Optimization of low ring polycyclic aromatic biodegradation

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Abstract. Polycyclic aromatic hydrocarbons (PAHs) are recalcitrance and persistence that finally turn into problematic environmental contaminants. Microbial degradation is considered to be the primary mechanism of PAHs removal from the environment due to its organic criteria. This study is carried out to optimize degradation process of low ring PAHs. Bacteria used in this study was isolated from sludge collected from Kolej Mawar, Universiti Teknologi MARA, Shah Alam, Selangor. Working condition namely, substrate concentration, bacteria concentration, pH and temperature were optimized. PAHs in the liquid sample was extracted by using solid phase microextraction equipped with a 7µm polydimethylsiloxane (PDMS) SPME fiber. Removal of PAHs were assessed by measuring PAHs concentration using GC-FID. Results from the optimization study of biodegradation indicated that maximum rate of PAHs removal occurred at 100 mgL⁻¹ of PAHs, 10% bacteria concentration, pH 7.0 and 30°C. These working condition had proved the effectiveness of using bacteria in biodegradation process of PAHs.

Keywords: bioremediation, persistent organic pollutant, degradation percentage

1. Introduction
PAHs represent a large family of organic compounds that are considered environmental pollutants. These compounds are widespread in environment and it is believed as the most abundant organic compounds in the universe that might pose a threat to the environment and mankind [1,2,3]. Significant levels are detected in air, water, soils and sediments [1,4]. PAHs impact the aquatic environment by targeting the bottom feeder such as polycates, nematodes, bivalves and crustaceans [5], thus creating a disturbance in the ecological balance of the aquatic systems. PAHs may be released into the environment when the soils or sediments are disturbed.

Nowadays, PAHs can also be found in many household products such as mothballs, cosmetics and hair dyes. In addition, some food products such as roasted coffee, peanut and barbecued meat also constitute other source of PAHs in domestic setting. These products at the end of their used would eventually end up in the wastewater treatment plants or landfill thus contributing to PAHs presence in water, sludge and soil.

Microbial degradation is among favorabale method in removing PAHs from the contaminated sites. Previous studies had proved successful process of PAHs degradation using several type of microbes isolated from different sources namely, riverine sediment, sludge, petrochemical sludge and soil [6,7,8,9]. Information on optimization condition is serve as an important parameter for ensuring complete biodegradation and bioremediation of PAHs on site. This study is carried out to optimize degradation process of low ring PAHs. Parameter such as substrate concentration, bacteria concentration, pH and temperature were optimized.
2. Method

2.1 Bacteria Strain
Strain of bacteria *Corynebacterium uroalyticum* was isolated from domestic municipal sludge. The sludge was sampled by placing a long hose tube at the bottom of the clarifier and pumping it out to the drying bed. Sludge sample was collected using the scoop method and stored in an air tight container. Samples for bacterial isolation was stored in air tight plastic container and was stored in a refrigerator at 4°C at the Microbiology Laboratory, Faculty of Applied Science, UiTM to inhibit microbial activities. Two different sources of PAHs, single PAHs and mix PAHs were used as carbon source during isolation process.

2.2 Preparation of Bacterial Strain and PAH Degradation
A liquid inoculum was prepared from a pure culture of bacteria. A sterile swab was used to transfer bacterial colonies from minimal agar to sterile nutrient broth. Then the inoculum was incubated at 30°C until the broth became turbid. The incubation time used depended on the turbidity test.

2.3 Design Experiment for Optimization study
Four parameters including the concentration of bacteria, concentration of PAH, pH and temperature were selected to determine their effects on the degradation of PAHs by the isolated bacteria strain. Experiments were conducted with 50 ml sterile minimal media subjected to specific parameters to be investigated. The flasks were placed in an incubated shaker at 150 rpm for two weeks. Summary of the variables on effects of abiotic factors are presented in Table 1 [7,9].

| Abiotic factors | Range of variables |
|-----------------|--------------------|
| Bacteria concentration | 1 % to 12 % |
| Substrate concentration | 1 ppm, 10 ppm, 50 ppm, 100 ppm and 500 ppm |
| pH | 5, 6, 7, 7.5 and 8 |
| Temperature | 20°C, 25°C, 30°C, 35°C and 40°C. |

2.4 PAHs Extraction
An innovative solid phase microextraction (SPME) method optimized by [10] was adopted to extract PAHs. 20 ml of sample was kept in 25 ml glass bottle with a septum cap and placed in an ultrasonic water bath. The sample volume and vial size were kept constant in order to reduce effects of external factors on extraction of the analytes. Besides that, to ensure consistency and uniformity in adsorption and desorption, the period and depth of immersion, time between immersion and exposure to the GC and GC desorption parameters including temperature and time were kept constant [11,12].

2.5 Analytical Instruments for PAHs
Gas chromatography was performed on a Perkin Elmer Clarus 500 system coupled to flame ionization detection (FID) system. In order to analyze naphthalene, acenaphthene, acenaphthene, fluorene, anthracene and phenanthrene, Elite Column 5MS with 30m long 0.25mm internal dimension and 0.25µm thickness was used to separate the compounds. The injector was operated at 250°C in the splitless mode with a two minute splitless period. Helium was used as the carrier gas with 1ml/min constant flow rate. The column temperature was initially set at 50°C for 1 min, increased to 150°C at a rate of 15°C/min and held at 1 min, and finally ramped at 5°C/min to 300°C and held constant until the end of the 35 min total run time. Identification of analytes in the chromatograms was based on retention time.
2.6 Abbreviations
A few abbreviations were used in the results to name the PAHs and mix of PAHs. N-napthalene, AC-acenaphylene, A-acenaphthene, F-fluorene, P-phenantherene. M1-mix pahs 1, M2-mix PAHs 2 and M3-mix PAHs 3.

3. Results and discussion
Four parameters including the initial concentration of bacteria, pH, temperature, concentration of substrate were tested for investigating their effects on the degradation of PAHs using the isolated bacteria strains.

The data in optimization study was analyzed using a general linear analysis of variance (ANOVA) model. For multiple comparisons of data means, Turkey’s honestly significant difference test with an $\alpha=0.05$ was performed.

The study on the optimal growth conditions for PAH oxidation by isolated bacteria species could increase the removal rate of PAHs from sludge, soil and other matrices. Apart from that these experiments will provide knowledge for further investigations.

3.1 Effect of Initial Bacteria Concentration on PAHs degradation
Degradation of PAHs by Corynebacterium uroalyticum (P1) will be used. PAHs degradation was investigated with 50 ml of aqueous media, two weeks (14 days) contact time at pH 7 and with bacteria concentration varied from 1% to 12% at 30°C. A typical graph on the effect of bacteria concentration on phenanthrene degradation is shown in Figure 1.

![Figure 1](image-url)  
**Figure 1.** Phenanthrene degradation at different concentration of Corynebacterium uroalyticum (P1) after 14 days of incubation

Figure 1 shows that after 14 days incubation, the degradation of phenanthrene increase as the initial concentration of bacteria increase. The PAHs degradation reaches the optimum level at 10% of bacteria concentration. The rate for 1% bacteria concentration is 3.9 ppm per day. The rate increased to 6.2 ppm per day, for 10% bacteria concentration and remains unchanged at 12% bacteria concentration. Therefore, the concentration of bacteria was selected as 10% for further experiments.

More enzymes could be synthesized with the increase in bacteria concentration. Each enzyme has a certain area on its surface that is known as active sites. The active site is the region at which the enzyme forms a loose association with PAHs as its substrate. Therefore, the more enzymes are secreted the more active sites will be provided for PAHs. Enzymes remain unchanged while they speed up degradation of PAHs.

The percentage of degradation increased as the concentration of bacteria increased and finally reached a steady state known as chemical equilibrium. At this point the enzyme’s active site or binding site is working at full capacity [13,7] reported the same range of bacteria concentration used for biodegradation of PAHs.

For the purpose of comparison among the different pair of bacteria strains and PAHs, a normalized graph on PAHs degradation for different bacteria concentrations were constructed as shown in Figure-2 for single PAH and Figure-3 for mixed PAHs.
Generally, it can be observed that all strains of PAHs degrading bacteria show increasing trend as the concentration of bacteria increase (as shown in Figures 2 and 3). Degradation of phenanthrene remain unchanged at 10% bacteria concentration.

Analysis of variance (ANOVA) performed on the results showed that at 5% level of significance, the degradation of phenanthrene was significantly affected by concentration of bacteria. Hence, higher concentration of bacteria result in faster degradation process.
3.1 Effect of pH on PAHs degradation

Degradation of phenanthrene using Corynebacterium uroalyticum (P1) was used for the purpose of discussion effect on pH. pH plays an important role in PAHs biodegradation. Effect of pH on PAHs degradation was evaluated at pH 5, 6, 7, 7.5 and 8. Like other proteins, enzymes in bacteria are affected by extreme pH such as strong acid or alkali. Thus, this study evaluated the effect of pH on PAHs degradation at pH value that is mildly acid and mildly alkali. Typical graph on effect of pH on PAHs biodegradation by Corynebacterium uroalyticum is shown in Figure-4. Based on Figure-4, degradation of phenanthrene by Corynebacterium uroalyticum shows increasing trend as pH increase. The degradation reaches optimum level at pH 7. At pH 7.5 and 8 degradation processes decrease.

![Figure 4. Effects of pH on phenanthrene degradation by Corynebacterium uroalyticum (P1)](image)

At equilibrium, biodegradation of PAHs on Corynebacterium uroalyticum as a function of pH is shown in Figure-4, which indicated that at pH 5 and pH 6, slower biodegradation were observed with the range of phenanthrene degradation 61% to 75% respectively. While at pH 7, 88% of phenanthrene was degraded. For the pH value higher than 7, biodegradation process is slower than optimum level. The result shows that the optimum degradation was accomplished at pH 7.

![Figure 5. Phenanthrene degradation by Corynebacterium uroalyticum (P1)](image)

Changes in pH can alter the electrical charge on various chemical groups in enzymes molecules. Changes in electrical charge probably alter the enzyme’s ability to bind its substrate and catalyze a reaction. Imbalance of the electrical charges in very acidic and alkali condition can disrupt hydrogen bonds and other weak forces that maintain enzyme structure. Such disruption of enzyme structure is called denaturation. Thus, this phenomenon resulted in poor biodegradation process. This explanation was also offered by [7]. They also had
pointed out that microbial enzymes have an optimum pH at which they function most effectively, thus it is related to an organism normal environment.

The results from this study suggest that the biodegradation of PAHs strongly depends on pH, as it affects the ionization state of the main functional group in PAHs which is benzene on bacterial cell [14]. Several studies reported by [15,7] indicated that optimum pH for PAHs biodegradation normally occurred at pH 7. In order to compare strength among pair of bacteria strains and PAHs at different pH, normalized graphs were constructed as shown in Figures 6 and 7.

![Normalized PAHs degradation](image)

**Figure 6.** Normalize PAHs degradation for pH value (single PAHs substrate)

Figures 6 and 7, show that all strains of PAHs degrading bacteria show increasing trend as pH values increase from pH 5 to pH 7. Optimum pH for all strains was established at pH 7. The graphs show decreasing trend after the optimum pH. This trend indicates that the activity of PAHs degrading bacteria is maximum under neutral condition.

Statistical analysis (ANOVA) performed on the results at 5% level of significance indicate that the degradation of phenanthrene was significantly affected by pH. Better degradation process was obtained with higher pH of substrate.

### 3.2 Effect of temperature on PAHs degradation

Degradation of phenanthrene using *Corynebacterium uroalyticum* as biodegrader is carried out at different initial temperature. Degradation of phenanthrene using *Corynebacterium uroalyticum* (P1) was used for the purpose of discussion effect of temperature. The temperatures chosen for temperature dependence study were 20°C, 25°C, 30°C, 35°C and 40°C. Figure-8 shows a typical graph on biodegradation of phenanthrene as a function of temperature.
Figure 7. Normalize PAHs degradation for pH value (mixed PAHs substrate)

Figure 8 shows biodegradation of PAHs by Corynebacterium uroalyticum at equilibrium condition as a function of temperature. It is shown that at low temperature such as 20°C low degradation process is observed, while at 25°C the biodegradation process start to increase. The results show that the optimum temperature was attained at 30°C. The equilibrium PAHs biodegradation is 87.2% at this temperature value.

Low degradation process at low temperature suggests that this temperature is not sufficient for the enzyme to speed up the reaction rate. Therefore, increased in temperature will result in faster reaction rate in degrading PAHs. In general enzymes as catalysts have certain temperature that it withstands and normally at optimum temperature will catalyze reaction most rapidly. Microbial enzymes likewise function best at optimum temperature which related to an organism’s normal environment. Thus, reaction such as degradation process is normally optimum at mild temperature in microbial cell. Above 40°C, however enzyme is rapidly denatured, and its activity decreased accordingly.

The results from this study suggest that the degradation of PAHs strongly depend on temperature. It affects the reaction rate to cleave binding in benzene as the main functional group. These observations were also reported by previous studies that indicated the optimum temperature for PAHs biodegradation occurred at 30°C.

Figure-8. Effects of temperature on phenanthrene degradation by Corynebacterium uroalyticum (P1)
A normalized graph for each pair of bacteria and PAHs were drawn at different temperatures as shown in Figure 9 and 10. Figures 9 and 10 indicate that all strains of PAHs degrading bacteria show increasing trend as temperature increased from 20°C to 30°C. Nevertheless, after the optimum temperature at 30°C, the graphs decrease. Statistical analysis (ANOVA) was conducted on the results for degradation of phenanthrene at different temperature. At 5% level of significance it was found that the degradation of phenanthrene was significantly affected by temperature. Better degradation was obtained with higher temperature.

![Figure 9. Normalize PAHs degradation for temperature (single PAHs substrate)](image)

![Figure 10. Normalize PAHs degradation for temperature (mixed PAHs substrate)](image)

3.3 Effects of Initial substrate concentration on PAHs degradation
The experiment on effect of initial substrate was conducted at optimized parameters, 10% bacteria concentration, pH 7.0 and temperature at 30°C. The initial PAHs concentrations were concentration adopted from characteristic study, 10ppm, 50ppm, 100ppm and 500ppm. The lowest concentration was chosen based on PAHs concentration in sludge sample analyzed. Other PAHs concentrations were chosen based on previous studies as reported by [13,14]. PAHs degradation by Corynebacterium uroalyticum is affected by the change in phenanthrene concentration as shown in Figure-11. The percentages of degradation increase as concentration of substrate increase up to 100ppm of substrate concentration (Figure-11). Thus, degradation process is optimum
at 100ppm of substrate or is known as a steady state. At this point the PAHs degrading bacteria have sufficient carbon sources for energy and growth.

There was about 87.3% phenanthrene degradation for cultures with the addition of 100 ppm PAHs but only 80.5% phenanthrene degradation at 500 ppm. The average degradation rate of phenanthrene is 6.00 ppm/day at 100ppm, 3.28 ppm/day at 500ppm, 0.98 ppm/day for 50ppm, 0.45 ppm/day at 10 ppm, 0.12 ppm/day for 5ppm and 0.01 ppm/day at 1ppm (as shown in Figure-11). 100 ppm phenanthrene shows a higher efficiency to remove phenanthrene compare to 500 ppm phenanthrene culture. This may be due to the fact that too high the concentration of phenanthrene would saturate the bacteria cell with the substrate. Therefore, the optimum degradation on phenanthrene at 86.3% was obtained at phenanthrene concentration of 100ppm.

![Figure 11](image1.png)

**Figure 11.** Effects of substrate concentration on phenanthrene degradation by *Corynebacterium uroalyticum* (P1)

Degradation of phenanthrene by *Corynebacterium uroalyticum* increased with substrate concentration indicates a great potential in application of *Corynebacterium uroalyticum* as a biodegrader to the treatment of sludge containing PAHs at high concentration.

![Figure 12](image2.png)

**Figure 12.** Average degradation rate for different concentration

Other researchers also reported that high concentration of PAHs were beneficial to the growth of isolated bacteria because PAHs could acts as a sole carbon source for bacterial growth. Therefore, low concentrations of PAHs would become a limiting factor for the bacterial culture to grow.

The degradation of PAHs at higher PAH concentration level shows an increasing trend because the increase of PAHs will increase the affinity of substrate toward formation of product. When neither substrate nor products are removed from the system, the reaction will ultimately reach a steady state known as chemical equilibrium. At equilibrium no net change was observed in the concentration of substrate or product. After the
steady state, the substrate saturated and degradation process start decreasing and resulted in lower rate of reaction as shown in Figure-12 for 500ppm phenanthrene concentration. Similar observation had been put forward by [15,13]. In order to compare strength of strains at different substrate concentration a normalized graph are constructed as shown in Figure 13 and 14 for single and mixed PAH respectively. Figures 13 and 14 indicate that all PAHs show increasing trend as concentration of substrate increased. This figures also show that at 100ppm of substrate concentration degradation of PAHs is more effective compared to 500ppm.

Figure 13. Normalize PAHs degradation for substrate concentration (single PAHs substrate)

Figure 14. Normalize PAHs degradation for substrate concentration (mixed PAHs substrate)

In this optimization study PAHs act as a sole source of carbon and energy for bacterial growth. Thus, high concentration of substrate is valuable to cell development of the culture. The results from this study and previous studies also showed that too high concentration of substrate would become a limiting aspect for culture expansion.

Statistical analysis using ANOVA conducted on the data at a 5% level of significance showed that a significance different between concentration substrate 1ppm, 5ppm, 10ppm, 100ppm and 500ppm exist at 5% level of significance. Hence, higher concentration results in faster degradation process. level of significance. Hence, higher concentration results in faster degradation process.
4. Conclusion
Results from the optimization study of biodegradation indicated that maximum rate of PAHs removal occurred at 100 mgL⁻¹ of PAHs, 10% bacteria concentration, pH 7.0 and 30°C. The bacteria isolated from single source of PAHs performed as well as bacteria isolated from mix PAHs.

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