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GRADUATE RESEARCH ARTICLE

Bioremediation of Spent Engine Oil on Selected Contaminated Soils within Ilorin Metropolis

Adeyinka, Hikmat*, Abdullahi

Department of Biosciences and Biotechnology, College of Pure and Applied Science, Kwara State University, Malete, Kwara State, Nigeria

ABSTRACT

The research aimed to investigate the bioremediation of spent engine oil on selected contaminated soils within Ilorin metropolis. To achieve this, soil samples were collected from three (3) mechanic workshops along Taiwo axis within the metropolis. The soil samples were then subjected to bioremediation using the land-farming approach. The physicochemical parameters of the soil samples before and after bioremediation were analyzed using standard methods. Bacteria were isolated using standard procedures and identified using biochemical tests and molecular methods. Results for the physicochemical parameters of the soil samples before bioremediation include particle size (all sandy in nature); pH (6.00 ± 0.14 - 6.20 ± 0.14); Organic carbon (14.65 ± 3.20 - 17.54 ± 1.87), Organic matter (33.50 ± 0.85 - 43.45 ± 9.12) and heavy metals (ND - 11.74 ± 0.07). Values after bioremediation for pH, organic carbon, organic matter and heavy metals were 8.25 ± 0.07 - 8.90 ± 0.14, 13.07 ± 0.05 - 13.25 ± 0.84, 37.25 ± 1.06 - 44.80 ± 1.13, ND - 9.40 ± 0.04 respectively. Values for bacterial count before and after bioremediation of the soil samples were 8.00 ± 1.41 - 67.50 ± 2.12 x 10^5 CFU/mL and 6.50 ± 2.12 - 164.00 ± 11.31 x 10^5 CFU/mL respectively. Bacterial isolates were identified as *Pseudomonas* sp., *Enterobacter* sp., *Acinetobacter* sp., and *Bacillus* sp. while the hydrocarbon-utilizing bacteria were identified as *Thalassospira mesophila* strain JCM 18969; *Pseudomonas fluorescens* F113; *Siccibacter turicensis* LMG 23730; *Pseudomonas Zeshuii* strain KACC 15471; *Pseudomonas stutzeri* strain CGMCC 1.1803 and *Marinobacter hydrocarbonoclasticus* strain ATCC 49840. In conclusion, the bacteria isolates effectively bioremediated the spent engine oil contaminated soils with a reduction of hydrocarbon pollutants.

Keywords: Bioremediation, Spent engine oil, Hydrocarbon-utilizing bacteria, Mechanic workshops, Contaminated Soils.

* Corresponding Author email:

sulaymanhikmat@gmail.com

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Student(s)

- Hikmat Omolara SULAYMAN

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Course Name: M.Sc. (Microbiology)
Course year: Final Year

Mentor(s)

- Adeyinka Elizabeth AJIBOYE
- Abdullahi Taiwo AJAO

1 Introduction

Engine oil is a thick mineral liquid applied to a machine or engine in order to reduce friction between the moving parts [1], [2]. Lands contaminated with spent engine oil are found mostly in developing countries of the world due to the ineffective environmental laws [3] which directly affects the rate at which the spent

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engine oil enters and pollutes the environment. Fatuyi *et al.* [4] defined engine oil as a pollutant in the environment that causes damage to the ecosystem as well as health hazard to human beings. On the account of any oil spillage, polycyclic aromatic hydrocarbons (PAHs) are important contaminant that are retained in the environment [5]. High percentage of aromatic and aliphatic hydrocarbons, nitrogen, sulphur compounds, and metals (Zn, Pb, Cr and Fe) are contained in spent engine oils than fresh oils. Some of the metals in the used engine oil could dissolve in water, move through the soil easily and may eventually be found in surface water and groundwater [6], [7].

Great damage to the soil and soil microflora has been accounted for by various researchers. This has been found to create an unsatisfactory condition for life in the soil due to poor aeration, immobilization of soil nutrients and lowering of soil pH [8]. Petroleum products with their widespread all over the world and rigorous use is strongly connected to heavy discharge of hydrocarbons into the environment [9]. Therefore, bioremediation a process by which microorganisms mediate the transformation or degradation of contaminants into non-hazardous or less hazardous substances is introduced into the environment to clean-up the hydrocarbons. It has being proven to be a simple technique, easy to maintain process, applicable over large areas, cost effective and leads to complete destruction of the contaminants [7], [10], [11]. However, some remediation approaches are energy-intensive, may require large areas of land (land-farming, soil vapour extraction, thermal desorption, etc.) and many depend on the introduction of inorganic fertilizers which rely on energy-intensive synthesis and mining of non-renewable resources, such as phosphorus [12]. The heart of bioremediation techniques are microbes like biopiles and it is advantageous to understand how soil amendments may influence the process. One approach commonly used in ecology for assessing microbial community profiles in soils is community-level physiological profiling (CLPP). Based on specific soil's heterotrophic bacterial community, different carbon sources are consumed at varying rates, thereby offering unique look at the functional carbon use in a given soil, which can lead to the statistical differentiation of soils based on EcoplatesTM data alone [13].

The effects of pollutants on the environment are detrimental and warrants immediate attention and action. When spent engine oil is released indiscriminately into free land space, and drainage systems, they could be carried about. An engine oil polluted land is unhealthy for plant proliferation, microbial survival and can be aesthetically unpleasant. Often times, people do not pay attention to the remediation of engine oil sites as more research on bioremediation has focused on crude oil, and diesel oil. Therefore, it becomes necessary to investigate the presence of natural microbial populations capable of degrading the engine oil polluted environments as well as isolate and characterize the microorganisms that are capable of degrading the contaminated soil environment. Similarly, it is important to estimate the biodegradative potential of the most promising strains as well as the factors which will influence the biodegradation potential of such pollutant. The research focuses on achieving the following:

i. Carrying out physicochemical analysis on the spent engine oil contaminated soil;
ii. Isolation and identification of naturally occurring bacteria in the engine oil contaminated soil;
iii. Determination of the total heterotrophic and hydrocarbon-utilizing bacteria in the engine oil contaminated soil;
iv. Carrying out biochemical and molecular characterization of the isolates;
v. Carrying out bioremediation of spent engine oil contaminated soil using bacterial isolates in soil supplemented with cow dung; and
vi. Determination of the physicochemical properties of the bioremediated soil.

2 Research Methodology

2.1 Preparation of Mineral Salt Medium (MSM)

This was prepared using the composition of 10 g/l of NaCl, 0.42 g/l of $MgSO_4.7H_2O$, 1.25 g/l of K_2HPO_4, 0.29 g/l of KCl, 0.83 g/l of KH_2PO_4, 0.42 g/l of $NaNO_3$, 1 % hydrocarbon source (engine oil) and 2.3 g

of agar-agar powder for mineral salt medium (MSM) agar with the same composition for mineral salt medium (MSM) broth while omitting the solidifying agent (agar-agar) [14].

2.2 Determination of Soil Parameters of the Soil Samples

Particle Size Analysis/Distribution: This was carried out by weighing 50 g of air-dried soil, after which it was passed through a 2 mm sieve and weight of the mass of sample > 2 mm taken. To 20 g of the weighed sieved soil, 25 mL of sodium diphosphate ($Na_2P_2O_7$) solution was added, left for about 8 hours to which 200 mL of water was added and shaken. The suspension was passed through a sieve set arranged in order of decreasing pore size, and placed directly above a 1 litre measuring cylinder. Sieves were then dried in an oven at 105 ^{0}C until a constant weight was achieved with each sieve weighed before and after sieving the suspension [15], [16]. **Bulk Density:** This was determined using an already oven-dried soil sample whose constant weight has been achieved. Known weight of the soil sample was then gently poured little at a time into a measuring cylinder while gently tapping the cylinder to compact it in order to measure the volume [15], [16]. **Exchangeable Cations:** Five (5) g of the soil was weighed and placed in a 100 mL polyethylene bottle. To this, (25) mL of ammonium acetate solution was added and the mixture shaken for 1 hour for filtration of the supernatant directly into a 100 mL volumetric flask through a filter paper held in a funnel inserted in the neck of the flask, leaving the soil in the bottle. Twenty (20) mL of 95 % ethanol was then added to the bottle and shaken. This was allowed to settle and the supernatant filtered into the same 100 mL flask as before. The extract was made-up to 100 mL with distilled water and the concentrations of exchangeable cations (Ca^{2+}, Mg^{2+}, Na^{+}, Mn^{2+} and K^{+}) determined using Atomic Absorption Spectrophotometer Model: BUCK Scientific ACCUSYS 211 [15], [16]. **Soil pH:** This was achieved by preparing a 10% (w/v) suspension of air-dried soil in double distilled water, mixed thoroughly, allowed to settle for 1 hour and filtered through the Whatmann filter paper. The pH for all the soil filtrates was then checked using a calibrated pH meter [17]. **Total Organic Carbon:** This was carried out using soil samples already sieved through 1 mm sieve. One (1) g of the sieved soil sample was placed in a 100 mL flask and to it, 10 mL potassium dichromate and 20 mL sulphuric acid was then added and shaken very well. The mixture was allowed to cool on asbestos sheet and the volume was made up of 100 mL with distilled water and kept overnight. The optical density was then measured at 660 nm wavelength using a spectrophotometer [18]. **Total Organic Nitrogen:** Nitrogen content of the contaminated soil was determined by Macro-kjeldahl method [19]. **Available Phosphorus:** This was achieved using Vanado-molybdo- phosphoric acid colometric method by using ammonium molybdate which forms molybdo-phoshoric acid under acidic condition. The intensity of the yellow colour was measured using spectrophotometer at 490 nm [20]**. Electrical Conductivity:** In achieving this, 10 g of the soil sample was weighed and dissolved in 100 mL distilled water. The conductivity cell was rinsed with three (3) portions of the sample and then immersed in sufficient volume of the sample. The conductivity meter was then turned on and the conductivity of the sample recorded [21]. **Heavy Metal Concentration:** Cd, Pb, Cu, Zn, Cr, and Fe were estimated by weighing 0.5 g of the dried soil and digested with conc. HNO_3, H_2SO_4 and H_2O_2 in the ratio 2:6:6. The blanks were run in a set, and the heavy metals present in the samples determined using Atomic Absorption Spectrophotometer Model: BUCK Scientific ACCUSYS 211 [22].

2.3 Determination of Hydrocarbon-utilizing Bacterial Counts

Enumeration for counts was carried out using oil agar (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 and 1 mL of spent engine oil in 1000 mL distilled water). The oil agar plates were then incubated at 30 ^{0}C for 7 days before visible growth was observed and the colonies were thereafter counted [23].

2.4 Determination of Total Heterotrophic Bacterial Counts

This was established using the pour plate method. Serial dilution was carried out on the soil sample collected from each site and 1 mL of the aliquot from each of the dilution was inoculated onto nutrient agar plates

in duplicates. The plates were then incubated at 37 ^{0}C for 24 hours for determination of the total heterotrophic bacteria counts [18].

2.5 Characterization and Identification of the Bacterial Isolates

The bacteria isolates obtained were characterized and identified by their Cellular Morphology (microscopically), Biochemical Test as well as by Molecular Characterization.

2.5.1 Cellular Morphology

This was observed under a compound microscope and in turn used for identification of the isolates. The cellular characteristics include:

Gram Staining: This was used to differentiate the bacteria isolates into either Gram positive or Gram negative. This was done by preparing thin smear of the bacteria isolates from the pure culture on clean, grease free-slides. The smears were air-dried and then heat fixed by passing the slide over flame a few times. The prepared smears were then flooded with crystal violet for 60 seconds, drained, flooded with Gram's iodine for 60 seconds and rinsed with distilled water. The smears were then decolourized using 95 % alcohol for 5 seconds and immediately rinsed with distilled water. The smears were thereafter counter-stained with safranin for 30 seconds, rinsed with distilled water and allowed to air-dry. The slide was then examined under oil immersion (X100) objective. Gram positive cells stained purple to blue while Gram negative cells stained pink to red. The cell arrangement as well as the cell shape was also observed in the course of viewing under the microscope [24]. **Spore Staining:** Thin smear of each isolate was prepared on clean, grease-free slides; air-dried and heat-fixed. The slides of the isolates were then arranged on staining racks over a pot of steaming water to stimulate spore production. On steaming, the smears were flooded continuously with malachite green for 10 minutes. The stain was then washed off with water and the smears further counter-stained with safranin for 20 seconds; which was in turn washed-off with distilled water. The slides were thereafter viewed under oil immersion objectives. Presence of spores was determined by the presence of green coloured spores inside pink coloured cells [24].

2.5.2 Biochemical Test

This was carried out for each isolate to identify them. The tests include:

Catalase Test: A loop-full of each isolate was emulsified on different slides and a drop of 3 % hydrogen peroxide was added to it. Effervescence, caused by the liberation of oxygen as gas bubbles indicated the production of catalase, thus such organism was catalase positive, otherwise catalase negative [24]. **Citrate Utilization Test:** The test was based on the ability of an organism to utilize citrate as its only source of carbon. Simmon citrate agar was prepared in McCartney bottles and autoclaved at 121 ^{0}C for 15 minutes following manufacturer's instruction. Using a sterile wire-loop, each of the bacterial isolate was picked and inoculated into the slanted citrate agar. This was then incubated at 37 ^{0}C for 24 hours. The utilization of citrate was indicated by a colour change from dark green to blue [24]. **Oxidase Test:** This test was used for the identification of organisms that produces the enzyme oxidase. For each bacterial isolate, a colony from pure culture slant was rubbed on filter paper after which a drop of 1 % tetramethyl–p–phenylenediamine dihydrochloride solution was placed on the colony. Presence of the enzyme oxidase was determined by the formation of purple colour within 1 - 30 seconds of application [24]. **Methyl Red and Voges Proskauer (MR-VP) Test:** Methyl red (MR) test was used to determine whether or not glucose produced can be converted to acidic products like lactate, acetate and formate due to increase in pH while Vogas Proskauer (VP) test was used to identify those bacteria that ferments glucose leading to the formation of 2,3-butanediol accumulation in the medium. Methyl red and Voges Proskauer (MR-VP) broth was prepared according to the manufacturer's specification and dispensed into different McCartney bottles and autoclaved. Each bacterial isolate was then inoculated aseptically into the two (2) bottles. After incubation for 72 hours at 37 ^{0}C, reagents for methyl red test and Voges Proskauer test was added respectively to the two (2) bottles for each of the organisms. For MR test, five (5) drops of methyl red indicator was added into the incubated isolates. Colour change from yellow to red indicated a positive result while no change in colouration indicated a negative result. For VP test; 1mL of 5 % α-naphthol, followed by 1 mL of 40 %

NaOH containing 0.3 g of creatinine was added respectively. Development of red colour in the culture medium 1 hour following the addition of the reagent represented a positive VP test; while the appearance of dirty brown was indicative of a negative VP result [24]. **Indole Test:** This test was used to detect those bacteria that could breakdown the amino acid tryptophan to release indole, pyruvic acid and ammonia catalysed by tryptophanase. Tryptone soy broth was prepared in McCartney bottles and autoclaved according to the manufacturer's specification. The broth medium was then inoculated with the isolates at 37 ^{0}C for 48 hours. After incubation, 2 mL of chloroform and 2 mL Kovac's reagent was added respectively to each McCartney bottle. After 20 minutes, those bacteria that produces a red ring in the tube were considered indole positive while absence of red ring in the broth was indicative of a negative result for indole production [24]. **Growth in 6.5% NaCl Broth:** To 100 mL of nutrient broth, 6.5 g of NaCl was added to make 6.5 % NaCl-nutrient broth complex. Bacterial isolates were then inoculated into each of the broth and incubated at 37 ^{0}C for 24 hours. Ability of each isolate to tolerate the NaCl was determined by turbidity of the broth after incubation [24]. **Growth in 7.5% NaCl Broth:** To 100 mL of nutrient broth, 7.5 g of NaCl was added to make 7.5 % NaCl-nutrient broth complex. Bacterial isolates were then inoculated into each of the broth and incubated at 37 ^{0}C for 24 hours. Ability of each isolate to tolerate the NaCl was determined by turbidity of the broth after incubation [24]. **Sugar Fermentation Test:** This test was used to determine the ability of bacterial isolates to ferment certain carbohydrates and produce acidic products and gas. The sugars tested for included lactose, glucose, maltose and mannitol. To every 100 mL of nutrient broth in different conical flasks for each sugar, 0.5 g of each sugar was added respectively as well as phenol red indicator, and then the broths boiled. Ten (10) mL of the mixture was then dispensed into different McCartney bottles and Durham tubes inverted into the bottles, and the broths then autoclaved. Thereafter, bacterial isolates were inoculated into the different broth for all the sugars which was then incubated at 37 ^{0}C for 48 hours. Colour change from red to yellow after incubation was indicative of positive result; implying the sugar was fermented. Displacement of broth in Durham tubes indicated gas production [24]. **Triple Sugar Iron Agar Test:** This test is generally used for the identification of enteric bacteria and also used to distinguish the *Enterobacteriaceae* from other gram-negative intestinal bacilli by their ability to catabolize glucose, lactose, or sucrose, and to liberate sulphides from ferrous ammonium sulphate or sodium thiosulfate. For the test, triple sugar iron agar was prepared according to the manufacturer's specification and dispensed into test tubes, autoclaved and slanted slightly to form a slope after autoclaving. The agar was then inoculated by streaking the surface of each slant with each isolate, then stabbed deep down into butt. Tubes were thereafter incubated and observed for 24 hours after incubation in order to detect the presence of sugar fermentation, gas production, as well as H_2S production. Colour change from red to yellow indicated acid production, red indicated alkaline reaction and black colour indicated the production of H_2S while pushing up or splitting of the agar from the butt indicated gas production [24]. **Coagulase test:** This test was done using the slide coagulase test method to differentiate Gram positive cocci. A loop-full of bacterial culture was placed on a clean, grease-free slide. Thereafter, a loop-full of plasma was placed on the culture and stirred to emulsify the mixture. The suspension was then observed for clumping which indicates a positive result otherwise, it was regarded as negative [24].

2.6 Bioremediation of Spent Engine oil in Soil Microcosms

In carrying this out, 250 mL of stock each for the isolated organisms was prepared using Mineral Salt Medium (MSM) broth [14]. Each of the hydrocarbon-utilizing bacteria kept as stock in the refrigerator was then inoculated into the individual broth and incubated at 37 ^{0}C for 8 hours after sterilization. On incubation, the broths were introduced collectively into the combined contaminated soil samples collected from the three (3) mechanic workshop and mixed thoroughly. A control sample was set aside containing only the contaminated soils. The soil samples were then monitored for parameters which included pH, total bacterial count, percentage moisture content and percentage organic matter for 15 days at 3 days interval.

2.7 Enhancing Treatment through the Addition of Organic Waste – Biochar

Biochar Production: Collection of cow dung used for the enhancement was from the Kwara State University (KWASU), Malete premises. In producing the biochar, a furnace at the University of Ilorin,

Microbiology Laboratory was subjected to a pyrolysis temperature of 600 ^{0}C for 3 hours based on the recommendation of Lehmann *et al.* [25]. **Biochar pH:** In determining this, a biochar to water ratio of 1:5 was prepared (appropriate for organic materials because of their ability to absorb more water) and the pH was determined using a standardized pH meter calibrated at pH 4, 7 and 9 [26]. **Calcium (Ca), Magnesium (Mg), Sodium (Na) and Potassium (K) Content of Biochar:** In doing this, 0.5 g of the biochar sample was added into a crucible and then placed into a furnace for 1 hour at a temperature of 600 ^{0}C. The ash was later digested with 10 mL of nitric acid, accompanied by heating to speed up the rate of the reaction. This was then transferred into a 100 mL flask and topped up with distilled water to the 100 mL mark. The exchangeable cation contents were then determined on the Atomic Absorption Spectrophotometer (AAS) Model: BUCK Scientific ACCUSYS 211 [26]. **Amendment of Soil with Biochar:** Amendment of the contaminated soil was done with cow dung; biochar at 0 (control soil) and 5 g/pot (bioremediated soil), and then replicated. Thereafter, the set up was left to stand under a shade at ambient temperature and samples taken at an interval of 3 days consecutively for 15 days. The set-up was used for the determination of parameters like total bacterial count, pH, percentage organic matter content as well as percentage moisture content. Both the bioremediated and control samples were moistened daily in other to avoid the soil from drying-up.

2.8 Monitoring of Parameters

pH: Changes in pH of the amended soil containing biochar as well as the control soil was monitored over the course of the bioremediation period. In doing this, known gram of the soil samples were taken and distilled water introduced into them. The mixtures were then shaken vigorously to obtain homogenized solution. The pH electrode was standardized using buffer solutions of pH 4, 7 and 9 after which pH readings were taken on the pH meter scale by dipping the glass electrode into the soil solution [27]. **Total Bacterial Count:** This was determined quantitatively by taking 1 g each of the control and bioremediated soil samples and then making serial dilutions up to 10^{-5}. Sterile molten nutrient agar was then poured aseptically into each Petri dish and the plates swirled gently to mix the inoculum and agar properly. On solidification the plates were incubated upside down at 37 ^{0}C for 24 hours. The numbers of colonies on each plate was then counted with the aid of colony counter and expressed as CFU/mL [28]. **Percentage Moisture Content:** This was carried out by making use of a machine referred to as Moisture Analyzer (RADWAG PCM 50/1 402496). Each of the soil sample (control and bioremediated) was weighed into a pre-weighed aluminum dry-dish used for the analyzer. On completion, the percentage moisture content of the soil sample was displayed on the screen of the machine and the result recorded [29]. **Percentage Organic Matter:** This was achieved by determining the mass of an empty, clean and dry porcelain dish and recorded. Thereafter, the entire oven-dried test specimen from the moisture content experiment was placed in the porcelain dish, and the mass of the dish and soil specimen was determined and recorded. The dish was then placed in a muffle furnace and the temperature in the furnace gradually increased to 440 ^{0}C and left overnight. The porcelain dish was then carefully removed using tongs and allowed to cool at room temperature after which the mass of the dish containing the ash was determined and recorded. The dish was then emptied and cleaned [30].

2.9 Statistical Analysis

Each set of the experimental data was collected in replicates and the analytical results were taken as the mean of the replicated measurements. The standard deviations (error bars) and statistical significance (5% level of significance) was determined with IBM SPSS Statistic 20 using (analysis of variance) ANOVA (One-way ANOVA).

3 Results and Discussion

The physicochemical parameters of the soil samples before bioremediation is presented in Table 1. One critical factor for microbial growth and survival is soil pH. A pH of near neutral is suitable for growth of

diverse bacterial populations [31]. The pH values for the three (3) soil samples before undergoing bioremediation indicated all the samples to be slightly acidic which could be due to the fact that hydrocarbons contain many free cations causing them to have properties of a weak acid. pH also influences the solubility and accessibility of soil components which indirectly influence biological activity in the soil [32].

Table 1: Physicochemical Parameters of the Soil Samples before Bioremediation

Soil Parameters (Units)	Sampling Sites / Values of Physicochemical Parameters		
	A	B	C
Particle Size:			
Silt (%)	5.04 ± 0.00^{a}	5.04 ± 0.00^{a}	5.00 ± 0.00^{a}
Clay (%)	ND	ND	ND
Sand (%)	91.96 ± 0.00^{a}	91.96 ± 0.00^{a}	91.99 ± 0.00^{a}
Bulk Density (g/cm^3)	1.38 ± 0.00^{c}	1.20 ± 0.00^{b}	1.03 ± 0.00^{a}
Soil pH	6.20 ± 0.14^{a}	6.15 ± 0.21^{a}	6.00 ± 0.14^{a}
Total Organic Carbon (%)	14.65 ± 3.20^{a}	16.87 ± 0.07^{a}	17.54 ± 1.87^{a}
NO_3^{2-} (mg/kg)	17.85 ± 0.07^{a}	27.80 ± 0.00^{c}	24.90 ± 0.14^{b}
PO_3^{2-} (mg/kg)	23.80 ± 0.14^{a}	39.05 ± 0.07^{c}	24.60 ± 0.14^{b}
Exchangeable Cations:			
Ca^{2+} (mg/kg)	8.15 ± 0.94^{a}	13.94 ± 0.72^{b}	10.25 ± 0.58^{a}
Mn^{2+} (mg/kg)	8.25 ± 0.07^{c}	7.44 ± 0.00^{b}	4.02 ± 0.16^{a}
Mg^{2+} (mg/kg)	6.82 ± 0.07^{b}	6.92 ± 0.07^{b}	6.66 ± 0.00^{a}
Na^{2+} (mg/kg)	14.25 ± 0.00^{b}	14.15 ± 0.00^{b}	6.66 ± 0.43^{a}
K^{+} (mg/kg)	3.49 ± 0.00^{c}	3.08 ± 0.00^{b}	2.51 ± 0.07^{a}
Electrical Conductivity(ms/cm)	0.58 ± 0.00^{b}	0.33 ± 0.00^{a}	0.42 ± 0.01^{c}
Heavy Metals:			
Cd (mg/kg)	0.02 ± 0.00^{a}	0.02 ± 0.00^{a}	0.02 ± 0.00^{a}
Pb (mg/kg)	0.87 ± 0.07^{a}	0.87 ± 0.07^{a}	0.62 ± 0.00^{a}
Cu (mg/kg)	11.74 ± 0.07^{c}	1.27 ± 0.04^{b}	0.80 ± 0.01^{a}
Zn (mg/kg)	2.45 ± 0.00^{a}	2.67 ± 0.00^{c}	2.49 ± 0.00^{b}
Cr (mg/kg)	ND	ND	ND
Fe (mg/kg)	12.20 ± 0.00^{a}	12.86 ± 0.07^{b}	12.04 ± 0.07^{a}
Organic Matter (%)	38.30 ± 2.40^{a}	33.50 ± 0.85^{a}	43.45 ± 9.12^{a}

Key: Site A = Galadima 1 Site B = Oko-Erin 1 Site C = Oko-Erin 2 ND = Not Detected

Values are means of duplicate reading and standard deviation of soil parameters of the soil samples from the three (3) sampling sites.

Values in the same row with different superscript are significantly different at < 0.05 level of significance.

The soil pH value obtained was similar to that detected by [33] who reported a range of pH values (5.96 – 8.88) for top soil samples. The pH values also fall within the optimal soil pH range recommended to support bacterial growth which is from 6 to 8 [31], [34], [35] as well as the optimum soil pH for efficient bioremediation which is 5.5 to 8.8 [36]. Soil pH is of importance because most microbial species can survive only within a pH range. In addition, soil pH can affect availability of nutrients [23].

Physical properties of soil like soil texture and bulk density have been considered to be very important for bioremediation because several factors affecting the degradation process like soil aeration, movement of nutrients through soil pores, water holding capacity etc. are under the direct and indirect influence of soil physical properties [37]. Increased ventilation has a direct impact on microbial growth, which can enhance the biodegradation of petroleum compounds [38]. Result of the particle size analysis for the three (3) sampling sites was indicative that all the soil was sandy in nature. The particle size distribution is known to have an influence on the bacteria diversity of soils [39].

Physicochemical property of the spent engine oil contaminated soil collected from the mechanic workshops revealed that some heavy metal content of the soil was high and above the National Environmental Regulation of soil quality standard. According to European Committee for Standardization (ECS), most of these heavy metal content in used engine oil contaminated soil are high above the safe limit for lead, nickel, copper, zinc and cadmium respectively. Generally, all the heavy metals (Cd, Pb, Cu, Zn, Cr and Fe) concentrations recorded for the respective soil samples have values ranging from ND to 12.81 mg/kg. Ipeaiyeda and Dawodu [40] reported values ranging from 0.05 mg/kg for zinc to 184 mg/kg for lead. Ilembayo and Kolade [33] also reported values ranging from 730.97 mg/kg for zinc to 217 mg/kg for lead.

Counts for the total heterotrophic as well as hydrocarbon-utilizing bacteria found in the soil samples is presented in Table 2. The average total heterotrophic bacterial count supports the statement that high polycyclic aromatic hydrocarbon (PAHs) concentration usually results in low moisture content which in turn inhibits microbial growth, and hence low CFU count which correlates with previous findings with Yuan *et al.* [41].

Table 2: Total Heterotrophic Bacteria and Hydrocarbon-utilizing Bacteria Counts of the Soil Samples

Sampling Sites	Total Heterotrophic Bacteria (10^5 CFU/mL)	Hydrocarbon-utilizing Bacteria (10^5 CFU/mL)
A	16.50 ± 0.71^b	13.50 ± 7.78^a
B	23.50 ± 12.02^b	18.50 ± 0.71^a
C	67.50 ± 2.12^c	8.00 ± 1.41^a

Key: Site A = Galadima 1 Site B = Oko-Erin 1 Site C = Oko-Erin 2

Values are means of duplicate reading and standard deviation of bacteria counts from the soil samples collected from three (3) mechanic workshops. Values in the same column with different superscript are significantly different at < 0.05 level of significance.

The cellular morphology and biochemical characteristics of the total heterotrophic bacteria isolated from the soil samples of the three (3) mechanic workshops under study is presented in Table 3. The isolates were *Enterobacter* sp., *Pseudomonas* sp., *Acinetobacter* sp. and *Bacillus* sp. with *Pseudomonas* sp. been isolated twice.

Table 3: Cellular Morphology and Biochemical Characteristics of the Total Heterotrophic Bacteria

Bacterial Isolates	Cellular Characteristics		Biochemical Characteristics											Tentative Identification
	Gram's Reaction	Cell Shape	Catalase Test	Citrate Test	Oxidase Test	Indole Test	Methyl Red (MR) Test	Voges Proskauer (VP) Test	Growth in 6.5% NaCl	Growth in 7.5% NaCl	Coagulase Test	Triple Sugar Iron (TSI) Test	Sugar Fermentation Test	
												H_2S	Mannitol	
T_1	-	Rod	+	+	+	-	-	+	-	-	-	-	+	*Enterobacter* sp.
T_2	-	Rod	+	+	+	+	ND	ND	-	-	-	+	+	*Pseudomonas* sp.
T_3	-	Cocci-Rod	+	+	+	+	-	+	+	+	-	+	+	*Acinetobacter* sp.
T_4	-	Rod	+	+	+	+	-	+	+	+	-	+	+	*Pseudomonas* sp.
T_5	+	Rod	+	+	+	+	+	+	-	-	-	+	+	*Bacillus* sp.

Key: + = Positive - = Negative ND = Not Determined

T_1 to T_5 represent the total heterotrophic bacterial isolates

Result for the hydrocarbon-utilizing bacteria isolated from the soil samples of the three (3) mechanic workshops under study is presented in Table 4. The isolates were identified as: *Thalassospira mesophila* strain JCM 18969; *Pseudomonas fluorescens* F113; *Siccibacter turicensis* LMG 23730; *Pseudomonas Zeshuii* strain KACC 15471; *Pseudomonas stutzeri* strain CGMCC 1.1803; and *Marinobacter hydrocarbonoclasticus* strain ATCC 49840 based on their percentage identity with the organisms in the GenBank.

Table 4: Cellular Morphology, Biochemical Characteristics and Molecular Identification of the Hydrocarbon-utilizing Bacteria

Bacterial Isolates	Cellular Characteristics				Biochemical Characteristics										Molecular Identification
	Gram's Reaction	Cell Shape	Arrangement	Spore Staining	Catallase Test	Citrate Test	Oxidase Test	Indole Test	Growth in 6.5% NaCl	Growth in 7.5% NaCl	Coagulase Test	Sugar Fermentation Test			
												Glucose	Lactose	Maltose	
H_1	-	Rod	Chain	-	+	+	+	-	+	-	-	+ No Gas	+ No Gas	+ No Gas	*Thalassospira mesophila* strain JCM 18969
H_2	-	Rod	Chain	-	+	-	+	-	+	-	-	+ No Gas	+ No Gas	+ No Gas	*Pseudomonas fluorescens* F113
H_3	-	Cocci	Chain	-	+	+	+	+	+	+	-	+ Gas	+ No Gas	+ No Gas	*Siccibacter turicensis* LMG 23730
H_4	-	Rod	Cluster	-	+	-	+	-	+	+	-	+ No Gas	- No Gas	+ No Gas	*Pseudomonas Zeshuii* strain KACC 15471
H_5	-	Rod	Chain	-	+	-	+	-	+	+	-	- No Gas	- No Gas	- No Gas	*Pseudomonas stutzeri* strain CGMCC 1.1803
H_6	-	Rod	Singly	-	+	-	-	-	+	-	-	- No Gas	- No Gas	- No Gas	*Marinobacter hydrocarbonoclasticus* strain ATCC 49840

Key: + = Positive - = Negative

H_1 to H6 represent the hydrocarbon-utilizing bacterial isolates

Hydrocarbon degraders may be expected to be readily isolated from an oil associated environment; the same degree can be isolated from a totally related environment such as soil [42]. The isolation of these organisms from the environment shows that these organisms have evolved strategies of adapting to the environment and/or utilizing these substances as energy sources [43]. Other studies by [44], [45], [46] reported some bacteria species belonging to the genera in hydrocarbon degradation as a sole source of carbon and energy.

The parameters tested for in the biochar used for amendment of the soil sample used in bioremediation is presented in Table 5. From the result, the biochar was found to be neutral in nature.

Table 5: pH, Calcium (Ca), Magnesium (Mg), Sodium (Na), Potassium (K) Content and Percentage Ash Content of Biochar

Parameters (Unit)	Value
pH	7.00 ± 0.28
Calcium (Ca) (mg/kg)	7.53 ± 1.66
Magnesium (Mg) (mg/kg)	4.09 ± 0.01
Sodium (Na) (mg/kg)	3.84 ± 0.07
Potassium (K) (mg/kg)	5.07 ± 0.21
Ash Content (%)	77.68 ± 1.07

Values are means of duplicate reading and standard deviation of pH, Calcium (Ca), Magnesium (Mg), Sodium (Na), Potassium (K) Content and Percentage Ash Content of Biochar to be supplemented into the bioremediated soil.

The counts for the total bacterial during the course of bioremediation is presented in Table 6. From the result, there was a steady increase in counts of the total bacteria from day 0 to day 9 for the bioremediated soil sample with a sharp decrease in count in day 12 and day 15. This was unlike the result of the control soil sample whose count decreased steadily from day 0 to day 6, had a pick on day 9 and decreased back in day 12 and day 15 respectively. The higher counts of bacteria in the amended soil as compared to the control might also be due to the fact that the organic waste was able to neutralize the toxic effects of the oil on the microbial population by rapid improvement of the soil physicochemical properties [47], [48].

Table 6: Total Bacterial Count of Soil Samples during Bioremediation

Soil Samples	Period of Bioremediation (Days) / Total Bacterial Count (10^5 CFU/mL)					
	0	**3**	**6**	**9**	**12**	**15**
Control	13.50 ± 6.36^{a}	11.00 ± 5.66^{a}	6.50 ± 2.12^{a}	60.00 ± 9.90^{a}	37.50 ± 10.61^{ab}	36.50 ± 23.33^{a}
Bioremediated	7.00 ± 2.83^{a}	42.50 ± 10.6^{b}	105.50 ± 65.76^{a}	164.00 ± 11.31^{b}	95.50 ± 34.65^{b}	36.50 ± 31.82^{a}

Values are means of duplicate reading and standard deviation of bacteria counts from the soil samples for the control and bioremediated soils. Values in the same column with different superscript are significantly different at < 0.05 level of significance.

The pH, percentage organic matter content and percentage moisture content of soil samples during the course of bioremediation is presented in Table 7. From the result, a steady decrease in the pH of the bioremediated soil sample was observed from day 0 to day 9. The decrease in pH is a pointer to the fact that bioremediation of engine oil in contaminated soil increases with increase in bioremediation period. This reduction could also be as result of acidic metabolites resulting from engine oil bioremediation. Also, change in pH has been found to have an effect on the biodegradative activity of microbial populations, as well as on the solubilisation and absorption/desorption of ions and pollutants [49].

Table 7: pH, Percentage Organic Matter Content and Percentage Moisture Content of Soil Samples during Bioremediation

Day of Bioremediation	pH		% Organic Matter		% Moisture Content	
	Control	Bioremediated	Control	Bioremediated	Control	Bioremediated
0	6.25 ± 0.07[a]	7.15 ± 0.07[b]	91.30 ± 0.14[b]	58.48 ± 0.01[a]	2.59 ± 0.28[a]	6.59 ± 0.29[b]
3	6.50 ± 0.28[a]	6.35 ± 0.21[a]	28.80 ± 0.85[a]	32.56 ± 1.63[a]	2.25 ± 0.07[a]	3.33 ± 0.19[b]
6	6.20 ± 0.28[a]	6.30 ± 0.00[a]	32.25 ± 0.07[a]	33.35 ± 0.35[b]	3.71 ± 0.05[b]	1.75 ± 0.01[a]
9	6.10 ± 0.00[b]	5.75 ± 0.07[a]	72.60 ± 0.85[a]	73.65 ± 0.07[a]	4.69 ± 0.14[b]	3.58 ± 0.02[a]
12	8.80 ± 0.14[a]	8.80 ± 0.00[a]	23.90 ± 0.14[a]	24.65 ± 0.35[a]	5.69 ± 0.04[b]	4.52 ± 0.44[a]
15	8.10 ± 0.14[b]	7.75 ± 0.07[a]	25.25 ± 0.35[a]	31.65 ± 1.78[b]	2.63 ± 0.05[a]	6.44 ± 0.29[b]

Values are means of duplicate reading and standard deviation of physicochemical parameters determined after bioremediation. Values along the same row for each parameter at different days with different superscript are significantly different at < 0.05 level of significance.

The physicochemical parameters of the soil samples after bioremediation is presented in Table 8. There was significant difference among the physicochemical parameters observed in the study and the activities of the microbes on the samples. The pH of the sample was significantly different as the day progressed between the control and bioremediated soil except for the pH of day 12 which showed no significant difference. Okonokhua *et al.* [50] reported that there was no significant difference between the pH of the control and spent oil treated soil in their study while Shahida *et al.* [51] reported pH of neutral from 7.02 - 7.5 in different points and slightly alkaline in one point of soil contaminated with hydrocarbons.

Table 8: Physicochemical Parameters of the Soil Samples after Bioremediation

Soil Parameters (Units)	Soil Sample	
	Control	Bioremediated
Soil pH	8.90 ± 0.14[b]	8.25 ± 0.07[a]
Total Organic Carbon (%)	13.25 ± 0.84[a]	13.07 ± 0.05[a]
NO_3^{2-} (mg/kg)	26.57 ± 0.12[a]	26. 61 ± 0.02[a]
PO_3^{2-} (mg/kg)	13.95 ± 0.07[a]	15.55 ± 0.07[b]
Heavy Metals:		
Cd (mg/kg)	ND	ND
Pb (mg/kg)	0.308 ± 0.00	ND
Cu (mg/kg)	1.066 ± 0.00	ND
Zn (mg/kg)	0.103 ± 0.00[a]	0.103 ± 0.00[a]
Fe (mg/kg)	9.40 ± 0.04[b]	0.021 ± 0.00[a]
Organic Matter	44.80 ± 1.13[b]	37.25 ± 1.06[a]

Values are means of duplicate reading and standard deviation of physicochemical parameters after bioremediation. Values in the same row with different superscript are significantly different at < 0.05 level of significance.

The decrease in total organic carbon recorded for the bioremediated soil samples compared with the control sample is an indication of effective hydrocarbon degradation as a result of increased microbial activities. Use of hydrocarbon as the sole source of carbon by hydrocarbon degraders helps in the clean-up of oil component in the process [49]. There was a gradual and significant reduction in the content of heavy metals

present in the bioremediated soil as compared to the control soil. Adenipekun *et al.* [52] also observed decrease in Mn, Pb, Ni and Cu in soil contaminated with fresh and spent cutting fluid after 2 months even in the control while Adams *et al.* [3] reported a gradual decrease in the different metals analyzed in the spent oil contaminated site as the experimental days progressed. Asadu *et al.* [53], pointed out that build-up of these metals (Zn, Cu, Pb and Cd) to critical levels could be phytotoxic and might result in reduced plant growth and increase within the food chain.

Significant difference at $P < 0.05$ in the statistical analysis carried out between the amended soil and the un-amended polluted soil (control) is an indication of the positive contribution of organic waste (biochar) to the biodegradation of spent engine oil in the soil. There was significant difference between the pH, PO_3^{2-} as well as the organic matter content of the control and bioremediated soil. There was no significant different between total organic carbon and NO_3^{2-} of the control and bioremediated soil.

4 Conclusions

The result obtained from the research revealed that soils within the premises of the mechanic workshops are good sources of hydrocarbon-utilizing bacteria namely: *Thalassospira mesophila* strain JCM 18969, *Pseudomonas fluorescens* F113, *Siccibacter turicensis* LMG 23730, *Pseudomonas Zeshuii* strain KACC 15471, *Pseudomonas stutzeri* strain CGMCC 1.1803 and *Marinobacter hydrocarbonoclasticus* strain ATCC 49840. These bacteria isolates can be effectively utilized in bioaugmentation aimed at the removal of hydrocarbon pollutants from contaminated environments especially those located within the residential vicinity. It is recommended that more research be done by introducing the hydrocarbon-utilizing bacterial isolates singly and then in combination randomly to treat the spent engine oil contaminated soil. Also, the introduction of the bacterial isolates on individual soils rather than on a combination of soil could be further researched on to ascertain their efficiency. Further studies need to also be done to confirm that these organisms can be used for bioremediation of engine oil contaminated soil. Although many recommendations have been issued in various research papers on this subject, education, awareness and sensitization of people will help to reduce problems associated with engine oil contamination. Also, the development of effective regulatory policies on the environment in order to prevent contamination of soil by spent engine oil will go a long way in addressing this issue of public health concern.

5. Competing Interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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