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PLASMID PROFILES OF BACTERIAL ISOLATES FROM KEROSENE, DIESEL AND CRUDE OIL POLLUTED SOILS

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ABSTRACT

Plasmid profile of bacterial isolates from kerosene, diesel and crude oil polluted soils was investigated. Serial dilution, culture based method and biochemical tests were used in isolation and identification of bacteria. E**ight groups of bacteria were identified; Gram-positives (*Bacillus* spp.*, Micrococcus* sp., *Lactobacillus* sp., *Enterococcus* spp.) and Gram-negatives (*Enterobacter* spp.*, Pseudomonas* spp.*, Klebsiella* spp., *Alcaligenes* sp., *Escherichia coli*, *Salmonella* sp., *Serratia* sp.). The presence of plasmids was detected using the Alkaline lysis method. Four out six bacteria (66.7%) from kerosene polluted soils harboured plasmid DNA. Diesel polluted soil recorded four out of seven bacteria with plasmid DNA (57.1%). Crude oil polluted soil recorded eighteen out of twenty-four bacteria with plasmid DNA (75%). Results showed 50% of Gram positives (*Bacillus* spp.) and Gram negatives (*Enterobacter* and *Pseudomonas* sp.) respectively from kerosene polluted soil habour plasmid DNA; 25% of Gram positive (*Lactobacillus* sp.) and 75% of Gram negatives (*Enterobacter* sp., *Pseudomonas* sp., *Alcaligenes* sp.) have plasmid DNA from diesel polluted soil; 61% of Gram positives (*Bacillus* spp., *Enterococcus* spp.) and 39% of Gram negatives (*Enterobacter* spp., *Pseudomonas* sp., *Klebsiella* sp., *Salmonella* sp.) isolated from crude oil polluted soil harbour plasmid DNA. The overall percentage occurrence of the bacteria isolated from kerosene, diesel and crude oil polluted soils that have plasmid DNA is 26/37 (70%). Gel electrophoresis was conducted to determine the sizes of plasmids as 10 kbp, 11 kpb and 12 kbp except *Micrococcus* sp. *E. coli* and *Serratia* sp., that have no plasmids. These bacteria can be employed in bioremediating kerosene, diesel and crude oil polluted soils.**

Introduction

The growth and survival of the microbes are indications that wherever oil spill occurs those native to the environment develop metabolic capabilities able to utilize the hydrocarbon substrates. The microbes move in the medium either by floating or suspending in the medium while making use of the contaminant. They make use of the pollutants by secreting bioemulsions/biosurfactant that

facilitates the dissolution of the hydrocarbons. Some bacteria are mobile and exhibit chemotactic response that is they sense the contaminant and moving towards it. The ability of microorganisms to degrade an organic compound is the result ultimately of the genetic makeup of the organisms. Plasmids are used by their host organism to cope with stress-related conditions like the hydrocabon spills in their environments.

A plasmid is a small double-stranded unit of DNA, usually circular but sometimes linear, that exists independent of the chromosome i.e extrachromosomal genetic elements and is capable of self-replication [1]. Each plasmid carries only a few genes. Plasmids come in many different sizes are used for many different purposes in biotechnology. Their ability to self-replicate enables the plasmids to be picked up from the environment and transferred between bacteria [2]. Many plasmids, for example, carry genes that code for the production of enzymes to inactivate antibiotics or poisons. Others contain genes that help a host organism digest unusual substances or kill other types of bacteria. Plasmid-encoded qualities include virulence factors, resistance to antibiotics, production of antimicrobials, degradation of xenobiotics, and functions involved in bacteria–host interactions [1]. Moreover, those conferring conjugative capabilities facilitate horizontal gene transfer. Hence, plasmids are considered to play key roles in evolutionary events of a given microbial community. Several characteristics of plasmids make them easy to be modified genetically: Firstly, they have relatively small DNA sequences, between 1,000 and 20,000 DNA base pairs. Secondly, they are easy to cut open, without falling apart, and snap back into shape. This makes it easy to insert new DNA into plasmids. Once a new DNA is inserted, the modified plasmid can be grown in bacteria for self-replication to make endless copies. The ease of manipulation and reproduction of plasmids, as well as their long-term stability, has made them indispensable tools in genetics and biotechnology laboratories. One of their most important functions is as a delivery vehicle, or vector, to introduce foreign DNA into bacteria, a fundamental step for genetic engineering and many other biotechnology applications. Multiple antibiotic resistance can be transfered between bacteria by plasmids. Today, plasmids are widely recognized not only as important factors facilitating genome restructuring but also as vehicles for the dissemination of beneficial characters within bacterial communities [1]. Plasmid diversity has been uncovered by means of culture dependent or -independent approaches and in this current research we applied the culture dependent approach to isolate and identify plasmids from bacteria in kerosene, diesel and crude oil polluted soils, to acertain the sizes and the diversity in the bacteria isolates.

Methods

Sample collection

Kerosene (DPK) polluted soil samples 1_{kero} and 2_{kero} were collected from two different kerosene depots at Umuerim Nekede, Owerri West, Imo State. Diesel (AGO) contaminated soil samples 3_{Diesel}, 4_{Diesel} and 5_{Diesel} were collected from three different diesel plants in Federal Polytechnic Nekede Owerri, Imo State. The soil sample were collected with sterile spatula into sterile labelled sampling bottles and immediately transported to the laboratory for bacteriological analysis. Agriculture farm soil samples ($6_{Crude\ oi}$, $7_{Crude\ oi}$, $8_{Crude\ oil}$) (1.5 kg) was collected from Federal University of Technology Owerri using a polythene bag and was polluted with Bonny Light Crude oil (100 ml) from Akin Ebocha Ohaji Local Government Area. It was vigorously mixed with 100 ml of water and left to stabilize for 21 days before the study. This grouping is based on the arrangement on the plasmid profile pictures (soil samples 1_{kero} and 2_{kero}; soil samples 3_{Diesel}, 4_{Diesel} and $5_{Diesel;}$ $6_{Crude\ oi}$, $7_{Crude\ oi}$, $8_{Crude\ oi)}$

Culture media preparation

The different culture media, nutrient agar(NA), MacConkey agar, Bushnell agar, cetrimide agar (CTAB) were all prepared according to the manufacturer's instructions; Mineral Salt agar (MSA; supplemented with DPK, AGO and crude oil prepared according to the method of Abiodun, [3] were all autoclaved at 121°C for 30 minutes.

Isolation of bacteria

One gram (1 g) of crude oil, diesel and kerosene contaminated soil samples were weighed into sterile test tubes each containing 9 ml of sterile water, then serial dilutions 10^{-4} for the samples were prepared. Thereafter aliquots (0.1 ml) of each (10^{-4}) of the samples were used to inoculate on nutrient media plates by the spread plate method. These were then incubated at 37^0C for 24 hours. Thereafter discrete colonies were picked and sub-cultured onto series of freshly prepared nutrient media plates then incubated at 37^0C for 24 hours. The pure colonies were inoculated on minimal salt agar supplemented with kerosene, diesel and crude oil according to the method of [3]. These were incubated at 25^oC for 14 days. The bacteria isolates were further sub cultured on the surface of already prepared MSM before subculturing on nutrient agar. Then after incubation, colonies were stored in nutrient agar slants at 4^oC and used for Gram staining, biochemical tests and plasmid DNA extraction.

Plasmid DNA extraction using alkaline lysis method

Buffers and Solutions

Alkaline lysis solution I: 50 mM glucose, 25 mM Tris-Cl (pH 8.0), 10 mM EDTA (pH 8.0), deionized water; Alkaline lysis solution II : 0.2 N NaOH, 1% (w/v) SDS, deionized water; Alkaline lysis solution III : 5 M potassium acetate, glacial acetic acid, deionized water; Ethanol 70% (v/v); Isopropanol; TE-RNAase pH 8.0

Steps:
Pour overnight grown bacterium culture to 1.5 ml labeled falcon tube then centrifugate at 4.000 rpm for 1 min after remove the supernatant from the tube. Repeat the above, until it leaves bacterial pellet as dry as possible. Thereafter add 150 µL resuspension buffer, resuspend the bacterial pellet properly by vortexing then add 200 µL lysis solution to bacterial suspension (freshly made), close the tube tightly and mix contents thoroughly by inverting the tube 4-6 times until the solution becomes viscious. Add 300 µL neutralization solution and mix contents thoroughly by inverting the tube 4-6 times then centrigufe at 14.000 rpm for 5 min. Collect the supernatant and transfer to a new 1.5 mL falcon max 300 µL. Then add equal volume of isopropanol in the supernatant (300 µL) and mix it by inverting the tube couple of times, after incubate in -80°C for 30 min. Centrifuge at 14.000 rpm for 5 min. Remove the supernatant and add 600 µL EtOH 70%. Centrifuge at 14.000 rpm for 5 min. Remove the supernatant and dry the pellet for 10-30 min. Dissolve the pellet in 20-50 µL TE-RNAase pH 8.0. Confirm the plasmid with 5 µL DNA solvent by Agarose Electrophoresi [4].

Gel Electrophoresis of Extracted Plasmid DNA of isolates
The gel electrophoresis of extracted plasmid DNA was conducted to determine the size of the extracted plasmid DNA based on their electrical charges (positive or negative chargers). Then 0.8 g of agarose powder was weighed and 100 ml of 1X TBE buffer was added to it. The mixture was dissolved by heating for 5 minutes using microwave oven and allowed to cool to 50°C, then 10 µl of ethidium bromide was added, mixed gently by swirling. The mixture was poured into electrophoresis tray with comb in place to obtain a gel thickness of about 5 mm. It was allowed to stand for 50 mins to solidify. The comb was removed and the tray placed in the electrophoresis tank. Thereafter 1X TBE buffer was poured into the tank ensuring that the buffer covers the surface of the gel. Then 20 µl of the extracted DNA was mixed with 2 µl of loading dye which was carefully loaded into the wells. The electrodes were connected to the power pack and electrophoresis ran at 60-100 volts. Electrodes were turned off and the Gel observed on UV trans-illuminator and results were recorded [4,5].

RESULTS
Heterotrophic bacterial counts (HTB) in pristine soil samples was 1,97±0.4x105 (cfu/g) colony forming unit per gram of soil sample in chronic kerosene contaminated soil samples. The heterotrophic bacterial (HTB) count ranged from 1.74 ± 0.3x104 cfu/g to 2.13 ± 0.6x105 cfu/g in nutrient agar and minimal salt agar (MSA). There is more bacteria count in the kerosene polluted soil than in the control soil, this may be that keroene encourages bacteria population.

Table 1: Total Heterotrophic count of bacterial isolated from kerosene soil

Kerosene contaminated soil samples	Heterotrophic bacterial count (cfu/g)
Pristine soil sample/control	$1.97 \pm 0.4 \times 10^3$
Soil Sample 1_{kero} in Nutrient agar	$2.00 \pm 0.3\ 10^5$
Soil Sample 1_{kero} in Mineral Salt Agar	$2.13 \pm 0.6 \times 10^5$
Soil sample 2_{kero} in Nutrient agar	$1.74 \pm 0.3 \times 10^4$
Soil Sample 2_{kero} in Mineral Salt Agar	$2.08 \pm 0.4 \times 10^5$

The kerosene pollution limits the types of bacteria that can degrade it to grow. There is equal occurrence of the few Gram positive and Gram negative bacteria found in kerosene polluted soil. The Gram positive in the kerosene polluted soil are *Bacillus* sp. with occurrence 4/10, *Micrococcus* sp. 1/10, this gives Gram positive a percentage occurence of 50%. The Gram negative bacteria from kerosene polluted soil are *Enterobacter* 2/10, *Pseudomonas* sp. 2/10, *Serratia* sp. 1/10. This makes the Gram negatives 50% present in kerosene polluted soil. There is a balance in the diversity of Gram positive and Gram negative bacteria in kerosene polluted soil. The reason may be that both groups of bacteria can be used for the bioremediation of kerosene polluted soils (Table 2 and 3).

Table 2: Biochemical identification of bacterial isolated from kerosene polluted soil

Soil Sample 1_{kero} Isolate identity	Morphology	Grams rxn	Citrate	Oxidase	Catalase	Methyl red	Voges Praskauer	Motility	Indole	Urease	Isolate identity
$NKSC_1$	Large mucoid pink spreading colony on nutrient agar	- rod	+	-	+	-	+	+	-	-	*Enterobacter*
$NKSC_2$	Small pink colony on MacConkey agar	- rod	+	-	+	-	+	+	-	-	*Enterobacter* sp.

$NKSC_3$	Large white colony on nutrient agar	-rod	+	+	+	-	-		+	-	-	*Pseudomonas* sp.
$NKSC_4$	Large transparent colony on nutrient agar	+rod	+	+	+	-	+		+	_	_	*Bacillus* sp.
$NKSC_5$	Small milkish colony on nutrient agar	+rod	+	+	+	-	+		+	-	-	*Bacillus* sp.
$NKSC_6$	Small milkish colony on nutrient agar	+rod	+	-	+	-	+		+	-	-	*Bacillus* sp.

Table 3: Biochemical identification of bacterial isolates from kerosene polluted soil

Soil sample 2_{kero} Isolates code	Gram stain	Citrate	Oxidaze	Catalase	Methyl red	VP	Motility	Indole	Urease	Isolate
$NKSA_1$	+ rod	+	-	+	-	+	+	-	-	*Bacillus* sp.
$NKSA_2$	- rod	+	+	+	-	-	+	-	-	*Pseudomonas* sp.
$NKSA_3$	- rod	+	-	+	-	+	+	-	+	*Serratia* sp.
$NKSA_4$	+coccus	+	+	+	-	+	-	-	+	*Micrococcus* sp.

Soil contamination with diesel recorded increased total microbial population (Table 4), but greatly restricts the number of species, favouring only the development of microorganisms that can degrade diesel into simple compounds (Table 5 and 6). Table 4 below shows the total bacteria count and morphology of bacteria isolated from diesel polluted soil samples. The number of colonies on MSA medium was lower when compared to the colonies on nutrient agar medium and this result also shows that the bacteria grown on enriched medium were able to degrade diesel. The few bacteria species isolated from diesel polluted soil include both Gram positive and Gram negative bacteria. The Gram positive bacteria are *Bacillus* specie (2/13), *Lactobacillus* sp. (1/13) and their percentage occurrence is low 23.1 % in the diesel polluted soil, maybe because diesel oil is more complex for them to breakdown; Table 10 also assents to this as only *Lactobacillus* sp., has plasmid DNA of 12 kbp size. The Gram negative bacteria include *Klebsiella* sp. (1/13), *E. coli* (2/13), *Pseudomonas* species (4/13), *Alcaligenes* (1/13) and *Enterobacter* species (2/13). The percentage occurrence of the Gram negative bacteria is higher in the diesel oil polluted soil (76.9%) (Table 5 and 6). The reason maybe that the Gram negative bacteria can degrade the diesel and have the necessary genetic makeup. This is confirmed in Table 10, that shows three of the Gram negatives (*Alkaligenes* sp., *Enterobacter* and *Pseudomonas* sp.) possessing the plasmid DNA size 12 kbp. This result shows the diversity of bacteria that degrade diesel.

Table 4: Total bacteria count and morphology of bacteria isolated from diesel polluted soil

SAMPLE CODE	Colour	Size (mm)	Shape	Elevation	Tcfu/g
Soil sample 3_{Diesel}					
Total bacteria count, cfu/g (nutrient agar)	Creamy	1 – 3	Irregular	raise	2.80×10^6
Total diesel degradation bacteria count, cfu/g (Mineral Salt Agar)	Creamy	1 – 3	Irregular	raise	1.10×10^3
Soil sample 4_{Diesel}					
Total bacteria count, cfu/ml (nutrient agar)	Creamy	1 – 3	irregular	raise	2.70×10^6
Total diesel degradation bacteria count, cfu/g (Mineral Salt Agar)	Creamy	1 – 3	Irregular	raise	$1.60\ 10^3$

Table 5: Bacterial isolated from diesel oil polluted soil

Soil sample 5_{Diesel} Isolate identity	Gram reaction	Citrate test	Oxidase test	Catalase test	Methyl red	Voges proskauer	Motility test	Indole test	Urease test	Isolate identity
NKS_1	-Rod	+	+	+	-	-	+	-	-	*Alcaligenes*
NKS_2	-Rod	+	+	+	-	-	+	-	-	*Pseudomonas* sp
NKS_3	-Rod	+	-	+	-	+	+	-	-	*Enterobacter* sp
NKS_4	-Rod	+	-	+	-	+	+	-	-	*Enterobacter* sp.
NKS_5	-Rod	+	+	+	-	-	+	-	-	*Pseudomonas* sp
NKS_6	+Rod	-	-	-	-	-	-	-	-	*Lactobacillus* sp
NKS_7	-Rod	+	+	+	-	-	+	-	-	*Pseudomonas* sp

Table 6: Biochemical identification of some bacteria isolated from diesel polluted soil

Soil Sample/ Isolate Code	Colony Morphology	Microscopic Characteristics	Gram Stain	Slant	Butt	Glucose	Lactose	Gas	H_2S	Indole	Motility	Oxidase	Citrate	Urease	Catalase	V.P	Metyl Red	Most Probable Organism
Soil sample 3_{Diesel} NKI	Creamy colony on Nutrient agar	Rod	-	A	A	+	+	+	-	-	-	-	+	+	+	-	-	*Klebsiella* sp.
NBa	Shiny creamy colony on Nutrient agar	Rod	+	A	A	+	+	-	-	-	+	+	+	-	+	+	-	*Bacillus* sp.
NEc	Shiny creamy colony on Nutrient agar	Rod	-	A	A	+	+	+	-	+	+	-	+	-	+	+	-	*Escherichia coli*
Soil Sample 4_{Diesel} NPs	Shiny creamy colony on Nutrient agar	Rod	-	B	B	-	-	-	-	-	+	+	+	-	+	-	-	*Pseudomonas* sp.
NBa	Shiny creamy colony on Nutrient agar	Rod	+	A	A	+	+	+	-	-	+	+	+	-	+	+	-	*Bacillus* sp.
NEc	Shiny creamy colony on Nutrient agar	Rod	-	A	A	+	+	+	-	+	+	-	+	-	+	+	-	*Escherichia coli*

Key: A = Acidic condition; **B** = Basic condition

The tables 7, 8 and 9 below show the presence of other bacterial isolates that degrade crude oil. There are the Gram posi-

tives and Gram negatives, this shows the diversity of bacteria that can breakdown crude oil. The Gram positives are *Enterococcus* spp. (9/24) *a*nd *Bacillus* spp. (5/24). The percentage occurrence of Gram positive bacteria is 58%. The Gram negative bacteria are *Klebsiella* spp. (2/24), *Enterobacter* spp. (5/24), *Salmonella* sp. (1/24) *Escherichia coli* (1/24) and *Pseudomonas* species (1/24). The percentage occurence of Gram negative bacteria is 50%. The Gram positive bacteria are two (*Bacillus* spp., and *Enterococcus* spp.) but had the highest percentage of occurrence which may be because the substrate crude oil is denser and few bacteria have the degrading mechanism. The Gram negative bacteria are more diverse in the crude oil polluted soil

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Table 7: Biochemical characteristics of the bacterial isolates from crude oil soil samples

Soil sample $6_{Crude\ oil}$ Isolate identity	Gram reaction	Citrate test	Oxidase test	Catalase test	Methyl red	Voges proskau-	Motility test	Indole test	Urease test	Isolate identity
$NKSD_1$	+cocci	-	-	-	-	+	-	-	-	*Enterococcus* sp
$NKSD_2$	-Rod	+	-	+	-	+	-	-	+	*Klebsiella* sp.
$NKSD_3$	-Rod	+	-	+	-	+	+	-	-	*Enterobacter* sp
$NKSD_4$	-Rod	+	-	+	-	+	+	-	-	*Enterobacter* sp.
$NKSE_1$	+cocci	-	-	-	-	+	-	-	-	*Enterococcus* sp
$NKSE_2$	+cocci	-	-	-	-	+	-	-	-	*Enterococcus* sp
$NKSE_3$	-Rod	+	-	+	+	+	+	-	-	*Salmonella* sp
$NKSE_4$	+cocci	-	-	-	-	+	-	-	-	*Enterococcus* sp.
$NKSG_1$	-Rod	+	-	+	-	+	+	-	-	*Enterobacter* sp.
$NKSG_2$	-Rod	+	-	+	-	+	+	-	-	*E. coli*

Table 8: Biochemical characteristics of the bacterial isolates from crude oil polluted soil

Soil Sample $7_{Crude\ oil}$ Isolate code	Gram reaction	Citrate	Oxidase	Catalase	Methyl red	**Voges-Proskauer**	Motility	Indole	Urease	Isolate identity
$NKSH_1$	-Rod	+	+	+	-	-	+	-	-	*Pseudomonas* sp.
$NKSH_2$	+ Rod	+	+	+	-	+	+	-	-	*Bacillus* sp.
$NKSD_1$	+Rod	+	+	+	-	+	+	-	-	*Bacillus* sp.
$NKSD_2$	+ Rod	+	+	+	-	+	+	-	-	*Bacillus* sp.
$NKSH_3$	+Rod	+	+	+	-	+	+	-	-	*Bacillus* sp.
$NKSH_4$	+ Rod	+	+	+	-	+	+	-	-	*Bacillus* sp.

Table 9: Bacterial isolated from crude oil polluted soil

Soil sample $8_{Crude\ oil}$ Isolate identity	Gram reac-tion	Citrate test	Oxidase test	Catalase test	Methyl red	Voges proskauer	Motility test	Indole test	Urease test	Isolate identity
NKa1	+cocci	-	-	-	-	+	-	-	-	*Enterococcus* sp
NKa2	-Rod	+	-	+	-	+	-	-	+	*Klebsiella* sp.
NKa3	-Rod	+	-	+	-	+	+	-	-	*Enterobacter* sp.
NKa4	+cocci	-	-	-	-	+	-	-	-	*Enterococcus* sp
NKa5	+cocci	-	-	-	-	+	-	-	-	*Enterococcus* sp
NKa6	+cocci	-	-	-	-	+	-	-	-	*Enterococcus* sp.
NKa7	- Rod	+	-	+	-	+	+	-	-	*Enterobacter* sp.
NKa8	+cocci	-	-	-	-	+	-	-	-	*Enterococcus* sp.

The plasmid profiling of bacterial isolates from kerosene polluted soil (soil sample 1_{kero}) is shown in Fig. 1. There was a total of ten bacteria isolated from kerosene polluted soil and only four of these bacteria harbour plasmid DNA. The iso-

lates A/NKSC$_1$ and B/NKSC$_2$ are both *Enterobacter* species. C/NKSC$_3$ is *Pseudomonas* sp. D/NKSC$_4$, E/NKSC$_5$ and F/NKSC$_6$ are three different strains of *Bacillus* species. A/NKSC$_1$ and B/NKSC$_2$ are of different strains since result showed that A had no plasmid whereas strain B had plasmid band of 12kbp (Fig. 1; Table 10). The isolate D/NKSC$_4$ has no plasmid band. E/NKSC$_5$ and F/NKSC$_6$ have plasmid bands of 12 kbp respectively (Fig. 1; Table 10).

The plasmid profile of bacteria from diesel polluted soil (soil sample 5$_{diesel}$) is shown in Fig. 2. The total of 7 bacteria were isolated from soil sample 5$_{Diesel,}$ and only four of these bacteria have plasmid DNA of molecular size 12 kbp each. They are *Alcaligenes* sp. (A/NKS1), *Enterobacter* sp. (C/NKS$_3$), *Lactobacillus* sp. (F/NKS$_6$), and *Pseudomonas* sp. (G/NKS$_7$), (Fig. 2; Table 10).

The plasmid profile of bacteria isolated from crude oil polluted soil (soil sample 6$_{crude\ oil}$) is shown in Fig. 3 and are ten bacteria species in all. This study has shown that seven out of the ten have plasmid DNA. They are: *Enterococcus* sp. (5/NKSD$_1$), *Enterbacter* sp. (7/NKSD$_3$), *Enterobacter* sp. (8/NKSD$_4$), *Enterococcus* sp. (1/NKSE$_1$), *Enterococcus* sp. (2/NKSE$_2$), *Salmonella* sp. (3/NKSE$_3$), and *Enterococcus* sp. (4/NKSE$_4$). They have plasmid DNA of molecular size 11 kbp each (Fig. 3; Table 10).

The plasmid profile of bacteria isolated from crude oil polluted soil (soil sample 7$_{crude\ oil}$) is shown in Fig. 4 and are six bacteria species in all. The outcome of this study has shown that only four out of the six have plasmid DNA, they are: A/NKSH$_1$ is *Pseudomonas sp.* and B/NKSH$_2$, C/NKSH$_3$, D/NKSH$_4$ are all different strains of *Bacillus* species. The *Pseudomonas* sp., and all *Bacillus* species harbor plasmid DNA of molecular size 12 kbp each, that are capable of degrading crude oil (Fig. 4; Table 10).

There was a total of eight bacteria species isolated from crude oil polluted soil (soil sample 8$_{crude\ oil}$) (Fig. 5). It was observed that seven out of the eight bacteria isloates habour plasmid DNA of size 10 kbp each. The bacteria isolates are *Enterococcus* sp. (1/NKa1), *Klebsiella* sp. (2/NKa2), *Enterobacter* sp. (3/NKa3), *Enterococcus* sp. (4/NKa4), *Enterococcus* sp. (5/NKa5), *Enterobacter* sp. (7/NKa7) and *Enterococcus* sp. (8/NKa8) (Fig 5; Table 10).

The plasmid DNA size of *Pseudomonas* sp. isolated from kerosene polluted soil is 12 kbp, that from diesel polluted soil is 12 kbp and that from crude oil polluted soil is 12 kbp. The occurrence of *Bacillus* spcies that have plasmid DNA in kerosene polluted soil is higher 2/4 (50%) than the other bacteria isolated. The occurrence of *Enterococcus* species that have plasmid DNA is higher (8/18; 44%) in crude oil polluted soil. The occurrence of *Enterobacter* species that have plasmid DNA in crude oil polluted soil is 4/18 (22%) while *Bacillus* species that have plasmid DNA in crude oil polluted oil is 3/18 (16.7%). Gram positive and Gram negative bacteria showed presence of plasmid DNA size 10 kbp, 11 kbp and 12 kbp. The presence of *Pseudomonas* species and *Enterobacter* species in all the hydrocarbon contaminated soil that habour plasmid DNA revealed their kerosene, diesel and crude oil utilizing potential and hence should be considered the first choice in bioremediation of hydrocarbon polluted sites (Table 10).

M- The total of four out six bacteria (66.7%) from kerosene polluted soils harboured plasmid DNA. Diesel polluted soil recorded four out of seven bacteria isolates harbour plasmid DNA (57.1%). The crude oil polluted soil recorded eighteen out of twenty -four bacteria isolates harbour plasmid DNA (75%). Table 10 shows that 50% of Gram positives (*Bacillus* spp.) and Gram negatives (*Enterobacter* and *Pseudomonas* sp.) respectively habour plasmid DNA from kerosene polluted soil; 25% of Gram negative (*Lactobacillus* sp.) harbour plasmid DNA while 75% of Gram negative (*Enterobacter* sp., *Pseudomonas* sp., *Alcaligenes* sp.) have plasmid DNA from diesel polluted soil; 61% of Gram positive bacteria (*Bacillus* spp., Enterococcus spp.) isolated from crude oil polluted soil harbour plasmid DNA whereas 39% of Gram negative bacteria (*Enterobacter* spp., *Pseudomonas* sp., *Klebsiella* sp., *Salmonella* sp.) harbour plasmid DNA from same soil. The overall percentage occurrence, in all the bacteria isolated from kerosene, diesel and crude oil polluted soils that have plasmid DNA is 26/37 (70%) (Table 10).

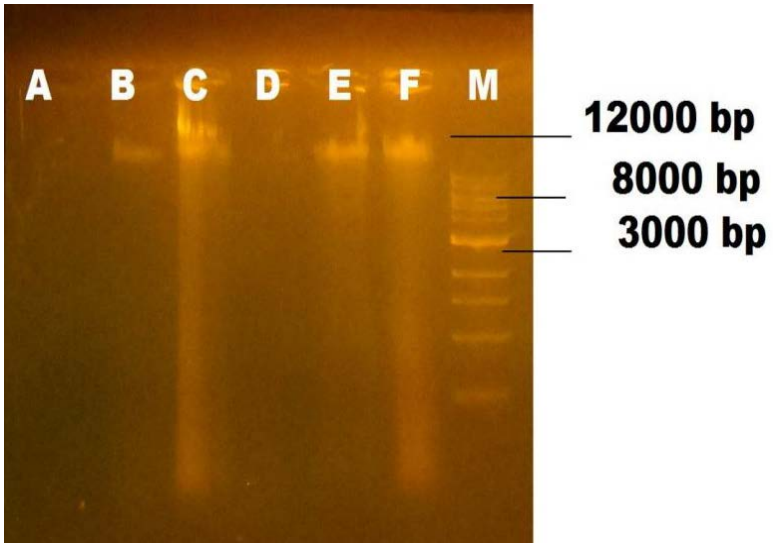

Fig. 1: Plasmid profiling of bacterial isolates from soil polluted with Kerosene
Key: M: Marker, A - F = NKSC 1- 6, A – B = *Enterococcus* sp., C = *Pseudomonas* sp., D – F = *Bacillus* sp.

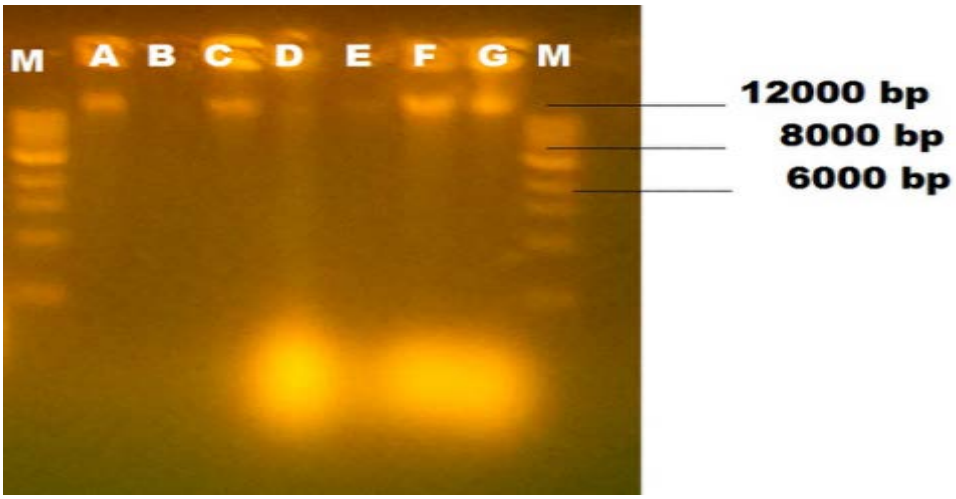

Fig 2: Plasmid profiling of of bacterial species isolated from soil polluted with Diesel
Key: M= DNA Marker; *Alcaligens* (A); *Pseudomonas* sp. (B, E, and G), *Enterobacter* (C &D) and *Lactobacillus* sp (F)

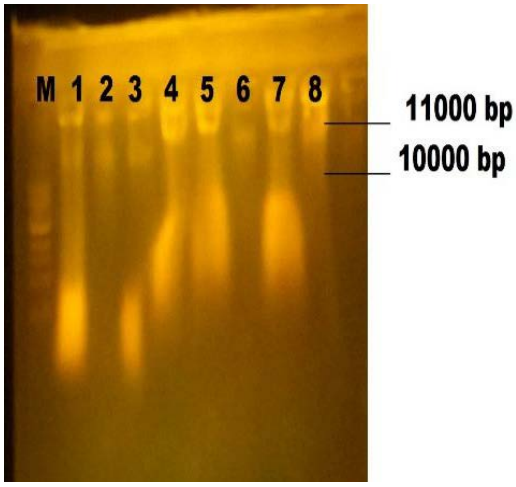

Fig 3:Plasmid profiling of bacterial isolates that degrade crude oil. Key: M: Marker; 1-4 = NKSE 1-4; 5-8 = NKSD 1-4.

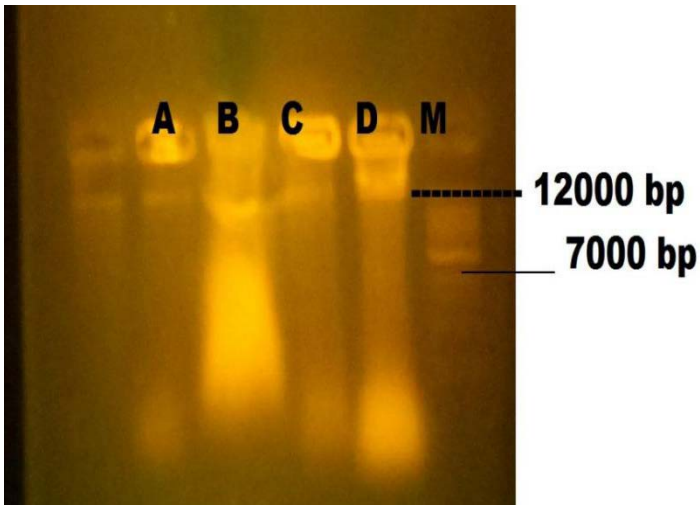

Fig 4: Plasmid profiling of bacterial isolates that degrade crude oil.
Key: M - Marker; A- D = NKSH 1-4

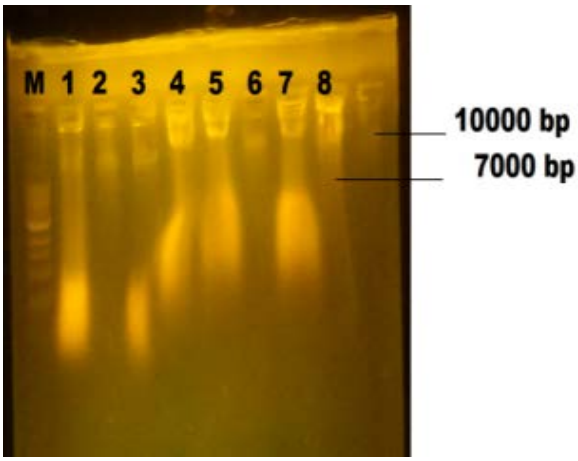

Fig 5: Plasmid profile of bacteria from crude oil contaminated soil
M-Marker; 1-8 NKa1- NKa8; *Enterobacter* sp. (1,3,7); *Klebsiella* sp. (2); *Enterococcus* (4,5,6,8)

Table 10: Bacterial isolates with their plasmids and sizes

Soil sample	Number of iso-lates	Designation of iso-lates/Name of bacteria	Codes on plasmid profiles	Those with Plasmids/ Name of bacteria	No. of Plasmid bands	Molecular wight (kbp)
1_{Kero}	6	$NKSC_1$/*Enterobacter* sp. $NKSC_2$ /*Enterobacter* sp. $NKSC_3$ /*Pseudomonas* sp. $NKSC_4$ / *Bacillus* sp. $NKSC_5$ / *Bacillus* sp. $NKSC_6$ / *Bacillus* sp.	A B C D E F	**No plasmid** $NKSC_2$ /*Enterobacter* sp. $NKSC_3$ /*Pseudomonas* sp. **No plasmid** $NKSC_5$ / *Bacillus* sp. $NKSC_6$ / *Bacillus* sp.	- 1 1 - 1 1	- 12 kbp 12 kbp - 12 kbp 12 kbp
2_{Kero}	4	$NKSA_1$, $NKSA_2$, $NKSA_3$, $NKSA_4$	-	NONE	-	-
3_{Diesel}	3	NKl, NKBa, NEc,	-	NONE	-	-
4_{Diesel}	3	NPs, NBa, NEc	-	NONE	-	-
5_{Diesel}	7	NKS_1 / *Alcaligenes* sp. NKS_2 /*Pseudomonas* sp. NKS_3 /*Enterobacter* sp. NKS_4 /*Enterobacter* sp. NKS_5 /*Pseudomonas* sp. NKS_6 / *Lactobacillus* sp. NKS_7 /*Psedomonas* sp.	A B C D E F G	NKS_1 / *Alcaligenes* sp. **No plasmid** NKS_3 /*Enterobacter* sp. **No plasmid** **No plasmid** NKS_6 / *Lactobacillus* sp. NKS_7 /*Psedomonas* sp.	1 - 1 - - 1 1	12 kbp - 12 kbp - - 12 kbp 12 kbp
$6_{Crude\ oil}$	10	$NKSD_1$ /*Enterococcus* sp. $NKSD_2$ /*Klebsiella* sp. $NKSD_3$ /*Enterobacter* sp. $NKSD_4$ /*Enterobacter* sp. $NKSE_1$ /*Enterococcus* sp. $NKSE_2$ /*Enterococcus* sp. $NKSE_3$ /*Salmonella* sp. $NKSE_4$ /*Enterococcus* sp.	5 6 7 8 1 2 3 4	$NKSD_1$ /*Enterococcus* sp. **No plasmid** $NKSD_3$ /*Enterobacter* sp. $NKSD_4$ /*Enterobacter* sp. $NKSE_1$ /*Enterococcus* sp. $NKSE_2$ /*Enterococcus* sp. $NKSE_3$ /*Salmonella* sp. $NKSE_4$ /*Enterococcus* sp.	1 - 1 1 1 1 1 1	11 kbp - 11 kbp 11 kbp 11 kbp 11 kbp 11 kbp 11 kbp

$7_{Crude\ oil}$	6	$NKSH_1$ /*Pseudomonas* sp.	A	$NKSH_1$ /*Pseudomonas* sp.	1	12 kbp
			B		1	12 kbp
		$NKSH_2$ /*Bacillus* sp.	-	$NKSH_2$ /*Bacillus* sp.	-	-
		$NKSD_1$/*Bacillus* sp.	-	**No plasmid**	-	-
		$NKSD_2$/*Bacillus* sp.	C	**No plasmid**	1	12 kbp
		$NKSH_3$ /*Bacillus* sp.	D	$NKSH_3$ /*Bacillus* sp.	1	12 kbp
		$NKSH_4$ /*Bacillus* sp.		$NKSH_4$ /*Bacillus* sp.		
$8_{Crude\ oil}$	8	NKa1/*Enterococcus* sp.	1	NKa1/*Enterococcus* sp.	1	10 kbp
		NKa2/*Klebsiella* sp.	2	NKa2/*Klebsiella* sp.	1	10 kbp
		NKa3/*Enterobacter* sp.	3	NKa3/*Enterobacter* sp.	1	10 kbp
		NKa4/*Enterococcus* sp.	4	NKa4/*Enterococcus* sp.	1	10 kbp
		NKa5/*Enterococcus* sp.	5	NKa5/*Enterococcus* sp.	1	10 kbp
		NKa6/*Enterococcus* sp.	6	**No plasmid**	-	-
		NKa7/*Enterobacter* sp.	7	NKa7/*Enterobacter* sp.	1	10 kbp
		NKa8/*Enterococcus* sp.	8	NKa8/*Enterococcus* sp.	1	10 kbp

Discussion

Microorganisms are extremely diverse and can adapt to survive in inhospitable environments. Microbes are capable of breaking down many complex molecules by adaptation of their degradative enzyme system. Microorganisms play important role in the natural environment; they contribute to the geological cycle of elements and transformation of natural chemicals. Contaminated sites often habour a vast array of microbial flora that is capable of utilizing the contaminant as an energy and carbon source.

All isolates in this study have been commonly reported as hydrocarbon degraders in various environments [3,6,7,8,9,10]. Few studies have reported on the roles of *Bacillus* spp. in hydrocarbon remediation; although there are several reports of bioremediation of pollutants by the action of *Bacillus* spp. occurring in extreme environments. Most reported *Bacillus* spp. as being the predominant isolate of all the crude oil utilizing bacteria characterized from highly polluted soil samples. It was postulated that *Bacillus* spp. are more tolerant to high levels of hydrocarbons in soil due to their resistant endospores. There is growing evidence that isolates belonging to the *Bacillus* spp. could be effective in clearing oil spills [3,6,7,8,9,10].

The ability of isolated species *klebsiella* spp, *Baccillus* sp, *Pseudomonas* spp, *Salmonella sp., Enterococcus spp., Enterobacter spp. Lactobacillus sp., Alcaligenes sp.*and *Escherichia coli* as bio-degraders of crude oil could be very useful for bioremediation of such petroleum hydrocarbon. The isolates from crude oil polluted soil is in agreement with finding of [3,6,8,9,10] who isolated *Escherichia coli*, *Pseudomonas aeruginosa, Bacillus species, Micrococcus* sp., *Pseudomonas, Alcaligenes, Sphingomonas, Rhodococcus,* and *Mycobacterium* among others from crude oil contaminated soil and recognized for their degradative abilities. These microbes have often been reported to degrade pesticides and hydrocarbons, both alkanes and polyaromatic compounds. Many of these bacteria use the contaminant as the sole source of carbon and energy. The report of these authors is similar to the outcome of this study. Therefore, there is need to harness these bacterial species for their biodegradative capacities on crude oil contaminated soil.

Researches have shown that genetic factors play important roles in conferring biodegradation potentials on microorganisms and plasmids found in these organisms play a leading role in this aspect. Cerniglia [11] reported that the ability of microorganisms to degrade more recalcitrant component of petroleum products like polycyclic aromatic hydrocarbons are sometimes plasmid mediated. Plasmids that have been found to harbour genes encoding for the transformation of environmental pollutants are known as catabolic plasmids. The incidence of plasmids in oil degrading bacteria had been reported by many workers [10,11,12,13,14,15,16,17,18]. The result obtained by Abiodun [3] from plasmid analysis showed that four bacterial isolates out of the ten isolates harbour plasmids with different molecular weight. *Providencia* sp and *Bacillus* sp harboured two plasmids each with molecular weight of 1,876 bp and 1,261 bp, and 2,577 bp and 2,031 bp respectively, while *Bacillus* sp and *Providencia* sp harbour just a single plasmid each of molecular weight of 1,366 bp and 32,707 bp respectively. Devereux and Sizemore [13] reported the incidence of plasmids in 21% of the strains isolated on crude oil and 17% on polynuclear aromatic hydrocarbons, multiple plasmids in 50% of the plasmids containing strains were also similar to what was obtained in the multiple plasmids obtained in *Providencia* sp. and *Bacillus* sp., Thavasi *et al*. [17] also reported the presence of multiple plasmid in *P. aeruginosa* isolated from their work. Small plasmid was obtained from *Pseudomonas* strain with a molecular weight of 3.2 MDa in sediments from Campeche Bank (Leahy *et al*., 1990). Thavasi *et al*. [17] also reported molecular weight of 3.8 to 4.2 kb in oil degrading bacteria, which also agreed with the results obtained in this work in which *Providencia* sp. and *Bacillus* sp. also harboured a single plasmid each of molecular weight of 3.3kb and 1.4kb respectively.

Bacteria isolated from oil polluted environments have been shown to be more effective in degrading hydrocarbons than bacteria from unpolluted environments [8] because exposures of a microbial community to hydrocarbons have been shown to increase the incidence of different types of plasmids in isolated bacteria [12,14,15,19,20,21].

Researches have shown that plasmid is very important from single step reaction to multi step pathways in degradation pathway, and they appear to be a versatile means by which microorganisms can gain metabolic capacities in the exploitation of otherwise unavailable resources [1,2,19,20,22,23]. Presence of catabolic genes responsible for the degradation of naphthalene in plasmid found in *Pseu-*

domonas putida was reported by Park *et al*. [16]. Akeredolu *et al*., [18] isolated from kerosene polluted soil, plasmid DNA bands from Gram positive bacteria: *Bacillus subtilis, Micrococcus varians*, *Bacillus* specie with molecular weights corresponding to 23,130 bp and DNA plasmids in Gram negative bacteria: *Pseudomonas aeroginosa, Klebsiella aerogenes, Alcaligenes* specie and *Pseudomonas* specie corresponding to 23 kpb. The results obtained from this work showed that *Pseudomonas* sp. *Enterobacter* sp. harboured different sizes of plasmids (12 kbp, 11 kbp, 10 kbp) able to degrade kerosene, diesel and crude oil. The plasmid DNA size of bacteria isolated in this current study is in the range (10 kbp - 12 kbp) of plasmid DNA sizes These sizes are ideal for genetic manipulations and introduction into other bacteria.

Conclusion

The study revealed that some bacterial strains were able to grow effectively on kerosene, diesel and crude oil polluted soil. The utilizing capabilities in some bacterial strains appear to be plasmid encoded. The results from this work affirms the increasing awareness that bacteria harbouring plasmids can be used in bioremediation to clear oil spill and hydrocarbon contaminants. There were eight groups of bacteria identified in this study and they fall into two groups namely: Gram-positive (*Bacillus* spp., *Micrococcus* sp., *Lactobacillus* sp., *Enterococcus* spp.) and Gram-negative (*Enterobacter* spp., *Pseudomonas* spp., *Klebsiella* spp., *Alcaligenes* sp., *Escherichia coli*, *Salmonella* sp., *Serratia* sp.). They all harboured plasmids of sizes 10 kbp, 11 kpb and 12 kbp except *Micrococcus* sp. *E. coli* and *Serratia* sp., that had no plasmids. Conclusively 70% of the isolated bacteria haboured these plasmid sizes. These bacterial strains can therefore be employed in remediating kerosene, diesel and crude oil polluted soil if their potential is maximally harnessed by process control and optimization. This involves factors that affect their ability and efficiency in kerosene, diesel and crude oil degradation. These factors include types of nutrients, the concentration of the nutrients, oxygen requirements, pH, amongst others.

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