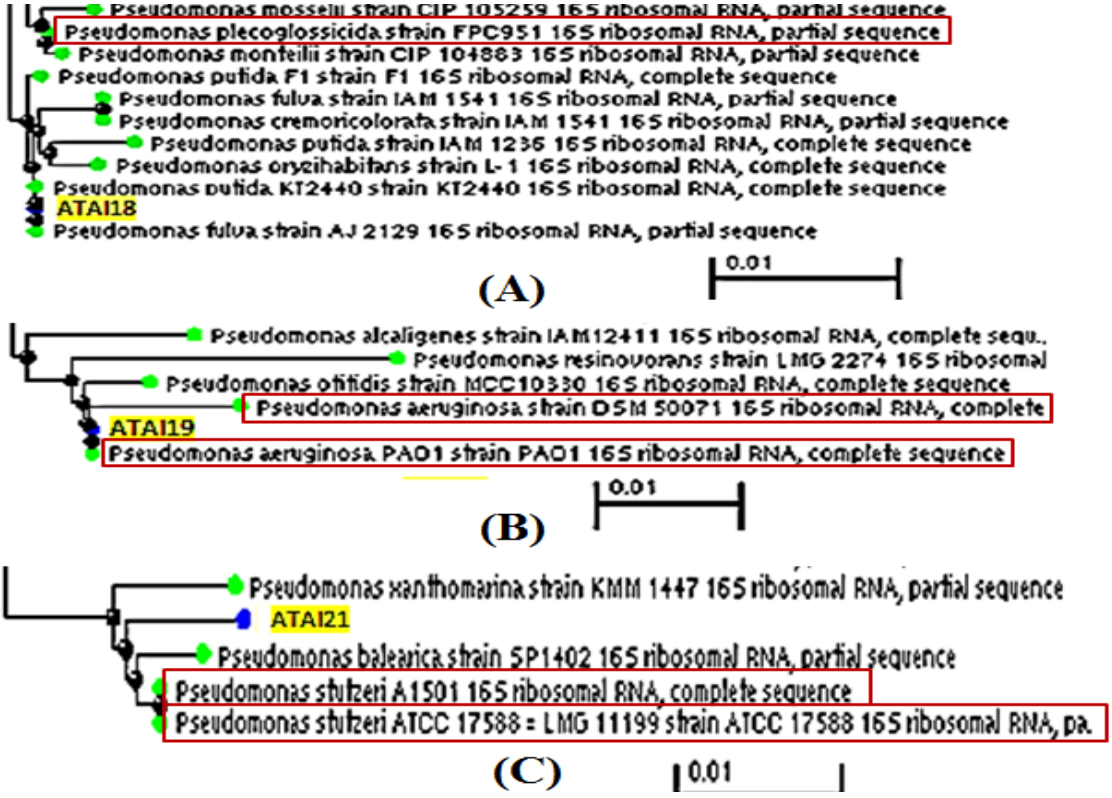

Fig. 5. Phylogenetic trees of *Pseudomonas plecoglossicida* ATAI18 (A), *Pseudomonas aeruginosa* ATAI19 (B) and *Pseudomonas stutzeri* ATAI21 (C)

Biodegradation Assay

In the present study, *Pseudomonas plecoglossicida* ATAI18 has decreased the concentration of pyrene from its initial concentration of 50 mg/L to 34.44, 32.20 and 27.34 mg/L within 9 days. *Pseudomonas aeruginosa* ATAI19 also has decreased pyrene concentration in its medium from 50 mg/L to 42.45, 40.17 and 34.39 mg/L. What is more, the initial concentration of 50 mg/L phenanthrene has been decreased by *Pseudomonas stutzeri* ATAI21 to 36.30, 33.70 and 30.31 mg/L within a 3-day interval (Fig. 6). Abiotic loss of phenanthrene and pyrene in the present study were 10.55% and 5.43%, respectively. *Pseudomonas plecoglossicida* ATAI18 and *Pseudomonas aeruginosa* ATAI19 degraded 45.32% and 31.23% of pyrene with initial concentration of 50 mg/L, respectively. Also the biodegradation rate of phenanthrene (50 mg/L) by *Pseudomonas stutzeri* ATAI21 was 39.38% after 9 days.

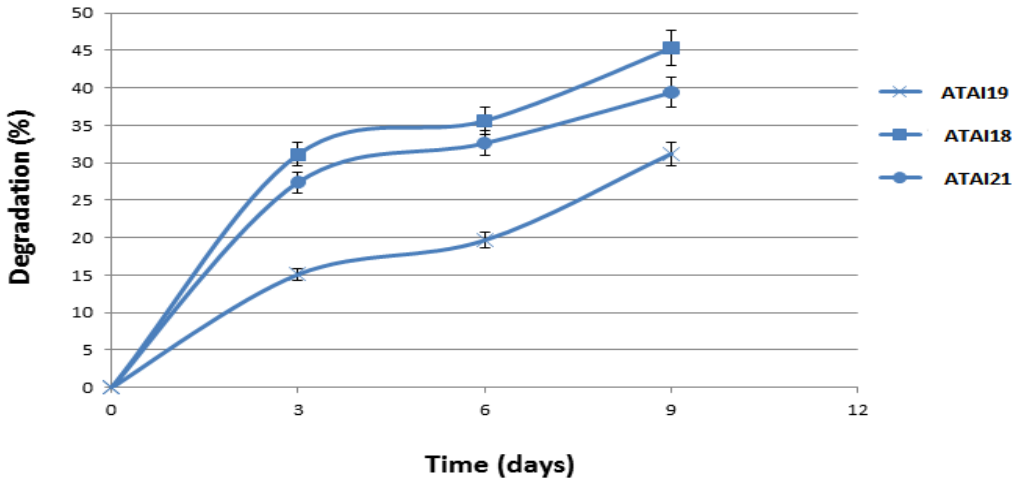

Fig. 6. Biodegradation of PAHs by three strains during 9 days

Several halotolerant bacteria have been isolated from an oil production skimmer pit by Okoro et al. (2012) identified as *Pseudomonas* sp., *Pseudomonas aeruginosa* and *Pseudomonas stutzeri*, able to produce biosurfactants and degrade petroleum hydrocarbons simultaneously by removing about 92% of the residual TPH in the skimmer pit within 2 weeks of exposure. *Pseudomonas aeruginosa* have been previously described as a diesel-degrading bacterium (Niazy et al., 2016). *Pseudomonas* sp has been isolated from landfills around the Shiraz too, being responsible for the degradation of 68.2% pyrene after 10 days (Kafilzadeh and Hoshyari pour, 2012). In a study by Ping et al. (2011) 50% of initial pyrene concentration (50 mg/L) has been mineralized by *Pseudomonas putida* in 6 days, while Obayori et al. (2008) isolated three pyrene-degrading *Pseudomonas* sp, capable of degrading 67.79, 66.61, and 47.09% of pyrene with initial concentration of 100 mg/L after 30 days incubation. *Pseudomonas stutzeri* sp ZP2 has been identified as phenanthrene-degrading bacterium with catechol-2,3-dioxygenase activity. The phenanthrene with an initial concentration of 250 ppm has been completely degraded by strain ZP2 at around day 5 (Zhao et al., 2011). In a study by Zhou et al. (2007) a phenanthrene-degrading *Pseudomonas* sp. ZJF08, isolated from petroleum-polluted-soil, degraded 97.1% of the phenanthrene with initial concentration of 0.1% in one week. Strain ZJF08 also produced catechol 2,3-dioxygenase during phenanthrene degradation. The differences between the biodegradation rate of this study and other studies are due to the initial concentration of PAHs, bacterial strains, and optimization of some conditions such as pH and temperature. The present study demonstrated in vitro degradation of PAHs by selected bacteria isolated from contaminated soils. Such indigenous bacteria are adapted to the conditions of Isfahan refinery contaminated soils, so they can be useful in biodegradation of such contaminated sites.

CONCLUSION

The current study dealt with the degradation of polycyclic aromatic hydrocarbons by three isolated *Pseudomonas* sp. After nine days, the degradation rate of pyrene (50 mg/L) by *Pseudomonas plecoglossicida* ATAI18 and *Pseudomonas aeruginosa* ATAI19 were 45.32% and 31.23%, respectively. The degradation rate of phenanthrene (50 mg/L) by *Pseudomonas stutzeri* ATAI21 was also determined 39.38% after nine days. The consortium, consisted of indigenous bacteria of a specific environment, has been adapted to the conditions of that environment; therefore, such isolated bacteria can be used

in bioremediation strategies for cleaning the original environment. They can also improve microbial population of other hydrocarbon polluted soils for faster bioremediation of such areas.

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