Biodegradation Potentials of Fungi Isolated from Hydrocarbon-Polluted Soil of Umungede, Owaza in Ukwa West Local Government, Abia State, Nigeria

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DOI: 10.22178/pos.117-37

LCC Subject Category: QH1-278.5

Received 26.04.2025
Accepted 24.05.2025
Published online 31.05.2025

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Abstract. Petroleum hydrocarbon contamination from the oil and gas industry's operations, including oil spills, tank leaks, lubrication, petroleum extraction, transportation, and services, is one of the major environmental issues affecting the world today. The bioremediation and degradation of hydrocarbon pollutants from the environment have been accomplished using various methods, including mechanical and chemical procedures; however, some of these processes are typically costly and may have negative environmental impacts. This research is designed to isolate, characterise, identify and determine the ability of fungi to degrade hydrocarbons present in crude oil while quantifying their degree of effectiveness. The Soil sample used in this research was aseptically collected from Umungede, Igiriukwu, in Owaza village of Ukwa West LGA, Abia State, and sent to the research laboratory. Growth and degradation studies were conducted over a time course using standard methods, including the gravimetric method of estimation and gas chromatography-mass spectrometry (GC-MS) analysis. The biodegradation studies were conducted over a 15-day period, and the initial and final total petroleum hydrocarbon concentrations in the various setups, which contained the isolated fungi, crude oil, and growth media, were calculated. The results of the screening revealed that the yeast *Pichia kudriavzevii/Issatchenkia orientalis, more commonly known as Candida krusei, had the highest percentage of degradability potential, at* 98.23%. The Aspergillus niger closely followed these strains, XM_MG659649.1, MW_186673.1, and MT_729936.1, which also showed a very high degradation potential of 96.56%, 91.66%, and 94.26%, respectively. *Aspergillus terreus* also demonstrated a good degradation ability, but it was the least effective among the organisms studied in this research setup. Based on this study, it can be summarised that fungi are highly effective and efficient at removing hydrocarbon pollutants from sediments, oil, and water. However, since the potential of fungus has not yet been fully realised, further research is advised, particularly in the contemporary proteomic and genomic era.

Keywords: Bioremediation; Biodegradation; mycoremediation; petroleum hydrocarbon; petroleum pollution; fungal purification; remediation; hydrocarbon breakdown

INTRODUCTION

Petroleum hydrocarbons (PHs) have naturally contaminated the environment since ancient times, but artificial oil spills have increased in frequency recently [5]. Many creatures, including humans, are extremely poisoned by hydrocarbon molecules and petroleum compounds in high concentrations. Ninety percent of the estimated 1.7 to 8.8 million metric tons of oil that are released into the world's soil and water each year are directly tied to human actions, such as the intentional disposal of waste. Additionally, according to [8], 30% of oil spills are thought to end up in freshwater systems.

Although several physical, chemical, and biological processes can be employed to remove hydrocarbons, including dispersion, dilution, sorption, abiotic transformation, and volatilisation, biodegradation treatment remains the most promising method for cleaning up pollutants [17, 21, 27]. The type of pollutants and their properties (physicochemical nature and toxicity), the characteristics of the contaminated site (the source of the pollution, the site's nature, and the type of pollution (old or recent) are some of the factors that influence the choice of remediation techniques. Only after assessing the type of pollutant, associated risks to humans and the environment, treatment feasibility, and anticipated efficacy is the polluted site treated [15, 19, 22].

The application of chemicals such as cleaners, dispersants, biosurfactants, demulsifiers, and soil oxidisers is the most popular chemical method for cleaning up hydrocarbons in polluted areas. However, due to their high cost, potential for secondary pollution, and toxicity to various ecosystems, many people are concerned about their use [25].

The most effective way to remove various xenobiotic pollutants, including hydrocarbons, from the environment is the use of natural microorganism populations in bioremediation procedures [28]. This method has several advantages over other approaches. It is an economical, effective, and natural approach [22]. Additionally, it converts hydrocarbons into less hazardous molecules through metabolic and enzymatic processes.

The process of bioremediation converts petroleum hydrocarbon spills into innocuous substances by utilising the metabolic capacities of microorganisms, such as bacteria and fungi [30]. The pollutant in sediment, water, or soil is either broken down, bioaccumulated, absorbed, or bioadsorbent by these bacteria [20].

The application of microorganisms that break down petroleum hydrocarbons has drawn the greatest attention of all the methods for cleaning up petroleum pollution that have been explored [16]. The ecosystem and public health are at risk due to petroleum hydrocarbon pollution [12]. The toxicity of petroleum hydrocarbons is the reason why the cleanup of the spill requires rapid and urgent care. Crude oil has a detrimental impact on soil organisms and agricultural output, according to [1]. Additionally, it has been demonstrated that crude oil lowers plant productivity and germination [2].

Isolating and identifying possible hydrocarbon-degrading microorganisms, such as fungi, in crude oil-polluted areas in Owaza, the southeastern part of Abia State, Nigeria, has become crucial due to the economic feasibility and efficacy of bioremediation. For example, eight fungal isolates from petroleum-polluted soil were found to be hydrocarbon-degrading in a study by [29]. These isolates include *Aspergillus Versicolor, Aspergillus niger, Aspergillus Flavus, Syncephalastrum spp., Tricoderma spp., Neurospora Sitophila, Rhizopus arrhizus,* and *Mucor spp.*

According to earlier reports, some of these fungi degrade hydrocarbons. Because they can grow in stressful environments and secrete extracellular enzymes, fungi have shown promise in the bioremediation of contaminated environments [6]. Additionally, there is a specialisation in the hydrocarbon components that bacteria break down. Fungi can break down certain hydrocarbon components that bacteria cannot break down, whereas some bacteria can break down hydrocarbon components that fungi may find challenging. According to [6], fungi are beneficial to the field of bioremediation because they can release extracellular enzymes that facilitate the biodegradation of petroleum hydrocarbons. The isolation and identification of hydrocarbon-utilising (degrading) fungi in soil that has been contaminated by oil in the southeast of Nigeria is the main goal of this investigation.

Laccases, manganese peroxidases, and lignin peroxidases are examples of active broad-specificity oxidoreductase enzymes that are released into the environment during the mostly extracellular process of fungal hydrocarbon breakdown [7]. Although lignin, a cross-linked phenolic polymer, is the primary phenolic component that these enzymes break down in nature, their poor specificity also enables them to break down other phenolic compounds, including those present in petroleum hydrocarbons.

Petroleum pollution is becoming a major global environmental issue that can have detrimental effects on both the environment and human health. Both natural and human-made sources contribute to environmental pollution. For industrial productivity, almost every industry uses clean water from rivers, seas, boreholes, and/or the ocean. Industrial facilities like electricity, desalination, nuclear, and water treatment facilities

are at risk if oil spills contaminate a large number of these water bodies. This is due to the high cost and complexity of treating water contaminated by heavy oil [24].

Spills of highly concentrated oil are very difficult to clean up. Similarly, cleaning oil spills requires substantial amounts of money to achieve a specific level of cleaning efficacy [3]. As a result, the final product of the cleaning process could not be entirely efficient. The loss of the actual crude oil, as well as the money that could have been made economically from the oil spill, are some consequences of oil spills [24]. As the majority of wildlife reserves, ranches, mangrove and swamp areas, water springs, and water-related leisure activities essentially disappear, tourism is also severely impacted. The environment's safety and health are also in danger. These factors have necessitated the conduct of this research to explore alternative methods for removing this contamination from the soil and preserving the environment in its original state beyond the application of traditional techniques. These traditional techniques frequently cause this pollutant to change from one harmful form to another [24]. The overall goal of this study was to find, define, isolate, and quantify the extent to which fungi are capable of hydrocarbon degradation.

METHODS

Sample collection. Crude oil-polluted soil samples were collected from the oil field in Umungede village, Owaza community, within Ukwa West Local Government Area. Owaza lies between Latitude 4°57'14.44" N and Longitude 7°14'21.37"E. This soil has historically been contaminated by petroleum oil spills resulting from industrial activity in the area. This community has been host to Shell Petroleum Development Company (SPDC) since 1958 and, as such, holds records of long-term crude oil spillage and pollution. The soil sample was collected randomly from different sites on the location, from a depth of approximately 0 to 5 cm in the top layer of soil, placed into a clean and sterile black polythene bag, and transported to the Labdx laboratory at Ahiaeke, Umuahia, Abia State, for chemical analysis.

Isolation of the fungi organisms. The Sabouraud Dextrose Agar (SDA) was prepared according to the manufacturer's instructions, and serial dilution was carried out on the soil sample to a concentration of 10^{-5}. The diluted sample from the third and fourth test tubes was used for the inoculation and subsequent isolation of the fungal organisms. The spread plate method of enumeration was employed in this isolation, and it was performed cautiously in a laminar flow hood to ensure a sterile environment. 0.1ml of the diluted oil-polluted soil sample from the 3rd and 4th tubes were placed on the centre of the already prepared SDA plate, four plates to each tube. Two plates were left as control. A sterile L-shaped glass spreader was used to distribute evenly across the surface of the agar in a circular motion. The plates were rotated in both clockwise and anticlockwise positions to ensure a uniform spread of the inoculum. They were then allowed to set for a while and subsequently incubated in an upside-down position at 36 °C for 5 days. After incubation, the plates were observed for fungal growth, and those showing distinct colonies were recorded.

Subculturing of the fungal organisms. The subculturing of the resultant fungal organisms was done to obtain a pure culture of each. Eight fresh SDA media plates were prepared as previously described and labelled appropriately, ready for subculture. In the laminar flow hood, a Bunsen burner was lit, and a sterile loop was used in this inoculation. The loop was flamed in the Bunsen burner, allowed to cool slightly, and then used to touch the surface of the individual fungal organism gently. It was transferred to a fresh plate by streaking gently in a zig-zag motion on the surface. This procedure was repeated for each of the individual colonies obtained. The plates were inverted and incubated at 28°C for 3 days, after which the plates were observed and used for further examinations. This procedure was replicated, stored in slants and sent out for biodegradation examinations.

Cultural characterisation identification. Fungal isolates were characterised and identified by their cultural features. To identify isolated fungal organisms, the colonies were observed after incubation for their size, growth rate, texture, colour, shape, exudate formation, odour and elevation. The observations were recorded.

Morphological characterisation and identification. This was carried out under a microscope. On a glass slide, a portion of the well-grown, three-day-old pure culture was submerged in 0.1 ml of 95% ethanol. After letting the ethanol evaporate, the specimen was covered with a cover slide and

stained with lactophenol blue. Following a microscope examination, the specimen's morphological characteristics were noted and contrasted using the reference materials supplied by [23].

Screening of Fungi for hydrocarbon degradation. Hydrocarbon Biodegradation Studies and TPH (Total Petroleum Hydrocarbons) Extraction: Growth and degradation studies over a time course were carried out using the [18] method, 2 ml of raw crude oil sample (as a sole source of carbon and energy)/ 98 ml sabouraud Dextrose Broth was put in a 250 ml flask. After that, a 5 mm disk from the mycelia of the isolated 7-day-old fungal colony was added to the liquid broth. The mycelia of the separated fungi were not added to the control flasks, and the initial total hydrocarbon content (TPH) was measured and noted.

All flasks were placed in an incubator set at 25°C and coated with cotton wool that was not absorbent. At regular intervals, the flasks were physically shaken to ensure that the contents were sufficiently mixed and uniform. The ultimate total petroleum hydrocarbon (TPH) content was determined by monitoring the experimental setup for 15 days [15]. On the 15th day, the flasks were removed, and 1% 1N HCL was added to cease microbial activity. 50 ml of culture broth and 50 ml of petroleum ether: acetone (1:1) were combined in a separating funnel to extract crude oil, and the mixture was shaken quickly to create a single emulsified layer.

Three layers formed after acetone was added, and the mixture was gently shaken to disrupt the emulsification. Petroleum ether, crude oil, and acetone will make up the top layer; clumping cells will form the intermediate layer; and soluble acetone water will be present in the bottom aqueous layer. The top layer, which contained acetone and petroleum ether, was removed into a clean beaker, while the bottom two layers were sorted out. To eliminate moisture, the extracted oil was run through anhydrous sodium sulfate. On a water bath set at 70°C, the petroleum ether and acetone were evaporated to around 1 millilitre. Weighing the amount of oil in a tared beaker allows for the gravimetric measurement of the amount of oil that remains after biodegradation. The percentage deterioration of the crude oil was calculated using the methodology outlined in [13]. The percentage of degradation is equal to a-b / an x 100, where an is the weight of the crude oil under control and b is the weight of the crude oil that is left in each instance.

Once the amount of oil in a tared beaker was weighed, two millilitres of methylene chloride were used to rinse the beaker twice. After the rinses were poured into the vial, gas chromatography using a Tc-5 capillary column (length: 30 m, id: 0.24 mm) was used to measure the amounts of total n-alkanes and aromatics. Helium was supplied as the carrier gas at a steady rate of 1.5 ml min-1, with an interface temperature of 280°C and a column pressure of 100 KPa. To allow lake eluting chemicals to leave the column, the temperature program was maintained at 280°C for 10–20 minutes after starting at 60°C and increasing by 10°C every minute. The injector temperature was kept at 280°C, and the injection volume was 2 μL.

RESULTS AND DISCUSSION

Hydrocarbon Biodegradation Studies and TPH (Total Petroleum Hydrocarbons) studies. The results of the study on the degrading ability of fungi isolated from hydrocarbon-polluted soil on crude oil, analysed using gas chromatography, are presented in Tables 1–2. Table 3 shows the initial and final concentrations of selected hydrocarbons.

Table 1 – Isolation and Identification of Microorganisms; Cultural Identification

	1F	2F	3F	4F	5F	6F
Size	Moderate growth	Moderate growth	Moderate growth	Moderate	Heavy (Large surface)	Non-heavy
growth rate	Moderate	Moderate	Moderate	Moderate	Rapid	Slow
Texture	Powdery	Powdery	Smooth	Watery	Thread-like	Powdery
Colour	Blue-green	Blue-green with smooth white edges	Pale brown with smooth white edges	Creamy-white with translucent edges	Brown	Creamy white

	1F	2F	3F	4F	5F	6F
Shape	Circular	Irregular	Irregular	Circular	Circular	Circular
Exudates	Absent	Absent	Absent	Present	Absent	Absent
Odour	Absent	Mild	Slightly pungent	Absent	Mild	Mild
Elevation	Raised	Raised and rough	Flat	Absent	Dome-like	Raised centre

Table 2 – Morphological/microscopic identification

	1F	2F	3F	4F	5F	6F
Hyphae	Septate	Septate	Aseptate	Aseptate	Aseptate	Aseptate
Conidia	Blastospores	Chlamydospores	sporangiospores	Sporangiospores	Blastospores	conidiospores
Budding	Absent	Absent	multilateral	Present	Absent	Absent
Suspected organisms	Mould	Mould	Yeast	Yeast	Mould	Mould

Table 3 – Initial and final concentrations of selected hydrocarbons

Sample code	Day one TPH concentration, ppm	Day 15 TPH concentration, ppm
Setup 1	1538.54	88.30
Setup 2	513.08	51.99
Setup 3	1221.61	21.59
Setup 5	901.00	75.13
Setup 6	1388.97	47.79
Setup control	1043.99	172.16
Raw crude oil	7.09×10^4	7.09×10^4
MEAN	1101.20	76.16
MEAN DIFFERENCE		1025.04
	t-value	6.747
	p-value	0.001

With the t-value = 6.747 (greater than the critical value of 4.587at df =10) and the p-value =0.001(<0.05), there is a significant difference in TPH concentration between day 1 and day 14 treatments. Raw crude oil is excluded from the analysis because it has a mean difference of zero and is also considered an outlier in both groups. The degree of freedom (df) for the paired test was calculated using the formula df=n1 + n2 -2.

Table 4 shows that sample setup 3 had the maximum percentage degradability potential of 98.23%, while raw crude oil had a value of zero. Additionally, all setups, excluding raw crude oil, had a high percentage degradability potential of greater than 80%.

In this study, six fungal isolates were isolated from the oil-contaminated soil samples. Aspergillus represented the highest percentage of appearance, at 90%, as evident by the percentage of the species. The rest of the species appeared in low percentages.

Table 4 – Percentage degradability potential of isolates on TPH after 15 Days

No	Sample code	Degradability potential of isolates on TPH (%)
1	1F	94.26
2	2F	89.87
3	3F	98.23
4	5F	91.66
5	6F	96.56
6	Control	83.51
7	Raw crude oil	0.0

The isolated fungi are then identified based on their morphological characteristics, including colony colour, texture, and growth patterns in Table 1. For more precise identification, a microscopic examination is conducted to observe features such as spore structures and hyphal characteristics, as seen in Table 2.

The study on the degrading ability of fungi isolated from hydrocarbon-polluted soil provides valuable insights into their potential for bioremediation. Gas chromatography analysis revealed significant variations in hydrocarbon degradation efficiency among the fungal isolates. The results indicate that certain fungal strains are particularly effective in reducing the concentration of hydrocarbons in crude oil. As seen above, the t-value of 6.747 is greater than the critical value of 4.587 at df=10, and the p-value of 0.01 (≤0.05) indicates a significant difference in the TPH concentration between the day 1 and day 15 treatments.

The observed differences in degradation rates seen in Table 1 align with previous research, which has shown that fungal species can vary widely in their ability to degrade hydrocarbons [7]. For instance, the high degradation efficiency of setup 3 (sample 3), which achieved an over 98% reduction in hydrocarbon concentration, is consistent with findings [26], who noted that certain fungi possess robust enzymatic systems capable of extensive hydrocarbon degradation. Aspergillus spp. are among the most frequently reported filamentous fungi that can grow on hydrocarbons [14] and significantly degrade it and, therefore, should be employed in hydrocarbon-contaminated site remediation [10]. The high degradative capacity of Aspergillus spp. also aligns with the research results [9].

The fungi Pichia kudriavzevii/Issatchenkia orientalis, also known as Candida krusei, as determined by molecular analysis, was shown to be the fungus with the highest bioremediation efficiency in this research work, as seen in Figures 3 and 4. The research by [4] on the biodegradation of candida strains strongly agrees with the findings of this work. Candida krusei was seen to be one of the organisms with the highest ability to degrade hydrocarbon.

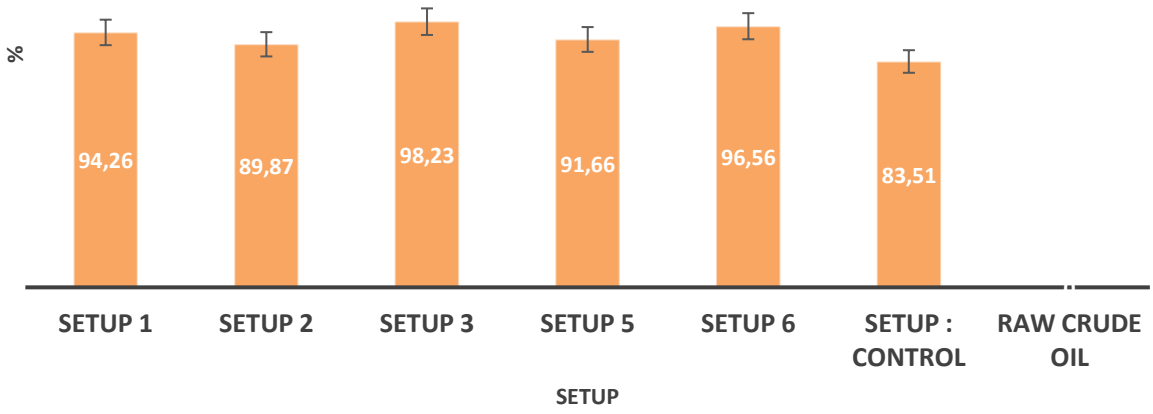

Figure 1 – Bar Chart Showing Percentage Degradability Potential of the Samples on TPH After 15 Days

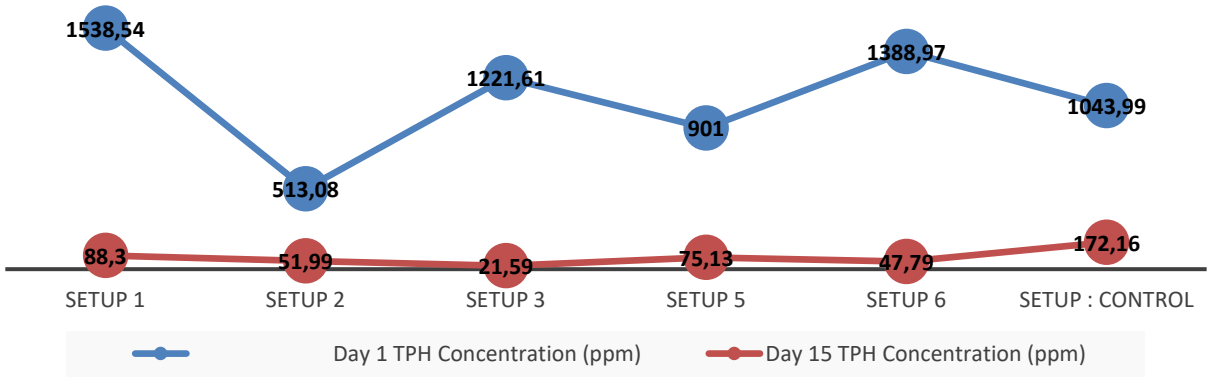

Figure 2 – Line Graph Showing Summary Result Of Tph Concentration Of The Samples On Day 1 (Initial, 23/5/2024) and Day 15 (Final, 05/6/2024)

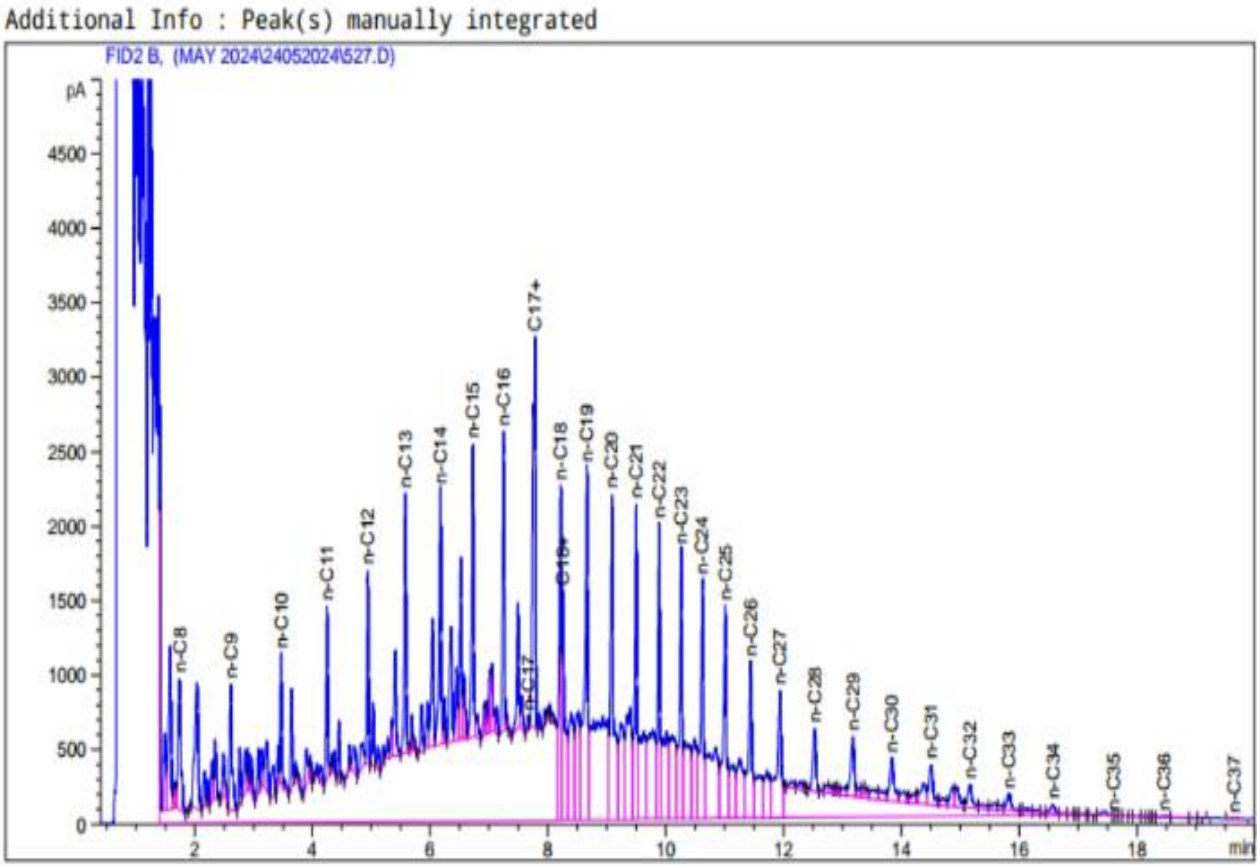

Figure 3 – Gc-Ms Result Showing The Initial Degradation Of Another Isolated Fungus (Sample 3)

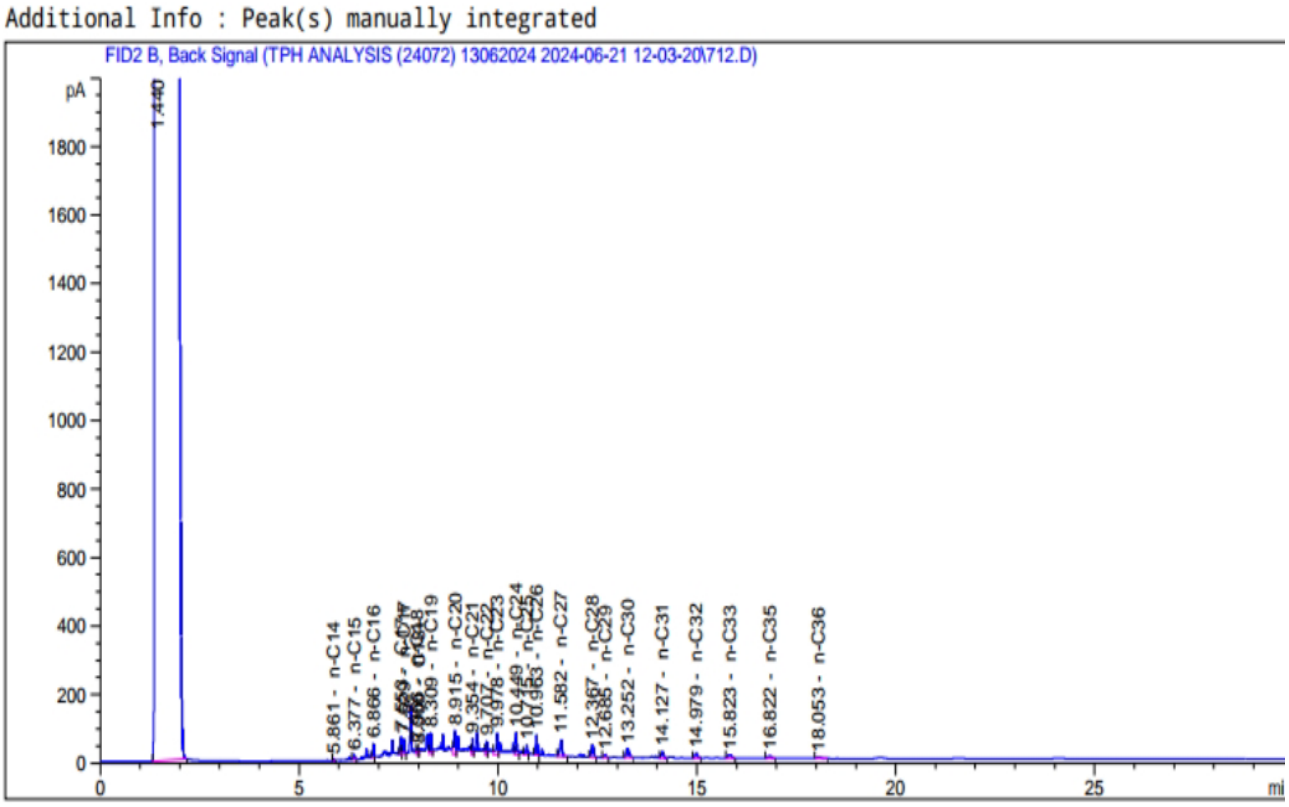

Figure 4 – GC-MS Result Showing the Final Degradation of Another Isolated Fungus (Sample 3)

Although sample 3 organism proved to have the highest degradation efficiency, every other organism isolated in this research work exhibited a very high level of effectiveness, with over 80% degradation. This goes a long way toward presenting these organisms as possible substitutes for their synthetic and harmful counterparts if properly optimised.

The findings suggest that the isolated fungi can potentially be used in bioremediation strategies for oil-contaminated environments. However, further research is needed to optimise conditions and enhance the degradation capabilities of less effective strains. Understanding the mechanisms of degradation and the specific pathways utilised by these fungi will be crucial for improving bioremediation techniques. Overall, this study contributes to the growing body of knowledge on fungal bioremediation, supporting the use of fungi in managing hydrocarbon pollution and emphasising the need for continued research to maximise their effectiveness.

CONCLUSIONS

The study demonstrated that fungi isolated from hydrocarbon-polluted soil were effective in degrading crude oil, as evidenced by gas chromatography results. Various fungal isolates exhibited different levels of degradation efficiency, with some strains showing significant reductions in hydrocarbon concentrations. The use of gas chromatography was crucial for accurately assessing the extent of hydrocarbon removal and identifying the most effective fungal strains.

In conclusion, the results provided valuable insights into the capabilities of fungi for bioremediation of hydrocarbon pollutants, supporting their use in environmental management strategies. The effective use of gas chromatography in this study reinforced its role in accurately measuring and comparing the degradation abilities of different fungal strains..

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