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Research Article

Fungal Flora Associated with Petroleum-Impacted Farmland in Borobara Community, Ogoni-Land, Niger Delta Area of Nigeria

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Abstract

Indigenous fungal species of hydrocarbon-impacted soil were isolated and characterized using standard techniques. Soil samples were collected from Borobara community, Tai L.G.A, Rivers State, an oil exploration zone of Nigeria's Niger Delta and transported to the laboratory for analyses. Nine (9) fungal isolates were screened for hydrocarbon biodegradation potentials in a shake-flask culture incorporated with 1% crude oil (hydrocarbon) and redox reagent (2% 2,6-dichlorophenol indophenols) for 14 days. Eight fungal isolates showed potentials for hydrocarbon degradation employing colour change, optical density and total hydrocarbon content (THC) depletion as monitoring parameters for 14 days. Five of the isolates exhibited the fastest onset and highest extent of biodegradation and were identified as: *Aspergillus* sp., *Yarrowia* sp. (1), *Yarrowia* sp. (2), *Zygorrhinchus* sp. (1) and *Zygorrhinchus* sp. (2). In this study, it was observed that the highest THC biodegradation efficiency was exhibited by Yarrowia sp. (1) and *Zygorrhinchus* sp. (2). This indicated that these fungal species are efficient hydrocarbon degraders. Thus, they can be considered in strain development programme for bioremediation of hydrocarbon-polluted farmlands (soil).

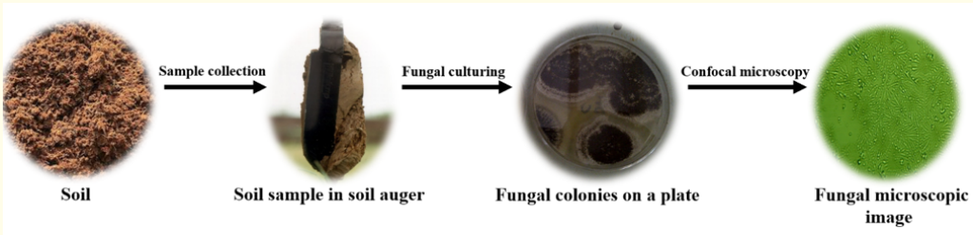
Keywords: Hydrocarbon; Fungal Isolates; Borobara Community; Niger Delta

Introduction

The exploration and exploitation practices and vandalization of oil installations in Nigeria lead to environmental pollution, especially in the Niger Delta and Southern parts of the country (Salu, 1999). These spills have the largest economic impact as they harm, to a large extent, the ecosystem. The ability of microorganisms to transform and degrade many types of pollutants in different matrices (soil, water, sediments, and air) has been widely recognized during the last decades. In the last years, various ecosystems have been changed by the growing influence of human activity. As a result, many people have become aware of the need to protect ecosystems as well as to evaluate the damage caused by contamination. During the previous years, the frequency and risk of oil pollution has led to extensive research. Most of the hydrocarbon pollutants go into the ecosystem via leaks from coastal oil refineries. This fact increased the interest of scientists in investigating oil distribution and its fate in the environment. Approximately five million tons of crude oil and refined oil enter the environment each year because of anthropogenic activities, such as oil spills. Past analysis of reported oil spills indicated that most of the oils come from tankers, barges, and other vessels as well as from land pipeline spills. Extensive changes in marine, as well as terrestrial ecosystems resulting from the grounding of the Exxon Valdez (1989), the Nahodka oil spill, the Erica spill (1999) and the Prestige spill (2002), have recently increased the attention of environmentalists, chemists, biotechnologists, and engineers.

Promoting environmental methods in the process of cleaning oil-polluted sites is an intense research area in today's research space as these methods are less expensive and do not introduce additional chemicals to the environment. Compared to physiochemical methods, bioremediation offers a very feasible alternative for an oil spill response. This technique is considered an effective technology for the treatment of oil pollution. One reason is that most of the molecules in crude oil and refined products are biodegradable. Biodegradation is most often the primary mechanism for contaminant destruction including petroleum contaminants (Leahy and Colwell, 1990). Bioremediation, the enhancement of natural biological degradation processes, has been proposed for cleanup of oil-spills in soils (Kerry, 1993) as cost-effective technology of removing contaminants. There have been many studies on microbial oil degradation in soils in which hydrocarbon-degrading microbes have been detected (Kerry, 1990; 1993; Aislabie., *et al*. 1998). The activity of microbial types naturally present can be enhanced by bioremediation techniques which include increased aeration of the polluted area and nutrient additions (Ivshina., *et al*. 1998; Christofi and Ivshina, 2002). The purpose of the study was to isolate the indigenous fungal flora associated with crude oil-contaminated soil

in Borobara Community, Tai Local Government Area, Ogoniland, Rivers State of Nigeria and evaluate them for hydrocarbon degradation potential. Soil samples were collected with soil auger, and cultured with appropriate media in plates, followed by visualization of the fungal colonies by confocal microscopy (Scheme 1). Indigenous fungal isolates capable of degrading hydrocarbon were then identified. A baseline data for on-site remediation of a hydrocarbon polluted soil using mould strains was obtained to create a biodata on our indigenous fungal isolates capable of hydrocarbon degradation and ultimately determine conditions that mediate accelerated attenuation.



Scheme 1: Illustration of soil sample collection, fungal culturing, and visualization of the fungal colonies by confocal microscopy.

Materials and Methods

Source of soil sample

Two hundred grams of the oil-contaminated soil sample used for the isolation was collected from Borobara community, Tai Local government area, Ogoniland, Rivers State of Nigeria using a soil auger. Ogoniland region covers 1,000km^2 in Rivers State, southern Nigeria and it has been the site for oil industry operations since the late 1950s, Ogoniland also has a tragic history of pollution from oil spills. Samples from the site were collected from the polluted area, 500 and 1000m away from the polluted area and transported in plastic ziplock bags to the laboratory for analysis.

Media and chemicals

Sabouraud's dextrose agar (SDA) and Czapek medium containing sucrose (30g/1), $NaNO_3$ (3g/1), K_2HPO_4 (1g/1), $MgSO_4.7H_2O$ (0.5g/1), KCl (0.5g/l), $FeSO_4.7H_20$ (0.01g/l) and agar (15g/l) were used for the isolation of the fungi. Bushnell-Haas broth containing $MgSO_4$ (0.2g/l), $CaCl_2$ (0.02g/l), KH_2PO_4 (1g/l), K_2HPO_4 (1g/l), $FeCl_2$ (0.05g/l) and NH_4NO_3 (1g/l) was used for the screening test. Redox reagent (2% 2, 6-dichlorophenol indophenols), Tween 80 (0.1%) and crude oil (1%) were all incorporated into the broth. This method was previously used by and adopted from George-Okafor., *et al*. (2009).

Isolation of fungi

The oil-contaminated soil sample was homogeneously mixed and carefully sorted to remove stones and other unwanted soil debris using a 2.5 mm sieve. A 1g mass of the sorted soil sample was introduced into a test tube. After which 10ml of sterile water was added and the tube was shaken vigorously to obtain soil solution representing 1:10. A 1ml volume of the supernatant of the 1:10 solution was transferred into 9ml of distilled water representing 1:100. In the same way, 1:1000, 1:10000 and 1:100000 were made. A 0.1ml volume of the last three dilutions was spread onto Sabouraud's dextrose agar + chloramphenicol and Czapek agar + chloramphenicol plates respectively using a glass rod. The Petri dishes were rotated by hand to disperse the medium and the soil suspension (Soil-dilution Plate Method). The plates were incubated at room temperature (28 - 30ºC) for 3 to 5 days for Sabouraud's dextrose agar and 5 to 7 days for Czapek agar plates. The colonies were carefully and aseptically sub-cultured onto fresh Sabouraud's dextrose agar plates to obtain pure cultures for biodegradation screening. The pure isolates were maintained on Sabouraud's Dextrose agar slants.

Screening for biodegradation potential

A modified method by Desai., *et al*. (1993) was used for the screening test. Two agar plugs (1 cm^2) of a pure growth of each isolate were inoculated onto Bushnell - Haas broth (50ml/250ml Erlenmeyer flask) incorporated with sterile crude oil (1% v/v) and redox indicator (2%v/v). The control flask had no organism. Incubation was done at room temperature (28^{o}C - 30^{o}C) with constant shaking for 14 days. The aliquots in the flasks were monitored daily for color change, optical density, and THC concentration loss for 14 days.

Identification of isolates

Pure cultures of the potential strains maintained on Sabouraud's Dextrose agar slants were identified at the Environmental microbiology laboratory of the Department of Microbiology, University of Port Harcourt, Rivers State, using colonial morphologies, and microscopic characteristics.

Results and Discussion

Nine (9) fungal isolates were obtained from SDA and Czapek media, eight isolates showed potential for hydrocarbon degradation. Five of these potential microbial degraders exhibited the fastest onset and highest extent of biodegradation, and were identified as *Aspergillus japonicus*, *Yarrowia lipolytica* sp. (1), *Yarrowia lipolytica* sp. (2), *Zygorrhynchus* sp. (1) and *Zygorrhynchus* sp. (2) using their respective colonial morphologies and microscopic characteristics (Figure 1 - 6). This result conforms to the findings of Chaillana., *et al*. [5].

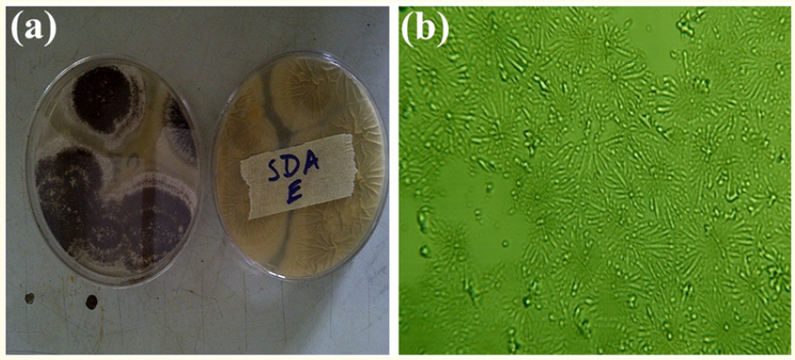


Figure 1: Photomicrograph and colonial appearance of *Aspergillus japonicas* x10 (2048 x 1536) on (a) a plate and (b) under a digital microscope.

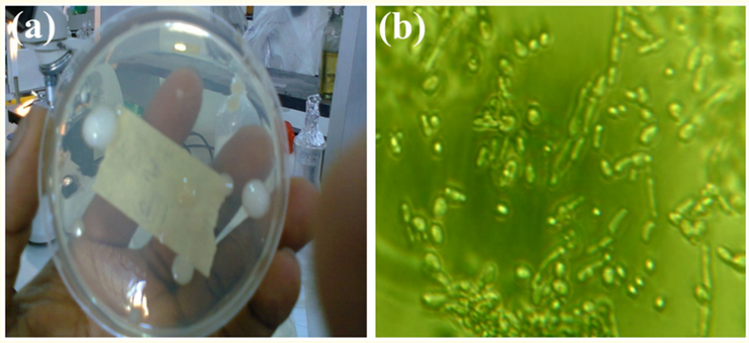


Figure 2: Photomicrograph and Colonial appearance of *Yarrowia lipolytica* sp. (1)x10 (2048x1536) on (a) a plate and (b) under a digital microscope.

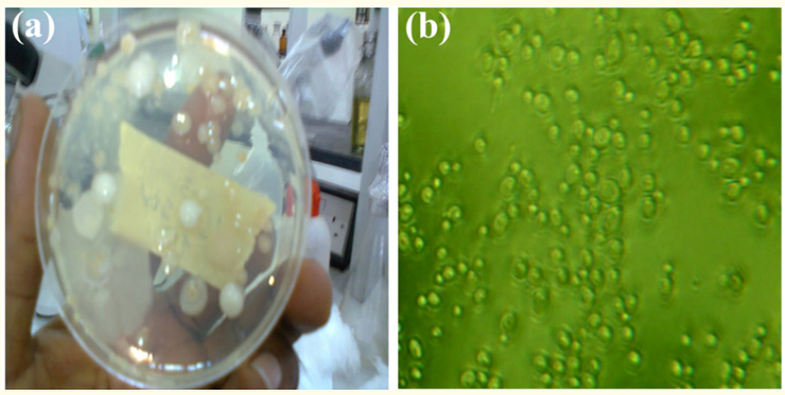


Figure 3: Photomicrograph and Colonial appearance of *Yarrowia lipolytica* sp. (2)x40 (640x480) on (a) a plate and (b) under a digital microscope

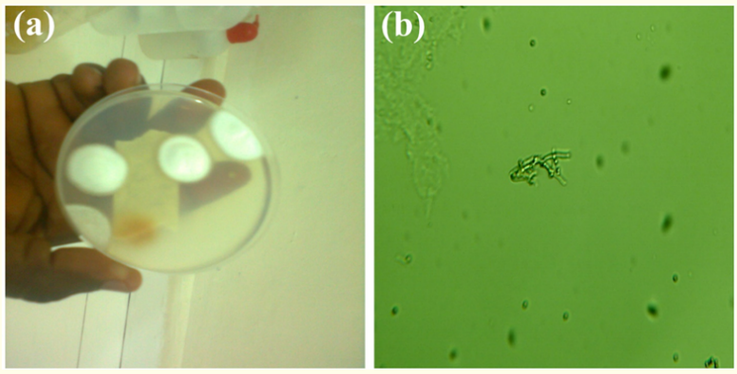


Figure 4: Photomicrograph and Colonial appearance of *Zygorrhynchus* sp. (1)x10 (2048x1536) on (a) a plate and (b) under a digital microscope.

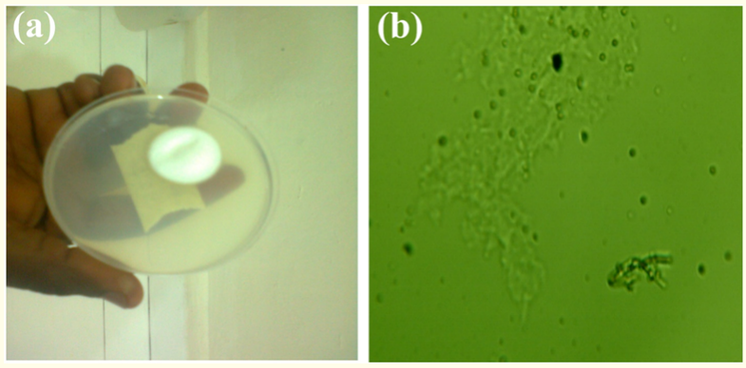


Figure 5: Photomicrograph and Colonial appearance of *Zygorrhynchus* sp. (2)x10 (2048x1536) on (a) a plate and (b) under a digital microscope.

There was progressive rise in the optical density of different experimental setups apart from the control, which was not inoculated (Table 1). It was observed that within 6 days of microbial inoculation, the increase in biomass as shown by optical density was highest in the option containing *Yarrowia lipolytica* sp. (1), followed by *Zygorrhynchus* sp. (2), *Geotrichum* sp. (1), *Yarrowia lipolytica* sp. (2), *Zygorrhynchus* sp. (1), *Aspergillus japonicus*, *Geotrichum* sp. (2), *Cladosporium* sp., and control was the least (Figure 7).

The biodegradation efficiency (>81%) exhibited by *Yarrowia lipolytica* sp. (2) (Table 2) within 14 days of incubation showed that this microbe posses specialized enzyme system thus synthesis of biosurfactant which enhanced hydrocarbon degradation.

High Biodegradation efficiency (>79%) exhibited by *Zygorrhynchus* sp. (1) (Table 2) within 14 days of incubation showed that cultural condition was appropriate for its growth as nutrients were readily available.

In this study, it was observed that a high biodegradation efficiency was exhibited by *Yarrowia lipolytica* sp. (1) and *Zygorrhinchus* sp. (2). This proved these fungal species to be efficient hydrocarbon degraders. Thus, they can be considered in the strain development programme for the degradation of oil polluted soil especially those located within the vicinity of the sampled sites.

Color change in the medium was due to microbial activities during the biodegradation screening as a result of the reduction of the indicator by the oxidized products of hydrocarbon degradation.

The reduction of the indicator also indicates increase in optical density following increase in absorbance (Figure 7).

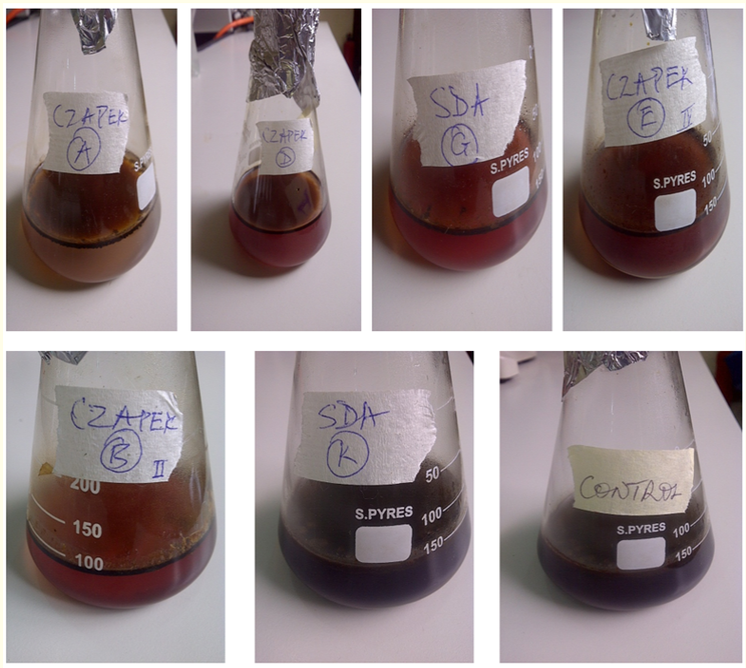


Figure 6: Photomicrograph of colour changes during degradation screening; *Yarrowia lipolytica* sp.1, *Zygorrhynchus* sp. (2), *Yarrowia lipolytica* sp. (2), *Zygorrhynchus* sp. (1), *Aspergillus japonicas*, *Fusarium* sp. respectively.(a) a plate and (b) under a digital microscope.

The high rate of hydrocarbon degradation by five fungal isolates (Figure 1 - 5) was as a result of their exponential growth and enzyme production responses during their active growth phases. This could be supported by the reports of Bogan and Lamar[4], which showed that extracellular ligninolytic enzymes of white rot fungi are produced in response to their growth phases. The result of total hydrocarbon content, which showed that the five isolates exhibited biodegradation efficiency above 80% also, confirmed their high degradation potentials. The utilization of hydrocarbons resulted to increase in cell densities[16] and optical densities with a concomitant gradual visual reduction in the oil layer and redox indicator (2,6 Dichlorophenol indophenols) and gradual disappearance of the oil with prolonged incubation. The color changes in the culture fluids in the experimental flasks within the 14-day incubation period further confirmed chemical changes of the hydrocarbon substrates which must have been precipitated by microbial enzymes[3]. It is evident from this investigation that hydrocarbon-degrading microorganisms could readily be isolated from soil (Scheme 1), without the need for time consuming traditional enrichment protocols. Further understanding of the metabolic processes of these organisms on the hydrocarbons will increase possibilities of developing models and strategies for removing hydrocarbon pollutants from oil-impacted environments.

Isolate	Day 1	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14
Fusaruim sp.	9.2	9.4	9.7	10.2	10.4	10.8	11.0	11.2
Zygorrhynchus sp. (1)	9.2	10.0	10.4	10.8	11.8	13.4	15.2	16.4
Geotrichum sp.	9.2	9.6	10.8	11.4	13.0	15.2	16.4	17.0
Cladosporium sp.	9.2	10.2	10.6	11.2	12.0	13.0	13.4	14.0
Aspergillus japonicas	9.2	9.8	10.2	10.8	11.4	11.8	13.0	14.4
Geotrichum sp. (2)	9.2	9.6	10.4	10.8	11.6	12.4	13.8	14.4
Yarrowia lipolytica sp. (1)	9.2	9.8	10.6	11.4	12.8	14.6	15.2	17.6
Zygorrhynchus sp. (2)	9.2	9.2	9.4	11.6	14.0	15.8	16.6	17.2
Yarrowia lipolytica sp. (1)	9.2	9.2	10.0	10.8	13.6	14.0	15.2	16.6
Control	9.2	9.2	9.2	9.2	9.2	9.2	9.2	9.2

Table 1: Optical density during fungi-mediated hydrocarbon degradation from 1-14 days.

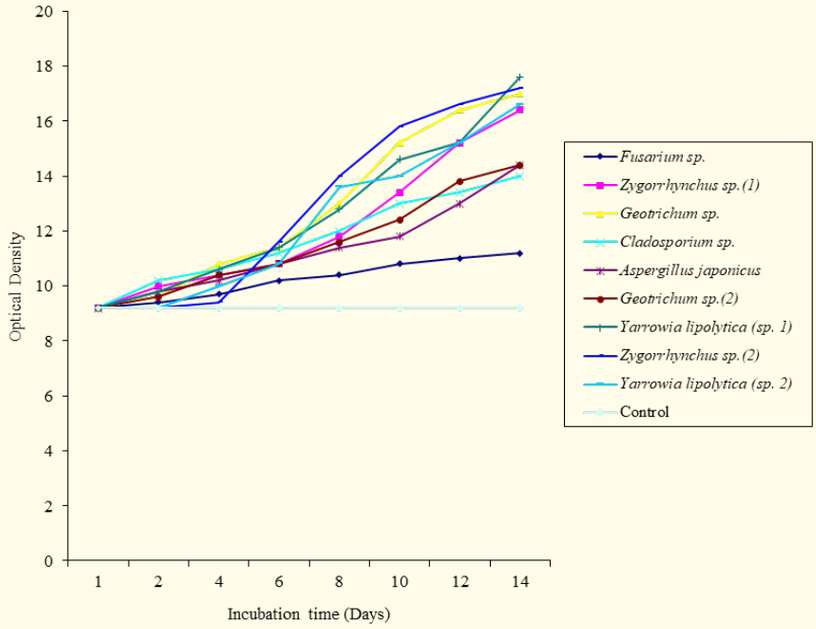


Figure 7: Optical density profile of fungal hydrocarbon degradation simulation from 1-14 days.

The increase in biomass is an indication that the biodegradative processes were driven by the inoculated microorganisms and available nutrients in the Bush-nell Hass medium (Nitrogen, phosphorus and petroleum hydrocarbon). From biodegradation experiment, it was observed that at baseline, the concentration of the total hydrocarbon in the medium was 8100 mg/ml (Table 2). In addition, on the 14th day following microbial inoculation, different percentages of biodegradation were observed. High biodegradation efficiency (>83%) exhibited by *Yarrowia lipolytica* sp. (1) and (>82%) by *Zygorrhynchus* sp. (2) as seen in Table 2 (Figure 8) within 14 days of incubation showed that the cultural conditions were very appropriate for their growth and biodegradation. In addition, 35%, 79%, 80.2%, 78.3%, 80% and 80% reduction in total hydrocarbon content was observed with *Fusarium* sp., *Zygorrhynchus* sp. (1), *Geotrichum* sp., *Cladosporium* sp., *Aspergillus japonicas*., *Geotrichum* sp. (2) respectively.

Isolate	Day 1	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14	% Deg.
Fusaruim sp.	8100	7850	7300	6905	6510	5900	5400	5265	35.0
Zygorrhynchus sp. (1)	8100	6130	5800	4800	2800	2100	1805	1665	79.4
Geotrichum sp.	8100	7500	6580	5860	4200	2605	1680	1597	17.0
Cladosporium sp.	8100	7080	6645	5775	4820	3900	3210	1755	78.3
Aspergillus japonicas	8100	7525	7010	6400	5877	5215	3688	1620	80.0
Geotrichum sp. (2)	8100	7680	6600	5540	4415	3533	2980	1620	80.0
Yarrowia lipolytica sp. (1)	8100	7600	8515	5912	4799	2945	1740	1350	83.0
Zygorrhynchus sp. (2)	8100	8100	7635	5757	3589	2680	1887	1440	82.2
Yarrowia lipolytica sp. (2)	8100	8100	7335	6519	3911	2813	1990	1485	81.6
Control	8100	8100	8100	8100	8100	8100	8100	8100	0

Table 2: THC values (mg/kg) from 1-14 days incubation of 9 fungal isolates in crude oil.

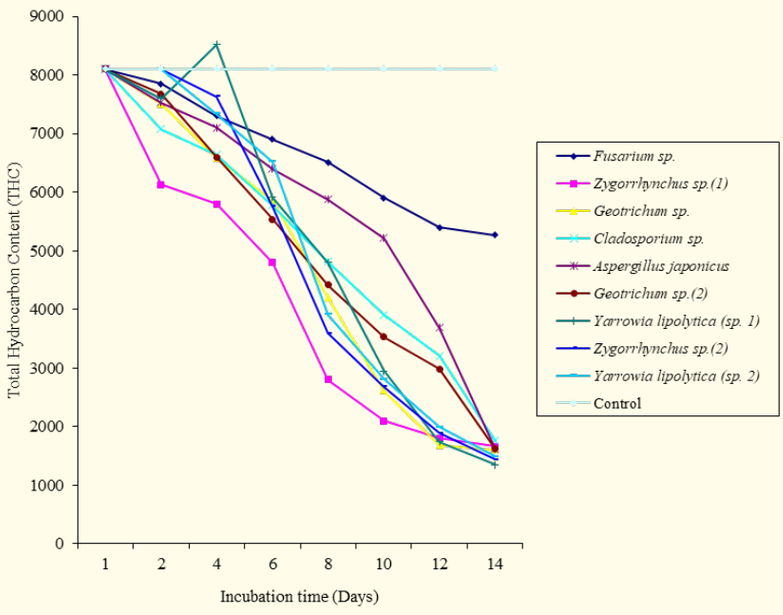


Figure 8: Hydrocarbon degradation profile of 9 fungal isolates from 1-14 days incubation in crude oil.

The hydrocarbon degradation ability of *Aspergillus japonicas* (>78%) was similar to the findings of George-Okafor., *et al.* [8] and April., *et al.* [2], which showed that this organism was among the sixty-four species of hydrocarbon-degrading filamentous fungi isolated from flare pit soils in northern and western Canada. It was observed from the 1st to the 14th day that the control set up which was not inoculated with microorganism recorded no significant reduction in total hydrocarbon content (figure 8). Similar results have been reported in previous studies by Nkwocha and Odokuma [15] as well as findings of Iheanacho., *et al.* [10].

Conclusion

Most existing studies have concentrated on evaluating the factors affecting oil bioremediation or testing favored products and methods through laboratory studies. The scope of current understanding of oil bioremediation is also limited because the emphasis

of most of these field studies and reviews has been on the evaluation of bioremediation technology for dealing with large-scale oil spills on marine shorelines. For now, our cultural method of identification cannot be over-emphasized. It serves as a tool in checkmating our molecular method of identification and our sources of genetic analysis. With the information obtained from this study, and the already established ploidy nature of our yeast species. Genetic engineering can be employed to increase the efficiency of our hydrocarbonoclastic microorganisms by exploiting the advantage of understanding of their individual features and capabilities, thereby increasing their gene dosage through hybridization or gene transfer mechanisms resulting to the production of more efficient hydrocarbonoclastic microbes. This research illustrates how microbiological methods significantly contribute to the knowledge of wide occurrence of effective hydrocarbon-degrading indigenous fungi that are not considered in bioremediation of environments highly contaminated by crude oil and petroleum products. Because of the complexity and new challenging methodologies involved in implementing bioremediation processes, many opportunities exist to further elucidate the application of our fungal isolates on an industrial scale. The biodegradation component of this study primarily focused on microbial isolation and investigation of biodegradation ability. Nonetheless, no research has been performed on detailed characterization of fungal nutrition and oxygen requirements. To increase the feasibility of the fungal isolates as possible commercial strains, future studies need to clarify the factors affecting their ability and efficiency of hydrocarbon and crude oil degradation, such as nutrient concentration, optimum temperature range, oxygen content, salinity and physical state of the oil.

Authors' Contributions

- CHINEDU CHRISTIAN IHEANACHO conceptualized/instigated the research idea and carried out all laboratory experiment.
- IKENNA LIGHT NKWOCHA developed the manuscript and proof-read the work.
- TEGA LILIAN ATAIKIRU assisted with laboratory analyses and supported during the monitoring programme.
- ALL authors' reviewed the manuscript and approved it.

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No funding was obtained for this research.

Competing Interest

There are no competing interests among the authors on this study.

Availability of Data and Material

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary material. Raw data that support the findings of this study are available from the corresponding author, upon request.

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