Evaluation of Organic Nutrient Supplements and Bioaugmenting Microorganisms on Crude Oil Polluted Soils

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Authors' contributions

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

Article Information

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ABSTRACT

Aim: The aim of the study was to evaluate the impact of organic nutrient supplements and bioaugmenting microorganisms on crude oil polluted soils.
Place and Duration of Study: Faculty of Agriculture Demonstration Farm, Rivers State University, Port Harcourt Nigeria.
Methodology: Baseline study of a deliberately polluted agricultural soil was investigated for its microbiota from which selected fungal and bacterial isolates were obtained. Microbial analyses of goat manure, fish wastes and crude oil polluted soil were investigated. Using the Randomized Complete Block Design (RCBD) the land was partitioned into nine (9) blocks of 100 cm x 50 cm x 20 cm (Length x Breath x Height) giving 100,000 cm^3 each. Two of these plots were designated as

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pristine (Unpolluted soil) and crude oil polluted soil without nutrient organics and bioaugmenting microbes to serve as controls respectively. Each of the experimental plots, except the control, was contaminated with 1,700 g of crude oil giving initial Total Petroleum Hydrocarbon (TPH) value of 9.296.825 mg/kg. The plots were left for 21 days to ensure even distribution and soil-oil bonding. All plots except Control 1 (plot 1) were separately and deliberately contaminated with 1,700 g of crude oil given Total Petroleum Hydrocarbon (TPH) value of 9.296.825 mg/kg. Sampling was done at seven day interval (Day 1, 7, 14, 21, 28, 35, 42, 49, 56). Parameters monitored were Nitrate, Sulphate, Phosphate, Total Organic Carbon (TOC) and Total Petroleum Hydrocarbon (TPH), 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). Percentage (%) Bioremediation was estimated from percentage (%) reduction of Total Petroleum Hydrocarbon (TPH) from day 1 to day 56 in relation to control plots.

Results: Two fungal; *Aspergillus niger* (Asp), *Mucor racemosus* (Muc); and two bacterial species – *Bacillus armyloliqquefaciens strain FJAT-45825* (Bac) and *Pseudomonas aeruginosa strain CL 9* (Pse) isolated from a baseline study showed biodegradability potentials. The physicochemical characteristics of organic nutrient supplement - goat manure (GM) and fish wastes (FW) employed in the study showed that it contained valuable sources of organic nutrients which enhanced the bioremediation process of the Crude oil polluted soil (PS). Mean counts of Hydrocarbon Utilizing Bacteria (HUB) and Hydrocarbon Utilizing Fungi {HUF} (Log10 CFU/g) respectively were: Control 2 which is crude oil Polluted soil (PS) (5.52±0.47, 4.92±0.29); PS+GM (5.832±0.68, 5.33±0.54); PS+FW (5.76±0.40, 5.10±0.40); PS+GM+FW (5.706±0.61, 5.17±0.27); PS+Asp+Muc (5.722±0.48, 5.08±0.49); PS+Bac+Pse (5.34±0.69, 5.01±0.52}; PS+Asp+Muc+Bac+Pse (5.652±0.48, 5.10±0.52}; PS+Asp+Muc+Bac+Pse+GM+FW (5.212±0.40, 4.76±0.53}. Evaluation of Bioremediation revealed the actual amount of crude oil (hydrocarbon) reduction and its percentage value from the initial contamination value of 9296.83 mg/kg during the period of 56days as PS+Asp+Muc+Bac+ Pse+GF+FW (9152.315 mg/kg; 98.45%) > PS+Bac+Pse+GF+FW (8032.825 mg/kg; 86.40%) > PS+GF+FW (6867.825mg/kg; 73.87%) > PS+Asp+Muc+GF+FW (6599.825 mg/kg; 70.99%) > PS+Bac+Pse (6587.825mg/kg; 70.86%) > PS+FW (6441.825 mg/kg; 69.29%) > PS+GM (5909.825 mg/kg; 63.57%) > PS+Asp+Muc+Bac+Pse (5081.825 mg/kg; 54.66%) > CTRL 2 (Polluted soil without amendment) (3604.825 mg/kg; 38.78%).

Conclusion: Results obtained from this study has shown that goat manure and fish wastes due to their high moisture and nutrient content properties makes them appropriate agents for enhanced bioremediation. It further revealed that a combination of biostimulating and bioaugmentating agents creates more favorable conditions for biological activity to thrive and has shown to be effective, economical, eco-friendly and sustainable in remediating organic pollutants from polluted soils.

1. INTRODUCTION

The release of Petroleum Hydrocarbons products into the environment is a major global environmental challenge due to its adverse impacts on the ecosystem. The process of crude oil exploration, production, refining, transportation and storage of petroleum products most often results in oil leaks and accidental spills causing environmental pollution. The severity of such impact is determined by the characteristics of the oil, weather conditions, tidal current, soil properties and duration of exposure [1,2]. Kvenvolden and Cooper [3] estimated annual crude oil seepage of 600,000 metric tons into the environment due to accidental or anthropogenic activities of hydrocarbon exploitation and exploration into the environment. This eventually results towater and soil pollution which is a major platform for life performance. Crude oil contamination on soils or water environment causes reduction in plant growth, biomass production, and microbial diversity and eventually affects human health.

Nigeria is the third largest producer of petroleum hydrocarbon in Africa, the sixth in the world. The Niger Delta region as of 2015 produced on average 1.7million barrels per day [4]. Nigeria has recorded over 6,000 oil spills mostly in the Niger Delta in the last 40 years of oil exploitation [5].The Niger Delta environment is reported to be

one of the most heavily petroleum hydrocarbon impacted region in the world due to decades of oil exploration and poor management practices [6]. The amount of hydrocarbon spilled into the Niger delta environment is estimated at 13 million tons as a result of pipeline vandalism, well blowout, engineering failures, sabotage [7,8] leading to massive contamination of land [9]. Crude oil pollution adversely affects the Niger delta soil properties such as clogged pore space, reduction in soil aeration, infiltration of water into the soil, increase bulk density of soil which affects plant growth reduces soil fertility [10] reduction in agricultural productivity and distorts aesthetic value of the ecosystem [11]. The socio-economic and livelihood of the Niger Delta people who depend to a large extend on farming and fishingare adversely affected [5,12,13]. With the increasing demand for food orchestrated by rising population, the spate of pollution of arable land is unacceptable risk to agricultural production, ecosystem, human health, good practices as well as sustainable development [5].

Conventional physical and chemical methods used for soil remediation such as incineration, soil vapor extraction, containment, burial at landfills, evaporation, dispersion and washing are prohibitively expensive and might not be an option for developing countries like Nigeria [14] can lead to incomplete decomposition of contaminants [5]. Global attention is turning towards a more prospective biotechnological approach in the treatment of petroleum hydrocarbon contaminated sites. Biological methods have been used to remediate crude oil contaminants from the environment to promote health and safety of the environment. Biological methods could either be used singly or a combination approach to degrade, brake down, transform, and/or essentially remove contaminants or impairment of quality also known as bioremediation from the ecosystem. Biological methods include; bioreactor, composting, phytoremediation, land farming and bioremediation [5].

Okpokwasili [15] defined bioremediation as an assisted, augmented, accelerated or enhanced biodegradation. Bioremediation process involves the use of microorganisms and their products to degrade and detoxify organic contaminants from the environment to harmless compounds such as carbon dioxide and water. The field of bioremediation as a biotechnological approach to remediation of hydrocarbon polluted sites has continued to elicited scientific research with proven successes [16,17,18,19,20,21,22].

Microorganisms actively depends on certain factors, including the type and concentration of the pollutants, toxicity, bioavailability, mobility, availability of macro and micronutrients and activated enzymes and the ability of the enzymes to degrade the contaminants [23,24].

Biostimulation has been reported to enhance the removal of crude oil from polluted soil [15,25]. Sang-Hwan et al. [26] reported that microbes when stimulated by the addition of nutrients lead to large quantity of carbon source which resulted in a rapid depletion of available nitrogen and phosphorus.

Ayotamono et al. [17] carried out bioremediation of crude oil polluted agricultural soil using fertilizer and goat manure as biostimulating agents and reported that the total heterotrophic bacteria count increased with time in all the treatment cells. Nrior and Mene, [21] carried out an assessment of bioaugmentation efficiency of *Penicillium chrysogenum* and *Aspergillus nudilans* in bioremediation of crude oil spill soil. They reported that comparatively, *Penicillium chrysogenum* (36%) expressed higher bioremediation potential than *Aspergillius nudilans* (35%).

Though different researchers have applied a wide range of organic and inorganic materials in bioremediation of hydrocarbon polluted soil, only a few of these nutrients were found to be effective and efficient [6,27,28]. It is therefore imperative to test the efficacy of these nutrients so as to enhance their utilization for effective bioremediation.

In this study, Goat manure, fish waste, *Aspergillusniger.*, *Mucor racemosus*; *Bacillus armyloliquefaciens strain FJAT-45825* and *Pseudomonas aeruginosa strain CL 9*were evaluated for their bioremediation potentials for 8 weeks.

2. MATERIALS AND METHODS

2.1 Study Area

The study was carried out in a pristine patch of land at Rivers State University demonstration farmland, Port Harcourt, Rivers State. The piece of land is situated at Longitude $4^o48'18.50''N$ and Latitude $6^o58'39.12''E$ (Fig. 1) measuring 5.4864m x 5.1816m with a total area of $28.4283m^2$ was cleared and sub-partitioned into 9 blocks of 100 cm x 50 cm x 20 cm giving $100,000cm^3$ each (Randomized Complete Block Design).
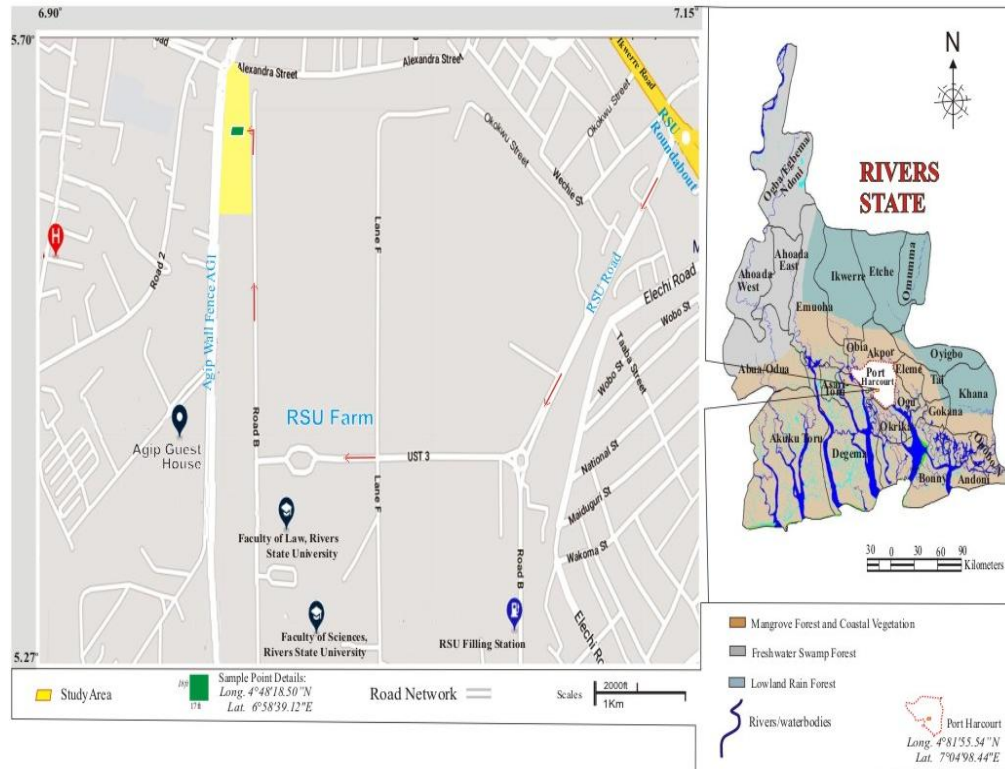
Fig. 1. Map of Experimental plots at Rivers State University Faculty of Agriculture Demonstration Farm, Longitude 4o48'18.50''N and Latitude 6o58'39.12''E

2.2 Biodegradation Process

Each experimental plot was polluted with 1700 grams of crude oil except plot 1 and allowed to stay for 21 days undisturbed. This is to allow for volatilization and sorption of crude oil before amendment with different treatment options with different concentrations applied accordingly. Two (2) of the plots were designated as pristine and crude oil polluted soil without treatments to serve as controls while other seven (7) plots received different types of treatments respectively (Table 1).

2.3 Soil Sample Collection

The soil samples used for laboratory analysis were collected from the various treatment plots in sterile sample bottles from a depth of 0-15cm after tilling using soil spatula. Soil samples were collected from 4-10 random points per plot and bulked to form a composite sample. Small portions (5 g) of the composite samples were collected into sterile bottles using sterile spatula for microbiological and physicochemical analyses. All microbiological analysis was carried out in the Microbiology laboratory of the Rivers State University while physicochemical analysis was carried out at Pollution Control and Environmental Management (POCEMA) and Giolee Global Resources laboratories both in Port Harcourt. Soil samples were stored at 14±2ºC for future analysis [29].

2.4 Sampling Period

Samplings were subsequently collected and analyzed for a period of 56 days viz: day7, 21, 28, 35, 42, 49 and 56 respectively.

2.5 Sources of Crude Oil, Experimental Organic Nutrients and Applications

The crude oil (Bonny light) black in clour was collected into 25 liter sterile plastic container from Shell Petroleum Development Cooperation (SPDC) Alakiri flow-station, Rivers State. Goat manure and Fish wastes were obtained from the goat and fish markets at Mile 3, Port Harcourt and taken to the Microbiology laboratory of the Rivers State University for further analysis. A total of 1700 g of crude oil was applied to all the

Table 1. Bioremediation set-up using different organic nutrients and bioaugmenting microorganisms

| Experimental plots | Organic nutrients (Biostimulating) | | | Microorganisms (Bioaugmenting) | | | |
	Crude oil (g)	Goat manure GM (g)	Fish waste FW(g)	*Bacillus (Bac)* (ml)	*Pseudomonas (Pse)* (ml)	*MucorMuc* (ml)	*AspergillusAsp* (ml)
CTRL 1 (Unpolluted soil)		-	-	-	-	-	-
CTRL 2 (Polluted soil)		-	-	-	-	-	-
CS+GM	1700	300	-	-	-	-	-
CS+FW	1700	-	200	-	-	-	-
CS+GM+FW	1700	300	200	-	-	-	-
CS+Asp+Muc	1700	-	-	-	-	150	150
CS+Bac+Pse	1700	-	-	150	150	-	-
CS+Asp+Muc+Bac+Pae	1700	-	-	150	150	150	150
CS+Asp+Muc+Bac+Pse+GM+FW	1700	300	200	150	150	150	150

P - Plot; CTRL: Controls; CS - Polluted soil; Asp -Aspergillus;Muc -Mucor;Bac - Bacillus armyloliquefaciens strain FJAT-45825;Pse -Pseudomonas aeruginosa strain CL 9; GM- Goat manure; FW-Fish waste

experimental plots except the control giving an initial 9296.825 mg/kg Total Petroleum Hydrocarbon concentration while 200g and 300g of fish wastes and goat manure respectively were applied to the plots.

2.6 Sources of Microbial Isolates and Applications

The microorganisms used in the evaluation studies were the fungi namely *Aspergillus niger*, *Mucor racemosus* and bacteria *Pseudomonas aeruginosa* and *Bacillus armyloliqquefaciens*. These isolates were obtained from the baseline crude oil polluted soil samples using appropriate selective media. Pure cultures obtained were inoculated onto Modified Sabouraud Dextrose broth in 500ml Erlenmeyer flask loosely plugged with sterile cotton wool for the growth of the augmenting test organisms. Broth cultures with optical density of 0.2 were used for augmentation. A total of 150 ml suspension of the microorganisms were applied to the amended plots accordingly.

2.7 Microbial Counts

The counts for total heterotrophic bacterial (THB), the hydrocarbon utilizing bacteria (HUB), total heterotrophic fungi (THF) and hydrocarbon utilizing fungi (HUF) were determined using the spread plate method on Nutrient agar plates [19,21,30,31].

Ten-fold serial dilution was carried out up to 10^{-6}, 10^{-5}, 10^{-4}, & 10^{-3} dilutions respectively. An aliquot (0.1ml) of each soil samples dilutions was spread plated onto sterile solidified Mineral Salt agar using the vapor transfer technique in triplicates. Distinct colonies were picked and cultured on well-dried Nutrient agar and Blood agar plates in triplicates respectively, incubated at 37^0C for 24 to 72 hours. The distinct bacterial and fungal colonies that grew on the plates were counted as number of colony forming units per gram culture (cfu/g) of the soil sample.

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. 0.1 ml
DF = dilution factor used for plating (10^6) that is 1/Dilution ($1/10^{-6} = 10^6$)

[Note: Dilution factor is the reciprocal of dilution]

Total Heterotrophic Bacterial count=

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

2.8 Identification of Bacterial and Fungal Isolates

The cultural, morphological, biochemical and physiological characteristics was used to identify discrete bacterial isolates according to the methods described by Nrior and Odokuma [32] and were compared with the recommendation in Bergey's Manual of Determinative Bacteriology [33]. The biochemical and morphological tests include; motility, gram staining, oxidase, catalase, citrate utilization, indole production, hydrogen sulphide production, methyl red and Voges proskauer. The bacterial isolates were further subjected to molecular identification. The presence or absence of septa in the mycelium, type of spore, presence of primary or secondary stigmata, and other microscopic characteristics and cultural characteristics were used in the identification of the fungal isolates of the bioremediation set up [34]. Pure cultures obtained were preserved in Sabouroud Dextrose broth in 500ml Erlenmeyer flask loosely plugged with sterile cotton wool and Nutrient agar broth for the growth of the augmenting test organisms at 37^0C for further tests.

2.9 Determination of Percentage (%) Crude Oil Reduction (% Bioremediation)

The method of Nrior and Echezolom [29] was used in calculating the percentage (%) bioremediation in the experiment at day 56. The process followed the steps stated below;

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

Bc = Ic – Fc   (2)

Where:

Bc = Amount of pollutant remediated
Ic = Initial Concentration of pollutant (week 1)
Fc = Final Concentration of pollutant (week8)

% Bioremediation = Bc x 100/Ic   (3)

2.10 Physicochemical Analysis

The physicochemical parameters analyzed include; soil texture, particulate size, moisture content, pH, temperature, phosphate, nitrate, sulphate and total organic carbon, using the methods prescribed by APHA [30] while residual Total Petroleum Hydrocarbons (TPH) was extracted from the soil samples and quantified using Gas chromatograph – flame ionization detector (GC-FID) in accordance with Nigerian requirements of Department of Petroleum Resources (DPR), National Oil Spill Detection Response Agency (NOSDRA) and Federal Ministry of Environment (FMEnv).

2.11 Statistical Analysis

Results were subjected to statistical analysis using Analysis of Variance (Two way ANOVA) to test whether the different nutrient amendments given to the crude oil polluted plots were statistically significant. Regression analysis of Physiochemical parameters during bioremediation of crude oil polluted soil showing regression equation of each parameter and their R^2 values.

3. RESULTS AND DISCUSSION

3.1 Microbial and Physicochemical Characteristics of Soil Before/After Crude Oil Contamination at 56 Day Biodegradation

Some of the microbiological and physicochemical properties of the soil polluted with crude oil and treated with different amendment options are shown in Table 2.The concentration of total petroleum hydrocarbon (TPH) in the experimental soil before application of amendment were 4.89 mg/kg; however, the value increased to 9296.85 mg/kg after crude oil application. This value is above the intervention value of 5000 mg/kg according (DPR) standard hence the soil is considered polluted and needs intervention/ remediation [35]. The total heterotrophic bacterial count and hydrocarbon utilizing bacteria were 6×10^7 cfu/g and 1.0 x

10^3 cfu/g respectively. The physicochemical characteristics of the soil were; sulphate: biodegradation (152.91 mg/kg) < unpolluted (2,376.97 mg/kg) <polluted soil (3,157.94 mg/kg).

3.2 Microbial Isolates

The bacterial and fungal isolates from this study were characterized based on their microscopic, biochemical, morphological properties. The bacteria belong to the genera: *Pseudomonas, Klebsiella, Norcadia, Staphylococcus, Corynebacterium, Flavobacterium* and *Bacillus* while the fungi genera include *Mucor, Aspergillus, Penicillum, Cladosporium,* and *Histoplasma.* This is in line with various reports from similar studies from crude oil polluted soil [19,21,29,36].

3.3 Microbial Count

It was observed from the experiment that the total heterotrophic bacterial and fungal counts increased progressively in the nutrient treated plots when compared with the controls. Time had a significant impact as degradation of petroleum hydrocarbon decreased with increase in time. This is evident in the results of the total heterotrophic bacteria, fungal and hydrocarbon utilizers (Tables 3 and 4; Figs. 2-5). The results shows: polluted soil + goat manure + fish waste (1.584×10^{10} cfu/g) >polluted soil + *Bacillus* + *Pseudomonas* (1.296×10^{10} cfu/g) >polluted soil + fish waste (9.76×10^9 cfu/g) >polluted soil + goat manure (8.64×10^9 cfu/g) for bacterial counts while the fungal counts showed; polluted soil + *Aspergillus* + *Mucor* + *Bacillus* + *Pseudomona* (6.6×10^6 cfu/g) >polluted soil + goat manure (6.2×10^6 cfu/g) >polluted soil + fish waste (6.0×10^6 cfu/g) >polluted soil + goat manure + fish wastes (5.7×10^6 cfu/g). The results obtained from plots treated with goat and fish nutrients indicated the highest microbial counts. This can be attributed to the high degradable organic matter in goat manure and fish wastes while the low organic matter content of the controls might be due to the impact of crude oil on soil microbial population and nutrients. Menkit and Amechi [14] reported high organic matter in goat manure while Anion et al. [37] reported that hydrocarbon polluted soils are deficient in organic matter with low microbial activity. Other researchers reported that the microbial counts during hydrocarbon bioremediation process is higher in nutrient treated plots than untreated plots [26,29]. They attributed the phenomenon to the abundance of nutrients for the microorganisms to feed on

during the first weeks and subsequently depleted due to acclimatization, competition with other microorganisms and reduction in available nutrients. It can be concluded that microbial count of crude oil polluted soil during bioremediation increases within the first 14 days.

There was a significant ($p \leq 0.05$) difference in the Total Petroleum Hydrocarbon (TPH), Nitrate, Sulphate, Phosphate and Total Organic Carbon (TOC) in the different treatments (Table 5). Total Petroleum Hydrocarbon (TPH) shows significant reduction with increase in time (day) when compared with the control (Table 6). The soil being left fallow for 6 days before contamination on the seventh day; after which it was allowed for 21 days for proper contamination and exposure to natural environmental factors to mimic crude oil spill site. The slight decrease in TPH results obtained from day 7 to day 21 could be attributed

to natural attenuation carried out by indigenous microorganism present in the soil. There was significant variation in TPH value after the application of nutrient organics/biostimulating agents (fish waste and goat manure) and bioaugmenting microorganisms from day 28 to day 56. This agrees with [29], that bioremediation of crude oil polluted soils with bacteria singly is less effective but a combination with other organic nutrients is a better palliative measure. Therefore, amendment with organic nutrients like Goat manure due to its high nutrient content as substrates for biostimulation of indigenous and augmenting biodegrading microbes is a better option. Regression analysis of Physiochemical parameters during bioremediation of crude oil polluted soil (Table 7) showed regression equation of each parameter and their R^2 values for variation level assessment and forcasting.

Table 2. Baseline parameters of soil before, after and 56 days crude oil contamination biodegradation

Parameters	(Units)	Unpolluted soil	Polluted soil	At 56 day of biodegradation
Total Heterotrophic Bacteria (THB)	cfu/g	6×10^7	2.2×10^8	$6.4 \times 10^{8*}$
Hydrocarbon Utilizing Bacteria (HUB)	cfu/g	1.0×10^3	1.5×10^3	$1.0 \times 10^{6*}$
Total Heterotrophic Fungi (THF)	cfu/g	4×10^3	7×10^3	$1.2 \times 10^{5*}$
Hydrocarbon Utilizing Fungi (HUF)	cfu/g	0	0	$1.5 \times 10^{4*}$
Total Petroleum Hydrocarbon (TPH)	mg/kg	4.8939	9296.8452	144.51^*
Nitrate (NO_3^{2-})	mg/kg	801.00	686.25	4761*
Sulphate (SO_4^{2-})	mg/kg	2,376.97	3,157.94	152.91*
Phosphate (PO_4^{3-})	mg/kg	0.28	5.78	121.57*
Total Organic Carbon (TOC)	%	0.21	0.93	0.32*
pH	None	7.0	5.0	7.1
Temperature	O^C	28	30	31*
Moisture content	mg/kg	200	206	206

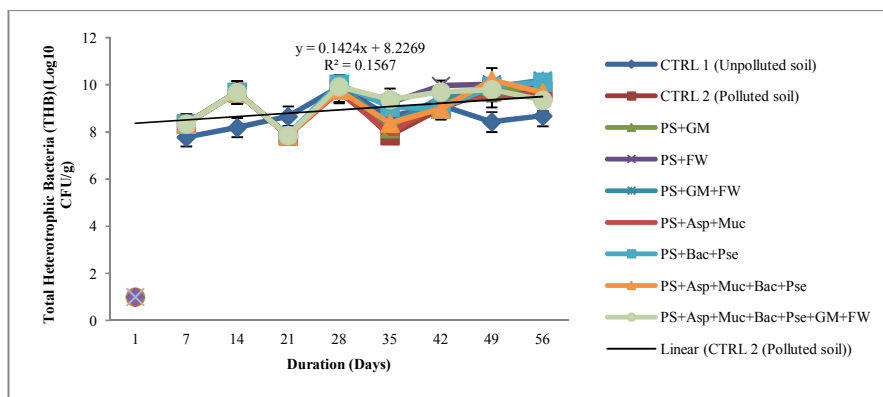*= lowest value from the different amendment plots*

Fig. 2. Variation in Total Heterotrophic Bacteria (THB –Log10 CFU/g) count during Bioremediation of crude oil polluted soil
CTRL: Controls; CS - Polluted soil; Asp -Aspergillus;Muc -Mucor;Bac - Bacillus armyloliquefaciens strain FJAT-45825;Pse -Pseudomonas aeruginosa strain CL 9; GM- Goat manure; FW- Fish waste

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Table 3. Total Heterotrophic Bacteria (CFU/g) count of controls and crude oil polluted soil during bioremediation of crude oil polluted soil

	Experimental plot	DAY1	DAY 7	DAY 14	DAY 21	DAY 28	DAY 35	DAY 42	DAY 49	DAY 56
P1	CTRL 1 (Unpolluted soil)	Plot forming and Crude oil application	5.89×10^7	1.55×10^8	4.57×10^8	8.0×10^9	4.6×10^8	1.34×10^9	2.7×10^8	4.8×10^8
P2	CTRL 2 (Polluted soil)		$2.19 \times 10^{8*}$	$4.79 \times 10^{8*}$	$7.08 \times 10^{8*}$	6.56×10^9	7.0×10^7	9.6×10^8	6.24×10^9	5.76×10^9
P3	PS+GM		$2.19 \times 10^{8*}$	$4.79 \times 10^{8*}$	$7.08 \times 10^{8*}$	8.32×10^9	1.4×10^8	2.91×10^9	5.04×10^9	8.64×10^9
P4	PS+FW		$2.19 \times 10^{8*}$	$4.79 \times 10^{8*}$	$7.08 \times 10^{8*}$	5.60×10^9	1.70×10^9	9.04×10^9	1.072×10^{10}	9.76×10^9
P5	PS+GM+FW		$2.19 \times 10^{8*}$	$4.79 \times 10^{8*}$	$7.08 \times 10^{8*}$	9.92×10^9	2.0×10^8	2.88×10^9	7.68×10^9	1.584×10^{10}
P6	PS+Asp+Muc		$2.19 \times 10^{8*}$	$4.79 \times 10^{8*}$	$7.08 \times 10^{8*}$	5.12×10^9	9.0×10^7	1.64×10^9	3.36×10^9	3.60×10^9
P7	PS+Bac+Pse		$2.19 \times 10^{8*}$	$4.79 \times 10^{8*}$	$7.08 \times 10^{8*}$	1.090×10^{10}	1.16×10^9	9.4×10^8	8.64×10^9	1.296×10^{10}
P8	PS+Asp+Muc+Bac+Pse		$2.19 \times 10^{8*}$	$4.79 \times 10^{8*}$	$7.08 \times 10^{8*}$	5.92×10^9	2.4×10^8	9.6×10^8	1.632×10^{10}	4.72×10^9
P9	PS+Asp+Muc+Bac+Pse+GM+FW		$2.19 \times 10^{8*}$	$4.79 \times 10^{8*}$	$7.08 \times 10^{8*}$	8.40×10^9	2.40×10^9	4.96×10^9	6.24×10^9	2.24×10^9

* Days during which plots were only polluted with crude oil without nutrient organics and augmenting microbes

CTRL: Controls; CS - Polluted soil; Asp -Aspergillus;Muc -Mucor;Bac - Bacillus amyloliquefaciens strain FJAT-45825;Pse -Pseudomonas aeruginosa strain CL 9; GM- Goat manure; FW- Fish waste

Table 4. Total heterotrophic fungi (CFU/g) count of controls and crude oil polluted soils plus treatments during bioremediation of crude oil polluted soil

	Experimental plot	Day 1	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 47	Day 56
P1	CTRL 1 (Unpolluted soil)	Plot forming and Crude oil application	3.02×10^3	5.01×10^4	1.00×10^5	2×10^5	1×10^5	2×10^5	3×10^5	5×10^5
P2	CTRL 2 (Polluted soil)		$7.24 \times 10^{3*}$	$7.59 \times 10^{4*}$	$1.51 \times 10^{5*}$	1.7×10^6	7×10^5	3×10^5	6.7×10^6	7.2×10^6
P3	PS+GM		$7.24 \times 10^{3*}$	$7.59 \times 10^{4*}$	$1.51 \times 10^{5*}$	3.6×10^6	1.7×10^6	1.3×10^6	6.5×10^6	6.2×10^6
P4	PS+FW		$7.24 \times 10^{3*}$	$7.59 \times 10^{4*}$	$1.51 \times 10^{5*}$	3×10^5	2.5×10^6	2.2×10^6	2.1×10^6	6.0×10^6
P5	PS+GM+FW		$7.24 \times 10^{3*}$	$7.59 \times 10^{4*}$	$1.51 \times 10^{5*}$	4×10^5	7×10^5	1.1×10^6	2×10^5	5.7×10^6
P6	PS+Asp+Muc		$7.24 \times 10^{3*}$	$7.59 \times 10^{4*}$	$1.51 \times 10^{5*}$	7×10^5	2.2×10^6	3.6×10^6	4.7×10^6	4.6×10^6
P7	PS+Bac+Pse		$7.24 \times 10^{3*}$	$7.59 \times 10^{4*}$	$1.51 \times 10^{5*}$	2×10^5	1.8×10^6	6×10^5	8.1×10^6	2.9×10^6
P8	PS+Asp+Muc+Bac+Pse		$7.24 \times 10^{3*}$	$7.59 \times 10^{4*}$	$1.51 \times 10^{5*}$	1.1×10^6	1.0×10^6	1.5×10^6	7×10^5	6.6×10^6
P9	PS+Asp+Muc+Bac+Pse+GM+FW		$7.24 \times 10^{3*}$	$7.59 \times 10^{4*}$	$1.51 \times 10^{5*}$	4×10^5	4×10^5	7×10^5	8×10^5	1.0×10^6

* Days during which plots were only polluted with crude oil without nutrient organics and augmenting microbes

CTRL: Controls; CS - Polluted soil; Asp -Aspergillus;Muc -Mucor;Bac - Bacillus armyloliquefaciens strain FJAT-45825;Pse -Pseudomonas aeruginosa strain CL 9; GM- Goat manure; FW- Fish waste

Table 5. Average values of physicochemical parameters for 56 days

Plot	Treatments	Physicochemical parameters						
		pH	Temperature	TPH	Nitrate	Sulphate	Phosphate	TOC
P1	CTRL 1 (Unpolluted soil)	6.66±0.29[a]	28.46±1.5[a]	2.80 ±1.55[a]	1265.4±831.74[a]	510.64±379.03[a]	33.11±9.82[a]	0.34±0.28[a]
P2	CTRL 2 (Polluted soil)	7.18±0.51[ab]	30.04±1.02[ab]	7071.76 ±1387.05[c]	1156.5±990.15[a]	383.68±336.20[a]	23.82±7.04[a]	0.54±0.21[a]
P3	PS+GM	7.3±0.32[b]	29.82±1.1[ab]	5601.77 2258.33[ab]	1113.3±1084.15[a]	305.982±226.98[a]	24.31±8.21[a]	0.47±0.20[a]
P4	PS+FW	7.28±0.13[b]	30.38±0.72[ab]	5240.17±2487.35[ab]	1080±1034.62[a]	500.824±504.45[a]	26.9±3.19[a]	0.47±0.23[a]
P5	PS+GM+FW	6.86±0.18[ab]	29.28±1.42[ab]	5856.97±2659.03[ab]	1246.05±909.62[a]	695.268±496.01[a]	28.76±4.13[a]	0.77±0.24[a]
P6	PS+Asp+Muc	7.08±0.14[ab]	29.98±1.16[ab]	6296.97±1924.97[c]	987.3±1097.5[a]	446.958±552.94[a]	27.06±4.24[a]	0.57±0.19[a]
P7	PS+Bac+Pse	7.06±0.089[ab]	30.16±0.47[ab]	5782.17±2700.24[ab]	952.2±1063.3[a]	382.88±322.54[a]	26.78±4.89[a]	0.57±0.14[a]
P8	PS+Asp+Muc+Bac+Pse	7.08±0.19[ab]	30.26±1.02[ab]	5231.36±2272.66[ab]	1015±1072.5[a]	843.854±794.47[a]	24.31±11.0[a]	0.57±0.15[a]
P9	PS+Asp+Muc+Bac+Pse+GM+FW	7.0±0.31[ab]	28.96±0.72[ab]	2107.58±4021.35[ab]	1402.65±843.74[a]	882.302±450.72[a]	32.45±17.6[a]	0.37±0.13[a]

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

CTRL: Controls; PS - Polluted soil; Asp -Aspergillus;niger, Muc -Mucor; racemosus, Bac - Bacillus armyloliquefaciens strain FJAT-45825;Pse -Pseudomonas aeruginosa strain CL 9; GM- Goat manure; FW- Fish waste

Table 6. Total petroleum hydrocarbon (mg/kg) and percentage reduction

S/n	Experimental plot	Day 1	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56	%Reduction
P1	CTRL 1 (Unpolluted soil)		4.8939	3.9661	3.9661	3.6839	3.6839	2.7561	1.56	1.11	49.72021
P2	CTRL 2 (Polluted soil)	Plot forming and Crude oil application	9296.83*	8747.83*	8198.83*	8195.49	7339	6790	6241	5692	14.8454
P3	PS+GM		9296.83*	8747.83*	8198.83*	8195.49	5969	4678	4678	3387	70.00295
P4	PS+FW		9296.83*	8747.83*	8198.83*	8195.49	5597	4683	3769	2855	37.54816
P5	PS+GM+FW		9296.83*	8747.83*	8198.83*	8195.49	6940	6519	4100	2429	62.73968
P6	PS+Asp+Muc		9296.83*	8747.83*	8198.83*	8195.49	6814	6102	4636	4636	81.62166
P7	PS+Bac+Pse		9296.83*	8747.83*	8198.83*	8195.49	7098	6334	3473	2709	18.00847
P8	PS+Asp+Muc+Bac+Pse		9296.83*	8747.83*	8198.83*	8195.49	4215	4215	4215	4215	31.45907
P9	PS+Asp+Muc+Bac+Pse+GM+FW		9296.83*	8747.83*	8198.83*	8195.49	523.39	333.95	239.23	144.51	55.98228

* Days during which plots were only contaminated with crude oil without nutrient organics and augmenting microbes

CTRL: Controls; PS - Polluted soil; Asp -Aspergillus;niger, Muc -Mucor; racemosus, Bac - Bacillus armyloliquefaciens strain FJAT-45825;Pse -Pseudomonas aeruginosa strain CL 9; GM- Goat manure; FW- Fish waste

Table 7. Regression analysis of Physiochemical parameters during bioremediation of crude oil polluted soil

Treatments	pH		Temperature		TPH		Nitrates		Sulphates		Phosphates		TOC	
	Regression equation (Y)	R²	Regression equation(Y)	R²	Regression equation (Y)	R²	Regression equation (Y)	R²	Regression equation	R²	Regression equation	R²	Regression equation	R²
CTRL 1 (Unpolluted soil)	0.18x + 6.12	0.92	0.16x + 27.98	0.028	-0.969x + 5.71	0.98	442.2x -61	0.71	9.22x + 483	0.0001	-5.58x + 49.86	0.81	-0.12x + 0.70	0.45
CTRL 2 (Polluted soil)	0.04x + 7.06	0.015	0.11x + 29.71	0.029	-830.7x + 9564	0.90	520.4x – 404.6	0.69	-118.7x +739.9	0.31	-3.19x + 33.39	0.51	-0.07x + 0.78	0.24
PS+GM	-0.07x + 7.51	0.116	0.48x + 28.38	0.427	-1311x+ 9535	0.84	639.2x – 804.1	0.87	-21.42x + 370.2	0.02	-4.46x + 37.67	0.74	-0.11x + 0.79	0.68
PS+FW	7.28	7.00E-29	0.19x + 29.81	0.157	-1471x + 9653	0.87	593.7x – 700.9	0.82	-172.7x + 1092	0.29	-1.84x + 32.42	0.83	-0.03x + 0.54	0.03
PS+GM+FW	0.05x + 6.71	0.189	0.45x + 27.93	0.242	-1657x + 10830	0.97	481.7x – 198.9	0.70	-246.1x + 1433	0.62	-2.45x + 32.20	0.67	-0.13x + 1.10	0.72
PS+Asp+Muc	-0.06x + 7.26	0.409	0.65x + 28.03	0.781	-1150x + 9746	0.89	630.8x – 905.1	0.83	-240.1x + 1167	0.47	-2.56x + 34.73	0.91	-0.02x + 0.51	0.04
PS+Bac+Pse	7.06	6.00E-28	-0.18x + 30.7	0.355	-1680x +10822	0.97	556.7x – 717.9	0.69	-159.2x + 860.4	0.61	-2.92x + 35.53	0.89	-0.06x +0.74	0.38
PS+Asp+Muc+Bac+Pse	0.07x + 6.87	0.331	0.58x + 28.52	0.794	-1016x + 8280	0.5	614.3x – 827.3	0.82	43.85x +712.2	0.01	-6.19x + 42.87	0.79	-0.08x + 0.75	0.61
PS+Asp+Muc+Bac+Pse+GM+FW	0.18x + 6.46	0.81	0.43x + 27.67	0.883	-1858x + 7684	0.53	403.1x – 193.5	0.57	-18.62 + 938.1	0.004	-9.52x +60.99	0.73	-0.07x +0.55	0.60

CTRL: Controls; CS - Polluted soil; Asp -*Aspergillus*;Muc -*Mucor*;Bac - *Bacillus armyloliquefaciens strain FJAT-45825*;Pse -*Pseudomonas aeruginosa strain CL 9*;
GM- Goat manure; FW- Fish waste
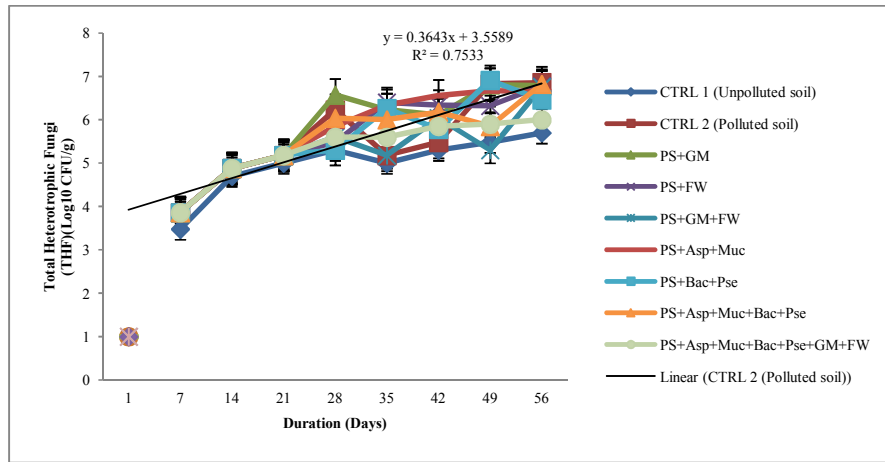
Fig. 3. Variation in total heterotrophic fungal (THF –Log10 cfu/g) count during Bioremediation of crude oil polluted soil

CTRL: Controls; CS - Polluted soil; Asp -Aspergillus;Muc -Mucor;Bac - Bacillus armyloliquefaciens strain FJAT-45825;Pse -Pseudomonas aeruginosa strain CL 9; GM- Goat manure; FW- Fish waste
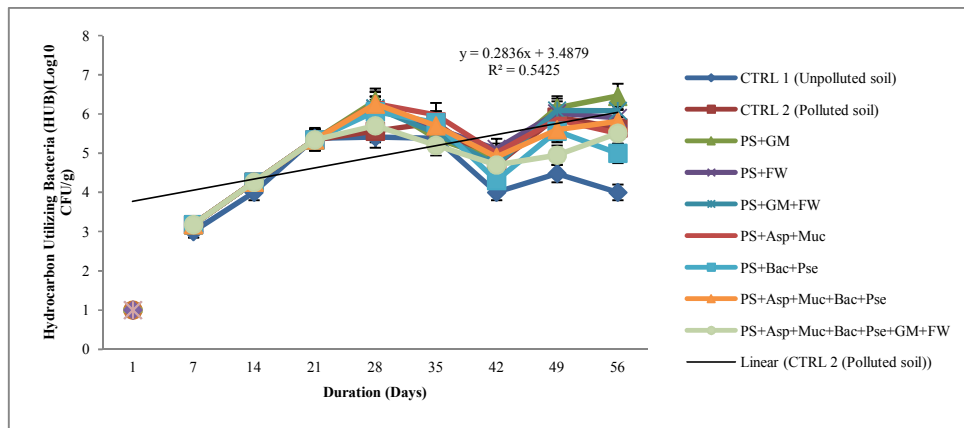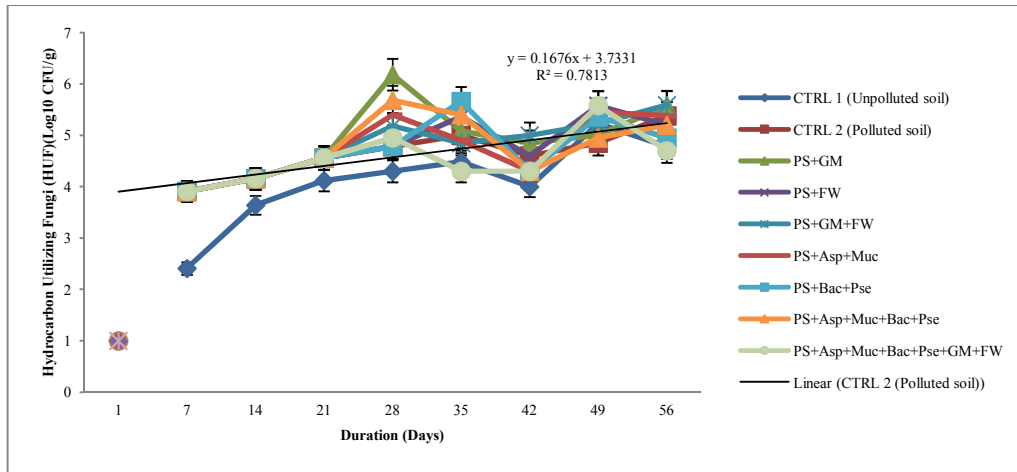

Fig. 4. Variation in hydrocarbon utilizing bacterial (HUB –Log10 cfu/g) count during bioremediation of crude oil polluted soil

CTRL: Controls; CS - Polluted soil; Asp -Aspergillus;Muc -Mucor;Bac - Bacillus armyloliquefaciens strain FJAT-45825;Pse -Pseudomonas aeruginosa strain CL 9;GM- Goat manure; FW- Fish waste

Figs. 2-8 and 6-9 represents the variations in microbial (Log10 cfu/g) counts during biodegradation and the mean values respectively. The plots treatment with bio stimulating agents and bio augmentingmicroorganisms showed a higher value than the controls. Comparatively the plot amended with a combination of bio-stimulating and bio-augmentating agents indicated the highest rate of biodegradation.

3.4 Percentage (%) Petroleum Hydrocarbon Bioremediation

Table 6, Figs. 10-11; shows the equations 2 & 3 resulting in the values obtained as reduction in total petroleum hydrocarbon in relation to percentage of biodegradation between the controls and treatment plots at day 56. The results indicated that polluted soil +*Aspergillus*+*Mucor* had (81.62%) > polluted soil + goat manure (70%) >polluted soil + goat manure + fish wastes (62.74%) >polluted soil+*Aspergillus*+*Mucor*+ *Bacillus*+*Pseudomonas* + goat manure+ fish wastes (55.98%) > un polluted soil (49.72%) >polluted soil + fish waste (37.55%) >polluted soil + *Aspergillus*+*Mucor* + *Bacillus* + *Pseudomonas* (31.46%) >polluted soil+*Bacillus*+*Pseudomonas* (18.01%) >polluted unamend soil (14.85%) The result showed that *Aspergillus niger* and *Mucor racemosus* had greater potential in enhancing bioremediation of

crude oil polluted soils followed by goat manure. This result is consistent with the results of Nrior and Echezolom [30] who reported that goat faeces had 21% remediation rate compared to fertilizer 12.95% amendment. A similar report was observed for fish wastes to have played significant role in this study with 37.54%.

3.5 Evaluation of Physicochemical Characteristics

Table 5 illustrates the reading for Nitrate shows Polluted soil + Aspergillius + Mucor +

Bacilliusamyloliquefaciens strain FJAT-45825+ Pseudomonas aeruginosa strain CL 9 +Goat manure + Fish waste (1402.65±843.74a) > CTRL 1 Unpolluted soil (1265.4±831.74a) > Polluted soil + Goat manure(305.982±226.98[a]) with the lowest while the Phosphate value showed that Polluted soil + *Mucor* + *Pseudomonas aeruginosa strain CL 9* had the highest (35.36±9.84[a]) and CTRL 2 polluted soil without treatment (23.82±7.04[a]) the lowest. The value for Total Organic Carbon (**TOC**) shows Polluted soil + *Mucor* + *Bacilliusamyloliquefaciens strain*

Fig. 5. Variation in hydrocarbon utilizing fungal (HUF –Log10 cfu/g) count during bioremediation of crude oil polluted soil
CTRL: Controls; CS - Polluted soil; Asp -Aspergillus;Muc -Mucor;Bac - Bacillus armyloliquefaciens strain FJAT-45825;Pse -Pseudomonas aeruginosa strain CL 9; GM- Goat manure; FW- Fish waste
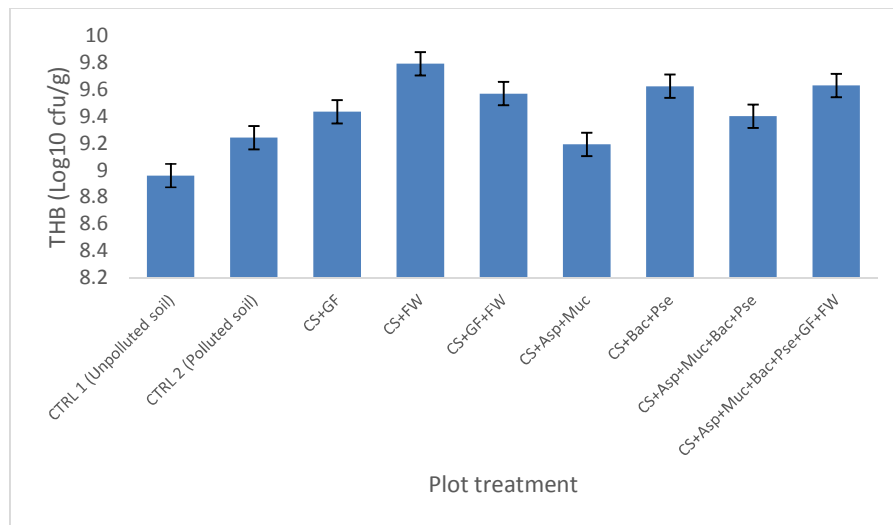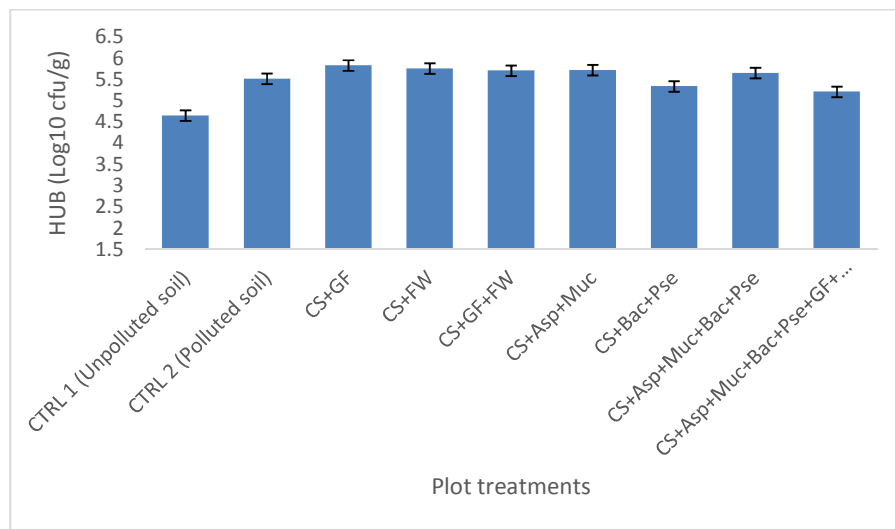

Fig. 6. Mean value of total heterotrophic bacteria (THB –Log10 CFU/g) count during bioremediation of the controls and crude oil polluted soil
CTRL: Controls; CS - Polluted soil; Asp -Aspergillus;Muc -Mucor;Bac - Bacillus armyloliquefaciens strain FJAT-45825;Pse -Pseudomonas aeruginosa strain CL 9; GM- Goat manure; FW- Fish waste
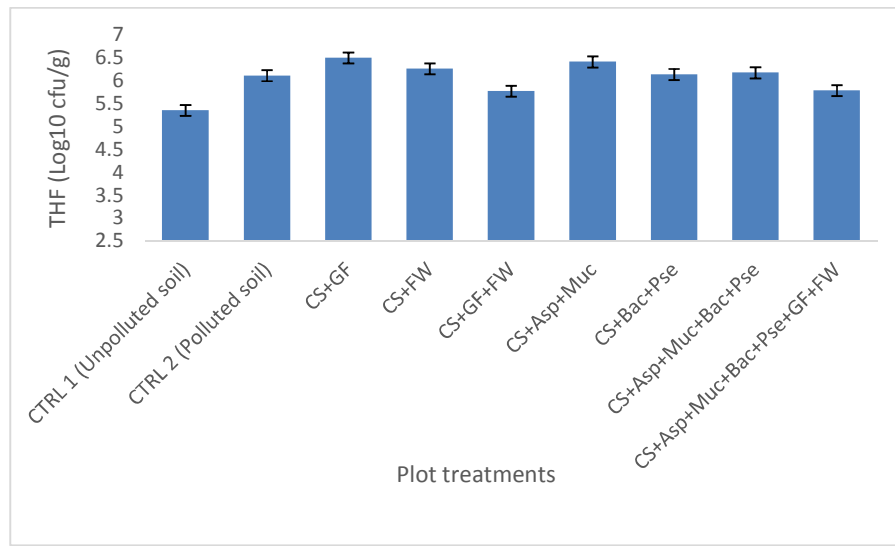
Fig. 7. Mean value of total heterotrophic fungal (THF –Log10 CFU/g) count during bioremediation of the controls and crude oil polluted soil
CTRL: Controls; CS - Polluted soil; Asp -Aspergillus;Muc -Mucor;Bac - Bacillus armyloliquefaciens strain FJAT-45825;Pse -Pseudomonas aeruginosa strain CL 9; GM- Goat manure; FW- Fish waste
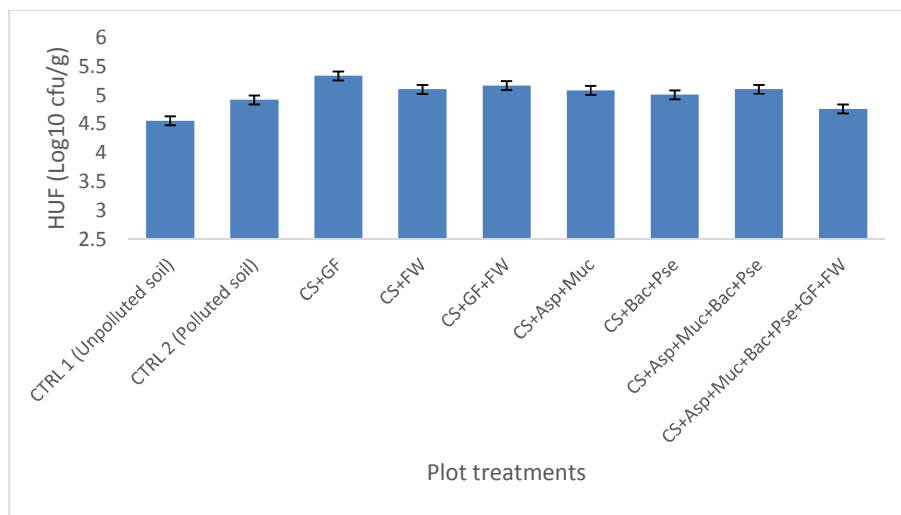

Fig. 8. Mean value of hydrocarbon utilizing bacterial (HUB –Log10 CFU/g) count during bioremediation of the controls and crude oil polluted soil
CTRL: Controls; CS - Polluted soil; Asp -Aspergillus;Muc -Mucor;Bac - Bacillus armyloliquefaciens strain FJAT 45825;Pse -Pseudomonas aeruginosa strain CL 9; GM- Goat manure; FW- Fish waste

FJAT-45825(0.87±0.66%) highest while Polluted soil +Mucor + *Pseudomonas aeruginosa strain CL 9* (0.37±0.13%) has the lowest percentage. Table 7 showed the regression analysis of the physicochemical parameters while Figs. 12-15 showed the variations in Nitrate, Sulphate, Phosphate and TOC respectively.

Total Organic Carbon (TOC), Nitrate, Sulphate and Phosphate as soil nutrients evaluators were analysed throughout the experimental period of 56 days at weekly intervals. Results obtained as shown in Figs. 12-15 revealed a supportive role in nutrient amendment dynamics using organic substrates (goat manure and fish waste) which was particularly evident in soil Nitrate values with increase in time. These suggest the positive impact nutrient amendment with organic substrates had on the augmenting microbes (*Bacillus amyloliquefaciens* FJAT-45825,

Pseudomonas aeruginosa strain CL-9, Aspergillus nier and Mucor racemosus) thereby increasing percentage (%) bioremediation; though fish wastes had a greater impact in relation to goat manure or augmenting microbes without organic substrates. Researchers have applied a wide range of organic and inorganic materials in bioremediation of hydrocarbon polluted soil, only a few of these nutrients were found to be effective and efficient [6,27,28]. This study observed that fish waste had a greater % bioremediation impact in relation to former nutrient application, thus could be preferred either singly or in combination with other organic

substrates or as augmenting microbes' enhancer.

More so, this study has shown that goat manure and fish wastes due to their high moisture and nutrient content properties makes them appropriate agents for enhanced bioremediation. It further revealed that a combination of biostimulating and bioaugmentating agents creates more favorable conditions for biological activity to thrive and has shown to be effective, economical, eco-friendly and sustainable in remediating organic pollutants from polluted soils.

Fig. 9. Mean value of hydrocarbon utilizing fungal (HUF –Log10 CFU/g) count during bioremediation of the controls and crude oil polluted soil
CTRL: Controls; CS - Polluted soil; Asp -Aspergillus;Muc -Mucor;Bac - Bacillus armyloliquefaciens strain FJAT-45825;Pse -Pseudomonas aeruginosa strain CL 9; GM- Goat manure; FW- Fish waste
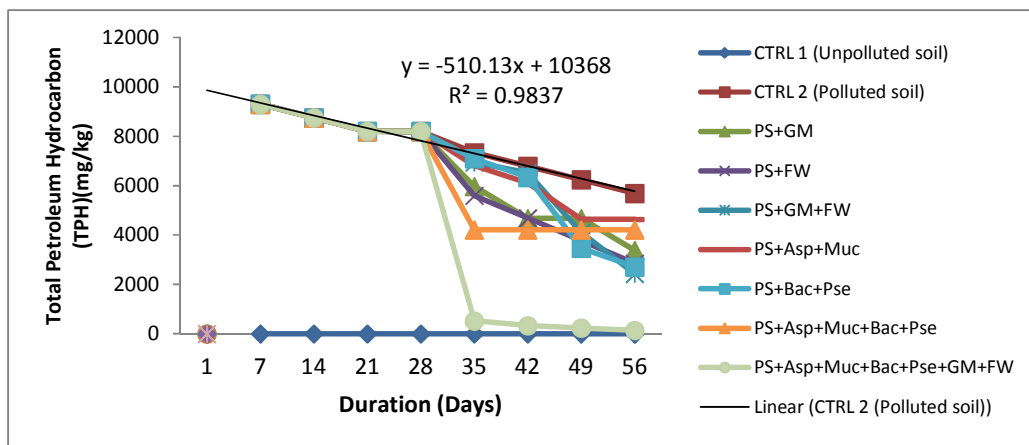

Fig. 10. Variation in Total Petroleum Hydrocarbon (TPH – mg/kg) count during Bioremediation of crude oil polluted soil
CTRL: Controls; CS - Polluted soil; Asp -Aspergillus;Muc -Mucor;Bac - Bacillus armyloliquefaciens strain FJAT-45825;Pse -Pseudomonas aeruginosa strain CL 9; GM- Goat manure; FW- Fish waste
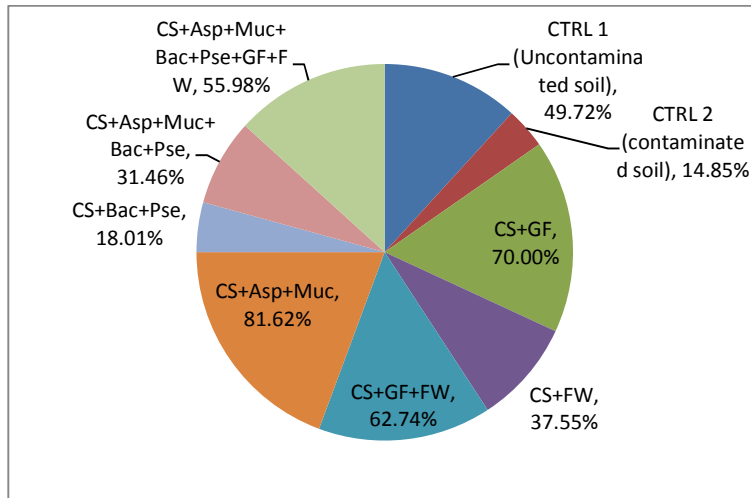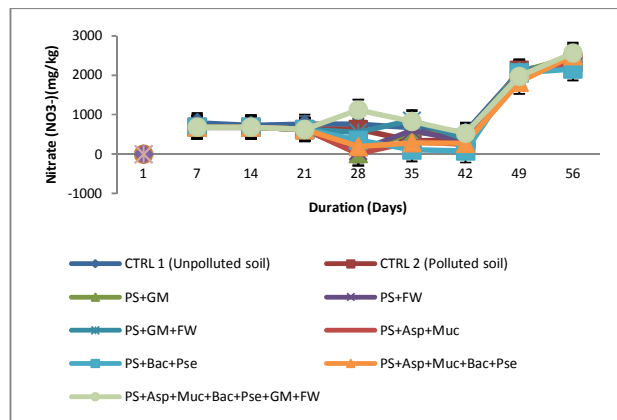
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Fig. 11. Bioremediation rate of nutrient amended crude oil polluted soil and controls
CTRL: Controls; PS - Polluted soil; Asp -Aspergillus;Muc -Mucor;Bac - Bacillus armyloliquefaciens strain FJAT-45825;Pse -Pseudomonas aeruginosa strain CL 9; GM- Goat manure; FW- Fish waste

Fig. 12. Variation in Nitrate (mg/kg) during Bioremediation of crude oil polluted soil
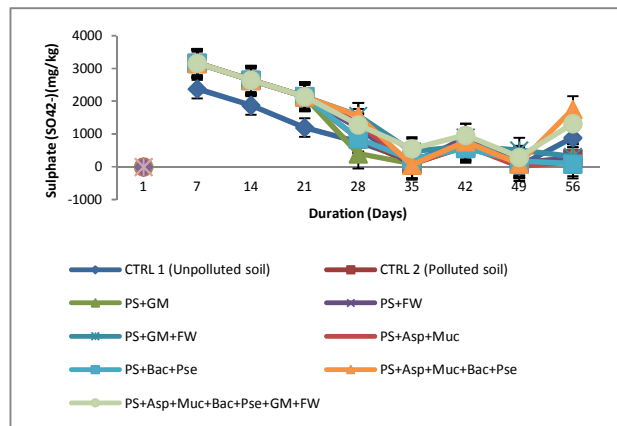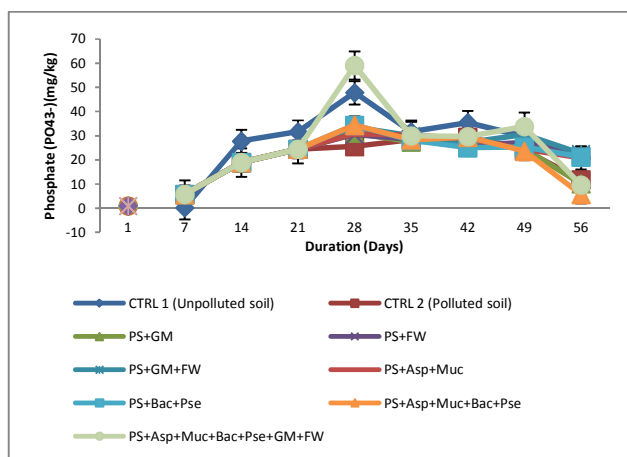

Fig. 13. Variation in sulphate (mg/kg) during bioremediation of crude oil polluted soil
CTRL: Controls; PS - Polluted soil; Asp -Aspergillus;Muc -Mucor;Bac - Bacillus armyloliquefaciens strain FJAT-45825;Pse -Pseudomonas aeruginosa strain CL 9; GM- Goat manure; FW- Fish waste

Fig. 14. Variation in phosphate (mg/kg) during BIOREMEDIATION of crude oil polluted soil
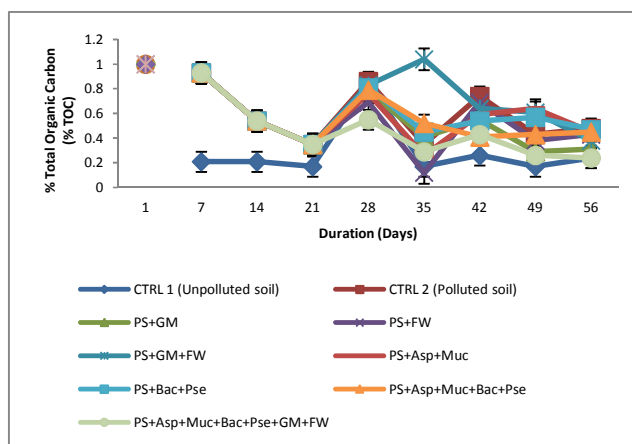

Fig. 15. Total organic carbon (TOC) (%) during bioremediation of crude oil polluted soil
CTRL: Controls; PS - Polluted soil; Asp -Aspergillus;Muc -Mucor;Bac - Bacillus armyloliquefaciens strain FJAT-45825;Pse -Pseudomonas aeruginosa strain CL 9; GM- Goat manure; FW- Fish waste

4. CONCLUSION

The use of goat manure and fish wastes as biostimulatingnutrients;The microorganisms such as*Aspergillus*sp., *Mucor* sp., *Bacillus armyloliquefaciens strain FJAT-45825*, and *Pseudomonas aeruginosa strain CL 9*asbioaugmentating agents singly has shown to increase the bioremediation of crude oil polluted soil. However, this study shows that a combination strategy of biostimulating and bioaugmenting agents in bioremediation processes produced a more effective and faster bioremediation, achieving a greater reduction in petroleum hydrocarbon hence recommended for environmental management and control for polluted environments with petroleum products in Nigeria. It is recommended that due to the significant biodegradability potentials of fish wastes, it could be employed as a potential stimulant/ nutrient organics during bioremediation even when bioaugmentation options are employed.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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