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Influence of Biostimulation Treatment Using Composted Plant Biomass on Bacterial Diversity of an Aged Petroleum Contaminated Soil as Determined by Culture-dependent and 16S rRNA Gene PCR-DGGE Based Identification Methods

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Authors' contributions

This work was carried out in collaboration between all authors. Author LS designed the study, performed the statistical analysis, wrote the protocol and the first draft of the manuscript. Author CJO managed the analyses of the study and literature searches. Author GCO proof-read the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Influence of biostimulation treatment using composted plant biomass on bacterial diversity of an aged crude oil contaminated soil (ACOCS) was determined using culture-dependent and 16S rRNA gene PCR-DGGE based identification methods. Seven treatment plots were designed and included treatments A (TPA) through G (TPG). Samples were collected bi-weekly from 7 treatment plots designed *in situ* during a 70-day study period that spanned 10 weeks. Composted (2,500 g each) Water hyacinth (EC), Mexican sunflower (TD) and Bermuda grass (CD) were used as nutrient supplements in 4,000 g of ACOCS *in situ*. TPA was un-amended while TPB, TPC, and TPD had EC,

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TD and CD added singly. TPE had EC and TD while TPF contained EC and CD in combination. TPG consisted of EC, TD and CD combined. Bacterial isolates were obtained on mineral salts medium and identified based on their morphological and biochemical characteristics. DGGE fingerprints of PCR-amplified 16S rRNA bacterial gene fragments were also determined using the universal primer set: 7F: 5'-GAGTTTGATCCTGGCTCAG-3' and 1492R:5'-GGTTACCTTGTTACGACT-3'that corresponded to positions 968 and 1401 of *Escherichia coli* 16S rRNA gene sequence. DGGE bands fell into corresponding operational taxonomic units based on a threshold of 91-100% sequence similarity. Dendrogram showed dominant DGGE bands in TPB though TPG over time with TPA having no band. The isolates are known crude oil utilizers and are closely related to *Gordonia* sp. BS261404 with 98% sequence similarity, *Aquitalea magnusonii* KG26145 (96%), *Sphingobacterium* sp. K1261411 (97%) and *Achromobacter* sp. HQ261417 (100%). Data indicated that pseudomonads are the dominant bacteria involved in hydrocarbon biodegradation after biostimulation with the composted plant materials. Cultural and molecular methods of cultivation of microorganisms are neither contradictory nor excluding and should be considered as complementary to interrogate the bacterial diversity in the natural soil environment.

Keywords: Plant biomass; biostimulation; 16S rRNA gene; pseudomonads; bacterial diversity.

1. INTRODUCTION

Hydrocarbons play a special role amongst the contaminants polluting the environment due to their wide-scale distribution and hazardous nature [1,2]. The adoption of biostimulation technique through the utilization of organic materials in aiding the biodegradation of hydrocarbons has huge potentials and prospects [3]. Interestingly, nutrients derived from these organic materials are easily accessible as they are mostly waste products and most importantly they are environmentally friendly products that can readily serve as needed nutrients for microbial metabolism thereby leading to a successful bioremediation project provided that environmental conditions and other important factors that aid optimum microbial growth are favourably enabled [4−7].

Several authors have reported the employment of various types of organic nutrients and waste products to attain biostimulation of hydrocarbon degading microorganisms [8,9,10]. Parameters such as bioavailability, nutrients supplementation and tilling to enhance aeration could be improved in soil by the use of organic substrates [11]. Abioye et al. [12] had earlier used organic wastes that comprised banana skin; brewery spent grain and spent mushroom compost while Abdulsalam et al. [13] focused on the use of nitrogenous fertilizer in biostimulation studies. The addition of plant-based organic manures as biostimulants to crude oil-contaminated soil could increase the organic contents of the soil thereby supporting the growth of resident bacterial in using the crude as the sole source of carbon [14−17]. In fact, it is necessary to point out that in Nigeria, fertilizers are not sufficient for agriculture, let alone for cleaning up oil spills that have ravaged the Niger Delta region. It, therefore, necessitates the search for cheaper, non-toxic and eco-friendly options of enhancing biodegradation of crude oil-contaminated soil. One of such option is the application of compostable plant-based manures to enhance the rate of crude oil biodegradation in soil via biostimulation technique [18−22]. Although microorganisms are present in the crude oil-contaminated soil environment, they cannot necessarily be there in the numbers required for bioremediation of the impacted site [23]. According to Chikere and Okpokwasili [24], their growth and activity could be stimulated by supplying them with the needed macronutrients. Bacteria need macronutrients like nitrogen and phosphorous to be able to grow optimally and carry out effective degradation of hydrocarbons [25,26].

The low culturability of bacterial species is well known [1]. Only a small fraction of naturally-occurring microbial assemblages can be cultured on conventional selective media and standard plating technique since these methods usually recover a very small proportion, 0.001% to 1% of the total assemblage [1,27,28]. It is nevertheless relevant to ask if those microorganisms that have been readily cultivated could be considered to be a representative sub-set of the whole microbial biodiversity [29,30]. Several reports have it that those bacteria that are readily cultured are not those that are abundant in 16S sequence databases for environmental media [31,32]. For example, species that would otherwise be "culturable" may fail to grow because their growth state in nature prevents adjustment to conditions

found in the medium. Also, many of the bacteria that dominate in natural settings are not adapted for growth in media containing high concentrations of complex organic carbon [33,34, 35,36]. The comprehensie characterization of hydrocarbon utilizing bacteria in an aged crude oil-contaminated-soil will enable the adequate and full description of the distinct species involved in hydrocarbon degradation.

Both cultural and molecular techniques have advantages and pitfalls associated with them and none provides complete access to the genetic and functional diversity of complex microbial communities in soil. However, both methods complement each other. Therefore, this study is aimed at determining the influence of biostimulation treatment using composted plant biomass on bacterial diversity of an aged petroleum contaminated soil using both culture-dependent and 16S rRNA gene PCR-DGGE based identification methods.

2. MATERIALS AND METHODS

2.1 Collection of Soil Samples

Crude oil-contaminated soil sample within 0-15 cm depth was obtained from each of 7 different bio-treatment plots set-up in Yorla farmland with the aid of an auger and transferred to the laboratory via polythene bags for microbiological, gas chromatographic and physicochemical analyses. The Yorla farmland study site is located at latitude 4° 39' N; longitude 7° 26'E [4]. It is a terrestrial environment with a patchy regenerating vegetation type and in close proximity to a brackish water source as reported by Solomon et al. [4,5]. Water hyacinth, Mexican sunflower and Bermuda grass were obtained, identified, composted and allowed to decay for two weeks before being used to biostimulate the resident hydrocarbon utilizing bacteria in the aged crude oil-contaminated soil treatment set-up *in situ*.

2.2 Determination of Macronutrients

The macronutrients in composted plants biomass were determined by Kjeldahl digestion method in continuous flow auto-analyzer (ChemLab, UK). The concentration of orthophosphate, as soluble reactive phosphorus was measured by the Malachite green–molybdate method (Olsen) while, nitrate was determined by direct ultraviolet spectrophotometry. The potassium content was analyzed by atomic absorption spectroscopy [37,38]. Each composted plant biomass was analyzed for nitrogen, phosphorus, potassium, calcium, and magnesium following the method of Atlas and Philip [39]. Composted plant parts were analyzed and dried at 60°C to constant weight. Thereafter, samples were ground through a 0·2- mm mesh size and stored for use.

2.3 BiostimulationTreatment Protocol

The treatment protocol is comprised of composted Water hyacinth (EC), Mexican sunflower (TD) and Bermuda grass (CD) biomasses which served as nutrient enhancers. EC, TD and CD (2,500 g each) were used as supplements for 4,000 g of an aged crude oil-contaminated soil (ACOCS) *in situ* in treatment plot A through G (TPA, TPB, TPC, TPD, TPE, TPF and TPG). TPA had no amendment and served as control, while TPB, TPC and TPD had EC, TD and CD being added singly. TPE was supplemented with EC and TD, TPF with EC and CD whereas; TPG contained EC, TD and CD combined. All treatments (Table 1) were tilled at a regular interval of 7 days to enhance soil aeration and microbial utilization of residual crude in the soil [4,5].

Table 1. Biostimulation treatment protocol

S/No.	Treatment plots	Content
1	TPA	ACOCS (4, 000 g), control
2.	TPB	ACOCS (4, 000 g) + EC (2500 g)
3.	TPC	ACOCS (4, 000 g) + TD (2500 g)
4.	TPD	ACOCS (4, 000 g) + CD (2500 g)
5.	TPE	ACOCS (4, 000 g) + EC (2500 g) + TD (2500 g)
6.	TPF	ACOCS (4, 000 g) + EC (2500 g) + CD (2500 g)
7.	TPG	ACOCS (4, 000 g) + EC (2500 g) + TD (2500 g) + CD (2500 g)

**g: gram. TPA-TPG: treatment plot A through G, ACOCS: Aged crude oil contaminated soil, EC: Composted Water hyacinth, TD: Composted Mexican sunflower, CD: Composted Bermuda grass*

2.4 Isolation of Crude Oil Utilizing Bacteria

Soil slurry was prepared and used for 10-fold serial dilution by mixing 1 g of wet soil with 9 ml of sterile physiological saline suspension in test tubes. Hydrocarbon utilizing bacteria (HUB) in soil was enumerated using a modified mineral salt medium of Mills et al. [40]. It contained $MgSO_4.7H_2O$ 0.40 g; KCl, 0.28 g; KH_2PO_4 0.80 g; Na_2HPO_4 1.20 g; NH_4NO_3 0.40 g; NaCl 15 g and agar No. 2 powder 20 g, all in 1 liter of de-ionized water. The pH of the medium was adjusted to 7.1 and subsequently sterilized at 121°C for 15 min at 15 psi (pounds per square inch).

Crude oil was introduced into the mineral salt medium through vapour phase transfer by soaking a 9 cm hatman No. 1 filter paper with 10 ml of fresh Bonny light. The flooded filter paper was then placed on the lid of the agar plate and incubated for 7 days at 25±8°C in an inverted position [41]. Hydrocarbon utilizing bacterial enumeration was carried out in triplicate and the titer obtained expressed as colony forming units per gram of original soil sample [42,43].

2.5 Morphological and Biochemical Characterization of Bacterial Isolates

The pure bacterial isolates were identified on the basis of their morphological and microscopic characteristics. The colonial morphologies that included the shape; margin and elevation were noted with reference to Bergey and Holt [44] and Cheesbrough [45].Gram staining and motility test were performed using the procedures described by Pala et al. [46]. Biochemical tests carried out to confirm the identity of isolated colonies included hydrogen sulphide, catalase, urease production, coagulase, nitrate reductase, oxidase, indole production, methyl red, Voges–Proskauer and citrate utilization tests. Spore and carbohydrate fermentation tests were also performed [47,48,27].

2.6 Molecular characterization of isolates

2.6.1 Amplification of 16S rRNA

Genomic DNA (gDNA) amplification of 16 subunits ribosomal RNA was performed and gDNA extracted from overnight grown bacterial cells using InstaGeneTM Matrix gDNA isolation kit [49,28,29]. The16S rRNA gene from the gDNA was amplified by polymerase chain reaction (PCR) using the primers set 7F: 5'-GAGTTTGATCCTGGCTCAG-3' and 1492R: 5'-GGTTACCTTGTTACGACT-3' corresponding to the forward and reverse primers respectively.

Amplification was done by initial denaturation of 94°C for 2 min followed by 35 amplification cycles of 94°C for 45 s, 55°C for 60 s; 72°C for 60 s and final extension at 72°C for 10 min in MJ Research Peltier Thermal Cycler [30,31]. The obtained PCR product was purified using Montage PCR Clean-up kit (Millipore) and the product sequenced using the 518F/800R primers [32].

2.6.2 Genomic DNA sequencing

DNA sequencing reactions were performed using an ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems).Single-pass sequencing was performed on each template using the aforementioned primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol [32,50]. The samples were re-suspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

2.6.3 Analysis of sequenced data

Sequenced data were analyzed using the 16S rRNA gene sequence which was compared with those from Genbank using the n-BLAST program. A phylogenetic tree was constructed by the neighbour-joining method using MUSCLE 3.7 and PhyML 3.0 aLRT program as earlier described by Edgar [30]. For tree rendering, Tree Dyn 198.3 was used [31].

2.6.4 Dendrogram analysis of isolates

A cluster of the DGGE fingerprints was constructed based on the Dice similarity coefficient using un-weighted pair group method clustering with the Quantity One software as reported by Galili [51].

3. RESULTS AND DISCUSSION

Biostimulating natural populations of indigenous bacteria in the crude oil-contaminated soil for the removal of hydrocarbon pollutants from the soil are cheaper than other remediation technologies [33]. The macronutrients present in composted Water hyacinth (EC), Mexican sunflower (TD) and Bermuda grass (CD) are as depicted in Fig.

1. Composted EC was found to contain 98.23 mg/kg, 68.12 mg/kg, 3.61 mg/kg and 4.38 mg/kg of total nitrogen, organic matter, total phosphorus and total potassium respectively. TD on the other hand, had total nitrogen (132.31 mg/kg), organic matter (43.31 mg/kg), total phosphorus (4.26 mg/kg) and total potassium (2.24 mg/kg) while CD had various concentrations of macronutrients of 102.12 mg/kg, 54.64 mg/kg, 3.62 mg/kg and 3.16 mg/kg respectively for total nitrogen, organic matter, total phosphorus and total potassium respectively.

Organic manures have been reported as potential biostimulants that aided bacterial growth and metabolism of residual crude oil in soil environment [34,52]. Hydrocarbon degradative process is widely distributed among numerous bacterial genera and biodegradation of hydrocarbons using bacteria has been established [53,54,55]. Preliminary characterization (Tables 2 and 3a-c) was performed to eliminate duplicate strains [36]. Molecular techniques have been extremely valuable in exploring the diversity of microbiota despite culture-based methods [35,56]. Phylogenetic relationships of the 16S rRNA sequences obtained from the dominant DGGE bands were performed using PHYLIP (phylogenetic inference package) version 3.67. Fig. 2 show the DGGE profiles of 16S rRNA gene fragments obtained from the various treatment plots.

Sequencing showed that *Pseudomonas* was the dominant bacterial isolate in the soil from various treatment plots and had similarity with isolates deposited in GenBank which ranged from 91% to 100%. The tentative taxa and phylogenetic affiliation of the 16S rRNA of purified bacterial isolates were amplified by polymerase chain reaction (PCR) [30] and the bacterial 16S rRNA sequences were aligned with basic local alignment search tool (BLAST) of the National Commission on Biotechnological Information (NCBI) databases [31,32].

The molecular identification and 16S rRNA sequence matches for bacterial isolates obtained from the different treatment plots during the study period are presented in Figs. 2−4. The numbers in the phylogenetic tree (LS1-LS33) branches represented the dominant DGGE bands (Fig. 2) while the maximum identities of these bands to their GenBank closest relatives are in percentages (94-100%). Partial 16S rRNA gene sequencing and database homology search for the isolates revealed their tentative close relationship to *Pseudomonas aeruginosa* strain LS26 (KR261431), a hydrocarbon degrading bacterium identified in this research.

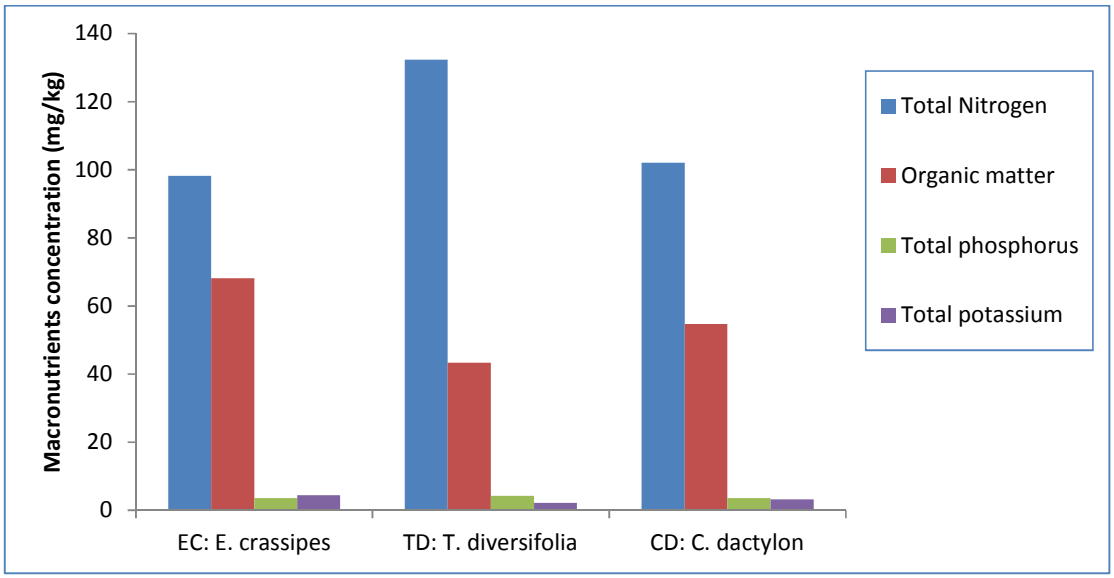

Fig. 1. Concentration of macronutrients present in composted water hyacinth, Mexican sunflower and Bermuda grass

Table 2. Cultural and colonial morphology of bacterial isolates obtained from ACOCS

Isolate code	Shape	Margin	Elevation	Colour	Size (mm)
S1.	Irregular	Entire	Flat	Green	2.4
S1	Irregular	Entire	Flat	Pink	2.0
S 2.	Circular	Entire	Convex	Yellow	3.5
S 3.	Irregular	Entire	Raised	Golden yellow	4.5
S 4.	Irregular	Entire	Flat	Green	2.4
S 5.	Rhizoid	Erose	Convex	Dark blue	3.2
S 6.	Circular	Pin point	Convex	Blue	1.3
S 7.	Circular	Entire	Umbonate	Mucoid, red	1.5
S 8.	Circular	Irregular	Flat	Brown	3.0
S 9.	Circular	Entire	Flat	Yellow	3.5
S 10.	Circular	Irregular	Convex	White	1.3
S 11.	Irregular	Entire	Flat	Cream	4.5
S 12.	Circular	Entire	Umbonate	Mucoid red	1.5
S 13.	Circular	Irregular	Convex	Red	2.3
S 14.	Irregular	Entire	Flat	Green	2.4
S 15.	Circular	Entire	Flat	Yellow	3.5
S 16.	Irregular	Lobate	Flat	Brown	4.4
S 17.	Circular	Entire	Convex	Yellow	3.5
S 18.	Rhizoid	Undulate	Umbonate	Yellow	2.0
S 19.	Rhizoid	Erose	Convex	Dark blue	4.2
S 20.	Circular	Irregular	Flat	Brown	3.0
S 21.	Irregular	Entire	Flat	Green	2.4
S 22.	Circular	Pin point	Convex	Blue	1.3
S 23.	Rhizoid	Erose	Convex	Deep yellow	3.5
S 24.	Circular	Punctiform	Umbonate	Tan	2.5
S 25.	Circular	Entire	Convex	Bright pink	4.2
S 26.	Circular	Undulate	Convex	White opaque	1.8
S 27.	Circular	Entire	Raised	Opaque	3.4
S 28.	Rhizoid	Erose	Convex	Yellow	4.5
S 29.	Circular	Irregular	Convex	Deep red	3.2
S 30.	Circular	Entire	Umbonate	Pink	3.0
S 31.	Circular	Entire	Flat	Light yellow	4.3
S 32.	Rhizoid	Erose	Raised	Yellow	3.5
S 33.	Irregular	Entire	Flat	Green	2-4

Fig. 3 shows cluster analysis of the banding pattern obtained from DGGE profiles of 16S rRNA gene fragments (Fig. 2) using a simple matching group average setting to separate communities according to species sequence differences. Two main clades were observed with a simple matching algorithm comprising: (1) aged crude oil-contaminated soil baseline (TPAB), pristine soil baseline (PCSB), aged crude oil-contaminated soil day 0 (TPA0D), aged crude oil-contaminated soil day 14 (TPA14D), (2) Water hyacinth-amended treatment day 28 (TPB28D), water hyacinth-amended treatment day 0 (TPB0D) and Mexican sunflower-amended treatment day 42 (TPC42D). The aged crude oil-contaminated soil baseline (TPAB) clustered with pristine soil baseline (PCSB) and aged crude oil-contaminated soil day 0 (TPA0D). Others are aged crude oil-contaminated soil day 14 (TPA14D), Mexican sunflower-amended treatment day 14 (TPC14D), Bermuda grass amended treatment day 28 (TPD28D) and Bermuda grass amended treatment plot day 56 (TPD56). However, TPB28D, TPB0D and TPC42D amended treatments clustered separately.

This meant that the treatments selected similar bacterial groups involved in the ACOCS degradation *in situ*. Treatment containing EC – TD day 0 (TPE0D), EC − TD day 14 (TPE14D), EC− CD day 14 (TPF14D) and EC− CD day 14 (TPF56) also formed a distinct cluster. More so, *EC− TD − CD* day 70 (TPG70D) and EC−D− CD day 42 (TPG42D) also clustered together. This implied that the amendments supplied to the treatment plot resulted in the selection of a similar genetic community profile across the days of remediation whence the genomic DNA samples were extracted from the aged crude oil-contaminated soil.

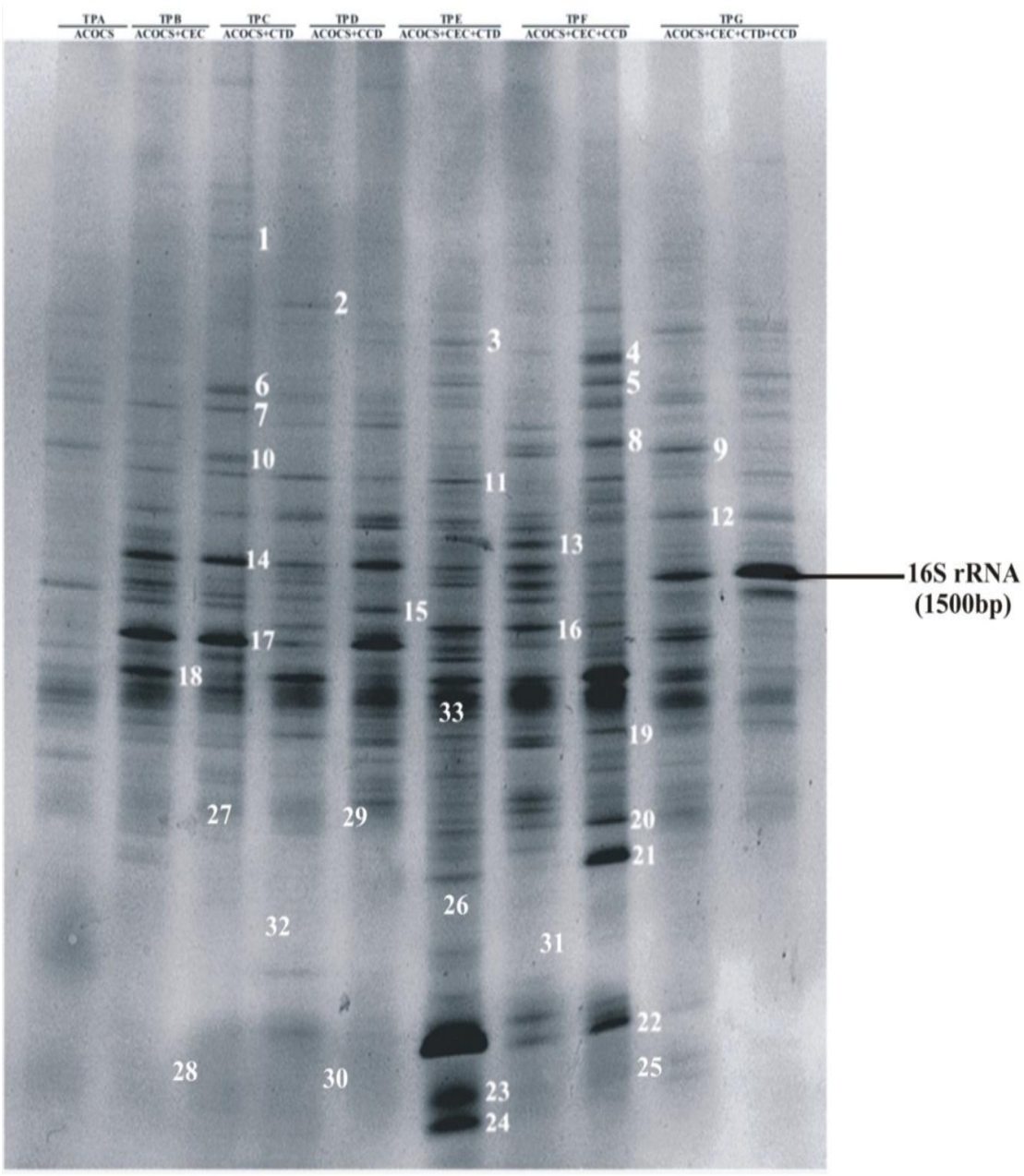

Fig. 2. DGGE profiles of PCR-amplified 16S rRNA gene fragments of bacterial communities from the seven different treatment plots (TPA, TPB, TPC, TPD, TPE, TPF and TPG)

Table 3a. Biochemical characteristics of hydrocarbon utilizing bacterial isolates obtained from crude oil-contaminated soil in Yorla

Isolate code	Gram stain	Morphology	Oxidase production	Citrate utilization	Methyl red	VogesProskauers	Catalase	Indole	Urease production	Coagulase	Nitrate reductase	Hydrogen sulphide	Spore test	Gas production	Glucose	Lactose	Maltose	Mannitol	Salicin	Sucrose	Trehalose	Motility test	Probable Organisms
S1	-	Rods, straight	+	+	+	-	+	-	-	-	+	-	-	-	A/G	-	-	-	-	-	-	+	*Pseudomonas* sp.
S 2.	+	Cocci, singly	+	-	+	-	+	-	+	-	+	-	-	-	A	-	-	-	-	+	-	-	*Micrococcus* sp.
S 3.	+	Rods, singly	-	-	+	+	+	-	+	-	-	-	-	-	A/G	-	-	-	-	-	-	-	*Corynebacterium* sp.
S 4.	+	Cocci, cluster	-	+	-	+	+	-	+	+	-	-	-	-	A	+	+	+	+	+	+	+	*Staphylococcus* sp.
S 5.	-	Rods, straight	+	+	+	-	+	-	-	-	+	-	-	-	A/G	-	-	-	-	-	-	+	*Pseudomonas* sp.
S 6.	-	Rods, straight	+	+	+	-	+	-	-	-	+	-	-	-	A	-	-	-	-	-	-	+	*Achromobacter* sp.
S 7.	-	Rods, singly	+	-	+	+	+	+	-	-	+	-	-	-	A	+	+	+	-	+	-	-	*Klebsiella* sp.
S 8.	-	Rods, singly	+	+	+	-	+	+	-	-	+	-	-	-	A	+	+	-	-	+	-	+	*Serratia* sp.
S 9.	-	Rods, singly	-	-	-	+	+	-	-	-	+	-	-	-	A	-	-	-	+	+	+	+	*Arthrobacter* sp.
S 10.	+	Rods, singly	-	-	-	+	+	-	-	-	-	-	+	-	A/G	-	+	-	-	-	-	+	*Bacillus* sp.
S 11.	-	Rods, pairs	+	-	+	-	+	+	-	-	-	-	-	-	A	+	+	-	-	-	-	+	*Proteus* sp.

Table 3b. Biochemical characteristics of hydrocarbon utilizing bacterial isolates obtained from crude oil-contaminated soil in Yorla contd

Isolate code	Gram stain	Morphology	Oxidase production	Citrate utilization	Methyl red	VogesProskauers	Catalase	Indole	Urease production	Coagulase	Nitrate reductase	Hydrogen sulphide	Spore test	Gas production	Glucose	Lactose	Maltose	Mannitol	Salicin	Sucrose	Trehalose	Motility test	Probable Organisms
S 12.	+	Rods, singly	-	-	+	-	+	+	-	-	-	-	-	-	A	+	+	-	-	-	-	-	*Lactobacter* sp.
S 13.	-	Rods, singly	+	+	+	-	+	+	-	-	-	-	-	-	A	+	+	-	+	+	-	+	*Citrobacter* sp.
S 14.	-	Cocci, chains	-	-	-	-	-	+	-	-	-	-	-	-	G	+	+	-	-	-	-	+	*Alcaligenes* sp.
S 15.	-	Rods, straight	+	+	+	-	+	-	-	-	+	-	-	-	A/G	-	-	-	-	-	-	+	*Pseudomonas* sp.
S 16.	+	Rods, singly	-	-	-	+	+	-	-	-	-	-	+	-	A/G	-	+	-	-	-	-	+	*Bacillus* sp.
S 17.	-	Rods, singly	-	+	-	-	-	-	+	-	-	-	-	-	A/G	-	+	-	-	-	-	+	*Acetobacter* sp.
S 18.	+	Rods, singly	-	-	+	+	+		+	-	-	-	-	-	G	-	-	-	-	-	-	-	*Corynebacterium* sp.
S 19.	-	Rods, straight	+	+	+	-	+	-	-	-	+	-	-	-	A/G	-	-	-	-	-	-	-	*Flavobacterium* sp.
S 20.	+	Rods, straight	+	+	+	-	+	-	-	-	+	-	-	-	A/G	-	-	-	-	-	-	+	*Nocardia* sp.
S 21.	-	Rods, singly	-	-	-	+	+	-	-	-	+	-	-	-	A	-	-	-	+	+	+	+	*Arthrobacter* sp.
S 22.	-	Rods, straight	+	+	+	-	+	-	-	-	+	-	-	-	A/G	-	-	-	-	-	-	+	*Pseudomonas* sp.

Table 3c. Biochemical characteristics of hydrocarbon utilizing bacterial isolates obtained from crude oil-contaminated soil in Yorla contd

Isolate code	Gram stain	Morphology	Oxidase production	Citrate utilization	Methyl red	VogesProskauers	Catalase	Indole	Urease production	Coagulase	Nitrate reductase	Hydrogen sulphide	Spore test	Gas production	Glucose	Lactose	Maltose	Mannitol	Salicin	Sucrose	Trehalose	Motility test	Probable Organisms
S 23.	-	Rods, singly	+	-	+	+	+	+	-	-	+	-	-	-	A	+	+	+	-	+	-	+	*Klebsiella* sp.
S 24.	+	Rods, straight	+	+	+	-	+	-	-	-	+	-	+	-	A/G	-	-	-	-	-	-	-	*Mycobacterium* sp.
S 25.	-	Rods, singly	-	-	-	+	+	-	+	-	+	-	-	-	G	-	+	-	+	+	+	+	*Aquitalea* sp.
S 26.	-	Rods, straight	+	+	+	-	+	-	-	-	+	-	-	-	A/G	-	-	-	-	-	-	+	*Shewanella* sp.
S 27.	-	Rods, straight	+	-	+	-	+	-	-	-	+	-	-	-	A	-	-	+	-	-	-	+	*Halomonas* sp
S 28.	-	Rods, singly	-	-	+	+	+	-	+	-	-	-	-	-	G	-	-	-	-	-	-	-	*Brevundimonas* sp
S 29.	+	Cocci, straight	+	+	+	-	+	-	-	-	+	-	-	-	A/G	-	-	-	-	-	-	-	*Rhodococcus* sp.
S 30.	-	Rods, singly	-	-	+	+	+	+	+	-	-	-	-	-	G	-	-	-	-	-	-	-	*Sphingobacterium* sp.
S 31.	-	Rods, singly	-	+	-	+	+	-	-	-	+	-	-	-	A/G	-	-	-	+	+	+	+	*Erwinia* sp.
S 32.	-	Spiral, singly	-	-	+	+	+	-	+	-	-	-	-	-	A/G	-	-	-	-	-	-	+	*Azospirillum* sp.
S33.	-	Rods, straight	+	+	+	-	+	-	-	-	+	-	-	-	A	-	-	-	-	-	-	+	*Gordonia* sp.

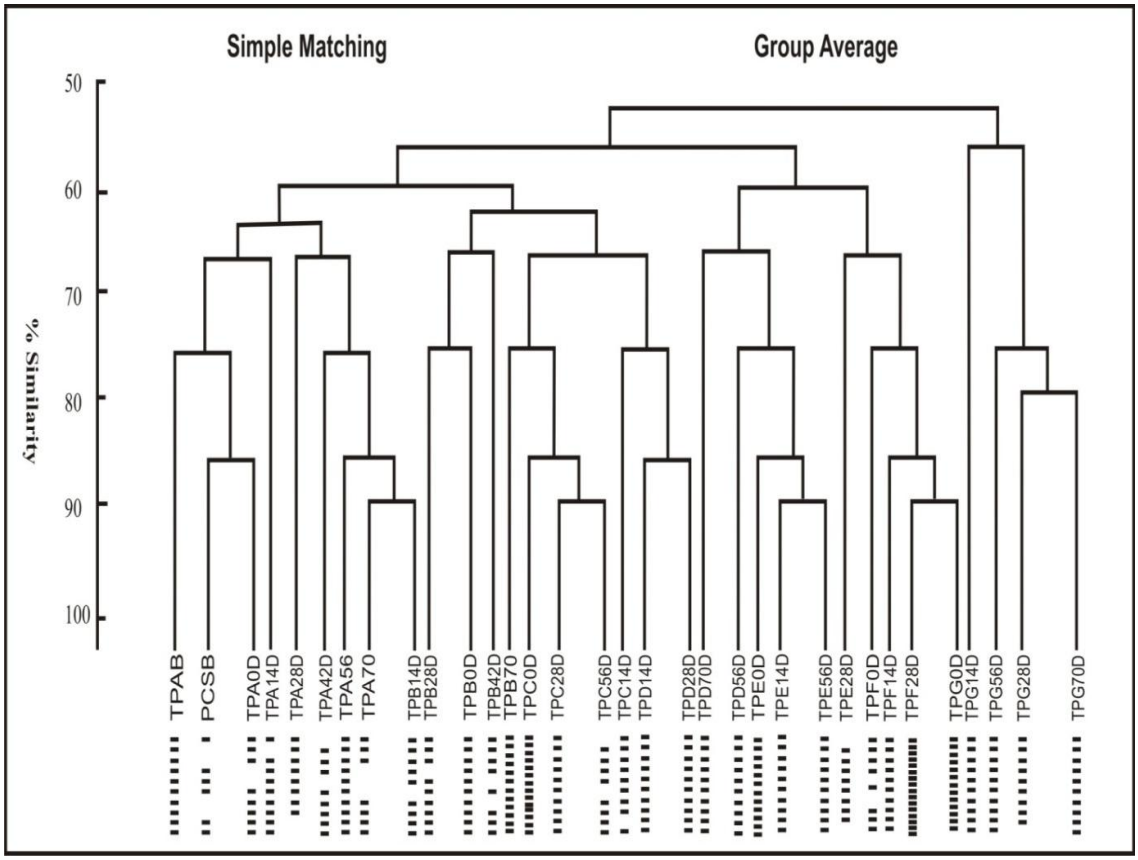

Fig. 3. Dendrogram of DGGE profiles of the bacterial communities in Fig. 2 using a simple matching group average setting to separate communities according to species sequence differences. Biotypes are defined as organisms having 91-100% sequence imilarity

The phylogenetic tree (Fig. 4) constructed was rooted with 16S rRNA gene sequence of *Pseudomonas aeruginosa* strain LS25 (KR261431). *Pseudomonas* spp. were the dominant bacterial isolates found that was successfully sequenced and had similarities > 97%. The 16S rRNA sequences ranging from 91 to 100% (Fig. 4) were deposited in GenBank with nucleotide sequence database containing accession numbers which ranged between KJ179809 to KJ179832. Isolates were identified as microbes closely related to *Gordonia* sp. BS261404 with 98% sequence similarity.

Sequencing data indicated that *Aquitaleamagnusonii* KG26145 had 96% sequence similarity, *Bacillus* sp. TC26142 (98%), *Sphingobacterium* sp. K1261411 (97%), *Shewanella* sp. NR261408 (99%) and *Achromobacter* sp. HQ261417 (100%).Other bacterial isolates obtained were *Halomonas* sp. UT261418 with 94% sequence similarity, *Achromobacter* sp. BK261409 (96%), *Pseudomonas* sp. LK261410 (91%), *Pseudomonas aeruginosa* VC261413 (93%), *Staphylococcus* sp. NC261413 (99%), *Corynebacterium* sp. NS261405 (98%) and *Brevundimonas* sp. KN261406 (99%).

Pseudomonas sp. BU261414 also showed 100% sequence similarity while *Acinetobacter*sp. BJ261432 had 87% and *Alcaligenes* sp. GK2614430 (83%). *Nocardia* sp. CM261428 had 92% sequence similarity. *Mycobacterium* sp. HB261425 (95%), *Flavobacterium*sp. JK261423 (99%), *Erwinia* sp. ZC261427 (98%), *Arthrobacter* sp. JQ261420 (96%), *Rhodococcus* sp. CB261407 (98%), *Azospirillum* sp. LG261412 (86%) and *Bacillus* sp. KL261431 (96%).

Furthermore, *Shewanella* sp. KJ261419 had 98% sequence similarity, *Achromobacter* sp. BJ261421 (99%), *Corynebacterium* sp. IC261422 (97%), *Pseudomonas* sp. B1261424 (98%),

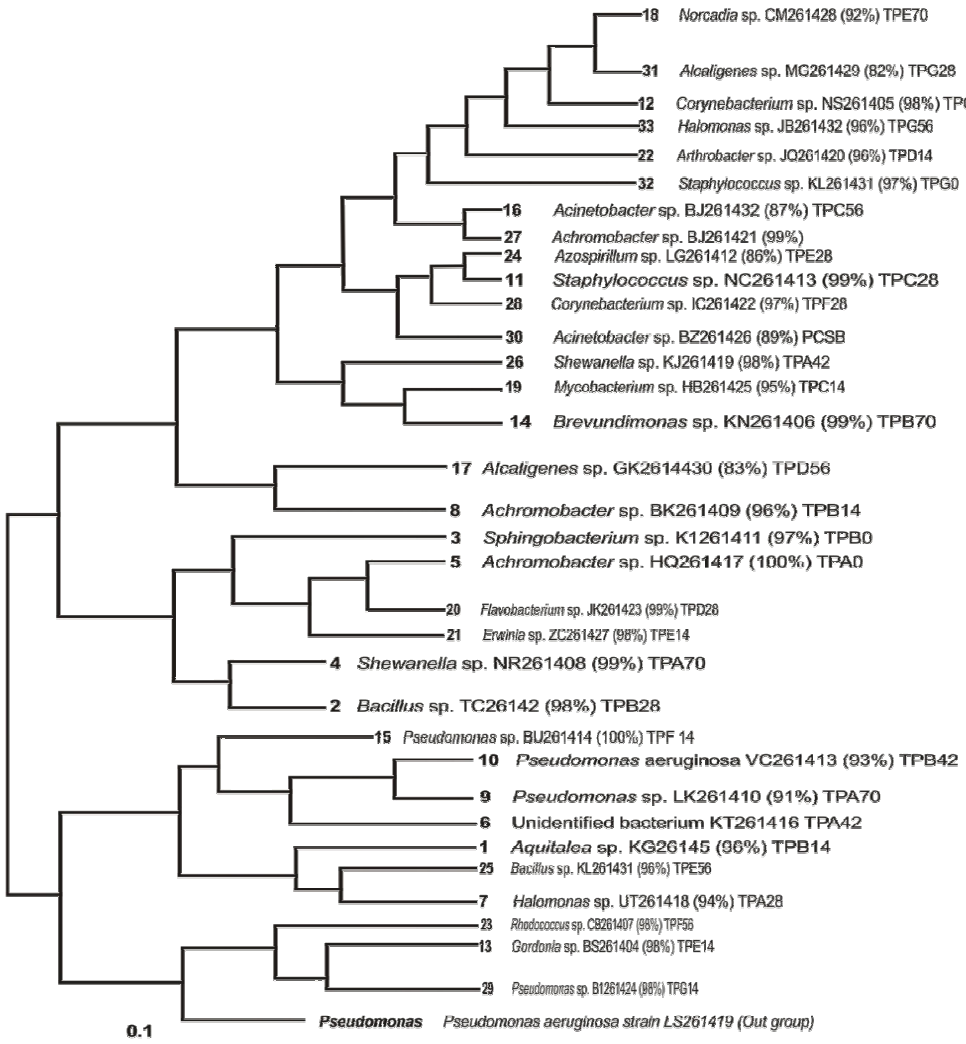

Fig. 4. Phylogenetic relationships of 16S rRNA gene sequences of bacterial species obtained from the various treatment plots during the enhanced bioremediation study period

*Acinetobacter*sp. BZ261426 (89%), *Alcaligenes* sp. MG261429 (82%), *Staphylococcus* sp. KL261431 (97%) and *Halomonas* sp. JB261432 (96%). The un-identified bacterium strain KT261416 (Fig. 3) obtained could be a novel species. There has been increased quest by researchers to isolate novel microorganisms within the terrestrial and moderately saline media that possess enzymes adapted to crude oil degradation as that may be of specific importance to the petroleum industries [57,58]. Majority of the isolates including *Pseudomonas* sp. and *Bacillus* sp., have been isolated from natural soil environments and have species recognized as halotolerant that have the potential for hydrocarbon biodegradation in various environmental media [59,60]. These organisms have been referred to as generalist hydrocarbon degraders since it constituted a significant proportion of hydrocarbon degraders that have played significant roles in hydrocarbon biodegradation [61,60]. The unidentified bacterium strain including the uncommon species of bacteria such as *Aquitalea magnusonii* and *Brevundimonas naejangsanensis* recovered from the natural ACOCS environment during the study may require further screening to ascertain their biodegradative capabilities.

The study further supports an earlier report by Oforibika et al. [62] on the proliferation of potential halotolerant microbes capable of degrading and/or utilizing environmental contaminants [63] including hydrocarbons in the natural soil system. The type and number of hydrocarbon utilizing bacterial species successfully sequenced indicated the biostimulatory activity of the plant manures on their growth and subsequent metabolism of residual crude oil in the contaminated soil thus, affirming their capacity for contaminated site restoration.

4. CONCLUSION

The 16S rRNA gene-based analysis of bacterial isolates used in this study provided a rapid and efficient culture independent approach to elucidate the bacterial diversity during bioremediation. This technique demonstrated that pseudomonads and related species maybe the dominant bacteria involved in crude oil biodegradation after biostimulation with organic nutrient ources. The mineralization of organic matter in soil enhances nutrient concentrations that can alleviate the nutrient limitation of the hydrocarbon contaminated soil leading to greater biodegradation of hydrocarbons. The analysis of bacterial diversity in an aged crude oil-contaminated soil could help us in discovering the black box of adapted bacterial consortia in response to the presence of hydrocarbons. Data obtained provided substantial evidence on the suitability of compost manures for crude oil-bioremediation. Further studies are ongoing to define the bioremediation capability of each identified strain in order to elucidate the metabolic pathways involved in hydrocarbon metabolism. Culture-based-traditional and culture-independent molecular techniques are neither contradictory nor excluding and should be considered as complementary. Both methods of cultivation should be adopted to interrogate the bacterial diversity in the soil environment.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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