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Degradation of Phenanthrene by Bacterial Strains Isolated from Soil in Oil Refinery Fields in Korea

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Abstract The degradation of phenanthrene, a model PAH compound, by microorganisms either in the mixed culture or individual strain, isolated from oil-contaminated soil in oil refinery vicinity sites, was examined. The effects of pH, temperature, initial concentration of phenanthrene, and the addition of carbon sources on biodegradation potential were also investigated. Results showed that soil samples collected from four oil refinery sites in Korea had different degrees of PAH contamination and different indigenous phenanthrene-degrading microorganisms. The optimal conditions for phenanthrene biodegradation were determined to be 30°C and pH 7.0. A significantly positive relationship was observed between the microbial growth and the rate of phenanthrene degradation. However, the phenanthrene biodegradation capability of the mixed culture was not related to the degree of PAH contamination in soil. In low phenanthrene concentration, the growth and biodegradation rates of the mixed cultures did not increase over those of the individual strain, especially IC10. High concentration of phenanthrene inhibited the growth of microbial strains and biodegradation of phenanthrene, but was less inhibitory on the mixed culture. Finally, when non-ionic surfactants such as Brij 30 and Brij 35 were present at the level above critical micelle concentrations (CMCs), phenanthrene degradation was completely inhibited and delayed by the addition of Triton X100 and Triton N101.

Key words: Biodegradation, PAHs, phenanthrene, mixed culture

Polycyclic aromatic hydrocarbons (PAHs) represent a large group of organic pollutions, which have contaminated the environment through improper disposal of materials such as creosote, coal tar, and hydrocarbon fuels [21, 22]. PAHs are ubiquitous pollutants found in soil at wood preservation plants, gas works, oil refineries, runoff from asphalt pavements, and combustion processes. Their physicochemical properties, which include low water solubility and high adsorption coefficient, make soils and sediments environmental sinkers for PAHs. Since PAHs are genotoxic and carcinogenic, they represent considerable environmental concerns [25, 35]. Their mutagenicity varies with the number of aromatic rings. PAHs released into the environment could be removed by many processes, including volatilization, photo-oxidation, chemical oxidation, bioaccumulation, and adsorption on soil particles. However, the principal process for successful removal and elimination of PAHs from the environment is microbial transformation and degradation [37]. In PAHs-contaminated soil, microorganisms capable of utilizing and degrading hydrocarbons would be present and could be employed for PAH elimination [5]. Potential biodegradation strains isolated from hydrocarbon-contaminated environments have been found as active as or even higher than those originating from noncontaminated soil, since certain bacteria could acclimatize and adapt to the contaminated environments [7, 36]. Several different bacterial genera, including species of *Pseudomonas*, *Alcalgenes*, *Mycobacterium*, *Rhodococcus*, *Sphingomonas* [6, 13, 21], and *Cycloclasticus* [10], are capable of degrading PAHs. Numerous fungal species have also been found to degrade PAHs of both low and high molecular weight [21]. For bacterial isolates, the majority have enriched their ability to grow on low molecular PAHs (2- or 3-ring PAHs). Nevertheless, some studies have shown that bacteria such as *Mycobacterium*, *Rhodococcus*, *Alcaligenes*, *Pseudomonas*, and *Sphingomonas* are able to grow on the four-ring PAHs [4, 9, 15, 19]. Moreover, numerous other lower molecular weight PAHs facilitated to degrade higher molecular weight PAHs, when lower and higher PAHs were co-metabolized [10, 21, 28]. The interaction between different microorganisms under mixed-culture conditions such as co-metabolism or antagonism might also be important, and biodegradation of toxic organic compounds such as PAHs by mixed culture could be different from that of a single culture [16]. In addition, the use of microorganisms

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isolated from soil in oil refinery sites could offer advantages for several reasons: (i) most of them are adapted to this contaminated environment to allowing the inoculum to survive, and (ii) as they are able to extend through the soil by propagation, bacteria can access xenobiotics. Therefore, the present study aimed to investigate the biodegradation potential of the enriched mixed bacterial cultures obtained from different soils, which were contaminated with petroleum hydrocarbons in oil refinery fields, and to test any relationship between PAHs contamination in soil and phenanthrene biodegradation. Moreover, the biodegradation abilities between the mixed culture and individual isolate enriched from the same soil were compared. The effects of temperature, pH, initial phenanthrene concentrations, and treatment with certain carbonate or non-ionic surfactants on phenanthrene biodegradation by the mixed culture were also examined. Phenanthrene itself is not genotoxic or carcinogenic, and is generally less toxic than other PAHs of higher molecular weights; nevertheless, it represents a threat to the environment [32]. Phenanthrene is also more amenable to degradation than other higher molecular PAHs [20].

MATERIALS AND METHODS

Chemicals

Phenanthrene (99.0% analytical standard) as the model PAH compound and surfactants such as Brij 30, Brij 35, Triton X100, and Triton N101 were purchased from Sigma-Aldrich Chemicals.

Soil Samples and Analysis of PAHs

Surface soil samples (0–2 cm) 8–12 were collected from four petroleum refineries sites in Korea; Yeocheon, Incheon, Ulsan (A), and Ulsan (B). The samples were disposed in zip-bags and transferred back to the laboratory. Subsamples were immediately used for enrichment and isolation of bacterial cultures. The remaining samples were freeze-dried, ground to powder, filtered through a 2-mm sieve, and analyzed for concentrations of total PAHs, while sixteen individual PAH compounds were used as the standard protocol, modified by Tam *et al.* [30]. The soil samples were extracted by trichloromenthane and methanol after addition of M-terphenyl as the internal standard. The trichloromethane phase was concentrated, saponified in KOH, and serially extracted with mixtures of hexane and diethyl ether in sequence of 9:1, 8:2, 7:3, 6:4, and 5:5 (v/v). The extract was then dried by pure nitrogen, and PAHs fractions were eluted with benzene through a chromatographic column consisting of deactivated silica gel. The concentrations and profiles of sixteen PAH compounds were analyzed by gas chromatography (Hewlett Packard 6890) equipped with a HP-5MS fused silica capillary column (0.25 mm×30 m) coated with 0.25-μm film and connected to a flame ionization detector (GC-FID). The oven temperature program was from 60°C for 2 min to 120°C at a rate of 10°C min^{-1}, and from 120°C to 300°C at a rate 3°C min^{-1}, and then held at 300°C for 10 min. The injector and detector temperatures were set at 280°C and 300°C, respectively.

Isolation and Enrichment of Microorganisms

Mineral basal salt medium (MBSM) containing 1.0 g of $(NH_4)_2SO_4$, 0.1 g of $CaCl_2 \cdot 2H_2O$, 0.8 g of K_2HPO_4, 0.2 g of KH_2PO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, and trace elements made up of 12 mg of $FeSO_4 \cdot 7H_2O$, 3 mg of $MnSO_4 \cdot 7H_2O$, 3 mg of $ZnSO_4 \cdot 7H_2O$, 1 mg of $CoSO_4 \cdot 7H_2O$, and 1 mg of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ per one liter was used as the culture medium. The pH of the medium was around 7.0±0.2. Stock phenanthrene solution was prepared in a brown bottle at a concentration of 1.0 mg ml^{-1} acetone at 4°C, and the bottle was wrapped to avoid any light illumination before dilution. The original phenanthrene-enriched mixed culture was obtained by adding 25 g of fresh soil sample immediately after field collection in 500 ml sterilized MBSM containing 50 mg ml^{-1} phenanthrene in Erlenmeyer flasks, and shaken in an orbital shaker with 150 rpm at 30°C in the dark. Two weeks later, 10-ml aliquots were transferred to 100 ml of fresh MBSM containing 50 mg ml^{-1} phenanthrene, and the flasks were shaken for another two weeks. This step was repeated three times to isolate and purify microbial strains to degrade phenanthrene. For long-term maintenance, single phenanthrene-degrading microorganism was transferred to MBSM agar at 30°C and preserved in MBSM, containing 5 mg l^{-1} of phenanthrene and 10% (v/v) glycerin, at -80°C. An aliquot of the enriched mixed culture obtained from each soil sample was kept at 4°C for biodegradation studies.

Identification of Isolates

The chromosomal DNA was isolated by using a method described by Yoon *et al.* [41]. 16S rDNA was amplified using two primers according to Stackebrandt and Liesack [29]; 5'-GAGTTTGATCCTGGCTCAG-3' and 5'-AGAA-AGGAGGTGATCCAGCC-3'. A PCR was run for 35 cycles in a DNA thermal cycler, (Genetic analyzer 377; Perkin-Elmer, Boston, U.S.A.), employing the thermal profile described by Yoon *et al.* [41]. The 16S rDNA sequences of the strains were aligned using CLUSTAL W software [23], and the evolutionary distance matrices were calculated with the DNADIST program within the PHYLIP package. The homology values of 16S rDNA similarity were calculated from the alignment, while the evolutionary distances were calculated using a Kimura two-parameter correction. A phylogenetic tree was constructed using the neighbor-joining method [27], based on the calculated distance matrix.

Biodegradation of Phenanthrene

For biodegradation studies, bacteria strains were pre-inoculated into 100 ml of MBSM containing 50 mg ml^{-1}

Table 1. Concentrations of total PAH and phenanthrene (Phe), and ratios of phenanthrene to anthracene (Ant) and fluoranthene (Flu) to pyrene (Pyr) in soil samples collected from four petroleum refineries vicinity sites in Korea.

Soil samples	Sample size	Total PAH concentration (ng g^{-1} dry wt)	Phenanthrene concentration (ng g^{-1} dry wt)	4-6 rings PAH (%)	Phe/Ant ratio	Flu/Pyr ratio
Yeocheon	10	2,515±1726	374±467	49.3±29.0	10.0	0.36
Incheon	10	1,460±467	104±19	66.1±14.0	7.0	0.32
Ulsan (A)	8	750±56	80±26	81.1±8.2	1.3	0.90
Ulsan (B)	12	2,063±1052	115±53	90.1±3.3	1.7	0.92

phenanthrene in Erlenmeyer flasks. The flasks were incubated for 24 h at room temperature, while shaking at 150 rpm, in dark. Then, 10 ml of each pre-culture were utilized as inoculum, which contained 1.5-2.0×10 cells ml^{-1} of the isolated bacterial strains. Phenanthrene biotransformation by the bacteria was determined in parallel flasks by using 2-l Erlenmeyer flasks containing 500 ml of sterile liquid MBSM plus four different concentrations of phenanthrene including 25, 50, 100, and 200 mg l^{-1}. The flasks in three replicates were incubated in darkness at 30°C, and an initial pH 7.0± 0.2 was maintained during the experiment. Uninoculated medium flasks and autoclaved controls were also run in duplicate for each treatment. All biodegradation experiments were carried out at four different concentrations of phenanthrene as the sole carbon and energy source, except the experiments in which carbon sources such as yeast extract, glucose, acetate, or pyruvate at 250 mg l^{-1} were added. Non-ionic surfactants such as Brij 30, Brij 35,

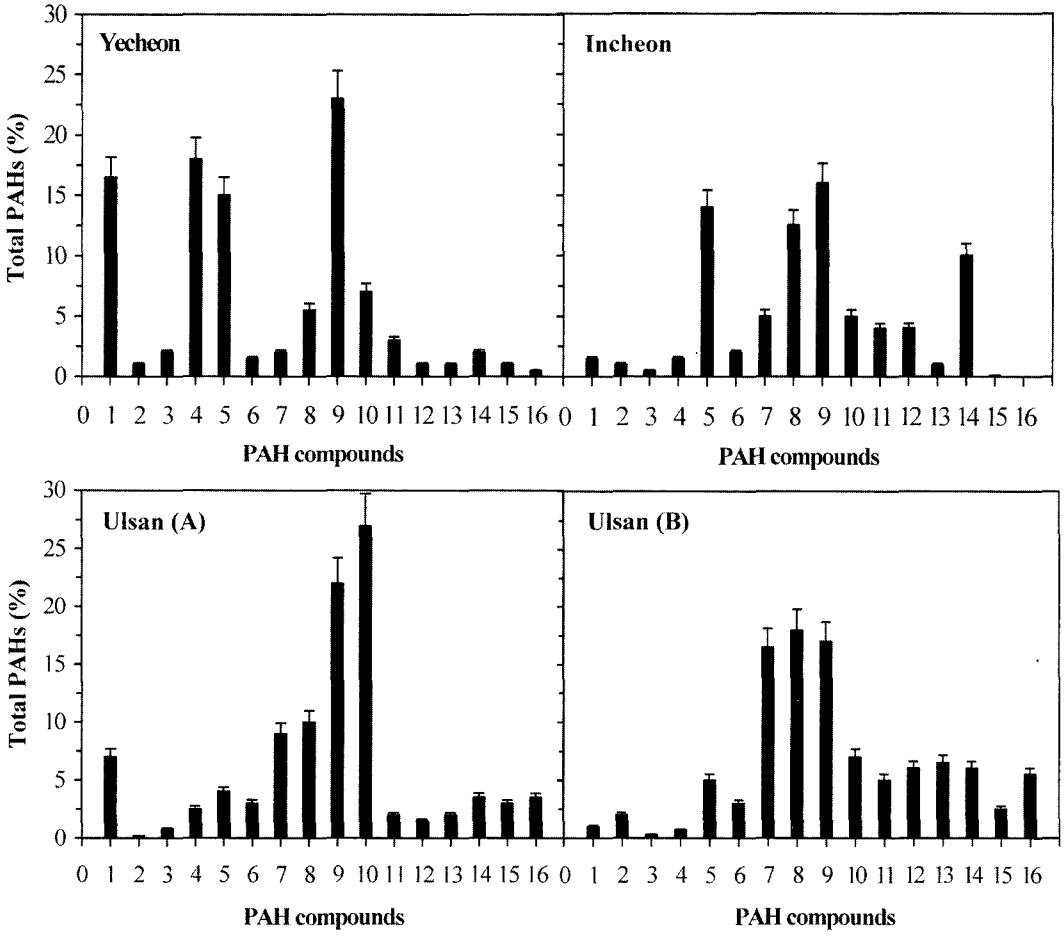

Fig. 1. The profiles of sixteen PAH compounds in four soil samples contaminated with petroleum. Mean percentage of each PAH compound to total PAHs is shown.

1, Naphthalene; 2, Acenaphthylene; 3, Acenaphthene; 4, Fluorine; 5, Phenanthrene; 6, Anthracene; 7, Fluoranthene; 8, Pyrene; 9, Benzo(*a*)anthracene; 10, Chrysene; 11, Benzo(*b*)fluoranthene; 13, Benzo(*a*)pyrene; 14, Indenol(1,2,3-*cd*)pyrene; 15, Dibenzo(*ah*)anthracene; 16, Benzo(*ghi*)perylene.

Table 2. Bacteria isolated from phenanthrene-enriched soil cultures and percentages of phenanthrene remaining in the mixed culture at the end of 14 days of enrichment.

Soil sample	Description of isolates from soil samples enriched with phenanthrene	Phenanthrene remaining (%)[a]
Yeocheon	YC13 (yellow colony, 1-5 mm)	68.3±2.3
	YC15 (pink colony, 0.5-1 mm)	
	YC33 (yellow colony, 1-3 mm)	
Incheon	IC10 (pink colony, cocci-shaped, 0.5-1 mm)	42.2±12.2
	IC28 (yellow colony, rod-shaped, 0.5-1 mm)	
Ulsan (A)	UA01 (white colony, rod-shaped, 3-4 mm)	48.2±6.9
	UA20 (green colony, rod-shaped, 0.5-1.5 mm)	
Ulsan (B)	UB18 (white colony, 1-15 mm)	70.9±6.2
	UB36 (green colony, 0.5-1.5 mm)	
	UB 41 (pale-yellow colony, 2-3 mm)	

[a]Phenanthrene remaining (%)=(initial phenanthrene concentration-phenanthrene at the end of the experiment)/initial phenanthrene concentration.

Triton X100, and Triton N101 at 1, 20, and 100 critical micelle concentrations (CMC) were employed. Other factors tested were pH (5.0, 6.0, 7.0, 8.0, and 9.0) and temperature (20°C, 30°C, and 40°C). Aqueous samples were periodically taken from treatment flasks for measuring residual concentrations. Control flasks with phenanthrene-containing MBSM were also prepared to determine abiotic losses of phenanthrene during the study. The growth of bacteria on phenanthrene was quantified daily by measuring absorbance at 660 nm. After six days, the media from bacterial and control flasks were extracted for the residual phenanthrene. The residual phenanthrene in the media was extracted with diethyl ether two times, and the extract was dried with anhydrous Na_2SO_4. Then, the extract was analyzed by GC-FID using the same setup as mentioned before.

Results and Discussion

Analysis of PAHs Contamination in Soil

The concentration of PAHs in the soil samples collected from four oil refiner vicinity sites in Korea varied significantly, with the highest concentration found in Yeocheon, followed by Ulsan (B) and Incheon, while Ulsan (A) showed the lowest concentrations (Table 1). The soil samples from Ulsan (A) and (B) had significantly higher percentages of high molecular weight (4-6 rings) PAHs, compared to total PAHs, and higher fluoranthene to pyrene (Flu/Pyr) ratios but lower ratios of phenanthrene to anthracene (Phe/Ant) than Yeocheon and Incheon (Table 1). In addition, phenanthrene concentrations in the soil samples differed among the four oil refinery sites in a similar order. The profiles of PAHs in the soil samples were also different (Fig. 1). Identification and quantification of 16 PAH compounds were based on matching their retention time (±5%) with a mixture of PAH standards (Fig. 1). The co-extractive interferences were minimal, and it was not difficult to identify the PAH peaks from soil extracts. These results suggest that the soil samples from the four different sites showed different degrees of PAH contamination [2]. Oil refinery fields are very organic-rich environments and accumulate large quantities of PAHs. It is not surprising, therefore, that microorganisms have evolved and developed abilities to utilize organic compounds such as PAHs and other petroleum hydrocarbons [1, 2].

Characteristics of Mixed Cultures and Individual Isolates for Degradation of Phenanthrene

The mixed culture enriched from the soil samples had size and color of microbial colonies (Table 2), indicating that soil samples collected from different locations give different phenanthrene-degrading microbial communities. The numbers of different hydrocarbon-degrading microbial isolates are highly significant in oil-contaminated soil and have considerable potential to degrade oil components [24, 26].

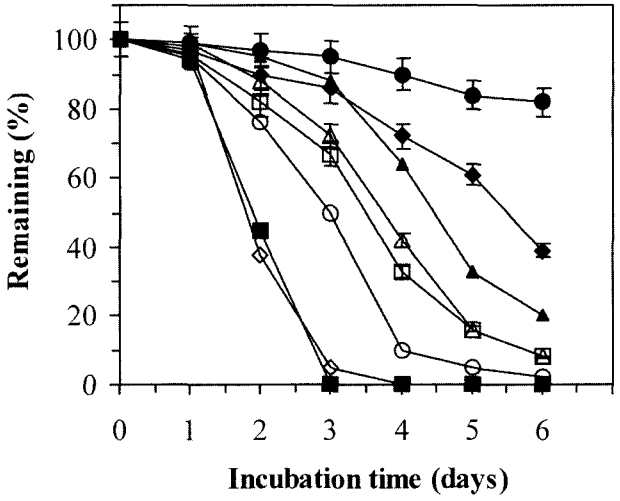

Fig. 2. Comparison of phenanthrene biodegradation in MBSM containing 25 mg l^{-1} at 30°C.
Symbols are (●), autoclaved control; (○), IC10; (□), IC28; (△), UA01; (▲), UA20; (■), IC10+IC28; (◆), UA01+UA20; (◇), IC10+IC28+UA01+US20.

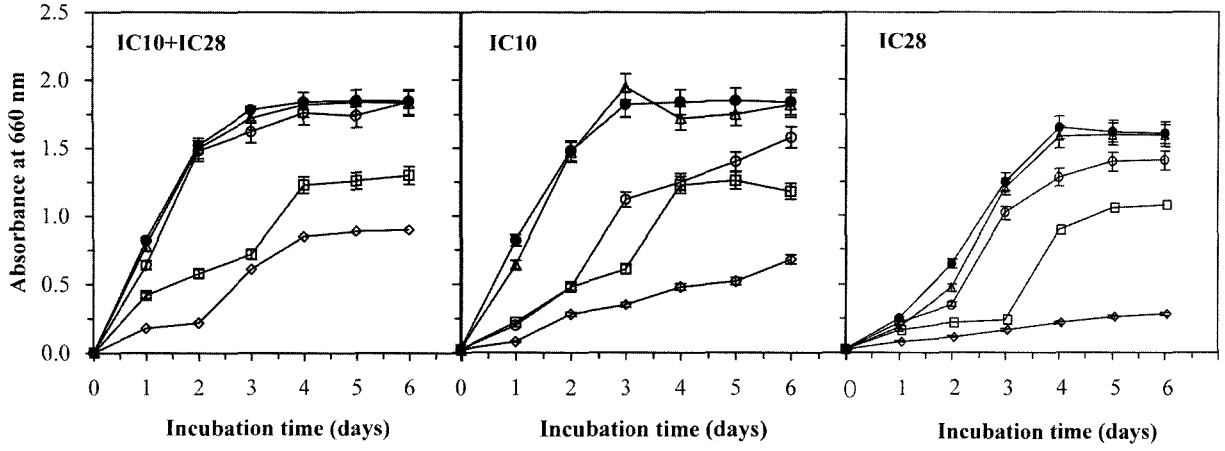

Fig. 3. Effects of phenanthrene concentrations on growth of microbial strains and mixed cultures.
Symbols are (●), control without phenanthrene; (∧), 25 mg l^{-1} of phenanthrene; (○), 50 mg l^{-1} of phenanthrene; (□), 100 mg l^{-1} of phenanthrene; (◇), 200 mg l^{-1} of phenanthrene.

The biodegradation potential of strains isolated from contaminated environments has been found to be either similar to or higher than strains originated from noncontaminated biotopes [7, 8, 36].

The degradation of phenanthrene in the medium for 6-days incubation by the enriched mixed culture also varied, ranging from 29.1% in Ulsan (B) to 57.8% in Incheon, suggesting that mixed cultures from different samples had different potentials to degrade phenanthrene. The interaction between different bacterial species on biodegradation of phenanthrene is an important consideration in developing bioremediation strategies for phenanthrene removal.

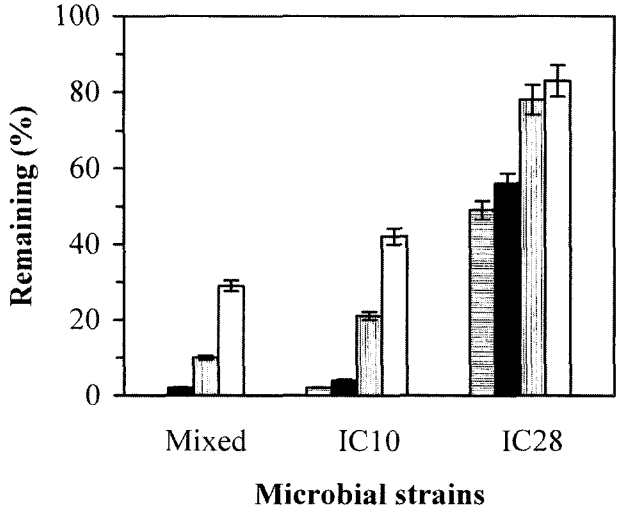

Fig. 4. Biodegradation of phenanthrene by bacterial isolates and enriched mixed culture from Incheon soil samples under different concentrations of phenanthrene after 6 days of incubation.
Symbols are (▯), 25 mg l^{-1} of phenanthrene; (■), 50 mg l^{-1} of phenanthrene; (▨), 100 mg l^{-1} of phenanthrene; (□), 200 mg l^{-1} of phenanthrene.

As shown in Fig. 2, following an initial log phase, the mixed culture degraded the phenanthrene completely in a 10 mg l^{-1} solution within 24 h. The growths of two individual isolates, IC10 and IC28, and mixed culture of IC10 and IC28 in four concentrations of phenanthrene were different (Fig. 3). Under MBSM culture containing 25 mg l^{-1} concentration of phenanthrene, the growth of isolate IC10 was similar to that of the mixed culture of IC10 and IC28. However, a longer lag phase was found in the IC28 culture. At high concentration of phenanthrene (100 and 200 mg l^{-1}), the growth of IC28 was strongly inhibited, but no inhibition was observed in the mixed culture of IC10 and IC28. The highest phenanthrene degradation was observed in the mixed culture of IC10 and IC28 at 25 mg l^{-1}, while the lowest degradation was recorded in IC28 cultures at 200 mg l^{-1} (Fig. 4). Microbial degradation of phenanthrene by the mixed culture of IC10 and IC28 was not higher than that by the individual strain IC10, although one individual strain IC28 was unable to grow and degrade phenanthrene at high concentration of phenanthrene (Figs. 3 and 4). It has been reported that isolated pure strain could not grow and metabolize organic pollutants, and that substances might be degraded or transformed under mixed culture conditions, but not in pure cultures [14].

Trzeiscka-Mlynartz and Ward [31] suggested that a mixture of four different fluoranthene-degrading PAH bacteria were more effective than any of the individual isolates alone. Numerous studies have been conducted on the capabilities of pure bacterial strains found in PAH-contaminated environments to degrade PAHs, and there is general agreement on the biochemical pathway and physiological aspects of PAHs degradation [2]. Individual microorganisms may metabolize only a limited range of substrates, therefore, it is probable that assemblages of different bacterial strains with broader enzymatic capabilities have a greater ability

to degrade complex PAH mixtures [11, 21, 31, 38]. In the present study, however, degradation of phenanthrene was not related to the degree of PAH contamination in soil, and no interrelationship could be found between PAH or phenanthrene concentrations and degradation ratio of phenanthrene by the mixed cultures. Thus, we used the mixed culture of IC10 and IC28 for all of our experiments.

The mixed culture consisted of four strains (IC10, IC28, UA01, and UA20) having distinct colony morphologies (Table 2). Each isolate was identified based on their sequence homology of 16S rDNA. The sequences of the strain IC10 showed higher homology to the sequence of *Rhodococcus* species; 95.0% to *R. rhodochrous*, 93.8% to *R. euqi*, 93.7% to *R. glovelurus*, and 93.5% to *R. fascines*, suggesting that the isolate IC10 was a *Rodococcus* sp. This strain, however, can not be accommodated into the same special species, since an exact same sequence was not found in the DNA databases, such as EMBL, GenBank, and DDBJ. The sequences of the strain IC28 exhibited very high homology to the sequence of *Pseudomonas fluorescens*, having 99.4% similarity. As the value of DNA-DNA homology is a conclusive factor in identifying bacterial strains, values higher than 70% are included in the same species [34]. According to the reports on the correlation between the DNA-DNA homology and 16S rDNA sequence homology [12, 16], strains reveal DNA-DNA homology higher than 70% when strains show sequence homology higher than 99.5%. Consequently, the strain IC28 should not be identified as *P. fluorescens*, but recognized as a *Pseudomonas* species.

Environmental Conditions for Degradation of Phenanthrene

A series of phenanthrene-degradation tests were carried out at various incubation temperatures, ranging from 20°C to 40°C and pH values from 50 to 9.0. As shown in Figs. 5(A) and 5(B), optimal conditions were determined to be 30°C and pH 7.0. This result is similar to those reported by Bauer and Capone [3]. As seen in Fig. 5(C), the slower degradation rate was observed due to increased toxicity at higher phenanthrene concentrations, in agreement with the report of Yuan *et al.* [39, 40] that biodegradation rate decreased at higher phenanthrene concentrations due to increased toxicity of phenanthrene metabolites. Figure 6 shows the effects of different carbon sources on phenanthrene microbial degradation. In general, microbial degradation of phenanthrene was improved by the addition of carbon

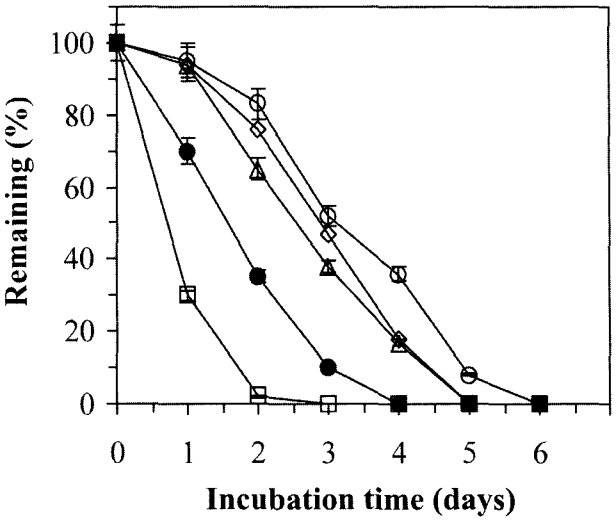

Fig. 6. Effects of various carbon sources on biodegradation of phenanthrene.
Symbols are (○), 50 mg l^{-1} of phenanthrene without adding any carbon sources; (□), adding yeast extract; (△), adding glucose; (◇), adding pyruvate; (●), adding acetate.

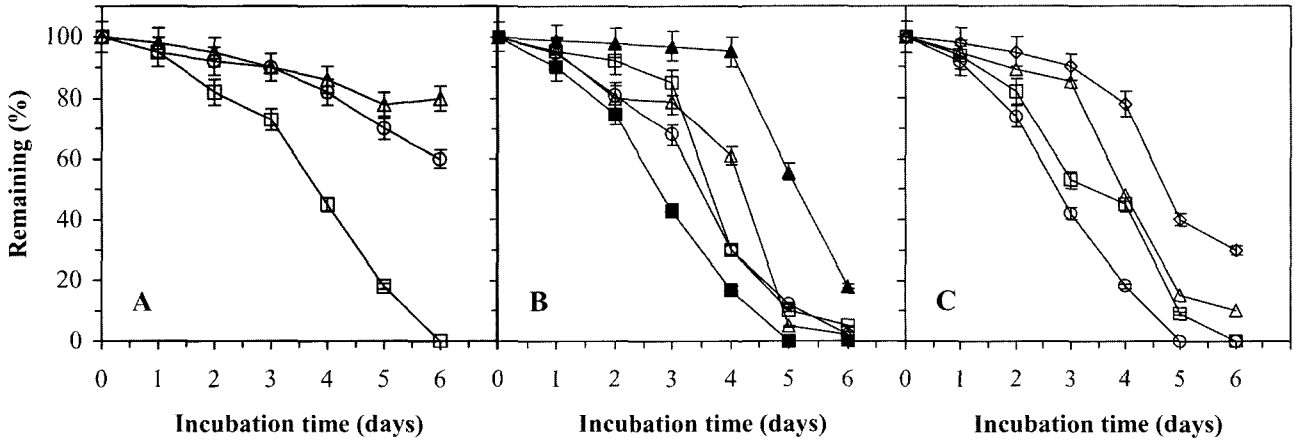

Fig. 5. Effects of changes in temperature (A), pH value (B), and phenanthrene concentration (C) on biodegradation of phenanthrene.
Symbols are (○), 20°C; (□), 30°C; (△), 40°C in (A): (▲), pH 5.0; (△), pH 6.0; (■), pH 7.0; (○), pH 8.0; (□), pH 9.0 in (B): (○), 25 mg l^{-1} of phenanthrene; (□), 50 mg l^{-1} of phenanthrene; (△), 100 mg l^{-1} of phenanthrene; (◇), 200 mg l^{-1} of phenanthrene in (C).

sources. The order of improvement of phenanthrene-degradation rates was as follows; yeast extract>acetate>glucose> pyruvate. Specifially, microbial growth and phenanthrene removal were increased by the treatment with yeast extract. This result is in accord with previous reports of yeast extract supplementation, which elevated the transformation of PAHs in soil [17]. Several other researchers have shown that phenanthrene biodegradation is influenced by a number of environmental factors such as salinity, phenanthrene concentrations, and additions of carbon sources [1]. The effects of carbon on PAH degradation are complicated and vary from microorganism to microorganism, depending on their specificity to environmental requirements. Supplementation in media with high glucose levels diminished specific fluoranthene degradation [31]. Zaidi and Iman [42] also indicated that supplementation of glucose did not improve degradation of phenanthrene, as carbon was not a limiting factor. In the present study, however, phenanthrene degradation by mixed cultures obtained from the sites contaminated with petroleum hydrocarbons was not improved by individually adding yeast extract, acetate, glucose, or pyruvate as carbon sources.

Effects of Adding Surfactant

In general, the overall degradation rate of phenanthrene was significantly improved by the addition of non-ionic surfactants (Fig. 7). We found that phenanthrene biodegradation was delayed by the four surfactants individually at 20 CMC and was completely inhibited by the addition of 100 CMC Brij 30 and Brij 35. Triton X100 and Triton N101 also slowed phenanthrene biodegradation at either 20 or 100 CMC. No significant difference was observed in naphthalene biodegradation in the presence of Brij 30 or Triton X100 at concentrations higher or lower than the CMC [18]. These results indicate that the higher the CMC of surfactants added, the greater the inhibition of phenanthrene biodegradation. Surfactants are known to produce toxicity and decrease the activity of microorganisms

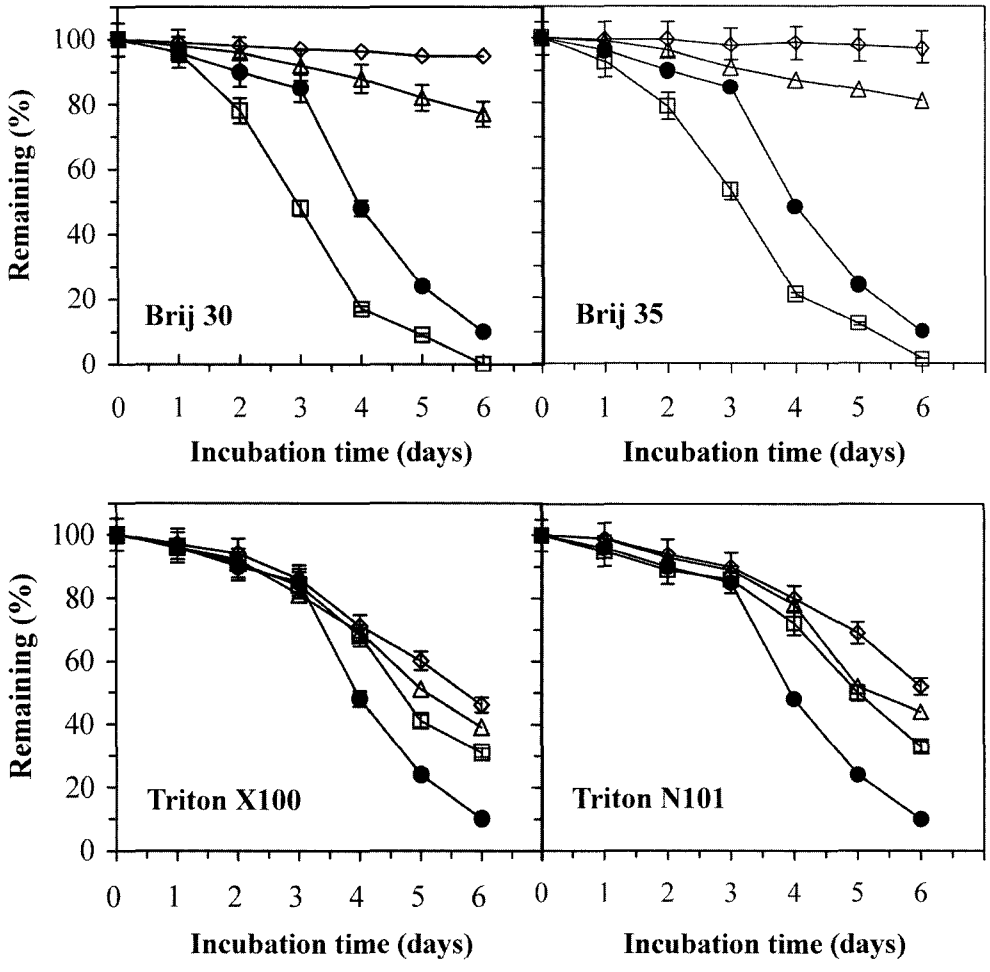

Fig. 7. Phenanthrene degradation by mixed culture in the presence of various surfactants.
Each surfactant was added to the medium to achieve the following concentrations; control (●), 1 CMC (□), 20 CMC (∧), and 100 CMC (◇). The control cultures contained no added surfactants. The incubations were carried out in MBSM at 30°C and pH 7.0.

[33]: Phenanthrene located in micelles is available only partly for degradation by microorganisms [12]. In addition, non-ionic surfactants contain polyoxyethylene molecules, an indication of the greater presence of hydrophilic molecules and a known inhibitor of microorganism activity [12].

In conclusion, phenanthrene biodegradation is affected by changes in pH, temperature, substrate concentration, and addition of carbon sources or non-ionic surfactant. Based on the results, the biodegradation potential of microorganisms isolated from petroleum hydrocarbons-contaminated soil should further be examined and optimized for bioremediation purpose. More studies on the interaction between different microorganisms, mixtures of PAHs, and effects of different environmental factors on biodegradation are essential.

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