Phenanthrene and Naphthalene Biodegradation by Soil Fungi in Agricultural Fields using Mycoremediation Techniques

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Abstract

Mycoremediation is a sustainable and environmentally friendly biotechnological approach used to remediate the environment by eliminating organic contaminants such as polycyclic aromatic hydrocarbons (PAHs), pesticides, and heavy metals. This strategy promotes soil health while improving crop productivity. The goal of this study is to identify the soil fungi that have the ability to degrade PAHs, particularly phenanthrene and naphthalene, and to evaluate the degradation efficacy and associated toxicity of identified fungi for sustainable food production. Soil samples were collected from Galle, Matara, Colombo, and Jaffna, among other urbanised areas of Sri Lanka. To isolate fungal colonies, the collected soil samples were serially diluted and plated using the spread plate method on potato dextrose agar (PDA) medium. The isolated colonies were subjected to primary screening in Bushnell Hass (BBH) medium to assess their degradation capacity. After demonstrating positive activity, colonies were selected to assess their capacity to degrade particular PAHs. Fungal strains that showed efficient degradation were identified at the molecular level. Ten morphologically different strains of fungi were identified in the first step. Nine strains showed better PAH degradation in primary screening. Out of them, the 6 best degraders were chosen, and 4 strains (SM1A, SC1, SJ1A, SJIC) showed an intense band in agarose gel electrophoresis. NCBI GenBank accession numbers were obtained for *Trichoderma virens* (PQ877924), *Talaromyces verruculosus* (PQ878088), *Aspergillus terreus* (PQ877925), *Penicillium citrinum* (PQ878072). The fungus *T. verruculosus* exhibits the highest degradation efficacy for phenanthrene, achieving 88.13%. *Penicillium citrinum* showed the highest degradation ability for naphthalene, achieving 81.84% of reduction. The results concluded that these fungal strains can be used as potential biological agents to degrade PAH pollutants such as phenanthrene and naphthalene.

Keywords: Mycoremediation, naphthalene, phenanthrene, *Penicillium citrinum*

https://doi.org/10.54389/VOYQ4310

Introduction

Soil pollution caused by diverse organic compounds such as PAHs is a worldwide problem that needs immediate attention. Rapid human activities such as urbanization, vehicle emissions, and the overuse of agricultural pesticides are the major causes of environmental pollution (Roy *et al*., 2025). PAHs are a category of persistent organic pollutants characterized by carcinogenic, teratogenic, and mutagenic effects (Luo *et al*., 2024). The Environmental Protection Agency has designated 16 PAHs as priority pollutants. Because of their hydrophobic properties, PAHs are prevalent environmental contaminants of great concern on a global scale and have a tendency to bioaccumulate. Among them, the two-ring naphthalene and the three-ring phenanthrene are frequently found in the environment, which makes them challenging to break down. Because of their varied metabolic capacities, soil fungi specifically found in agricultural fields have recently come to light as prospective candidates for the bioremediation of PAHs. To evaluate the degradation capacity of different fungus, this study uses UV vis spectrophotometric analysis to understand the best degraders of PAHs.

Mycoremediation efficiently and cost-effectively converts complex ring structures of PAHs into oxidized/hydroxylated intermediates through a fungal consortium (Bokade and Bajaj, 2023). The mycelium of the fungi used in mycoremediation adheres to the soil particles to form a filamentous body that can withstand contaminants and adjust its growth to temperature, pH, and nutrient variations. Because of their unique hyphal network, biomass, and long lifecycle, fungi are preferred over bacteria in the bioremediation of polluted settings (Akpasi *et al*., 2023). According to earlier research, adding trace amounts of organic waste materials to soil can improve the microbial breakdown of phenanthrene by acting as a co-substrate or microbial activity booster. Furthermore, it has been demonstrated that applying pre-treated or immobilised organic matter with white-rot fungi increases catabolic efficiency while favourably affecting the physicochemical and biological characteristics of modified soils, which could be helpful in agricultural applications (Omoni and Taghoghor, 2021). Although *Pleurotus ostreatus* exhibits potential in the degradation of PAHs by spectrophotometric analysis, it is yet unknown how laccase gene expression and degradation efficacy are related (Elhusseiny *et al*., 2018).

The primary objective of this study is to determine the PAH degrading soil fungi, evaluate the toxicity of fungi and their by-products, ultimately for improving agricultural soil quality to promote sustainable food production.

Materials and Methodology

Sample collection and fungal isolation

Approximately 50 g of paddy soil was collected from Galle, Matara, Colombo, and Jaffna with a single sample without replicated samples. A 5g soil sample was mixed with 50 mL of 0.9% Saline solution. Serial dilution was carried out by transferring 1 mL from one test tube to the next sequentially up to 10^{-10}. To isolate fungal strains, 100 µL from 10^{-1}, 10^{-4}, and 10^{-8} dilutions were added to PDA plates. The plates were incubated for 72 hours at 30℃. Morphologically different fungal strains were chosen for further experiments (Dharmasiri *et al*., 2022).

Primary screening

Each isolated strain was cultured in separate BBH plates for starvation. The plates were incubated for 3 days at 30℃. To identify the fungus with PAH degrading ability, primary screening was performed. BBH plates were divided into 4 compartments and spiked separately with 100 ppm of phenanthrene and naphthalene using a cotton swab (Dharmasiri *et al*., 2019). Then, 4 colonies from each starved fungal strain

were inoculated into 2 compartments each in one side of the plate separately for both PAHs. The plates were incubated for 3 days at 30℃ (Dharmasiri *et al*., 2023).

Molecular identification

The genomic purification DNA kit from Promega was used to extract DNA from isolated pure fungal colonies. The extracted DNA was amplified using polymerase chain reaction (PCR), using the primer pair, ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′TCCTCCGCTTATTGATATGC-3′) in the presence of Gotaq Green Mater Mix form Promega to further identify the isolated fungi at the molecular level (Dharmasiri *et al*., 2022. Successful amplification of DNA was confirmed by the presence of intense bands observed in AGE. Sanger sequencing was used to obtain the sequences, which were then submitted to the NCBI GenBank and assigned an accession number. ITS sequences of fungi isolates were aligned using MEGA software, and a maximum likelihood phylogenetic tree was constructed using 1000 bootstrap replicates.

Analysis of the degradation of PAHs

Following test tubes sterilization 10 mL of Bushnell Hass broth (BHB) was added to each test tube. For each fungal strain, 16 test tubes with BHB were prepared, 8 supplemented with 100 ppm of naphthalene and eight with 100 ppm of phenanthrene. Then 500 µL of methylene blue was added. Fungal colonies were inoculated for each test tube. The UV vis spectrophotometric analysis was performed after 24 hours' incubation for each test tube sequentially for 8 days. Absorbances were taken as triplicates. Degradation percentage was taken using the formula below (Senevirathna *et al*., 2025).

$$\text{Degradation Percentage} = \frac{(\text{Absorbance of control} - \text{Absorbance of sample})}{\text{Absorbance of control}}$$

Then the time-dependent PAH removal of each strain was calculated using the formula below.

$$N_t = N_0(1/2)^{t/t(half)}$$

Where, N_t = the quantity that remains and has not yet decayed after a time t, N_0 = the initial quantity of the substance that will decay, and t(half) = the time taken for the decaying quantity.

Brine shrimp lethality assay

Test tubes were prepared using 10 mL of BBH media mixed with both 100 ppm of phenanthrene and naphthalene. Each fungal strains were inoculated in the test tubes and then the test tubes were incubated for 8 days. Brine shrimp eggs were soaked on the 6th day in seawater beaker, and it was allowed for hatch for 48 hours with the supply of an aerator. On the 8th day test tubes were centrifuged and the supernatant was transferred into petri plates. Ten hatched brine shrimps were taken from a Pasteur pipette and then transferred to each petri plate. Alive brine shrimp count was taken in the first 4 hours, after 24 hours, and after 48 hours.

Results

Fungal isolation

Morphologically different 10 fungal colonies were isolated (Figure 1).

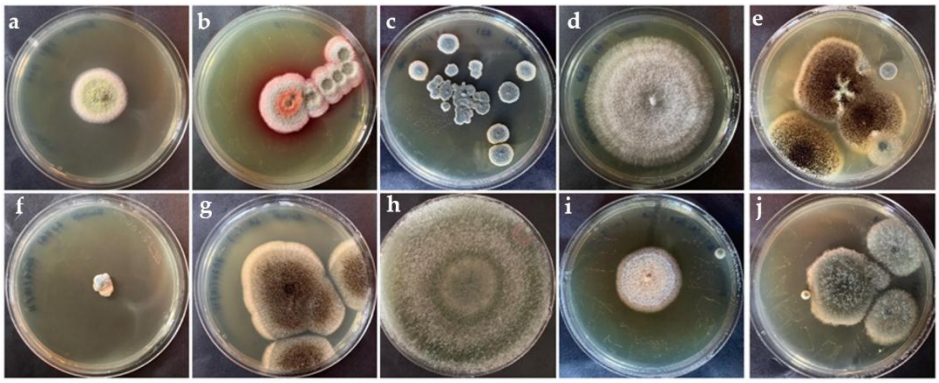

Figure 1: *Morphologically distinct pure fungal cultures isolated from initial spread plate techniques. a) SM1A, b) SM8, c) SJIB, d) SGIB, e) SJB, f) SJIC, g) SG1A, h) SC1, i) SJIA, j) SMIB*

Primary screening

Out of 10 fungal strains tested, 9 exhibited positive growth, indicating their ability to utilize PAHs as the only carbon source, while 1 strain showed no growth (SGIB) in phenanthrene.

Table 1: *Growth response of 10 fungal strains on phenanthrene and naphthalene as sole carbon sources.*

Bacterial strain		**SC1**	**SGIB**	**SMIA**	**SM8**	**SJ1A**	**SG1A**	**SJ1B**	**SJ1C**	**SJ8**	**SMIB**
Number of grown colonies	Phenanthrene	2/2	0/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2
	Naphthalene	2/2	1/2	2/2	1/2	2/2	2/2	1/2	2/2	2/2	2/2

Molecular identification

GenBank accession numbers of the four most efficient PAH-degrading fungal strains (Table 2). Six best strains were selected from secondary screening, and DNA extraction and PCR amplification were performed. Four strains were which showed an intense band in AGE subjected to Sanger sequencing.

Table 2: *NCBI accession numbers*

Code name	Fungal strain	Accession number
SC1	*Trichoderma virens*	PQ877924
SM1A	*Talaromyces verruculosus*	PQ878088
SJ1A	*Aspergillus terreus*	PQ877925
SJ1C	*Penicillium citrinum*	PQ878072

Phylogenetic analysis

Sequences were submitted to NCBI and the accession number was used to infer the evolutionary relationship (Figure 2).

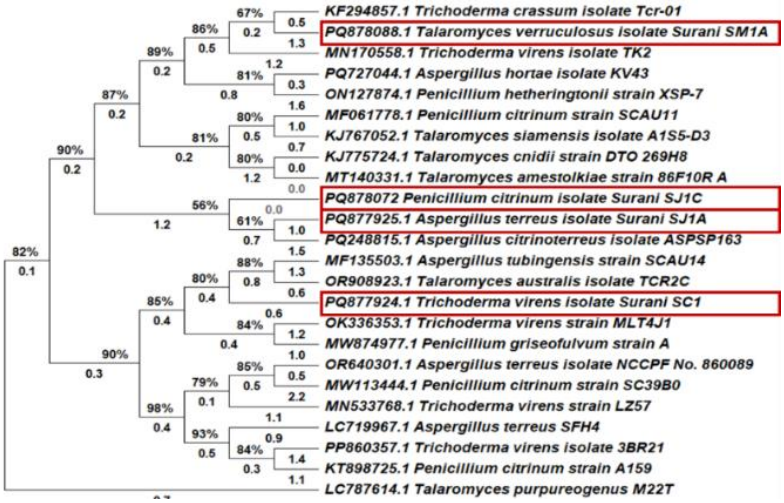

Figure 2: *Phylogenetic tree of the 4 best degraders. Constructed using maximum likelihood in MEGA.*

Degradation analysis

The graph (Figure 3) shows varying degradation rates among the strains, indicating differences in their metabolic efficiency toward PAHs.

Figure 3: *Degradation analysis of naphthalene and phenanthrene degradation by different fungal strains over 8 days*

Estimation of biodegradation half-life

The table indicates the days required by each strain to degrade 50% of the PAHs (Table 3).

Table 3: *Half-life values of PAH degradation by different fungal strains*

Fungal strain	Half life	
	Naphthalene	Phenanthrene
SC1	3.652 $days^{-1}$	3.093 $days^{-1}$
SM1A	3.841 $days^{-1}$	2.601 $days^{-1}$
SJ1A	3.869 $days^{-1}$	3.084 $days^{-1}$
SJ1C	3.250 $days^{-1}$	2.861 $days^{-1}$

Discussion

Paddy soils were chosen due to their exposure to petroleum-based compounds through agricultural practices. By creating anaerobic settings that restrict oxygen availability, rice fields' wet conditions also raise the bioavailability of PAHs. Furthermore, the sampling locations are close to harbours, which are frequent sources of PAH contamination due to shipping, transport, and industrial emissions. In primary screening with 100 ppm of PAHs, the key molecular characteristic of fungal activity is that they usually synthesise a variety of bioactive substances, especially the extracellular enzymes that are involved in the metabolism of PAHs, such as laccase, cytochrome P450 monooxygenase, lignin peroxidase, and dye-decolorizing peroxidases. Therefore, mycoremediation is a sustainable strategy to achieve a healthy and green ecosystem (Gupta *et al*., 2023). The findings of the primary screening showed that 9 fungal strains can grow in PAH supplemented media, using PAH as the sole carbon source (Table 1). From these 6 most efficient PAH degrading fungi selected for molecular identification. Only 4 species were identified up to the species level. After successful submission, to NCBI GenBank accession number was obtained for the

fungal strains (Table 2). The relationship between species was shown via a phylogenetic tree (Figure 2). SJ1A and SJ1C show a close evolutionary association with 4% difference. According to the spectrophotometric analysis (Figure 3), *Penicillium citirinum* (SJ1C) shows the highest degradation rate, which is 81.83% for degrading naphthalene. *Aspergillus terreus* (SJ1A), *Trichoderma virens* (SC1), and *Talaromyces verruculosus* (SM1A) showed degradation rates for naphthalene of 76.15%, 78.1% and 76.39% respectively. The amount of degrading phenanthrene in each strain was increased as time elapsed. The highest rate of degradation is the *Talaromyces verruculosus* (SM1A), which is 88.13%. Other strains *Trichoderma virens* (SC1), *Penicillium citirinum* (SJ1C), and *Aspergillus terreus* (SJ1A) have the rate of 83.34%, 85.60% and 83.44% respectively. The *Talaromyces* is useful for both biotransformation and the removal of pollutants. For instance, *Talaromyces verruculosus* can release secretory enzymes during cellulosic biomass degradation, which requires a more complex enzymatic system for initial oxidation and ring cleavage (Gao *et al*., 2019). Research demonstrated that *Penicillium* sp., can break down a variety of PAHs via catechol 1,2-dioxygenase activity, with metabolites including salicylic acid and catechol attesting to actual biodegradation. Its ability to effectively remove PAHs from soil and improve crop uptake is further supported by its potent naphthalene action, even at low temperatures (Gerginova *et al*., 2023). As for the ANOVA single factor results, the phenanthrene and naphthalene degradation rates were not significantly different, indicating that the degradation rate of all fungal strains is similar to each other. All the brine shrimp survived the first 4 hours, suggesting that the fungal strains do not produce any harmful substances.

Conclusion

This study determines fungal strains which has the ability to degrade naphthalene and phenanthrene, and their degradation percentage. These finding supports the possible efficacy of mycoremediation of phenanthrene and naphthalene. Identified strains, therefore, can be used as biological agents to lower PAH contamination of the soil, improve soil quality to promote plant growth, and lower the risk of exposure to these contaminants. These findings offer several chances for additional study to investigate the application of these degraders to remediate soil effectively.

Acknowledgment

The authors would like to acknowledge the BMS campus, Colombo 06, for financial assistance.

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