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Molecular Characterisation of Culturable Aerobic Hydrocarbon Utilising Bacteria and Fungi in Oil Polluted Soil at Ebubu-Ejama Community, Eleme, Rivers State, Nigeria

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Authors' contributions

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

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ABSTRACT

The research work aims to isolate and identify culturable hydrocarbon utilising bacteria and fungi, from an aged oil impacted soil and these organisms would be used as inoculants in bioremediation of petroleum hydrocarbon pollution. Culturable hydrocarbon utilising bacteria and fungi were harvested from aged oil impacted soil in Ebubu-Ejama Community, Eleme, Rivers State, Nigeria. The hydrocarbon utilising fungi and bacteria were isolated by using mineral salt agar, and petroleum hydrocarbon was supplied to the inoculated plates using the vapour phase technique. Genomic DNA of hydrocarbon utilising bacteria and fungi were extracted and subjected to Polymerase Chain Reaction (PCR). PCR amplified DNA of fungi and bacteria were sequenced by using BIG Dye Terminator Kit on a 3510 ABI sequencer. Internal Transcribed Spacer (ITS) sequence for fungi and 16S rRNA sequence for bacteria were identified using Basic Local

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Alignment Search Tool (BLAST) algorithm of National Center for Biotechnology Information (NCBI). ITS sequences for fungi shows proximal relatedness to *Aspergillus aculeatus* strain LrBF25 and *Penicillium citrinum* strain XQ39. The 16S Sequence for bacteria shows proximal relatedness to *Alcaligenes faecalis* strain VC-10, *Alcaligenes faecalis* strain 4339 and *Bacillus cereus* strain GOAA7MS06. Sequences of the hydrocarbon utilising bacteria and fungi were submitted to GenBank and their Accession numbers were F10: *Aspergillus aculeatus* MG738329; F11: *Penicillium citrinum* MG738328; B8: *Alcaligenes faecalis* MG738324; B9: *Bacillus cereus* MG738325 and B10: *Alcaligenes faecalis* MG738326. These organisms could be used to bioaugment the cleanup of petroleum hydrocarbon from the environment.

Keywords: Hydrocarbon utilising bacteria and fungi; Ebubu-Ejama; 16S ribosomal ribonucleic acid; internal transcribed spacer; polymerase chain reaction; GenBank.

1. INTRODUCTION

Since the inception of petroleum exploration and exploitation in the Niger Delta of Nigeria, the region has been constantly polluted with hydrocarbons and other materials related to petroleum exploration, exploitation and processing. The Niger Delta is now being listed among the Five most severely petroleum-damage ecosystems in the world [1]. Petroleum hydrocarbon pollution has created significant health and environment problems; hence there has been an increasing agitation by its inhabitants for cleanup, reclamation and restoration of oil polluted environment within the Niger Delta [2]. The use of hydrocarbon utilising microbes in the cleanup of petroleum hydrocarbon is a potential solution [3] because effective bioremediation relies primarily on microbial consortia rather than a single species. Several studies have shown that there is a synergy between bacteria and fungi in the biodegradation of petroleum hydrocarbon in soil [4,5]. Enumeration and monitoring of hydrocarbon utilising bacterial and fungi populations in contaminated environments using culture-dependent techniques provide a tentative identification of cultured isolates. Recent studies results in microbial ecology recommend the combination of molecular and culture-dependent approaches to describe bacterial and fungal diversity and their degradative ability in petroleum hydrocarbon polluted environments [6]. For the past 20 years, scientists have relied on polymerase chain reaction in identifying and classifying microbes. The 16S rRNA sequencing is used in identification and classification of bacterial diversity, while the Internal Transcribed Spacer sequencing is used in the identification and classification of fungi diversity.

The objective of this study is to isolate and identify culturable hydrocarbon utilising fungi and bacteria present in an aged oil impacted soil in Ebubu-Ejama, Rivers State, Nigeria.

2. MATERIALS AND METHODS

2.1 Site Description

Hydrocarbon utilising bacteria and fungi were harvested from an aged oil-impacted soil in Ebubu-Ejama Community of Eleme Local Government Area in Rivers State, Nigeria during 2017. Oil spill occurred in this area in 1970 from the Agbada-Bomu 28" Trans Niger Pipeline (TNP) belonging to Shell Petroleum Development Company (SPDC).

2.2 Sample Collection

Caked oil impacted soil sample were collected from 0 to 15 cm depth of the surface of the soil by using sterile polythene bags. The sample was then transported to Applied and Environmental Biology Laboratory, Rivers State University, Nigeria and stored at 4°C for 24 hours.

2.3 Preparation of the Sample

The caked soil sample was homogenised by using ceramic mortar and pestle, sterilised with 100% Ethanol and air dried. The soil sample was sieved with 2 mm mesh sieve to remove debris.

2.4 Enumeration of Total Culturable Heterotrophic and Hydrocarbon Utilising Bacteria and Fungi

Bacterial population was estimated by using colony forming unit per gram (CFU/g) method and fungi population was estimated using spore forming unit per gram (SFU/g) method. After a tenfold serial dilution [7], spread plate method was used to enumerate total culturable heterotrophic bacteria on nutrient agar and total

culturable heterotrophic fungi on Sabouraud dextrose agar; total culturable hydrocarbon utilising bacteria was enumerated by using spread plate method on mineral salt medium incorporated with fungisol to inhibit fungi growth, the plate was supplied with hydrocarbon through vapour phase transfer technique [8,9]. The plates were incubated for 9 days when discrete colonies were visible [10,11]. Total culturable hydrocarbon utilising fungi was enumerated by using spread plate method on mineral salt medium incorporated with tetracycline to inhibit bacterial growth. The plates were supplied with petroleum hydrocarbon through vapour phase transfer technique and incubated for 3 days when spores were observed.

2.5 Molecular Characterization of Bacterial Isolates

Genomic DNA of hydrocarbon utilising bacteria isolates was extracted using the boiling method endorsed by [12]. The extracted DNA sample was quantified by using Nano drop 1000 spectrophotometer. Two Microlitres (2 µl) of the extracted bacterial DNA were subjected to polymerase chain reaction (PCR) using the 16S rRNA sequence. The 16S region of the rRNA gene of bacterial isolates was amplified using 27F 5^{1}AGAGTTTGATCCTGGCTCAG3^{1} and 1492R 5^{1}GGTTACCTTGTTACGACTT3^{1} synthesised primers. During amplification, 0.5 µm of the synthesised primer was used. Single strength master mix of 12.5µl comprise of Taq Polymerase, DNTPs (deoxyribonucleotide Triphosphate), buffer and $MgCl_2$ (Magnesium Chloride) was used during amplification. Nuclease free water was used during amplification, but its quantity was dependent on the number of isolates to be amplified. The PCR final volume was set at 25 µl.

2.6 Molecular Characterization of Fungi Isolates

Genomic DNA of hydrocarbon utilising fungi isolates was extracted using ZR fungi/bacterial DNA mini prep extraction kit. The extracted fungi DNA sample was quantified by using Nanodrop 1000 spectrophotometer. Two Microlitres (2 µl) of the extracted fungi DNA were subjected to PCR method using sequencing known as the internal transcribed spacer (ITS). The ITS region of the rRNA gene of the fungi isolates was amplified using ITS1 5^{1}TCCGTAGGTGAACCTGCGC3^{1} and ITS4 5^{1}TCCTCCGCTTATTGATATGC3^{1} synthesised primer. During amplification, 0.5 µm of the synthesised primer was used. Single strength master mix of 12.5 µl comprised of Taq Polymerase, DNTPs (deoxyribonucleotide Triphosphate), buffer and $MgCl_2$ (Magnesium Chloride) was used during amplification. Nuclease free water was used during amplification, but its quantity is dependent on the number of isolates to be amplified. The PCR final volume was set at 25 µl.

2.7 Agarose Gel Electrophoresis

The amplified PCR gene was subjected to agarose gel electrophoresis to ascertain whether the PCR was successful. Most of the 16S rRNA sequence for bacteria is ~ 1500 base pair in length [13], while ITS sequence for fungi are mostly within 500 to 800 base pair in length [14]. The gel was placed on a UV transilluminator for the bands to fluoresce and pictures were taken. Sequencing of the amplified 16S rRNA and ITS was done by using Big Dye Terminator Kit on a 3510 ABI sequencer.

2.8 Phylogenetic Analysis

Obtained sequences were edited by using bioinformatics algorithm trace edit, and both the 16S gene sequence and ITS sequence were compared with the database of the National Center for Biotechnology Information, using Basic Local Alignment Search Tool (BLAST). The evolutionary history of the obtained sequence was inferred using neighbor-Joining method [15]. The percentage of replicates trees in which the association taxa clustered together in bootstrap test was 1000 replicates. The tree was drawn to scale with branch length in the same limit as those of the evolutionary distance used to infer the phylogenetic tree. The evolutionary distances were computed by using the Jukes-Cantor method [16]. The evolutionary analysis was conducted in MEGA 7 [17]. Obtained sequences were submitted to GenBank to obtain their accession numbers.

3. RESULTS AND DISCUSSION

As seen from Tables 1 and 2, the mean density of hydrocarbon utilising fungi is high in compared to hydrocarbon utilising bacteria. The isolation of high number of hydrocarbon utilising microbes from petroleum polluted environment can be taken as an evidence that these organisms are the active hydrocarbon degraders [18]. Generally, Fungi can withstand harsh

environmental conditions and play an essential role in the degradation of petroleum hydrocarbon [19]. Due to the low substrate specificity of their degradative enzyme machinery (e.g. lignin peroxidase, laccase and Mn peroxidase), fungi can breakdown a wide range of pollutants including hydrocarbons in the contaminated environment [20,21].

Hydrocarbon utilising fungi and bacteria can survive in such harsh environment as they have developed enzymatic and physiological responses that allows them to use petroleum hydrocarbon as a substrate [22].

Molecular based technique was performed to affirm the identities of the hydrocarbon utilising fungi and bacteria. Bands from PCR amplified ITS of fungi isolates showed 550bp and 750bp (Plate 1). While Plate 2 showed band from PCR amplified 16S rRNA of bacterial isolates having 1500 bp.

Table 1. Total culturable heterotrophic and hydrocarbon utilising fungi

	Mean density (SFU/g) and standard deviation	Percentage $\frac{\text{TCHUF}}{\text{TCHF}}$
TCHF	$1.8\times10^{5}\pm5.657$	6.11%
TCHUF	$1.1\times10^{4}\pm4.243$	

Key: TCHF – Total Culturable Heterotrophic Fungi
TCHUF – Total Culturable Hydrocarbon Utilising Fungi

Table 2. Total culturable heterotrophic and hydrocarbon utilising bacteria

	Mean density (SFU/g) and standard deviation	Percentage $\frac{\text{TCHUB}}{\text{TCHB}}$
TCHB	$9.6\times10^{4}\pm2.121$	1.58%
TCHUB	$1.5\times10^{3}\pm0.707$	

Key: TCHB –Total Culturable Heterotrophic Bacteria
TCHUB – Total Culturable Hydrocarbon Utilizing Bacteria

Table 3. Identified fungi isolates ITS sequences relatedness and their assigned GenBank accession numbers

Isolates	Tentative Identity	GenBank accession number	GenBank closest cultured organism	Accession number/%
F10	*Aspergillus aculeatus*	MG738327	*Aspergillus aculeatus*	LC102114.1 100%
F11	*Penicillium citrinum*	MG738328	*Penicillium citrinum*	MG659617.1 100%

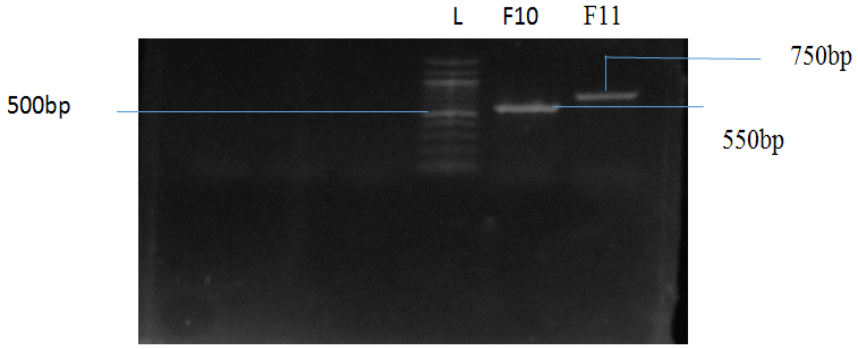

Plate 1. Agarose gel electrophoresis showing the amplified ITS bands of the fungal isolates. Lane L represents the 500bp molecular ladder, Lanes F10 and F11 indicate the ITS bands

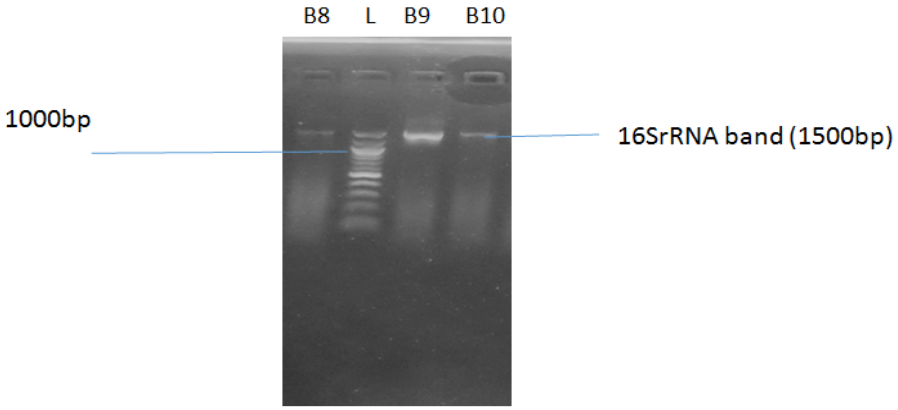

Plate 2. Agarose gel electrophoresis showing the amplified 16S rRNA gene bands of bacteria isolates. Lanes B8, B9, B10 represent the 16S rRNA bands while L represents the 1000bp molecular ladder

Table 4. Identified bacterial isolates 16S rRNA sequences relatedness and their assigned GenBank accession numbers

Isolates	Tentative identity	GenBank accession number	GenBank closest cultured organism	Accession number/%
B8	*Alcaligenes faecalis*	MG738324	*Alcaligenes faecalis*	KX817227.1 90%
B9	*Bacillus cereus*	MG738325	*Bacillus cereus*	MG725733.1 100%
B10	*Alcaligenes faecalis*	MG738326	*Alcaligenes faecalis*	KX828568.1 77%

Bacterial 16S rRNA Sequencing and fungi ITS sequence were aligned using BLAST algorithm of National Centre for Biotechnology Information (NCBI) database. Table 3 shows the percentage relatedness of the fungi isolate to those deposited in GenBank while Table 4 displays the percentage relatedness of the bacterial isolate to those deposited in GenBank. Several studies have demonstrated that *Alcaligenes, Bacillus, Penicillium* and *Aspergillus* spp. are potential petroleum hydrocarbon degraders [23,24,25,10]. Strains of *Bacillus cereus* has been identified as petroleum hydrocarbon degraders [26,27]. Although strains of *Alcaligenes feacalis* was first discovered in feces, later strains of *Alcaligenes feacalis* was commonly found in soil, water and other environments [28]. Recent studies have also shown that strains of *Alcaligenes feacalis* have been used as petroleum hydrocarbon degraders [29,30].

4. CONCLUSION

The present study reveals that *Alcaligenes feacalis*, *Bacillus cereus*, *Aspergillus aculeatus* and *Penicillium citrinum* are aerobic culturable hydrocarbon degraders in oil impacted soil at Ebubu Ejama, Rivers State, Nigeria. These hydrocarbon degraders could be used to bioaugment the degradation of petroleum hydrocarbon in the environment.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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