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Abstract
The contamination of soil originated from evolutional activities such as transportation and spillage, as in the case of Ogoni land in the Niger Delta area of Nigeria, and it is a well-known environmental problem in Nigeria. This study examined the capacity of two indigenous bacteria, Pseudomonas aeruginosa, and Bacillus subtilis to remediate the hydrocarbon contaminated soil. The focus of the work was to isolate, identify, and determine the extent of bioremediation achieved in the soil contaminated with crude oil in Ogoni land, which was used as a case study for the slurry-phase bioremediation process. These organisms earlier mentioned were identified based on their morphological and biochemical characteristics. The bacteria species were considered separately and in combination in a Laboratory-scale slurry-phase in a 14-day treatment period using 30 g of soil for each case. The performance of each batch bioreactor was compared to the sterile control. The slurry phase bio-reactor, R_A, treated with Pseudomonas sp., had an actual degradation of 31%, the slurry phase bio-reactor, R_B, treated with Bacillus sp., had a real degradation of 35%. The slurry phase bio-reactor Rc, which contain both microorganisms had an actual degradation of 36%, the slurry phase bio-reactor R_D had a real degradation of 0%. The results obtained confirm that the isolated bacterial from the soil can effectively utilize contaminating crude oil as a source of carbon and energy. The decrease of hydrocarbon in the sterile control was used as a basis for comparison of the petroleum hydrocarbons lost through the extraction process as microbiological activity was not possible. Results from the study have shown that P. *aeruginosa* and B. *subtilis* strains were effective for the slurry-phase bioremediation of hydrocarbon contaminated soil.

Keywords: Oil Spillage, Bioremediation, Slurry-phase Reactor, Hydrocarbon Degrading Bacteria.

1. Introduction
Soil and water contamination with petroleum is one of the major contamination problems we have today. It affects both plant and animal life and causes severe short term and long-term negative impacts on the environment [1]. It occurs as a result of the introduction of crude oil or its refined petroleum products (benzene, toluene, xylene (BTX), gasoline, and diesel fuel), and acid mine drainage to the naturally occurring soil. Oil contamination arising from pollution emission activities such as petroleum drilling processes and its transportation, such as in the case in Ogoni land in Niger Delta, Nigeria, is a well-known environmental problem in Nigeria. Therefore, safer and economical treatment is necessary. An environmentally friendly and cheap way to do this is the use of bioremediation techniques. Bioremediation means the use of

microbes or microorganisms to breakdown environmental pollutants or contaminants into less toxic products. It makes use of plants, microorganisms such as fungi or bacteria, or a consortium of microorganisms to detoxify or breakdown hazardous substances to the environment and or human health [2]. Bioremediation technology, which is based on the application of living organisms to remove environmental contaminants, has proven to be a promising and effective means of getting rid of petroleum-derived products from the environment [3]. Microorganisms contributed a lot to the success of cleaning nature from contamination [4]. This technology can be applied either by stimulating microorganisms present in the contaminated site, known as biostimulation, or by the addition of genetically grown microorganisms known as bioaugmentation [4]. During the bioremediation process, the control of environmental factors such as moisture contents, pH, temperature, and oxygen is essential to achieve higher rates of biodegradation [5]. Many research works have reported on the bioremediation of crude oil contaminated soil using the biostimulation approach (where either organic or inorganic biostimulants were employed). However, the former has attracted better attention in recent times because of its relatively low cost and environmental safety [6].

The exportation of crude oil accounts for 80% of the budgetary revenues and 95% of the country's foreign exchange earnings. In the recent past, large-scale coastal and marine pollution have been occasioned primarily by off-shore drilling activities and accidents caused by oil tankers' transportation of fuels on the sea [7]. On the average, approximately twenty (20) oil spillage mishaps occur annually, resulting in the accidental discharge of hundreds or thousands of metric tons of crude oil or its products into the seas [8]. Studies from available literature revealed that about 1200 oil spills have occurred between 1967 and 2010 [9]. The total volume of crude oil spills, most of the time, far exceeds the available bioremediation capacity in any particular environment, and it usually results in negative ecological impacts [10]. Oil exports account for 95% of the foreign exchange earnings and 80% of the budgetary revenues. And also, the quality of the extracted oil is considered good, as it has a low content of sulfur (0.14%) and a high content of lighter fractions [11, 12]. The type of oil involved accounts for the severity of the contamination and the oil seeps through the soil surface easily if it is light oil. For heavier oil, it takes slower speed to spread. Subsequently, concern has risen about petroleum contamination and its damaging environmental side effect in the world at large as a result of continuous use of petroleum as a major source of energy and as an important feedstock to the chemical and petrochemical industries. Several methods have been adapted for treatment of petroleum-contaminated soil to meet the strict environmental control regulations. Among the widely used and most promising techniques for remediating hydrocarbon polluted soil is bioremediation, which has several inherent advantages, including in-situ treatment possibility, environmentally friendly nature, and low total cost) compared to the other physical or chemical treatment methods [13]. It is a fact that there is a wide variation in the ratios of crude oil organic molecules constituents, but its elemental compositions are almost fixed as follows: 83-87 % carbon, 10-14 % hydrogen, 0.1-2 % nitrogen, 0.05-1.5 % oxygen, 0.05-6.0 % Sulfur, and < 0.1 % Metals [14].

This study investigates the nature and extent of the biodegradation of crude oil contaminants during the slurry-phase bioremediation of hydrocarbon-polluted soil in order to identify common factors that limit effective bioremediation. A major advantage of these methods is that they are less harmful to the environment with minimum or no by-products. Moreover, conventional physical and chemical treatments are expensive and inefficient and cause more harm than good [1]. Thus, by evaluating research undertaken in bioremediation so far, more efficient and feasible

bioreactors may be designed. Furthermore, these systems could be capable of completely removing contaminants from the environment.

2. Material and Methods

2.1 Experimental Methodology

The bioremediation experiment was performed using four reactors and aerated for four (4) hours per day throughout the treatment period. The mixing of the slurry was done using a manual mechanical stirrer before supplying air. Air was supplied using an air compressor to enable microbial activities. The compressor was then stopped, and solid particles were allowed to settle after the experiment. After treatment, the slurry was removed and dewatered to separate the treated soil from the aqueous phase.

2.2 Collection of Soil Samples used for Bioremediation Study

Soil samples were collected from a contaminated site in Ogoni, River State (4º38'21"N 7º16'8"E). The soil samples were collected in pre-sterilized sample containers to avoid alteration of physical and chemical properties at a depth of 5 cm-15 cm. The collected samples were duly labeled then taken for analysis in the laboratory. Plate I is the crude oil-contaminated soil.

Plate 1*:* Contaminated Site, Bodo community, Ogoni

2.3 Microbiological Analysis of Oil Polluted Soil

Chemical Reagent: The chemical reagent used for the isolation were of analytical grade and therefore required no further purification.

They include: Nutrient agar was used for the estimation of total heterotrophic aerobic bacteria, purification of hydrocarbon utilizers and pure culture. In addition, modified Mineral Salt agar without antibiotics was used for the isolation of hydrocarbon utilizing bacteria.

2.4 Enumeration of Total Heterotrophic Bacteria in the Oil Polluted Soil Sample

10 g each of the soil sample was measured into a conical flask and 90 mL of sterile water was mixed with the sample. The samples were then shaken to dislodge and homogenize the solution.

2.5 Serial Dilution of the Samples

1 mL of the sample stock was aseptically transferred into sterile test tubes containing 9 mL of diluent to give a dilution factor of 10^{-1}. The procedure was repeated several times until a dilution factor of 10^{-7} was achieved [15, 16].

2.6 Inoculation of Culture Medium

1 mL from 10^{-4} dilution from each soil sample were transferred into sterile Petri dishes in duplicates. About twenty millimeters (20 mL) of sterile nutrient agar was aseptically poured into the seeded plates (pour plate technique). 0.1 mL each from the 10^{-4} dilution was transferred into nutrient agar plates in duplicates. The inoculum was then evenly spread on to the surface of the plates using a hockey stick, which is the spread plate technique, as previously described by [17]. All nutrient agar plates were incubated at 28 ^{o}C for 24 hrs. Colonies that emerged after incubation were enumerated.

2.7 Enumeration of Hydrocarbon Degraders

The estimation of densities of hydrocarbon-degrading bacteria was determined following the method of [18]. The Vapour phase transfer method of the sterile crude oil from the lid of the plates was used as the source of carbon and energy. 1 mL of 10^{-4} dilution from each of the soil samples was used to seed the sterile Petri dishes in duplicates. Sterile molten mineral salt medium (MSM) supplemented with 0.5 mL of chlorophenicol to suppress the bacterial on the different plates. These media were poured into the seeded plates and swirled to mix with the inocula. All plates were inoculated for fourteen (14) days at 28^{o}C. Plates that developed colonies were counted and recorded as colony-forming unit/g.

2.8 Purification and Maintenance of Microbial Isolates

Discrete colonies from culture plates were picked for characterization. Bacteria colonies were repeatedly sub-cultured into freshly prepared nutrient agar by streaking method and incubated for growth before transferring to agar slant. Pure isolates of the bacteria were maintained as stock on the agar slant and then preserved in the refrigerator.

2.9 Characterization and Identification of Bacteria Isolates

Bacteria isolates were characterized and identified presumptively based on their morphological, cultural, and physiological characterization, while confirmatory identification was based on biochemical reactions. The biochemical tests performed were; Gram staining, motility, catalase, spore staining, oxidase, urease, citrate, starch hydrolysis, methyl red - Voges Proskauer (MR-VP) test, hydrogen sulphide production test, and sugar fermentation. The results from the tests for various isolates were collected, and the identification carried out by comparing with the standard manual [19].

3. Soil Pre-Treatment

A non-contaminated Soil sample was pretreated and later contaminated with crude oil. For effective bioremediation, some pre-treatment is required to remove all oversize material [20]. The soil sample was first sieved through a two millimeter (2 mm) sieve to remove objects, such as rocks or bricks. To further remove the contaminant present, the soil was washed with distilled water 20 times and thereafter, autoclaved at 100^{o}C for 2hr to kill any microbe present.

3.1 Preparation of Contaminated Soil Sample

Contaminated soil samples used for this experiment consisted of pretreated soil and contaminant (crude oil). The results of the sieved soil and crude oil were weighed using an analytical balance, mixed and allowed to stand for three (3) days to allow the crude oil to mix and saturate the soil properly. For this research, 10 mL of crude oil was used to contaminate 30 g of the soil sample.

3.2 Preparation of Slurry Phase/Bioremediation Process

The slurry phase sample used in this experiment consisted of contaminated soil, distilled water, inoculated bacteria, and nutrient.

3.3 Slurry-Phase Bio-Reactor

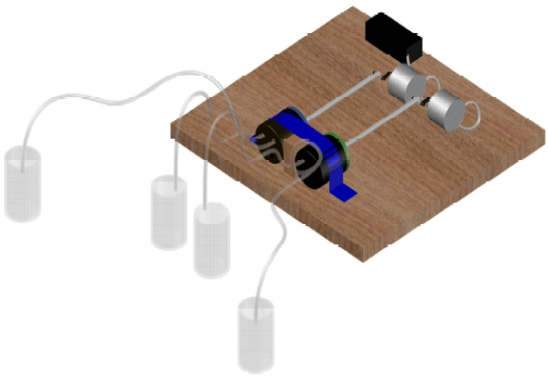

Plate II: Slurry-Phase Bio-Reactor

A slurry phase batch reactor used for this experiment was designed following the method previously described by [21]. 300 mL slurry phase batch reactor was used for the treatment of the contaminated soil in the laboratory at an average room temperature of 30 ^{0}C. The slurry was then aerated using a compressor to enhance and boost its microbial activities. The compressor was operated for three (3) hours per day of the experiment. Mechanical mixing was performed before the compressor was turned on. Table 2.1 shows the detail of the reactor composition.

Table 1**:** Detail Showing Reactor Compositions

Reactor	Volume of Reactor (mL)	Soil(g)	Water(mL)	Bacterial	Crude oil Concentration (mLs)	Nutrient (g)
A	300	30	100	Present	10	1

B	300	30	100	Present	10	1
C	300	30	100	Present	10	1
D(control)	300	30	100	Absent	10	1

3.4 Determination of Percentage of Hydrocarbon Biodegraded by the Inoculated Microorganisms

The samples were shaken and allowed to settle for three (3) hours such that the biomass settled at the bottom of the containers and the aqueous phase containing the bulk of the oil was stored in a beaker. The biomass was washed with 20 mL of dichloromethane twice and decanted into the beaker containing the aqueous phase. The aqueous mixture in the beaker was separated using a separating funnel as a result of two immiscible layers of liquid formed. Oil and dichloromethane was formed at the bottom while water was formed on top due to difference in liquid density. The extract containing crude oil and dichloromethane was separated out using a soxhlet extractor at a temperature of 39 ^{O}C, and the residual oil was measured.

Percentage reduction of hydrocarbon was calculated using equation (1):

$$\%\ reduction = \frac{initial\ TPH - final\ TPH}{initial\ TPH} \times 100 \quad \text{... (1)}$$

4. Result and Discussion

4.1 Morphological and Biochemical Test Result

Table 2: Microbial density of the indigenous microbes

		Microbial density	
Samples	**Medium**	**HUB (**cfu/g)	**TNO**
A	MSA_1	2.0 X 10^5	21
	MSA_2	1.1 X 10^5	12
B	MSA_1	1.3 X 10^5	11
	MSA_2	1.5 X 10^5	22

HUB – Hydrocarbon Utilizing Bacteria, TNO – Total Number of Organism

Table 3**:** Morphological characteristics of the indigenous microbes

Biochemical Characteristics

Samples	Gram reaction	Shape	Catalase	Coagulase	Motility	Starch hydrolysis	Citrate	Urease	Methyl red	Voge Proskauer	Spore	H_2S	Oxidase	Glucose	Maltose	Lactose	Fructose	Sucrose	Manitol	Gal. & Dex.	Probable Organism
A	+ve	Rod	+	-	+	+	-	-	-	+	+	-	-	AG	A	-	A	A	-	$A/_A$	B. cereus
	+ve	Cocci	+	-	-	+	+	+	+	-	-	+	+	A	-	A	-	A	A	$A/_A$	Micrococcus sp.
	+ve	Rod	+	-	+	+	-	-	-	+	+	-	-	AG	A	-	A	A	-	$A/_A$	B. cereus
B	+ve	Rod	+	-	+	+	+	-	-	+	+	-	-	AG	A	-	A	-	-	$A/_-$	B. substilis
	+ve	Rod	+	-	+	-	+	-	-	+	-	-	+	A	A	-	AG	AG	-	$AG/_{AG}$	Pseudomonas aeruginosa
	+ve	Rod	+	-	+	+	+	-	-	+	+	-	-	AG	A	-	A	-	-	$A/_-$	B. substilis

The bacterial strains from the mineral salt agar used the crude petroleum hydrocarbon substrates as their only source of carbon and energy [22]. This is clearly evident, as shown in Table 3.1, where the selected bacteria strains, Pseudomonas Aeruginosa and Bacillus Subtilis, utilized for the remediation study increased tremendously in their population or density after they were incubated [23,24]. The rate of the bioremediation of the hydrocarbon contaminated soil is dependent on the survival or growth of microorganisms after their inoculation [25]. The application of the isolated microorganisms as hydrocarbon degraders for the bioremediation experiment conforms with the results of other researchers [25,26]. Table 3.3 shows the detail of the crude oil properties used.

Table 4: Showing crude oil properties used

	Property	Value	Unit
1.	Dynamic Viscosity	528.9 @ 12rmp	Kg/m.s
2.	Specific Gravity	0.8866	-
3.	Density	886.65	kg/m^3
4.	Kinematic viscosity	528900	m^s/s
5.	API gravity	28.08	-

4.2 Estimation of Hydrocarbon Biodegradation

This research aimed to investigate the potential of the two selected bacterial isolates for the biodegradation of the hydrocarbon contaminated soil sample obtained from Ogoni land. The isolates obtained from the Ogoni contaminated soil sample were identified as Bacillus *subtilis* and Pseudomonas *aeruginosa*. They were assessed in terms of their ability to biodegrade the petroleum-contaminated soil using the hydrocarbon as a carbon source for the microcosms in slurry phase bioreactors.

Table 5: Amount of crude oil left after extraction with dichloromethane

Slurry Bioreactor	Initial amount (mL)	Final amount (mL)	Degradation	True Degradation
R_A	10	6.2	38%	31%
R_B	10	5.8	42%	35%
R_C	10	5.3	43%	36%
R_D	10	9.3	7%	0%

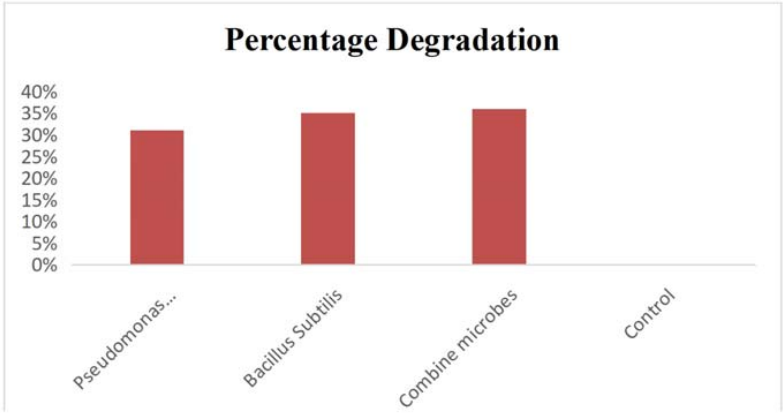

Figure 1: Graph showing the percentage of degradation of crude oil after bioremediation

Figure 1.0 illustrates the decrease in total petroleum hydrocarbon concentration after a 14-day treatment period. The performance of each reactor was compared to the sterile control. The decrease in the total petroleum hydrocarbon from the sterile control was used to measure the petroleum hydrocarbons lost through the extraction process as microbiological activity was not possible. The soil slurry treated with Pseudomonas sp. had a true degradation of 31%. Soil slurry treated with Bacillus has a true degradation of 35%. Reactor Rc, which contains both microorganisms, had a true degradation of 36%; the sterile control sample (microcosm) had a true degradation of 0%. The extent of biodegradation obtained by the bacterial isolates revealed that the microorganisms utilize carbon as food and energy source. This finding is similar to other literature, as reported by [2, 27]. In addition, The results obtained further confirm that the soil's microorganisms have the ability to utilize the hydrocarbon content of the crude oil contaminant as a source of carbon and energy [28, 29]. This is because microorganisms generally possess systems which aid and enable them to utilize crude oil contaminants as carbon source. The pattern of biodegradation varies considerably for different microorganisms, and this may be attributed to the differences in the catabolizing enzymes they possess [30]. The research findings reported here measure specifically the total petroleum hydrocarbon (TPH), rather than the individual components.

5. Conclusion

The purpose of this study was to carry out the Slurry-Phase Bioremediation of a hydrocarbon-contaminated soil sample from Ogoni Land and determine its extent of bioremediation by two indigenous microorganisms, Pseudomonas and Bacillus species. The bacterial isolates were selected based on their ability to utilize the hydrocarbon in the crude oil contaminated soil as a source for their food and energy. During the study period, four (4) bioreactors containing 30 g of contaminated soil samples were successfully used to access the ability of isolates to utilize hydrocarbon at room temperature (30 ^{0}C). The soil slurry treated with *Pseudomonas sp*. and Bacillus sp. had a true degradation of 31 and 35 %, respectively. Reactor Rc, which contain both microorganisms, had a true degradation of 36 %, whereas the sterile control sample had a true degradation of 0 %. Bacillus specie was found to have performed better in the biodegradation of the crude oil contaminated soil than the Pseudomonas species, whereas the combined approach using the two species proved to be more effective. This study confirms the advantage of the remediation approach-the the Slurry-Phase Bioremediation and the ability of Bacillus and Pseudomonas isolates to drastically lower the Total Petroleum Hydrocarbon **(**TPH**)** content in crude oil contaminated soil sample. The bioactivities of these bacteria could enhance their advantages and use in the bioremediation of crude oil contaminated soils in Ogoni land and tropical countries.

Recommendation

This study contributed potent strains that can be further exploited in the ex-situ treatment process of hydrocarbon polluted land. The approach used in this study may help provide answers to the questions on the various microbial communities that are present in the soils within Ogoni land and their uniqueness in certain biological activities. However, it is recommended that a GC-MS analysis be carried out in further experiments to determine the classes and components of hydrocarbon that are affected by slurry-phase bioremediation treatment. The relatively short duration of treatment could have limited the effectiveness of all three treatments because of the chemical nature of the weathered contaminant in the soils. However, the bioremediation result could have been improved if a longer period was observed. A future study that would allow full-term treatment could offer a more representative comparison.

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