Biodegradation of PAH-polluted soil by indigenous bacteria

A Fazilah 1*, N Ismail 2 and I Darah 3

1Faculty Science and Marine Environment, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia
2Institute Marine Biotechnology, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia
3Industrial Biotechnology Research Laboratory (IBRL), School of Biological Sciences, Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia

defazilah@umt.edu.my

Abstract. Polycyclic aromatic hydrocarbons (PAHs) are environmental pollutants and phenanthrene is one of the PAH compounds shown to be toxic, mutagenic and carcinogenic. The efficient way to clean up and minimize the PAH pollution in the environment is by bioremediation process. Therefore, the objective of this study was to determine the degradation activity of phenanthrene-degrading bacteria by inoculating the bacterial culture into soil treated with phenanthrene as a carbon source. Ultrastructure morphology of bacterial cells during degradation process in soil were observed using Scanning Electron Microscope. Addition of Acinetobacter sp. P3d, Bacillus sp. P4a, Pseudomonas sp. P6 to soil microcosms supplemented with phenanthrene resulted in phenanthrene degradation. The degradation resulted in more visible, particularly when all the three bacterial cultures were mixed together. The degradation rate for Consortium A was 100% followed by Consortium B (87.45%), Consortium D (76.58%) and Consortium C (76.13%) for only 30 days of cultivation. Based on this study, pure culture of the Acinetobacter sp. P3d and mixed cultures of consortia A; Acinetobacter sp. P3d, Bacillus sp. P4a and Pseudomonas sp. P6 were selected as a potential bacterial culture to carry out bioremediation study in the phenanthrene contaminated soil.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) can be found everywhere in air, soil, water and sediments. PAHs are widespread environmental pollutants derived from incomplete combustion which produces toxic by-products such as carbon monoxide. These derivative products are discharged mostly from human activities [1]. The presence of PAH pollutant could give adverse effects to the environment and human because of their toxic and carcinogenic characteristics [2]. Phenanthrene is a low molecular weight PAH which consists of three fused benzene rings with phenyl and anthracene compound attached together. It is a toxic pollutant and occurs as a major component of PAH compounds in the environment [3]. Therefore, biodegradation of this compound necessary in order to bioremediate the contaminated soils. Microorganisms such as bacteria is known as a key successful factor in the degradation of organic pollutants from contaminated sites. Although a variety of bacterial capable of degrading PAH have been isolated, most PAH-degraders are more adapted in an in situ bioremediation of PAH-contaminated soil [4]. In situ bioremediation treats the contaminated soil in the location in which it was found. Whereas, ex situ bioremediation processes require excavation of contaminated soil before they can be treated. Usually, in bioremediation of contaminated sites, inoculating of microorganisms or mixed micro biomes effectively metabolized the high-molecular-weight polycyclic aromatic hydrocarbons such as PAHs in the soil [5]. Various studies have been done to evaluate potencies of bioaugmentation of PAH-polluted soil with PAH-degrading bacterial or mixed culture [6, 7]. Therefore, the objective of this study was to examine the degradation activity of phenanthrene and survival of phenanthrene-degrading bacteria by inoculating the bacterial culture into soil treated with phenanthrene as a carbon source.
2. Material and Methods

2.1 Isolation and characterization of soil bacteria
Three bacterial strains, *Acinetobacter* sp. P3d, *Bacillus* sp. P4a and *Pseudomonas* sp. P6 previously isolated from soil contaminated hydrocarbon [8] and maintained in the laboratory culture collection.

2.2 Inoculation bacteria culture into tray system
The ability of bacterial cultures to degrade phenanthrene was performed by carrying the biodegradation experiment in seven different systems at regular interval of every 5 days beginning from the incubation day 0 until 30 days (Table 1). Each system has been designed for triplicate which contains 1 kg of soil sample placed in plastic trays (30 × 30 x 15 cm). The trays were covered with sterilized aluminium foil. Inoculum size of 10% (v/v) was used in this experiment. In order to inoculate 10% of culture into 1 kg of soil, 100 ml of cultures were needed. The bacterial cultures (40 ml) were transferred into centrifuge tubes and were centrifuged at 5000 rpm for 10 min. The pellets formed were collected and added to the system accordingly. Two controls (C1 and C2) were set up: C1 consisted of the soil and bacterial cultures without phenanthrene inoculated into soil whereas C2 consisted of soil with phenanthrene only and without bacterial culture [8]. The experiments were set up in triplicates for 30 days.

| Systems          | Inocula                                      |
|------------------|----------------------------------------------|
| Mono Cultures:   | *Acinetobacter* sp. P3d                      |
|                  | *Bacillus* sp. P4a                           |
|                  | *Pseudomonas* sp. P6                         |
| Mixed cultures:  | *Acinetobacter* sp. P3d + *Bacillus* sp. P4a |
|                  | *Acinetobacter* sp. P3d + *Bacillus* sp. P4a |
|                  | *Acinetobacter* sp. P3d + *Pseudomonas* sp. P6|
|                  | *Bacillus* sp. P4a + *Pseudomonas* sp. P6    |

2.3 Biodegradation phenanthrene concentration in soil spiked with phenanthrene
Phenanthrene which was spiked into soil samples was determined by sequential extraction of 10 g of soil sample with 100 ml of hexane. The extracted portion was filtered through Whatman No. 1 filter paper into a beaker. Next, the filtered solvent hexane was evaporated in a fume hood until dried. The samples were then dissolved with equal volume of hexane (100 ml) and the phenanthrene was quantified by a Clarus 500 Gas Chromatography- Flame Ionization Detector (GC-FID) [8]. Phenanthrene degradation was detected by observing any decrease of the phenanthrene concentration during the experimental period. The phenanthrene degradation percentage was calculated as below:

Phenanthrene degradation percentage (%):\[
\frac{\text{Initial concentration} - \text{Final concentration}}{\text{Initial concentration}} \times 100\%
\]
2.4 The ultrastructure morphology of cell during degradation process using Scanning Electron Microscope (SEM)

Freeze drying method was used for the observation of bacteria in the soil sample. Glue was placed on a planchette to stick soil sample. The planchette that contained the soil sample was placed in a Petri dish containing filter paper, and a few drops of 1% osmium tetroxide (OsO₄) and distilled water were added to the filter paper. The soil sample was exposed to the OsO₄ vapor for 1 hr. Then, the soil sample was plunged in liquid nitrogen (-210°C) for 1 min before transferred to freeze drier (EMI TECH K750X) for 7 hrs. After freeze drying, the soil samples together with the planchette were then stuck on a SEM specimen stud using a double-sided tape. The specimen was placed in platinum coater (EMI TECH K575X) around 3 min to coat with platinum before viewing with SEM (Leo Supra 50VP Field Emission SEM equipped with Oxford INCA 400 energy dispersive x-ray microanalysis system).

3. Result and Discussion

3.1 Biodegradation of phenanthrene by bacteria culture system

The phenanthrene degradation percentages (%) are shown in Figure 1. The seven systems consisted of three monocultures and four mixed cultures consortia were calculated and plotted at 30 days of cultivation. Among the monoculture systems, *Acinetobacter* sp. P3d exhibited the highest degradation percentage of 78.08% after 30 days of cultivation period, followed by *Pseudomonas* sp. P6 (72.91%) and *Bacillus* sp. P4a (68.14%). Among mixed cultures, Consortium A that consist of three bacterial strains exhibited the highest phenanthrene degradation which was 100%, followed by Consortium B, Consortium D and Consortium C with 87.45%, 76.58% and 76.13% of degradation, respectively. Overall, the monoculture system had lower phenanthrene degradation percentage as compared with mixed culture system after 30 days of cultivation.

![Figure 1](image-url)

**Figure 1.** Phenanthrene degradation percentage of different bacteria culture systems after 30 days cultivation.

The average degradation percentage of these three monoculture systems were 73.04%, while the degradation percentage for four mixed culture systems were 85.04%. Microbial consortium may be
effective in improving the PAH degradation compared with the single pure bacterial cultures due to the syntrophic contribution among the diverse members in the consortium [7]. Therefore, it can perform a complete degradation pathway of PAH. The present study determined that mixture of three bacterial cultures showed high phenanthrene degradation efficiency (100%) compared with two mixed bacterial cultures and also single pure culture. This was supported by Kim et al. [2] where three combinations of three different bacterial strains (Acinetobacter baumannii, Klebsiella oxytoca and Stenotrophomonas maltophilia) could degrade phenanthrene rapidly and could achieve about 80% of phenanthrene degradation [9]. Furthermore, Chang et al. [10] studied the potential of a microbial consortium in the soil to degrade phenanthrene efficiently at pH 7 and at 30°C same parameter used in the present study [10]. Wan et al. [11] reported that the removal of PAH from spiked soil showed 90% degradation of anthracene, phenanthrene and pyrene after 30 days [10]. It gives quite similar result with the present study.

Results from present study indicate that mixed cultures of microorganisms may effectively degrade target chemicals, even if the microorganisms showed quite low degradation activity in pure culture [9]. Furthermore, phenanthrene was mineralized more slowly in soils (30 days) than in liquid media (20 days to 28 days).

3.2 The ultrastructure morphology of cell during degradation process using Scanning Electron Microscope (SEM)

Based on the SEM micrographs, results showed that mixed cultures of Consortium A consisted of three bacterial strains: Acinetobacter sp. P3d, Bacillus sp. P4a and Pseudomonas sp. P6 were able to grow in phenanthrene-contaminated soil. Figure 2 (a) and (b) clearly show the soil particles and the bacteria growing at surface of particle soil saturated with phenanthrene at Day 0, respectively.
Figure 2. Scanning electron micrographs of Consortium A in soil medium. (a) Soil particles (b) Cells grown at surface of particle soil saturated with phenanthrene at Day 0 (c) Sample after 15 days of incubation (d) Sample after 30 days of incubation (e) Control without phenanthrene at 0 day (f) Control without phenanthrene after 30 days of incubation. The dimensions of bars are given in micrometer.

The cells were compacted and gathered together and adhered to the crystal surface of phenanthrene and soil particle. However, three types of bacteria in the forms of coccus, short bacilli and long rod can be seen in Fig. 2(c). During this stage, the bacteria attached to the surface of phenanthrene that may serve as a carbon and energy sources. This is supported by [12], where Pseudomonas putida were immobilized and formed biofilm on the phenanthrene crystal that acted as their carbon source.

Figure 2 (c) also shows micrograph of the cells during prolonged incubation time to day 15, where some of the cells underwent binary fission growth. The cell cultures grew well indicated that phenanthrene was used as a sole source of carbon and performed their decomposition and degradation process of phenanthrene.

Most of the cells bursted and wrinkled due to deficiency of phenanthrene substrate in the soil on day 30 (Figure 2(d). One common reason of this condition was that the essential nutrient became completely used up by the bacteria cells. The production of metabolites or toxic waste product during metabolic activity by the bacteria cells may resulted in death of some of the bacteria in the medium. The number of bacteria increased rapidly when nutrients are added to the soil but their metabolic activity diminishes abruptly when the nutrient sources are removed [13].

In control systems (Figure 2 (e) and (f), the bacteria cells cannot be seen clearly even on day 0 incubation. This may be due to the bacteria cells cannot be able to stick to the soil particle and was not visible under SEM analysis. Moreover, the physiological adaptation which involves some time requirement for the bacteria cells to adapt to new conditions and synthesizing carriers to absorb nutrients available in the soil. However, at day 30, some of the cells grew and were visible under SEM, which may due to the utilization of the organic matter contained in the soil. Some cells turn to wrinkle shaped caused by of the organic matter being used by the cells and no phenanthrene was supplied to the medium as an energy source. The cell was not able to survive and no binary fission of the cells was observed in the control medium. In order to grow in the medium, cells need a constant supply of energy to generate and maintain the biological activity in the cell [14].
4. Conclusion
Three bacterial strains of Acinetobacter sp. P3d, Bacillus sp. P4a and Pseudomonas sp. P6 showed the capability of degrading phenanthrene. The efficiency of phenanthrene degradation was measured using GC-FID. The decrease in the concentration of this compound resulted in increasing potential degradation of the bacterial culture over the cultivation period. When a pure bacterial culture was tested against phenanthrene, the degradation rate was much lower which were 78.08%, 68.14% and 79.21% for Acinetobacter sp. P3d, Bacillus sp. P4a and Pseudomonas sp. P6, respectively, at 28 days of cultivation. However, when these bacterial cultures were mixed together, the phenanthrene efficiency was enhanced. The degradation resulted in more visible, particularly when all the three bacterial cultures were mixed together. The degradation rate for Consortium A was 100% followed by Consortium B (87.45%), Consortium D (76.58%) and Consortium C (76.13%) for only 30 days of cultivation. More studies must be conducted to determine the environmental factors (abiotic) that may account for the slow biodegradation in soil. However, the single bacterial cultures and mixed cultures combination resulted in effective degradation of phenanthrene and it may use other related compounds in the bioremediation process particularly in the contaminated environment.

5. References
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