

# Biodegradation of Polycyclic Aromatic Hydrocarbons by Aerobic Mixed Bacterial Culture Isolated from Hydrocarbon Polluted Soils

*Shafiee, Parviz; Shojaosadati, Sayed Abbas \*<sup>+</sup>; Charkhabi, Amir Hossein*

*Chemical Engineering Department, Biotechnology Group, Tarbiat Modarres University,  
P.O. Box 14155-143 Tehran, I.R. IRAN*

**ABSTRACT:** *In this study, the degradation potential of five polycyclic aromatic hydrocarbons (PAHs) by aerobic mixed bacterial cultures was investigated. Microorganisms were isolated from hydrocarbon contaminated soils of Shadegan wetland located in southwest of Iran. The degradation experiments were conducted in liquid cultures. PAH or PAHs concentration was 100 mg/L at the beginning of degradation experiments. After ten days incubation, the mixed culture was capable of degrading phenanthrene completely and anthracene 80%, pyrene 60%, fluorene 30%, and fluoranthene 20%, individually. Optimal temperature and pH were as 30 °C and 7.0 respectively. Results showed that in samples containing a mixture of the five PAHs, fluoranthene could degrade cometabolically and the rate of degradation increased from 20 to 44%. It was observed also that PAHs degradation could be enhanced by the individual addition of yeast extract or glucose. The isolated mixed culture is valuable in bioremediation of PAH-contaminated environments.*

**KEY WORDS:** *Polycyclic Aromatic Hydrocarbons (PAHs), Mixed culture, Biodegradation, Bioremediation, Shadegan wetland.*

## INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are compounds composed of two or more fused benzene rings. The inertness of these compounds, their low water solubility and strong lipophilic character lead to very high accumulation levels in the environment [1]. PAHs occur as common constituents of petroleum, and coal tar but are most frequently formed by incomplete combustion of fossil fuels, waste incineration, or as by-products of industrial processes [2-4]. Some PAHs are toxic,

mutagenic and carcinogenic, and represent a potential threat to human health. The US Environmental Protection Agency (USEPA) has identified 16 PAH compounds as priority pollutants whose levels in industrial effluents require monitoring [5].

Recently, there is increased interest in developing biotechnologies to detoxify PAH contaminated sites. Bioremediation which is based on microbial transformation and degradation is one of the most promising

---

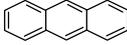
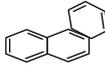
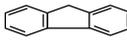
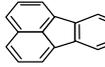
\* To whom correspondence should be addressed.

+ E-mail: [shoja\\_sa@modares.ac.ir](mailto:shoja_sa@modares.ac.ir)

1021-9986/06/3/73

6/\$/2.60

**Table 1: Structural and Chemical Characteristics of 5 PAHs used in this study**

Compound	Molecular Weight	Molecular Formula	Molecular Structure	Aqueous Solubility <sup>a</sup>
Anthracene	178	C <sub>14</sub> H <sub>8</sub>		73
Phenanthrene	178	C <sub>14</sub> H <sub>8</sub>		1290
Fluorene	166	C <sub>13</sub> H <sub>10</sub>		1980
Pyrene	202	C <sub>16</sub> H <sub>10</sub>		135
Fluoranthene	202	C <sub>16</sub> H <sub>10</sub>		260

a) Concentrations are in units of  $\mu\text{g/l}$  (ppb) at 25 °C.

methods applied in the field of environmental biotechnology for clean up of contaminated environments [6,7]. Until now, various bacteria have been isolated that are capable of degrading individual PAHs [8,9]. However, the success of bioremediation projects has been limited by the scarcity of microorganisms capable of degrading a broad range of PAHs.

In this study, we isolated a mixed bacterial culture by liquid enrichment with phenanthrene as sole source of carbon and energy from hydrocarbon polluted soils. Soil samples were collected from Shadegan wetland, which is an international wetland located in southwest of Iran. High level of hydrocarbon pollutions occurred in this wetland during the Gulf War of 1991. Degradation potential of five selected PAHs was measured by the mixed culture. The five selected PAHs used in this study are described in Table 1. In addition, comparisons were made to measure the effects of the presence of yeast extract and glucose on PAH degradation by the mixed culture.

## EXPERIMENTAL

### Media and Culture Conditions

A mixed bacterial population from the contaminated soil of the Shadegan wetland was enriched in a medium containing phenanthrene as the sole source of carbon and energy. Soils were collected from the upper 20 cm of known sites of continuous hydrocarbon effluent discharge

in Shadegan wetland. Soil samples were transported on ice back to the laboratory.

The minimal basal salts (MBS) medium used for enrichment and further experiments contained per liter [10]: 1.0 g of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 5.0 g KH<sub>2</sub>PO<sub>4</sub>, 0.1 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 5 mg of Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub> and 1.0 mL of trace elements solution. The trace element solution contained per liter [11]: 23 mg MnCl<sub>2</sub>·2H<sub>2</sub>O, 30 mg MnCl<sub>4</sub>·H<sub>2</sub>O, 31 mg H<sub>3</sub>BO<sub>3</sub>, 36 mg CoCl<sub>2</sub>·6H<sub>2</sub>O, 10 mg CuCl<sub>2</sub>·2H<sub>2</sub>O, 20 mg NiCl<sub>2</sub>·6H<sub>2</sub>O, 50 mg ZnCl<sub>2</sub>, and 30 mg Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O.

Culture media and trace element solutions were autoclaved (20 min, 121 °C, 10<sup>5</sup> Pa) separately. After sterilization, the trace element solution was slowly poured into the stirred media. Bacterial enrichment cultures were set up in 1 liter cotton-plugged Erlenmeyer flasks containing 500 mL of MBS medium. Collected soil samples were mixed and were used as source of inoculum and 20 g was added to the flask.

Flask was supplemented with 100 mg of phenanthrene per liter as the sole source of carbon and energy. Phenanthrene was added as a solution (5%) in acetone. The pH of the culture solution was adjusted to 7.0 with sodium hydroxide. The culture was incubated on a rotary shaker at 180 rpm (30°C) in the dark. Adaptation was performed after several transfers of 10% enrichment culture to a new fresh sub-enrichment culture at 7 day intervals for 1 month.

For preparation of solid medium, 20 g of agar were added per liter of above medium and 20 mL portions were poured into Petri dishes. Employing a spray-plate technique [12], the phenanthrene-acetone solution was sprayed to product a solid film of phenanthrene on the plate. Agar plates were incubated at 30°C for 3-5 days. Phenanthrene-utilizing bacteria were identified by the presence of a clear zone around the colonies.

### **Biodegradation of PAHs**

For the degradation experiments, 500-mL Schott flasks were filled with 250 mL culture media containing 100 mg/L single PAH or PAH mixture, which was added to mineral salts medium as a solution in acetone. Inoculation was prepared by scratching colonies from the surface of agar slants and preparing a suspension in 0.1 M phosphate-buffer, pH 7, to give an optical density of  $E_{630\text{ nm}} = 1.0$ . The flasks were inoculated with 250  $\mu\text{L}$  concentrated enrichment culture. The flasks were shaken at 180 rpm on rotary shakers in the dark at 30°C. At intervals, 10 mL samples were removed for extraction to determine the amount of PAH remaining in suspension.

### **PAHs Extraction and Analysis**

Residual PAHs in 1 mL sample media were extracted by adding 10 mL each of dichloromethane and acetone, and was shaken for 24 h at 30°C. The resulting mixture was transferred to centrifuge tube and centrifuged for 10 min at 12000 g. After removing excess water (upper layer) by pipetting, 4g of anhydrous sodium sulfate were mixed with the PAH-containing solvent to remove residual water completely from solvent. The extract was then concentrated to 1-2 mL using a rotary-evaporator and filtered with 0.45  $\mu\text{m}$  PTFE syringe filters.

Extracts were analyzed by high performance liquid chromatography (HPLC) using a fluorescence detector (model FL-1), pump (model 125), and system gold (all from Younglin). The HPLC apparatus was also equipped with a polymeric bound silica column (Phenomenex). The excitation level for the fluorescence detector was set at 254 nm and emission level at 390 nm. The mobile phase used was mixture of acetonitrile and water (ratio 80: 20).

## **RESULTS AND DISCUSSION**

### **Mixed culture characteristics**

The isolated mixed bacterial culture consisted of six

strains with distinct colony morphologies. Each of the isolates was identified by a variety of biochemical, bacteriological, and growth tests by Microbiology Department of Tehran University. Based on standard tests such as Gram reaction, morphology and fatty acid methyl ester profile, the bacteria were identified as the genus *Pseudomonas*, *Mycobacterium*, *Dienococcus*, *Eikenella*, *Oligella*, and *Corynebacterium* with a similarity value of 0.89.

Several studies show that PAHs with more than three aromatic rings are mainly degraded with *Mycobacterium sp.* [13-16] but degradation by some *Sphingomonas sp.* [17] has also been reported. The genera *Sphingomonas* and especially *Mycobacterium* therefore seem to be specialized in degrading such less-bioavailable compounds.

The six isolated strains showed optimal growth on YEPD agar at 26-28°C; appreciable growth was observed at room temperature (approximately 24°C), while growth was slow at 35-36°C. The strains failed to grown at 40°C. Incubations at 28°C yielded visible colonies after 24 to 30 h and heavy growth within 4 to 5 days. Colonies of the *Mycobacterium* strain were medium-size, smooth, and shiny with yellow-gold pigment, which darkens as colonies age. *Pseudomonas*, *Dienococcus*, *Eikenella*, *Oligella*, and *Corynebacterium* strains yielded green, orange, brown, bright yellow, and dark orange colonies, respectively.

### **Biodegradation of Polycyclic Aromatic Hydrocarbons**

Figs. 1 to 5 present data on the degradation of five PAHs in mixture, individually, and supplemented with glucose and yeast extract. The mixed bacterial culture degraded phenanthrene, completely, anthracene to 60%, pyrene to 80%, fluorene to 30%, and fluoranthene to 20%. The results show that as the number of benzene rings in PAH compounds increases the rate of degradation decreases. Time-course studies generally have shown that compound with low molecular weights tend to degrade at faster rates than those with high molecular weights. The biodegradability primarily depends on the complexity of chemical structures and physico-chemical properties of PAHs [8].

When mixture of five PAHs was present in aqueous solution, the degradation rates were delayed for phenanthrene, and decreased for anthracene, fluorene,

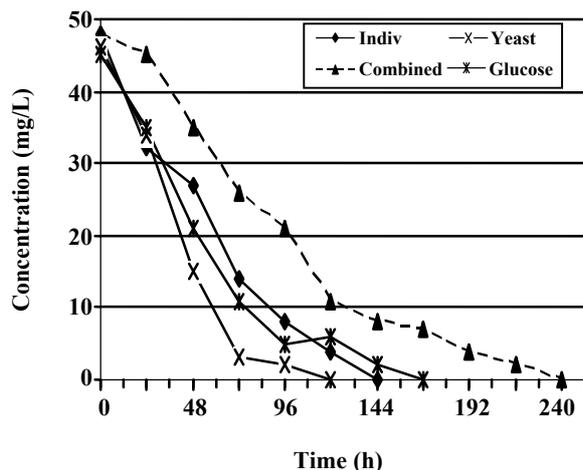


Fig. 1: Biodegradation of phenanthrene by a mixed culture.

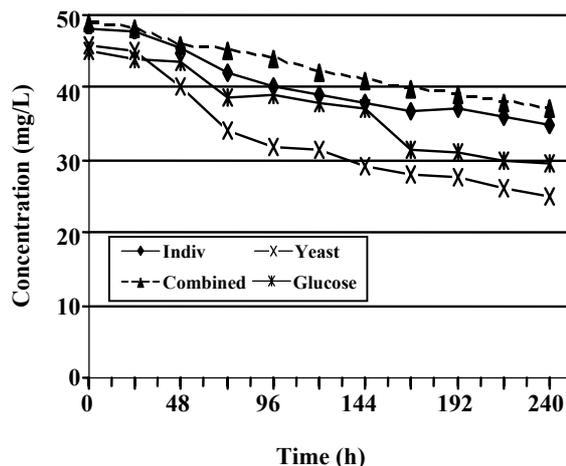


Fig. 3: Biodegradation of fluorene by a mixed culture.

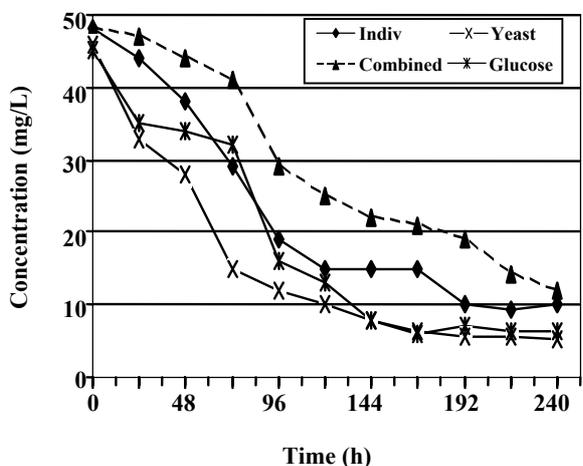


Fig. 2: Biodegradation of anthracene by a mixed culture.

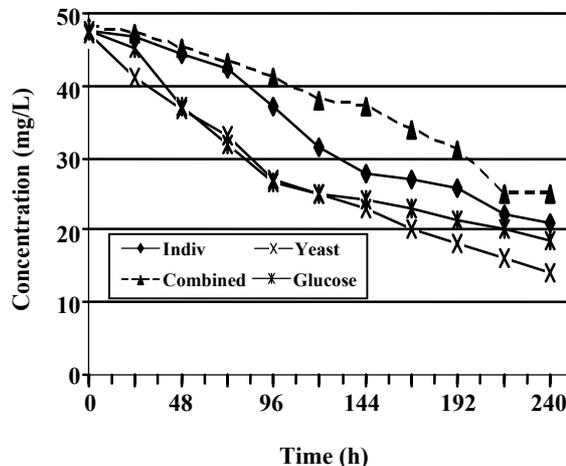


Fig. 4: Biodegradation of pyrene by a mixed culture.

and pyrene; however, fluoranthene degradation rate was increased from 20% to 44%. These results, which are similar to those reported by Tiehm and Fritzsche [18], may indicate that fluoranthene was only degraded cometabolically by the mixed culture.

Also bacterial growth, and PAH degradation were enhanced by addition of yeast extract and glucose. Several other researchers have also shown that when co-substrates were present, enhancement of PAH degradation occurred [19].

The suggestion is that the isolated aerobic mixed culture be added for PAH detoxification in PAH-polluted environments such as Shadegan wetland.

## CONCLUSIONS

In the present study, the degradation potential of five

polycyclic aromatic hydrocarbons (PAHs) by aerobic mixed bacterial cultures was performed. Optimal temperature and pH in experiments were obtained as 30 °C and 7.0, respectively. In optimal condition, the mixed culture was capable of complete degradation of phenanthrene after ten days. The results for other PAHs show that as the number of benzene rings in PAH compounds increases the rate of degradation decreases. Fluoranthene could degrade cometabolically in samples that contain all PAHs and the rate of degradation increased from 20 to 44%. According to results, the isolated mixed cultures could be valuable in bioremediation of PAH-contaminated environments.

## Acknowledgements

The authors would like to acknowledge the financial

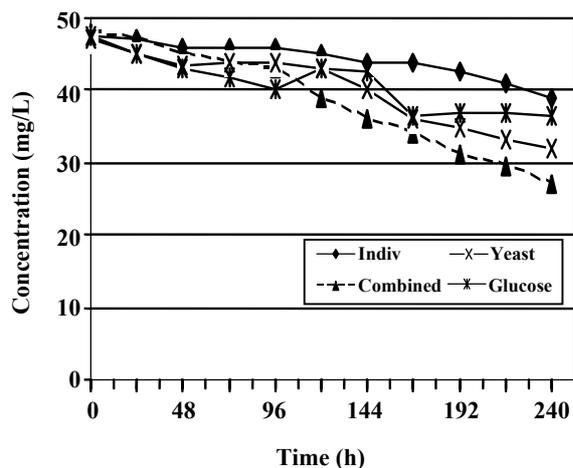


Fig. 5: Biodegradation of fluoranthene by a mixed culture.

support from Monitoring Project No. 500425. The kind help of N. Nadian throughout the research and the help of P. Hejazi and R. Panahi are appreciated.

Received: 23<sup>th</sup> September 2004 ; Accepted: 26<sup>th</sup> December 2005

## REFERENCES

- [1] Muller, J. G., Chapman, P. J., Blattmann, B. O., Pritchard, P. H. Isolation and characterization of a fluoranthene-utilizing strain of *Pseudomonas paucimobilis*, *Appl. Environ. Microbiol.*, **56**, 1079 (1990).
- [2] Pothuluri, J.V., Cerniglia, C.E., Microbial metabolism of polycyclic aromatic hydrocarbons, In: Chaundry GR (ed.), Biological degradation and bioremediation of toxic chemicals, *Dioscorides Press*, Portland, Ore., pp. 92-124 (1994).
- [3] Jones, K. C., Stratford, J. A., Waterhouse, K. S., Furlong, E. T., Giger, W., Hites, R. A., Schaffner, C., Johnston, A. E., Increases in the polynuclear aromatic hydrocarbon content of an agricultural soil over the last century. *Environ. Sci. Technol.*, **23**, 95 (1989a).
- [4] Jones, K.C., Stratford, J.A., Tidridge, P., Waterhouse, K. S., Polynuclear aromatic hydrocarbons in an agricultural soil: long-term changes in profile distribution. *Environ. Pollut.*, **56**, 337 (1989b).
- [5] Keith, L. H., Telliard, W. A., Priority pollutants I-a perspectives view, *Environ. Sci. Technology*, **13**, 416 (1979).
- [6] Wilson, S. C., Jones, K. C., Bioremediation of soil contaminated with polynuclear aromatic hydrocarbons (PAHs): a review., *Environ. Pollut.*, **81**, 229 (1993).
- [7] Atlas, R. M., Cerniglia, C. E., Bioremediation of petroleum pollutants, *Bioscience*, **45**, 332 (1995).
- [8] Cerniglia, C. E., Biodegradation of polycyclic aromatic hydrocarbons, *Biodegradation*, **3**, 351 (1992).
- [9] Yaghmaei, S., Safekordi, A., Biodegradation of polycyclic aromatic hydrocarbons (PAHs) by some bacteria isolated from coal tar contaminated soil, *Scientia Iranica*, **8**, 197 (2001).
- [10] Churchill, S. A., Harper, J. P., Churchill, P. F., Isolation and characterization of a *Mycobacterium* species capable of degrading three- and four-ring aromatic and aliphatic hydrocarbons, *Appl. Environ. Microbiol.*, **65**, 549 (1999).
- [11] Bastiaens, L., Springael, D., Wattiau, P., Harms, H., Dewachter, R., Verachtert, H., Diels, L., Isolation of adherent polycyclic aromatic hydrocarbon (PAH)-degrading bacteria using PAH-sorbing carriers, *Appl. Environ. Microbiol.*, **66**, 1834 (2000).
- [12] Kiyohara, H., Nagao, K., Yana, K., Rapid screen for bacteria degrading water-insoluble, solid hydrocarbons on agar plates, *Appl. Env. Microbiol.*, **43**, 454 (1982).
- [13] Boldrin, B., Thiem, A., Fritzsche, C., Degradation of phenanthrene, fluorene, fluoranthene, and pyrene by *Mycobacterium sp.*, *Appl. Environ. Microbiol.*, **59**, 1927 (1993).
- [14] Bouchez, M., Blanchet, D., Vandecasteele, J. P., Degradation of polycyclic aromatic hydrocarbons by pure strains and by defined strain associations: inhibition phenomena and cometabolism, *Appl. Microbiol. Biotechnol.*, **43**, 156 (1995).
- [15] Dean-Ross, D., Cerniglia, C. E., Degradation of pyrene by *Mycobacterium flavescens*, *Appl. Microbiol. Biotechnol.*, **46**, 307 (1996).
- [16] Jimenez, I. Y., Bartha, R., Solvent-augmented mineralization of pyrene by a *Mycobacterium sp.*, *Appl. Environ. Microbiol.*, **62**, 2311 (1996).
- [17] Weissenfels, W. D., Beyer, M., Klein, J., Rehm, H. J., Microbial metabolism of fluoranthene: isolation and identification of ring fission products, *Appl. Microbiol. Biotechnol.*, **34**, 528 (1991).
- [18] Tiehm, A., Fritzsche, C., Utilization of solubilized and crystalline mixtures of polycyclic aromatic hydrocarbons by *Mycobacterium sp.*, *Appl. Microbiol. Biotechnol.*, **42**, 964 (1995).

- [19] Grosser, R. J., Warshawsky, D., Vestal, J. R.,  
Indigenous and enhanced mineralization of pyrene,  
benzo[a]pyrene, and carbazole in soils, *Appl.*  
*Environ. Microbiol.*, **57**, 3462 (1991).