



Culture-Dependent and Metagenomic Profiling of Eukaryal Diversity in Petroleum Hydrocarbon-Polluted Soil from B-Dere, Gokana, Rivers State, Nigeria

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JAMB/2021/v21i1230414

Editor(s):

(1) Prof. Hung-Jen Liu, National Chung Hsing University, Taiwan.

Reviewers:

(1) Saurabh Chhotalal Norris, Sumandeep Vidhyapeeth University, India.

(2) Rebah N. Algafari, Al-Nahrain University, Iraq.

Complete Peer review History, details of the editor(s), Reviewers and additional Reviewers are available here:
<https://www.sdiarticle5.com/review-history/75752>

Original Research Article

Received 20 August 2021
Accepted 30 October 2021
Published 07 December 2021

ABSTRACT

B-Dere is one of the communities in Ogoniland and a major oil producing area in Rivers State where oil exploration and production activities commenced 50 years ago and is now characterized by oil fields and installations that have remained dormant for several decades. Past spills, lack of maintenance, oil trapping and damage to oil infrastructures have been common sight in this region and the environment has been without remediation over the years. B-Dere community has suffered surface water pollution throughout its creeks with massive hydrocarbons for years without remediation. The aim of this study was to determine the Culture- dependent and Metagenomic studies of fungal diversity in petroleum polluted soils in B-Dere community in Gokana LGA of Rivers State, Nigeria. This is to profile fungal communities through next-generation techniques by shotgun sequencing of total DNA isolates directly from the oil polluted environment. Soil samples were collected aseptically with hand auger at a depths of 0-15 and 15-30 cm and made up to a composite sample and transported to the laboratory for analysis using standard microbiological methods for culture- dependent analysis while the Metagenomic studies was carried out at the Microbial Insights,

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Incorporated; United State of America. In this study next-generation sequencing techniques by shotgun sequencing of total DNA methods were used for identification of fungal isolates from the crude oil polluted soils in B-Dere. Deoxyribonucleic acid (DNA) extraction from crude oil polluted soil samples was performed using ZymoBiomix DNA extraction kits (Zymo Research, Inc; USA). DNA sequencing was performed by the next generation sequencing technique to determine the nucleotide sequence of all eukaryal community structure present in the polluted soil sample using ITS region. Results of the culture-dependent technique showed that only two fungal genera namely *Penicillium* sp and *Aspergillus* sp were isolated and identified while the soil was mainly dominated by the genera *Penicillium* (73.33%), followed by the *Rhodotorula* (6.54%), *Dactylellina*(5.09%), *Kalmanozyma*(2.56%), *Fereydounia*(1.89%), *Xerochrysium*(1.36%), *Arthrobotrys* (1.14%) and *Diutina* (0.77%) by the metagenomic analysis. However the three major groups were classified as Ascomycota, Basidiomycota and Mucoromycota with Ascomycota having the highest taxonomic reads of 86.76%. However, a total of 60 eukaryal species were identified, in the metagenomic study. In conclusion, these fungal strains can be used in bioremediation process and oil pollution reduction in soil ecosystems because of their high activity in aliphatic hydrocarbon degradation and cell surface hydrophobicity. The next-generation techniques by shotgun sequencing assays appear to be suitable alternatives for rapid identification of the above mentioned fungal isolates.

Keywords: *Culture-dependent method; metagenomics profiling; eukaryal diversity; B-Dere; soil; petroleum hydrocarbons.*

1. INTRODUCTION

Petroleum hydrocarbons are the most widespread environmental pollutants, including n-alkanes, cycloalkanes, and polycyclic aromatic hydrocarbons (PAHs) that have been regarded as a serious ecological and public health concerns [1] Petroleum hydrocarbon exploration causes great environmental problems in Nigeria as a result of oil spillages both onshore and offshore. It is known that greater degradation of oil pollutants in the soil is carried out in-situ by a consortium of microorganism and more than 200 species of bacteria and fungi biodegrade [2]. Hydrocarbon-degrading microorganisms are widely distributed in the ecosystem but their characterization is difficult to achieve, mainly because their ability to metabolize hydrocarbons depends largely on a particular substrate. With the advent of molecular biological tools, the abundance and distribution of microorganisms in natural environments can be studied.

However, petroleum is the world's most important derived energy source and it's the major income earning of the Nigerian economy [3]. The Niger Delta is among the ten most significant wetlands and marine ecosystems in the world in which ecosystem has been severely damaged by petroleum pollution due to unsustainable oil exploration activities and they eventually constitute as environmental pollutants. The most common are petroleum hydrocarbons which includes-alkanes and other aliphatics, aromatic compounds and other minor constituents [4,5]. Petroleum is a complex

mixture of hydrocarbons and non-hydrocarbons from metallic porphyrins, acid and organ metallic compounds origin [6]. The refining, storage and distribution of crude oil and allied petroleum products are all point sources of soil and water pollution [6,7,8] Polycyclic aromatic hydrocarbons (PAHs) are among the toxic components of petroleum oil spills considered to be the main contributor of mutagenicity and teratogenicity in humans [9]. Globally, there is a growing concern about the risks of environmental contamination from the exploration, transportation and storage of petroleum products. Due to growth and activities of petroleum and petroleum associated industries in Nigeria and world over, there have been increased occurrences of pollution in our environment. The characteristics of petroleum hydrocarbon has made it one of the most significant pollutant in the environment as it is capable of causing serious changes to humans and the ecosystem [10].

In the oil-producing areas of Nigeria, especially the Niger Delta region have devastating experiences of oil spills on both the terrestrial and aquatic environments in the past 50 years of petroleum hydrocarbon exploration and production [11]. Udotong [12] presented for the first time, a 43-years oil spills statistics in Nigeria. This statistics shows that oil spills occur almost daily and in most cases more than once in a year. Contamination of surface water with petroleum hydrocarbon is reportedly among the

most severe environmental problems facing Nigeria [13,14,15].

The introduction of metagenomics play a vital role in unearthing and monitoring the microbial communities by providing access to the taxonomic and functional gene composition. Most of the metagenomic analysis tools have opened new windows of opportunities for researchers to analyze the microbial community as a whole (whole-genome sequencing) and the genetic diversity, which facilitates active metabolic pathways in any given environment. With the advent of massive DNA sequencing technologies, several methods have been developed to assign shotgun reads to microbial taxonomic categories. These methods aim to perform a microbial community profiling that infers its relative structure, and they are very important to understand how microbiomes work in nature, their phylogenetic composition, and even their dynamics and evolutionary history. However, currently most metagenomics studies use a range of a short-read sequencing instruments between 100 and 600 bp in order to maximize counting reads and lower costs. These short-reads contain the genomic, phylogenetic, and functional information of the microbiome into millions of discrete DNA fragments, which are sufficient to make a reliable estimate of the phylogenetic diversity present in a microbial sample. Previous studies have focused on species composition of a community, metagenomic studies enable assessment of the biological function of the gene rather than the taxonomic identify. Microbial diversity is measured as a function that depends on the richness and abundance of distinct taxons among any community [16]. Obtaining representative DNA sequences from the entire community is essential to make valid inferences. Profiling a microbial community through 16S gene analysis generally consists of four steps. To date, several computational tools have been developed to analyze microbial communities through the 16S gene marker; however, estimating the total microbial diversity in any environment is a still a major challenge [17,18,19,20].

Profiling microbial communities from massive sequencing data constitutes a breaking point in the understanding of population structure and dynamics, their ecological functions and the complex relationships established between non-cultivable microorganisms. The study of microbial biodiversity and whole-genome sequencing and

analysis of any complicated samples with numerous microorganisms, which are not pure and un-culturable in any given laboratory, have made shot gun metagenomics a more efficient tool than most of the already existing conventional techniques. Owing to the paucity of information regarding the microbial community composition of hydrocarbon impacted sites at B-Dere in Gokana Local Government Area of Rivers State, Nigeria, this study is therefore aimed at comparing the culture dependent and metagenomics study of the fungal diversity of an oil polluted soil.

2. MATERIALS AND METHODS

2.1 Soil Sample Collection

Soil samples were collected from B-Dere polluted sites in Gokana L.G.A of Rivers State with the aid of a hand auger using the method by. In each sampling point, samples were collected from two different points at different depths of 0-15 cm and 15-30 cm and made up into composite samples. The soil samples were put in sterile polyethylene bags and transported to the Microbiology Research Laboratory for analyses within 2 hours of sample collection.

2.2 Isolation of Fungal Isolates from Soil Sample by Culture Dependent Technique

Mineral Salt Agar (MSA) amended with 250mg of tetracycline to inhibit the growth of bacteria was compounded as described by [21]. Sterilization was done using the autoclave at 15psi (121°C) for 15 minutes. MSA was composed of 0.29 g of KCl, 10 g of NaCl, 0.42 g of MgSO₄·7H₂O; *King agar B base 6g/l*.

King B base was added to the Mineral Salt Agar as a bio stimulant to enhance the growth of the hydrocarbon utilizing fungi which did not grow on the ordinary Mineral Salt Agar as a results of mutation and recombination due to heavy contamination of soil over a period of time, thus King B base serves as limiting nutrient for the growth of a recombinant species.

The soil sample was diluted using 10-fold serial dilution. The dilution was carried out to a dilution of 10⁻⁸ in which 1g of the soil sample was weighed and dispensed into a sterile test tube containing 9ml of sterile normal saline. Crude oil serves the carbon and energy source for the

hydrocarbon utilizers). All the inoculated Mineral salts Agar plates optimized with king agar B base were incubated at room temperature (28°C) in an inverted form for 7 days.

2.3 Morphological Characterization of Fungal Isolates

The fungal isolates were identified morphologically based on conidial morphology and pigmentation. The technique described by [22]. was also adopted for the identification of the isolated fungi using cotton blue in lactophenol stain. This was done by placing a drop of the stain on clean slide with the aid of a mounting needle, where a small portion of the aerial mycelia from the representative fungal cultures was removed and placed in a drop of lactophenol. The mycelium was spread on the slide with a needle. A cover slip was gently placed with little pressure to eliminate air bubbles. The slide was then mounted and viewed under the light microscope using $\times 10$ and $\times 40$ objective lenses. The morphological characteristics and appearance of the fungal isolates seen were identified in accordance with standard scheme for identification of fungi as adopted by [23].

2.4 Metagenomics Study of Eukaryal Diversity DNA Extraction from Soil Samples

DNA was extracted from soil samples using an extraction kit from ZYMO Research, microbial insights, United State of America. Extraction was carried out according to manufacturer's instruction. About 0.25g of soil was introduced into a Zymo Bashing Bead Lysis bottle and 750

μ l Lysis solution was also introduced. This was fixed unto bead beater (Gene disruptor) and processed at a high speed for 5 minutes. Centrifugation was done at 10,000xg for 60 seconds (1 minute). About 400 μ l of the supernatant was transferred into ZymoSpin IV Spin Filter in a collection tube and centrifuged at 7,000xg for 60 seconds; 1200 μ l of soil DNA Binding Buffer was added in a collection bottle to wash the filtrate; 800 μ l of the mixture was put in a ZymoSpin IIC Column in a collection tube and centrifuged at 10,000xg for 60 seconds; 200 μ l of DNA prewash Buffer was then added to the filtrate in a ZymoSpin IIC Column and then

centrifuged at 10,000xg for 60 seconds. 500 μ l Soil DNA Wash Buffer was added and centrifuged at 10,000xg for 60 seconds. The filtrate obtained was transferred into a clean

1.5ml microcentrifuge tube, 100 μ l of DNA Elution Buffer was added and centrifuge at exactly 10,000xg for 30 seconds to elute the DNA. The eluted DNA is now transferred into a prepared ZymoSpin Filter in a clean 1.5ml microcentrifuge tube and centrifuged at exactly 8,000xg for 60 seconds. The eluted DNA is now suitable for PCR assay.

2.5 DNA Sequencing

Next Generation Sequencing of pure DNA amplicons was carried out at Microbial Insights in the United State of America. The illumine Miseq machine was used for the analysis.

2.6 Processing of Illumina Miseq Sequenced Data Reads

Next Generation Sequencing reads were trimmed and filtered using the FASTX TRUNCATE program with a minimum length of 250 nucleotide to remove low-quality regions and short reads. Chimeras were detected with UCHIME2. To form operational taxonomic units, the sequence reads were clustered at 97% sequence identity with UCLUST and the most abundant unique read within each cluster was as its representative sequence. Initial phylogenetic identification was made using BLAST. The BLAST search program was used to check for close relatives and phylogenetic affiliation. The search results were used as a guide for phylogenetic tree construction using MEGA7.

Rarefaction diversity analysis, Inverse Simpson species diversity analysis, heatmaps of sample structure and abundance using the Jaccard index and thetaYC coefficient, Venn diagrams of shared microbial species, community study, population, abundance, similarity and distance tree of the samples and microbial species were all determined using MOTHUR [24].

3. RESULTS

3.1 Colonial Morphology of Isolates

Two (2) different colonies were identified on improved Mineral Salt Agar. The isolates showed creamy white colonies with smooth surfaces

while the other was yellowish with green mycelium and white lawn at the periphery and were identified as *Aspergillus* and *Penicillium* species respectively (Table 1). However inoculation of crude oil contaminated soil samples on the conventional Mineral Salt Agar used for Hydrocarbon utilizing fungi did not produce any growth until a limiting factoring agar B base was added which now showed growth of two fungal colonies that were isolated and identified as *Aspergillus* and *Penicillium* species.

3.2 Metagenomic Identification of Autochthonous Fungal group

Metagenomic approach was used for identification of fungal population in the hydrocarbon impacted soil sample. The 16S

fungal metagenomic report revealed the taxonomic classification as follows:

Phylum Classification: The result showed that the soil had Ascomycota (86.76%) as the predominant group. This was followed by Basidiomycota (12.73%), Mucoromycota (0.34%) while 0.10% of the taxa that belonged to this phylum were unknown.

Genus Classification: The metagenomic analysis of the polluted soil showed that the dominant genera were the *Penicillium* (73.32%), followed by *Dotorula* (6.54%), *Dactylellina* (5.09%), *Kalmanozyma* (2.56%), *Fereydounia* (1.89%), *Xerochrysium* (1.36%), *Arthrobotrys* (1.14%), *Malassezia* (0.94%), *Aspergillus* (0.85%), *Diutina* (0.77%) (Table 2).

Table 1. Morphology of fungal isolates obtained from crude oil contaminated soil

Isolates	Cultural/Colonial morphology	Microscopy	Presumptive Organisms
Si1	Creamy white colonies with smooth surface.	Non-septate hyphae with unbranched conidiophores and swollen vesicles, round conidia singly and some in chains.	<i>Aspergillus</i> sp.
Si 2	Yellowish green mycelium, white lawn at the periphery. Smooth powder colonies with greenish grey surface	Non septate branched hyphae with spores arranged in chains. Has short filaments with conidiophores	<i>Penicillium</i> sp.

Table 2. Top genus classification

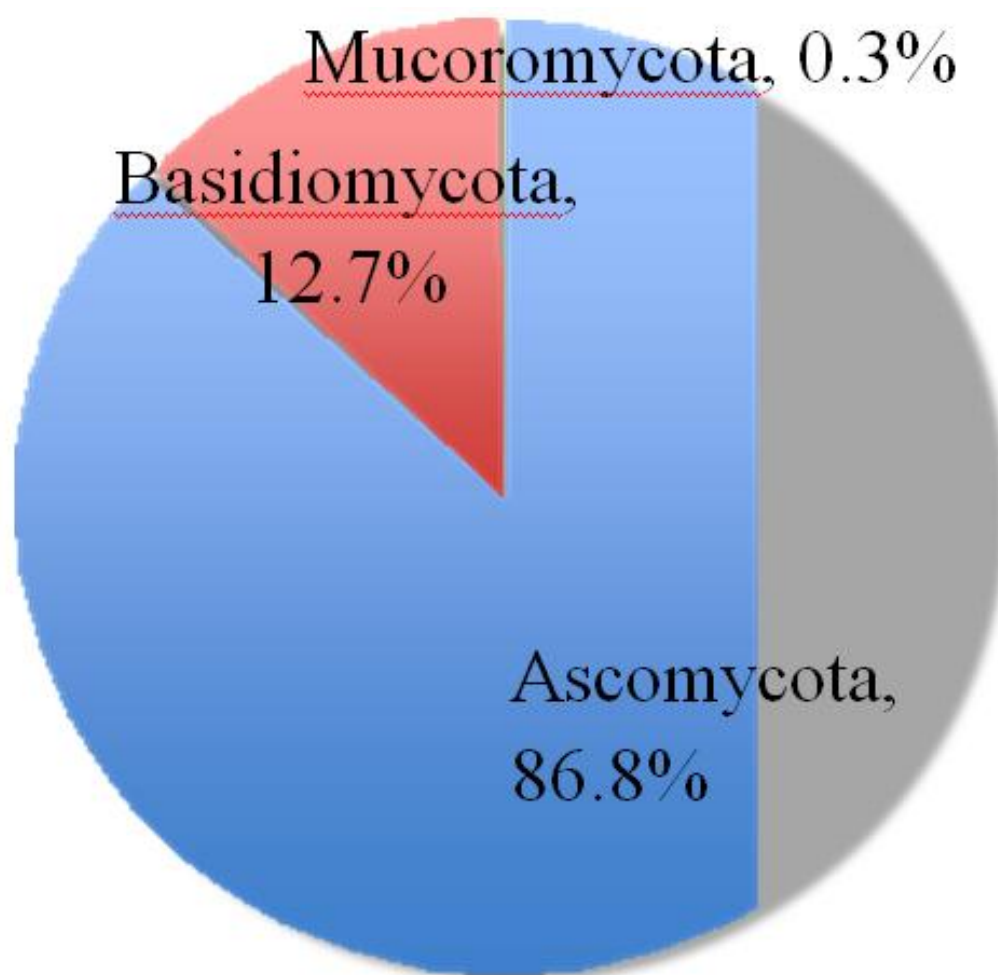
Genus	Reads	Percent %
Penicillium	20,496	73.32%
Rhodotorula	1,828	6.54%
Dactylellina	1,422	5.09%
Kalmanozyma	716	2.56%
Fereydounia	528	1.89%
Xerochrysium	380	1.36%
Arthrobotrys	318	1.14%
Malassezia	264	0.94%
Aspergillus	238	0.85%
Diutina	214	0.77%

Table 3. Taxonomic classification of reads

Taxonomic Level	Reads classified to Taxonomic level	% Total Reads Classified to Taxonomic Level
Kingdom	27,952	100.00%
Phylum	27,926	99.91%
Class	27,926	99.91%
Order	27,926	99.91%
Family	27,900	99.81%
Genus	27,862	99.68%
Species	27,480	98.31%

Table 4. Phylum classification of DNA sequences (Reads)

Classification	Number of Reads	% Classified Reads
Ascomycota	24,254	86.76%
Basidiomycota	3,558	12.73%
Mucoromycota	94	0.34%

**Fig. 1. Phylum Classification of DNA sequences (Reads)**

4. DISCUSSION

Phylogenetic relationships among microbial taxa in natural environments provide key insights into the mechanisms that shape community structure and functions. This study was carried out to profile the microbial distribution in natural ecosystems particularly in crude oil contaminated soil. Soils provide adequate and optimal conditions for various activities of microorganisms in the soil, particularly in the enhancement of biogeochemical cycles of organic and inorganic compounds. This method involved the culture-independent genomic

analysis of microbiomes on a particular environment [24]. From the study, three major Phyla, Ascomycota, Basidiomycota, Mucoromycota were identified with the following genera as dominant fungi namely, *Penicillium*(73.32%), followed by *Dotorula*(6.54%), *Dactylellina*(5.09%), *Kalmanozyma*(2.56%), *Fereydounia*(1.89%), *Xerochrysium*(1.36%), *Arthrobotrys*(1.14%), *Malassezia*(0.94%), *Aspergillus*(0.85%), *Diutina* (0.77%)

Most of the strains isolated after the metagenomic based phylogeny belonged to the

phylum Ascomycota, although few Basidiomycota were isolated. The dominance of Ascomycota in polluted soil has been extensively acknowledged. Zhang [25] reported that Ascomycota represented up to 73–96% of the total 18S sequences of a coking area soil, this is in consonance with reports obtained from this study with 86.76% reads. Ascomycetes have been reported to remove anthracene [26], from crude oil polluted soils. An oilfield with a history of 50 years pollution like the B-Dere case where this fungi was found to be abundant, demonstrates that Ascomycetes is less sensitive to soil PAHs [27]. However the Ascomycetes possess a Sclerotia which are firm aggregations of modified hyphae that serve as resting bodies to overcome adverse conditions with mycelial strands which are linear hyphal aggregates capable of unlimited growth.

Similarly, only a small fraction of the isolates belonged to Basidiomycota (12.73%) [25]. From other studies, it is observed that Basidiomycota represented only 5.3% of the total isolates. Besides *Penicillium*, *Rhodotorula*, *Dactylellina*, *Kalmanozyma*, *Fereydounia*, *Xerochrysium*, *Arthobotrys*, *Malessezia*, *Aspergillus* and *Diutina* were among the isolates identified in this study. However, in the culture dependent technique, all samples were dominated by *Penicillium* and *Aspergillus* species only. The identification of these genera as polluted soil inhabitants has been reported already. *Penicillium* have been implicated in hydrocarbon degradation [28,29,30]. Mycelial organisms can penetrate soluble fractions of petroleum hydrocarbon thus increasing the surface area available for bacterial attack. Besides, fungi can grow in environmentally stressed conditions such as low pH and poor nutrient status. The potential of fungi in the utilization and degradation of petroleum hydrocarbon and eventual oil spill cleanup has been established and polluted soils are vast reservoirs for these groups of beneficial microorganisms [31]. On the other hand, Mucoromycota was relatively small with 0.34% of the contaminated soil. However, Moraese *et al* [32], found more than 50% of Mucoromycota in a petroleum contaminated coastal site, but very small fraction was obtained in this study as a negligible fraction of the mycoflora. Mucoromycota represented a small fraction of the isolates also in an estuarine sediment contaminated mainly by PAHs [33], and in heavy crude oil-contaminated soil [34]. Similar results have been reported earlier [35]. The isolation of

these strains opens to intriguing solutions for their application in bioremediation processes.

On the contrary in the present study, only two fungal isolates *Aspergillus* and *Penicillium* was isolated using the culture dependent technique. Initially there was no growth of any fungi on the conventional Mineral salt agar used for Hydrocarbon utilizing fungi until the limiting nutrient *king agar B base* was added to the medium to boost the growth of only two genera of fungi. It is possible that the nutrient added increased their growth or metabolic activity which was measured by their metabolic response to produce growth which was proportional to the amount of the specific nutrient added to the assay medium [36]. However, *Aspergillus* and *Penicillium* strains have been already isolated in several polluted samples [37,33,34]. Data on soil isolation of *Aspergillus niger* [37], *fumigatus* [33] *Aspergillus terreus* [34], *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus nomius* [34], *Aspergillus sydowii* [38], *Aspergillus versicolor* [39]. have been reported. Many of the species of *Aspergillus*, however has been isolated as air-born contaminants or of clinical relevance [40,41] or have been only associated to not-contaminated lands [42,43], or as part of the rhizosphere of tussock [44].

5. CONCLUSION

This study was carried out to profile fungal communities through next-generation sequencing techniques by shotgun sequencing of total DNA isolated directly from the crude oil contaminated environment. The study have contributed substantially to the discovery of totally new microbial diversity in such distinctive environments in B-Dere community of Ogoniland. In the present study, we evaluated the evolution of fungal communities from polluted soil by culture independent and dependent methods. The results showed that the soil depth did not influence the evolution of microbial communities. The strains isolated reflected the microbial composition of the enriched consortia. It is expected that future studies will focus on the biodegradation abilities of the isolates in the bioremediation of oil polluted environments.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and

producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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