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Impact of Bioremediation on the Microbial Community of Crude Petroleum Polluted Agricultural Soil

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ABSTRACT

The effect of bioremediation on sterile agricultural soil polluted with crude petroleum was investigated with a view to developing a suitable technique for the rehabilitation of similar environments when polluted with crude oil. Axenic cultures of non-pathogenic hydrocarbon utilizing bacteria namely; Pseudomonas aeruginosa and Bacillus subtilis were inoculated into the polluted soil to enhance the biodegradation action. Soil samples from the polluted soil were analyzed on a weekly basis for physicochemical and microbiological characteristics over sixty-three days study period. Hydrocarbon utilizing bacteria (HUB) isolated from the polluted soil include; Bacillus spp, Micrococcus spp, Pseudomonas spp, Acinetobacter spp, Flavobacterium spp, Alkaligenes spp, while the fungi include; Penicillum spp, Aspergillus spp, and Fusarium spp. Total Pseudomonas recovered from the experimental soil samples ranged from an average value of $0.6 \pm 0.10 \times 10^{-7}$ cfug $^{-1}$ at week zero reaching up to $0.90 \times 10 \pm 0.15$ cfug $^{-1}$ at the middle of the sampling period, and finally to $0.7 \pm 0.2 \times 10^{-7}$ cfug $^{-1}$ at week nine. There was a marked reduction in the residual oil concentration especially in week nine which recorded a high 6.10 ± 0.02g/kg (93.90%) reduction and 7.60 ± 0.01g/kg (92.40%) reduction in experimental samples A and B respectively, when compared to an initial 88.0 ± 0.05 (12.00 %) and 81.6g/kg (18.40%) reduction in both samples at the beginning of the

experiment. At 5% level of significant, almost equal ability was exhibited by both organisms in removing the oil pollutants.

Keywords: Bioremediation**,** Crude oil pollution, Microbial community, Agricultural soil

INTRODUCTION

One of the major issues facing the world today is soil pollution brought on by crude petroleum. Everyday operations such as oil exploration, waste disposal (fuel and oil disposal), and accidental spills produce major environmental problems that cause oxidative stress and alter the chemical composition of soils with limited nutrient availability [1]. Crude petroleum hinders seed germination, diminishes photosynthetic pigments, slows absorption, restricts root growth, produces leaf deformities, and causes cellular damage. Others include the destruction of biological membranes, the disruption of metabolic signalling, and the modification of plant root structure [2]. Low molecular weight hydrocarbons have been shown in studies to infiltrate plant cells and kill them.

Petroleum, frequently referred to as crude oil, is a combustible fossil fuel that occurs naturally beneath the earth's crust. Alkane, cycloalkane, aromatic hydrocarbon, asphaltene, and resin combinations make up its composition[3]. Petroleum hydrocarbons are a necessary energy resource for a country's economic and social development. Humans rely on petroleum hydrocarbons in their daily lives. Petroleum hydrocarbons are utilised to make fuels, petrochemicals, polymers, and chemical synthesis precursors, as well as to meet other daily demands [4]. Excessive usage of petroleum hydrocarbons pollutes the soil, air, and water, and generates environmental toxicity, posing a major threat to human health and other species[5]. It is detrimental to varying degrees, including mutagenic, genotoxic, carcinogenic, cardiotoxic, hepatotoxic, nephrotoxic, immunotoxic, neurotoxic, and cytotoxic. They have the potential to harm our essential organ systems, including the neurologic, circulatory, immunological, and endocrine systems, as well as induce metabolic and hormonal disorders.

Oil spill contamination of soil is a serious global concern today. The toxicity of petroleum hydrocarbons is well documented, with consequences for our ecosystem, agricultural practises, and human health. Soil contaminated with crude oil is a major risk and produces organic pollution of ground water, limiting the soil's agricultural production. The pervasiveness of petroleum hydrocarbons has a severe influence on soil and water ecosystems. It changes the biological characteristics of soil through influencing microbial diversity and enzymatic activity. They also have a phytotoxic effect on seed germination and plant growth by limiting mineral and water intake, lowering plant root length and leaf area [6].

Biodegradation is the primary mechanism through which hydrocarbonoclastic microorganisms are employed to degrade hydrocarbon pollutants, as hydrocarbons are a natural source of energy and carbon. Because of their catalytic activities, indigenous microorganisms or consortiums can attenuate petroleum hydrocarbons and increase the rate of pollutant degradation [7]. More research is needed to better understand microorganisms' modes of action and proliferation in polluted environments [8]. As a result, additional study on basic and applied components of

microbial synergistic effects is required to determine the biodegrading capacity of bacteria in contaminated areas, as well as changes in their structure and metabolic processes.

METHODS

Isolation And Indentification Of Microorganisms

Two hydrocarbon utilizing bacteria; Pseudomonia aeruginosa and Bacillussubtilis isolated, characterized and identified from oil impacted agricultural soil were employed in the bioremediation process. The bacteria in pure culture were further culture in the developed Beef extract medium (UDBB) before application on the Petroleum impacted soil during research.

Collection And Processing Of Samples

The crude oil used in this research was the Escravos light crude with specific gravity 0.08467 obtained from Chevron Nigeria Limited (CNL). The soil was collected from a farmland within the biological garden of the University of Lagos, Akoka, Nigeria. Soil samples from both experimental and control soils were collected randomly and aseptically into sterile screw-capped bottles for microbiological analyses.

Media Preparation

Laboratory prepared microbiological media made from locally available raw material (beef broth) designated UDBB was used throughout the research for the Innoculum development and for dispersing on the experimental and control soils.

Innoculum Development

The bacterial species were resuscitated by sub-culturing on nutrient agar plates and incubated for 24h at 37^{o}C. 250ml of UDBB broth was prepared in triplicates and the organisms were inoculated into them. These cultures were gently rocked to homogenize before incubation. The optical densities of the cultured broth were measured using spectrophotometer-model: (Jenway 6305 Uv/Vis) at 620mn wavelength. Turbidity was measured at 6 hourly intervals for 72hrs respectively. Values for the optical density (OD) were plotted against growth time to determine the exponential growth period. The growth of the microorganisms was monitored using optical density and standard plate count. To achieve this 1.0ml of each broth culture was aseptically inoculated into a tube containing 9.0ml sterile distilled water (SDW) to give 10^{-1} dilution and a ten-fold serial dilution was carried out. Aliquots (0.1ml) from dilutions 10^{-2}, 10^{-4} and 10^{-6} were inoculated in duplicates on nutrient agar plates using the spread plate technique. The plates were incubated for 24 hours at 37^{0}C, after which the observed colonies on each plate were counted and expressed as colony forming unit/ml (cfu/ml).

Bioremediation Protocols

Experiments to achieve ex-situ biomediation were set up in sizable plastic containers of equal dimensions. 5% pollution of the freshly obtained garden soil sample was prepared in the laboratory set ups designated as follow:

- Experimental set up (ESU) which contained polluted agricultural soil (5% v/w) and 250ml of the Pseudomonas aeruginosa broth at its exponential growth phase evenly dispersed all over the set up.
- A second but similarly designed representing control 1 (CSU 1) which contained the polluted agricultural soil (5%v/w) and 250ml sterile prepared microbiological media (UDBB), but devoid of any microorganism and evenly dispersed in the soil.
- A third also similarly designed representing control 2 (CSU 2) which contained the polluted agricultural soil (5%v/w) and 250ml sterile distilled water (SDW) evenly dispersed all over the soil.

The above designs were set up for both Pseudomonas aeruginosa denoted sample A and for Bacillus subtiles denoted sample B. Each set up was replicated thrice.

The above set-ups were watered (150 ml Sterile and distilled/deionized water) at weekly intervals. At week three however, 150ml sterile distilled/deionized water containing mineral salts (Raymondismedivm) was added to all the set-ups respectively to further enhance the remediation [9].

An exact duplicate set ups similar to ESU A for P. aerginosea and ESU B for B. subtilis were also instituted but with the application of a bioventing procedure.

Soil samples from the above set ups were analyzed on weekly basis for changes in physicochemical and microbiological characteristics over a period of 63 days.

Microbiological Analysis

Plate count technique was employed in the enumeration of the various microbial groups. Serial (10-fold) dilutions of each sample were prepared and the required dilutions were inoculated.

Determination Of Microbial Populations In The Soil

Before pollution of the soil samples with crude petroleum and throughout the study period both the total heterotrophs (TH) and total hydrocarbon utilizers (THCU) present in the soil were estimated on a weekly basis.

To achieve this, 1.0g soil sample was transferred aseptically put into a test containing 9ml sterile distilled water (10^{-1} dilution). Aliquots (0.1ml) from both low (10^{-3}, 10^{-5}) and high (10^{-7}, 10^{-9}) dilutions of the soil samples were plated out on nutrient agar (NA) and on potato dextrose agar (PDA) plates for isolation of bacteria and fungi respectively. For bacteria, 6 replicates were made; 3 were incubated at 37^0Cfor 2 – 4 days, while the remaining 3 were incubated at room temperature – 28$\pm$ 2^0C for 2 – 6 days. Three replicates were made for fungal isolation and incubation was at room temperature 28$\pm2^0$C for 3 – 6 days [10].

At the end of incubation, the colonies were screened, counted and identified based on the taxonomic schemes and descriptions by [11] for bacteria and by [12] for fungi. The relative abundance (population density) of the organisms in

the soil samples were estimated by multiplying the plate count per ml for each organism by the dilution factor used. The counts obtained from bacteria and fungi were then summed up, to obtain the total heterotrophs (TH).

Similarly, for the estimation of the total hydrocarbon utilizers (THCU), 0.1ml from 10^{-1}, 10^{-3} and 10^{-4} dilutions of the soil samples were plated out in 6 replicates in minimal salt medium containing (g/e): NaCl, 10.00; $MgSO_4$. $7H_2O$, 0.42; Kcl, 0.29; KH_2PO_4, 0.83; $Na_2HPo_4.H_2O$, 1.25; $NaNo_3$, 0.42; Agar, 15.00; distilled / deionized water to 1000ml mark (PH, 7.2). Crude oil sterilized by filtration (Millipore size 0.25µm) served as the carbon and energy source in this vapour phase technique culture. 3 of the replicates were incubated for bacterial isolation at 37^0C for 3 – 5 days, while the remaining 3 were incubated for fungal isolation at room temperature 28 $\pm$ 2^0C for 2 – 5 days. At the end of incubation, the THCU was estimated and identified as described above.

Estimation of Pseudomonas Species

To determine the population of Pseudomonas species, present in the test soil samples before pollution and throughout the study period, standard plate count on Pseudomonas selective medium was employed. Duplicate 1.0g of both experimental and control soils were transferred aseptically to different test-tubes containing 9.0ml sterile physiological saline each to give 10^{-1} dilution. From these other dilutions were then made. Aliquots (0.1ml) from 10^{-2}, 10^{-4}, 10^{-6} and 10^{-8} dilutions were then aseptically transferred onto the selective medium plates in triplicates and spread using a sterile L-shaped glass rod spreader. The plates were incubated at 37^0C for 24 – 48h. Colonies observed were counted, sub-cultured and identified according to [13], including pigmentation to differentiate Pseudomonas species [14].

Estimation of Bacillus Species

The population density of Bacillus species was determined by standard plate count on Bacillus special agar. Aliquots (0.1ml) from 10^{-3}, 10^{-4} and 10^{-6} dilutions were plated out on the selective agar medium in duplicates and a sterile glass spreader was used to spread the inoculum. The agar plates were incubated for 24 – 48h at 37^0C. The colonies that developed after incubation were counted.

Identification Tests for Bacterial Isolates

Pure cultures of bacteria isolated from the soils were presumptively identified on the basis of their morphological and biochemical characteristics. The observed organisms were characterized and identified according to. The following tests were carried out in duplicates.

Colonial Morphology

The shape, size, colour and elevation of the bacterial colonies were observed on nutrient agar plates which had been incubated for 48 hours at room temperature. Production of pigments was noted.

Gram Reaction

To carry out the Gram stain technique, thin smear of each of the isolates was prepared on clean glass slide. It was air dried and heat-fixed by passing it horizontally across the Bunsen flame. The smear was then flooded with crystal violet stain for one minute and rinsed in slow running tap water. Lugol's iodine solution was then poured on the smear for 30 seconds, and washed off gently under slow running tap water. The smear was then decolourized by flooding with 70% ethyl alcohol until no more crystal violet was washed off – 10 – 15 seconds. It was immediately rinsed under slow running tap water. The smear was counter stained with safranin solution for 30 seconds and rinsed with tap water. The slide was blotted dry with a piece of filter paper and finally allowed to dry in air. It was then examined under the oil immersion objective lens of a light compound microscope. Gram positive organisms were characterized by retention of the purple colour of the basic stain- crystal violet, while Gram negative organisms showed the pinkish colour of the counter stain - safranin.

Motility Test

This was carried out using the semi-solid agar method as described by. 5.0g of Nutrient Agar was dissolved in 250ml of distilled water and heated to dissolve properly. The medium was dispensed into test tubes and properly stoppered before autoclaving at 15psi and 121^{o}C for 15 minutes to sterilize. The autoclaved test tubes were left to solidify at room temperature. The test organisms were inoculated by the stab techniques before incubation at 37^{o}C for 24h. Observation of zigzag growth along the line of inoculation signified motility while only a straight-line growth meant no motility.

Catalase Test

This test is dependent on the presence of an enzyme, catalase, which breaks down hydrogen peroxide to release oxygen. A suspension of the organism was made on a clean glass slide containing few drops of hydrogen peroxide. The occurrence of effervescence with production of gas bubbles indicated a positive result, while the absence of the above reaction indicated a negative result.

Indole Test

This test is a demonstration of the ability of certain bacteria to decompose an amino acid, tryptophan to indole. The organisms were grown in peptone water for 48 hours at room temperature. Kovac's reagent (0.5ml) was added to the culture. Development of a rose pink colour was an indication of indole production.

Methyl Red Test

Methyl Red Test is used to test for organisms that produce acid. This test shows the production of acid during fermentation of glucose such that the pH of a culture is sustained below a value of 4.5 as shown by the colour change of the methyl red indicator added at the end of the period of incubation. Each isolate was inoculated into Methyl-Red Voges Proskauer (MRVP) medium (5ml) and incubated for 3 days at room temperature. After incubation, solution two drops of methyl red (0.4%) were added. A positive result was observed by a change in

colour from yellow to red showing that the pH of the medium has been reduced to the acidic range due to acid production by the isolate. A yellow colouration showed a negative result.

Voges – Proskauer Reaction

This reaction is dependent on the production of acetoin from glucose which is then oxidized by the addition of alkali to diacetyl which gives a pink colour.

Isolates were inoculated into McCartney bottles containing MRVP medium and incubated for 2 days at room temperature. One milliliter of Potassium hydroxide (10%) was added and the bottles were left at room temperature for 15 minutes. A pink colour indicated a positive reaction while negative reaction was indicated by no colour change.

Gelatin Liquefaction

Tubes of sterile nutrient gelatin medium were inoculated with the isolates and incubated at room temperature for 48 hours. The tubes were then put in the refrigerator for 24 hours at 4^{O}C. Failure of the medium to solidify indicated production of gelatinase.

Citrate Utilization

This test identifies organisms that utilize citrate as their sole carbon source for growth and metabolism. Slants of Simmon's citrate agar were inoculated by streaking the bacterial isolates on the surface. The agar slants were incubated at room temperature for 24 hours. Positive reaction was shown by a colour change from green to blue. Organisms unable to utilize citrate as their carbon source did not grow, therefore left a negative result of persistent green colour.

Oxidase Test

A filter paper was soaked with KOVAC's reagent (a dye-P-dimethylaminobenzaldehyde), which is colour less in its reduced state. Fresh culture of a test organism was rubbed on the filter paper. A positive reaction was indicated by the appearance of a purple colour along the line of streaking within 10 seconds.

Starch Hydrolysis

Starch agar was inoculated and incubated at room temperature for 48 hours, after which it was flooded with dilute iodine solution. Hydrolysis was indicated by clear zones around the colonies while unchanged starch due to lack of hydrolysis gave blue black colour.

Urease Activity

Slants of Christensen's urea medium were inoculated with the isolates and incubated at room temperature for three days. The production of a red colour showed a positive urease activity while no colour change meant a negative result.

Sugar Fermentation (Bromocresol Purple Indicator)

Five milliliters of sugar-indicator peptone water base in test tubes were sterilized by autoclaving. The tubes were inoculated with 18 - 24hour old bacterial cultures and incubated at room temperature for 48 hours. Positive reaction was shown by colour change from purple to yellow. The Durham tubes in the bottles were also observed for presence of gas.

Determination of Residual Oil Concentration

The mean changes in residual oil content (ROC) of the soil samples over the 90 days study period were determined both gravimetrically and chromatographically using the method described in [15].

Gravimetric Analysis

Three samples of 10.0g each were randomly and aseptically collected from each of the three set ups and their replicates at biweekly intervals and analyzed. The samples were collected as previously described. Oil was extracted from each sample by mixing 10.0g soil with 30.0ml volume of n- Hexane**:** Dicloromethane solvent system (1**:**1) and stirred for five minutes in a beaker at room temperature (28±2^{0}C). The mixture was filtered through glass wool in a funnel. The extraction procedure was repeated three times and the extracts were pooled together. The solvent system was evaporated from the extract in an oven at 80^{0}C for 5- 10 minutes. Cessation of effervescence in the beaker indicated complete evaporation of the solvent system. The beaker containing the oil extract was allowed to cool at room temperature inside desiccators and then weighed. The weight of the ROC was obtained by differences in weights. Results reported were Mean Residual Oil Content (ROC) obtained from the nine soil samples - three replicates for each set up [15].

Gc Analysis

For the gas chromatographic analysis, the Residual oil Extract (ROE) was diluted with n-Hexane (1**:**5) before it was run under the following conditions**:** System model, Hewlette and Packard 5890 Series II**:** column caplillary DB – 1 (30m x 0.25mm (i.d) x 0.25um); carrier gas**:** Helium**;** temperature programme, 50- 305^{0}C at 3.5^{0}C per min for 90 mins; injector, splittles 45 s valve closure; quality injected, 1ul; detector, flame ionization detector (FID) at 350^{0}C. The peaks corresponding to the different aliphatic hydrocarbon fractions were identified by reference to standards (nC4 - nC40) obtained by computer – optimized calibrations. Also, biomarkers namely nC17/pristine and nC18/phytane ratios indicative of disappearance of crude oil due to microbial activity were extrapolated from GC profiles.

RESULTS

The results for inoculum development showed growth patterns of Pseudomonasaeruginosa and Bacillussubtilis in the microbiological media (UDBB) prepared in the laboratory for the bioremediation experiment. Pure cultures of these microorganisms in UDBB broth were in their exponential phase of growth: 0.756 x 10^{6}cful/ml for Pseudomonasaeruginosa, and 0.625 x 10^{6}cful/ml for Bacillus subtilis as shown by the optical density measurement

before inoculation into the polluted soil samples **(Figure 1)**. Population density of these microorganisms as observed during inocula development are also shown in **(Figure 2)**.

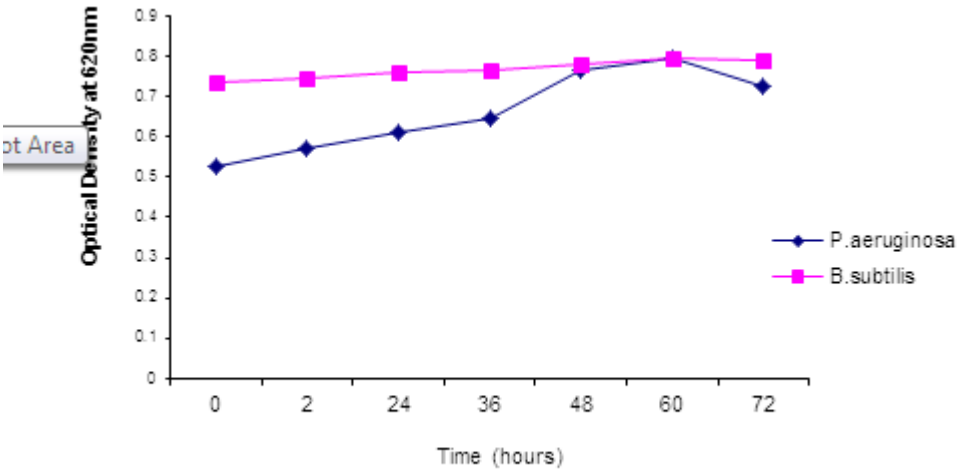

Figure. 1: Growth pattern of Pseudomonas aeruginosa and Bacillus subtilis during inoculum development in UDBB broth for seventy-two hours (Optical density) OD620nm

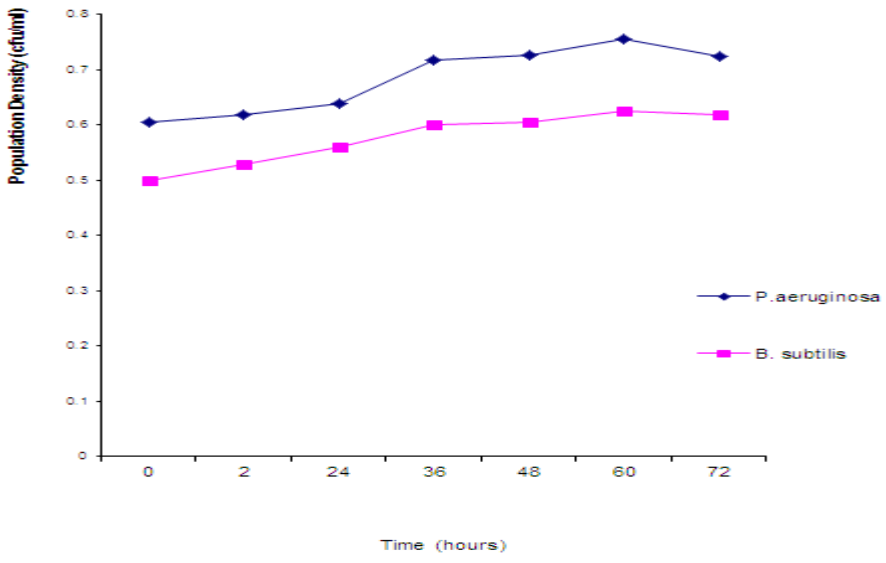

Figure. 2: Population density of Pseudomonas aeruginosa and Bacillus subtilis during Inoculum development in UDBB broth for seventy-twohours [cfuml^{-1}]

The mean population density of the different microbial groups enumerated in the crude oil polluted agricultural soil during bioremediation using Pseudomonas aeruginosa (sample A), and Bacillus subtilis (sample B) showed that Pseudomonas and Bacillus species were observed to be higher in the experimental soil than in the control soils. Similar observations were made in the population levels of hydrocarbon utilizing fungi and bacteria. Total heterotrophs consequently recorded the same trend.

For total Pseudomonas, a mean population range of 0.7±0.10-0.5±0.20cfug^{-1} was recorded for the experimental soil while the control soils ranged between 0.40±0.10 – 0.20±0.00x10^{7}cfug^{-1} for control 1 and 0.40± 0.00 – 0.10 ± 0.10x10^{7}cfug^{-1} for control 2. Mean population density recorded for total bacillus subtilis, ranged between 0.90±0.10 – 1.00±0.00x10^{7}cfug^{-1} for the experimental set up, while the controls ranged from between 0.70±0.00 and 0.80±0.00 for control 1 and 0.70±0.01 – 0.80±0.01x10^{7}cfug^{-1} for control 2. For the total hydrocarbon utilizing fungi, mean population levels were between 1.4±0.00 and 3.00±0.20x10^{5}cfug^{-1} for the experimental soil; and 1.60±0.10 – 2.60±0.20; 1.80±0.00-2.41±0.30x10^{5}cfug^{-1} for controls 1 and 2 respectively. Total hydrocarbon utilizing bacteria recorded a mean population level between 3.20±0.20-5.10±0.00x10^{7}cfug^{-1} for experimental soil; and 2.9±0.20-3.50±0.01; 2.50±0.30-3.0±0.1x10^{7}cfug^{-1} for control 1 and 2 respectively; while mean population level for total heterotrophic organisms ranged between 6.20±0.04 and 9.60±0.30x10^{7}cufg^{-1} for the experimental soil and 5.6±0.40-7.10±0.30; 5.4±0.40-6.24±0.40x10^{7}cfug^{-1} for the control 1 and 2 respectively. A profile of mean population densities as observed throughout the bioremediation experiment on the various microbial groups are shown on Figures 3 to 7 respectively.

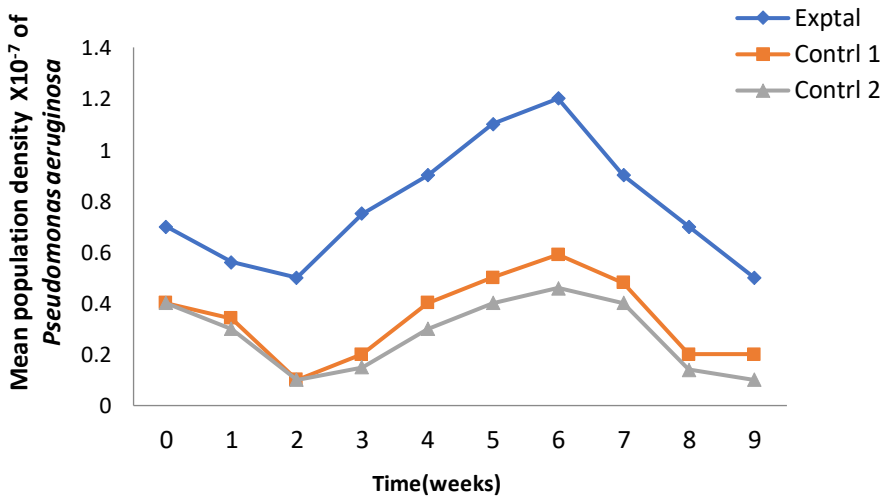

Figure 3. Mean population density [x 10^{-7}cfu/g] of Pseudomonas aeruginosa during bioremediation of crude Petroleum polluted agricultural soils

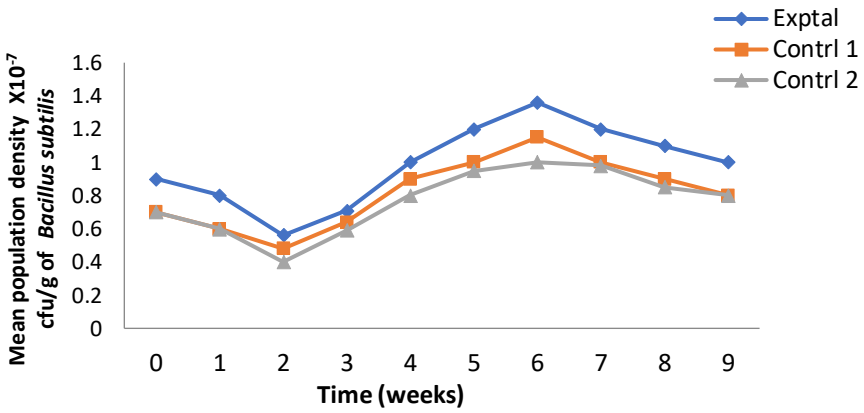

Figure 4. Mean population density [x 10^{-7}cfu/g] of Bacillus subtilis during bioremediation of crude Petroleum polluted agricultural soils

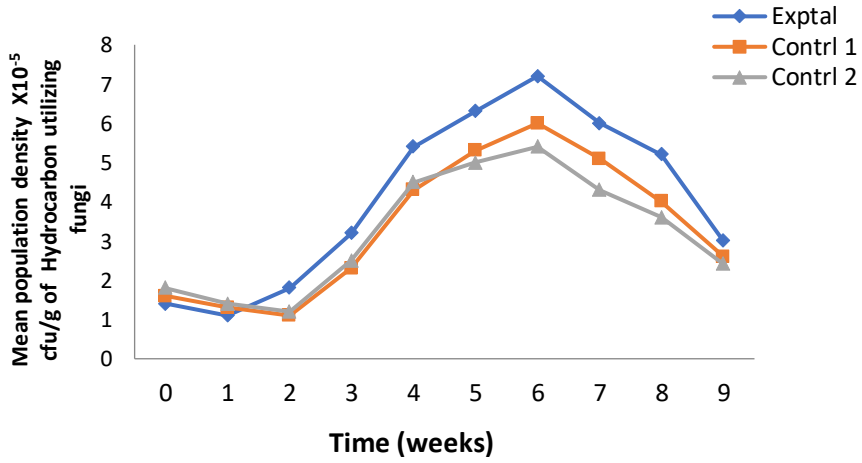

Figure 5. Mean population Density [x10^{-5} cfu/g] of Hydrocarbon utilizing Fungi during bioremediation of crude Petroleum polluted agricultural soils

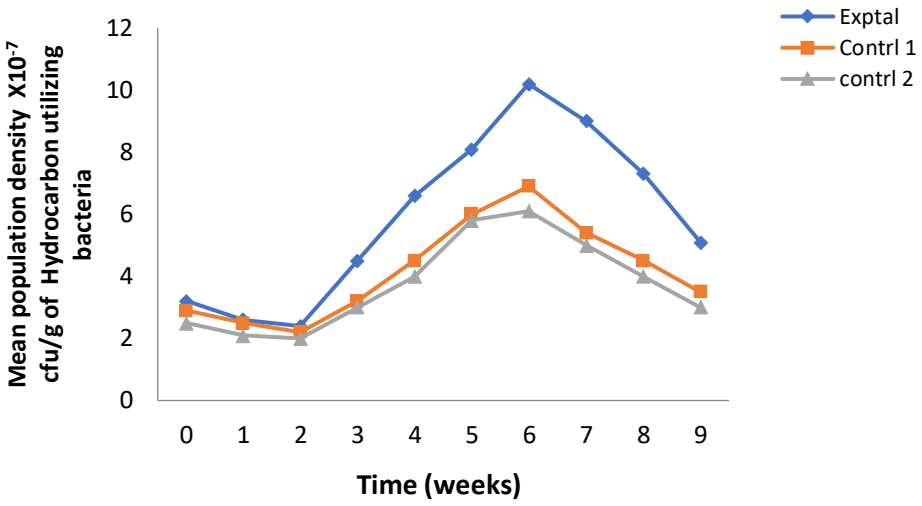

Figure 6: Population Density [x10-7cfu/g] of Hydrocarbon utilizing Bacteria during bioremediation of crude Petroleum polluted agricultural soils

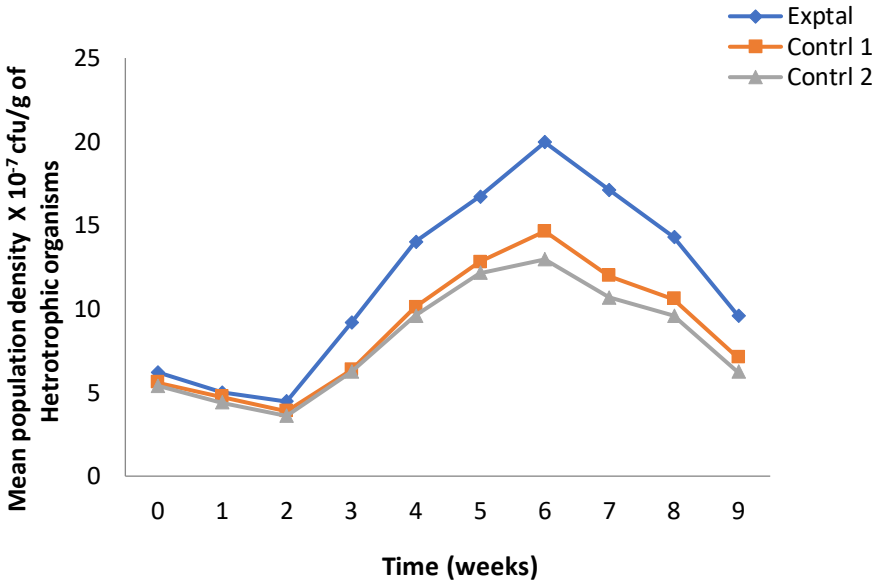

Figure 7. Mean population Density [x10^{-7}cfu/g] of Heterotrophic Organisms during bioremediation of crude Petroleum polluted agricultural soils

Isolation and identification of microorganisms in the experimental and control soils revealed bacteria colonies based on their morphological, cellular and biochemical characteristics. Nine genera were found present in the soil. Among these nine heterotrophic bacterial genera identified, Micrococcus was Gram positive along with Bacillus, while the others were Gram negative. Micrococus is also the only coccal cells present. Alongside Pseudemonas aeroginosa, two other species of Pseudomonas were encountered; they were identified as P. putida and P. fluorescens. They were all gram-negative rods, catalase positive, motile, urease negative and oxidase positive.

Gravimetric analysis of both experimental and control soils revealed the mean changes in residual oil concentration as shown in figures 8 and 9. Results obtained indicated a progressive loss of the oil content of both the experimental and control soils down the weeks. A much higher percentage decrease was however recorded from the experimental soils than the control soils. Residual oil concentration decreased from 88.0 ± 0.05 at week zero to 6.0±0.02g/kg at week 9 from experimental soil A (treated with Pseudemonas aeruginosa) representing a removal difference of 82.0% and a total removal of 94.0% content of the oil pollutant from the soil during the bioremediation experiment. From experimental soil B (treated with Bacillus subtilis) the values ranged from 86.0±0.7 at week 0 (zero) to 6.9±0.2g/kg at week 9; this represents a percentage removal difference of 79.1% and a total removal of 93.1% content of the oil pollutant from the soil at the end of the monitoring period.

The control soils recorded value ranges as follows: for sample A; control 1 from 87.8±0.10 to 34.9±0.03g/kg, representing a percentage removal difference of 52.9% and total removal of 65.1% of the crude petroleum pollutant at the end of the bioremediation experiment. Control 2 showed a mean range of residual oil concentration, from 84.9±0.00 to 35.4±0.06g/kg which represents a percentage removal difference of 51.5% and total removal of 64.6% at the end of the experiment.

Similarly, for sample B controls, the mean residual oil concentration values recorded ranged from 84.4±0.05 to 36.0±0.02g/kg for control 1 representing a percentage removal difference of 47.4% and a total removal of 63.0% from this set-up at the end of the bioremediation process. Control 2 ranged from 84.9±0.03 to 36.9±0.07g/kg which gave a percentage removal difference of 48.0% and a total removal of 63.1% of the oil pollutant from this set-up at the end of the monitoring period. The above results obtained from this study revealed that in all the designs, the reduction in residual oil concentrationin the experimental soils all the time is much more pronounced when compared to the control soils.

Duncan's Multiple Range Test indicated that for sample A, residual oil concentrations obtained for experimental soil (144.4) is significantly lower than that of the control soils (240.2 for control 1, and 242.7 for control 2). For sample B, residual oil concentration for the experimental soil (150.6) is significantly lower than of the controls (245.4 for control 1, and 250.9 for control 2).

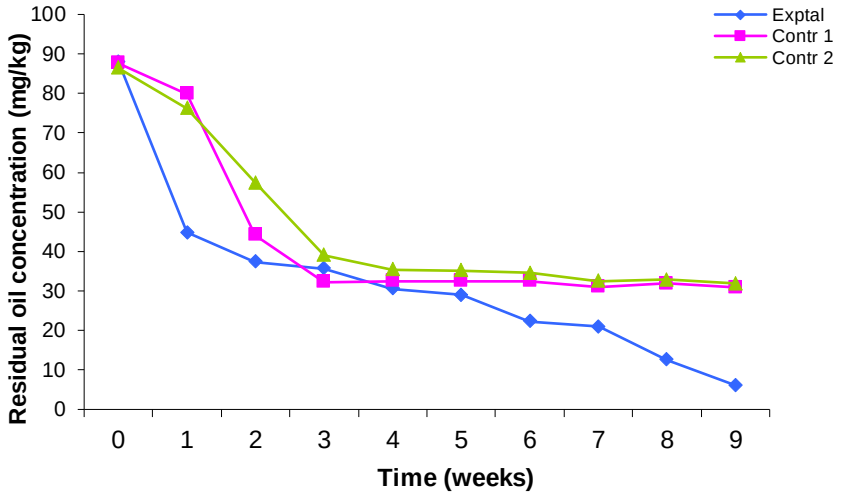

Figure 8: Mean Residual Oil concentration of experimental and control soilsusing Pseudomonas aeruginosa [SAMPLE A]

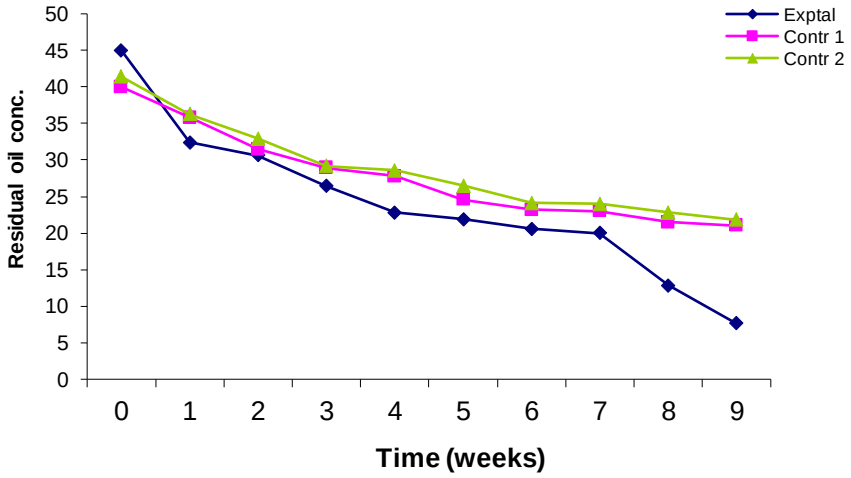

Figure 9. Mean Residual Oil concentration of the experimental and control soil Bacillus subtilis (SAMPLE B)

DISCUSSION

In a bioremediation procedure, microorganisms capable of degrading hazardous chemicals are used to restore polluted soil. The findings of this study revealed that the bacterial population in the test soils dropped in the first several weeks following crude petroleum-based pollution. This decline is attributed to the toxicity effect of crude petroleum. The same decline has been documented by other researchers working on crude oil breakdown in soils,

using various microbes and procedures: [16,17]. The main crude oil degraders significantly reduced the negative impacts of the crude oil, making the contaminated soil more bearable for the growth of further oil degraders [18]. The addition of Raymond medium, a microbial growth stimulant, enhanced the organisms' growth even further. The medium contains growth nutrients as well as other nutrients required for bacterial growth.

The oil removal assessment revealed a highly successful remediation result using the bioaugmentation and bio-stimulation techniques implemented. The experimental results of the residual oil concentration observed in the analysis indicated that the polluting crude oil was removed more effectively than the control soils. This finding is consistent with the findings of [19]. The bio-stimulation and bio-augmentation procedures aided in the remediation process, hastening the removal or remediation of the polluting crude oil. Bio-stimulation provides the additional nutrients and metabolites essential for increased and improved microbial growth. This indicates co-metabolism because the carbon required was supplied by the crude oil, while the other growth components required were supplied by the nutrients added.

The GC analysis results demonstrate that the biodegradation of crude oil begins with the lighter and straight-chain hydrocarbons. Before the heavy C chain hydrocarbons, the C1-C6 and C7-C10 hydrocarbons were eliminated. Aromatic and para-aromatic hydrocarbons are the leftover components in petroleum degradation, according to.

Due to their complexity, those high C-hydrocarbons appear to be more difficult for microbes to degrade due to oxidation. On the basis of the microbial infallibility principle, these residual components are later degraded. This study's observations, which show that the straight-chain and light-chain hydrocarbons decomposed first before the high-C-hydrocarbon components, support the preceding findings.

Higher crude oil metabolism was detected after the introduction of the generated culture - Psadominas aeruginosa and Bacillus subtilis. This observation is also consistent with the bioaugmentation principle. The introduced organisms, in conjunction with the autochthonous microbes, improved the metabolism of the influencing crude oil. Because the addition of these species increased the number of petroleum-degrading bacteria in the soil, more organisms got involved in the petroleum metabolism. According to [20],a growth in oil degraders resulted in greater oil pollutant metabolism.

CONCLUSION

The results obtained in this work showed that the crude petroleum pollution affected the agricultural soil adversely. This adverse effect was however remediated by the test microorganisms. The augmentation with Pseudomonas aeruginosa gave a better result than Bacillius subtilis. A combination of bio stimulation, and bioaugmentation yielded a better recovery rate both in efficiency and time. The microbial metabolism of the oil pollutants, and the oxidation of dead organic remains caused the increase in the soil fertility index. It is important to state here that the research proved the effectiveness of both bioaugmentation and bio stimulation in the bioremediation of crude oil impacted agricultural soil.

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