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Screening and Characterization of Polycyclic Aromatic Hydrocarbons Tolerant Fungi from Petrochemical Refinery Effluent

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Abstract

Fungi were isolated from effluent released by petrochemical refinery and screened for their tolerance to 50 mg/L concentration of naphthalene, phenanthrene and pyrene under agitation condition of 150 rpm and ambient temperature over a period of 21 days. Samples were analysed for residual PAH concentrations using HPLC and tolerance ability were calculated in percentages. Fungal growth in mineral salt medium supplemented with PAH was measured in dry weight of mycelial biomass was used as index for assessing their tolerance ability. Out of the 22 fungi isolated, only four were identified as Aspergillus, Talaromyces, Fusarium and Trichoderma species to exhibit tolerance to naphthalene (94.6, 96.9, 99.7 and 99.8%), phenanthrene (91.1, 92.2, 99.5 and 99.8%) and pyrene (89.4, 90.5, 92,6 and 94.2%) respectively. Low molecular weight PAHs (naphthalene and phenanthrene) were better tolerated compared to high molecular weight pyrene. Based on findings made in this study, the four isolates with higher tolerance to PAHs could be recommended for bioremediation of PAH contaminated environments.

Keywords: Fungi; PAHs; Mycoremediation; petrochemical refinery effluent

INTRODUCTION

In the past few decades, human activities have led to the release of large amounts of hydrocarbons, making them one of the most common and important environmental pollutants on the planet (Ukaogo *et al.*, 2020). Among these are the polycyclic aromatic hydrocarbons (PAHs) which encompass a cluster of organic compounds containing two or more fused aromatic rings within their molecular makeup (Al-hawash *et al.*, 2018). These PAHs are discharged into the surroundings as a result of the incomplete combustion of organic materials (Al-hawash *et al.*, 2018). The rise in auto mobile workshops mostly in urban centres have contributed immensely to soil pollution with PAHs. This is as a result of indiscriminate disposal of spent engine oil and consequently, surface and ground water are contaminated with these pollutants through surface runoffs. The levels of priority organic pollutants known as PAHs have shown a consistent rise in recent times (Ontiveros-Cuadras *et al.*, 2019). These compounds are now found in various sources, including food, air, water, soil, and sediments (Olayinka *et al.*, 2018).

Petroleum refining activities or processes contributes to PAHs in refinery wastewater (Kuyukina *et al.*, 2020). The quality of discharged waste bearing PAHs and other pollutants into lands and rivers from various industries in Nigeria is far cry from the acceptable levels. There is also a high degree of negligence regarding the harmful effects associated with such releases directly into the environment without adequate treatment (Samaila and Iyeri, 2017). Sixteen kinds of PAHs including naphthalene, phenanthrene and pyrene are of major environmental and human concern due to their wide spread occurrence, persistence and toxicity potentials on living systems. Hence, are listed by the European Union (EU) and the Environmental Protection Agency (EPA) as priority pollutants (Rodrigues and Sette, 2018) in ecosystem. Polycyclic aromatic hydrocarbons (PAHs) are a large group of pollutants whose fate and distribution in the environment are of increasing interest. This is as a result of their recalcitrant and hydrophobic nature which enables them bind strongly to soil and sediment, making them less available for biological uptake (Maletic *et al.*, 2019).

Over the recent decades, addressing the presence of PAHs in the environment has gained worldwide attention due to their intrinsic characteristics (Patel *et al.*, 2020). Various strategies for remediation, including physical, chemical, biological, and more recently, integrated approaches, have been consistently employed to differing levels of effectiveness (Patel *et al.*, 2020). Amid these numerous remediation techniques, approaches that

involve microorganisms (including bacteria, fungi, and algae) for the ecological recovery of PAH-contaminated environments have been extensively assessed as other methods often encounter several limitations (Lazzem *et al.*, 2022).

Bioremediation is a technology that aids in the removal/conversion of contaminants into products that are non-toxic and comparatively safer to humans and the environment, such as carbon dioxide and water (Medaura *et al.*, 2021). Mycoremediation which involves the use of fungi have proven useful in remediation of PAHs, among other features which enable them play great role in bioremediation is secretion of extracellular enzymes (Tomer *et al.*, 2021).

Numerous studies have been conducted globally, exploring the isolation and identification of effective microbial strains capable of either transforming or assimilating PAHs (Stoyanova *et al.*, 2022). The majority of these investigations have concentrated on bacteria, with limited emphasis on fungi as a means of remediation of PAHs (Stoyanova *et al.*, 2022). Among fungal strains, studies are often focused on the use of ligninolytic fungi (Basidiomycetes), of which PAH degradation is limited to certain species of white rot fungi such as; *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, *Crinipellis stipitaria* due to their ability to secrete three major classes of extracellular enzymes; namely, laccase, manganese and lignin peroxidase. However, these fungi are not frequent species in polluted habitats (Mahmud *et al.*, 2022), hence, the need to sort for alternative fungal genera/species. The filamentous fungi known as ascomycetes, which frequently dominate industrially polluted regions of the environment, remain inadequately researched concerning their potential to degrade hazardous environmental pollutants, including PAHs (Stoyanova *et al.*, 2022). Hence, the need to screen filamentous fungi in order to guarantee that species with promising potentials for bioremediation programs are obtained with respect to PAH degradation.

MATERIALS AND METHODS

Culture media and chemicals

Low molecular weight (naphthalene and phenanthrene) and high molecular weight (pyrene) with purity >99.8% were purchased from Sigma-Aldrich Chemicals, (Germany). Stock solution of 1000mg/L of each PAH was prepared in acetone and sterilized using 0.22 µm filter. HPLC grade n-hexane and acetone were also procured from Sigma-Aldrich Chemicals, (Germany). Growth of fungal isolates on PAHs was determined 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 $MgSO_4.7H_2O$, 5.0g glucose, pH 5±0.2. Potato dextrose agar (PDA) (g/L): 4g of potato extract, 20g of dextrose, 15g of agar-agar (Oxoid ltd, Basinstoke, United Kingdom). The media were sterilized by autoclaving at 121°C under 15 psi for 15 minutes.

Collection of refinery effluent

Effluent samples were collected from the waste retention pond at Kaduna Refining and Petrochemical Company (KRPC) in Kaduna state, Nigeria. The samples were carefully collected in sterile amber sample bottles. After sample collection, outer surfaces of sample bottles were rinsed with sterile distilled water and transported in ice box to the laboratory for fungal isolation and assessment of hydrocarbon contents (Ezeonuegbu *et al.*, 2022).

Isolation of fungi from refinery effluent

The effluent samples were allowed to stand at ambient temperature on a disinfected laboratory work bench. Duplicate samples, each measuring 10 ml, were dispensed into sterile centrifuge tubes under aseptic conditions. These tubes were then centrifuged at 250 rpm for 10 minutes to concentrate the samples. Following the removal of the supernatant, 0.1 ml of sediment from each sample was evenly spread onto two freshly prepared PDA plates containing 50µg/L of chloramphenicol, using a sterile bent glass rod (Ezeonuegbu *et al.*, 2022). The plates were incubated aerobically at room temperature for a duration of 7 days (Ezeonuegbu *et al.*, 2022).

Morphological and microscopic characterization of fungal isolates

Colonies emerging on PDA medium were distinguished on the basis of cultural characteristic features, such as size (diameter), colours (surface and reverse), sporulation, zonation and texture.

To conduct microscopic characterization of the isolates, a small portion of the growing region was mounted onto a clean, grease-free slide. A drop of lactophenol cotton blue was added, and the slide was covered with a cover slip. Examination was carried out using a ×40 objective lens. The isolates were then characterized and identified with reference to a taxonomic guide (Ezeonuegbu *et al.*, 2022). The pure isolates were maintained in PDA slants and stored in refrigerator at 4°C.

Screening of isolates for tolerance to PAHs

The test isolates (5 mm plugs of fungal mycelia) taken from the pure fungal culture were aseptically inoculated onto MSM amended with 50 mg/L of the individual PAHs (naphthalene, phenanthrene and pyrene) separately and incubated at ambient temperature on a rotary shaker at 150 rpm in the dark for a period of 21 days. Un-inoculated MSM broth amended with PAH served as abiotic negative control samples. All experiments were carried out in triplicate.

Extraction and quantification of residual PAHs from cultures

After the incubation period, residual PAHs were extracted from the medium and mycelium by adding 50 ml n-hexane into cultures. After which, the content in each flask was shaken vigorously for five minutes to remove adsorbed PAH from fungal mycelia. The content was filtered to separate fungal mycelia from the culture. Another 50 ml n-hexane was added to the harvested mycelia to re-extract any adsorbed PAH. The filtrate was combined in a conical flask (Agrawal *et al.*, 2018). The harvested mycelia were dried in hot air oven at a temperature of 60°C for 10 minutes and weighed using a weighing balance (Agrawal *et al.*, 2018). Extraction of residual PAHs in the filtrate was carried out according to the method described by Olayinka *et al.* (2018). Analysis of residual PAHs was carried out using HPLC as described according to the method of Agrawal *et al.* (2018).

Tolerance ability of each isolate to individual PAH/biodegradation efficiency was assessed using the following formula;

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).

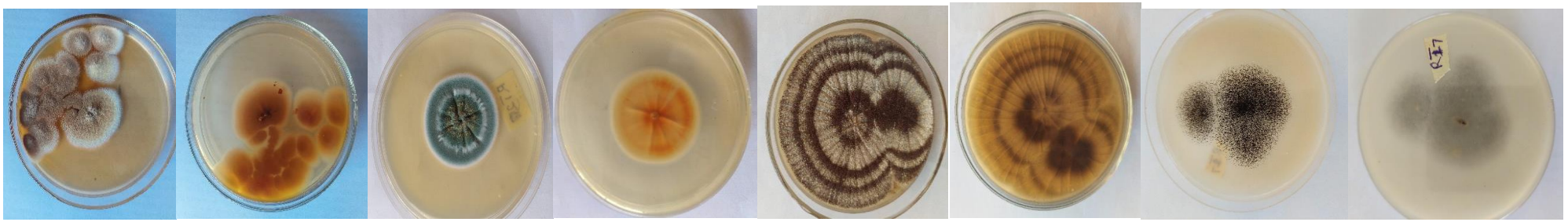
RESULTS

A total of 22 fungal isolates consisting of ten genera with *Aspergillus* as the predominant genus were isolated. Cultural characteristics of fungi on potato dextrose agar in terms of colour, surface texture, reverse, edge and colony diameter at seven days incubation period are presented in table 1. The pictorial depictions as well as microscopic features are shown on plate 1a ,1b 11a to 11b. Microscopic features of the isolates reveal features such as conidia (asexual spores), conidiophores, vesicle and phialides.

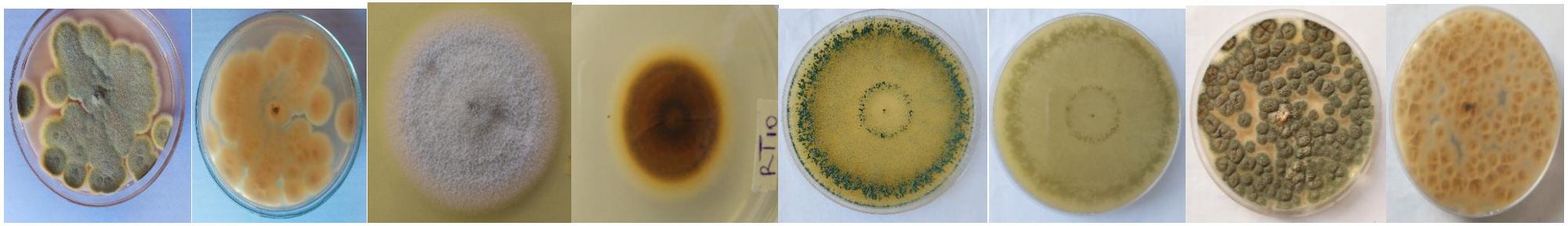
Table 1: Cultural characteristics of fungi isolated from refinery effluent

Isolate Code	Surface colour	Surface texture	Edge	Reverse colour	Colony diameter (mm)	Identity of isolates
R1	pink/white	cottony	white/circular	pink	43.0	*Rhizoctonia* spp.
R2	green	granular	white/irregular	cream	41.8	*Aspergillus* spp
R3	pinkish-	cottony	white/irregular	cream	31.8	*Rhizoctonia* spp.
R4	white	powdery	green/circular	orange	21.3	*Talaromyces* spp
R5	army green	powdery	brown/irregular	yellow	16.0	*Aspergillus* spp
R6	brown	velvety	white/circular	orange	29.3	*Penicillium* spp.
R7	teal green	granular	white/irregular	cream	44.0	*Aspergillus* spp.
R8	dark brown	granular	black/irregular	cream	35.2	*Aspergillus* spp
R9	black	velvety	green/irregular	cream	33.3	*Penicillium* spp.
R10	lemon green	velvety	white/circular	brown	33.8	*Rhizoctonia* spp.
R11	grey	powdery	green/circular	cream	78.0	*Trichoderma* spp.
R12	green	powdery	white/irregular	cream	49.0	*Aspergillus* spp.
R13	green	cottony	grey/circular	cream	48.3	*Rhizopus* spp.
R14	grey	granular	cream/circular	cream	29.7	*Phoma* spp.
R15	brown	powdery	cream/irregular	orange	12.0	*Penicillium* spp.
R16	green	powdery	orange/circular	cream	78.0	*Trichophyton* spp.
R17	light orange	velvety	brown/circular	cream	44.5	*Curvularia* spp.
R18	dark brown	cottony	white/irregular	purple	44.0	*Fusarium* spp.
R19	white	powdery	white/irregular	green	10.8	*Penicillium* spp.
R20	green	powdery	white/circular	cream	21.0	*Penicillium* spp.
R21	bluish-green	granular	white/irregular	pale	21.2	*Aspergillus* spp.
R22	green	powdery	green/circular	yellow	44.5	*Aspergillus* spp.
	green			cream		

R1: *Rhizoctonia* spp. R2: *Aspergillus* spp. R3: *Rhizoctonia* spp. R4: *Talaromyces* spp.

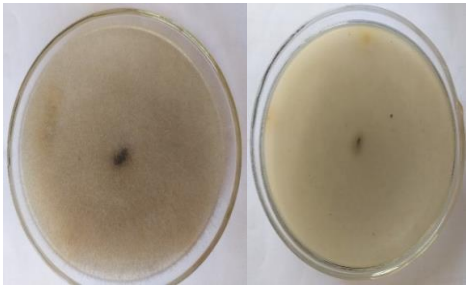


R5: *Aspergillus* spp. R6: *Penicillium* spp. R7: *Aspergillus* spp. R8: *Aspergillus* spp.

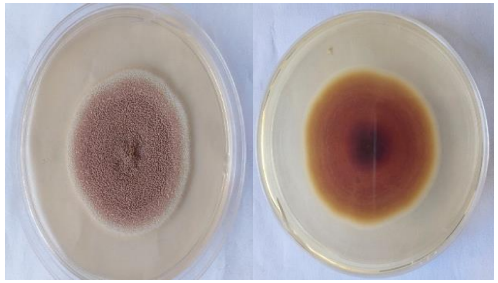


R9: *Penicillium* spp. R10: *Rhizoctonia* spp. R11: *Trichoderma* spp. R12: *Aspergillus* spp.

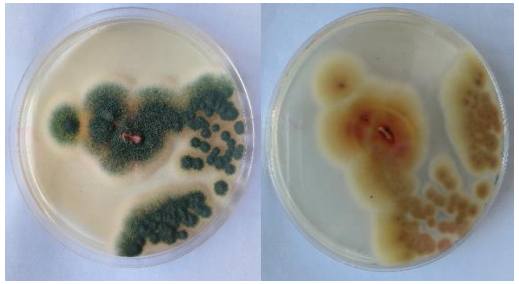
Plate 1a: Surface and reverse cultural morphology of fungi grown on PDA medium at 7 days incubation



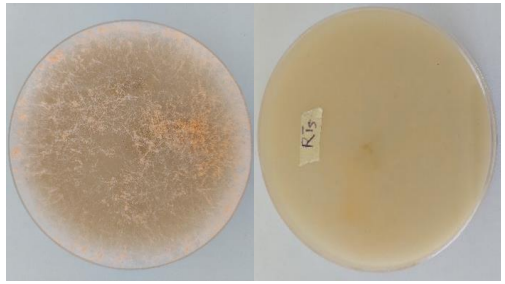
R13: *Rhizopus* spp.



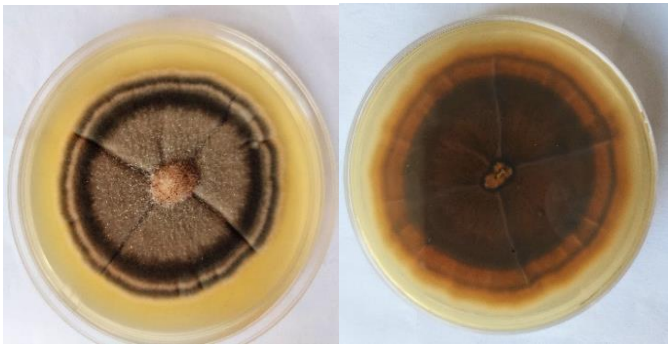
R14: *Phomas* pp.



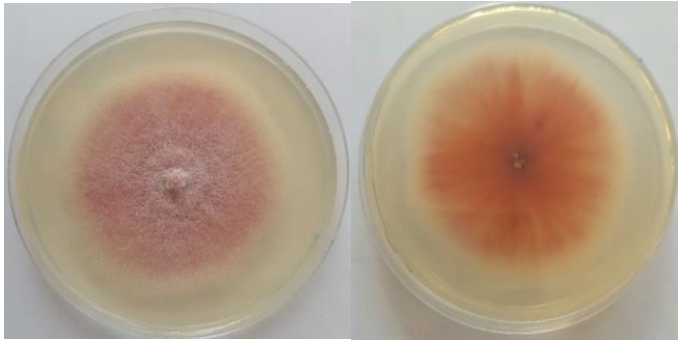
R15: *Penicillium* spp.



R18: *Trichophyton* spp.

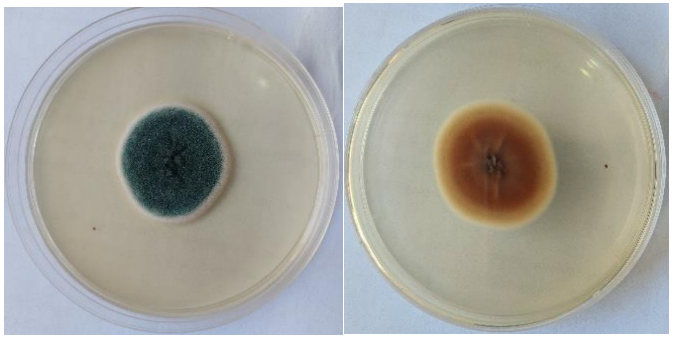


R17: *Curvularia* spp.

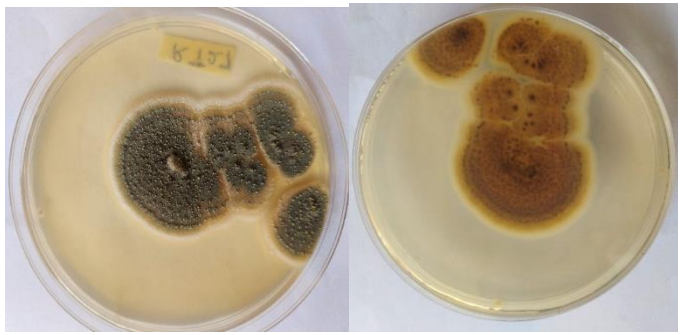


R18: *Fusarium* spp.

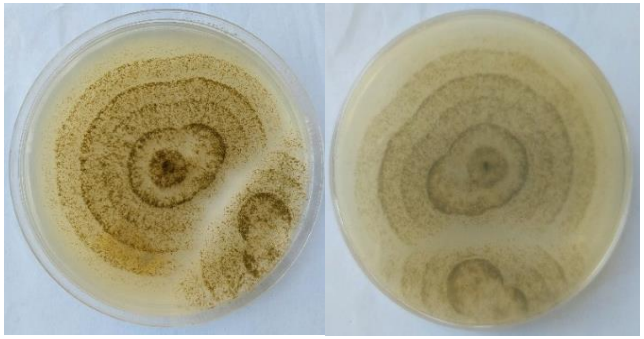
R19: *Penicillium* spp.



R20: *Penicillium* spp.

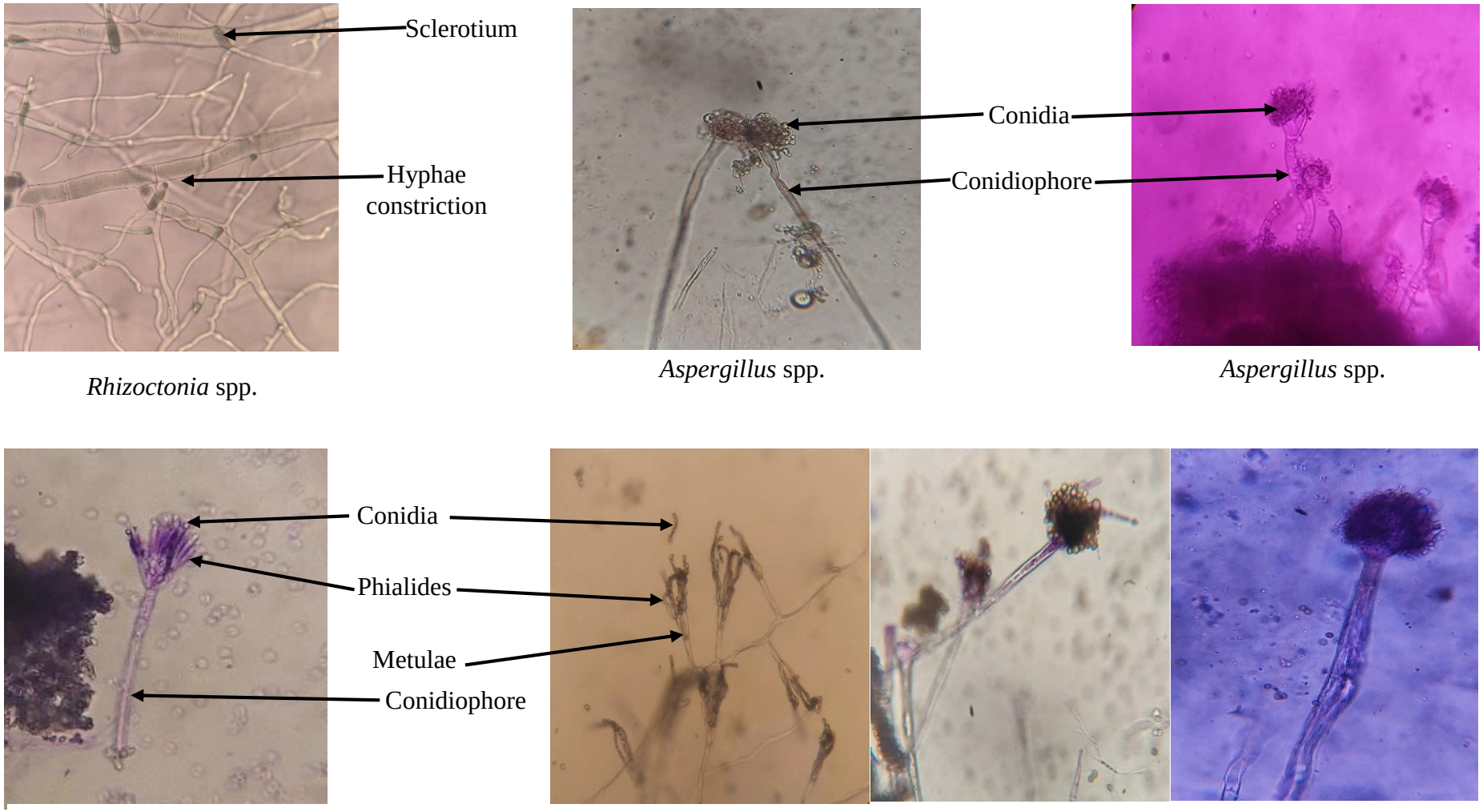


R21: *Aspergillus* spp.



R21: *Aspergillus* spp.

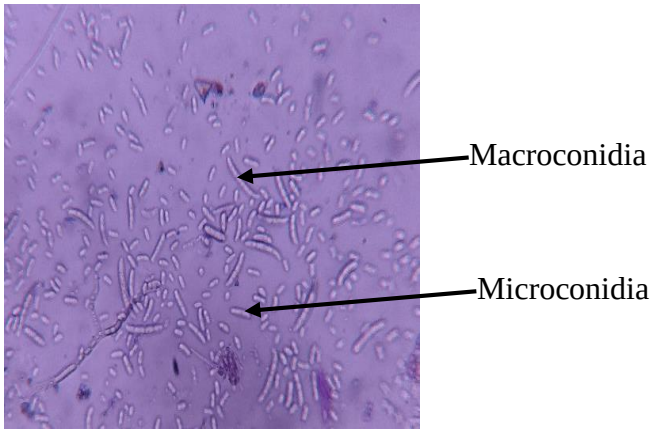
Plate 1b: Surface and reverse cultural morphology of fungi grown on PDA medium at 7 days incubation



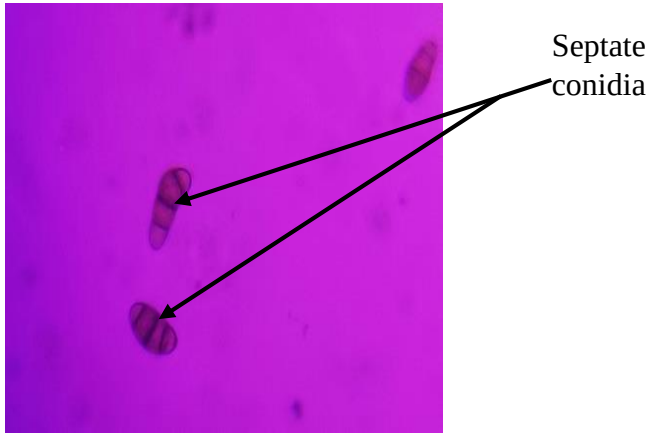
Rhizoctonia spp. *Aspergillus* spp. *Aspergillus* spp.

Talaromyces spp. *Penicillium* spp. *Aspergillus* spp. *Aspergillus* spp.

Plate 11a: Microscopic morphology of fungal isolates

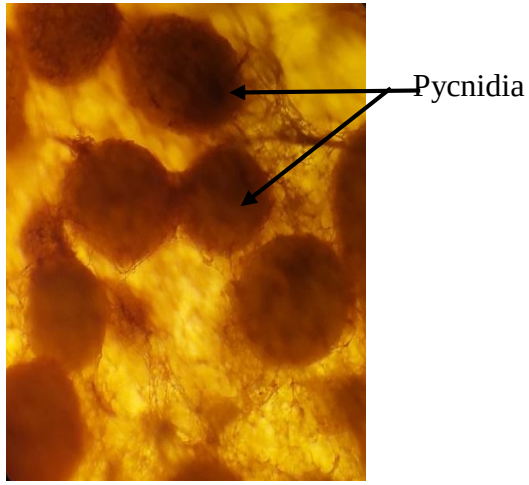


Fusarium spp.

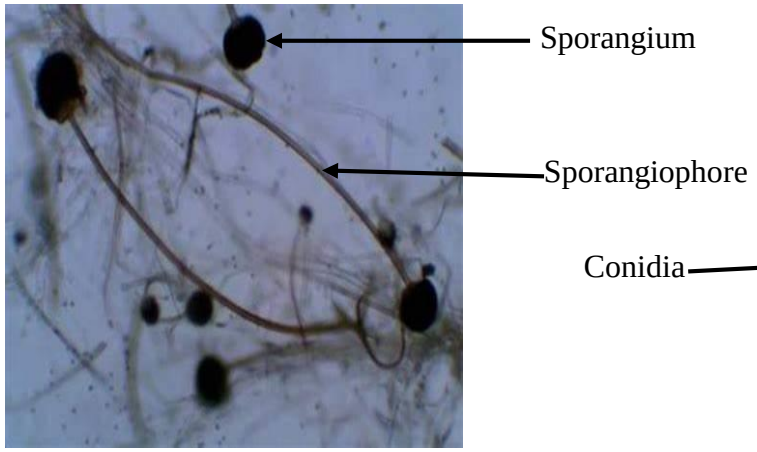


Culvularia spp.

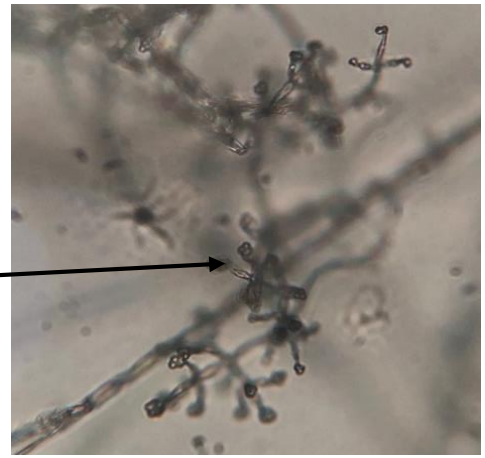
Trichophyton spp.



Phoma spp.



Rhizopus spp.



Trichoderma spp.

Plate 11b: Microscopic morphology of fungal isolates

Out of 22 fungal isolates tested, only four isolates tolerated 50 mg/L concentration with higher degradation percentages across the three PAHs (table 2). These four isolates were of the genera *Talaromyces* (R4), *Trichoderma* (R11), *Aspergillus* (R12) and *Fusarium* (R18). Their tolerance to naphthalene ranged from 94.6 to 99.8% while tolerance to phenanthrene ranged from 91.1 to 99.8% and tolerance to pyrene ranged from 89.4 to 94.2% with *Aspergillus* spp. having the least and *Trichoderma* spp. having the highest tolerance ability (*Aspergillus* > *Talaromyces* > *Fusarium* > *Trichoderma* spp.) in all cases. Lowest weight of dry fungal biomass was recorded during pyrene degradation as seen in table 4.

Table 2: Tolerance of isolates to 50 mg/L of naphthalene over 21 days incubation period

Isolate code	Dry weight fungal biomass (mg)	Tolerance ability (%)
Rhizoctonia spp. (R1)	151±0.333	57.1
Rhizoctonia spp. (R3)	160±0.667	60.8
Rhizoctonia spp. (R10)	150±0.333	58.5
Aspergillus spp. (R2)	200±0.577	78.1
Aspergillus spp. (R5)	180±0.577	65.4
Aspergillus spp. (R7)	190±0.882	70.4
Aspergillus spp. (R8)	200±1.155	74.2
Aspergillus spp. (R12)	240±0.577	94.6
Aspergillus spp. (R21)	210±0.333	87.0
Aspergillus spp. (R22)	199±0.577	75.3
Penicillium spp. (R6)	180±0.882	64.1
Penicillium spp. (R9)	150±0.333	55.4
Penicillium spp. (R15)	200±0.577	73.2
Penicillium spp. (R19)	110±0.577	50.2
Penicillium spp. (R20)	179±0.667	63.1
Talaromyces spp. (R4)	240±0.882	96.9
Trichoderma spp. (R11)	270±0.577	99.8
Rhizopus spp. (R13)	199±0.333	74.6
Phomas pp. (R14)	190±0.333	71.1
Trichophyton spp. (R16)	210±0.333	86.7
Curvularia spp. (R17)	160±1.155	60.5
Fusarium spp. (R18)	270±0.333	99.7
Abiotic control	-	0.3

Table 3: Phenanthrene tolerance at 50 mg/L by test fungi at 21 days incubation period

Isolate code	Dry weight fungal biomass (mg)	Tolerance ability (%)
Rhizoctonia spp. (R1)	140±0.333	55.2
Rhizoctonia spp. (R3)	160±0.333	60.0
Rhizoctonia spp. (R10)	150±0.667	55.6
Aspergillus spp. (R2)	190±0.882	73.2
Aspergillus spp. (R5)	169±0.577	65.1
Aspergillus spp. (R7)	179±0.577	70.4
Aspergillus spp. (R8)	190±0.333	71.4
Aspergillus spp. (R12)	230±0.333	91.1
Aspergillus spp. (R21)	190±0.577	73.6
Aspergillus spp. (R22)	180±0.667	68.3
Penicillium spp. (R6)	160±0.333	59.2
Penicillium spp. (R9)	140±0.333	54.2
Penicillium spp. (R15)	180±0.333	70.5
Penicillium spp. (R19)	110±0.333	52.2
Penicillium spp. (R20)	160±0.577	61.9
Talaromyces spp. (R4)	270±0.882	92.2
Trichoderma spp. (R11)	270±0.577	99.8
Rhizopus spp. (R13)	190±0.577	72.2
Phomas pp. (R14)	190±0.333	68.8
Trichophyton spp. (R16)	199±0.333	80.0
Curvularia spp. (R17)	110±1.453	50.8
Fusarium spp. (R18)	270±1.155	99.5
Abiotic control	-	0.3

Table 4: Pyrene tolerance at 50 mg/L by test fungi at 21 days incubation period

Isolate code	Dry weight fungal biomass (mg)	Tolerance ability (%)
Rhizoctonia spp. (R1)	90±1.155	48.8
Rhizoctonia spp. (R3)	139±0.667	55.7
Rhizoctonia spp. (R10)	100±0.333	48.5
Aspergillus spp. (R2)	170±0.577	65.4
Aspergillus spp. (R5)	150±0.333	57.3
Aspergillus spp. (R7)	170±1.155	64.0
Aspergillus spp. (R8)	170±0.333	64.7
Aspergillus spp. (R12)	210±0.333	89.4
Aspergillus spp. (R21)	160±0.667	60.1
Aspergillus spp. (R22)	160±0.882	59.1
Penicillium spp. (R6)	90±0.333	48.6
Penicillium spp. (R9)	111±0.577	50.7
Penicillium spp. (R15)	161±0.577	61.7
Penicillium spp. (R19)	90±0.333	48.2
Penicillium spp. (R20)	150±0.882	58.7
Talaromyces spp. (R4)	230±1.453	90.5
Trichoderma spp. (R11)	241±0.577	94.2
Rhizopus spp. (R13)	150±0.882	57.3
Phomas pp. (R14)	140±0.333	54.7
Trichophyton spp. (R16)	110±0.333	50.2
Curvularia spp. (R17)	99±0.577	48.5
Fusarium spp. (R18)	230±1.732	92.6
Abiotic control	-	0.3

DISCUSSION

The study indicates that members of the genera isolated, have the capacity to grow and survive in environments that are extremely contaminated with hydrocarbons. Furthermore, the ability of the fungi to secrete a wide range of extracellular enzymes into their growth environments have been advanced as an explanation for their capacity to grow on a wide range of carbon sources. These results were similar to those of Machido *et al.* (2014) who isolated ten fungal genera consisting of *Aspergillus*, *Penicillium*, *Fusarium*, *Curvularia*, *Trichoderma*, *Nigrospora*, *Microsporum*, *Rhizoctonia*, *Trichophyton* and *Geotrichum*. Ezeonuegbu *et al.* (2022) also isolated four genera consisting of *Aspergillus*, *Penicillium*, *Fusarium* and *Tricoderma* from refinery effluent while Uzo *et al.* (2009) also reported similar results while working with petroleum-contaminated soils.

In this study, it was observed that *Talaromyces*, *Aspergillus*, *Trichoderma* and *Fusarium* species tolerated the 50 mg/L concentration each, of naphthalene, phenanthrene and pyrene within the 21 days degradation period compared to other isolates. The dry weight of fungal biomass obtained at the end of the degradation period was also evidence to the tolerance ability of these isolates to the PAHs, as appreciable amount of dry fungal biomass was obtained. The ability of these fungal species to grow and produce measurable amounts of biomass in the presence of PAHs, might indicates their ability to utilize the PAHs as source of carbon and energy for their cellular growth. The rate at which the isolates degraded these PAHs ranged from naphthalene > phenanthrene > pyrene. Low molecular weight (LMW) PAHs are typically more readily degraded or metabolized by wider range of fungi than high molecular weight (HMW) PAHs due to their smaller molecular size, simpler chemical structure and high solubility in water compared to HMW PAHs. These make them easily accessible to enzymes, allowing for quicker and more efficient degradation (Patel *et al.*, 2020). The ability of these fungal species to effectively tolerate PAHs could be due to a combination of several factors such as ability to effectively colonize and persist in contaminated environments by strong biofilm formation on various surfaces which can protect the cells from environmental stress and increase substrate availability, high metabolic diversity including the ability to produce extracellular enzymes including laccase, manganese and lignin peroxidase as well as cytochrome p450 mono-oxygenase and di-oxygenases and secondary metabolites that can further enhance PAH degradation (Mohammed *et al.*, 2014), adaptability to a wide range of environmental conditions including low pH, high salinity, and high temperatures, ability to degrade PAHs through multiple pathways including oxidative and reductive pathways and higher growth rate (Deshmuk *et al.*, 2016). The loss of minute amounts of PAH over time in control samples observed, could be due to abiotic processes including photo-degradation and evaporation (Seopela *et al.*, 2021).

CONCLUSION

The study provides original data about the potential of 22 ascomycete's fungi isolated from a petrochemical refinery effluent to tolerate 50 mg/L each of naphthalene, phenanthrene and pyrene. Out of the 22 fungi isolates screened, only 4 were found to have high tolerance to this concentration of PAHs. They were identified as *Talaromyces*, *Trichoderma*, *Aspergillus* and *Fusarium* species. The isolates have proven their usefulness in bioremediation of PAHs. Hence, the current study has established and extended knowledge of the degradation of PAHs by fungi from the Ascomycota division. It has also provided information that can be useful in developing and improving bioremediation technologies aimed at decontaminating PAH-contaminated environments including soil and water.

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