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

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Abstract

The aim of this research work is to isolate and identify culturable hydrocarbon utilizing 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 utilizing bacteria and fungi were harvested from aged oil impacted soil in Ebubu-Ejama Community, Eleme, Rivers State, Nigeria. The hydrocarbon utilizing fungi and bacteria were isolated using mineral salt agar and petroleum hydrocarbon was supplied to the inoculated plates using the vapour phase technique. Genomic DNA of hydrocarbon utilizing bacteria and fungi were extracted and subjected to Polymerase Chain Reaction (PCR). PCR amplified DNA of fungi and bacteria were sequenced 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 Alignment Search Tool (BLAST) algorithm of National Center for Biotechnology Information (NCBI). ITS sequences for fungi shows close relatedness to *Aspergillus aculeatus* strain LrBF25 and *Penicillium citrinum* strain XQ39. The 16S Sequence for bacteria shows close related to *Alcaligenes faecalis* strain VC-10, *Alcaligenes faecalis* strain 4339 and *Bacillus cereus* strain GOAA7MS06. Sequences of the hydrocarbon utilizing 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 can be used to bioaugment the cleanup of petroleum hydrocarbon.

Keywords: Hydrocarbon utilizing 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 listed among the Five most severely petroleum-damage ecosystems in the world (FME, 2006). Petroleum hydrocarbon pollution has created major health and environment problems, hence there have been an increasing agitation by its inhabitants for cleanup, reclamation and restoration of oil polluted environment within the Niger Delta (UNEP, 2011). The use of hydrocarbon utilizing microbes in the cleanup of petroleum hydrocarbon is a potential solution (Akinde *et al*., 2012), because effective bioremediation relies mainly on microbial consortia rather than a single species. Several studies have shown that there is a synergy between bacterial and fungi in the biodegradation of petroleum hydrocarbon in soil (Chaillan *et al*., 2004; Pozdnyakava, 2012). Enumeration and monitoring of hydrocarbon utilizing bacterial and fungi populations in contaminated environments using culture-dependent techniques provides tentative identification of cultured isolates. Recent research results in microbial ecology recommends the combination of molecular and culture-dependent approaches in order to describe bacterial and fungi diversity and their degradative ability in petroleum hydrocarbon polluted environments (Brito *et al*., 2012). 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 (ITS) sequencing is used in the identification and classification of fungi diversity.

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

2. Methodology

2.1. Site Description

Hydrocarbon utilizing bacteria and fungi were harvested from an aged oil-impacted soil in Ebubu-Ejama Community of Eleme Local Government Area in Rivers State, Nigeria, in the year 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 obtained from 0 to 15cm depth of the surface of the soil and the sample were collected in sterile polythene bags. The sample was then transported to Applied and Environmental Biology Laboratory, Rivers State University, Nigeria and stored at 4^0C for 24 hours.

2.3. Preparation of the Sample

The caked soil sample was homogenized using ceramic mortar and pestle that is cleaned with 100% Ethanol and air dried. The soil sample was sieved with 2mm mesh sieve in order to remove debris.

2.4. Enumeration of Total Culturable Heterotrophic and Hydrocarbon Utilizing Bacteria and Fungi

Bacteria population was estimated 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 (Harrigan & McCance, 1990), 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 utilizing bacteria was enumerated 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 (Ebuehi *et al*., 2005; Ibiene *et al*., 2011). The plates was incubated for 9 days when discrete colonies were observed (Ariyo & Obire, 2006; Atagana, 1996), total culturable hydrocarbon utilizing fungi was enumerated using spread plate method on mineral salt medium incorporated with tetracycline in order to inhibit bacterial growth. The plates was 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 utilizing bacteria isolates was extracted using boiling method according to Queipo-ortuno *et al*., (2008). The extracted DNA sample was quantified using Nano drop 1000 spectrophotometer. Two Microlitres (2µl) 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 the bacterial isolates were amplified using 27F 5^1AGAGTTTGATCCTGGCTCAG3^1 and 1492R 5^1GGTTACCTTGTTACGACTT3^1 synthesized primers. During amplification, 0.5µm of the synthesized primers was used. Single strength master mix of 12.5µl which comprises of Taq Polymerase, DNTPs (deoxyribonucleotide Triphosphate), buffer and $MgCl_2$ (Magnessium 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 25µl.

2.6. Molecular Characterization of Fungi Isolates

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

2.7. Agarose Gel electrophoresis

The amplified PCR gene were subjected to agarose gel electrophoresis to ascertain whether the PCR was successful. Most of the 16S rRNA sequence for bacteria are approximately 1500 base pair in length (Mitra & Roy, 2010), while ITS sequence for fungi are mostly within 500 to 800 base pair in length (Schoch *et al* 2012). The gel was place on a UV transilluminator for the bands to fluoresce and pictures were taken. Sequencing of the amplified 16S rRNA and ITS was done using Big Dye Terminator Kit on a 3510 ABI sequencer.

2.8. Phylogenetic Analysis

Obtained sequences were edited using bioinformatics algorithm trace edit and both the 16S gene sequence and ITS sequence were compared with the sequences that is in 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 (Saitou & Nei, 1987). 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 using the Jukes-Cantor method (Jukes & Cantor, 1969). The evolutionary analysis was conducted in MEGA 7 (Kuma *et al*., 2016). Obtained sequences were submitted to Genbank in order to obtain their accession numbers.

3. Results and Discussion

As seen from table 1 and 2 below, the mean density of hydrocarbon utilizing fungi is high compared to hydrocarbon utilizing bacteria. The isolation of high number of hydrocarbon utilizing microbes from petroleum polluted environment is commonly taken as evidence that these organisms are the active hydrocarbon degraders in that environment (Chikere & Ekwuabu, 2014). Fungi generally withstand harsh conditions and play an important role in the degradation of petroleum hydrocarbon (Chikere & Obieze, 2017). Due to the low substrate specificity of their degradative enzyme machinery (e.g. lignin peroxidase, laccase and Mn peroxidase), fungi are able to breakdown a wide range of pollutants including hydrocarbons in contaminated environment (D'Annibale *et al*., 2006; Prenafeta-Boldu *et al*., 2006).
Hydrocarbon utilizing fungi and bacteria are able to survive in such harsh environment because they have developed enzymatic and physiological responses that allow them use petroleum hydrocarbon as substrate (Thenmozhi *et al*., 2012)

Table 1. Total Culturable Heterotrophic and Hydrocarbon Utilizing Fungi

	Mean Density (sfu/g) and Standard Deviation	Percentage $\frac{TCHUF}{TCHF}$
TCHF	$1.8\times10^{5}\pm5.657$	6.11%
TCHUF	$1.1\times10^{4}\pm4.243$	

Key: TCHF – Total Culturable Heterotrophic Fungi
TCHUF – Total Cuiturable Hydrocarbon Utilizing Fungi

Table 2. Total Culturable Heterotrophic and Hydrocarbon Utilizing Bacteria

	Mean Density (cfu/g) and Standard Deviation	Percentage $\frac{TCHUB}{TCHB}$
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

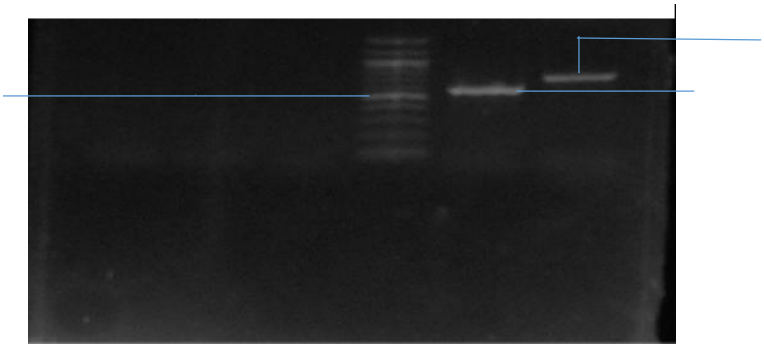

Plate 1.

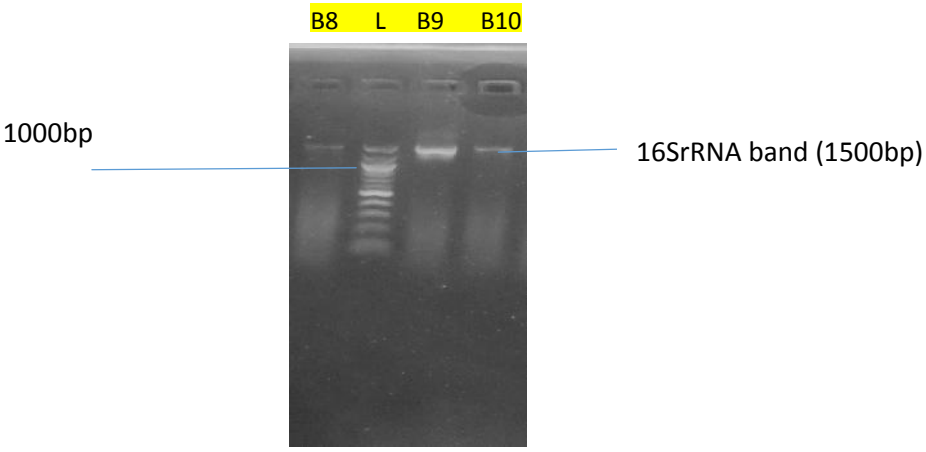

Plate 2. Agarose gel electrophoresis showing the amplified 16S rRNA gene bands of bacteria isolates. Lanes B8, B9, B10 represent the 16S rRNA bands while L represents the 1000bp molecular ladder.

Molecular based technique was employed to affirm the identities of the hydrocarbon utilizing fungi and bacteria. As shown in Plate 1 above, bands from PCR amplified ITS of fungi isolates shows 550bp and 750bp while Plate 2 above, shows band from PCR amplified 16S rRNA of bacterial isolates having 1500bp.

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%

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 shows the percentage relatedness of the bacterial isolate to those deposited in Genbank. Several studies have shown that *Alcaligenes, Bacillus, Penicillium* and *Aspergillus* are potential petroleum hydrocarbon degraders (Oboh *et al*., 2006; Nync *et al*., 1968; Sandrin & Maier, 2003; Ariyo & Obire, 2016).

4. Conclusion

This study reveals that *Alcaligenes feacalis*, *Bacillus cereus*, *Aspergillus aculeatus* and *Penicillium citrinum* are aerobic culturable hydrocarbon degraders in Ebubu Ejama, Rivers State, Nigeria. These hydrocarbon degraders can be used to bioaugment the degradation of petroleum hydrocarbon in an environment.

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