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Phylogenetic Relationship of Bacterial Species Involved in Bioremediation of Hydrocarbon Polluted Soils

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Authors' contributions

This work was carried out in collaboration between the authors. Author SPA designed the study and wrote the protocol. Author RBA performed the Statistical analysis, wrote the first draft of the manuscript, managed the analyses of the study and managed the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

The bio-stimulation of hydrocarbon degrading microbial population in soil using agricultural wastes was carried out. Top soil (0-25 cm depth) from three points were bulked to form composite soil samples, 6 kg each of the composite soil sample was weighed and transferred into 150 plastic buckets with drainage holes at the base. The soil in each plastic bucket was spiked with 300 ml crude oil and amended with different concentrations of agro-wastes and allowed for duration of 30, 60 and 90 days. The soil samples were then collected and analysed for both total heterotrophic bacterial counts and crude-oil utilizing bacterial counts. Data collected were subjected to a three-way analysis of variance and significant means were separated using Least Significant Difference Test at 5% probability level. The result showed that the application of the amendments increased the bacterial counts in the soil at different treatment levels. However, a higher proliferation rate was observed with bacteria counts exposed to higher waste concentrations compared to their counterparts exposed to lower waste concentrations. The phylogenetic relationship of the

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hydrocarbon degrading bacterial species shows that the identified bacteria were in two clusters: cluster 1 consist of *Bacillus cereus, Bacillus thuringiensis, Bacillus altitudinis, Pseudomonas aeruginosa, Proteus mirabilis* and *Proteus penneri,* while cluster 2 consist of *Serratia marcescens, Providencia rettgeri and Enterobacter asburiae*. The bacterial species obtained shows a greater relationship, this imply that the similarity of the bacterial species could be the reason for their high proficiency in degrading the hydrocarbons in the soil.

Keywords: Phylogenetic; soil; bio-stimulation; hydrocarbon; microbial; agro-waste; degradation.

1. INTRODUCTION

Oil exploration activities in producing communities have inadvertently affected the natural land occupied by a rich biodiversity of plants and animal species. The lost in biodiversity of plants and animal's species have resulted in abandonment of waste land, with an alarming outcry by the occupancy of those community. At present the waste land at Ogoni is receiving remediation attention by the federal government. The reduction in the soil microorganisms which are not hydrocarbon utilizers result in the rapid increased in the population of hydrocarbon utilizing microorganisms in the polluted environment [1]. Hofman et al. [2] reported that though the number of soil microorganisms increase in hydrocarbon polluted soils, species richness often decreases over time. Petroleum hydrocarbons may interfere with plant-fungus relationship by altering the soil environment so that movement of diffusible chemical signals such as auxins is prevented. It may also affect this relationship by altering the root exudation pattern [3]. Bioremediation involves the application of microorganism in contaminated site to reducing or removing the contaminant from the soil, water or air. Bioremediation is a mechanism for removing pollutants from polluted environment thus restoring the original natural environment [4]. The treatment of polluted environment is necessary because it helps to protect the soil, water and the people in the ecosystem. Bioremediation by means of stimulating the growth of microorganisms have achieved a wide success, in that many waste lands has been restored. Biostimulation over the years have exploited the potential use of agricultural wastes in the enhancement of microbial growth in the soil. Agbor et al. [5] in their study reported the potentials of plantain peels and cocoa pod husks in increasing the microbial population of the soil which culminated in the reduction of hydrocarbon content of the soil. Biostimulation using inorganic fertilizer has been extensively employed worldwide in reclaiming oil polluted soil [6,7,8,9] Ekpo et al. 2012, [5]. This current research examined the biostimulation potentials of various agro-wastes in enhancing microbial growth in the soil. Phylogenetic analysis revealed the evolutionary relatedness among different species of organisms found in soil, air and water environment. Molecular phylogenetic utilizes sequence data to compare the similarities or relationship of organisms and the different genes they possess. Agbor et al. [5] reported that fungi species obtained from a bioremediated soil shows a little evolutionary divergence in the different identified fungi species. Olukunle and Boboye [10], Mahesh and Kalpana [11] reported that phylogenetic analysis is a reliable tool in comparing sequenced genes. These study was aimed at comparing the evolutionary relatedness among bacterial species isolated from hydrocarbon polluted soils enhanced with agro-wastes.

2. MATERIALS AND METHODS

Microbial analysis was done in the Department of Microbiology (University of Calabar) and Nigerian Institute of Medical Research (Lagos State), while sequencing analysis was done at Inqaba Biotechnology Pty South Africa. Nigerian light crude oil was obtained from NAOC (Nigerian Agip Oil Company), located in Port Harcourt, Rivers State, Nigeria. Agro-wastes such as groundnut husks (GH), maize cobs (MC), empty fruit bunch of oil palm (EFBOP) and cassava peels (CP) were collected from local farmers and processing industries in Cross River State, Nigeria. The collected agro-wastes (GH, MC, EFBOP, CP) were sun-dried for 10 days and blended to powder using electric blender (Model 4250, Braun, Germany). The dispersants were sieved to pass through 2 mm sieve. They were labelled and stored in containers. Top soils (0-25 cm depth) were evenly obtained from three (3) points, using a Dutch auger, then homogenized to form composite soil sample. Six kilograms (6 kg) of the soil samples was weighed and transferred into each of the hundred and fifty

(150) labelled plastic buckets (PB) with drainage holes at the sides and based. The arrangement of the PB was in triplicate using completely randomized design (CRD). Artificial pollution was done by introducing 300 ml (0.3 litters) of crude oil into PB containing the soils, except the pristine soils samples that served as the positive control. The PB containing the polluted soils were mixed thoroughly and allowed to stand for 14 days (these was to allow indigenous microorganisms to become acclimatized with the new soil condition). The amendments were applied in single and combined forms using the following concentrations: pristine control (unpolluted (positive), 0%), crude oil control (polluted (negative), 0%), 3.33% and 6.67% and 10% of the amendments (5 levels). Soil samples for bacterial analysis were collected at 30, 60 and 90 days.

Calculation of treatment in percentage

$$PT = \frac{QOW}{QS} \text{ X } 100$$

Where

PT = Percentage of treatment
QOW = Quantity of organic wastes
QS = Quantity of soil

Substitute in the equation

$$0\% \text{ treatment} = \frac{0g}{6000g} \text{ x } 100$$

$$3.33\% \text{ treatment} = \frac{200g}{6000g} \text{ x } 100$$

$$6.67\% \text{ treatment} = \frac{400g}{6000g} \text{ x } 100$$

$$10\% \text{ treatment} = \frac{600g}{6000g} \text{ x } 100$$

2.1 Soil Microbial Analysis

Soil samples were taken to the Laboratory of Department of Microbiology for determination of bacterial population in the soil using surface plating method.

Determination of total heterotrophic bacteria: The spread plate method using nutrient agar (NA) was used in determining the total heterotrophic bacteria count in the soil according to APHA (1998). Serial dilutions were prepared with one gram of soil on a ten-fold. From the test tube of 10^{-6} and 10^{-7} 0.1 ml of the dilutions were spread on the plates in duplicate. Discrete bacterial colonies were counted after incubation at 28°C for 24 hours and the counts were calculated based on colony forming unit per gram of soil.

Determination of crude oil-utilizing bacteria (CUB): The procedure of Hamamura et al. [12] was adopted. The viable count method using the surface spreading technique was used. About 1.5% agar was added to the mineral salts medium to solidify. A ten (10) fold serial dilutions with 1 g of soil was prepared and 0.1 ml of 10^{-5} and 10^{-6} dilution were spread on the plates in duplicates. After agar plates inoculation, a sterile Whatman No.1 filter paper was dipped into crude oil, allowed to dry and was placed on the inside of the lid (cover) of the Petri dishes. The saturated filter paper containing crude oil acted as a source of carbon and energy for growth of the bacterial through vapour phase transfer (Okpokwasili & Amanchukwu, 1988). The plates were inverted and incubated at normal temperature for 5 days, during which the CUB were counted from the duplicate plates and expressed as colony forming unit per gram of soil samples.

Purification and maintenance of microbial isolates: The bacterial isolates obtained from mineral salt medium were purified by repeated sub-culturing. The isolates were subjected to series of transfers unto fresh medium. The bacterial isolates were transferred onto fresh nutrient agar medium and incubated at 28°C for 24 hours. Pure colonies of bacterial were maintained on slopes of nutrient agar (NA) and stored in a refrigerator at 4°C till needed for studies.

Characterization and identification of the isolates using traditional methods: Standard inocula was prepared from the preserved stock culture by taking a loopful of the isolates and aseptically inoculating onto sterile nutrient agar (NA) plates. The plates were incubated at 28°C for 24 hours. The characterization of the isolates was performed, by employing Gram staining reaction, oxidase test, catalase, citrate test, urease test, coagulase test, TSI (triple sugar iron agar) test, MIO (motility indole ornithine) test and methyl red and Voges Proskauer test as described in Bergey's Manual of Determinative Bacteriology, 9th edition (1994).

2.2 DNA Extraction

DNA Extraction was performed at the Anaerobe Laboratory, Molecular Biology and biotechnology Division, Nigerian Institute of Medical Research

Yaba Lagos. Methodology was based on PCR and metagenomics analysis. While sequencing analysis was done at a Inqaba Biotechnology Pty South Africa.

DNA extraction was from a 24 hours' growth of microbial isolates in Nutrient broth harvested by centrifugation at 14, 000 x g for 10 minutes. The cells were washed three times in 1 ml of Ultra-pure water by centrifuging at 12,000 rpm for 5 min. DNA extraction and purification was done using ZR Bacterial DNA MiniPrep™50 Preps. Model D6005 (Zymo Research, California, USA). 50-100 mg of bacterial cells was re-suspended in 200 µl of sterile water. This was transferred into a ZR Bashing Bead™ Lysis Tube. Exactly 750 µl Lysis Solution was added to the tube. The bead containing the solution was secured in a bead beater fitted with a 2 ml tube holder assembly and process at maximum speed for 5 minutes. The ZR Bashing Bead™ Lysis Tube was Centrifuged in a microcentrifuge at 10,000 x g for 1 minute. 400 µl of the supernatant was pipeted into a Zymo-Spin™ IV Spin Filter in a Collection Tube and centrifuged at 7,000 x g for 1 minute. This was followed by the addition of 1,200 µl of Bacterial DNA Binding Buffer into the filtrate in the Collection Tube. After this 800 µl of the mixture was transferred into a Zymo-Spin™ IIC Column in a Collection Tube and centrifuge at 10,000 x g for 1 minute.

The flow through was discarded from the Collection Tube and the process was repeated to obtain the remaining products. The 200 µl DNA Pre-Wash Buffer was added into the Zymo-Spin™ IIC Column in a new Collection Tube and centrifuge at 10,000 x g for 1 minute. This was followed by the addition of 500 µl Bacterial DNA Wash Buffer into the Zymo-Spin™ IIC Column and centrifuged at 10,000 x g for 1 minute. The Zymo-Spin™ IIC Column was transferred into a clean 1.5 ml microcentrifuge tube and 100 µl of DNA Elution Buffer was then added directly to the column matrix. This was centrifuged at 10,000 x g for 30 seconds to elute the DNA. The Ultra-pure resulting filtrate (DNA) obtained was used as a template during the assay. This was transported in ice to the laboratory for sequencing.

2.3 DNA Sequencing

DNA sequencing was performed by Sanger (dideoxy) Sequencing Technique to determine the nucleotide sequence of the specific microorganism isolated using automated PCR cycle- Sanger Sequencer™ 3730/3730XL DNA Analyzers from Applied Biosystems (Russell, 2002; Metzenberg 2003). This results were obtained as nucleotides when amplified using sequencing primer -16S: 27F: 5'-GAGTTTGATCCTGGCTCAG-3' and 518R: 5'-ATTACCGCGGCTGCTGG-3' (Weisburg et al. 1991). Sequence data from resultant nucleotides base pairs was downloaded and read using FinchTv software followed by direct nucleotide blasting on http://blast.ncbi.nlm.nih.gov. For every set of isolate, a read was BLASTED and the resultant top hits for every BLAST result showing species name was used to name the specific organism. Corresponding gene back accession number and query length of sequences blasted was also recorded.

2.4 Statistical Analysis

Data collected were subjected to a three-way analysis of variance and significant means were separated to least significant difference test at 5% probability level.

3. RESULTS

3.1 Total Heterotrophic Bacterial (THB) Counts of the Soil Enhanced with Agro-wastes

Microbial population in any polluted soil environment is an important tool in determining the degradation process of the petroleum hydrocarbon in the soils. The results obtained for the bacterial population of the soil showed that soil treated with 3%, 6% and 10% $CasP_{14}P$ + $EFBOP_{14}P$ and polluted soils treated with 6% and 10% $EFBOP_{14}P$ had significantly high ($P<0.05$) bacterial counts with insignificant difference ($P>0.05$) in the average THB counts as compared to the positive and negative control and also the other treatment groups (Table 1). These were followed by polluted soil treated with 3%, 6%, 10% $GnH_{14}P$ + $MaC_{14}P$, and $GnH_{14}P$ + $EFBOP_{14}P$, 10% $CasP_{14}P$, 6% $EFBOP_{14}P$, 10% $GnH_{14}P$ + $CasP_{14}P$ and 6%, 10% $CasP_{14}P$ + $MaC_{14}P$ which had insignificant difference ($P>0.05$) in the average THB counts of the soil. The next were the counts obtained from soil treated with 10% $GnH_{14}P$, 6% $CasP_{14}P$, 3% $CasP_{14}P$ + $MaC_{14}P$ and 6% $GnH_{14}P$ + $CasP_{14}P$ which had no variation in the mean THB counts of the soils but the counts were greater than the counts obtained from soil amended with 3% $CasP_{14}P$, 3% $GnH_{14}P$ + $CasP_{14}P$, 10% $MaC_{14}P$ and 6% $MaC_{14}P$ + $EFBOP_{14}P$ which had no

variation in the mean THB counts of the soils (Table 1). The soils treated with 3% GnH14P and 3% MaC14P + EFBOP14P had significantly reduced ($P<0.05$) mean THB counts in the soils but significantly increase ($P<0.05$) than the average THB counts obtained in the pristine control and the crude oil control soils. However, the crude-oil control soil was observed to produce the lowest bacterial population compared to the pristine control and the treated groups (Table 1). Fig. 1 present the results of the THB counts obtained at different duration of study and the results showed that the average THB counts of the soil treated with $EFBOP_{14}P$ and $CasP_{14}P$ + $EFBOP_{14}P$ at 30days and 60days were significantly increase ($P<0.05$) than other amended groups with no variation in the mean values. These were followed by soil treated with $CasP_{14}P$ at 60 days, $CasP_{14}P$ + $EFBOP_{14}P$ at 90 days, $GnH_{14}P$ + MaC14P at 60 days, $GnH_{14}P$ + $EFBOP_{14}P$ and $CasP_{14}P$ + $MaC_{14}P$ at 30^{th} and 60^{th} day with insignificant difference ($P>0.05$) in the average THB counts of the soils, these counts were more than the counts obtained from the soil treated with $EFBOP_{14}P$ and $GnH_{14}P$ + MaC14P at 90 days with no variation in the mean values obtained. These were also more than the mean THB counts obtained from soils treated with $GnH_{14}P$ + $MaC_{14}P$, $CasP_{14}P$ at 30 days, and $GnH_{14}P$ + $EFBOP_{14}P$ at 90 days with no variation the mean values obtained, more than the counts obtained from the soil treated with $GnH_{14}P$, $MaC_{14}P$, $MaC_{14}P$ + $EFBOP_{14}P$ and $GnH_{14}P$ + $CasP_{14}P$ at 30^{th}, 60^{th} and 90^{th} day of treatment duration. Fig. 1 showed that the total heterotrophic bacterial counts at 60 days were higher than the count obtained at 30 days, while the counts obtained for 30 days were more than the THB counts at 90 days. However, from the result obtained it was observed that the soil amended with $CasP_{14}P$ + $EFBOP_{14}P$ had higher ($P<0.05$) mean THB counts in the soil than other treatment groups but followed by soil treated with $EFBOP_{14}P$ (Fig. 1). It was also observed that the combined treatment produced higher ($P<0.05$) THB counts than the single amended groups. These imply that polluted soil amelioration is best achieved with the combined treatments.

3.2 Crude Oil-utilizing Bacterial (CUB) Counts in Soils Amended With Agro-wastes

The determination of crude oil utilizing bacteria was also assessed, to ascertain the populations of the bacteria that are purely hydrocarbon degraders in the soils. The results as presented in Table 1 showed that the soil treated with 10% $GnH_{14}P$ + $MaC_{14}P$ and $CasP_{14}P$ + MaC14P had more CUB counts, with no variation in mean values obtained. These were followed by CUB counts soils treated with 6% $GnH_{14}P$ + $MaC_{14}P$ and 10% $MaC_{14}P$ + $EFBOP_{14}P$ with no variation in the average values but higher than the count obtained from soil treated with 6% $CasP_{14}P$ + $MaC_{14}P$. These were also followed by soil treated with 10% $GnH_{14}P$, 10% $MaC_{14}P$, 3% $GnH_{14}P$ + $MaC_{14}P$, 10% $GnH_{14}P$ + $EFBOP_{14}P$ and 3% $CasP_{14}P$ + MaC14P, with no variation in the average values but higher than the counts obtained from soil treated with 6% $GnH_{14}P$, 6% $MaC_{14}P$ 10% $CasP_{14}P$ + $EFBOP_{14}P$, 6% $GnH_{14}P$ + $EFBOP_{14}P$ and 3% $MaC_{14}P$ + $EFBOP_{14}P$ with insignificant difference in the average CUB counts of the soils. The CUB counts obtained from the agro-wastes treated soils were observed to be higher than the count obtained from the pristine and crude oil control soils. However, the pristine soil had higher CUB counts than the crude oil controls.

The result as presented in Fig. 2 showed that the soil treated with $GnH_{14}P$ + $MaC_{14}P$ and $CasP_{14}P$ + $MaC_{14}P$ at 60 days had significantly increase ($P<0.05$) in CUB counts, with no variation in the average values obtained. These were higher than the counts obtained in soil amended with $CasP_{14}P$ + $MaC_{14}P$ at 30 days, $MaC_{14}P$ + $EFBOP_{14}P$ and $GnH_{14}P$ + $CasP_{14}P$ at 60 days with insignificant differences ($P>0.05$) in the average CUB counts obtained in the soils. The counts obtained from $GnH_{14}P$ + $MaC_{14}P$ at 90 days was next, higher than the counts obtained from the soil treated with $GnH_{14}P$ + $MaC_{14}P$ at 30 days of soil treatment. It was also observed that the counts obtained from the soils treated with $GnH_{14}P$, $MaC_{14}P$, $CasP_{14}P$ + $GnH_{14}P$, $GnH_{14}P$ + $GnH_{14}P$, $CasP_{14}P$ + $MaC_{14}P$ at 60 days and $MaC_{14}P$ + $EFBOP_{14}P$ at 30 days' soil treatment had insignificant difference ($P>0.05$) in mean CUB counts of the soils. The results for CUB counts in the soil at 90 days of soil remediation were decreased as compared to the high counts recorded at 60 days, followed by the count obtained at 30 days of soil remediation. The results as presented on Fig. 3 also showed that the CUB counts of the soil treated with $GnH_{14}P$ + $MaC_{14}P$ and $CasP_{14}P$ + $MaC_{14}P$ were significantly increased than other amended soils.

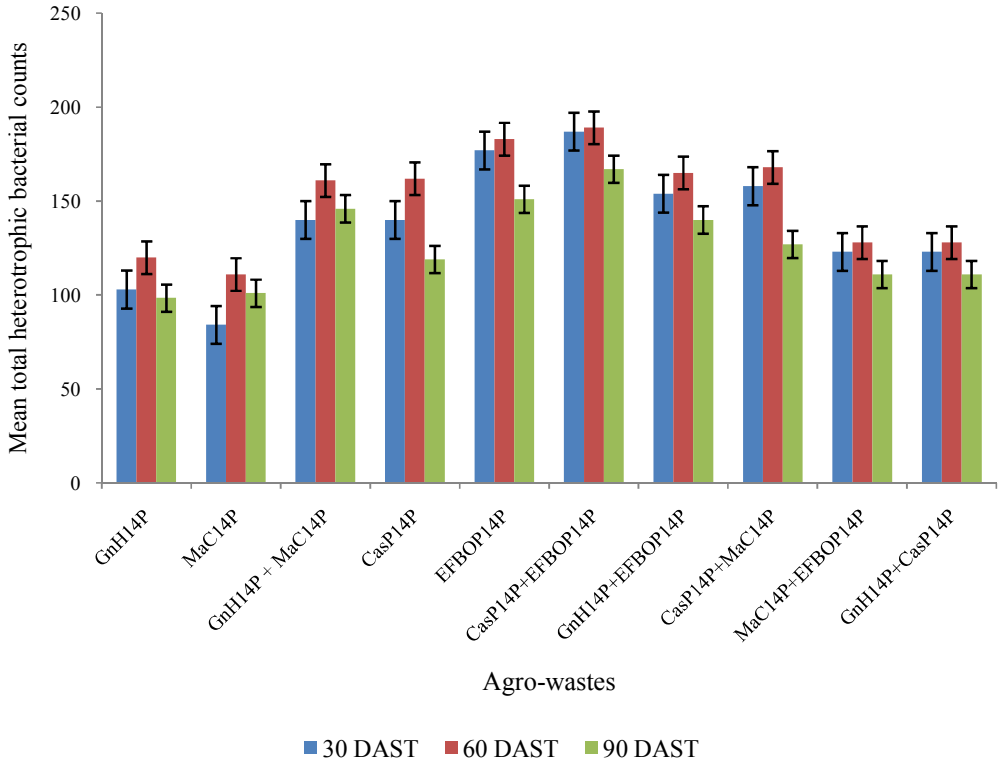

Fig. 1. Total heterotrophic bacterial counts at different durations of soil treatment
Legend: $MaC_{14}P$ Maize cob 2014 powder; $EFBOP_{14}P$ Empty fruit bunch of oil palm 2014 powder $CasP_{14}P$ Cassava peels 2014 powder; DAST Days after soil treatment

3.3 Identification of Bacterial in the Soils

The bacterial species identified using the traditional methods of identification (biochemical test) were as follows: *Bacillus* spp, *Proteus* spp, *Chromobacterium* sp, *Serratia* sp, *Pseudomonas* sp, *Streptococcus* sp, *Escherichia coli, Micrococcus* sp, *Achromobacter* sp, and *Providennia* sp (Table 2).

3.4 Molecular Identification of Microbial Isolates

The molecular analyses method of identifying bacterial to species level have been found to be more reliable than the traditional approaches because the technique depends on the examination of genetic diversity of isolates (Table 3). The maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. The scale bar of the trees represents a 0.1% difference in nucleotide sequences. The phylogenetic relationship of the hydrocarbon degrading bacterial species shows that the identified bacteria were in two clusters: cluster 1 consist of *Bacillus cereus, Bacillus thuringiensis, Bacillus altitudinis, Pseudomonas aeruginosa, Proteus mirabilis* and *Proteus penneri,* while cluster 2 consist of *Serratia marcescens, Providencia rettgeri and Enterobacter asburiae*. Between cluster 1 and 2 the results imply that there is genetic variability between the hydrocarbon degrading bacteria isolated from the soil environment. The *Bacillus cereus*, *Bacillus thringiensis* and *Bacillus altitudinis* are closely related by 99% similarity. *Pseudomonas aeruginosa, Proteus mirabilis* and *Proteus penneri* by are related to each other by 94% similarity. While *Proteus mirabilis* and *Proteus penneri* are 100% similar. *Serratia marcescens* in cluster 2 are related by 100% with *Providencia rettgeri* and *Enterobacter asburiae*. While *Enterobacter asburiae* and *Providencia rettgeri* are closely related by 36% (Fig. 4). The high percentage similarities that the bacteria isolated have with the blast revealed that they are closely related to each other.

Table 1. Effect of treatment levels on the bacterial population of crude-oil polluted-soils

Parameters	Treatment levels	THBC (CFU/g)	CUBC (CFU/g)
	PC	$6.71^{n}\pm1.97 \times 10^{6}$	$5.60^{n}\pm1.56 \times 10^{6}$
	COC	$4.48^{l}\pm1.64 \times 10^{6}$	$4.07^{l}\pm2.27 \times 10^{6}$
$GnH_{14}P$	3%	$1.01^{g}\pm4.9 \times 10^{7}$	$9.81^{f}\pm4.79 \times 10^{6}$
	6%	$1.38^{e}\pm5.32 \times 10^{7}$	$1.11^{e}\pm3.75 \times 10^{7}$
	10%	$1.84^{c}\pm8.67 \times 10^{7}$	$1.32^{d}\pm5.31 \times 10^{7}$
$MaC_{14}P$	3%	$9.69^{g}\pm5.83 \times 10^{6}$	$8.16^{g}\pm4.13 \times 10^{6}$
	6%	$1.27^{f}\pm10.46 \times 10^{7}$	$1.11^{e}\pm8.78 \times 10^{7}$
	10%	$1.58^{d}\pm6.5 \times 10^{7}$	$1.26^{d}\pm8.64 \times 10^{7}$
$GnH_{14}P+MaC_{14}P$	3%	$1.97^{bc}\pm4.10 \times 10^{7}$	$1.40^{d}\pm6.25 \times 10^{7}$
	6%	$2.12^{b}\pm4.21 \times 10^{7}$	$1.63^{b}\pm5.96 \times 10^{7}$
	10%	$2.27^{b}\pm8.2 \times 10^{7}$	$1.77^{a}\pm6.95 \times 10^{7}$
$CasP_{14}P$	3%	$1.72^{d}\pm7.77 \times 10^{7}$	$5.91^{h}\pm3.53 \times 10^{6}$
	6%	$1.94^{c}\pm11.66 \times 10^{7}$	$7.56^{g}\pm5.35 \times 10^{6}$
	10%	$2.24^{b}\pm3.16 \times 10^{7}$	$8.84^{g}\pm5.18 \times 10^{6}$
$EFBOP_{14}P$	3%	$2.18^{b}\pm6.44 \times 10^{7}$	$6.29^{h}\pm2.13 \times 10^{6}$
	6%	$2.57^{a}\pm10.3 \times 10^{7}$	$8.64^{g}\pm2.13 \times 10^{6}$
	10%	$2.64^{a}\pm8.77 \times 10^{7}$	$1.01^{f}\pm8.34 \times 10^{7}$
Parameters	Treatment levels	THBC (CFU/g)	CUBC (CFU/g)
$CasP_{14}P+ EFBOP_{14}P$	3%	$2.57^{a}\pm5.63 \times 10^{7}$	$8.78^{g}\pm7.89 \times 10^{6}$
	6%	$2.66^{a}\pm5.77 \times 10^{7}$	$1.03^{f}\pm8.49 \times 10^{7}$
	10%	$2.73^{a}\pm6.19 \times 10^{7}$	$1.18^{e}\pm8.35 \times 10^{7}$
$GnH_{14}P+ EFBOP_{14}P$	3%	$1.97^{bc}\pm6.73 \times 10^{7}$	$8.87^{g}\pm3.13 \times 10^{6}$
	6%	$2.21^{b}\pm6.59 \times 10^{7}$	$1.12^{e}\pm5.07 \times 10^{7}$
	10%	$2.36^{b}\pm6.43 \times 10^{7}$	$1.29^{d}\pm7.16 \times 10^{7}$
$CasP_{14}P+MaC_{14}P$	3%	$1.88^{c}\pm7.61 \times 10^{7}$	$1.32^{d}\pm9.22 \times 10^{7}$
	6%	$2.19^{b}\pm11.87 \times 10^{7}$	$1.52^{c}\pm10.95 \times 10^{7}$
	10%	$2.37^{b}\pm13.1 \times 10^{7}$	$1.74^{a}\pm14.18 \times 10^{7}$
$MaC_{14}P+ EFBOP_{14}P$	3%	$1.43^{e}\pm5.57 \times 10^{7}$	$1.10^{e}\pm9.4 \times 10^{7}$
	6%	$1.66^{d}\pm5.32 \times 10^{7}$	$1.35^{d}\pm10.18 \times 10^{7}$
	10%	$1.84^{c}\pm5.72 \times 10^{7}$	$1.65^{b}\pm9.83 \times 10^{7}$
$GnH_{14P}+CasP_{14}P$	3%	$1.70^{d}\pm6.31 \times 10^{7}$	$6.41^{h}\pm2.81 \times 10^{6}$
	6%	$1.88^{c}\pm6.65 \times 10^{6}$	$8.39^{g}\pm4.33 \times 10^{6}$
	10%	$2.07^{b}\pm7.02 \times 10^{6}$	$1.00^{f}\pm4.85 \times 10^{7}$

Mean with the same superscript along the vertical arrays indicate no variation
Legend: $GnH_{14}P$: Groundnut husks 2014 powder; $MaC_{14}P$: Maize cob 2014 powder; $EFBOP_{14}P$: Empty fruit bunch of oil palm 2014 powder; $CasP_{14}P$: Cassava peels 2014 powder; PC: Pristine control
COC: Crude oil controlin the mean THB counts of the soils (Table 1). The soils treated with 3% $GnH_{14}P$ and 3% $MaC_{14}P$ + $EFBOP_{14}P$ had significantly reduced ($P<0.05$) mean THB counts

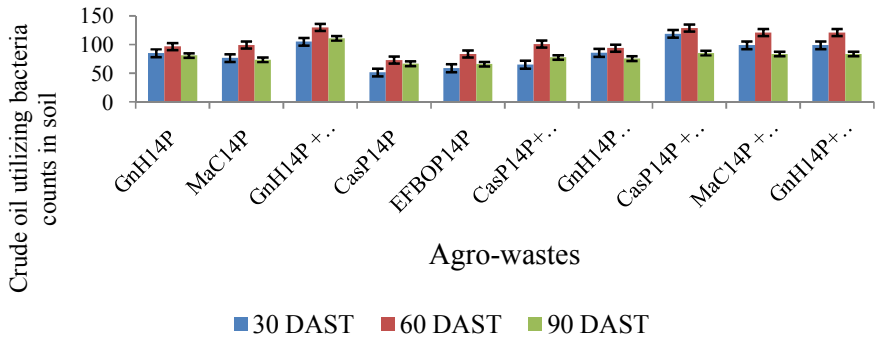

Fig. 2. Crude oil utilizing bacterial counts in polluted soils amended with agro-wastes
Legend: $MaC_{14}P$: Maize cob 2014 powder; $EFBOP_{14}P$: Empty fruit bunch of oil palm 2014 powder; $CasP_{14}P$: Cassava peels 2014 powder; DAST: Days after soil treatment

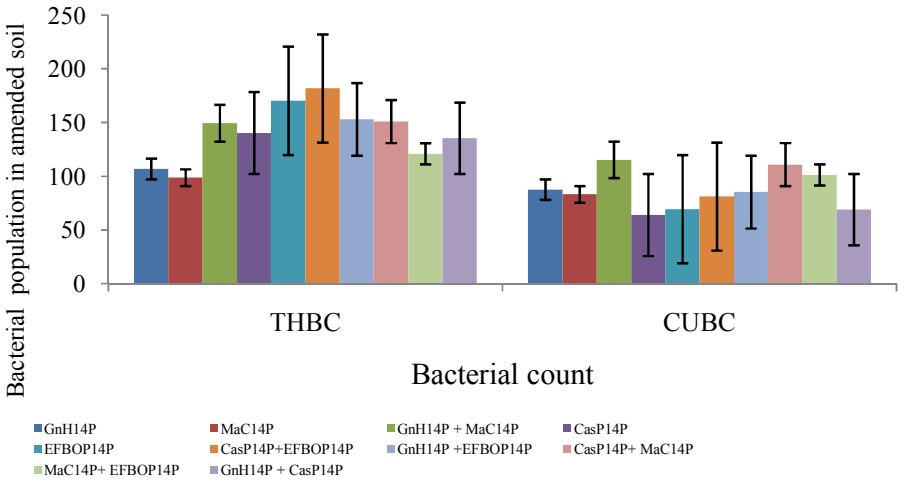

Fig. 3. Bacterial population in soils enhanced with different agro-wastes

Legend: $MaC_{14}P$ Maize cob 2014 powder; $EFBOP_{14}P$ Empty fruit bunch of oil palm 2014 powder; $CasP_{14}P$ Cassava peels 2014 powder

Table 2. Probable bacterial identified in soil using traditional methods

Agro-wastes	Bacterial
PS	*Bacillus* sp
	Proteus
	Chromobacterium sp
COPS	*Bacillus* sp
	Serratia sp
$GnH_{14}P$	*Pseudomonas* sp
	Bacillus sp
	Streptococcus sp
$MaC_{14}P$	*Escherichia coli*
	Proteus spp
	Pseudomonas sp
	Enterobacter sp
$GnH_{14}P+MaC_{14}P$	*Bacillus* spp
	Micrococcus sp
	Achromobacter sp
	Pseudomonas sp
$CasP_{14}P$	*Bacillus* sp
	Serratia sp
$EFBOP_{14}P$	*Pseudomonas* sp
	Proteus sp
$CasP_{14}P+ EFBOP_{14}P$	*Providennia* sp
	Pseudomonas sp
$GnH_{14}P+ EFBOP_{14}P$	*Bacillus* sp
	Proteus sp
$CasP_{14}P+MaC_{14}P$	*Bacillus* sp
	Serratia sp
$MaC_{14}P+ EFBOP_{14}P$	*Proteus* sp
	Enterobacter sp
	Bacillus sp
$GnH_{14P}+CasP_{14}P$	*Pseudomonas* sp
	Bacillus sp

3.5 Molecular Identification of Microbial Isolates

The molecular analyses method of identifying bacterial to species level have been found to be more reliable than the traditional approaches because the technique depends on the examination of genetic diversity of isolates (Table 3). The maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. The scale bar of the trees represents a 0.1% difference in nucleotide sequences. The phylogenetic relationship of the hydrocarbon degrading bacterial species shows that the identified bacteria were in two clusters: cluster 1 consist of *Bacillus cereus, Bacillus thuringiensis, Bacillus altitudinis, Pseudomonas aeruginosa, Proteus mirabilis* and *Proteus penneri,* while cluster 2 consist of *Serratia marcescens, Providencia rettgeri and Enterobacter asburiae*. Between cluster 1 and 2 the results imply that there is genetic variability between the hydrocarbon degrading bacteria isolated from the soil environment. The *Bacillus cereus*, *Bacillus thringiensis* and *Bacillus altitudinis* are closely related by 99% similarity. *Pseudomonas aeruginosa, Proteus mirabilis* and *Proteus penneri* by are related to each other by 94% similarity. While *Proteus mirabilis* and *Proteus penneri* are 100% similar. *Serratia marcescens* in cluster 2 are related by 100% with *Providencia rettgeri* and *Enterobacter asburiae*. While *Enterobacter asburiae* and *Providencia rettgeri* are closely related by 36% (Fig. 4). The high percentage similarities that the bacteria isolated have with the blast revealed that they are closely related to each other.

4. DISCUSSION

Petroleum hydrocarbon pollution has been known and reported by various scientists to produce adverse effect on biodiversity and abundance of soil microbes [13]. Microbial degradation is an important aspect in bioremediation of polluted soil. The degradation potentials of microorganisms depend solely on their abundance in the soils. The reduction or removal of petroleum hydrocarbons from soils depend on the ability and the metabolic capability

Table 3. Sequence identification of bacteria species detected in agro-wastes amended soil samples

Sample No.	Query Length	Gene bank accession No	Identity of Isolate obtained
1	1064		No- significant similarity found
2	1122	JQ308547.1	*Bacillus cereus*
3	1022	EF633995.1	*Bacillus cereus*
4	993	EF434507.1	*Pseudomonas aeruginosa*
5	696	FR717839.1	*Proteus mirabilis*
6	1136	KC150144.1	*Proteus mirabilis*
7	1144	KC344360.1	*Proteus mirabilis*
8	1117	KC150144.1	*Proteus mirabilis*
9	1130	LC002198.1	*Proteus mirabilis*
10	1054		No- significant similarity found
11	1137	KF933674.1	*Bacillus pumilus*
		Same as	Same as
		KF933678.1	*Bacillus altitudinis*
12	1104	HQ259936.1	*Proteus penneri*
13	1141	KF938667.1	*Serratia marcescens*
14	1148	KC172019.1	*Providencia rettgeri*
15	1116	JX941572.1	*Bacillus thuringiensis*
16	Failed PCR Amp.		*DNA may not be present*
17	1138	KJ877656.1	*Enterobacter asburiae*
18	1152	KJ398213.1	*Proteus penneri*
		Same as	Same as
		JN384144.1	*Proteus vulgaria*
19	1076	EU161995.1	*Bacillus thuringiensis*
			Same as
		GU329917.1	*Bacillus cereus*
20	1130	KC150144.1	*Proteus mirablis*

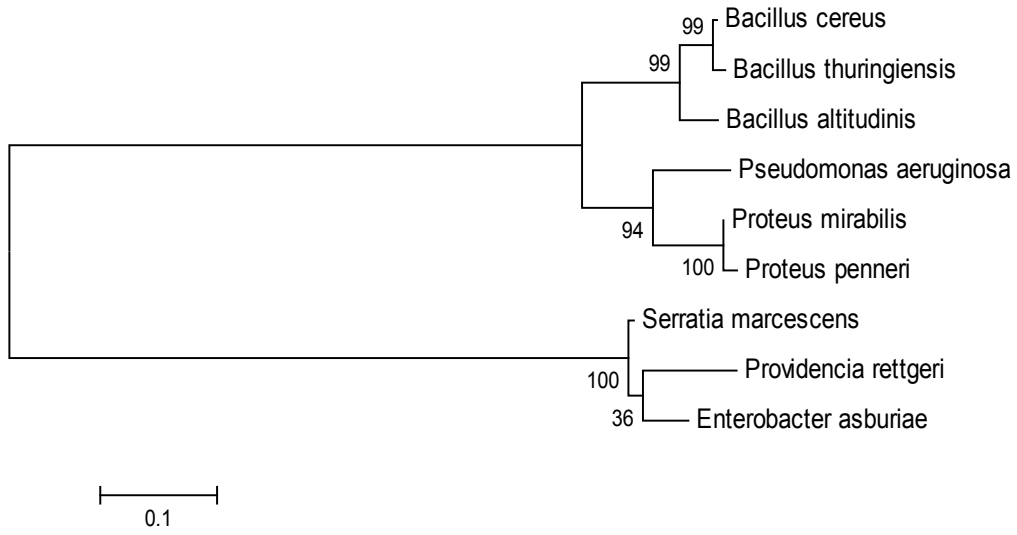

Fig. 4. Phylogenetic relationship among hydrocarbon degrading bacterial species

of the microorganisms to break down the hydrocarbon components in the polluted soils (Not all microorganisms can degrade hydrocarbons in soil but all hydrocarbon utilizing organisms possessed specific abilities to degrade hydrocarbon components in polluted soils). The results of the baseline analysis showed that the abundance of the bacteria and fungi in soil after contamination were significantly higher than the population of the microorganisms in soils before pollution with crude oil.

The bacterial genera present in the pristine soil were as follows: *Bacillus* sp, *Chromobacterium* sp and Proteus sp. While the fungal genera present in the soil were: *Aspergillus* sp and *Mucor* sp. Soil contamination produces higher bacterial and fungal counts than the natural soil without crude oil. The abundance of soil microbes in the contaminated soil compared to the low counts obtained in the ordinary soil may be due to the presence of crude oil that may serve as energy and carbon source to microbes. Atlas [14] in a similar study observed that the availability of petroleum product in the soil could cause a high proliferation of microorganisms. Amending the contaminated soil with agricultural wastes bio-stimulated the growth of the soil microbes to breakdown hydrocarbons in soil. Evans et al. [15] reported the diversity of bacteria community present in soil microcosms through an effective application of bio-stimulation principle. The most populated microbial species in the soils were Gram positive bacteria during the study. Chikere et al. [16] reported that gram positive bacteria contribute largely to the bioremediation of polluted soils. The findings of Olabisi et al. [17] also showed that the most frequently occurring bacterial genera in hydrocarbon polluted soils treated with melon shell were; *Bacillus, Micrococcus, Pseudomonas* and *Acinetobacter*. The Gram positive bacteria, due to their metabolic capabilities in degrading environmental pollutant could be considered as best organisms in bioremediation studies. The high advantage attributed to the gram positive bacteria could be due to their metabolic abilities, resilience in highly polluted environments and ability to produce bio-surfactants [12].

The high content of nitrogen and phosphorus in the agro-wastes used in the study could be one of the major factors that contributed to the high bacterial and fungal population in the soil. Agbor et al. [5] reported that availability of adequate quantity of nitrogen and phosphorus in the soil, bio-stimulated microbial degradation of hydrocarbon products. The compatibility of the soil due to the presence of hydrocarbons could be reduced through the application of adequate levels of agro-wastes which could serve as bulking agent. The loosening of the compacted soil may produce soil aeration, thus providing suitable environmental condition for aerobic microorganisms to attack the hydrocarbons in the soil. Stephen et al. [18] observed significant difference ($P>0.05$) in microbial counts in diesel-polluted soil amended with cowpea chaff. Akpe et al. [19] reported that hydrocarbon degrading bacteria were more in soils with amended plantain peels and guinea corn shaft than the un-amended soil samples. Bossert & Bartha [20]

reported a significant reduction in microbial counts in polluted soils and attributed the reduction to toxic compounds and certain harmful metabolites present in the pollutant. The success of bio-stimulation of microbial population in the degradation of contaminants in soil enhanced with organic manure such as NPK, urea fertilizer and chicken droppings was reported by Chikere et al. [16]. The high levels of the treatment applications resulted in an increased proliferation of microbial counts in the amended soil compared to those with lower treatment levels. However, among the single amendment used, groundnut husks powder showed a higher microbial population counts compared to other single amendment used, but the combined form of cassava peel powder + maize cob produced higher microbial counts than the single amendment. This revealed that the combined form of the agro-wastes used possessed high bio-utilization capabilities in the degradation of pollutants in soil. However, the efficiency of the combined wastes could be attributed to the acclimated microorganisms or nutrients found in the single amendments. The report by Okolo et al. [21] and Ebere et al. [22] supports that the combined forms of agricultural wastes in the appropriate measures could results in an effective in biodegradation of hydrocarbon products in terrestrial environment. The soil treated with the agricultural wastes at different levels showed significant increases ($p< 0.05$) in the hydrocarbon utilizing bacterial and fungal population in the soil. The soil treated with 6% of $CasP_{14}P$ + $MaC_{14}P$ produced higher microbial population in the soil compared to the single amendment at higher treatment application levels. This result is an indication that high treatment levels of the agro-wastes influences higher microbial proliferation in the soil than the single lower treatment levels. The results from this study suggest that microbial degradation is achievable at a faster rate when agro-wastes are applied at the right proportion, especially in a combined form.

4.2 Microbial Degradation of Crude Oil Polluted Soil

The molecular techniques used in the identification of microorganisms have been found to be more appropriate than the traditional approaches because these involve culture-independent analyses such as genotyping the 16S rRNA genes, fluorescence *in situ* hybridization (FISH) [23] the use of genetic probes, polymerase chain reaction (PCR) and metagenomics [24]. Molecular identification involving rRNA enables identity of novel sequences and diversity. Maslow et al. [25] reported that 16S rRNA analysis for characterization of bacteria is excellent because the technique depends on the examination of genetic diversity of isolates. Olukunle and Boboye [10] reported that molecular methods for rapid detection of diverse strains of oil-degrading bacteria for bioremediation process is of great relevance in promoting a sustainable development of our environment with low environmental impact. Although a wide phylogenetic diversity of microorganisms is capable of aerobic degradation of contaminants, *Pseudomonas* species and closely related organisms have been the most extensively studied owing to their ability to degrade many different contaminants. Walker et al. [26] reported that *Pseudomonas* possessed some more competent and active hydrocarbon-degradative enzymes than other biodegraders.

The high percentage similarities that the bacterial and fungal isolates have with the BLAST means that they are closely related to each other. Some of the bacteria obtained in this study have been isolated by other researchers such as Ojo, [27] and Boboye et al. [28] by means of traditional techniques. The 16S rRNA comparison reveals that *Bacillus cereus* and *B. thuringiensis* are closely related to each other, Delvecchio et al. [29]. Annweiller et al. (2000) reported that *Bacillus cereus* is more tolerant to high level of hydrocarbon in soil due to their resistance endospore. Muthuswamy et al. (2008) reported that *Bacillus* genera is capable of degrading short carbon chain length in hydrocarbon contaminated soils. The bacterial species identified in the soils during the bioremediation study using molecular identification methods were identified as *Bacillus cereus, Pseudomonas aeruginosa, Proteus mirabilis, Bacillus pumilus* or *Bacillus altitudinis, Proteus penneri, Serratia marcescens, Providencia rettgeri, Bacillus thuringiensis, Enterobacteria asburiae* and *Proteus penneri* same as *Proteus vulgaria.*

5. CONCLUSION

It is therefore concluded that the agricultural wastes used during the study were effective in enhancing bacterial population in the polluted soils. The phylogenetic analysis of the identified bacteria shows that the bacterial species are closely related with no much evolutionary divergence.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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