Archives of Agriculture and Environmental Science 9(1): 14-22 (2024)
https://doi.org/10.26832/24566632.2024.090103

e-ISSN: 2456-6632

This content is available online at AESA

Archives of Agriculture and Environmental Science

Journal homepage: journals.aesacademy.org/index.php/aaes

ORIGINAL RESEARCH ARTICLE

Evaluation of the degrading potentials of plasmid and non–plasmid borne soil bacterial strains on bonny light crude oil

Marcel Chikelue Ifediegwu[1], Michael Uchenna Orji[2], Samuel Chinedu Onuorah[2] and Bright Obidinma Uba[3*]

[1]Lecturer, Department of Applied Microbiology and Brewing, Nnamdi Azikiwe University, P.M.B. 5025 Awka, Anambra State, NIGERIA
[2]Professor, Department of Applied Microbiology and Brewing, Nnamdi Azikiwe University, P.M.B. 5025 Awka, Anambra State, NIGERIA
[3]Senior Lecturer, Department of Microbiology, Chukwuemeka Odumegwu Ojukwu University, P.M.B.02 Uli, Anambra State, NIGERIA
*Corresponding author's E-mail: bo.uba@coou.edu.ng

ARTICLE HISTORY

Received: 12 December 2023
Revised received: 29 January 2024
Accepted: 10 February 2024

Keywords

Bioremediation
Crude oil
Gas chromatography
n –Alkanes
Soil pollution

ABSTRACT

This study was undertaken to evaluate the degrading potentials of plasmid and non–plasmid borne soil bacterial strains on Bonny light crude oil. Enrichment technique, turbidometric test, plasmid curing test as well as gas chromatographic flame ionization detection technique was adopted for isolating and evaluating the oil degrading capabilities of the selected bacterial strains. The preliminary physicochemical results revealed that pH was recorded slightly neutral, higher conductivity (0.41 to 0.44 µS/cm), higher organic carbon (2.32 to 4.34 %) but lower nitrogen and phosphorus contents (0.27 % and 10.11 kg) as well as lower water holding capacity was noted, respectively. The results indicated that 22 out of the 60 isolated bacterial strains had high crude oil degrading potentials (A600nm > 0.3). The result further indicated that bacterial strains belong to various species which are *Bacillus cereus* C12, *Pseudomonas aeruginosa* KAVK01, *Bacillus licheniformis* 126, *Ochrobacterium intermedium* E85b, *Bacillus subtillis* SDDlas, *Bacillus subtillis* LK4.5, *Enterobacter cloacae* GEBRI III and *Bacillus cereus* So24. Plasmid borne *P. aeruginosa* strain KAVK01 was the best degrader with 88 % remediation efficiency within the period while the plasmid cured *P. aeruginosa* strain KAVK01 had 65 % degradation with the order of n – alkane hydrocarbon degradation: (n-C8 - n-C17) > (n-C18 - n -C25) > (n-C26 - n-C32). The data obtained from the current study could help in the selection of bacterial species, most especially plasmid borne bacteria that can be employed in the restoration of oil contaminated soil ecosystem in the Niger Delta region of Nigeria.

Citation of this article: Ifediegwu, M. C., Orji, M. U., Onuorah, S. C., & Uba, B. O. (2024). Evaluation of the degrading potentials of plasmid and non–plasmid borne soil bacterial strains on bonny light crude oil. *Archives of Agriculture and Environmental Science*, *9*(1), 14-22, https://dx.doi.org/10.26832/24566632.2024.090103

INTRODUCTION

The primary constituents of bonny light crude oil are various hydrocarbon molecules, primarily aliphatic (from C1 to C30), aromatic (from C6 to C8), cyclohexane, and other compounds containing sulfur, nitrogen, and oxygen (Santisi *et al*., 2015). The majority of the degradable components found in petroleum pollutants will eventually be used by the environment when microorganisms use them as a source of energy and carbon (Hassanshahian *et al*., 2014). The components, concentration of petroleum products, and environmental factors influencing the growth of microorganisms at the time of pollution all have a significant impact on the toxicity of crude oil (Ifelebuegu *et al*., 2017). Used oil spills from cars and motorcycles can also be a significant cause of environmental oil pollution. One of the primary methods of bioremediation is the biodegradation of crude petroleum by naturally occurring bacterial strains (Nwinyi *et al*., 2017). The fact that petroleum product dominates Nigerian economy also creates a lot of conditions for release of large amounts of pollutants into the environments (Isibor and Imoobe, 2017). Whenever crude oil is released into the environment, serious pollution problems occur which can be deliberate

or by mistake (Obafemi *et al*., 2018). Crude oil pollution may release high oil fractions which may culminate in malfunctioning of the liver, kidney and central nervous system (Blanca *et al*., 2016; Abdel-Shafy *et al*., 2016). The Breast Cancer Fund (BCF, 2013) reported that heavy PAHs such as benzo[a]pyrene damage the DNA of living organisms. They are genotoxic and are implicated in human breast cancer (Macaulay and Rees, 2014).

It is worthy to note that, in addition to chromosomal DNA, a large number of bacteria also have extra-chromosomal DNA known as plasmids that bear catabolic genes encoding some alkane degrading enzymes. These plasmids are classified into three main groups: a) the plasmids containing alkane degradation genes (as the OCT plasmid), b) the plasmids containing naphthalene and salicylate degradation genes (as the NAH plasmids), and c) the plasmids containing toluene- and xylene-oxidizing genes (as the TOL plasmids). Additionally, plasmids are highly mobile form of DNA, which could transmit new phenotypes, such as alkane degradation ability to the recipient organisms (Esmaeili *et al*., 2020). Catabolic plasmids deserved special attention as powerful tools for dissemination of catabolic genes in the bacterial community. Therefore, research on catabolic plasmid genes and their degrading potentials is important because of environmental oil-contamination threat and its degradation.

There is paucity of literatures on the applicability of plasmid and non – plasmid borne soil bacterial strains on crude oil biodegradation. To the best of our knowledge, the existing facts especially in Nigeria largely centered on the applications of the non – plasmid borne soil bacterial strains to oil and oil - products remediation with limited study on applicability of the plasmid borne bacterial strains to oil remediation and hence justifies the current study. Thus, considering the environmental mishaps and public health difficulties associated with oil substrate and its products, this study was undertaken to initially isolate, screen, and establish the plasmid profile of the selected soil bacterial strains with high potential for crude degradability isolated from four automobile workshops having history of age long oil contamination. The study further described the molecular features and degrading capabilities of the selected soil bacterial strains using gas chromatographic index which contributes to the attempts to understand the evolutionary and phylogenetic perspectives of the bacteria as well as elucidate the mechanisms of the crude oil biodegradation. The current study was undertaken to evaluate the degrading potentials of plasmid and non–plasmid borne soil bacterial strains on Bonny light crude oil.

MATERIALS AND METHODS

Procurement of Bonny light crude oil

For this study, Bonny light crude oil (API gravity=32.15) was acquired from the Port Harcourt Refinery of the Nigerian National Petroleum Corporation (NNPC) at Alesa-Eleme, Rivers State, Nigeria.

Sampling site

Samples of soil were taken from four locations in Anambra State—Awka, Aguleri, Onitsha, and Ekwulobia—where hydrocarbon contamination dates back fifteen years. The Awka sampling site, which is situated near Aroma Junction in the Awka South Local Government Area of Anambra State, is situated between latitude N06.22677° and longitude E007.07602°. Its mean elevation is 133 meters above sea level. The Onitsha sample site is located at the Upper Iweka Axis of the Onitsha South Local Government of Anambra State and is latitude N6.13378° and longitude E6.79393°, with a mean elevation of 43 meters above sea level. The Ekwulobia area is located at latitude N5.99053° and longitude E7.17018°, with a mean elevation of 88 meters. The sample site is Ekwulobia Motor Park in the Aguata Local Government Area of Anambra State.

Sampling method

The georeferenced point in the center of the site and two additional points eight meters distant were used to gather soil samples at depths ranging from 0 to 10 cm. Additionally, three subsamples, spaced six meters apart, were taken from each point (Nakamura *et al*., 2014). From the four sample locations, a total of 75 composite samples were gathered. With the aid of a soil auger, the sample was taken and placed in a plastic bag. The composite sample's polyethylene bag was kept cold in a refrigerator for microbiological and chemical analysis after being placed in a plastic bucket filled with dry ice (Uba, 2018). It was then promptly transferred to the Microbiology Laboratory of Nnamdi Azikiwe University in Awka, Anambra State.

Soil sample processing

Composite soil samples were bulked together in the laboratory, crushed to break the large soil aggregates and air dried under room temperature. The samples were sieved through a 2 mm sieve to take out big grains, debris and stones. The sieved soil was ground to pass through a 0.5 mm sieve and kept for the physico-chemical analysis (Uba *et al*., 2018).

Physico-chemical properties of petroleum hydrocarbon contaminated soil samples and pristine soil

The physiochemical analysis of sampled soils and pristine soil (unpolluted agriculture soil) was done at Soil Testing Laboratory, Anambra State Ministry of Agriculture, Awka by adopting the standard of AOAC (2012). This was to determine the effects of hydrocarbon on the oil contaminated sites (sampling environments) using uncontaminated soil as a control. The soil characteristics studied were pH, electrical conductivity, moisture content, water holding capacity, organic carbon, nitrogen and available phosphorous, respectively.

Isolation of hydrocarbon degrading bacteria

According to Gayathri *et al*. (2014), the enrichment culture approach was utilized to isolate bacterial strains that could use crude oil as their only source of carbon and energy. When the media were about to harden (between 45 and 50 °C), different quantities of crude oil (1%, 2%, 5%, and 10%) were carefully mixed with 100 mL of media (Luria Bertani, Mineral Salt, and

Nutrient broth). This process produced crude oil mixed media. After being suspended for 24 hr. at 27±°C temperature in 9 mL of distilled water, 1 g of each soil sample was used. The following day, 250 µL of the supernatant was applied to crude oil-containing Nutrient agar, LB, and MS Petri dishes in order to isolate bacteria that break down hydrocarbons, and it was then incubated at room temperature. The following day and up to the fourth day, the Petri dishes were checked for the development of bacterial colonies (Uba, 2018). Seven transfers in a series were used to randomly select the cultures. In preparation for additional examination, the purified bacterial strains were refrigerated at 4 °C on agar slants.

Preliminary screening of bacterial isolate for hydrocarbon degradation

Inoculum preparation: As stated by Nwanyanwu *et al.* (2016), the inoculum was prepared. For 48 hrs., the test isolates were cultured in nutrient broth medium in 100 mL Erlenmeyer flasks at room temperature. The cells were then extracted using centrifugation for 10 min at 6000 rpm, followed by rinsing in sterile deionized water. The cell suspensions were utilized throughout the investigation, unless otherwise noted, and were standardized by adjusting the turbidity to optical density of 0.1 at absorbance of 540 nm.

Turbidometric test: The ability of the bacterial isolates to use the hydrocarbon fraction (4% v/v) was also evaluated using the MS medium. In this test, 50 mL of the MS medium were added to a 100 mL conical flask, which was then sterilized to make the medium. Each of the 60 bacterial isolates was added separately to a flask for inoculation. For seven days, the flasks were incubated at room temperature at 120 rpm on an orbital shaker. Crude oil degradation capability was measured by cell growth as measured by optical density at 600 nm (OD_{600}) (Nwanyanwu *et al.*, 2016). Bacteria that break down crude oil and have a high potential for degradation were chosen and were used for future studies.

Plasmid isolation and curing: In this experiment, plasmids were detected or not in 22 bacterial isolates that showed rapid growth on the modified mineral salt media. As a result, all 22 isolates underwent plasmid isolation using techniques from Isiodu *et al.* (2016) and Uba (2019a). Plasmid profiling was done by agar gel electrophoresis. The DNA bands were matched with those for lambda DNA Hind III digest kb molecular weight marker. The approximate molecular weight of each plasmid was obtained by extrapolation on graphical plots of molecular weight of marker against the distance travelled by the respective plasmid band (Ajao, 2013). Eight out of the twenty-two bacterial isolates that underwent plasmid curing after being screened for the presence or absence of plasmid were discovered to be plasmid-borne. Nutrient broth (NB) was used to grow bacterial cells overnight. A 0.1 mg/mL acridine orange supplement was added to five (5) milliliters of nutritional broth. Zero-point one milliliter (0.1 mL) of newly made culture suspension of the test strains from a 10^{-5} dilution was added to nutrient broth that contained acridine orange. The mixture was then incubated for four days at 37 °C (pH 7.6) in the dark before being played out on nutrient agar. Acridine orange was not added to control cultures when they were prepared in nutrient broth. Isolated and regarded as cured were colonies that could grow on nutrient agar but not on modified solid mineral salt medium.

Molecular identification of crude oil degrading organism: Molecular studies were done for the eight bacterial isolates (B_1, C_3, D_1, H_4, I_6, J_3, K_4, and L_2) that had catabolic plasmid genes following DNA extraction, PCR amplification of the bacterial 16S rRNA genes and gel electrophoresis of the isolate were done at FOWM Biotechnology LTD, Jibowu, Yaba, Lagos and the Molecular Biology Laboratory of National Institute for Medical Research (NIMR), Yaba, Lagos, Nigeria. The PCR product was sent to Epoch Life Science, Texas, USA where the Sanger Sequencing was carried and blasting of the generated sequences was done at National Centre for Biotechnology Information (NCBI) websites where organisms of similar origin were identified from their closest relatives in the GenBank as described by Uba (2018) and Uba (2019b).

Bioremediation of crude oil in liquid media: The study was carried out in 250 mL Erlenmeyer flasks containing 100 mL mineral salt broth amended with 3 % crude oil. Control containing 100 mL of the medium with 3% crude oil only was set up. Four bacterial isolates were used in this study and the isolates were selected based on their bioremediation potentials in the previous tests. The isolates selected were plasmid borne and cured cells of *B. cereus* C12, *P. aeroginosa* K0VK01, *B. subtilis* SDDlas and *O. intermedium* E85b.

Experimental design: The flasks were grouped into 2 experimental set-ups of 4 flasks each in triplicates labeled Ex. A, Exp. B, Exp. C, Exp. D, Exp. E, Exp. F, Exp. G, Exp. H and control sample tagged CS. The plasmid cured bacterial strains were inoculated into the plasmid cured set-ups and plasmid borne bacterial cells were inoculated into plasmid borne experimental set-ups while the control remained un-inoculated as shown in Table 1. The biodegradability test of the plasmid cured and plasmid borne were done by inoculating each of the categories of the isolate (24 hr. old culture of bacteria) into a separate 250 mL Erlenmeyer flasks containing 100 mL of mineral salt medium (MSM) supplemented with 3 % v/v crude oil as sole carbon source. The inoculated flasks were incubated at 37 °C and at 150 rpm in a rotary shaker for 30 days and the extent of biodegradation determined by total residual hydrocarbons in the flask after 30 days determined with gas chromatography (Malik and Ahmed, 2012).

Analysis of crude oil samples with Gas chromatography (GC): This test was done using methods described by Nkanang *et al.* (2017) and Obafemi *et al.* (2018), respectively.

Table 1. Experimental design.

S. No.	Codes	Experimental variants
1	Exp. A	Plasmid borne *B. cereus* C12 + 100 mL MSM + 3% crude oil
2	Exp. B	Plasmid cured *B. cereus* C12 + 100 mL MSM + 3% crude oil
3	Exp. C	Plasmid borne *P. aeroginosa* KAVK01 + 100 mL MSM + 3% crude oil
4	Exp. D	Plasmid cured *P. aeroginosa* KAVK01 + 100 mL MSM + 3% crude oil
5	Exp. E	Plasmid borne *O. intermedium* E85b + 100 mL MSM + 3% crude oil
6	Exp. F	Plasmid borne *O. intermedium* E85b + 100 mL MSM + 3% crude oil
7	Exp. G	Plasmid borne *B. subtilis* SDDlas + 100 mL MSM + 3% crude oil
8	Exp. H	Plasmid cured *B. subtilis* SDDlas + 100 mL MSM + 3% crude oil
9	Exp. CS	100 mL MSM + 3% crude oil

Sample (2 mL) was taken from flask with disposable pipette. The flasks were shaken vigorously to suspend the solid materials in order to obtain homogenous sample. The samples were extracted ultrasonically with mixture of methanol, di-chloromethane (DCM) and water (1:5:4) and centrifuged for 15 min. The extracts were dried over with Na_2SO_4 and concentrated in rotary evaporator to final volume of 5 mL. A 1 mL aliquot was separated and was diluted to concentration (1 µL) appropriate for Gas chromatography (GC). Individual components of the aliphatic hydrocarbon were used as standards (standard match method). A commercial standard mixture, AccuStandard was used to calibrate the GC column. N-alkane identification and quantification was done based on retention time and peak area of the C8 - C40 standards. The concentrations of the crude oil components were calculated from the peak area chromatograms and the total aliphatic components were calculated to compare the percentage degradation at different times and intervals of the biodegradation.

Statistical analysis

Statistical calculations were made using SPSS version 25 software (SPSS Inc., Chicago, IL, USA). The data obtained was subjected to descriptive statistics using mean and standard deviation of mean. One-way analysis of variance (ANOVA) test under Completely Randomized Design (CRD) was used in interpreting the results. Post-Hoc test using Duncan Multiple Range Test (DMRT) was used to determine the averages that are conspicuously different from the other. *P* value of less than 0.05 was considered to indicate statistical significance. Excel plots were used for the charts (Obafemi *et al*., 2018).

RESULTS AND DISCUSSION

Microorganisms are incredibly varied and have the ability to adapt to live in harsh conditions. By modifying their degradative enzyme system, microbes can degrade a wide range of complex molecules. In the natural world, microorganisms are vital because they change natural chemicals and participate in the elemental cycle of the geological formation. Many different types of microbiota can be found in contaminated areas, and they can use the contaminant as a source of carbon and energy (Okore *et al*., 2021).

In this study, the degrading potentials of plasmid and non – plasmid borne soil bacterial strains on Bonny light crude oil was evaluated and Table 2 depicts the results of the physicochemical properties of the hydrocarbon contaminated soil samples and pristine soil. The result revealed an average pH of 7.2 for hydrocarbon contaminated soils while the pristine soil had pH of 7.11. There was no significant difference in the pH of the contaminated soil samples and agreed with the published works of Bada *et al*. (2019) and Ughala *et al*. (2019). However, the electrical conductivity was higher (0.39 to 0.44 µS/cm) in contaminated soil samples than the uncontaminated, which might be due to the presence of metal or other ions. This observation is in agreement with the work of Ayogu *et al*. (2020) which documented that hydrocarbon contaminated soil had higher electrical conductivity than pristine soil. The organic carbon content in all the contaminated soil samples was significantly higher than the uncontaminated pristine soil and ranges from 2.32 to 4.34 %. The possible reason for this difference may be due to the presence of hydrocarbons in the contaminated soil samples. This agreed with the reports of research conducted by Ughala *et al*. (2019) and Ayogu *et al*. (2020) which stated that petroleum hydrocarbon (PHC) pollution has negative effects on soil conditions, microorganisms and plants. Hydrocarbon pollution in the soil gives rise to deterioration of soil structure, loss of organic matter contents, loss of soil mineral nutrients such as potassium, sodium, calcium, magnesium, nitrogen, sulphate, phosphate and nitrate. The nitrogen and available phosphorous contents of the soil samples were less than that of pristine soil. Comparatively, pristine soil had higher nitrogen and phosphorus contents of 0.77 % and 14.70 kg, respectively than that of contaminated soil which nitrogen and phosphorus contents averaged 0.27 % and 10.11 kg, respectively. Lower concentration of nitrogen, phosphorous and other mineral nutrients had been reported as limiting factors for the growth of microorganisms in PHC polluted environments and agreed with the published works of Bada *et al*. (2019) and Ayogu *et al*. (2020). The water holding capacity which determines the extent of water retention and aeration in the soil was less in petroleum hydrocarbon contaminated soils than that of pristine soil. The water holding capacity which determines the extent of water retention and aeration in the soil was also less in PHC contaminated soils than that of pristine soil (Table 2). Ayogu *et al*. (2020) reported that the presence of PHC in the soil increases the soil hydrophobicity, reducing the water holding capacity of the soil. These two properties are essential for the growth of biotic components in the soil.

Table 2. Physicochemical properties of petroleum hydrocarbon contaminated soil and pristine soil samples.

Soil property	Polluted soil				Pristine soil
	A	B	C	D	
pH	7.25	7.31	7.18	7.28	7.11
Electrical conductivity (µs/cm)	0.41	0.44	0.42	0.41	0.32
Organic Carbon (%)	3.37	2.32	4.34	3.33	0.82
Nitrogen Content (%)	0.28	0.41	0.24	0.18	0.77
Phosphorus content (kg/mL)	9.46	11.22	9.29	10.46	14.70
Moisture content (%)	1.41	0.91	1.71	1.84	9.24
Water holding capacity (%)	29.45	30.63	35.40	40.67	62.27

Key: A = Soil samples collected from Awka; B = Soil samples collected from Ekwulobia; C = Soil samples collected from Onitsha; D = Soil samples collected from Aguleri.

Table 3. Preliminary screening of isolates for hydrocarbon degradation.

Isolate code	OD600	Isolate code	OD600	Isolate code	OD600	Isolate code	OD600
A1	0.35	E1	0.31	G4	0.18	J2	0.12
A2	0.37	E2	0.28	G5	0.11	J3	0.40
A3	0.28	E3	0.35	H1	0.15	J4	0.28
B1	0.35	E4	0.18	H2	0.34	J5	0.20
B2	0.27	E5	0.19	H3	0.12	K1	0.33
B3	0.29	F1	0.31	H4	0.31	K2	0.12
B4	0.19	F2	0.36	I1	0.25	K3	0.14
C1	0.31	F3	0.29	I2	0.11	K4	0.30
C2	0.27	F4	0.15	I4	0.30	K5	0.10
C3	0.42	F5	0.32	I5	0.17	L1	0.13
D1	0.38	G1	0.16	I5	0.31	L2	0.37
D2	0.22	G2	0.37	I_6	0.36	L3	0.15
D3	0.24	G3	0.14	J1	0.10	L4	0.14
M1	0.35	M2	0.21	M3	0.17	M4	0.23
N1	0.28	N2	0.19	N3	0.15	N4	0.32

An indicator of petroleum hydrocarbon (PHC) biodegradation capacity has been the absorbance (A600nm) of cells cultured in a medium containing petroleum as the only carbon source. Uba *et al*. (2018) had differentiated growth of the alkane degraders based on A_{600nm} using the following criteria: + + + Heavy growth: OD 600 nm > 0.2; + + Moderate growth: OD 600 nm 0.1 ≤ 0.2 + Poor growth: OD 600 nm 0.02 ≤ 0; - No growth: OD 600 nm < 0.02. In this study, sixty hydrocarbon utilizing bacteria were isolated from crude oil supplemented mineral salt agar medium and the bacterial isolates' optical densities (A_{600} nm) values varied from 0.10 to 0.42 in the growth medium containing crude oil, as indicated by the preliminary screening test results (Table 3). With an optical density of 0.42, isolate C3 had the fastest growth, while isolate K5, which had an optical density of 0.10, had the slowest growth. The other isolates showed variations in their growth on the substrate and the isolate with least growth has optical density of 0.11. Thereafter, 22 isolates designated with the strain codes A1, A2, B1, C1, C3, D1, E1, E3, F2, F5, G2, H2, H4, I3, I5, I6, J3, K1, K4, L2, M1, and N4, were chosen for further research after exhibiting noticeable growths (OD_{600} nm > 0.30) as shown in Table 3. The ability to isolate high numbers of certain oil degrading microorganisms from an environment is commonly taken as evidence that those organisms are the active degraders of the constituents of that environment (Ebakota *et al*., 2017). The efficient utilization of crude oil by these selected bacterial strains also revealed that they possess the physiological capabilities to metabolize crude oil hydrocarbon as carbon and energy sources. Nwanyanwu *et al*. (2016) isolated *Micrococcus* sp. RS38 which showed impressive level of growth during screening in crude oil and other petroleum products where the organisms grew at equal optical densities of > 0.2 within 14 days of incubation. Uba *et al*. (2018) reported that the abilities of the poly aromatic degrading marine bacterial strains to degrade different hydrocarbons including crude oil were found to vary. *P. vermicola* strain ANT1 had moderate/heavy growth on all the substrates while the other isolates had heavy, moderate, poor and no growth on the hydrocarbon's substrates. The reports of these authors corroborated with the findings of the present study.

Table 4 showed the closest relative of the isolates in Gen Bank and specific area of isolations. It was observed that 2 of the bacteria (C_3 and D_1) were isolated from soil sample collected from Awka, 3 organisms (G_2, K_4 and I_6) were isolated from Aguleri while Ekwulobia and Onitsha samples had one organism each (Table 4). The result in Table 4 of the blasted sequences indicated that isolates belong to various species which are *B. cereus* C12 (100 % sequence similarity), *P. aeruginosa* KAVK01 (100% sequence similarity), *B. licheniformis* 126 (96 % sequence similarity), *O. intermedium* E85b (97% sequence similarity), *B. subtillis* SDDlas (100 % sequence similarity), *B. subtillis* LK4.5 (97 % sequence similarity), *E. cloacae* GEBRI III (97 % sequence similarity) and *B. cereus* So24 (100 % sequence similarity). *Pseudomonas* and *Bacillus* species are the most abundant and potent bacteria hydrocarbon–degraders reported in the literatures (Osarumwense *et al*., 2019).

Table 4. Closest genera and percentage sequence homology of the 16S rRNA of the isolates.

Isolate code	Closest relative in Gen Bank	Max. score	Total score	Query cover	Per. identity	Accession number	Isolation site
D1	*B. cereus* C12	1768	2999	94%	100%	MF800952	Awka
C3	*P. aeruginosa* KAVK01	2745	2745	100%	100%	KC119195	Awka
L2	*B. subtilis* SDDlas	1866	1866	100%	100%	HQ262546	Onitsha
J3	*O. intermedium* E85b	1291	1291	90%	96.79%	KM894187	Ekwulobia
H4	*B. licheniformis* 129	2641	2641	100%	99.93%	KU922363	Onitsha
G2	*B. cereus* So24	1829	1829	100%	100%	MG00925	Aguleri
K4	*B. subtilis* LK4.5	1602	1602	95%	96.89%	KY083700	Aguleri
I6	*E. cloacae* GEBRI III	1555	1555	94%	97.20%	MH473593	Aguleri

Baig *et al.* (2021) reported that three dominant bacterial strains, *P. putida* and two *P. aeruginosa* (accession numbers ATCC-27853, BAA -427, and ATCC-49128), were isolated from three different oil-contaminated sites in order to evaluate their bioremediation potential. Ahda *et al.* (2018) reported that isolation and identification of two species of bacteria, i.e., *Bacillus* sp. and *Alcaligenes* from waste lubricant oil contaminated soil of workshops and examined its degradation capacity *in vitro*. Uba *et al.* (2018) identified polyaromatic and crude oil hydrocarbon degrading bacteria belonging to both the Gram positive and Gram-negative groups, although, the Gram-negative bacteria predominate in all the samples. Saha *et al.* (2022) in their published work documented the comparison between bacterial strains (Grampositive *B. stratosphericus* A15 a nd Gram-negative *O. pseudintermedium* C1) on the utilization of pure straight chain hydrocarbons, waste mineral lubricating oils as s ole carbon source and chemical characterization of the synthesized surface-active compounds. Olukunle (2019) reported *A. faecalis* SH179a, *A. faecalis* subsp. Phenolicus, *B. thuringiensis* serovar konkukian, *O. anthropi*, *A. faecalis* SH179b, Uncultured soil bacterium clone and *A. faecalis* IVN45 as hydrocarbon-degrading bacteria associated with oil polluted soil samples collected from Mesogar community of Delta State, Nigeria. The implications of these bacterial strains especially the Gram negatives in the soil oil hydrocarbon degradation by these authors in their previous studies supported the findings in the present study.

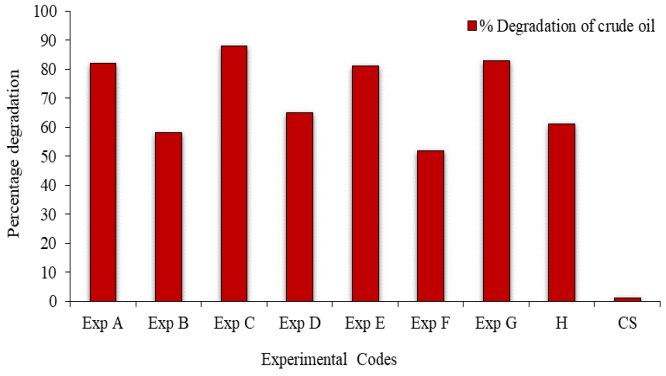

Figure 1. *Degradation of crude oil by the bacterial selected isolates*

NB: Isolates were inoculated into experimental set up as follows:
Exp A = Plasmid borne B. cereus C12; Exp E = Plasmid borne B. subtilis SDDlas; Exp B= Plasmid cured B. cereus C12; Exp F = Plasmid cured B. subtilis SDDlas; Exp C= Plasmid borne P. aeruginosa K0VK01; Exp G = Plasmid borne O. intermedium E85b; Exp D =Plasmid cured P. aeruginosa K0VK01 Exp H = Plasmid cured O. intermedium E85b; CS = Control sample

Figure 1 depicted the crude oil degradation percentage in liquid media by the selected bacterial isolates. Gas chromatography analysis indicated that both plasmids borne and cured isolates could utilize crude oil range *n*-alkanes, however, the efficiency of hydrocarbon utilization varied among the bacterial isolates. Moreso, it was observed that each of the plasmid borne wild-type bacteria degraded the crude oil substrate better than its cured strains in all the tests conducted. All the selected isolates significantly ($P<0.05$) reduced the concentration of crude oil. Moreover, plasmid borne *P. aeruginosa* strain KAVK01 was the best degrader with 88 % remediation efficiency within the period under study while the plasmid cured *P. aeruginosa* strain KAVK01 had 65 % degradation. In the same vein, plasmid borne *O. intermedium* strain E85b had degradation efficiency of 83 % and its plasmid cured strains recorded 62 % degradation. Wild-type *B. cereus* strain C12 and its plasmid cured strains degraded the oil substrates to 82 % and 54 %, respectively. Plasmid borne and cured strains of *B. subtilis* SDDlas were the least degraders and degraded the substrate to 71 % and 46 % of the petroleum hydrocarbon, respectively. The result also revealed that there was 1 % loss of the crude oil in the control. These differences in their biodegradation capabilities could be attributed to the presence and role of the genetic element catabolic plasmid which mediated the crude oil degradation mechanism and agreed with previous studies by Fagbemi and Sanusi (2017), Obafemi *et al.* (2018). Similarly, *B. subtilis* SDDlas used in this study did not completely loose its degradative ability but had a remarked reduction in hydrocarbon degradation after curing. This goes further to prove that not all the genes for degradation are located in plasmid but few are located on bacterial chromosome (Fagbemi and Sanusi, 2017). The evidence for a plasmid being involved in crude oil degradation stems from the fact that all the cured cells of isolates studied were able to degrade crude oil but at lower level when compared with the wild type. Several other reports documented that catabolic pathways that encode different aromatic hydrocarbon degradation routes are frequently located on plasmids. But localization of degradative genes can be on either chromosome or plasmid (Ndubuisi-Nnaji *et al.*, 2014; Fagbemi and Sanusi, 2017; Obafemi *et al.*, 2018; Uba, 2019a). Plasmids are important in the overall physiology and survival of many bacteria as they carry genes that confer a selective advantage to their host in a specific environment (Ndubuisi-Nnaji *et al.*, 2014).

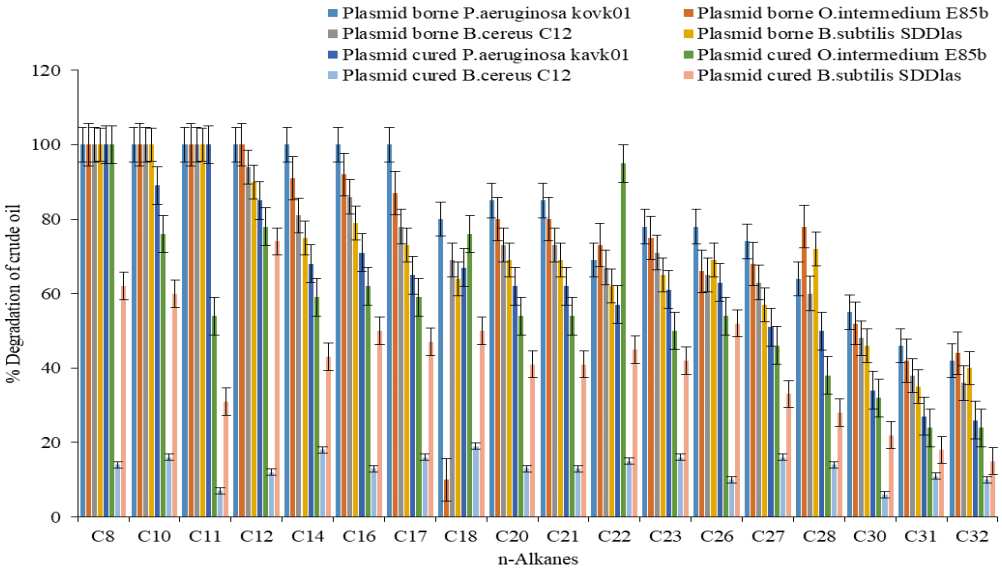

Figure 2. *Remediation of n-alkanes (C8 - C32) by the selected bacterial isolate.*

Comparison of the individual *n*-alkanes degradation profile of the plasmid borne and plasmid cured isolates showed similar trend of hydrocarbon degradation for plasmid borne and cured cells. *B. subtilis* strain SDDlas had the least degradation percentage for individual *n*-alkanes while the degradation percentages of *B. cereus* strain C12 and *O. intermedium* E85b were comparable. Plasmid borne *P. aeruginosa* strain KAVK01 was found to have 100 % degradation of C_8, C_{10}, C_{11}, C_{12}, C_{14}, C_{16} and C_{17} which were short chain n-alkanes and less toxic whereas its plasmid cured strains had 65 % remediation of C_8, C10, C_{11} and 60 %, 56 %, 53 %, 51 % remediations of C_{12}, C_{14}, C_{16},$C_{17,}$ respectively (Figure 2). The remediation percentages of individual n-alkanes by test organisms followed similar patterns with the growths of test of the organisms as reported earlier. The toxic long chain *n*-alkanes $C_{30,}$ C31 and C_{32} were observed to be toxic and degraded in varying percentage proportions which were 55 %, 46 % and 42 %, respectively by plasmid borne *P. aeruginosa*. The rest of the isolates also followed the same degradation trends. The result in Figure 2 of the microcosm studies further showed that the lower molecular weight n-alkanes (n-C8 - n-C17) were degraded prior to the medium (n-C18 - n-C25) molecular weight hydrocarbon, which was degraded preferentially to the high molecular weight n-alkanes (n-C26 - n-C32). This observation is consistent with the general knowledge of aerobic biodegradation of hydrocarbons where lower molecular weight hydrocarbons are preferentially degraded prior to the degradation of high molecular weight hydrocarbons. Increased biodegradability is inhibited with increased complexity of hydrocarbons due to the fact that accessibility by microbes/attack by enzymes is hindered. Obafemi *et al.* (2018) in their study reported that 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. 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, respectively. Nkanang *et al.* (2017) also reported that *C. amalonaticus* was able to degrade more than 75 % of the Bonny light crude oil fractions including C14, C17, C24, C26 and C27 except C10 and C20 which recorded only 20 % degradation. The observations of these authors corroborated with the findings obtained in this present study although our bacterial strains had higher degradation capabilities than theirs based n –alkane aliphatic hydrocarbon chains.

Conclusion

On the whole study, the sampling sites are good reservoirs of hydrocarbon bacterial degraders. The identified bacteria degraders consist of both Gram positive and negative groups in the genera *Bacillus*, *Pseudomonas* and *Ochrobacterium*. The plasmid borne *P. aeruginosa* strain KAVK01 was the best degrader with 88 % remediation efficiency within the period under study while the plasmid cured *P. aeruginosa* strain KAVK01 had 65 % degradation. In the same vein, plasmid borne and cured strains of *B. subtilis* SDDlas were the least degraders and degraded the substrate to 71 % and 46 % of the petroleum hydrocarbon, respectively. The plasmid borne *P. aeruginosa* strain KAVK01 was found to have 100 % degradation of C_8, C_{10}, C_{11}, C_{12}, C_{14}, C_{16} and C_{17} which were short chain n-alkanes and less toxic whereas its plasmid cured strains had 65 % remediation of C_8, C10, C_{11} and 60 %, 56 %, 53 %, 51 % remediations of C_{12}, C_{14}, C_{16},$C_{17,}$ respectively. On the general note, the consortium of plasmid borne isolates were the best degraders of crude oil compared to that of plasmid cured counterpart and individual isolates which was confirmed by Gas chromatography and growth studies. The highest rate of degradation occurred using the consortium suggests synergistic effects or interactions. The results of this study showed that the consortium is a promising technology for the elimination of hydrocarbon from contaminated soil ecosystem.

Authors contribution

Conceptualization, M.C.I., M.U.O. and S.C.O.; Methodology, M.C.I., M.U.O. and S.C.O.; Software, M.C.I.; Validation, M.C.I., M.U.O. and S.C.O.; Formal analysis, M.C.I.; Investigation, M.C.I and B.O.U.; Resources, M.C.I.; Data curation, M.C.I. and B.O.U.; Writing—Original draft preparation, B.O.U; Writing—Review and editing, M.C.I. and B.O.U.; Visualization, M.C.I. and B.O.U.; Supervision, M.U.O. and S.C.O.; Project administration, S.C.O.; Funding acquisition, M.C.I. and B.O.U. All authors have read and agreed to the published version of the manuscript. All authors have read and agreed to the published version of the manuscript

Conflict of interest: The authors declare no conflict of interest.

Ethical approval: Not applicable.

Data availability: The data that support the findings of this study are available on request from the corresponding author.

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