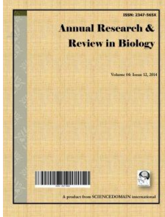

Annual Research & Review in Biology

25(5): 1-11, 2018; Article no.ARRB.38152
ISSN: 2347-565X, NLM ID: 101632869

Plasmid Profiling of Crude Petroleum Degrading Bacterial Strains Isolated from Polluted Soils in Ota, Nigeria

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Authors' contributions

This work was carried out in collaboration among all authors. Author OYD designed the study. Authors OYD and IPO wrote the first draft of manuscript and performed the statistical analysis. Authors AAO and TOS wrote the protocol and managed the analyses of the study. All authors managed the literature searches, read and approved the final manuscript.

Article Information

DOI: 10.9734/ARRB/2018/38152
Editor(s):
(1) Ibrahim Farah, Professor, Jackson State University, Mississippi, USA.
(2) J. David Puett, Professor, Department of Biochemistry and Molecular Biology, University of Georgia, Athens, USA .
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Reviewers:
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(3) Hanan E. S. Ali, Egyptian Petroleum Research Institute, Egypt.
(4) Mohamed M. El Nady, Egyptian Petroleum Research Institute, Egypt.
Complete Peer review History: http://www.sciencedomain.org/review-history/24206

Original Research Article

Received 14th November 2017
Accepted 17th March 2018
Published 19th April 2018

ABSTRACT

Pollution from petroleum products is of public health concern because of its attendant health and environmental impacts.

Aims: To study the biodegradation of Bonny light crude petroleum by bacteria isolated from soils of three different automobile mechanic workshops in Ota, Ogun State.

Study Design: Contaminated soils from three (3) different auto-mechanic sources were enriched with Bonny light crude oil for a period of twenty-one (21) days after which the culture was changed

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and further enriched using crude oil as the only source of carbon and energy.
Place and Duration of Study: Department of Biological Sciences (Microbiology Unit), Covenant University, Ota, Ogun State Nigeria, between June 2016 and March 2017.
Methodology: Bacteria were isolated using standard microbiological techniques from enrichment of the soil samples in minimal salt medium (MSM) supplemented with 1% (v/v) crude petroleum as the only source of carbon and energy. The petroleum utilizing bacteria belonging to the genera *Bacillus* sp. (SB4), *Pseudomonas* sp. (SC8), *Serratia* sp. (SC11), and *Acinetobacter* sp. (SC12) were screened and subjected to oil degradation procedures. Gas Chromatographic (GC) analysis was used to analyze the component and percentage of the petroleum utilized. Plasmid curing and profiling were performed to determine whether the ability to utilize carbon is plasmid or chromosomally encoded.
Results: Four (4) bacterial strains out of thirty-six (36) bacterial isolates were able to utilize petroleum as energy source. The GC fingerprints showed that both the aliphatic and aromatic components of crude petroleum were reduced to varying degree with the exception of nonadecane C19. Strain SC11 could not reduce anthracene, chrysene, benzo(a)pyrene and pyrene components of the crude petroleum. Strain SB4 depleted 24% - 57% of the aliphatic and 20% - 42% of the aromatics components while strain SC8 depleted 38% - 67% of the aliphatic and 30% - 79% of the aromatics components. However, strain SC11 only depleted 12% - 46% of the aliphatic and 13% - 29% of the aromatics components of the crude petroleum used.
Conclusion: All organisms harbored plasmid which suggests that petroleum degradation capabilities could be plasmid encoded. This indicates that the petroleum utilizing bacteria which are part of the ecosystem could be used for natural remediation of petroleum polluted environments.

Keywords: Biodegradation; bioremediation; plasmid profiling; gas chromatography.

1. INTRODUCTION

The most widely used primary source of energy is the hydrocarbons; this is because they are source of large amounts of energy. Hydrocarbons molecules that make up crude petroleum and other oil components are highly toxic to microorganisms, plants, animals and humans [1]. Oil pollution results from routine normal operations of crude oil exploration, exploitation, refining, and transportation [2]. Whenever crude oil is released into the environment; serious pollution problems occur which can be deliberate or by mistake. Small amount of oil spill into the ecosystem may exceed acceptable levels [3]. The issues often cause a lot of problems to both the living and non-living components of the environments, more so that some hydrocarbon components have been identified as carcinogenic [3] and neurotoxic. Crude oil pollution may release high oil fractions which may culminate in malfunctioning of the liver, kidney and central nervous system [4,5]. This could also lead to bone marrow cancer and cancer of the different part of the body [6].

There are other ways through which petroleum also penetrates into the environments; these could be through waste disposal, accidental spills, leakage in tanks, oil bunkering, vandalization of oil pipes and losses during storage [7]. The fact that petroleum product dominates Nigerian economy also creates a lot of conditions for release of large amounts of these toxins into environments [3,8].

Bonny light crude petroleum is mainly composed of different components of hydrocarbon molecules, mainly Aliphatic from C1 to C30; Aromatic from C6 – C8, cyclohexane and other compounds containing nitrogen, oxygen and sulfur [9]. Major components in the petroleum pollutants are degradable, and they will become utilized from the environment as microorganisms use them for source of carbon and energy [10]. Crude oil toxicity varies greatly with the components, concentration of the petroleum products [11,12], and environmental factors affecting the growth of the microorganisms at the time of the pollution [13]. Discharge of used oil from vehicles or motorcycles can also be a major source of oil pollution in the environment [14]. Biodegradation of crude petroleum by natural strains of bacteria is one of the main processes of bioremediation [15].

2. MATERIALS AND METHODS

2.1 Some Physicochemical Properties of Soil Samples

Three soil samples were taken or could be captioned as written thus: three soil samples

from the depth of 0-10 cm were taken in duplicates from three petroleum polluted soils in Sango Ota, Ogun State, Nigeria. The three locations were A (Obasanjo Farm); B (The Bells area) and C (Canaan Land City).

2.2 Enumeration and Identification of Crude Oil Degrading Bacteria

The total population of the heterotrophic bacteria (THB) count was determined by pour plate method, each soil collected was diluted using sterile distilled water. A dilution of 10^7 was plated onto nutrient agar and plate count agar. The inoculated plates were incubated at 35°C and examined for bacteria growth at 24 hours. Total population of the hydrocarbon utilizing bacteria (HUB) was obtained by pour plate method on minimal salts medium (MSM) with the crude petroleum as the carbon source only.

2.3 Growth of Bacteria Strains on Crude Oil

The medium was compounded using the following quantity in g/l of distilled water; NaCl 10.0; KCl, 0.29; $MgSO_4$.$7H_2O$, 0.42; KH_2PO4, 0.83; $NaNO_3$, 0.42; and Na_2HPO_4, 1.25 respectively. The medium was solidified with 20g of Agar. The pH was modified to 7.27 and autoclaved at 121°C for 15 minutes. Isolation of crude petroleum utilizing bacteria species was obtained by enrichment of the samples in the crude petroleum mixed with the MSM. One gram of soil sample was poured in 200 ml conical flask containing 50 ml of the media. The MSM was supplemented with 1% (v/v) crude oil as the sole source of carbon and energy. Incubation was done by shaking on rotatory shaker incubator at 180 rpm at room temperature for 21 days. After incubation, 1 ml of the enrichment culture was taken and serially diluted to 10^4 and plated using spread plate method on the mineral salt agar (MSA) in duplicates with vapor phase supply of crude petroleum and inoculated plated and incubated for seven days at 35°C. Selected isolated bacteria were identified using the basic microbiology morphological, and biochemical techniques using taxonomic scheme of Bergey's manual of determinative Bacteriology [16].

2.4 TPH and PAH Depletion Analysis (Gas Chromatography Analysis)

The process of extracting the residual oil in the flask from the above experiments was done by adding of 20 ml of hexane to resulting culture in a flask and shaking was done vigorously. Removal of the aqueous phase was achieved using a funnel to separate the oil from the medium and the residual oil concentrations were then measured using Gas Chromatography (GC) analysis. The same process of oil extraction was repeated for the broth in the control flask and the values obtained in the GC result was expressed as a percentage of the control result. 1µl of the resulting hexane extracts was achieved using the Hewlett Packard 6890 powered HP Chemo station (Rev A 09.01 1206) software. The Gas chromatographic machine was equipped with a flame ionization detector FID and 30 m long HP-6 column (the diameter of the internal column is 0.25 mm and the thickness of the film is 0.25 µm). Nitrogen was the carrier gas used in this experiment and the temperatures of the injector and detectors were controlled to be at 250°C and 350°C respectively. The column was programmed at start up temperature of 100°C; this temperature was maintained for about 3 minutes, and then ramped at 10°C per minute to 250°C and for another 5 minutes.

2.5 Plasmid Analysis (Profiling and Curing)

Plasmid DNA was extracted from the isolated bacterial strains using the conventional method and the nucleic acid concentration and purity was checked using Thermo Scientific Nano Drop 2000 Spectrophotometer machine. The extracted plasmids were then loaded on the wells of the gel electrophoresis on a 0.7% horizontal Agarose gel. Electric current was supplied at 70 V for 3hours; the gels were marked with ethidium bromide. The bands were then viewed with Ultraviolet Trans illuminator. The molecular sizes of the different plasmids were compared with RPI plasmid from E coli strain JC 3272 using 1-Kbp weight as the marker.

Isolated bacterial strains were then inoculated into lactose broth amended with the crude petroleum and incubated for 18 to 24 hours. After 18 hours of incubation, 1 µl of this culture was added to 5 ml of lactose broth containing 5ml of the ethidium bromide as the curing agent and this was incubated at 37°C for 72 hours. After incubation, the culture was diluted and inoculated onto nutrient agar (NA) plates using pour plate method and incubated at 37°C for 24 hours. Colonies were counted and the colonies were then sub cultured onto minimal salt agar amended with crude petroleum and onto NA. The plates were incubated at 37°C for 72 hours.

Colonies that were able to grow on NA, but not on MSM supplemented with crude petroleum were then isolated and considered as cured.

2.6 Statistical Analysis

The experiments were all carried out in triplicate and the data were subjected to a standard analysis of variance. Means were compared using Duncan's multiple range test (P<0.05). Statistical calculations were made using SPSS v21.0 software (SPSS Inc., Chicago, IL, USA). Data are the mean ± Standard Error (S.E). Also the measured values were expressed as median ± range.

3. RESULTS AND DISCUSSION

3.1 Some Physicochemical Properties of Soil Samples

The physicochemical properties which include the pH, temperature, nitrate, phosphate, potassium, conductivity, moisture content and total organic contents and in the oil polluted soil samples were analyzed and presented in Table 1.

3.2 Enumeration and Identification of Crude Petroleum Degrading Bacteria

The total heterotrophic bacteria count was within the range of 2.6 x 10^7- 4.5 x 10^7 cfu/g while the crude petroleum utilizing bacteria population ranges 1.2 - 1.5 x 10^4 cfu/g. Soil samples enrichment with 1% (v/v) crude oil resulted in the isolation of four bacteria strains. The bacteria isolates which could grow and utilize on crude oil were picked randomly and identified using the morphological and biochemical techniques with reference to the taxonomic scheme of Bergey's Manual of Determinative Bacteriology as shown in Table 2. Growth of bacteria strains on the medium were monitored over a period of 21 days. *Bacillus* sp (SB4) exhibited best growth between the 12th and the 18th day. The bacterial population increases from 1.0 x 10^7 (cfu/g) on day 3 to 5.5 x 10^9 (cfu/g) on day 18 before dropping to 5.0 x 10^9 (cfu/g). There was a decrease in pH from 7.27 to 6.4. *Pseudomonas* sp (SC8) showed increase in the bacterial population from 1.0 x 10^7 (cfu/g) on day 3 to 7.5 x 10^9 (cfu/g). pH also decreases from 7.27 to 6.02. The optical density increases considerably from 0 on day 1 to 1.41 on day 21. For the *Serratia* sp, increase in growth was slow ranging from 1.0 x 10^9 (cfu/g) to 4.5 x 10^9 (cfu/g) on the day 15 to day 18 with a decrease in the pH from 7.27 to 6.4. *Acinetobacter* sp also showed an increase in the bacterial population and optical density with a decrease in pH. Bacteria and fungi which are part of the ecosystem are responsible for the biodegradation of environmental pollutants. Biodegradation of crude oil often occur as a result of the action of bacteria on both the aliphatic and the aromatic components of crude oil. The ratio of hydrocarbon utilizing bacteria relative to the heterotrophic bacteria was below 0.5%. This shows that only a small fraction of the bacteria population sampled in this study was capable of utilizing hydrocarbons.

3.3 TPH and PAH Depletion Analysis

The percentage depletion profile of the total petroleum hydrocarbon (TPH) and polycyclic aromatic hydrocarbon (PAH) for each bacterial strain were identified (Fig. 1). The amount of aliphatic hydrocarbon depleted by *Bacillus* sp. (SB4) was 36% while the aromatic hydrocarbon was 42%. *Pseudomonas* sp. (SC8) was able to deplete 43% aliphatic hydrocarbon and 49% aromatic hydrocarbons. Hence, the highest

Table 1. Some physicochemical properties of the soil samples

Parameters	Concentration		
	Soil A	**Soil B**	**Soil C**
Soil structure	Packed silt loam	Packed silt loam	Loose sandy loam
Moisture content (%)	13.67	13.53	7.79
pH in Water(H_2O)	6.83	6.64	6.62
Temperature (°C)	29.80	29.40	29.40
Electrical conductivity (µS/cm)	1.082	1.064	1.079
Potassium (mg/kg)	1.54	1.62	10.87
Phosphate (mg/kg)	1.65	1.87	1.95
Total organic carbon (TOC) (%)	14.0	15.5	24.1
Nitrate (mg/kg)	2.65	3.05	2.87

Table 2. Identification of the bacterial isolates

Biochemical Test	*Bacillus* sp. (SB4)	*Pseudomonas* sp.(SC8)	*Acinetobacter* sp. (SC12)	*Serratia* sp. (SC11)
Gram's Reaction	+ve Rod	-ve Rod	-ve short Rods	-ve short Rods
Mobility	Motile	Motile	Non motile	-ve
Indole	-ve	-ve	-ve	+ve
Methyl Red	-ve	-ve	-ve	+ve
Voges- Proskauer	+ve	-ve	+ve	+ve
Citrate	+ve	+ve	-ve	-ve
Urease	-ve	+ve	-ve	+ve
Nitrate	+ve	+ve	-ve	+ve
Triple Sugar Iron	*	*	K/K	A/A
Catalase	+ve	+ve	+ve	+ve
Oxidase	+ve	+ve	+ve	-ve
Mannitol	F	NF	NF	+ve
Maltose	+ve	NF	NF	NF
Lactose	NF	NF	NF	NF
Glucose	NF	-ve	+ve	F with gas
Sucrose	-ve	+ve	-ve	+ve
Starch Hydrolysis	+ve	-ve	*	-ve
Lysin	-ve	+ve	-ve	+ve
Arginine	-ve	+ve	-ve	+ve
Omithine	-ve	+ve	-ve	-ve

*Key: +ve - positive, -ve - negative, A - Acid, K - Alkali, F - Fermentative, NF - Non Fermentative, * - Not Determined*

depletion rate for this study. *Serratia* sp. (SC11) has the lowest depletion rate of aliphatic hydrocarbon 12% and 13% for the aromatic hydrocarbon. For the *Acinetobacter* sp. (SC12), the percentage depletion was 26% for the aliphatic hydrocarbon and 16% for the aromatic hydrocarbon.

In this study, nearly all component of crude petroleum ranging from C9 to C30 were reduced by actions of bacteria strains with over 50% degradation of the aliphatic component of the crude petroleum. *Bacillus* sp (SB4) had a percentage degradation of between 24% and 57% for the aliphatic component of the crude petroleum. Highest percentage of degradation of the aromatic was achieved by *Pseudomonas* sp. 38% - 67% while the *Serratia* sp. had 12% - 29% and *Acinetobacter* sp. had 20% - 40% percentage degradation. All the bacterial strains could not degrade nonadecane C19. *Pseudomonas* sp. (SC8) reduced all the components of C9 to C30 with the exception of the C19 likewise the *Bacillus* sp. (SB4) however the *Serratia* sp. (SC11) could not degrade C12 and C19. *Acinetobacter* sp. (SC12) also reduced all the C9 to C30 with exception of C19 (Fig. 2).

The polycyclic aromatic components of the crude petroleum and their rate of degradation (Fig. 3) revealed that *Bacillus* sp. (SB4) was able to reduce all the aromatic components of the petroleum; Benzo(b)flouranthene was best degraded (68%) while the Flourene (20%) and Anthracene (20%) were least degraded. *Pseudomonas* sp. (SC8) was also able to reduce all the aromatic components of the petroleum; Benzo(b)flouranthene was also best degraded (73%) while Acenaphthylene (30%) was least degraded by *Pseudomonas* sp. *Serratia* sp. (SC11) was not able to degrade Benzo(a)anthracene (0%), Chrysene (0%), Benzo(a)pyrene (0%). Pyrene was best degraded (29%) by *Serratia* sp. *Acinetobacter* sp. was able to reduce all the components of the petroleum used; Dibenzo (a,h) anthracene (54%) was best degraded by this strain and the least degraded was Acenaphthylene (12%). The result obtained during the substrate specificity and growth studies are similar to the findings of [17] who found that the two organisms (*Bacillus* sp. and *Pseudomonas* sp.) can degrade crude oil under optimum growth conditions. The crude oil utilizing bacteria identified under this study have also been isolated and observed by several

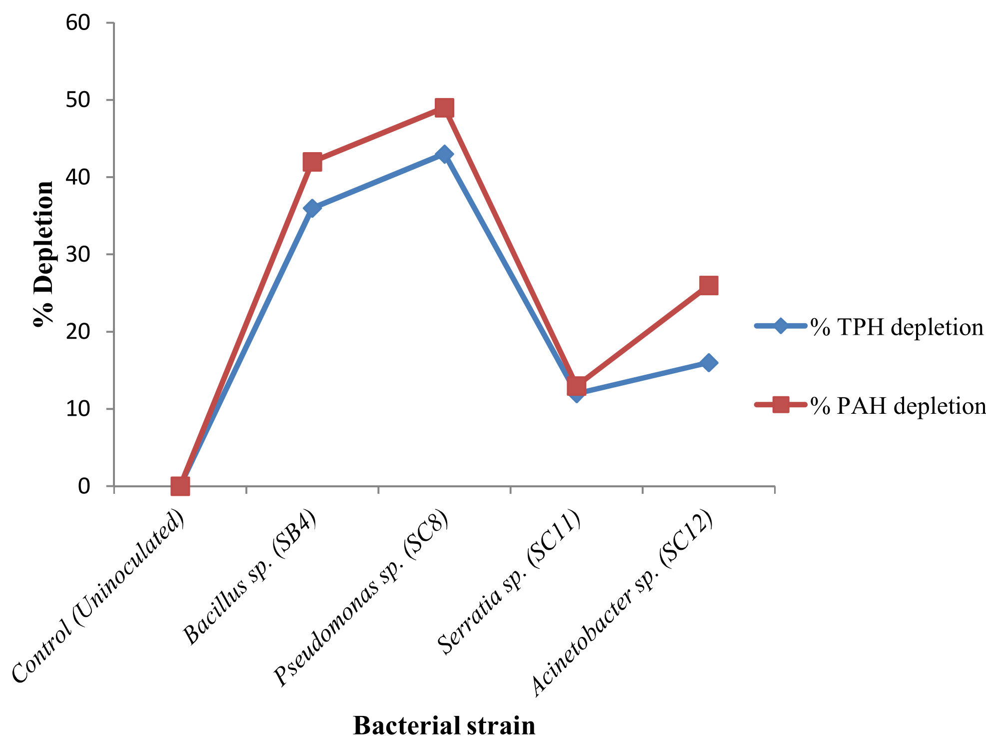


Fig. 1. Percentage depletion of crude petroleum by isolated bacteria strains

Key: Control (uninoculated flask), SB4- MSM inoculated with Bacillus sp., SC8- MSM inoculated with Pseudomonas sp., SC11- MSM inoculated with Serratia sp., SC12- MSM inoculated with Acinetobacter sp.

researchers [18,19]. There was no significant difference in mean comparison of degradation of the bonny light crude petroleum between the four bacterial strains (Table 3). High capability of *Bacillus* sp. isolated from the soil in degrading crude oil in soils was earlier observed by Chandankere et al. [20,21].

3.4 GC Fingerprints of the Residual Oil

Comparing the GC fingerprints of residual oil from the uninoculated control flask and the flasks inoculated with the isolated bacteria strains (Fig. 4) showed some reduction and in some cases disappearance of the main alkane peaks. The percentage of biodegradation of crude petroleum by the bacterial strains in this study was up to 70% which was seen in *Bacillus* sp. (SB4) which degraded benzo(b) flouranthene by 68% and *Pseudomonas* sp. (SC8) which degraded same component by 73% using 1% (v/v) crude oil. Analysis of the crude petroleum after biodegradation showed that the aliphatic components are more easily degraded than aromatic components (Fig. 2 and Fig. 3). These results are in agreement with those obtained previously by [20]. The aliphatic component C19 resisted degradation while the other straight chain alkanes were utilized at varying degrees regardless of the number of carbon atoms present, similar to the earlier reports of [21,22]. It is common that both bacteria strains were able to grow on all aromatics tested with the exception of anthracene, chrysene and benzo(a)pyrene which could not be used by *Serratia* sp. as sole sources of carbon and energy.

3.5 Plasmid Analysis

The Agarose gel electrophoretic separation profiles of plasmids from bacterial strains used in the study (Fig. 5). Curing experiments demonstrated that cured bacterial strains that lost their plasmids. These bacterial strains also lost their hydrocarbon biodegradation ability, so the capacity to degrade hydrocarbons could be plasmid related in all the strains studied as previously supported by the reports of [23,24].

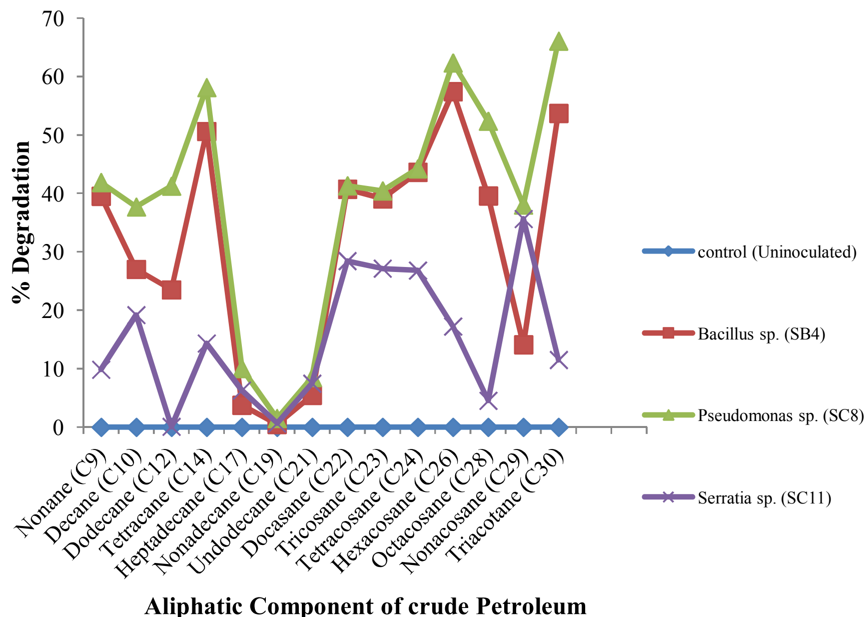


Fig. 2. Percentage degradation of the aliphatic component of crude petroleum by isolated bacterial strains

Key: Control (uninoculated flask), SB4- MSM inoculated with Bacillus sp., SC8- MSM inoculated with Pseudomonas sp., SC11- MSM inoculated with Serratia sp., SC12- MSM inoculated with Acinetobacter sp.

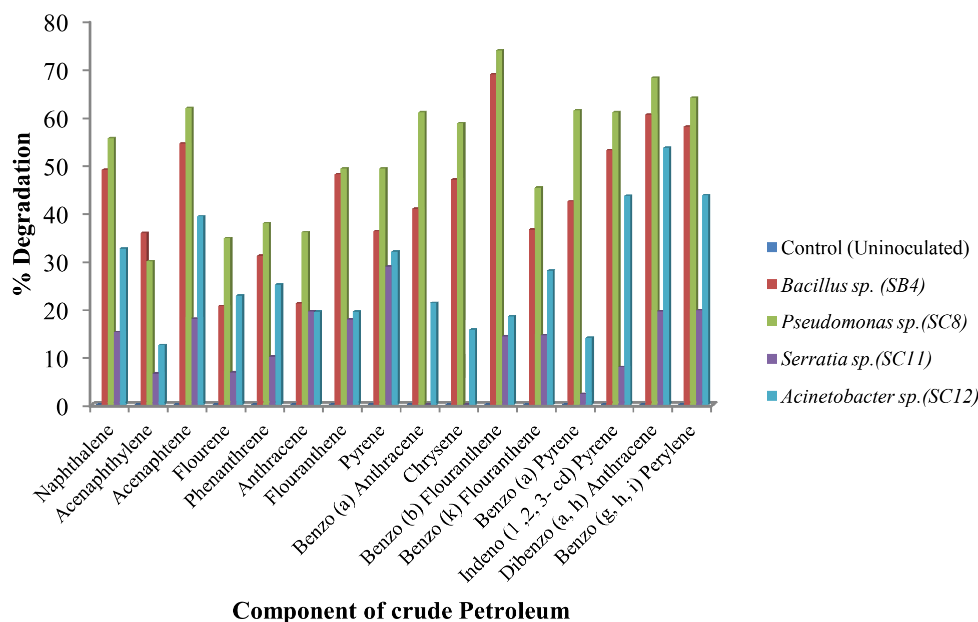


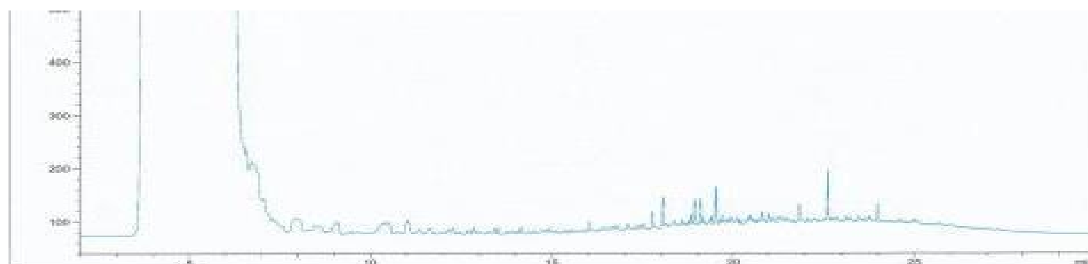
Fig. 3. Percentage degradation for polyaromatic hydrocarbon by isolated bacterial strains

Key: Control (uninoculated flask), SB4- MSM inoculated with Bacillus sp., SC8- MSM inoculated with Pseudomonas sp.;SC11- MSM inoculated with Serratia sp., SC12- MSM inoculated with Acinetobacter sp.

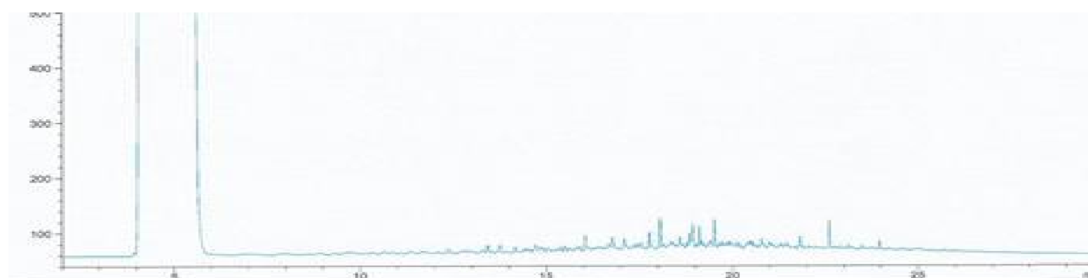
Table 3. Mean comparison of degradation capabilities of crude petroleum (n=5)

ANOVA					
Parameters	**Sum of squares**	**df**	**Mean square**	**F**	**Sig.**
Between groups	3913.163	15	260.878	.477	.944
Within groups	34972.776	64	546.450		
Total	38885.940	79			

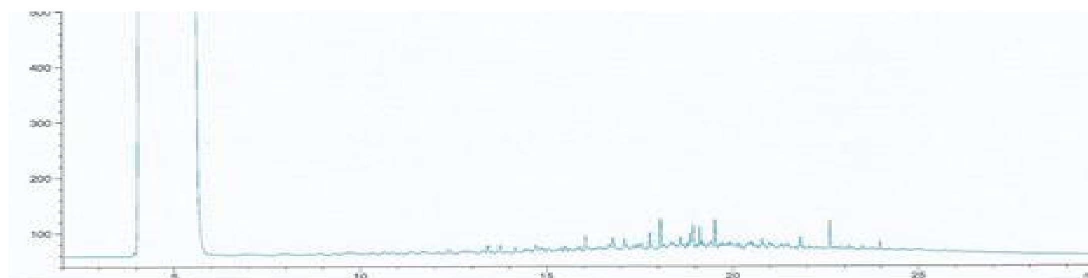
Key: P>0.05 - There is no significant difference



(CONTROL)



(SB4)



(SC8)

(SC11)

(SC12)

Fig. 4. Gas Chromatographic fingerprints of residual oil recovered from crude petroleum
Key: Control (uninoculated flask), SB4- MSM inoculated with Bacillus sp., SC8- MSM inoculated with Pseudomonas sp., SC11- MSM inoculated with Serratia sp., SC12- MSM inoculated with Acinetobacter sp.

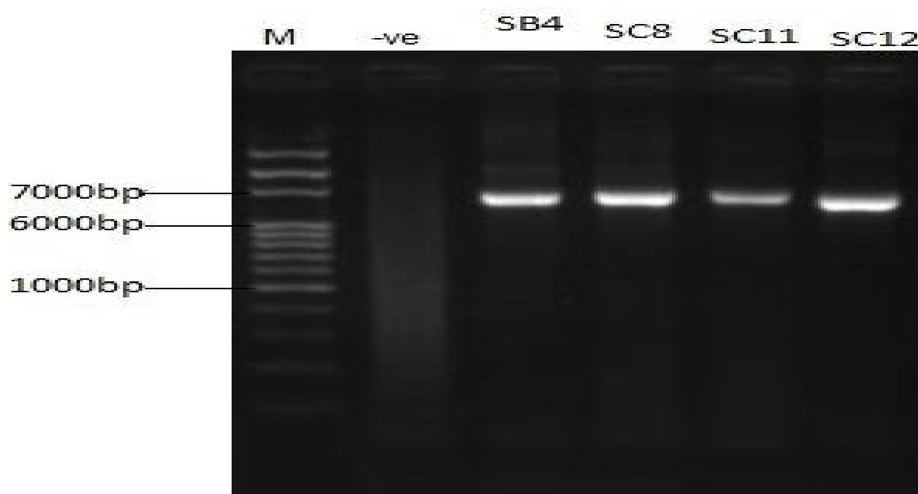


Fig. 5. Electrophoretic separation profiles of plasmids from cured and non-cured bacterial isolates
Key: M – 1000bp Plasmid Ladder, -ve – Bacterial Control, SB4 – Bacillus species, SC8 – Pseudomonas species, SC11 – Serratia species, SC12 – Acinetobacter species

4. CONCLUSION

The result of this study revealed that some bacterial strains were able to grow effectively on crude oil, metabolizing both the aliphatic and aromatic components. The utilizing capabilities in some bacterial strains appear to be common and plasmid encoded. These bacterial strains can therefore be employed in large scale oil spill clean-up if their potential is maximally harnessed.

ACKNOWLEDGEMENT

We acknowledge the financial support of Covenant University, Ota, Ogun State, Nigeria.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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