Original Research Article

Determination of the Bacterial Community Structure in a Crude oil-inundated Tropical Soil using Next Generation Sequencing Technique

ABSTRACT

Aim: To identify the community composition of a crude oil impacted soil in Gbarain kingdom of Bayelsa State, Nigeria

Study design: A crude oil-impacted soil sample (0-10 cm depth) was collected from Etelebuo-Ogboloma, flow station in Yenagoa L.G.A. of Bayelsa State and taken to the laboratory for various microbiological analyses.

Place and duration of study: The study was carried out at the Environmental Microbiology Laboratory, University of Port Harcourt, Nigeria, for 35days.

Methodology: Cultural morphology of the isolates was studied based on their physical appearance such as colour, shape, size, elevation and margin. While catalase test, oxidase test, indole test, motility test, Methyl-Red Voges-Proskauer's (MRVP) test and citrate utilization test were used for the biochemical identification of the isolates. Deoxyribonucleic acid (DNA) eextraction from crude oil polluted soil sample was performed using ZYMO soil DNA extraction Kit (Model D 6001, Zymo Research, USA) following the manufacturer's instructions. DNA sequencing was performed by the Next Generation Sequencing Technique to determine the nucleotide sequence of all microorganisms present in the soil sample using sequencing primer -16S: 27F: 5'-GAGTTTGATCCTGGCTCAG-3' and 518R: 5'- ATTACCGCGGCTGCTGG-3'.

Results: Five different bacterial genera were isolated and identified using the cultural techniques, and they include *Acetobacter* sp*., Pseudomonas* sp., *Arthrobacter* sp., *Bacillus* sp. and *Micrococcus* sp. However, the molecular characterization revealed that the soil was mainly dominated by the Alphaproteobacteria (54.64%), followed by the Actinobacteria (9.67%), Gammaproteobacteria (6.55%) , Betaproteobacteria (2.27%) and Bacilli (0.95%) as well as Clostridia (0.34%); as the most dominant class of bacteria. The unknown group accounted for 25.28%. A total of one hundred and four (104) diverse bacterial species were identified, in the overall metagenomics.

Conclusion: This study has shown the bacterial community composition of the crude oil polluted soil obtained from Gbarain Kingdom. These findings are fundamental in understanding the biological fate of crude oil in these oil rich regions.

Keywords: Metagenomics*; bacterial* composition*;* Gbarain Kingdom*; crude oil.*

1.0 INTRODUCTION

Crude oil spill has remained a major source of environmental pollution in the Niger Delta area of Nigeria. These spills impact on the environment at varying degrees, depending on the volume spilled and physicochemical properties of the oil. The deleterious effects of crude oil contamination on the flora and fauna of the impacted media usually result in biodiversity loss, as exposure to crude oil fractions reduces bacterial population in the affected media, leading to loss in species diversity [1].

Bioremediation has remained an effective impact mitigation strategy (alongside other physical and chemical methods of site remediation). The process of bioremediation involves the interplay of various factors meant to achieve an efficient breakdown of these highly complex crude oil products in the environment, in which case the synergistic activities of microbial consortium are required; where hydrocarbonoclastic and hydrocarbon degrading microorganisms work together [2]. *Bacillus, Pseudomonas, Rhodococcus, Arthrobacter and Corynebacterium* spp. are a group or bacterial consortium having the ability to degrade hydrocarbons [3]. However, for bioremediation to be very effective, the involvement of well adapted species to the prevailing environmental/site conditions is very necessary with the following hydrocarbonoclastic bacteria; *Arthrobacter, Flavobacterium*, *Sphingomonas* (a novel *Pseudomonas* sp), and *Pseudomonas* spp., well known for their adaptation to petroleum contaminated environments [4]. The use of well adapted species helps in facilitating a better and faster rate of hydrocarbon biodegradation in the contaminated site. However, not all microorganisms found in the environment can degrade a particular hydrocarbon due to genetic factors. Various authors have reported the efficacy of these adapted species. For instance, [5] conducted a plasmid curing experiment and established that *Nitrosomonas and Nitrobacter* degraded crude oil by the aid of crude oil degrading plasmid. Also, *Staphylococcus* sp. has been shown to have the ability to utilize drilling fluid base oil and is therefore, a good agent for the remediation oil from drilling fluid [6]. Fungal species such as *Candida* sp. and *Schizosaccharomyces pombe* have been also found to be involved in the biodegradation of hydrocarbon [7; 8].

Recent advancement in bioremediation studies relies on molecular methods. These methods are useful in characterizing microorganisms associated with biodegradation of crude oil contaminated sites or media. The advantages of these molecular techniques are owed to their ability to identify autochthonous bacterial group *in situ* and also provide a rapid as well as efficient non-culture dependent methods of studying the bacterial composition (structure and function) of a site undergoing bioremediation [9].

Several molecular techniques are available to study microbial communities in nature, especially the non-culturable ones which have not been identified in the laboratory via cultural approach. Comparative studies and analysis involving culture dependent and molecular metagonomic approach have revealed that only about 1% of the total microorganisms are amenable to culture [10]. Currently, nucleic acids, lipids, and proteins are harnessed to provide primary information that aid in the identification of these uncultured bacterial groups. Whole genomes or selected genes analysis which

includes 16S for prokaryotes and 18S rRNA for eukaryotes are molecular techniques that are non-culture dependent. Analytical studies of these genomes help to group microorganisms into three (3) primary categories: two prokaryotic groups such as bacteria and archaea as well as one eukaryotic group (eukarya) [11]. The development of techniques to characterise or identify functional diversity as well as phylogenic differences has been of enormous importance in microbiology. Approaches such as partial community analysis and whole community analysis are methods used to probe into bacterial genetic properties which help in identifying microbial population based on their structure as well as functional attributes.Modern molecular techniques have made way for an increased understanding of bacterial diversity as well as functionality during crude oil degradation.

Sequence-based and function-based sequencing are two conventional paths metagenomic screening has followed. The analysis of genome sequence data that has been recovered from the environment is motivated by many objectives, which include the establishment of gene inventories and natural product discovery [12].

Various researchers have used different molecular techniques to characterise and identify functional genes in hydrocarbon degradation. However, the study of microbial diversity is somewhat complex and requires several approaches, used in combination. This will provide more useful information regarding diversity of the organisms. Owing to the paucity of information regarding the bacterial community composition of hydrocarbon impacted sites in Gbarain kingdom, Bayelsa State, Nigeria, this study was therefore carried out to identify the community composition using metagenomics and culture dependent techniques. Data from this investigation could provide useful information for potential bioengineering of the species through targeting specific genes of interest for efficient bioremediation of crude oil contaminated sites.

2.0 MATERIALS AND METHODS

2.1 Soil Sample Source and Collection

A crude oil-impacted soil (0–10 cm depth) was collected from Etelebuo-Ogboloma, flow station in Yenagoa L.G.A. of Bayelsa State using an Elderman auger into a Ziploc bag. Thereafter, the samples were kept in an ice box and immediately transported to the laboratory for microbiological analyses.

2.2 Culture-dependent Isolation, characterization and identification of hydrocarbon utilizing bacteria.

2.2.1 Place and duration of study: The study was carried out at the Environmental Microbiology Laboratory, University of Port Harcourt, Nigeria, for 35days.

2.2.2 Isolation of bacterial strains from soil sample

The soil sample was diluted using 10-fold serial dilution, which was carried out to a dilution of 10^{-5} by weighing 1 g of the soil sample into a sterile test tube containing 9ml of sterile physiological saline.

The heterotrophic bacteria were isolated by spreading 0.1 ml of the diluted soil sample on Nutrient Agar (NA) plates and incubated at 35 ^{0}C for 24hours. In the same manner, the hydrocarbon utilizing bacteria were isolated by culturing the diluted soil samples on Mineral Salts Agar (MSA). A Whatman's filter paper was saturated with crude oil and placed on the lid of each glass Petri dish using sterile forceps. The crude oil served as the sole source of hydrocarbon (that is carbon and energy source for the hydrocarbon utilizers). The inoculated Mineral Salts Agar (MSA) plates were inverted and placed over the lid containing the saturated filter paper, and incubated at room temperature for seven (7) days.

2.2.3 Morphological identification of isolates.

Representative colonies of the different morphological types that appeared on the plates after incubation were carefully picked with a sterile inoculating loop and sub-cultured to obtain pure cultures. This was done by streaking aseptically, onto freshly prepared nutrient agar plates. Cultural morphology of the isolates was studied based on their physical appearance such as colour, shape, size, elevation and margin

2.2.4 Biochemical characterization of isolates

Isolated colonies were further purified by sub-culturing and identified using standard biochemical test methods [13].

2.3 Molecular Analyses

2.3.1 Deoxyribonucleic acid (DNA) extraction from soil sample

Deoxyribonucleic acid (DNA) extraction from crude oil polluted soil sample was performed using ZYMO soil DNA extraction Kit (Model D 6001, Zymo Research, USA) following the manufacturer's instructions. According to this method, genomic DNA was extracted by weighing out 0.25 grams of soil sample using an analytical balance (Ohaus, Germany). The sample was then added into a ZR Bashing Bead ™ lyses tube followed by the addition of 750 µl lyses solution to the tube. The content of the 2 ml tube was disrupted by mixing in a vortex mixer at maximum speed for 5 minutes. The ZR Bashing Bead ™ lyses tube was centrifuged in a micro centrifuge at ≤ 10,000 x *g* for 1 minute. After this, 400 µl of the filtrate was added to a Zymo-Spin ™ IV spin filter in a collection tube and centrifuged at 7,000 rpm for 1 minute. This was followed by the addition of 1,200 µl of DNA binding buffer to the filtrate in the collection tub after which 800 µl of the mixture from above was added to a Zymo-Spin ™ IIC column in a collection tube and centrifuged at 10,000 x *g* for 1 minute. Flow through from the collection tube was discarded and this particular step was repeated with the remaining

filtrate. This was followed by the addition of 200 µl of DNA pre-wash buffer into the Zymo-Spin ™ IIC Column in a new collection tube and centrifuged at 10,000 x *g* for 1 minute, and then 500 µl DNA wash buffer was added to the Zymo-Spin ™ IIC column and centrifuge at 10,000 x *g* for 1 minute. The Zymo-Spin ™ IIC column was transferred into a clean 1.5 ml micro centrifuge tube and 100 µl DNA elution buffer was directly added to the column matrix. This was centrifuged at 10,000 x *g* for 30 seconds to elude the DNA. The eluded DNA was transferred into a filter unit of Zymo-Spin ™ IV-HRC Spin Filter in a clean 1.5 ml micro centrifuge tube and centrifuged at exactly 8,000 x *g* for 1 minute. The filtered DNA was then used for PCR and DNA sequencing.

2.3.2 DNA Sequencing

All sequencing analysis was carried out at Inqaba Biotechnical Pty Ltd, South Africa. DNA sequencing was performed by Next Generation Sequencing Technique to determine the nucleotide sequence of all microorganisms present in the soil sample using sequencing primer -16S: 27F: 5'-GAGTTTGATCCTGGCTCAG-3' and 518R: 5'- ATTACCGCGGCTGCTGG-3'. The sequencing was carried out using an automated PCR cycle-Genome Sequencer™ MiSeq (Illumina). Analysis and alignment was performed using Vecton NTI suit 9 (InforMax, Inc.). Overall bioinformatics analysis was done using NCBI-BLAST-2.2.24 and CLC bio Genomics workbench v7.5.1, for every sample set: every read was BLASTed and the result file saved. The top 5 hits for every BLAST result (that is, species name) was counted and a record was kept of how many times each species appeared as a hit. The number in the last column is the number of times a read hit/matched that species. The frequency (i.e count/total number of reads) and absolute count of each species were reported and used to name the specific organism.

Sequencing Codons were finally saved in fasta format and output of results showing the kingdom, phylum, class, order, family, genus, species present in the sample given. The percentage of each variable was also enumerated. The names of species of culturable and non culturable organisms present in the sample, the corresponding accession number in NCBI data base, the number of hits and the e score were also given.

3.0 RESULT AND DISCUSSION

3.1 Colonial Morphology of Isolates

All the isolates were circular in shape. The margin of the isolates were irregular, regular and entire. While the elevation was either flat or convex (Table 1).

Table 1: Colonial Morphology of bacterial isolates obtained from crude oil contaminated soil.

S/N	Isolate code	Colour	Shape	Size (mm)	Elevation	Margin
1	A	Brown	Circular	3.0	Flat	Irregular
2	B	Cream	Circular	3.5	Flat	Entire
3	C	Cream	Circular	3.2	Flat	Entire
4	D	Yellow	Circular	1.0	Flat	Entire
5	E	Cream	Circular	1.4	Convex	Entire
6	F	Brown	Circular	5.0	Flat	Regular
7	G	Yellow	Circular	1.0	Flat	Entire
8	H	Cream	Circular	1.5	Convex	Entire
9	I	Yellow	Circular	1.0	Flat	Entire
10	J	Cream	Circular	3.5	Flat	Entire
11	K	Cream	Circular	1.2	Convex	Entire
12	L	Yellow	Circular	1.0	Flat	Entire
13	M	Brown	Circular	3.0	Flat	Irregular
14	N	Cream	Circular	1.2	Convex	Entire
15	O	Cream	Circular	4.0	Flat	Entire
16	P	Brown	Circular	3.0	Flat	Irregular
17	Q	Brown	Circular	4.5	Flat	Regular
18	R	Cream	Circular	3.5	Flat	Entire
19	S	Cream	Circular	3.2	Flat	Entire
20	T	Yellow	Circular	1.0	Flat	Entire
21	U	Cream	Circular	3.5	Flat	Entire
22	V	Yellow	Circular	1.0	Flat	Entire
23	W	Cream	Circular	1.2	Convex	Entire
24	Y	Cream	Circular	3.0	Flat	Entire

3.2 Culture based identification of Isolates

Five different bacterial genera (*Acetobacter* sp*., Pseudomonas* sp., *Arthrobacter* sp., *Bacillus* sp. and *Micrococcus* sp.) were isolated and identified (Table 2). Most of the organisms isolated were gram-negative rods except *Bacillus* sp. and *Micrococcus* sp. that were gram-positive rod and cocci, respectively.

All the organisms isolated were indole negative and also showed an inability to produce hydrogen sulphide (H_2S) and gas as well as an inability to ferment lactose. *Bacillus* sp. was the only spore former isolated. All the bacterial isolates were urease negative except *Acetobacter* sp which was urease positive (Table 2).

Table 2: Morphological and biochemical characteristics of bacteria isolated from crude oil contaminated soil

S/N	Isolate Code	Gram reaction	Cell morphology	Oxidase	Citrate utilization	Methyl Red	Voges Proskauer	Catalase	Indole	Urease	Motility	Endospore	H_2S production	Spore test	Gas production	Glucose	Lactose	Maltose	Sucrose	Mannitol	Probable organism
1	A	-	Rod	-	-	-	-	+	-	-	+	-	-	-	-	A	-	-	+	-	*Arthrobacter* sp.
2	B	+	Rod	-	-	-	+	+	-	-	+	+	-	+	-	AG	-	-	-	-	*Bacillus* sp.
3	C	+	Rod	-	-	-	+	+	-	-	+	+	-	+	-	AG	-	-	-	-	*Bacillus* sp.
4	D	-	Rod	+	-	+	+	-	-	-	+	-	-	-	-	AG	-	-	-	-	*Pseudomonas* sp.
5	E	+	Cocci	+	+	+	-	+	-	-	-	-	-	-	-	A	-	-	+	-	*Micrococcus* sp.
6	F	-	Rod	-	+	-	-	-	-	+	+	-	-	-	-	AG	-	+	-	-	*Acetobacter* sp.
7	G	-	Rod	+	-	+	+	-	-	-	+	-	-	-	-	AG	-	-	-	-	*Pseudomonas* sp.
8	H	+	Cocci	+	+	+	-	+	-	-	-	-	-	-	-	A	-	-	+	-	*Micrococcus* sp.
9	I	-	Rod	+	-	+	+	-	-	-	+	-	-	-	-	AG	-	-	-	-	*Pseudomonas* sp.
10	J	+	Rod	-	-	-	+	+	-	-	+	+	-	+	-	AG	-	-	-	-	*Bacillus* sp.
11	K	+	Cocci	+	+	+	-	+	-	-	-	-	-	-	-	A	-	-	+	-	*Micrococcus* sp.
12	L	-	Rod	+	-	+	+	-	-	-	+	-	-	-	-	AG	-	-	-	-	*Pseudomonas* sp.
13	M	-	Rod	-	-	-	-	+	-	-	+	-	-	-	-	A	-	-	+	-	*Arthrobacter* sp.
14	N	+	Cocci	+	+	+	-	+	-	-	-	-	-	-	-	A	-	-	+	-	*Micrococcus* sp.
15	O	+	Rod	-	-	-	+	+	-	-	+	+	-	+	-	AG	-	-	-	-	*Bacillus* sp.
16	P	-	Rod	-	-	-	-	+	-	-	+	-	-	-	-	A	-	-	+	-	*Arthrobacter* sp.
17	Q	-	Rod	-	+	-	-	-	-	+	+	-	-	-	-	AG	-	+	-	-	*Acetobacter* sp.
18	R	+	Rod	-	-	-	+	+	-	-	+	+	-	+	-	AG	-	-	-	-	*Bacillus* sp.
19	S	-	Rod	-	-	-	+	+	-	-	+	+	-	+	-	AG	-	-	-	-	*Bacillus* sp.
20	T	-	Rod	+	-	+	+	-	-	-	+	-	-	-	-	AG	-	-	-	-	*Pseudomonas* sp.
21	U	+	Rod	-	-	-	+	+	-	-	+	+	-	+	-	AG	-	-	-	-	*Bacillus* sp.
22	V	-	Rod	+	-	+	+	-	-	-	+	-	-	-	-	AG	-	-	-	-	*Pseudomonas* sp.
23	W	+	Cocci	+	+	+	-	+	-	-	-	-	-	-	-	A	-	-	+	-	*Micrococcus* sp.
24	Y	+	Rod	-	-	-	+	+	-	-	+	+	-	+	-	AG	-	-	-	-	*Bacillus* sp

These organisms have been reported by several researchers to be involved in the bioremediation of petroleum hydrocarbons [14; 15]. As previously reported by [16], in a bioremediation study of a crude oil polluted mangrove soil in Port Harcourt, using NPK as source of limiting nutrients, *Acetobacter* sp., *Pseudomonas* sp., *Arthrobacter* sp., *Bacillus* sp., *Alcaligenes* sp., *Flavobacterium* sp., *Citrobacter* sp., *Vibrio* sp. and *Corynebacterium* sp. are members of hydrocarbon utilizing bacterial population. The findings of [16] were in consonance with the hydrocarbon utilizing bacterial population isolated in this study, from Etelebou- Ogboloma Community (Gbarain Kingdom), an oil rich area in the Niger Delta region of Nigeria.

3.2 Molecular identification of autochthonous bacterial group

Metagenomics approach was used for *in situ* identification of bacterial population in the hydrocarbon impacted soil sample. The 16s bacterial metagenomic report revealed the taxonomic classification as follows:

Kingdom Classification: The report revealed bacteria as the predominant group (99.87%) and protozoa was the next group with 0.09%, followed by fungi and archaea group with 0.02% each.

Phylum Classification: The result showed that the soil had Proteobacteria (63.5%) as the predominant group. This was followed by Actinobacteria (9.67%), Firmicutes (1.29%), Chlamydiae (0.07%) and Ciliophora (0.05%). However, 25.28% of the taxa that belonged to this phylum where unknown whereas others (Bacteroidetes, Chloroflexi, Tracheophyta, Fusobacteria, Cyanobacteria) made up 0.11% of this phylum classification. Also, groups not assigned had 0.04% (Fig. 1).

Class Classification: The class taxonomy shows the prevalence of the different groups in the following order: Alphaproteobacteria (54.64%) > Unknown (25.28%) > Actinobacteria (9.67%) > Gammaproteobacteria (6.55%) > Betaproteobacteria (2.27%) > Bacilli (0.95%) > Clostridia (0.34%) while the rest of the groups (others) made up 0.31% of the population (Fig 2).

Order Classification: Rhizobiales (54.29%) were found to be the more dominant group. While Actinomycetales, Pseudomonadales, Bacillales, Xanthomonadales, Hydrogenophilales as well as other members of the group, were 9.61%, 1.33%, 0.88%, 0.85%, 0.62% and 1.8% of the population, respectively, the unknown group had 30.63% (Fig. 3).

Family Classification: The metagenomic analysis of the polluted soil showed that the family level taxonomical groups were mostly of the Methylobacteriaceae family (45.93%). Those that belonged to the Mycobacteriaceae family were8.92% in population whereas, the Bradyrhizobiaceae family made up 8.06% of the population. The family of Pseudomonadaceae accounted for 1.33% of this taxonomical classification. Hydrogenophilaceae and Xanthomonadaceae constituted0.88% and 0.62% of the population,

respectively while the unknown group was 30.67% and others made up 3.58% of the population (Fig. 4).

BLAST output result: Data from the basic local alignment search tool (BLAST) showed (in Table 3) the most dominant bacteria to be *Methylobacterium* sp. which constituted 45.90% of the population. The uncultured bacterium was 24.63% and *Mycobacterium* sp., 8.86%. Also, *Bradyrhizobium* sp. was 7.99% whereas, uncultured gamma, *Pseudomonas aeruginosa* and betaproteobacterium accounted for 3.96, 1.29 and 1.05% of the bacterial population respectively.

Phylogenetic relationships: A phylogenetic tree based on the sequence analysis obtained from the soil metagenomics is shown in Figs. 5a and b.

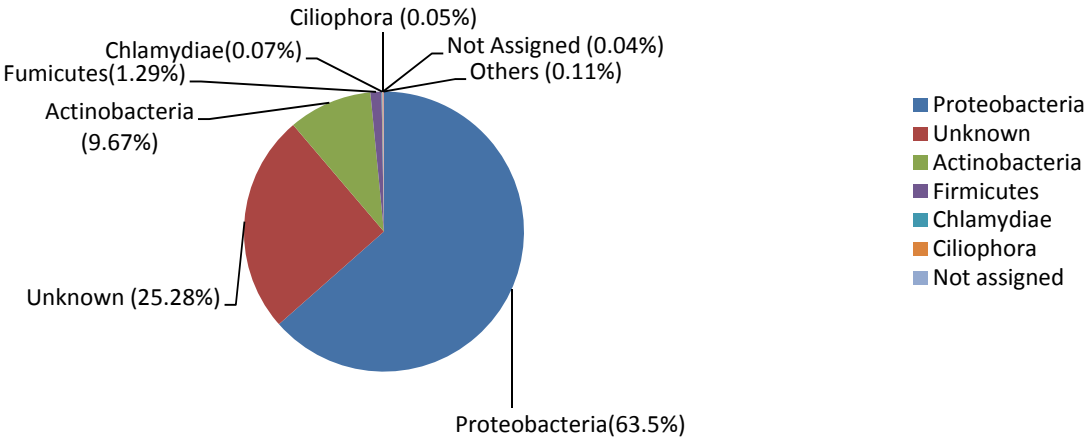


Fig. 1: Top phylum classification of bacterial isolates obtained from the crude oil contaminated soil.

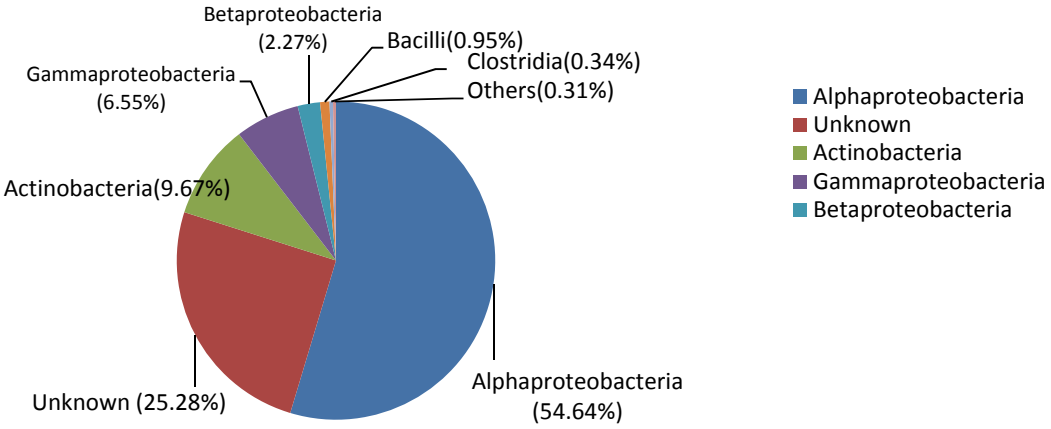


Fig. 2: Top class classification of bacterial isolates obtained from the crude oil contaminated soil

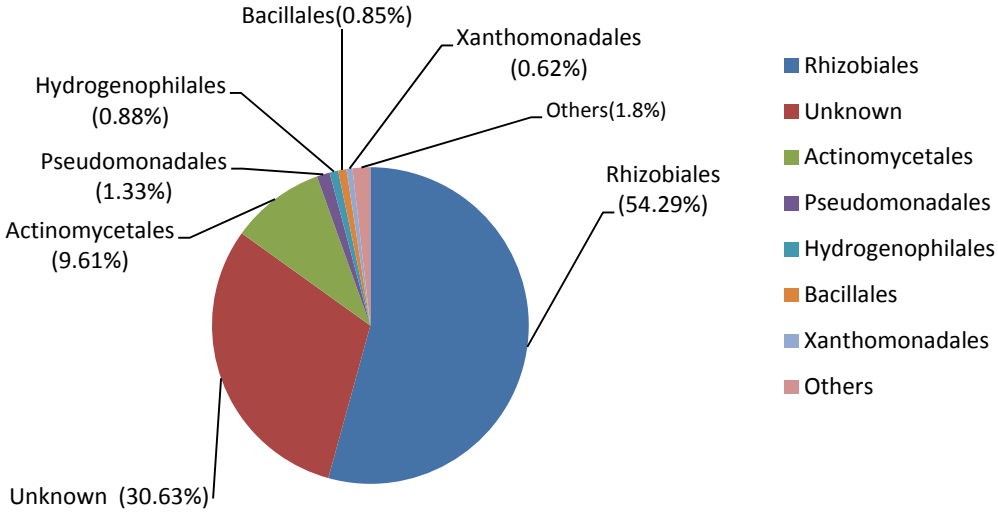


Fig. 3: Top order classification of bacterial isolates obtained from the crude oil contaminated soil.

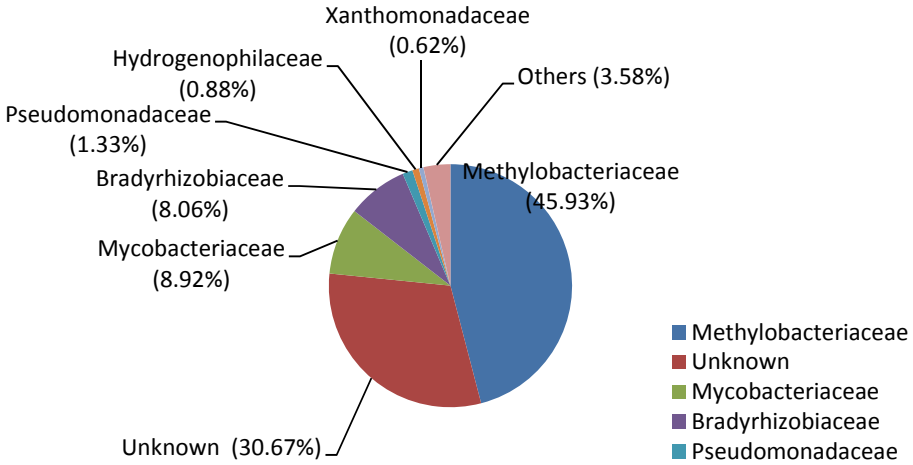


Fig. 4: Top family classification of bacterial isolates obtained from the crude oil contaminated soil.

Table 3: BLAST output results

BLAST HIT	Read	Count %
1. *Methylobacterium* sp.	5857	45.90
2. uncultured bacterium	3143	24.63
3. *Mycobacterium* sp.	1130	8.86
4. *Bradyrhizobium* sp.	1020	7.99
5. uncultured gamma	505	3.96
6. *Pseudomonas* aeruginosa	165	1.29
7. beta *proteobacterium*	134	1.05
8. *Thiobacillus prosperus*	106	0.83
9. *Stenotrophomonas maltophilia*	79	0.62
10. *Enterobacter* sp.	69	0.54
11. *Brachybacterium paraconglomeratum*	54	0.42
12. bacterium btn	46	0.36
13. *Alicyclobacillus* hesperidum	35	0.27
14. *Ochrobactrum* anthropi	32	0.25
15. unidentified eubacterium	26	0.20
16. *Bacillus* sp.	24	0.19
17. uncultured alpha	23	0.18
18. *Staphylococcus* aureus	22	0.17
19. *Achromobacter* sp.	11	0.09
20. uncultured beta	11	0.09
21. No hits	10	0.08
22. uncultured chlamydia	9	0.07
23. *Geobacillus thermoleovorans*	9	0.07
24. *Anaerobic bacterium*	9	0.07
25. *Spirulina subsalsa*	9	0.07
26. *Bradyrhizobium japonicum*	8	0.06
27. *Dietzia* sp.	8	0.06
28. *Mycobacterium* heidelbergense	8	0.06
29. uncultured streptococcus	7	0.05
30. *Alicyclobacillus acidiphilus*	7	0.05
31. uncultured eubacterium	7	0.05

32.	*Clostridium* sp.	7	0.05
33.	alpha proteobacterium	7	0.05
34.	*Providencia vermicola*	6	0.05
35.	*Thiobacillus* sp.	6	0.05
36.	*Chroococcidiopsis* sp.	6	0.05
37.	uncultured rothia	6	0.05
38.	*Acidovorax delafieldii*	6	0.05
39.	*Bacterium 'smarlab*	5	0.04
40.	*Ralstonia pickettii*	5	0.04
41.	*Grimontella senegalensis*	5	0.04
42.	*Staphylococcus epidermidis*	5	0.04
43.	*Leuconostoc mesenteroides*	5	0.04
44.	*Sphaerobacter thermophilus*	4	0.03
45.	*Streptomyces* sp.	4	0.03
46.	*Actinomycete* species	4	0.03
47.	*Comamonas testosteroni*	4	0.03
48.	*Paracoccus* sp.	4	0.03
49.	*Sphingomonas* sp.	3	0.02
50.	*Micrococcus lylae*	3	0.02
51.	*Microcoleus vaginatus*	3	0.02
52.	*Bacillaceae bacterium*	3	0.02
53.	uncultured brevundimonas	3	0.02
54.	*Aeromonas* sp.	3	0.02
55.	*Pseudonocardia yunnanensis*	3	0.02
56.	uncultured hyphomicrobium	3	0.02
57.	*Sulfuricurvum kujiense*	3	0.02
58.	*Bacillus soli*	3	0.02
59.	*Micrococcus luteus*	3	0.02
60.	uncultured chloroflexi	2	0.02
61.	*Acidovorax* sp.	2	0.02
62.	uncultured soil	2	0.02
63.	*Oscillatoria amphigranulata*	2	0.02
64.	*Bacteroidetes bacterium*	2	0.02
65.	uncultured fusobacterium	2	0.02
66.	*Kocuria* sp.	2	0.02
67.	*Alcaligenes* sp.	2	0.02
68.	*Clostridium indolis*	2	0.02
69.	*Acidocella* sp.	2	0.02
70.	uncultured pseudomonas	2	0.02
71.	uncultured actinobacterium	1	0.01
72.	*Shewanella putrefaciens*	1	0.01
73.	*Bacillus sphaericus*	1	0.01
74.	*Morganella* sp.	1	0.01
75.	uncultured scenedesmus	1	0.01
76.	agricultural soil	1	0.01
77.	uncultured chloroflexus	1	0.01
78.	*Pseudomonas stutzeri*	1	0.01
79.	*Rhodopila globiformis*	1	0.01
80.	unidentified bacterium	1	0.01
81.	uncultured archaeon	1	0.01
82.	*Bifidobacterium* sp.	1	0.01
83.	uncultured rubrobacteridae	1	0.01
84.	*Dysgonomonas* sp.	1	0.01
85.	*Chondromyces crocatus*	1	0.01
86.	*Pseudomonas pertucinogena*	1	0.01
87.	*Agrobacterium tumefaciens*	1	0.01
88.	*Saprospira* sp.	1	0.01
89.	*Delftia* sp.	1	0.01
90.	uncultured candidate	1	0.01
91.	*Rhizobium* sp.	1	0.01
92.	uncultured gloeothece	1	0.01

93.	uncultured delta	1	0.01
94.	*Marinospirillum alkaliphilum*	1	0.01
95.	uncultured rhodospirillaceae	1	0.01
96.	*microbulbifer* sp.	1	0.01
97.	uncultured sphingomonas	1	0.01
98.	*Clostridium subterminale*	1	0.01
99.	*Cetobacterium somerae*	1	0.01
100.	*Mobiluncus curtisii*	1	0.01
101.	uncultured methanogenic	1	0.01
102.	uncultured syntrophorhabdaceae	1	0.01
103.	*Aquaspirillum peregrinum*	1	0.01
104.	*Dellovibrio bacteriovorus*	1	0.01

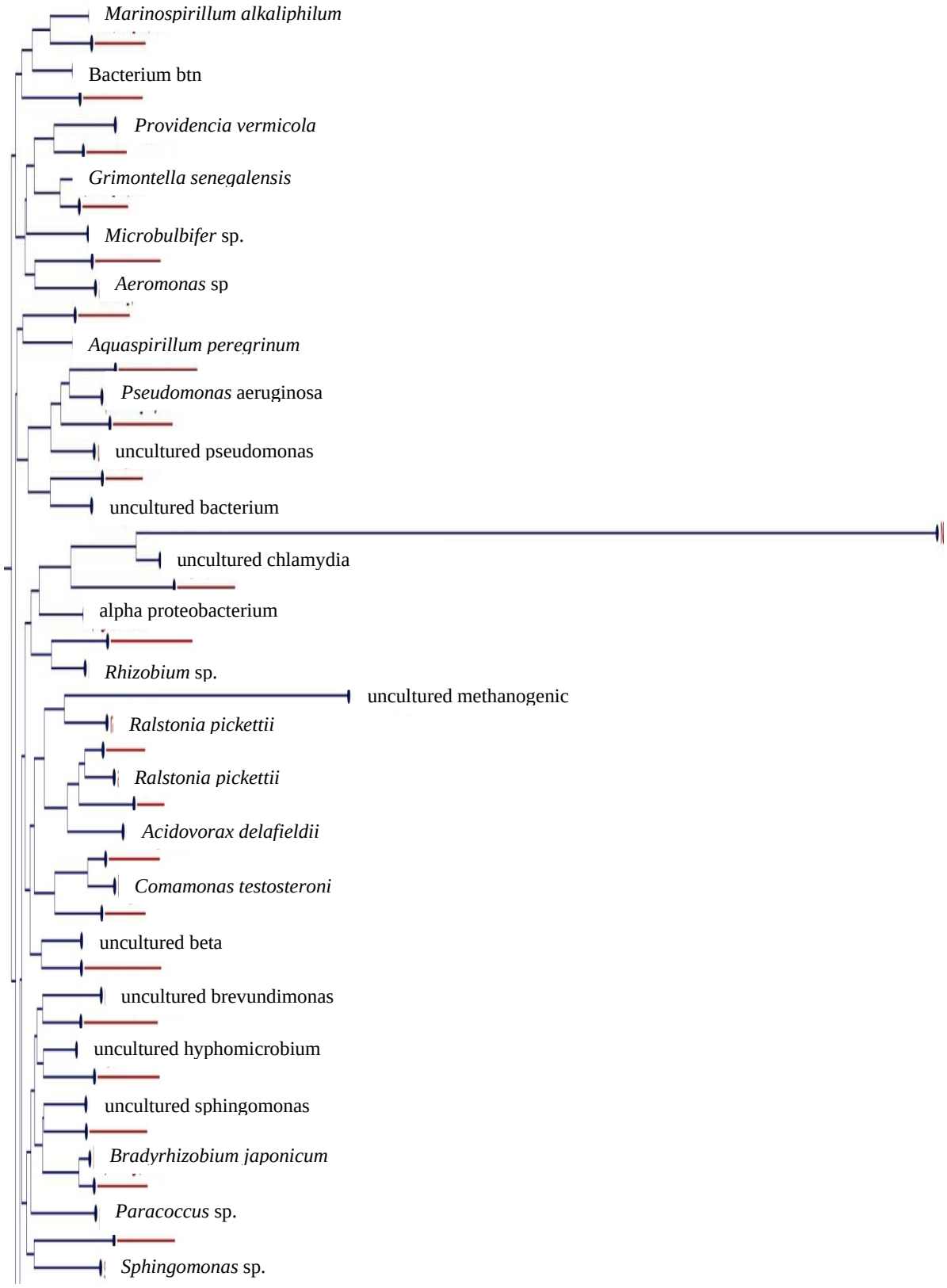
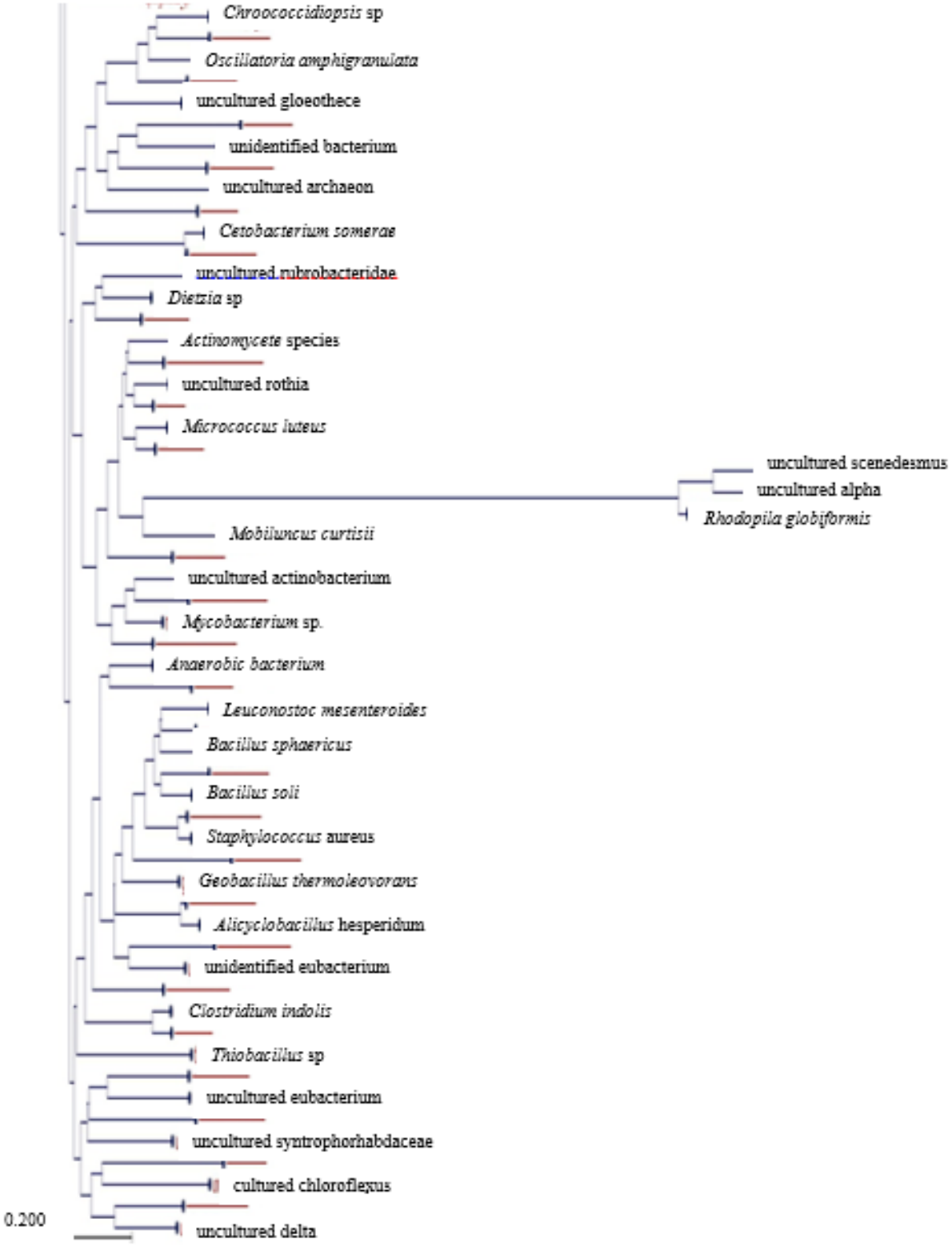


Fig. 5: Phylogenetic tree of 16S rRNA gene sequences of bacterial isolates obtained from crude oil contaminated soil sample.



385

386 Fig. 5 cont'd: Continuation of phylogenetic tree of 16S rRNA gene sequences of bacterial isolates
387 obtained from crude oil contaminated soil.

Metagenomic approach was used to identify the autochthonous bacterial population in the polluted soil sample used in the investigation and results show that bacteria were predominant in the crude oil impacted soil. Proteobacteria were the predominant bacterial population which may be indicative of the major role played by the proteobacteria group in a crude oil polluted soil ecosystem. Proteobacteria are Gram negative group of bacteria. This probably accounts for the predominance of gram negative rods identified via culture-dependent approach described above. The data also showed that the soil was mainly dominated by the Alphaproteobacteria (54.64%), followed by the Actinobacteria (9.67%), Gammaproteobacteria (6.55%), Betaproteobacteria (2.27%) and Bacilli (0.95%) as well as Clostridia (0.34%); as the most dominant classes of bacteria.

The order taxonomical classification showed that Rhizobiales (54.29%) were the most dominant group followed by the Actinomycetales, Pseudomonadales, Bacillales, Xanthomonadales and Hydrogenophilales.

The BLAST data indicated the dominant bacteria to be *Methylobacterium* sp. making up 45.90 % of the population. The uncultured bacterium was 24.63% and *Mycobacterium* sp., 8.86%. Also, *Bradyrhizobium* sp. was 7.99% and uncultured gamma, *Pseudomonas aeruginosa* and beta proteobacterium accounted for 3.96, 1.29 and 1.05% of the bacterial population respectively. These organisms have been identified by other researchers to be associated with biodegradation [17; 18]. Molecular studies on microbial diversity have interestingly been related to degradation potentials of these organisms by various researchers [17; 18; 19]. The research of [17] examined the degradation of benzo[α]pyrene by a group of bacteria by using the DGGE technique, in order to determine their dynamics during the degradation process, and identified *Sphingomonas paucimobilis EPA505*, *Mycobacterium str.PYR-1,* and *Alcaligenes denitrificans WW1* to be present in the consortium. These organisms are well known to be associated with the degradation of aromatic compounds, as *Mycobacterium* and *Pseudomonas* species have been also reported to be able to degrade PAH [18]. In crude oil, carbazole is often present with its alkylated derivatives which often have monomethyl, dimethyl, trimethyl, and tetramethyl side chains on different carbon positions of carbazole (called C1-, C2-, and C3-, C4-carbazoles, respectively). Researchers have isolated strains of *Pseudomonas* species, which could not only degrade carbazole efficiently, but also showed nitrification and denitrification ability [19].

In the overall, one hundred and four (104) diverse bacterial species were identified as shown in table 3. The five (5) different bacterial genera (*Acetobacter* sp., *Pseudomonas* sp., *Arthrobacter* sp., *Bacillus* sp. and *Micrococcus* sp.) isolated via cultural approach were similar to the bacterial species identified using metagenomics. However, a comparison of the number of isolates obtained using both methods clearly indicates that molecular techniques such as metagenomics increases the understanding of bacterial diversity as well as functionality during bioremediation. Cultural techniques only help to culture and identify minute population of microorganisms [20], representing about 1% of the total microorganism found in a crude oil contaminated site [10].

The phylogenetic tree showed the predominant bacterial community in the hydrocarbon polluted soil in relation to each other, using a scale of 0.2 and uncultured delta (0.01) at the root of the tree. The phylogenetic sequence relationship shows that the most distant groups/species are the uncultured scenedesmus (0.01%), uncultured alpha (0.18%) and *Rhodpila globiformis* (0.01%). These groups however, belong to the same clade with *Mobiluncus curtisii* (0.01%). The *no hits* (0.08%) were also seen to be distantly related to the uncultured *Clamydia* (0.07%) which is related to the alpha proteobacteria (0.05%). The tree shows numerous clades for the 104 BLAST output result. This shows the crude oil contaminated soil is composed of diverse groups of microorganisms and thus, implies the hydrocarbon polluted soil is a reservoir for diverse bacterial groups.

Similar study by [21] involving the excision, amplification and sequencing of dominant DGGE bands in biostimulated soils revealed the presence of distinct hydrocarbon degraders like *Corynebacterium* spp., *Dietzia* spp., low G+C Gram positive bacteria and some uncultured bacterial clones. Phylogenetic analysis of the 16S rRNA gene sequences of these dominant bacterial communities was conducted using the neighbour joining method of PHYLIP. The researcher observed two distinct clades appearing in the tree with members of the *Actinobacteria* and *Firmicutes* clustering separately [21]. In another study, the analysis of 16S rRNA of the isolated isolates from a crude oil impacted soil in the Niger Delta region of Nigeria showed the species belonged to eight bacterial genera namely: Achromobacter, Alcaligenes, Azospirillus, Bacillus, Lysinibacillus, Ochrobactrum, Proteus, and Pusillimonas, with Alcaligenes as the dominant genus [22].

The variations in the number of hits of bacterial species observed in this study conform to the fact that the polluted soil has diverse bacterial groups with different levels of adaptation. The ability of these bacteria to metabolize or adapt to crude oil hydrocarbons is relative to the possession of degradative genes or resistance genes [23]. These studies have revealed the phylogeny of the polluted soil used in this study, which shows the diverse species of bacteria. These bacterial species differ in their structure and function. For example *Thiobacillus* sp. is known to be associated with iron oxidation and inorganic sulphur reduction (*Thiobacillus ferroxidans*). Some others are associated with heavy metal reduction. This implies the polluted soil ecosystem harbours diverse bacterial population having different structures and function which aid the process of bioremediation. While some of these organisms are hydrocarbonoclastic, others (like *Pseudomonas* sp.) are known for biosurfactant production. Biodegradation of petroleum hydrocarbon pollutants is therefore feasible in such ecosystem. However, the fate of these crude oil pollutants will partly depend on the ecological/site characteristics of the impacted soil. Gbarain kingdom is an oil rich region in the Niger Delta area of Nigeria. This area is replete with crude oil pipe lines traversing the land and water body. This may be partly responsible for *Thiobacillus* sp. ranking among the top 8 in the BLAST output report. This finding does not only make a case for bioremediation but also presents the critical need for pipeline coating and biomonitoring.

4.0 CONCLUSION

The bacterial community composition of a crude oil polluted soil obtained from Gbarain Kingdom of Bayelsa State, Nigeria suggests the presence of a rich and versatile group of hydrocarbon utilizing bacteria adapted for crude oil degradation. These hydrocarbonoclastic bacterial species are involved in the utilization of the petroleum hydrocarbons spilled at the site sampled and they have the potentials to facilitate the bioremediation of the contaminated soil through natural attenuation or enhanced bio-treatment protocols. Data obtained in this study make it obvious that only a very small proportion of the bacterial population in the soil can be isolated and identified through cultural techniques. Therefore, a detailed insight into the agents and dynamics of crude oil degradation in soil thus, requires in-depth metagenomics which provide the structure and function of the diverse microbial genera for the purpose of exploring and harnessing the potentials of these autochthonous bacterial populations. The emergence of next generation gene sequencing (NGS) technique has enabled the identification of a wide range of bacteria which were hitherto, unidentifiable via culture-based methods. The NGS method is performed independent of PCR products and boycotts the limitations associated with such techniques and is suitable for the study of the bacterial community structure of a crude oil impacted media. However, cultural methods are not to be sacrificed for identification using molecular characterization as both methods are important in bioremediation monitoring.

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