Effect of Agro-Waste on Bioremediation of Soil Polluted with Spent Hydrocarbon in Keffi, Nasarawa State, Nigeria.

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ABSTRACT

Petroleum hydrocarbon is a major environmental pollutant throughout the world. This study was aimed at the effect of agro-wastes on bioremediation of soil polluted with spent hydrocarbon in Keffi**.** Hydrocarbon contaminated soils were collected from three (3) different auto mechanic workshops in Keffi, Nasarawa State Nigeria. The agro wastes used as stimulants were corncobs, fruits peels and melon shells. The hydrocarbon utilization was determined using gravimetric analysis. The hydrocarbon utilization bacteria were isolated and identified using standard microbiological method. The hydrogen ions (pH) of the contaminated soil sample were 4.5. pH of corncobs (CB) was 6.2, melon shells (MS) was 5.6 and fruits peels (FP) was 7.6. The value of total nitrogen for contaminated soil was 0.45 %, when amended with CB was 6.2%, MS was 1.26% and FP was 3.56%. Organic carbon in contaminated soil prior to study was 5.80 %, when amended with CB was 9.03%, MS was 7.55% and FP was 10.12%. The Phosphorous in contaminated soil was 8.41 mg/kg, when amended with CB was 13.40 mg/kg, MS was11.82mg/kg and FP was 15.61 mg/kg. Effect of time in reduction of hydrocarbon from contaminated soil amended with CB recorded highest after 40days (44.98 mg/kg) and the least (10.31 mg/kg) after 10days. Contaminated soil amended with CB the highest reduction was at 30 ℃ (48.92 mg/kg) and the least was at 35℃ (19.21mg/kg). From FP the highest reduction was at 30℃ (48.04 mg/kg). The highest degrading bacteria count was recorded from Keffi garage mechanic workshop (4.04 x10^6 cfu g/ml) and the lowest was from pyanko road mechanic workshop (1.18 x10^6 cfu g/ml). The highest bacteria isolated from none amended contaminated soil was *Flavobacterium* sp (33.3 %) and the lowest was *Bacillus subtilis* and *Proteus* sp (33.3%). From amended soil with CB the highest were *Corynebacteria* spp and *Flavobacterium* sp (100%) and the lowest were *Bacillus subtilis*, *Pseudomonas flourescens*, and *Proteus* sp (66.6%). From contaminated soil amended with MS the highest bacteria isolated were *Flavobacterium* sp and *Corynebacteria* sp (33.3%) and the lowest were *Pseudomonas flourescens* and *Proteus* sp (33.3 %). From contaminated soil amended with FP the highest bacteria isolated were *Pseudomonas flourescens* and *Proteus* sp (100%) and the lowest were *Bacillus subtilis*, *Flavobacterium* sp and *Corynebacteria* sp (66.6%). It is recommended that the environmental agencies in the country give consideration to agricultural waste products for bioremediation of hydrocarbon polluted soil in Nigeria.

Keywords: environmental pollutant, amended, contaminated soil, reduction, hydrocarbon

INTRODUCTION

Petroleum hydrocarbon is a major environmental pollutant throughout the world today because exploration and downstream utilization are associated with economic development [1]. Mechanic workshops within Nigeria are poorly managed and can be sources of constant release of spent hydrocarbon discharged from the crank cases of cars and motorcycles which can be aesthetically unsightly and cause serious environmental pollution. Cleanup of mechanic sites is still elusive as operators of such sites are usually ignorant of the deleterious effects of the spent hydrocarbon on the environment. There is also a likelihood of percolation to ground water and a pint of spent hydrocarbon can contaminate 100,000liters of ground water [2].

Bioremediation refers to the use of naturally occurring microorganisms or genetically engineered microorganisms by man to detoxify man-made pollutants [3]. Since bioremediation is a microbial process, it requires the provision of nutrients among other factors or requirements. The addition of organic waste

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materials such as poultry litter (PL), Cow dung (DG) and Coir pith (CP) to the soil facilitates aeration through small pores and increases the water holding capacity of the soil, thus enhancing bioremediation [4]. It allows natural processes to clean up harmful chemicals in the environment. Microscopic “bugs” or microbes that live in soil and groundwater use certain harmful chemicals such as those found in gasoline and oil spills. Crude oil is a complex mixture of diverse hydrocarbons including alkanes, aromatics, alicyclics, branched hydrocarbons, and non-hydrocarbon compounds including polar fractions containing hetero-atoms of nitrogen, sulfur and oxygen (NSO fraction), and asphaltens [5]. The high demand for petroleum products in the form of cooking gas, aviation fuel, gas oil, engine lubricating oil, asphalt and coal tar means increase in production and this eventually results in oil spills and hydrocarbon contamination of the environment especially through oil well blow out, tanker accidents, accidental rupture of pipelines and routine clean-up operations. Current technologies for cleaning hydrocarbon contaminated soil include soil washing, solvent extraction, thermal treatment, composting, chemical oxidation (Fenton's reagent, permanganate, ozone etc.) and bioremediation (bioaugmentation, biostimulation and phytoremediation) [6,7]. The most widely used bioremediation procedure is biostimulation of the indigenous microorganisms by the addition of nutrients, as input of large quantities of carbon sources tends to result in rapid depletion of the available pools of major inorganic nutrients, such as nitrogen and phosphorus [7]. Biostimulation is the addition of nutritional amendments to increase microbial metabolism and to encourage bioremediation [7]. When microbes completely digest these chemicals, they change them into water and harmless gases such as carbon dioxide. Nutrient is one factor that can hinder biodegradation if not handled properly and could limit the rate of hydrocarbon degradation in the terrestrial environment [8]. This study focuses on effect of agro-waste on bioremediation of soil polluted with spent hydrocarbon in Keffi, Nasarawa State.

METHODS

Study Area

The Study area was different auto mechanic workshops in Keffi, a fast-growing cosmopolitan town geographically located on longitude $7^{\circ}50^{`E}$ and latitude $8^{\circ}3^{`N}$, North West of Lafia (The capital of Nasarawa State, Nigeria), and is situated on an altitude of 850M above sea level. Keffi, though in Nasarawa State is about 68km from Abuja, the Federal Capital of Nigeria [9].

Sample Collection

Hydrocarbon contaminated soil was collected from three (3) different auto mechanic workshops at Old Nasarawa Road, Abuja Road, and Pyanko Road, all in Keffi, Nasarawa State, Nigeria, at a depth of 10cm; then put in well labeled black polythene bags and immediately transported to the laboratory for analysis as described by Makut and Majekodunmi, [10].

Collection and Processing of Amendment Materials (Agricultural Wastes)

The organic materials used as stimulants were corncobs, fruits peels and melon shells. Organic materials were sourced from Keffi Ultra-Modern Market Keffi, Nasarawa State. The agricultural wastes were collected using rake and shovel, air dried, grinded and kept in clean polythene bags. All processed samples were transported to the laboratory for further analysis

Sterilization of Organic Amendment Materials

Two hundred gram of each organic waste such as corncobs, fruits peels and melon shells were mixed with water to about half its water holding capacity and sterilized in the autoclave at 121^{o}C for 15 minutes. Each organic waste was mixed carefully with distilled water inside glass conical flask and covered with a lid, then wrapped with foil paper before placing in the autoclave.

Soil Amendment and Bioremediation

All treatments were carried out in flowerpot reactors filled with 100g unsterilized contaminated soil. The

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treatments were as follows: (1) the soil without the corncobs and melon shell and bacteria addition was used as the control (CK); (2) the bacteria were amended at a 10% v/w ratio to the soil to assess the bio-stimulatory effect of the bacteria alone (BF); (3) the sterilized corncobs and melon shell were amended at a 5% w/w ratio to the soil to assess the bioaugmentation effect of the corncobs and melon shell alone (BC); (4) the sterilized corncobs and melon shell were added in a 5% w/w ratio, while the bacteria were applied in a 10% v/w ratio in soil to assess the effect of corncobs and melon shell -free degrading bacteria (BMF). Five treatments were carried out in three replicates and incubated at 30℃, 60% relative humidity (RH) for 60 days. Deionized water were added every two days to keep the moisture content at 70% of the maximum water holding capacity, and the indexes of soil physicochemical properties, enzyme activities and microbe quantity were measured every 10 days [11].

Effect of Duration on Utilization of Spent hydrocarbon from Amended Polluted Soil

Effects of duration were carried out following a method described by Adams *et al*. [12]. One hundred (100) g of the polluted soil was amended with ratio 5% w/w corncobs, fruits peel and melon shell and the bacteria were applied in a 10% v/w ratio in polluted soil and was transferred into different pots and incubated at 26^{o}C, 30^{o}C and 35^{o}C respectively

Effect of Temperature on Utilization of spent Hydrocarbon from Amended Polluted Soil

Effect of temperatures was carried out following a method described by Adams *et al*. [12] One hundred (100) g of the polluted soil was amended with ratio 5% w/w corncobs, fruits peel and melon shell and the bacteria was applied in a 10% v/w ratio in polluted soil and was transferred into different pots and incubated for 40 days

Determination of Soil Physicochemical Properties

Determination of pH

The pH of the polluted and non-polluted soil was carried out using a method described by Andreolli *et al.* [8]. Ten gram (10g) of 2mm sieved air-dry soil in a 50ml plastic beaker, 25ml of distilled water was added and the suspension was stirred several times for about 30 minutes. Then, it was allowed to stand for about 30 minutes undisturbed. The pH meter was calibrated using pH buffer 4, 7 and 9. The electrode was immersed into the soil, and was careful not to allow it touch the bottom of the beaker. The pH was read after 30 seconds. The procedure was repeated using 0.01M $CaCl_2$ solution. The readings were carefully recorded as pH in water and pH in $CaCl_2$.

Determination of Total Nitrogen

The total Nitrogen of the polluted and non-polluted soil was carried out using a method described by Andreolli *et al.* [8]. One gram (1g) of the fine mesh soil was weighed into the Kjeldahl flask (digestion tube). Some few drops of water were added and were allowed to stand for about 30 minutes. 5g of the Kjeldahl catalyst mixture (mixture of 500g of Na2SO4 + 50g of CUSO4 + 0.5g of selenium catalyst grinded to fine powder) was added. Then 20ml of the concentrated sulphuric acid was added. It was heated on a digestion block until frothing ceased, and the temperature was increased until the digest was clear. The flask was allowed to cool, and a little water with care was gently added before washing the content into 100ml volumetric flask. The content was also allowed to cool and was made to mark. 10ml was introduced from the digest into a distillation flask. 20ml of Boric acid was measured into the 100ml conical flask and was introduced to the bottom of the condenser with 3 drops of the mixed indicator. 10ml of the NaOH was added with the 10ml of the digest in the distillating flask, and was immediately introduced in the distillating unit in order to distil off the digest, and about 60ml of the distillate was collected. The distillate was then titrated with 0.01N H_2SO_4 with colour change from green-blue to purple which indicated the end point.

Determination of Organic Carbon

The total organic carbon of the polluted and non-polluted soil was carried out using a method described by

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Andreolli *et al.* [8]. One gram (1g) of the soil was weighed into 250ml Erlenmeyer flask, but the organic matter was too high, so the soil measurement was then reduced to 0.5g. 10ml of 1N $K_2Cr_2O_7$ was introduced into a flask. The flask was swirled gently in order to disperse the soil. Exactly 10ml of concentrated H_2SO_4 was rapidly added from a measuring cylinder and was swirled again for one minute. The flask was allowed to stand on asbestos sheet for about 30 minutes. 100ml of distilled water was added to the flask, and was allowed to cool. 3 drops of the indicator (phenanthroline) was added, and was then titrated with ammonium ferrous sulphate solution with a white background. Blank determination was made in the same manner but without soil.

Determination of Phosphorus

The Phosphorus of the polluted and non-polluted soil was carried out using a method described by Andreolli *et al.* [8]. One gram (1g) of the soil sample was weighed using the electrical weighing balance. 7ml (pipette) of the extraction solution was added. The container was seriously shaken for exactly one minute and was centrifuged to about 5 minutes in order to obtain clear supernatant (clear solution). It was then decanted into a clean vial. 2ml of the extract was taken into another clean separate vial. 4ml of the ascorbic acid (molybdate solution) was added, and was diluted with distilled water to the mark (25ml), the colour was allowed for about 30 minutes to develop. Then it was read on a calorimeter to detect the colour intensity or the concentration of phosphorus.

Determination of Total Petroleum Hydrocarbons in Soil

After the incubation, the soil samples were extracted and analysed. Total petroleum hydrocarbons (TPHs) extracted from the soil was determined using the gravimetric method [13]. Five gram (5g) of the soil wrapped in a filter paper were extracted using 30 mL of methylene chloride by ultrasonication for 15 min, and then the paper was transferred into the Soxhlet Extractor at 54℃, Water Bath for 12h. After that, the extracts were concentrated on a rotary evaporator, and then made up to a constant volume of 50 mL for measurement. The amount of residual TPHs was quantified gravimetrically and the concentration of n-alkanes (C 12–18) was measured by a gas chromatograph mass spectrometer (GC-MS). The TPHs removal (%) was calculated using the following formula:

TPHs removal (%) ¼ [(w0 - wt)/w0] x100 where w0 is the initial soil TPHs concentration (g kg^{-1}), w t stands for the residual TPHs concentration at time t (g kg^{-1}), and t is the remediation time (day).

Enumeration of Hydrocarbon Degrading Bacteria

Three replicate samples from each experiment soil were withdrawn every 7 days for the enumeration of total aerobic heterotrophic bacteria (AHB). Serial dilution was carried out by weighing 1g of the experiment soil into a test tube containing 9ml of sterile water, 1ml of the soil suspension was picked and transferred to 2^{nd} test tube containing 9ml of sterilized water this step was repeated till 10^{th} tube. Then 0.1ml of the serially diluted samples picked from the 6^{th} tube was plated on nutrient agar medium supplemented with 50µg/ml nystatin to suppress the growth of fungi. Triplicate plates were incubated at 30°C for 24 h before the colonies were counted. Hydrocarbon-utilizing bacteria (HUB) in the soil samples were enumerated using mineral salt medium of Zajic and Supplission [14] supplemented with used engine oil and 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, 1% used engine oil in 1,000 ml distilled water, pH7.4). The oil agar plates were incubated at 30°C for 5 days, and the colonies were counted and randomly picked, and pure isolates were obtained by repeated sub-culturing on nutrient agar [13].

Characterization and Identification of Bacterial Isolates

The identification of bacteria was done based on morphological characteristics and biochemical tests carried out on the isolates. Morphological characteristics observed for each bacteria colony after 24hours of growth include colony appearance; shape, elevation, edge, optical characteristics, consistency, colony surface and pigmentation. Identification and characterization of isolates were examined and recorded as described by Cheesbrough [15]

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Biochemical Characterization of Bacterial Isolates

In order to identify the purified cultures tentatively, biochemical tests were performed as described by Cheesbrough [15] catalase test, indole test, citrate utilization test, urease test, motility test, oxidase test, starch hydrolysis test, voges proskauer test, carbohydrate fermentation test.

Molecular Identification of Bacteria Isolates Using 16S rRNA

DNA Extraction

The DNA extraction of bacteria species was carried out as described by Makut *et al*. [16]. Two hundred and fifty millilitres (250ml) of pure colonies of the suspected organism, dissolved in 10ml of Luria-Bertani Broth, was added to a Bashing Bead Lysis Tube (0.1 and 0.5) and 750 µl Lysis Solution was added to the tube. A bead beater fitted with a 2 ml tube holder assembly was secured and processed at a maximum speed for 5 minutes.

The Bashing Bead Lysis Tube was centrifuged in a microcentrifuge at 10,000 x g for 1 minute. 400µl supernatant was transferred to a Zymo-Spin IV Spin Filter in a collection tube [16]. Eight hundred microliters (800 µl) of the mixture was transferred to a Zymo-Spin IIC column in a collection tube and was centrifuged at 10,000 x g for 1 minute. The flow through from the collection tube was discarded. 200 µl DNA Pre-Wash Buffer was added to the Zymo-Spin IIC Column in a new collection tube and was centrifuged at 10,000 x g for 1 minute. Five hundred microliters (500 µl) DNA Wash Buffer was added to the Zymo-Spin IIC Column and was centrifuged at 10,000 x g for 1 minute 800 µl of the mixture was transferred to a Zymo-Spin IIC Column in a collection tube and was centrifuged at 10,000 x g for 1 minute.

The flow through was discarded from the collection tube. 1, 200 µl of DNA Binding Buffer was added to the filtrate in the collection tube. The Zymo-Spin IIC Column was transferred to a clean 1.5 ml microcentrifuge tube and100 µl (35 µl minimum) DNA Elution Buffer was added directly to the column matrix and centrifuged at 10,000 x g for 30 seconds to elute the DNA [16].

The base of the Zymo-Spin IV-HRC Spin Filter was snapped off and placed into a collection tube and was centrifuged at 8,000 x g for 3 minutes. The eluted DNA was transferred to a prepared Zymo-Spin IV-HRC Spin Filter in a clean 1.5 ml microcentrifuge tube and was centrifuged at 8,000 x g for 1 minute. Extracted DNA was stored at -20^{o}C until use.

The DNA Amplification

DNA amplification was conducted by using a forward primer and a reverse primer specific for the bacteria specific for the 16S rRNA. The primer amplified a product of 1 078 base pairs (bp) in the 16SrRNA genome. The PCR was performed in a 10 µl reaction volume containing 3.5 µl of 2× Master Mix from Promega; 3.0 of 25ng/µl DNA and 2.0µl of water and 0.5 µl of each oligonucleotide primer (5pMol forward primer and 5pMol reverse primer Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa).

The mixtures were subjected to initial denaturation at 94 ºC for 5 minutes, followed by 36 cycles of amplification involving denaturation at 94 ºC for 30 seconds, primer annealing at 56 ºC for 30 seconds, and primer extension at 72 ºC for 45 sec; a final primer extension at 72 ºC for 7 minutes, using a DNA thermal cycler (Gene Amp PCR system 9700, Applied Biosystems, South Africa).

PCR Product Purification

The PCR product purification was carried out using a method described by Makut *et al*. [16]. In this method, 20µl of absolute ethanol was added to the PCR product and incubated at room temperature (±) for 15minutes after which it was spun at 10,000rpm for 15minutes. The supernatant was decanted and spun at 10, 000 rpm for 15 minutes. Twenty microliter (20µl) of 70% ethanol was added and again the supernatant was decanted and air-dried. About 10µl of ultrapure water ordered from Oxford, the United Kingdom was added. Amplicon on 1.5% agarose was checked for**.** The product from the purification process was loaded on the 3130xl genetic

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analyzer from Applied Biosystems to give the sequences.

Sequencing Analysis

PCR DNA was sent to Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa for sequencing using the primer pair. Sequencing data obtained were assembled and edited to a total length of 1078 bp using free sequencing software (BioEdit) to view and analyse the data.

BLAST (Basic Local Alignment Search Tool) searches of the sequences were conducted using the techniques developed by the National Centre for Bioinformatics, Islamabad, Pakistan, to determine the similarity between sequencing data obtained from local strains and those available in GenBank. Data were recorded as percentage similarity to related species.

Statistical Analysis

Data collected from the study were analyzed using general descriptive statistics, one Way Analysis of Variance (ANOVA) at 95% probability level of significance.

RESULTS

Soil Physicochemical Properties

The physical and chemical properties of the contaminated soil and soil amended with agro wastes used for study are shown in Table 1. The hydrogen ions (pH) of the polluted soil sample were 4.9. pH of corncobs (CB) was 6.2, melon shells (MS) was 5.6 and fruits peels (FP) was 7.6 respectively. The value of total nitrogen for polluted soil was 0.45 %, CB was 6.2%, MS was 1.26% and FP was 3.56%. Organic carbon in polluted soil prior to study was 5.80 %, CB was 9.03%, MS was 7.55% and FP was 10.12%. The Phosphorous in contaminated soil was 8.41 mg/kg, CB was 13.40 mg/kg, MS was11.82mg/kg and FP was 15.61 mg/kg. (Table 1)

Reduction of Spent Hydrocarbon

Total reduction of spent Hydrocarbon (TSH) in soil assessed for 60 days is shown in Table 2. The concentration of TSH present in soil at the beginning of this study was 51.12mg/kg. The result revealed that the polluted soil recorded highest reduction after 40days (2.01mg/kg) followed by 30days (1.62 mg/kg), 20days (1.11 mg/kg) and the lowest was 10days (0.87 mg/kg). Polluted soil amended with CB recorded highest after 40days (44.98 mg/kg) followed by 40.01 mg/kg after 30days, 35.02 mg/kg after 20days and the least 10.31 mg/kg after 10days. Polluted soil amended with MS recorded highest 39.90 mg/kg after 40days followed by 33.14 mg/kg after 30days, 21.93 mg/kg after 20days and 5.02 mg/kg after 10days. Polluted soil amended FP recorded highest reduction 48.11 mg/kg followed by 40.1 mg/kg after 30 days, 38.04 mg/kg after and the lowest 12.00 mg/kg.

Effect of Temperature on Total Petroleum Hydrocarbon Reduction

Effect of temperature on reduction of spent petroleum hydrocarbon in polluted soil and agro waste-amended soil from mechanic workshops is as shown in Table 3. The highest reduction of spent hydrocarbon was observed at 26 ℃ (2.84 mg/kg) followed by 30 ℃ (1.95mg/kg) and the lowest were at 35℃ (1.37 mg/kg) for polluted soil. From polluted soil amended with CB the highest was observed at 30 ℃ (48.92 mg/kg) followed by 26℃ (28.11mg/kg) and the least was at 35℃ (19.21mg/kg). From MS the highest was at 30℃ (33.03 mg/kg) followed by 26 ℃ (22.82 mg/kg) and the lowest were at 35℃ (13.08 mg/kg). From FP the highest reduction was at 30℃ (48.04 mg/kg) followed by 26℃ (31.01 mg/kg) followed by 35℃ (23.88mg/kg) respectively. (Table 3)

Table 4 shows the cultural, morphological and biochemical characteristics of bacteria isolated Polluted and Agro-wastes Amended soils.

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Table 1: Physicochemical Properties of Hydrocarbon Polluted Soil and Agro-Wastes Amended Soil

Parameter	**Polluted Soil**		**Agro Wastes**	
		CB	**MS**	**FP**
pH	4.9	6.2	5.6	7.6
Total Nitrogen (%)	0.45	2.98	1.26	3.56
Organic Carbon (%)	5.80	9.03	7.55	10.12
Phosphorous (mg/kg)	8.41	13.40	11.82	15.61

* CB = Corn Cobs; MS = Melon Shell; FP = Fruit Peels

Table 2 Effect of Duration on Reduction of Total Petroleum Hydrocarbon in Polluted Soil and Agro-Wastes Amended Soils

Time (Days)	Polluted Soil (mg/kg ±SD)	Mean Reduction of total petroleum hydrocarbon (mg/kg ±SD)		
		CB	MS	FP
0	51.12±1.03	51.12±1.03	51.12 ±1.03	51.12±1.03
10	0.87±0.62	10.31±0.23	5.02±0.20	12.00±0.21
20	1.11±0.15	35.02±0.23	21.93±1.43	38.04±0.23
30	1.62±0.92	40.01±0.23	33.14±0.03	40.041±0.13
40	2.01±0.18	44.98±0.11	39.90±0.10	48.11±0.40

* CB = Corn Cobs; MS = Melon Shell; FP = Fruit Peels

Table 3: Effect of temperature on reduction of Total Petroleum Hydrocarbon in Polluted and Agro-wastes Amended soils

Temperature (°C)	Polluted Soil (mg/kg)	Mean Reduction of total petroleum hydrocarbon (mg/kg ±SD)		
		CB	MS	FP
26	2.84±0.22	28.11±0.13	22.82±0.10	31.01±0.41
30	1.95±0.11	41.92±0.21	33.03±1.43	48.04±0.13
35	1.37±0.42	19.21±0.24	13.08±0.33	23.88±0.17

* CB = Corn Cobs; MS = Melon Shell; FP = Fruit Peels

Table 4 Cultural, Morphological and Biochemical Characteristics of bacteria isolated

Cultural Morphology	GR	Biochemical characteristic				Sugar fermentation			Inference
		Cat	Ox	In	Nit	Fru	Mal	Glu	
grey coloured with a shiny surface and entire margin;	+	+	-	-	+	+	-	+	*Bacillus subtilis*

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mucoid or rough colonies									
smooth none elevated colonies green pigment on NA	-	-	+	-	+	-	-	+	*Flavobacterium* sp
smooth none elevated colonies green pigment on NA	-	-	+	-	+	-	-	+	*Pseudomonas* sp
smooth none elevated colonies green pigment on NA	-	-	-	-	+	-	-	+	*Corynebacterium* sp
Grey coloured with a shiny surface and entire margin; mucoid or rough	-	-	+	-	+	+	-	+	*Proteus* sp

GR= Gram Reaction; Cat=Catalase; Ox=Oxidase; In=Indole; Fru=Fructose; Mal=Maltose; Glu=Glucose

Total Number of Degrading Bacteria Count

The total number of hydrocarbon degrading bacteria from polluted soil and amendment soil polluted is as given in Table 5. The highest degrading bacterial count was recorded from Keffi garage mechanic workshop (4.04 $x10^6$ cfu) followed by Old Nasarawa road mechanic workshop (3.11 $x10^6$ cfu), Abuja road mechanic workshop (2.07 $x10^6$ cfu) and the lowest was from pyanko road mechanic workshop (1.18 $x10^6$ cfu). From polluted soil amended with CB the highest was from Abuja road mechanic workshop (7.10 $x10^6$ cfu) followed by Old Nasarawa road mechanic workshop (6.03 $x10^6$ cfu), Keffi garage mechanic workshop (5.24 $x10^6$ cfu) and the lowest was from Pyanko road mechanic workshop (4.88 $x10^6$ cfu). From MS the highest was from Pyanko road mechanic workshop (5.08 $x10^6$ cfu) followed by Keffi garage mechanic workshop (4.32$x10^6$ cfu g/ml), Abuja road mechanic workshop (4.18 $x10^6$ cfu) and Old Nasarawa road mechanic workshop (3.93 $x10^6$ cfu). From polluted soil amended with FP the highest degrading bacterial count recorded was from Keffi garage mechanic workshop (9.12 $x10^6$ cfu) followed by Abuja road mechanic workshop (7.88 $x10^6$ cfu), Old Nasarawa road mechanic workshop (6.11 $x10^6$ cfu) and the least was from Pyanko road mechanic workshop (5.12 $x10^6$ cfu).

Percentage (%) Occurrence of Bacteria in Polluted and Agro-wastes Amended Soils

Table 6 shows the bacterial occurrence from non-amended and amended polluted soil with spent hydrocarbon from mechanic workshops in Keffi. The highest frequency of occurrence of bacteria isolated from non-amended polluted soil was *Pseudomonas* sp (66.6%) while *Flavobacterium* sp, *Bacillus subtilis* and *Proteus* sp had a frequency of occurrence of (33.3%) respectively. From amended soil with CB the highest were *Corynebacteria* spp and *Flavobacterium* sp (100%) and the lowest were *Bacillus subtilis, Pseudomonas flourescens,* and *Proteus* sp (66.6%). From polluted soil amended with MS the highest bacteria isolated were *Flavobacterium* sp and *Corynebacteria* sp (33.3%) and the lowest were *Pseudomonas flourescens* and *Proteus* sp (33.3 %). From polluted soil amended with FP the highest frequency of occurrence of bacteria isolated were *Pseudomonas flourescens Corynebacteria* sp and *Proteus* sp (100%) and the lowest were *Bacillus subtilis,* and *Flavobacterium* sp (66.6%) respectively (Table 6).

The Agarose gel electrophoresis of the 16S rRNA gene of bacteria isolated from amended soil contaminated with spent hydrocarbon is as shown in Plate 1, where *Pseudomonas flourescens, Bacillus firmus, Proteus mirabilis, Flavobacterium columnare* and *Priestia flexa* 16SrRNA gene bands shown at 1000bp.

Figure 1 – 5 shows the phylogenetic tree showing the evolutionary distance between each of the bacteria isolated from amended soil contaminated with spent hydrocarbon.

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Table 5: Bacterial Load of Polluted and Agro-wastes Amended Soil

Sample	Polluted Soil (cfu g x10^6)	Amended Soil (cfu x10^6)		
		CB	MS	FP
Old Nasarawa Road	3.11±0.21	6.03±0.11	3.93±0.71	6.11±0.31
Abuja Road	2.07± 0.44	7.10± 0.64	4.18± 0.37	7.88± 0.17
Pyanko Road	1.18 ±0.06	4.88 ±0.26	5.08 ±0.61	5.12 ±0.01
Keffi Garage	4.04±0.31	5.24±0.09	4.32±0.82	9.12±0.12

* CB = Corn Cobs; MS = Melon Shell; FP = Fruit Peels

Table 6: Frequency of Occurrence (%) of Bacteria from Polluted and Agro-wastes Amended Soils

Bacteria	No. Sample	Polluted soil No. Isolated (%)	Amended Soil No. isolated (%)		
			CB	MS	FP
Bacillus sp	3	1(33.3)	2(66.6)	0(00)	2(66.6)
Pseudomonas sp	3	2(66.6)	2(66.6)	1(33.3)	3(100)
Flavobacterium sp	3	1(33.3)	3(100)	2(66.6)	2(66.6)
Corynebacterium sp	3	0(00)	3(00)	2(66.6)	3(100)
Proteus sp	3	1(33.6)	2(66.6)	1(33.3)	3(100)

* CB = Corn Cobs; MS = Melon Shell; FP = Fruit Peels

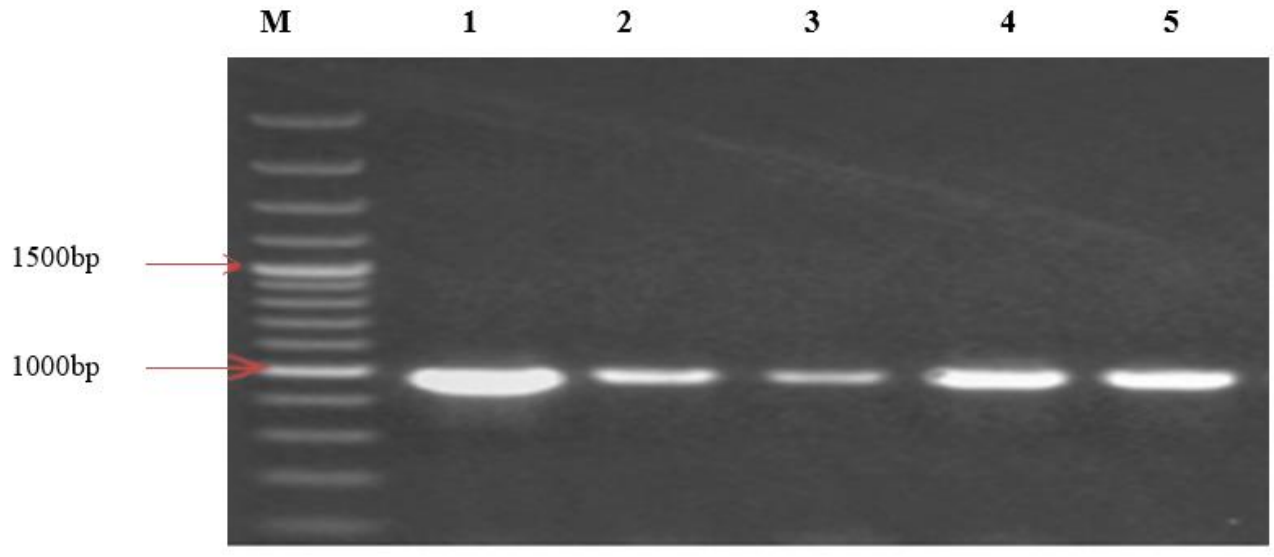

Plate 1: Agarose gel electrophoresis of the 16S rRNA gene of bacteria isolated from amended soil contaminated with spent hydrocarbon. Lanes 1-5 represent the *Pseudomonas flourescens, Bacillus firmus, Proteus mirabilis*, *Flavobacterium columnare* and *Priestia flexa* 16SrRNA gene bands of 1000bp. Lane M represents the 1500bp molecular ladder

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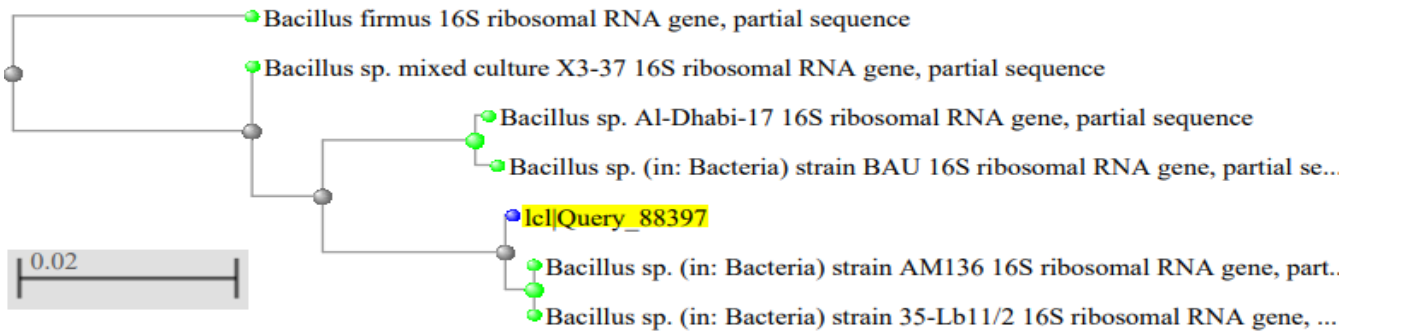

Figure 1: Phylogenetic tree showing the evolutionary distance between *Bacillus firmus* isolated

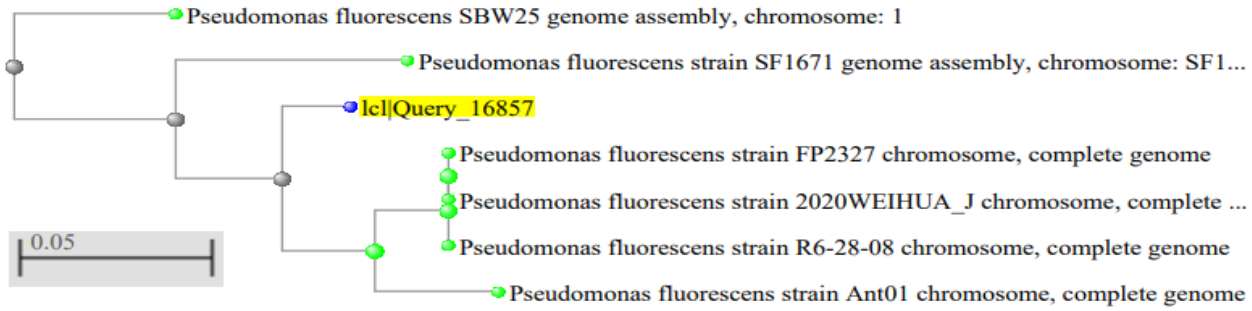

Figure 2: Phylogenetic tree showing the evolutionary distance between *Pseudomonas florescens* isolated

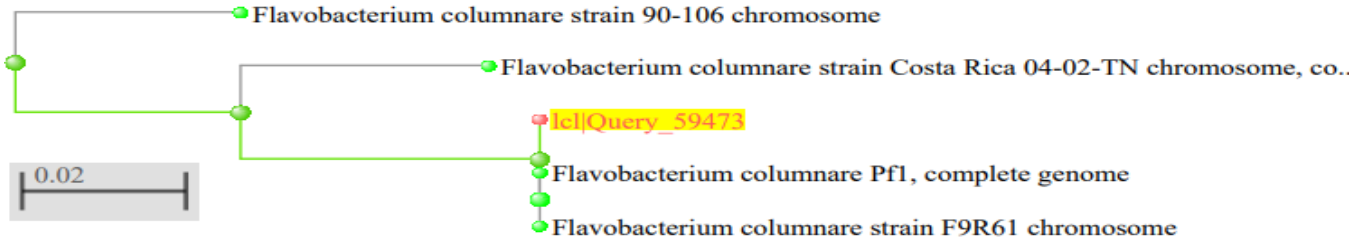

Figure 3: Phylogenetic tree showing the evolutionary distance *flavobacterium columnare* isolated

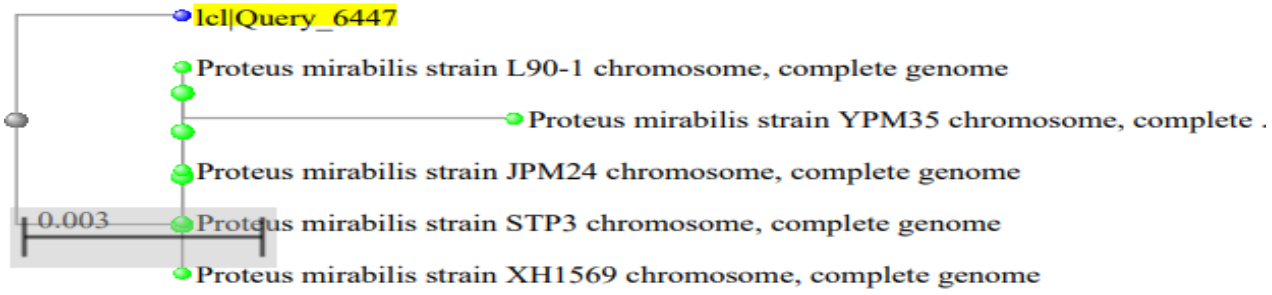

Figure 4: Phylogenetic tree showing the evolutionary distance between *Proteus mirabilis* isolated

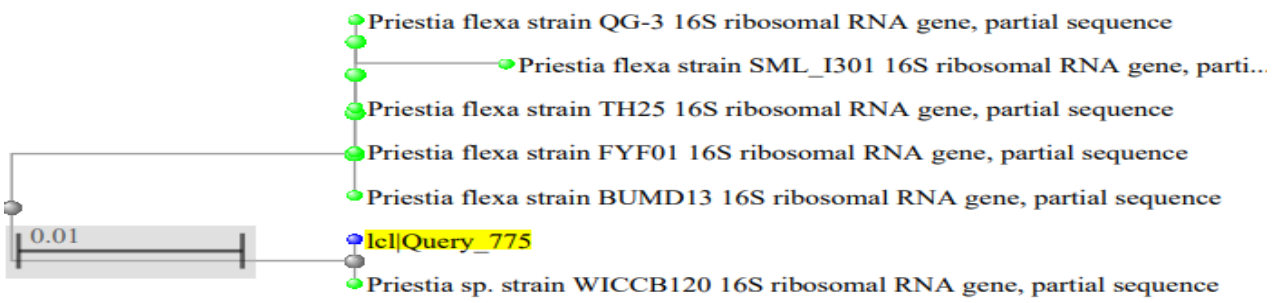

Figure 5: Phylogenetic tree showing the evolutionary distance between *Priestia flexa* isolated

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DISCUSSION OF THE FINDINGS

Spent hydrocarbon contains high concentration of petroleum hydrocarbon which slows biodegradation rate and high rate of bioaccumulation [17]. Presence of Spent hydrocarbon in soil can affect its physico-chemical properties as well as the population of natural flora. The finding of this study is similar of Muhammad *et al*. [17] and Ezeigbo *et al.* [18] who recorded drop in pH on addition of spent hydrocarbon to the polluted soil as observed in this study but when amended with agro waste there was increase in the pH level ranging from 5.6 to 7.2 after 40 days of the amendment. The findings of this study showed that polluted soil was low in essential nutrients such as organic carbon had 0.45 %, nitrogen had 5.80 %, and phosphorous 8.41 mg/kg, making the polluted soil unable to meet the requirement for efficient degradation of spent hydrocarbon. As the polluted soils were amended with agro wastes the essential nutrients increased ranging from 1.26% to 6.2% in nitrogen, the total organic carbon ranged from 7.55% to 10.12% and phosphorous range from 11.82mg/kg to 15.61 mg/kg which were expected to be more for effective bioremediation. This is also similar to studies reported by Abioye *et al*. [11]; Anjana and Meenal [19] and Muhammad *et al*. [17], who reported the use of some organic wastes such as wood chips, sewage sludge, banana peel and spent mushroom compost as amendments to arouse the degradation of spent engine oil in soil.

The amount of spent hydrocarbon reduction during degradation process observed in this study varied with different treatments. Highest reduction was recorded in polluted soil amended with FP followed by CB and MS as shown in Table 2 and 3. The amount of reduction in non-amended polluted soil was lower when compared to the amended polluted soil. Highest reduction in polluted soil amended with FP may be attributed to high essential nutrients it contains. This may be due to high percentage of organic carbon present and bioavailability of the nutrients in FP to different bacteria species in the polluted soil [11]. The findings of this study revealed high degradation/reduction of spent hydrocarbon from polluted soil amended with agro wastes compared to concentration degraded in non-amended polluted soil. However, highest reduction was recorded in polluted soil amended with fruits peel after 40days (48.11 mg/kg) of the amendment followed by corn cobs (44.98 mg/kg) and melon shell (39.90 mg/kg). This result showed that duration play an important role in degradation of soil polluted with hydrocarbon when right nutrients are made available for indigenous bacteria to degrade the hydrocarbon. The high reduction of hydrocarbon at different temperature as observed in this study is similar to studies reported by Makut *et al*. [16] who reported high reduction of spent hydrocarbon at 30°C by different bacteria species. This is clear evidence that agro waste is a better stimulant of indigenous bacterial growth owing to its high organic carbon and nitrogen composition as given in Table 1 which is essential for the degradation of spent hydrocarbon by the indigenous bacteria. The differences in the concentration of spent hydrocarbon reduction within different period and temperature of this study might be due to differences in the composition of nutrients in agro wastes and number of hydrocarbon degrading bacteria in the polluted soil [11].

The counts of hydrocarbon degrading bacteria from non-amended polluted soil and amended during this study ranged 1.18 $x10^6$ cfu/g to 4.04 $x10^6$ cfu/g for none amended contaminated soil and 4.88 $x10^6$ cfu/g to 9.12 $x10^6$ cfu/g for amended polluted soil from different mechanic workshops in Keffi, this shows that hydrocarbon degrading bacteria can strive even in extreme conditions and high concentration of spent hydrocarbon [20,12].

Spent hydrocarbon degrading bacteria isolated and identified from non-amended polluted soil and amended soil were *Bacillus*, *Flavobacterium*, Corynebacteria, *Proteus* sp and *Pseudomonas*. The isolation of these species is an indication of their tolerant to the hydrocarbon contents of the soil and thus can be supported with agro wastes to stimulate their bioremediation potential. This is in agreement with an earlier study by Adam *et al* [12] who isolated *Pseudomonas* and *Corynebacteria* from hydrocarbon polluted soil. In the study of Ajao *et al* [21], bacterial species of *Flavobacterium*, *Bacillus*, *Pseudomonas* and *Serratia* were isolated from used motor oil. From the results of this study, there is need to use agro waste in reclaiming abandoned mechanic workshop in Keffi and soil polluted with hydrocarbons.

CONCLUSION

In this study there was drop in pH level to 4.6 of soil polluted with spent hydrocarbon as observed but when amended with agro waste the pH level rose from 5.6 to 7.2 after 40 days of the amendment. The findings of

this study showed that hydrocarbon polluted soil is low in essential nutrients such as organic carbon had 0.45 %, nitrogen had 5.80 %, and phosphorous 8.41 mg/kg. After amended with agro waste the essential nutrients increased ranging from 1.26% to 6.2% in nitrogen, the total organic carbon ranged from 7.55% to 10.12% and phosphorous ranged from 11.82mg/kg to 15.61 mg/kg. Highest reduction of Total Petroleum Hydrocarbon (TPH) was recorded in polluted soil amended with fruits peel after 40days (48.11 mg/kg) followed by corn cobs (44.98 mg/kg) and melon shell (39.90 mg/kg). It was observed that temperature and time played important role in spent hydrocarbon degradation when amended with organic waste. The hydrocarbon utilizing bacteria isolated and identified from non-amended polluted soil and amended soil include *Bacillus*, *Flavobacterium*, Corynebacterium, *Proteus* sp and *Pseudomonas.*

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