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UTILIZATION OF PETROLEUM HYDROCARBONS BY INDIGENOUS FUNGI ISOLATED FROM A PETROLEUM REFINERY EFFLUENT SITE IN NIGERIA

Odili[1], U.C., Ibrahim[1], F.B., Shaibu-Imodagbe[2], E.M. and Atta,[3*]H.I.
[1]Department of Water Resources and Environmental Engineering, Ahmadu Bello University, Zaria
[2]Division of Agricultural College, Ahmadu Bello University, Zaria
[3]Department of Microbiology, Ahmadu Bello University, Zaria
*Corresponding author: hiatta@abu.edu.ng

ABSTRACT
Soil contaminated with hydrocarbons harbors microorganisms that can utilize these hydrocarbons for their nutritional requirements. A large number of such microbes are fungi and bacteria. In this study, the potential of fungi isolated from soil contaminated with effluent from a petroleum refinery was assessed for the ability to degrade crude oil. The ability of the isolates to degrade crude oil was evaluated using standardized spore suspensions of the isolates in liquid and solid mineral salt media containing crude oil as the sole carbon source. The growth of the fungi on crude oil was monitored by determining the percentage degradation and hydrocarbon utilizing fungal counts. The fungal species isolated from the study site included: Madurella grisea, Monocillium sp., Aspergillus niger, Trichophyton megnini, Trichophyton tansurans, Aspergillus flavus, Fusarium* **sp., Actinomadura* **sp.***, Chrysosporium* **sp.***; two strains of Actinomadura sp. were isolated. The most efficient hydrocarbon degrader was found to be Monocillium sp., with a percentage degradation of 80% and the highest spore count of 8.5 x 10^7 spore/mL; Aspergillus niger performed almost as well as Monocillium* **sp.** *with a corresponding value of 70% and 6.1 x 10^7 spore/mL respectively. Based on the findings of this study, Monocillium* **sp.** *and Aspergillus niger are potential candidates for bioremediation of soils polluted with petroleum hydrocarbons.***
Keywords: Fungi, Petroleum, Biodegradation, Hydrocarbons, Isolation.

INTRODUCTION

Over the past several decades, man has grown more and more reliant on fossil fuels. Many of the comforts that man enjoy today are possible largely because of fossil fuels such as petroleum. This increase in use has resulted not only in an increased dependence but also an increase in petroleum-related pollution (McDonald, 2001; Onifade *et al*., 2007; Nwachi *et al*., 2013). The rate of oil spillage reported in the country since the commencement of oil exploration and development has been rising with a corresponding increase in petroleum production (Onifade *et al.,* 2007; Olabisi *et al*., 2009), there is, therefore, the need to remediate contaminated sites to protect human health and environmental ecosystem (McDonald, 2001).

Bioremediation refers to the use of organisms to degrade contaminants that pose environmental and human risks. Due to its safety, it has become an accepted remedy for cleaning polluted soil and water (Gupta and Pawan, 2016). Bacteria, fungi, yeast, and some algae are known to degrade aromatic hydrocarbons (Nilanjana and Preethy, 2011; Al-Hawash, *et al*., 2018), It is known that more than two hundred species of bacteria, fungi, and even algae can biodegrade hydrocarbon (Onifade *et al*., 2007). Compared to most bacteria, fungi adapt more readily to adverse environmental conditions and it constitutes more of the soil biomass than bacteria, depending on the soil depth and nutrient conditions and is an important component of the soil microbiota (Dayalan *et al*., 2011). Microorganisms produce enzymes in the presence of carbon sources that are responsible for attacking the hydrocarbon molecules, but lack of an appropriate enzyme will either prevent the attack or will act as a barrier to complete hydrocarbon degradation (Bijay *et al*., 2012). Many studies have been conducted on isolation and characterization of hydrocarbon degraders from oil spill sites (Onifade *et al.,* 2007; Olabisi *et al.,* 2009; AbdelRahman, 2011; Nilanjana and Preethy, 2011; Isaac, 2018; Orjiude, 2018), this study, therefore, aims at assessing the hydrocarbon-degrading ability of fungi isolated from soil contaminated with petroleum refinery effluent.

MATERIALS AND METHODS

Soil sample

Soil samples contaminated with crude oil were collected randomly from five different points from the effluent point of the Kaduna Refinery at depths of 0 - 15cm. The samples were collected using a sterile spatula and placed in clean Ziplock bags, then stored in an icebox to preserve the samples. In the laboratory, stones and debris were removed using a 2mm sieve (Prenafeta-Boldu *et al.,* 2001).

Crude oil

Approximately 1.5 L of crude oil sample was collected from the Oil Movement Department of the Kaduna Refining and Petrochemical Company (K.R.P.C) in the morning hours. It was collected in a clean plastic bottle and transported to the Department of Microbiology, Ahmadu Bello University, Zaria, Nigeria.

Determination of physicochemical properties of the crude oil contaminated soil

The contaminated soil samples collected were dried and sieved using a 2mm mesh and the soil sample was mixed thoroughly and stored in McCartney bottles. The sample was analyzed for pH, total organic carbon, organic matter, total nitrogen, available phosphorus, moisture content, particle size analysis, electrical conductivity. Standard methods were used in determining the physicochemical properties of the soil (ISRIC, 2002). pH and temperature were determined using HANNA combo tester (H198130, Denver, USA) and a thermometer respectively. Organic carbon and organic matter were determined using the Walkley-Black method, available phosphorus by Olsen method, total nitrogen by Kjeldahl method, electrical conductivity was determined using a whitstone bridge conductivity meter, particle size and textural class (Joel and Amajuoyi, 2009), and moisture content using the evaporation method.

Preparation of mineral salt medium (MSM)

Fungi capable of utilizing petroleum hydrocarbons were isolated from the soil sample using mineral salt medium (MSM) with the following composition: Na_2HPO_4 (0.2 g), K_2SO_4 (0.017 g), NH_4NO_3 (0.4 g), KH_2PO_4 (0.053 g), $MgSO_4.7H_2O$ (0.05 g) as described by (Nwachukwu, 2000). The salts were dissolved in 100 mL of distilled water and sterilized by autoclaving at 121ºC for 15 minutes (Ekundayo *et al*., 2012).

Preparation of oil agar

One hundred and fifty millilitres (100 mL) of MSM supplemented with 1% (v/v) crude oil and 3 g of agar (Oxoid, United Kingdom) was prepared and was sterilized by autoclaving at 121℃ for 15minutes at 15 psi (Abdel Rahman, 2011).

Microbial analysis of the crude oil contaminated soil sample

Isolation and identification of fungal isolates

Hydrocarbon degrading fungi were enriched and isolated using a mineral salts medium with the composition stated above but supplemented with 1 % (v/v) crude oil as the sole carbon source and 0.025 g of chloramphenicol added to prevent the growth of bacteria. The flask was incubated for 7 days at ambient temperature on a rotary shaker (SHA-C, China) at 130 rpm (Prenafeta-Boldu *et al.,* 2001). After the period of incubation, tenfold dilution was carried out and one milliliter aliquots of each dilution were poured into duplicate Potato Dextrose Agar (PDA) plates for the isolation of crude oil-degrading fungi. The plates were incubated at 28℃ for 7 days. Pure fungal cultures were obtained by subsequent transfers to fresh PDA plates. The pure isolates were preserved in PDA slants and stored at 4℃ in a refrigerator until required for further use (AbdelRahman, 2011). The microscopy technique was employed for the identification of the isolates. They were characterized using colonial morphology such as type and colour of mycelial, shape and kind of spores, presence of foot cell, conidiophores; and the microscopic characteristics of the spores. The identification process was completed by comparing these characteristics mentioned above with those of known taxa as described by Larone (2002). A wet preparation was made using an inoculating needle to pick small portions of the colonies and placed on a clean grease-free slide. Two (2) drops of phenol cotton blue were added and covered with a coverslip. It was then viewed under the microscope using the x10 and x40 objectives to view their cellular morphology (Larone, 2002).

Inoculum preparation

Two milliliters (2 mL) of the standardized spore suspensions of each isolate was inoculated into 5mL of mineral salt medium supplemented with 1% v/v crude oil and 0.1% (v/v) Tween 80. Incubation was done at 130 rpm on a rotary shaker at 28^0C for 96 hrs (Nwankwegu *et al.,* 2016).

Determination of spore count in suspension

This was done using the method described by Bekada *et al.* (2008) with a slight modification. Spores grown on PDA slants at ambient temperature for seven days were washed by scraping and shaking in 5mL of Tween 80 for 15minutes.

After shaking, one milliliter of each spore suspension was used to make a tenfold dilution up to 10^{-2}. The spore size of each isolate's suspension was evaluated by direct counting using a hemocytometer and the standardized spore suspensions were adjusted to obtain a population density of 3.0×108 spore/ ml and stored at 4℃ in the refrigerator until required.

Screening the isolates for crude oil-degrading potential

The fungal isolates were tested for their ability to grow on solid and broth mineral salts medium with crude oil as the sole carbon source. Their growth on broth mineral salts medium was taken as an indication of the isolates' ability to degrade crude oil (AbdelRahman, 2011).

Ability to grow on oil agar medium

Oil agar was prepared as previously described and each fungal isolate was inoculated on the medium using a sterile inoculating needle. The plates were incubated at ambient temperature for 10days. The fungal growth was monitored and determined by visual observation. The diameter of the colony for each isolate was measured using a ruler (Al-Jawhari, 2014).

Screening the isolates for crude oil-degrading potential in liquid mineral media

The ability of the fungal isolates to degrade crude oil was determined. This was achieved by a screening test used for determining the biodegradation ability. One millilitre (1 mL) of a 96hr cell suspension containing 1×106spores/mL of each isolate was inoculated into a 50 mL Erlenmeyer flask containing MSM and 1 % (v/v) of crude oil. A separate flask containing MSM and crude oil with no inoculum was prepared as the control. Incubation was carried out at an ambient temperature in a rotary shaker set at 130 rpm for 7 days (Prenafeta-Boldu *et al.,* 2001). The fungal growth was determined by measuring the optical density using a spectrophotometer. The spectrophotometer was standardized using sterile MSM and a wavelength of 600 nm (Nwiyi and Olutubo, 2014). The absorbance reading of each isolate and control was taken and recorded. Weight loss of crude oil was then calculated after determining the amount of residual oil from a prepared standard curve using known amounts of crude oil in Equation 1 (AbdelRahman, 2011; Barnes *et al.,* 2017).

$$\%\text{weight loss of crude oil} = \frac{\text{Concentration control} - \text{Concentration final Concentration control}}{\times 100} \quad (1)$$

RESULTS

Physicochemical properties of the crude oil contaminated soil sample

The physicochemical parameters of the soil are presented in Table 1. The texture of the soil is sandy loam; the pH was alkaline; and the moisture content was relatively low. The carbon to nitrogen ratio is high (15.5:1) as well as the oil and grease content.

Table 1: Physicochemical properties of crude oil contaminated soil from Kaduna Refinery

Property	Mean value	Standard Error (±)
pH	8.5	0.065
Electrical Conductivity (EC) µS/m	0.80	0.005
Total Organic Carbon (TOC) (%)	1.43	0.040
Organic Matter (%)	2.46	0.025
Nitrogen Content (%)	0.092	0.0025
Oil and grease (mg/L)	840	1.5
Available Phosphorus (mg/kg)	5.95	0.03
Moisture Content (%)	1.57	0.025
Particle size distribution (%)		
Silt	14	
Clay	7	
Sand	79	
Textural Class	Sandy Loam	

Isolation and identification of hydrocarbon utilizing fungi from crude oil contaminated soil

A total of ten (10) fungi capable of growing on crude oil as the sole carbon source were isolated from the soil samples collected from the study site (Table 2). The isolates were given codes based on their source (Kaduna Refinery) from REF-1 to REF-10. The macroscopic and microscopic characteristics of the fungal isolates revealed that they were all moulds.

Table 2: Colonial morphology and microscopic characteristics of the fungal isolates

Isolate	Identity	Macroscopic characteristics	Microscopic characteristics
REF-1	*Madurella grisea*	Surface is folded in the center with radial grooves toward the periphery. Colonies grow slowly, the texture of the colonies are wooly. Color varies from white to yellow-brown, dark gray or olive brown.	Septate hyphae, mostly wide, the hyphae sometimes appear to be made of rounded cells, cylindrical, branched hyphae also present. Conidia are not commonly formed.
REF-2	*Monocillum* sp.	Colonies are moderately growing, surface is white, then becomes bluish green and powdery with white boundary. Substrate mycelium is red with diffused pigment	Septate hyphae with branched conidiophores; branches are flask-shaped phialides that bear unbranched chains of rough round conidia arranged in whorls. The entire structure forms a characteristic brush appearance
REF-3	*Aspergillus niger*	Grows rapidly, mature within 3 days, initially white and then any shape of yellow, orange, green, brown or black depending on the species. Texture is velvety.	Hyphae are septate, unbranched conidiophore. The conidiophore is enlarged at the tip, forming a swollen vesicle.
REF-4	*Trichophyton megnini*	Surface is velvety, at first white and then pink to violet with widely spaced radial grooves. Possible red pigmentation.	Septate hyphae with tear drop-shaped microconidia along the sides. Microconidia infrequently produced, are long, and narrow.
REF-5	*Trichophyton tansurans*	Surface was white and grayish; suede-like with concentric folds.	Hyphae are septate with many variably shaped microconidia. Microconidia are club shaped.
REF-6	*Aspergillus flavus*	Colonies are moderately growing; dark green to grayish color and wooly texture, irregular shape and milky colour in bottom of media	Septate hyphae with branched conidiophores that branch at wide angles and have flask-shaped phialides. Conidia are round or oval, single celled and clustered together at the end of each phialide.
REF-7	*Fusarium* sp.	At first white and cottony, but often quickly develops a pink or violet center with a lighter periphery.	Hyphae are septate, conidiophores bearing small 1-or-2- celled conidia singly or in clusters.
REF-8	*Actinomadura* sp.	Red and orange colonies grow slowly under aerobic conditions. The colony has a waxy, mucoid, and folded appearance.	Narrow abundantly branched filaments, with short chains of round conidia.
REF-9	*Chrysosporium* sp.	White colonies grow moderately rapidly; surface is powdery, flat and spreading.	Septate hyphae, conidia are one celled, clavate, with rounded apex and broad flattened base.
REF-10	*Actinomadura* sp.	Colonies grow slowly; with a waxy, folded appearance.	Narrow abundantly branched filaments.

Screening the isolates for crude oil-degrading potential

Table 3 shows the utilization of crude oil by the isolates. *Monocillium* sp*., Aspergillus niger, Trichophyton megnini* showed high growth while *Aspergillus flavus, Chrysosporium* sp*., Actinomadura* sp., showed moderate growth and *Madurella grisea Trichophyton tansurans, Fusarium* sp., showed low growth on crude oil.

Table 3: Ability of Fungi to Grow on Oil Agar (solid mineral medium)

S/N	Isolates	Colony Diameter (mm)
REF-1	*Madurella grisea*	2.6
REF-2	*Monocillium* sp.	7.5
REF-3	*Aspergillus niger*	7.0
REF-4	*Trichophyton megnini*	6.9
REF-5	*Trichophyton tansurans*	2.9
REF-6	*Aspergillus flavus*	5.5
REF-7	*Fusarium* sp.	2.6
REF-8	*Actinomadura* sp.	5.1
REF-9	*Chrysosporium* sp.	5.3
REF-10	*Actinomadura* sp.	5.1

Screening the isolates for oil degrading potential in crude-oil-MSM broth

The removal of hydrocarbons recorded varied from 518mg - 886mg of (47 - 80 %). All the isolates were able to degrade crude oil in seven days with *Monocillium* sp. showing the highest removal at 80% and the least by *Aspergillus flavus* and *Chrysosporium* sp. at 47% respectively. Table 4 and Figure 2 show the removal of hydrocarbons by each fungal species. In Table 4, the isolate with the code, REF-10 was excluded because it is the same species as REF-8 (*Actinomadura* sp.), and they both had the same colony diameter and rate of growth during screening on solid media.

Table 4: Quantitative Measurement of Reduction of Hydrocarbon during biodegradation studies

Code	Isolate	Absorbance	Concentration (mg/mL)	Weight loss (mg/mL)	Percentage loss (%)
REF-1	*Madurella grisea*	0.211	370	739	67
REF-2	*Monocillium* sp.	0.120	223	886	80
REF-3	*Aspergillus niger*	0.190	336	773	70
REF-4	*Trichophyton megnini*	0.307	526	583	53
REF-5	*Trichophyton tansurans*	0.316	541	568	51
REF-6	*Aspergillus flavus*	0.347	591	518	47
REF-7	*Fusarium* sp.	0.235	409	700	63
REF-8	*Actinomadura* sp.	0.222	388	721	65
REF-9	*Chrysosporium* sp.	0.347	591	518	47

Absorbance for Control was 0.666 and initial concentration was 109 mg/mL

3.5 Hydrocarbon Utilizing Fungal Counts

All fungi isolated showed resistance to crude oil pollution as visible growth was recorded. Among the isolates, *Monocillium* sp. recorded the highest counts of 8.5 x 10^7 spore/mL at the end of the biodegradation experiment. *A.niger* equally performed very well with a count of 6.1 x 10^7 spore/mL (Figure 1).

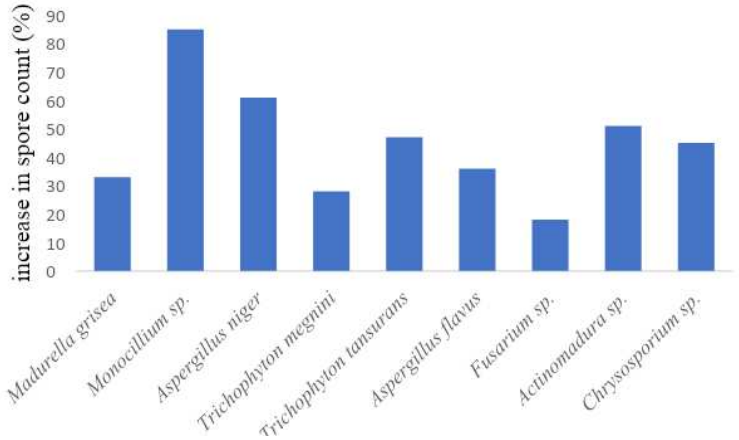

Figure 1: Percentage Increase in Spore Counts of Fungi during biodegradation

DISCUSSION

Microbial growth and activities are affected by some physicochemical properties such as pH, moisture, temperature, and nutrient availability. The pH of the soil was observed to be alkaline which shows its suitability for bioremediation as supported by the work done by Bijay *et al.* (2012) where it was stated that the rate of microbial activity falls within a pH of 5.5 – 8.8. The overall rate of biodegradation of hydrocarbons is generally higher under alkaline conditions (Saadoun, 2004). Also, acidification of soil depletes important nutrient elements such as potassium, magnesium and calcium (Onojake and Osuji, 2012). The moisture content in this study was found to be very low; fungi tend to thrive in dry environment as their ability to produce spores enables them to withstand harsh environmental conditions. However, excess moisture can be detrimental because it reduces the quantity of accessible oxygen for aerobic respiration (Onojake and Osuji, 2012). A wide variety of morphological characteristics was observed with possible pigmentation in some of them. Species such as *Aspergillus niger* grew more rapidly than the others while, *Madurella grisea* and *Actinomadura* spp. grew slowly. Genera such as *Actinomadura*, *Madurella* and *Monocillum* have not been featured commonly in biodegradation studies of petroleum unlike *Aspergillus*, *Fusarium* and *Trichophyton*. Species of *Aspergillus* have been isolated from petroleum contaminated soils and are known hydrocarbon degraders (Fatuyi *et al*., 2012; Usman *et al*., 2019). A limitation in the identification of moulds is the fact that some genera share certain characteristics as such, it is best to use more than one atlas during the process as was the case in this study. However, molecular methods are more superior in identifying soil fungi.

The presence of these fungi in the petroleum contaminated soil is an indication that they utilize the hydrocarbons for their metabolic needs. Fungi produce extracellular enzymes which enable them to breakdown many organic substances in the terrestrial environment. They also produce enzymes such as peroxidases, laccases, cytochrome P450 monooxygenase, which are utilized in the degradation of hydrocarbons (Raji, 2016). The microbial community in petroleum contaminated terrestrial and aquatic habitats comprise of bacteria, fungi, archaea and algae.

The diameter of the colonies is an indication of the rate of growth of the fungi, and it provides a more precise estimation of their ability to utilize crude oil. Thus, the greater the colony diameters the more efficient the fungus is in breaking down the hydrocarbons. *Monocillium* sp. had the highest colony diameter and thus, is the most efficient oil degrader based on this preliminary testing while the colonies of *Fusarium* sp. had the least diameter.

The absorbance of the broth culture of microorganism during utilization of hydrocarbons has been used in similar studies to monitor the removal of the hydrocarbons (Atta, 2009; Saidu *et al*., 2018; Usman *et al*., 2019). In this study, the optical density typically increases as the fungi utilizes the hydrocarbons, thus there is an increase in growth and subsequent removal of hydrocarbons. The increase in spore counts of the fungi is directly proportional to the breakdown of the hydrocarbons in crude oil, for the simple reason that the fungi are able to use the hydrocarbons as a source of Carbon and energy. In a study by Usman *et al*. (2019) on the degradation of monoaromatics by fungi, a spore count of 8.2×10^7 spore/mL was reached by the most efficient fungus, *Aspergillus terreus*. Among the microorganisms capable of hydrocarbon utilization, the fungi have the advantage of being able to reach into soil particles with their hyphae thus enhancing the influence of their enzymes. Studies focused on the ecology of polluted soils often determine the abundance of the gene of interest to determine the rate of degradation of toxic compounds (Higashioka *et al*., 2009; Ding *et al*., 2010; Korotkevych *et al*., 2011; Raji, 2016). Other studies that are based on culture dependent methods of enumerating hydrocarbon degrading microorganisms include those of Atta (2009) who monitored the counts of bacteria during enhancement of crude oil degradation by biostimulation; and Usman *et al*. (2019) examined the counts of hydrocarbon utilizing fungi during the degradation of BTEX compounds. Studying the abundance of microorganisms during pollutant degradation is important in monitoring the rate at which the process occurs, and it also confirms the viability of the cells.

CONCLUSION

The soil pH of 8.5 was in alkaline range which aided the growth of the fungi. The fungi isolated from hydrocarbon contaminated site could possibly be used for oil spill cleanup as they readily utilized hydrocarbons as the sole carbon and energy source. Ten (10) fungi were isolated from the contaminated soil which include: *Madurella grisea, Monocillium* sp., *Aspergillus niger, Trichophyton megnini, Trichophyton tansurans, Aspergillus flavus, Fusarium* sp., *Actinomadura* sp. (two strains), and *Chrysosporium* sp. *Monocillium sp.* and *Aspergillus niger* showed the highest

degradative ability of 80 % and 70 % based on screening on liquid and solid mineral media. The metabolic capacities of hydrocarbon degrading fungi can be affected when they are grown with other species, as such it is pertinent to conduct pilot studies before carrying out bioremediation studies with hydrocarbon degrading fungal species.

RECOMMENDATIONS

1. More research should be carried out on biodegradation analysis to assess the interactions between the native degraders both singly and in consortium that demonstrate good growth in crude oil to remediate hydrocarbon contaminated water and soil.
2. Research on molecular characterization of the isolates obtained in this study should be carried out in order to detect the functional genes responsible for degradation of petroleum hydrocarbons.

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