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Bioremediation of Phenanthrene by Monocultures and Mixed Culture Bacteria Isolated from Contaminated Soil

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***Abstract*—**Three different bacteria capable of degrading phenanthrene were isolated from hydrocarbon contaminated site. In this study, the phenanthrene-degrading activity by defined monoculture was determined and mixed culture was identified as *Acinetobacter* sp. P3d, *Bacillus* sp. P4a and *Pseudomonas* sp. P6. All bacteria were able to grow in a minimal salt medium saturated with phenanthrene as the sole source of carbon and energy. Phenanthrene degradation efficiencies by different combinations (consortia) of these bacteria were investigated and their phenanthrene degradation was evaluated by gas chromatography. Among the monocultures, *Pseudomonas* sp. P6 exhibited 58.71% activity compared to *Acinetobacter* sp. P3d and *Bacillus* sp. P4a which were 56.97% and 53.05%, respectively after 28 days of cultivation. All consortia showed high phenanthrene elimination which were 95.64, 79.37, 87.19, 79.21% for Consortia A, B, C and D, respectively. The results indicate that all of the bacteria isolated may effectively degrade target chemical and have a promising application in bioremediation of hydrocarbon contaminated soil purposes.

Keywords*—Acinetobacter* sp. P3d, *Bacillus* sp. P4a, consortia, phenanthrene, *Pseudomonas* sp. P6.

I. Introduction

IN recent years, the fast development occured in various fields, especially in industrial technology. Due to this, development requires the constant exploitation in terms of human energy and natural resource. Malaysia is one of the vast developing countries that shows a rapid urban development with establish lots of industrial areas. Indirectly, it will cause adverse impact to the environment. Most of the environmental pollutions caused by polycyclic aromatic hydrocarbon (PAH). This PAH pollution becomes global environmental problem and it is found in environment such as through oil spills and transportation activities.

Phenanthrene is one of a group of PAH composed of three fused benzene rings. It has physical characteristics which are hydrophobicity and insoluble in water but is soluble in most organic solvents. It is one of the 16 PAH on the U.S Environmental Protection priority pollutant list. The presence of PAH pollutant such as phenanthrene compound creates harm to the environment and human because of their toxic and carcinogenic characteristic. Therefore, phenanthrene is always chosen to be used as a model substrate in most studies on the biodegradation and metabolism of PAH [1]. The degradation of PAH is important, concerning the toxicity of PAH compound to human and environment. In addition, microorganisms play an important role in biodegradation process. Microbes will degrade the pollutants in beneficial ways and produce the end products which is non-toxic compounds such as water and carbon dioxide [1]. Therefore, bioremediation techniques are very important and considered as promising environmental friendly treatment to remediate PAH compounds. Considerable effort has been focused on the isolation and identification of bacteria that have the capability to degrade phenanthrene.

The goal of the present work is to study the degradation activity of phenanthrene by monocultures and mixed bacterial culture (consortia) in shake flask system.

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II. Materials and Methods

A. Collection of Soil Samples

Seven sampling sites were selected along the East Coast Peninsular of Malaysia. PAH-contaminated soil samples were collected in the state of Pahang, Terengganu and Kelantan at the depth of 1-10 cm. The samples were collected aseptically using a stainless spatula, stored in plastic bags and transferred back to the laboratory. Samples were stored at 4 ^{o}C before the analysis is done. All of these samples were further used for isolation of potential PAH- degrading bacteria

B. Enrichment Culture Preparation

Minimal Salts Medium (MSM) was used for isolation and screening. The MSM consisted of the following compositions: 1.0 g of $(NH_4)_2SO_4$, 0.1 g of $CaCl_2.2H_2O$, 0.8 g of K_2HPO_4, 0.2 g of KH_2PO_4, 0.012 g of $FeSO_4.7H_2O$, 0.003 g of $MnSO_4.7H_2O$, 0.003 g of $ZnSO_4.7H_2O$ in 1 L of distilled water. The pH of the medium was around 7 ± 0.2. The hexane stock solutions of phenanthrene at concentration 10 g/l were prepared and stored in brown bottles placed at 4 ^{o}C.

C. Isolation and Screening of Phenanthrene-Degrading Bacteria

The phenanthrene-degrading bacteria were enriched in cotton-plugged Erlenmeyer Flasks containing 150 ml sterilized MSM, 15 g of soil and 5 ml of phenanthrene (330 mg/l)). The flasks were incubated in an orbital shaker at 150 rpm at 30 ^{o}C. A week later, 5 ml aliquots were transferred to 150 ml of fresh MSM containing 5 ml phenanthrene. The

flasks were shaken for another two weeks. This step was repeated for four times to isolate and purify bacteria to degrade phenanthrene. Bacteria were isolated by serial dilutions made in 10 ml sterile distilled water and then spread inoculating 0.1 ml of each dilution onto nutrient agar (NA). The inoculated NA plates were then incubated at 37 ^{o}C for 48 hours. Colonies which were appeared on the plates were picked based on different morphological appearance and pure isolates obtained by repeated sub-culturing on NA. The macro-morphology and microscopic observations of the isolated colonies were recorded. Phenanthrene degrading bacteria cultures were preserved in MSM agar and NA slant containing phenanthrene and stored at 4 ^{o}C.

D. Identification of Bacterial Degrading Phenanthrene

Identification and characterization of selected bacterial isolate were based on macro-morphology and microscopic observation. Further characterization was performed using the BBL Crystal Identification Kit and molecular characterization using 16SrRNA sequencing.

E. Biodegradation of Phenanthrene by Flasks System

The bacteria isolates were used to evaluate their ability to degrade phenanthrene as sole carbon and energy source. Minimal Salt Medium 50 ml at pH 7.2 were autoclaved for 20 minutes at 121 ^{o}C and phenanthrene with a final concentration of 330 mg/L was aseptically added into 250 ml Erlenmeyer flasks. The culture flasks were incubated aerobically at 30 ^{o}C with agitation at 150 rpm. The experiment set up as described above was designated as A. Two controls (C1 and C2) were set up; C1 consisted of the same material present in A but without phenanthrene while C2 consisted all the materials in A with no test isolate inoculated. The ability of cultures to degrade phenanthrene was performed by carrying the biodegradation experiment in seven different systems for 28 days at room temperature (Table I). The system contained 1 ml of pure culture and mixed cultures (O.D 0.5) in 47.5 ml Minimal Salt Medium consisted 1.5 ml phenanthrene (300 mg/l) as the sole source of carbon and made up a total mixture 150 ml. The experiment was set up in triplicate for 28 days. Analysis of phenanthrene concentration in the culture medium was conducted at the regular interval time of every 4 days.

F. Gas Chromatography Analysis

Biodegradation of phenanthrene was assayed by determining the residual phenanthrene using Gas Chromatography analyses. The sample analyzed with Perkin Elmer Clarus 500 with DB-5HT column (30 m x 0.317 mm x 0.10 µm) equipped with a flame ionization detector (FID). A series of standard solutions were prepared by dissolving phenanthrene stock solution (1000 mg/l) with hexane. The prepared standard solutions (0, 100, 200, 300, 400, 500 mg/l) was injected in triplicate onto GC-FID column. The standard solution was prepared to produce the calibration graph of phenanthrene to determine the limit of degradation. The concentration of phenanthrene versus the peak ratio was plotted and a line of best fit obtained (R2= > 0.986). Phenanthrene degradation was detected by observing any decrease of the phenanthrene concentration during the experimental period. The GC readings obtained (uV*s) were converted into concentration (mg) using the respective standard curves.

TABLE I
SEVEN DIFFERENT SYSTEMS CONDUCTED IN PHENANTHRENE BIODEGRADATION STUDY

Systems	Inocula
Mono Culture:	*Acinetobacter* sp. P3d
	Bacillus sp. P4a
	Pseudomonas sp. P6
Mixed cultures:	
Consortia A	*Acinetobacter* sp. P3d + *Bacillus* sp. P4a + Pseudomonas sp. P6
Consortia B	*Acinetobacter* sp. P3d + *Bacillus* sp. P4a
Consortia C	*Acinetobacter* sp. P3d + *Pseudomonas* sp. P6
Consortia D	*Bacillus* sp. P4a + *Pseudomonas* sp. P6

III. RESULTS AND DISCUSSION

Soil samples were taken from seven different locations in Pahang, Kelantan and Terengganu, Malaysia (Table II). Fourteen indigenous phenanthrene-degrading bacterial strains were isolated from different PAH-contaminated soil sites. The temperatures obtained from all soil samples collected were in the range of 28-34 ^{o}C while the pH in the range of 5.65-9.20. It also shows that the type of soils was mineral soil. This type of soil is commonly available in Malaysia. Table III shows the microscopic and macroscopic observations on NA of 14 isolated bacteria. These bacterial strains were six Gram-positive while eight were Gram-negative. This finding suggested that Gram-negative bacteria were the most abundantly isolated bacteria compared to Gram-positive bacteria in the hydrocarbon polluted sites. The degradation analysis was demonstrated by phenanthrene removal from MSM broth supplemented with phenanthrene. The phenanthrene concentration decreased within 30 days from 330 mg/L to a level not detected by GC-FID analysis. Fig. 1 shows the percentage of degradation of phenanthrene by all 14 bacterial isolates. GC analysis showed that Isolate P3d produced the highest degradation of 85.10%, followed by Isolate P4a and P6 which were 52.88% and 48.48%, respectively. However, Isolate P3b, P4b, P5a and P5c showed degradation of phenanthrene with no significant difference of 38.37%, 22.84%, 28.60% and 33.11%, respectively. While the other six isolates which were Isolate P1, P2a, P3a, P3c and P5b showed no degradation at all during the experimental processes. The results obtained from biochemical characteristic analysis using BBL Crystal Identification Kit showed similarity with the result from 16S rRNA gene sequence analysis. This study identified three species of bacterial isolate, which belong to *Acinetobacter* sp. P3d, *Bacillus* sp. P4a and *Pseudomonas* sp. P6.

TABLE II
LOCATIONS AND TYPE OF SOIL SAMPLES COLLECTED FROM PAHANG, TERENGGANU AND KELANTAN, MALAYSIA

NO	LOCATION	TEMP (°C)	pH	SOIL TYPE	TEXTURE	COORDINATE
A	**PAHANG STATE**					
i.	Cargill Palm Industry, Gebeng	29	5.65	Mineral Soil	Coarse Sandy Clay	3° 58' 32.46" N 103° 23' 31.66" E
ii.	Beach Side, Kuantan Port	29	7.46	Mineral Soil	Coarse Sand	3° 59' 09.60" N 103° 25' 31.85" E
iii.	Beach Side, Taman Gelora, Kuantan	30	7.87	Mineral Soil	Coarse Sand	3° 48' 23.50" N 103° 20' 50.45" E
iv.	Tanjung Api Port, Kuantan	30	7.67	Mineral Soil	Silty Clay	3° 48' 32.28" N 103° 20' 33.50" E
v.	Neram Palm Industry (FPISB)	32	9.20	Mineral Soil	Sandy Clay	4° 00' 16.99" N 103° 17' 21.71" E
B	**KELANTAN STATE**					
i.	Tok Bali Port	28	8.75	Mineral Soil	Coarse Sand	5° 53' 21.33" N 102° 28' 53.83" E
C	**TERENGGANU STATE**					
i.	Petronas Oil Refinery Plant, Kerteh	31-34	7.89	Mineral Soil	Coarse Sand	4° 33' 59.26" N 103° 27' 44.63" E

TABLE III
MORPHOLOGICAL CHARACTERISTICS OF BACTERIA ISOLATED FROM VARIOUS SITES OF KELANTAN, TERENGGANU AND PAHANG, MALAYSIA

No. of isolates	Gram-stain	Shape	Colony colour	Surface	Form	Elevation	Margin
P1	+	long rod	White	Dry	Irregular	Raised	Undulate
P2a	-	rod	Light Yellow	Smooth	Circular	Flat	Entire
P2b	+	long rod	White	Dry	Irregular	Raised	Undulate
P3a	+	rod	Cream	Glistening	Circular	Raised	Entire
P3b	+	cocci	Cream	Smooth	Irregular	Raised	Entire
P3c	-	cocci	Light Green	Sticky & Glistening	Circular	Convex	Entire
P3d	-	rod	Cream	Glistening	Circular	Raised	Entire
P4a	+	rod	White	Dry	Filamentous	Flat	Filiform
P4b	-	cocci	White	Dry	Irregular	Raised	Undulate
P5a	+	rod	Cream	Glistening	Irregular	Raised	Undulate
P5b	-	rod	Cream	Glistening	Circular	Raised	Entire
P5c	-	cocci	Cream	Glistening	Circular	Raised	Entire
P6	-	rod	Cream	Glistening	Circular	Raised	Entire
P7	-	rod	Cream	Glistening	Irregular	Raised	Undulate

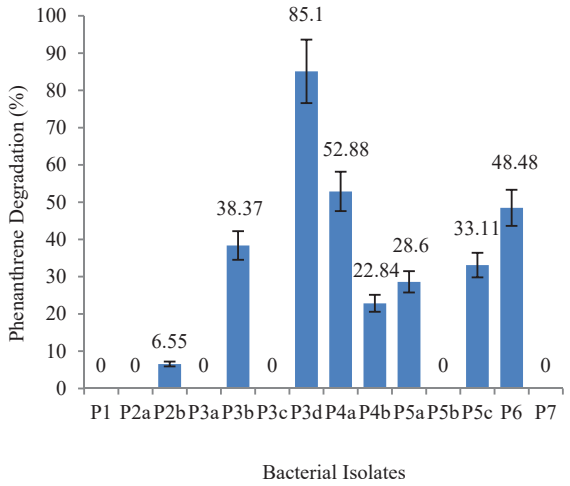

Fig. 1 Phenanthrene degradation of 14 bacterial isolates after 30 days incubation in MSM

In order to determine the best phenanthrene degrader among the three monocultures and four mixed cultures consortia, the phenanthrene degradation percentage (%) for these seven systems were calculated. The results are shown in Fig. 2. Comparison of the phenanthrene degradation percentage between these monocultures and mixed cultures were based until the 20th day of cultivation in order to determine the potential phenanthrene degraders. Among the monocultures, *Pseudomonas* sp. P6 exhibited 58.71% activity compared to *Acinetobacter* sp. P3d and *Bacillus* sp. P4a which were 56.97% and 53.05%, respectively. Whilst, among the mixed cultures, Consortium A which consisted of three bacterial strains exhibited 95.64% activity compared to Consortium B, C and D which were 87.19%, 79.21% and 79.37% after 20 days of cultivation, respectively. Generally, monoculture systems had lower phenanthrene degradation activity compared with mixed culture systems after 20 days of incubation. The average degradation percentage for three monoculture systems were 58.19% only while the degradation percentage for four mixed culture systems were 85.35%. This is because most PAH degradations do not depend on the metabolism of a particular microbial species, but it requires the capabilities of the entire microbial communities [1].

Co-metabolism and cooperation among associations of bacteria are very important. The enzymatic capacities of most bacteria have the capability to degrade a certain range of

hydrocarbons but when with assemblages of mixed culture with overall broad enzymatic capacities, the efficiency of a biodegradation process can be improved [2]. This could be due to the synergistic effects among the microorganisms [3].

Although various monocultures have been proven to degrade phenanthrene and degradation efficiency may improve when the microorganisms are combined to form of consortium [4], [5]. This is due to the synergistic interactions among bacteria. The combinations of the bacteria give the cooperative effects because different bacteria may create different biochemical degradation pathways [6]. By the reaction of this synergistic interaction, some of the consortia may remove the toxic pollutant completely or also can degrade more complex of PAH compounds [7]. Through this, a significant positive relationship can be found between bacterial growth and percentages of phenanthrene degradation. The increasing cell biomass indicated that the utilization of phenanthrene as a carbon source [7]. This hypothesis was supported by [8] who stated that the degradation of PAH contaminants by bacteria or fungi studied in MSM medium might increase. It was probably related to the presence of metabolic complementary which produced intermediate metabolites in the MSM medium among the microorganisms [9].

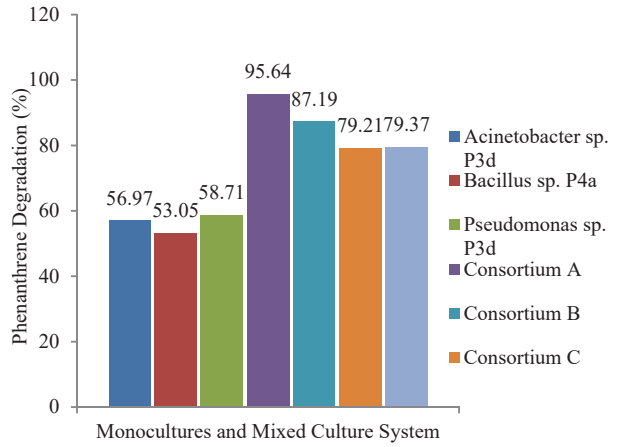

Fig. 2 Phenanthrene degradation percentage of three monoculture systems and four mixed culture systems of consortia after 20 days cultivation

IV. Conclusion

The present study showed that the isolated bacteria could be able to utilized phenanthrene as their sole of carbon and energy source. Based on the result, the combination of these three bacterial cultures (Consortium A) gave a great potential to be applied in bioremediation of environments polluted by phenanthrene. These potential bacterial cultures can be highly recommended for bioremediation of hydrocarbon contaminants due to their capability to degrade phenanthrene.

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