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Synergistic bioremediation of crude oil contaminated soil: Role of bioreactor and pigeon droppings

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Abstract
Aim: The aim of study was to assess the role of Bioreactor and Pigeon Droppings in the synergistic bioremediation of crude oil contaminated soil.
Study Design: This study employs experimental designs, Randomized Block Design Treatment set up, Statistical analysis of data and Interpretation.
Place and Duration of Study: The area used for this study is within the Rivers State University Demonstration Farmland in Nkpolu-Oroworukwo, Mile 3 Diobu area of Port Harcourt, Rivers State. The piece of land is situated at Longitude 4.80620^{o}N and Latitude 6.97752^{o}E measuring 5.4864 m x 5.1816 m with a total area of 28.4283 m^{2}. This was cleared and sub-partitioned into 9 blocks of 100 cm x 50 cm x 20 cm giving 150kg soil in each plot using a digital standing scale (Fig 3.1), with each block 65 cm apart. Two of these plots were designated as pristine and crude oil soil to serve as negative and positive controls.
Methodology: Crude oil used in this experiment was obtained from Nigerian Agip Flow Station Ebuocha. The stock culture was prepared by weighing out 4000g (PCE analytical weighing balance PCE-6000 UK), and dissolving in 1.0 l of distilled water to give initial crude oil concentration of 2500 g/l. The soil was artificially contaminated by spiking the prepared crude oil concentration on the plots and allowed to stay for 21 days (To ensure volatilization and sorption of crude oil into the soil matrix) before application of various treatments.
The plots were amended with 750 g of pigeon droppings, 375 ml of *Pseudomonas* and 375 ml of *Bacillus* broth, respectively. Plot one was uncontaminated and plot 2 was contaminated. These two plots served as controls. Other plots were amended with different concentrations of treatment. These were allowed for 7 days with the objective of assessing the microbial activities within week 1 of treatment (Bioremediation) sampling.
Results: The microbiological (THB, F, HUB, HUF) analysis for uncontaminated and contaminated soil showed average counts of $4.77\pm0.47\times10^{6}$CFU/g and $2.40\pm2.80\times10^{7}$ CFU/g for THB, $4.33\pm0.96\times10^{4}$SFU/g and $5.10\pm1.11\times10^{5}$SFU/g for F, $2.43\pm0.10\times10^{3}$CFU/g and $3.69\pm0.59\times10^{3}$CFU/g for HUB, $1.85\pm0.59\times10^{3}$SFU/g and $4.00\pm0.53\times10^{3}$SFU/g for HUF, respectively. The physicochemical results revealed that the pH was acidic with value of 6.3±0.13 for uncontaminated soil and 6.9±0.02 for contaminated soil. The temperature was 27.47 ± 0.35^{O}C for uncontaminated while the contaminated was 27.80 ± 0.10^{O}C. Nitrogen was 15.67±0.03 for uncontaminated whereas the contaminated was 12.7±0.04 mg/kg, phosphorus revealed 0.75±0.04 mg/kg and 0.597±0.25 mg/kg for uncontaminated and contaminated. The potassium was 1.825±0.05 mg/kg for uncontaminated and 1.813±0.15 mg/kg for contaminated. THC for uncontaminated was 44.0±2.01 mg/kg and contaminated revealed value of 5785±0.063mg/kg. The bacterial genera isolated from crude oil contaminated soil were: *Pseudomonas* sp, *Bacillus* sp, *Micrococcus* sp, and *Serratia* sp while the fungal isolates were: *Aspergillus* sp, *Penicillium* sp *Alternaria* sp, *Candida* sp and *Fusarium* sp.
Conclusion: The study showed that the bioreactor was effective in multiplying the microbial isolates used in the various treatment plots which in turn enhanced the degradation of the contaminated soil. The isolation and identification of microorganisms from the soil samples indicated an abundance of hydrocarbon utilizing microorganisms, thus, indicating that the isolates possess the functional ability for hydrocarbon degradation.
From this study, the synergistic bioremediation of crude oil contaminated soil using *Pseudomonas aeruginosa, Bacillus safensi* and pigeon droppings with 51.17% hydrocarbon reduction offers a potential option for crude oil remediation. This is cost-effective and a tool for the preservation of the agricultural ecosystem.

Keywords: Crude oil, contaminated soil, bioreactor, bioremediation, uncontaminated

1. Introduction
The environment is currently in danger of ecological damage due to the sporadic rise in

population, urbanization, and industrialization (Cherniwchan, 2012) [7]. The ecosystem is in risk due to the ongoing harm that humans bring to it (Goudie, 2013) [13]. The production of industrial chemicals, excessive use of petroleum and its derivatives, polyethylene, pesticides, and organic herbicides which are mainly used to prevent fungal, insect, and weed attacks have contaminated and polluted nature's elements, including aquatic and terrestrial ecosystems (Mrozik & Piotrowska-Seget, 2010; Tyagi *et al.*, 2010; Federici 2012; Schultz-Jansen *et al.*, 2016) [20, 30, 11, 28]. The petrochemical and pharmaceutical industries, gas stations, oil refineries, seepage of water from agricultural lands treated with pesticides and herbicides, and gas stations are the main sources of these pollutants in the soil and water resources.

The direct toxicity of hydrocarbons from light oil fractions and the altered hydrophobicity and other physical and chemical properties of soil caused by heavy oil fractions are the main mechanisms by which petroleum negatively affects living organisms (Abosede, 2013; Adewuyi & Oluremi, 2015; Ossai *et al.*, 2020) [1, 2, 26]. Compared to the effects of heavy oil, the effects of light oil on plants are more potent but also more transient (Korshunova *et al.*, 2019) [18]. However, the light petroleum fractions are rapidly migrated from the soil or broken down by microbial destructors; as a result, when contamination is severe, it is primarily the heavy petroleum fractions that harm living things, including microbes, plants, and animals.

According to Azubuike *et al.* (2016) [4] and Williams and Amaechi (2017) [31], bioremediation is a process that uses living things, primarily microorganisms and green plants and their enzymes, to remove, degrade, mineralize, transform, and detoxify the hazardous and environmentally polluting components into less toxic or harmless forms while treating contaminated sites to restore them to their original state. The kind of contamination, an electron acceptor, and the microorganisms are all factors that must be taken into account during the bioremediation process. Due to their genetic potential, microorganisms have a wide range of degradation capabilities and can break down petroleum hydrocarbons by using them as a source of carbon and energy (Nrior & Mene, 2017; Williams & Inweregbu, 2019) [32].

This process results in the formation of carbon dioxide, water, biomass, and simple compounds that have no negative environmental effects. The hydrocarbon is oxidized by losing electrons while the oxygen is reduced by gaining electrons (Artin-Hatzikioseyian, 2010) [3]. This is accomplished either by adding exogenous microbes to improve bioremediation or by stimulating natural activities and other environmental modifications that use fertilizers or organic nutrients as substrates to increase rates of biodegradation. To accelerate the biodegradation of the pollutants, certain exogenous microorganisms are added to the soil through the process of bioaugmentation (Joo *et al.*, 2007; Juwarkar *et al.*, 2010) [16, 17]. Numerous factors affect the effectiveness and success of bioaugmentation, such as the inoculated microorganisms' capacity to grow and survive in the new environment, their ability to retain their degradative potentials, their interaction and contact with the contaminant, the availability of electron donors and acceptors, and the availability of enough nutrients to eliminate the target contaminants (Artin-Hatzikioseyian, 2010) [3]. Additionally, the ability of exogenous microorganisms to survive and catabolize, as well as their resistance to other co-contaminants found in the soil and the contaminants' bioavailability, are taken into account (Beskoski *et al.*, 2012) [5]. According to Beskoski *et al.* (2012) [5], using microorganisms that have been isolated from the soil to be remediated is the most practical way to get around these problems. Several pilot, field, and laboratory-scale applications of the principles of biodegradation have been conducted, with differing degrees of success. (Ogbonna *et al.*, 2007; Chikere *et al.*, 2012; Erenee *et al.*, 2017; Shivendra *et al.*, 2017) [24, 8, 9].

Since microbial bioreactors provide a controlled environment in which critical process parameters can be optimized to maximize the microbial bioremediation process, their use in remediation is highly attractive. One additional benefit is that the bioreactor's size and configuration can be easily customized to fit the reactor's intended use or application (Pino-Herrera *et al.*, 2017; Gargouri *et al.*, 2011; Chikere *et al.*, 2012; Azubuike *et al.*, 2016) [27, 12, 8, 4]. For thousands of years, fungi and bacteria have been collected and utilized in bioremediation processes.

2. Materials and Methods

2.1 Description of Study Area

The area used for this study is within the Rivers State University Demonstration Farmland in Nkpolu-Oroworukwo, Mile 3 Diobu area of Port Harcourt, Rivers State. The piece of land is situated at Longitude 4.80620^{o}N and Latitude 6.97752^{o}E measuring 5.4864m x 5.1816 m with a total area of 28.4283 m^{2}. This was cleared and sub-partitioned into 9 blocks of 100cm x 50cm x 20cm giving 150 kg soil in each plot using a digital standing scale with each block 65cm apart. Two of these plots were designated as pristine and crude oil soil to serve as negative and positive controls

2.2 Isolation of the Test Organisms

The bio-augmenting organisms (*Bacillus* and *Pseudomonas* species) were isolated from the soil using standard microbiological methods (Spread plate method) as described by Prescott *et al.* (2005) [35]. One gram of soil sample was aseptically transferred into 9 ml of sterile normal saline and subjected to tenfold serial dilution. Then an aliquot (0.1 ml) of the sample was inoculated into properly dried nutrient agar plates (15 ml in each plate) in duplicate, spread evenly using flamed bent rod and incubated at 37 °C for 24 hours, after incubation, the bacterial colonies that grew on the plates were sub-cultured unto fresh nutrient agar plates using the streak plate technique to obtain pure culture of the bacterial isolates as adopted by Williams and Inweregbu (2019) [32].

2.3 Characterisation and Identification of Test Organisms

Bacterial isolates were characterized on the basis of their colonial morphology, microscopic and biochemical characteristics (Table 1) and by making reference to the identification manual by Cheesebrough (2000) [6].

2.4 Construction of Bioreactor

A bioreactor was constructed in order to obtain the right cultivation of the two organisms, *Pseudomonas* (Pse) and *Bacillus* (Bac), which were used to carry out degradation of

the contaminant (crude oil) under controlled conditions. Seventeen (17) litres of broth were prepared and poured into two containers labelled Pse and Bac and *Pseudomonas* and *Bacillus* were inoculated in the broth. Continuous stirring was carried out every 1hr for 5 days. Buffer was added to both organisms to give the required pH needed.

2.5 Preparation of Broth Culture and Standardization of Inoculum

Five colonies from the pure culture of each isolate were inoculated into nutrient broth in 500 ml conical flask separately, and incubated at 37 °C for 18 to 24 hours. After incubation, an aliquot of 0.1 ml was inoculated on a pre dried nutrient agar to determine the total viable counts of the broth culture. Turbidity of the bacterial suspension (i.e. overnight nutrient broth (250 ml) with population density) was adjusted to match that of 0.5 McFarland Standard (30 to 300 colonies mostly 200 colonies) by making a dilution of 1:100 in sterile nutrient broth (Cheesebrough, 2000) [6].

2.6 Media Used and Preparation

The media used during the analysis were Nutrient agar (NA), Sabouraud dextrose agar (SDA), Mineral salt agar (MSA). All the media were sterilized and prepared according to Manufacturer's instruction. Table 3.2 shows the composition of MSA media.

2.7 Sterilization

Glass wares were sterilized at 160 °C for 1hr in hot air oven, including petri dishes, Bijou bottles, test tubes, conical flasks, pipettes, beakers. Culture media was sterilized by autoclaving at 121 °C at 15 psi for 15 minutes**.** The hockey stick (spreader glass) was disinfected by dipping in alcohol and passing over flame. Other materials, including the wire loop were sterilized by heating to red hot using a Bunsen burner flame.

2.8 Treatment and Application of Crude Oil and Nutrients

Crude oil used in this experiment was obtained from Nigerian Agip Flow Station Ebuocha. The stock culture was prepared by weighing out 4000 g (PCE analytical weighing balance PCE-6000 UK), and dissolving in 1.0 l of distilled water to give initial crude oil concentration of 2500 g/l. The soil was artificially contaminated by spiking the prepared crude oil concentration on the plots and allowed to stay for 21 days (to ensure volatilization and sorption of crude oil into the soil matrix) before application of various treatments.

The plots were amended with 375 ml of *Pseudomonas* and 375 ml of *Bacillus* broth respectively (Nrior & Echezolom, 2016) [22]. Plot one was uncontaminated and plot 2 was contaminated but unamended. These two plots served as controls. Plot 3 to 9 were amended with different concentrations of treatment as indicated in table 1 Plot 9 was specifically amended with mixture of biostimulating and bioaugmenting nutrients concentrations. These were allowed for 7 days with the objective of assessing the microbial activities within week1 of treatment (Bioremediation) sampling.

Table 1: Treatment of Experimental Plots using Nutrient Amendments and Bioaugmenting Organisms

			Bioaugmenting Organisms in Broth		Biostimulating Nutrient
Sample ID	**Plot Code (g)**	**Crude oil (L)**	**PSE (ml)**	**BAC (ml)**	**PD (1500g)**
P1	US	-	-	-	-
P2	CS	4	-	-	-
P3	CS + Pse	4	750	-	-
P4	CS + Bac	4	-	750	-
P5	CS + Pse + Bac	4	375	375	
P6	CS + PD	4	-	-	750
P7	CS + Pse + PD	4	375	-	750
P8	CS + Bac + PD	4	-	375	750
P9	CS + Pse + Bac + PD	4	375	375	750

Keys: P – Plot, US – Uncontaminated Soil, CS – Contaminated soil; Pse – *Pseudomonas*; Bac- *Bacillus*; PD – Pigeon Droppings

2.9 Sample Collection for Analysis

Soil samples for laboratory analysis were collected on days 1, 14, 28, 42, and 56 in sterile sample containers from a depth of 0 to 15 cm after tilling using hand auger. Two samples collected were made from 4 to 10 random points per plot and bulked to form a composite sample. Small portions (5 g) of the composite samples were collected into sterile bottles using sterile hand auger for microbiological and physiochemical analysis. Microbiological analysis was carried out in the Microbiology Laboratory of the River State University within two hours of sample collection while chemical analysis was carried out at Pollution Control and Environmental Management (POCEMA) Port Harcourt Rivers State. Soil samples were stored at 14±2 °C for futher analysis (Nrior & Mene, 2017) [23].

2.10 Microbiological Analysis

2.10.1 Enumeration of Total Heterotrophic Bacterial Count (THB): Total heterotrophic bacterial count was carried out on both the contaminated and uncontaminated soil by weighing 1 g of sample which was serially diluted in 9 ml normal saline up to ten-fold. Using spread plate method, an aliquot of 0.1ml from 10^{-6} test tube was inoculated into solidified nutrient agar in duplicates. The culture plates were incubated at 30 °C for 24 hrs for count. Discrete colonies that developed were counted and expressed in cfu/g (Williams *et al*., 2019) [32].

2.10.2 Enumeration of Fungal Count (TFC)

Fungal count was carried out on both the contaminated and uncontaminated soil by weighing 1g of sample and was serially diluted up to ten-fold. Using spread plate method, an aliquot of 0.1ml from 10^{-4} test tube was inoculated into solidified PDA plate in duplicates. The culture plates were incubated for 48hrs at room temperature 28 °C for count. Discrete colonies that developed were counted and expressed in cfu/g (Morikawa *et al.*, 2000) [19].

2.11 Isolation of Hydrocarbon Utilizing Microorganisms for Bioremediation Treatment

2.11.1 Enumeration and Isolation of Hydrocarbon Utilizing Bacteria (HUB)

The culture medium used for the isolation of hydrocarbon utilizing bacteria was mineral salt agar (MSA). Hydrocarbon utilizing bacteria (HUB) were isolated and enumerated as adopted from (Ibiene *et al*., 2011) [15] using vapour phase transfer method. Using a spread plate method, an aliquot of 0.1ml of the 10^{-4} dilution was plated into a solidified MSA plates medium supplemented with a nystatin (Fungosol) to inhibits the growth of fungi in duplicates and the plates were inverted and filter papers were placed inside the inverted plates cover and was flooded with 1ml of sterile crude oil as source of carbon and energy. It was then incubated at 30 ^{0}C for 5 to 7 days. Discrete colonies that developed were counted and expressed in cfu/g.

To obtain a pure culture, the bacterial isolates were sub-cultured using the accepted techniques, the pure cultures were characterized and identified to ascertain the bacterial species.

2.11.2 Serial Dilution

crude oil contaminated soil was homogenized, 1.0 g of soil mix samples were measured using electric weighing balance and aseptically transferred into a sterile test tube containing 9.0 ml of normal saline. The dilution was carried out serially in a stepwise fashion until a dilution of 10^{-6} was reached.

2.11.3 Isolation of Pure Culture

Colonies and spores that grew on NA and SDA from the baseline and biostimulation setup after incubation were enumerated. Similarly, colonies and spores were picked for subculture to obtain pure cultures. Pure cultures of fungi were stored on SDA slants while the bacterial isolates were stored in 10% glycerol in Bijou bottles. The colonies counted were expressed as colony forming unit (CFU) per gram or SFU/g of soil for bacteria and fungi using the formula:

$$T = N/V \times DF \quad (1)$$

Where:
T = total number of colonies or spores in CFU/g/SFU/g soil
N = number of colonies or spores counted on the plate
V = volume of inoculum plated i.e. 0.1ml
DF = dilution factor used plating (10^6)

Total Heterotrophic Bacterial counts = number of colonies/volumes plated (0.1ml) × Dilution factor (2)

Fungal count = number of spores/volumes plated (0.1ml) × Dilution factor (3)

2.11.4 Inoculation and Incubation

Isolates were inoculated into bioreactor tanks for the multiplication of organisms before bioremediation of crude oil contaminated soil.

Using a pipette, 1ml aliquot of 10^{-1}, 10^{-3} and 10^{-4} dilutions were spread plated into sterile solidified SDA and MSA containing tetracycline in replicate and incubated for 5 to 7 days at 28 °C while 10^{-1}, 10^{-5} and 10^{-6} dilutions were spread plated on solidified nutrient Agar and MSA containing fluconazole in duplicate and incubated at 37° C for 24 hours. Vapor Phase transfer method was used during incubation of the MSA plates with crude oil being the sole source of carbon and energy.

2.11.5 Identification and Characterization of Isolates

Each isolate obtained after isolation was characterized based on colonial, microscopic and macroscopy. Bacterial colonies were identified based on their color, morphology, elevation, size, and margin etc. Discrete bacterial colonies were purified through sub culturing. The isolates were identified according to descriptions in the Bergeys Manual of Systematic (Holt, 2000) and recorded accordingly.

2.12. Bioremediation Studies

2.12.1 Bioremediation Field Application

Nine Randomized Complete Block Design (RCBD) degradative plots according to the method adopted by Williams *et al*., (2025) [34] were set-up with the aim of monitoring bioremediation of crude oil contaminated soil, which include US, CS, CS + Pse, CS + Bac, CS + Pse + Bac, CS + PD, CS + Pse + PD, CS + Bac + PD and CS + Pse + Bac + PD

2.12.2 Bioremediation Assessment Process

All plots were tilled twice weekly to ensure proper aeration and even distribution of crude oil and treatment agents. Samples were taken at regular interval of days 1, 14, 28, 42, & 56, respectively for microbiological and physicochemical analyses according to the method of (Okpokwasili, & Ibiene, 2006) [25].

2.12.3 Percentage (%) Bioremediation Analyses

The amount of soil containing pollutant remediated and the % remediation in the experiment were determined using the approach of Williams *et al*., (2025) [34].

For the amount of pollutant degraded:

Pollutant remediated amounts are equal Initial Pollutant Concentration (day 1) minus Final Pollutant Concentration (day 56)

$$Ba = Ic - Fc \quad (4)$$

Where:
Ba= Amount of soil contains pollutant degraded
Ic = Initial Concentration of pollutant (day 1)
Fc = Final Concentration of pollutant in plot x (day 56)

For percentage remediation:

The percentage (%) remediation equals Amount of pollutant remediated divided by the Initial Concentration of pollutant (day1), multiplied by 100.

$$\% \text{ remediation} = (Bc/Ic) \times 100 \quad (5)$$

2.13 Physicochemical Analysis

The physicochemical property of the soil sample was determined before experimental contamination/contamination of the soil to establish the baseline parameters and subsequently after crude oil contamination and nutrient application for the duration of bioremediation process for physicochemical parameters. They include; pH, temperature, phosphorus, nitrogen, potassium total hydrocarbon content (THC) following standard procedures.

2.14 Analysis of Microbiological Parameters after Treatment for Bioremediation Monitoring

2.14.1 Enumeration of Total Heterotrophic Bacterial (THB)

Total heterotrophic bacteria count was determined by weighing 1g of sample and was serially diluted in to 9ml normal saline up to ten-fold, using spread plate method as described by Chikere *et al. (*2012). An aliquot of 0.1ml from 10^{-4} test tube was inoculated into solidified nutrient agar plate. The culture plates were incubated for 24hrs for count. Discrete colonies that developed were counted and expressed in cfu/g.

2.14.2 Enumeration of Hydrocarbon Utilizing Bacteria (HUB)

According to Ezekoye *et al.* (2015) [10], the vapour phase transfer technique was used to enumerate hydrocarbon-utilizing bacteria (HUB). In order to prevent the growth of fungi, the media was supplemented with cicatrin. Aliquot of 0.1 ml of the 10^{-3} dilution was spread onto solidified MSA plates using the spread plate technique. The plates were then inverted, filter paper was placed inside the inverted plate cover, and 1 ml of sterile crude oil was added as a source of carbon and energy. The inoculated plates were incubated for 5 to 7 days at 37^0C. The number of discrete colonies that formed were counted and given in cfu/g.

2.15 Statistical Analysis

In order to determine if there was a significant difference in mean value between different treatments and the study's data, analysis of variance (ANOVA) with P-values test of significance was conducted at a 95% level of confidence.

3. Results and Discussion

Microbiological and Physicochemical Properties of the Soil Before Application of Various Treatments for Bioremediation Analysis

The microbiological and physicochemical properties of the soil before the application of various bioremediation treatments are shown in Tables 2 and 3.

The microbiological (THB, F, HUB, HUF) analysis for uncontaminated and contaminated soil showed average counts of $2.40\pm2.80\times10^7$ CFU/g and $4.77\pm0.47\times10^6$CFU/g for THB, $5.10\pm1.11\times10^5$SFU/g and $4.33\pm0.96\times10^4$SFU/g for F, $3.69\pm0.59\times10^3$CFU/g and $2.43\pm0.10\times10^3$CFU/g for HUB, $4.00\pm0.53\times10^3$SFU/g and $1.85\pm0.59\times10^3$SFU/g for HUF, respectively.

The physicochemical results revealed that the pH was acidic with value of 6.3±0.13 for uncontaminated soil and 6.9±0.02 for contaminated soil. The temperature was 27.47 ± 0.35^OC for uncontaminated while the contaminated was 27.80 ± 0.10^OC. Nitrogen was 15.67±0.03 for uncontaminated whereas the contaminated was 12.7±0.04 mg/kg, phosphorus revealed 0.75±0.04 mg/kg and 0.597±0.25 mg/kg for uncontaminated and contaminated. The potassium was 1.825±0.05 mg/kg for uncontaminated and 1.813±0.15 mg/kg for contaminated. THC for uncontaminated was 44.0±2.01 mg/kg and contaminated revealed value of 5785±0.063 mg/kg.

Table 2: Microbiological Analysis of Uncontaminated and Contaminated Soil

Parameters	Uncontaminated Soil	Contaminated Soil
THB(CFU/g)	$6.2\pm2.80^b\times10^8$	$3.7\pm0.47^a\times10^8$
F (SFU/g)	$4.1\pm1.11^a\times10^5$	$3.3\pm0.96^a\times10^6$
HUB (CFU/g)	$3.7\pm0.59^b\times10^5$	$3.4\pm0.10^a\times10^6$
HUF (SFU/g)	$3.2\pm0.53^b\times10^5$	$2.8\pm0.59^a\times10^6$

*Means with same alphabet shows no significant difference across the column ($p\geq0.05$)

Key: THB= Total heterotrophic bacteria, F= Fungi, HUB= Hydrocarbon utilizing bacteria, HUF= Hydrocarbon utilizing fungi, Cfu/g – Colony forming Unit per gram, SFU/g – Spore forming Unit per gram,

Table 3: Physicochemical Parameters of Uncontaminated and Contaminated Soil

Parameters	Uncontaminated Soil	Contaminated Soil
pH	5.8	6.9
Temperature ^{o}C	27.8	27.80
Nitrogen mg/kg	13.3.	12.70
Phosphorus mg/kg	0.65	0.597
Potassium mg/kg	3.41	1.813
Total Hydrocarbon Content (THC) mg/kg	53.41	5785

Key ^{o}C – Degree Celsius, mg/kg – Milligram per kilogram

4.2 Morphological and Biochemical Characteristics of the Isolates: The morphological and biochemical characterization of the isolates and the probable organisms in the study are presented in Table 4.2-4.3. The bacterial genera isolated from crude oil polluted soil were: *Pseudomonas* sp, *Bacillus* sp, *Micrococcus* sp, and *Serratia* sp while the fungal isolates were: *Aspergillus* sp, *Penicillium* sp *Alternaria* sp, *Candida* sp and *Fusarium* sp.

Table 4: Biochemical Identification of Bacterial Isolates

Isolate codes	Gram Rxn	Cell Shape	Catalaqse	Citrate	Oxidase	Indole	Motility	Lactose	Glucose	Mannitol	Sucrose	MR	VP	Elevation	Margin	Shape	Texture	Colour	Size (mm)	Isolated Organism
1	-ve	Rods	+	+	+	-	+	-	-	-	-	-	-	Raised	Entire	Round	Moist	Green	3	*Pseudomonas* sp
2	-ve	Rods	+	+	+	-	+	-	AG	AG	AG	-	+	Raised	Entire	Round	Moist	Red	2	*Serratia* sp
2	+ve	Rods	+	+	+	-	-	-	AG	A	AG	+	-	Flat	Serrated	Round	Dry	Creamy	3	*Bacillus* sp
4	+ve	Rods	+	+	+	-	+	A	AG	-	-	+	-	Raised	Entire	Round	Dry	Creamy	5	*Bacillus* sp
5	+ve	Rods	+	-	+	-	+	-	AG	AG	AG	-	-	Flat	Irregular	Round	Dry	Whitish	6	*Bacillus* sp
6	+ve	Cocci	+	+	-	-	-	AG	AG	AG	AG	-	-	Raised	Entire	Round	Moist	Milky	2	*Micrococcus* sp
7	+ve	Rods	+	+	+	-	-	-	A	A	A	+	-	Flat	Serrated	Round	Dry	Milky	4	*Bacillus* sp
8	+ve	Rods	+	+	+	-	-	-	A	A	-	+	-	Flat	Filamentous	Irregular	Dry	Milky	6	*Bacillus* sp
9	+ve	Rods	+	-	+	-	+	A	A	-	A	+	+	Flat	Serrated	Round	Dry	Creamy	5	*Bacillus* sp
10	+ve	Rods	+	-	+	-	+	A	A	A	A	-	-	Flat	Undulate	Round	Moist	Whitish	3	*Bacillus* sp

4.6 Microbial Counts in the Bioremediation Evaluation of Crude Oil Polluted Soil

The results of the microbial counts obtained during bioremediation of crude oil contaminated soil are shown in Figures 1-2.

Significant counts for total heterotrophic bacteria were recorded on days 14 and 56. The counts for each set up during the monitoring were as follows; CS+PD (1.50×10^{9}Cfu/g), CS+PSEU+PD (2.30×10^{9}Cfu/g) , CS (1.04×10^{9}Cfu/g), CS + BAC (1.03×10^{9}Cfu/g), CS + PSEU (9.7×10^{8}Cfu/g), CS+BAC+PD+PSEU (8.97×10^{8}Cfu/g), CS + BAC + PD (8.76×10^{8}Cfu/g) and CS+PSEU+BAC (9.40×10^{8}Cfu/g) (Figure 1).

Hydrocarbon utilizing bacteria; had its peak count on day 28. The counts for each set up during the monitoring were as follows; US (1.35×10^{6}Cfu/g), CS+PSEU+PD (5.16×10^{5}Cfu/g), CS+PSEU+BAC (4.16×10^{5}Cfu/g), CS (5.90×10^{5}Cfu/g), CS+PSEU (4.73×10^{5}Cfu/g), CS+PSEU+PD (5.16×10^{5}Cfu/g) , CS+BAC (6.20×10^{5}Cfu/g) , CS+BAC+PD (5.30×10^{5}Cfu/g) (Figure 2).

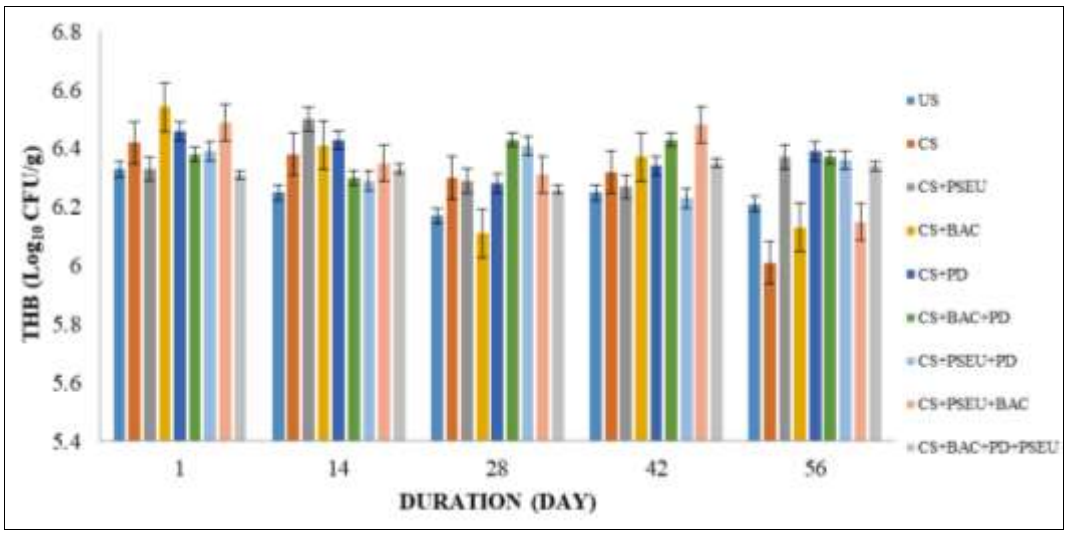

Fig 1: Mean Changes in Total Heterotrophic Bacteria Counts during Bioremediation of Crude Oil Polluted Soil

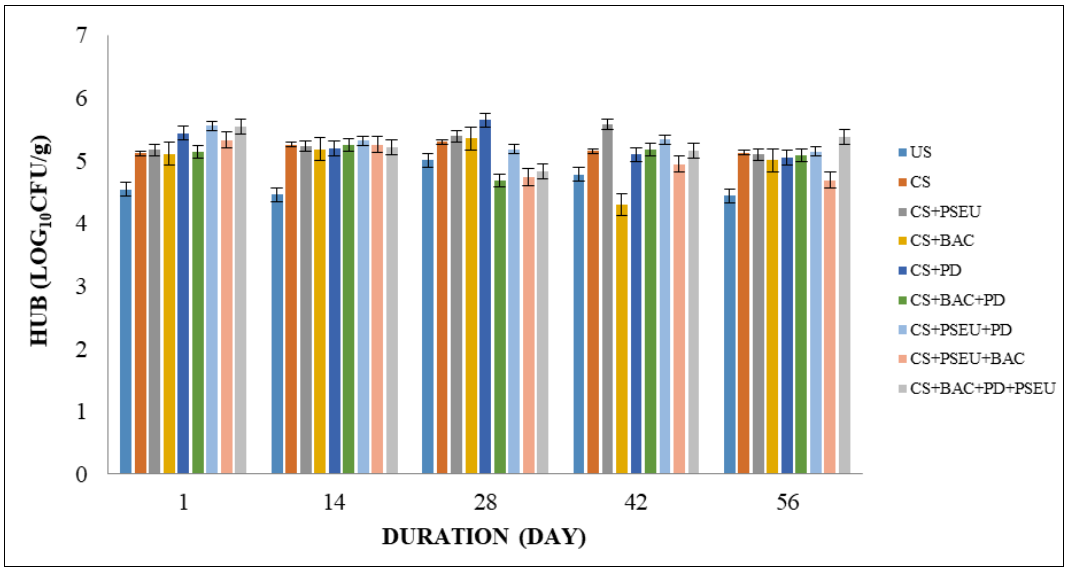

Fig 2: Mean Changes in Hydrocarbon Utilizing Bacteria Counts during Bioremediation of Crude Oil Polluted Soil

4.7 Physicochemical Parameters of the Crude oil - contaminated Soil Samples during Bioremediation Monitoring

The physicochemical changes of the crude oil-contaminated soil with various bioremediation treatments are shown in Figures 3 -10.

pH values obtained from the set up during bioremediation is shown in Figure 3. The values ranged from 5.94 (CS+PD) to 7.39 (US)

Temperature values obtained from the set up during bioremediation is shown in Figure 4. The values obtained from the study were relatively same between the bioremediation set up during the monitoring which ranged from 26.35 °C (CS+PD) to 27.40 ˚C (US).

Nitrogen values obtained from the set up after 56 days during bioremediation as shown in Table 5. It ranged from 6.90 mg/kg (CS+PSEU+PD) to 15.86 mg/kg (US).

Phosphorus values recorded is shown in fig. 6 after 56 days of monitoring. Day 1 recorded a high value, 0.640 mg/kg (US) and the lowest value, 0.137 mg/kg was obtained from treatment, CS+PSEU+PD.

Potassium values recorded during bioremediation monitoring is shown in Figure 7. It ranged from 1.002 mg/kg (CS+BAC+PD+PSEU) to 1.835 mg/kg (US).

Total Hydrocarbon Content (THC) value obtained during bioremediation of crude oil contaminated soil is recorded in Figures 8 & 9. The amount of hydrocarbon removed and % bioremediation efficiency after 56 days of monitoring with different treatments in the setups is given in their decreasing order as follows: initial THC value of 9296.83 mg/kg, CS + Pse + Bac + 2976 mg/kg; 51.17% (PD) to 1120 mg/kg: 19.36% (CS).

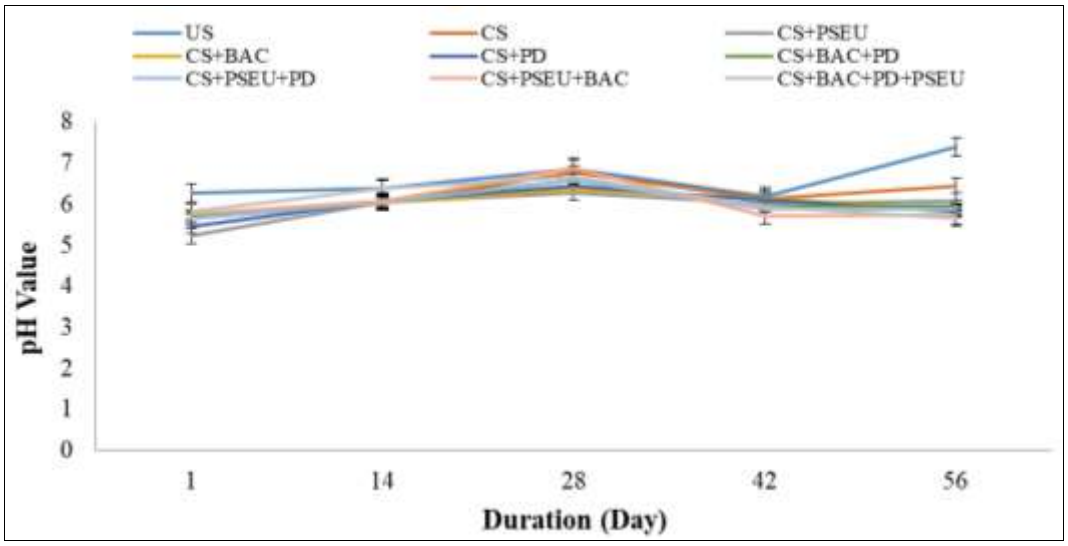

Fig 3: Trends in pH during Bioremediation of Crude of Crude Oil Contaminated Soil

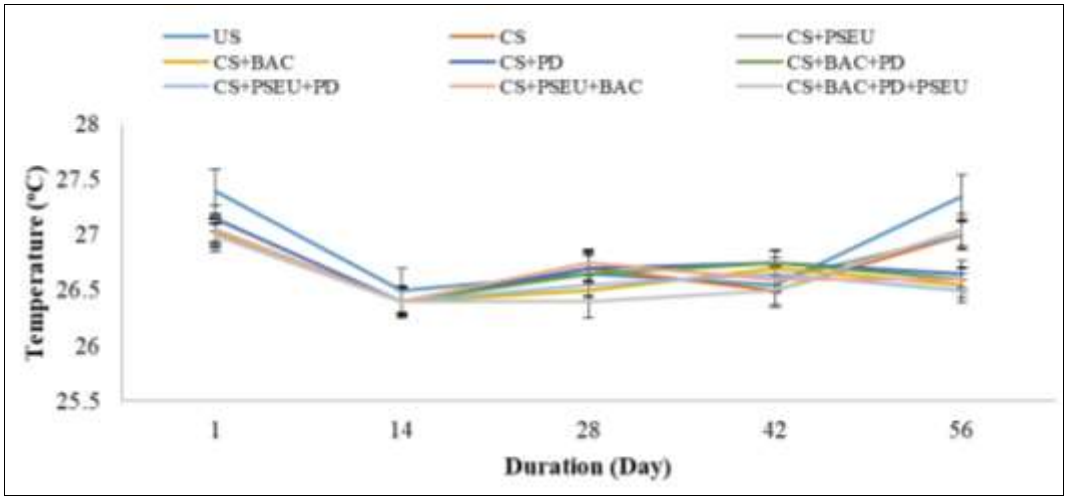

Fig 4: Trends in Temperature during Bioremediation of Crude Oil Contaminated Soil

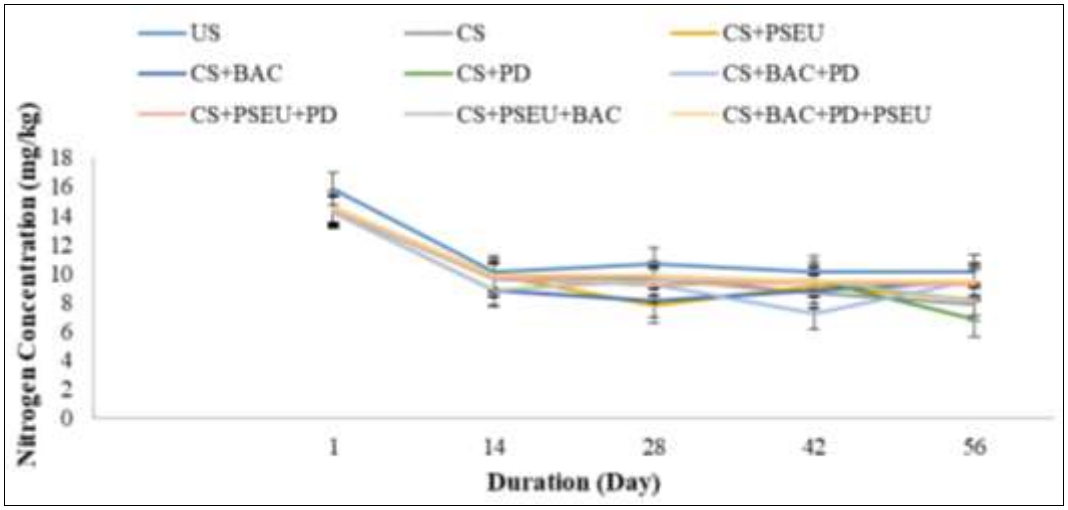

Fig 5: Trends in Nitrogen during Bioremediation of Crude of Crude Oil Contaminated Soil

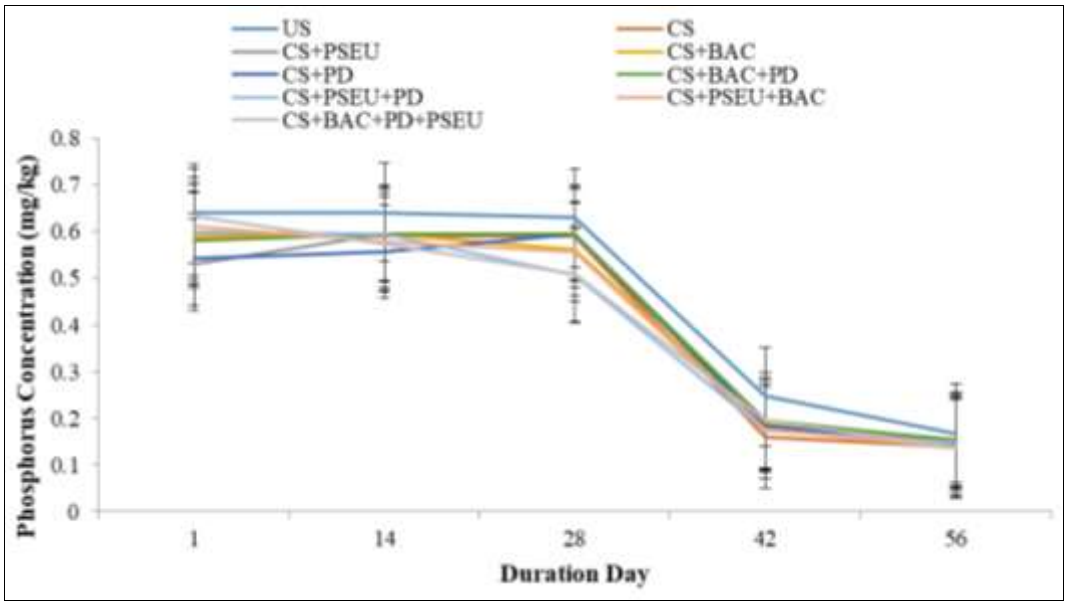

Fig 6: Trends in Phosphorus during Bioremediation of Crude of Crude Oil Contaminated Soil

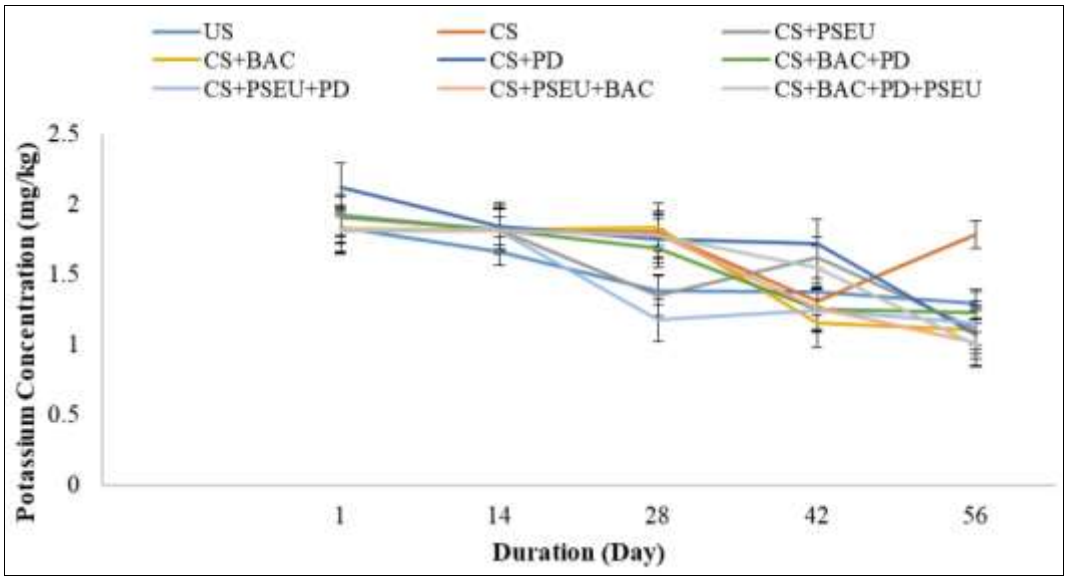

Fig 7: Trends in Potassium during Bioremediation of Crude of Crude Oil Contaminated Soil

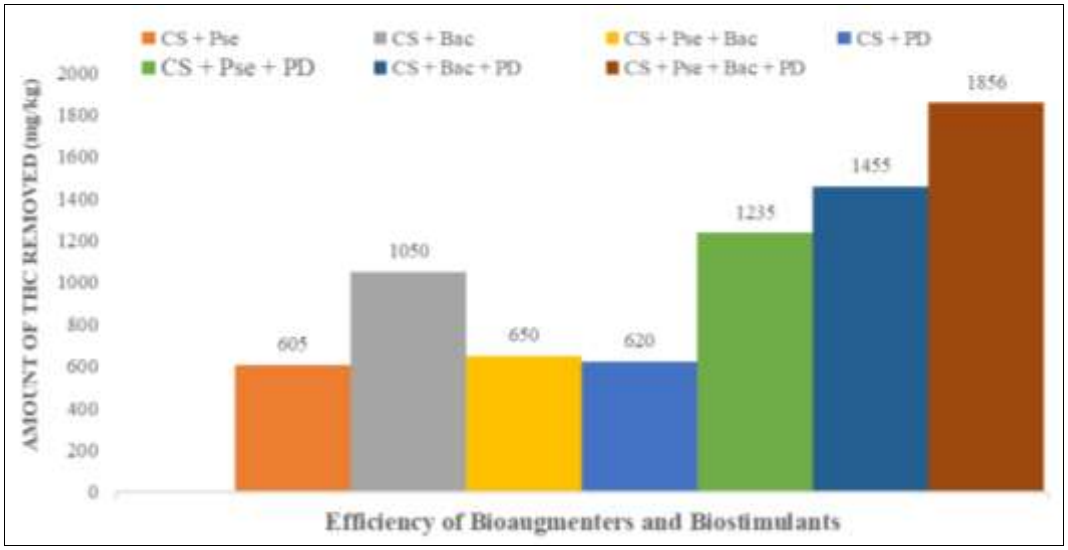

Fig 8: Amount of THC (mg/kg) Removed During Bioremediation of Crude Oil Contaminated Soil

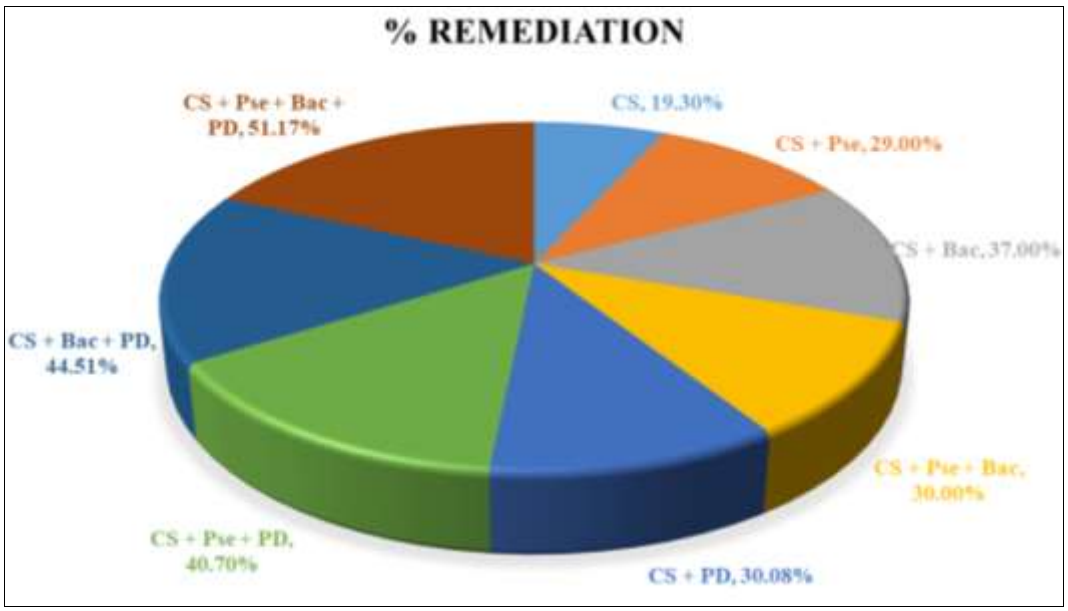

Fig 9: % THC (mg/kg) Percentage Remediation during Bioremediation of Crude Oil Polluted Soil

The results of the microbiological analyses from uncontaminated and crude oil - contaminated soil showed that total heterotrophic bacterial count was the highest compared to the other microbial populations and crude oil utilizing bacterial counts followed closely. The increase in the counts may be as a result of the abundant nature of bacteria in soil and their ability to utilize hydrocarbon substances as carbon sources (Williams & Amaechi 2017) [31].

The bacterial isolates from the experimental soil used in this study belong to the genera: *Bacillus, Pseudomonas, Aeromonas, Escherichia* and *Micrococcus* species. The result is in correlation with the work reported by Williams and Akemi (2020) [36] that isolated *Pseudomonas, Klebsiella, Bacillus, Micrococcus,* and *Proteus* species. The result of this study showed that these microorganisms could be used in bioremediation of crude oil contaminated soil.

A significant difference of crude oil biodegradation in soil amended with pigeon droppings and unamended soil treatment occurred between 14 and 28 days, where bio stimulation resulted in significant increase of oil biodegradation. Addition of nutrients stimulate the degradative capabilities of the indigenous microorganisms, thus, allowing the microorganisms to break down the organic pollutants at a sporadic rate (Ausma *et al.*, 2002; Williams *et al*., 2025)) [34].

The samples amended with Pigeon droppings degraded the crude oil contaminated soil more than the unamended (Williams *et al*., 2024) [33]. This may possibly be due to a higher nutrient level present in Pigeon droppings. Amendment with organic nutrients like goat manure, fish wastes and pigeon droppings are recommended for contaminated soils due to their high nutrient content as substrates for bio stimulation of indigenous and augmenting biodegrading microbes (Williams *et al*., 2024; Nnemelu *et al*., 2024) [33, 21]. Research has shown that organic waste harbors hydrocarbon utilizing bacteria (Agarry *et al*., 2012) [37]. The biodegradation recorded in the unamended soil sample could be due to non-biological factors such as evaporation, photo-degradation volatilization, adsorption, abiotic factors (Temperature and pH). Reduction of petroleum hydrocarbon in unamended sample has also been reported by previous studies.

The results of the total hydrocarbon content (THC- mg/kg)

of the bioremediation set up suggested that there was a reduction on the 56th day of monitoring.

5. Conclusion

The study showed that the bioreactor was effective in multiplying the microbial isolates used in the various treatment plots which in turn enhanced the degradation of the contaminated soil. The isolation and identification of microorganisms from the soil samples indicated an abundance of hydrocarbon utilizing microorganisms, thus, indicating that the isolates possess the functional ability for hydrocarbon degradation.

From this study, the synergistic bioremediation of crude oil contaminated soil using *Pseudomonas aeruginosa, Bacillus safensi* and pigeon droppings with 51.17% hydrocarbon reduction offers a potential option for crude oil remediation. This is cost-effective and a tool for the preservation of the agricultural ecosystem.

6. Conflict of Interest: Not available

7. Financial Support: Not available

8. References

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