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Degradation of Polycyclic Aromatic Hydrocarbons (PAHs) by Indigenous Mixed and Pure Cultures Isolated from Coastal Sediments

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ABSTRACT

The goal of this paper was to quantify and characterize microorganisms (bacteria) in sediment samples contaminated with polycyclic aromatic hydrocarbons (PAHs: fluorene and naphthalene). The isolated organisms were evaluated for their ability to degrade PAHs compounds. The results indicated that the total number of recovered heterotrophic colony forming units was higher than zone forming units produced by the PAHs compounds. There was a relationship between the biomass of the bacteria recovered from the sediment and the degradation of the compounds. This indicated the utilization of the compounds by the bacteria as a carbon source. Two bacterial species were isolated from the contaminated sediments and identified as *Pseudomonas* sp. and *Ochrobactrum* sp.

Index Entries: Polycyclic aromatic hydrocarbons (PAHs); indigenous cultures; degradation; mixed species; single species.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are priority pollutants, and some are known to be chemical carcinogens and mutagens *(1,2)*. PAHs are widespread in the environment. Because of their hydrophobic nature, PAHs sorb to organic-rich soils and sediments, and can accumulate in fish and other aquatic organisms. Microbial degradation of PAHs is considered

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to be the major decomposition process for these contaminants in nature and is of great practical interest for implementation of bioremediation. Microorganisms capable of degrading PAH compounds are common in soil and sediments previously contaminated with PAHs. A great deal of effort has been directed to the use of indigenous microorganisms to accomplish bioremediation of sites contaminated by such compounds. The use of specially selected microorganisms to enhance bioremediation efforts has proven effective in a number of applications *(3,4)*. Aquatic sediments act as important sinks for PAH derivatives that are discharged either directly or indirectly into the aquatic environment. In this article, degradation of PAH compounds was tested with indigenous mixed and pure cultures isolated from coastal sediments.

PROCEDURE AND METHODS

Chemicals were obtained from Sigma Chemical Company (St. Louis, MO).

Sediment Sampling and Extraction

Sediment samples were obtained from the basin of Houston Ship Channel. Sediment samples were taken with sterilized shovels after removing 20 cm of the surface layer. The materials were collected in sterilized 1-L jars. The jars were transferred to the laboratory within a few hours, stored aerobically at 4°C, and were shipped from Rice University. The microorganisms were isolated from the sample by mixing 1 g of sediment with 10 mL of sterile sodium pyrophosphate solution ($Na_2P_2O_7$, 2.8 g/L) and 3-g glass beads (3 mm diameter) in a 50-mL plastic centrifugation tube. The tube was closed and shaken for 2 h in a horizontal position on a rotary shaker (350 rpm). The solid particles were allowed to settle for 30 min, and aliquots of the supernatant phase were used as inoculum. Dilutions of the inoculum were prepared with pyrophosphate solution. The sample extract (0.1 mL) was plated on the media, which were either blank (reference plates) or coated with one PAH compound/plate as a sole carbon source. The plates were incubated for up to 14 d at 25°C, and monitored regularly for growth or zone formation.

Isolation and Purification of Single Species

The isolation of single species was started by a repeated streaking of individual zone-forming colonies on solidified nutrient broth medium without PAH. Then the nutrient-grown colonies were transferred to the original PAH containing mineral medium. Colonies that were still able to grow and to form clearing zones on PAH were picked again and checked for purity by microscopical means. The purification steps were repeated several times. Species were identified by using the Biolog Microbial Identification System.

Culture Conditions and Degradation Experiment

Batch cultures were carried out in shake flasks. The composition of the mineral salt medium (4) was as follows: (mg/L) $(NH_4)_2 SO_4$ (1000), K_2HPO_4 (800), KH_2PO_4 (200), $MgSO_4$ (1000), $CaCl_2$-H_2O (100), sea salt (400), $FeCl_3$-$6H_2O$ (5) and $(NH_4)_6M\ o_7O_{24}$-$4H_2O$. A 200-mL sample of culture medium in a 500-mL conical flask was placed in a shaker at 30°C, 125 rpm, in the dark for growth and degradation of PAH components. The flasks were treated with PAH compounds in the range of 50 mg/L in acetone. Twelve conical flasks were inoculated, and three were taken at certain periods for analysis of dry weight (dry biomass) and residual PAH component. Parallel sterile controls were prepared similarly, except that no culture was added. Killed cell controls were obtained by autoclaving the flask containing the added culture. After an appropriate period of incubation time, three replicate cultures and controls were sampled following the introduction of 10 mL of acetonitrile (HPLC-grade, Fisher Scientific, Norcross, GA) into each of the Erlenmeyer flasks to stop growth and to solubilize the PAH. The flasks were placed back on the shaker and shaken overnight.

Gas Chromatography

The samples were centrifuged, and supernatants were analyzed by using HPLC, according to specifications of Tsomides et al. (2). Aqueous samples were solvent-extracted by shaking 10–15 mL of aqueous solution with 1 mL of dichloromethane for 30 min. The solvent phase was then removed and analyzed on the Gas Chromatograph (GC). A Hewlett Packard Model 5890 Series II Gas Chromatograph equipped with a photoionization detector and HP-5 column was used to perform the organic analysis of fluorene, phenanthrene, and naphthalene. The run parameters for the GC were as follows: initial temperature of 75°C, held isothermally for 1 min, increased at 20°C/min to 230°C, and held for 2 min. Injection port temperature is 250°C, and the detector temperature is 250°C. The carrier gas was helium at a flow rate of 40 cm/s. A series of standards was prepared by dissolving PAH crystals in dichloromethane. The standards were then analyzed on the GC, and a linear calibration line was plotted. This plot was used to measure the concentration of the experimental samples. Standards were run prior to analysis of each set of experimental samples.

Dry Weight

For estimation of dry biomass, the total batch (200 mL culture) was centrifuged at 2500 rpm for 15 min. The supernatant was decanted into another flask for analysis of the PAH residual. The residue was quantitatively transferred to a thin aluminum foil of known weight, dried in an oven overnight at 105°C, and then transferred to a desiccator at room temperature. It was weighed again, and from the difference between the final and initial weights, the amount of dry biomass was estimated. All analyses were in triplicate and averaged.

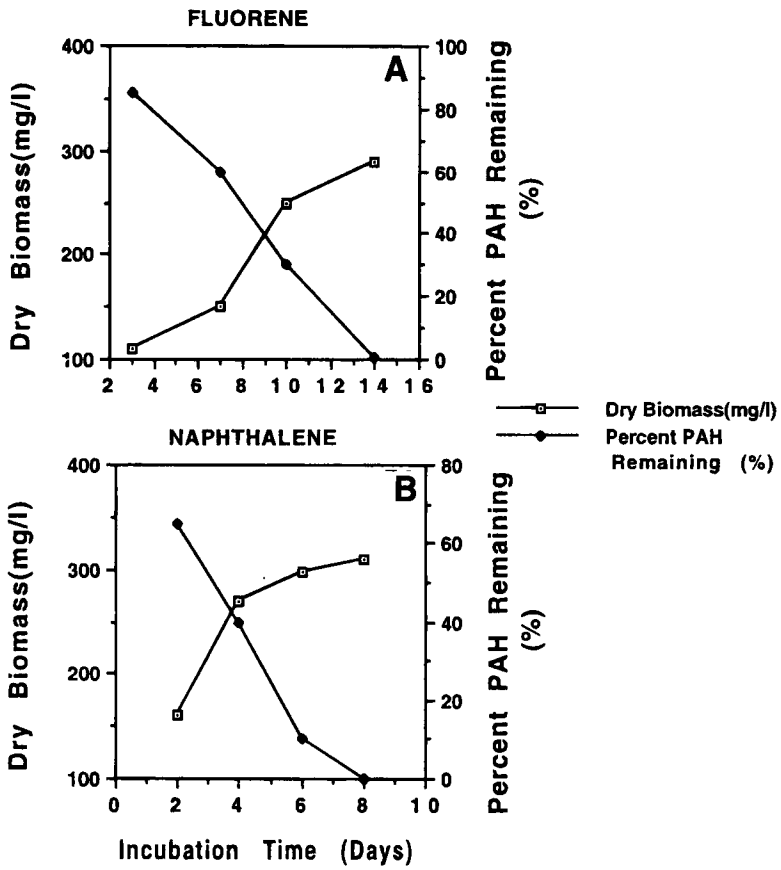

Fig. 1. Degradation of fluorene and naphthalene (0.05 mg/mL) and biomass of microflora in 200 mL of mineral medium. Standard deviation did not exceed 2% of the mean for three replicates.

RESULTS AND DISCUSSION

The ability of mixed organisms isolated from the sediments to degrade the PAH compounds is shown in Fig. 1. The biomass of the microorganism in the media containing PAH compound as sole carbon was also determined. The degradation percentage of the compounds and the biomass of microflora expressed, as a dry weight, are both shown as a function of the time. The degradation of the PAH components as well as the biomass of the sediment bacterial consortium was investigated during the growth of cultures as media containing 0.05 mg/mL naphthalene, phenanthrene, and fluorene as sole carbon sources (Fig. 1A, B). The biomass of the microflora grew with increased degradation percentage of the PAH component, which served a as source of carbon. The degradation of fluorene took a longer time than naphthalene. In sterile control tests containing no inoculum, the concentration of PAH's chemicals remained unchanged during incubation.

Table 1
Degradation of PAH compounds Fluorene and Napthalene by mixed and Isolated Microorganisms Enriched from the Sediments[a]

Culture	Fluorene	Napthalene
Mixed	90.4	98.7
Ochrobactrum sp.	85.3	77.2
Pseudomonas sp.	74.7	87.4

[a]Individual PAH degradation % used as sole growth substrate, by indigenous mixed and isolated cultures after 8 days of incubation.

The individual strains and the mixed cultures were compared for their ability to remove the PAH compounds (Table 1) after 8 d of incubation period. Individual strains were adjusted to the same optical density. The mixed cultures were prepared by mixing equal volumes of the individual strains, so that the mixture has the same optical density of the individual components. The results indicated that the degradation by the mixed culture was faster than the isolated strains. The degradation percentage of PAH components was almost complete when mixed indigenous cultures were tested, but when the isolated pure cultures were tested, the degradation percentage was slow and less than that mediated with mixed cultures. The isolated strains were able to degrade all the tested components, but each species degraded certain components at a higher rate when compared to the other components. For example, *Ochrobactrum* degraded fluorene at a much higher percentage than the other components. As is typically observed. *(6,7)*, low-mol wt compounds were degraded at faster rates than high-mol wt structures in time-course studies. The bioavailability and biodegradability of these compounds depend mainly on the complexity of the chemical structures and the corresponding physicochemical properties *(8)*.

Two bacterial strains were isolated from the original mixed culture, identified as *Pseudomonas* sp, and *Ochrobactrum* sp. *Pseudomonas* and *Flavobacterium* species have been found to degrade different PAHs in numerous studies. Mueller and coworkers *(3)* demonstrated that *Pseudomonas paucimobilis* EPA 505 degraded a range of aromatic compounds, including naphthalene, fluorene, anthracene, phenanthrene, and fluoranthene. *P. paucimobilis* and *Pseudomonas versicularis* have been shown to metabolize phenanthrene and fluorene, respectively. A *Flavobacterium* species was found in a mixed culture degrading anthracene oil *(8)*.

In biodegradation studies with either fluoranthene alone or a defined PAH mixture, better degradation was observed using the mixed culture than using individual strains. Walter and coworkers *(8)* demonstrated that degradation of anthracene oil, which contains a complex mixture of PAHs resulting from fractional distillation of coal tar, involved a 15-member mixed culture, including species of *Pseudomonas, Achromobacter, Alcaligenes,* and *Flavobacterium.*

An interesting and significant conclusion from these studies is that bioaugmentation of remediation systems should focus on the addition of mixed cultures and not pure ones. As evidenced in these studies, individual strains could degrade several PAHs, but preferred one. Thus, bioaugmentation with pure culture may not be particularly effective in field systems, where multiple contaminants are present. The collective metabolism by mixed cultures of microorganisms may result in an enhanced PAH utilization, since intermediary biotransformation products from one microorganism may serve as substrates for catabolism and growth by others *(9–11)*. Future research should focus on identifying the factors that enhance the biodegradation of PAH's compounds by the indigenous species of the sediments. Such an understanding of these limitations will lead to ecologically based and cost-effective strategies for stimulating indigenous microbial species to degrade pollutants, unless special circumstances demand inoculation with specific degrading organisms *(12)*. Several reports have been successful with *in situ* bioremediation using inoculation with specific biodegrading organisms. Biodegradation of added pollutants is often more rapid in samples from sites that have previously been exposed to pollutants than from uncontaminated sites *(2, 12–14)*.

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REFERENCES

1. Wilson, C. S. and Jones, C. K. (1993), *Environ. Pollut.* **81,** 229–249.
2. Tsomides, H. J., Hughes, J. B., Thomas, J. M., and Ward, C. H. (1995), *Environ. Toxicol. Chem.* **14,** 953–959.
3. Mueller, J. G., Chapman, P. J., Blattmann, B. O., and Pritchard, P. H. (1990), *Appl. Environ. Microbiol.* **56,** 1079–1086.
4. Mueller, J. G., Chapman, P. J., and Pritchard, P. H. (1989), *Environ. Sci. Technol.* **23,** 1197–1201.
5. Weissenfels, W. D., Beyer, M., and Klein, J. (1990), *Appl. Microbiol. Biotechnol.* **32,** 479–484.
6. Bossert, I. D. and Bartha, R. (1986), *Bull. Environ. Contam. Toxicol.* **37,** 490–495.
7. Wodzinski, R. S. and Johnson, M. J. (1968), *Appl. Microbiol.* **16,** 1886–1891.
8. Walter, U., Beyer, M., Klein, J., and Rehm, H. J. (1991), *Appl. Microbiol. Biotechnol.* **34,** 671–676.
9. Kiyohara, H., Nagao, K., and Yana, K. (1982), *Appl. Environ. Microbiol.* **43,** 454–457.
10. Bhatnagar, L. and Fathepure, B. (1991), in *Mixed cultures in Biotechnology*, Zeikus, J. G. and Johnson, E. A., (eds.), McGraw-Hill, New York, pp. 293–340.
11. Keck, J., Sims, R. C., Coover, M., Park, K., and Symons, B. (1989), *Water Res.* **23,** 1467–1476.
12. Spain, J. P., Pritchard, P. H., and Bourquin, A. W. (1980), *Appl. Environ. Microbiol.* **40,** 726–734.
13. Thomas, J. M., Lee, M. D., Scott, M. J., and Ward, C. H. (1989), *Indust. Microbiol.* **4,** 109–120.
14. Cerniglia, C. E. and Heitkamp M. A. (1989), in *Metabolism of Polycyclic Aromatic Hydrocarbons in the Aquatic Environment*, Varanasi, U. ed., CRC, Boca Raton, FL., pp. 41–68.