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BIOREMEDIATION OF DIESEL CONTAMINATED SOIL USING BACTERIAL COCKTAIL AND ORGANIC NUTRIENTS

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ABSTRACT

Bioremediation is a process of contaminant degradation in the environment using microorganisms. Bioremediation of diesel contaminated soil was studied using bacterial cocktail and organic nutrients from cow dung and poultry droppings at interval of 21 days for a total period of 84 days. Two hundred grams (200g) of soil were weighed into clay pots polluted with 10% (w/w) diesel oil and left undisturbed for 48 hours in an open field. After 48 hours, the clay pots were inoculated with bacterial cocktail (two bacteria isolates from diesel contaminated soil), 10% (w/w) of cow dung (CD), 10% (w/w) of poultry droppings (PD) and 5% (w/w) of sodium azide (NaN_3). The two bacterial isolates were identified as *Micrococcus luteus* trpE16 and *Bacillus subtilis* DNK UT 02 after screening for hydrocarbon utilization using standard methods. The counts of hydrocarbon utilizing bacteria (THB) in the amended soil ranged from 20.2×10^7 and 63.5×10^7 cfu/g while unamended soil had the least count of THB ranging between 8.4×10^7 and 19.0×10^7 cfu/g. Soil bioremediated with bacterial cocktail (BC) + 10% (CD+PD) recorded highest total petroleum hydrocarbon (TPH) degradation of 48.76%, 56.32%, 72.89% and 96.80% at the end of days 21, 42, 63 and 84 respectively while autoclaved soil with 5% NaN_3 recorded the least (10.03%, 13.38%, 14.02% and 18.42%) respectively. First order kinetic model showed that soil bioremediated with BC with 10% (CD+PD) recorded highest biodegradation rate constant of 0.2096 day^{-1} and half-life of 3.31 days. Statistical analysis indicated that the results obtained were significantly ($P < 0.05$) different during the 84 days of this study. Amendment of diesel contaminated soil with bacterial cocktail and organic wastes caused changes in the soil physiochemical properties and accelerated the rate of biodegradation in the soil. However, poultry droppings and cow dung can serve as a potential and viable biostimulant for enhanced biodegradation of diesel in soil.

Keywords: Bioremediation, *Micrococcus luteus*, *Bacillus subtilis*, organic compound

INTRODUCTION

Over the years, the activities of Oil and Gas industry have turned out to be a threat to the environment due to the several occurrences of oil spillage into soil and water environment. Oil spillage has caused untold hardship on those residing in areas where this natural resource is in abundance, as it has deprived them of portable water being that the surface and ground water down the soil layers gets contaminated when the spillage occurs (**Olabemiwo *et al.*, 2014**).

Diesel changes the physico-chemical properties of soil. It increases the level of toxins such as zinc and iron in the soil and reduces the amount of nutrients available. There is high accumulation of aluminium and manganese ions which are toxic to plant growth due to the anaerobic condition in the soil, the water logging and acid metabolites created by the diesel fuel. Furthermore, there is reduction in the amount of oxygen diffused to the root system if the oil is stranded on the plant shoot, thus affecting the soil indirectly (**Ebere *et al.*, 2011; Chen *et al.*, 2015**).

Due to high demand on land and water, it is imperative that the soil and water body affected by the diesel spillage has to be rehabilitated in time for uses. Therefore, natural methods like bush fallowing for soil cannot always be relied on. This has given rise to several techniques of remediation. Among the various techniques that have been utilized in replenishing soil nutrients over the years, bioremediation seems to be most thriving (**Tariq*et al.*, 2016**).

Bioremediation is the process by which pollutants in the environment is converted into less toxic or non-toxic compounds, using naturally occurring microorganisms or genetically modified microorganisms. It is a natural degradation process by which microorganisms chemically alter or break down organic molecules into innocuous substances such as carbon dioxide, fatty acids and water in order to obtain energy and nutrients (**Adaba, 2013**).

Bioaugmentation and biostimulation are two approaches to bioremediation geared toward enhancing and speeding up the process. Bioaugmentation involves the addition of external microbial population (endogenous or exogenous) to the polluted site. Bacteria such as *Bacillus* sp, *Micrococcus* sp, *Pseudomonas* sp, *Streptomyces* sp, *Methanobacterium* sp, *Thiobacillus* sp are the most common microbes used (**Castro-Gutiérrez *et al.*, 2012; Okoh, 2013**). Biostimulation involves the addition of appropriate nutrients such as organic manure, inorganic fertilizers (NPK), provision of oxygen to a polluted site to increase microbial activities of indigenous microbial flora (**Odu *et al.*, 2015**). Previous study (Ibiene *et al.*, 2011), reported the use of cow dung and poultry droppings for bioremediation of hydrocarbon contaminated soil. However, to the best of my knowledge on literature search, there is little or no information on the use of bacteria cocktail with cow dung and poultry droppings.Therefore, the aim of this study was to remediate diesel contaminated soil using bacterial cocktail (containing *Micrococcus luteus*and *Bacillus subtilis* isolated from contaminated soil) and alongside cow dung and poultry droppings.

MATERIALS AND METHODS

Collection of samples

Soil sample was collected from 0-10cm depth using soil auger from mechanic workshop beside Bosso primary school Minna and brought to Microbiology Department Federal University of Technology Minna. Diesel was purchased from Filling Station in Bosso, Minna Niger State. Cow dung and poultry droppings were collected from animal farm and poultry farm in Bosso Minna, Niger State.

The isolation of microorganisms

The aerobic heterotrophic bacteria were isolated from soil sample in mechanic workshop using methods described by (**Henrick, 2004**) while mineral salt medium (MSM) in the soil was used to enumerate the hydrocarbon-utilizing bacteria (HUB). The mineral salt medium contains 1.8 g K_2HPO_4, 4.0 g NH_4Cl, 0.2 g $MgSO_4.7H_2O$, 1.2 g KH_2PO_4, 0.01 g $FeSO_4.7H_2O$, 0.1 g NaCl, 20 g agar,

1% diesel (as the only carbon source) in one liter of deionized water. The pH was adjusted to 7.4 with 0.1M NaOH. The oil agar plates were inoculated with 0.1mL of serially diluted soil samples (10^{-3}) and were incubated at 30ºC for five days and observed. The identities of the isolates were determined by comparing their characteristics with those of known taxa as described in Bergy's manual of determinative bacteriology. All chemicals (analytical grade) were purchased from Sigma-Aldrich, St Louis, MO, USA.

Screening of bacterial isolates for biodegradation potential

Nutrient broth was prepared and 9mL of the broth wasdispensed into test tubes and autoclaved. The colonies of the pure isolates were inoculated into the test tubes containing the broth and incubated at 37ºC for 24 hours. Mineral Salt medium (broth) was prepared by adding 1.2 g KH_2PO_4, 1.8 g K_2HPO_4, 4.0 g NH_4Cl, 0.2 g $MgSO_4.7H_2O$, 0.1 g NaCl and 0.01 g $FeSO_4.7H_2O$ in one liter of deionized water. The prepared broth (100 mL) was mixed with 0.1 % of diesel as the only source of carbon and autoclaved. When cooled, 1 mL of the test organisms in the nutrient broth was transferred into test tubes containing 9 mL of the sterile mixtures (MSM broth and diesell), and were incubated at 37ºC for 5 days. At the end of incubation, a UV spectrophotometer (model 752 Guang-Zhou Co. Ltd, China) was used to determine the optical density at 620 nm wavelength. The best bacterial isolates were selected for the cocktail used for the bioremediation process.

Preparation of inoculums for bioremediation

The two bacterial isolates that recorded higher hydrocarbon degradation were inoculated into bijou bottles, each containing 5 mL of nutrient broth and were incubated at 37ºC for 24 to 48 hours. The microbial count was carried out by measuring absorbance using a spectrophotometer at an absorbance of 560 nm wavelengths, until a cell concentration of 1.5×10^7 colony forming units (cfu)/mL (McFarland Standard) was achieved. The 5 mL culture was transferred into 1 liter sterile nutrient broth and incubated at 37ºC for 24 hours. The bacterial cocktail was prepared by mixing equal volumes (500 mL) of the culture of the above cell concentration of each isolate.

Microcosm set up

Complete Randomized Block Design (CRBD) was used in this study. Two hundred (200) grams of air-dried soil sieved with 2-mm mesh size was placed in clay pots polluted with 10% w/w diesel and left undisturbed for 48 hours. After 48 hours, treatment one was diesel polluted soil without any amendment with 10 mL of cell concentration 1.5×10^7 cfu/mL bacterial cocktail and 10% of each organic wastes (cow dung and poultry droppings) serve as control, Treatment two: diesel polluted soil + 10 mL of broth culture of bacterial cocktail, Treatment three: diesel polluted soil + 10mL of broth culture of bacterial cocktail + 10% (w/w) of cow dung, Treatment four: diesel polluted soil + 10mL of broth culture of bacterial cocktail + 10% (w/w) of poultry droppings, Treatment five: diesel polluted soil + 10mL of broth culture of bacterial cocktail + 10% (w/w) of cow dung and poultry droppings, Treatment six: diesel polluted soil + 10% (w/w) of cow dung, Treatment seven: diesel polluted soil + 10% (w/w) of poultry dropping, Treatment eight: autoclaved soil + 10% (w/w) diesel + 5% sodium azide (NaN_3) (to eliminate all life forms during bioremediation process) and was thoroughly mixed as shown in Table 1. The moisture content was adjusted to 60% water holding capacity and set up under natural environment exposed to sunlight and at room temperature 28ºC ±2. The content of each vessel was mixed twice a week for aeration, and the moisture content was maintained at 60% water holding capacity by addition of sterile distilled water. This experiment was set up in duplicate.

Table 1 Experimental layout for bioremediation

Design	Treatment
A	200g of Soil + 10% diesel (Control)
B	200g of Soil + 10% diesel oil + 10 mL of BC
C	200g of Soil + 10% diesel oil + 10 mL of BC + 10% PD
D	200g of Soil + 10% diesel oil +10 mL of BC + 10% CD
E	200g of Soil + 10% diesel oil + 10 mL of BC + 10% (PD+ CD)
H	200g of Autoclaved soil + 10% diesel oil + 5% NaN_3

Key BC= bacterial cocktail (two bacterial isolates from diesel contaminated soil); PD= Poultry dropping; CD= Cow dung: NaN_3= Sodium azide

Determination of physicochemical properties of soil (unpolluted & polluted) and organic wastes

Soil pH was determined with pH meter (Model 511) on 1:2.5 (w/v) soil/ distilled water after 30 minutes equilibration. The content of nitrogen in the soil and organic wastes utilized for bioremediation was determined using the Kjeldahl method, and the contents of phosphorus and carbon were determined using ICP-OES and furnace method, respectively. Moisture content was determined using the gravimetric method.

Microbial counts

Changes in microbial population were determined by inoculating 0.1 mL of serially diluted soil sample onto nutrient and diesel oil agar for total heterotrophic and hydrocarbon utilizing bacteria counts respectively. The nutrient agar plates were incubated at 37ºC for 24hrs and diesel oil agar plates at 37ºC for 5 days. The colonies developed after incubation were counted and expressed as colony forming units per gram (cfu/g) of soil sample.

Total petroleum hydrocarbon determination

Gravimetric method was used to determine the hydrocarbon content of the soil samples using tuolene cold extraction method described by **Adesodun and Mbagwu (2008)**. This was done by adding 10 g of soil sample into 20mL of toluene (Analar grade). The mixture was shaken for 30 minutes using an orbital shaker and the supernatant was measured using spectrophotometer at 420 nm. To determine the total petroleum hydrocarbon in the soil, values were estimated with reference to standard curve obtained from new diesel oil diluted with toluene. The total petroleum hydrocarbon data was fitted to first-order kinetics model of **Yeung *et al.* (1997)** as follows: $y = ae^{-kt}$ In this equation, y is the residual soil hydrocarbon content (g kg^{-1}), a is the soil initial hydrocarbon content (g kg^{-1}), k is the biodegradation rate constant (day^{-1}), and t is the treatment time. Half- life was calculated as: Half life = 1n (2) / *k*. The assumption in this model was that the rate of hydrocarbons degradation positively correlated with the soil hydrocarbon pool size **(Yeung *et al.*, 1997**).

Gas chromatographic mass- spectrophotometric analysis of diesel extract

Gas chromatography - Mass spectroscopy analysis of the diesel extracts from the soil samples was analyzed using GC-MS model 7890A with Mass Selective Detector model: 5975C (MSD). The diesel oil extraction was carried out by dissolving 5g of the soil samples in 10mL (99.99%) pure dichloromethane in a well corked reagent bottle. This was thoroughly mixed using an ultra sonicator for a period of five hours. The mixture was allowed to stand for 72 hours and filtered into a beaker; the mixture was rewashed with 20mL dichloromethane for two more consecutive times. The combined aliquots was evaporated on a steam berth to 5mL and filtered through a pipette stocked with glass wool (membrane) with packed anhydrous sodium sulfate silica gel to remove the left over moisture and other impurities. The filtrate was concentrated to 1mL in the vial bottle and was analyzed on Gas chromatography.

Statistical analysis of data

The data generated from this study was subjected to analysis of variance ($P \leq 0.05$) using SPSS version 20 and the averages were compared by Duncan Multiple Range Tests (DMRT) $P \leq 0.05$. The effect of studied factors were considered significant when $P \leq 0.05$.

RESULTS

Physicochemical properties of soil (unpolluted) and the organic waste

The physicochemical properties of soil and organic wastes that were used for bioremediation are presented in Table 2.

Table 2 The physicochemical properties of soil and organic wastes

Parameter	**Soil**	**Organic wastes**	
		CD	PD
pH	6.70±0.25	7.10±0.19	7.40±0.25
Nitrogen (%)	0.34±0.02	0.74±0.01	3.26±0.25
Phosphorus (mgkg^{-1})	23.80±1.52	35.37±2.42	87.58±0.25
Organic C (%)	1.30±0.09	2.79±0.20	6.82±0.25
Moisture (%)	8.20±0.10	36.20±3.15	15.40±0.25
Sand (%)	44.24± 3.67	____	____
Silt (%)	30.28± 2.29	____	____
Clay (%)	25.48±1.09	____	____
Texture	Sandy loam	____	____

Key: CD = Cow dung, PD = Poultry dropping

Nutrients Composition of bioremediated soil

The pH of the bioremediated soils ranged from 7.1 to 7.8 during the 84 days study period. Soil bioremediated with bacterial cocktail (BC) with 10% poultry droppings (PD) + Cow dung (CD) recorded highest pH of 7.8 while autoclaved

remediated soil with 5% sodium azide (NaN_3) recorded the least pH of 7.1 as shown in Fig. 1

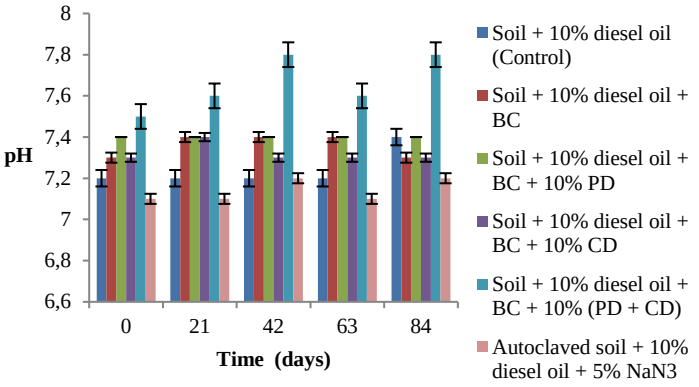

Figure 1 pH values of diesel contaminated soil during bioremediation

The minerals (nitrogen, phosphorus, and carbon) content in all experimental soil decreases with increase in bioremediation time (Fig. 2-4). However, the autoclaved remediated soil with 5% sodium azide (NaN_3) had the highest nitrogen (3.59±0.21 - 3.42±0.02%) while unamended soil (control) had the least (0.85±0.02%) on day zero (0) whereas soil bioremediated with bacterial cocktail had the least (0.43±0.02%) on the last day (day 84). Also, soil bioremediated with bacterial cocktail (BC) + 10% Poultry droppings (PD) + Cow dung (CD) had the highest phosphorus (39.84±0.67mg/kg) on day zero (0) whereas autoclaved control soil with 5% (NaN_3) had the highest phosphorus (24.00±0.26 mg/kg) on the last day (day 84) meanwhile unamended soil (control) had the least phosphorus (23.56±0.47-14.62±0.45 mg/kg) content. The highest organic carbon content was observed in soil bioremediated with BC + 10% PD + CD (3.61±0.04%) on day zero whereas autoclaved control soil with 5% (NaN_3) had the highest organic carbon (2.07±0.02%) on day 84 meanwhile remediated control soil had the least organic carbon (1.90±0.02-1.03±0.05%).

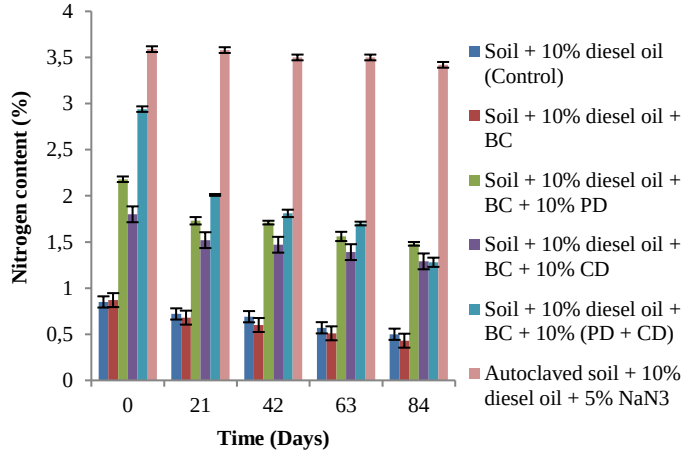

Figure 2 Nitrogen contents of diesel contaminated soil during bioremediation

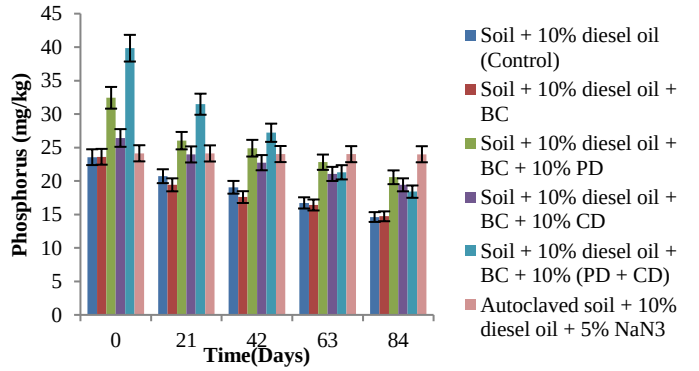

Figure 3 Phosphorus contents of diesel contaminated soil during bioremediation

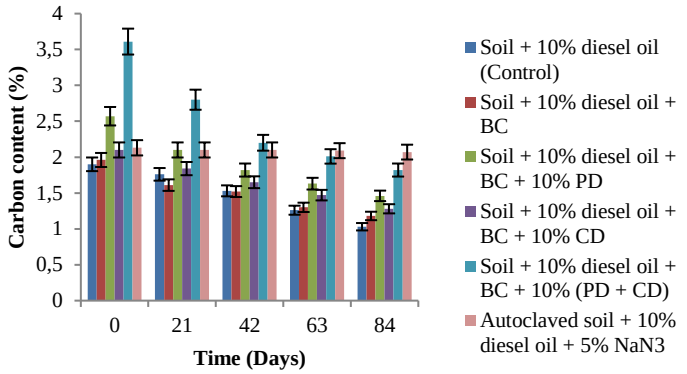

Figure 4 Carbon contents of diesel contaminated soil during bioremediation

Microbial counts

The counts of total heterotrophic bacteria (THB) in all experimental soil increased with increase in bioremediation time. However, soil bioremediated with bacterial cocktail (BC) + 10% poultry droppings (PD) + cow dung (CD) had the highest THB (between 20.2×10^7 and 63.5×10^7 cfu/g) while unamended soil (control) had the least count of THB (8.4×10^7 and 19.0×10^7 cfu/g) (Fig. 5). Similarly, soil bioremediated with BC + 10% (PD + CD) had the highest hydrocarbon utilizing bacteria (HUB) ranging between 15.4×10^6 and 86.4×10^6 cfu/g, while the autoclaved remediated soil with 5% NaN_3 had the least (1.00×10^6 cfu/g) as shown in Fig. 6.

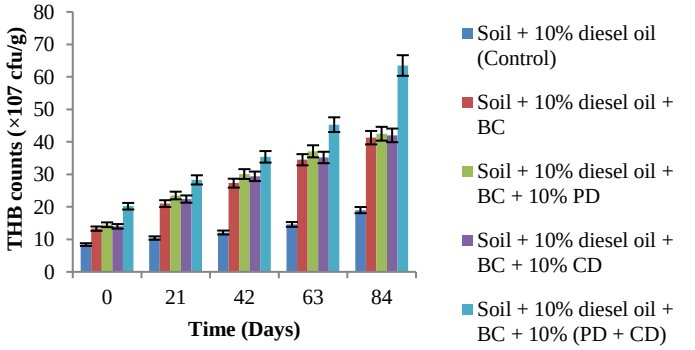

Figure 5 Total heterotrophic bacteria (THB) count in diesel contaminated soil during bioremediation

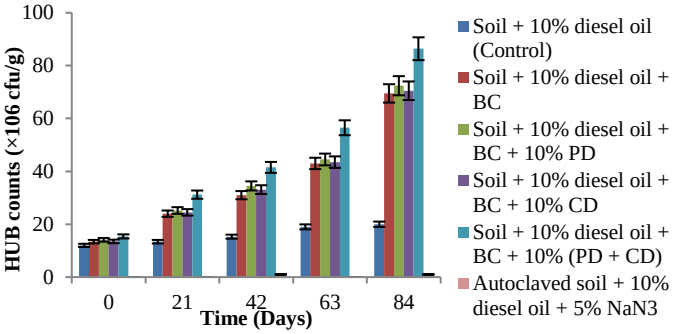

Figure 6 Hydrocarbon-utilizing bacterial (HUB) counts in diesel contaminated soil during bioremediation

Biodegradation of diesel oil

The biodegradation rate of diesel during the period of study is presented in Fig 7. There was a progressive decline in total petroleum hydrocarbon during the process of remediation in all the soil bioremediated with the cow dung and poultry droppings compared to that of unamended soil and autoclaved remediated soil with 5% NaN_3. Soil bioremediated with bacterial cocktail (BC) + 10% Poultry droppings (PD) + Cow dung (CD) had the highest total petroleum hydrocarbon (TPH) degradation of 48.76%, 56.32%, 72.89% and 96.80% at the end of days 21, 42, 63 and 84 respectively while soil remediated with 5% NaN_3 had the least TPH degradation of 10.03%, 13.38% 14.02% and 18.42% at the end of days 21, 42, 63 and 84 respectively.

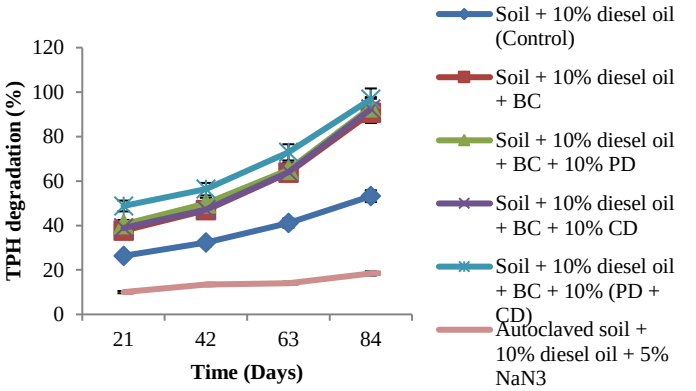

Figure 7 Total petroleum hydrocarbon degradation in diesel contaminated soil during bioremediation

Net percentage loss of diesel contaminated soil

The efficiency of bioremediation was investigated by determining the net % loss of diesel oil in contaminated soil. Soil remediated with BC + 10% (PD + CD) had the highest net percentage loss of 22.44±0.30%, 24.05±0.49%, 31.83±0.30% and 43.62±0.65% at the end of days 21, 42, 63 and 84 respectively. The least net percentage loss of diesel oil of 11.33±0.25%, 14.59±0.20%, 22.70±0.33% and 37.46±0.02% was recorded for soil remediated with BC at the end of days 21, 42, 63 and 84 (Table 2).

Table 2 Net percentage loss of total petroleum hydrocarbon in diesel contaminated soil

Treatment	Time (days)			
	21	42	63	84
Soil + 10% diesel oil + BC	11.33±0.25	14.59±0.20	22.70±0.33	37.46±0.02
Soil + 10% diesel oil + BC + 10% PD	14.16±0.23	17.66±0.20	23.98±0.25	39.90±0.55
Soil + 10% diesel oil + BC + 10% CD	12.54±0.22	14.83±0.40	22.89±0.20	39.18±0.25
Soil + 10% diesel oil + BC + 10% (PD + CD)	22.44±0.30	24.05±0.49	31.83±0.30	43.62±0.65

Key: BC = bacterial cocktail (two bacterial isolates from diesel polluted soil),PD = Poultry droppings CD = Cow dung

Biodegradation rate constant and half-life

The biodegradation rate constant (k) and half-life ($t_{1/2}$) for the different treatments within the 84 days of study is shown in Table 3. Soil remediated with BC + 10% (PD + CD) shows the highest biodegradation rate of 0.2096 day^{-1} and least half-life of 3.31 days; while autoclaved soil + 10% diesel oil + 5% NaN_3 had the least biodegradation rate of 0.0097day^{-1} and highest half-life of 71.46 days. However, the biodegradation rate of unamended soil (control) was 0.0916 day^{-1} and half-life of 7.57 days.

Table 3 Biodegradation rate and half-life of hydrocarbon in diesel contaminated soil

Treatment	Biodegradation rate constant (*k*) day^{-1}	Half-life ($t_{1/2}$) days
Soil + 10% diesel oil (Control)	0.0916	7.57
Soil + 10% diesel oil + BC	0.1732	4.00
Soil + 10% diesel oil + BC + 10% PD	0.1848	3.75
Soil + 10% diesel oil + BC + 10% CD	0.1772	3.91
Soil + 10% diesel oil + BC + 10% (PD + CD)	0.2096	3.31
Autoclaved soil + 10% diesel oil + 5% NaN_3	0.0097	71.46

Key: BC = bacterial cocktail (two bacterial isolates from diesel polluted soil),PD = Poultry droppings CD = Cow dung

Gas chromatography and mass spectroscopy

Gas Chromatography and Mass Spectroscopy (GCMS) analysis revealed a total number of 67 individual hydrocarbons in undegraded diesel oil (Fig. 8) consisting of n-alkane of carbon chain (C_9 - C_{31}), carboxylic acid (C_2H_3O - $C_{23}H_{44}O_3$), naphthalene, akyl group of naphthalene ($C_{10}H_8$ – $C_{15}H_{28}$), aromatic and polycyclic aromatic compounds at different peak numbers, retention time and percentage Area or height of abundance using 60 percent match quality **NIST library (1999)** as shown in Table 4.

Soil bioremediated with bacterial cocktail (BC) + 10% poultry dropping (PD) and cow dung (CD) had the highest reduction in the hydrocarbon compounds (Fig. 9) in which the odd molecular number of n- alkane (C_{11}-C_{31}) except C_{23}, carboxylic acid (C_2H_3O – $C_{23}H_{44}O_3$), naphthalene, akyl group of naphthalene ($C_{10}H_8$ – $C_{15}H_{28}$), aromatic and polycyclic aromatic compounds had been degraded at the end of the study period (Table 5). There is also a significant reduction in individual hydrocarbons with soil bioremediated with BC + 10% PD (Fig. 10) having degraded a total number of 48 out 67 individual hydrocarbons identified in undegraded diesel oil. Individual hydrocarbon like decane (C_{10}), undecane (C_{11}), dodecane (C_{12}), pentadecane (C_{15}) at different peak numbers 1, 6, 11, 12, respectively (saturated alkane), nonyl vinyl ester ($C_{12}H_{22}O$) (carboxylic acid), 4,6,8-Trimethylazulene ($C_{13}H_{14}$) (polycyclic aromatic compound) at peak numbers 4,19 and many more compounds had been degraded. However, soil bioremediated with BC + 10% CD (Fig. 11) had more higher molecular number of n-alkane (C_{36}-C_{54}), carboxylic acid (C_2H_3O – $C_{23}H_{44}O_3$), aromatic and polycyclic aromatic compounds than soil bioremediated with BC + 10% PD but degraded all naphthalene and akyl group of naphthalene ($C_{10}H_8$ – $C_{15}H_{28}$) as shown in Tables 6-7.

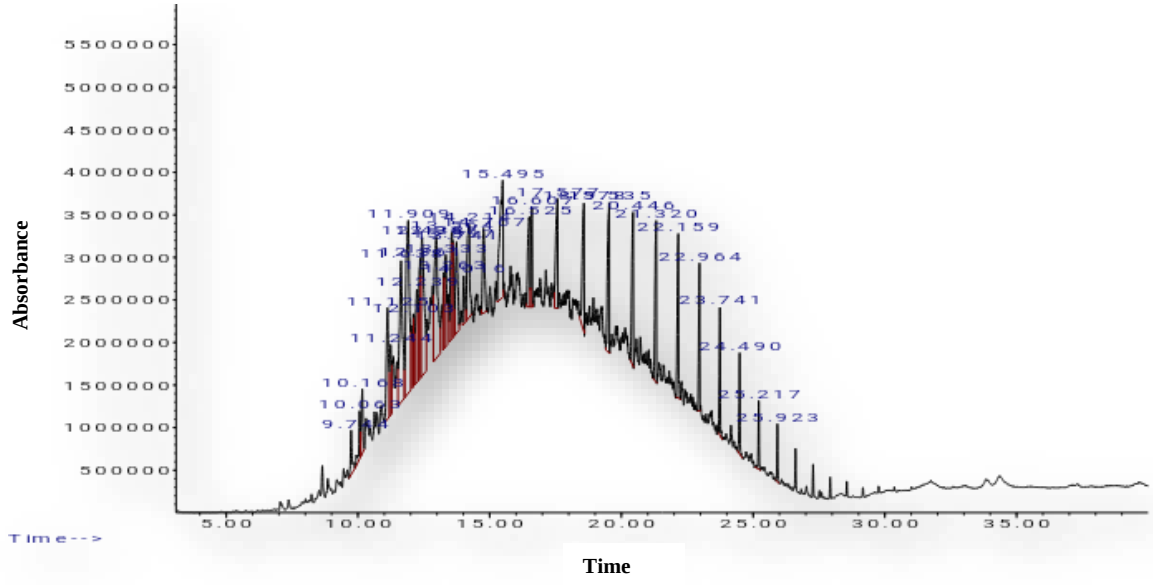

Figure 8 Chromatogram charts of undegraded diesel oil used for bioremediation

Table 4 Individual hydrocarbon identified in undegraded diesel oil

S/N	PK	RT	%AREA	COMPOUNDS	M.F	M.Q
1	1	9.741	0.98	Dodecane, 4,6-dimethyl-	$C_{12}H_{26}$	87
2				Hexadecane, 2,6,11,15-tetramethyl-	$C_{18}H_{32}$	93
3				Nonane, 3,7-dimethyl-	$C_{13}H_{32}$	91
4	2	10.062	1.08	Naphthalene, 2-methyl-	$C_{10}H_8$	97
5	3	10.165	1.46	Tridecane	$C_{14}H_{31}$	86
6				Undecane	$C_{11}H_{24}$	75
7				Carbonic acid, nonyl vinyl ester	CH_2CO_3	69
8	4	11.126	3.60	Decahydro-1,1,4a,5,6-pentamethyl naphthalene	$C_{15}H_{28}$	91
9				Neopentylidenecyclohexane	$C_{11}H_{20}$	89
10				Bicyclo[3.1.1]heptane, 2,6,6-trimethyl-	C_7H_{12}	92
11	5	11.246	1.63	Dodecane, 2,6,11-trimethyl-	$C_{12}H_{26}$	90
12				Docosyl pentyl ether	$C_{10}H_{20}O$	87
13	6	11.635	6.50	Tetradecane	$C_{14}H_{30}$	89
14				Decane	$C_{10}H_{22}$	91
15	7	11.910	8.67	Naphthalene, 2,6-dimethyl-	$C_{10}H_8$	97
16	8	12.105	0.97	Naphthalene, 2,7-dimethyl-	$C_{12}H_{14}$	89
17	9	12.242	2.94	6-Octen-1-ol, 3,7-dimethyl-, (R)-	$C_{10}H_{20}O$	94
18				2-Octen-1-ol, 3,7-dimethyl-	$C_{10}H_{20}O$	95
19				Citronellol	$C_{11}H_{23}O$	93
20	10	12.362	3.34	Decahydro-1,1,4a,5,6-pentamethylnaphthalene	$C_{15}H_{28}$	87
21				2-Anthracenamine	$C_{14}H_{11}N$	69
22				Benzo[f]quinoline, 3-methyl-	$C_{13}H_9N$	84
23	11	12.436	6.46	Dodecane, 2,6,11-trimethyl-	$C_5H_{11}O_3$	87
24				Carbonic acid, eicosyl vinyl ester	$C_{23}H_{44}O_3$	91
25				Undecane	$C_{11}H_{24}$	89
26	12	12.969	6.64	Dodecane	$C_{12}H_{26}$	65
27				Pentadecane	$C_{15}H_{32}$	82
28				Methoxyacetic acid, 2-tridecyl ester	$C_3H_6O_3$	87
29	13	13.260	1.10	Naphthalene, 1,6,7-trimethyl-	$C_{10}H_8$	89
30				3-(2-Methyl-propenyl)-1H-indene	$C_{17}H_{27}$	92
31	14	13.335	2.88	Naphthalene, 1,4,6-trimethyl-	$C_{13}H_{17}$	83
32	15	13.564	3.04	Naphthalene, 1,6,7-trimethyl-	$C_{13}H_{17}$	96
33	16	13.598	1.80	Naphthalene, 1,6,7-trimethyl-	$C_{13}H_{17}$	96
34				4,6,8-Trimethylazulene	$C_{13}H_{14}$	89
35	17	13.741	3.13	4,6,8-Trimethylazulene	$C_{13}H_{14}$	89
36				Naphthalene, 2,3,6-trimethyl-	$C_{10}H_8$	92
37	18	14.016	1.18	Naphthalene, 2,3,6-trimethyl-	$C_{13}H_{17}$	91
38				4,6,8-Trimethylazulene	$C_{13}H_{14}$	79
39	19	14.216	3.77	Hexadecane	$C_{16}H_{34}$	75
40	20	14.765	2.67	Hentriacontane	$C_{31}H_{64}$	83
41				Methoxyacetic acid, 2-tridecyl ester	$C_3H_6O_3$	67
42				Tetradecane	$C_{14}H_{30}$	83
43	21	15.498	5.48	Dodecane, 2,6,11-trimethyl-	$C_{12}H_{26}$	78
44				Octadecane, 2,6-dimethyl-	$C_{21}H_{42}$	92
45				Pentadecane, 2,6,10,14-tetramethyl	$C_{17}H_{34}$	91
46	22	16.522	2.96	Octadecane	$C_{18}H_{38}$	89
47	23	16.608	1.71	Dodecane, 2,6,10-trimethyl-	$C_{10}H_{22}$	94
48				Hexadecane, 2,6,10,14-tetramethyl-	$C_{19}H_{42}$	69
49				2,6,10-Trimethyltridecane	$C_{16}H_{36}$	87
50	24	17.575	3.17	Nonadecane	$C_{19}H_{40}$	91
51	25	18.576	3.28	Eicosane	$C_{20}H_{42}$	94
52				Octadecane, 2-methyl-	$C_{18}H_{38}$	97
53	26	19.537	3.73	Heneicosane	$C_{22}H_{42}$	89
54	27	20.447	3.28	Docosane	$C_{22}H_{46}$	78
55	28	21.323	3.16	Tricosane	$C_{23}H_{48}$	65
56				Hexacosane	$C_{26}H_{54}$	91
57	29	22.158	2.94	Tetracosane	$C_{24}H_{50}$	87
58	30	22.965	2.10	Pentacosane	$C_{25}H_{52}$	68
59	31	23.743	1.61	Hexadecane, 2,6,10,14-tetramethyl-	$C_{26}H_{54}$	93
60	32	24.493	1.22	Hexadecane, 1-iodo-	$C_{29}H_{68}$	91
61				Heptadecane	$C_{17}H_{36}$	79
62	33	25.219	0.86	Octacosane	$C_{28}H_{58}$	92
63				Hexadecane, 1-iodo-	$C_{16}H_{34}$	97
64				3-Eicosene, (E)-	$C_{20}H_{32}$	85
65	34	25.923	0.66	Hexadecane, 1-iodo-	$C_{16}H_{34}$	91
66				Octadecane, 1-iodo-	$C_{18}H_{38}$	84
67				1-Octadecene	$C_{18}H_{37}11$	67

Key: PK= Peak number, RT= Retention time, M.F=Molecular formula, M.Q=Match Quality

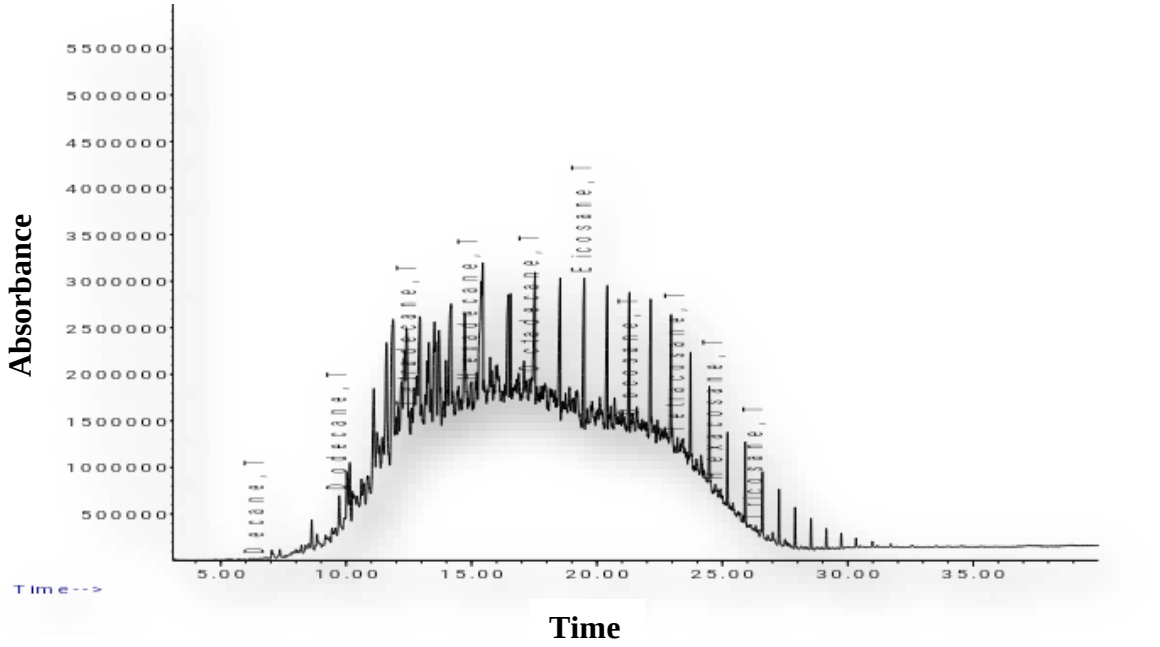

Figure 9 Chromatogram of diesel contaminated soil remediated with bacterial cocktail + 10% cow dung + poultry dropping after of 84 days

Table 5 Individual hydrocarbon identified in diesel contaminated soil remediated with BC + 10% (PD + CD) after of 84 days

S/N	PK	RT	%Area	COMPOUNDS	M. F	M.Q
1	1	6.428	0.03	Decane	$C_{10}H_{22}$	91
2	2	9.724	3.32	Dodecane	$C_{12}H_{26}$	89
3	3	12.505	1.17	Tetradecane	$C_{14}H_{30}$	93
4	4	14.954	1.06	Hexadecane	$C_{16}H_{34}$	85
5	5	17.403	1.35	Octadecane	$C_{18}H_{38}$	69
6	6	19.497	8.62	Eicosane	$C_{20}H_{42}$	92
7	7	21.346	0.39	Docosane	$C_{22}H_{44}$	63
8	8	23.205	0.62	Tetracosane	$C_{24}H_{50}$	87
9	9	24.716	0.33	Hexacosane	$C_{26}H_{54}$	79
10	10	26.329	0.15	Tricosane	$C_{23}H_{48}$	65

Key: BC = bacterial cocktail (two bacterial isolates from diesel polluted soil), CD = Cow dung
PD =Poultry dropping PK= Peak number, RT= Retention time, M.F=Molecular formualar, M.Q=Mash Quality

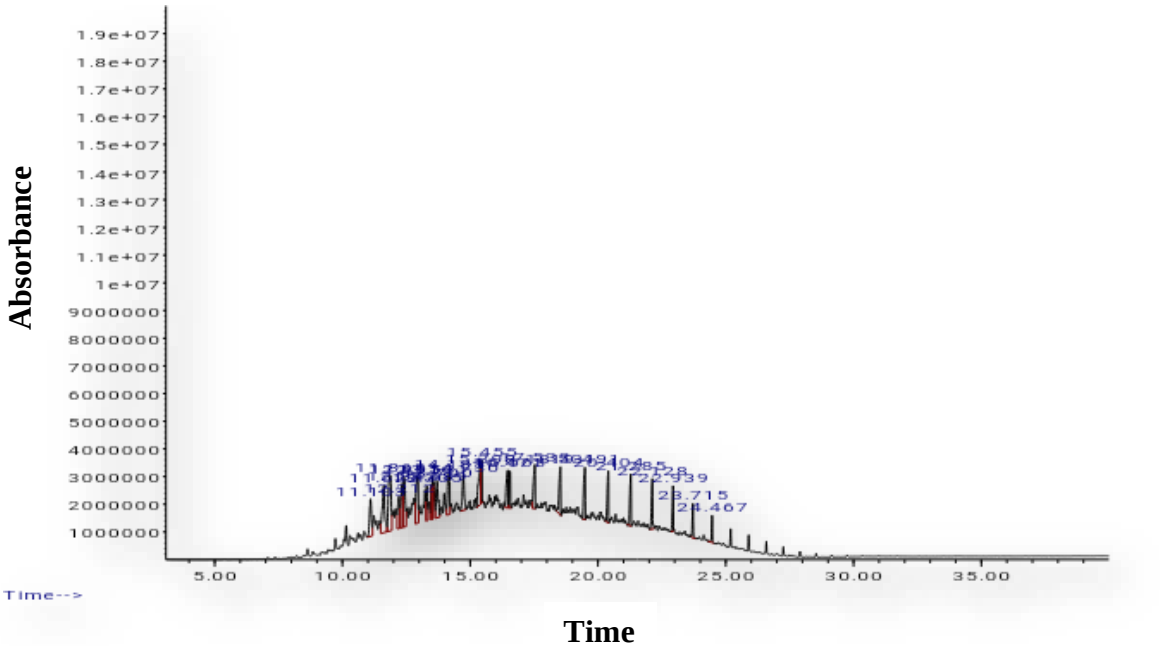

Figure 10 Chromatogram of diesel contaminated soil remediated with bacterial cocktail + 10%poultry dropping after of 84 days

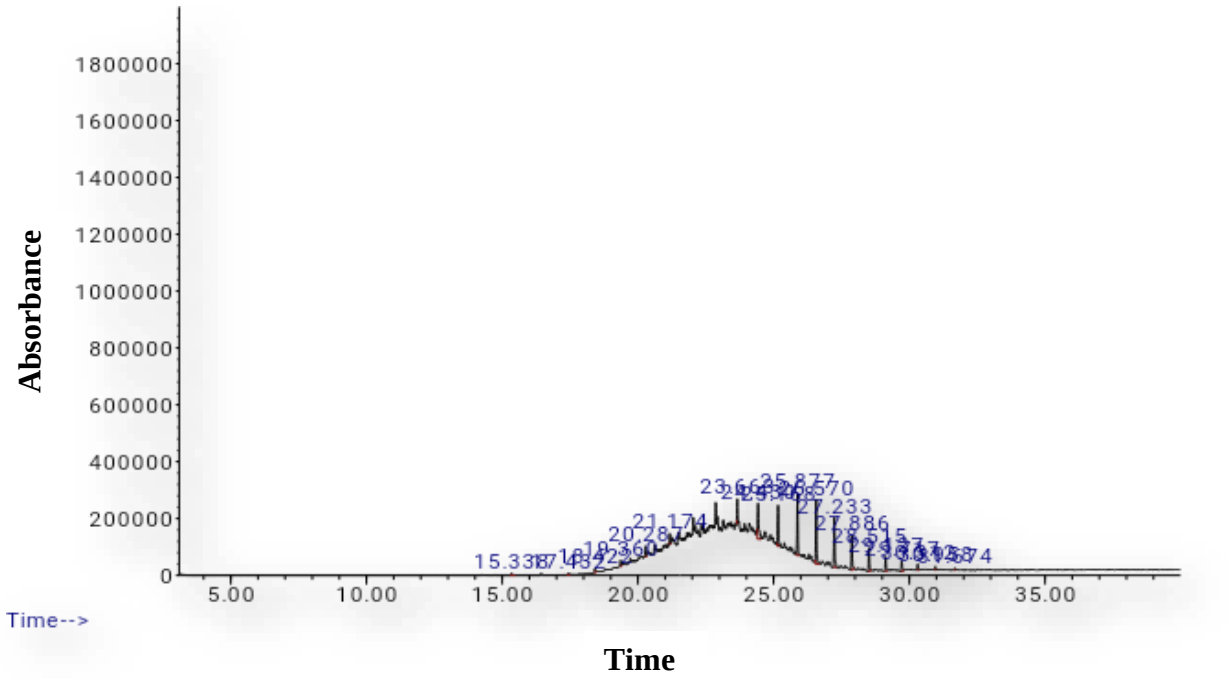

Figure 11 Chromatogram of diesel contaminated soil remediated with bacterial cocktail + 10% cow dung after of 84 days

Table 6 Individual hydrocarbon identified in diesel contaminated soil remediated with BC + 10% PD after of 84 days

SN	PK	RT	%AREA	COMPOUNDS	M.F	MQ
1	1	11.103	4.01	Decahydro-1,1,4a,5,6-pentamethylna phthalene	$C_{15}H_{23}10H$	97
2				2-Pentanone, 4-cyclohexylidene-3,3 -diethyl-	$C_{15}H_{24}O$	95
3				Bicyclo[2.2.1]heptane, 2,2,3-trimethyl-, endo-	C_7H_{12}	96
4	2	11.870	8.59	Naphthalene, 1,6-dimethyl-	$C_{10}H_{22}$	87
5	3	12.213	3.68	Citronellol	$C_{10}H_{22}$	89
6				6-Octen-1-ol, 3,7-dimethyl-	$C_{10}H_{22}$	91
7	4	12.339	4.29	Decahydro-1,1,4a,5,6-pentamethylna phthalene	$C_{10}H_{22}$	84
8				2(1H)-Naphthalenone, octahydro-4a, 7,7trimethyl	$C_{10}H_{22}$	65
9	5	13.306	2.43	Naphthalene, 1,4,6-trimethyl-	$C_{12}H_{12}$	76
10	6	15.400	4.81	Dodecane, 2-methyl-8-propyl-	$C_{12}H_{26}$	84
11				Methoxyacetic acid, 2-tetradecyl ester	$C_{12}H_{22}O_2$	92
12	7	16.562	2.30	2,6,10-Trimethyltridecane	$C_{13}H_{28}$	90
13	8	17.529	4.12	Heptadecane	$C_{17}H_{36}$	88
14	9	18.530	4.17	Eicosane	$C_{20}H_{42}$	75
15	10	19.492	4.26	Heneicosane	$C_{21}H_{44}$	87
16	11	20.401	3.65	Docosane	$C_{22}H_{46}$	93
17	12	22.936	2.11	Pentacosane	$C_{25}H_{52}$	97
18	13	23.715	1.67	Hexadecane, 1-iodo-	$C_{16}H_{34}$	89
19	14	24.464	1.14	Tetracosane	$C_{24}H_{50}$	82

Key: BC = bacterial cocktail (two bacterial isolates from diesel polluted soil), PD =Poultry dropping, PK= Peak number, RT= Retention time, M.F=Molecular formualar, M.Q=Match Quality

Table 7 Individual hydrocarbon identified in diesel contaminated soil remediated with BC + 10% CD after of 84 days

S/N	PK	RT	%AREA	COMPOUNDS	M.F	M.Q
1	1	15.338	1.04	1-Heptadecanamine	$C_{17}H_{37}N$	79
2				Carbonic acid, prop-1-en-2-yltridecyl ester	CH_2CO_3	67
3	2	17.432	0.69	1-Octanol, 2-butyl-	$C_8H_{18}O$	91
4				Carbonic acid, prop-1-en-2-yltetradecyl ester	$C_{19}H_{36}O_3$	89
5	3	18.422	1.32	Sulfurous acid, pentadecyl 2-propy l ester	H_2SO_3	93
6	4	19.360	1.42	Oxalic acid, cyclobutyl pentadecyl ester	$C_{21}H_{38}O_4$	69
7	5	20.287	2.42	Docosyl isobutyl ether	$C_{16}H_{34}O$	94
8	6	21.174	2.09	Octatriacontyl pentafluoropropionate	$C_{41}H_{75}F_5O_2$	87
9	7	23.663	4.65	Tetrapentacontane, 1,54-dibromo-	$C_{54}H_{108}Br_2$	83
10	8	26.570	15.78	Triacontane	$C_{30}H_{62}$	91
11				Docosane, 9-octyl-	$C_{22}H_{46}$	87
12	9	27.233	12.63	3-Eicosene, (E)-	$C_{20}H_{40}$	83
13				Heptacosane, 1-chloro-	$C_{27}H_{56}$	69
14	10	27.886	8.20	Tritetracontane	$C_{43}H_{88}Cl$	87
15				Heptacosane, 1-chloro-	$C_{12}H_{22}O_3$	95
16	11	28.515	5.69	Carbonic acid, eicosyl vinyl ester	$C_{12}H_{26}$	91
17				Heptacosane, 1-chloro-	$C_{27}H_{56}$	93
18	12	30.312	1.40	Carbonic acid, eicosyl vinyl ester	$C_{12}H_{22}O_3$	89
19				Sulfurous acid, 2-propyl tetradecyl ester	$C_{12}H_{26}$	87
20	13	30.958	0.92	Octadecane	$C_{18}H_{38}$	85
21	14	31.674	0.51	Cyclotrisiloxane, hexamethyl-	$C_{36}H_{78}O_3$	93

Key: BC = bacterial cocktail (two bacterial isolates from diesel polluted soil), CD = Cow dung, PK= Peak number, RT= Retention time, M.F=Molecular formular, M.Q=Match Quality

DISCUSSION

Species of *Micrococcus*, *Bacillus*, *Rhodococcus*, *Staphylococcus*, and *Pseudomonas* have been reported by different authors in hydrocarbon degradation (**Abioye *et al.*, 2010; Dadrasnia and Agamuthu 2013**). Of all the isolates identified, *Micrococcus luteus* strain trpE16 *and Bacillus subtilis strain* DNK UT 02 demonstrated higher ability in utilizing hydrocarbon when inoculated directly into mineral salt medium (broth) containing diesel oil as the sole source of carbon and energy (results not shown). This may be due to the ability of these organisms to secrete hydrocarbon degradative enzymes and catabolic genes for hydrocarbon degradation (**Abioye *et al.*, 2010; Dadrasnia and Agamuthu 2013; Nwogu, *et al*., 2015**).

The pH of the soil indicates that the soil was alkaline in nature. Soil bioremediated with bacterial cocktail (BC) with 10% poultry droppings (PD) + Cow dung (CD) recorded highest pH of 7.8 while autoclaved remediated soil with 5% sodium azide (NaN_3) recorded least pH of 7.1. This might be a factor that contributes to the high bacteria counts recorded in this study because bacteria survives and develops within this pH range than any other microorganisms.

Studies have reported that microbial growth in soil is controlled not by the total amount of resource available but by the scarcest resources (limiting factor), which are, in this case, Nitrogen (N) and Phosphorus (P) (**Akpoveta *et al.*, 2011; Gorban *et al*., 2011; Abioye *et al.*, 2012**). In this study, the soil bioremediated with bacterial cocktail (BC) + 10% cow dung and poultry droppings (PD + CD) had the second highest nitrogen (2.94±0.19%), highest phosphorus (39.84±0.67 mg/kg) and carbon (3.61±0.04%) content than others in the bio-remediated soil samples. These nutrients (N, P and C) in the appropriate ratio favored the proliferation of hydrocarbon-utilizing bacteria in the bio-remediated soil which in turn increase total petroleum hydrocarbon (TPH) degradation. This observation is in agreement with previous studies that reported enhanced degradation of crude oil using other animal manures such as goat manure (**Nwogu, *et al*., 2015**), poultry manure (**Odu *et al.*, 2015**). However, the autoclaved control soil with 5% NaN_3 that recorded highest amount nitrogen content (3.59±0.21-3.42±0.02 %) throughout the study period. The increase in nitrogen content is due to the addition of NaN_3 which is a nitrogenous compound. This neither favored nor stifled the activities of the hydrocarbon-utilizing bacteria (HUB) in the remediated soil. This may be due to the antimicrobial activities of NaN_3 which limit the growth and biodegradative activities of HUB in soil (**Rahmoun *et al.*, 2013**).

The HUB counts in all amended bioremediated soils were gave a higher count when compared to the control, this result is in agreement with **Abioye *et al.* (2010)** who reported the count of $\times 10^6$ cfu/g for hydrocarbon degraders in used oil polluted soil. This is found to be higher than those obtained by **Nwogu, *et al*. (2015)** who reported a count of $\times 10^5$ cfu/g for HUB in hydrocarbon contaminated soil; this could be as a result of differences in adaptability and ecological nature of the microorganisms in the experimental soils. Higher bacterial counts recorded in amended bioremediated soils might be as a result of high quantities of nitrogen and phosphorus recorded in poultry droppings and cow dung, which are essential nutrients needed for bacterial biodegradative activities (**Abioye *et al.*, 2010; Baruah and Das, 2014; Adeleke *et al.*, 2016**).

There was a progressive reduction in the total petroleum hydrocarbon during remediation in all the bioremediated soil with bacterial cocktail (BC), Poultry droppings (PD) and cow dung (CD) compared to unamended soil (control) and autoclaved remediated soil with 5% NaN_3. Although microorganisms are present in contaminated soil, their numbers might not be sufficient to initiate remediation of contaminated sites (**Nwogu *et al*., 2015**). The growth and activities of HUB must be stimulated and require essential nutrients like nitrogen, phosphorus, and carbon as building blocks. This study shows that the rate of biodegradation of diesel oil in soil bioremediated with BC + 10% (PD + CD) had the highest total petroleum hydrocarbon (TPH) degradation of 96.80% at the end of remediation (day 84).

This is consistence with kinetic parameters observed in this study (Table 3) which show that the degradation rates of diesel in soil remediated with BC + 10% (PD + CD) was higher than other treatments. This may be due to the synergetic effect of cow dung and poultry droppings resulting in high percentage of nutrient especially nitrogen and phosphorus which are needed for optimum growth and performances of HUB thus facilitating the synthesis of the necessary enzymes needed to break down the petroleum hydrocarbon contaminants (**Gorban *et al*., 2011; Okoh, 2013**). However, the rate of hydrocarbon degradation in autoclaved remediated soil with 5% NaN_3 was lower (18.42%) than even the unamended soil (control) and this may be due to the antimicrobial nature of NaN_3 (**Rahmoun *et al.*, 2013**), which limit the biodegradative activities and growth of HUB in soil.

Although, there was slow and gradual decrease in the TPH concentration in unamended soil compared to the other amended bioremediated soils. This might be attributed to other processes such as volatilization, adsorption, and abiotic factors (temperature), which have been reported to contribute to decrease in TPH concentration (**Nwogu, *et al*., 2015**). Similarly, the control soil was able to record higher TPH degradation than the autoclaved soil remediated with 5% NaN_3 because autoclaving also could inhibit the growth of the organisms and reduce the rate of biodegradation whereas unamended soil (control) contains some level of bacteria which enhance biodegradation of hydrocarbon.

Gas chromatography and mass spectrometry (GCMS) are the methods used for identification of compounds in oil compounds. In this study, gas chromatography with mass spectrometer was used to provide insight into the hydrocarbon composition of diesel contaminated soil after bioremediation. GCMS chromatogram show significant hydrocarbon degradation in all sample tested compared to natural diesel oil and the chromatograms of the bio-remediated soil revealed some peaks.

Soil bio-remediated with bacterial cocktail (BC) + 10% poultry dropping (PD) and cow dung (CD) had the highest reduction in individual hydrocarbons odd molecular weight of n- alkane (C_{11}-C_{31}), carboxylic acid (C_2H_3O – $C_{23}H_{44}O_3$), naphthalene and akyl group of naphthalene ($C_{10}H_8$ – $C_{15}H_{28}$), aromatic and polycyclic aromatic compounds compared to other bio-remediated soil at the end of study period (day 84). This result correlates with total petroleum hydrocarbon (TPH) degraded (96.80%) and kinetic parameter observed in this study and it is similar to **Dadrasnia and Agamuthu (2013)**. This may be due to the ability of the organisms to secrete enzymes (dioxygenase, cytochrome P_{450}) capable of degrading the hydrocarbon compounds and also to the synergistic effect of cow dung and poultry droppings containing high percentage of nutrient especially nitrogen and phosphorus needed for optimum growth and developments (**Gorban *et al*., 2011; Nilanjana and Preethy, 2011**).

CONCLUSION

In this study, Hydrocarbon utilizing bacteria isolated from contaminated soil were *Micrococcus luteus* strain trpE16, *Bacillus subtilis strain* DNK UT 02, *Rhodococcus* sp, *Staphylococcus* sp, and *Pseudomonas* sp. *Micrococcus luteus* straintrpE16 a*nd Bacillus subtilis* strain DNK UT 02 were used as bacterial cocktail as a result of their higher ability to utilize diesel oil as source of carbon and energy than other organisms that were isolated. Bioremediation of diesel contaminated soil by bacterial cocktail (BC) and organic wastes (cow dung and poultry droppings) recorded above 90% of diesel degradation after of 84 days. However, soil bioremediated with BC + 10% Poultry droppings (PD) + Cow dung (CD) recorded the highest bacterial counts.

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