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Isolation and Characterization of Crude Oil Degrading Fungi Isolates from Soil Samples from Niger Delta

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Abstract:- Few fungi strains are known to degrade crude oil and other organic pollutants. The primary aim of this study was therefore to isolate crude oil degrading fungi from impacted soil samples from the Niger Delta region of Nigeria. Collected soil samples were analyzed for physicochemical parameters, total aerobic heterotrophic fungi, characterization of isolates and crude oil utilization using standard microbiology techniques. The results indicate that the physicochemical parameters of the soil and sediments samples varied significantly with iron and nickel above permissible limits. Aerobic fungi counts ranged from 1.9 to 4.0 (x10^5) CFU/g. Fungi isolates capable of degrading crude oil were identified using molecular techniques were *Aspergillus niger, Trichoderma asperellum, Aspergillus oryzae and Penicillium commune.*

Keywords:- *Crude Oil, Fungi, Biodegradation, Heavy Metals.*

I. INTRODUCTION

Niger Delta is home to Africa's largest and the most fragile Delta in the world (Ajibade and Awomuti, 2009; Udofia et al., 2018; UNEP, 2011). Over six decades of crude oil exploration and production has done unimaginable and devastating damage to the region's environment (Ajibade and Awomuti, 2009; Akpan, 2012). This damage is largely due to toxic components of crude oil. Crude oil is a well-known naturally occurring environmental pollutant as some of its hydrocarbon fractions are considered environmental pollutants and are recalcitrant (Leahy and Colwell, 1990). Crude oil hydrocarbons have been shown to affect the growth and performance of important crops and vegetables, can destroy vegetation, reduce diversity of aquatic and terrestrial lives (Ojimba and Iyaba, 2012; Ekunday *et al.*, 2001; Winter *et al.*, 1976).

The release of crude oil into an environment whether aquatic and terrestrial alters the composition of the microbial structure and function (Edet *et al.*, 2017). The alteration promotes the proliferation of hydrocarbon utilizing microorganisms which includes both bacteria and fungi. Incidentally, these hydrocarbon utilizing bacteria and hydrocarbon utilizing fungi are also the organisms that are responsible for the biodegradation and eventual cleanup of oil spills in our environment (Okpokwasili and Amanchukwu, 1988; Hamamura *et al.*, 2006). These microbial communities that are exposed to hydrocarbon contaminants become adapted, exhibiting selective enrichment and genetic changes (Leahy and Colwell, 1990). The rate of breakdown of crude oil hydrocarbon is driven by several factors such as their susceptibility to microbial attack (n-alkanes > branched alkanes > low-molecular-weight aromatics > cyclic alkanes ranked in the following order of decreasing susceptibility). Other factors include the type of microorganisms involved in the process and physicochemical parameters (Guo-liang *et al.*, 200, Leahy and Colwell, 1990; EL-Hanafy, 2017).

Most of the bioremediation studies have largely been centered on the use of bacteria and few on fungi. A few studies have outlined the ability of fungi and yeasts to utilize petroleum hydrocarbon as sole carbon and energy sources (Okpokwasili and Okorie, 1988; Al- Nasrawi, 2012; Dirisu, 2015). The aim of this study was therefore to isolate and characterise crude oil utilizing fungi from soil sample collected from an oil producing community in the Niger Delta using molecular identification.

II. MATERIALS AND METHODS

- *Sampling location*

Soil samples in triplicates were collected using a sterile hand-held auger from four different locations from depths of 0-15cm as described previously (Edet *et al.*, 2018). The triplicates samples from each of the locations were then made into four composite samples. The soil samples were first sieved using a 2mm pore sieve and then mixed thoroughly before making them into composite samples. The samples were the transported immediately to the laboratory for further analysis.

- *Physicochemical analysis of samples*

Temperature and pH were determined *in situ* according to methods already described (Verla *et al.*, 2012). Conductivity was determined with the Lovibond conductivity meter type cm-21 model. Total suspended solid and dissolved solids (mg/L), dissolved oxygen, biochemical oxygen demand, chemical oxygen demand were determined by adapted methods of American Public

Health Association (APHA, 1998). Total nitrogen, nitrate-nitrogen and oil and grease were differentially digested and measured according to Ahmad and co-workers (Ahmad *et al.*, 2005). Heavy metals (cadmium, nickel, copper, iron, chromium) were analyzed after digesting samples in mixture of acids and using Atomic Absorption Spectrophotometer (AAS) (APHA 301A) (Model: 5100 PC, Perkin-Elmer, Boston, USA).

- *Microbiological analysis*

Following preparation of media, the samples were subjected to a number of microbiological analysis and these included serial dilution, inoculation of the plates, enumeration of total heterotrophic fungi and screening for hydrocarbon utilizing fungi (Antai, 1990; Collin and Lyne, 1980; Udotong *et al*., 2015).

- *Screening for hydrocarbon utilizing fungi*

This was done following the pour plate method of Antai (2014) as modified by Edet *et al.* (2019). From the homogenized composite soil samples, 10g each were weighed out and dissolved in sterile 90ml of distilled water. From this, serial dilution was carried out as already explained. Following serial dilution, freshly prepared mineral salt medium (MSM) and plate count agar, crude oil and filter paper were autoclaved at 121^{o}C at psi for 15 minutes. After autoclaving, the media were dispensed (15ml/plate) and in triplicates. The filter paper were then soaked with 1ml of the sterile crude oil and carefully used to cover the lid of the plates and sealed with masking tape. The plates were then incubated inverted for 24 to 96hours. The growth of bacteria was inhibited by supplementing the MSM medium with 50 g/ml each of penicillin G and Streptomyces added aseptically to the media after autoclaving.

- *Molecular characterization of the hydrocarbon utilizing fungi*

The hydrocarbon utilizing fungi DNA were extracted using Z-R Fungal-Bacterial DNA MiniPrep™50 Preps Model D6005 (Zymo Research, California, USA) following manufacturer's instructions and as previously reported by (Edet *et al*, 2018). Amplification was done using the ITS1 TCC GTA GGT GAA CCT GCG G and ITS4- TCC TCC GCT TAT TGA TAT GC primer pair. Sequencing and electrophoresis were carried with the ABI V3.1 Big dye kit according to manufacturer's instructions. Following sequencing, identification was done using Basic Local Alignment Search Tool. They resulting isolates were *Aspergillus niger, Trichoderma asperellum, Rhizomucor variabilis*, and *A, aculeatus.*

III. RESULTS

Table 1 shows the total heterotrophic counts of the fungi from the various soil sample locations. The results show that soil sample from location 2 recorded the highest count of 40 (x 10^4 CFU/g) followed by location 5 with a count of 34 (x 10^4 CFU/g. The count came from location 4that recorded the least count of 19 (x 10^4 CFU/g).

Sampling type and location	Fungi count (x 10^4 CFU/g)
SS_2	40
SS_3	22
SS_4	19
SS_5	34

SS= composite soil sample, while 2, 3, 4 and 5 represent the various locations.

Table 1:- Total heterotrophic bacteria counts for composite sediment samples

The results of the rate of growth of the fungi utilizing crude oil showed that there was no growth until day 3 and by day 5 and 6 four isolates were already growing on the plates. These were identified using cultural and microscopic characteristics to be *Aspergillus oryzae, Penicillium, Rhizopus, and Trichoderma species*

Table 3 shows the heavy metal profile of the various soil samples as compared to the WHO/FEPA standards for sediments. For zinc and copper, the amount of these metals exceeded those of WHO/FEPA with values of 1.42 and 3.42 mg/L respectively in just one out of four locations. While iron and nickel were far above the standard allowable limits for them. Their values ranged from 5.80 to 6.15mg/L and 0.09-0.11 mg/L, respectively. Chromium levels were far lower than the 2.0mg/L. Cobalt standards were not available from WHO/FEPA but from our study sites its values ranged from 0.09 to 0.53mg/L.

Parameters (mg/L)	SS2	SS3	SS4	SS5	WHO/FEPA Standard in sediment
Copper	1.42	0.73	0.58	0.66	1.00
Zinc	3.42	0.72	1.99	2.18	3.00
Iron	6.15	6..05	5.80	6.14	0.30
Chromium	0.11	0.03	0.05	0.07	2.00
Nickel	0.10	0.09	0.11	0.09	0.02
Cobalt	0.23	0.11	0.53	0.09	NA

SS= soil sample; 2, 3, 4, &5 represent the various locations.
[a]Represents significant mean determinations that were significant across the rows

Table 2:- Heavy metal profile of the various soil samples in various locations

From Table 3, across the study locations, pH ranged from 6.60 to 7.00. Temperature ranged from 27.50 to 29.40^{o}C. Phosphorus levels ranged from 7.24 to 13.50 mg/L with locations 2 and 5 recording the highest values of 13.40 and 13.50mg/L. Electrical conductivity values ranged from 11.80 to 32.50 s/cm and location 2 recording the highest value. Base saturation ranged from 38.50 to 42.00mg/L. Sulphide values and those of nitrate were far higher than those of nitrite and ammonia. The levels of nitrates were the highest of all the physiochemical parameters examined in the study while ammonia levels were the least.

Parameter	SS_2	SS_3	SS_4	SS_5
pH	6.70[a]	7.00[a]	6.60[a]	6.70[a]
Temperature (℃)	29.40	27.50	28.70	28.00
Phosphorus (mg/L)	13.50	7.24	8.60	13.40
Electrical conductivity (s/cm)	32.50	27.30	18.50	11.80
BS (mg/L)	42.00	51.50	38.50	47.50
Sulphide (mg/L)	25.00	10.50	6.00	127.00
N-nitrate (mg/L)	87.00	98.80	78.00	81.00
N-nitrite (mg/L)	0.10	0.02	0.16	0.60
N-Ammonia	0.02	0.01	0.04	0.06

CCS= composite soil sample; 2, 3, 4, 5 &6 represent the various locations.
[a]Represents significant mean determinations that were significant across the rows.

Table 3:- Mean determinations of physicochemical characteristics of soil samples

IV. DISCUSSION

After bacteria, fungi remain the second most abundant group in soil ecosystem where they play important roles in maintaining the soil ecosystem health and balance. They are responsible for releasing nutrients contained decomposing organic matter thereby contributing to soil fertility. They also play an important role in degrading various organic pollutants (El Hanafy *et al.*, 2015). From the results of our studies, it can be seen that total heterotrophic fungi counts from our study location ranged from 1.9 to 4.0 (x 10^5 CFU/g). This was higher than the 0.41 ± 0.16 to 3333.33 ± 288.00 × 10^2 CFU/g soil reported by Dawoodi *et al* (2015), and 2.0 x 10^3 to 3.0 x $10^3$and 0.7 x 10^3cfu/g to 1.2 x 10^3cfu/g for hydrocarbon utilizing fungi by Chukwura *et al* (2016). Furthermore, it was also higher than the 2. 27 ± 0.30 × 10^3 to 4.2 ± 1.08 × 10^3 cfu/g reported by Dirisu (2015). These differences in the counts could be due to the age of the impacted samples used in these studies. The older the sample, the higher the number of microbial counts and vice versa.

Some heavy metals such as zinc, copper, iron and nickel in at least one location gave values higher than permissible for soil, they were however lower than values previously reported by Stephen & Oladele (2012) from top soil around the iron ore deposit at Itakpe North Central Nigeria. Our pH values were only slightly lower than the 7.5 to 7.9 earlier reported by Borkar (2015). However, the pH range of our stud samples is within range of bioremediation (Vidali, 2011). Our electrical conductivity was lower than the 0.14 µS to 0.23 µS Borkar (2015) reported.

A number of fungi species are capable of degrading hydrocarbon and other organic pollutants. Molecular identification revealed the isolates as *Aspergillus niger, Trichoderma asperellum, Rhizomucor variabilis*, and *A. aculeatus.* In an earlier study using ITS -1 and ITS-2 analysis of the hydrocarbon utilizing fungi, El-Hanafy (2015) reported A. *niger* and *Penicillium commune*, with 54%and 48% capacity to degrade crude oil and both Genus were also obtained in our study . In another study, *Trichoderma asperellum* H15, a previously isolated strain characterized by its high tolerance to low (LMW) and high molecular weight (HMW) polyaromatic hydrocarbon (PAHs), was tested for its ability to degrade 3–5 ring PAHs (phenanthrene, pyrene, and benzo[a]pyrene) in soil microcosms along with a biostimulation treatment with sugarcane bagasse. The results of the study showed that T. asperellum have the ability to remove large amounts of PAHs in soil (Zafra *et al.*, 2014).

Dirisu (2015) isolated *Mucor* and *Penicillium* species capable of utilizing hydrocarbon while El Hanafy et al (2017) isolated *Aspergillus* and *Penicillium.* Chukwura *et al* (2016) isolated *Aspergillus niger, Aspergillus fumigatus, Penicillium xingjiangense, Mucor racemosus,* and *Rhodotorula* sp. Furthermore, Atagan (2009), isolated *Fusarium flocciferum, Trichoderma spp., Trametes versicolor* and *Pleurotus ostreatus* capable of growing in high level of heavy metal contamination.

V. CONCLUSION

Fungi like bacteria are capable of degrading organic pollutants like crude oil thereby reducing them to less harmful substances. From the findings in our study, it can be seen that the study area is influenced by anthropogenic activities. Molecular characterization of the crude hydrocarbon utilization was more sensitive than the macro and microscopic methodologies.

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