VOL. 12 NO. 9,
MAY, 2026
E-ISSN 3026-958X
P-ISSN 3027-1169

African Scholar Publications & Research International
www.africanscholarpub.com

Journal of Science Innovation & Technology Research (JSITR)

Biodegradation of Low and High Molecular-Weight Polycyclic Aromatic Hydrocarbons by Lignolytic Enzyme Producing *Trichoderma harzianum*-OR485009

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DOI: https://doi.org/10.70382/ajsitr.v12i9.073

Abstract

Polycyclic aromatic hydrocarbons (PAHs) are toxic, persistent organic pollutants with significant ecological and health risks due to their widespread distribution and resistance to natural degradation. This study evaluates the potential of *Trichoderma harzianum*-OR485009, isolated from petrochemical refinery effluent, to degrade representative PAHs (naphthalene, phenanthrene, and pyrene) individually and in combination, while also analyzing associated enzyme activity and degradation intermediates. Biodegradation experiments were conducted over a 25-day period using standard microbiological and analytical protocols. The fungal strain was exposed to 150 mg/L of each PAH, both singly and in mixture. Degradation efficiency was monitored using chromatographic analysis. Enzymatic assays were performed to evaluate the activity of lignolytic enzymes (laccase, manganese peroxidase, lignin peroxidase), and GC-MS was employed to identify intermediate degradation products. *Trichoderma harzianum*-OR485009 completely degraded naphthalene and phenanthrene (100%), and achieved 95.5% degradation of pyrene. Degradation efficiency declined with increasing PAH ring complexity. In mixed-PAH treatments, higher degradation rates were observed for low molecular weight compounds. Laccase activity was highest among the enzymes assayed and also decreased as the number of aromatic rings increased. GC-MS analysis revealed key degradation intermediates, including butenoic acid, oxalic acid, diisooctyl phthalate, and eicosane.

The results confirm that *T. harzianum*-OR485009 is capable of efficiently degrading both individual and mixed PAHs, particularly low molecular weight compounds, through mechanisms involving lignolytic enzyme activity. The detection of specific metabolic intermediates indicates active enzymatic transformation pathways. This study proves the suitability of *T. harzianum*-OR485009 as a viable agent for mycoremediation of PAH-contaminated environments. Its efficiency, particularly in degrading persistent pollutants and producing environmentally benign metabolites, supports its inclusion in eco-friendly bioremediation strategies.

Keywords: Fungi, Polycyclic Aromatic Hydrocarbons, Mycoremediation, Biodegradation, Lignolytic Enzymes, Environmental Sustainability.

Introduction

Polycyclic aromatic hydrocarbons (PAHs), a group of organic pollutants with increasing global interest in the past few decades due to their inherent properties (Patel *et al.,* 2020; Zhang *et al.,* 2023). Human activities account for the considerable high amounts of these environmental pollutants continuously released into the environment (Mojiri *et al.,* 2019; Srivastava *et al.,* 2019). Polycyclic aromatic hydrocarbons are classified into two groups based on the number of aromatic rings in their structure: low molecular weight (LMW) and high molecular weight (HMW). Naphthalene, phenanthrene and pyrene have been listed by the European Union (EU) and the United State Environmental Protection Agency (USEPA) as priority pollutants in ecosystems due to their widespread, persistent and potential human and ecological health effect (Ameh *et al.,* 2023). HMW-PAHs have shown stronger hydrophobicity and more recalcitrant to degradation compared to LMW-PAHs (Hesham *et al.,* 2017).

Among the PAH remediation strategies including physical, chemical, biological, bioremediation (biological), has proven to be an environmentally sustainable method for the effective remediation of PAHs. The use of living systems including fungi, algae, and bacteria, remain an eco-friendly and cost-effective approach in the conversion of contaminants into non-toxic products compared to physical and chemical methods (Yin *et al.,* 2019; Medaura *et al.,* 2021). The ability of fungi to thrive in harsh environmental conditions such as PAHs polluted environments where they tolerate high levels of these persistent pollutants, makes them effective tools in bioremediation (Akpasi *et al.,* 2023).

Filamentous fungi, especially the ascomycetes, produce extracellular lignolytic enzymes including laccase (Lac), manganese peroxidase (MnP), and lignin peroxidase (LiP) that exhibit broad substrate specificity and can co-metabolize a wide range of recalcitrant aromatic pollutants (Mahmud *et al.,* 2022). The non-specific oxidative action of these enzymes enables the breakdown of both LMW and HMW PAHs, often surpassing the capabilities of bacterial

systems that primarily target smaller aromatic compounds (Zafra and Cortés-Espinos, 2015). LMW PAHs can increase the overall metabolic activity of microbial communities, leading to enhanced degradation of co-contaminating HMW PAHs through co-metabolism. The addition of availability enhancers such as cyclodextrins and biosurfactants can further increase the bioavailability of HMW PAHs, making them accessible to induced enzyme systems (Madrid *et al.,* 2022). Additionally, the presence of LMW PAHs can induce or upregulate enzyme systems that have broad substrate specificities. Research has demonstrated that LMW PAHs are not merely easier targets for degradation but actively participate in "pleiotropic" enzyme regulation where a single enzyme system responds to LMW PAHs and subsequently enables HMW PAH breakdown (Kweon *et al.,* 2014). A mutant strain defective in the pdoA2 gene (encoding an RHO) lost the ability to degrade HMW PAHs, confirming that the enzyme system induced by LMW PAHs is essential for HMW PAH metabolism (Jiménez, 2021).

Among diverse fungal species, *Trichoderma* species have gained considerable attention not only as biocontrol agents but also as proficient degraders of xenobiotics. Unlike the white rot fungi (*Phanerochaete chrysosporium*, *Pleurotus ostreatus*, and *Crinipellis stipitaria*) previously employed in PAH degradation, *Trichoderma harzianum* is of greater ecological relevance in polluted environments, has faster growth, and higher tolerance to environmental stresses. Additionally, it produces a broader range of degrading enzymes, excels in rhizosphere colonization, and benefits from established commercial production methods, making it more practical and adaptable for bioremediation applications.

However, while *Trichoderma harzianum* has been reported for pesticide and dye degradation, its potential for simultaneous degradation of LMW and HMW PAHs particularly in relation to specific lignolytic enzymes remains underexplored. Additionally, fungi from petrochemical refinery effluent offers an alternative means of obtaining fungal strains specifically adapted to hydrocarbon conditions since such effluent frequently contains these pollutants.

Therefore, this study was designed to evaluate the capacity of *Trichoderma harzianum*-OR485009, a strain isolated from petrochemical refinery effluent, to degrade both LMW (naphthalene and phenanthrene) and HMW (pyrene) PAHs under controlled liquid culture conditions. The findings are expected to illuminate the mechanistic basis of PAH mycoremediation by *T. harzianum* and support its application in developing sustainable, enzyme-driven bioremediation strategies for mixed PAH-contaminated environments, particularly in industrializing nations.

MATERIALS AND METHODS

Culture Media and Chemicals

PAHs (naphthalene (99.0%), phenanthrene, and pyrene (98.0%)) of analytical grade standards were purchased from Sigma-Aldrich Chemicals (Germany). All other chemicals used, such as n-hexane (HPLC grade), acetone, guaiacol, and Azure B, were of analytical grade and were also purchased from Sigma-Aldrich Chemicals (Germany). Potato dextrose agar (PDA) (g/L):

4g of potato extract, 20g of dextrose, and 15g of agar-agar (Oxoid Ltd., Basinstoke, United Kingdom). Sterilization process was carried out at 121°C at 15 psi for 15 minutes.

Fungus and Preparation of Inoculum

Trichoderma harzianum-OR485009 was isolated from a refining and petrochemical effluent. The inoculum was prepared by growing the fungus on freshly prepared PDA plates incubated at 25 ºC for approximately 7 days. Fungal spore suspension was prepared by flooding the PDA plates with sterile distilled water containing 0.05% Tween 80. Following the addition of the solution, spores were gently scraped from the surface of the newly formed mycelia using a sterile wire loop. Fragments of mycelia were removed from the spore suspension by filtration through a sterile cheese cloth. To determine the concentration of spores in solution, spores were enumerated using a Neubauer hemocytometer with the help of an optical microscope. One (1) mL of spore suspension containing approximately 1×10^6 spores/mL was used as inoculum for PAH degradation (Gopalakrishnan *et al.,* 2022).

Screening of Fungi for Lignolytic Enzyme

Production of lignolytic enzymes (laccase, manganese peroxidase, and lignin peroxidase) was qualitatively assessed using a plate assay. Production of laccase was based on the oxidation of guaiacol, while production of peroxidases was based on the oxidation of Azure B. Several fungi with the ability to secrete lignin peroxidase also have the ability to decolorize Azure B. Laccase screening was done using freshly prepared PDA plates supplemented with 0.01% guaiacol, while manganese and lignin peroxidase were screened using freshly prepared PDA plates supplemented with 0.01% w/v Azure B. The PDA plates were spiked with 50 mg/L each of naphthalene, phenanthrene, and pyrene following the method described by Sharma et al. (2017). PDA supplemented with 0.01% guaiacol and PDA supplemented with 0.01% w/v Azure B without PAHs were also prepared. The medium was autoclaved, transferred aseptically to petri dishes, and allowed to cool to room temperature. This was followed by inoculation with 6 mm of mycelial, taken from the periphery of a 7-day-old culture of the fungus. The plates were incubated at 30 ºC for 7-14 days. Cultures were examined for the development of reddish-brown color halo zones around laccase-producing *T. harzianum*-OR485009 and clearance of blue color in manganese peroxidase and lignin peroxidase-producing *T. harzianum*-OR485009.

PAH Degradation

PAH degradation was carried out in mineral salt medium (MSM) with the following composition (g/L distilled water): 1.0 $K_2HPO_4.3H_2O$, 0.4 KH_2PO_4, 0.5 NaCl, 0.1 $(NH_4)_2SO_4$, 0.2 $NaNO_3$, and 0.025 $MgSO_4.7H_2O$, 3.80 g/L glucose, and pH 7 in 250 mL Erlenmeyer flasks. The flasks were supplemented separately with 150 mg/L of each PAH: naphthalene, phenanthrene, and pyrene, and in combination: naphthalene + phenanthrene; naphthalene + pyrene; phenanthrene + pyrene; naphthalene + phenanthrene + pyrene. Fungal inoculum

(1×10^6 spores/mL) prepared from the pure culture of *T. harzianum*-OR485009 was aseptically inoculated into each flask. Flasks were incubated at 30 °C on a rotary shaker at 150 rpm in the dark for a period of 25 days. Un-inoculated MSM broth, amended with individual PAH separately, served as abiotic controls. Experiments were carried out in triplicate. Residual concentrations of PAHs were determined at intervals of 5 days until day 25.

Extraction and Quantification of Residual PAHs

Every 5 days, a 20-mL aliquot was collected from each flask and transferred to a 100-mL conical flask containing 50 mL of n-hexane. The content was transferred into a separatory funnel and shaken vigorously with periodic venting to release excess pressure at intervals. The sample was then allowed to stand until two distinct layers were formed in the flask. The organic layer of the sample was decanted into a 250-mL amber bottle and passed through a column loaded with anhydrous sodium sulfate and silica gel to remove traces of water and impurities. The aqueous phase was re-extracted three times using 50 mL of n-hexane. The extracts were combined and concentrated by evaporation at room temperature overnight in a fume cupboard by covering with perforated aluminum foil. The extracts were further concentrated to 1 mL using a rotary evaporator under a gentle stream of pure nitrogen and then transferred into a well-labeled vial and stored at 4°C (Olayinka *et al.,* 2018) prior to high-performance liquid chromatography (HPLC) analysis. The analysis of residual PAHs was carried out using C18 column HPLC. The mobile phase consisted of water and acetonitrile (1:1) at a flow rate of 1 mL/min. All the selected PAH compounds were detected by UV absorbance at 254 nm (Moghimi *et al.,* 2017; Agrawal *et al.,* 2018). The percentage of PAH removal by *Trichoderma harzianum*-OR485009 was calculated as

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

Where C_o represents initial concentration of PAHs (mg/L) in sample and Ce represents final concentration of PAHs in sample (mg/L) (Agrawal *et al.,* 2018).

Lignolytic Enzyme Activity Assay

One mL (1mL) of culture samples was centrifuged for 5 minutes at 8000 rpm, and the enzyme activity was determined directly in the supernatant. The enzymatic activities in all treatments were monitored using a Shimadzu UV-160 spectrophotometer. The enzyme activity was determined in triplicate, and all activities were expressed as units per liter of enzyme, where 1 unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmol product per minute under the experimental conditions (Umar *et al.,* 2022). Sweet potato infusion broth inoculated with *T. harzianum*-OR485009 without PAH was used as a control sample. Throughout the test, sterile distilled water was used as a blank.

Laccase activity

Laccase activity was measured by observing the oxidation of guaiacol in a reaction mixture containing sodium acetate buffer (3 mL of 10 mM (pH 5.0), 1 mL of 2 mM guaiacol), and 1

mL of enzyme source (culture supernatant). The mixture was incubated at 30°C for 15 min, and the absorbance was read at 450 nm following a study by Sharma et al. (2017).

Manganese Peroxidase Activity

Manganese peroxidase (MnP, E.C. 1.11.1.13) activity was determined by monitoring the reaction of guaiacol with manganese ions as described in a study by Sharma et al. (2017). The reaction mixture (2 mL) contained 0.5 mL of 10 mM citrate buffer (pH 5.5), 0.4 mL of 1 mM guaiacol, 0.5 mL of 1 mM $MnSO_4$, and an aliquot of culture supernatant, bringing the reaction mixture volume to 2 mL. The reaction was initiated by the addition of 0.2 mL of 50 M H_2O_2. The mixture was incubated at 30°C for 10 min, and the increase in absorbance due to the oxidation of guaiacol was measured at 460 nm.

Lignin Peroxidase Activity

Lignin peroxidase (LIP, E.C. 1.11.1.14) was determined by monitoring the oxidation of azure B in the presence of sodium acetate buffer and hydrogen peroxide. The reaction mixture contained 1 mL of 50 mM sodium tartrate buffer (pH 4.5), 500 µl of 400 mM hydrogen peroxide, 500 µl of 32 µM Azure B, and 500 µl of enzyme source (culture supernatant). The reaction was initiated by adding H_2O_2. The mixture was incubated at 30°C for 15 min, and the absorbance was read at 651 nm. One unit of enzyme activity was expressed as a decrease in absorbance per minute per liter (Jovic *et al.,* 2018).

Identification of Intermediate Products of PAHs Degradation

The GC-MS technique was applied for the detection of intermediate products formed during individual PAH degradation on day 10 and 25. The culture medium was extracted in an Ultra-Turrax homogenizer using 60 mL of n-hexane as the extractor agent. After two extractions, organic phases were pooled and dried in vacuum using a rotary evaporator in a water bath at 30 ºC. For each culture time, five replicas were grouped in order to obtain a higher concentration of the formed products. The final residue was dissolved in 2 mL of ethyl acetate, and 1 µL was injected into the GC-MS system. Sample components were separated on a HP-5MS capillary column (30 m × 0.25 mm × 0.25 µm) following a temperature program: from 60 ºC to 280 ºC, up 5 ºC per minute, kept at 280 ºC for 26 min, and from 280 ºC to 310 ºC, up 5 ºC per minute, and held at 310 ºC for 5 min. The injector temperature was 250 ºC (splitless injection), and the interface temperature was 300 ºC. Samples were analyzed in full scan mode in the 40–600 *m*/*z* range. Peaks were identified by comparing the mass spectrum and the data available in the library software NIST MS SEARCH version 2.0 and independently through fragmentation pattern interpretation (Vieira *et al.,* 2018).

RESULTS

Qualitative screening for lignolytic enzymes (lac, MnP, and LiP) using plate culture assays revealed that *T. harzianum*-OR485009 was positive for the three enzymes both in the presence

and absence of PAHs in the medium (Plate 1). Laccase-positive culture plates revealed a reddish-brown halo zone due to the oxidation of guaiacol. Manganese and lignin peroxidase-positive culture plates revealed a greenish color due to the oxidation of Azure B by manganese peroxidase and lignin peroxidase-producing *T. harzianum*-OR485009.

The degradation of PAHs and their mixtures by the fungus revealed a gradual increase in degradation efficiency as process progresses from day 5 to day 25. However, the rate of PAH degradation decreased with an increase in the number of aromatic rings. The fungus achieved 100% degradation of naphthalene and phenanthrene compared to 95.5% pyrene degradation at day 25 (Figures 1, 2, and 3). Abiotic control samples revealed values ranging from 0.01-0.1% for PAH degradation during the 25 days which could be due to volatilization and photodegradation.

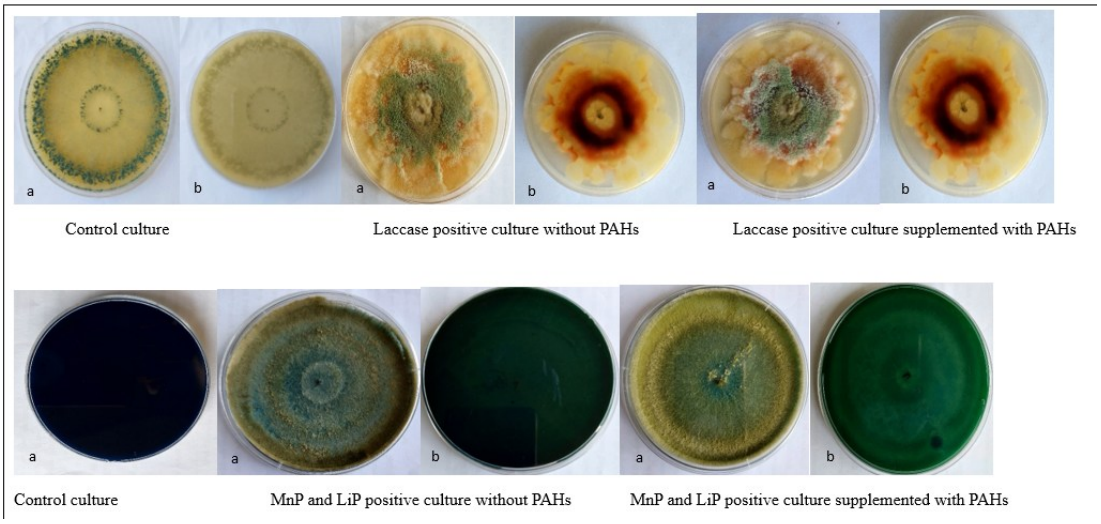


Plate 1: *Trichoderma harzianum*-OR485009 culture on PDA showing (a) surface and (b) reverse cultural morphology of ligninolytic enzyme production

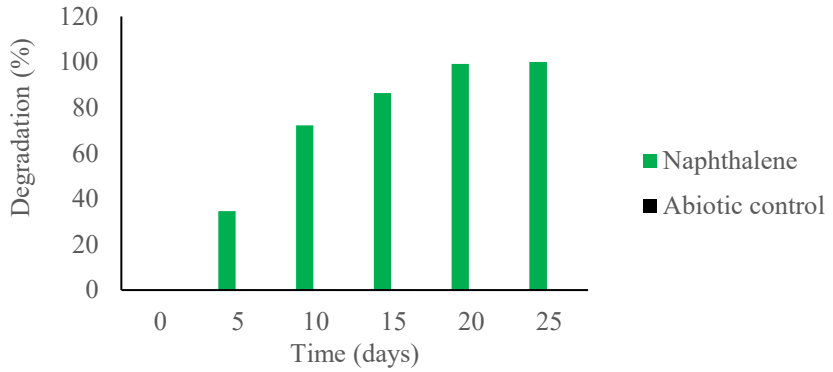


Figure 1: Degradation of naphthalene by *Trichoderma harzianum*-OR485009

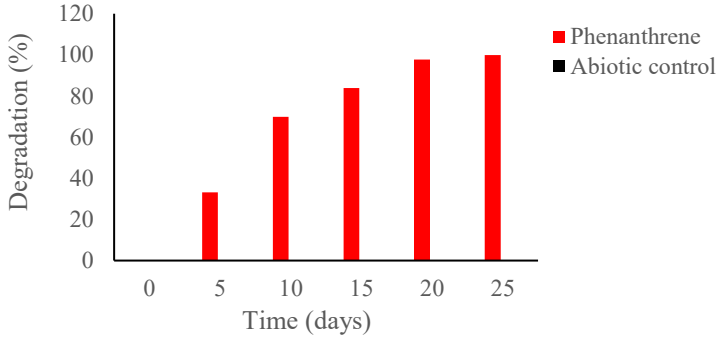


Figure 2: Degradation of phenanthrene by *Trichoderma harzianum* OR485009

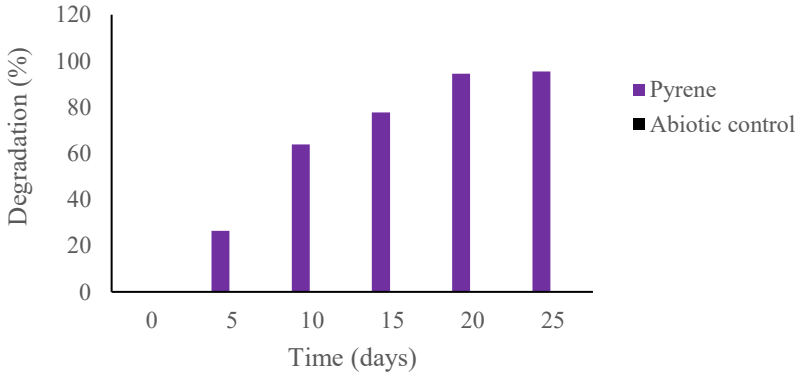


Figure 3: Degradation of pyrene by *Trichoderma harzianum*-OR485009

Moreso, degradation of the PAH mixture by the fungus showed 98.1/84.4% naphthalene/phenanthrene, 95.0/71.6% naphthalene/pyrene, 87.2/64.0% phenanthrene/pyrene, and 84.3/77.5/64.4% naphthalene/phenanthrene/pyrene, respectively, at day 25 (Figures 4, 5, 6, and 7).

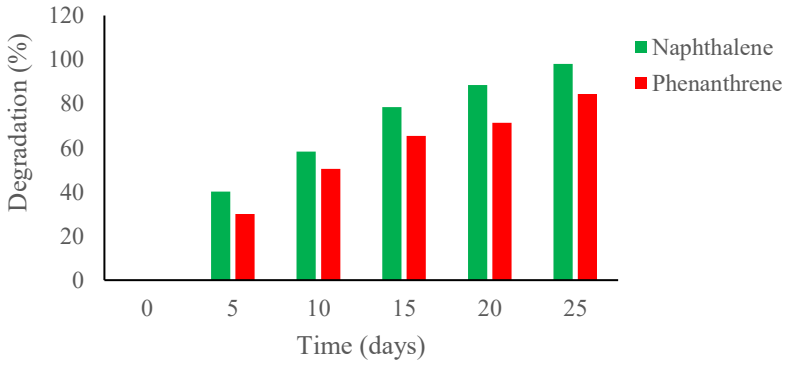


Figure 4: Degradation of naphthalene and phenanthrene by *Trichoderma harzianum*-OR485009

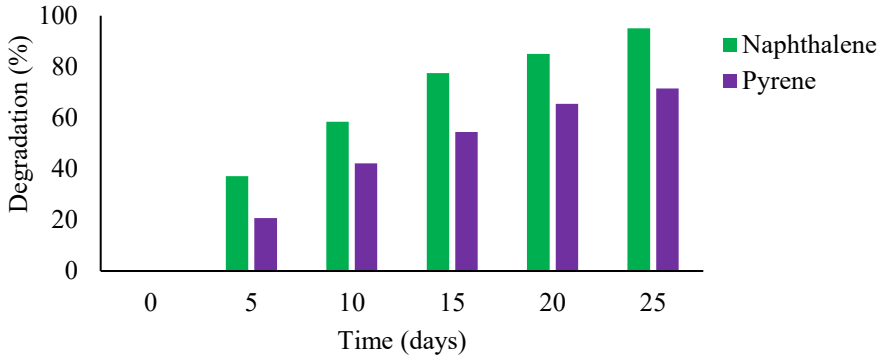


Figure 5: Degradation of naphthalene and pyrene by *Trichoderma harzianum* OR485009

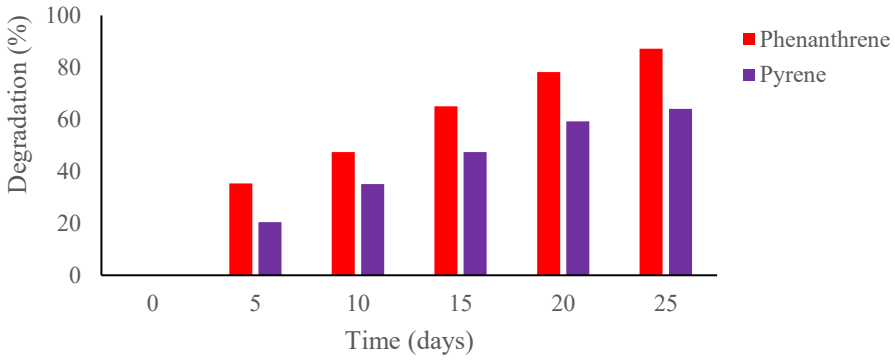


Figure 6: Degradation of phenanthrene and pyrene by *Trichoderma harzianum*-OR485009

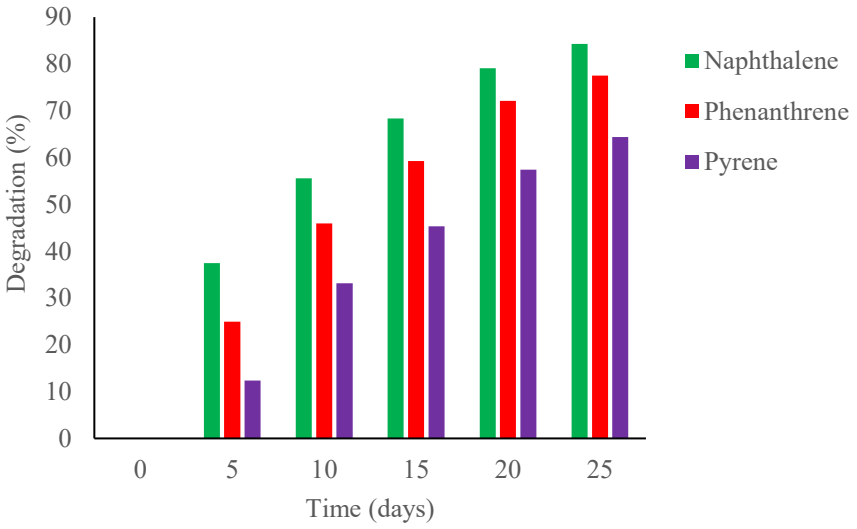


Figure 7: Degradation of naphthalene, phenanthrene and pyrene by *Trichoderma harzianum*-OR485009

Maximum enzyme activity (5.2, 3.5, and 2.6 U/L; 3.9, 2.8, and 2.0 U/L; 3.3, 2.5, and 1.8 U/L) were recorded for laccase, MnP, and LiP at day 15 of naphthalene, phenanthrene, and pyrene degradation, respectively (Figures 8, 9, and 10). Maximum enzyme activity (7.4, 5,2, and 3.5 U/L; 6.8, 4.5 and 3.5 U/L; 5.5, 3.4 and 2.4 U/L; 5.3, 3.2 and 2.2 U/L) was recorded for Laccase, MnP, and LiP at day 15 during naphthalene/phenanthrene, naphthalene/pyrene, phenanthrene/pyrene, and naphthalene/phenanthrene/pyrene degradation, respectively (Figures 11, 12, 13, and 14). For control samples, maximum enzyme activity (0.8, 0.6, and 0.2 U/L) was recorded for laccase, MnP, and LiP, respectively, on days 15 for laccase and 20 for MnP and LiP.

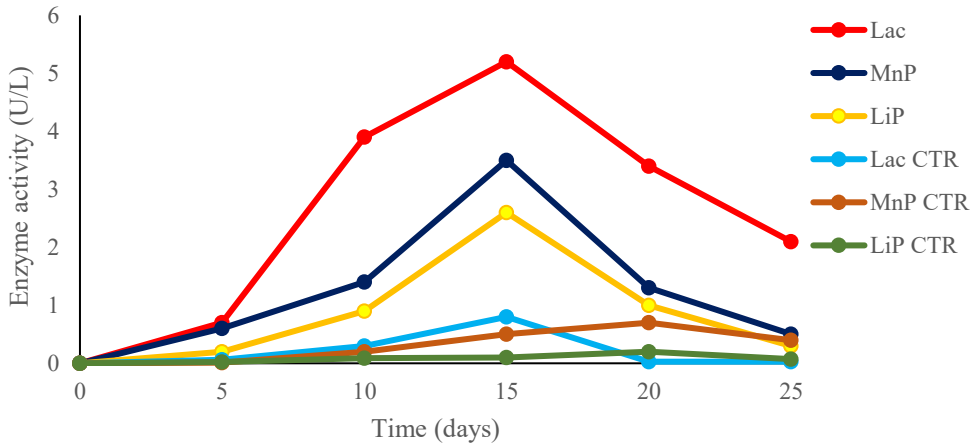


Figure 8: Lignolytic enzyme activity of naphthalene degradation by *T. harzianum*-OR485009
Key: Lac (Laccase); MnP (Manganese peroxidase); LiP (Lignin peroxidase); CTR (Control)

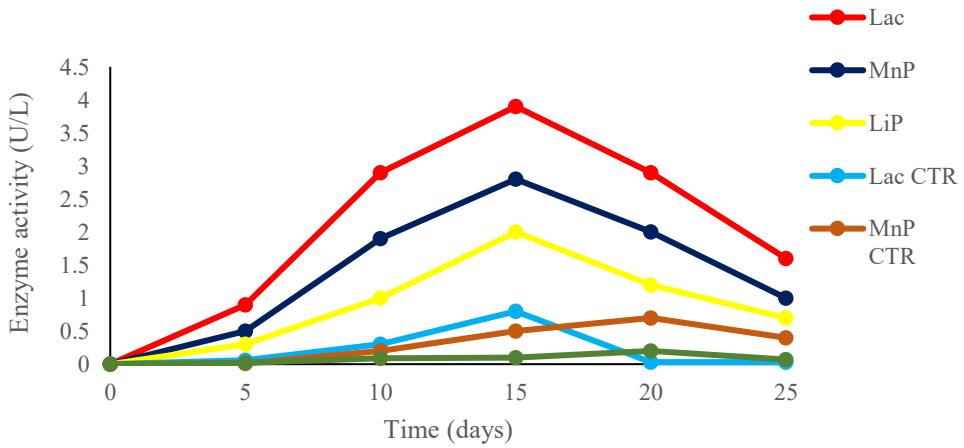


Figure 9: Lignolytic enzyme activity of phenanthrene degradation by *T. harzianum*-OR485009
Key: Lac (Laccase); MnP (Manganese peroxidase); LiP (Lignin peroxidase); CTR (Control)

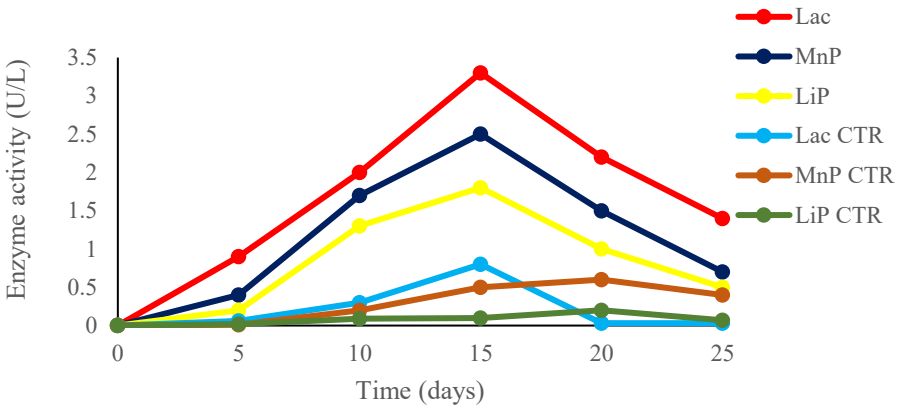


Figure 10: Lignolytic enzyme activity of pyrene degradation by *T. harzianum*-OR485009
Key: Lac (Laccase); MnP (Manganese peroxidase); LiP (Lignin peroxidase); CTR (Control)

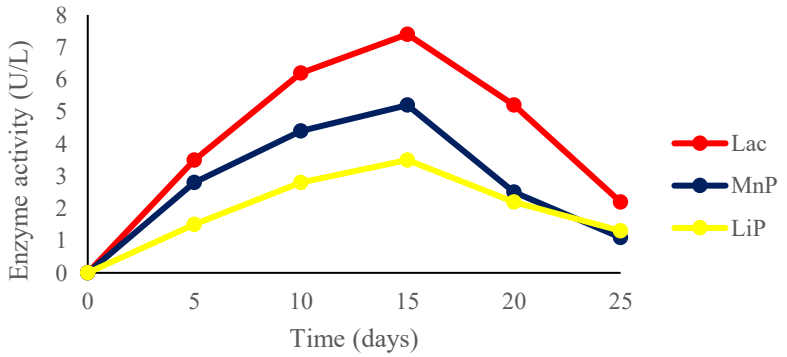


Figure 11: Lignolytic enzyme activity of naphthalene/phenanthrene degradation by *T. harzianum*- OR485009
Key: Lac (Laccase); MnP (Manganese peroxidase); LiP (Lignin peroxidase)

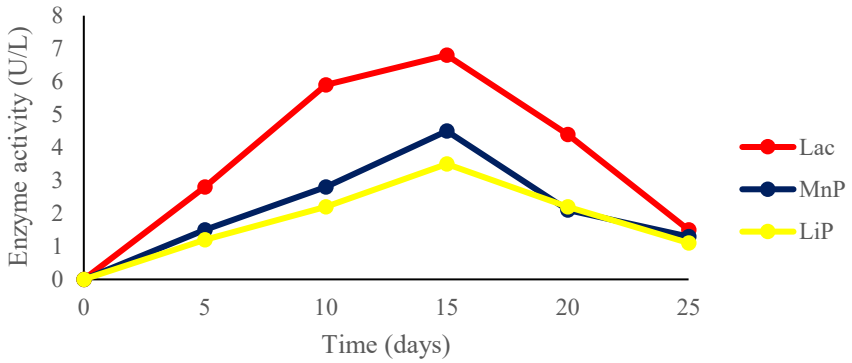


Figure 12: Lignolytic enzyme activity of naphthalene/pyrene degradation by *T. harzianum*-OR485009

Key: Lac (Laccase); MnP (Manganese peroxidase); LiP (Lignin peroxidase)

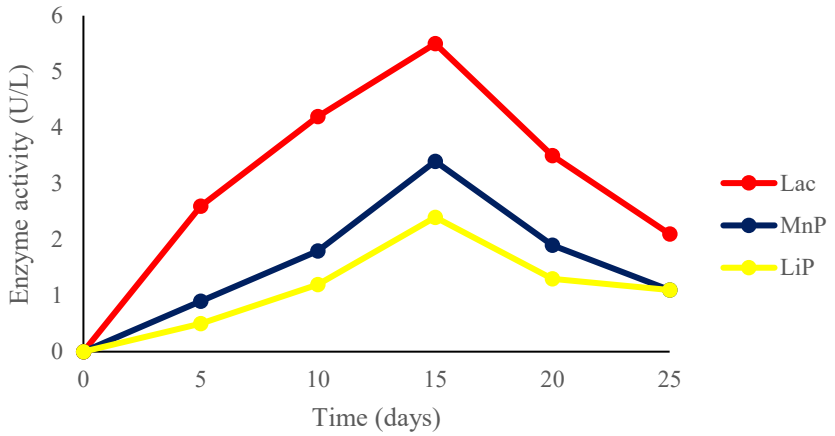


Figure 13: Lignolytic enzyme activity of phenanthrene/pyrene degradation by *T. harzianum* -OR485009
Key: Lac (Laccase); MnP (Manganese peroxidase); LiP (Lignin peroxidase)

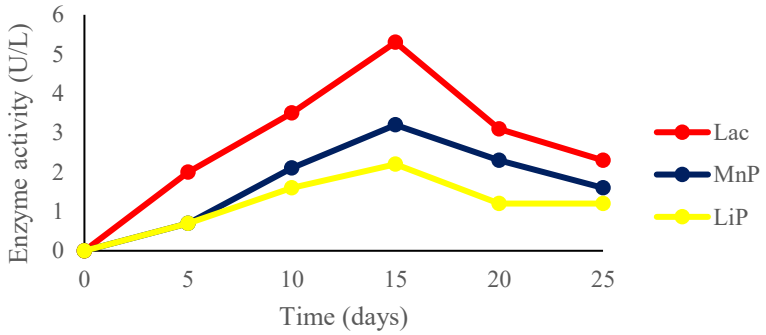


Figure 14: Lignolytic enzyme activity of naphthalene/phenanthrene/pyrene degradation by *T. harzianum*-OR485009

Key: Lac (Laccase); MnP (Manganese peroxidase); LiP (Lignin peroxidase)
Intermediate products of PAH degradation revealed (butenoic acid, oxalic acid, bis(2-ethylhexyl) phthalate), (diisooctyl phthalate, eicosane, cycloeicosane, oxalic acid) and (phenanthrene, diisooctyl phthalate and oxalic acid) during naphthalene, phenanthrene and pyrene degradation respectively (Tables 1, 2 and 3). These compounds were used to propose possible PAH degradation pathways for *T. harzianum*-OR485009 (Figures 15, 16, and 17).

Table 1: GC/MS spectra properties of intermediates generated by *T. harzianum*-OR485009 during naphthalene degradation

Compound name	Retention time	m/z of major ion fragments
3-Butenoic acid	9.8106	39, 45, 58, 68, 86, 29, 53, 63
Oxalic acid	10.8965	41, 57, 71, 29, 85, 171, 126, 97
Bis(2-ethylhexyl) phthalate	32.7095	149, 57, 279, 113, 83, 180, 390

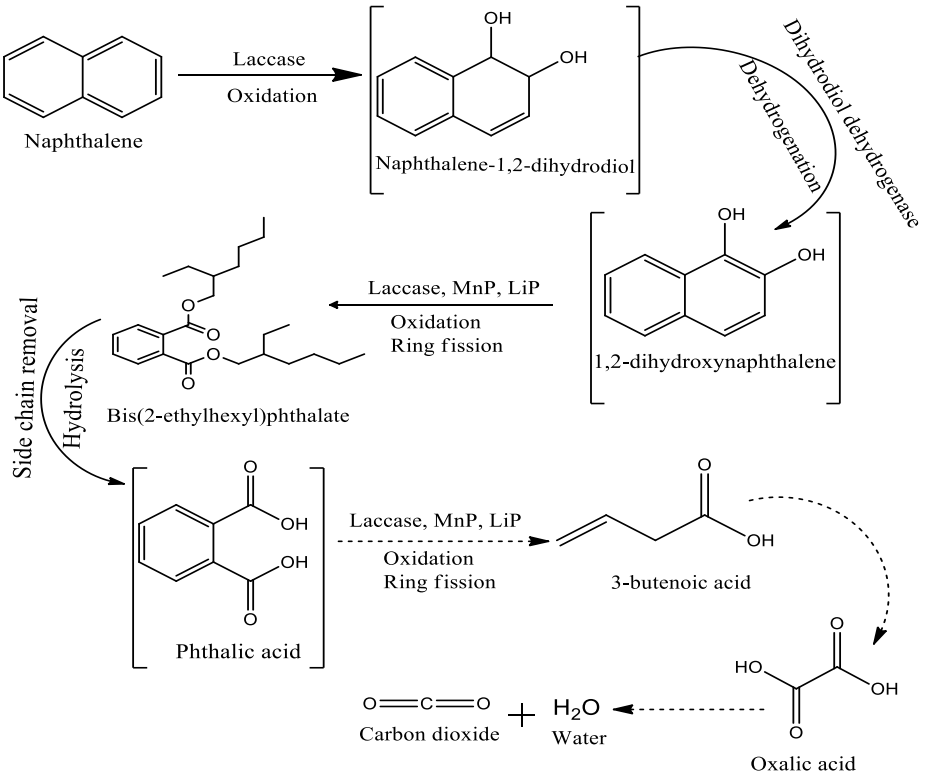


Figure 15: Proposed pathway for naphthalene degradation by *T. harzianum*-OR485009 from identified intermediate products

Note: Compounds in parentheses are intermediates not detected by GCMS in this study and dotted arrows represent series of enzymatic reactions

Table 2: GC/MS spectra properties of intermediates generated by *T. harzianum*-OR485009 during phenanthrene degradation

Compound name	Retention time	m/z of major ion fragments
Bis(2-ethylhexyl) phthalate	32.7103	149, 57, 279, 113, 83, 180, 390
Cycloeicosane	33.8072	55, 83, 29, 111, 139, 280, 252, 168, 196, 224
Oxalic acid	10.8965	41, 57, 71, 29, 85, 171, 126, 97
5-Eicosene	27.7078	57, 83, 111, 29, 139, 280, 167, 196, 252, 224

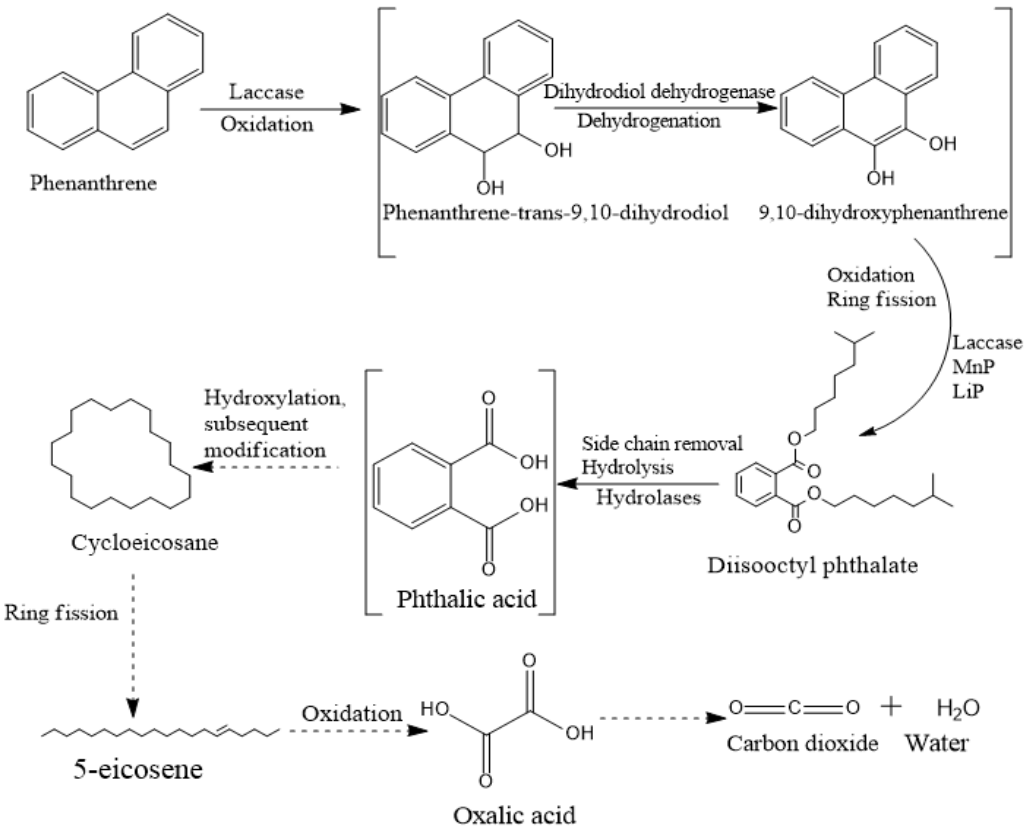


Figure 16: Proposed pathway for phenanthrene degradation by *T. harzianum*-OR485009 from identified intermediate products

Note: Compounds in parentheses are intermediates not detected by GCMS in this study and dotted arrows represent series of enzymatic reactions.

Table 3: GC/MS spectra properties of intermediates generated by *T. harzianum*-OR485009 during pyrene degradation

Compound name	Retention time	m/z of major ion fragments
Phenanthrene	18.666	178, 76, 152, 89, 63, 50, 126, 139, 102, 38
Pyrene	24.9071	202, 101, 174, 74, 150, 50, 122, 27, 12
Diisooctyl phthalate	32.7347	149, 57, 279, 83, 104, 29, 168
Oxalic acid	10.8965	55, 83, 111, 29, 283, 139

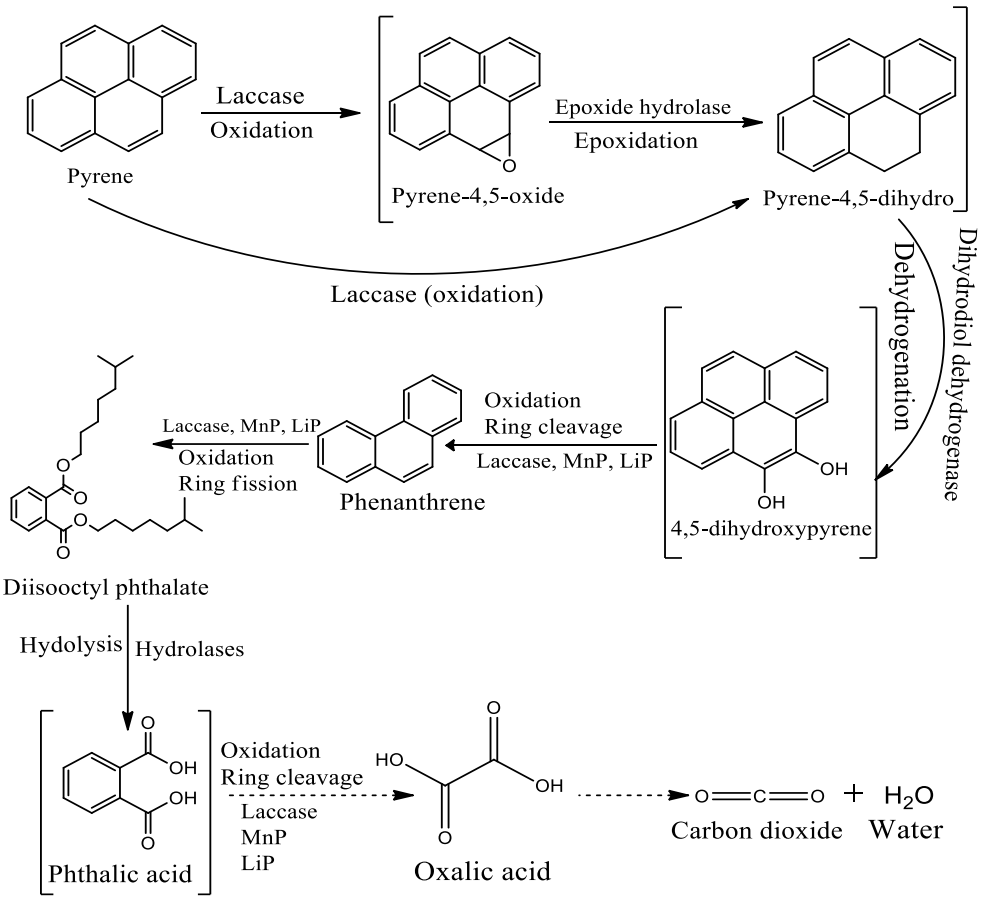


Figure 17: Proposed pathway for pyrene degradation by *T. harzianum*-OR485009 from identified intermediate products

Note: Compounds in parentheses are intermediates not detected by GCMS in this study and dotted arrows represent series of enzymatic reactions.

DISCUSSION

Lignolytic Enzyme Production

The production of ligninolytic enzymes by the test fungus both in the presence and absence of PAH in cultures confirms its ability degrade organic pollutants in the environment, such as PAHs. Filamentous fungi such as "Ascomycetes" are potential agents of degradation because they possess branched hyphae that can reach the deepest portions of contaminated substrates, digesting them through the secretion of extracellular enzymes (Daccò *et al.,* 2020). These extracellular enzymes, usually involved in the oxidation of lignin, have broad substrate-specificity and can therefore oxidize a wide range of organic pollutants, including pesticides, plastics, and hydrocarbons.

Daccò et al. (2020), Sijinamanoj et al. (2021), Jonathan et al. (2017) and Abd El Monssef et al. (2016) reported the presence of one, two or all three enzymes in *T. harzianum*. These differences could be due to genetic variability among fungal strains, which can lead to

differences in the presence or expression of genes encoding the enzymes. It could also be due to environmental conditions or factors such as nutrient composition, pH, temperature, and oxygen levels. Variations in the local environmental conditions where fungi grow have been shown to result in differences in the expression and regulation of lignolytic enzyme genes (Asemoloye *et al.,* 2018; Lee *et al.,* 2023).

***Degradation of individual and PAHs mixtures by T. harzianum*- OR485009**

Degradation of individuals as well as binary and ternary PAH mixtures, confirms the ability of this fungus to tolerate toxic environmental pollutants. This tolerance ability could be attributed to the production of lignolytic enzymes as well as other enzyme systems such as cytochrome P450 monooxygenase and dioxygenases. Additionally, its ability to degrade PAH mixtures can be attributed to the presence of diverse or multiple enzyme systems, including dehydrogenases and hydrolases, which can act on various PAH compounds by targeting the aromatic rings.

Fungi also exhibit metabolic plasticity, meaning they can adapt their degradation pathways in response to different environmental conditions and available substrates (Li *et al.,* 2021). This flexibility allows fungi to switch between different degradation pathways and utilize alternative enzymes when exposed to various PAH mixtures. Fungi can also utilize PAH compounds as co-metabolites for their growth and energy (Li *et al.,* 2021). This means that even when a specific PAH compound is not a direct carbon or energy source, fungi can still transform and degrade it. This co-metabolic capacity enables fungi to tolerate and degrade multiple PAH mixtures as they can utilize shared metabolic intermediates or enzymes (Li *et al.,* 2021). Elyamine et al. (2021) reported that *T. harzianum* metabolized 65% out of 50 mg/L initial concentration of pyrene as well as 33% out of 100 mg/L initial concentration of pyrene after a 28-day incubation period. This high performance was explained by the capacity of this strain to produce laccase, LiP, and MnP. Zafra and Cortés-Espinos (2015) reported that *T. asperellum* H15 rapidly adapted to PAH-contaminated soils, achieving up to 78% of phenanthrene degradation in soils contaminated with 1000 mg/kg after 14 days. The researchers also reported that *T. asperellum* was also able to simultaneously degrade a mixture of PAHs, achieving 74% phenanthrene, 63% pyrene, and 81% benzo(α)pyrene. The degradation ability of this fungus was linked to its ability to produce catechol 1,2-dioxygenase, laccase, and peroxidase enzymes. Andreolli et al. (2016) reported that *Trichoderma longibrachiatum* metabolized 95.2% phenanthrene, 96.8% anthracene, 89.7% pyrene, and 88.1% fluoranthene from the 25 mg/L initial concentration of each PAH after 14 days. However, a slight reduction in tolerance rate was observed when all four PAHs were present in the same medium. *Enzyme activity*

Lignolytic enzyme activity of *T. harzianum*-OR485009 during degradation of individuals as well as PAH mixtures showed that laccase had higher activity compared to the peroxidases. This could be attributed to their more versatile nature in terms of substrate specificity, which allows them to oxidize a wide range of organic compounds compared to MnP and LiP. The

fungus ability to degrade HMW pyrene could be attributed specifically to the high laccase enzyme activity. A study by Behera et al. (2024) reported that *Trichoderma* sp. CNSC-2 demonstrated 69.42 ± 0.4% pyrene removal at 100 mg L^{-1} over 12 days. Molecular docking studies revealed a significant binding affinity of −7.82 kcal mol^{-1} between pyrene and the Lac1 laccase protein's active site. Laccase activity peaked on day 6 of incubation, confirming laccase enzyme induction during degradation. Another study reported that a consortium of three basidiomycetes isolated from compost achieved approximately 56% pyrene removal from soil (100 mg kg^{-1}) over 28 days, with enzyme assays confirming laccase and manganese-independent peroxidase involvement (Anastasi *et al.,* 2009)

The activity of all three enzymes was significantly higher in the presence of PAHs compared to the control group. PAHs are known to induce the expression of genes encoding lignolytic enzymes in many fungi, including *Trichoderma harzianum*. The fungus might upregulate the synthesis of these enzymes as a response to the presence of PAHs (induction of enzyme production), leading to higher enzyme activity. It was also observed that the rate of enzyme activity decreased with an increase in the number of aromatic rings. This could be attributed to why LMW naphthalene, followed by phenanthrene, were degraded faster than HMW pyrene. The smaller molecular size and simpler structure of LMW-PAHs make them readily soluble in water and accessible to enzymes, allowing for faster and more efficient degradation (Kumar *et al.,* 2021).

Lignolytic enzymes (Lac, MnP, and LiP) play a crucial role in PAH degradation by catalyzing the oxidation of PAHs due to their similar aromatic structures. They act by generating reactive oxygen species, highly reactive intermediates, and free radicals that can initiate the degradation of or attack the aromatic rings and break down PAH molecules (Rashid *et al.,* 2015). Elyamine et al. (2021) reported that the rates of PAH degradation directly correlate with the rate of ligninolytic enzyme activity. This statement supports the results obtained in this study, as higher enzyme activities were recorded for higher rates of PAH degradation.

It is logical to say that, temperature (30 ºC), pH (7), and glucose (3.80 g/L) of the degradation medium favored the activities of lignolytic enzymes in the present study, as high degradation values were recorded at these environmental conditions. Nutritional as well as environmental factors (temperature and pH) have been shown to affect the production and activities of lignolytic enzymes (Andriani *et al.,* 2016). Prasher and Chauhan (2015) reported that glucose and ammonia oxalate were more effective carbon and nitrogen sources, respectively, for the highest MnP enzyme production and activity. Chowdhary et al. (2019) reported that MnP showed optimum production at pH 6 and a temperature of 30 ºC in *Pichia pastoris,* while Manawadi et al. (2019) reported maximum lac and LiP activity at pH 6 and 7.2, at a temperature of 45 and 40 ºC, respectively.

Intermediate Products of PAH Dgradation

The degradation process of PAH generally involves the breakdown or transformation of complex organic molecules into simpler units or compounds known as intermediate products

through a series of enzymatic reactions and different degradation pathways. These intermediates are subsequently broken down in the degradation pathway, leading to the generation of end products such as carbon dioxide and water.

Fungal species such as *Trichoderma harzianum* have the ability to utilize both lignolytic (lac, MnP, and LiP) and non-lignolytic (cytochrome P450 monooxygenase and dioxygenase) pathways in the degradation of PAHs. Extracellular enzymes oxidize PAHs and create PAH transient diphenols, which are automatically oxidized to quinones thereafter or may oxidize PAHs initially by epoxidation to form arene oxides using cytochrome P450 monooxygenase or laccase (Lladó *et al.,* 2013). In this study, *Trichoderma harzianum*-OR485009 produced lac, MnP, and LiP both in the presence and absence of PAHs. Despite these enzymes being much more induced when PAHs were present in the medium, not all theoretically expected intermediate products, such as quinones, which are the principal intermediates raised from ligninolytic enzymatic activities (Lladó *et al.,* 2013; Pozdnyakova, 2012), were detected during PAH degradation. In addition, dihydrodiols and their derivatives, "dihydroxy," which are products of arene oxides raised from non-ligninolytic enzymatic activities, were also not detected. Both quinones and arene oxides are highly reactive compounds and may quickly undergo further degradation, leading to the formation of other intermediates, hence the likely reason for their absence in this study.

CONCLUSION

The present investigation demonstrates the promising potential of *Trichoderma harzianum*-OR485009 for the bioremediation of PAH-contaminated environments. The fungus effectively degraded naphthalene and phenanthrene completely and showed significant degradation of pyrene. Furthermore, the fungus displayed the ability to degrade mixtures of PAHs, with a preference for lower molecular-weight compounds. This degradation ability was accompanied by the production of lignolytic enzymes, particularly laccase. Interestingly, the formation of intermediate products like butenoic acid, oxalic acid, and phthalate esters during degradation provided valuable insights into the possible metabolic pathway of PAHs by *T. harzianum*-OR485009. These findings highlight the potential of this specific strain as a powerful tool for the eco-friendly removal of PAHs from polluted environments. In other to bridge laboratory findings and real-world application, scale-up studies are recommended for practical field applications. It is also recommended that *Trichoderma harzianum*-OR485009 be incorporated into existing wastewater treatment plants as part of industrial application to ascertain its effectiveness in PAH degradation.

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