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# Microbiological Analysis of Phenanthrene and Naphthalene Degrading Soil Bacteria Isolated from Landfills and Filling Stations: Bioremediation Approach for a Green Environment

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## Abstract

Polycyclic Aromatic Hydrocarbons (PAH) are organic compounds with two or more fused benzene rings produced by the incomplete combustion of anthropogenic sources. Due to their ubiquitous nature, contribution to pollution and prominent health risks such as carcinogenicity and genotoxicity, they have been considered as persistent organic pollutants. With 90% of PAHs settling on soil, the primary purpose of this study is to isolate, identify and select the best phenanthrene and naphthalene degrading bacteria from contaminated soil of landfills and filling stations and study their degradation percentages. In this study, via quadrant streaking, a total of 3 morphologically different strains that can degrade the PAHs of interest above 25%, confirmed via plate assay and spectrophotometry were identified as potential biological degraders towards bioremediation. Via this study, a total of 3 morphologically different strains that can degrade the PAHs of interest above 25% were identified as potential biological degraders towards bioremediation.

**Keywords:** Bacterial Degraders, Bioremediation, Naphthalene, Phenanthrene

## 1. Introduction

Soil is one of the key environmental components that sustain most living organisms and maintain biodiversity, directly and indirectly.[1] However, soil fertility is rapidly declining amidst steady global population increase and persistent disposal of pollutants while failing to provide appropriate care. With the rapid global industrial advancement, this has created pressure on sustaining soil quality due to the resulting soil toxicity changing its integral properties.[2] Essentially, soil pollution is the presence of xenobiotics (pollutants or contaminants) in soil at a concentration higher than accepted levels which can cause adverse effects on flora and fauna diversity of the ecosystem.[3] Similarly, exceeding the accepted levels of naturally occurring soil contaminants is also considered as soil pollution. One of the major components that is primarily responsible for soil pollution is anthropogenically produced aromatic hydrocarbons (AHs).

Currently, AHs or arenes are considered as a primary pollutant that majorly contributes to environmental pollution. They are a class of unsaturated, cyclic and planer compounds that represents a six-carbon ring moiety termed benzene ($C_6H_6$) in electronic configuration and chemical behavior in its simplest form.[4] In a nutshell, AHs can be defined as any cyclic compound composed only of hydrogen and carbon atoms that fulfills the Hückel's Rule.[5] Monocyclic Aromatic Hydrocarbons (MAHs) consists of a central benzene ring with six carbon and hydrogen atoms and three double bonds.[6] Among MAHs, benzene, toluene, ethylbenzene, and the three xylene isomers (ortho-, meta-, and para-xylene) (BTEX), produced via substitution of the hydrogen atoms and considered volatile organic

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compounds (VOCs).[7] MAHs are often produced in gasoline service stations, motor vehicle exhaust and fuel evaporation, the burning of coal and oil, and various other sources including natural sources such as volcanoes and forest fires.[8]

On the other hand, Polycyclic Aromatic Hydrocarbons (PAHs) are a wide class of ubiquitous organic compounds that consist of two or more fused benzene rings in linear, cluster or angular arrangements and are classified as per their sources of emissions and molecular weight.[9] PAHs with four or fewer aromatic rings are considered low-molecular-weight (LMW) and often originates from petroleum sources, while those with five or more rings are considered high-molecular-weight (HMW) PAHs which originates from pyrogenic (combustion) sources.[10] They are rapidly released into the environment due to incomplete combustion of natural sources which attributes to PAH background values and accumulation via anthropogenic activities with the emergence of industrialization and progressive technologies contributing to the majority of PAH contamination as they occur naturally in coal, crude oil, and gasoline.

PAHs have a very strong stable index in soil as they are highly hydrophobic, have low volatility and vapor pressure while being thermodynamically stable due to their strong negative resonance energy.[11] Approximately, 90% of PAHs deposit in soil as a final reservoir which creates a path to the food chain. PAHs have been reported to alter grain size, porosity and hydrophilicity of soil resulting in adverse effects on the diversity and population of its habitual microbes required for its replenishment.[12] The effects of PAHs on soil are further exacerbated in combination with the impacts of global warming. Interestingly, reports evidence that PAHs could have played a vital role in the development of biological processes involved in the emergence of life.[13]

Human exposure is often via breathing contaminated air, automobile exhaust, consuming contaminated food, and cigar smoke. The effects of PAHs on human health greatly will differ with the extent of exposure, concentration, toxicity, and way of exposure.[14] Short-term, exposure to high amounts of PAHs mixed pollutants can result in eye irritation, nausea, vomiting, diarrhea, and confusion; while chronic impacts of PAH exposure on living organisms have been reported as carcinogenic, mutagenic, immunotoxic, genotoxic and teratogenic at different levels of development.[14]

Naphthalene can be primarily found in moth repellents, cigarettes, and deodorants. Its main exposure routes are dermal, inhalation and ingestion, commonly through clothing. For humans, primary exposure for phenanthrene is via inhalation of tobacco smoke apart from ingestion and dermal contact.[15]

Several remediation techniques attempt reduction to safe levels via transformation and degradation. Most physical methods such as soil washing and electrokinetic remediation, do not structurally change the compounds but simply transfer PAHs and are time-consuming. Alternatively, chemical methods such as ozone oxidation and photocatalysis predominantly involve redox reactions which could disturb soil quality due to the possibility of harmful secondary compounds apart from being costly and complex.[16] Hence, biological methods that are environment-friendly and efficient are favored.

The study was conducted to identify bacteria capable of degrading naphthalene and phenanthrene, select the best bacteria with effective degradation, and study their degradation percentage in order to investigated the cocktail effect of bacteria to address PAH contamination with PAH-degrading bacterial consortia, isolated in a porous bed medium. The final product will be a compost medium that contains bacteria that can degrade PAHs. With this, the ultimate goal is to utilize eco-friendly method such as bioremediation to address soil pollution by PAH contamination.

## 2. Methodology

*2.1. Sample Collection.* Approximately 50g of soil samples were collected from landfills and

filling stations located in 6 different locations (Table 1) of Sri Lanka, which are considered highly contaminated areas with PAHs, due to regular occurrence of oil spills, leakages, automobile exhaust, and waste disposal and incineration from frequent urban activities.

**Table 1.** Sample collection sites

| Landfills | filling stations |
|---|---|
| Negombo, Gampaha<br>7°6'59.924"N, 79°52'28.989"E | Wallawatta, Colombo<br>6°51'58.4"N 79°51'46.0"E |
| Meethotamulla, Colombo<br>6°56'12.592"N, 79°53'24.781"E | Kurunegala, Kurunegala<br>7°28'58.7"N 80°21'13.0"E |
| Kolonnawa, Colombo<br>6°54'8.375"N, 79°54'7.205"E | Beruwela, Kaluthara<br>6°28'27.3"N 79°59'02.4"E |

*2.2. Environmental Analysis.* Accurately, 4g of each sample was dissolved in 20ml of Hexane and redissolved in acetonitrile followed by nylon filtration. Standard solutions 500, 250, 100 and 50 ppm were used for phenanthrene and naphthalene. They were then analyzed using a High-Performance Liquid Chromatography (HPLC) (Agilent 1100 series Agilent Technologies, Waldbronn, Germany) and vacuum-filtered acetonitrile and distilled water was used as the mobile phase. The obtained results were then compared to the human hazardous levels available for Naphthalene and Phenanthrene.

*2.3. Isolation of Bacteria.* Five grams of soil collected from each location was measured and centrifuged at 13000rpm with saline water and the supernatant was collected and diluted from $10^{-1}$ to $10^{-10}$. $10^{-1}$, $10^{-5}$ and $10^{-10}$ from the dilution series were spread on Nutrient Agar (NA) and incubated for 24 hours at 25℃.

After the incubation period population density for each morphologically different strain identified from the spread plates was calculated using below equation.[17]

$$\text{CFU/ml} = \frac{\text{No.of Colonies}}{\text{Vol.of Sample Used x Dilution Factor}} \text{ x } 1\text{ml}$$

Morphologically different bacterial isolates were quadrant streaked onto NA plates under aseptic conditions and incubated for 24 hours and 25℃. The Gramm's Staining[18] was performed to observe the Gramm's positive and negative bacterial isolates.

*2.4. Primary and Confirmatory Degradation Screening.* In preparation for Plate Assay, the bacterial isolates were starved for 3 days. After which, BBH Agar petri plates supplemented with 25 mg of Naphthalene and Phenanthrene were divided into 25 equal quadrants. The isolates were placed onto each quadrant and incubated for 7 days at room temperature (25℃).[19]

For the spectrophotometric analysis each strain was inoculated in 25 mg of spiked PAHs in 10 ml of distilled water with 2-3 drops of methylene blue (MB). The tubes were incubated for 24 hours at 25℃ and the average absorbance was measured at 609nm following 7 days.[17,20] The percentage degradation of each strain was calculated using the following equation.[17]

$$\text{Degradation Percentage} = \frac{\text{Actual Absorbance} - \text{Sample Absorbance}}{\text{Actual Absorbance}} \text{ x } 100$$

*2.5. Molecular and Biological Identification of the Selected Strains.* In order to extract rDNA, the sub-cultured samples and 1 ml of 0.9% NaCl were centrifuged for 5 minutes at 13000 rpm. To the supernatant collected, 20 µl of TAE buffer was added and placed in ice for 15 minutes. Next, the samples were transferred to a 95℃ water bath for another 15 minutes. After which, it was centrifuged again at 13000rpm for 5 minutes. The supernatant was transferred to new Eppendorf tube with 20 µl of 100% ethanol

and stored at -20$^0$C. PCR amplification (Table 2) was performed to isolate 16s rDNA using Bacterial Universal Primers 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT) in 5'-3' direction.

**Table 1**. PCR reagents and their respective volumes.

| Reagents | Amount (Volume) |
|---|---|
| DNA Template | 2 µl |
| 27F primers | 1 µl |
| 1492R primer | 1 µl |
| GoTaq® Green Master Mix (M7122, Promega, USA) | 12.5 µl |
| Nuclease-Free Water | 8.5 µl |
| Total | 25 µl |

**Table 3.** PCR Procedure; temperature and time duration of each step.

| Step | Temperature | Duration |
|---|---|---|
| **Initial denaturation** | 94 °C | |
| **Denaturation** | 94 °C | 30 seconds |
| **Annealing** | 52 °C | 1 minute |
| **Extension** | 72 °C | 1 minute |
| **Final extension** | 72 °C | |

*2.6. Agarose Gel Electrophoresis.* In the agarose gel electrophoresis process, 1% agarose gel was made by mixing 1g of agarose powder with 100ml of 1x TAE buffer solution. The molecular weight marker of a 1 kb ladder was used. The gel was run at 55 V for 1 hour and the PCR bands were visualized using UV image analyzer.

The PCR products were purified via NGS bead purification prior to being sequenced via Sanger sequencing at GeneLabs, Sri Lanka. The sequences were trimmed between 550 - 680 bp and the best matching strains were identified as the respective species via NCBI BLASTn tool and the sequences were submitted to NCBI-GeneBank to accession numbers were obtained. A phylogenetic tree was constructed using the neighbor-joining method in MEGA 11 software and the pairwise alignment was conducted using EMBOSS watersmith online alignment tool.[20]

*2.7. Statistical Analysis.* Using Statistical Product and Service Solutions (SPSS 29.0 Command Syntax Reference, USA), the average percentages and means of the confirmatory screening results were analyzed via one-way analysis of variance (ANOVA) with Tukey's multiple comparison test.

## 3. Results and Discussion

*3.1. Environmental Analysis (HPLC).* The results obtained by HPLC analysis; the total PAH concentration ranged from 20.24 ppm to 66.37 ppm in landfills (Figure 1) and 22.32 ppm to 43.31 ppm in filling stations (Figure 1). The concentration level of PAHs vary depending on the location and how polluted the sites are. This was further confirmed by a study conducted by Oketola and Oyeleke (2017) that reported LMW PAH between 18.0 - 28.9% from landfills in Nigeria while Nganje *et al.,* (2006), demonstrates, that in soil samples collected from Petroleum Handling Facilities in Calabar (including filling stations), the total PAH concentration ranged from 1.80 mg/kg to 334.34 mg/kg (1 ppm: 1 mg/kg).[21,22] The results observed in this study demonstrate a higher occurrence of naphthalene in landfills (131.12ppm) however, a higher prevalence of phenanthrene was observed in filling stations (129.04ppm).

For humans, the hazardous exposure levels of Phenanthrene have been determined to be between 100 ppm orally and 300 ppm dermally.[23] For naphthalene, it was 250 ppm.[24] HPLC environmental analysis of soil samples

collected from landfills and filling stations determined values (Figure 1) were lower than the above mentioned levels however if pollution continues without effective remediation, they could rise exponentially within a few years.[22]

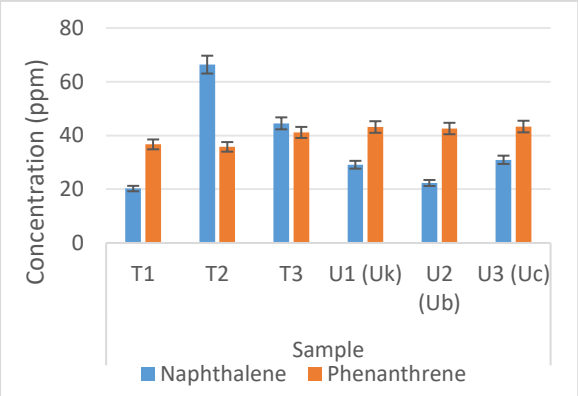


**Figure 1.** Concentration (ppm) of PAHs present in the soil samples collected as per HPLC analysis conducted (T=Landfills, U=Filling Stations)

*3.2. Primary Screening.* A total of 9 bacterial isolates were isolated from landfills and 8 isolates were isolated from filling stations.

**Table 4.** Plate Assay Results obtained for land fills

| Strain | Naphthalene | Phenanthrene |
|---|---|---|
| ST1-1 | 25/25 | 25/25 |
| ST1-2 | 3/25 | 4/25 |
| ST2-3 | 7/25 | 25/25 |
| ST2-4 | 25/25 | 19/25 |
| ST3-5 | 5/25 | 1/25 |
| ST3-6 | 25/25 | 11/25 |
| ST3-7 | 9/25 | 14/25 |
| ST3-8 | 2/25 | 3/25 |
| ST3-8/a | 25/25 | 25/25 |

**Table 5.** Plate Assay Results obtained for Filling Stations

| Strain | Naphthalene | Phenanthrene |
|---|---|---|
| SS2-U2 | 25/25 | 19/25 |
| SS2-U3 | 25/25 | 10/25 |
| SS3-U4 | 25/25 | 24/25 |
| SS3-U5 | 25/25 | 25/25 |
| SS2-U6 | 20/25 | 11/25 |
| SS2-U7 | 25/25 | 25/25 |
| SS2-U8 | 23/25 | 09/25 |
| SS2-U9 | 24/25 | 25/25 |

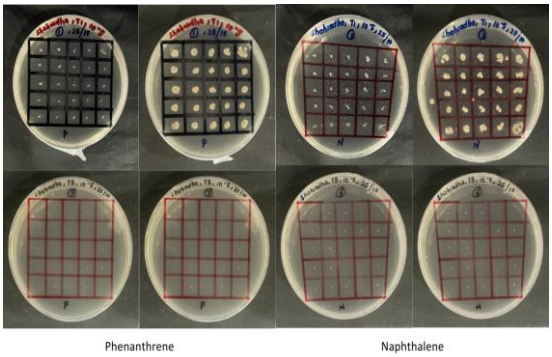


**Figure 2.** Following 3 days of starvation (Left) and following 7 days of incubation (Right)

Transferring individual colony isolates to BBH medium supplemented with PAHs allow bacterial growth by degrading PAHs. Each strain was able to successfully degrade both naphthalene and phenanthrene, but it shows some strains have higher proficiency than others (Table 4 & 5, Figure 2). From the strains isolated from landfills, a total of 127/225 squares on phenanthrene spiked plates and 126/225 squares on naphthalene spiked plates exhibited degradation. Meanwhile, all the strains collected from filling stations exhibited growth on over 20 squares for naphthalene unlike phenanthrene. It can be determined from the primary screening that the bacterial strains isolated from filling stations are better adapted towards PAH degradation and that strains collected from landfills are better adapted

towards degrading phenanthrene while for the strains that belonged to filling stations are better opted towards naphthalene degradation.

To analyze PAH degradation of each isolate, two screening processes were conducted using BBH mediums, due to a lack of carbon source – bacterial growth become inhibited, and enter a state of stress-induced dormancy, which can enhance their ability to degrade PAHs.[25]

In 2022, Dharmasiri *et al.*, reported *Bacillus sp.* and *Bacillus megaterium* isolated from ornamental plants around urban areas of Sri Lanka, that demonstrated growth on 25 squares for phenanthrene.[26] Walton and Buchan in 2023 also reported *Bacillus* strains isolated from contaminated marine environments that developed clear zones indicative of phenanthrene degradation.[27] Additionally, Ibrahim *et al.*, reported *Staphylococcus aureus*, isolated from oil polluted soil in Dammam, Saud Arabia that can degrade PAHs in lysogeny broth (LB) liquid medium.[28]

*3.3. Secondary Screening.* The above results were further elaborated by the spectrophotometry analysis in secondary screening, using the cationic dye MB sensitive to 609nm. The degradation was corroborated by measuring absorbance however, considerable decolorization of MB was also observed in all the tubes. Since the bacterial biodegradation of PAHs are generally initiated by oxidization, MB is reduced to colourless leuco-MB in this process.[29-30]

As per the results demonstrated by Figure 3, from landfills, ST1-1, ST8-8 and ST3-8/a demonstrated high degradation ability above 40%. ST1-2 and ST3-5 demonstrated similar degradation for naphthalene and ST3-6 for phenanthrene. The rest of the strains demonstrated moderate to low degradation below 30%. The statistical analysis generated a p-value of 0.332, which is higher than 0.05. Therefore, it was concluded that there are no significant differences between degradation percentages of both PAHs of interest.

From filling stations, majority of the strains demonstrated high degradation ability above 50%, with SS3-U5 and SS2-U9 demonstrating the highest ability for phenanthrene and naphthalene respectively. Strain SS3-U4 and SS2-U8 demonstrated the lowest degradation at 15% and 33% for phenanthrene and naphthalene respectively. With a p-value of 0.592, which is higher than 0.05 it was concluded that there are no significant differences between degradation percentages of both PAHs of interest.

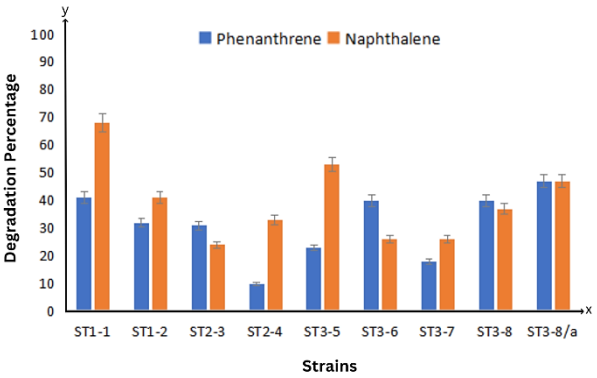


**Figure 3.** Percentage Degradation Rates for Landfills (T) and Filling stations (U) determined via Spectrophotometry

Previously conducted studies substantiate the results reported above. For instance, Sharma *et al.*, (2023) isolated *Providencia rettgeri* VMP5, *Bacillus tropicus* VMP4 and *Bacillus sp.* VMP2 that can degrade phenanthrene at a rate of 98.63%, 89.9% and 82.63% respectively from mined soils.[31] These strains presented biosurfactants and more defence compounds which could assist in their adaptability and degradation potential. Additionally, Dharmasiri *et al.*, (2019) reported isolation of 5 species of *Bacillus* from phyllosphere in Sri Lanka, that can degrade phenanthrene above 20% which were analyzed via UV-Vis spectrophotometry and HPLC.[32] Similarly, Kiamarsi *et al.*, (2018) reported *Bacillus subtilis, Staphylococcus pasteuri* and *Bacillus atrophaeus* isolated from contaminated soils that can degrade PAHs at rates of 55.5%, 39.0% and 49.9% respectively.[33] Chen *et al.*, (2023) identified a phenanthrene degrading consortium that consisted of *P. seudomonas sp., Stenotrophomonas sp., Delftia sp., Pseudomonas sp., Brevundimonas sp., Curtobacterium sp.*, and *Microbacterium sp.*, with an individual capacity of 15%, 12%, 16%, 38%, 13%, 8% and 21%, respectively.[34] They were isolated from long term phenanthrene

exposed soils in China and the degradation was assessed via GC analysis.

*3.4. Molecular Analysis.* Isolates ST3-6, ST3-8 and SS2-U3 were selected for further analysis considering their degradation kinetics.

Following sequencing (Table 6), ST3-6 was identified as *Bacillus anthracis* strain B3 with an accession number of PP340948. Belonging to the *B cereus* group, they are rod-shaped Gram-positive, aerobic, spore-bearing strains that are highly resistant to harsh treatment such as dehydration, elevated temperature, toxic chemicals, and enzymatic digestion.[35] ST3-8 was identified to be *Ureibacillus manganicus* strain SS4R-24 with accession number PP340961. It has not been widely investigated except for its basic details. It is an aerobic gram-positive, rod-shaped spore-forming bacteria that belongs to the *Caryophanaceae* family.[36]

SS2-U3 was identified as *Staphylococcus hominis*, with accession number PP340951. *Staphylococcus hominis* strain SS2-U3 is a coagulase-negative member of the bacterial genus *Staphylococcus*, aerobic, spherical shaped, consisting of Gram-positive, spherical cells in clusters.

**Table 6.** Identification results for selected strains

| Sample | Strain | Accession Number |
|---|---|---|
| ST3-6 | *Bacillus anthracis* strain FS-B3 | PP340961 |
| ST3-8 | *Ureibacillus manganicus* strain SI-SS4R-24 | PP340948 |
| SS2-U3 | *Staphylococcus hominis* strain SS2-U3 | PP340951 |

## 4. Conclusion

The following study was conducted to improve the efficacy of bioremediation as a solution for PAH contamination. This is the first wet laboratory research in Sri Lanka to address PAH contamination. From landfills, Bacillus anthracis strain B3 (PP340948) and *Ureibacillus manganicus* strain SS4R-24 (PP340961) were identified as Phenanthrene (both at 40%) and Naphthalene (26% and 37%) degraders. From filling stations, *Staphylococcus hominis* strain SS2-U3 was identified with a 48.78% of phenanthrene degradation percentage and a 50.68% naphthalene degradation percentage. With a degradation percentage of over 25% these strains, offer an eco-friendly bioremediation solution.

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