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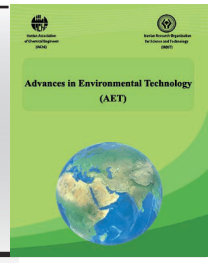

Characterization and kinetic study of PAH–degrading *Sphingopyxis ummariensis* bacteria isolated from a petrochemical wastewater treatment plant

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ABSTRACT

The expansion of a microbial bank for the degradation of polycyclic aromatic hydrocarbons (PAHs) is crucial for removal of these persistent pollutants. In this study, five gram–negative, aerobic, non–fermentative bacterial strains (III–R3, IV–P11, IV–P13, IV–R13, and V–P18) were isolated from the activated sludge of a petrochemical wastewater treatment plant using enrichment protocol based on phenanthrene. The isolates were capable of utilizing phenanthrene, anthracene, and pyrene as a sole carbon and energy source in an aerobic batch aqueous system. The PAHs biodegradation yields were evaluated by gas chromatography and the bacterial isolates were identified using the 16S rRNA sequencing method. A first–order kinetic model provided the best fit to the phenanthrene degradation profiles with a correlation coefficient value of 0.95–0.98. The phenanthrene biodegradation rate constants and half–lives were measured at the range 0.653–0.878 day^{-1} and 0.79–1.06 day, respectively. Lower values of Anthracene degradation resulted with the isolates of the current study, while a relatively high percentage of the removal of Pyrene was obtained by some of the isolates. The data obtained in this study shows that bacterial isolates have degradation preference over *Mycobacterium sp*. and *Pseudomonas aeruginosa;* and they are comparable with *Pseudomonas stutzeri*, *Sphingomonas sp.*, and microbial consortium applied by other researchers. Analysis of the 16S rDNA gene sequence, when compared with the GenBank, indicates that all the strains belong to the genus *Sphingopyxis* with the nearest type strain being *Sphingopyxis ummariensis* UI2 (MTCC 8591T). It is the first time that *Sphingopyxis ummariensis* is reported for its capability in the degradation of PAHs.

1. Introduction

Industrial wastewater treatment plants consistently receive complex mixtures which include a wide variety of organic pollutants. The compounds present in the wastewater of petrochemical plants include polycyclic aromatic hydrocarbons (PAHs), which have mutagenic and carcinogenic properties and are listed by the US–EPA and the EU as priority pollutants. The persistence of PAHs in the environment poses a potential threat to human health through bioaccumulation and biomagnification via food chains [1]. In past decades, PAH biodegradation processes and their mechanisms have been extensively studied [2]. Phenanthrene (Phen), Anthracene (Anth), and Pyrene (Pyr) are polycyclic aromatic hydrocarbon composed of three and four fused benzene rings and have been identified as priority pollutant by the US–EPA. Although PAHs may undergo adsorption, volatilization, photolysis, and chemical degradation, the principal process for successful removal and elimination of PAHs from the environment is microbial transformation and degradation [3,4].

The low rate of degradation could be associated with the microorganisms' inability to degrade, to low solubility of the contaminant, and to specific nutrient limitation [5]. Because of their hydrophobic nature, most PAHs tend to adsorb to the solid surfaces, making them less available for biological uptake. Since the solubility of most PAHs in aqueous media is generally low, different techniques have been proposed to increase PAH mass transfer rates such as the addition of surfactants and organic solvents, thermal treatment, and mechanical dispersion [3,4,6]. However, the success of PAH bioremediation experiments mainly depends on microbial activities. In the last decade, a variety of bacterial and fungal

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species capable of degrading PAHs, particularly low–molecular weight compounds such as phenanthrene, have been discovered. Most of them are isolated fromcontaminated soil or sediments. *Pseudomonas sp*., *Mycobacterium sp.*, *Haemophilus sp*., *Rhodococcus sp*., *Paenibacillus sp.*, *Alcaligenes sp.*, *Arthrobacter sp.*, *Sphingomonas sp.*, *Burkholderia sp., Phanerochaete chrysosporium, Bjerkandera adusta,* and *Pleurotus ostreatus* are some of the studied PAH–degrading bacteria and fungi [7–14].

The isolation and identification of new bacterial and fungal species capable of PAHs biodegradation is still being investigated by researchers to achieve higher rates of biodegradation. Therefore, there would be no need to add other chemicals (e.g. surfactants) to the culture, as the augmentation of the chemicals makes the bioremediation process expensive. In addition to microbial activity and population, the rate of biodegradation depends on pH, temperature, oxygen, accessibility of nutrients (bioavailability), chemical structure of the compound, and cellular transport properties [7]. Petrochemical waste harbours bacteria capable of degrading PAH to a considerable extent.

The goal of this study was to investigate five PAH degraders (belong to the *Sphingopyxis* genus), isolated from the wastewater treatment plant of a petrochemical plant. The Abadan Petrochemical Company (APC), a petrochemical complex located in southern Iran, mainly produces poly–vinylchloride (PVC) and chlorinated intermediate compounds. The wastewater discharge of APC contains chlorinated chemicals in addition to different hydrocarbons and sulfides. Phen, Anth, and Pyr were selected as PAH models in biodegradation studies. The biodegradation kinetic of the isolated microorganisms was also studied for the selected PAHs. The capability of *Sphingopyxis ummariensis* in PAH degradation was not reported in previous studies.

2. Materials and methods

2.1. Materials

The purity of analytical–grade anthracene, phenanthrene, and pyrene were 96, 97, and 96%, respectively (Merck Co.). All materials were analytical–grade and obtained from the Merck Company. The materials for PCR amplification of the 16S rRNA gene were procured from the Cinnagen Company. (Tehran, Iran).

Three PAHs (Phen, Anth, and Pyr) were selected as model substrates due to their prevailing presence in environments. The selected properties of the PAHs are presented in Table 1.

2.2. Microorganisms

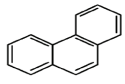
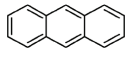
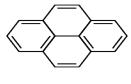
The microorganisms used in this study were isolated from the activated sludge of the wastewater treatment plant of the Abadan Petrochemical Company in Iran. The Phen–degrading bacteria were isolated from activated sludge based on the method of double–layer plates [15]. Briefly, bacteria that are capable of degrading PAHs were isolated by incorporating activated sludge diluted with water in combination with solutions of Phen into an agarose overlayer and pouring the mixture over a mineral salts underlayer. The Phen–degrading bacteria embedded in the overlayer were recognized by a halo of clearing in the opaque Phen layer. Twelve bacterial species were isolated based on their growth rate on a culture containing phenanthrene as a sole carbon and energy source.

Furthermore, the higher growth of isolates in the mineral medium after 230 hours of incubation was the basis of isolate selection in the liquid culture. Five isolates, namely III–R3, IV–P11, IV–P13, V–R13, and V–P18, showed high growth rate and were applied for biodegradation studies.

2.3. Growth studies of isolated species on PAHs

Selected bacterial isolates were grown in a mineral medium containing each of the polycyclic aromatic hydrocarbons (Phen, Anth, and Pyr) at concentrations of 100 mg L^{-1}. The aqueous mineral medium was composed of (mg L^{-1}) 1000 $(NH_4)_2SO_4$, 800 Na_2HPO_4, 200 K_2HPO_4, 200 $MgSO_4.7H_2O$, 100 $CaCl_2.2H_2O$, 5 $FeCl_3.6H_2O$, and 1 $(NH_4)_6Mo_7O_{24}.4H_2O$. The micronutrients used were (mg L^{-1}) 0.2 $MnCl_2.2H_2O$, 0.05 $ZnSO_4.7H_2O$, 0.015 $CuSO_4.5H_2O$, 0.1 $CaCl_2.6H_2O$, and 0.01 $NiCl_2.6H_2O$ [2]. The *p*H was adjusted to 7.0. Predetermined amounts of Phen, Anth, and Pyr were dissolved

Table 1. Some physico–chemical parameters of Phenanthrene, Anthracene and Pyrene.

PAHs	Formula	Molecular Weight	Chemical Structure	Density (g L^{-1})	Melting Point (°C)	Boiling Point (°C)	Vapor Pressure at 25 °C (Pa)[a]	Aqueous Solubility at 25 °C (mg L^{-1})[a]
Phenanthrene	$C_{14}H_{10}$	178.2		1.182	100.5	340	$2x10^{-2}$	1.00
Anthracene	$C_{14}H_{10}$	178.2		1.250	216.4	342	$1x10^{-3}$	0.015
Pyrene	$C_{16}H_{10}$	202.1		1.271	393	150.4	$6x10^{-4}$	0.12

[a] [7]

in dichloromethane (DCM) solvent, filtered by a 0.25 µm filter, and stored in a refrigerator as stoke solution. A sufficient volume of the stoke solution was added to the flask and released under sterile conditions, where the solvent was evaporated at room temperature prior to the medium addition. A medium of 50 cm^3 containing PAH was inoculated by 2% preculture in 250–ml Erlenmeyer followed by incubation in an orbital shaker at a temperature of 30 °C and a rotational shaking of 200 rpm. Cell density (optical density at 600 nm) was monitored by a Perkin–Elmer UV/VIS spectrophotometer, model Lambda 35, during cultivation time and reported as cell growth. For terminating the growth, the medium was acidified by 1N HCl until the pH reached 2.5 and the residual polycyclic aromatic hydrocarbon was measured. All experiments were carried out in three replications and the average values were reported.

2.4. Extraction and determination of PAHs

The biodegradation yield of Phen, Anth, and Pyr was evaluated by gas chromatography (GC). The growth medium was extracted twice by 10 mL DCM at ambient temperature. The extraction phase was stripped of water droplets by the addition of sodium sulphate, concentrated in a vacuum evaporator to 2 mL and analyzed by a gas chromatography. The 0.5 µL portion of the solution was injected into a GC (Philips Model PU4500) equipped with a FID detector and a capillary column (TRB5: 25 m x 0.53 mm x 1.5 µm). The column was maintained at 85–280 °C with an increase rate of 8 °C min^{-1}. The injection port and detector temperatures were 290 °C. Nitrogen was conducted as the carrier gas.

2.5. Biochemical test

The microorganisms were grown on an R2A agar medium for 48 hours at 37 °C. The characteristics of the colonies were studied visually. The morphology of the cells was examined with an optical microscope (x1000 magnification). Phenotypic identification of the Phen–degrading bacteria was done by biochemical tests including catalase reaction, oxidase reaction, oxidation or fermentation of carbohydrates (OF), nitrate reduction, urea hydrolysis, motility, esculin hydrolysis, ortho–nitrophenyl–β–D–galactopyranoside (ONPG) test, indole production, and citrate use by standard methods [16].

2.6. Identification of phenanthrene–degrading bacteria

The bacterial isolates were identified by the 16S rRNA sequencing method. The bacterial DNA was extracted by a Qiagen kit according to the method described in the DNeasy Blood and Tissue Handbook [17]. For PCR amplification of the 16S rRNA gene, a solution of 10 µL incubation buffer (x10), 20 µL dNTPs (deoxyribonucleotide triphosphates) (1mM), 1 µL 27f primer (5'– GAGTTTGATCCTGGCTCAG–3') (0.5 µg μL^{-1}), 1 µL 1541r primer (5'– AAGGAGGTGATCCAGCCGCA–3')(0.5 µg μL^{-1}), 66.6 µL distilled water, and 1 µL extracted DNA were mixed in a microtube. All the materials were prepared by the Cinnagen Company (Tehran, Iran). A Thegenomic DNA template (99.6 µL) was amplified using a 30–cycle PCR (52 °C for 1min; 72 °C for 1min; 92 °C for 1min; and final extension, 72 °C for 10 min). A Qiaquick PCR Purification Kit (QIAGEN, Germany) was used to purify PCR products according to the manufacturer's instructions and analyzed by gel electrophoresis. The gene sequence of the purified products of the chain polymerase reaction was determined using the primers mentioned below by a genetic analyzer based on the fluorescent deoxynucleotides labeling technique (3130XL Genetic Analyzer, ABI, America):

27f : 5'–GAGTTTGATCCTGGCTCAG–3'

16R339: 5'–ACTGCTGCCTCCCGTAGGAG–3'

704f: 5'–GTAGCGGTGAAATGCGTAGA–3

16S rRNA Nucleotide sequences of the species was compared to the sequences in the Gen–Bank database.

2.7. Biodegradation rate and kinetic study

The yield of biodegradation was calculated from initial and final concentrations of the PAH during growth time. A reference sample (control) containing individual PAHs without any cells was used in each experiment to exclude other origins of PAH deterioration from the biodegradation calculation.

The isolated strains were kinetically studied for Phen degradation. The isolates were cultivated in a 100 mg L^{-1} liquid mineral medium for 9–11 days. The growth conditions were the same as before (described in section 2.3). The growth of the strains was monitored and samples were taken intermittently for determination of residual Phen concentration and biodegradation yield. The first–order kinetic model was selected to determine the Phen consumption. The rate of the first–order reaction and Phen half-life were determined by the following equations:

$$R = -(dC/dt) = kt \quad (1)$$

$$ln\,(C/C_o) = -kt \quad (2)$$

$$C = C_o\, exp\,(-kt) \quad (3)$$

$$t_{1/2} = ln2 / k \quad (4)$$

Where C_o is the initial concentration of Phen, C is the Phen concentration, t is the time, k is the biodegradation rate constant, and $t_{1/2}$ is the half life time of Phen. The kinetic constant of Phen biodegradation was then calculated from the slope of $ln(C/C_o)$ line versus time.

3. Results and discussion

3.1. Phenotypic characterization of Phen–degrading bacteria

A total of 12 bacterial strains were isolated from

the activated sludge of a petrochemical company, which mainly produces PVC, essentially on the basis of the growth on solid medium containing Phen as the sole carbon and energy source. Five bacteria, namely III–R3, IV–P11, IV–P13, V–R13 and V–P18, were selected for further studies based on their high growth rate in the mineral medium. All isolated bacteria were gram–negative, rod–shaped, or coccobacillus which formed yellow, round, and convex colonies in 1–3 mm diameter, when grown at 30 °C for 48 hours (Fig. 1).

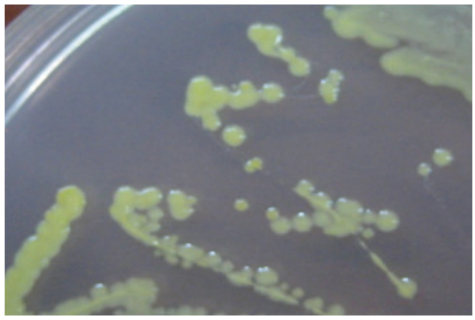


Fig. 1. Colony formation of the isolates.

The results of biochemical tests for the isolates are summarized in Table 2. The phenotypic tests showed that all isolates were aerobic and non–fermentative bacteria. They revealed oxidase and weak catalase activity.

Table 2. Results of biochemical tests on isolated bacteria.

Test	III–R3	IV–P11	IV–P13	V–R13	V–P18
OF	NF	NF	NF	NF	NF
Oxidase	+	+	+	+	+
Catalase	W	W	W	W	W
Indole	–	–	–	–	–
Motility (SIM)	–	–	–	–	–
Nitrate reduction	–	–	–	–	–
Citrate	–	–	–	–	–
Urea hydrolysis	–	–	–	–	–
Esculin hydrolysis	–	–	–	–	–
ONPG	–	–	–	–	–

3.2. Utilization of carbon substrates by isolated strains

Three sources of PAHs including Phen, Anth, and Pyr were tested as carbon substrates for the isolates in liquid culture. Five isolates were tested for removing the selected PAHs at100 mg L^{-1} in 257 h. During all sampling periods, the content of PAH–inoculated with living cells decreased, whereas most of the PAH remained in the control sample. This indicates that the disappearance of PAHs is related to microbial degradation.

The most extensive degradation was apparent with the 3–ring Phen, with a decrease of 92–98.4% by all isolates (Fig. 2). This could be attributed to the enrichment process in which Phen was used as the sole carbon source and also relatively high Phen bioavailability due to its higher solubility (1.0 mg L^{-1}) compared to Anth and Pyr (0.015 and 0.12 mg L^{-1}, respectively) [7].

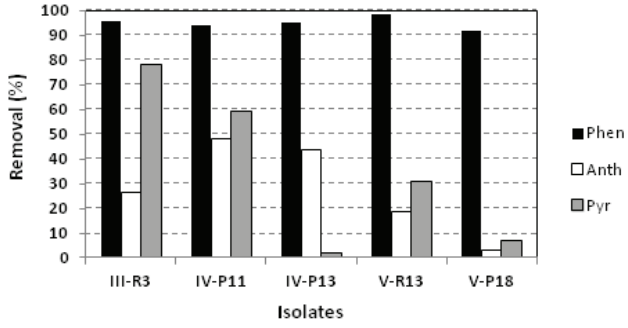


Fig. 2. Biodegradation of individual PAH in the liquid medium at100 mg L^{-1} for 257 h.

The isolate V–R13 showed the highest removal of Phen (i.e. 98.4%). The degradation of anthracene and pyrene by this isolate was observed at lower values of 18.5% and 30.8%, respectively. It can be concluded that the biodegradation ability of a microorganism toward one PAH cannot be extrapolated to other PAHs. It was also found that strain V-P18 could not use other tested PAHs as its sole carbon and energy source except for Phen. The degradation yields for Phen, Anth, and Pyr were 92.0, 3.1 and 7.0%, respectively for this isolate. The limited carbon–source utilization of strain V–P18 suggests its potentially different and interesting Phen–degrading pathway.

A three–ring Anth was degraded the least with reductions of 3–48%. The highest Anth removal was observed by IV–P11 and IV–P13 isolates with a degradation yield of 47.9% and 43.9%, respectively. In spite of the structural similarity of Phen and Anth, a great difference has been observed between their biodegradation potential using the selected isolates due to the very low solubility of Anth. The 4–ring Pyr were degraded at slower rates, with a reduction of 7–78%, compared to Phen. Both III–R3 and IV–P11 showed higher pyrene degradation (78.3 and 59.1%, respectively).

Although the biodegradation of PAHs are influenced by the number of rings, it should be noted that structural characteristics, solubility, and thermodynamic stability of the compounds also plays an important role.

The limitation of bioavailability caused by low aqueous solubility of PAHs, which results in low efficiency of a bioremediation process, was well understood during the last decade and different methods have been applied to overcome this limitation [4,6,18].

Fig. 3 represents the effect of Phen concentration on the microbial growth of V–P18 after 236 hours which shows the

highest level of bacterial growth at 500 mg L^{-1} of Phen, suggesting that this bacterial strain can grow at higher Phen concentrations. Most of the studies have experienced a lower concentration of Phen (see Table 2). The residual concentration of Phen in this condition was calculated as 12mg L^{-1} (i.e. 98.8% Phen removal).

The biodegradation rate depends on the type of microorganisms used (bacteria or fungi), initial concentration, chemical structure of the pollutant, and the environmental conditions (pH, Temp., nutrients). The comparison of different biodegradation studies using bacterial isolates is presented in Table 3. To compare our results to the other investigations, only data for biodegradation of bacterial species in liquid mineral medium in shake flasks without addition of surfactants has been demonstrated. However, there are other reports on PAH degradation (in solid phase, using fungal species, addition of surfactants, and application of bioreactors) which is not considered in this comparison. However, the experimental conditions including concentration and cultivation time are not the same in studies presented in Table 3.

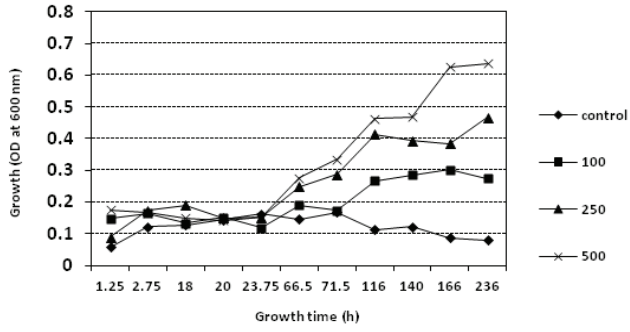


Fig. 3. Growth of V–P18 isolate at different Phen concentrations (mgL^{-1}).

The data obtained in this study clearly shows that bacterial isolates have degradation preference over *Mycobacterium sp.* as reported by Moody et al. [19] and *Pseudomonas aeruginosa* applied by Jacques et al. [20] and are comparable with *Pseudomonas stutzeri* [4,21], *Sphingomonas sp.* [22] and microbial consortium [23] in Phen degradation. Lower values of Anth degradation resulted with the isolates of the current study, while a relatively high percentage of the removal of Pyr was obtained by some of the isolates.

Increasing the cultivation time and controlling the process parameters in bioreactors (e.g. aeration) might increase the biodegradation rate for less–soluble Anth and Pyr.

In addition to Phen, Anth and Pyr, isolate V–P18 was tested for growth on dichloromethane adjoining Phen (Table 4).

Table 4. Growth of V–P18 isolate on Phen (500 mg L^{-1}) and mixture of Phen and DCM (500 mg L^{-1} Phen dissolved in DCM).

Substrate	Growth (OD_{600})	Residual Phen Conc. (mg L^{-1})	Phen Rmoval (%)
Phen	0.64	12	98.8
Phen+DCM	1.17	449	10.2

A liquid mineral medium containing the appropriate Phen or Phen+DCM was inoculated with the tested strain and incubated in an orbital shaker for 343 h. The results reveal that this strain preferably consumes DCM, which is a chlorinated solvent, in the presence of Phen. This might be due to the natural habitat of the strain (petrochemical industry) which mostly contains chlorinated hydrocarbons; also, the enzymatic pathway of the isolate has been evolved in this strain to metabolize chlorinated hydrocarbons. This will be an interesting subject for further investigations. The next section will show that the isolates belong to the type strain *Sphingopyxis ummariensis* that have been isolated from a hexachlorocyclohexane dump site [24], and this might be the reason these isolates influence the degradation of chlorinated hydrocarbons.

3.3. Taxonomic identification of the isolates

The analysis of the 16S rDNA gene sequence of selected isolates (III–R3, IV–P11, IV–P13, V–R13, and V–P18), when compared to the Gen-Bank, indicates that all strains belong to the genus *Sphingopyxis* with nearest type strain *Sphingopyxis ummariensis* UI2 (MTCC 8591^T) (99% identity). Fig. 4 represents the 16S rRNA gene sequence of Strain III–R3 as an example. Gel electrophoresis indicated that the prod

Table 3. Comparisons of different bacterial species in biodegradation efficiency of three PAHs in laboratory shake flasks.

Reference	Microorganism	Time (day)	PAH Conc. (mg L^{-1})	PAH degradation %		
				Phen	Anth	Pyr
[4]	*Pseudomonas stutzeri*	6	250–1000	96–90	–	–
[19]	*Mycobacterium sp.*	14	89–178	90	92	–
[23]	*River sediment*	1.6–16	2–10	100	–	–
[20]	*Pseudomonas aeruginosa*	2	250	48	71	–
[21]	*Pseudomonas stutzeri*	7–15	17.8–35.6	99–92	–	–
[22]	*Sphingomonas sp.*	2	10	–	–	42.6
Current study	*Sphingopyxix ummariensis*	10	100	98.4	47.9	78.3

ucts of PCR for all isolated strains were composed of 1500 nucleotides (Fig. 5). The genetics and enzymology of *Sphingopyxis ummariensis* UI2, which was isolated from a hexachlorocyclohexane dump site in India for the first time, has been elucidated in some detail [24].

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   1 TGATTCTGGC TCAGAACGAA CGCTGGCGGC ATGCCTAACA CATGCAAGTC
  51 GAACGAGATC TTCGGATCTA GTGGCGCACG GGTGCGTAAC GCGTGGGAAT
 101 CTGCCCTTGG GTACGGAATA ACTCAGAGAA ATTTGTGCTA ATACCGTATA
 151 ATGTCTTCGG ACCAAAGATT TATCGCCCAA GGATGAGCCC GCGTAGGATT
 201 AGCTAGTTGG TGAGGTAAAA GCTCACCAAG GCGACGATCC TTAGCTGGTC
 251 TGAGAGGATG ATCAGCCACA CTGGGACTGA GACACGGCCC AGACTCCTAC
 301 GGGAGGCAGC AGTGGGGAAT ATTGGACAAT GGGCGAAAGC CTGATCCAGC
 351 AATGCCGCGT GAGTGATGAA GGCCCTAGGG TTGTAAAGCT CTTTTACCCG
 401 GGATGATAAT GACAGTACCG GGAGAATAAG CTCCGGCTAA CTTCGTGCCA
 451 GCAGCCGCGG TAATACGAGG GGAGCTAGCG TTGTTCGGAA TTACTGGGCG
 501 TAAAGCGCGC GTAGGCGGTT TTTAAGTCA GAGGTGAAAG CCCGGGGCTC
 551 AACCCCGGAA TAGCCTTTGA AACTGGAAAA CTAGAATCTT GGAGAGGTCA
 601 GTGGAATTCC GAGTGTAGAG GTGAAATTCG TAGATATTCG GAAGAACACC
 651 AGTGGCGAAG GCGACTGACT GGACAAGTAT TGACGCTGAG GTGCGAAAGC
 701 GTGGGGAGCA AACAGGATTA GATACCCTGG TAGTCCACGC CGTAAACGAT
 751 GATAACTAGC TGTCCGGGCT CATAGAGCTT GGGTGGCGCA GCTAACGCAT
 801 TAAGTTATCC GCCTGGGGAG TACGGTCGCA AGATTAAAAC TCAAAGGAAT
 851 TGACGGGGGC CTGCACAAGC GGTGGAGCAT GTGGTTTAAT TCGAAGCAAC
 901 GCGCAGAACC TTACCAGCGT TTGACATCCT GATCGCGGTT ACCAGAGATG
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1001 AGCTCGTGTC GTGAGATGTT GGGTTAAGTC CCGCAACGAG CGCAACCCTC
1051 ATCCCTAGTT GCCATCATTA AGTTGGGCAC TCTAAGGAAA CTGCCGGTGA
1101 TAAGCCGGAG GAAGGTGGGG ATGACGTCAA GTCCTCATGG CCCTTACGCG
1151 CTGGGCTACA CACGTGCTAC AATGGCGGTG ACAGTGGGCA GCAACCTCGC
1201 GAGAGGTAGC TAATCTCCAA AAGCCGTCTC AGTTCGGATT GTTCTCTGCA
1251 ACTCGAGAGC ATGAAGGCGG AATCGCTAGT AATCGCGGAT CAGCATGCCG
1301 CGGTGAATAC GTTCCCAGGC CTTGTACACA CCGCCCGTCA CACCATGGGA
1351 GTTGGTTTCA CCCGAAGGCA GTGCTCTAAC CCGCAAGGGG GGAAGCTGAC
1401 CACGGTGGGA TCAGCGACTG GGGTGAAGTC GTAACAAGGT AGCCGTAGGG
1451 GAACCTGC
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Fig. 4. 16S rRNA gene sequence of Strain III–R3 (1458 bp)

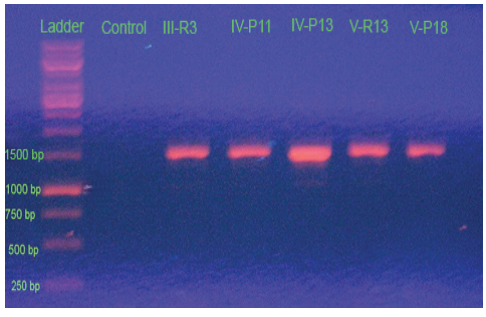

Fig. 5. Gel electrophoresis of PCR product of isolated bacterial species.

Hydrocarbon–degrading members of the *Sphingomonadaceae* (*Sphingomonas, Sphingobium, Novosphingobium, and Sphingopyxis*) are common gram–negative, aerobic organisms that have been isolated from a wide variety of environments. They degrade a broad range of polycyclic aromatic compounds and are potent candidates for bioremediation applications. The following are investigations concerning the PAH biodegradation ability of these genera: *Sphingomonas paucimobilis* for decreasing high molecular weight PAHs [25], *Sphingomonas yanoikuyae JAR02* for removal of benzo–pyrene [26], *Sphingomonas paucimobilis* for bioaugmentation of PAH contaminated soil [13], and *Sphingomonas sp.* for degradation of Pyr [22]. To our knowledge, this is the first time that *Sphingopyxis ummariensis* is reported for its capability for PAHs degradation. These strains can be attractive for bioremediation of sites which are polluted with both chlorinated hydrocarbons and PAHs.

3.4. Kinetic of Phen biodegradation by Sphingopyxix strains

The time–course of aerobic degradation of Phen and the cell concentration in liquid media for five *Sphinopyxix* isolated strains are shown in Fig. 6. The Phen removal data in this study is well fitted to the first order kinetics, with the correlation coefficient (R^2) values between 0.944–0.975 (Table 5).

Table 5. The first–order kinetic constant of Phen degradation by isolated *Sphingopyxix* strains.

Strains	Correlation coefficient (R^2)	Kinetic constant (day^{-1})	Half life of Phen (day)
IV–P11	0.944	0.653	1.06
IV–P13	0.969	0.677	1.02
III–R3	0.975	0.878	0.79
V–R13	0.945	0.698	0.99
V–P18	0.975	0.768	0.90

However, due to the limited amount of kinetic data concerning the consumption of PAHs, a first order kinetic equation was applied for bioremediation of PAH–contaminated soils with the fungi *Cunninghamella echinulata* [27] and for biodegradation of phenanthrene in river sediment (Yuan et al., 2001). The results of the current study revealed the sequence of Phen biodegradation rates of isolates (from high to low) as: III–R3, V–P18, V–R13, IV–P13, and IV–P11. Phen biodegradation rate constants were measured in the range 0.653–0.878 day^{-1} and half–lives of 0.79–1.06 day.

Biodegradability rate constants have been previously reported to be dependent on the concentration so that the specific degradation rate of Phen by *Pseudomonas stutzeri* in cultures containing 100 µM of Phen was 1.6 times higher than that obtained for cultures with 200 µM of Phen (Moscoso et al., 2012). This is also reported by Deive et al. [28] for ionic liquid bioremediation. It can be explained by considering inhibitory effects of higher Phen concentrations on growth and enzymatic biodegradation of microorganisms. In this regard, some higher values of rate constants reported by Yuan et al. [23], i.e. 0.12–1.13 day^{-1}, have been obtained in lower concentration of 0.5–10 mg L^{-1} by the microbial consortium of river sediment (1.13 and 0.21 day^{-1} for 0.5 and 10 mg L^{-1}, respectively). Therefore, the *Sphingopyxix* isolates in this study exhibit higher rates of Phen compared to Yuan et al.' work if the concentration has been taken into account.

Due to the limitations in controlling the reaction parameters in laboratory flasks (e.g. aeration, agitation, mass transfer), higher PAH biodegradation efficiencies with the *Sphingopyxix* isolates are expected in large–scale bioreactors. As an example, comparison between the flask and bioreactor cultivation of *Pseudomonas stutzeri*, demonstrated 6 times

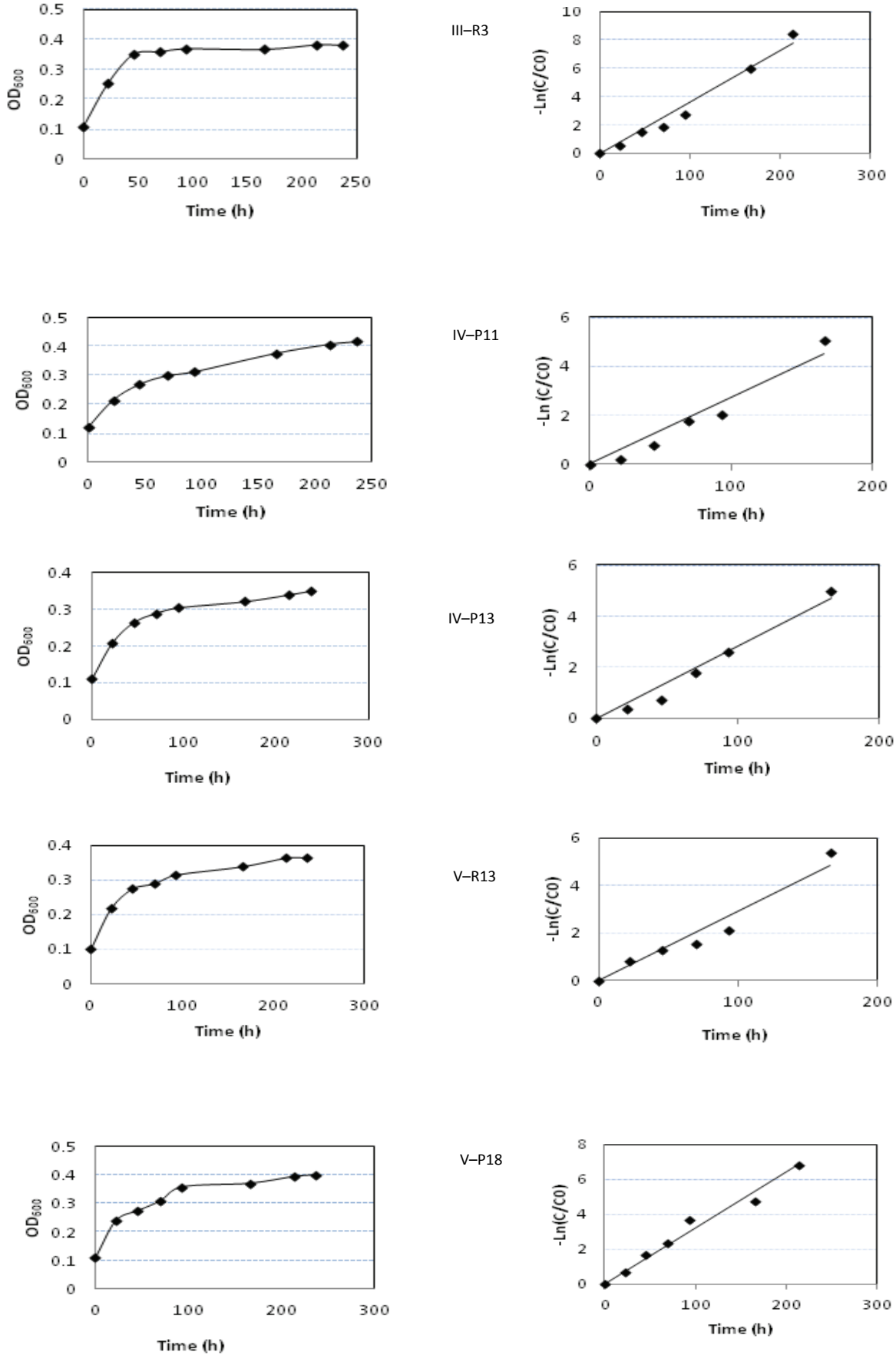

Fig. 6. Growth and kinetics of Phen consumption by five isolated *Sphingopyxis* strains.

higher specific Phen degradation rates in the bioreactor [21]

4. Conclusions

Polycyclic aromatic hydrocarbons (PAHs) are among the most persistent pollutants that accumulate in the natural environment mainly as a result of anthropogenic activities. Therefore, the improvement of the available bank of microbial resources and information is very important to the proper management of PAHs–polluted sites and effluents. In recent years, a wide diversity of bacteria genera has been reported for degradation of PAHs. In this study, five strains of *Sphingopyxis ummariensis* have been isolated from the activated sludge of a petrochemical wastewater treatment plant in Iran. The isolates have shown Phen, Anth, and Pyr biodegradation capability for the first time. The experimental results of the isolates showed over 95% Phen biodegradation by all of these strains, while some of them were also able to degrade Anth and Pyr as less–soluble PAHs. The Phen removal in this study fitted well to the first order kinetics with rate constants of 0.653–0.878 day–1, which are profitable for biodegradation purposes.

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References

[1] Sponza, D. T., & Gök, O. (2010). Effect of rhamnolipid on the aerobic removal of polyaromatic hydrocarbons (PAHs) and COD components from petrochemical wastewater. *Bioresource technology*, *101*(3), 914-924.

[2] Sang, L. Z., Wei, X. Y., Chen, J. N., Zhu, Y. X., & Zhang, Y. (2009). Simultaneous fluorimetric determination of the biodegradation processes of dissolved multi-component PAHs. *Talanta*, *78*(4), 1339-1344.

[3] Li, J. L., & Chen, B. H. (2009). Surfactant-mediated biodegradation of polycyclic aromatic hydrocarbons. *Materials*, *2*(1), 76-94.

[4] Zhao, H. P., Wu, Q. S., Wang, L., Zhao, X. T., & Gao, H. W. (2009). Degradation of phenanthrene by bacterial strain isolated from soil in oil refinery fields in Shanghai China. *Journal of hazardous materials*, *164*(2), 863-869.

[5] Boopathy, R. (2000). Factors limiting bioremediation technologies. *Bioresource technology*, *74*(1), 63-67.

[6] Pantsyrnaya, T., Blanchard, F., Delaunay, S., Goergen, J. L., Guédon, E., Guseva, E., & Boudrant, J. (2011). Effect of surfactants, dispersion and temperature on solubility and biodegradation of phenanthrene in aqueous media. *Chemosphere*, *83*(1), 29-33.

[7] Haritash, A. K., & Kaushik, C. P. (2009). Biodegradation aspects of polycyclic aromatic hydrocarbons (PAHs): a review. *Journal of hazardous materials*, *169*(1), 1-15.

[8] Weissenfels, W. D. Beyer. M., and Klein. J. (1990). Deg radation of phenanthrene, fluorine and fluoranthene by pure culture bacterial strains. *Applied microbiology and biotechnology*, *32*, 479-484.

[9] Guo, W., Li, D., Tao, Y., Gao, P., & Hu, J. (2008). Isolation and description of a stable carbazole-degrading microbial consortium consisting of Chryseobacterium sp. NCY and Achromobacter sp. NCW. *Current microbiology*, *57*(3), 251-257.

[10] Dean-Ross, D., Moody, J., & Cerniglia, C. E. (2002). Utilization of mixtures of polycyclic aromatic hydrocarbons by bacteria isolated from contaminated sediment. *FEMS microbiology ecology*, *41*(1), 1-7.

[11] Kim, T. J., Lee, E. Y., Kim, Y. J., Cho, K. S., & Ryu, H. W. (2003). Degradation of polyaromatic hydrocarbons by Burkholderia cepacia 2A-12. *World journal of microbiology and biotechnology*, *19*(4), 411-417.

[12] Mohamed, M. E., Al-Dousary, M., Hamzah, R. Y., & Fuchs, G. (2006). Isolation and characterization of indigenous thermophilic bacteria active in natural attenuation of bio-hazardous petrochemical pollutants. *International biodeterioration & biodegradation*, *58*(3), 213-223.

[13] Madueño, L., Coppotelli, B. M., Alvarez, H. M., & Morelli, I. S. (2011). Isolation and characterization of indigenous soil bacteria for bioaugmentation of PAH contaminated soil of semiarid Patagonia, Argentina. *International biodeterioration & biodegradation*, *65*(2), 345-351.

[14] Zeng, J., Lin, X., Zhang, J., & Li, X. (2010). Isolation of polycyclic aromatic hydrocarbons (PAHs)-degrading Mycobacterium *sp*. and the degradation in soil. *Journal of hazardous materials*, *183*(1), 718-723.

[15] Bogart, A.H., Hemmingsen, B.B. (1992). Enumeration of phenanthrened–degrading bacteria by an overlayer technique and its use in evaluation of petroleum–contaminated sites. *Applied and environmental microbiology, 8*, 2579–2582.

[16] Boone, D. R., Castenholz, R. W., Garrity, G. M., Brenner, D. J., Krieg, N. R., & Staley, J. T. (Eds.). (2005). *Bergey's Manual® of Systematic Bacteriology* (Vol. 2). Springer Science & Business Media.

[17] DNeasy®, 2006. Blood & Tissue Handbook, DNeasy blood & tissue kit for purification of total DNA from animal blood, animal tissue, rodent tails, ear punches, cultured cells, fixed tissue, bacteria, insects. Available via QIAGEN Group. www.QIAGEN.com

[18] Johnsen, A. R., & Karlson, U. (2004). Evaluation of bacterial strategies to promote the bioavailability of polycyclic aromatic hydrocarbons. *Applied microbiology and biotechnology*, *63*(4), 452-459.

[19] Moody, J. D., Freeman, J. P., Doerge, D. R., & Cerniglia, C. E. (2001). Degradation of phenanthrene and anthracene by cell suspensions of Mycobacterium sp. strain PYR-1. *Applied and environmental microbiology*, *67*(4), 1476-1483.

[20] Jacques, R. J., Santos, E. C., Bento, F. M., Peralba, M. C., Selbach, P. A., Sá, E. L., & Camargo, F. A. (2005). Anthracene biodegradation by Pseudomonas sp. isolated from a petrochemical sludge landfarming site. *International biodeterioration & biodegradation*, *56*(3), 143-150.

[21] Moscoso, F., Deive, F. J., Longo, M. A., & Sanromán,

M. A. (2012). Technoeconomic assessment of phenanthrene degradation by Pseudomonasstutzeri CECT 930 in a batch bioreactor. *Bioresource technology*, *104*, 81-89.

[22] Kobayashi, T., Murai, Y., Tatsumi, K., & Iimura, Y. (2009). Biodegradation of polycyclic aromatic hydrocarbons by Sphingomonas sp. enhanced by water-extractable organic matter from manure compost. *Science of the total environment*, *407*(22), 5805-5810.

[23] Yuan, S. Y., Chang, J. S., Yen, J. H., & Chang, B. V. (2001). Biodegradation of phenanthrene in river sediment. *Chemosphere*, *43*(3), 273-278.

[24] Sharma, P., Verma, M., Bala, K., Nigam, A., & Lal, R. (2010). Sphingopyxis ummariensis sp. nov., isolated from a hexachlorocyclohexane dump site. *International journal of systematic and evolutionary microbiology*, *60*(4), 780-784.

[25] Straube, W. L., Jones-Meehan, J., Pritchard, P. H., & Jones, W. R. (1999). Bench-scale optimization of bioaugmentation strategies for treatment of soils contaminated with high molecular weight polyaromatic hydrocarbons. *Resources, conservation and recycling*, *27*(1), 27-37.

[26] Rentz, J. A., Alvarez, P. J., & Schnoor, J. L. (2008). Benzo [a] pyrene degradation by Sphingomonas yanoikuyae JAR02. *Environmental pollution*, *151*(3), 669-677.

[27] Cutright, T. J. (1995). Polycyclic aromatic hydrocarbon biodegradation and kinetics using Cunninghamella echinulata var. elegans. *International biodeterioration & biodegradation*, *35*(4), 397-408.

[28] Deive, F. J., Rodríguez, A., Varela, A., Rodrígues, C., Leitao, M. C., Houbraken, J. A., ... & Pereira, C. S. (2011). Impact of ionic liquids on extreme microbial biotypes from soil. *Green chemistry*, *13*(3), 687-696.