IMMOBILIZATION OF PAH DEGRADING SOIL BACTERIA FROM URBAN COASTS, ROADSIDES, AND FARMLANDS: ECO-FRIENDLY BIOREMEDIATION

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Polycyclic aromatic hydrocarbons (PAHs) are organic compounds comprised of carbon and hydrogen molecules. These compounds have two or more linked aromatic rings formed due to natural and anthropogenic environmental sources. Studies have recorded PAH's tendency to cause genotoxic and cancer-causing impacts in recent years. This research focuses on the degradation of these PAHs: Naphthalene and Phenanthrene using soil bacteria for effective bioremediation. The main objective is to identify and isolate bacteria that inhibit the soil in urbanized coasts, roadsides, and farmlands that have the potential to degrade these PAHs and to develop a compost consisting of this bacterial consortium. Strains of bacteria with different morphologies were identified from each location. Moreover, the selected locations were established to be impaired with PAHs using High-Performance Liquid Chromatography (HPLC) analysis. Furthermore, a plate assay was performed to identify the ability of soil bacteria to degrade PAHs, and spectrometric analysis was carried out with methylene blue to determine the PAH degradation percentage of the strains. The molecular identification results disclosed that *Staphylococcus kloosii* strain SP1-01 and *Bacillus cereus* SV3-03 showed the highest growth in both PAHs degradation. In secondary screening *Staphylococcus kloosii strain* SP1-01, *Pseudomonas fulva* strain SP2-02 showed less than 70% degradation for naphthalene whereas *Bacillus velezensis* strain SV2-02, *Bacillus cereus* strain SV3-03 were capable of degrading naphthalene by over 40% and *Pseudomonas fulva* strain Ss2-D6 were able to degrade more than 50%. However, *Staphylococcus kloosii* strain SP1-01 and *Pseudomonas fulva* strain SP2-02 degraded not more than 20%. In contrast, the strains *Bacillus velezensis* strain SV2-02, and *Bacillus cereus* strain SV3-03 degraded less than 30% and the *Pseudomonas fulva* strain Ss2-D6 degraded more than 70% in phenanthrene. Toxicity assays that were performed proved that no hazardous substances were released into the environment. Thus, no harmful effects on the surroundings and the ecosystem. The immobilization of the bacterial consortium in the soil was confirmed by scanning electron microscope imaging (SEM). In conclusion, these selected strains*: Staphylococcus kloosii* strain SP1-01, *Pseudomonas fulva strain* SP2-02, *Bacillus velezensis* strain SV2-02, *Bacillus cereus* strain SV3-03 and *Pseudomonas fulva* strain Ss2-D6 can be possible biological agents to degrade PAHs like phenanthrene and naphthalene.

Keywords: bioremediation, degradation percentage, polycyclic aromatic hydrocarbons, toxicity assay

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INTRODUCTION

Worldwide, one of the major problems is soil pollution. Soil pollution is brought about by the direct or indirect presence of dangerous chemicals or other materials in the soil that can endanger ecosystems and living beings (Singh and Singh, 2020). This major issue arises from two primary sources: natural and anthropogenic (or man-made) causes. The great majority of pollutants originate from anthropogenic origins. Soil contains pollutants that might be dangerous and have negative consequences that present a hidden risk to people as well because we are frequently unable to evaluate and detect them directly. The majority of human activities that pollute soil include garbage disposal, chemicals, construction, industrial processes, and petroleum by-products (Xu Wang Zhu, 2019).

Polycyclic aromatic hydrocarbons (PAHs) are one of the main substances that can contaminate soil. PAHs are broadly dispersed substances with two or more fused benzene rings organized in various configurations. These PAHs are carcinogenic and genotoxic, which means that they can alter genes and cause cancer in both people and animals due to their availability and toxicity (Habe and Omori, 2003). PAHs are made up of carbon and hydrogen atoms joined in linear, cluster, or angular configurations by two or more benzene rings. They lack charge, are non-polar, and have planar structures because they are the result of incomplete combustion of organic materials. Previous research has demonstrated that PAHs are readily maintained in soil because of their strong hydrophobicity and low water solubility (Wen *et al.,* 2021). The two PAH pollutants used in this study are phenanthrene and naphthalene. This research focuses on the bacterial consortium degradation process, which is successful.

Among the PAHs, naphthalene is produced artificially (mothballs) as well as naturally through the burning of biomass and fossil fuels. It is made up of the benzene rings fused together, and it is produced by burning diesel fuel, petrol, tobacco, and the coal-tar sector. If the naphthalene is exposed to humans through ingestion and inhalation, it is linked to both cancer and noncancer health effects. The human body's respiratory, ocular, and circulatory systems are initially affected by modest levels of toxicity (Yost *et al.,* 2021).

Phenanthrene is also categorized as a PAH due to its capacity to form an epoxide in its bay and k regions with its three fused benzene rings. In large quantities, it poses a serious risk to human health and is one of the organic contaminants that need to be removed from soil because long-term exposure can cause respiratory irritation and may provide various carcinogenic hazards to people (Huang *et al.,* 2021).

Bioremediation is recommended because it is more efficient and less expensive than chemical and physical remediation methods. Bacteria that are important in the breakdown of PAHs include ***Pseudomonas aeruginosa, Pseudomonas fluorescens, Mycobacterium spp., Haemophilus spp.,*** and ***Rhodococcus spp.,*** Urban areas with significant levels of pollution are home to bacteria that are highly effective at breaking down harmful substances into less harmful substances.

METHODOLOGY

1. Sample collection and preparation
Soil samples were collected from 9 different locations Island-wide: 3 samples from the coasts (P1: Crow Island, P2: Mount-Lavina, P3: Prithipura), roadsides (Ss1: Colombo, Ss2: Gampaha, Ss3-Kurnegala), farmlands (Vc: Colombo, Vk: Kandy, Vj: Jaffna). Three replicate samples were used from each location. All samples were stored at 2°C-8°C until the serial dilution process. Following this, the samples were washed with saline water.
2. Bacterial isolation
Soil bacteria were cultured using the spread plate method on nutrient agar. The soil sample was diluted up to 10^{-9} dilution factor. Bacterial colonies that were morphologically different were isolated by using the streak plate technique. These isolated colonies were observed through Gram staining and the primary screening was done.
3. Plate assay
The isolated colonies were starved on BBH agar for 3 days and were transferred to Naphthalene and Phenanthrene-supplemented (100 ppm) BBH agar plates which had been divided into 25 squares. Following 7 days of incubation at room temperature, bacteria that degrade PAH were identified by counting the grown colonies out of 25 squares.
4. Spectrophotometric analysis
To evaluate the PAH degradation percentage secondary screening was performed using spectrophotometry analysis with methylene blue (MB). The bacterial colonies from the streak plates were inoculated into Naphthalene and Phenanthrene (100ppm) supplemented BBH broth with 2 drops of MB. During, the research negative controls were also sustained. In the end, the PAH degradation percentage was calculated using the absorbance at 609nm.
5. Immobilization of bacteria into compost
Bacterial strains that were selected were added to 1 mL of distilled water in addition to 100ppm phenanthrene and naphthalene. This composition was transferred to the compost and mixed well which was used to grow 6 *Vigna radiata*. By, maintaining standard conditions the growth of the *Vigna radiata* was observed along with the controls.
6. Toxicity assay
Naphthalene and phenanthrene (100ppm) were supplemented to the BBH broth medium from which 10mL was added into test tubes to which a bacterial colony was inoculated and incubated for 7 days at 37°C. Following, the incubation period the supernatant was obtained and transferred into a petri-plate to which 10 *Artemia salina* were added and the count of viable *Artemia salina* was calculated for each hour following 24 hours.
Vigna radiata was grown in the compost containing the bacterial strains. The growth was measured from shoot to root after 7 days of growth.
7. Scanning electron microscope (SEM)
Bacterial strains which were selected were inoculated into 2mL of distilled water. This mixture was added to 1.5g of the compost and allowed to evaporate. At the same time, the controls were observed at the highest magnification using SEM.

RESULTS AND DISCUSSION

Morphologically different nine bacterial strains were isolated from coasts, roadsides, and farmlands. The spread plate technique was used to obtain morphologically different bacterial strains. The population densities of the bacterial strains which were selected were calculated and shown in Table 1.

Table 1: Population density of selected morphologically different bacterial strains

Bacterial code	Number of Isolated colonies	Dilution factor	Population factor
SV1-01	1	10^{-9}	1×10^{10}
SV2-02	1	10^{-5}	1×10^{6}
SV3-03	9	10^{-9}	9×10^{10}
SV4-04	1	10^{-9}	1×10^{10}
SV5-05	1	10^{-9}	1×10^{10}
SV6-06	1	10^{-5}	1×10^{6}
SV7-07	1	10^{-5}	1×10^{6}
SV8-08	1	10^{-5}	1×10^{6}
SP1-01	4	10^{-9}	4×10^{10}
SP2-02	3	10^{-5}	3×10^{6}
SP2-03	1	10^{-5}	1×10^{6}
SP2-04	1	10^{-5}	1×10^{6}
SP2-05	1	10^{-9}	1×10^{10}
SP3-06	3	10^{-5}	3×10^{6}
SP3-07	26	10^{-5}	26×10^{6}
SP3-08	1	10^{-9}	1×10^{10}
Ss1-D1	1	10^{-5}	1×10^{6}
Ss1-D2	1	10^{-5}	1×10^{6}
Ss1-D3	1	10^{-5}	1×10^{6}
Ss2-D4	21	10^{-5}	21×10^{6}
Ss2-D5	1	10^{-5}	1×10^{6}
Ss2-D6	1	10^{-5}	1×10^{6}
Ss2-D7	1	10^{-9}	1×10^{9}
Ss3-D8	1	10^{-5}	1×10^{6}

Ss3-D9	1	10^{-5}	1×10^{6}

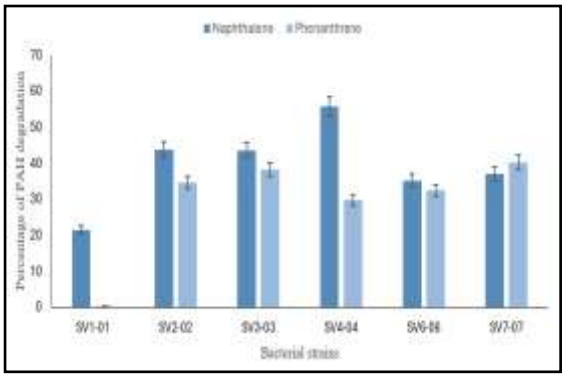
Samples with code SV were collected from farmlands, SP were collected from coasts, and samples with code Ss were collected from roadsides.
As shown in Figure 2 plate assay results show the bacterial growth which was observed to help identify the PAH with the best degrading ability as tabulated in Table 2.
Table 2: Growth of bacterial strains in plate assay

Bacteria	Naphthalene	Phenanthrene
SV1-01	25/25	25/25
SV2-02	07/25	20/25
SV3-03	25/25	25/25
SV4-04	10/25	05/25
SV6-06	06/25	05/25
SV7-07	25/25	25/25
SP1-01	25/25	25/25
SP2-02	25/25	25/25
SP2-04	25/25	08/25
SP2-05	25/25	25/25
SP3-06	0/25	0/25
SP3-07	0/25	0/25
SP3-08	20/25	25/25
Ss1-D2	20/25	15/25
Ss1-D3	5/25	10/25
Ss2-D4	25/25	25/25
Ss2-D5	25/25	15/25
Ss2-D6	25/25	25/25
Ss2-D7	25/25	25/25
Ss3-D8	25/25	25/25
Ss3-D9	25/25	25/25

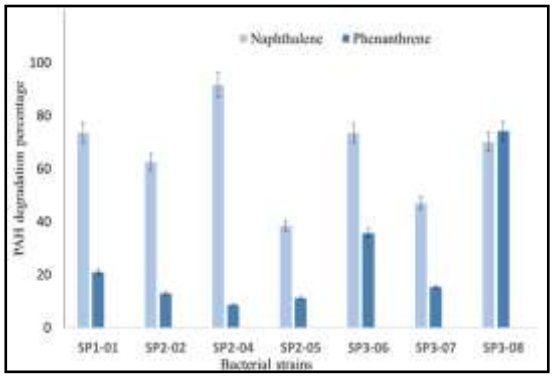
A significant number of anthropocentric processes, such as incomplete fossil fuel burning, waste incarceration, and agricultural emissions, lead to polycyclic aromatic hydrocarbons. According to Patel et al. (2020), soil is classified as extremely contaminated if PAH is more than 1000ng/g and mildly polluted if PAH is less than 200-600 ng/g. Therefore, the soil that was collected around roadsides, farmlands, and coasts was contaminated with PAH posing a risk to the environment. However, from these locations around 24 morphologically different bacterial strains were isolated. During, primary screening it can be observed that most of the strains can degrade the PAHs.The growth of the bacterial strains in BBH agar indicates that they utilize the PAHs as the source of carbon. Secondary screening was carried out using spectrophotometry analysis which uses 609nm for the absorbance of the samples as it standardized for the absorbance of MB. The results show that the values below the negative control revealed the degradation ability of the PAHs used (Kannangara and Undugoda, 2016).

$$PAH\ Degradation\ Percentage = \left[\frac{(Mn - Ms)}{Mn}\right] \times 100$$

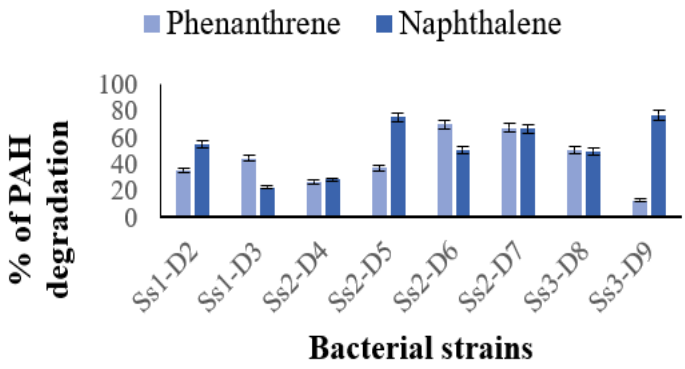
Mn - Absorbance of Negative control
Ms - Absorbance of sample



Graph A



Graph B



Graph C

Graphs: A (Farmlands), B (Coast), and C (Roadsides) display the degradation percentage by isolated bacterial strains which was determined by the spectrophotometer. Following this, the highest degraders of PAHs were recognized and a molecular identification process was carried out

(DNA extraction and PCR), which led to the identification of the species, and accession number was obtained.

Table 3: Identified species and the obtained accession numbers

Sample	Identified species	Accession number
SV2-02	*Bacillus velezensis*	PP340952
SV3-03	*Bacillus cereus*	PP345790
SP1-01	*Staphylococcus klossii*	PP340950
SP2-02	*Pseudomonas fulva*	PP373775
Ss2-D6	*Pseudomonas fulva*	PP346392

A toxicity assay was performed to observe the effects of the bacterial consortium in the environment and whether it poses harmful effects. It was carried out in plants and animals which revealed that these strains don't produce any toxic byproducts.

SEM is used to scan an object by its surface and generates a detailed and highly magnified image. This is carried out by using electrons in a focused beam and is commonly used to delve into the microstructure and chemistry of many materials (Singh, 2015)). Figure 1 shows an identified and magnified image of the compost of the control (A) and (B) compost which captures the adherence of the bacteria to the soil particles as shown in the image. Therefore, it can be concluded that the bacterial consortium does bind to the compost and help in the degradation process as mentioned.

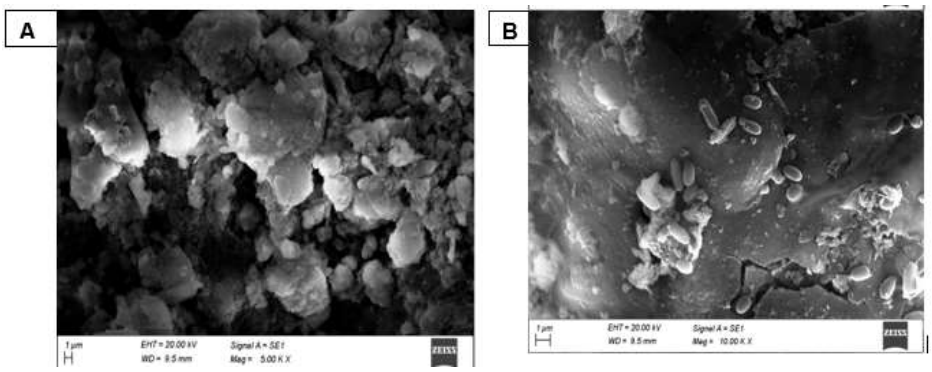


Figure 1: (A) SEM image of control (B) Compost containing bacteria

CONCLUSION

In conclusion, the bacteria isolated from the soil samples from coasts, roadsides, and farmlands: *S. kloosii* strain, *P. fulva* strain, *B. velezensis*, and B. *cereus* strains are potential PAH degraders as they can degrade hydrocarbons without forming harmful substances that can endanger the environment. The high percentage of degradation they reveal makes them suitable for bioremediation of PAH-contaminated environments.

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