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Myco-enhanced Bioremediation in Open Field Crude Oil Contaminated Soil Using *Mucor racemosus* and *Aspergillus niger*

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Authors' contributions

This work was carried out in collaboration among all authors. Authors DNO, RNO 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 RNO and OU managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To assess the Mycoremediation potential of *Mucor racemosus* and *Aspergillus niger* in open field crude oil contaminated soils in Rivers State, Nigeria.
Study Design: The study employs experimental design, statistical analysis of the data and interpretation.
Place and Duration of Study: Rivers State University demonstration farmland in Nkpolu-Oroworukwo, Mile 3 Diobu area of Port Harcourt, was used for this study. The piece of land is situated at Longitude 4°48'18.50" N and Latitude 6°58'39.12" E measuring 5.4864 m x 5.1816 m with a total area of 28.4283 square meter. Mycoremediation process monitoring lasted for 56 days, analyses were carried out weekly at 7 days' interval.
Methodology: Five (5) experimental plots were employed using a Randomized Block Design each having dimensions of 100 x 50 x 30 cm (Length x Breadth x Height) and were formed and mapped out on agricultural soil, each plot was contaminated with 22122.25g of Crude Oil except Control 1

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and left fallow for 6 days after contamination for proper contamination and exposure to natural environmental factors to mimic crude oil spill site. On the seventh day bio-augmentation process commenced using two (2) fungal isolates namely *Aspergillus niger* [Asp] and *Mucor rasemosus* [Muc]). Two (2) control plots (P1: Uncontaminated and unamended soil - CTRL 1 US) and P2: Crude Oil contaminated but unamended soil - CTRL 2 CS); P3 = P5 were contaminated and amended/bioaugmented (P3: CS+Asp, P4: CS+Muc, P5: CS+Asp+Muc respectively. Soil profile before and after contamination was assayed while parameters like Temperature, pH, Nitrogen, Phosphorus, Potassium and Total Petroleum Hydrocarbon (TPH) contents were monitored throughout the experimental period. Microbial analyses such as Total Heterotrophic Bacteria (THB), Total Heterotrophic Fungi (THF), Hydrocarbon Utilizing Bacteria (HUB) and Hydrocarbon Utilizing Fungi (HUF) were recorded. Bioremediation efficiency was estimated from percentage (%) reduction of Total Petroleum Hydrocarbon (TPH) from day 1 to the residual hydrocarbon at day 56 of bio-augmentation/ biostimulation plots with the control.

Results: Results revealed actual amount of remediated hydrocarbon and % Bioremediation Efficiency at 56 days in the different treatment plots (initial TPH contamination value of 8729.00mg/kg) in a decreasing order as follows: CS+Muc (8599.19mg/kg; 33.66%) > CS+Asp+Muc (8357.31mg/kg; 33.04%) > CS+Asp (8341.58mg/kg; 32.98%) > CTRL 2 -CS (Polluted soil without amendment) (81.06mg/kg; 0.32%). Microbiological results After fifty-six (56) days of bioremediation monitoring; %HUB were as follows; CS+Asp+Muc (45.30%) > CS+Asp (40.32%) > CS+Muc (35.01%) > CTRL 2 –CS (30.43%) > CTRL 1 – US (0%). These results indicate that the presence of the contaminated crude oil stimulated and sustained the growth of Hydrocarbon Utilizing Bacteria (HUB) in the contaminated plots (P2 - P3); more so, the higher growth in the enhanced bio-augmented plots (P3 – P5) shows the positive impact of fungal bio-augmentation in bioremediation of crude oil polluted soil. It was further observed that treatment plots with higher HUB or HUF had higher percentage (%) bioremediation efficiency; that is, the higher the sustained HUB and HUF population, the higher the %Bioremediation process. Hydrocarbon Utilizing Bacteria (Log10 CFU/g): CS+Asp (4.20) (Day 35) > CS+Muc+Asp (4.18) (Day 35) > CS+Muc (4.08) (Day 28) > CTRL 2 – CS (3.95) (Day 21) > CTRL 1 – US (3.78) (Day 35). (Fig. 3). Hydrocarbon Utilizing Fungi (Log10 CFU/g): CS+Asp (4.68) (Day 35) > CS+Muc+Asp (4.58) (Day 35) > CS+Muc (4.48) (Day 35) > CTRL 2 – CS (4.23) (Day 21) > CTRL 1 – US (2.85) (Day 42).

Conclusion: Study showed that bioremediation of crude oil-contaminated soils with Bioaugmenting fungus singly may be more effective than combination with others depending on the type of substrate used, nature of the hydrocarbon utilizing organism and environmental conditions prevalent as seen in *Mucor racemosus* having higher bioremediation potential than when combined with *Aspergillus niger.* Notably, Hydrocarbon Utlilizing Bacteria (HUB) and Hydrocarbon Utilizing Fungi (HUF) which are the key players in Bioremediation has its peak count value on Day 35, this confers that nutrient renewal on bioremediation site should be at interval of 35 days for continuous effective bioremediation of hydrocarbon pollutants. It is therefore recommended that single microbes of high bioremediation potential could be used since its more effective than consortium of many hydrocarbon utilizing microbes. Also, nutrient or bio-augmenting microbes' renewal on bioremediation site should be at an interval of 35 days for continuous effective bioremediation of hydrocarbon pollutants.

Keywords: Bioremediation; Bioaugmentation; Mycoremediation; petroleum hydrocarbon; Aspergillus niger; Mucor racemosus; crude oil contamination; Soils.

1. INTRODUCTION

The release of hydrocarbons into the environment whether accidentally or due to human activities is a main cause of water and soil pollution [1,2]. Soil contamination with hydrocarbons causes extensive damage of ecosystems through the food chain since accumulation of pollutants in animals and plant tissue may cause death or mutations [3].

During the past century, industrial production, urbanization, energy consumption, transportation and human population have expanded exponentially, resulting in increased soil, water and air pollution, which in turn has placed the environment under substantial pressure [4]. These factors produced a large number of highly polluted sites all over the planet, usually containing complex mixtures of toxic and carcinogenic, organic and inorganic compounds. Organic contaminants such as total petroleum

hydrocarbons (TPH) and polycyclic aromatic hydrocarbons (PAHs) are known mutagens and carcinogens that enter the food chain together with lipophilic compounds [5,6,7]. According to the United States Environmental Protection Agency the very hazardous chemicals like benzene, toluene, ethylbenzene, xylenes, and naphthalene are included in the petroleum hydrocarbons [8-12]. These pollutants can affect soil physical characteristics like soil texture and structural status, compaction, saturated hydraulic conductivity and penetration resistance [13]. When released on the surface soil, petroleum hydrocarbons, with specific physico-chemical characteristics [14] pushes soil toward a condition undesirable for proper and sustainable growth of plant and rhizosphere organisms activity [5,15]. Sources of crude oil/hydrocarbon release into the environment may include storage tank leakages. In 2018, storage tanks leakage and spill accounted for around 116,000 tonnes discharge of hydrocarbons and crude oil into the environment. This means that the presence of these contaminants in soil significantly reduce the quality of soil and thus minimize the germinating, growth and health of plants [16]. Therefore, remediation and removing of these pollutants from soil is necessary for sustainable environmental health [17,18].

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 [19,20]. 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 [21]. Petroleum hydrocarbon contamination may occur through pipelines and oil wells leakages, wrong methods of disposal of petroleum wastes and accidental oil spills [22]. 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 [23]. Notably, different oil products like gasoline diesel or heavy oils can cause soil contamination [24].

Mycoremediation is defined as a natural or artificial process in which fungi are used to degrade contaminants to less toxic or nontoxic forms, thereby reducing or eliminating environmental contamination. Ligninolytic fungi (white rot fungi) can degrade petroleum hydrocarbons by extracellular lignin modifying enzymes [25]. These enzymes have very low substrate specificity, making them suitable for degradation of a wide range of highly recalcitrant compounds that is structurally similar to lignin. The ligninolytic enzymes consist of lignin peroxidase, manganese peroxidase and laccase [26]. 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 [27]. According to Okerentugba *et al* [25] Spent Mushroom Compost can be effective in the degradation of petroleum hydrocarbon because of its degrading and ligninolytic properties. Most studies about hydrocarbon and petroleum degradation have been conducted on groundwater aquifers [28] and in laboratory and/or field studies; however little research has been carried out on soils. Wegwu *et al* [29] in their study indicated that attenuation method is one of the best techniques for soil refinement in contaminated soils with crude oil. There are three methods of attenuation which include; natural attenuation, biostimulation and bioaugmentation which are introduced as effective methods for removal of Total Petroleum Hydrocarbons (TPHs) from soils [30]. Studies have been conducted to isolate and characterize hydrocarbon degraders from oil spill sites but little have been done to determine the changes in soil nutrients and TPHs as bioremediation of the spill site progresses, thus the aim of this research is to assess the potential of myco-remediating microbes Mucor and Aspergillus species in bioremediation of crude oil contaminated soil and their effects in key soil nutrient (NPK).

2. MATERIAL AND METHODS

2.1 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 $4^{\circ}48'18.50"$ N and Latitude $6^{o}58'39.12"$ E

measuring 5.4864m x 5.1816m with a total area of 28.4283m^2. This was cleared and sub-partitioned into 9 blocks of 100cm x 50cm x 30cm 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 (according to method described by Ogbonna et al [31]. The soil is of sandy clay texture with specific gravity of 2.57. From these plots; unpolluted, crude oil polluted and nutrient amended soil samples were taken for bioremediation analysis. The study area is shown in Fig. 1. The choice of the Rivers 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.

2.2 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 0-15cm depth [32].

2.3 Sources of Microbial Isolates

The microorganisms used were fungi specifically *Aspergillus nudilans* and *Mucor racemosus*. These organisms were isolated from the soil samples using Sabouroud Dextrose Agar as selective media for fungi. After which pure cultures obtained were inoculated onto Modified Sabouraud Dextrose broth in 500 ml Erlenmeyer flask loosely plugged with sterile cotton wool for the growth of the augmenting test organisms. Broth cultures with an optical density of 0.2 were used for augmentation.

2.4 Treatment/ Field Application

Five Randomized Complete Block Design (RCBD) degradative plots according to the method of Toogood [32] 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. They are as follows.

2.5 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 distilled 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.

The plots were amended with 750ml of *Aspergillus* and 750ml of *Mucor* accordingly [31,33]. 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 (Table 1).

2.6 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.

Table 1. Treatments of Experimental plots using Nutrient amendments and bio-augmenting organisms

Sample ID	Plot Code	Crude oil (g)	Aspergillus(Asp) (ml)	Mucor (Muc) (ml)
P1	CTRL 1 (Uncontaminated soil -US)	0	-	-
P2	CTRL 2 (Contaminated soil - CS)	2122.25	-	-
P3	CS+Asp	2122.25	750	-
P4	CS+Muc	2122.25	-	750
P6	CS+Asp+Muc	2122.25	375	375

P=- Plot; US = Uncontaminated soil; CS = Contaminated soil; Asp = Aspergillus nidulans; Muc = Mucor racemosus

2.7 Watering

The plots were watered to 65% water holding capacity [34] before experimental crude oil contamination and subsequently at two days' interval with 600ml of water per plot as required.

2.8 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 samples collected were made from 4-10 random points per plots and bulked to form a composite sample. Small portions (5g) of the composite samples were collected into sterile bottles using sterile spatula for microbiological and physicochemical analysis. All microbiological analysis were carried out in the Microbiology laboratory of the Rivers State University within 2 hours after sample collection while physicochemical analysis was carried out at Pollution Control and Environmental Management (POCEMA) and Giolee Global Resources Laboratories both in Port Harcourt, Rivers State. Soil samples were stored at 14±2^{o}C for future analysis [34].

2.9 Microbiological Analysis of Soil Samples

The following Media were used for microbial enumeration and isolation

2.9.1 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 1000ml of distilled water and then sterilized/autoclaved at 121^{0}C for 15 minutes according to the manufacturer specification

2.9.2 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 [34]; Nrior and Echezolom [35]. The medium was prepared with a composition of K_2HPO_4 (0.5g), $MgSO_4.7H_2O$ (0.3g), $NaCl_2$ (0.3g), $MnSO_4.H_2O$ (0.2g), $FeSO_4.6H_2O$ (0.02g), $NaNO_3$ (0.03g), $ZnCl_2$ (0.3g) and agar (15g) 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 bacteria (oil degraders). The medium was then prepared by the addition of 1% (v/v) crude oil sterilized with 0.22Millipore filter paper to sterile MSM cooled to 45^{o}C under aseptic condition. The MSM and crude oil were then mixed thoroughly and dispensed into sterile Petri dishes to set.

2.9.3 Sabouroud dextrose agar

Sabouroud Dextrose Agar (SDA) was used for the isolation of fungi isolates. Media was prepared by weighing out 65g into 100ml of distilled water and using the manufacturer's specification, depending on the number of plates used. After the preparation it was autoclaved at 121^{0}C for 15 minutes and then the media was aseptically poured into plates for inoculation.

2.10 Glassware and Media Sterilization

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

2.11 Microbiological Analyses

2.11.1 Microbial estimation

The total heterotrophic bacteria (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 [36] as cited by Chikere *et al.,* [37]; Oliveira *et al.,* [38] and Nrior and Mene [2].

2.11.2 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 so were those that grew on MSA plates. Pure culture of fungi were stored on SDA slants, while those of bacteria isolates were stored in 10% glycerol, all in Bijou bottles.

2.11.3 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).

2.11.4 Wet mount preparation

A flamed 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 [39] and other microscopic and cultural characteristics were further used in the identification of the fungal isolates of the bioremediation set up [40].

2.11.5 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 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^{o}C until sporulation occurred [39].

2.11.6 Purification and Preservation of Pure Cultures

The Fungal isolates were inoculated onto Sabouraud Dextrose Broths in 500ml Erlenmeyer flask loosely plugged with sterile cotton wool respectively. The broth cultures were incubated for 5 days at 28^{o}C. Serial dilution was made to determine the number of cells per 0.1 ml aliquot.

2.11.7 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:

$$T = \frac{N}{V} \; x \; DF$$

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)

Total Heterotrophic Bacterial count =

$$\frac{Number\ of\ colonies}{Volume\ plated\ (0.1ml)} \; x \; Dilution\ factor$$

2.11.8 Bioremediation evaluation procedure

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

2.9 Bioremediation Analysis

2.9.1 Percentage (%) bioremediation analysis

The method of Nrior and Mene [2] was used in calculating the percentage of Bioremediation in the experiment. The process followed the steps stated.

Step i: The amount of pollutant remediated equals to Initial Concentration of pollutant (Week 1) minus the Final Concentration of pollutant at the end of experiment (Last day or Week 8)

Bc = Ic – Fc same as AR*x* = IC*x* – FC*x*

Where:

Bc (ARx) = Amount of pollutant remediated in plot x
Ic (ICx) = Initial Concentration of pollutant in plot x (week 1)
Fc (FCx) = Final Concentration of pollutant in plot x (week 8)

Step ii: The percentage (%) Bioremediation equals Amount of pollutant divided by the Initial Concentration of pollutant (week 1), multiplied by 100

% Bioremediation = (Bc/Ic) x 100 same as **% Bioremediation = (ARx/ICx) x 100** (Nrior and Mene), [2]

2.9.2 Actual %bioremediation

Step 1: Calculate Amount of Hydrocarbon (Crude oil) remediated in Control without experimental Contamination (CTRL 1 – US) *[**Note**: This is essential where there are heavy activities of oil companies, exploration, spillage, marketing of crude oil etc, that makes almost impossible for any piece of land to be completely free from residual crude oil contamination; example, Niger Delta Region of Nigeria]*

For Control without experimental contamination CTRL 1 – US

$$AR_{CTRL1\text{-}US} = IC_{CTRL1\text{-}US} - FC_{CTRL1\text{-}US}$$

Step 2: Actual Amount Remediated (AAR) equals Amount Remediated in each Treatment (ARx) minus Amount Remediated in Control (ARc) (Experimental Uncontaminated soil CTRL 1 - US)

$$AAR = ARx - ARc$$

$$\%AAR = (ARx/\sum ARx) * 100$$

2.9.3 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 [36]. Soil texture was determined using sieves of different sizes – Master Sizer 2000 (Malner International), while moisture content was determined by drying 10g of the soil sample in an oven at 80^oC. Then 10g 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%).

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.

2.9.4 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.* [37] 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 duplicates while ensuring precision and reliability of results through standard quality assurance and control procedures.

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

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

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

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

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

25ml of the extracting solution was added to 5g of soil sample and shaken for 30minutes and the suspension was filtered through a filter paper. 5ml of the extract was transferred into 50ml volumetric flask. 5ml 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 50ml with distilled water, and the absorbance was measured at 425nm.

2.9.8 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 shows the baseline physico-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 microbial analysis 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 contaminated soil. The electrical conductivity was 500µS/cm for uncontaminated soil and 590µS/cm for contaminated soil. TPH value was as low as 87.89mg/kg in the uncontaminated soil and 8729mg/kg in the contaminated soil.

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	$^{\circ}C$	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

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 [41]. 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 [42].

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 [43].

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.

The results of the microbial evaluation of the study are shown in Fig. 2-5. Counts for 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 all determined in this study. Significant microbial counts for Total Heterotrophic Bacteria (Log10 CFU/g) were recorded on day 42, 49 and 56 of the bioremediation; The highest count for each plots were as follows; CTRL 2 – CS (9.86) (Day 56) > CTRL 1 – US (9.24) (Day 49) > CS+Muc (9.12) (Day 49) > CS+Muc+Asp (9.03) (Day 42) = CS+Asp (9.03) (Day 28) (Fig. 1). Generally, there seems to be peak count on day 49 and a decline in the THB count on Day 56.

Total Heterotrophic Fungi (Log10 CFU/g): CTRL 2 –CS (5.20) (Day 28) > CS+Muc+Asp (4.95) (Day 56) > CS+Muc (4.93) (Day 35) > CS+Asp (4.78) (Day 42) > CTRL 1 – US (3.95) (Day 48). (Fig. 2). Hydrocarbon Utilizing Bacteria (Log10 CFU/g): CS+Asp (4.20) (Day 35) > CS+Muc+Asp (4.18) (Day 35) > CS+Muc (4.08) (Day 28) > CTRL 2 – CS (3.95) (Day 21) > CTRL 1 – US (3.78) (Day 35). (Fig. 3). Hydrocarbon Utilizing Fungi (Log10 CFU/g): CS+Asp (4.68) (Day 35) > CS+Muc+Asp (4.58) (Day 35) > CS+Muc (4.48) (Day 35) > CTRL 2 – CS (4.23) (Day 21) > CTRL 1 – US (2.85) (Day 42). (Fig. 4) Notably, Hydrocarbon Utlilizing Bacteria (HUB) and Hydrocarbon Utilizing Fungi (HUF) which are the key players in Bioremediation has its peak count value on Day 35, this confers that nutrient renewal on Bioremediation site should be at interval of 35 days for continuous effective bioremediation of hydrocarbon pollutants.

Evaluation of Percentage (%) Hydrocarbon Utilizers during enhanced Bioremediation of Crude Oil Contaminated soil showed Significant growth in plots contaminated with crude oil while at day 56 the Uncontaminated plot used as Control 1 recorded zero percent (Table 3). The 0% HUB and HUF on Day 56 is a clear indication of the absence of Crude oil as their carbon source.

After fifty six (56) days of bioremediation monitoring; %HUB were as follows; CS+Asp+Muc (45.30%) > CS+Asp (40.32%) > CS+Muc (35.01%) > CTRL 2 –CS (30.43%) > CTRL 1 – US (0%), the mean value has same trend (Table 3-4). This result indicates that the presence of the contaminated Crude Oil stimulated and sustained the growth of Hydrocarbon Utilizing Bacteria (HUB) in the contaminated plots (P2 - P3); more so, the higher growth in the enhanced bio-augmented plots (P3

– P5) shows the positive impact Myco (fungal) bio-augmentation in bioremediation of Crude oil polluted soil. It was further discovered that treatment plots with higher HUB or HUF had higher percentage (%) bioremediation; that is, the higher the sustained HUB and HUF population, the higher the %Bioremediation. (Table 3-4, Fig 3-5).

Assessment of Hydrocarbon Utilizing Fungi showed similar trend to that of HUB, with the enhanced treatment plots (Mycobio-augmented plots P3 – P5)) having higher counts, though CS+Muc (91.87%) (Day 56) with Net %HUF (12.18%) > CS+Asp+Muc (88.32%) (Net %HUF 12.03%)

Biodegradation mediated by indigenous microbial communities is a key process by which petroleum hydrocarbons are mineralized and removed from contaminated environments. Thus, microbial oil biodegradation is recognized as one of the most important methods for petroleum hydrocarbon remediation. Most petroleum hydrocarbons are biodegradable under aerobic conditions. Hydrocarbon-oxidizing bacteria capable of growth on aliphatic and aromatic hydrocarbons are found in many genera. In the presence of O_2, the initial steps in the bacterial degradation of hydrocarbons rely on oxygenases. These oxygenases are membrane-bound, the cell must come into direct contact with their water-insoluble substrates. The oxygenases are group-specific for example, therefore some degrade specific fractions of alkanes, whereas others work on aromatics or cyclic hydrocarbons, it follows that only a mixture of different microorganisms can efficiently degrade crude oil and petroleum fractions [2,43]

3.3 Physico-Chemical Properties of Soil during Bioremediation

The physico-chemical characteristics of the bio-remediated soil was duly conducted. This was done by determining the pH, temperature, nitrogen, phosphorus and potassium concentrations. The pH ranged between 5.68 – 7.19 with its mean peak value 6.95±0.20 recorded in the First Control: Uncontaminated soil without Bio-amendment (CTRL 1 – US) plot (Fig. 6, Table 5). The Crude Oil contaminated plots had relatively lower pH; this implies that crude oil had a reductive effect on the soil pH tending toward acidity. Temperature also ranged between 27.62±0.81 - 28.77±0.96^{o}C with its peak in the Second Control plot - Crude Oil Contaminated soil without Bio-amendment (CTRL 2 - CS). Temperature range were relatively same between the bioremediation group and the control group but were higher in the Crude Oil Contaminated plot than the Uncontaminated Control plots (Fig. 7, Table 5). Two things seemed clear; that the presence of Crude oil in soil tends to lower soil pH and increase its Temperature.

Nitrogen value in the experimental plots ranged from 344.32 – 549.22mg/kg with its mean peak value as 494.39±24.14mg/kg. Similar trends were observed with phosphorus and potassium (Table 5-6, Fig. 8-10). Noteworthy, the control groups varied significantly from the CS+Asp, CS+Muc and CS+Asp+Muc as featured in Fig. 10, for Potassium day 14 (CS+Asp+Muc) and (CS+Muc); while Total Petroleum Hydrocarbon (TPH) (mg/kg) in Fig. 11 and 12, Day 1-56 for Control 2 (Crude Oil Contaminated soil without amendment CTRL 2- CS) varied significantly from the Contaminated and amended plots (CS+Asp, CS+Muc and CS+Asp+Muc). In a study on the effects of organic manures on the physico-chemical properties of crude oil polluted soils, the percentage 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 [27,44]. 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 [31]. 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 [45].

As depicted in Table 7 and Fig. 12-13, the total petroleum hydrocarbon concentration in the contaminated soil CS ranged between 8562.46 - 8729.00 mg/kg with the peak concentration being recorded in day 1 and a very slight negligible decline between day 7 and 56. 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 87.89mg/kg for the uncontaminated soil used as control, the 8729.00 mg/kg, the value at day 56 for all bioremediation option had a range of 2.41 - 779.99 mg/kg. The least TPH level at day 56 was recorded in CS+Muc and CS+Asp+Muc+SMS with values of 129.81 mg/kg and 258.40 mg/kg respectively.

The findings of the present study conforms with the findings of a study by Benyahia & Embaby [46] 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. [47] reported TPH concentration of 1.1004 $x10^4$ mg/kg of the sandy soil was achieved after spiking and tilling. In this same study, there was a reduction in the TPH level from 300mg/kg after 8 weeks, to 282mg/kg after 10 weeks.

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 [43]. 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 these PAHs by the bioremediation process was achieved via aerobic process [48].

Actual Amount of remediated hydrocarbon and % Bioremediation Efficiency at 56 days in the different treatment plots (initial TPH contamination value of 8729.00mg/kg) in a decreasing order as follows: CS+Muc (8599.19mg/kg; 33.66%) > CS+Asp+Muc (8357.31mg/kg; 33.04%) > CS+Asp (8341.58mg/kg; 32.98%) > CTRL 2 -CS (Polluted soil without amendment) (81.06mg/kg; 0.32%). Microbiological results After fifty six (56) days of bioremediation monitoring; %HUB were as follows; CS+Asp+Muc (45.30%) > CS+Asp (40.32%) > CS+Muc (35.01%) > CTRL 2 –CS (30.43%) > CTRL 1 – US (0%) (Table 7 and Fig. 13).

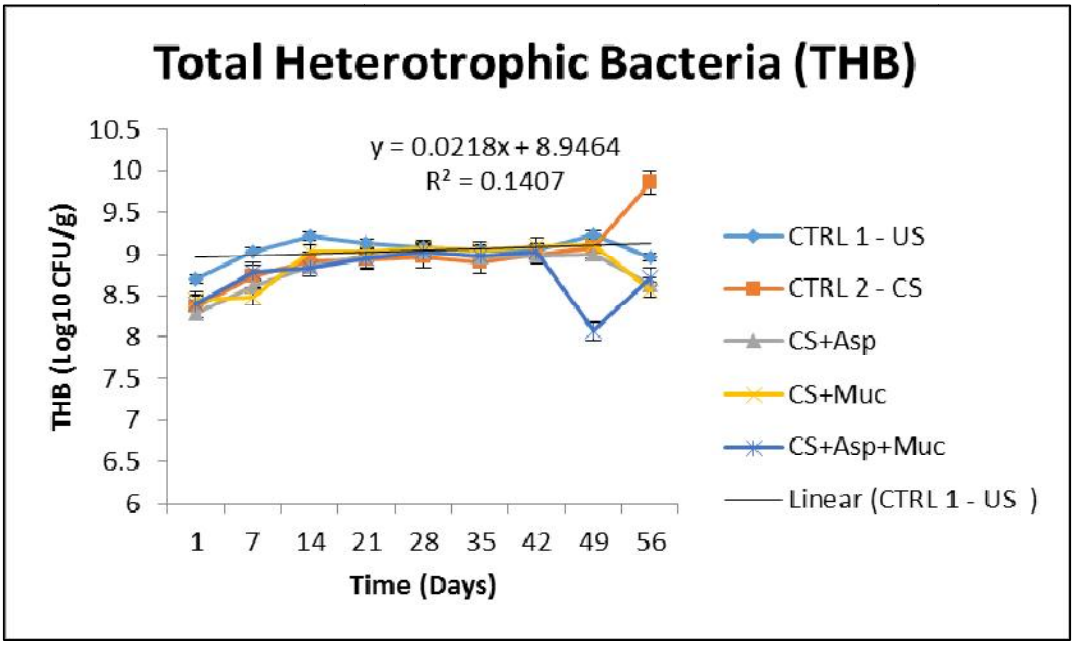

Fig. 1. Total Heterotrophic Bacteria (THB – Log10 CFU/g) during enhanced bioremediation of Crude Oil contaminated soil

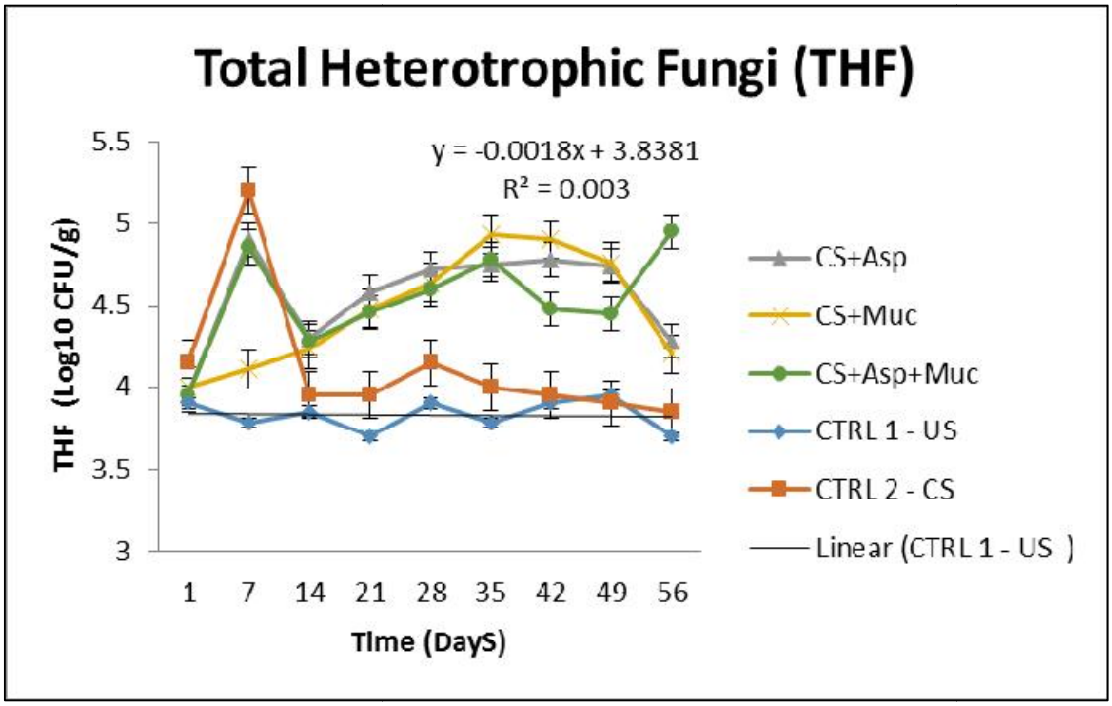

Fig. 2. Total Heterotrophic Fungi (THF – Log10 CFU/g) during enhanced bioremediation of Crude Oil contaminated soil

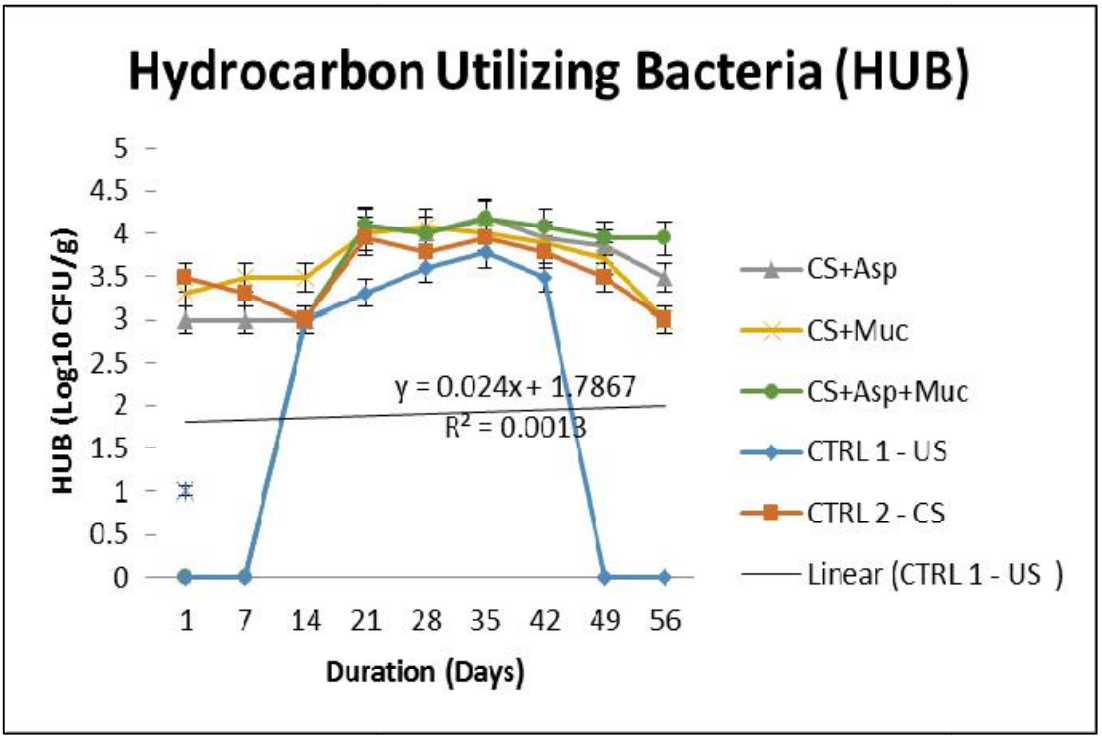

Fig. 3. Hydrocarbon Utilizing Bacteria (HUB – Log10 CFU/g) during enhanced bioremediation of Crude Oil contaminated soil

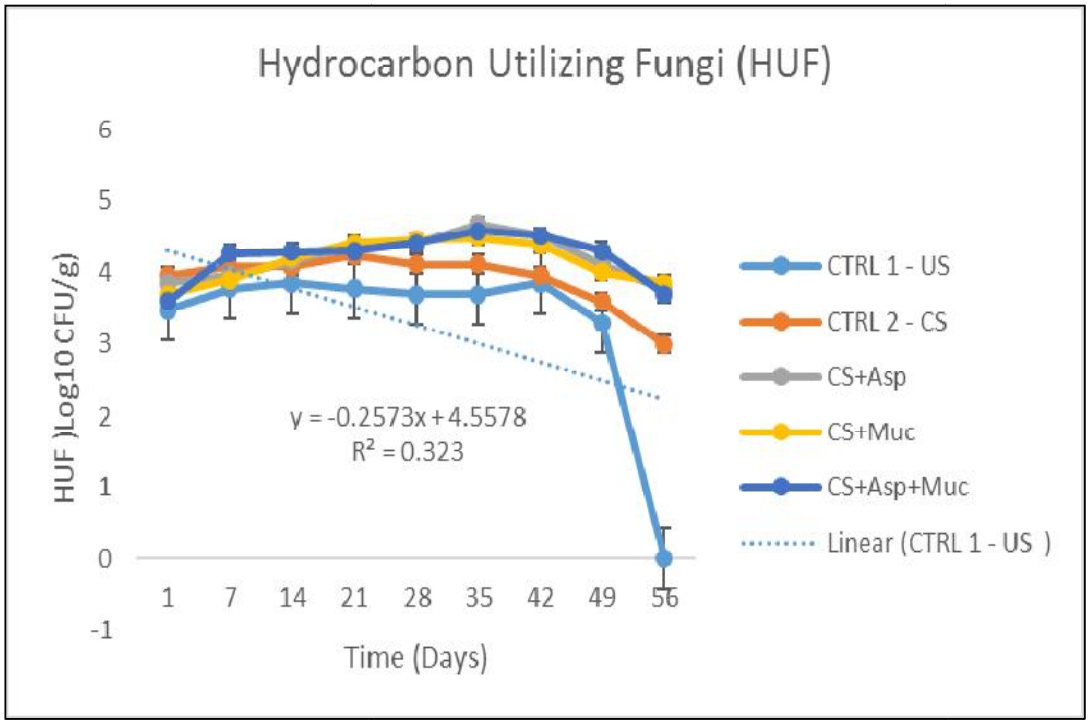

Fig. 4. Hydrocarbon Utilizing Fungi (HUF – Log10 CFU/g) during enhanced bioremediation of Crude Oil contaminated soil

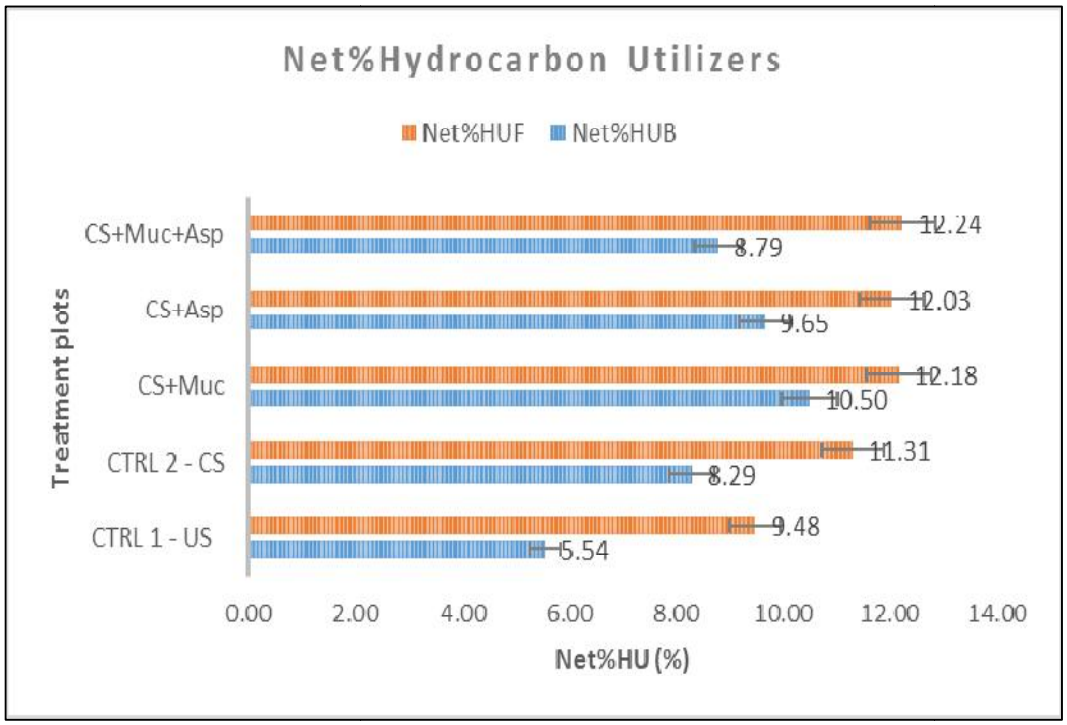

Fig. 5. Net percentage of hydrocarbon utilizers

Table 3. Percentage (%) Hydrocarbon utilizers during enhanced Bioremediation of crude oil contaminated soil

%HUB		Experimental plot	Day 1	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56
%HUB	P1	CTRL 1 - US	0	0	32.54	36.11	39.65	41.72	38.41	0.00	0.00
	P2	CTRL 2 - CS	41.63	37.80	33.67	44.18	42.14	44.38	41.95	38.33	30.43
	P3	CS+Asp	36.23	34.84	33.90	45.38	44.30	46.82	43.94	42.73	40.32
	P4	CS+Muc	39.05	41.04	38.54	44.25	44.93	44.20	42.81	40.57	35.01
	P5	CS+Asp+Muc	0	0	33.94	45.92	44.25	46.50	45.18	48.95	45.30
%HUF	P1	CTRL 1 - US	63.59	73.54	74.03	75.14	69.23	71.43	73.08	58.23	0
	P2	CTRL 2 - CS	95.18	78.46	103.29	107.09	99.04	102.75	100	92.31	77.92
	P3	CS+Asp	97.47	80.61	96.51	95.63	93.43	98.53	94.35	86.71	88.32
	P4	CS+Muc	92.5	94.89	99.29	98.44	96.12	90.87	89.39	84.03	91.67
	P5	CS+Asp+Muc	91.13	87.84	100	96.41	95.87	95.82	100.67	96.63	74.75

Table 4. Mean Standard Deviation and Percentage Microbial (Log10 cfu/g) counts during Bioremediation of Crude Oil Contaminated Soils+

		Microbial populations (log_{10}cfu/g)							
Plot	**Treatments**	**THB**	**THF**	**HUB**	**HUF**	**%HUB**	**%HUF**	**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}	5.54	9.48
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}	8.29	11.31
P3	CS+Asp	8.82 ± 0.26^{ab}	4.56 ± 0.31^{b}	3.62 ± 0.50^{a}	4.20 ± 0.31^{a}	40.94 ± 4.84^{a}	92.40 ± 5.95^{ab}	10.50	12.18
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}	9.65	12.03
P5	CS+Asp+Mc	8.76 ± 0.33^{ab}	4.53 ± 0.31^{a}	3.03 ± 1.75^{a}	4.22 ± 0.34^{a}	34.45 ± 19.97^{a}	93.24 ± 8.00^{a}	8.79	12.24

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

THB = Total Heterotrophic Bacteria, THF = Total Heterotrophic Fungi, HUB = Hydrocarbon Utilizing Bacteria, HUF = Hydrocarbon Utilizing Fungi, P=- Plot; US = Uncontaminates soil; CS = Contaminated soil; Asp = Aspergillus niger; Muc = Mucor racemosus

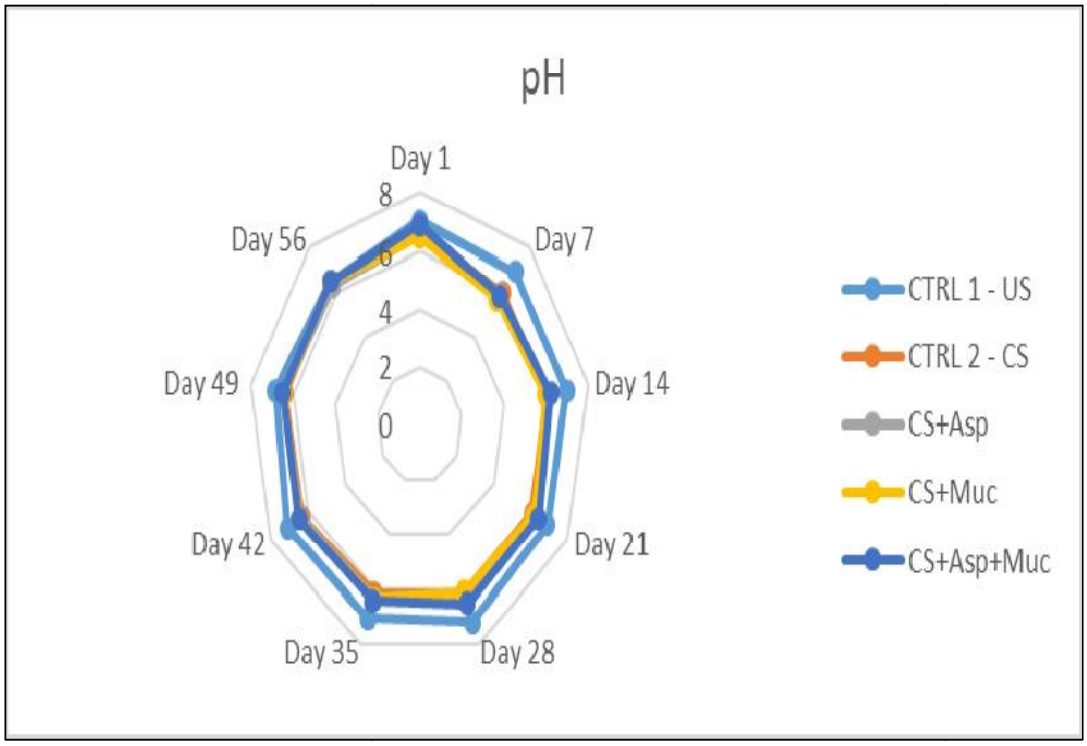

Fig. 6. Variation in pH during bioremediation of crude oil contaminated soil

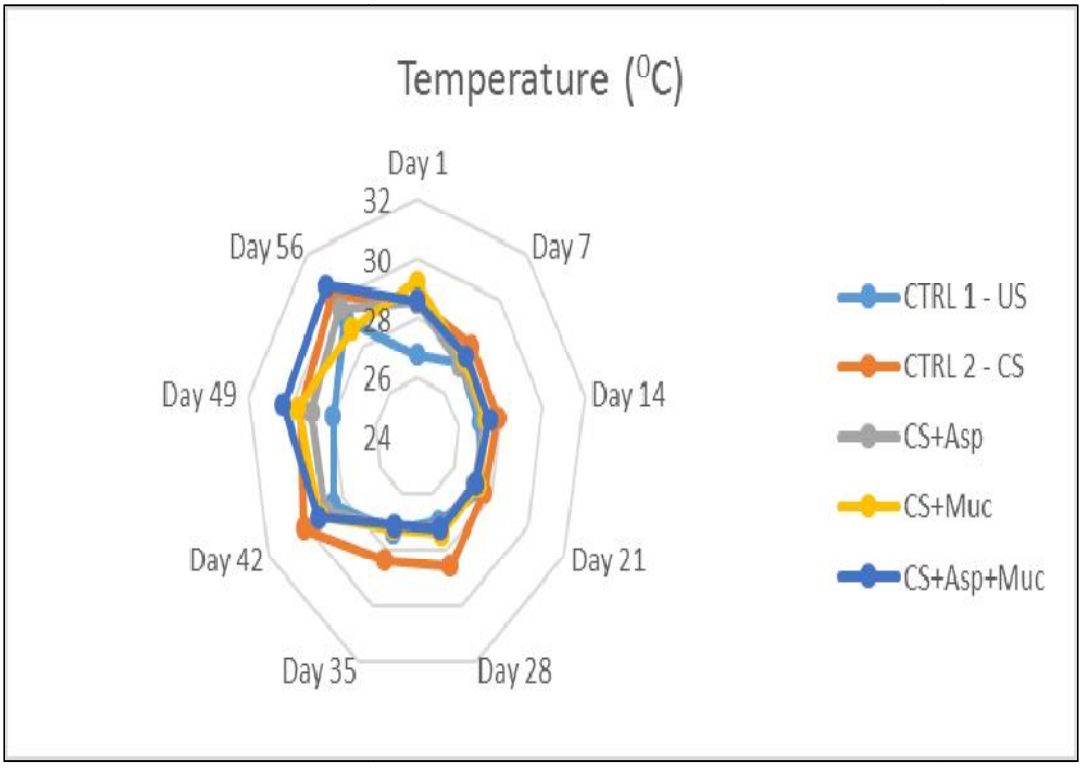

Fig. 7. Variation in temperature (˚C) during bioremediation of crude oil contaminated Soil

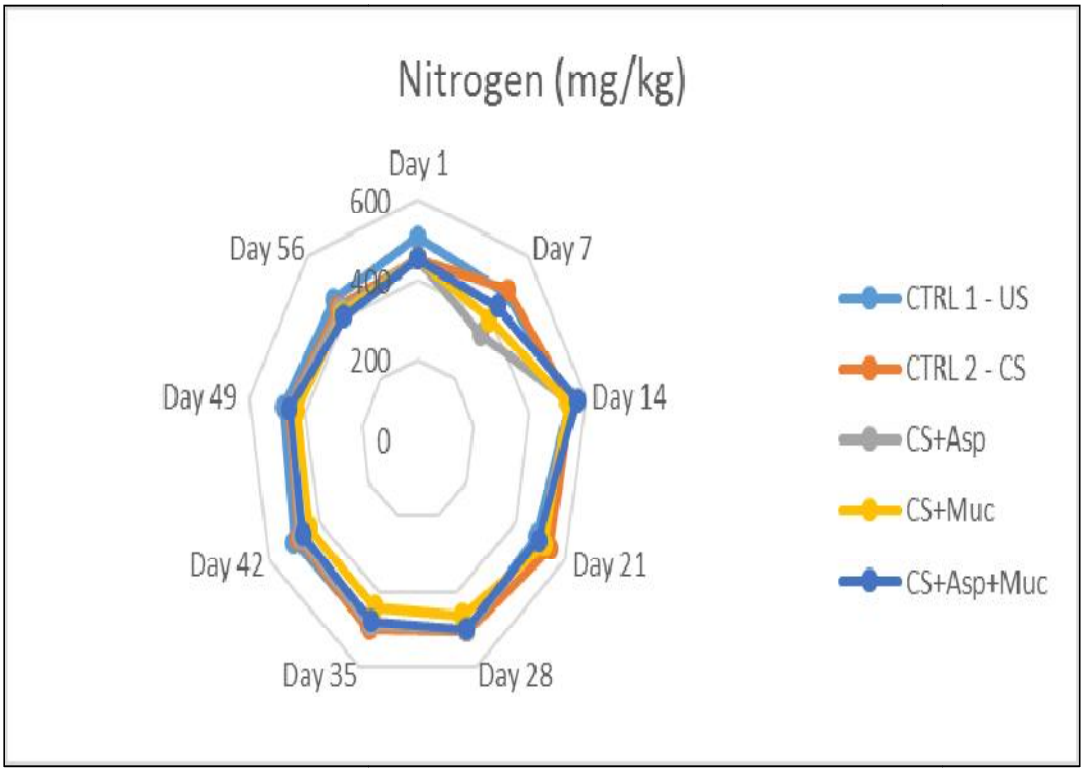

Fig. 8. Variation in nitrogen (mg/kg) during bioremediation of crude oil contaminated soil

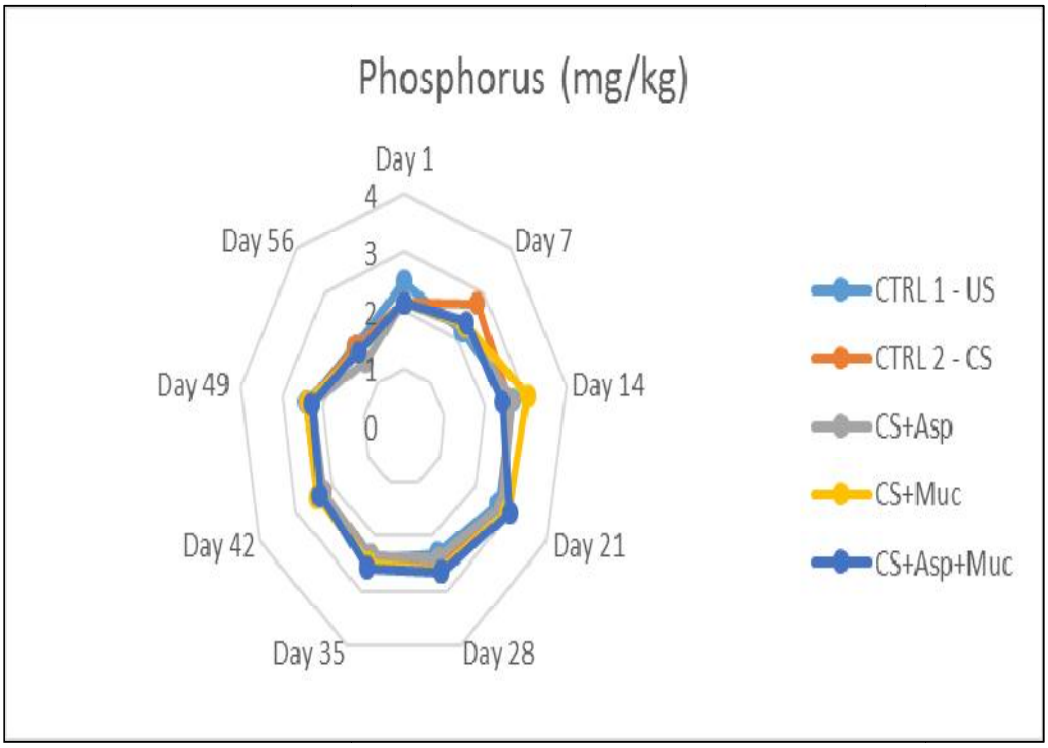

Fig. 9. Variation in phosphorus (mg/kg) during bioremediation of crude oil contaminated soil

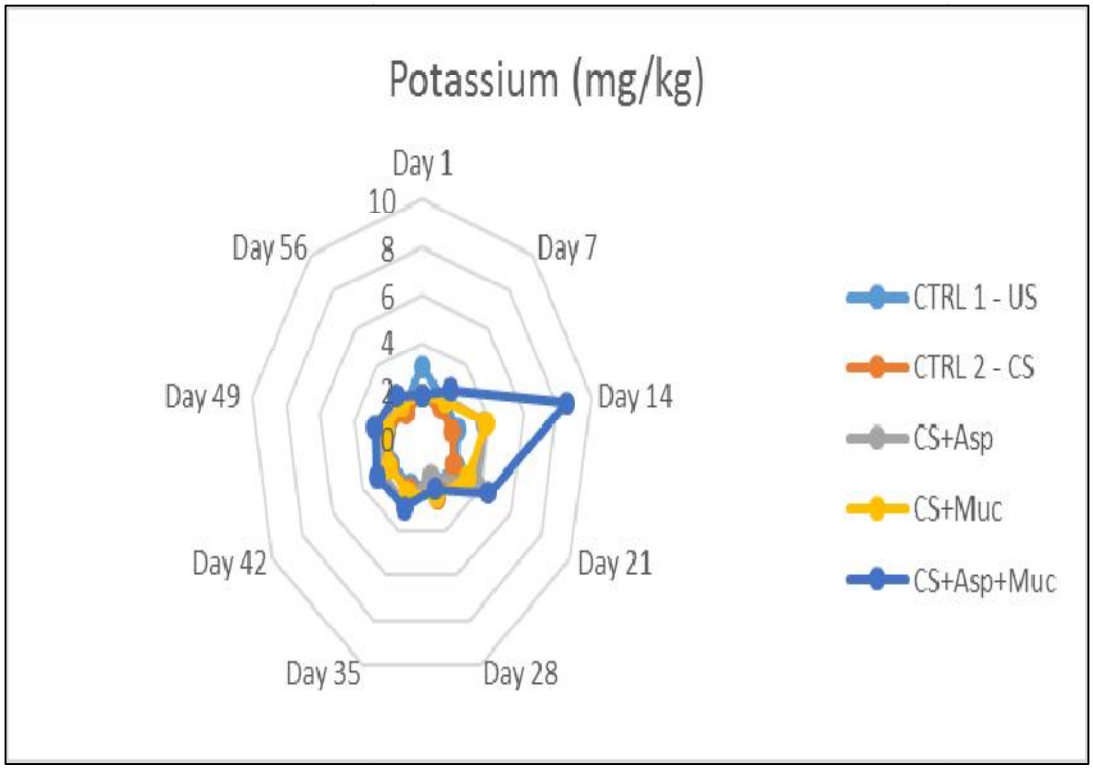

Fig. 10. Variation in potassium (mg/kg) during bioremediation of crude oil contaminated soil

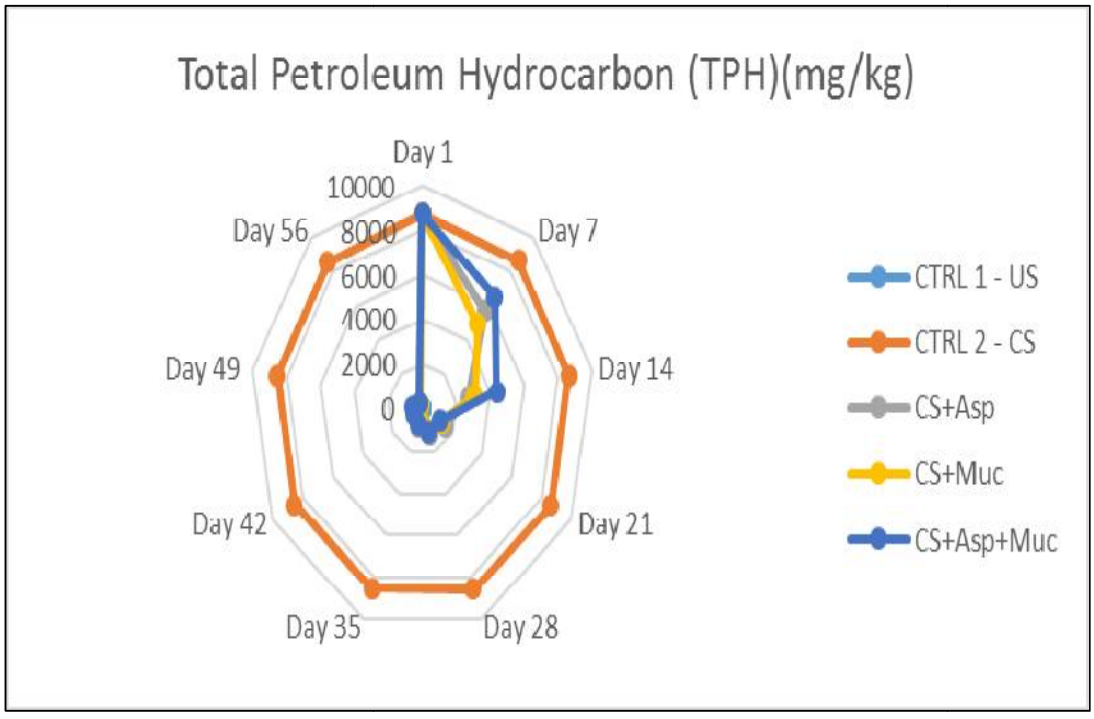

Fig. 11. Variation in total petroleum hydrocarbon (tph) (mg/kg) during bioremediation of crude oil contaminated soil

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±0.20^{a}	27.62±0.81^{a}	494.39±24.14^{b}	2.38±0.26^{a}	2.11±0.39^{a}	28.27±28.09^{a}
P2	CTRL 2 - CS	6.28±0.25^{a}	28.77±0.96^{a}	491.06±37.92^{ab}	2.39±0.31^{a}	1.99±0.34^{a}	8612.76±57.80^{b}
P3	CS+Asp	6.30±0.20^{b}	28.01±1.03^{a}	464.44±56.55^{ab}	2.27±0.39^{a}	2.61±0.73^{a}	2418.41±2918.83^{ab}
P4	CS+Muc	6.25±0.29^{a}	28.18±1.02^{a}	453.90±46.57^{ab}	2.46±0.40^{a}	2.43±0.64^{a}	2238.25±2922.68^{ab}
P5	CS+Asp+Muc	6.43±0.27^{a}	28.39±1.41^{a}	472.93±46.52^{ab}	2.38±0.35^{a}	3.44±2.07^{a}	2678.06±3124.12^{ab}

***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
P3	CS+Asp	0.022x + 6.191	0.093	0.229x + 26.86	0.371	1.034x + 459.2	0.002	-0.065x + 2.615	0.240	0.001x + 2.600	1E-05	-19.49x + 8710	0.737
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
P6	CS+Asp+Muc	0.013x + 6.366	0.017	0.342x + 26.68	0.440	-6.041x + 503.1	0.126	-0.042x + 2.595	0.109	-0.171x + 4.294	0.051	-998x + 7668	0.765

P=- Plot; US = Uncontaminated soil; CS = Contaminated soil; Asp = Aspergillus niger; Muc = Mucor racemosus

Table 7. Analysis of bioremediation

Sample ID	Treatments	Initial Conc (mg/kg)Day 1	Final Conc. (mg/kg) Day 56	Amount Remediated (mg/kg)	Actual Amount Remediated (mg/kg)	%Bioremediation (%)
P1	CTRL 1 - US	87.89	2.41	85.48	-	-
P2	CTRL 2 - CS	8729.00	8562.46	166.54	81.06	0.32
P3	CS+Asp	8729.00	301.94	8427.06	8341.58	32.98
P4	CS+Muc	8729.00	129.81	8599.19	8513.71	33.66
P6	CS+Asp+Muc	8729.00	286.21	8442.79	8357.31	33.04

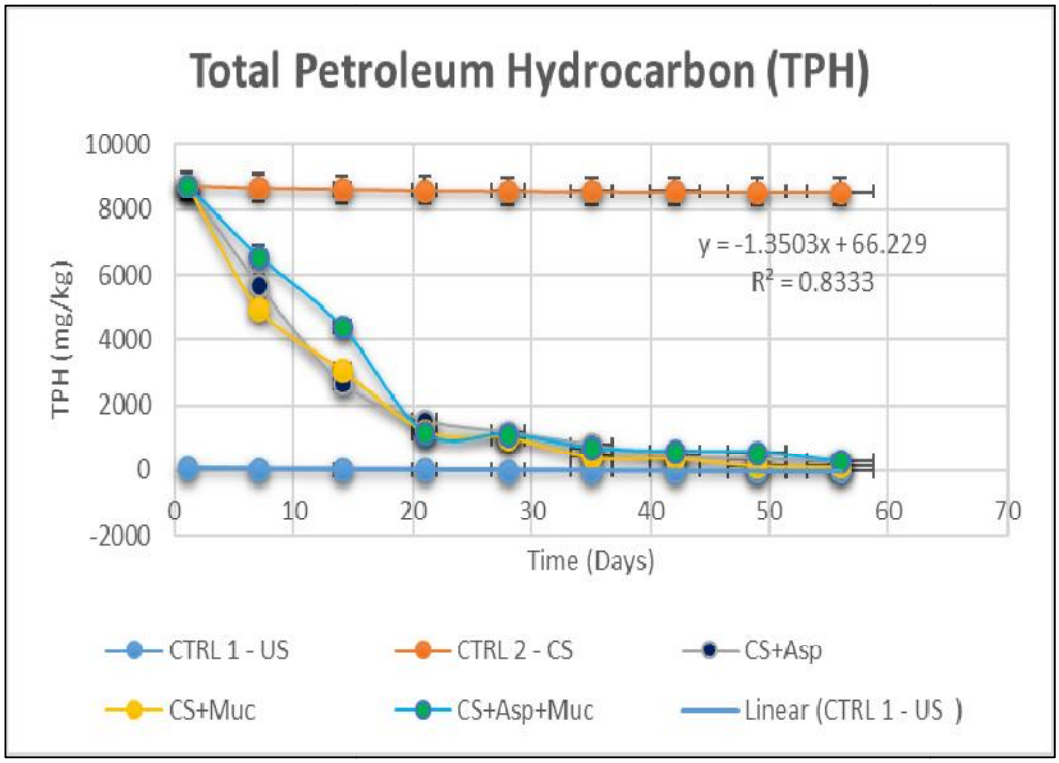

Fig. 12. Variation in nitrogen (mg/kg) during bioremediation of crude oil contaminated soil

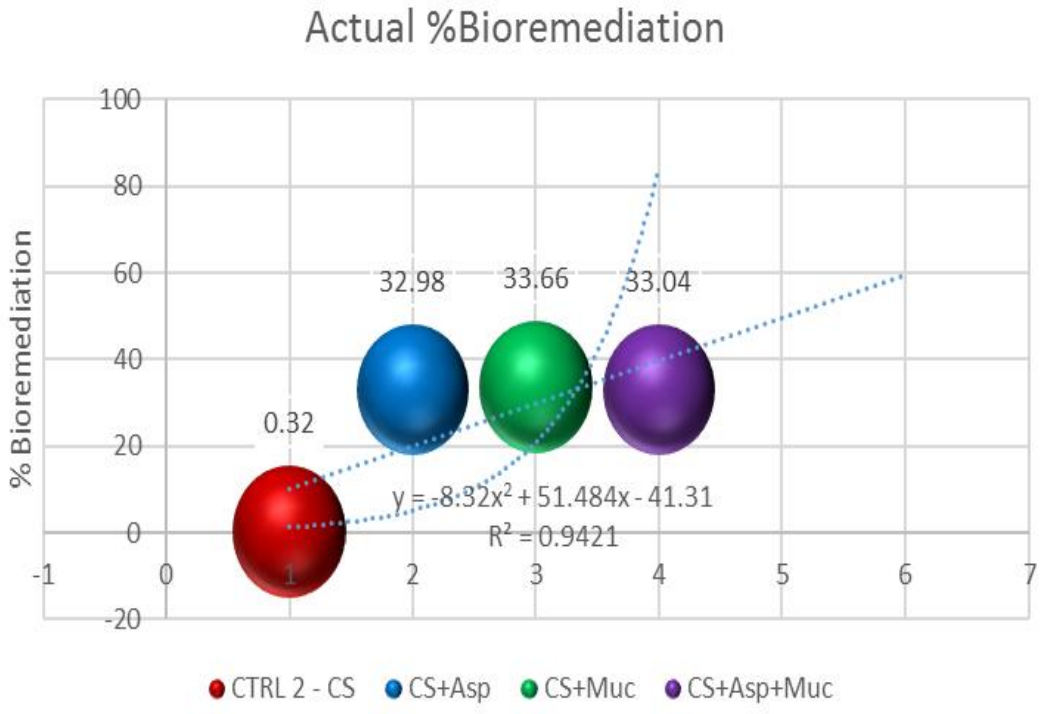

Fig. 13. Actual % bioremediation assessment during bioremediation of crude oil contaminated soil

4. CONCLUSION AND RECOMMENDATION

Study showed that bioremediation of crude oil-contaminated soils with Bioaugmenting fungus singly may be more effective than combination with others depending on the type of substrate used, nature of organism and environmental conditions prevalent as seen in *Mucor racemosus* having higher Bioremediation potential than when combined with *Aspergillus niger.* Notably, Hydrocarbon Utilizing Bacteria (HUB) and Hydrocarbon Utilizing Fungi (HUF) which are the key players in Bioremediation have peak count values on Day 35, this confers that nutrient renewal on bioremediation sites should be at interval of 35 days for continuous effective bioremediation of hydrocarbon pollutants. Also, it was found that the presence of the crude oil in the contaminated soil stimulated and sustained the growth of Hydrocarbon Utilizing Bacteria (HUB) in the contaminated plots; more so, the higher growth in the enhanced bio-augmented plots showed the positive impact Myco (fungal) bio-augmentation in bioremediation of crude oil polluted soil. It was further discovered that treatment plots with higher HUB or HUF had higher percentage (%) bioremediation; that is, the higher the sustained HUB and HUF population, the higher the %Bioremediation.

Summarily, it is therefore recommended that nutrient renewal on bioremediation site should be at interval of 35 days for continuous effective bioremediation of hydrocarbon pollutants. Also, microbes of high bioremediation potential could be more effective than consortium of many hydrocarbon utilizing microbes.

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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