Physicochemical Properties and Diversity of Crude Oil Degrading Fungi Isolated from Crude Oil Contaminated Soil

(Study in Ologbo, Edo State, Niger Delta region of Nigeria)

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Abstract: Crude oil pollution is a widespread environmental problem of major concern, oil spill due to oil pipeline rupture, tank failures various pollution and storage problem, and transportation accident are the major causes. This study was aimed at determining the physico-chemical, Microbiological potential of fungi isolated from soil polluted with crude oil. Crude oil impacted soil from Nigeria Petroleum Distribution Company (NPDC) exploration site in Ologbo, Edo State was analyzed for Microbiological profiles using standard methods. Physicochemical properties which include, electrical conductivity, pH, carbon, phosphorus and nitrogen were analyzed using standard methods. Total Petroleum hydrocarbon (TPH) and Polynuclear aromatic hydrocarbon (PAH) was elucidated using gas chromatography (GC). Identification of fungi isolates with 18S rRNA and phylogenetic analysis were elucidated. There was no significant change in the pH of the soils, however the electrical conductivity was higher (0.4 to 1.11 µs/cm) in contaminated soil samples. Carbon content in all the contaminated soil samples was significantly higher than normal soil and ranged from 2.67 to 7.33%. The TPH contents in the contaminated soils were found to be in the range of 684.86 mg/kg to 5,991.69 mg/kg of soil compared with the control 37.8 mg/kg. The PAH content in the contaminated soil ranged from 1.841 mg/kg to 13.913 mg/kg of soil with the control being 0.059 mg/kg. A total of 15 fungi were isolated from crude polluted soil 18S rRNA revealed the following genera of Fungi viz: Scedosporium, Penicillium, Candida, Aspergillus, Pseudalescheria, and Byssoschlamys.

Keywords: Phylogenome, Physicochemical, Gas-chromatography, Polynuclear aromatic hydrocarbon PAH & Total petroleum hydrocarbon.
1. Introduction

Crude Oil product are continually used as a main source of energy in industry and daily life (Kvenvolden and cooper 2003). Crude oil is transported via pipelines road ships and rail posing great danger to the environment in case of spills. This necessitate the need for developing techniques that are eco-friendly to clean up oil spill one of such method is the use of biological agent due to its efficiency and cost effectiveness, compared to physicochemical methods, Ojo 2006.

Crude oil consists of naturally occurring hydrocarbon that are considered environmental pollutants, its recalcitrance and abundance has been reported in most polluted sites. The degradability of individual hydrocarbon component is influenced by the quality of the hydrocarbon content present in the crude oil. Oil containing large amount of high –molecular weight compounds is difficult to degrade biologically because of the complexity of their structure Guo-Lin et al 2005. The chemical composition of oil influence the growth of microbial population. Microbes that use hydrocarbon as a source of energy thrive under high temperature and salinity conditions Westlake et al 1974. Fungi are known to play a significant role in eliminating hazardous compounds from soil and water contaminated with oil spill as they inhabit such substrate and utilize hydrocarbons as a source of carbon.

Scientists all over the world are searching for inexpensive and environmentally safe approaches to remediation of crude oil polluted soils. Microbes naturally selected in soils impacted by crude oil spills are increasingly being tested for their use in bioremediation purposes (Amirlatifi et al 2013). We believe that indigenous microbes selected in soils impacted by crude will be most suitable for application in bioremediation in the Niger Delta region of Nigeria. This report represents our initial study to determine the physicochemical parameters, microbial diversity and isolation of candidate fungi capable of using crude oil as sole carbon source.

Fungi are known to be one of the best oil degrading organism (Ojo, 2006; Batelle 2000). Different studies have identified numerous fungi genera capable of utilizing crude oil as source of carbon and energy this fungi include *Cephalosporium, Rhizopus, Paecilomyces, Alternaria, Mucur Talaromyces, Gliocladium, Aspergillus, Penicilium, Torulopsis* and *Pleurotus* (Ameen et al 2016; Dawoodi et al 2015; Zhang et al 2016). The objectives of this work was to investigate population of fungi from crude oil polluted soil in Ologbo, Edo State Niger Delta Region Of Nigeria.

2. Materials and Methods

2.1 Study Area and Geo-mapping of the Study Area

NPDC exploration site is situated at Ologbo (host) community, Ikpoba – Okha Local Government Area of Edo State South – South Nigeria. the communities play host to oil fields and flow stations as well. The vegetation is typical of the rainforest except for drainage streams where swampy areas exists NPDC geographical location is at $5^0 39' 17''$ E. $6^0 3' 30''$ N located within $6^0 20' 0''$ - $6^0 4' 30''$ N and $5^0 39' 0''$ - $5^0 41' 0''$ E with elevation below sea level.
2.2 Sample Collection

Crude oil contaminated soil where obtained from the Nigeria petroleum Distribution company (NPDC with exploration activities for 16 years) Exploration site in Ologbo Using a soil Auger at 15cm and 30cm depth into sterile polyethene bags, thereafter they were transported to the laboratory for Microbiological Examination.

2.3 Physicochemical Analysis

The physicochemical analysis of the samples were carried out. The following parameters were analyzed: pH, Nitrogen, phosphate, Electrical conductivity, Total organic carbon, total petroleum hydrocarbon, Polyaaromatic hydrocarbon.

2.4 Total Petroleum Hydrocarbon/Polyaromatic Hydrocarbon in Soil samples

2.4.1 Extraction

Soil samples were weighed (2gm) into a clean extraction tube. 10ml of extraction solvent (Hexane) was weighed added into the samples and mixed thoroughly and allowed to settle. The mixture was carefully filtered into clean solvent rinsed extraction bottle using filter paper fitted into Buchner funnels. The extracts were concentrated to 2ml then transferred for clean up/ separation

2.4.2 Clean up/ Separation

A 1cm of moderately packed glass wool was placed at the bottom of 10mm ID×250mm long chromatographic column, at the top of the column was added 0.5cm of sodium sulphate. The column was rinsed with additional 10ml of methylene chloride. The column was pre-eluted with 20ml of hexane this was allowed to

Figure 1: Map of Edo State highlighting the study area of Ologbo in Ikpoba-Okha LGA Edo State
flow through the column at a rate of about two minutes until the liquid in the column was just above the sulphate layer. Immediately 1ml of the extracted sample was transferred into the column. The extraction bottle was rinsed with 1ml of hexane and added to the column as well. The stop clock of the column was opened and the eluent was collected with a 10ml graduated cylinder. Just prior to exposure of the sodium sulphate layer to air, hexane was added to the column 1-2ml increment. Accurately measured volume of 8-10ml of the eluent was collected and was labeled aliphatic (API 1994).

2.4.3 Gas Chromatographic Analysis
The concentrated aliphatic fraction were transferred into labeled glass vials with Teflon or rubber crimp caps for GC analysis. 1µl of the concentrated sample was injected by means of hypodermic syringe through a rubber septum into the column. Separation occur as the vapour constituent partition between the gas and liquid phases. The samples were automatically detected as it emerges from the column (at a constant flow rate) by the FID detector whose response is dependent upon the composition of the vapour. (INRCC 1997).

2.4.4 Cultivation counting and isolation of indigenous fungi
The enumeration of fungi was done using the dilution plate technique. Potato dextrose agar (PDA) medium was used for isolating and enumerating heterotrophic soil fungi and Mineral Salt Medium (MSM) supplemented with 1% crude oil was used for isolation, enumeration and preliminary identification of fungi isolates from soil. mineral salt medium (MSM) which contain per liter 5.0g NaCl; 1.0gK2HP04; 5.0gKH2P04; 1.0gNaNO3; 0.25g Mgso4 7H2O; 0.02gCaCl2, 2H2O; 0.02gFeCl3 5g of yeast extract and 10g of Agar in 500ml of deionized water. Streptomycin antibiotics was added to the media to prevent bacteria growth. A 0.1ml aliquot of appropriate dilutions of sample was inoculated into three replicate. The plates were incubated for 2-3days at room temperature and colonies formed were counted, the mean was taken and expressed in cfu/g.

2.4.5 DNA Extraction for Fungi isolates
Genomic DNA for Fungi isolate was done using CTab, 25mg of growth culture was transferred into 1.5ml eppendorf tube and stored overnight at -80°C. Add 300µl of CTab solution into tube and crush sample with pistol, add an additional 300µl of CTab (CTab 600µl). Add 60µl of 20% SDS (sodium dodecyl sulphate) mix gently thereafter place on a water bath at 60°C for 1h. Add 660µl of phenol chloroform, isoamyl alcohol (25:24:1) invert to mix, centrifuge at 11500 xg for 20mins.transfer 450µl of supernatant to a new tube, add 450µl of chloroform, isoamyl alcohol (24:1). Centrifuge at 11500xg for 15mins, transfer 300µl to a new tube add 0.6ml (300×0.6) i.e 180µl of ice cold isopropanol and invert to mix. Centrifuge at 12000xg for 10mins, decant supernatant add 300µl of ice cold 70% ethanol and centrifuge at 12000xg for 5mins decant supernatant, centrifuge at 12000xg for 1mins to remove the remaining ethanol. Air dry tubes for 30-40mins. Add 40µl (0.1MTE) to elute DNA and store at -20°C freezer for downstream application.

2.4.6 Purification of Cultured Fungi Isolates
GeneJET PCR purification kit (Thermo scientific) was used to purify post pcr proct of bacteria and fungi isolates. 40µl of Binding buffer was added to 40µl of post PCR product and mix by pipetting,(1:1 of binding buffer and PCR mixture). Add 40µl of isopropanol and mix (1:2 i.e 40µl of isopropanol in 80µl in step 1). Place column and load the entire solution into the column and spin at 12000x g for 1mins. Add 700µl of wash buffer (9ml of concentrated wash buffer and 45ml of Ethanol) spin at 12000xg for one minutes and transfer column to a clean 1.5ml microcentrifuge tube. Add 50µl of Elution buffer to the center of the GeneJET purification column membrane and centrifuge for 1min. Discard the GeneJET purification column and store the purified DNA at -20°C.
2.4.7 Identification and characterization of Fungi isolates

Total genomic DNA was extracted from growth cultures of fungi isolates, the 18S rRNA gene fragment was amplified with a pair of universal primers ITS1F 5’ CTT GGTCAATTCAGAGGAAGTAA 3’ and ITS4R 5’TCCCTCGCTTATTGATATGC 3’ using the extracted DNA as template.19µl of PCR water, 25µl of Gotaq, 2.5µl forward primer, 2.5µl reverse primer and 1µl of template DNA. Polymeric chain reaction (PCR) was performed in a thermal cycler using the following conditions: pre-heating for 3mins at 95°C followed by 30 cycles of 30s at 95°C; 45s at 55°C; 90s at 72°C with a final extension of 10 min at 72°C. The size of the PCR product was confirmed with electrophoresis using a 1.5% Agarose Gel. The PCR product were purified by using GeneJET PCR purification kit. The amplicon was sequenced at Eurofins Genomics (USA) the raw sequences were aligned, edited manually and Blast searched using a Blast search option of NCBI GenBank to estimate the degree of similarities to other species in the GeneBank.

3. Results and Discussion

Table 1 shows the results of soil analysis as well as population of total heterotrophic fungi count after enumeration of indigenous fungi the average count of total heterotrophic fungi on PDA agar were expressed as ×10²CFU/g soil. In the soil sample heterotrophic count were 3.60± 1.00 for well 1, 37.14± 3.50 for well 2, 5.65±0.85 well 3 with control 0.50±0.10.

Organic carbon for well 1,2, and 3 range from 2.67%-7.33% were as that of control was 0.85%, as shown in table 2. Since the entrance of crude oil into the environment increase the amount of organic carbon in soil owning to the desirable amount of phosphorus and neutral pH, however well 1 and well 3 had the highest pH value of 7.60% when compared with well 2 with 7.30% pH value. An excess of dissolved salt (anions and cations) in the soil is detected by electrical conductivity (Grisso et al 2007). Electrical conductivity is one of the characteristics that promote antimicrobial activity excessive heavy metal concentration in the soil was reported to cause decrease in microbial population (Al-kadeeb and Siham 2007). most metal ions have the ability to create oxygen radicals thus forming molecular oxygen which is highly toxic to bacteria and fungi Dastje and Montazer (2010). The electrical conductivity varies depending on the amount of moisture held by soil particles, increasing soil solution salinity (Ec) will decrease available water, this will have a negative effect on crop yield and microorganism such as fungi also may affect their survival (Bauder 2001) the discharge of large quantities of oil to this areas has affected most of the microorganisms and has reduce the microbial population.

<table>
<thead>
<tr>
<th>Soil Sample ID</th>
<th>Sample Description</th>
<th>TPH (mg/kg)</th>
<th>PAH (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Well #</td>
<td>Depth</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Well 1, 15 cm</td>
<td>2,531.74</td>
<td>5.647</td>
</tr>
<tr>
<td>2</td>
<td>Well 1, 30 cm</td>
<td>897.08</td>
<td>1.996</td>
</tr>
<tr>
<td>3</td>
<td>Well 2, 15 cm</td>
<td>5,991.69</td>
<td>13.913</td>
</tr>
<tr>
<td>4</td>
<td>Well 2, 30 cm</td>
<td>1516.76</td>
<td>3.364</td>
</tr>
<tr>
<td>5</td>
<td>Well 3, 15 cm</td>
<td>1585.83</td>
<td>3.502</td>
</tr>
<tr>
<td>6</td>
<td>Well 3, 30 cm</td>
<td>684.86</td>
<td>1.541</td>
</tr>
<tr>
<td>Control</td>
<td>Control T15cm</td>
<td>37.98</td>
<td>0.059</td>
</tr>
<tr>
<td></td>
<td>control B30cm</td>
<td>35.25</td>
<td>0.045</td>
</tr>
</tbody>
</table>
Table 2: Physicochemical properties of crude oil polluted soil

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Well 1</th>
<th>Well 2</th>
<th>Well 3</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.6±0.02</td>
<td>4.5±0.02</td>
<td>7.3±0.05</td>
<td>2.2±0.02</td>
</tr>
<tr>
<td>Electrical conductivity (mhos)</td>
<td>0.4±0.05</td>
<td>1.11±0.01</td>
<td>0.42±0.06</td>
<td>0.32±0.02</td>
</tr>
<tr>
<td>Carbon</td>
<td>2.6±0.02</td>
<td>0.29±0.03</td>
<td>7.3±0.02</td>
<td>0.16±0.01</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>0.19±0.04</td>
<td>0.18±0.02</td>
<td>0.16±0.02</td>
<td>0.24±0.05</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>0.17±0.02</td>
<td>0.15±0.01</td>
<td>0.30±0.06</td>
<td>0.33±0.03</td>
</tr>
<tr>
<td>Particle Size</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sand (%)</td>
<td>0.38±0.03</td>
<td>0.39±0.03</td>
<td>0.33±0.03</td>
<td>0.16±0.01</td>
</tr>
<tr>
<td>Silt (%)</td>
<td>0.15±0.01</td>
<td>0.15±0.01</td>
<td>0.16±0.01</td>
<td>0.33±0.03</td>
</tr>
<tr>
<td>Clay (%)</td>
<td>0.27±0.05</td>
<td>0.28±0.02</td>
<td>0.27±0.05</td>
<td>0.34±0.03</td>
</tr>
<tr>
<td>Calcium (meq/100g)</td>
<td>0.16±0.02</td>
<td>0.17±0.01</td>
<td>0.20±0.04</td>
<td>0.15±0.01</td>
</tr>
<tr>
<td>Sodium (meq/100g)</td>
<td>0.14±0.02</td>
<td>0.16±0.02</td>
<td>0.15±0.01</td>
<td>0.42±0.06</td>
</tr>
<tr>
<td>Potassium (meq/100g)</td>
<td>0.14±0.03</td>
<td>0.16±0.03</td>
<td>0.20±0.04</td>
<td>0.15±0.03</td>
</tr>
<tr>
<td>TPH (mg/kg)</td>
<td>1780.56</td>
<td>2070.61</td>
<td>1672.77</td>
<td>37.98</td>
</tr>
<tr>
<td>PAH (mg/kg)</td>
<td>5.647</td>
<td>13.913</td>
<td>3.502</td>
<td>0.059</td>
</tr>
<tr>
<td>Heavy Metal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Manganese (mg/kg)</td>
<td>0.42±0.02</td>
<td>0.21±0.03</td>
<td>0.16±0.01</td>
<td>0.12±0.08</td>
</tr>
<tr>
<td>Zinc (mg/kg)</td>
<td>0.24±0.02</td>
<td>0.28±0.02</td>
<td>0.27±0.05</td>
<td>0.42±0.02</td>
</tr>
<tr>
<td>Copper (mg/kg)</td>
<td>0.22±0.04</td>
<td>0.24±0.05</td>
<td>0.38±0.03</td>
<td>0.16±0.02</td>
</tr>
<tr>
<td>Iron (mg/kg)</td>
<td>0.33±0.03</td>
<td>0.15±0.04</td>
<td>0.32±0.02</td>
<td>0.12±0.02</td>
</tr>
<tr>
<td>Cadmium (mg/kg)</td>
<td>0.20±0.04</td>
<td>0.15±0.05</td>
<td>0.22±0.04</td>
<td>0.21±0.04</td>
</tr>
<tr>
<td>Chromium (mg/kg)</td>
<td>0.17±0.02</td>
<td>0.29±0.03</td>
<td>0.16±0.01</td>
<td>0.14±0.03</td>
</tr>
<tr>
<td>Lead (mg/kg)</td>
<td>0.22±0.04</td>
<td>0.15±0.03</td>
<td>0.16±0.02</td>
<td>0.14±0.02</td>
</tr>
</tbody>
</table>

The Total Petroleum Hydrocarbon (TPH) and Poyaromatic hydrocarbon (PAH) contents in the contaminated soils were shown in (figure 2-13), with well 2 having the highest TPH and PAH content compared with control 37.98mg/kg. The level of PAH in contaminated soil ranged from 1.996mg/kg – 13.913mg/kg with well 2 having the highest PAH content compared with control 0.059mg/kg. (Table 2). TPH concentration in surface and subsurface soil samples collected from crude oil polluted site, exceeded the 50mg/kg compliance baseline limit set for petroleum industries in Nigeria. Total Petroleum Hydrocarbon levels in the soil samples were higher than the global average permissible limit of TPH for soil (50 mg/kg; Lotfinasabasl et al., 2013), indicating high PHC pollution. Chukwujindu et al (2008) observed the level of TPH in three sites and reported high concentration ranging from 486-4438.7; 116.3-433.4 and 54.5-244.2mg/kg for 0-15, 15-30, and 30-60cm. In agreement with this study similar TPH values was reported from all the wells studied and ranged from 1585.83-5991.69 and 684.86-1516.76mg/kg for 0-15 and 15-30cm depth respectively. These report were higher compared to those reported by Chukwujindu et al 2008.

Figure 2: Diversity of hydrocarbon compounds in well 1 T15cm TPH

Figure 3: Diversity of hydrocarbon compounds in well 1 T15cm PAH

Figure 4: Diversity of hydrocarbon compounds in well 1 B30 cm TPH

Figure 5: Diversity of hydrocarbon compounds in well 1 B30 cm PAH

Figure 6: Diversity of hydrocarbon compounds in well 2 T15cm TPH

Figure 7: Diversity of hydrocarbon compounds in well 2 T15cm PAH

Figure 8: Diversity of hydrocarbon compounds in Well 2 B 30cm TPH

Figure 9: Diversity of hydrocarbon compounds in Well 2 B 30cm PAH

Figure 10: Diversity of hydrocarbon compounds in Well 3 T15cm TPH

Figure 11: Diversity of hydrocarbon compounds in Well 3 T15cm PAH
Figure 12: Diversity of hydrocarbon compounds in Well 3 B30cm TPH

Figure 13: Diversity of hydrocarbon compounds in Well 3 B30cm PAH
Figure 14: Diversity of hydrocarbon compounds in control

Figure 15: Diversity of hydrocarbon compounds in control soil PAH
4.1 Identification of fungi isolates using 18SrRNA

Polymeric chain reaction was done using the following internal transcribe sequences ITS1F CTTGGTCATTTTAGAGGAAGTTAA and ITS4R TCC TCC GCT TAT TGA TAT GC for amplification. Identification with molecular characterization and complete sequencing of fungal isolates reveal that the isolates were from the following genera: *Aspergillus*, *Pseudallescheria*, *Scedosporium*, *Candida*, *Byssochlamys*, *Penicillium*, sequences of all the fungi isolates have been deposited in the GenBank database under accession numbers MH973242 to MH973256. The fungal isolates obtained from this study were similar with those earlier reported from an oil polluted sample collected around Uzere flow stations in Warri (Chikere and Chijioke-osuji, 2006). The fungal genera: *Trichoderma*, *Penicillium*, and *Aspergillus* are major species that were reported in crude oil polluted soil (Jobson *et al*. 1972; Nwankwo, 1984; Odu, 1981; Chikere and Chijioke-osuji 2006). These were similar to those obtained in the present study. The fungi identified in this study belong to the following genera such as *Scedosporium*, *Penicillium*, *Aspergillus*, *Pseudallescheria*, *Byssochlamys* sp *Candida*, with *Pseudallescheria*, *Byssochlamys* never been reported in crude oil degradation studies.

Zhang *et al*. 2016 studied the biodegradation of crude oil by *Apergillus* spp. And their potential use for oil recovery, in agreement with this study *Apergillus terreus* grew on mineral salt agar containing crude oil base, indicating their ability to utilize crude oil for growth and reproduction.

Nkwelang *et al*. (2008) showed that the main active fungi genera of crude oil contaminated soil were *Aspergillus*, *Penicillium*, and *Mucur*. In this study *Scedosporium* apiospermum, *Penicillium* critinum, *Candida annelisea*, *Pseudallescheria* boydii *Aspergillus terreus* *Pseudallescheria* ellipsoidea *Byssochlamys* spectabilisi were isolated from crude oil impacted soil with *Pseudallescheria* boydii, *Aspergillus* terreus and *penicillium* being the most dominant genera. Also the presence of these isolate in crude oil contaminated soil suggest that they could utilize fractions of petroleum hydrocarbon for growth. Conceição *et al*. (2005) concluded that the genera of Microorganism found in crude oil polluted soil Definitely possesses mechanism to resist adverse environmental conditions and some of them have the ability to degrade oil residue.

Fungi biotransform a wide range of hydrocarbons using both extracellular peroxidases and laccases and the intracellular P-450 detoxification system. However, while fungal-bacterial interactions play an important role in the biodegradation of recalcitrant hydrocarbons or mixtures of contaminants, fungal hydrocarbon biodegradation is often non-specific, incomplete and can even result in the formation of more toxic metabolites (Cerniglia 1997).

![Figure 2 : Abundance of fungal isolates in crude oil impacted soil](image-url)
From figure 9 sequences of CYF11, CYF12, and CYF1 were having 99% similarity to sequence of Scedosporium boydii already present in GeneBank. Sequences of CYF 4 having 98% similarity to sequences of Aspergillus terreus.

5. Conclusion
This study was successfully managed to isolate and identify fungi strains that were able to degrade hydrocarbon present in crude oil, further studies need to be done to isolate and identify other group of microorganism present in the region that are able to utilize long chain hydrocarbon in the shortest period of time to help expedite the process of bioremediation.

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