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Phenanthrene-Degrading Bacteria, *Acinetobacter* sp. P3d from Contaminated Soil and Their Bioactivities

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ABSTRACT

This study was conducted to identify the indigenous active phenanthrene-degrading bacteria from phenanthrene-contaminated soil and to determine phenanthrene degradation activity of the isolated bacteria in liquid system. The minimal salt medium supplement with phenanthrene was the selective medium to isolate phenanthrene degrading bacteria. A total of nine isolates of bacteria were successfully isolated using the spread plate method. All isolates were tested for their ability to degrade phenanthrene by cultivation in a minimal salt medium supplemented with 330mg/L phenanthrene, incubated at 30°C and 150 rpm for 28 days. The biodegradation study was analyzed by Gas Chromatography-Flame Ionization Detector (GC-FID) and the result showed that *Acinetobacter* sp. P3d was capable to degrade about 93.58% phenanthrene in the selective medium. The *Acinetobacter* sp. P3d has the capability to degrade the phenanthrene as a sole source of carbon and energy. The results revealed that this bacterial isolate was able to degrade phenanthrene significantly and it may prove to be promising bacteria for bioremediation of PAH-containing pollutant from contaminated site.

INTRODUCTION

One of the most serious problems affecting our world nowadays is related to the Polycyclic Aromatic Hydrocarbons (PAH) pollution. This PAH pollution becomes global environmental problem. PAHs are found in environment through oil spills, leaks and inefficient management of industrial wastes, transportation, wood processing products, and combustion of fossil fuels (Majumdar et al. 2017). Therefore, it gives an adverse effect on the environment and human health. In Malaysia, soil contaminated with PAHs had occurred during the last 50 years resulted from agriculture and industrial activities (Abidin 2016). There are many industrial plants discharging their effluents and disposing their untreated chemicals into the environment. Due to the increasing number of environmental issues and decrease in the environmental quality, it actually brings negative impact to environment and humanity. It is very important to look after and restore a safe and clean environment. Therefore, bioremediation of soil contaminated with PAHs has received increasing attention internationally. Biodegradation is one way using microorganisms to eliminate PAHs from contaminated sites. It refers to the application of biodegradation reactions to convert toxic compounds to non-toxic compounds. Moreover, bioremediation is considered as promising environmental friendly treatment to remediate hydrocarbon compounds.

In Malaysia, there are many cases of phenanthrene-contaminated soil, especially in Terengganu and Pahang where the petroleum processing plants are located. In some cases, phenanthrene has contaminated the soil for more than eight years (Lee et al. 2011). Phenanthrene has been listed as one of the 16 PAHs listed in the US EPA as a priority pollutant. Some studies have shown that phenanthrene can be degraded by microorganisms such as bacteria, fungi, yeast and microalgae (Bundy 2004). However, bacteria play an important role in this degradation. Degradation of individual PAHs, especially phenanthrene, by single and mixed bacterial cultures has been reported by several researchers (Heitkamp et al. 1988, Cerniglia 1992, Boldrin et al. 1993, Wilson & Jones 1993, Kanaly & Harayama 2000). Bacteria will degrade this organic pollutant using their own metabolic activity to convert contaminants into different compounds. In other words, the metabolic processes of bacteria have the capability to utilize the chemical contaminants as an energy source and typically convert it to a less toxic form than the original contaminants. Therefore, the aim of this study is to isolate the potential bacteria from PAHs contaminated soil to remediate phenanthrene in the shake flasks system.

MATERIAL S AND METHODS

Soil sample collection: Soils contaminated with PAHs were collected along the East Coast Peninsular of Malaysia in the State of Pahang, Terengganu and Kelantan. PAHs contaminated soil was collected at the depth of 1-10 cm. The samples were kept into sterile plastic bags and placed in a box containing ice to be brought to the laboratory. All of these samples were further used for isolation of potential PAH degrading bacteria.

Enrichment culture preparation: Mineral salts medium (MSM) was used for isolation and screening works. 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, 0.2g of KH_2PO_4, 0.012g of $FeSO_4.7H_2O$, 0.003 g of $MnSO_4.7H_2O$, 0.003g of $ZnSO_4.7H_2O$ in 1 L of distilled water (Coral & Karagoz 2005). The pH of the medium was 7 ± 0.2. The hexane stock solutions of phenanthrene at concentration of 10 g/L were prepared and stored in brown bottles placed at 4°C.

Isolation and screening of phenanthrene degrading bacteria: The PAH-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 with 150 rpm at 30°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 (modified from Zhao et al. 2009). This step was repeated 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°C for 48 h. Colonies which appeared on the plates were picked based on different morphological appearance, and pure isolates obtained by repeated sub-culturing on NA. The morphology and microscopic observations of the isolated colonies were recorded. Phenanthrene degrading bacteria cultures were preserved in MSM agar and NA slant containing 1% phenanthrene and stored at 4°C.

Identification of bacterial degrading phenanthrene: Identification of selected bacterial isolate was based on morphology and microscopic observation using Gram staining. Further characterization was performed using the BBL Crystal Identification Kit and molecular characterization using 16S rRNA gene sequence analysis.

Phenanthrene degradation analysis by isolated bacteria: Phenanthrene-degradation experiments were carried out using Erlenmeyer flasks containing 50 mL of MSM and added phenanthrene with a final concentration of 330 mg/L. The inoculum was prepared by culturing concentrated strains in 0.05× Nutrient Broth (NB) medium at 30°C for 24 hours. Subsequently, after centrifugation at 5,000 rpm for 10 minutes, the cells were harvested and washed twice with sterile MSM solution (modified from Zhao et al. 2009). The optical density of the cell was adjusted by using a UV-visible spectrophotometer (UV-1700 Model TCC-240A, Shimadzu, Japan) at 600 nm to exponential phase OD of 0.5 (Fazilah 2015). The initial colony forming unit (CFUs) was calculated through the growth on the NA plates. The cell inoculum of 1 mL was added to 50 mL of MSM and the culture was shaken and incubated under aerobic conditions in an orbital shaker at 30°C at a speed of 150 rpm. Abiotic and growth control were performed by adding phenanthrene into MSM without inoculum, and inoculum into MSM without phenanthrene, respectively. The experiment was set up in triplicate for 28 days and samples taken at every 4 day interval. The samples were taken and examined for the remaining amount of phenanthrene and bacterial growth CFU/mL. The remaining PAH concentration was analysed using the GC-FID method (Zhao et al. 2009), and the growing cells were measured by standard plate count (Kafilzadeh et al. 2011).

Measurement of PAHs: Biodegradation of phenanthrene was assayed to determine the residual phenanthrene using gas chromatography analyses. The samples were analyzed by Perkin Elmer Clarus 500 with DB-5HT column (30 m × 0.317 mm × 0.10 µm) equipped with a flame ionization detector (FID). A series of standard solutions was prepared by dissolving stock solution with hexane of the phenanthrene ranging in concentration from 0-500 mg/L. Standards were analyzed by GC-FID and the peak ratio of the phenanthrene to the standard was calculated. 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 curve. In order to determine the phenanthrene degradation percentage, data from the GC analysis were taken into consideration too. The phenanthrene degradation percentage was calculated as below:

$$\text{Phenanthrene degradation percentage } (\%) = \frac{\text{Initial concentration} - \text{Final concentration}}{\text{Initial concentration}} \text{ mg/L} \times 100$$

RESULTS AND DISCUSSION

Identification of bacterial isolates of phenanthrene degrading bacteria: Soil samples were obtained from seven

locations which had been exposed to hydrocarbons several years ago. The results of physical parameters of each different sampling site are given in Table 1. The average temperature at the sampling sites was at 30°C while the pH values were at 7.7. The type of soil was analysed and identified as mineral soil. Mineral soil is defined as soil derived from minerals or rocks and containing little humus or organic matter. It consists primarily of mineral (sand, silt and clay) materials rather than organic matter (Plante et al. 2008). Referring to this table, there were a few textures of the mineral soil recorded such as coarse sandy clay, coarse sand, silty clay and sandy clay. Generally, microbial populations are extensively high, probably due to their better aeration, high moisture content and high organic matter in the soil itself. Thus, these factors influence the microbial activity of the particular microorganisms in soil such as some are playing an important role in bioremediation.

PAH-degrading bacteria were isolated through enrichment cultures from soils contaminated with oil or hydrocarbon itself. After cultivating these isolates over one month in Minimal Salt Medium (MSM) with phenanthrene resulted in nine phenanthrene-degrading bacteria to have been isolated. There were four Gram positive bacteria while five were Gram negative bacteria (Table 2). These isolated bacteria were preliminarily identified by morphology characteristics and based on the differences in the biochemical activities. From the screening using GC-FID analysis, nine isolates showed degrading potential and isolate P3d gave the highest in phenanthrene decomposition of 85.1% compared to other isolates (Table 3). Isolate P3d was preliminary identified as Genus *Acinetobacter* based on the result of biochemical tests (BBL Crystal ID Kit). Isolate P3d was characterized as coccobacillus Gram negative, pale yellowish to creamy, glistening surface, circular, raised and entire when grown on nutrient agar plate. It was supported by Jung & Park (2015) who stated that *Acinetobacter iwoffii* had the capability of degrading broad range of PAHs especially in petroleum hydrocarbons. Referring to Holt (2000), the genus of acinetobacter is strictly Gram negative bacteria and occur in coccobacilli morphology on non-selective agar. The morphology is generally encapsulated and non-pigmented with non-motile bacteria. This bacterium form is

Table 1: Location and characteristics of soil samples collected from the east coast peninsular of Malaysia in the state of Pahang, Terengganu and Kelantan.

No	Location	Temp. (°C)	pH	Texture	Coordinate
A	**Pahang State**				
	i. Cargill Palm Industry, Gebeng	29	5.65	Coarse Sandy Clay	3°58'32.46"N 103°23'31.66"E
	ii. Beach Side, Kuantan Port	29	7.46	Coarse Sand	3°59'09.60"N 103°25'31.85"E
	iii. Beach Side, Taman Gelora, Kuantan	30	7.87	Coarse Sand	3°48'23.50"N 103°20'50.45"E
	iv. TanjungApi Port, Kuantan	30	7.67	Silty Clay	3°48'32.28"N 103°20'33.50"E
	v. Neram Palm Industry (FPISB)	32	9.20	Sandy Clay	4°00'16.99"N 103°17'21.71"E
B	**Kelantan State**				
	i. Tok Bali Port	28	8.75	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	Coarse Sand	4°33'59.26"N 103°27'44.63"E

Note: Soils were sampled 1-10 cm from soil surface and located at various positions on the site. Soils 0.5-1.0 kg were collected in 1.5 L plastic bag and stored at 4°C prior to use.

Table 2: Microscopic and morphology observation of the nine isolated bacteria on nutrient agar.

Isolate Bacteria	Microscopic Observation		Morphology Observation					Bacterial Identification using BBL Crystal Identification Kit
	Gram Stain	Cell Shape	Colour	Surface	Form	Elevation	Margin	
P2b	+	Long rod	White	Dry	Irregular	Raised	Undulate	*Corynebacterium genitalium*
P3b	+	Cocci	Cream	Smooth	Irregular	Raised	Entire	*Staphylococcus saprophyticus*
P3d	-	Coccobacilli	Pale Yellow	Glistening	Circular	Raised	Entire	*Acinetobacter baumannii*
P4a	+	Rod	White	Dry	Irregular	Raised	Undulate	*Corynebacterium* sp.
P4b	+	Rod	White	Dry	Filamentous	Flat	Filiform	*Bacillus* sp.
P5a	-	Rod	Cream	Glistening	Irregular	Raised	Undulate	*Flavimonas oryzihabitans*
P5b	-	Rod	Cream	Glistening	Circular	Raised	Entire	*Enterobacter cloacae*
P5c	-	Cocci	Cream	Glistening	Circular	Raised	Entire	*Klesiella pneumoniae*
P6	-	Rod	Cream	Glistening	Circular	Raised	Entire	*Flavimonas oryzihabitans*

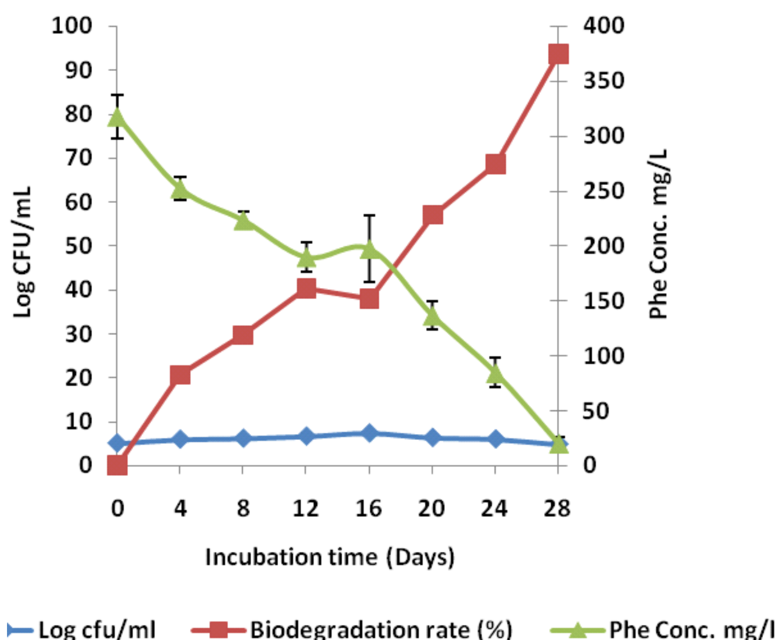

Fig. 1: The rate of degradation for phenanthrene by *Acinetobacter* sp. P3d compared with incubation time (days). Growth was monitored by estimated bacterial count (log CFU/mL) while phenanthrene concentration (mg/L) was estimated by GC analysis of the residue phenanthrene in flask system.

Table 3: Degradation analysis using GC-FID by nine bacterial isolates after 30 days incubation in minimal salt medium containing phenanthrene as a sole source of carbon and energy.

Bacterial isolates	Log CFU/mL at 0 day of incubation	Log CFU/mL after 30 days of incubation	Phenanthrene degradation rate (%)
P2b	5.8	7.65	6.55
P3b	5.9	8.05	38.37
P3d	5.65	9.2	85.10
P4a	6.3	8	52.88
P4b	5.7	7	22.84
P5a	5.55	6.9	28.60
P5b	5.85	6.9	0
P5c	6	7.3	33.11
P6	6.2	8.4	48.48

circular with cream in colour and slightly glistening, convex, raised and entire. Isolate P3d was further identified by 16S rRNA sequencing and the DNA sequence analysis of PCR-amplified 16S rDNA were used for the similarity search against GenBank database in NCBI using the BLAST program. It was confirmed that isolate P3d indeed belongs to the genus *Acinetobacter* (Table 4). The genus *Acinetobacter* is considered ubiquitous bacteria and virtually obtained from soil and surface water. It is a common bacterium capable of degrading aromatic hydrocarbons. A study done by Hamzah et al. (2010), *Acinetobacter* sp. P3d showed capability with high degrading activity towards a variety of crude oil samples such as Sumandak and Angsi oils and also Arabian and Indian oils.

Degradation of phenanthrene by *Acinetobacter* sp. P3d: Isolate P3d is known as *Acinetobacter* sp. P3d, possessed the highest degradation within 28 days of incubation in MSM containing phenanthrene as a sole source of carbon and energy. Gas chromatography analysis of phenanthrene degradation showed *that Acinetobacter* sp. P3d degraded 93.58% of phenanthrene (Fig. 1).

According to Fig. 1, the growth patterns coincided with the degradation of the phenanthrene. Bacterial growth was well over time of incubation in minimal salt medium containing phenanthrene. During day 0 of incubation it showed that the bacterial growth was slow after contacting with phenanthrene due to the lack of adapting to the new environment (lag phase). In this condition it usually show no immediate increase in cell number due to the medium might be different with the nature that the bacteria growing in previously. But after day 4, the bacterial growth was increased because the bacteria had adapted with this phenanthrene as a substrate and as a sole growth source (log phase). During this condition, bacteria are growing and dividing at the maximal rate. The bacterium slowly degraded the phenanthrene to about 20.56%. After day 12 of incubation, the bacterial growth entered into the stationary phase and the growth was slightly constant over the time of incubation.

Table 4: Similarity percentage of 16S rDNA sequences for Isolate P3d compared to those obtained from BLAST database.

Sample	Match	Accession No.	Score (bits)	Identify (%)
Isolate P3d	*Acinetobacter* sp. KL1(2010)	GU566317	2758	99
	Acinetobacter sp. KL5(2010)	GU566321	2752	99
	Acinetobacter sp. JD2 (2010)	GU566314	2752	99
	Acinetobacter sp. MB1 (2010)	GU566332	2747	99
	Uncultured *Acinetobacter* sp. clone MHP_1Pitesti	DQ3661	2747	99

This might be result of a balance between cell division and cell death. It may also be due to the growth simply cease to divide. The main factor is nutrient limitation itself. Phenanthrene may be severely depleted causing the growth population becoming slow in the medium, while the phenanthrene was degraded faster on day 12 to 40.23%.

At day 28, the growth curve was slightly decreased. In these conditions, viable cells declined due to the detrimental environmental changes such as phenanthrene deprivation in the medium. Another reason may be the accumulation of toxic wastes that cause harm and loss of viability of the cells. Some of the metabolites produced during the degradation process can exert the growth rate of bacteria (Maigari & Maigari 2015). As shown in Fig. 1, following the death phase, the bacteria degraded 93.58% of phenanthrene effectively which was 20.41mg/L within 28 days of incubation. This isolate was able to utilize phenanthrene as a sole source of carbon and energy.

There are various enzymes responsible in the PAH biodegradation. According to (Krishnaswamy 2017), two types of enzymes are PAH dioxygenase (PDO) and Catechol 2, 3 dioxygenase (C23O) which showed high enzyme activity during phenanthrene biodegradation and the C23O plays an important role in the metabolism of aromatic hydrocarbons. Catechol 1,2 dioxygenase has been purified by Gou et al. (2009) from extracts of *Sphingomonas xenophaga* QYY that showed the ability to degrade various aromatic compounds. Therefore, the bacteria that have capability to degrade various hydrocarbons have a potential to be used for bioremediation of hydrocarbon-contaminated environment.

CONCLUSION

Phenanthrene degrading bacterial strain was isolated and identified from contaminated soil collected from Petronas Oil Refinery Plant, Terengganu State. *Acinetobacter* sp. P3d showed the maximum degradation rate of 93.58% after 28 days cultivation. The degradation was determined by chromatography analysis through Gas Chromatography-Flame Ion Detector. The performance of *Acinetobacter* sp. P3d to degrade phenanthrene will be beneficial for the bioremediation of PAH contaminated environment. Further research will be directed towards understanding the role of *Acinetobacter* sp. P3d in influencing the effectiveness of *in situ* degradation processes.

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