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Percentage Bioremediation Assessment of Spent Mushroom Substrate (SMS) and *Mucor racemosus* in Hydrocarbon Contaminated Soil

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Authors' contributions

This work was carried out in collaboration among all authors. Authors DNO, SAN and RRN designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors RRN, RNO and OU managed the analyses of the study. Authors SAN, RNO and OU managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The aim of the study was to assess Percentage Bioremediation of Spent Mushroom Substrate (SMS) and *Mucor racemosus* in hydrocarbon contaminated soil
Place and Duration of Study: A portion of Rivers State University demonstration farmland in Nkpolu-Oroworukwo, Mile 3 Diobu area of Port Harcourt, Rivers State was used for this study. The piece of land is situated at Longitude 4°48'18.50''N and Latitude 6o58'39.12''E measuring 5.4864 m x 5.1816 m with a total area of 28.4283 m2. Bioremediation monitoring lasted for 56 days, analysis carried out weekly (per 7 days' interval).
Methodology: Five (5) experimental plots employing the Randomized Block Design were used each having dimensions of 100 x 50 x 30 cm (Length x Breadth x Height) = 150,000cm^3. Baseline study of the uncontaminated and the deliberately contaminated agricultural soil was investigated for its microbiota and physico-chemical properties. Two of these plots were designated as pristine (Unpolluted soil) (CTRL 1) and crude oil contaminated soil without nutrient organics and bioaugmenting microbes (CTRL 2); these two serve as controls. Each of the experimental plots,

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except the control (CTRL 1), was contaminated with 2500 cm^3 (2122.25 g) of crude oil giving initial Total Petroleum Hydrocarbon (TPH) value of 8729.00 mg/kg. The crude oil polluted soil in Plot 3 was further treated with 750 ml of *Mucor racemosus* broth (CS+Muc), Plot 4 was treated with 3000 g of Spent Mushroom Substrate (CS+SMS) while plot 5 was treated with the combination of both (CS+Muc+SMS). The plots were left for 7 days to ensure even distribution and soil-oil bonding. Sampling was done at seven-day interval (Day 1, 7, 14, 21, 28, 35, 42, 49, 56). Physicochemical parameters monitored were pH, Temperature, Nitrogen, Phosphorus, Potassium, and Total Petroleum Hydrocarbon (TPH) throughout the experimental period. Microbial parameters monitored were Total Heterotrophic Bacteria (THB), Total Heterotrophic Fungi (THF), Hydrocarbon Utilizing Bacteria (HUB) and Hydrocarbon Utilizing Fungi (HUF). Percentage (%) Bioremediation was estimated from percentage (%) reduction of Total Petroleum Hydrocarbon (TPH) from day 1 to day 56 in relation to control plots. Net % Bioremediation were also assessed to ascertain the actual potential of treatment agents singly or combined.

Results: Total Heterotrophic Bacteria (THB) (CFU/g) recorded on day 7 and day 56 of the bioremediation were; day 7; CTRL 1 – US (1.07 $x10^9$), CTRL- CS (5.4 $x10^8$), CS+Muc (3.0 $x10^8$), CS+SMS (4.6 $x10^8$) and CS+Muc+SMS (5.0 $x10^8$). On day 56, data obtained were CTRL 1 –US (9.4 $x10^8$), CTRL 2 –CS (7.2 $x10^9$), CS+Muc (3.7 $x10^8$), CS+SMS (8.1$x10^8$) and CS+Muc+SMS (6.8 $x10^8$). The increase in number in the treated plots is a depiction of an increase in activity of the organism and the stimulating effect of bio-organics SMS while the untreated plot CTRL 1-US showed decrease in population at day 56. Similar trend showed for Total Heterotrophic Fungi. Generally, it was observed that the highest growth/ count was recorded at the 7^{th} and 8^{th} week (day 42 or day 49), at the 9^{th} week there was an observable decrease; probably due to depletion of nutrients and other factors such as rainfall and seepage. The Net Percentage Hydrocarbon Utilizing Bacteria and Fungi (Net %HUB and Net %HUF) were highest in Crude Oil contaminated plot treated with Spent Mushroom Substrate (SMS) singly; that is (CS+SMS) (11.02% and 12.07%) and lowest in the uncontaminated soil – Control (CTRL 1 –US) (5.41% and 9.26%) respectively. The trend in decreasing order of Net % Hydrocarbon Utilizing Bacteria were as follows: CS+SMS (11.02%) > CS+Muc+SMS (10.14%) > CS+Muc (9.43%) > CTRL 2 –CS (8.1%) > CTRL 1 –US (5.41%) while Net % Hydrocarbon Utilizing Fungi followed similar trend and were: CS+SMS (12.07%) > CS+Muc+SMS (11.76%) = CS+Muc (11.76%) > CTRL 2 –CS (11.05%) > CTRL 1 –US (9.26%). Evaluation of Amount of Crude Oil or Hydrocarbon remediated and Net %Bioremediation revealed Crude Oil contaminated plot augmented with *Mucor racemosus* broth singly (CS+Muc) as having the highest bioremediation potential while the least is the untreated soil. The trend is as follows: CS+Muc (8599.19 mg/kg; 33.93%) > CS+Muc+SMS (8298.95 mg/kg; 32.74%) > CS+SMS (8197.03 mg/kg; 32.34%) > CTRL 2 –CS (166.54 mg/kg; 0.66%) > CTRL 1 –US (85.48 mg/kg; 0.34%)

Conclusion: This shows that a single nutrient substrate or augmenting microorganism applied appropriately may have a more positive result, that is; higher bioremediation potential than combined or multiple mixed treatments. It was further observed that microbial counts decreased with time in treatments with augmenting organisms alone but increased considerably in treatments supplement with organics having its peak on the 49^{th} day. It is therefore recommended that bioremediation of crude oil-polluted soil using bio-augmenting microorganism should be applied appropriately noting the volume: area ratio and be supplemented with efficient nutrient organics after every 49-day interval.

Keywords: Bioremediation; spent mushroom substrate; Mucor racemosus; crude oil contaminated soil.

1. INTRODUCTION

Bioremediation is defined as a process, which relies on biological mechanisms to reduce (degrade, detoxify, mineralize or transform) concentration of pollutants to an innocuous state [1]. The process of pollutant removal depends primarily on the nature of the pollutant, which may include: agrochemicals, chlorinated compounds, dyes, greenhouse gases, heavy metals, hydrocarbons, nuclear waste, plastics, and sewage. Apparently, taking into consideration site of application, bioremediation techniques can be categorized as: ex situ or in situ. The nature of pollutants, depth and degree of pollution, type of environment, location, cost,

and environmental policies are some of the selection criteria that are considered when choosing any bioremediation technique [2].

There are many ways petroleum hydrocarbon contamination occurs. This may be through pipelines and oil wells leakages, wrong methods of disposal of petroleum wastes and accidental oil spills [3]. The contamination caused by petroleum hydrocarbon leads to various carcinogenic and neurotoxic effects. Therefore, to reduce the hazardous effect of petroleum hydrocarbon, their control and treatment strategies through bioremediation are required [4]. Notably, different oil products like gasoline diesel or heavy oils can cause soil contamination [5].

The spent mushroom compost (SMC) contains a consortium of hydrocarbon degrading bacteria and ligninolytic fungi. The SMC contains large amounts of different types of ligninolytic enzymes. It seems that SMC can be effective in the degradiation of petroleum hydrocarbon [6].

Spent mushroom compost is the residual compost waste generated by the mushroom production industry. The spent mushroom substrate (SMS) is released after button mushroom cultivation and it contains all the essential nutrients needed for raising a healthy field crop in addition to harbouring fungal biomass and large population of heterotrophic microbes. SMS also has the ability to chemically adsorb the organic and inorganic pollutants, while the diverse category of microbes it harbours have the capability of biological breakdown of the organic xenobiotic compounds present in soil and water [7].

Polluted sites may be cleaned by physico-chemical strategies including excavation and storage, washing, and chemical treatments [8,9], Physical containment and recovery of bulk or free oil is the primary response option of choice in most parts of the world for cleanup of oil spills in marine and freshwater shoreline environments. Chemical methods, particularly dispersants, have been routinely used in many countries as a response option. However, chemical methods have not been extensively used in most parts of the world due to the disagreement about their effectiveness and the concerns of their toxicity and long-term environmental effects [10].

Most studies about hydrocarbon and petroleum degradation have been conducted on groundwater [11], aquifers and in laboratory and/or field studies; however little research has been carried out on soil samples especially using fungal isolates to ascertain their bioremediation potential and the influence of organic bio-stimulating agent such as Spent Mushroom Substrate on its effectiveness in the spill site bioremediation; thus the essence of this study.

2. MATERIALS AND METHODS

2.1 Baseline Studies of Crude Oil Contaminated Soils

Soils used for baseline studies were obtained from an uncontaminated environment around the Faculty of Agriculture of the Rivers State University Demonstration farmland. Several portions of the composite soil were emptied into 10L buckets used as sample containers and contaminated with different concentrations of crude oil. The process was to determine the rate of survival of indigenous microorganisms in the soils deliberately contaminated with crude oil. This set up was monitored for 3 months to determine its effect on the microbiota of the soil. The organisms isolated were then used for the field application on site contaminated with crude oil for bioremediation evaluation.

2.2 Description of area of Study

The area used for this study is a pristine patch of land 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 $4^{o}48'18.50''N$ and Latitude $6^{o}58'39.12''E$ 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 30 cm giving 214.905 kg of soil in each plot. Two of these plots were designated as pristine and crude oil polluted soil to serve as controls respectively. The soil is of sandy clay texture with specific gravity of 2.57. From these plots; unpolluted, crude oil polluted and nutrient amended soils samples were taken for bioremediation analysis.

The choice of River State University demonstration farm was premised on the following factors; enough space, relatively flat topography, accessibility, availability of water and secured environment. The site also demonstrated adequate safeguards for the protection of human health and the environment.

2.3 Experimental Design

The Randomized Complete Block Design (RCBD) was used for the study. Each unit of block or plot measured 100cm x 50cm x 30cm. The volume of each block gives 214.905 kg volume of soil taken into consideration the microbial influence on agricultural soils is in the range of 0-15 cm depth [12].

2.4 Treatment/ Field Application

Five Randomized Complete Block Design (RCBD) degradative plots according to the method of Toogood [12] were set-up for the aim of monitoring bioremediation of crude oil polluted soil Table 1. The bioremediation protocol consists of five RCBD. Two plots of the RCBD act as control (CTRL 1 for Uncontaminated soil without treatment while CTRL 2 is for Crude Oil Contaminated soil without treatment); the other three plots were treated singly or combined with bioaugmenting microorganism and spent mushroom substrates (SMS). They are as follows:

- **Plot 1:** Control 1 – Uncontaminated soil (pristine)
- **Plot 2:** Control 2 – Contaminated soil (no amendment)
- **Plot 3:** Contaminated soil + 750 ml *Mucor racemosus* broth.
- **Plot 4:** Contaminated soil + 3000 g Spent Mushroom Substrate (SMS)
- **Plot 5:** Contaminated soil + 750 ml *Mucor racemosus* broth + 3000 g Spent Mushroom Substrate (SMS).

All plots except Control 1 (plot 1) were deliberately contaminated with 2122.25 g/l (2500 ml or cm^3) of crude oil.

The baseline microbiological, chromatographic and physicochemical analysis of the soil before the crude oil contaminate on was determined using APHA [13] method Table 2.

2.5 Source of Organic Manure

Spent Mushroom Substrate used as organic nutrients were obtained from River State University demonstration farm, Mushroom Production Section, Port Harcourt, Rivers State.

2.6 Treatment and Application of Crude Oil and Nutrients

Crude oil used in this experiment was obtained from AGIP flow station. The stock culture was prepared by weighing out (PCE analytical weighing balance PCE-6000), 2122.25g and dissolve in 1.0 L of distill water to give initial crude oil concentration of 2122.25g/l. The soil was artificially contaminated by spiking the prepared crude oil concentration on the plots and allowed to stay for 7 days (to ensure volatilization and sorption of crude oil into the soil matrix) before application of various treatments to ensue.

The plots were amended with 750ml of *Aspergillus,* 750 ml of *Mucor* and 3000g of Spent Mushroom Substrate (SMS) respectively were added accordingly [14,15,16]. Plot 1 was uncontaminated (pristine) and Plot 2 was Contaminated but un-amended. These two plots served as controls. Plots 3-5 were amended with different concentration of treatments as indicated in Table 1. Plots 3–5 were specifically amended with bioaugmenting organism, biostimulating nutrients and a combination of both, seven (7) days after experimental crude oil pollution. These were allowed for 7 days with the objective of assessing what happens to microbial activities within one week of treatment (bioremediation) before sampling.

2.7 Tilling

The experimental plots were slightly tilled once a week. This optimizes the transfer of oxygen into contaminated soils and promotes aerobic degradation of the organic contaminants.

2.8 Watering

The plots were watered to 65% water holding capacity [14] before experimental crude oil contamination and subsequently at two days' interval with 600ml of water per plot as required.

2.9 Sample Collection for Analysis

Soil samples for laboratory analysis were collected on day 1, 7, 14, 21, 28, 35, 42, 49 and 56 in sterile sample container from a depth of 0-15cm after tilling using soil spatula. Soil sample collected were made from 4-10 random points per plots and bulked to form a composite sample. Small portions (5 g) of the composite samples were collected into sterile bottles using sterile spatula for microbiological and physicochemical State University within 2 hours after sample collection while physicochemical analysis was carried out at Pollution Control and Environmental Management (POCEMA) and analysis. All microbiological analysis was carried out in the Microbiology laboratory of the Rivers

Table 1. Treatments of experimental plots using nutrient amendments and bio-augmenting organisms

Sample ID	Plot Code	Plot Dimension/ Volume of Soil	Crude Oil	Mucor broth (Muc)	Spent Mushroom Substrate (SMS)
P1	CTRL 1 -US	100x50x30cm/150,000cm^3	0	-	-
P2	CTRL 2 - CS)	100x50x30cm/150,000cm^3	2122.25g(2500cm^3)	-	-
P4	CS+Muc	100x50x30cm/150,000cm^3	2122.25g(2500cm^3)	750ml	-
P5	CS+SMS	100x50x30cm/150,000cm^3	2122.25g(2500cm^3)	-	3000g
P8	CS+Muc+SMS	100x50x30cm/150,000cm^3	2122.25g(2500cm^3)	750ml	3000g

P=- Plot; US = Uncontaminated soil; CS = Contaminated soil; Muc = Mucor racemosus broth; SMS = Spent Mushroom Substrate

Giolee Global Resources laboratories both in Port Harcourt, Rivers State. Soil samples were stored at 14±2°C for future analysis [14].

2.10 Microbiological Analysis of Soil Samples

The following Media were used for microbial enumeration and isolation.

2.10.1 Nutrient agar

Nutrient agar (NA) was used as a general-purpose medium because it supports the growth of a wide range of non-fastidious microorganisms. Nutrient agar of Becton Dickson and Company, USA was used for the isolation of Total Heterotrophic Bacteria (THB) by preparing/weighing out (with a normal calibration) 28 grams of the Nutrient agar into 1000 ml of distilled water and then sterilized/autoclaved at 121°C for 15 minutes according to the manufacturer specification.

2.10.2 Oil agar medium

Oil agar medium was prepared for the isolation of hydrocarbon utilizing bacteria. Oil-agar medium was prepared by the method of Modified Salts Medium (MSM) of Nrior and Odokuma [17]; Nrior and Echezolom [18]. The medium was prepared with a composition of K_2HPO_4 (0.5 g), $MgSO_4.7H_2O$ (0.3 g), $NaCl_2$ (0.3 g), $MnSO_4.H_2O$ (0.2 g), $FeSO_4.6H_2O$ (0.02 g), $NaNO_3$ (0.03 g), $ZnCl_2$ (0.3 g) and agar (15 g) into 1litre of distilled water. 1% of pure Bonny light crude oil was added to the mixture and then autoclaved at 121°C for 15 minutes. The medium was used for the isolation, enumeration and preliminary identification of petroleum utilizing bacterial (oil degraders). The medium was them prepared by the addition of 1% (v/v) crude oil sterilized with 0.22Millipore filter paper to sterile MSM cooled to 45°C under aseptic condition. The MSM and crude oil were then mixed thoroughly and dispensed into sterile Petri dishes to set.

2.10.3 Sabouroud dextrose agar

Sabouroud Dextrose Agar (SDA) is use for the isolation of fungi isolates. Media is prepared by weighing out 65 g into 100 ml of distill water and using the manufacturer specification, depending on the number of plates to be used. After the preparation it was autoclaved at 121°C for 15 minutes and then aseptically poured the media into plates for inoculation.

2.11 Glassware and Media Sterilization

The glassware used for the laboratory analysis were sterilized in a hot air oven at 160°C for 1-3hours. The sterilization for the media and water used for the serial dilutions were carried out in an autoclave at 120°C and 15 pounds per square inch (psi) for 15 minutes while sugars for fermentation and metabolism testes were sterilized in the autoclave for 5-10 minutes.

2.12 Microbiological Analyses

2.12.1 Microbial estimation

The total heterotrophic bacterial (THB), the hydrocarbon utilizing bacteria (HUB), total heterotrophic fungi (THF) and hydrocarbon utilizing fungi (HUF) were determined using the spread plate count method on nutrient agar according to APHA [13] as cited by Chikere et al. [19]; Oliveira et al. [20] and Nrior and Mene [14].

2.12.2 Serial dilution

1.0 g of homogenized mixed and crude oil contaminated soil samples were measured using electric weighing balance and aseptically transferred into a sterile test tube containing 9.0 ml of normal saline.10-fold serial dilution was done to 10^{-1}, $10^{-3,}10^{-4,}$ and 10^{-6} dilutions.

2.12.3 Inoculation and incubation

0.1 ml aliquot of 10^{-1}, $10^{-3}$and $10^{-4,}$ dilutions were spread plated into sterile solidified SDA (Sabouraud Dextrose Agar) and MSA (Mineral Salt Agar), containing tetracycline in triplicate and incubated for 5-7 days at 28°C, 10^{1}, 10^{5}, and 10^{6} dilutions were spread plated on solidified Nutrient agar (NA) 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.

2.12.4 Enumeration and isolation of pure culture

Colonies and spores that grew on NA and SDA from the baseline and bioremediation set-up after incubation were enumerated. Similarly, colonies and spores were picked for subculture to get pure cultures and were those that grew on MSA plates. Pure culture of fungi was stored on SDA slants, while those of bacteria isolates were stored in 10% glycerol, all in Bijou bottles.

The colonies counted were expressed as Colony Forming Unit (CFU) per gram of soil using the formula:

$$T = \frac{N}{V} \; x \; DF \qquad (1)$$

Where,

T = total number of colonies in cfu/g soil
N = number of colonies counted on the plate
V = volume of inoculum plated i.e. 0.1ml
DF = dilution factor used for plating (10^{6}) [DF =1/Dilution used]

Total Heterotrophic Bacterial count = $\frac{Number\ of\ colonies}{Volume\ plated\ (0.1ml)} \; x\ Dilution\ factor$

2.13 Identification of Fungal Isolates

Two fungal spores that utilized petroleum hydrocarbons as their sole carbon energy source were viewed macroscopically and microscopically (using Lactophenol Cotton Blue Stain and the slide culture technique).

2.13.1 Wet preparation

A flame needle was used to pick spores with mycelium from SDA plate and aseptically placed onto two drops of Lactophenol Cotton Blue (LPCB) on a grease-free slide. The spores were thinned out to enable easy identification. A cover slip was placed on the slide and the stained fungi viewed using X40 magnification [21] and other microscopic and cultural characteristics was further used in the identification of the fungal isolates of the bioremediation set up [22].

2.13.2 Slide culture method

From the sterile SDA, a small square shaped piece was cut and placed to fit onto a grease-free slide under a cover slip. Using a flamed needle, a growing fungal spore was picked from a SDA plate and embedded into the four sides of the piece of agar and a cover slip placed on top of the embedded piece of agar. Moistened filter paper was placed in a petri-dish under the glass slide. The petri-dish was covered and incubated at 37°C until sporulation occurred [21].

2.14 Maintenance/Preservation of Pure Cultures

Bacteria and Fungi isolates were inoculated onto Nutrient and Sabouraud Dextrose Broths in 500 ml Erlenmeyer flask loosely plugged with sterile cotton wool respectively. The broths cultures were incubated for 5 days at 37° C for bacterial and at 28°C for fungal cultures respectively. Serial dilution was made to determine the number of cells per 0.1 ml aliquot.

2.15 Bioremediation Evaluation Procedure and Analysis

All plots were tilled twice weekly to ensure proper aeration and even distribution of crude oil and the treatment agents. Samples were taken at regular interval of days 1, 7, 14, 21, 28, 35, 42, 49 and 56 for microbiological and selected physicochemical analyses.

The method of Nrior and Mene [14] was used in calculating the percentage of Bioremediation in the experiment. The process followed the steps stated below;

Step i: The amount of pollutant remediated (B_A) equals to Initial Concentration of pollutant (Week 1) minus the Final Concentration of pollutant at the end of experiment (Last Week or Week 9) (Equation 2)

$$\text{Amount of pollutant Remediated } B_A = Ic - Fc \qquad (2)$$

Where:

B_A = Amount of pollutant remediated
I_C = Initial Concentration of pollutant (week 1)
F_C = Final Concentration of pollutant (Last week of Experiment)

Step ii: The percentage (%) Bioremediation equals Amount of pollutant remediated in each treatment divided by the Initial Concentration of pollutant in same treatment (week 1), multiplied by 100 (Equation 3)

$$\% \text{ Bioremediation} = ((B_A / I_C) \times 100) \quad (3)$$

(Nrior and Mene, [14])

Step iii: The Net percentage (%) Bioremediation equals Amount of pollutant remediated in each treatment divided by the Sum total of Amount of pollutant remediated in all treatment, multiplied by 100 (Equation 4)

$$\text{Net \%Bioremediation} = ((B_C/\sum B_T) * 100) \quad (4)$$

Where:

B_C = Amount of pollutant remediated in each treatment
$\sum B_T$ = Sum total of Amount of pollutant remediated in all treatment [18]

Calculating % HUB & %HUF of same treatment (Equation 5 and 6)

$$\% \text{ HUB} = ((\text{HUB}/\text{THB}) * 100) \quad (5)$$
$$\%\text{HUF} = ((\text{HUF}/\text{THF}) * 100) \quad (6)$$

Nrior and Odokuma [17]
Calculating NET % HYDROCARBON UTILIZERS (HU); that is Net %HUB and Net %HUF (Equation 7,8 and 9)

Stage 1:

$$HU_T = ((\sum \text{HUB} + \sum \text{HUF}) \quad (7)$$

Where $\sum$HUB = Sum total of HUB values in all treatment
Where $\sum$HUF = Sum total of HUF values in all treatment
HU_T = Sum total of Hydrocarbon Utilizers (HUB & HUF) in all treatments

Stage 2:

Calculating Net %HUB and Net %HUF in each treatment.

$$\text{Net \%HUB} = ((HUB_X/HU_T) * 100) \quad (8)$$

$$\text{Net \%HUF} = ((HUF_X/HU_T) * 100) \quad (9)$$

Where

HUB_X = Hydrocarbon Utilizing Bacteria in each treatment
HUF_X = Hydrocarbon Utilizing Fungi in each treatment
HU_T = Mean of Hydrocarbon Utilizers (Nrior and Mene [14])

2.16 Physicochemical Analysis of Selected Parameters

The Physicochemical property of the soil sample was determined before experimental contamination/pollution of the soil to establish the baseline parameters and subsequently after crude oil contamination and nutrient application for the duration of bioremediation process for selected parameters. The following selected parameters including; soil texture, particulate size, moisture content, pH, temperature, phosphate, nitrate (NO_3^-), sulphate, total organic carbon, electrical conductivity, and moisture content were determined using the methods from APHA [13]. Soil texture was determined using sieves of different sizes – Master Sizer 2000 (Malner International), while moisture content was determined by drying 10 g of the soil sample in an oven at 80°C. Then 10 g of oven dried soil was placed on filter papers (Whatman No. 42) and filtered into Buchner funnels. De-ionized water was added slowly until the water level was just above the soil surface, then saturated and dipped into the flask. The funnel was removed and left to dry overnight. The soil was left for 24hrs, rewetted and the whole apparatus reweighed. The percentage moisture content of the soil in triplicate was then determined and calculated as water holding capacity (100%).The soil particulate size was determined using the hydrometer method.

Soil pH was determined using a pH meter (pH-911 Pen type). The temperature of the soil was determined using a mercury thermometer, by inserting the thermometer into the tilled soil for a period of 3-5 minutes and taking the reading immediately the thermometer is removed from the soil.

2.16.1 Total petroleum hydrocarbon (TPH)

Residual Total Petroleum Hydrocarbons (TPH) was extracted from the soil samples and quantified using Gas Chromatograph – Flame

Ionization Detector (GC-FID) Agilent 7890A, according to the methods of ASTDM 3921 and US EPA 8015 analytical protocol (TPI, 2007) as reported by Chikere et al. [19] and in accordance with Nigerian requirements of Department of Petroleum Resources (DPR), National Oil Spill Detection Response Agency (NOSDRA) and Federal Ministry of Environment (FMEnv). Samples were collected in a sealed sample container from Giolee Global Resources laboratory. Samples were kept in a cooler with icepack at 4°C, labeled appropriately and sent to the laboratory for analysis. All samples were analyzed in duplicated while ensuring precision and reliability of results through standard quality assurance and control procedures.

2.16.2 Determination of nitrate (NO_3^{2-}) in soil sample

5 g of soil sample was weighed into a shaking bottle.125 ml of distilled water was added and shaken for 10 minutes on a rotary shaker and then filtered to obtain the extract. 1 ml of the extract was transferred into 10 ml volumetric flask. 0.5 ml of Brucine reagent was then added. 2 ml of conc. sulphuric acid was rapidly added and mixed for about 30 seconds. The flask was allowed to stand for 5 minutes; 2 ml of distilled water was added and mixed for about 30 seconds. Flask was allowed to stand in cold water for about 15 minutes. The absorbance was measured at wavelength of 470 nm.

2.16.3 Determination of phosphate (PO_4^{3-}) in soil sample

25 ml of 2.5% Acetic acid was added to 1 g of soil sample and shaken for 30 minutes. The suspension was filtered through a filter paper. 10 ml of the extract was transferred into 50 ml volumetric flask. Extract was diluted with distilled water until the flask is about 2/3 full. 2 ml of Ammonium Molybdate reagent was added and mixed with extract. 2 ml of stannous chloride was also added and mixed; the solution was diluted to 50 ml mark with distilled water. The flask was allowed to stand for 30 minutes, and the absorbance was measured at wavelength of 690 nm.

2.16.4 Determination of sulphate (SO_4^{2-}) in soil sample

25 ml of the extracting solution was added to 5 g of soil sample and shaken for 30minutes and the suspension was filtered through a filter paper. 5 ml of the extract was transferred into 50 ml volumetric flask. 5 ml of 50% acetic acid was added and 1ml of H_3PO_4 was added and mixed. The solution was diluted with distilled water to about ¾ of the flask. 1g of Barium chloride was added and mixed. The solution was left to stand for 10 times, then 1ml of 0.5% gum acacia was added to the solution and made up to 50 ml with distilled water, and the absorbance was measure at 425 nm.

2.17 Statistical Analysis

Data obtained from the bioremediation set up were subjected to statistical analysis using computer based program, SPSS version 22 for Analysis of Variance (ANOVA) and Excel on microbiological, Total petroleum hydrocarbons and physicochemical parameters to compare data between soils in all treatments and controls and test whether the different nutrient amendments given to the crude oil contaminated soils were statistically significant at a confidence level of 95% or P>0.05. The results expressed as Mean±SD and regression analysis.

3. RESULTS AND DISCUSSION

3.1 Microbial and Physico-Chemical Properties of the Soil Prior to Application of Various Treatments for Bioremediation Evaluation

Baseline Physico-chemical and Morphological properties of the soil prior to Bioremediation.

Table 2 depicts the baseline physic-chemical and microbiological properties of the soil before the application of various bioremediation treatment approaches. Notably, key parameters determined were pH, electrical conductivity, Nitrate, potassium, phosphorus, sulphate, phosphate, moisture content, total organic carbon and particle size. The microbials determined in this study were; Total Heterotrophic Bacteria (THB), Total Heterotrophic Fungi (THF), Hydrocarbon Utilizing Bacteria (HUB) and Hydrocarbon Utilizing Fungi (HUF) while the concentration of total petroleum hydrocarbon TPH was also determined. The baseline results revealed that the pH was 7.01 for uncontaminated soil and 6.80 for contaminate soil. The electrical conductivity was 500 µS/cm for uncontaminated

soil and 590 µS/cm for contaminated soil. TPH value was as low as 87.89 mg/kg in the uncontaminated soil and 8729 mg/kg in the contaminated soil.

Soil physical properties define movement of air and water/dissolved chemicals through soil, as well as conditions affecting germination, root growth, and erosion processes. Soil physical properties form the foundation of several chemical and biological processes. The physical, chemical, and biological properties collectively determine the quality of the soil [23]. The soil's chemical properties are inherited from the processes of soil formation, during weathering and transport of the parent material from which the soil has formed. Thus the chemical nature of the rocks and minerals and the intensity of the weathering processes are fundamental in determining the chemical properties of the soil [24].

In soil, electrical conductivity (EC) is a measure of the ability of the soil to conduct an electrical current. Most importantly to fertility, EC is an indication of the availability of nutrients in the soil. The higher the EC, the more negatively charged sites (clay and organic particles) there must be in the soil, and therefore the more cations (which have a positive charge) there are that are being held in the soil. Sodium (Na^+), ammonium (NH_4^+), potassium (K^+), calcium (Ca^{2+}), magnesium (Mg^{2+}), hydrogen (H^+), iron (Fe^{2+}), aluminum (Al^{3+}), copper (Cu^{2+}), zinc (Zn^{2+}) and manganese (Mn^{2+}) are some examples of these cations that are beneficial to plants. As with most things in the soil, it is important that the EC does not get too high either, as too many of these nutrients, especially Na and Mg, can be detrimental to soil health. Optimal EC levels in the soil therefore range from 110-570 milli Siemens per meter (mS/m). Too low EC levels indicate low available nutrients, and too high EC levels indicate an excess of nutrients. Low EC's are often found in sandy soils with low organic matter levels, whereas high EC levels are usually found in soils with high clay content [25].

3.2 Microbiological Evaluation During Bioremediation of Crude Oil Polluted Soil

The bacteria genera isolated form crude oil polluted soil were: *Bacillus, Micrococcus, Comamonas, Klebsiella, Chryseobactrium, Pseudomonas, Pseudomona, Staphylococcus and Nitrosomonas* while fungal isolates were: *Aspergillus* sp.*, Penicillium* sp., *Cladosporium* sp., *Mucor* sp., *Microsporium* sp.

Table 2. Baseline physico-chemical and microbiological properties of the soil prior to application of various treatments for bioremediation evaluation

S/N	Parameter	Unit	Uncontaminated soil	Contaminated soil
1	pH	-	7.01	6.80
2	Temperature	^{0}C	26.78	28.56
1	Electrical Conductivity	µS/CM	500.00	590.00
2	Nitrate	mg/kg	506.95	454.72
3	Potassium, K	mg/kg	3.01	1.85
4	Phosphorus,P	mg/kg	2.49	2.14
5	Sulphate SO_4^{2-}	mg/kg	0.026433	0.020025
6	Phosphate PO_4^{3-}	mg/kg	0.00156	0.00167
7	Moisture Content	%	15.95	18.67
8	Total Organic carbon (TOC)	%	0.88	0.28
9	Particle size (>75µm)	%	81.10	50.90
10	Total Petroleum Hydrocarbon (TPH)	mg/kg	87.89	8729
11	Total Heterotrophic Bacteria (THB)	CFU/g	5.0×10^8	2.3×10^8
12	Total Heterotrophic Fungi (THF)	CFU/g	8.0×10^3	1.4×10^4
13	Hydrocarbon Utilizing Bacteria (HUB)	CFU/g	0	3.0×10^4
14	Hydrocarbon Utilizing Fungi (HUF)	CFU/g	3.0×10^3	9.0×10^4

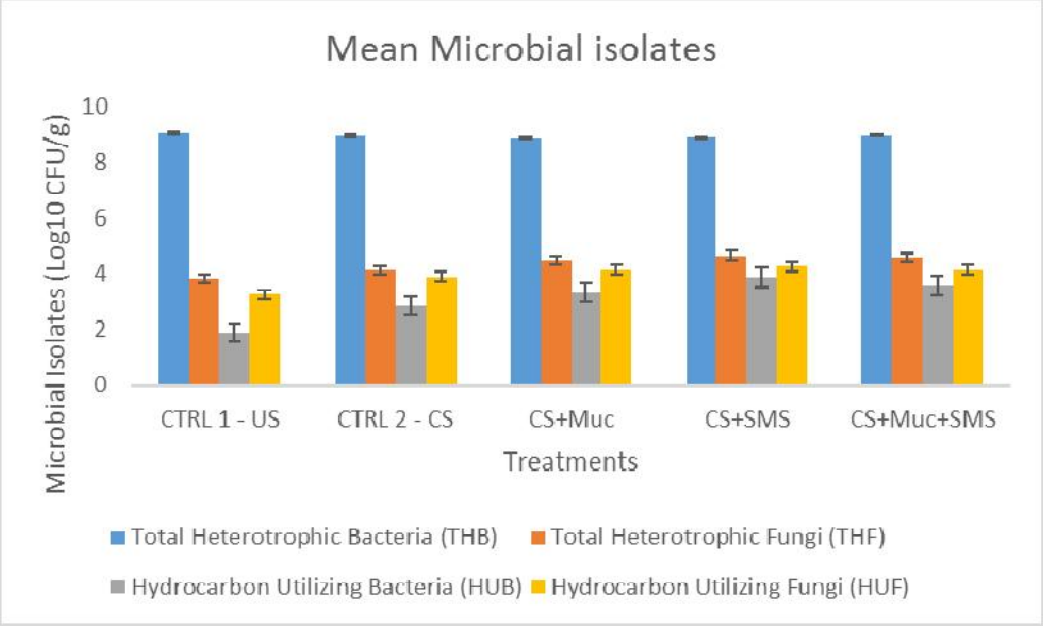

Fig. 1. Mean microbial groups in the different treatments and controls

Total Heterotrophic Bacteria (THB), Total Heterotrophic Fungi (THF), Hydrocarbon Utilizing Bacteria (HUB) and Hydrocarbon Utilizing Fungi (HUF) during bioremediation of crude oil polluted soil were indicated in Table 3-6. The Total Heterotrophic Bacteria counts recorded on day 7 and day 56 of the bioremediation were; day 7, the THB counts: 1.07 $x10^9$, 5.4 $x10^8$, 3.0 $x10^8$, 4.6 $x10^8$ and 5.0 $x10^8$ (CFU/g) for CTRL 1 - US, CTRL 2 – CS, CS+Muc, CS+SMS, CS+Muc+SMS. On day 56, data obtained for the same bioremediation group showed that the THB counts in CFU/g were 9.4 $x10^8$, 7.2 $x10^9$, 3.7 $x10^8$, 8.1$x10^8$ and 6.8 $x10^8$ respectively. The increase in number in the treated plots is a depiction of an increase in activity of these organisms while the untreated plot CTRL 1-US showed decrease in population at day 56. Similar results trend showed for Total Heterotrophic Fungi. Generally, it was observed that highest growth/ count was recorded at the 7th and 8th week (day 42 or day 49), at the 9th week there was an observable decrease Table 3; probably due to depletion of nutrients and other factors such as rainfall and seepage.

Notably, similar observation was made for the THF and HUF. However, fungal count increased significantly with increase in the number of bioremediation days. In characterizing hydrocarbon-degrading microorganisms isolated from crude oil contaminated soil and remediation of the soil by enhanced natural attenuation. Generally, the THB counts were higher in crude oil free soil than in crude oil polluted soil. There were higher counts of hydrocarbon utilizing bacteria (HUB) in crude oil polluted soils (3.60$x10^3$ to 9.0$x10^4$ cfu/g of soil) than crude oil Uncontaminated soil (1.0 $x10^3$ to 6.0 $x10^3$ cfu/g of soil). The value of crude oil uncontaminated HUB result up to 6.0 $x10^3$ cfu/g shows that most soils in the Niger Delta of Nigeria have been previously exposed to crude oil contamination either mildly or heavily due to the numerous crude oil exploration, drilling, refining, its transportation etc. by different companies as earlier observed by Nrior and Mene [14]; Ogbonna et al [16].

3.3 Microbial Evaluation Mean Values

This study also compares with a study on the bioremediation efficiency of *Mucor racemosus* with the nutrient organics (Spent Mushroom Substrate SMS) on crude oil polluted the soil revealed that the microbial colonial values increases with increase in time of exposure. The results observed on day 56 indicate that Uncontaminated soil (9.06±0.16 Log10 CFU/g) > CS+Muc+SMS (8.98±0.31 Log10 CFU/g) while

the least was CS+Muc (8.88 Log10 CFU/g). Total Heterotrophic Fungal count showed an alternate trend with Uncontaminated soil (CTRL 1-US) having the lowest fungal load while Plot treated with Spent Mushroom Substrate (CS+SMS) had the highest fungal load (4.65±0.34 Log10 CFU/g) Table 4 and Fig. 1. Similar trend was observed by Ogbonna et al. [16].

Hydrocarbon Utilizing Bacteria (HUB) and Hydrocarbon Utilizing Fungi (HUF) showed similar trend with CS+SMS having highest value 3.89±0.46 and 4.26±0.33 Log10 CFU/g respectively while least values of 1.91±1.82 and 3.27±1.24 Log10 CFU/g were obtained from the uncontaminated soil (CTRL 1 –US) used as control Table 4 and Fig. 1.

In a study of heterotrophic and crude oil-utilizing soil fungi in crude oil contaminated regions in Iran, the total heterotrophic fungi ranged from 0.41 ± 0.16 to 3333.33 ± 288.00 × 10^2 CFU/g soil while counts of crude oil-utilizing fungi ranged from 0.26 ± 0.10 to 2860.00 thirteen isolated fungal genera namely; Aspergillus, Penicillium, Fusarium, Acremonium, Candida, Rhodotorula, Mucor, Aureobasidium, Cunninghamella, Rhizopus, Alternaria, Beauveria and Paecilomyces, among which Beauveria, Paecilomyces and species of Aspergillus were isolated in that study [26].

It was also observed generally that Total Heterotrophic Bacteria (THB) were higher than Total Heterotrophic fungi (THF) while assessment of Hydrocarbon utilizers showed alternate trend with Hydrocarbon Utilizing fungi (HUF) having higher colonial load than Hydrocarbon Utilizing Bacteria (HUB) Table 4 and Fig. 1.

The Net Percentage Hydrocarbon Utilizing Bacteria and Fungi (Net %HUB and Net %HUF) were highest in Crude Oil contaminated plot treated with Spent Mushroom Substrate (SMS) singly; that is (CS+SMS) (11.02% and 12.07%) and lowest in the uncontaminated soil – Control (CTRL 1 –US) (5.41% and 9.26%) respectively Table 4 and Fig. 2. This shows that a single nutrient substrate applied appropriately may have a more positive result, that is higher bioremediation potential than combined or multiple mixed treatments. The trend in decreasing order of Net % Hydrocarbon Utilizing Bacteria were as follows: CS+SMS (11.02%) > CS+Muc+SMS (10.14%) > CS+Muc (9.43%) > CTRL 2 –CS (8.1%) > CTRL 1 –US (5.41%) while Net % Hydrocarbon Utilizing Fungi followed similar trend and were: CS+SMS (12.07%) > CS+Muc+SMS (11.76%) = CS+Muc (11.76%) > CTRL 2 –CS (11.05%) > CTRL 1 – US (9.26%).

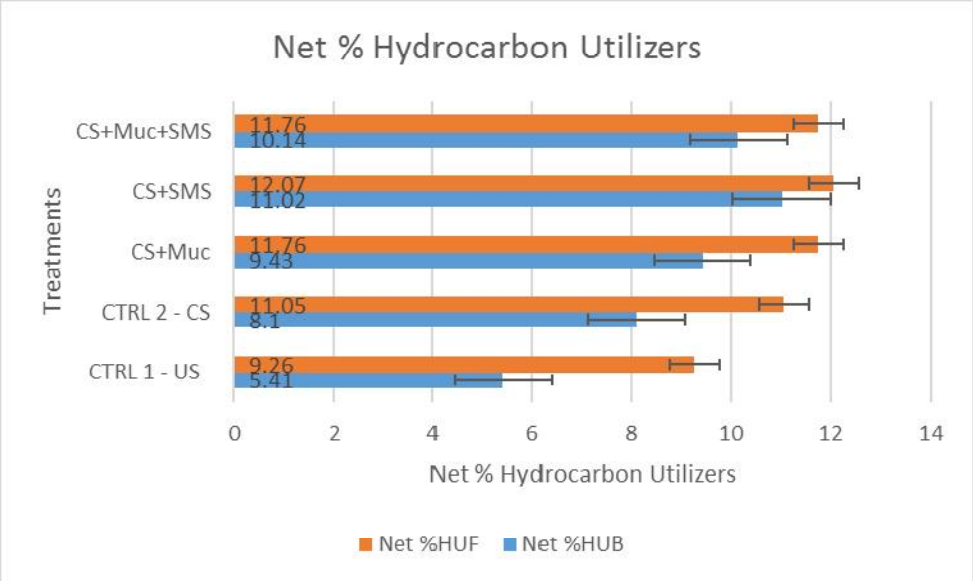

Fig. 2. Net % Hydrocarbon utilizers (hydrocarbon utilizing bacteria and hydrocarbon utilizing fungi)

Table 3. Variation in microbial population (CFU/g) during bioremediation of crude oil polluted soils

	Plot Code		Day 1	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56
THB	P1	CTRL 1 - US	5.0×10^{8}	1.07×10^{9}	1.65×10^{9}	1.37×10^{9}	1.20×10^{9}	1.14×10^{9}	1.14×10^{9}	1.74×10^{9}	9.4×10^{8}
	P2	CTRL 2 - CS	2.3×10^{8}	5.4×10^{8}	8.1×10^{8}	8.7×10^{8}	9.4×10^{8}	8.0×10^{8}	$1.0\ 2 \times 10^{9}$	1.20×10^{9}	7.2×10^{9}
	P3	CS+Muc	2.8×10^{8}	3.0×10^{8}	1.06×10^{9}	1.10×10^{9}	1.20×10^{9}	1.11×10^{9}	1.29×10^{9}	1.31×10^{9}	3.7×10^{8}
	P4	CS+SMS	1.7×10^{8}	4.6×10^{8}	8.4×10^{8}	1.18×10^{9}	1.16×10^{9}	1.21×10^{9}	1.15×10^{9}	1.21×10^{9}	8.1×10^{8}
	P5	CS+Muc+SMS	2.0×10^{8}	5.0×10^{8}	1.25×10^{9}	1.35×10^{9}	1.51×10^{9}	1.73×10^{9}	1.52×10^{9}	1.43×10^{9}	6.8×10^{8}
THF	P1	CTRL 1 - US	8×10^{3}	6×10^{3}	7×10^{3}	5×10^{3}	8×10^{3}	6×10^{3}	8×10^{3}	9×10^{3}	5×10^{3}
	P2	CTRL 2 - CS	1.4×10^{4}	1.6×10^{5}	9×10^{3}	9×10^{3}	1.4×10^{4}	1.0×10^{4}	9×10^{3}	8×10^{3}	7×10^{3}
	P3	CS+Muc	1.0×10^{4}	1.3×10^{4}	1.7×10^{4}	3.0×10^{4}	4.4×10^{4}	8.6×10^{4}	7.9×10^{4}	5.8×10^{4}	1.6×10^{4}
	P4	CS+SMS	1.1×10^{4}	1.5×10^{5}	1.8×10^{4}	3.8×10^{4}	5.7×10^{4}	7.0×10^{4}	6.8×10^{4}	6.2×10^{4}	3.8×10^{4}
	P5	CS+Muc+SMS	8×10^{3}	1.2×10^{5}	2.7×10^{4}	3.5×10^{4}	5.7×10^{4}	9.0×10^{4}	6.4×10^{4}	5.9×10^{4}	9×10^{3}
HUB	P1	CTRL 1 - US	0	0×10^{3}	1×10^{3}	2×10^{3}	4×10^{3}	6×10^{3}	3×10^{3}	0	0
	P2	CTRL 2 - CS	3×10^{3}	2×10^{3}	1×10^{3}	9×10^{3}	6×10^{3}	9×10^{3}	6×10^{3}	3×10^{3}	1×10^{3}
	P3	CS+Muc	2×10^{3}	3×10^{3}	3×10^{3}	1.0×10^{4}	1.2×10^{4}	1.0×10^{4}	8×10^{3}	5×10^{3}	1×10^{3}
	P4	CS+SMS	3×10^{3}	2×10^{3}	2×10^{3}	2.4×10^{4}	2.2×10^{4}	2.2×10^{4}	1.6×10^{4}	1.2×10^{4}	4×10^{3}
	P5	CS+Muc+SMS	3×10^{3}	1.0×10^{4}	9×10^{4}	1.9×10^{4}	1.6×10^{4}	1.9×10^{4}	1.3×10^{3}	8×10^{3}	1×10^{3}
HUF	P1	CTRL 1 - US	3×10^{2}	6×10^{2}	7×10^{2}	6×10^{2}	5×10^{2}	5×10^{2}	7×10^{2}	2×10^{2}	0
	P2	CTRL 2 - CS	9×10^{3}	1.2×10^{4}	1.2×10^{4}	1.7×10^{4}	1.3×10^{4}	1.3×10^{4}	9×10^{3}	4×10^{3}	1×10^{3}
	P3	CS+Muc	5×10^{3}	8×10^{3}	1.6×10^{4}	2.6×10^{4}	2.9×10^{4}	3.0×10^{4}	2.4×10^{4}	1.0×10^{4}	7×10^{3}
	P4	CS+SMS	7×10^{3}	5×10^{3}	1.7×10^{4}	2.7×10^{4}	3.0×10^{4}	4.8×10^{4}	3.6×10^{4}	2.2×10^{4}	1.2×10^{4}
	P5	CS+Muc+SMS	5×10^{3}	9×10^{3}	1.4×10^{4}	2.5×10^{4}	2.8×10^{4}	2.4×10^{4}	2.2×10^{4}	1.2×10^{4}	8×10^{3}

THB = Total Heterotrophic Bacteria, THF = Total Heterotrophic Fungi, HUB = Hydrocarbon Utilizing Bacteria, HUF = Hydrocarbon Utilizing Fungi, P = Plot; CTRL = Control, US = Uncontaminates soil; CS = Contaminated soil; Asp = Aspergillus niger; Muc = Mucor racemosus; SMS = Spent Mushroom Substrate

Table 4. Mean, standard deviation and percentage microbial counts (Log10 CFU/g) and % hydrocarbon utilizers during bioremediation of crude oil contaminated soils

		Microbial populations (Log_{10}CFU/g)				% Hydrocarbon Ulilizers			
Plot	**Treatments**	**Total heterotrophic bacteria**	**Total heterotrophic fungi**	**Hydrocarbon utilizing bacteria**	**Hydrocarbon utilizing fungi**	**% hydrocarbon utilizing bacteria**	**% hydrocarbon utilizing fungi**	**Net %HUB**	**Net %HUF**
P1	CTRL 1 - US	9.06±0.16^{b}	3.83±0.09^{b}	1.91±1.82^{a}	3.27±1.24^{a}	20.94±20.02^{a}	62.02±23.91^{a}	5.41	9.26
P2	CTRL 2 - CS	8.97±0.40^{a}	4.12±0.42^{a}	2.86±1.63^{a}	3.90±0.38^{a}	32.27±4.79^{a}	95.12±10.54^{a}	8.10	11.05
P4	CS+Muc	8.88±0.29^{a}	4.47±0.35^{a}	3.33±1.28^{a}	4.15±0.30^{ab}	41.16±3.27^{a}	93.02±4.78^{b}	9.43	11.76
P5	CS+SMS	8.90±0.29^{a}	4.65±0.34^{ab}	3.89±0.46^{a}	4.26±0.33^{a}	43.67±4.38^{a}	91.94±8.30^{a}	11.02	12.07
P8	CS+Muc+SMS	8.98±0.31^{a}	4.58±0.42^{a}	3.58±1.44^{a}	4.15±0.26^{ab}	43.52±6.48^{a}	91.08±6.47^{ab}	10.14	11.76

***means with the same superscript along the columns are not significantly different (p>0.05)*

P=- Plot; US = Uncontaminated soil; CS = Contaminated soil; Muc = Mucor racemosus; SMS = Spent Mushroom Substrate, HUB = Hydrocarbon Utilizing Bacteria, HUF = Hydrocarbon Utilizing Fungi

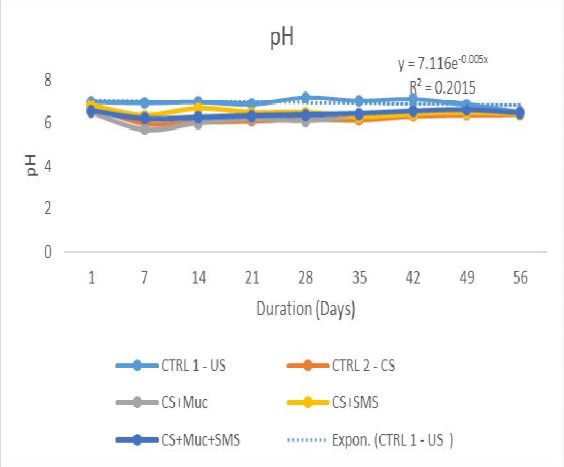

Fig. 3. Changes in pH during bioremediation of crude oil contaminated soil

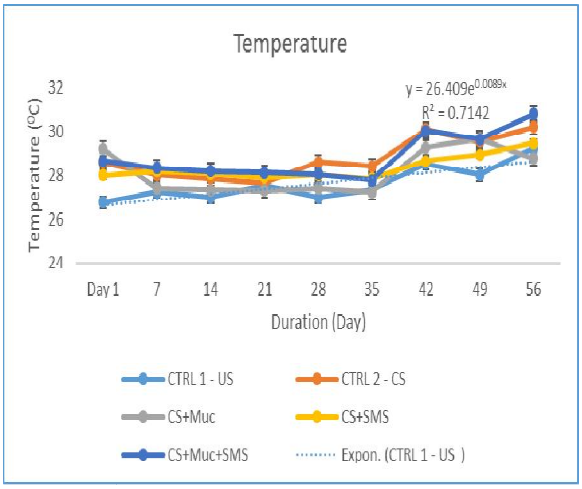

Fig. 4. Changes in temperature (^{0}C) during bioremediation of crude oil contaminated soil

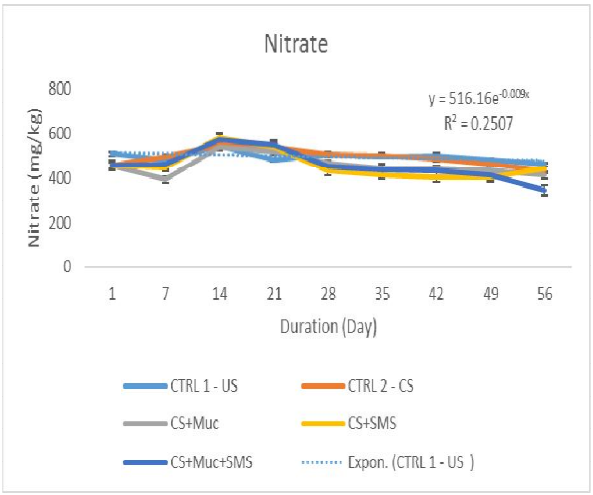

Fig. 5. Changes in nitrate (mg/kg) during bioremediation of crude oil contaminated soil

Table 5. Mean and Standard deviation of Physicochemical parameters during bioremediation of crude oil polluted soil

Plot	Treatments	Physicochemical parameters					
		pH	Temperature	Nitrate	Phosphorus	Potassium	TPH
P1	CTRL 1 - US	6.95±0.20a	27.62±0.81a	494.39±24.14b	2.38±0.26a	2.11±0.39a	28.27±28.09a
P2	CTRL 2 - CS	6.28±0.25a	28.77±0.96a	491.06±37.92ab	2.39±0.31a	1.99±0.34a	8612.76±57.80b
P4	CS+Muc	6.25±0.29a	28.18±1.02a	453.90±46.57ab	2.46±0.40a	2.43±0.64a	2238.25±2922.68ab
P5	CS+SMS	6.49±0.17b	28.33±0.56a	456.45±62.85ab	2.19±0.31a	4.65±2.27a	2928.36±2957.38ab
P8	CS+Muc+SMS	6.42±0.14b	28.84±1.06a	456.31±68.32ab	2.43±0.24a	3.66±1.41a	2686.41±2892.97ab

***means with the same superscript along the columns are not significantly different (p>0.05).*

P=- Plot; US = Uncontaminates soil; CS = Contaminated soil; Asp = Aspergillus niger; Muc = Mucor racemosus; SMS = Spent Mushroom Substrate

Table 6. Regression analysis of physiochemical parameters during bioremediation of crude oil polluted soil

Plot	Treatment	pH		Temp		Nitrate		Phosphorus		Potassium		TPH	
		Regression equation (Y)	R^2	Regression equation (Y)	R^2	Regression equation (Y)	R^2	Regression equation (Y)	R^2	Regression equation (Y)	R^2	Regression equation (Y)	R^2
P1	CTRL 1 - US	-0.032x + 7.111	0.195	0.248x +26.38	0.710	-4.360x +516.2	0.244	-0.047x +2.612	0.248	-0.105x + 2.633	0.550	-9.417x +75.35	0.842
P2	CTRL 2 - CS	0.005x + 6.252	0.003	0.274x + 27.39	0.613	-5.755x +519.8	0.172	-0.060x +2.696	0.294	0.006x + 1.961	0.002	-19.49x + 8710	0.853
P4	CS+Muc	0.059x + 5.955	0.324	0.147x + 27.44	0.156	-5.381x +480.8	0.100	-0.053x +2.723	0.132	-0.059x + 2.729	0.064	-917.5x + 6826	0.739
P5	CS+SMS	-0.033x + 6.653	0.305	0.154x + 27.55	0.577	-11.5x + 513.9	0.251	-0.039x +2.389	0.115	-0.041x + 4.860	0.002	-969.6x + 7776	0.806
P8	CS+Muc+SMS	0.023x + 6.300	0.221	0.265x + 27.51	0.472	-16.43x +538.5	0.434	-0.000x +2.431	1E-05	0.020x + 3.555	0.001	-931.4x + 7343	0.777

P=- Plot; US = Uncontaminates soil; CS = Contaminated soil; Asp = Aspergillus niger; Muc = Mucor racemosus; SMS = Spent Mushroom Substrate

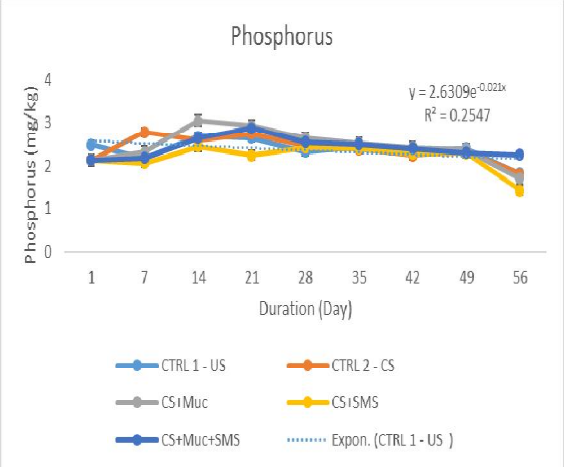

Fig. 6. Changes in Phosphorus (mg/kg) during bioremediation of crude oil contaminated soil

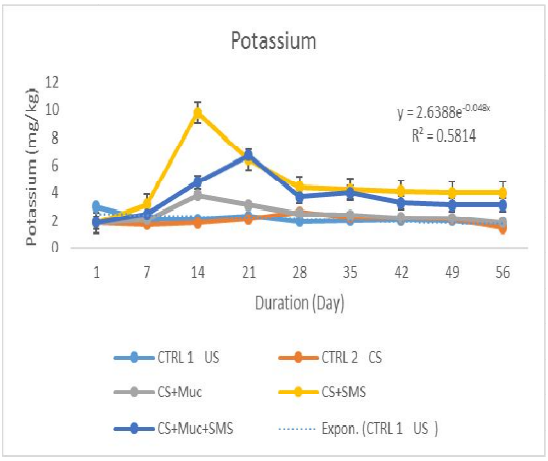

Fig. 7. Changes in potassium (mg/kg) during bioremediation of crude oil contaminated soil

3.4 Physico-Chemical Properties of Soil during Bioremediation

The physic-chemical characteristics of the bio-remediated soil was duly conducted. This was done by determining the pH, temperature, nitrate, phosphorus and potassium concentrations respectively. As depicted in the Table 5 and Fig. 3, the pH ranged between 6.25±0.29 - 6.95±0.20 with the peak pH being recorded in the CTRL 1 - US control plot.

Temperature also ranged between 27.62±0.81-28.84±1.06^{o}C (Table 5-6 and Fig. 4). Temperature range were relatively same between the bioremediation group and the control group. Similar trend was observed with nitrate ranging from 453.90±46.57 to 494.39±24.14 mg/kg, phosphorus and potassium showed slight variations (Table 5-6, Fig. 5-7). Noteworthy, the control groups varied significantly from the CS+Muc, CS+SMS and CS+Muc+SMS. In a study on the effects of organic manures on the physic-chemical properties of crude oil polluted soils, the percentage the pH, percentage total nitrogen, phosphorus and exchangeable bases (Ca, K and Mg) significantly decreased along with a decrease in the hydrocarbon content of the soil in that study [27]. Elsewhere, a study on the physicochemical properties of crude oil contaminated soils as influenced by cow dung and showed that the percentage of Nitrogen, Phosphorus, Potassium and pH significantly decreased two weeks after crude oil contamination, thereby suggesting that the addition of crude oil may have adverse effect on the physicochemical properties of soil [28].

The physicochemical parameters of the bioremediation study of a contaminated soil resulted in a decrease of the total organic carbon (56.64%), sulfate (57.66%), nitrate (57.69%), phosphate (57.73%), sodium (57.69%), potassium (57.68%), calcium (57.69%) and magnesium (57.68%) except pH (3.90%) that slightly increased [29].

Notably, compost additions (like the spent mushroom substrate) can also have extremely beneficial effects on soil chemical properties other than nutrient availability. These include buffering of soil pH, increases in soil cation exchange capacity, and the reduced activity of potentially toxic substances. Generally, compost will increase the soil pH for acid soils and reduce the soil pH for alkaline soils. Composts produced from recycled waste material is generally near neutral or slightly alkaline in pH, though compost pH from 5.5 to 8.0 is commonly observed. Soil pH near neutral is ideal for most plants, though some plant species, particularly ericaceous species, need more acid soils, and will suffer micronutrient deficiencies at neutral pH levels. Higher pH levels may aggravate certain plant diseases as well, though there is no general rule for all plants, and this is not a common observation. One of the most important benefits of addition of organic matter is an increase in the ability of soils to retain nutrients in an available or slowly available form [30].

Evaluation of Amount of Crude Oil or Hydrocarbon remediated and Net %Bioremediation revealed Crude Oil contaminated plot augmented with *Mucor racemosus* broth singly (CS+Muc) as having the highest bioremediation potential while the least is the untreated soil. The trend is as follows: CS+Muc (8599.19 mg/kg; 33.93%) > CS+Muc+SMS (8298.95 mg/kg; 32.74%) > CS+SMS (8197.03 mg/kg; 32.34%) > CTRL 2 – CS (166.54 mg/kg; 0.66%) > CTRL 1 –US (85.48 mg/kg; 0.34%) (Table 6-7, Fig. 8).

Table 7. Bioremediation analysis

Bioremediation analysis						
TPH	**Plot code**	**Initial crude oil conc.**	**Final crude oil conc.**	**Amount remediated**	**% bioremediation**	**Net %bioremediation**
P1	CTRL 1 - US	87.89	2.41	85.48	97.25	0.34
P2	CTRL 2 - CS	8729.00	8562.46	166.54	1.91	0.66
P4	CS+Muc	8729.00	129.81	8599.19	98.51	33.93
P5	CS+SMS	8729.00	531.97	8197.03	93.91	32.34
P8	CS+Muc+SMS	8729.00	430.05	8298.95	95.07	32.74

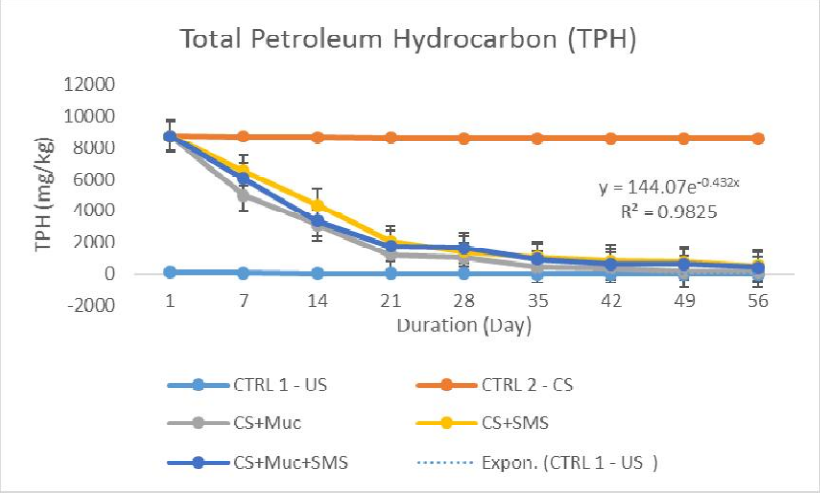

Fig. 8. Changes in total petroleum hydrocarbon (TPH)(mg/kg) during bioremediation of crude oil contaminated soil treated with bio-organics and augmenting microbe *Mucor racemosus*

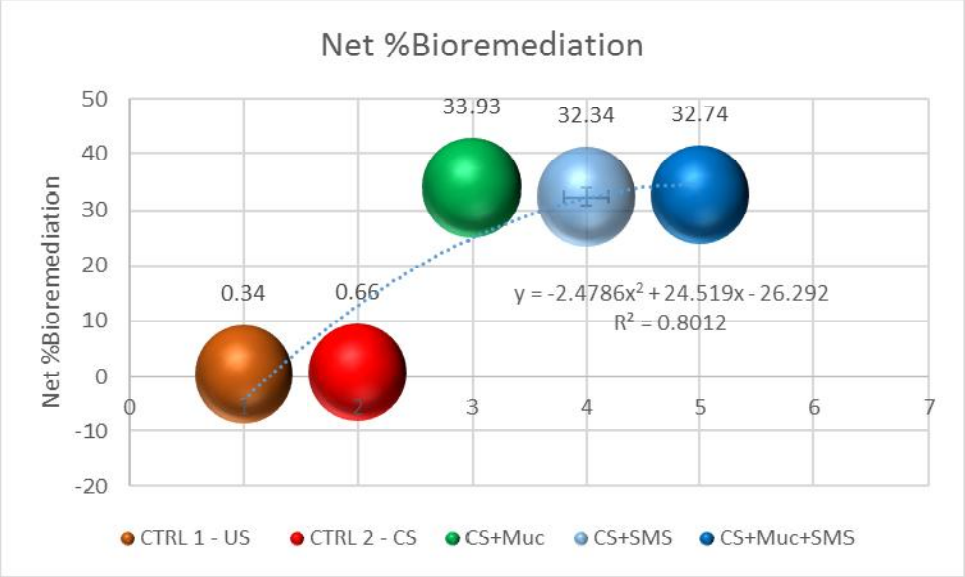

Fig. 9. Net %Bioremediation of crude oil contaminated soil treated with bio-organics and augmenting microbe

Notably, no particular trend of decline was observed. TPH levels during bioremediation showed that a progressive decline in the concentration was observed from day 7 to day 56 with the highest decline being recorded at the end of the bioremediation at day 56. While the values in day 1 was 8729.00 mg/kg, the value at day 56 for all bioremediation option had a range of 129.81 - 779.99 mg/kg. The least TPH level at day 56 was recorded in CS+Muc and CS+Muc+SMS with values of 129.81 mg/kg and 430.05 mg/kg respectively. The findings of the present study conforms with the findings of a study by Benyahia and Embaby [31]) who reported a total petroleum hydrocarbon (TPH) reduction of 77% over 156 days longer than the bioremediation period in the present study. In another related study, Ebuehi et al. [32] reported TPH concentration of 1.1004 $x10^4$ mg/kg of the sandy soil was achieved after spiking and tilling. In this dame study, there was a reduction in the TPH level from 300mg/kg after 8 weeks, to 282mg/kg after 10 weeks.

Assessment of Net %Bioremediation revealed Crude Oil contaminated plots showed that Crude Oil contaminated soil augmented with *Mucor racemosus* broth singly (CS+Muc) as had the highest bioremediation potential while the least is the untreated soil. The trend is as follows: CS+Muc (33.93%) > CS+Muc+SMS (32.74%) > CS+SMS (32.34%) > CTRL 2 –CS (0.66%) > CTRL 1 –US (0.34%) (Table 6, Fig. 9)

Typically, Petroleum hydrocarbons are complex substances formed from hydrogen and carbon molecules and sometimes containing other impurities such as oxygen, sulfur, and nitrogen. They are highly lipophilic and unless they are of high viscosity (e.g., tar and motor oil), they are generally readily absorbed through skin and intact mucosae [33]. TPH is a mixture of chemicals, but they are all made mainly from hydrogen and carbon, called hydrocarbons. Scientists divide TPH into groups of petroleum hydrocarbons that act alike in soil or water. These groups are called petroleum hydrocarbon fractions. Also, PAHs are constituents of petroleum hydrocarbons that have become ubiquitous in the environment because of the persistent exploitation of crude oil and its derivatives. Such pollutants may undergo photolysis, chemical oxidation, volatilization, leaching, bioaccumulation, and/or adsorption in soil. The degradation of this PAHs by the bioremediation process was achieved via aerobic process [34].

4. CONCLUSION AND RECOMMENDATION

This study shows that a single nutrient substrate or augmenting microorganism applied appropriately may have a more positive result, that is; higher bioremediation potential than combined or multiple mixed treatments. This has produced more effective and faster

bioremediation, achieving a greater reduction in petroleum hydrocarbon. It was further observed that microbial counts decreased with time in treatments with augmenting organisms alone but increased considerably in treatments supplement with organics having its peak on the 49th day.

It is therefore recommended that bioremediation of crude oil-polluted soil using bio-augmenting microorganism should be applied appropriately noting the volume: area ratio and be supplemented with efficient nutrient organics after every 49-day interval.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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