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Research Article

Biodegradation of naphthalene, phenanthrene, and pyrene by four indigenous fungal species isolated from petrochemical refinery effluent

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are persistent organic pollutants of global environmental concern due to their toxicity and resistance to degradation. Traditional remediation approaches are often costly and unsustainable. This study investigated the potential of four fungal species isolated from petrochemical refinery effluent in the biodegradation of selected PAHs (naphthalene, phenanthrene, and pyrene). Molecular identification of fungi was conducted by PCR amplification and sequencing of the internal transcribed spacer (ITS) region. The fungal isolates were evaluated for PAH degradation capabilities at concentrations of 100, 150, and 200 mg/L. The residual PAHs were analysed using UV spectroscopy analysis and the percentage of each PAH removal was calculated. The four fungi were identified as *Talaromyces purpureogenus*, T*richoderma harzianum*, *Aspergillus flavus*, and *Fusarium oxysporum*. *Trichoderma harzianum* showed the highest degradation efficiency across all concentrations, followed by *Fusarium oxysporum*, *Aspergillus flavus*, and *Talaromyces purpureogenus*. Degradation ranges were 92.6 ± 0.12% – 99.6 ± 0.06%, for naphthalene, 78.3 ± 0.12% – 99.1 ± 0.06% for phenanthrene, and 39.1 ± 0.06% – 95.3% ± 0.15% for pyrene. The results demonstrate that these fungi, especially *Trichoderma harzianum* and *Fusarium oxysporum* are potentially valuable species for environmentally friendly bioremediation of PAH-contaminated sites. This study underscores the potential application of indigenous fungal species in sustainable remediation of PAH-contaminated environments particularly in developing countries like Nigeria and supports their inclusion in future environmental clean-up strategies.

Keywords: Fungi; Biodegradation; Environmental sustainability; Mycoremediation; Polycyclic aromatic hydrocarbons

Introduction

Polycyclic aromatic hydrocarbons are environmental pollutants of significant importance due to their persistence and toxicity and are widely spread in the environment [1]. While PAHs can naturally occur in the environment, human activities significantly accelerate their presence [2]. In the atmosphere, PAHs attach to dust particles and can be inhaled or deposited onto surfaces like soil and plants [3]. They undergo chemical transformations through photolysis and reactions with atmospheric compounds including hydroxyl radicals, nitrogen oxides, ozone, sulphur dioxide, and peroxyacetyl nitrate [4]. These reactions produce derivatives such as oxygenated, nitrated, and sulphonic acid PAHs, some of which are more toxic and mutagenic than the parent compounds [5]. Consequently, PAHs contribute to air pollution and associated health hazards depending on exposure levels and duration [6]. Soil acts as a major sink for PAHs, accumulating up to 90% of environmental PAHs with longer persistence than in air or plants [7]. PAHs enter soils via sewage sludge application and industrial effluent from petrochemical and agricultural chemical industries, which poses risks to soil organisms, crop safety, and ultimately human health [8]. Lower molecular weight PAHs can leach into groundwater,

increasing the scope of contamination [9]. In aquatic systems, PAHs originate from atmospheric deposition, storm runoff, sewage discharge, and petroleum spills [10]. Their fate in water depends on molecular weight and substrate attachment, with lighter PAHs more prone to evaporation and degradation, while heavier PAHs tend to adsorb onto sediments and organisms, leading to bioaccumulation [11]. These persistent pollutants threaten ecosystem stability and biodiversity, passing toxic effects up the food chain [12].

Human exposure to PAHs occurs mainly via inhalation, ingestion, and dermal contact, often through multiple simultaneous routes [13]. While complex mixtures make attributing specific health effects difficult, chronic exposure to PAHs is linked to eye and skin irritation, respiratory issues, immunosuppression, developmental and reproductive toxicity, and carcinogenesis [14]. The U.S. Environmental Protection Agency has identified sixteen priority PAHs, including naphthalene, phenanthrene, and pyrene, for focused monitoring due to their high toxicity [15]. To address PAH contamination, physical and chemical remediation methods such as electrokinetics, thermal treatment, solvent extraction, and photocatalysis are employed [16]. However, these methods often encounter challenges including substantial expenses, suboptimal effectiveness, and the production of harmful secondary compounds. [17]. In contrast, microorganisms like bacteria, fungi, and algae have the ability to break down PAHs, ultimately converting them into innocuous substances such as carbon dioxide and water providing environmentally friendly and cost-effective alternatives [18].

Among fungi, white-rot fungi have been widely studied for PAH degradation due to their broad enzyme capabilities, including species like *Phanerochaete chrysosporium* and *Pleurotus ostreatus* [19]. However, these are not commonly abundant in polluted habitats, prompting research into other fungal genera. Filamentous ascomycetes, frequently found in industrially polluted sites, are increasingly recognized for their potential in degrading complex PAHs but remain underexplored [20]. Therefore, the current study focuses on evaluating the PAH degradation efficiency of four indigenous fungi, *Talaromyces purpureogenus, Trichoderma harzianum*, *Aspergillus flavus*, and *Fusarium oxysporum* isolated from petrochemical refinery effluent.

The use of filamentous ascomycetes stems from their ability to tolerate various environmental conditions and production of both intracellular and extracellular enzymes that aid in PAH degradation [21]. Intracellular enzymes such as Cytochrome P450 (CYP450) monooxygenase system requires the PAH to first diffuse or be transported into the cell. Once inside, the CYP450 enzyme performs a regio-selective oxygenation (adding a hydroxyl group) as a Phase I detoxification reaction [22]. Extracellular enzymes including Lignin Peroxidase (LiP), Manganese Peroxidase (MnP), and Laccase generate highly reactive radicals that oxidize PAHs outside the cell. This allows them to break down high-molecular-weight (HMW) PAHs without the compound needing to enter the cell first. This process can lead to the complete mineralization of the compound yielding carbon dioxide and water [22].

Materials and Methods

Culture Media andChemicals

Analytical grade PAHs (naphthalene, phenanthrene and pyrene with purity >99.8% were purchased from Sigma-Aldrich Chemicals (Germany). PAH stock solutions were prepared in acetone and sterilized on a 0.22 µm filter. Mineral Salt medium (MSM) was used for the growth of fungal isolates on PAHs [23].

Fungal isolates

The fungi employed in this study were isolated from a petrochemical refinery effluent. They were characterized, and screened for tolerance to naphthalene, phenanthrene, and pyrene at 50 mg/L in a study by [23]. The four PAH-tolerant fungi were identified as *Talaromyces*, *Aspergillus*, *Trichoderma*, and *Fusarium* species based on phenotypic characteristics.

Molecular identification of PAH tolerant fungi

Extraction of genomic DNA

Prior to DNA extraction, fungi were grown on freshly prepared potato dextrose agar and incubated at 25 ^{o}C for 5 days. Fungi were later sub-cultured into a 250 mL Erlenmeyer flask containing 100 mL potato dextrose broth and incubated for another 5 days at 25 ^{o}C on a rotary shaker. The mycelial mass of fungus was separated from the media by filtration using sterile Whatman No. 1 filter paper. The harvested fungal mycelia were crushed using a porcelain mortar and transferred to Eppendorf tubes (1.5 mL). The genomic DNA extraction was carried out using Zymo Research Fungal/Bacterial DNA MiniPrep Kit (Zymo Research, Irvine, CA, USA) following manufacturer's instructions [24].

Amplification of ITS regions

The ITS regions were amplified using primers specific for the internal transcribed spacer region, ITS1 (5′–TCC GTA GGT GAA CCT GCG G–3′) and ITS4 (5′–TCC TCC GCT TAT TGA TAT GC–3′). Amplification of the extracted DNA was performed in a 20 µL reaction mixture consisting of 1 µL gDNA template, 0.2 µL DNA polymerase, 0.5 µL each of forward and reverse primers, 1 µL dNTPs, and sterile double-distilled water to a final volume of 20 µL. DNA Amplification and amplicon purification was performed using the Wizard® SV Gel and PCR Clean-up System kit (Promega) and quantified using the NanoDrop® (Thermo Scientific) [24]. DNA sequencing was performed using the ABI 3500XL Genetic Analyzer sequencing machine. All PCR products were sequenced by the Sanger method according to [25]. The resulting sequences were compared to homologous sequences available in the National Center for Biotechnology Information (NCBI) database.

Biodegradation analysis of PAHs

Enumerated fungal spores (1×10^6 spores/ml) were aseptically introduced into Mineral Salt Medium (MSM) with pH of 6.0 and

0.5% glucose and supplemented with individual PAHs at concentrations of 100, 150, and 200 mg/L. The inoculated media were placed on a rotary shaker at 150 rpm and incubated at ambient temperature (25-28°C) in the dark for 49 days. Uninoculated MSM supplemented with PAHs served as abiotic controls. Experiments were conducted in triplicate. Every 7 days, a 10-mL aliquot was collected from each flask and transferred to a 100-mL conical flask. To extract residual PAHs, 50 mL of analytical grade n-hexane was added to the 10-ml aliquot and agitated. Extraction of residual PAHs was carried out following the method by [23]. The residual PAHs were analyzed using UV spectroscopy and absorbance was taken at 273nm, 274nm and 261nm wavelengths for naphthalene, phenanthrene and pyrene respectively. Replication of UV spectroscopy measurements for PAHs using individual fungi were carried out as described by [26]. For naphthalene and phenanthrene, the limit of detection (LOD) and limit of quantitation (LOQ) for UV-vis spectroscopy were 0.58 ng mL^{-1} and 3.52 ng mL^{-1} respectively [27] while for pyrene LOD (0.010 mg L^{-1}) and LOQ (0.020 mg L^{-1}) were recorded [28]. Residual concentrations were calculated from a prepared standard curve for each PAH using their known concentrations. Percentage of PAH removal (biodegradation efficiency) by each fungus was calculated using the following equation by [29].

$$Biodegradation\ efficiency\ (\%) = \frac{Co - Ce}{Co} \times 100$$

Where C_o represents initial concentration of PAHs (mg/L) in sample and Ce represents final concentration of PAHs in sample (mg/L).

Results and Discussion

The amplified ITS regions of fungal isolates revealed amplicon sizes of 550-600 base pairs (Figure 1). The four fungal isolates were identified as *Talaromyces purpureogenus, Trichoderma harzianum*, *Aspergillus flavus*, and *Fusarium oxysporum* after comparison with reference sequences in GenBank (Table 1). The phylogenetic analysis of these fungi included 16 nucleotide sequences (Figure 2).

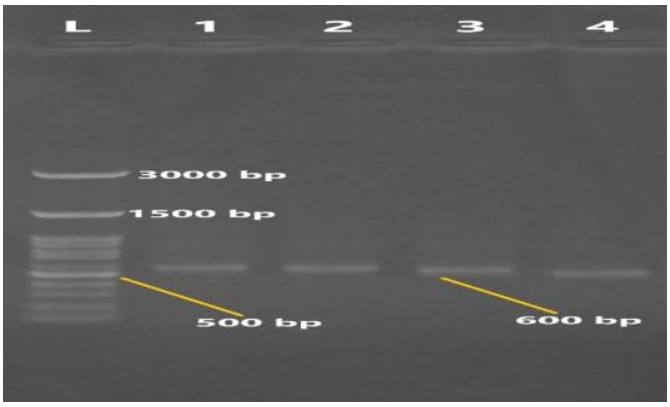

Figure 1. Agarose gel electrophoresis of PCR products of fungal ITS regions

Lane L= 100bp DNA Ladder; Lane 1= Talaromyces purpureogenus (R4); Lane 2=Trichoderma harzianum (R11); Lane 3= Aspergillus flavus (R12); Lane 4= Fusarium oxysporum (R18)

Table 1. Molecular identification of PAH tolerant fungi based on ITS sequences

Isolate code	Percentage identity (%)	Fungi identity	GenBank Accession No.
R4	98.3	*Talaromyces*	MN907642
R11	99.8	*purpureogenus*	MT065755
R12	99.7	*Trichoderma*	OQ248215
R18	99.3	*harzianum*	MN545304
		Aspergillus flavus	
		Fusarium oxysporum	

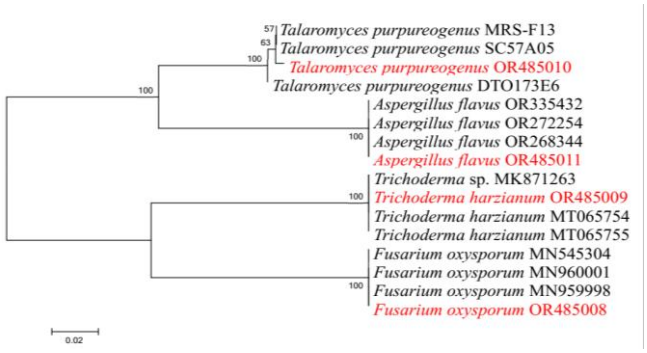

Figure 2. A maximum likelihood tree of ITS gene sequences of PAH-tolerant fungi from petrochemical refinery effluent and other related fungi from the NCBI database.

Note: Numbers at branch nodes represent bootstrap percentages (1,000 replicates); values ⩾50% are shown. The scale bar (0.02) indicates genetic distance (substitutions per site).

The test fungi tolerated the different concentrations of PAHs to varying levels, as indicated by increasing degradation percentages in charts (Figures 3–5). Rapid degradation of PAHs was observed within the first seven days even in the presence of glucose. The rapid decline could be attributed to a combined effect including fungal metabolic versatility, enzyme induction, rapid growth dynamics, substrate utilization preferences, and redox balancing. PAH degradation by microorganisms including fungi, requires the presence of various nutrients such as nitrogen, phosphorus, sulfur, and even additional carbon sources, which are essential for microbial growth and metabolic processes. The presence of glucose in the medium provided an additional source of carbon which may have acted as a stimulus for the growth and activity of fungal species, consequently leading to the rapid utilization of PAHs. [30] reported that the addition of glucose significantly enhanced the degradation of PAHs by fungi, resulting in a rapid decrease in PAH concentrations within seven days.

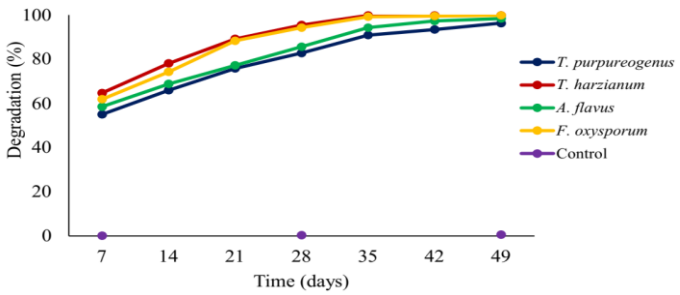

Figure 3a. Naphthalene degradation at 100 mg/L by PAH tolerant fungi

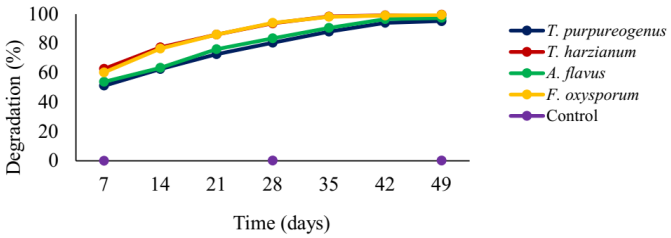

Figure 3b. Naphthalene degradation at 150 mg/L by PAH tolerant fungi

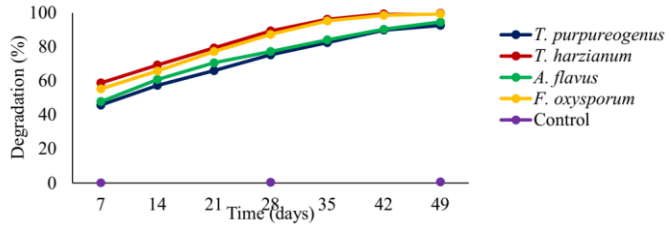

Figure 3c. Naphthalene degradation at 200 mg/L by PAH tolerant fungi

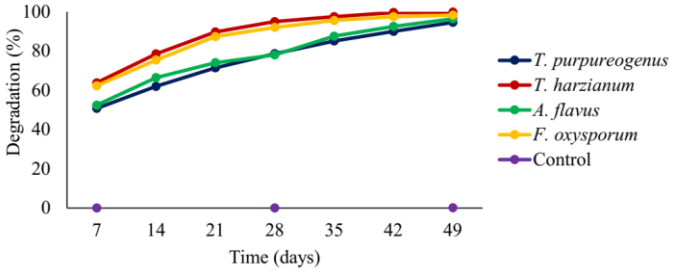

Figure 4a. Phenanthrene degradation at 100 mg/L by PAH tolerant fungi

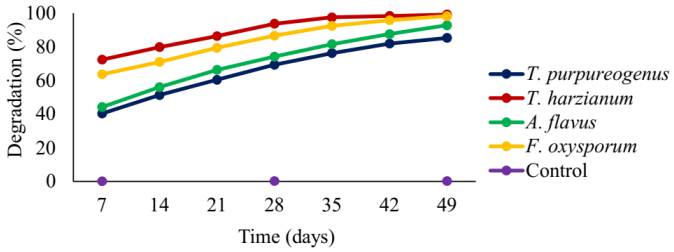

Figure 4b. Phenanthrene degradation at 150 mg/L concentration by PAH tolerant fungi

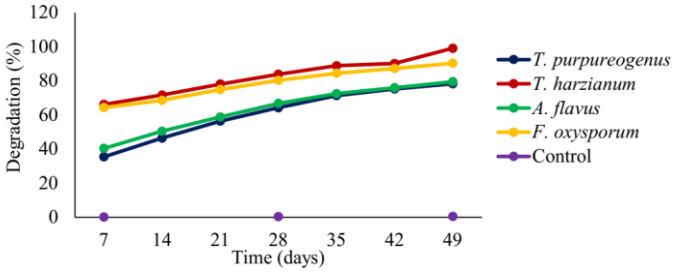

Figure 4c. Phenanthrene degradation at 200 mg/L concentration by PAH tolerant fungi

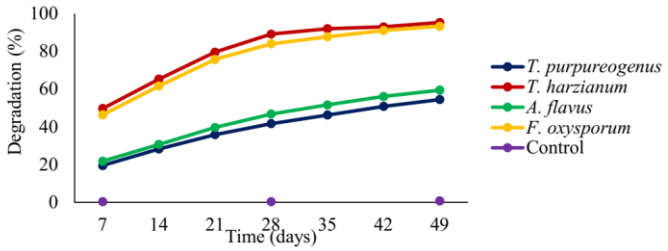

Figure 5a. Pyrene degradation at 100 mg/L concentration by PAH tolerant fungi

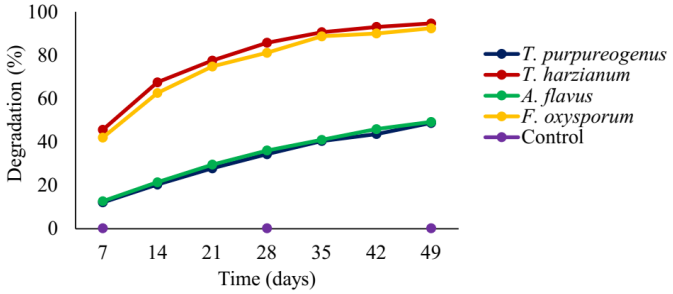

Figure 5b. Pyrene degradation at 150 mg/L concentration by PAH tolerant fungi

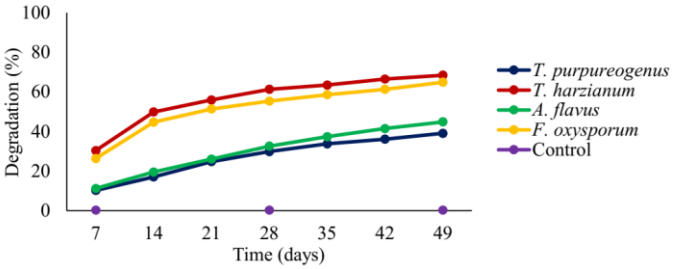

Figure 5c. Pyrene degradation at 200 mg/L concentration by PAH tolerant fungi

Another study by [31] reported that *Fusarium solani* dissipated approximately 30% of 500, 500, and 50 mg/L phenanthrene, pyrene, and dibenz(a,h)anthracene respectively after 28 days when glucose was supplied as a carbon source. Fungal metabolism of glucose has been found to produce biosurfactants, that increase the solubility and accessibility of hydrophobic PAHs, making them more available for fungal uptake and degradation [32].

The rate of PAH degradation towards increasing concentrations of PAHs by the fungi ranged from 92.6 ± 0.12% – 99.6 ± 0.06%, 78.3 ± 0.12% – 99.1 ± 0.06% and 39.1 ± 0.06% – 95.3% ± 0.15% for naphthalene, phenanthrene, and pyrene respectively. PAH degradation efficiencies by the various fungi were found to increase in the following order: *Trichoderma harzianum* > *Fusarium oxysporum* > *Aspergillus flavus* > *Talaromyces purporeogenus*. The degradation rates observed for these isolates varied among different PAHs, with naphthalene being degraded at the highest rate, followed by phenanthrene, and then pyrene. This trend is consistent with the general principle that low-molecular-weight (LMW) PAHs are typically more easily degraded or metabolized by a broader range of fungi compared to high-molecular-weight (HMW) PAHs [13]. The

molecular weight and size of HMW PAHs make the molecules less accessible to microbial enzymes compared to LMW PAHs that are more soluble in water increasing their bioavailability to microorganisms [11].

The capacity of fungal species to tolerate varying concentrations of PAHs is intricately linked to their ability to produce extracellular enzymes including laccases and peroxidases [33]. By oxidizing and solubilizing PAHs, these enzymes transform them into intermediates that can be further degraded intracellularly [34]. Fungal species often induce the production of specific extracellular enzymes in response to the presence of PAHs. These compounds act as inducers, triggering the expression of genes encoding degradative enzymes [35]. Oxidative enzymes such as laccases catalyze the oxidation of phenolic and non-phenolic substrates, leading to the breakdown of complex PAH structures [36]. Peroxidases including lignin, manganese, and versatile peroxidase use hydrogen peroxide to oxidize PAHs, making them more accessible for further degradation [19]. Extracellular enzymes such as dioxygenases and monooxygenases act by incorporating oxygen into the PAH molecules, leading to ring cleavage and the formation of less complex, more water-soluble compounds that are easier for the fungi to metabolize [37]. Fungi are known for their ability to generate a diverse array of metabolites during PAH degradation that can further enhance the degradation of PAHs in polluted environments [38].

Various studies have reported the implications of these fungi in PAH degradation. [19] reported that *Aspergillus glaucus* degraded 66% and 44% of 300 mg/L initial concentration of naphthalene and phenanthrene respectively after 15 days. [39] found that *Talaromyces spectabilis* degraded (23 and 21%), *Fusarium* sp. (25 and 19%) and *A*spergillus *terreus* (25 and 18%) of phenanthrene and pyrene respectively after 10 days. [40] found that *Aspergillus terreus* degraded about 98.5% of 150 ppm naphthalene concentration after four weeks in soil. [41] reported that *Trichoderma* sp. degraded 74% out of 100 mg/L of phenanthrene in the presence of glucose in the degradation medium after 30 days incubation. [42] reported that phyllosphere fungi T*richoderma harzianum* P4M-16 and *Fusarium solani* P11M-46 efficiently degraded naphthalene, phenanthrene and pyrene. [43] reported a decrease in phenanthrene half-life from 24.64 days in control soil to 4.61–6.37 days following inoculation with a consortium containing *Aspergillus sydowii* which represented an approximately 4- to 5-fold reduction in persistence. The differences in variation observed in PAH degradation ability values in this study compared to others could be attributed to differences in environmental conditions, incubation period, initial PAH concentration, and species of fungi used.

Comparing the degradation efficiency of the four fungi it was evident that *Trichoderma harzianum*, followed by *Fusarium oxysporum*, displayed the best degradation capacity towards higher concentrations of PAHs. This could be attributed to various factors including rapid growth rate, wide metabolic versatility and adaptability to different environmental conditions [41].

The mean values recorded for abiotic control samples ranged from 0.01 to 0.7% on days 7 and 49, respectively. The decreased concentration values for control samples observed could be due to abiotic dissipation such as photolysis and volatilization. These processes are influenced by various environmental factors, including light intensity, temperature, vapor pressure, and surface area [44].

Conclusion

This study confirms that indigenous fungi isolated from petrochemical refinery effluent; *Talaromyces purpureogenus*, *Trichoderma harzianum*, *Aspergillus flavus*, and *Fusarium oxysporum* are effective degraders of naphthalene, phenanthrene, and pyrene at concentrations ranging from 100–200 mg/L. *Trichoderma harzianum* exhibited the highest degradation efficiency, followed closely by *Fusarium oxysporum*, *Aspergillus flavus*, and *Talaromyces purpureogenus*. Degradation efficiency decreased with increasing PAH molecular weight and concentration, yet all four species demonstrated substantial PAH removal capabilities. These findings support the application of these ascomycete fungi, particularly *T. harzianum* and *F. oxysporum*, as cost-effective and environmentally sustainable bioresources for the bioremediation of PAH-contaminated sites, especially in developing nations where affordable remediation strategies are urgently needed.

Author Contributions

LEOA: Conceptualization, Methodology, Investigation, Formal Analysis; Data Curation, Writing – Original Draft, Review & Editing. DAM: Conceptualization; Methodology; Formal Analysis, Visualization, Validation; Writing – Review & Editing. ODA: Visualization, Validation, Writing – Review & Editing. HA: Visualization, Validation, Writing –Review & Editing. All authors read and approved the final version of the manuscript and its submission and have given consent for publication.

Declaration of Generative AI and AI-Assisted Technologies in Scientific Writing

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Data Availability Statement

All relevant data are available within the manuscript

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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