# Isolation and Characterization of some Hydrocarbon Degrading Bacteria Isolated from Soil Contaminated with Auto Mechanic Used Oil

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***Abstract*: The disposal of used hydrocarbon can be more environmentally damaging than crude oil pollution itself. This study focused on the isolation and characterization of some hydrocarbon degrading bacteria isolated from soil contaminated with auto mechanic used oil in Keffi. The bacteria were isolated using standard microbiological technique and identified using Real-time quantitative polymerase chain reaction. The highest total bacteria count was recorded from worrkshop 4 ($3.02 \pm 0.08$) and the least was from shop 2 ($1.61 \pm 0.01$). The different bacteria species identified were *Bacillus thuringiensis, Proteus terrae, Pseudomonas synxantha, Flavobacterium columnare* and *Acinetobacter beijerinckii.* The total viable count of hydrocarbon utilizing bacteria was *Pseudomonas synxantha* with ($1.88 \pm 0.03$ cfu/g). The effect of temperature on utilization of used hydrocarbon from mechanic workshops showed that highest utilization was observed at temperature of 32℃ were *Pseudomonas synxantha* utilized 14.01±2.01mg/ml. At temperature of 34℃, the highest utilization was also by *Pseudomonas synxantha* with 9.38±0.28mg/ml and the lowest was by *Proteus terrae* 3.00±0.14mg/ml. At 38℃ the highest utilization was observed by *Bacillus thuringiensis* 3.95±0.01mg/ml and the lowest was *Pseudomonas synxantha* 2.11±0.08mg/ml. the utilization of used hydrocarbon after one month showed that highest utilization was obtained after 3 weeks by *Bacillus thuringiensis* 9.60±0.18mg/ml and the lowest utilization were recorded by *Proteus terrae* 4.20±1.01mg/ml. From this study it was observed that different bacteria can utilize used hydrocarbon that contaminate soil.**

***Keywords*: Hydrocarbon, Bacteria, utilization, contaminated soil and isolation**

## I. INTRODUCTION

As the world's population keeps increasing, there is a concurrent increase in the demand for hydrocarbons (petroleum and petroleum products), which apparently constitutes a source of environmental pollution [1]. Pollution caused by hydrocarbons, like oil from auto-mechanic workshops is a major environmental concern in Nigeria; as the oil contains compounds from engine wear which include iron, steel, copper, zinc, lead, barium, cadmium, sulfur, dirt, ash and heavy polycyclic aromatic hydrocarbons (PAHs) and because of the additives and contaminants, disposal of used hydrocarbon can be more environmentally damaging than crude oil pollution [2]. Microbial degradation studies have shown that susceptibility of hydrocarbons ranged from linear alkanes to branched alkanes small aromatics cyclic alkanes [3]. Major products of petroleum hydrocarbons include liquefied petroleum gas, gasoline or petrol, naphtha, kerosene, diesel oil, heavy fuel oil, lubricating oils that include engine oil, paraffin wax, asphalt, tar, petroleum coke, diesel and transmission oil are used daily in various forms in auto-mechanic workshops. These products tend to harden and change the color of the soil, which may have untold health hazard on the technicians and artisans [4].

Hydrocarbons are organic compounds that composes of two major elements which are carbon and hydrogen, and they are the dominant components of crude oil with small quantities of molecules like Sulphur, nitrogen, metals, oxygen and others [5]]. The carbon atoms join together to form the framework of the compound, and the hydrogen atoms attach to them in many different configurations. They are the world's most widely used primary energy and fuel resources, due to the energy they produce [6]. Over 17,000 organic compounds have been identified in crude oil, and subdivided into four main classes; saturates, aromatics, asphaltenes and resins [7].

Hydrocarbon polluted soil can be remedied, managed and restored in order to prevent damage to human health or the environment and to restore all or part of the contaminated soil to a useful purpose through a process called Remediation. As reported by Abioye [2], Many techniques have been developed in remediating soil that have been polluted by hydrocarbons. Such techniques include physical degradation, chemical degradation and photodegradation. However, some of these methods have some limitations in completely remediating hydrocarbon contaminated soil as they leave behind daughter compounds which are more toxic to the environment than the parent compounds. Biological treatment offers the best environmentally friendly method for remediating hydrocarbon and heavy metal contaminated soil because it utilizes the capability of the indigenous microorganisms in the soil environment to break down the hydrocarbons and heavy metals into innocuous substances.

Numerous techniques are used in restoring hydrocarbon contaminated soils, but bioremediation technique is being considered here because it is effective and an environmentally friendly alternative to physical and thermal clean ups. Bioremediation as described by Adams *et al*. [8], includes the addition of biological agents like microorganisms (bacteria, cyanobacteria, algae, fungi, protozoa) or enhancement of microorganism already present, to degrade or clean up the

pollutants completely or transform them into harmless substances using biological activities. But the process can be altered due to some factors influencing the rate of microbial growth like soil moisture, temperature, population diversity, pH, oxygen supply and nutrient levels.

For effective remediation of polluted soil, some basic microbial requirements like concentrations of carbon, nitrogen and other essential elements have to be present for the process to be a success. And this research looks to isolating and characterization of bacteria that have to degrade hydrocarbons in the study area.

## II. MATERIALS AND METHODS

### *A) Sample collection*

Twenty grams (20g) each of auto-mechanic oil contaminated soils were collected from 6 different auto-mechanic workshops situated at different locations in Keffi. These locations include; Nasarawa road mechanic shop, Kaduna Road mechanic shop and Abuja Road mechanic shop. The top soil sample were collected aseptically at depths of 0-15cm down the horizon with a spatula into well labelled, sterile sample collection bottles from each site and transported in cold storage containers to the laboratory.

### *B) Sample Preparation*

According to the method described by Eze *et al*., [9], ten grams (10g) of auto-mechanic oil contaminated soil samples were aseptically weighed into 90 ml of sterile distilled water in a 100 ml conical flask. The samples were vortexed to homogenize and allowed to stand for 10 minutes. From this initial dilution, 10-fold serial dilutions were carried out in clean sterile test tubes containing 9 ml of sterile distilled water.

### *C) Isolation of Total Heterotrophic bacteria*

As described by Makut and Ishaya [10], 0.1ml of prepared samples, $10^{-3}$ and $10^{-5}$ were spread and plated in duplicates into nutrient agar, brilliant green agar, desoxycholate citrate agar, supplemented with 50µg/ml of nystatin to inhibit the growth of fungi. Plates were incubated at $35^{O}$C for 24 hours and the bacterial colonies observed were identified.

### *D) Identification of Isolates*

The identification of bacteria was done based on morphological characteristics and biochemical tests. Morphological characteristics observed for each bacteria colony after 24-48 hours 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 Fahad [11].

### *E) Gram staining*

The Gram staining technique was carried out as described by Abiola and Oyetayo [12]. A thin smear of each of the pure 24hours old culture was prepared on a clean grease-free glass slide, and emulsified in a drop of distilled water until a thin homogeneous film is obtained, then the wire loop was` re-sterilized and the thin homogeneous film was allowed to air-dry, and heat-fixed by passing through the flame. The slide was then flooded with crystal violet for 1 minute, and then rinsed with distilled water. The stain was again flooded with Lugol's iodine for 1 minute, and rinsed with distilled water and then decolorized, rapidly with acetone alcohol until no more colour appeared to flow from preparation and rinsed appropriately with distilled water. The stain was then counter-stained with neutral red for 1 minute, and rinsed with distilled water and allowed to air dry and viewed microscopically using x100 oil immersion objective. Gram positive organism retains the dark blue colour inferred by the iodine/crystal violet complex, while Gram negative organisms appears red; maintaining the colour of the secondary dye

### *Biochemical characterization*

In order to identify the purified cultures tentatively, biochemical tests were performed on the suspected bacteria species isolates such as: Catalase test, Indole, Methyl red, Vorges-Proskauer tests, Nitrate reduction, Urease production, Citrate utilisation, and glucose fermentation tests

### *F) Molecular Characterization using Sequence analysis of the 16S rRNA DNA*

Polymerase chain reaction (PCR) was used to amplify the target 16S rRNA region of the DNA in bacterial cells. The process was performed by picking a single colony of bacteria isolates from the nutrient agar medium using the tip of a sterile pipette and placing it in 100 µl of sterile distilled water in a 1.5 ml microcentrifuge tube. The tube was incubated at between 94 and 95 °C for 10 min using a digital dry bath. A volume of 2 µl was then used as a DNA template for the amplification reaction. The 16S rRNA region was amplified by PCR using the forward primer, 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and reverse primer 1492R (5′-CGG CTA CCT TGT TAC GAC TT-3′). The amplification reaction was prepared using 10 µl of 2× PCR Master Mix (Thermo Scientific Phusion Flash High-Fidelity), 1 µl of each forward and reverse primer (10 µM), 2 µl of the DNA template and 6 µl of sterile distilled water resulting in a 20 µl reaction volume. The negative control was set up without genomic DNA. The amplification reaction was performed in a thermal cycler as follows: one cycle at 98 °C for 10 seconds, followed by 34 cycles at 98 °C for one second, 53 °C for 1 minute and 72 °C for 15seconds. A final extension step at 72 °C for 1 min was performed for 1 cycle. The reaction was held at 4 °C until the amplicons were removed from the thermal cycler. The amplicons were then assessed by running 1% agarose gel electrophoresis and viewed in the Gel Doc imager. The amplified 16S rRNA gene sequences was aligned using the Bio-edit and CLUSTALW software. The Basic Local Alignment Search Tool (BLAST) program of the National Centre for Biotechnology Information (NCBI) was used to search and identify the closest species. The Mothur 1.25.1 software program was then used to cluster similar sequences into OTUs (operational taxonomic units). Finally, Simpsons Index of Diversity was used to define the community structure.

### *G) Screening for Hydrocarbon utilization by isolates*

The microbial isolates were screened for their ability to utilize sole used hydrocarbon as carbon source using mineral salt medium as described by Mills *et al*. [14] and adopted by Ekanem and Ogunjobi [15]. The medium (9.0ml) were dispensed into test tubes. Into each of the test tubes, 1.0ml mixture of petrol, engine oil, diesel and transmission oil were added respectively and capped. After capping, all the test tubes were sterilized at 121$^{o}$C for 15 minutes and allowed to cool. On cooling, the test tubes were inoculated with 1ml of standardized bacterial cell suspension of the respective bacterial isolates. The test tubes which served as control was not inoculated. All the test tubes were incubated at 30$^{o}$C for 14 days after which each tube was standardized using serial dilution and spread on nutrient agar plate and incubated at 30 $^{o}$C for 24 hours and colony counter was used to count the colonies and expressed as $x10^{7}$.

*H) Determination of hydrocarbon utilization*

utilization of hydrocarbon was carried out by using Turbidometry method as described by Gayathiri [16], to determine bacterial utilization of hydrocarbon, a mineral salt medium broth was prepared with (1% mixture of petrol, engine oil, diesel and transmission oil) given as carbon source. The medium contained K2HPO4 (1.8g/l); NH4Cl (4g/l); MgSo4.7H2O (0.2g/l); NaCl (0.1g/l); Na2So4.7H2O (0.01g/l); and distilled water (1L). A MSM medium without hydrocarbons was sterilized by autoclaving at 121°C for 15 min. The degrading activities of each isolate were obtained by using mineral salt broth (MSB) in which 1% of hydrocarbon (mixture of petrol, engine oil, diesel and transmission oil) was added and incubated at room temperature for 30 days. The growth of the bacterium was measured using spectrophotometer (Eppendorf Biophotometer 8.5mm, Lichtstrahihohe, England) by taking the O.D readings at 600nm from 0 hrs– 30 days at regular intervals of 1 week against mineral salt medium as control.

*Effect of Temperature on utilization of used hydrocarbon substrate from contaminated soil*

Effect of temperatures was carried out following a method described by Boochan *et al.* [17]. One hundred (100) ml of the degradation media was transferred into different conical flasks and the degradation media was incubated at 32$^{o}$C, 34$^{o}$C, 36$^{o}$C and 38$^{o}$C as described by Muhammad *et al*. [17].

## III. RESULTS

The Total mean viable count of bacteria isolated from contaminated soil of hydrocarbon in Mechanic workshops Keffi is as given in Table 1. The total bacteria count from contaminated soil of hydrocarbon from shop 4 ($3.02 \pm 0.08$) and the least was from shop 2 ($1.61 \pm 0.01$) respectively

The cultural, morphological and biochemical characteristics of bacteria isolated is as shown in Table 2

Plate 1 shows the agarose gel electrophoresis of the 16S rRNA bands of bacteria isolated from auto-mechanic workshop in Keffi, Lanes 1 represents *Bacillus* sp, Lanes 2 represent *Pseudomonas* sp, Lanes 3 represent *Proteus* sp and Lanes 4 represent *Acinetobacter* sp the 16SrRNA bands (1500bp), Lane M represents the 1500bp molecular ladder.

The phylogenetic tree of the bacteria isolated from auto-mechanic workshop in Keffi sequenced and blasted showing the evolutionary *Bacillus thuringiensis, Proteus terrae, Pseudomonas synxantha, Flavobacterium columnare* and *Acinetobacter beijerinckii* as shown in Fig 1-4.

The total viable count of hydrocarbon utilizing bacteria is as shown in Table 3 where the highest count was *Pseudomonas synxantha* ($1.88 \pm 0.03$) followed by *Bacillus thuringiensis* ($1.05 \pm 0.01$), *Flavobacterium columnare* ($0.81 \pm 0.04$), *Proteus terrae* ($0.12 \pm 0.01$) and *Acinetobacter beijerinckii* ($0.00 \pm 0.00$).

The effect of temperature on utilization of used hydrocarbon from mechanic workshops is as shown in Table 4. The highest utilization of used hydrocarbon was observed at temperature of 32°C were *Pseudomonas synxantha* had the highest utilization of 14.01±2.01mg/ml and the least was *Proteus terrae* 3.51±0.45mg/ml. At temperature of 34°C the highest utilization was *Pseudomonas synxantha* 9.38±0.28mg/ml and *Proteus terrae* 3.00±0.14mg/ml. At 36°C the highest was observed by *Bacillus thuringiensis* 5.18±1.12 mg/ml. At 38°C the highest utilization was observed by *Bacillus thuringiensis* 3.95±0.01mg/ml and the lowest was *Pseudomonas synxantha* 2.11±0.08mg/ml.

The determination of utilization of used hydrocarbon after four weeks is as given in Table 5. The highest utilization was obtained after 3 weeks by *Bacillus thuringiensis* 9.60±0.18mg/ml and the lowest utilization was recorded by *Proteus terrae* 4.20±1.01mg/ml. After 4 weeks *Bacillus thuringiensis* utilized 7.55±0.78mg/ml used hydrocarbon and lowest utilization was recorded by *Proteus terrae* 2.00±0.12mg/ml. After Week 2 *Bacillus thuringiensis* utilized 4.40±0.88mg/ml of used hydrocarbon and the least was recorded from *Proteus terrae* 1.22±0.24mg/ml. after week 1 the highest utilization was recorded from *Pseudomonas synxantha* 2.21±0.58mg/ml utilization of used hydrocarbon.

Table 1 Total mean viable count of bacteria isolated from soil contaminated with hydrocarbon in auto-mechanic workshops Keffi.

| Sample location | Total viable count (cfu/g) $x10^{7}$ |
|---|---|
| shop 1 | $2.11 \pm 0.03$ |
| shop 2 | $1.61 \pm 0.02$ |
| Shop3 | $2.20 \pm 0.03$ |
| shop 4 | $3.02 \pm 0.08$ |
| shop 5 | $1.70 \pm 0.02$ |
| shop 6 | $2.54 \pm 0.05$ |
| Control | $6.01 \pm 0.12$ |

Table 2: Cultural, Morphological and Biochemical characteristics of Bacterial Isolates

| | | | Biochemical characteristics | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Cultural Morphology | Shape | Gram stain | Cat | Nit | Coa | Gel | Ure | Glu | Suc | Inference |
| Brown on Mac and Greenish Colonies on NA | Rod | - | + | + | - | + | - | + | - | *Pseudomonas synxantha* |
| Smooth, mucoid, pale yellow to greyish white colonies on NA | ovoid | - | + | + | - | + | - | + | + | *Acinetobacter beijerinckii* |
| Bulky milkfish and dried colonies on NA | Rod | + | + | - | - | - | + | - | - | *Bacillus thuringiensis* |
| Mucoid and brownish on NA | Rod | - | - | + | - | - | - | + | - | *Proteus terrae* |
| Colonies are translucent and pigmented yellow to orange on NA | Rod | - | + | + | - | + | + | + | + | *Flavobacterium columnare* |

KEY: NA- nutrient agar, Cat-catalase, Nit- nitrate, Coa-coagulase, Gel-gelatin, Ure- urea, Glu- glucose, Suc- sucrose

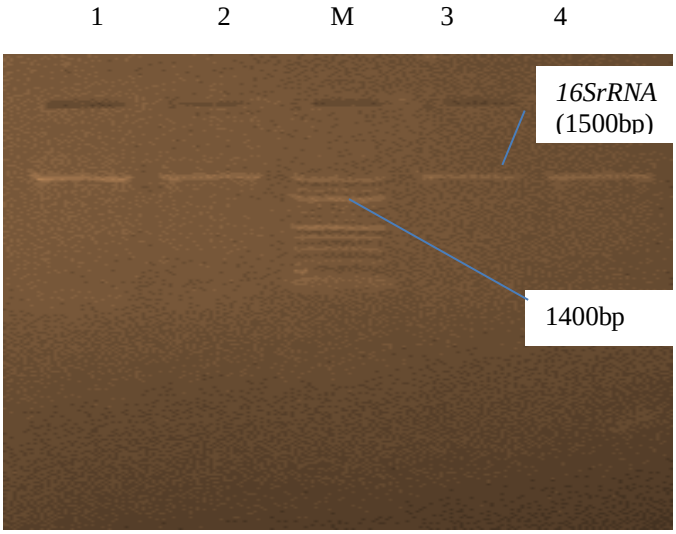


*Plate 1*: Agarose gel electrophoresis of the 16S rRNA band of bacteria isolates. Lanes 1 represents *Bacillus thuringiensis*, 2 represent *Pseudomonas synxantha*, 3 represent *Proteus terrae* and 4 represent *Acinetobacter beijerinckii* the 16SrRNA bands (1400bp), Lane M represents the 1500bp molecular ladder.

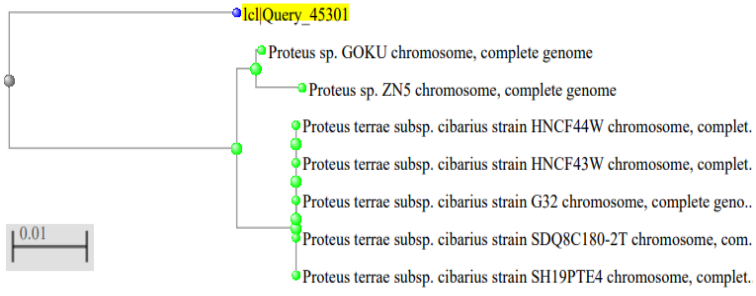


Fig1: Phylogenetic tree showing the evolutionary distance between the bacteria isolates.

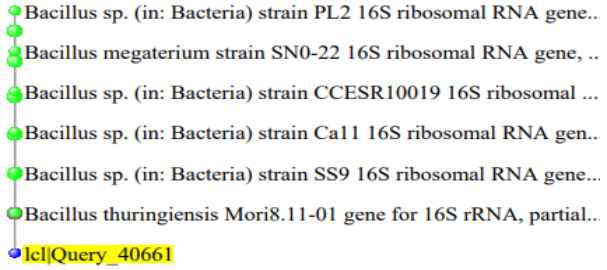


Fig2: Phylogenetic tree showing the evolutionary distance between the bacteria isolates.

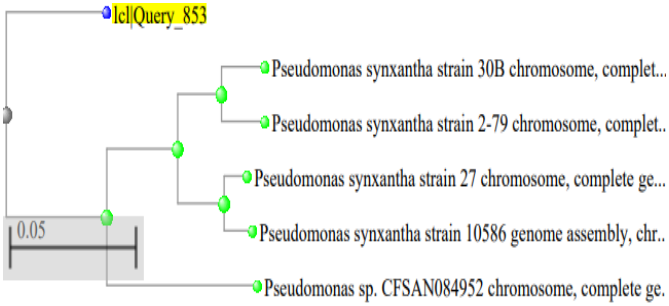


Fig3: Phylogenetic tree, showing the evolutionary distance between the bacteria isolates.

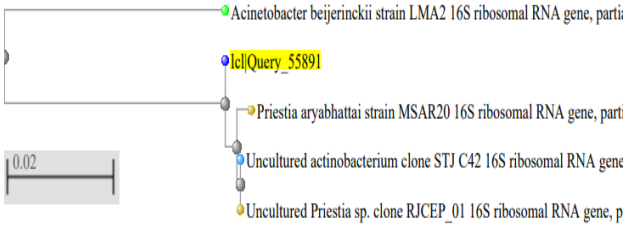


Fig5: Phylogenetic tree, showing the evolutionary distance between the bacteria isolates.

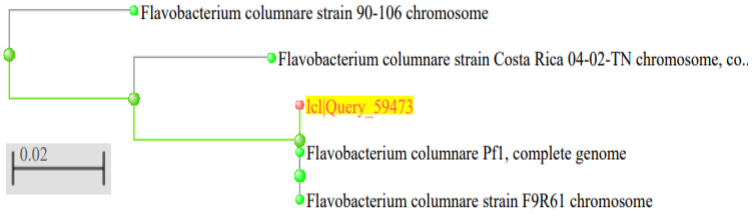


Fig6: Phylogenetic tree showing the evolutionary distance between the bacteria isolates.

Table 3 Total viable count (TVC) of bacterial isolates in hydrocarbon medium

| Organisms | Total HUB count (cfu/g) $x10^7$ |
|---|---|
| *Flavobacterium columnare* | 0.81 ± 0.04 |
| *Pseudomonas synxantha* | 1.88 ± 0.03 |
| *Bacillus thuringiensis* | 1.05 ± 0.02 |
| *Proteus terrae* | 0.12 ± 0.01 |
| *Acinetobacter beijerinckii* | 0.14 ± 0.02 |

Key: HUC- hydrocarbon utilization bacteria

Table 4 Effect of temperature on utilization of hydrocarbon by bacteria isolated from soil contaminated with auto-mechanic oil

| | Amount of used hydrocarbon utilized in different temperature | | | |
|---|---|---|---|---|
| Isolates | 32°C mg/ml | 34°C mg/ml | 36°C mg/ml | 38°C mg/ml |
| *Acinetobacter beijerinckii* | 8.11 ± 0.59 | 6.79±0.48 | 4.00 ± 0.16 | 3.10± 0.11 |
| *Pseudomonas synxantha* | 14.01±2.01 | 9.38±0.28 | 4.08±0.14 | 2.11±0.08 |
| *Bacillus thuringiensis* | 12.21±0.72 | 6.13±0.88 | 5.11±1.12 | 3.95±0.01 |
| *Proteus terrae* | 3.51±0.45 | 3.00±0.14 | 0.79±0.01 | 0.0±0.00 |
| Control | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ±0.00 | 0.00±0.00 |

Table 5 Hydrocarbon utilized in different weeks by bacteria isolated from from soil contaminated with auto-mechanic oil

| | Amount of hydrocarbon utilized in different weeks | | | |
|---|---|---|---|---|
| Bacteria | Week 1 Mg/ml | Week 2 Mg/ml | Week 3 Mg/ml | Week 4 Mg/ml |
| *Flavobacterium columnare* | 1.40 ± 0.11 | 2.70±0.28 | 7.13 ± 0.40 | 5.10 ± 0.10 |
| *Pseudomonas synxantha* | 2.21±0.58 | 3.21±0.25 | 7.59±0.26 | 6.55±0.01 |
| *Bacillus thuringiensis* | 2.11±0.12 | 4.40±0.88 | 9.60±0.18 | 7.55±0.78 |
| *Proteus terrae* | 0.41±0.05 | 1.22±0.24 | 4.20±1.01 | 2.00±0.12 |
| Control | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |

## IV. DISCUSSION

Biodegradation or utilization of used hydrocarbons is a complex process that depends on the nature and number of different hydrocarbons present in the contaminated soil. This study aimed at isolation and characterizing of some bacteria that utilized used hydrocarbon in some selected mechanic workshops in Keffi. The total viable count of bacteria from contaminated soil with used hydrocarbon was impressive showing the presence of some bacteria that utilized or biodegrade this harmful pollutant in the environment. The highest viable count was observed from shop 4 with 3.02 ± 0.08 mg/ml the high number of bacteria present in the polluted soil may be due to the fact that the bacteria had adjusted to their present environment which contain high hydrocarbon which they use as their source of carbon similarly to the low bacteria count which may be due to the high amount of used hydrocarbon that is present in the soil. This result is similar to findings of Udeani *et al*. [18]. The occurrence of different bacteria isolated from soil contaminated with hydrocarbon in auto-mechanic workshops in Keffi is similar to the finding earlier reported by Ekanem and Ogunjobi, [19], the bacterial isolated were *Pseudomonas synxantha*, *Acinetobacter beijerinckii*, *Bacillus thuringiensis*, *Proteus terrae* and *Flavobacterium columnare*. the high isolation rate of these bacteria maybe due to their ability to withstand harsh environmental conditions because they have extra mechanism to adapt to any condition to make it favorable for them to grow and multiply as reported by Boboye *et al*. [20].

The survival rate of *Pseudomonas synxantha* and *Bacillus thuringiensis* in solely used hydrocarbon medium as observed in this study was encouraging it showed that these bacteria utilized the used hydrocarbon as their source of carbon which helps in reducing contaminate from the soil in auto-mechanic workshops in Keffi and is in agreement with the studies earlier reported by Sebiomo *et al*. [21].

The utilization of used hydrocarbon recorded in this study from different temperature showed that these bacteria have the ability to utilize the used hydrocarbon if the right temperature is maintained when cleaning up the polluted soil. In this study, it was observed that at temperature of 32°C favorable these bacteria to utilize the used hydrocarbon that contaminate soil in different auto-mechanic workshops. It was observed that *Pseudomonas synxantha* had the highest utilization of used hydrocarbon at 32°C with 14.01±2.01mg/ml and *Bacillus thuringiensis* 12.21±0.72mg/ml this shows that physical factors such as temperature plays an important role in biodegradation of hydrocarbons by directly affecting the chemistry of the pollutants as well as affecting the physiology and diversity of the microbial flora in the polluted area [22].

Similarly, the effect of duration on utilization of the used hydrocarbon as observed in this study showed the highest utilization after 3 weeks were *Bacillus thuringiensis* have the highest utilization rate with 9.60±0.18mg/ml and *Pseudomonas synxantha* 7.59±0.26 mg/ml, the drop in the utilization in week 4 maybe due to the amount of the used hydrocarbon in the environment and lack of other nutrient or the medium becoming toxic due to acidity which was not studied and not determined. But research have shown that changes in occur when bacteria use hydrocarbon as source of carbon in a medium, the medium tend to become highly acidity which affect the bacteria and reduce their population. This is similar to the study reported by Ilori *et al*. [23, 24, 25]. As observed in this study environmental factors affect the microorganism will help in utilization of used hydrocarbon in our environment.

## V. CONCLUSION

In this study it was observed that used hydrocarbon affect the microbial population in the soil as we recorded low viable count of bacteria from the contaminated soil in the study area. The different bacteria isolated and molecularly identified are namely: *Pseudomonas synxantha, Bacillus thuringiensis, Flavobacterium columnare* and *Proteus terrae*. These bacteria were able to utilize used hydrocarbon at different temperature and the highest utilization was recorded at temperature of 32℃. The findings suggest that temperature and duration plays important role in used hydrocarbon degradation or clean up from soil that is contaminated

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