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Study of *Sphingopyxis* Isolates in Degradation of Polycyclic Aromatic Hydrocarbons

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Phenenahtrene, anthracene and pyrene are polycyclic aromatic hydrocarbons (PAHs) which have been used in this study as model for degradation studies due to their relative toxicities. The ability of bacterial species isolated from a petrochemical wastewater in Iran, on degradation of polycyclic aromatic hydrocarbons was evaluated in aerobic batch aqueous system. Six isolates were selected from 12 by the best growth in phenanthrene. Optimum growth of the isolates on phenanthrene was achieved at 30 ºC. Phenanthrene, anthracene and pyrene were used separately as sole carbon and energy source. Initial concentration, temperature and biodegradation time was selected as 100 mgL^{-1}, 30 ºC, and 257 hours. Biodegradation yield of selected PAH, quantified by gas chromatography, revealed high phenanthrene degradation yield by all six bacterial species (i.e. up to 98%), while maximum 48% and 78% degradation was obtained for anthracene and pyrene, respectively. Phenanthrene was degraded to a greater extent than anthracene and pyrene by the isolates, possibly because it had been used as the sole carbon source during the earlier enrichment process. The results also show that enzymatic degradation of PAH depends on structural and thermodynamic characteristics of the compounds.
The order of PAHs degradation by the bacterial species was: Phenanthrene>pyrene>anthracene. Biochemical studies showed all of the selected species were gram-negative, aerobic and non-fermentative bacillus.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs), which are produced by incomplete combustion processes of organic carbon-based material, are hydrophobic compounds and their persistence in the environment is chiefly due to their low water solubility. Their solubility in water decreases with an increase in molecular weight. These compounds are of increasing interest because of their toxic, mutagenic and carcinogenic properties, their presence in all components of environment (air, water, soil), resistance towards biodegradation and potential to bio-accumulate.
Bioremediation, expected to be an economic and efficient alternative method to other remediation processes such as chemical or physical ones, has been developed as a soil and water clean-up technique. However, the success of PAH bioremediation experiments mainly depends on microbial activities. Bioremediation is not a new strategy for PAH removal (Shuttleworth and Cerniglia, 1995; Potin et al., 2004), but most of the results show low rate of degradation. This could be associated with the microorganisms' inability to degrade, to low solubility of the contaminant and to specific nutrient limitation (Boopathy, 2000). One of the problems is their low bioavailability to microbial degradation.

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Bioavailability is determined by the rate of substrate mass transfer into microbial cells relative to their intrinsic catabolic and excretion activity. PAH-degrading bacteria may enhance the bioavailability of PAHs by excreting biosurfactants (Johnsen and Karlson, 2004).
In our previous study, some bacterial species capable of degrading phenanthrene, were isolated from activated sludge of a petrochemical wastewater treatment plant. The objective of this study was determination of the growth and degradation potential of bacterial isolates on phenanthrene, anthracene and pyrene as models of PAHs.

2. Materials and Methods

2.1 Materials

The purity of analytical-grade phenanthrene, anthracene and pyrene were 97, 96 and 96%, respectively. The solvent dichloromethane was analytical grade and more than 99.5% pure. All other materials were analytical grade and obtained from Merck Company.

2.2 Isolation of phenanthrene-degrading bacteria and growth studies

Samples were collected from activated sludge of wastewater treatment plant of a petrochemical company in southern part of Iran. For the isolation of phenanthrene-degrading bacteria, Bogart and Hemmingsen's (1992) method was used.
Selected bacterial isolates were grown in mineral medium containing polycyclic aromatic hydrocarbons (phenanthrene, anthracene, pyrene) at concentration 100 mg L^{-1}. Mineral medium was composed of (mgL^{-1}) 1000 $(NH_4)_2SO4$, 800 Na_2HPO_4, 200 K_2HPO_4, 200 $MgSO_4.7H_2O$, 100 $CaCl_2.2H_2O$, 5 $FeCl_3.6H_2O$, 1 $(NH_4)_6Mo_7O_{24}.4H_2O$. Micronutrients used were (mgL^{-1}) 0.2 $MnCl_2.2H_2O$, 0.05 $ZnSO_4.7H_2O$, 0.015 $CuSO_4.5H_2O$, 0.1 $CaCl_2.6H_2O$, 0.01 $NiCl_2.6H_2O$ (Sang et al., 2009). Predetermined amounts of PAHs were dissolved in dichloromethane solvent and stored in refrigerator as stoke solution after filtration by 0.25 micrometer filter. Sufficient volume of the stoke solution was added to the flask and the solvent was evaporated at room temperature prior of the medium addition. 50 cm^3 of medium containing PAH was inoculated by 2% preculture followed by incubation in orbital shaker at predefined temperature and rotational shaking of 200 rpm. Cell density (optical density at 600 nm) was monitored by Perkin-Elmer UV/VIS spectrophotometer, model Lambda 35, during cultivation and reported as cell growth. For terminating the reaction, pH of the medium was decreased to 2.5 by HCl addition and the residual polycyclic aromatic hydrocarbon was measured.

2.3 Determination of PAH biodegradation yield

The biodegradation yield of phenanthrene, anthracene and pyrene was evaluated by gas chromatography. The growth medium was extracted twice by 10 mL dichloromethane (DCM) at ambient temperature. The extraction phase was stripped of water droplets by addition of sodium sulphate, concentrated in a vacuum evaporator to around 2ml, then cold-dried by flow of nitrogen gas. The residue was diluted in DCM and analyzed by gas chromatography (GC). 0.5 µL portion of the residue solution was injected into a GC (Philips Model PU4500) equipped with FID detector and a capillary column (TRB5:25mx0.53mmx1.5µm). The column was maintained at 85-280 ºC with increase rate of 8 ºC/min. The injection port and detector temperatures were 290 ºC. Nitrogen was conducted as the carrier gas. The yield of biodegradation was calculated from initial and final concentrations of the PAH.

3. Results and Discussion

3.1 Isolation and selection of phenanthrene-degrading bacteria

By sampling from activated sludge of a petrochemical wastewater treatment plant in Iran, 12 bacterial species were isolated in regard to their higher growth rate on culture containing phenanthrene as sole

carbon and energy source (Bogart and Hemmingsen, 1992). All 12 isolates grew in mineral medium containing phenanthrene. The higher optical density of culture after incubation for 230 hours (Figure 1) was the basis of isolate selection. Six isolates, namely III-R3, IV-P11, IV-P13, V-R13, V-P18, V-Ph5/1, showed high rate of growth and were selected for further biodegradation studies.

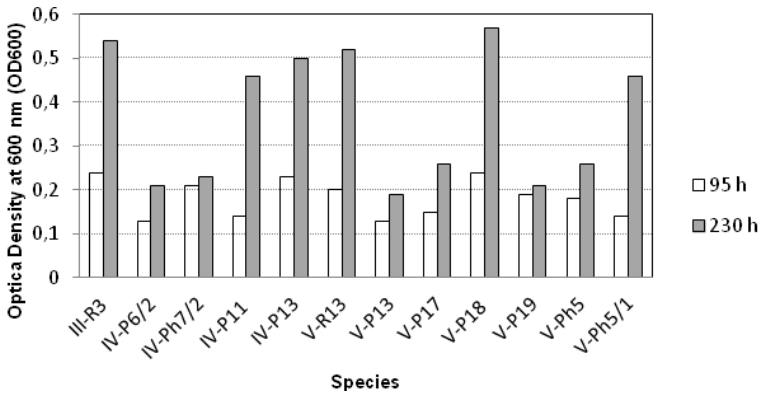

Figure 1: Growth of bacterial isolates on media containing phenanthrene as sole carbon and energy source, Initial phenanthrene concentration: 100 mgL^{-1}, temperature: 30 °C

All the bacterial isolates were aerobic, non-fermentative gram-negative bacillus. The results revealed that all isolates were species of *Sphingopyxis* proteobacteria.

3.2 Optimization of growth temperature

The growth of all the six isolates on phenanthrene was monitored at 20, 30 and 40 °C for 200 hour. The results of four isolates have been shown in Figure 2. For all isolates the highest growth rate was achieved at 30 °C, while lowest growth has been observed at 40 °C. The natural habitat of the microorganisms was activated sludge of wastewater treatment plant, where the temperature varied between 27-32 °C at different seasons. Therefore, the enzymes of the microorganisms have been adapted to this temperature. The biodegradation experiments were conducted at optimized temperature of 30 °C.

3.3 Biodegradation of phenanthrene, anthracene and pyrene

Biodegradation of polycyclic aromatic hydrocarbons (Phenanthrene, anthracene, pyrene) by six isolates were individually tested at initial concentration of 100 mgL^{-1} for 257 h. The results have been shown in Table 1. Phenanthrene was degraded more than 92% by all six isolates. Isolate V-R13 showed the highest degradation yield. The degradation of anthracene and pyrene was observed at lower values compared to phenanthrene. Isolates IV-P11, IV-P13 and V-Ph5/1 showed highest anthracene biodegradation within the range 44-48%. Amongst the bacterial species, III-R3 and IV-P11 revealed higher pyrene degradation of 60-78% pursued by V-R13 with degradation yield of 30%. Phenanthrene was degraded to a greater extent than anthracene and pyrene by the isolates, possibly because it had been used as the sole carbon source during the earlier enrichment process. Also, the higher solubility of phenanthrene (1.2 mgL^{-1}) compared to the solubility values of 0.076 and 0.077 mgL^{-1} for anthrecene and pyrene (ToxProbe Inc., 2010) might increase the bioavailability of this component to the bacterial isolates. The low aqueous solubility of PAHs limits their bioavailability and thus the efficiency of a bioremediation process. Therefore, surfactant-mediated bioremediation has been a research focus in recent years (Li and Chen, 2009).

It can be concluded that biodegradation ability of a microorganism toward one PAH cannot be extrapolated to other PAHs. In spite of structural similarity of phenanthrene and anthracene, a great difference has been observed between their biodegradation by the selected isolates. 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 has great role.

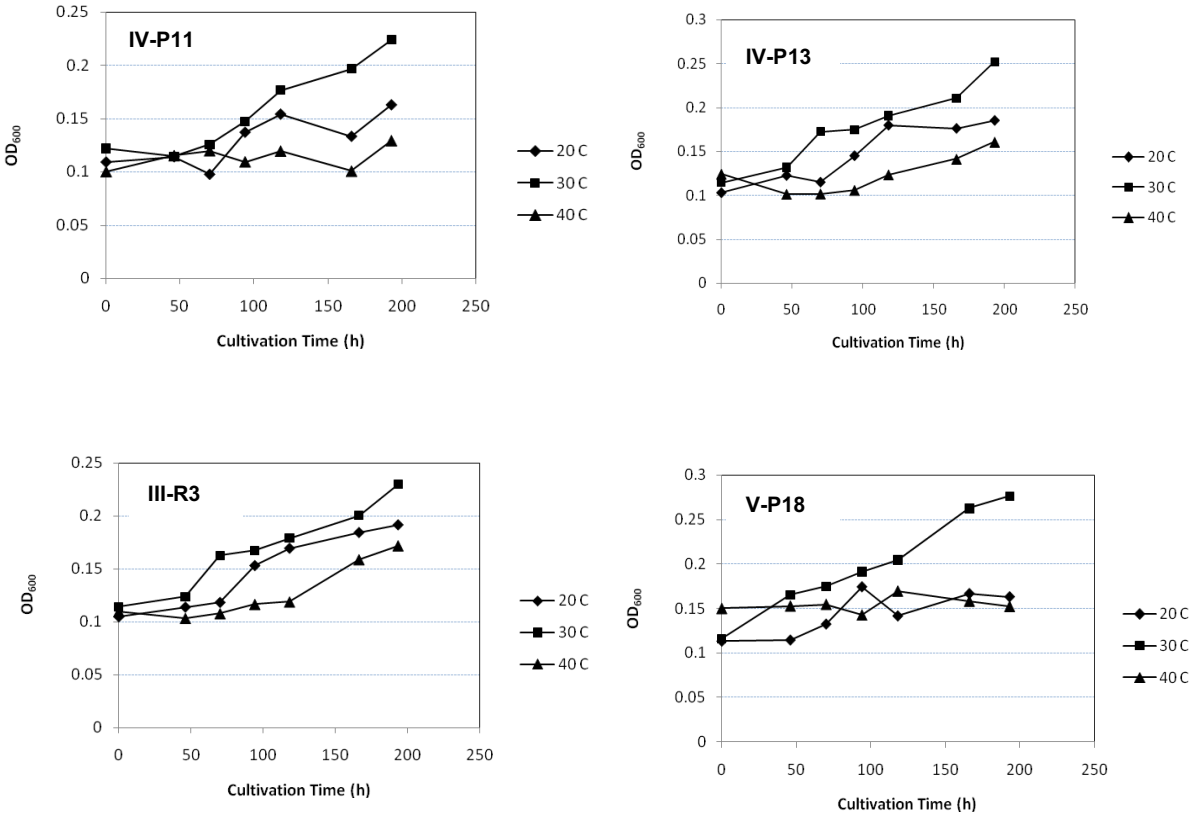

Figure 2: Effect of Temperature on selected bacterial Growth at initial phenanthrene concentration of 100 mgL^{-1}

Table 1: Biodegradation of polycyclic aromatic hydrocarbons by selected Sphingopyxis isolates after incubation for 257 h containing 100 mgL^{-1} of individual PAH

Isolate	Phenanthrene Degradation (%)	Anthracene Degradation (%)	Pyrene Degradation (%)
III-R3	95.7	26.6	78.3
IV-P11	94.1	47.9	59.1
IV-P13	95.0	43.9	1.8
V-R13	98.4	18.5	30.8
V-P18	92.0	3.1	7.0
V-Ph5/1	95.7	44.6	3.1

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