

Biodegradation of Polycyclic Aromatic Hydrocarbons (PAHs) by Some Bacteria Isolated from Coal Tar Contaminated Soil

A. Safekordi¹ and S. Yaghmaei*

Microbiological analyses of soil chronically exposed to coal tar of an industrialized area in Iran resulted in isolation of some bacteria capable of degrading naphthalene, phenanthrene and anthracene in an aqueous solution. The initial PAH concentrations were at the water solubility level and degradation proceeded to a nondetectable level in some cases. The degradation rate decreased with an increase in the number of fused benzene rings. For PAHs with the same number of fused rings, however, fewer clustered molecules appeared to degrade faster. The kinetic parameters of hydrocarbon (substrates) degradation and microorganism growth rates were determined using the logistic mathematical model. The calculated data based on the proposed model were in good agreement with the experimental ones.

INTRODUCTION

Polycyclic Aromatic Hydrocarbons (PAHs) are a class of ubiquitous environmental pollutants which have been detected in numerous aquatic and terrestrial ecosystems. There is concern about the presence of these compounds in the environment as they have been shown to exhibit toxic, mutagenic and carcinogenic effects [1]. PAHs may enter the environment through many routes. However, anthropogenic processes such as the combustion of fossil fuels, accidental spilling of hydrocarbons and oils, coal gasification and liquifaction, wood treatment processes, open burning and incineration of wastes are the major sources of PAHs in the environment [2-7]. Thus, the potential carcinogenic effect of PAHs in the environment and the health risks to human have led to the development of remedial technologies to detoxify PAH contaminated waters [8].

In contrast to chemical methods, biodegradation technology has received considerable attention. The so-called bioremediation, in addition to its high selectivity and mild operating condition, neither alters the intrinsic soil properties nor involves the transport of contaminants off [9].

In recent years, numerous reports have been published on isolation of potent microorganisms that detoxify low (two or three aromatic rings) and high (having four or more aromatic rings) molecular weight PAHs using pure or mixed cultures of isolated microorganisms [10-14].

This report is the first attempt on screening and isolating of potent microorganisms from Iranian soils for which the detoxifying potentials were examined and characterized. Additionally, the kinetic properties of the isolated strain were then investigated and the kinetic parameters were estimated.

MATERIALS AND METHODS

Source of PAH-Degrading Microorganisms

A series of soil samples was collected near the coal tar disposal site of Isfahan Iron Foundry Unit, located 45 km away south west of Isfahan. The samples were taken from the soil surface of six different locations near the coal tar disposal site [15-17].

Materials

Naphthalene, phenanthrene and anthracene (each used as PAH model) were purchased from Fluka AG, Buchs, Switzerland and dichloromethane (proanalysis) was obtained from Merck Company. Other chemicals used in this study were of analytical or higher grade.

1. Department of Chemical Engineering, Sharif University of Technology, Tehran, I.R. Iran.

*. Corresponding Author, Department of Chemical Engineering, Sharif University of Technology, Tehran, I.R. Iran.

Culture Media

Mineral medium contained, g/l: NaH_2PO_4 , 5.32; NH_4Cl , 2.67; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.06; mg/l: $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, 0.6; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.09; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 2.4. The medium was adjusted at pH of 7.10. To prepare mineral medium agar, 6 g agar was added to 300 ml of mineral medium.

Isolation and Purification of PAH-Degrading Bacteria

All microorganisms were isolated from the soil near the coal tar disposal site. One gram of each soil sample was suspended in 10 ml distilled water and then allowed to sit for enough time to settle soil particles. The supernatant was assayed for the presence of PAH-degrading bacteria.

The technique used for isolation was based on that of Kiyohara [18]. The surfaces of the 18 agar plates (six soil samples for three chemicals) were coated lightly with dichloromethane spray of dilute PAHs (naphthalene, phenanthrene, anthracene) separately and dried for 24 h at 35°C to volatilize the carrier solvent. Inocula from each soil sample supernatant liquid were spread with sterile rods onto agar surface of the petri dishes and were incubated for 3 weeks at 30°C.

Although many heterotrophic microorganisms grew on these media, PAH-degrading bacteria were distinguished as colonies surrounded by clear zones due to PAH utilization. These plating assays resulted in the isolation of some pure cultures which were able to utilize PAHs during growth. Therefore, at proper time and suitable temperature, colonies representative of each of the different morphological types were removed and single colonies were repeatedly purified on mineral medium agar.

The isolates were subsequently maintained on NA (Nutrient Agar) plates and stored as slant cultures under oil at 4°C.

Characterization of Isolates

Morphological characteristics were observed by a research photomicrographic microscope (Olympus, Vanox, AHB3). Gram reaction, oxidase and catalase tests were conducted using conventional microbiological procedures.

Determination of PAHs Degradation

To screen the isolates capable of degrading two and three ringed PAHs, first naphthalene (a two-ringed PAH) was considered. The naphthalene mineral medium solution was prepared by adding crystalline naphthalene to autoclaved mineral medium containing

low levels of peptone and filtering the solution through a filter paper, after enough shaking. The concentration of naphthalene in this solution was approximately at the water solubility (25 ppm). A portion of 250 ml of the solution was poured into a sterile 500 ml Erlenmeyer flask fitted with a teflon lined screw cap. The flask was inoculated with 0.1 ml of bacterial isolate suspension with uniform density and then was incubated and shaken (125 cycle/min) in the dark at 30°C.

Control incubation with no microbial inoculum was prepared to monitor the abiotic loss of naphthalene. Samples from flasks were periodically removed in duplicate for analysis. The filtrate samples were used to study the degradation of naphthalene by measurement of UV absorption-spectrum. The calculation of concentration in the samples was based on standard curves. This procedure was done for all of bacterial isolates identically.

To select the bacterial isolates to degrade phenanthrene and anthracene, as two models of three-ringed PAHs, all the experiments described for naphthalene degradation were separately repeated for them also.

Mathematical Modeling to Determine Kinetic Parameters

Many biochemical processes such as the shake flask culture in this research involve batch growth of cell populations. After seeding a liquid medium with an inoculum of living cells, nothing (except gases) is added to the culture or removed from it as growth proceeds. Typically in such a reactor, the concentrations of substrates and cells vary with time as growth proceeds.

Many batch growth models have been proposed to predict the treatment of these biochemical processes. Pearl and Reed contributed to a theory which included an inhibiting factor to population growth [19]. Assuming that inhibition is proportional to x^2 , they used:

$$\frac{dx}{dt} = kx(1 - \beta x), \quad x(0) = x_0. \quad (1)$$

This is a Riccati equation which can be easily integrated to give the logistic model for x (the cell concentration). β and k are two constants for this model. In this case, the rate of substrate utilization is:

$$\frac{ds}{dt} = \frac{1}{y} \frac{dx}{dt} + mx, \quad s(0) = s_0, \quad (2)$$

where m is the maintenance coefficient and y is the cell yield based on substrate utilization [19].

By integrating Equation 2 and replacing x from integration of Riccati equation, the substrate concentration can be expressed by an algebraic equation.

Three kinetic parameters m , k and y can be related to each other by the definition of the maintenance energy parameter as:

$$S_c = \frac{m}{k/y + m}, \quad (3)$$

where S_c is the fraction of substrate utilization for energy production.

In this research, three equations (Equation 3 and two integrated forms of Equations 1 and 2) have been used to model the substrate biodegradation and microorganism growth rate for one of the bacterial isolates, B:3. For this purpose, the substrate and cell concentrations have been measured at different time intervals.

It should be mentioned that cell concentration has been measured by hemacytometer technique [19] as the number of cells per unit of volume. The maintenance energy parameter which is not a sensitive parameter has been considered equal to 0.09 for these hydrocarbons.

Kinetic parameters for naphthalene, phenanthrene and anthracene have been separately determined by fitting the logistic mathematical model to the experimental data by nonlinear optimization. This has been carried out by minimizing the sum of squares by Quatro Pro software using optimizer method [20].

RESULTS AND DISCUSSION

Among the isolated bacteria, five strains with the degrading potential of PAHs were selected and characterized. The results of microscopic observations (cell morphology) and bacteria's gram reaction oxidase and catalase tests are shown in Table 1. For each of the five selected strains, the rate and extent of the degradation of naphthalene, anthracene and phenanthrene were examined in pure culture and batch scale at the initial concentration equal to their water solubility. The results of these aerobic degradation experiments are presented in Figures 1 to 3 for the three PAHs, respectively.

The level of PAHs in control incubations (no microbial inoculum) remained approximately constant over the duration of the test period, indicating that

no significant loss of PAHs occurred due to abiotic processes.

These figures show that almost all five selected bacteria were able to degrade the two- and three-ringed PAHs from their water solubility limits to nondetectable levels, but with different rates.

Figure 1 illustrates that for naphthalene, the bacteria B:3 and B:7 had higher degradation rate than others and could degrade 60% of naphthalene in 20 h. Complete degradation was observed after 90 h for all five bacteria.

According to Figure 2, B:14 showed the highest

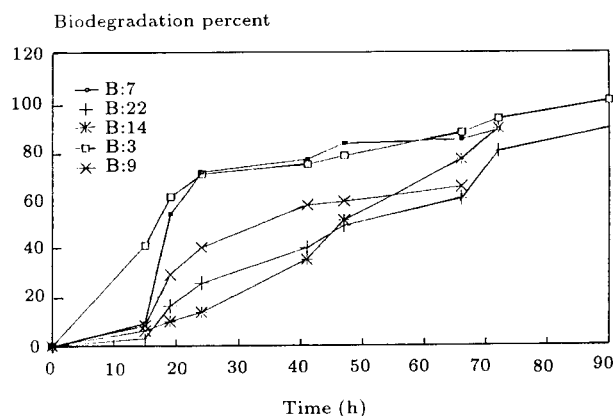


Figure 1. Comparison of naphthalene biodegradation by bacterial isolates.

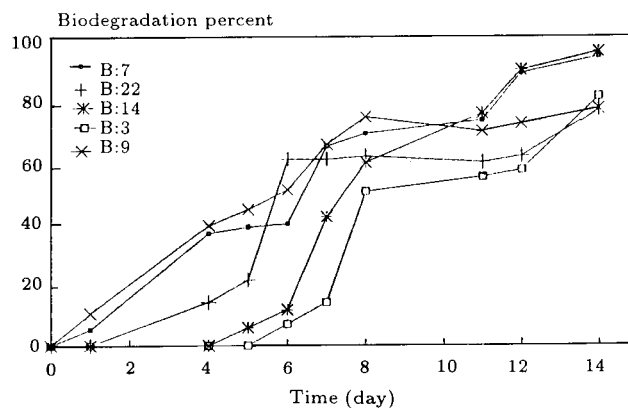


Figure 2. Comparison of phenanthrene biodegradation by bacterial isolates.

Table 1. Partial characterization of bacterial isolates.

Bacterial Isolates	Cell Morphology	Gram Reaction	Catalase	Oxidase
B:22	Polyomorphic	+	-	-
B:3	Rod shaped	+	+	-
B:7	Club-shaped	+	+	-
B:14	Cocci	+	+	-
B:9	Cocci	+	+	+

degradation rate of anthracene and complete degradation was observed after five days, whereas complete degradation took eight days for the other four bacteria with similar behavior.

The degrading potentials of the bacteria were investigated for phenanthrene, which are illustrated in Figure 3. All the bacteria consumed phenanthrene at almost the same rate, however, a lag period was observed for B:22, B:14 and B:3. The bacteria B:7 and B:9 could degrade phenanthrene completely after 14 days without lag.

These findings are quite similar to the observation of Dan L. McNally et al [21] who have recently studied the aerobic degradation of anthracene and phenanthrene by some bacteria isolated from a creosote contaminated hazardous waste site.

The observation that the degradation rate decreases with an increase in the number of fused benzene rings as for naphthalene (two-ringed PAH), phenanthrene and anthracene (three-ringed PAH) reveals that the molecular structure and size of a PAH are among the factors that can influence the biotransformation of a given chemical [22].

In addition to the size of the PAH molecule, the shape of the molecule also appears to have an influence on the microbial degradation of PAHs as seen for the phenanthrene with the same number of fused rings.

Comparison of the biodegradation of the two three-ringed PAHs demonstrates that phenanthrene is degraded at a slower rate than anthracene. These data appear to suggest that among the PAHs with the same number of fused rings, those which are more condensed or clustered (phenanthrene) are less susceptible to biodegradation than those which are less clustered (anthracene).

The available information on the biodegradation of two- and three-ringed PAHs suggests that in addition to molecular size, steric and electronic factors also

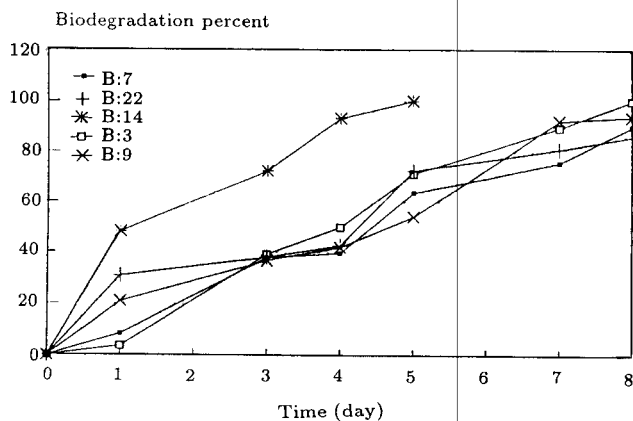


Figure 3. Comparison of anthracene biodegradation by bacterial isolates.

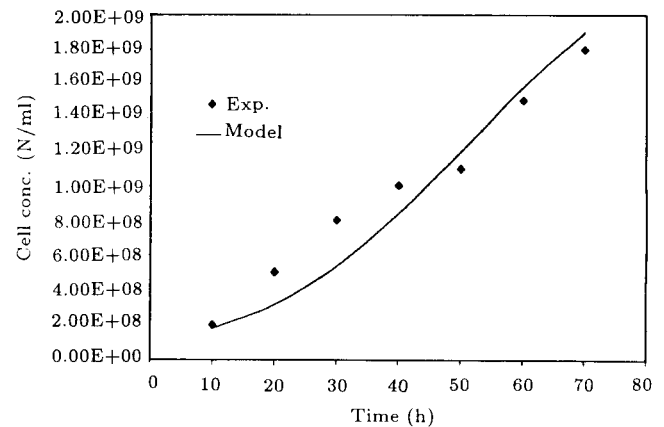


Figure 4. Comparison of model predicted and measured cell concentration for naphthalene.

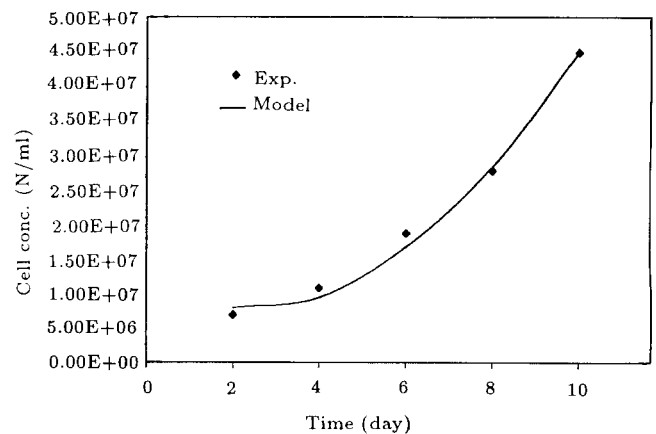


Figure 5. Comparison of model predicted and measured cell concentration for phenanthrene.

contribute to the overall rate at which various PAHs are degraded by microorganisms.

For determination of kinetic parameters, the degrading potential of one of the bacterial isolates, B:3, was examined for all three PAHs.

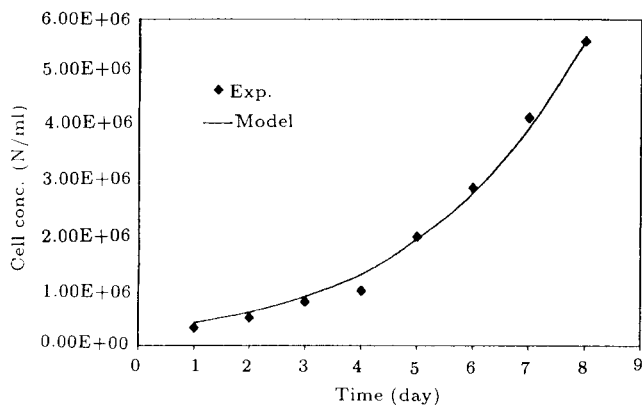
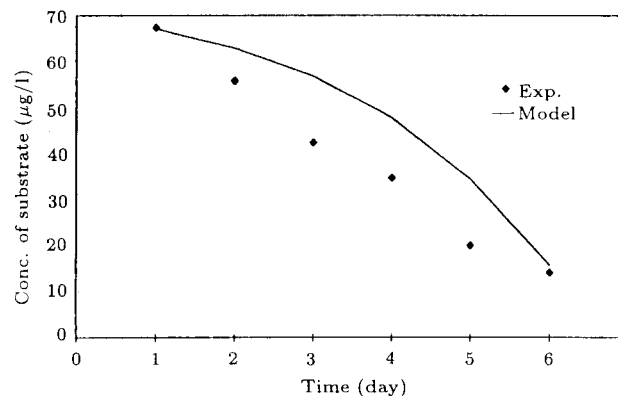
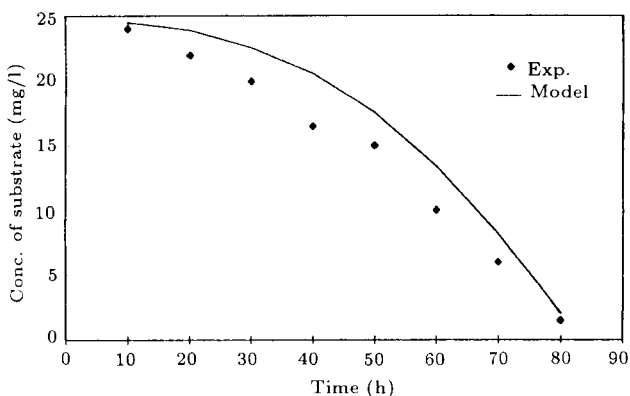
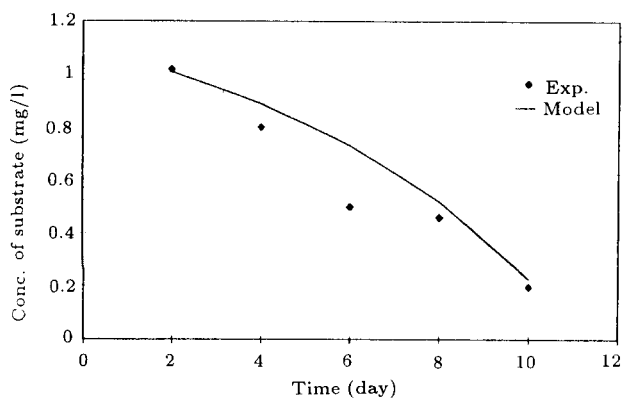
For each substrate, four kinetic parameters have been determined by fitting the logistic mathematical model to the experimental data as described previously, which are listed in Table 2. Figures 4 through 6 depict the growth of the bacterial isolate (B:3) and Figures 7 to 9 show the substrate degradation for naphthalene, phenanthrene and anthracene, respectively. In these figures, the solid lines are the logistic model prediction using kinetic parameters shown in Table 2. The comparison of model prediction and experimental data demonstrates a good agreement.

CONCLUSION

It has been established that microbial metabolism of specific organic compounds is increased by prior exposure to the compounds. Thus, the rate of pollutant

Table 2. Kinetic parameter values driven from batch experiment for B:3.

Constant	Unit	Naphthalene	Phenanthrene	Anthracene
k	1/h	0.062	0.013	0.016
β	ml/cells	4×10^{-10}	7.5×10^{-9}	2.95×10^{-8}
y	cells/mg	2.27×10^{-9}	2×10^{-8}	5.3×10^{-9}
m	mg/cells.h	2.7×10^6	6.4×10^4	2.96×10^2

**Figure 6.** Comparison of model predicted and measured cell concentration for anthracene.**Figure 9.** Comparison of model predicted and measured substrate concentration for anthracene**Figure 7.** Comparison of model predicted and measured substrate concentration for naphthalene.**Figure 8.** Comparison of model predicted and measured substrate concentration for phenanthrene.

degradation is more rapid in contaminated environments compared to pristine sites. This suggests that greater number of bacteria capable of utilizing PAH as a sole source of carbon and energy can be isolated from the site of greatest PAHs contamination in the coal tar polluted soil.

Furthermore, this study demonstrates that some bacterial isolates from Iranian coal tar contaminated soil with different specific growth rates have the ability to degrade two- and three- ringed PAHs varying in molecular size, shape and chemical structure.

A mathematical expression for cell growth and substrate utilization was proposed and the kinetic parameters were determined. Evaluation of the kinetic parameters may be useful in comparing the degrading characteristics of the microbial strains toward PAHs compounds and also can be applied for successful commercial scale design. Further studies are needed to examine whether these bacteria can degrade high molecular weight, e.g., five or six aromatic rings, and whether they can degrade PAHs in soil at concentrations higher than water solubility.

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