



Comparative Evaluation of Crude oil Degradability Efficiency of *Bacillus amyloliquefaciens* and *Comamonas testosteroni* in Soil

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Authors' contributions

This work was carried out in collaboration among all authors. Author VGA designed the study and performed the statistical analysis. Authors NPA, SID and VGA wrote the protocol and wrote the first draft of the manuscript managed the analyses of the study and literature searches under the strict supervision of author DNO. All authors read and approved the final manuscript.

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ABSTRACT

Comparative evaluation of crude oil degradability efficiency of *Bacillus amyloliquefaciens* and *Comamonas testosteroni* with nutrient amendment were investigated in Crude oil Contaminated Soil. The bacteria species used in this study were isolated from the soil collected from Rivers state university research farm using standard microbiological methods. Evaluation and monitoring of bioremediation were done for a period of 56 days while analyses were carried at a constant interval of seven (7) days. Seven (7) experimental set-ups were employed using black polythene bag, The bags were perforated to enhance aeration, each containing 5 kg of agricultural soil and left to fallow for 6 days, on the seventh day each of the experimental set-ups (5kg of soil) except the control (CTRL) were contaminated with crude oil (COCS) giving initial Total Petroleum Hydrocarbon (TPH) value of 10328.03 mg/kg; after which it was allowed for 21 days to ensure even distribution and soil-oil bonding to mimic crude oil spill site before application of augmenting bacteria; *Bacillus amyloliquefaciens* (BC) and *Comamonas testosteroni* (CM) and the stimulant; Goat manure (GM). Soil profile before and after contamination were analyzed while parameters like Sulphate, pH, Nitrate and Total Petroleum Hydrocarbon (TPH), as well as microbial analyses

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such as Total Heterotrophic Bacteria (THB), Total Heterotrophic Fungi (THF), Hydrocarbon Utilizing Bacteria (HUB) and Hydrocarbon Utilizing Fungi (HUF) were monitored and evaluated throughout the experimental period. Bioremediation efficiency was estimated from percentage (%) reduction of Total Petroleum Hydrocarbon (TPH) from day 1 to the residual hydrocarbon at day 56 of bioaugmented/biostimulation set-ups with the control. The microbiological result of the soil before and after contamination revealed that *Bacillus sp* had the highest percentage for bacterial isolates while *Mucor sp.* had the highest percentage for fungal isolates in both uncontaminated and contaminated soil. Colonial count of uncontaminated soil ranged from 5×10^4 (HUB) $< 7 \times 10^4$ (HUF) $< 1.6 \times 10^5$ (THF) to 2.58×10^8 (THB) while contaminated soil colonial count ranged from 8×10^4 (HUB) $< 9 \times 10^4$ (HUF) $< 2.0 \times 10^5$ (THF) to 2.10×10^8 (THB). Microbial evaluation of the bioremediation set-ups showed increased colonial values with increase in time but slightly decreased on the last day. Results of total petroleum hydrocarbon revealed the actual amount of hydrocarbon reduction after the experiment and its percentage hydrocarbon remediated from the initial concentration in the various treatment setups in the following decreasing order; (CTRL) 125.71 mg/kg; 1.21% $<$ (COCS + BC) 1855.74 mg/kg; 17.96% $<$ (COCS + CM) 2261.01 mg/kg; 21.89% $<$ (COCS + CM + BC) 3321.23 mg/kg; 32.15% $<$ (COCS + GM + BC) 4983.81 mg/kg; 48.25% $<$ (COCS + GM + CM) 7313.47 mg/kg; 70.81%. Conclusively, the results obtained indicate that *Comamonas testosteroni* with nutrient amendment had the more degradability efficiency compared to *Bacillus amyloliquefaciens*. It is therefore recommended that bioremediation crude oil polluted soil using bioaugmentation technique should be amended with organic nutrient to enhance efficiency.

Keywords: Comparative; crude oil degradability; *Bacillus amyloliquefaciens*; *Comamonas testosteroni*; soil.

1. INTRODUCTION

Crude oil has proven to be a vital economic resource for oil producing nations and has ignited fierce competition among nations [1]. Indeed, anthropogenic activity is reliant on oil to meet its energy demands, which causes the petrochemical industry to flourish. However, the use of crude oil and its derivatives results in environmental deterioration [2]. Pollution of soil environment by hydrocarbons has become prevalent over the years across the globe. This has been linked directly to increased dependence on crude and its products as a major source of energy throughout the world, rapid industrialization, population growth and complete disregard for the environmental health. The stressed environment can proffer solution to the production of bioactive substance like enzymes which are of great industrial purposes and contributes to the local content value of Nigeria economy [3].

The volume of natural crude oil introduced into the environment was estimated to be about 600,000 metric tons per year with a range of uncertainty of 200,000 metric tons per year [4]. Release of hydrocarbons into the environment whether accidentally or due to human activities is a main cause of water and soil pollution [4].

Hydrocarbon pollutants usually cause disruptions of natural equilibrium between the living species and their natural environment. Hydrocarbon components have been known to belong to the family of carcinogens and neurotoxic organic pollutants [5]. Oil spills during the refining, transportation, storage of crude oil and its products ought to be recycled or eliminated to a great degree as possible, but in some cases, it is difficult to recover the spilled materials, its remaining results in contaminating the affected areas and posing persistent risks to the environment. Although pollution is a difficult matter and can be very costly when using conventional methods of oil clean-up such as; use of dispersants, manual removal, use of chemicals, burning, cutting of vegetation, passive collection sorbents, debris removal, trenching, removal of sediment, slurry and blasting and others [6]. Petroleum hydrocarbon-degrading microorganisms have evolved as a result of existing in close proximity to naturally occurring petroleum hydrocarbons in the environment. Such organisms are candidates for the treatment of oil pollutants [7,8].

Due to the detrimental drawbacks of oil spills incidents which are becoming more rampant in developing countries scientists, researchers and environmentalists have developed new methods and forms of technology to facilitate the tasks of cleaning up oil spills [9]. One promising treatment

method is to exploit the ability of microorganisms to remove these organic pollutants from contaminated sites [10]. According to Finley et al. [10], it is an alternative treatment strategy that is effective, minimally hazardous, economical, versatile and environment-friendly in the clean-up of pollutants from the environments.

The ability of microorganism to transform organic molecules into another form is termed microbial remediation; the mechanism of this process is that the microbial organisms transform the substance through metabolic or enzymatic processes. It is based on two processes: growth and cometabolism. In growth, an organic pollutant is used as sole source of carbon and energy. This process results in a complete degradation (mineralization) of organic pollutants and can also be referred to as mineralization. It is a transformation which may proceed to the total conversion of organic molecules to simple inorganic compounds like carbon dioxide, water and biomass [11]. In cometabolism, these microorganisms utilize organic compounds for their metabolism and hence serve as an alternative to chemical remediation which over the years has been linked to several toxic constraints and reduction in microbial diversity. Bioremediation methods are currently receiving favorable publicity as promising environmental friendly treatment technologies for the remediation of hydrocarbons [12].

Moreover, biological methods can have an edge over the physicochemical treatment regimes in removing spills as they offer cost effective *in-situ* biodegradation of oil fractions by the microorganisms [13]. According to Ogbonna [11], bioremediation offers an alternative method to detoxify contaminants and is being used as an effective means of mitigating hydrocarbons, halogenated organic solvents and compounds, non-chlorinated pesticides and herbicides, nitrogen compounds, metals (lead, mercury, chromium) and radionuclides. Bioremediation functions basically on biodegradation, which involves complete mineralization of organic contaminants into carbon dioxide, water, inorganic compounds, and cell protein or transformation of complex organic contaminants to other simpler organic compounds by biological agents like microorganisms [11].

Comamonas testosteroni is a gram-negative, aerobic, motile, pink-pigmented, oxidase-positive bacilli. This organism is called *testosteroni* because it can grow on media containing *testosteroni* as the sole carbon source [14]. *C.*

testosteroni rarely infects humans and commonly lives in environments such as soil, water, plants, and animals. *Comamonas testosteroni* is previously known as *Pseudomonas testosteroni*. *Comamonas testosteroni* has also been routinely reported for bioconversion of different steroids and heavy metal removal and have also shown to possess the capability of polycyclic aromatic hydrocarbons (pahs) degradation [15]. *Bacillus amyloliquefaciens* is a ubiquitous gram-positive bacterium and highly successful in colonising a diversity of environments [13]. *Bacillus* species groups of microorganisms have been found to show appreciable numerical increase in hydrocarbon polluted sites and have also been reported as a core petroleum hydrocarbon degrader [13]. Hence, this paper therefore focuses on the comparative evaluation of degradability potential of *Bacillus amyloliquefaciens* and *Comamonas testosteroni* in crude oil contaminated soil.

2. MATERIALS AND METHODS

2.1 Study Area

The Rivers State University research farmland in Nkpolu-Oroworukwo, Mile 3 Diobu area of Port Harcourt, Rivers State was used for this study. The experimental design was cited on the portion of land situated at Longitude 4°48'18.50"N and Latitude 6°58'39.12"E.

2.2 Experimental Setup and Application of Crude Oil

Seven (7) experimental setups were employed using black polythene bag. The bags were perforated to enhance aeration [16], each containing 5 kg of agricultural soil and left to fallow for 6 days, on the seventh day each of the experimental set-up except the control were contaminated with 10% crude oil giving initial Total Petroleum Hydrocarbon (TPH) value of 10328.03 mg/kg, after which it was allowed for 21 days to ensure even distribution and soil-oil bonding to mimic crude oil spill site before application of augmenting bacteria and the stimulant ((Goat manure) [17].

2.3 Source of Bacteria

The bacteria used in this study were: *Bacillus amyloliquefaciens* MN273757 and *Comamonas testosteroni* MN273753 isolated using standard microbiological methods from the top 5cm of homogeneously soil sample collected from the study site. The two distinct colonies with varying

cultural characteristics, suspected to be *Bacillus* and *Comamonas* species were picked and sub-cultured onto freshly prepared nutrient agar plates incubated at 37°C for 24 hours. The bacteria were then identified using biochemical tests including: Gram staining reactions, coagulase test, oxidase test, spore test, motility test, indole test, methyl red test, vogues proskauer test, citrate utilization test, sugar fermentation test (sucrose, lactose and glucose). The bacterial isolates were also identified by comparing their characteristics with those of known taxa and molecular characterization as described by Cheesbrough [16]. Pure cultures obtained were inoculated onto nutrient broth in 500 ml Erlenmeyer flask loosely plugged with sterile cotton wool for the growth of the augmenting test organisms. Broth cultures adjusted to 0.5 McFarland standards were then used for augmentation.

2.4 Application of Bioaugmenting Microbes and Nutrient Amendment for Biostimulation on Experimental Setups

Two hundred and fifty millilitres (250 ml) of the broth cultured bacterial isolates were added to each setup except the controls. These were properly stirred with a sterile spatula to ensure the microorganisms thrive and have sufficient oxygen. Four (4) litres of water was added to each setup weekly, tilted slightly to enhance moisture content and microbial activity [17,18]. Illustrative representations of the experimental plots are shown in Table 1.

2.5 Sampling Methods and Monitoring of Bioremediation

From each plot, 8-12 random points from 0-15 cm were collected to form a composite sample after tilling using soil spatula. Small portions measuring 5 g of the composite samples were

collected into sterile bottles using a sterile spatula for microbiological and physiochemical analysis. Sampling and monitoring were done at a constant interval of seven days for 56 days after contamination of the various setups (at day 7, 14, 21, 28, 35, 42, 49 and 56) [15].

2.6 Microbiological Analyses

2.6.1 Isolation and enumeration of total heterotrophic bacterial

Total heterotrophic bacteria sample were enumerated using spread plate technique as described by Prescott [19]. An aliquot (0.1 ml) of the dilution 10^{-7} dilution was aseptically transferred unto properly dried nutrient agar plates in duplicate, spread evenly using bent glass rod and incubate at 37°C for 24 hour, after incubation, the bacterial colonies that grew on the plates were counted and sub-cultured unto fresh nutrient agar plate using the streak plate technique. Discrete colonies on the plates were aseptically transferred into 10% (v/v) glycerol suspension, well label and stored as stock cultures for preservation and identification [20]. Total Heterotrophic Bacteria (THB) Counts were calculated from the mean value of colonies counted from the duplicate plates using the below formula:

$$\text{THB (cfu/g)} = \frac{\text{Number of Colonies}}{\text{Dilution (10}^{-7}\text{) x Volume plated (0.1ml) Prescott}} [19]$$

2.6.2 Isolation and enumeration of total heterotrophic fungal

The total Heterotrophic fungi were enumerated using spread plate method as described by Prescott, [19]. An aliquot (0.1 ml) of the dilution of 10^{-3} dilution was aseptically transferred unto properly dried Sabouraud Dextrose Agar plates containing antibiotic (tetracycline and penicillin) to inhibit bacterial growth in duplicate [15].

Table 1. Illustrative experimental layout for bioremediation setup

S/N	Treatment	5 kg soil	Crude oil	250 ml broth of <i>Bacillus</i> sp.	250 ml broth of <i>Comamonas</i> sp.	Goat manure
1	UCS (CTRL 1)	+	-	-	-	-
2	COCS (CTRL 2)	+	+	-	-	-
3	COCS + BC	+	+	+	-	-
4	COCS + CM	+	+	-	+	-
5	COCS + GM + CM	+	+	-	+	+
6	COCS + GM + BC	+	+	+	-	+
7	COCS + CM + BC	+	+	+	+	-

key: UCS = Uncontaminated soil, COCS = crude oil contaminated soil, BC = *Bacillus amyloliquefaciens*. CM = *Comamonas testosteroni*, GM = Goat manure

Plates were spread evenly using bent glass rod and incubated at 28°C for 3 days. Fungal spores were sub-cultured onto Sabouraud Dextrose Agar slant in bijou bottle for preservation [21]. Total Heterotrophic Bacteria (THB) Counts were calculated from the mean value of colonies counted from the duplicate plates using the below formula:

$$\text{THF (cfu/g)} = \frac{\text{Number of Colonies}}{\text{Dilution (10}^{-3}\text{) x Volume plated (0.1ml)}} [19]$$

2.6.3 Isolation and enumeration of hydrocarbon utilizing bacterial count

The population of hydrocarbon utilizing bacteria was determined by inoculating 0.1 ml aliquot of the serially diluted samples onto mineral salt agar media as shown in (Table 2) using vapour phase transfer method according to Brusseau, [22]. The mineral salt agar used for enumeration of hydrocarbon utilizing bacteria was amended with fungisol (Miconazole Nitrate B.P. 2%) [23]. The plates were inverted and incubated at 28°C for 5 days. The filter paper saturated with sterile crude oil served as the sole source of carbon in the mineral salt agar. Colonies formed in the duplicate plates were counted and the mean values were recorded and expressed as colony forming unit (cfu/g).

2.6.4 Isolation and enumeration of hydrocarbon utilizing fungal count

The population of hydrocarbon utilizing fungi was determined by inoculating 0.1 ml aliquot of the serially diluted samples onto mineral salt agar media using vapour phase transfer method according to Odokuma [21]. For hydrocarbon utilizing fungi the mineral salt medium used was amended with 250mg of tetracycline to inhibit the growth of hydrocarbon utilizing bacteria. The plates were inverted and incubated at 28°C for 5 days. The counts of fungi were expressed and recorded as colony forming unit per gram (cfu/g) [19].

2.7 Determination of Percentage Occurrence of the Isolates

This was done to determine the incidence of occurrence of the different isolates. The frequency of occurrence of the isolates from the soil was determined. The total number of each isolate in the soil sample was obtained against the total number of all the isolates in the sample screened. The mean value of this yielded the percentage of occurrence using the following equation:

$$\% \text{ of occurrence} = X/N \times 100;$$

Where;

X = Total number of each isolate in the sample and N = Total number of all the isolates in the sample [19].

2.8 Physiochemical Analyses of the Treated Set-ups

2.8.1 Determination of pH

The pH of soil sample was determined using a pH meter (H-19811-5, Romania). The meter was switched on and allowed for some time. It was then calibrated with buffer solutions of higher pH range between 8 and 9 as well as a lower pH range between 1 and 6 by dipping the electrode into the buffer solutions as produced by the manufacturer. Ten grams of soil was weighed into 100 ml beaker, 25 mls of distilled water was then added to allow immersion of the electrode, mixing was carried out by stirring frequently for few minutes. Then beaker was allowed to stand for 15 minutes. The electrode was immersed into the slurry and the pH values of the samples were recorded accordingly [24,25].

2.8.2 Determination of temperature

The Temperature for each sample was determined using a thermometer (H-19811-5, Romania). The thermometer was immersed into the samples such that the mercury bulb was well covered by the samples. The final readings were considered and the actual readings were taken after it was allowed to stabilize [25].

2.8.3 Determination of nitrate

The nitrate levels for the samples were determined using an ultraviolet (UV) spectrophotometer method. Five grams of samples were weighed into a shaking bottle. 125 ml of distilled water was added and shaken for 10 minutes on a rotary shaker and then filtered to obtain the extract, 1 ml of the extract was transferred into 10ml volumetric flask, 0.5 ml of Brucine reagent was added. Subsequently, 2ml of concentrated sulphuric acid was rapidly added and mixed for about 30 seconds. The flasks were allowed to stand for 5minutes. Two milliliters of distilled water was then added and mixed for about 30 seconds. Flasks were allowed to stand in cold water for about 15 minutes. The absorbance of the samples was measured using the spectrophotometer (S-HP10012414-50, 721D-UK) at wavelength of 470 nm [24,25].

2.8.4 Determination of phosphate

The phosphate levels were determined using the sulphuric acid - nitric acid digestion method. Twenty five milliliters of 2.5% Acetic acid was added to 1 g of sample and shaken for 30 minutes. The suspension was filtered through a filter paper after which 10 ml of the extract was transferred into 50 ml volumetric flask. Extract was diluted with distilled water until the flask was about two third full. 2 ml of ammonium molybdate reagent and 2 ml of stannous chloride were added and mixed, the solution was diluted to 50 ml mark with distilled water. The flask was allowed to stand for 30minutes and the absorbance was measured at wavelength of 690nm using a UV Spectrophotometer (S-HP10012414-50, 72ID-UK) [25].

2.8.5 Determination of sulphate

The sulphate levels of the samples were determined. Twenty five milliliters of the extracting solution was added to 5 g of sample and shaken for 30 minutes and the suspension was filtered through a filter paper. 5ml of the extract was transferred into 50ml volumetric flask. 5 ml of 50% acetic acid and 1ml of H_3PO_4 were added and mixed. The solution was diluted with distilled water to about $\frac{3}{4}$ full of the flask. One gram of barium chloride was added and mixed. The solution was left to stand for 10 minutes, 1 ml of 0.5% gum acacia was added to the solution and made up to 50 ml with distilled water. The absorbance of each sample was measured at 425 nm using UV Spectrophotometer (S-HP10012414-50, 72ID-UK) [25].

2.8.6 Determination of heavy metals (calcium, magnesium, potassium and sodium)

Heavy metals including calcium, magnesium, potassium and sodium were determined. The Atomic Absorption Spectrophotometric (AAS) method by APHA (2005) was adopted. The equipment absorbs the digested sample and gives the concentration of the metals present in the sample. A hollow cathode lamp for the desired metal was installed in the AAS (201VGP-UCK) and the wavelength dial was set accordingly. The slit width was also set for the element to be measured. It was then turned on and allowed to warm up until the energy source was stabilized. The current was readjusted as required after it was warmed up and the wave length was optimized such that optimum energy

gain was obtained, the lamp was then aligned accordingly. A suitable burner head was installed and position was adjusted to the equipment. Air was turned on and the flow rate adjusted such that maximum sensitivity for the metal being measured was obtained. Acetylene was turned on and flame ignited. Standard solution was aspirated. The absorbance of the standards were recorded. At least three concentrations of each standard metal solution were selected to bracket the expected metal concentration of the sample, each in turn was aspirated into flame and the absorbance recorded. One gram of the soil sample was transferred into a Kjeldahl flask, 20 ml of concentrated nitric acid (HNO_3) was added and the sample pre-digested by heating gently for 20mins. More acid was thereafter added and digestion was continued for 30-40mins. Digestion was stopped when a clear digest was obtained. The flask was cooled and the content transferred into 50 ml volumetric flask and made to the mark with distilled water. The resulting solution was analyzed for accordingly for the respective heavy metals. Concentrations of each metal ion were calculated in milligrams per litre by referring to the appropriate calibration curve accordingly.

2.8.7 Determination of Total Petroleum Hydrocarbon (TPH)

Total Petroleum Hydrocarbon (TPH) of the experimental setups were analysed according to APHA standard methods (APHA) [25] at constant interval of 7 days for 56 days. The soil samples were extracted using a gas chromatograph (HP 5890, Hewlett Packard and Avondale, USA) equipped with a flame ionization detector (FID) and column (30 m by 0.2 mm) with helium carrier gas, hydrogen gas, air flow at flow rates of 2 ml/min, 30 ml/ min and 300 ml/min. The oven temperature was programmed from 50°C for 10 min to 340°C for 20 min. Retaining this temperature for 10 min, the residual TPH in the different treatment setups were extracted with 40 ul of n-pentane (HPLC grade) by sonicating the samples 5min at each extraction for 3 times. The pentane extract was centrifuged at 3000 g for 5 min, the three organic phases were oven dried over anhydrous sodium sulphate (Na_2SO_4), pooled and adjusted to 150 ml after which 32ul of cumene (isopropyl benzene) was added as internal standard. Analyses were carried out using a GC-FID (Avondale, USA). The extractable TPH was identified and quantified by comparison with a sample chromatogram with standard calibration [25].

2.9 Percentage (%) Bioremediation Evaluation

The percentage (%) bioremediation rate was calculated from the formula adopted by Nrior and Echezolom [15] as follows:

Step 1: Amount of total petroleum hydrocarbon remediated is equals to Initial concentration of TPH (mg/kg (Day 1) minus final concentration of pollutant at end of experiment (last day).

Step 2: Percentage (%) bioremediation equals to amount of oil and grease remediated divided by initial concentration of pollutant (Day 1) multiplied by 100.

Thus;

$$B_c = I_c - F_c$$

$$B_x = I_c - I_o$$

$$\% \text{ Bioremediation} = \frac{B_c}{B_x} \times 100$$

Where;

B_c = amount of Hydrocarbon degraded

I_c = initial concentration of Hydrocarbon at the beginning of experiment (Day 7)

F_c = final concentration of hydrocarbon at the end of experiment (day 56)

I_o = initial concentration value of control at day 7

B_x = actual amount of hydrocarbon in the setup [15]

2.10 Statistical Analysis

The data obtained during the study was analyzed statistically using a computer based program, SPSS version 22 for analysis of variance (ANOVA) of the data in the respective setup.

3. RESULTS AND DISCUSSION

The Microbial status of soil samples which include; total heterotrophic bacteria (THB), total fungi (TF), hydrocarbon utilizing bacteria (HUB) and hydrocarbon utilizing fungi (HUF) as well as physiochemical status including: nitrate, phosphate, sulphate, pH, temperature, electrical conductivity, moisture content, total petroleum hydrocarbon (TPH), calcium, sodium,

magnesium, and potassium were determined before and after contamination in order to characterize the indigenous organisms present in the soil and also their percentage of occurrence. The results of the indigenous organisms and their percentage of occurrence in the soil before and after contamination are presented in Figs. 1-4 while Table 2 shows the results of the physiochemical status of the soils. The result microbiological analysis carried on the soil before and after contamination revealed that *Bacillus* sp. had the highest percentage for bacterial isolates while *Mucor* sp. had the highest percentage for fungal isolates in both uncontaminated and contaminated soil, colonial count of uncontaminated ranged from 5×10^4 (HUB) $< 7 \times 10^4$ (HUF) $< 1.6 \times 10^5$ (THF) to 2.58×10^8 (THB), while contaminated soil colonial count ranged from 8×10^4 (HUB) $< 9 \times 10^4$ (HUF) $< 2.0 \times 10^5$ (THF) to 2.10×10^8 (THB). Microbial evaluation of the bioremediation set-ups showed increased in colonial values with increase time but slightly decreased on the last day.

Results of physiochemical analyses of the soil before and after contamination are presented in (Table 2). The pH, nitrate, calcium and magnesium values decreased after contamination. following physicochemical parameters; temperature, moisture content, electrical conductivity, sulphate, phosphate, potassium and sodium increased slightly after crude oil contamination while TPH concentration increased drastically from 88.1 mg/kg to 10328.03 mg/kg after contamination with crude oil. This is above the intervention value of 5,000 mg/kg as recommended by Department of Petroleum Resources (DPR) standard [24].

It has been established that the physicochemical features of soil can be affected by the type of soil (unpolluted or polluted) due to the quantity of petroleum content and the extent of pollution [22]. Hence, the physicochemical parameters of the uncontaminated and contaminated soil samples were estimated and compared statistically. In this study, five out of the physicochemical parameters investigated showed significant variation between the uncontaminated and contaminated soil samples. From the results of soil nutrients analyzed, nitrate (mg/kg) was significantly higher ($P < 0.0001$) in the uncontaminated soil (811.5 ± 0.70) than the contaminated soil sample (791.5 ± 0.65) also magnesium level was significantly higher

($p=0.0327$) in the uncontaminated soil (3.38 ± 0.07 mg/kg) than the contaminated soil sample (3.18 ± 0.07 mg/kg). This could be attributed to the fact that the higher number of hydrocarbon utilizing bacteria and hydrocarbon utilizing fungi as evidenced in the microbial counts of the

contaminated soil could be using up some of the essential nutrients in the soil. This report is in accordance with Chaillan, [23] who reported higher concentration of magnesium in soil sample collected from an unpolluted site than in the soil collected from a crude oil polluted site.

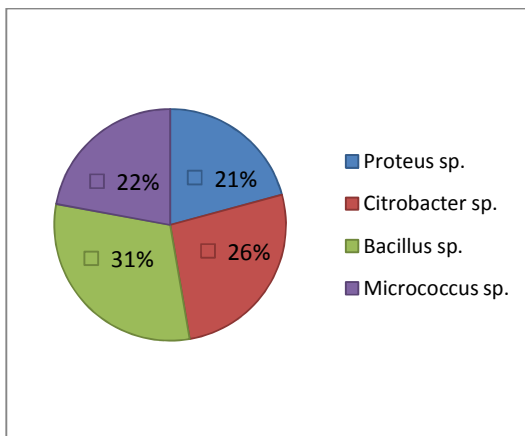


Fig. 1. Percentage occurrence of bacterial Isolates in the uncontaminated soil

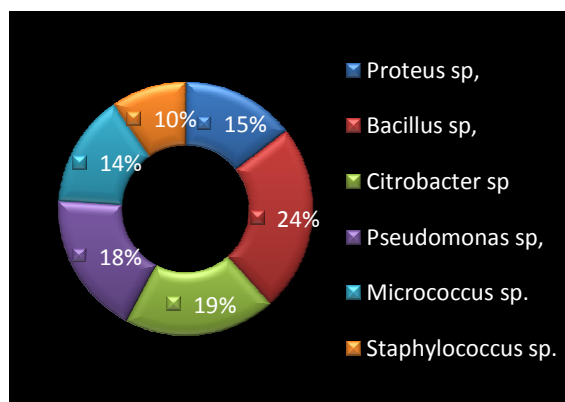


Fig. 2. Percentage occurrence of bacterial Isolates in the contaminated soil

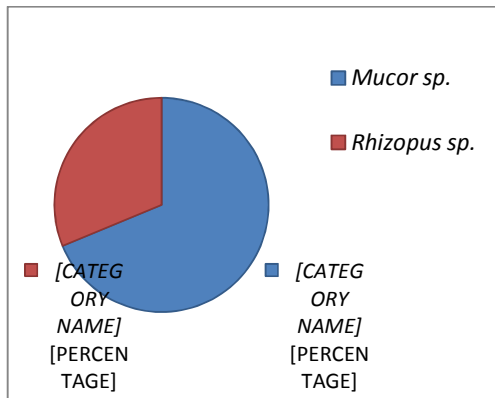


Fig. 3. Percentage occurrence of fungal isolates in the uncontaminated soil

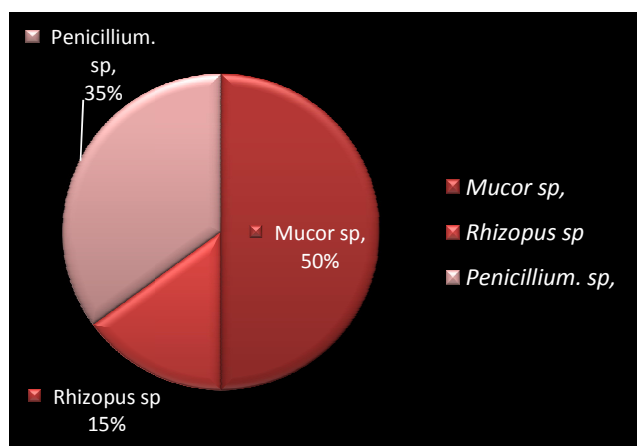


Fig. 4. Percentage occurrence of fungal isolates in the contaminated soil

Table 2. The physicochemical and microbiological analysis of the soil sample before and after crude oil contamination

Parameter	Uncontaminated soil (Mean±SD)	Contaminated soil (Mean±SD)	P-value
Nitrate (mg/kg)	811.50±0.70	791.50±0.65	<0.0001
Phosphate (mg/kg)	15.782±8.09	15.982±8.46	0.0057
Sulphate (mg/kg)	734.13±12.10	735.94±15.59	0.0003
pH	6.85±0.31	5.95±0.26	0.9895
Temperature (°C)	27.33±0.47	28.10±0.36	0.0892
Electrical Conductivity (µS)	0.07 ±0.02	0.08 ±0.01	0.5879
Moisture Content (%)	11.52±0.63	12.28±0.44	0.1657
Total Petroleum Hydrocarbon (mg/kg)	88.1±0.02	10328.03 ±5.74	<0.0001
Calcium (mg/kg)	0.94±0.07	0.87±0.04	0.2581
Sodium (mg/kg)	1.97±0.04	1.98±0.08	0.7844
Magnesium (mg/kg)	3.38±0.07	3.18±0.07	0.0327
Potassium (mg/kg)	1.09±0.02	1.11±0.03	0.3305

On the contrary, the concentration of the phosphate and sulphate was significantly higher in the contaminated soil than the uncontaminated soil. Phosphate (mg/kg) (15.782±8.09: uncontaminated) and (15.982±63.84: contaminated) (P=0.0057), Sulphate (mg/kg) (734.13±20.10: uncontaminated) and (735.94±15.59: contaminated), (P=0.0003), TPH increased drastically in the contaminated soil sample (88.1±0.02 mg/kg: uncontaminated) and (10328±5.74 mg/kg: contaminated) (P=<0.0001). This confirms the presence of crude oil in the contaminated sample. Other parameters analysed which did not show significant difference between the uncontaminated and contaminated soil were: temperature, pH, electrical conductivity, moisture content, calcium, sodium and potassium. The pH of the contaminated soil sample (5.95±0.26) was lower than that of the uncontaminated soil sample (6.85±0.26). This result is in accordance with

work done by Menkit and Amechi, [17] who observed that the presence of crude oil makes contaminated soil more acidic due to the availability of contaminants in the contaminated soil when compared with the control soil samples.

The results of analysis carried out to evaluate crude oil degradability efficiency of *Bacillus amyloliquefaciens* and *Comamonas testosteroni* with nutrient amendment using biostimulating agent: goat manure (GM) on crude oil contaminated soil revealed that these organisms helped in bioremediation rate as well as reducing the contaminant caused by crude oil in the soil with time during the monitoring analyses carried out on weekly intervals; Day 7, 14, 21, 28, 35, 42, 49 and 56. Microbial evaluation of the bioremediation setups showed increased colonial values with increase time but slightly decreased on the last day. Table 3 shows

the means of the microbial population and percentage of the hydrocarbon utilizing microbes during the study period. Total heterotrophic bacterial counts ranged between 7.39 log₁₀cfu/g (control) – 8.25 log₁₀cfu/g (CS+CM+BC), total fungal counts ranged between 4.11 log₁₀cfu/g (CS+CM) – 4.18 log₁₀cfu/g (CS+CM+BC), hydrocarbon utilizing bacterial counts ranged between 3.68 log₁₀cfu/g (control) – 4.17 log₁₀cfu/g (CS+CM+GM) and hydrocarbon utilizing fungal counts ranged between 3.57 log₁₀cfu/g (control) – 4.10 log₁₀cfu/g (CS+CM+GM). Ogbonna et al., [26], also made a similar observation and concluded that hydrocarbon microbial population increased rapidly on the first day of 20 days testing period. From this study, it was observed that the Total Heterotrophic Bacterial and Total Heterotrophic Fungal counts generally increased during the study as the treatment progressed resulting in corresponding decrease in total petroleum hydrocarbon concentration with time in the bio augmented soil compared to the controls. The hydrocarbon utilizing bacterial counts as well as the hydrocarbon utilizing fungal counts were comparatively observed to decrease with time (in days) especially at the last day of the experiment as bioremediation progressed. This can be attributed to the abundance of nutrients for the microorganisms to feed on during the first week, but started to deplete with acclimatization and competition for nutrients by the microorganisms. Shang-Hawan et al. [27] and Nrior and Echezolom [15] made similar observations and concluded that the microbial count of crude oil contaminated soils during bioremediation increases within the first three weeks of bioremediation work.

The Total Heterotrophic bacteria and Total fungi were observed to show highest counts with set up containing; contaminated soil + *Bacillus amyloliquefaciens* + *Comamonas testosteroni*. Similar observations were also made for the Hydrocarbon utilizing bacterial and fungal counts in the same treatments setup. The result is consistent with the reports of Nrior and Echezolom, [15]; Chikere et al., [28] who observed that Total Heterotrophic Bacterial and Hydrocarbon Utilizing Bacterial counts increased over time and were highest in plots amended with consortium of mixed augmenting bacterial isolates during crude oil contaminated soil undergoing bioremediation with time.

Tables 4 and 5 shows the mean values of the physicochemical analysis carried out during the

bioremediation study. Parameters including the nitrate concentrations, pH and temperature were monitored. The nitrate level ranged between 789.0 mg/kg (control sample) – 813.2 mg/kg (treated sample); the pH of the medium in the various set up ranged between 6.60 (control sample) – 6.92 (treated sample) and the temperature ranged between 27.66°C (control sample) – 28.53°C (treated sample). It was observed that the pH and temperature of the medium maintained similar values and were not affected by the various treatments during the study period. The result in Table 6 show the differences in the Total Petroleum Hydrocarbon of the different experimental set-up, indicating the decrease in the total petroleum hydrocarbon with increasing time even in the control that was not augmented with organisms or stimulated with stimulant.

Results of total petroleum hydrocarbon revealed amount of hydrocarbon remediated at 56 days in the various treatment set-ups in the following increasing order; (Control 1 uncontaminated soil without treatment); 5.66mg/kg > (Control 2 contaminated soil without treatment); 125.71 mg/kg > (COCS + BC); 1855.74 mg/kg > (COCS + CM); 2261.01mg/kg > (COCS + CM+ BC); 3321.23 mg/kg > (COCS + GM + BC); 4983.81 mg/kg > (COCS + GM + CM); 7313.47 mg/kg. From our results, the percentage of TPH bioremediated in the various treatments set ups followed the decreasing order accordingly: (COCS + GM + CM); 70.81% < (COCS + GM + BC); 48.25% < (COCS + CM+ BC) < 32.15% < (COCS + CM); 21.89% < (COCS + BC); 17.96% < (Control 1); 6.43% < (Control 2); 1.21%. These results indicate that *Comamonas testosteroni* has more degradability efficiency especially when amended with goat manure compared to *Bacillus amyloliquefaciens* due to the high percentage of TPH remediated when compared with the other treatments.

This observation is in agreement with the report of Ogbonna et al., [26] who worked on “Bioremediation Efficiency of *Bacillus amyloliquefaciens* and *Pseudomonas aeruginosa* with the nutrient amendment on crude oil polluted the soil” and made similar observations. From the result, we observed that the consortium with combination of the microorganisms had more potential to degrade the quantity of TPH in the soil samples than the single microorganism as seen in Table 6.

Table 3. Mean and percentage microbial counts of soil samples during bioremediation

Treatments	Microbial populations		Hydrocarbon utilizing bacteria (log ₁₀ cfu/g)	Hydrocarbon utilizing fungi (log ₁₀ cfu/g)	Percentage hydrocarbon utilizing bacteria (%)	Percentage hydrocarbon utilizing fungi (%)
	Total heterotrophic bacteria (log ₁₀ cfu/g)	Total fungi (log ₁₀ cfu/g)				
US (control1)	7.39 ±0.71	4.17 ±0.07	3.68 ±0.15	3.57 ±0.15	49.79	85.61
COCS (control 2)	7.56 ±1.37	4.15 ±0.08	4.13 ±0.08	4.06 ±0.10	54.63	97.83
COCS+BC	7.89 ±1.65	4.14±0.21 ^{ab}	4.16 ±0.28 ^a	3.92 ±0.11 ^a	52.72	94.69
COCS+CM	7.76 ±1.54	4.11 ±0.14	4.06 ±0.07 ^a	4.01 ±0.10 ^a	52.32	97.57
COCS+GM+CM	8.11 ±1.83	4.16 ±0.16	4.17 ±0.10 ^a	4.10 ±0.09 ^a	51.42	98.56
COCS+GM+BC	8.22 ±1.92	4.17 ±0.14	4.11 ±0.04 ^a	4.04 ±0.09 ^a	50.00	96.88
COCS+CM+BC	8.25 ±1.94	4.18±0.16	4.15 ±0.08 ^a	4.04 ±0.09 ^a	50.30	96.65

Key: US = Uncontaminated soil, COCS = contaminated soil, BC = *Bacillus amyloliquefaciens*. CM = *Comamonas testosteroni*, GM = Goat manure

Table 4. Means of physicochemical characteristics of soil samples during bioremediation

Set up	Treatments	Nitrate (mg/kg)	pH	Temp (°C)
SU 1	Control 1	789.0	6.60	27.66
SU 2	Control 2	792.4	6.61	28.53
SU 3	COCS + BC	808.5	6.74	27.86
SU 4	COCS + CM	813.2	6.84	27.84
SU 5	COCS + GM + CM	804.6	6.92	28.24
SU 6	COCS + GM + BC	791.7	6.68	28.04
SU 7	COCS + CM + BC	798.2	6.67	28.26

Key: SU = Set up, COCS = Contaminated soil, BC = *Bacillus amyloliquefaciens*. CM = *Comamonas testosteroni*, GM = Goat manure

Table 5. Regression analysis of physicochemical characteristics of soil samples

Set up	Treatments	Nitrate		pH		Temperature	
		Regression equation (Y)	R ²	Regression equation (Y)	R ²	Regression equation (Y)	R ²
SU 1	Control 1	-0.46x + 791.55	0.271	-0.0331x + 6.784	0.462	-0.017x+ 27.76	0.714
SU 2	Control 2	-13.99x + 869.35	0.458	-0.0339x + 6.805	0.714	-0.088x+ 29.01	0.410
SU 3	COCS + BC	-10.67x + 867.16	0.758	-0.0339x + 6.932	0.063	-0.258x+ 29.28	0.910
SU 4	COCS + CM	-8.66x + 860.82	0.657	-0.0077x + 6.888	0.003	-0.277x+ 29.36	0.918
SU 5	COCS + GM + CM	-11.95x + 870.38	0.776	0.0089x + 6.872	0.003	-0.191x+ 29.29	0.839
SU 6	COCS + GM + BC	-16.14x + 880.50	0.747	-0.0446x + 6.929	0.227	-0.227x+ 29.28	0.798
SU 7	COCS + CM + BC	-11.12x + 868.47	0.778	-0.0792x + 7.106	0.224	-0.189x+ 29.30	0.760

Key: SU = Set up, COCS = contaminated soil, BC = *Bacillus amyloliquefaciens*. CM = *Comamonas testosteroni*, GM = Goat manure

Table 6. Changes in the Total Petroleum Hydrocarbon (TPH) contents during the bioremediation process

Setup	Experimental set-up treatments	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56	Amount remediated	% remediated
SU 1	UCS (CTRL 1)	88.1	86.49	86.39	86.0729	87.3975	85.1483	83.5418	82.4338	5.66	6.431555
SU 2	COCS (CTRL 2)	10328.03	10309.58	10291.64	10282.55	10247.8	10229.35	10211.41	10202.32	125.71	1.217173
SU 3	COCS + BC	10328.03	10309.58	10291.64	10123.34	10093.29	9487.613	8918.525	8472.285	1855.74	17.96804
SU 4	COCS + CM	10328.03	10309.58	10291.64	10120.97	9817.375	9130.589	8491.543	8067.011	2261.01	21.89206
SU 5	COCS + GM + CM	10328.03	10309.58	10291.64	10193.22	8970.116	6818.884	4636.718	3014.557	7313.47	70.81189
SU 6	COCS + CM+ BC	10328.03	10309.58	10291.64	10121.56	9312.477	8381.518	7375.282	7006.796	3321.23	32.15748
SU 7	COCS + GM + BC	10328.03	10309.58	10291.64	10139.53	9126.782	7667.517	6287.471	5344.219	4983.81	48.25519

Key: UCS = Uncontaminated soil, COCS = crude oil contaminated soil, BC = *Bacillus amyloliquefaciens*. CM = *Comamonas testosteroni*, GM = Goat manure

Furthermore, [13] suggested that a mixed population of microbial community is necessary for complete biodegradation of oil pollutants as the required enzymes for petroleum degradation are better found in a combination organism than in a single organism, this may be attributed to the higher microbial counts observed as stated in Table 3. However, the addition of the organic nutrients like the goat manure applied in this study stimulated the activity of the indigenous microbial population and resulted in lesser TPH values at the last day of the bioremediation process as evident in our results on the amounts of TPH remediated as illustrated in Table 6 [17].

4. CONCLUSION AND RECOMMENDATION

This study has revealed that *Comamonas testosteroni* had more crude oil biogradability potential for bioremediation of hydrocarbon contaminated soil than *Bacillus amyloliquefaciens* but much more effective when amended with organic nutrient such as goat manure. It is therefore recommended that bioremediation of crude oil polluted soil using bioaugmentation technique should be amended with organic nutrient to enhance the bioremediation efficiency.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Ekanem J, Nwachukwu I. Sustainable agricultural production in degraded oil producing and conflict prone communities of Niger Delta, Nigeria. *Journal of Agriculture and Sustainability*. 2015;8(1): 14-28.
2. Kvenvolden KA, Cooper CK. Natural seepage of crude oil into the marine environment. *Book of Geology and Marine Environment*. 2003;23(3-4):140–146.
3. Awari VG, Ibiene AA, Ariole CN. A broad-range pH/temperature-stable cellulase from a novel hydrocarbon contaminated mangrove soil bacterium, *Bacillus licheniformis* VVA21. *Microbiology Research Journal International*. 2018;24 (2):1-14.
4. Thapa B, Ajay-Kumar K, Ghimire A. Review on bioremediation of petroleum hydrocarbon contaminants in soil. *Kathmandu University Journal of Science, Engineering and Technology*. 2012;8(1): 164-170.
5. Das N, Chandran P. Microbial degradation of hydrocarbon contaminants; An Overview. *Journal of Biotechnology Research International*. 2011;1:1–13.
6. Ron EZ, Rosenberg RE. Biosurfactants and oil bioremediation. *Current Opinion. Biotechnology Journal*. 2002;13(3):249–52.
7. United Nations. The adverse impacts of oil pollution on the environment and wellbeing of a local indigenous community: The Experience of the Ogoni People of Nigeria; 2011.
8. Akani NP, Obire O. Bacterial population of *Clarias gariepinus* (Burchell 1822) exposed to an oilfield wastewater in Rivers State, Nigeria. *Asian Journal of Biological Science*. 2014;7(5):208-216.
9. Obire O, Akinde SB. Comparative efficiency of cow dung and poultry manure in bioremediation of oil polluted soil. *Niger Delta Biologia*. 2006;5(2):82-91.
10. Finley SD, Broadbelt LJ, Hatzimanikatis BV. In silico feasibility of novel biodegradation pathways for 1, 2, 4-trichlorobenzene. *Journal of Biology and Medical Century*. 2010;4(1):1–14.
11. Ogbonna DN. Application of biological methods in the remediation of oil polluted environment in Nigeria. *Journal of Advances in Biology and Biotechnology*. 2018;17(4):1-10.
12. Chikere CB, Okpokwasili GC, Chikere BO. Bacterial diversity in tropical crude oil polluted soil undergoing bioremediation. *African Journal of Biotechnology*. 2009;8 (11):2535-2540.
13. Sathishkumar M, Arthur B, Sang-Ho B, Sei-Eok Y. Biodegradation of crude oil by individual bacterial strains and a mixed bacterial consortium isolated from hydrocarbon contaminated areas, soil, air, water. *Advance Journal of Applied and Environmental Microbiology*. 2008;36:92-96.
14. Abu GO, Moro KO. Glucose and hydrocarbon utilization by bacteria isolated from diesel impacted soil in the Niger delta. *Global Journal of Pure and Applied Science*. 2004;11:205-208.
15. Nrior RR, Echezolom EC. Assessment of bioremediation of petroleum contaminated soil with biostimulating agents. *Current Studies in Comparative Education*,

- Science and Tehnology. 2016;3(1):203-215.
16. Cheesebrough M. District laboratory practices in tropical countries (Second edition). Cambridge: Cambridge University Press; 2006.
 17. Menkit MC, Amechi AK. Evaluation of first and second order degradation rates and biological half-lives in crude oil contaminated soil. Asian Journal of Biotechnology and Genetic Engineering. 2019;2(1):1-11.
 18. Douglas SI, Barisi SP. Bioremediation of crude oil polluted terrestrial soil using *Aspergillus clavatus* and *Pichia* spp. International Journal of Current Microbiology and Applied Sciences. 2019; 8(3):733-744.
 19. Prescott LM, Harley JP, Klein DA. Microbiology. 6th edition, McGraw Hill, London. 2005;23-67.
 20. Bento FM, Carmago AO, Okeke BC, Frankenberger WT. Comparative bioremediation of soil contaminated with diesel oil by natural attenuation, biostimulation and bioaugmentation. Bioresource Technology Journal. 2005; 96:1049-1055.
 21. Odokuma LO, Dickson AA. Bioremediation of crude oil polluted tropical mangrove environment. Journal of Applied Science Environmental Management. 2003;7(2): 23-29.
 22. Brusseau ML. The impact of physical, chemical and biological factors on biodegradation, in proceedings of the International Conference on Biotechnology for Soil Remediation: Scientific Bases and Practical Applications R. Serra, Ed., C.I.P.A. S.R.L., Milan, Italy. 1998;81-98.
 23. Chaillan F, Chaîneau H, Point V, Saliot A. Oudot J. Factors inhibiting bioremediation of soil contaminated with weathered oils and drill cuttings, Environmental Pollution. 2006;144(1):255-265.
 24. Environment Guidelines and Standard for the Petroleum Industry in Nigeria (EGASPIN). Standard guideline through Department of Petroleum Resources (DPR). Revised Edition. 2002;227-288.
 25. American Public Health Association (APHA). Standard methods for the examination of water and wastewater. 21st edition, American Public Health Association/American Water Works Association, Washington, DC., USA. 2005; 12:45-54.
 26. Ogbonna DN, Iwegbue CMA, Sokari TG. Effect of bioremediation on the growth of Okro (*Abelmoshus esculetus*) in the Niger Delta soil. Environmentalist. 2007;27:303-309.
 27. Shang HA, Ilori MO, Amund OO, Teniola OD, Olatope SO. Microbial degradation of petroleum hydrocarbons in a polluted tropical stream. World Journal of Microbiology and Biotechnology. 2007;23(8):1149-1159.
 28. Chikere CB, Ekwuabu CB. Molecular characterization of autochthonous hydrocarbon utilizing bacteria in oil-polluted sites at Bodo Community, Ogoni land, Niger Delta, Nigeria. Nigerian Journal of Biotechnology. 2014;27:28-33.

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