Microbial Degradation of Polycyclic Aromatic Hydrocarbons in Soil by Bacterium-Fungus Co-cultures

Jeong-Dong Kim and Choul-Gyun Lee*

Institute of Industrial Biotechnology, Department of Biological Engineering, Inha University, Incheon 402-751, Korea

Abstract Two fungi and the phenanthrene-degrading bacterial strain *Rhodococcus* sp. IC10 were used as inocula for the bioremediation of petroleum hydrocarbon-contaminated soil from a manufactured gas plant area. The two fungi, which were previously isolated from different hydrocarbon-contaminated soil samples, were identified as *Aspergillus terreus* and *Penicillium* sp. In addition, two types of co-cultures which consist of fungal species including *A. terreus* or *Penicillium* sp. with *Rhodococcus* sp. IC10 were applied. After a 10-week incubation period, the concentrations of anthracene, phenanthrene, and pyrene were totally biodegraded by days 68, 54, and 64, for the 16 polycyclic aromatic hydrocarbons (PAH's) tested. The ecotoxicity of the soil after bioremediation did not show any effect on the survival of *Daphnia magna* (24 h-old-daphnids). However, the toxicity on seed germination of *Brassica alba* and the oxidoreductase activity of *Bacillus cereus* declined after 5- and 10-weeks of incubation, respectively. Co-cultures of *Penicillium* sp. and *Rhodococcus* sp. IC10 revealed the best efficiency at reducing ecotoxicity. © KSBB

Keywords: bioremediation, PAH, Rhodococcus, Penicillium

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAH's) represent a large group of organic pollutants which have contaminated the environment through the improper disposal of materials such as creosote, coal tar, and hydrocarbon fuels [1-3]. PAH's 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 of PAH's. PAH's represent a considerable environmental concern [4,5] because they are genotoxic and carcinogenic. Their mutagenicity varies with the number of aromatic rings and the PAH's released into the environment could be removed by many processes including volatilization, photo-oxidation, chemical oxidation, bioaccumulation, and adsorption on soil particles. However, the most successful process for removal and elimination of PAH's from the environment is to use the microbial transformation and degradation [6,7].

***Corresponding author**
Tel: +82-32-860-7518 Fax: +82-32-872-4046
e-mail: leecg@inha.ac.kr

In PAH contaminated soil, the microorganisms capable of degrading and utilizing hydrocarbons would be present and could be employed for PAH elimination [8]. Potential biodegradation microorganisms isolated from hydrocarbon-contaminated environments have been found as active as or even more active than those originating from uncontaminated soil, since certain bacteria could acclimate themselves to the contaminated environments [9,10]. Some different bacterial genera, including species of *Pseudomonas*, *Alcaligenes*, *Mycobacterium*, *Rhodococcus*, *Burkholderia* [1,11-13], and *Cycloclasticus* [14,15], are able to degrade PAH's. For the bacterial isolates, the majority have enriched their ability to grow on low molecular weight PAH's (2- or 3-ring PAH's). Nevertheless, some studies have shown that some bacteria such as *Mycobacterium*, *Rhodococcus*, *Alcaligenes*, *Pseudomonas*, and *Sphingomonas* can grow on the four-ring PAH's [16-19]. Moreover, several other lower molecular weight PAH's (LMW-PAH's) facilitate the degradation of higher molecular weight PAH's (HMW-PAH's) when lower and higher molecular weight PAH's were co-metabolized [1,14,15,20]. For fungi, the white rot fungi are able to degrade PAH's [21] and other aromatic compounds through peroxide enzymes [20,22,23]. Several fungal species have also been found to degrade PAH's of both low and high molecular weight [1].

As a result, the interaction between different microorganisms under co-culture conditions such as co-metabolism or antagonism may also be important, and the biodegradation of toxic organic compounds such as PAH's by co-culture could be different from those of a single culture [24]. In addition, the use of microorganisms isolated from petroleum hydrocarbon-contaminated soil demonstrate several advantages in the performance of bioremediation [25,26]. Despite this, few reports illustrate both, fungi and bacteria applied together for the biodegradation of PAH's in contaminated soil [27]. Furthermore, the elimination of pollutants such as PAH's from the contaminated soil does not necessarily result in the reduction in the hazardous characteristics of the contaminated soil [28].

Accordingly, this work pertains to the isolation of fungi from diverse hydrocarbon-polluted soils, which were contaminated with petroleum hydrocarbons in oil refinery fields, and examines the single and combined (fungal-bacterial) PAH-degradation potential of the isolated fungal species combined with a phenanthrene-degrading bacterium *Rhodococcus* sp. IC10. The ecotoxicity of the polluted soil was also determined on three living bio-indicators.

MATERIALS AND METHODS

Soil Samples

Several surface soil samples (0~5 cm depth) were collected from petroleum refinery sites in Incheon, Korea. Immediately after collection, sub-samples were used to enrich and isolate fungal cultures. The remaining samples were freeze-dried, ground to a powder, filtered through a 2-mm sieve, and analyzed for concentrations of total PAH's, while 16 individual PAH compounds described by United States Environmental Protection Agency were tested. The soil was kept at 4°C in the dark until used in the experiment.

Bacterium

The bacterial culture of *Rhodococcus* sp. IC10 was isolated from petroleum refinery sites by our laboratory [25] and was maintained on malt extract agar slants. The bacterial seed culture was prepared in mineral basal salt medium (MBSM) [25] with 0.05 g/L of yeast extract, which was incubated at 30°C for 24 h.

Isolation and Identification of Fungi

Fungi were isolated from the different hydrocarbon polluted soil samples according to the method of Ross *et al.* [29]. Next, the soil samples were air-dried, passed through a 2-mm sieve, and homogenized. Following this, 10 g of dry soil was added to 200 mL of sterile 0.05% agar in deionized water in a 500 mL-Erlenmeyer flask, which was shaken at 150 rpm for 30 min on a reciprocation shaker. After the solid particles were allowed to settle for 1 h, serial dilutions were prepared and 100 μL aliquots of each dilution were spread homogeneously on the surface of three replicates of Rose Bengal medium plates, which were incubated at 28°C for 3~5 days. The hyphal tips of representative isolates were transferred onto potato dextrose agar (PDA) media with no PAH's added.

Colonies were replated until pure cultures were obtained. Several isolated fungi from hydrocarbon-contaminated soil samples were selected based on their growth ability in MBSM [25] containing 50 mg/L of phenanthrene. In addition, the growth ability at several pH's including 5.0, 5.5, 6.0, and 6.5 were tested as a means for primary selection because *Rhodococcus* sp. IC10 showed the highest activity on PAH degradation at pH 5~6 in a previous study [24]. The identification of fungal strains was carried out according to the general principles of fungal classification as described previously [30-34] using a PDA and Biolog FF MicroPlate™ (Biolog Catalog #1006; Biolog Inc., Hayward, CA, USA). Moreover, the fungal strains were examined macroscopically and microscopically (40 × and 100 × with cotton blue).

Preparation of Bacterial and Fungal Inocula

Mineral basal salt-modified medium, used to grow *Rhodococcus* sp. IC10, contained 2.0 g of glucose, 2.0 g of polypeptone, 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 $MgSO_4 \cdot 7H_2O$, 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 liter. A bacterial suspension was prepared by resuspending a previously seeded culture of *Rhodococcus* sp. IC10 in 5 mL of the described medium. This suspension was added to 250 mL of mineral basal salt-modified medium in 500 mL-Erlenmeyer flasks. The flasks were shaken at 150 rpm for 2 days and the cells were harvested by centrifugation at 10,000 × g for 20 min at 4°C. In addition the cells were resuspended in the mineral basal salt-modified medium for further use as an inoculum.

The fungal inoculum was prepared by growing each fungus on PDA plates at 30°C for 3 days. The inoculum was then transferred to each flask containing MBSM medium at a 55% moisture composition, a temperature of 30°C, and daily aeration with sterile air for about 5 min every day during the 10 days. The fungi culture broth was filtered through filter paper No. 4 (Whatman Inc., Clifton, NJ, USA) at sterile conditions and a 1.0 g per wet weight of mycelia as an inoculum.

PAH Degradation in Soil

The experimental study was carried out for 10 weeks. The experimental setup is shown in Table 1. Erlenmeyer flasks (500 mL) were filled with 25 g of soil and two isolates were selected for their ability to degrade PAH's. Before inoculation, a suspension of *Aspergillus terreus/Penicillium* sp. spores was prepared by a 7-day-old Petri dish culture of each isolate on a PDA plate with 5 mL of sterile deionized water. The fragments of mycelia were removed from the spore suspension by filtration through sterile glass wool. Furthermore,

the spore suspension was estimated using a hemocytometer. To evaluate the effect of fungal inoculum on the degradation of PAH's in soil, a volume of spore suspension was added to yield a final concentration of 1×10^4 spores per gram of soil. A solution of glucose and sucrose (5 mg per gram of soil for each) was added to the sample as the carbon source. The bacterial culture of *Rhodococcus* sp. IC10 (5 mL) was inoculated repeatedly at 5 week intervals. Every inoculation had a cell concentration of 1×10^7 cfu/mL. Moreover, the humidity of the soil was adjusted to 70% of its maximum water holding capacity and maintained by the regular addition of sterilized water throughout the experiment. Each treatment had three replicates and after inoculation, the flasks were incubated in the dark at room temperature for 10 weeks.

Extraction and Analysis of the PAH's

After 10 weeks of incubation, the PAH-contaminated soil samples, with or without inocula, were homogenized and dried with sodium sulfate (analytical grade; Merck, Damstadt, Germany). The homogenized mixtures were subjected to a Soxhlet extraction using acetone-hexane (1:3, v/v) mixture for 12 h. The extracted content was evaporated by a Rotary Evaporator (N-1000, EYELA, Tokyo, Japan), redissolved in acetonitrile and analyzed by HPLC (1090, Hewlett-Packard, Palo Alto, CA, USA) equipped with a Hewlett-Packard photo-diode array model 1040A detector at 254 nm. The compounds were eluted using a linear gradient of 40 to 95% methanol/water over 40 min at 1 mL/min with a 4.6 × 250 mm 5 μm C_{18} Inertsil ODS-3 column (Meta-Chem Technologies, Torrance, CA, USA). Ultraviolet (UV) absorbance spectra were acquired over the internet (http://www.intute.ac.uk/sciences/cgi-bin/search.pl?). The solvents for extraction were of analytical grade and purchased from Merck, Darmstadt, Germany. The standards of the individual PAH's were obtained from Sigma (St. Louis, MO, USA).

Ecotoxicity Test

The ecotoxicity of the PAH-contaminated soil samples was determined at inoculation (0 week) as well as at the middle (5 weeks) and the end (10 weeks) of the bioremediation using three bioassays including (a) survival of *Daphnia magna*, (b) inhibition of *Brassica alba* seed germination, and (c) a decrease in oxidoreductase activity in *Bacillus cereus*. The survival of *D. magna* and seed germination of *B. alba*, were tested with water elutriates. The elutriates obtained from the control and treated soil samples with microorganisms were prepared by adding 50 mL of deionized water to each replicate, which was shaken at 50 rpm for 24 h. Next, the soil-slurry was centrifuged at 5,000 × g for 10 min and the supernatant was collected as an elutriate.

The acute ecotoxicity tests were performed according to the Organization for Economic Cooperation and Development (OECD) standard protocol [35]. The toxicity tests on *D. magna* were carried out in 100 mL polystyrene vessels, with 50 mL water elutriates, by observing the immobility of 24 h-old-daphnids. Twenty neonates aged less than 24 h were transferred to vessels containing different water elutriate concentrations, and the vessels were closed with a polyethylene cap. The inhibition of mustard seed germination was determined on Whatman filter paper No.1 which was moistened with 5 mL of elutriates. The method for seed germination testing followed the study by Petukhov *et al.* [36], and the toxicity tests on *B. cereus* were performed according to Ronnpagel *et al.* [37].

RESULTS AND DISSCUSSSION

Soil Analysis

The total concentration of PAH's, which is a more important parameter than the concentrations of individual PAH's as reported by the USEPA, was found to be 589.3 mg per kg of dry soil. The concentrations of individual PAH's are shown in Table 2. Furthermore, the soil was analyzed for cyanides (1.2 mg per kg of dry soil), and was characterized for pH (6.8), total dry mass (80.93%), and maximum water holding capacity (0.436 g H_2O per gram of total dry mass). Several heavy metals viz., cadmium, copper, mercury, and lead were contained as < 0.5, 1.4, 0.2, and 39.8 mg/kg, respectively.

Removal of PAH's by Fungal Strains and by Fungal-Bacterial Co-cultures

Penicillium sp. ICF117 and *A. terreus* ICF101 were isolated from PAH-contaminated soil and were tested for their capability to grow in medium added with 50 mg per kg of phenanthrene in agar for 7 days. A 58% phenanthrene removal was achieved by *Penicillium* sp., whereas a 35% removal of phenanthrene was achieved by *A. terreus*. The two other fungi achieved removal to the order of 20% (data not shown).

Six different combinations (Table 1) of the three microorganisms, *A. terreus*, *Penicillium* sp., and *Rhodococcus* sp. IC10, were tested for their ability to remove PAH's in soil originating from PAH-contaminated sites with two negative controls. PAH removal after 5 weeks of exposure to fungi, *A. terreus* and *Penicillium* sp., and bacterium *Rhodococcus* sp. IC10 from PAH-polluted soil is shown in Fig. 1A. *Penicillium* sp. alone (setup D in Table 1), which was co-cultured with *Rhodococcus* sp. IC10 (setup G in Table 1) was more efficient in the removal of four PAH's: 45 and 66.7% in anthracene, 15 and 16% in fluoranthene, 64 and 72.8% in phenanthrene, and 46.5 and 68.4% in pyrene, respectively (initial concentration = 100% of individual PAH in the soil for the experiment). Moreover, the concentrations of chrysene were also reduced by 11~16% of the same treatments. Boonchan *et al.* [38] found that *Penicillium janthinnellum* and *Stenotrophomonas malthophilia*, isolated from soil also removed the chrysene. Interestingly, the removal rate of chrysene and fluoranthene in the latter 5-week period (Fig. 1B) were slower than those of the two PAH's in the first 5-week period (Fig. 1A). The

Table 1. Biodegradation experimental setup

(A)	Uninoculated soil control
(B)	Soil inoculated with heat-killed inocula of the fungi and *Rhodococcus* sp. IC10
(C)	Modified mineral basal salt medium grown *Aspergillus terreus*
(D)	Modified mineral basal salt medium grown *Penicillium* sp.
(E)	Modified mineral basal salt medium grown *Rhodococcus* sp. IC10
(F)	Co-culture involving *A. terreus* and *Rhodococcus* sp. IC10
(G)	Co-culture involving *Penicillium* sp. and *Rhodococcus* sp. IC10

Table 2. Concentration of PAH's in initial soil before any treatment

PAH's	mg/kg of dry soil
Acenaphthene	3.8
Acenaphthylene	7.6
Anthracene	121.4
Benzo(*a*)anthracene	15.2
Benzo(*a*)pyrene	30.3
Benzo(*b*)fluoranthene	30.3
Benzo(*g,h,i*)perylene	1.2
Benzo(*k*)fluoranthene	11.4
Chrysene	30.3
Dibenzo(*a,h*)anthracene	75.9
Fluoranthene	38.0
Fluorine	7.6
Indenol(1,2,3-*cd*)pyrene	7.6
Naphthalene	7.7
Phenanthrene	106.2
Pyrene	94.8
Total PAH's	589.3

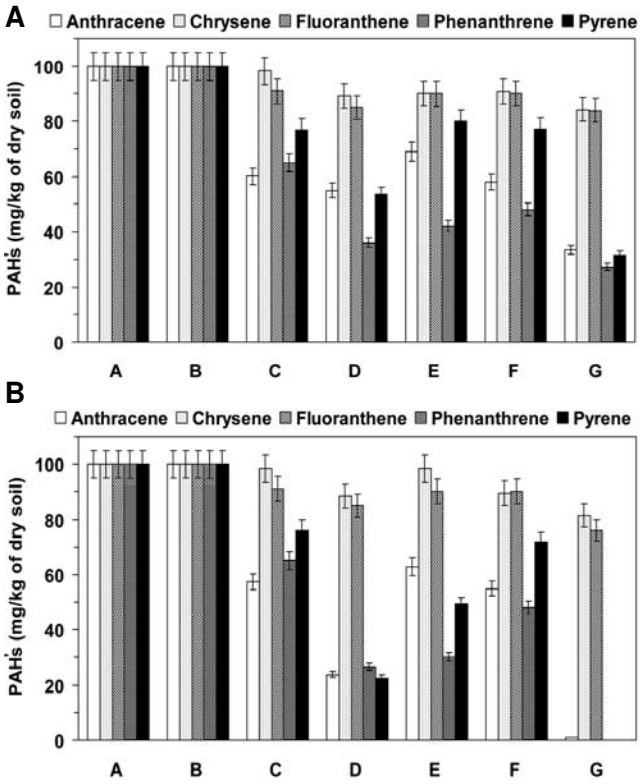

Fig. 1. Remaining concentration of PAH's after 5 (A) and 10 (B) weeks of treatment with fungal and bacterial cultures. A, Uninoculated soil control; B, Heat killed control; C, *Aspergillus terreus*; D, *Penicillium* sp.; E, *Rhodococcus* sp. IC10; F, Co-culture of *Aspergillus terreus* and *Rhodococcus* sp. IC10; G, Co-culture of *Penicillium* sp. and *Rhodococcus* sp. IC10.

treatment of *Penicillium* sp. (D in Figs. 1A and 1B) and its co-culture with *Rhodococcus* sp. IC10 (G in Figs. 1A and 1B); did not lessen the concentration of the 13 different PAH's (except for anthracene, phenanthrene, and pyrene, which were completely biodegraded by days 68, 54, and 64, respectively. In the mean time, the remaining percentage of chrysene and fluoranthene were 81.5 and 76.3%, respectively, with the same treatment. The degradation rates for PAH's in soil samples were observed as phenanthrene > pyrene > anthracene > fluoranthene > chrysene for both treatments D and G. *Achromobacter* sp. [39] and *Cunninghamella echinulata* [40] reduced the concentration of PAH's by nearly 100% in polluted soil after 8 weeks of incubation. Ross *et al.* [29] reported similar results, using indigenous fungi such as *Penicillium* and *Trichoderma* and micro-flora of soil. Brodkorb and Legge [41,42] also emphasized that it was important to mention that the use of co-cultures enhances the degradation of contaminants and it was possible to enhance the mineralization of PAH's by the indigenous micro-flora from oil-contaminated soil. The results of the present study compared the *Penicillium* sp. fungus, which was comparable to or better than *Rhodococcus* sp. IC10 removing low molecular weight PAH's when the fungus was applied by itself or by co-culture with *Rhodococcus* sp. IC10.

We observed that the initial population of *Penicillium* sp. was 1×10^4 spores/g of soil and propagated to 7.6×10^6 spores/g of soil after co-culturing for 10 weeks. On the other hand, the population of *Rhodococcus* sp. IC10 was increased to 5.8×10^8 cfu/g of soil. This suggests that the degradation of PAH in soil is the result of fungal action, and that such action can be improved by the treatment with PAH-adapted fungi. The fungus, *Penicillium* sp., probably oxidizes phenanthrene, thus increasing its solubility in water, and the bacterium. *Rhodococcus* sp. IC10, continues the oxidation steps [43] and further addresses the interaction between the co-culture and their oxidation products which are required to understand this synergistic mechanism. The removal efficiency of PAH's for the both controls, uninoculated soil and the soil inoculated with heat killed inocula, were detected to be less than 1%.

Table 3. Ecotoxicity test results of inhibition of *Brassica alba* seed germination and reduction in oxidoreductase activity of *Bacillus cereus* at the end of 5 and 10 weeks of bioremediation in soil

Treatment of soil samples[a]	Total 16 PAH's (mg/kg)[b]		Inhibition of seed germination of *Brassica alba* (%)		Reduction in oxidoreductase activity of *Bacillus cereus* (%)	
			Test time (weeks)			
	5	10	5	10	5	10
(A)	588.1±43 (99.8%)	579.9±36 (98.4%)	57.1±0.1 (100.0)	57.3±0.3 (100.0)	52.1±0.1 (100.0)	57.6±0.1 (100.0)
(B)	584.0±53 (99.1%)	572.8±34 (97.2%)	56.9±0.2 (99.6)	55.1±0.9 (96.2)	61.7±0.6 (118.4)	54.2±0.7 (94.1)
(C)	540.9±44 (91.8%)	457.9±34 (77.7%)	54.8±0.9 (96.0)	52.7±0.7 (92.0)	49.5±0.7 (95.0)	47.4±0.9 (82.3)
(D)	358.3±18 (60.8%)	295.8±36 (50.2%)	55.1±1.2 (96.5)	40.1±1.8 (70.0)	50.1±1.3 (96.2)	38.1±1.0 (66.1)
(E)	450.2±38 (76.4%)	434.9±41 (73.8%)	53.2±0.4 (93.2)	47.1±0.2 (82.2)	42.4±0.9 (81.4)	43.7±0.9 (75.9)
(F)	508.6±29 (86.2%)	512.1±58 (86.7%)	52.9±1.3 (92.6)	51.4±0.6 (89.7)	47.1±0.8 (90.4)	46.1±0.1 (80.0)
(G)	280.1±18 (48.2%)	218.6±38 (37.1%)	43.3±0.3 (75.8)	28.9±0.9 (50.4)	40.2±1.1 (77.2)	25.2±0.9 (43.8)

[a](A), (B), (C), (D), (E), (F), and (G) are mentioned in Table 1.
[b]Values represent mean ± standard deviation (% PAH recovery) of three replicates.

Ecotoxicity

The results of the ecotoxicity test are shown in Table 3. All twenty juvenile crustaceans were motile in each of three replicates when exposed to water elutriates prepared from soil samples from 0, 5, and 10 weeks. Among the three tests, the ecotoxicity test examining the survival rate of *D. magna* did not generate significant resultsin terms of the resistance of *D. magna* to PAH's. Although the PAH's were detected in soil elutriates, many authors have indicated that PAH water elutriates leached from heavily PAH-contaminated soils had no toxic effect on *D. magna* [44].

The two other biological indicators (inhibition of mustard seed germination and reduction in oxidoreductase activity in *B. cereus)* resulted in a lower ecotoxicity after the treatments (Table 3). The significant decreases in toxicity were first observed in the D and G treatments after 10 weeks of inoculation (compared to the controls, A and B). The inhibitory effect on mustard seeds decreased from 70.0 to 50.4% by the 10th week of D and G treatment, respectively (Table 3).

In other words, a significant synergistic effect of the co-culture of the fungus and the bacterium was observed, (in comparison with the bacterial culture alone, E), which reduced the inhibitory effect by only 17.8% after a 10-week treatment. This result confirms the suggested synergistic mechanism. Only the fungus *Penicillium* sp. can degrade the PAH's in soil. Moreover, the bacterium *Rhodococcus* sp. IC10 utilizes the degraded products by oxidation and thus enhances the overall biodegradation process. The culture of *A. terreus* (C) and its co-culture with *Rhodococcus* sp. IC10 (F) did not significantly alter the germination inhibition of mustard seed (no more than 11%). The results obtained from the oxidoreductase activity test in *B. cereus* exhibited similar trends (Table 3) which identifies the co-culture of *Penicillium* sp. and *Rhodococcus* sp. IC10 as the most efficient combination of organisms which reduces the inhibitory effect on the enzyme to 43.8% of the negative control after a 10-week treatment. The pure culture of *Penicillium* sp. (D) was relatively efficient at reducing the inhibition of oxidoreductase to 66.1% of the control. Of particular note, the percentage of oxidoreductase activity reduction in *B. cereus* by the co-culture (D), 56.2%, is approximately the sum of individual oxidoreductase activity reduction, 33.9 and 24.1% for *Penicillium* sp. (D) and *Rhodococcus* sp. IC10 (E), respectively.

Again, the co-culture of *A. terreus* and *Rhodococcus* sp. IC10 alleviated the inhibition of oxidoreductase only by 20%. This suggests that there must be several types of interactions between fungus and bacterium: positive, neutral, and negative. The co-culturing of the bacterium *Rhodococcus* sp. IC10 with the fungus *A. terreus* was not stable for PAH degradation. However, further studies are required to clarify the mechanisms of PAH removal as well as the synergistic mechanisms of microbial interaction and the ecology of these systems.

CONCLUSION

This study investigated the ability of PAH removal by fungi and bacteria isolated from petroleum hydrocarbon-contaminated soil. The co-culture of *Penicillium* sp. and *Rhodococcus* sp. IC10 was found to be the optimal combination for the removal of different PAH's from contaminated soil and for the reduction of soil toxicity, evaluated by mustard seed germination and the oxidoreductase activity of *B. cereus*. Despite this, the co-culture of the fungi *A. terreus* and *Rhodococcus* sp. IC10 did not show any synergistic effect in the degradation efficiency when compared to the axenic culture of the fungi. This suggests that selecting a proper set of strains for the co-culture is important for the optimal degradation with synergistic effects. This study found that the co-culture of two microorganisms, *Penicillium* sp. and *Rhodococcus* sp. IC10, isolated from polluted soils, could enhance the bioremediation processes in the treatment of contaminated soils with petroleum from creosote which contains PAH's.

Acknowledgements The authors would like to gratefully

thank the Korea Research Foundation Grant (KRF-2006 J00702), for the support of this study.

Received March 28, 2007; accepted June 12, 2007.

REFERENCES

1. Mueller, J., C. Cerniglia, and P. Pritchard (1997) Bioremediation of environments contaminated by polycyclic aromatic hydrocarbons. pp. 125-194. In: R. Crawford and D. Crawford (eds.). *Bioremediation: Principles and Practices.* Cambridge University Press, New York, NY, USA.
2. Mueller, J. G., P. J. Chapman, and P. H. Pritchard (1989) Creosote-contaminated sites. Their potential for bioremediation. *Environ. Sci. Technol.* 23: 1197-1201.
3. Leon, V. and M. Kumar (2005) Biological upgrading of heavy crude oil. *Biotechnol. Bioprocess Eng* 10: 471-481.
4. Pahlman, R. and O. Pelkonen (1987) Mutagenicity studies of different polycyclic aromatic hydrocarbons: The significance of enzymatic factors and molecular structure. *Carcinogenesis* 8: 773-778.
5. White, Jr., K. L. (1996) An overview of immunotoxicology and carcinogenic polycyclic aromatic hydrocarbons. *Environ. Carcinog. Rev.* 4: 163-202.
6. Wilson, S. C. and K. C. Jones (1993) Bioremediation of soil contaminated with polynuclear aromatic hydrocarbons (PAHs): A review. *Environ. Pollut.* 81: 229-249.
7. Ahn, T. S., G. H. Lee, and H. G. Song (2005) Biodegradation of phenanthrene by psychrotrophic bacteria from Lake Baikal. *J. Microbiol. Biotechnol.* 15: 1135-1139.
8. Catallo, W. J. and R. J. Portier (1992) Use of indigenous and adapted microbial assemblages in the removal of organic chemicals from soils and sediments. *Wat. Sci. Technol.* 25: 229-237.
9. Chaneau, C. H., J. Morel, J. Dupont, E. Bury, and J. Oudot (1999) Comparison of the fuel oil biodegradation potential of hydrocarbon-assimilating microorganisms isolated from a temperate agricultural soil. *Sci. Total Environ.* 227: 237-247.
10. Wild, S. R. and K. C. Jones (1993) Biological and abiotic losses of polynuclear aromatic hydrocarbons (PAHs) from soils freshly amended with sewage sludge. *Environ. Toxicol. Chem.* 12: 5-12.
11. Cerniglia, C. E. (1992) Biodegradation of polycyclic aromatic hydrocarbons. *Biodegradation* 3: 351-368.
12. Han, K., Y. T. Jung, and S. Y. Son (2003) Phylogenetic analysis of phenanthrene-degrading *Sphingomonas*. *J. Microbiol. Biotechnol.* 13: 942-948.
13. Lee, D. S., M. W. Lee, S. H. Woo, and J. M. Park (2005) Effects of salicylate and glucose on biodegradation of phenanthrene by *Burkholderia cepacia* PM07. *J. Microbiol. Biotechnol.* 15: 859-865.
14. Dyksterhouse, S. E., J. P. Gray, R. P. Herwig, J. C. Lara, and J. T. Staley (1995) *Cycloclasticus pigetti* gen. nov., and aromatic hydrocarbon-degradation bacterium from marine sediments. *Appl. Environ. Microbiol.* 45: 116-123.
15. Dyksterhouse, S. E., J. P. Gray, R. P. Herwig, J. C. Lara, and J. T. Staley (1995) *Cycloclasticus pugetii* gen. nov., sp. nov., an aromatic hydrocarbon-degrading bacterium from marine sediments. *Int. J. Syst. Bacteriol.* 45: 116-123.
16. Boldrin, B., A. Tiehm, and C. Fritzsche (1993) Degradation of phenanthrene, fluorene, fluoranthene, and pyrene by a *Mycobacterium* sp. *Appl. Environ. Microbiol.* 59: 1927-1930.
17. Dagher, F., E. Deziel, P. Lirette, G. Paquette, J. G. Bisaillon, and R. Villemur (1997) Comparative study of five polycyclic aromatic hydrocarbon degrading bacterial strains isolated from contaminated soils. *Can. J. Microbiol.* 43: 368-377.
18. Kastner, M., M. Breuer-Jammali, and B. Mahro (1994) Enumeration and characterization of the soil microflora from hydrocarbon-contaminated soil sites able to mineralize polycyclic aromatic hydrocarbons (PAH). *Appl. Microbiol. Biotechnol.* 41: 267-273.
19. Lloyd-Jones, G. and D. W. Hunter (1997) Characterization of fluoranthene- and pyrene-degrading Mycobacterium-like strains by RAPD and SSU sequencing. *FEMS Microbiol. Lett.* 153: 51-56.
20. Schocken, M. J. and D. T. Gibson (1984) Bacterial oxidation of the polycyclic aromatic hydrocarbons acenaphthene and acenaphthylene. *Appl. Environ. Microbiol.* 48: 10-16.
21. Kobayashi, H. and B. E. Rittmann (1982) Microbial removal of hazardous organic compounds. *Environ. Sci. Technol.* 16: 170-183.
22. Bumpus, J. (1998) Perspectives on the use of white rot fungi in bioremediation technologies. Vol. II. pp. 67-109. In: K. Subhas and R. L. Sikdar-Irvine (eds.). *Bioremediation: Principles and Practices Biodegradation Technology Developments.* Technomic Publishing Co., Lancaster, PA, USA.
23. Mester, T., M. Pena, and J. A. Field (1996) Nutrient regulation of extracellular peroxidases in the white rot fungus, *Bjerkandera* sp. strain BOS55. *Appl. Microbiol. Biotechnol.* 44: 778-784.
24. Fernandez-Sanchez, J. M., R. Rodriguez-Vazquez, G. Ruiz-Aguilar, and P. J. J. Alvarez (2001) PCB biodegradation in aged contaminated soil: Interactions between exogenous *Phanerochaete chrysosporium* and indigenous microorganisms. *J. Environ. Sci. Health A* 36: 1145-1162.
25. Kim, J. D., S. H. Shim, and C. G. Lee (2005) Degradation of phenanthrene by bacterial strains isolated from soil in oil refinery fields in Korea. *J. Microbiol. Biotechnol.* 15: 337-345.
26. Da Silva, T. L., V. Calado, N. Silva, R. L. Mendes, S. S. Alves, J. M. T. Vasconcelos, and A. Reis (2006) Effects of hydrocarbon additions on gas-liquid mass transfer coefficients in biphasic bioreactors. *Biotechnol. Bioprocess Eng.* 11: 245-250.

27. Kotterman, M. J., E. H. Vis, and J. A. Field (1998) Successive mineralization and detoxification of benzo[a] pyrene by the white rot fungus *Bjerkandera* sp. strain BOS55 and indigenous microflora. *Appl. Environ. Microbiol* . 64: 2853-2858.
28. Hauser, B., G. Schrader, and M. Bahadir (1997) Comparison of acute toxicity and genotoxic concentrations of single compounds and waste elutriates using the Microtox/Mutatox test system. *Ecotoxicol. Environ. Saf.* 38: 227-231.
29. Ross, B., Z. Marie, and E. Senior (1995) Bioremediation of an oil contaminated soil by fungal intervention. *Proceeding of 3rd International In Situ and on Site Bioreclam Symposium.* April 24-27. San Diego, CA, USA.
30. Deacon, J. W. (1997) *Modern Mycology.* 3rd ed., Blackwell Science, Oxford, USA.
31. Langeron, M. and R. Vanbreuseghem (1995) *Outline of Mycology.* 2nd ed., Sir Isaac Pitman & Sons, London, England.
32. Larone, D. H. (1993) *Medically Important Fungi a Guide to Identification.* 2nd ed., American Society for Microbiology, Washington DC, USA.
33. Onions, A. H. S., D. Allsopp, and H. O. W. Eggins (1981) *Smith's Introduction to Industrial Mycology.* John Willey & Sons, Inc., New York, NY, USA.
34. Von Arx, J. A. (1974) *The Genera of Fungi Sporulating in Pure Culture.* J. Cramer., Vaduz, Germany.
35. OECD (1998) *Daphnia magna* reproduction test No. 211. In: *OECD Guidelines for Testing of Chemicals*. Organization for Economic Co-operation and Development, Paris, France.
36. Petukhov, V. N., V. M. Fomchenkov, V. A. Chugunov, and V. P. Kholodenko (2000) Plant biotests for soil and water contaminated with oil and oil products. *Appl. Biochem. Microbiol.* 36: 564-567.
37. Ronnpagel, K., W. Lis, and W. Ahlf (1995) Microbial bioassay to asses the toxicity of solid-associated contaminants. *Ecotoxicol. Environ. Saf.* 31: 99-103.
38. Boonchan, S., M. L. Britz, and G. A. Stanley (2000) Degradation and mineralization of high-molecular-weight polycyclic aromatic hydrocarbons by defined fungal-bacterial cocultures. *Appl. Environ. Microbiol.* 66: 1007-1019.
39. Cutright, T. and S. G. Lee (1994) Remediation of PAH-contaminated soil using *Achromobacter* sp. *Energy Sources* 16: 279-287.
40. Cutright, T. J. (1995) Polycyclic aromatic hydrocarbon biodegradation and kinetics using *Cunninghamella echinulata* var. *elegans*. *Int. Biodeter. Biodeg.* 34: 397-408.
41. Brodkorb, T. S. and R. L. Legge (1992) Enhanced biodegradation of phenanthrene in oil-contaminated soils supplemented with *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* 58: 3117-3121.
42. Ohmiya, K., K. Sakka, and T. Kimura (2005) Anaerobic bacterial degradation for the effective utilization of biomass. *Biotechnol. Bioprocess Eng.* 10: 482-493.
43. Meulenberg, R., H. H. M. Rijnaarts, H. J. Doddema, and J. A. Field (1997) Partially oxidized polycyclic aromatic hydrocarbons show an increased bioavailability and biodegradability. *FEMS Microbiol. Lett.* 152: 45-49.
44. Gourlay, C., M. H. Tusseau-Vuillemin, J. Garric, and J. M. Mouchel (2003) Effect of dissolved organic matter of various origins and biodegradabilities on the bioaccumulation of polycyclic aromatic hydrocarbons in *Daphnia magna*. *Environ. Toxicol. Chem.* 22: 1288-1294.