Optimization parameters for *Mycobacteria confluentis* biodegradation of PAHs

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Abstract - In this study, the effect of process parameters (incubation period, temperature, initial pH-value, concentration of PAHs in the medium, and bacterial inoculum size) on the biodegradation of PAHs using *Mycobacteria confluentis* was studied using One-Factor-At-a-Time (OFAT) method. From the results of the study, it was observed that the studied parameters had significant effects on the degradation process. The capability of *Mycobacteria confluentis* on the degradation of PAHs was found to be maximum when the initial pH of the PAH was 7, temperature 40 °C, PAH concentration of 50 µL, bacterial concentration of 7.5 mL, and incubated for 3 to 5 days. This condition gave a PAH degradation percentage of 40 to 50 %, showing a similarity to most previous studies where the maximum percentage of degradation has been around 40 to 50 %. This study, therefore, concludes that *Mycobacteria confluentis* is a good PAHs degrader which can be of significant important in the management of oil polluted fields and environments.

Keywords: PAHs, Biodegradation, *Mycobacteria confluent, Optimization*

1 Introduction

The polycyclic aromatic hydrocarbons (PAHs) are a complex group of chemicals which are of great concern owing to their abundant environmental distribution, resistance to biodegradation, as well as their harmful effects. They are mainly implicated in the deterioration of water and soil quality. There have been series of treatment options to curb the economic dangers and ecological deterioration caused by the PAHs. Bioremediation is one of such recommended treatment options that holds a promise of delivering a lasting solution for the degradation of PAHs. Biodegradation is the process of degrading the high molecular chains of hydrocarbons using bacteria [1, 2] and fungi [3, 4]. Other microorganisms such as algae have also been reported for their capability of degrading hydrocarbons [5, 6]. Bacteria have been reported as the major and most active degraders of petroleum pollutant [7, 8].

The process of bioremediation involves microorganisms mitigating, degrading and reducing dangerous organic pollutants to the less harmful ones such as CO_2, CH_4, H_2O, without any adverse environment effect [9]. Biodegradation is a primary bioremediation technique where oleophilic microbes are employed for the removal of hydrocarbon pollutants from the environment [5, 10, 11]. The petroleum refinery effluents (PRE) are wastes which originate primarily from oil refining industries during the production of crude oil, lubricants, and petrochemical intermediates [12]. The PREs are complex matrices of organic pollutants; it has been established that oily and hydrocarbon-rich waste waters can be completely mineralized through photocatalytic bioremediation. Interestingly, all the organic substrate found in the PREs are mineralized; therefore, the PREs can be effectively remediated [13].

The ability of microorganisms to degrade hydrocarbons vary; some have the capability to degrade aliphatics, while others can degrade the monoaromatics, polyaromatics or resins. Some of the reported hydrocarbon pollutants degrading microorganisms in the literature include the genera *Achromobacter*, *Acinetobacter*, *Azoarcus, Brevibacterium, Cellulomonas, Corynebacterium, Flavobacterium, Marinobacter*, *Micrococcus, Nocardia, Ochrobactrum, Pseudomonas, Stenotrophomaonas* and *Vibrio* [10, 14]. The bacterial degradation of PAHs and other hydrocarbon pollutants depend on several environmental conditions such as the type of the microorganisms, nature and chemical structure of the compound to be degraded, the temperature of the process, the initial pH of the environment, as well as the number of organisms in the environment. The PAHs are biotransformed into less complex metabolites through mineralization into inorganic minerals such as H_2O, CO_2 under aerobic condition, and CH_4 under anaerobic condition.

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The biodegradation of PAHs has been studied using various microbes such as *Pseudomonas aeruginosa, Pseudomonas fluorescence, Mycobacterium spp., Haemophilus spp., Rhodococcus spp., Paenibacillus spp* isolated from the contaminated soil [15]. Although many studies have reported the use of various microbes for the degradation of PAHs, there has been no report on the use of *Mycobacteria confluentis* for the biodegradation od PAHs. This study was, therefore, initiated to study the effect of various parameters such as the period of incubation, the temperature of the process, the initial pH of PAHs, the concentration of PAHs in the medium as well as the concentration of the microbe in the medium using One-Factor-At-a-Time (OFAT) method. The outcome of the study will contribute to the pool of already identified microbes for the bioremediation of hydrocarbon polluted environments.

2 Materials and Methods

2.1 Sample collection and preparation

The refinery wastewater samples were collected in glass bottles, covered with screw caps, and sent to the laboratory for analysis and Liquid-Liquid extraction as described in [16] using Filters Fioroni paper (90 mm diameter), under vacuum pump. The extracted samples were stored at 4 ^{o}C for further use.

2.2 Media preparation

An enrichment medium was prepared in demineralized water containing the following constituents (g/ L): 0.2 $MgSO_4$; 0.02 $CaCl_2$; 1.0 NH_4NO_3; 1.0 KH_2PO_4; 0.05 K_2HPO_4; 0.05 $FeCl_3$ [17]. Nutrient agar for the bacterial culture was prepared by adding 23.0 g of nutrient agar (Hardy Diagnostics, USA) to 1000 mL of demineralized water. The nutrient broth was also prepared by adding 20.0 g of nutrient broth (Merck, Germany) to 1000 mL of demineralized water. All the media were autoclaved for 15 minutes at 121 ^{o}C and allowed to cool before use.

2.3 Biodegradation experiments

The bacterial suspension was prepared in nutrient broth contained in Falcon tubes. Into the tubes, 25 mL of the already prepared nutrient broth was added, followed by 5 mL of the bacterial broth culture containing about 3.0 x 10^8 CFU/mL. The tubes were incubated at 37 ^{o}C for 24 h. For the PAHs biodegradation assay, the prepared bacterial suspension (5 mL) was added into the enriched medium (45 mL), followed by 50 µL of PAHs.

2.4 Effect of each parameter

2.4.1 Effect of incubation period

To study the effect of the incubation period, the initial pH of the PAHs was adjusted to 7 with NaOH; 50 µL of the adjusted PAHs was added into 45 mL of the enriched medium, followed by 5 mL of the bacterial suspension. The medium containing the organism and PAHs was incubated at 37 ^{o}C for a total period of 7 days. At the end of every 24 h, an aliquot of the medium was sampled for turbidity check and PAHs degradation percentage.

2.4.2 Effect of temperature

To study the effect of temperature, the initial pH of the PAHs was adjusted to 7 with NaOH; 50 µL of the adjusted PAHs was added into 5 set tubes containing 45 mL of the enriched medium, followed by 5 mL of the bacterial suspension. One set of the tubes was incubated at 5oC, the second, third, fourth, and fifth tubes were incubated at room temperature (25 ± 2 ^{o}C), 30, 40, and 55 ^{o}C, respectively for 5 days. The samples were analyzed for turbidity and percentage PAHs degradation only after 5 days of incubation.

2.4.3 Effect of initial PAHs pH

To study the effect of pH, the initial pH of the PAHs was adjusted to pH 5, 7, and 9 with NaOH; 50 µL of each adjusted PAHs was added into 3 sets of tubes containing 45 mL of the enriched medium, followed by 5 mL of the bacterial suspension. The media containing the organism and pH-adjusted-PAHs were incubated at 37^{o} C for 5 days. At the end of 5 days, an aliquot of each medium from the three sets was sampled for turbidity check and PAHs degradation percentage.

2.4.4 Effect of bacterial inoculation concentration

To study the effect of bacterial concentration, the initial pH of the PAHs was adjusted to 7 with NaOH; 50 µL of the adjusted PAHs was added into 4 sets of tubes containing 45 mL of the enriched medium, followed by 1, 3, 5, and 7.5 mL of the bacterial suspension into the 1st, 2nd, 3rd, and 4th tubes, respectively. All the media containing the organism and PAHs were incubated at 37^{o} C for 5 days. At the end of 5 days, an aliquot of each of the medium was sampled for turbidity check and PAHs degradation percentage.

2.4.5 Effect of PAHs concentration

To study the effect of PAHs concentration, the initial pH of the PAHs was adjusted to 7 with NaOH; 50, 275, and 500 µL of the adjusted PAHs was added into 3 sets of the tube containing 45 mL of the enriched medium, followed by 5 mL of the bacterial suspension. The media containing the organism and PAHs were incubated at 37 ^{o}C for 5 days. At the end of 5 days, an aliquot of each set sampled for turbidity check and PAHs degradation percentage.

2.4.6 Cell growth estimation

The percentage of cell growth in each of the respective assays was turbidimetrically analysed at 600 nm using a microplate reader (Infinite M200 PRO). The percentage growth was calculated using Equation 1. A high cell growth indicates a high utilization of the PAHs as the source of carbon by the organism, indicating a high degradation of the hydrocarbon.

$$Cell\ growth = Abs\ of\ test - Abs\ of\ control \quad (1)$$

where Abs of the test is the absorbance of the tubes containing the media constituent, the PAHS, and the organism; while Abs of the control is the absorbance of the tubes containing only the media and PAHS without the organism.

2.5 PAHs degradation rate

The rate of PAHs degradation at the end of each incubation period was estimated from the turbidimetric analysis of the tubes. The degradation percentage was calculated using Equation 2.

$$PAHs\ \deg radation(\%)\left(\frac{Abs\ of\ test - Abs\ of\ control}{x100\ Abs\ of\ test}\right) \quad (2)$$

where Abs of test = Absorbance of the tubes containing all the media supplements, PAHs, and the organism, while Abs of control = the absorbance of the tubes containing all the media supplements and PAHs.

3 Results and Discussions

3.1 Effect of incubation period

The effect of the incubation period on the biodegradation of PAHs using *Mycobacteria confluentis* was studied for a period of 7 continuous days of incubation. The outcome of the study is shown in Table 1. It could be observed from the table that there was a steady utilization of the PAHs as the sole source of carbon by the *Mycobacteria confluentis*, indicated by the steady increase in the absorbance of the media from day 1 to day 3. However, from day 4, there was a progressive decrease in the absorbance of the media, indicating a decrease in the number of active bacteria in the media. The decrease in the number of bacteria could be because of the complete utilization of the carbon source (PAHs), and due to the accumulation of bacterial metabolic waste products which interfered with the growth of the microbes. Kachienga and Momba [18] have reported the biodegradation of hydrocarbon chains of crude oil by-product by selected protozoan isolated from waste water. From the results of that study, the organisms were reported to achieve up to 61 % of hydrocarbon degradation within 5 days of incubation at 30 ^{o}C. other studies have similarly reported a complete biodegradation of hydrocarbons within 3 to 5 days of incubation.

Table 1: Effect of incubation period on the biodegradation of PAHs using *Mycobacteria confluentis*

Incubation period (days)	Bacterial growth (mean ± SD)	PAHs degradation (%)
1	0.069 ± 0.001	5.3
2	0.076 ± 0.000	29.7
3	0.105 ± 0.003	51.61
4	0.092 ± 0.003	36.41
5	0.089 ± 0.004	36.0
6	0.076 ± 0.001	28.83
7	0.069 ± 0.000	25.92

n = 3

3.2 Effect of temperature

The effect of the different incubation temperatures on the biodegradation of PAHs using *Mycobacteria confluentis* was studied for a period of 5 days. The results of the study are presented in Table 2. From the table, it could be observed that the bacterial cell growth was affected by the temperature of incubation. A temperature range of less than 30 ^{o}C did not support the growth of the bacteria as witnessed in the corresponding rate of PAHs degradation. However, a temperature of 40 ^{o}C was better for the growth of the organism as indicated by the high degradation percentage observed. A temperature of more than 40 ^{o}C did not support the growth of the organism; the organisms were likely to be inactivated, resulting in a reduced degradation of the hydrocarbon. Although other studies have reported a lower temperature for the biodegradation of hydrocarbons, it should be noted that different organisms, even different species of a genus, have different levels of tolerance to physical factors such as temperature and pH. Shafiee, Shojaosadati, and Charkhabi [19] reported an ideal temperature of 30 ^{o}C for the biodegradation of polycyclic aromatic hydrocarbons by aerobic mixed bacterial culture. They reported that the rate of degradation of the PAHs-fluoranthene culture was increased from 20 to 44 % after 10 days of incubation at 30 ^{o}C and pH 7. These findings indicate that the right temperature for the effective growth of any organism intended to be used for bioremediation must be determined to achieve the best experience of using the organism.

Table 2: Effect of temperature on the biodegradation of PAHs using *Mycobacteria confluentis*

Temperature (^{o}C)	Bacterial growth (mean ± SD)	PAHs degradation (%)
5	0.078 ± 0.002	27.05
RT	0.077 ± 0.001	26.1
30	0.076 ± 0.001	25.1
40	0.089 ± 0.004	36.0
55	0.070 ± 0.003	18.7

n = 3

3.3 Effect of initial PAHs pH

The effect of the initial PAHs pH on its biodegradation using *Mycobacteria confluentis* was studied for 5 days. The ionic concentration of a medium can affect the growth and metabolic activities of the microorganism. Been that most microbes living in the soil thrive around a Ph range of 6 to 8, changes in the pH of the medium can also affect the bacterial activities of the inherent microbes
[20]. For the studied microorganism, a pH range of 5 to 9 was studied and the results shown in Table 3. It could be observed a neutral Ph was ideal for the bacterial growth and PAHs degradation even though a Ph range of 5 to 9 had a significant support on the growth of the organism. This maximum result achieved at Ph 7 indicated that this organism is a neutrophilic organism. Krishnaswamy and Namasivayam [20] when reporting the effect of Ph, nitrogen source and salts on the degradation of phenol by the bacterial consortium under saline conditions stated that a maximum degradation of phenol was observed at pH 7 using a consortium of bacteria. Similarly, Shafiee, Shojaosadati, and Charkhabi [19] reported an optimal pH of 7 for the biodegradation of polycyclic aromatic hydrocarbons by aerobic mixed bacterial culture. Kauselya and Narendiran [21] also reported that the degradation of benzene was fastest at a neutral pH. All these evidences supported the outcome of this study on the effect of pH on the biodegradation of PAHs using *Mycobacteria confluentis.*

Table 3: Effect of PAHs initial pH on the biodegradation of PAHs using *Mycobacteria confluentis*

Initial PAHs pH	Bacterial growth (mean ± SD)	PAHs degradation (%)
5	0.079 ± 0.001	16.4
7	0.118 ± 0.001	44.0
9	0.097 ± 0.001	31.9

n = 3

3.4 Effect of bacterial inoculation size

The result of the study on the effect of bacterial inoculation size on the biodegradation of PAHs using *Mycobacteria confluentis* is presented in Table 4. It could be observed from the table that the rate of PAHs degradation increases with an increase in the concentration of the microorganism. The result showed that the degradation of PAHs is directly related to the number of active microorganisms in the medium. The maximum observed degradation rate of 100 % was achieved with the maximum studied microbe concentration of 7.5 mL in a 50-mL reaction volume. The study reported by Kauselya and Narendiran [21] on the effect of pH and inoculation size on benzene biodegradation using mixed culture showed that the degradation of benzene increased with inoculation size. With this report, the outcome of this study is, therefore, validated.

Table 4: Effect of bacterial inoculation size on the biodegradation of PAHs using *Mycobacteria confluentis*

Bacterial concentration (mL)	Bacterial growth (mean ± SD)	PAHs degradation (%)
1	0.06 ± 0.001	5.1
3	0.095 ± 0.004	40.1
5	0.101 ± 0.005	43.6
7.5	0.106 ± 0.002	46.3

n = 3

3.5 Effect of PAHs concentration

The effect of the initial concentration of PAHs on its biodegradation using *Mycobacteria confluentis* is shown in Table 5. It could be observed from the table that the initial concentration of PAHs has an impact on the cell growth and rate of PAHs degradation. A moderate concentration of 50 µL favored the optimum growth of the organism and facilitated the degradation of the hydrocarbon; while a higher concentration of 500 µL had a negative inhibiting effect on the growth of the organism and its degradation of the hydrocarbon. This implies that the initial concentration of the hydrocarbon should be kept low for an efficient cell growth and biodegradation. A study on the optimization of growth condition for diesel oil-degrading bacterial strains reported by Mahalingam and Nithya [22] found that diesel at 1 % supported the growth of the studied bacteria better than when supplemented at 5 %.

Table 5: Effect of PAHs concentration on the biodegradation of PAHs using *Mycobacteria confluentis*

PAHs concentration (µL)	Bacterial growth (mean ± SD)	PAHs degradation (%)
50	0.118 ± 0.001	44.0
275	0.114 ± 0.002	42.1
500	0.078 ± 0.001	15.3

n= 3

4 Conclusions

The effect of five process parameters (PAHs concentration, pH, temperature, a period of incubation, and inoculation size) on the biodegradation of PAHs using *Mycobacteria confluentis* was studied using One-Factor-At-a-Time method. From the screening process, it was observed that these factors have a significant impact on the rate of the bacterial growth and its significance on the rate of PAHs degradation. The optimum condition for an effective growth of the cell and degradation of the hydrocarbon was found as follows: PAHs concentration 50 µL, pH 7, temperature 40 ^{o}C, inoculation size 7.5 mL, and an incubation period of 3 - 5 days, giving a degradation of about 40 to 50 %. This study concludes that a combination of parameters as stated will encourage the effective growth of *Mycobacteria confluentis* and enhance its capability in the biodegradation of PAHs.

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