

# Biodegradation of PAHs in Soil: Influence of Initial PAHs Concentration

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**Abstract:** Most studies on biodegradation of Polycyclic Aromatic Hydrocarbons (PAHs) evaluate the effect of initial PAHs concentration in liquid medium. There are limited studies on evaluation in solid medium such as contaminated soil. This study investigated the potential of the bacteria, *Corynebacterium urealyticum* isolated from municipal sludge in degrading phenanthrene contaminated soil in different phenanthrene concentration. Batch experiments were conducted over 20 days in reactors containing artificially contaminated phenanthrene soil at different concentration inoculated with a bacterial culture. This study established the optimum condition for phenanthrene degradation by the bacteria under non-indigenous condition at 500 mg/kg of initial phenanthrene concentration. High initial concentration required longer duration for biodegradation process compared to low initial concentration. The bacteria can survive for three days for all initial phenanthrene concentrations.

**Keywords:** Biodegradation, contaminated soil, *corynebacterium urealyticum*, phenanthrene.

## 1. Introduction

PAHs are highly toxic, mutagenic and carcinogenic that can be linked to other health problems which had attracted increasing interest in the research community (Li et al., [6]; Zhao et al., [10]). Accidental discharge of high concentration PAHs due to human activities has led to serious concerns on human health and ecological system well-being [2]. The concern for human health arises from the bioaccumulation of PAHs through plants or animals exposed to contaminated soil. PAHs can accumulate in food chains due to their affinity for fatty tissue [9]. PAHs was tested on laboratory animals and results had shown that it was it was responsible for tumor, cancer, reproductive problems, damage to skin and immune system [3]. In addition, the pregnant mice fed with PAHs also showed other harmful effects, such as birth defects and decreased body weight. Studies in animals have also shown that PAHs can cause harmful effects on the body system for fighting disease after exposure with PAHs.

The use of bacteria in PAHs bioremediation can be affected by different factors. These factors may lead the bioremediation process to become either more or less efficient. Thus, optimization of these factors is required to achieve high degradation efficiency in the bioremediation process. In addition, the optimization process can enhance the bacterial activity in soil.

Organic contaminants are utilized by bacteria for anabolism and metabolism [1]. High variation of substrate concentration is influenced either by natural or anthropogenic activities. Zhao et al. [10] reported that degradation rate was reduced with the higher phenanthrene concentration by inoculated *Tistrella* sp and *Sphingomonas* sp. The study reported the highest degradation was achieved at minimum PAHs concentrations, i.e., 250 ppm. Results from Zhao et al. [10] were consistent with the study reported by Janbandhu and Fulekar [5], where the highest degradation of phenanthrene was achieved at lowest concentration, i.e., 100 ppm. High



contaminant concentrations may have reduced the sorption behavior in microorganism's cells. Therefore, higher concentrations had increased toxicity of contaminants to microorganisms and subsequently reduce the bacterial activity.

Established studies show that high organic contaminant concentrations had reduced the degradation rates. Mostly established studies were conducted in liquid medium. However, there is limited report in solid medium such as contaminated soil. The specific aim of this present work is to evaluate the potential of *Corynebacterium urealyticum* in degrading phenanthrene in different initial phenanthrene concentration. This study also evaluated the growth rate of the bacteria at different initial phenanthrene concentrations.

## 2. Methodology

### 2.1 Materials

All chemicals used for extraction, preparation of minimal media, and bacteria culture were of analytical grade and supplied by Merck, Germany. The phenanthrene standard for the gas chromatography mass spectrometer (GCMS) analyses was also obtained from Merck, Germany. Ultra-pure water (UPW) used in this study was produced from Alga Purelab Ultra (18.2 MΩ, United Kingdom).

### 2.2 Sample Preparation

#### 2.2.1 Preparation of contaminated sand

Silica sand was sampled and washed three times in a sand:water mixture of 1:2 (w:v), air dried in the dark, and passed through a 1 mm sieve. The sand samples were stored in a 500 ml glass jar and dried at 60°C until a constant mass was recorded. Phenanthrene was dissolved in hexane to produce phenanthrene/hexane solution. The prepared sand samples were autoclaved (Hirayama, HVE-50, Japan) for 20 minutes at 121°C and spiked with the phenanthrene/hexane solution. Hexane was evaporated under continuous mixing to ensure homogenous distribution of phenanthrene in the sand samples. The samples were stored at 4°C until to be used in the biodegradation studies. The initial concentration of phenanthrene was verified in triplicates before being used for biodegradation study.

#### 2.2.2 Preparation of minimal media

Minimal media solution was prepared by dissolving 8.5 g Na<sub>2</sub>HPO<sub>4</sub>, 3.0 g KH<sub>2</sub>PO<sub>4</sub>, 0.5 g NaCl, 1.0 g NH<sub>4</sub>Cl, 0.5 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.0147 g CaCl<sub>2</sub>, 0.0004 g CuSO<sub>4</sub>, 0.001 g KI, 0.004 g MnSO<sub>4</sub>·H<sub>2</sub>O, 0.004 g ZnSO<sub>4</sub>, 0.005 g H<sub>3</sub>BO<sub>3</sub>, and 0.002 g FeCl<sub>3</sub> in 1L UPW. The prepared minimal media were autoclaved (Hirayama, HVE-50, Japan) for 20 minutes at 121°C.

#### 2.2.3 Preparation of bacteria strain

The strain, *Sphingobacterium spiritovorum*, was isolated from municipal sludge by Othman [7] preserved at -80°C in microbeads (microbank<sup>TM</sup>, Round Rock, Texas). The strain was revived by transferring a few of the frozen beads into universal bottles containing 20 ml of Nutrient Broth that was incubated at 30°C for three days. A series of dilution streaking was performed and the strain was sub-cultured three times to attain the active bacterium before it was used in biodegradation studies.

### 2.3 Growth Curve Test

The plate count method was used to establish the growth curve after the bacteria was inoculated in contaminated sand. The bacteria number in the sand samples were quantified by mixing 1 g of sand with 9 mL of sterile phosphate buffered saline and homogenized at high speed for 1 minute using a vortex mixer. Successive 1/10 dilutions were made by adding 1 mL of the sand suspension to 9 mL of phosphate buffered saline. An aliquot (0.1 mL) of each dilution was transferred to nutrient agar on Petri dish. The dishes were incubated at 30°C at an inverted position. After 4 days, the numbers of bacterial colonies were counted using a plate counter. Plates with different dilutions were prepared and those with colonies in the range of 30 to 300 were used to estimate the number of bacteria. This number of colonies was then multiplied by the dilution factor to find the total number of bacteria per 1g of the sand. The numbers of colonies was expressed as colony-forming units per gram of sand (CFU/g). All tests were conducted in triplicate.

#### 2.4 Biodegradation Study

Batch experiments were conducted in a 250 ml Erlenmeyer flask as the reactor. The reactor contained 20 g autoclaved sand, 10 mg/kg, 100 mg/kg, 250 mg/kg, 500 mg/kg and 1000 mg/kg initial phenanthrene concentration, 5.6 ml minimal media, and 1.4 ml bacterial inoculums. 5.6 ml of minimal media constituted 40 percent of the total volume. This volume was selected based on the quantity of inorganic nutrient required for bioremediation of PAHs in soil [6]. 1.4 ml of bacterial inoculum occupied 10 percent of the total volume and this quantity was selected based on an optimization study conducted by Othman [7]. The bacteria inoculated into the samples were collected at the middle of the exponential growth phase based on established growth curves, i.e., on day 4. This experiment was repeated three times and the average bacteria concentration on day 4 was  $1 \pm 1.2 \times 10^7$  cells/g soil. Then, the strain was inoculated into contaminated soil. After inoculation, all flasks were shaken in an incubator shaker at 150 rpm in the dark at 30°C. Sterile water was supplied at 2 percent remaining weight every day. All samples were analyzed in triplicate. Control samples consisted of 20 g autoclaved sand, 10 mg/kg, 100 mg/kg, 250 mg/kg, 500 mg/kg and 1000 mg/kg phenanthrene, and 5.6 ml minimal media without bacterial inoculums. The plate count method was performed on the control samples and no colony was found.

#### 2.5 Extraction and Analysis

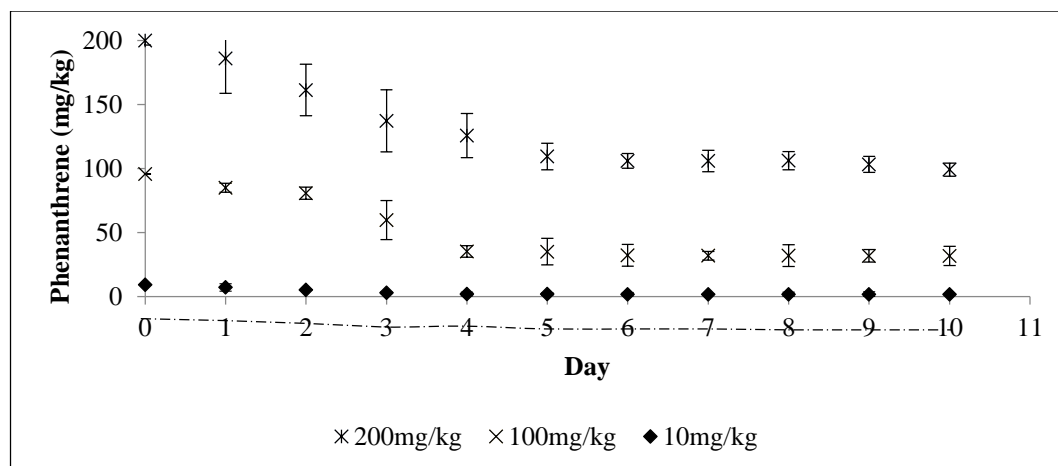
For sample analysis, 500 mg of the contaminated sand sample was dissolved in 25 ml of n-hexane and acetone 7:3 (v/v). The extractions were performed using the pressurized microwave extraction system (MAE) Multiwave 3000 (Rotor 8XF100 SOLV and solvent safety system; Graz, Austria). All samples that were placed in the MAE were extracted for 40 minutes under a pressure of 10 bars. When the extraction period was completed, the equipment was allowed to cool down to room temperature (20 minutes). Subsequently, the samples were filtered with a Whatman fiber filter with a pore size of 11  $\mu$ m and stored in a 25 ml universal bottle. The samples were concentrated by means of a rotary evaporator to 1 ml. The extraction method had been pre-tested for recovery efficiency for phenanthrene and demonstrated an efficiency of 99 percent. Phenanthrene concentrations were analysed using gas chromatography mass spectrometer (Perkin Elmer Clarus 600; Shelton, Connecticut), equipped with Elite Column 5MS of 30 m long X 0.25 mm internal dimension X 0.25  $\mu$ m thickness. The injector was operated at 250°C in the splitless mode with a 3 minute splitless period. Helium was used as the carrier gas with 1 ml/min constant flow rate. The column temperature was initially set at 50°C for 1 minute, increased to 250°C at a rate of 25°C/min, kept constant at 1 minute, and held constant until the end of the 22 minute total run time.

### 3. Results and Discussions

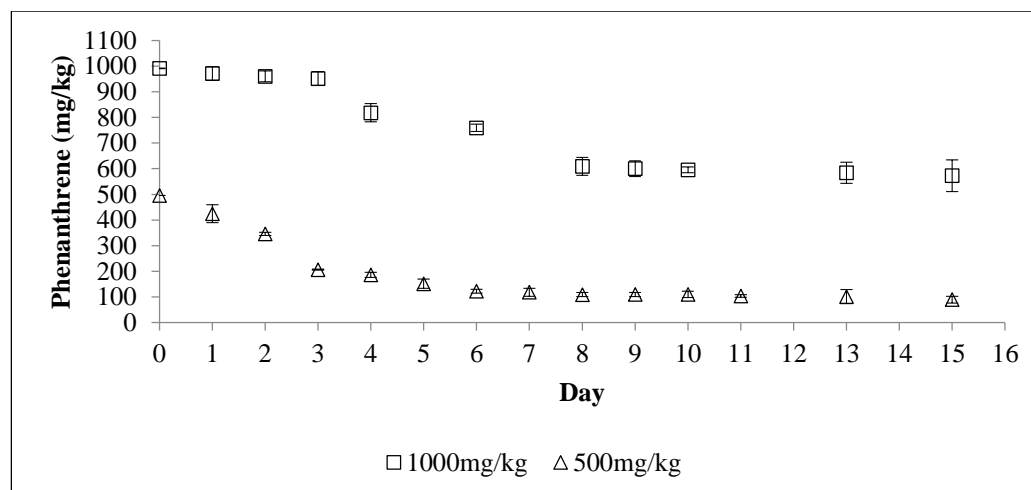
Phenanthrene degradation by the bacteria was evaluated at different initial phenanthrene concentrations, namely, 10 mg/kg, 100 mg/kg, 200 mg/kg, 500 mg/kg and 1000 mg/kg. Figure 1 and Figure 2 show the phenanthrene degradation for low concentrations that is, 200 mg/kg, 100 mg/kg and 10 mg/kg and higher concentrations, that is, 1000 mg/kg and 500 mg/kg, respectively.

Degradation trend can be divided into two phases, phase 1: rapid degradation and phase 2: very slow degradation. Experiment durations in phase 1 were different for each concentration. The durations in phase 1 were observed on day 4, day 5 and day 6 for low concentrations, that is, 10 mg/kg, 100 mg/kg and 200 mg/kg, respectively. On the other hand, for high concentrations, the durations in phase 1 were observed on day 9 and day 7 for 1000 mg/kg and 500 mg/kg, respectively. This result shows that the experiment durations were shorter for low concentrations compared to high concentrations, i.e., 10 days and 15 days for low and high concentrations, respectively.

Based on degradation rates, the optimum initial concentration for phenanthrene degradation was observed at 500 mg/kg. As initial phenanthrene concentration increased, the degradation rate was also increased except for 1000 mg/kg of initial phenanthrene concentration. For samples at high of phenanthrene concentration, such as 500 mg/kg, the bacteria have more carbon sources that can be metabolized to bring more energy.



**Figure 1.** Phenanthrene degradation by the bacteria for low initial concentrations, i.e., 200mg/kg, 100 mg/kg and 10 mg/kg.



**Figure 2.** Phenanthrene degradation by the bacteria for high initial concentrations, i.e., 1000mg/kg and 500 mg/kg.

However, very high phenanthrene concentration, such as 1000 mg/kg, had saturated the bacteria cell with the substrate. Therefore, the performance was reduced at 1000 mg/kg of initial phenanthrene concentration. Performance of the bacteria in degrading at different phenanthrene concentrations in this study was not consistent with the results from a study conducted by Othman [7]. The bacteria was previously used by Othman [7] to degrade phenanthrene with initial concentration of 1 ppm, 5 ppm, 10 ppm, 100 ppm and 500 ppm in minimal media. The study reported that the bacteria achieved a highest degradation rate for initial concentration of 100 ppm. Then, degradation rate was reduced at 500 ppm of initial phenanthrene concentration. In contrast, higher degradation rate was observed for initial concentration of 500 mg/kg in this study. Therefore, at high phenanthrene concentration, the bacteria performed better in a contaminated soil compared to minimal media.

Phenanthrene degradation by the bacteria increased with initial concentration, suggests a great potential in application of the bacteria in degradation of contaminated soil that is containing PAHs at high concentration. In chemical treatment, high contaminant concentration requires high of the chemical agent to remediate the contaminant [4]. This high concentration of chemical agent can result in toxicity and produces other problems such as health hazard [8].

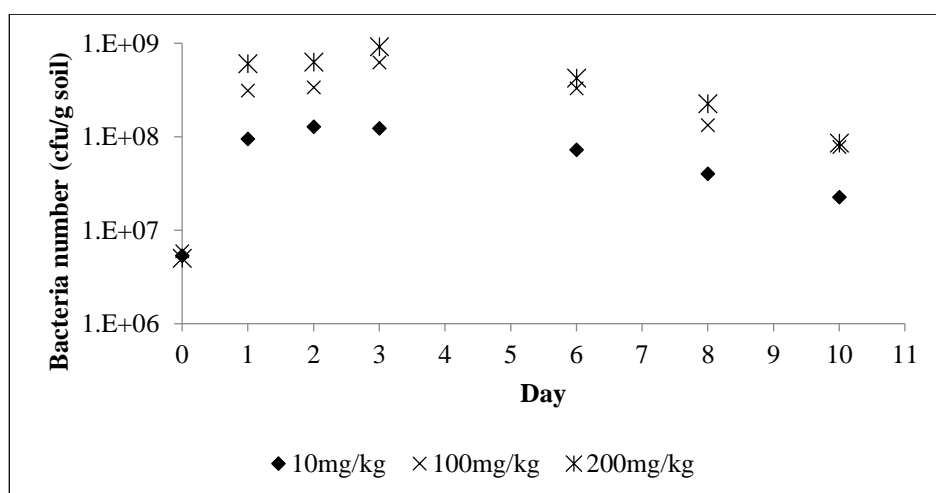
Statistical analysis using ANOVA was conducted to establish the correlation between initial phenanthrene concentrations and degradation rates. From ANOVA, at the 5% level of significance, it was found

that initial phenanthrene concentrations have significant effect to the degradation rates ( $p < 0.05$ ). This analysis shows that the degradation rates were influenced by initial phenanthrene concentrations.

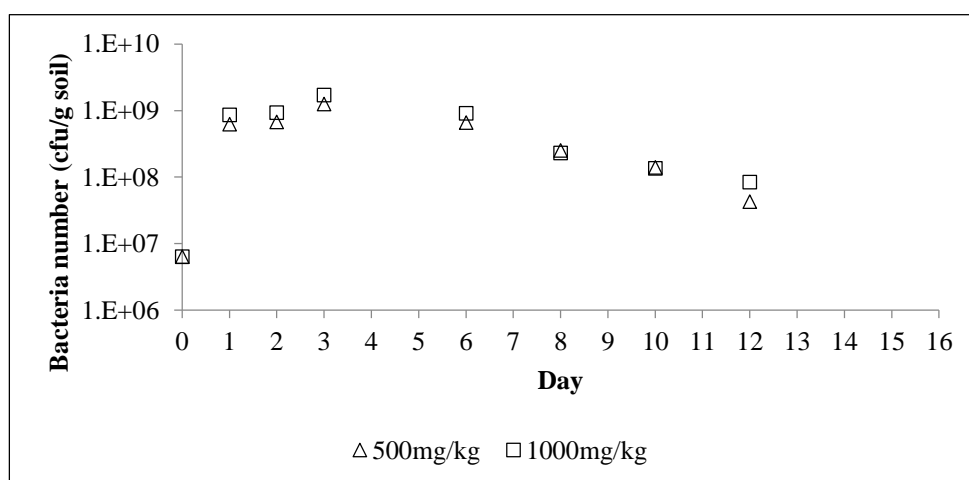
Figures 3 and Figure 4 show the growth of the bacteria at different initial phenanthrene concentrations, which indirectly indicates the strains survival. Bacteria numbers were increased until day 3 for all initial phenanthrene concentrations. After day 3, bacteria numbers were reduced until at the end of the experiment. This result indicates that the bacteria can survive for three days for all initial phenanthrene concentrations. As initial phenanthrene concentration increased, the growth rates were also increased. The growth rates for initial phenanthrene concentration of 10 mg/kg, 100 mg/kg, 200 mg/kg, 500 mg/kg and 1000 mg/kg were observed to be  $3.91 \times 10^7$  cfu/g soil/day,  $2.06 \times 10^8$  cfu/g soil/day,  $3.04 \times 10^8$  cfu/g soil/day,  $4.15 \times 10^8$  cfu/g soil/day and  $3.31 \times 10^8$  cfu/g soil/day, respectively.

As previously discussed in this section, the bacteria have high carbon sources to bring more energy in high initial phenanthrene concentration. This high energy resulted in better growth. Therefore, the bacteria grew better at high concentrations compared to low concentrations. For low concentrations, phenanthrene can be limited and retard the growth of the strain.

Statistical analysis using ANOVA was conducted to establish the correlation between phenanthrene concentrations and growth rates. At the 5% level of significance, it was found that initial phenanthrene concentrations have significant effect to the growth rates ( $p < 0.05$ ). This analysis shows that the growth rates were influenced by initial phenanthrene concentrations.



**Figure 3.** Growth curve of the bacteria for low initial concentrations, i.e., 200mg/kg, 100 mg/kg and 10 mg/kg.



**Figure 4.** Growth curve of the bacteria for high initial concentrations, i.e., 1000mg/kg and 500 mg/kg.

#### 4. Conclusions

Optimization studies provide optimum environmental condition for PAHs degradation and bacterial growth. This condition can be used in designing the soil bioremediation with a focus on PAHs as soil contaminants. This study established the optimum condition for phenanthrene degradation by the bacteria at 500 mg/kg of initial phenanthrene concentration. The growth optimum also obtained at 500 mg/kg of initial phenanthrene concentration. Optimization of PAHs biodegradation should also be conducted on different factors, e.g., soil types, salinity, moisture and organic matter. This is important to evaluate the effectiveness of potential bacteria as PAHs degrading bacteria for in-situ remediation.

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