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Full Length Research Article

BIODEGRADATION OF BONNY LIGHT CRUDE OIL BY PLASMID AND NON-PLASMID BORNE SOIL BACTERIAL STRAINS USING BIOSTIMULATION AND BIOAUGMENTATION TECHNIQUES

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ABSTRACT
A laboratory scale study was designed and conducted to assess the biodegradation of Bonny light crude oil by plasmid and non-plasmid borne soil bacterial strains using biostimulation and bioaugmentation techniques. The enrichment technique, turbidometric test, plasmid curing test, molecular identification method, biostimulation test, bioaugmentation test and gas chromatographic technique were carried out using standard analytical techniques. The physicochemical analysis result showed that the pH was slightly neutral, the organic carbon content was higher (2.32 to 4.34%), the conductivity was higher (0.41 to 0.44 µS/cm), and the water holding capacity was lower (0.27 percent and 10.11 kg, respectively). Based on their capacity to use crude oil, the results showed that 22 of the 60 isolated bacterial strains had higher pollutant degrading potentials (A_{600nm} > 0.3).The identified potent hydrocarbon degraders includes: *Bacillus* spp., *Pseudomonas aeruginosa* KAVK01 and *Ochrobacterium* E85b strains. The highest degradation efficiency of 91% was found in soil that had been contaminated with 3 % (v/w) crude oil, amended with inorganic salts, and inoculated with plasmid-borne mixed cultures. The result further indicated that the consortium of plasmid borne isolates enhanced the reduction of the crude oil from the initial concentration of 10,318 ppm to 501 ppm (95 %) whereas 64 % decontamination was facilitated by the consortium of plasmid cured isolates. The information gathered from this investigation may be useful in choosing bacterial species, particularly plasmid-borne ones that can be employed to biodegrade soil contaminated by crude oil in Nigeria's Niger Delta region as well as the sample collection locations.

Keywords: Bioaugmentation, Biostimulation, Catabolic plasmid, Crude oil, Soil contamination.

INTRODUCTION

Crude oil is presently a non-renewable energy source in everyday life. This fossil fuel is used for a variety of purposes including fuel for transportation and factories. Unfortunately, oil spills sometimes occur within the environment due to accidents, mishaps and unavoidable actions such as weather and earthquakes or through intentional spills from war and dumping (Bordenave *et al.,* 2007). Several techniques can be used to clean up oil spills and to prevent further destruction by this hazardous constituent. Cleanup and recovery of spilled oil is difficult and depends upon many factors, including the type of oil spilled, the climatic conditions of spilled site which includes temperature of the water, tidal intensity and the types of shorelines and beaches involved. In general, spilled oil can be cleaned using three methods namely physical, chemical and natural methods (Bordenave *et al.,* 2007).

Bioremediation is the process whereby organic wastes are biologically degraded under controlled conditions to an innocuous state, or to levels below concentration limits established by regulatory authorities. It involves the use of naturally occurring bacteria and fungi or plants to degrade or detoxify substances hazardous to human health and/or the environment (Abatenh *et al*., 2017). Bioremediation can be effective only where environmental conditions permit microbial growth and activity, its application often involves the manipulation of environmental parameters to allow microbial growth and degradation to proceed at a faster rate (Kumar *et al*., 2011). Bioremediation can occur naturally and can be encouraged with addition of living things and fertilizers. Bioremediation technology is principally based on biodegradation (Jain and Bajpai, 2012).

The organic carbon content in hydrocarbon contaminated site is found to be very high and is attributed to constant input of hydrocarbons. Because of the high carbon content of oil and the low level of other nutrients essential for microbial growth, the rate and extent of degradation are, in general, limited by the low availability of nitrogen and phosphorus (Sonawdekar, 2012). Consequently, growth of hydrocarbon-degrading bacteria and hydrocarbon degradation can be strongly enhanced by fertilization with inorganic N and P. In majority of the treatments, the C: N: P ratio is maintained as 120:10:1. The addition of nutrients adjusts the essential nutrient balance for microbial growth and reproduction as well as having impact on the biodegradation rate and effectiveness (Abatenh *et al.,* 2017). Nutrient balancing especially the supply of essential nutrients such as N and P can improve the biodegradation efficiency by optimizing the bacterial C: N: P ratio. To survive and continue their microbial activities, microorganisms need a number of nutrients such as carbon, nitrogen, and phosphorous (Abatenh *et al.,* 2017).

Bioaugmentation consists of the addition of selected microbial species, harboring specific catabolic abilities, into a contaminated environment (Mahjoubi *et al*., 2018). The addition of pollutant degrading microorganisms to augment the biodegradative capacity of indigenous microbial populations in the contaminated area is known as bioaugmentation. In order to rapidly increase the natural microorganism population growth and enhance degradation that

preferentially feed on the contaminants site, microbes are collected from the remediation site, separately cultured, culturally modified and returned to the pollution sites (Abatenh *et al*., 2017). Different studies confirmed the efficiency of autochthonous microorganisms in the decontamination of hydrocarbon-polluted sites based on the fact that environmental conditions are suitable for their growth and metabolism as well as contribute significantly to the biochemical activities in soil (Nikolopoulou *et al*., 2013; Fodelianakis *et al.,* 2015).

Remarkably, many bacteria possess extra-chromosomal DNA called plasmids, which contain catabolic genes encoding certain enzymes that break down alkanes, in addition to their chromosomal DNA. The plasmids can be categorized into three primary groups: a) the OCT plasmid, which contains genes for alkane degradation; b) the NAH plasmids, which contain genes for naphthalene and salicylate degradation; and c) the TOL plasmids, which contain genes for oxidizing xylene and toluene. Furthermore, plasmids are an extremely mobile form of DNA that can transfer novel phenotypes to the recipient organisms, like the capacity to degrade alkanes (Esmaeili *et al*., 2020). As effective vehicles for distributing catabolic genes throughout the bacterial community, catabolic plasmids merited particular consideration. Consequently, because of the danger of environmental oil contamination and its degradation, research on catabolic plasmid genes and their potential for degradation is crucial.

Numerous investigators have assessed bioremediation procedures through individualized methods, with minimal focus on integral technologies such as bioaugmentation and biostimulation. Furthermore, most of the literature that has been published on bioremediation, particularly in Nigeria, has concentrated on the efficiency of pollutant degradation and paid less attention to the plasmid genetic mechanisms underlying the technology, which supports the current investigation. The current study was undertaken to evaluate the biodegradation of Bonny light crude oil by plasmid and non – plasmid borne soil bacterial strains using biostimulation and bioaugmentation techniques.

MATERIALS AND METHODS

Materials

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^{o} and longitude E007.07602^{o}. 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^{o} and longitude E6.79393^{o}, with a mean elevation of 43 meters above sea level. The Ekwulobia area is located at latitude N5.99053^{o} and longitude E7.17018^{o}, 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. 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 (Ekpo and Thomas, 2007).

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 (LB, MS, and Nutrient broth). This process produced crude oil mixed media. After being suspended for 24 h at room 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 (Hyina *et al*., 2003). Seven transfers in a series were used to randomly select the cultures. In preparation for additional examination, the refined 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 h, 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 a rinse 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 isolate 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). 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 ^{o}C (pH 7.6) in the dark before being plated out on Nutrient agar. Acridine orange was not added to control cultures when they were prepared in Nutrient broth. Isolated and regarded strains as cured were colonies that could grow on Nutrient agar but not on modified solid mineral salt medium. At the end of the experiment, 8 out of the 22 bacterial isolates that underwent plasmid curing after being screened for the presence or absence of plasmid were discovered to be plasmid-borne.

Molecular Identification of Crude Oil Degrading Bacteria

The molecular identification of 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 carried out at FOWM Biotechnology LTD, Jibowu, Yaba, Lagos and the Molecular Biology Laboratory of National Institute for Medical Research (NIMR), Yaba, Lagos, Nigeria. The PCR reagents in each tube amounted to 50 µL containing: buffer (5 µL), MgCl2 (1.5 µL), universal primer 1(forward 16S - /P1 PCR 5-AGAGTTTGATCCTGGCTCAG-3/) (2 µL), primer 2 (reverse 16S/--P2 PCR 5 -AAGGAGGTGATCCAGCCGCA-3) (2 µL), dNTP mix (1 µL), Dream Taq (0.25 µL), sterile sabax water (35.25 µL) and DNA samples (3 µL) as previously described (Uba, 2018). The cycling conditions were set at (a) initial denaturation 10 min at 95°C for 1 cycle. (b) Denaturation at 95 °C for 30 seconds, (c) Annealing cycling at 94°C for 30 seconds, (d) Elongation at 54 °C for 2 min. All steps in denaturation, annealing and elongation were for 35 cycles and (e) final elongation 10 min at 72 °C for 1 cycle. The reactions were held at 4 °C for 1 h in the thermal cycler. The PCR products were separated electrophoretically with 1 %. 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 in our previous publication (Uba, 2018; Uba, 2019b).

Biostimulation studies

Soil treatment

The soil used for this study was obtained from a site free of petroleum hydrocarbon contamination at demonstration farm of Anambra State Agricultural Development Programme, Awka, Anambra State, Nigeria. It was collected in pre-cleaned plastic container, air dried, powdered and sieved through 2 mm mesh sieve and then subjected to steam sterilization (1 h at 120 °C) and repeated three times after a 24 h interval. The axenic condition of the sterilized soil was tested by incubating the soil particles in nutrient agar (3 days at 25 ±°C). There was no growth on any of the incubated Petri dishes.

Inoculum preparation for bacterial consortium

The bacterial inoculum consortium was prepared using method of Ghazali *et al*. (2004) with slight modification. Individual cultures of *Pseudomonas aeruginosa* and *Ochrobacterium intermedium* were separately grown on 3 % v/v crude oil- containing mineral salt medium (100 mL) in a shake flask for 2 days and subsequently transferred into another 1 % v/v of crude oil mineral salts solution for 12 h at 30 °C in an orbital shaker at 150 rpm prior to inoculation. The cells were harvested by centrifugation, rinsed three times with sterile saline before being re-suspended in sterile MSM to yield an absorbance reading of 0.5 at 540 nm.

Experimental design

Tests were carried out in 500 mL sealed sterile glass flasks, each containing 300 g of sterilized agricultural soil; mixed thoroughly with 3 mL of crude oil (1 % v/w) to reach a concentration level of 13,000 mg/kg or parts per million. Mineral salts $(NH_4)_2SO_4$ and KH_2PO_4 were added to adjust the nitrogen and phosphorus contents of the medium of two experimental set-ups. One hundred milligram (100 mg) of $(NH_4)_2SO_4$ and 45 mg of KH_2PO_4 were dissolved in 100 mL sterile distilled water and mixed with the contaminated soil to distribute the nutrients through the soil particles and also to enhance good aeration. The soil was moistened by the addition of 15 mL of sterile distilled water and adjusted to 70 % of field capacity. Ten millilitres (15 mL) of the standardized inoculum of both individual and co-bacterial cultures were used as inocula. The glass flasks were grouped into five experimental set-ups in Table 1. Triplicate samples of the various treatment containers were then incubated at room temperature and turned every week with sterile glass rod to provide the necessary aeration. All glass flasks were incubated at room temperature, in the dark for 90 days. Throughout the experiment, a sterile cotton wool and sterile aluminium foil covered the microcosms to minimize external microbiological contamination (Wu *et al*., 2016, Cisneros-de La Cueva *et al*., 2014).

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Table 1: Biostimulation experimental design

Set up	Description
Exp. 1	Mixed culture of plasmid borne bacteria + nutrient + soil
Exp. 2	Mixed culture of plasmid cured bacteria + nutrient + soil
Exp. 3	Mixed culture of plasmid borne bacteria without nutrient + soil
Exp. 4	Mixed culture of plasmid cured bacteria without nutrient + soil
Exp. 5	Un-inoculated control experiment

Determination of total petroleum hydrocarbon

This study was done using Gas chromatographic method as reported by Lin *et al*. (2005). A Buck 530 gas chromatography equipped with HP 88 capillary column, automatic injector and Flame ionization detector was used for the analysis of biodegradation products. Ten grams of each soil sample were removed from the soil microcosms and mixed with an equal mass of anhydrous sodium sulphate. The mixture was placed in a Whatman cellulose extraction thimble. The 10 mL of crude oil in each of the samples was extracted twice with equal volumes (200 mL) of dichloromethane (DCM). The mixture was separated using separating funnel and the dichloromethane layer was concentrated in rotary evaporator. The extract was dissolved in 1 mL of DCM and analyzed by gas chromatography. The chromatographic analysis was performed on HP 88 capillary column (100 m x 0.25 µm film thickness,) CA, USA with a flame ionization detector (FID) detector. Helium was used as a carrier gas with a flow rate of 2 mL/min and injection volume was 1 µL at 200 °C injection temperature. The initial column oven temperature was kept at 70 ^{0}C with a holding time of 10 min. The ramp1 was 15 °C per minutes to 220 °C and ramp 2 was 12 ^{0}C per min to 280 °C. This final temperature was held for 5 min with maximum temperature exceeding 280 °C. The total run time was 43 min. Individual components of the aliphatic hydrocarbon were used as standards (standard match method). A commercial standard mixture, AccuStandard (Hydrocarbon Window Defining Standard, USA) DRH-008S-R2 calibration mixture was used to calibrate the GC column. The N-alkane identification and quantitation were done based on retention time and peak area of the C_8 - C_{40} 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 time and intervals of the biodegradation experiment. The results presented are the average of duplicate experiments. The degradation percentage of the crude oil was calculated using the formula:

$$\%\text{Degradation} = \frac{Concentration\ of\ initial\ crude\ oil - Concentration\ of\ Crude\ oil\ Degraded}{Concentration\ of\ initial\ Crude\ oil} \times 100\ \%$$

Bio-augmentation studies

Soil used

The artificially crude oil contaminated agricultural soils used for this study were collected from demonstration farms of Anambra State Agricultural Development Programme (ADP) sited at ADP premises, Awka, Anambra State, Nigeria. The samples were collected using the hand auger at a depth of 0 - 15 cm into sterile plastic buckets, mixed thoroughly, air dried (48 h), and sieved to uniform consistency using 2 mm sieve.

Microcosm design

Crude oil contaminated soil environment was simulated in soil microcosms. The microcosms were plastic containers and each microcosm received 1 kg of soil contaminated with 30 mL of crude oil (3 %) and kept in the laboratory at room temperature. Aeration was accomplished through mechanical mixing three times per week with a glass rod previously sterilized. Soil moisture was periodically monitored and water was supplied to the microcosms to maintain the soil moisture content. Twenty millilitres (20 mL) of the standardized inoculum of both individual and co-bacterial cultures were used as inocula as described by Kawo and Faggo (2017). The microcosms were then incubated at room temperature for 20 weeks without shaking to mimic an oil spill situation (Table 2). The percentage of crude oil degradation was determined as stated below (Kawo and Faggo, 2017);

$$\%\ \text{Degradation} = \frac{Initial\ conc.\ of\ crude\ oil - Conc.\ of\ crude\ oil\ degraded}{Concentration\ of\ control\ crude\ oil}\ x\ 100\ \%$$

Table 2: Experimental outlay

S/N	Code	Experimental variants
1	S (Control)	1kg soil + 3 % crude oil
2	S + PBP	1kg soil + Plasmid-borne *P. aeruginosa* + 3 % crude oil
3	S + PCP	1kg soil + Plasmid-cured *P. aeruginosa* + 3 % crude oil
4	S + PBO	1kg soil + Plasmid borne *O. intermedium* + 3 % crude oil
5	S + PCO	1kg soil + Plasmid cured *O. intermedium* + 3 % crude oil
6	S + MCPB	1kg soil + Mixed culture of plasmid borne isolates + 3 % crude oil
7	S + MCPC	1kg soil + Mixed culture of plasmid cured isolates + 3 % crud

Key: S = Soil; S+ PBP = Soil + plasmid borne *Pseudomonas aeroginosa*; S + PCP = Soil + plasmid cured *Pseudomonas aeroginosa*; S + PCO = Soil + Plasmid cured *Ochrobacterium intermedium*, S + PBO = 1kg soil + Plasmid borne *Ochrobacterium intermedium*, MCPB = Mixed culture of plasmid borne isolates; MCPC = Mixed culture of plasmid cured isolates

Hydrocarbon estimation

The hydrocarbon content in soil microcosm samples was estimated by gas chromatographic (GC-FID) method as described previously above by Lin *et al*. (2005).

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

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RESULTS

Physicochemical properties of petroleum hydrocarbon contaminated soil samples and pristine soil

The results of the physico-chemical characteristics of the pristine soil and soil samples contaminated with hydrocarbons are shown in Table 3. The contaminated soil samples had higher electrical conductivity (0.41 to 0.44 µS/cm) than the uncontaminated soil samples, but there was no discernible difference in their pH. All of the polluted soil samples had higher organic carbon contents—which range from 2.32 to 4.34%—than the pure soil. The soil samples had lower levels of available phosphorus and nitrogen than pure soil. In contrast, the nitrogen and phosphorus contents of pristine soil were 0.77 % and 14.70 kg, respectively, higher than those of contaminated soil, which had an average of 0.27 % and 10.11 kg, respectively. In comparison to pristine soil, soil contaminated with petroleum hydrocarbons had a lower water-holding capacity, which controls the amount of water retention and aeration in the soil.

Table 3. Physicochemical properties of petroleum hydrocarbon contaminated soil and pristine soil samples

	Polluted soil				
Soil property	A	B	C	D	**pristine soi**
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, µS/cm = Microsiemens per centimeter, % = Percent, Kg/mL = Kilogram per milliliter

Preliminary screening of isolates for hydrocarbon degradation

The results of the preliminary screening test showed that the optical densities (A600 nm) of the bacterial isolates in the crude oil-containing growth medium ranged from 0.10 to 0.42, as shown in Table 4. Based on their noticeable growths (OD600 nm > 0.30), 22 isolates bearing 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 selected for further investigation. Table 4 illustrates this process. The fastest growing isolate was C3, with an optical density of 0.42; the slowest growing isolate was K5, with an optical density of 0.10.

Table 4. 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	**I3**	**0.30**	K5	0.10
C3	**0.42**	**F5**	**0.32**	I4	0.17	L1	0.13
D1	**0.38**	G1	0.16	I5	**0.31**	**L2**	**0.37**
D2	0.22	**G2**	**0.37**	**I6**	**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**

Key: OD600 = Optical density at 600 nanometer

Identification of crude oil degrading organisms

Table 5 displayed the isolates' closest relatives in Gen Bank along with the isolations' specific location. Table 4 showed that two bacteria (C3 and D1) were isolated from an Awka soil sample, three organisms (GN2, K4, and I6) were isolated from Aguleri, and one organism was isolated from each of the Ekwulobia and Onitsha samples

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Table 5: 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	*Bacillus cereus* C12	1768	2999	94%	100%	MF800952	Awka
C3	*Pseudomonas aeruginosa* KAVK01	2745	2745	100%	100%	KC119195	Awka
L2	*Bacillus subtilis* SDDIas	1866	1866	100%	100%	HQ262546	Onitsha
J3	*Ochrobacterium intermedium* E85b	1291	1291	90%	96.79%	KM894187	Ekwulobia
H4	*Bacillus licheniformis 129*	2641	2641	100%	99.93%	KU922363	Onitsha
G2	*Bacillus cereus* So24	1829	1829	100%	100%	MG00925	Aguleri
K4	*Bacillus subtilis* LK4.5	1602	1602	95%	96.89%	KY083700	Aguleri
I6	*Enterobacter cloacae* GEBRI III	1555	1555	94%	97.20%	MH473593	Aguleri

Key: % = Percentage

Effects of nutrient supplements on remediation of crude oil by selected isolates

Figure 1 depicted the effects of nutrient supplementation on the remediation of crude oil by particular isolates. In particular, the amount of n-alkanes was measured and separated in order to determine the level of petroleum degradation in microcosms. The majority of the n-alkanes found in the recovered oils fell between n-C8 and n-C32. The total amount of n-alkanes recovered per treatment microcosm was compared to undegraded crude oil and uninoculated controls. This was calculated by adding up all of the identified n-alkanes. Through statistical analysis, the percentage of the oil substrate's degradation was ascertained. As a result, the soil that had been contaminated with 3% (v/w) crude oil, amended with inorganic salts, and inoculated with plasmid-borne mixed cultures had the highest degradation efficiency at 91%. The soil that had not been amended and had been treated with a consortium of plasmid-borne cultures had the lowest degradation efficiency, at 85% (Figure 1). Nonetheless, the unamended soil seeded with the consortium of plasmid-cured bacteria and the nutrient-amended soil treated with plasmid-cured mixed bacterial cultures had the lowest degradation efficiency, at 69% and65%, respectively (Figure 1). Over the course of the investigation, the control microcosm's degradation percentage was 3.5%. Differences between recovered TPH of treatments and controls were statistically identified using ANOVA and the Newman-Keuls pairwise test. Remarkably, an ANOVA showed that the combination of plasmid-borne and cured isolates considerably decreased the total amount of n-alkanes ($P < 0.05$). The total amount of n-alkane degraded by the plasmid-borne bacterial isolates in the experimental microcosms was found to be significantly higher ($P < 0.05$) than the total amount of n-alkane degraded by the consortium of plasmid-cured bacterial isolates, according to a pairwise comparison using the Newman-Keuls test. Nevertheless, there were no significant differences between the plasmid-borne and plasmid-cured isolates ($p = 0.12$) according to the results of the Newman-Keuls Pairwise comparison tests for differences in means values of the percentage degradations among the test isolates.

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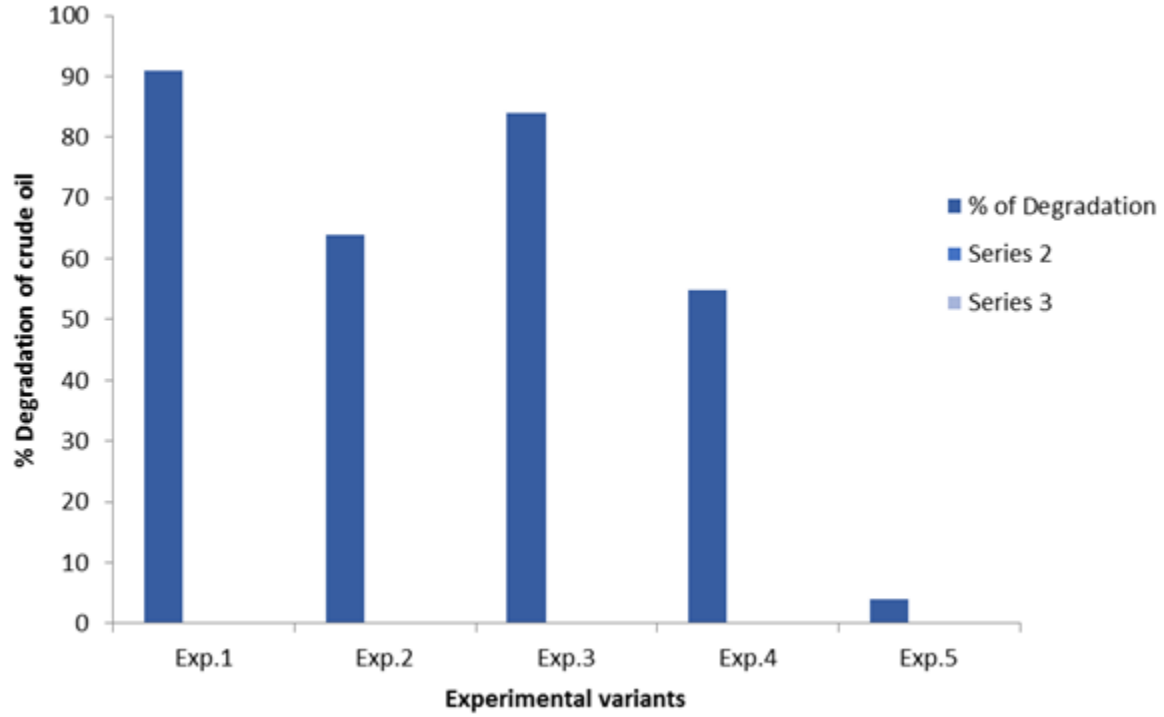

Figure 1: Effect of nutrient addition on degradation of crude oil in soil microcosm inoculated with mixed culture of *Pseudomonas aeruginosa and Ochrobacterium intermedium*

Key: Exp.1 = Mixed culture of plasmid borne bacteria + polluted soil + nutrient; Exp.2 = Mixed culture of plasmid cured bacteria + polluted soil + nutrient; Exp.3 = Mixed culture of plasmid borne bacteria + polluted soil without nutrient; Exp.4 = Mixed culture of plasmid cured bacteria + polluted soil without nutrient; Exp.5 = Un-inoculated control (Polluted soil only)

Bioaugmentation studies

Figure 2 indicated that the consortium of plasmid borne isolates enhanced the reduction of the crude oil from the initial concentration of 10,318 ppm to 501 ppm (95 %) whereas 64 % decontamination was facilitated by the consortium of plasmid cured isolates. The indigenous organisms in the uninoculated control soil microcosm had 21 % remediation percentage .On the individual capacities, plasmid borne *Pseudomonas aeroginosa* K0VK01 had greater effect on the oil remediation (80 %) than its plasmid free cells (62 %).Plasmid borne *Ochrobacterium intermedium* E85b improved hydrocarbon reduction (78 %) when compared with the 54 % crude oil reduction recorded by its plasmid cured cells. The mass of total n-alkanes were determined by the summation of individual n-alkane masses recovered from treatment soil microcosms, which was also compared with undegraded petroleum and control.

ANOVA and Newman- Keuls tests were used to statistically identify discrepancies between recovered TPH of treatments and controls. ANOVA revealed that the total n-alkanes were significantly ($P < 0.05$) reduced by both the consortium of plasmid borne and cured. Pair wise comparison using Newman-Keuls indicates that total degraded n-alkane by plasmid borne bacterial mixed cultures was significantly ($P < 0.05$) higher than the total n-alkanes degraded by plasmid cured bacterial mixed cultures. However, Newman-Keuls comparison tests for differences in mean values of the percentage degradations among the test isolates, revealed significant differences between the plasmid borne and plasmid cured isolates ($P < 0.05$) but no significant differences among the individual plasmid borne or plasmid cured isolates ($P = 0.12$).

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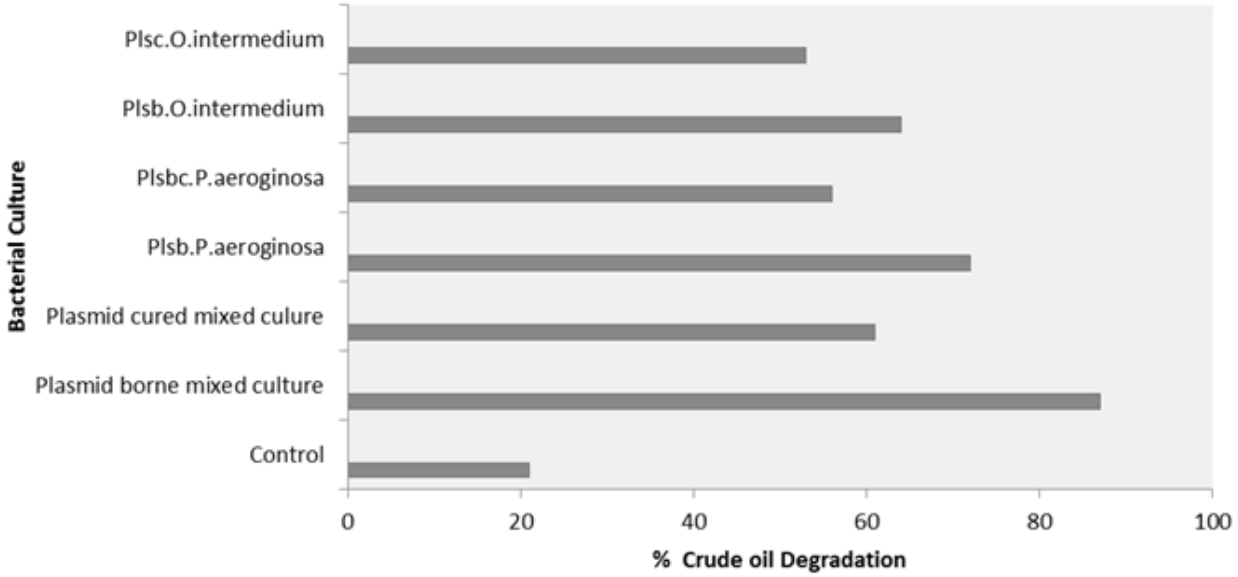

Figure 2: Bioaugumentation of crude oil contaminated soil with mixed culture and single culture of plasmid borne *Pseudomonas aeruginosa* and *Ochrobacterium intermedium*

NB: Plsb = Plasmid borne; Plsc = Plasmid cured

DISCUSSION

Microorganisms can adapt to live in harsh environments and are incredibly diverse. Microbes can break down a variety of complex compounds by altering their degradative enzyme system. Microorganisms are essential to the natural world because they participate in the elemental cycle of the geological formation and alter natural chemicals. Contaminated areas are home to a wide variety of microbiota species that can use the contaminant as a source of energy and carbon (Okore *et al*., 2021).

In this study, the biodegradation of Bonny light crude oil by plasmid and non – plasmid borne soil bacterial strains using biostimulation and bioaugmentation techniques was evaluated. Following physico-chemical analysis of both pristine and contaminated soil, Table 2 presented the findings regarding the impact of petroleum hydrocarbons on the soil ecosystem. For hydrocarbon-contaminated soils, the average pH was found to be 7.2, whereas the pH of pristine soil was 7.11. While the pH of the soils under investigation remained unchanged, the contaminated soil samples had a higher electrical conductivity (0.39 to 0.44 µS/cm) than the uncontaminated samples. This difference in conductivity could be attributed to the presence of metal or other ions, as indicated by Table 2. This finding aligned with the research conducted by Pathak *et al*. (2011), who found that soil contaminated with hydrocarbons had higher electrical conductivity than pristine soil and attributed this to the presence of metal or other ions in the contaminated soil. The presence of hydrocarbons in the contaminated soil samples may have contributed to the significantly higher organic carbon content in all of the hydrocarbon-contaminated soil samples compared to uncontaminated soil. This was in line with the research report by Uche *et al*. (2011), which claimed that harmful effects of petroleum hydrocarbon (PHC) pollution were seen in plants, microorganisms, and soil conditions. Degradation of soil structure, loss of organic matter contents, and loss of soil mineral nutrients like potassium, sodium, calcium, magnesium, nitrogen, sulphate, phosphate, and nitrate are all caused by hydrocarbon pollution in the soil (Akubugwo *et al*., 2009). The soil samples were found to have lower levels of available phosphorus and nitrogen than uncontaminated soil. In PHC-polluted environments, lower concentrations of phosphorous, nitrogen, and other mineral nutrients have been identified as growth-limiting factors for microorganisms (Rahman et al., 2002). In addition, PHC-contaminated soils had lower water-holding capacities than pure soil, which is a measure of the soil's ability to retain water and aerate it (Table 2). According to Osuji and Nwoye (2007), PHC increases the soil's hydrophobicity, which lowers the soil's ability to hold water. These two characteristics are necessary for the biotic components in the soil to grow.

Additionally, from mineral salt agar medium supplemented with crude oil, sixty hydrocarbon-utilizing bacteria were isolated (Table 3). Other authors have also reported that the bacteria in this study, which were all isolated from a soil sample contaminated by petroleum, readily flourished in the media supplemented with oil (Rahman et al., 2003). Table 3 showed that because of their capacity to use crude oil, 22 of the isolates were found to have high pollutant degrading potentials (A600nm > 0.3). An indicator of the potential for PHC biodegradation has been the absorbance (A600nm) of cells cultured in a medium containing petroleum as the only carbon source (Ciric *et al*., 2010; Husain *et al*., 2011). The other isolates showed variations in their growth on the substrate and the isolate with least growth has optical density of 0.11. Ciric *et al*. (2010) had differentiated growth of the alkane degraders based on A_{600nm} using the following criteria: No growth = A_{600nm} 0.00–0.019; +, minimal growth = A_{600nm} 0.02–0.099; ++, moderate

growth = A_{600nm} 0.1–0.2; +++, maximum growth = A_{600nm} > 0.2. 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. Vinothini *et al*. (2015) reported optical density of 0.55 by *Pseudomonas putida* which crude oil degradation ability was screened based on the growth efficiency on 2 % crude oil at the 7th day of incubation period.

The results of the blasted sequences in Table 4 showed that the isolates belonged to a number of species, including *Bacillus cereus* C12 (which had a 100% sequence similarity), *Pseudomonas aeruginosa* KAVK01 (100% sequence similarity), *Bacillus licheniformis* 126 (96% sequence similarity), *Ochrobacterium intermedium* E85b (97% sequence similarity), *Bacillus subtillis* SDDlas (which had a 100% sequence similarity), *Bacillus subtillis* LK4.5 (97% sequence similarity), *Enterobacter cloacae* GEBRI III (97% sequence similarity), and *Bacillus cereus* So24. For the forward and reverse reactions, primers 27F and 1492R were utilized, respectively. According to Macrogen (2013), the two primers were the most highly ranked bacterial universal primers. In their critical evaluation of these two primers for the bacterial 16S rRNA gene, Frank *et al*. (2008) found that the formulations of 27F and 1492R were straightforward. There was very little reduction in the overall amplification efficiency and specificity when seven different primer sequences were compared. Rajesh (2017) isolated *Pseudomonas* sp. strain IR1 from Chandigarh, India's vehicle service station site, and cultured it in BSM broth with 0.1% w/v naphthalene added as a substrate. In 2015, Eraky and colleagues isolated *Ochrobactrum* sp., which was identified as a biodegradation agent for BTEX hydrocarbons. According to Chai *et al*. (2015), an environmental isolate of *Ochrobactrum intermedium* has the ability to break down crude oil. Ikuesan *et al*. (2016) found that while Gram negative bacteria predominate in all of the samples, both Gram positive and Gram negative bacteria can degrade crude oil. The results of this study support the findings of Salam *et al.* (2011), who reported that the mineralization of hydrocarbon pollutants has been linked to both Gram positive and Gram negative bacteria.

Figure 1 depicted the effects of nutrient supplementation on remediation of the crude oil by the co-cultures of the selected isolates. The remediation percentages of the oil contaminated soil occasioned by the stimulation of the bacterial cells with inorganic salt was significantly ($P < 0.05$) higher in soil treated with plasmid borne cell than that treated with plasmid cured cells. This is in agreement with Breedveld and Sparrevik (2012) which reported that low inorganic nutrient concentrations in soil could reduce the number of active PAHs-degrading bacteria due to an insufficient level of nitrogen (N) and phosphorus (P) and amendment of soil with inorganic N and P enhances the level of PAHs degradation. The results have clearly indicated that inorganic salts play a vital role in bioremediation of PAHs contaminated soil by plasmid borne organisms; which may be due to the availability of sufficient nutrients for the metabolic activity of organisms. Kumar *et al*. (2010) reported that degradation activity of the isolate was diminished after plasmid curing with acridine orange. The total extent of crude biodegradation was 22 % higher in soil inoculated with plasmid borne co-cultures amended with inorganic salt than soil amended with plasmid cured strains and 6 % higher in un-amended soil inoculated with mixed cultures of plasmid borne bacterial cultures. Efficiency of plasmid borne organism to degrade crude oil suggests that plasmid possesses a substantial potential for the cleanup of pollutant. This result clearly indicates that both the genomic and the plasmid DNA of the strain have their more or less contributions in hydrocarbon degradation.

The potential of the bacterial isolates to augment the remediation of the crude oil was shown in Figure 2. It was observed that the consortium of plasmid borne isolates enhanced the reduction of the crude oil from the initial concentration of 10318 ppm to 501 ppm (95 %) whereas 64 % decontamination was facilitated by the consortium of plasmid cured isolates. The indigenous organisms in the uninoculated control soil microcosm had 21 % remediation percentage (Figure 2). Isiodu *et al*. (2016) documented efficient remediation of crude oil by both cured and uncured bacterial consortium and emphasized that uncured treatment had slightly more percentage of the crude oil degraded, 96.98 % loss of the PAH against 95.31 % loss of the PAH in the cured treatments. This means that the removal of plasmid from the consortium did not result in the loss of biodegradation potential but however led to slightly decreased degradation. Akpe *et al*. (2013) observed that loss of plasmids by *Klebsiella pneumoniae* and *Serratia marscencens* did not lead to complete loss of their degradative abilities. It only resulted in reduction in their degradation potential. Leahy and Colwell (1990) reported that exposure of natural microbial populations to oil or other hydrocarbons may impose a selective advantage to strains possessing plasmids encoding enzymes for hydrocarbon catabolism. Plasmid generally carries genes that confer a selective advantage to their host in a specific environment; they increase access to horizontal gene pool for adaptive traits that may be important in the overall physiology and survival of many bacteria (Obayori and Salam, 2010). Mirdamadian *et al*. (2010) reported that the catabolic gene which encode degradation routes of different aromatic and aliphatic hydrocarbons are frequently located on plasmids, although degradative genes can be located on either chromosome or plasmid. Different strategies to improve the remediation of crude oil contaminated environment have been reported by previous authors. Varjani *et al*. (2015) reported that *Stenotrophomonas maltophilia* was able to effectively degrade crude oil when used in consortium with *Pseudomonas aeruginosa* and *Ochrobactrum* sp. Kavynifard *et al*. (2016) reported that introduction of consortium of various bacterial genera leads to increase in gene diversity, which results in increase in the ability of consortium to consume more different compounds. According to other studies by Gharibzahedi *et al.,*2014; Bihari *et al*., 2011; consortium of *D. cinnamea* KA1 and *D. cinnamea* AP had a suitable efficiency in degrading a broad range of compounds contained in crude oil as sole carbon and energy source (97.26 %) in a shorter period (5 days). Wang *et al*. (2011) reported a novel *Dietzia* strain DQ12-45-1b, which was capable of using a broad range crude oil components, but the strain needed a long period of time (8 – 34 days). Furthermore, compared to other *Dietzia* species, consortium of *D. cinnamea* KA1 and *D. cinnamea* AP could use broader range crude oil components as the sole carbon sources, including saturated fraction, resins, and aromatic hydrocarbon in a shorter period (Wang *et al*., 2011).

Conclusion

On the whole study, the crude oil contaminated soils are good habitats for potent hydrocarbon degraders of the genera *Bacillus*

spp., *Pseudomonas aeruginosa* KAVK01 *and Ochrobacterium* E85b *strains.* Moreso, an enhanced bioremediation of crude oil polluted soil was achieved in the combined treatment of bacterial consortia supplemented with nutrients. This study raised very strong hope for cleanup of oil spill with reduced cost. The bacterial species isolated in this work have shown to be good degraders of crude oil. These bacteria could show greater biodegradating ability with further studies, since their degradative dependency (chromosome and plasmid) have been established in this study. The enhanced remediative potentials of the plasmid borne isolates suggested that there is complementary action by the plasmid DNA and chromosomal DNA. Therefore, degradation required an interaction between chromosomal and plasmid genes. The plasmid of *Pseudomonas aeruginosa* KAVK01 *and Ochrobacterium intermedium* E85b could be used in recombinant DNA technology to develop bacteria cell with potentials to effectively degrade crude oil. The information from this study can help in the selection of bacterial species, most especially plasmid borne bacteria that can be used for the bio-degradation of crude oil contaminated soil in the Niger Delta region of Nigeria and sample collection sites.

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