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Biodegradation of Crude Oil-Polluted Soil by Bacterial Isolates from Nigeria

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Abstract: Biodegradation of crude oil-polluted soils by bacterial isolates was carried out experimentally, to determine the ability of bacterial isolate to utilize and detoxify crude oil-polluted soils. Three soil samples were collected from crude oil-polluted sites of Owazangboko, Abia State, Aguleri-otu, Anambra State and Obi-igbo, Rivers State, Nigeria. Microbiological analysis and physico-chemical analysis were carried out in the Microbiology Laboratory of Chukwuemeka Odumegwu Ojukwu University, Uli. Physico-chemical analysis was carried out to determine the pH, moisture content, water holding capacity and total organic carbon of the soil samples. Microbiological analysis was carried out using serial dilution and spread plate methods to determine the total viable count, enumeration of hydrocarbon utilizing bacteria (HUB) was done using vapour phase method on mineral salt agar monitored at the wavelength of 600nm for 5 days. Adaptation test was carried out and monitored for 5 days using mineral salt broth containing 1ml and 2ml of sterile crude oil separately. A consortium of six effective isolates was taken for the biodegradation of the crude oil, which was monitored for 14 days. Biochemical tests were carried out on the isolates. Polymerase chain reaction (PCR) technique was used to confirm the identity of the isolates. Gas chromatographic flame ionization detector (GC-FID) was used to monitor the amount of the residual total petroleum hydrocarbon after the biodegradation study. Results of physic-chemical analysis showed; pH value of 3.7– 6.6, moisture content 1.6 – 4.4., water holding capacity 28.0 – 32.0., total organic carbon 3.4 – 7.1. Results of total viable count were in the range of 7.1×10^{6} - 5.9×10^{10}. Results of vapour phase method ranged from 0.16 – 0.66. Adaptability test revealed values, 0.11 – 0.54. Biochemical test results revealed *Bacillus spp., Pseudomonas spp., Serratia spp., Micrococcus spp., Arthrobacter spp., Proteus spp., Shigella spp*. PCR results gave the identity of the organisms to be *Lysinibacillus spp.* M2c, *Serratia marcescens* Mb4, *Bacillus aerius* TPM-23, *Proteus mirabilis* LS-3 and a new unidentified bacterium. TPH concentrations were in the range of 582.67 mg/l – 123.67 mg/l for *Lysinibacillus spp.* M2c and *Serratia marcescens* Mb4, 682.65 – 203.85 mg/l for *Bacillus aerius* TPM-23and 753.32 mg/l – 324.77 mg/l for *Proteus mirabilis* LS-3 and the new unidentified bacterium. The effect of bacterial growth across the days showed a p-value of 0.000 less than the α-value 0.01, this concluded that the growth of the bacteria across the days was significant for both vapour phase test and adaptability test. From this study it is evident that *Serratia marcescens* Mb4 and *Lysinibacillus spp.* M2c could be used to effectively treat crude oil-polluted environment.

Keywords: Crude Oil, Soil, Microbiological, Physico-Chemical, Biodegradation, PCR, Gas Chromatographic

1. Introduction

Crude oil pollution has been considered a global concern to both humans and the environment. In Nigeria, there is an average of 300 oil spills every year [1]. Environmentalists consider the Niger Delta region of the country as one of the world's most polluted regions [35]. The Niger Delta region consists entirely of nine States which include Abia, Akwa Ibom, Bayelsa, Cross Rivers, Delta, Edo, Imo, Ondo and

Rivers [2]. The Niger Delta region, the base of the Nigerian oil and gas industry, generates over 90 percent of the nation's foreign exchange earnings. Paradoxically, in spite of the increasing revenue from crude oil exploitation, the communities from which this resource flows in the Niger Delta continue to live in conditions of social deprivation and abject poverty.

All stages of oil exploitation impact negatively on the environment, and the greatest single intractable environmental problem caused by crude oil exploration in the Niger Delta region is oil spillage [40]. Apart from other anthropogenic emission sources, atmospheric pollution in the region is associated with emissions from flaring and venting of petroleum associated natural gas by petroleum industries [13, 25, 34]. Atmospheric contaminants from anthropogenic activities can be categorized into (i) gaseous pollutants, (ii) persistent organic pollutants, (iii) particulate matter and (iv) trace element and/or heavy metals [16]. Release of petroleum hydrocarbons into the environment, whether accidentally or due to anthropogenics activities, is a major cause of uncontrolled water and soil pollution [16], and may also contribute to regional atmospheric pollution [13].

Bioremediation is described as the use of living microorganisms or microbial processes to detoxify and degrade environmental pollution. In order words, it is a technology for removing pollutants from the environment thus restoring the original natural environment. Soil biological activities: microbial counts, soil respiration, soil biomass and enzymes activities can be used to ascertain the extent of bioremediation process of oil contaminated soil [22]. Diplock *et al.* [8], suggested that meeting a bioremediation end point could be used to evaluate and predict the bioremediation of a contaminated area. The biochemical potential for a contaminated soil to reach a target level is used to define the end point of the remediation process [4]. Quantification of the total culturable degrader population; an evaluation of hydrocarbon bioavailability and a measure of constraints to biodegradation are key parameters to predict the likely performance of a bioremediation strategy [32]. Lin *et al.* [21] used a bioprocess of bioaugmentation and biostimulation with a molecular monitoring microarray biochip integrated with land farming operation to effectively degrade approximately 70% and 63% of diesel oil and fuel oil respectively after a period of 28 days. The bioslurry assays yielded a total petroleum hydrocarbon (TPH) reduction efficiency of 57% and 65% in 28 days respectively, Radwan *et al.* [33], employed the technique of immobilizing oil degrading bacteria, nocardioforms and Acinetobacter in biofilms coating macroalgae to remediate noctadecane and phenanthrene while Kermanshahi *et al*. [19], used immobilized microbial cell airlift bioreactor for aerobic bioremediation of simulated diesel fuel contaminated groundwater. Gargouri *et al*. [11] used a continuously stirred tank bioreactor (CSTR) to optimize feasible and reliable bioprocess system to treat hydrocarbon-rich industrial wastewaters. The use of the mixed cultures in the studies demonstrated high degradation performance for hydrocarbons range n-alkanes (C10–C35).

These techniques for removing petroleum hydrocarbons in the soil are developed around strategies for delivering moisture, aeration and nutrients in order to optimize microbial activity and degradation of the pollutants [3, 17, 36]. This process involves treating the contaminant at the site of the contamination and is divided into bioattenuation, biostimulation and bioaugmentation. A study has revealed the high potency of *Pseudomonas* strains in bioremediation of petrochemical waste-waters [26]. Typical groups of bacterial known for their ability to degrade hydrocarbon include: *Micrococcus, Pseudomonas, Alcanivorax, Microbulbifer* and *Cellulomonas*. Hexadecane was degraded by *Pseudomonas putida, Rhodococcus erthroplolis* and *Bacillus thermoleoovorans* [1].

The long term aim of bioremediation designs is to present a cost effective strategy which will help to reduces pollutants to a level reasonably and practicably possible.

1.1. Statement of the Problem

Despite the availability and use of advanced technology in the petroleum industry, various forms of accidents such as blow-outs of production wells, explosions and pipeline rupture still occur, which are worsened by vandalization of oil installations and pipelines [14, 31] Irrespective of the numerous environmental laws enacted to protect the environment, gas flaring for example has also continued despite the Nigerian government's directive to end flaring by 2010. The Niger delta region claims that the activities of the oil companies instead of improving have impoverished its people by causing a serious decline in their marine and agricultural resources, which constitute their economic main stay. Since the latest escalation of violence, hundreds of people have been killed in clashes between militants and government security forces. Armed gangs have carried out numerous attacks on oil facilities kidnapping many oil workers and ordinary Nigerians for ransom. The Ogoni, Ijaw and other people in the Niger Delta, those who have been affected for decades have been trying to stand up for themselves, their environment and their basic human and economic rights. There is an urgent need to look into the ugly situation faced by the people of this region, to ensure environmental conservation of the Niger Delta by using microorganism to bioremediate their polluted environment. The goal of this research is to establish the ability of bacterial isolates to utilize and detoxify crude oil contaminated soil.

1.2. Study Area

The Niger Delta is described as a unique ecological zone by virtue of its size and geophysical configuration [23]. It is one of the world's largest wetlands covering an area of approximately 70,000 km. Located in the South-South geopolitical region of Nigeria. It lies between latitude 4º and 6º North of the equator and longitude 5º and 7º East of Greenwich. It stretches from the Benin river in the West to

Bonny river in East, while in land, it begins a few miles below Aboh at a point where river Niger splits into river Nun and Forcados into the Atlantic West at the South, stretching over 257,495 metres[15]. The region consists entirely of nine States which include Rivers, Bayelsa, Cross Rivers, AkwaIbom, Delta, Edo, Ondo, Abia, and Imo [2].

1.3. Scope

This study has been able to examine crude oil-polluted soil samples from Abia, Anambra and Rivers States of Nigeria. The biodegradation studies were conducted from September, 2016 to May, 2017.

2. Materials and Method

2.1. Sample Collection

Methods of Wood [41] were used in this study. Three composite soil samples were collected from three different locations of crude oil contamination. The locations include: Owazangboko, Ukwa west in Abia, Aguleriotu in Anambra and Obi-igbo, Oyigbo in River State. One soil sample was collected from a non-contaminated site in Awka, Anambra State, as control. Clean shovel was used to dig about 20cm of the soil before sterile soil auger was used to collect the contaminated soil samples. Collected soil samples were passed through a sterile 2mm sieve into a plastic zip-lock bags, which was immediately transferred into a cooling box (4-10°C) containing ice. Samples were properly labelled; the cooling box was used to transfer the samples to the laboratory. Samples were then stored in the refrigerator at 4ºC according to [24]. Then physicochemical analysis was immediately carried out on the soil samples.

2.1.1. Determination of Soil pH

pH of soil samples was analyzed using an electronic pH meter, 10g of the soil sample was air-dried after sieving through 2-mm mesh size and transferred into a 50ml-beaker containing 20ml of distilled water. The soil suspension was stirred several times for about 30 seconds using a glass rod. The suspension was allowed to stand for 2 minutes using the pH meter, Hana-digital conductivity meter model 98107 inserted into the partly settled suspension to measure the pH, until a steady reading was obtained [18].

2.1.2. Determination of Soil Moisture Content

The soil moisture content was done to determine the amount of moisture in the soil sample. Four crucible covered with lid were oven dried at 105°C for about 2 hours. They were then cooled in a desicator and their weight was taken separately. 10g of soil samples were weighed into each of the crucible and their initial weights taken. The soil samples in the crucible were transferred into oven with lid off to dry for 24 hours. Then crucible and the samples were transferred to a desiccator before weighing again until a constant weight is obtained [18]. The loss in weight was calculated, using the formula below:

$$\text{Moisture Content} = \frac{W_2 - W_3}{W_3 - W_1}\ x\ 100$$

Where: W_1 = weight of empty crucible (g)
W_2 = weight of crucible + moist soil (g)
W_3 = weight of crucible + oven dry soil (g)

2.1.3. Determination of Water Holding Capacity

Four small plastic containers with one opened end and closed end were used. A medium sized nail was used to make holes at the closed end of the containers. Whatman's no 1 filter paper that sized the perforated end was placed on the inside of the sealed end through the opened end. The filter paper was moistened with a jet of distilled water from a wash bottle. The weight of the container with the wet filter paper was recorded. Then the container was filled with oven dried soil sample of 24 hours compacted by dropping the container from a height until the soil surface was levelled. The weight was taken again and recorded. The container was put in a bowl containing water until the surface of the soil is moistened. The container was removed and put in empty Petri dish to allow the excess water to drain off. The container was then removed and weighed [18]. The quantity of moisture retained per unit weight of the oven dried soil contained in each of the container was determined using the formula below:

Mass of dry soil = weight of plastic container with filter paper and soil (W_2) – weight of plastic container with filter paper (W_1)

Mass of saturated soil = weight of plastic container with wet soil (W_3) – weight of plastic container with filter paper (W_1)

Mass of water contained in saturated soil = mass of saturated soil – mass of dry soil.

% of water holding capacity = mass of water contained in the saturated soil x 100
mass of saturated soil.

2.1.4. Determination of Total Organic Carbon (TOC)

Ten (10) ml 1N k_2CrO_7 was added into 5 conical flasks of 250 ml, 1g of each of the soil samples to be analyzed was crushed and sieved with 0.5mm sieve which was transferred into each of the conical flask containing the potassium dichromate solution. 20ml of conc. $H_2SO_4$was added into each of the conical flasks; the whole sample was then swirled gently to disperse the heat generated for 30 minutes on asbestos before cooling down. Then 100ml of distilled water was gradually added to each of the flask and mixed, 5ml of phosphoric acid was added with 3 drops of ferroin indicator to each of the samples. Titration was done using ferrous sulfate against the test samples. During titration the solution changed from a bluish-green colour upon addition of ferrous sulfate drop-wise, to a reddish grey colour or maroon colour. This was taken as the end point of the titration, the percentage carbon in the samples were calculated using the equation:

$$\frac{(\text{Blank titre} - \text{Actual titre})}{\text{Wt of air dried soil sample}} \times 0.3 \times \text{M} \times \text{F}$$

Where M = concentration of FeS04
F = correction factor = 1.33

2.2. Sterilization of Glassware and Media

Materials which included conical flasks, funnels and test tubes were sterilized in a hot air oven at 160°C for about 1 hour while the media was sterilized by autoclaving at 121°C for 15mins. All pipettes and other heat-resistant glassware's were wrapped in Aluminum foil to protect the items from recontamination during handling and storage before sterilization was done at 160°C for 1hr in the hot air oven. The Petri dishes used were also sterilized. Water was used to wash all the equipment's, detergents were used where necessary and 70% ethyl alcohol which is bactericidal was used to swab the top of the working bench in the laboratory where the inoculations were done.

2.3. Microbiological Analysis and Isolation of Bacteria

Collected soil samples transferred to the laboratory in a cooling box with ice were stored at the temperature of 4°C until lab process and analysis takes place. Soil from each crude oil contaminated site was serially diluted using distilled water. Total heterotrophic bacteria were enumerated using the spread plate method on nutrient agar according to methods of [5]. Ten grams of each of the soil sample was weighed and transferred into 250ml flask containing 100ml of distilled water to form the neat. The solution was mixed properly and allowed to stand for 2-3 minutes after which the suspension was decanted into another 250 ml flask. Then 1ml of each of the soil suspension was serially transferred into a test tube containing 9ml of distilled water. The suspensions were shaken intermittently for about 60 seconds. Ten-fold serial dilution was set up from the soil suspension. 0.1ml of the one in 104, 106 and 108 were spread in duplicate. One freshly prepared nutrient agar was poured and allowed to set. This was used as control. Plates were incubated in an inverted manner at temperature of 37°C for 24 hrs. Total plate counts were carried out using the colony counter. Organisms were sub-cultured in sterile nutrient agar for total heterotrophs [38]. Mineral salt agar was used for hydrocarbon utilizing organism (HUO) according to methods previously modified by Chikere*et al.,*[7] and also improved by American type culture collection.

2.4. Enumeration of Hydrocarbon Utilizing Bacteria

Culturable hydrocarbon degraders, also known as hydrocarbon utilizing bacteria (HUB) were assayed for. Here crude oil was used as the sole source of energy and carbon. Sterilized crude oil was used to soak sterilized filter paper. This was placed in the lid of inverted plates. The plates were incubated at room temperature for 6 days and HUB were counted [7]. mineral salt agar (per litre: 2.0g $NaNO_3$, 0.5g $MgSO_4$, 0.5g KCL, 0.01g, $Fe_2(SO_4)_3H_2O$, 0.14g KH_2PO_4, 1.2g K_2HPO_4, 15g Agar, 0.02g yeast extract and pH 7.2) was used to culture the serially diluted samples. Plates were incubated in the dark at room temperature. 100mg of Nyzoral was added to make the media selective for bacteria only.

2.4.1. Determination of Bacterial Growth Curve

Optical densities readings were taken by taking a loopful of the bacterial growth on mineral salt agar into a test tube containing saline, by adjusting the turbidity of the bacterial suspension to match with the turbidity of 0.5% McFarland which is approximately 1×10^8CFU/ml. The 0.5% of McFarland solution was prepared by mixing 0.5 ml of 1.175% barium chloride dehydrate, with 99.5 ml of 1% sulfuric acid. Readings were taken for a period of 5 days. The optical density was measured at 600nm using the Spectrophotometer.

2.4.2. Determination of Bacterial Adaptation Using Strain Improvement Test

Bacterial isolates which showed optimal growth curve were further subjected to growth adaptation, using mineral salt broth containing 1ml and 2ml of sterile crude oil in a test tube covered with sterile cotton plug, incubation was done at 37°C in an incubator for a period of 5 days. Optical density and pH of the bacterial broth was measured with a pH meter and spectrophotometer at everyday interval throughout the period of 5 days [6].

2.5. Characterization and Biochemical Identification of Isolates

Pure isolates were examined for their cultural appearance. Endospore and Grams staining techniques were performed to establish the cell morphology of the isolates. motility, citrate utilization, oxidase, triple sugar iron test, catalase and indole tests were carried out according to methods of Umeaku[39] and Cheesbrough[5] Microbial identification was performed using the keys provided by Bergey's Manual of Determinative Bacteriology (1994).

2.6. Biodegradation Studies

The biodegradation of the crude oil was carried out in the laboratory using liquid medium. Method of Ekpo and Ekpo[10] was adopted, 1 litre of mineral salt broth was prepared and autoclaved, 99ml of the liquid medium was dispensed each into four different sterile 250ml amber bottle then 1ml of sterile crude oil was added into each of the amber bottles, and inoculated with five millilitre of improved bacterial strain, one was left uninoculated, it served as the control. The bottles were then incubated at an ambient temperature (30°C) in a dark cupboard for 14 days, with manual shaking of 100 strokes per minute for 30 minutes, each day for 14 days. Bacterial growth rate and pH were determined for the initial day and every 7 days [10]. The total petroleum hydrocarbon was monitored and measured for changes using gas chromatography [30].

2.7. Gas Chromatographic Analysis

Total petroleum hydrocarbon analysis was done using the four samples from the biodegradation studies. Before the biodegradation studies isolates which showed ability to

grow and use the crude oil present in mineral salt agar using vapour phase method were transferred to the mineral salt broth for optimal growth and adaptability. The residual total petroleum hydrocarbons (TPHs) were extracted from the mineral salt broth containing organisms isolated from different soil samples collected from Anambra, Abia and River state. Quantification was done using gas chromatograph-flame ionization detector (GC- FID) at seven days' interval for 14 days according to the methods of ASTM D3912(2013) and USEPA (2007). At Anal Concept Laboratory Port-Harcourt. A 250ml of liquid sample was measured out into a separating funnel, the container was rinsed with dichloromethane then 25ml of dichloromethane was added to the 250ml of liquid sample. The separating funnel was then mixed vigorously to allow homogenization of the organic solvents. The organic extract was collected into a receiving container, and then the organic extract was passed through a column containing cotton plug silica gel and anhydrous sodium sulphate. The collected organic extract about (1µl) was injected into the gas chromatograph machine model 5890 series II (using a micro syringe) through a rubber septum into a flash vaporizing port at the head of the column. The temperature of the sample port is about 60°C higher than the boiling point of the least volatile component of the sample. Inside the GC the carrier gas (hydrogen) was allowed to move each analyte through a capillary column with the length and temperature of 30m and 278°C. Each analyte required a different amount of time to pass through the column. The sample's outlet stream was monitored using a flame ionization detector; results of different hydrocarbon were shown in chromatograms [38].

2.8. Molecular Analysis

DNA extraction and purification – Zymo research kit was used.

DNA quantity and quality analysis by nanodrop spectrophotometer.

DNA Amplification – Eppendorf thermocycler was used.

Gel electrophoresis of DNA and PCR products.

PCR products purification and DNA sequencing of 16s rDNA gene.

2.8.1. DNA Extraction and Purification – Zymo Research Kit Was Used

The DNA extraction and purification was carried out by adding 100mg (wet weight) of bacterial cells into a bashing tube containing 200 µl of isotonic buffer, then 750 µl of lysis solution was added to the tube. Tube was then secured in a bead fitted with 2 ml tube holder assembly and process at maximum speed for > 5 minutes, centrifugation of the ZR bashing bead TM lysis tube was carried out in a micro-centrifuge at > 10,000 x g for 1 minute. Then 400 µl supernatant was transferred to a Zymo-spinTM IV spin filter (orange top) in a collection tube and centrifuged at 7,000 x g for 1 minute., prior to this the base of the Zymo-spin TM spin filter was snapped off. After that, 1,200 µl of bacterial DNA binding buffer was added to the filtrate in the collection tube and 800 µl of the mixture was transferred to a Zymo-spinTM IIC column in a collection tube and centrifuged at 10,000 x g for 1 minute. This step was repeated twice because the Zymo-spinTM IIC column has a maximum capacity of 800 µl. the flow through from the collection tube was discarded, 200 µl DNA pre-wash buffer was added to the Zymo-spin TM IIC column in new collection tube and centrifugation was done at 10,000 x g for 1 minute and 500 µl bacterial DNA wash buffer was also added to the Zymo-spinTM IIC column and it was also centrifuged at 10,000 x g for 1 minute. The Zymo-spinTM IIC column was then transferred to a clean 1.5 ml micro-centrifuge tube and 100ulDNA elution bufferwas added directly to the column matrix before centrifuging at 10,000 x g for 30 seconds to elude the DNA. DNA was now suitable for PCR.

2.8.2. Preparation of Agarose Electrophoresis

Agarose gel electrophoresis was prepared by weighing out the appropriate amount of 1.5g of agarose into a 100ml of tris borate EDTA from a 10x TBE into a 250ml flask. This was melted in the microwave oven for 1 minute and 20 seconds, the solution was visually checked to see that all the agarose has melted. Unmelted agarose which looked like tiny refractive lenses floating around was heated a little longer. Then the electrophoretic tank was set up. The agarose was brought out of the oven and allowed to cool to 55°C, 5µl of GR green was then added after cooling the gel. The TBE/Gel was poured onto the gel tray; pouring was done up to half the height of the teeth of the comb inserted on the gel tray. Bubbles were chased out on the surface with a plastic micropipette tip. The gel was allowed to cool until it turns slightly white; this usually takes at least 20 minutes at room temperature, the comb was removed, and enough 1X TAE was poured into the buffer chamber to cover the top surface of the gel, then 10ml of each of the extracted DNA was mixed with 5ml of GR green, micropipette was used to load 2.0µl of the DNA mixture onto the wells on gel tray of the electrophoretic tank. The gel wells were positioned at the cathode end of the gel electrophoretic tank, each of the light sources was then plugged in with electric current switched on at 100 volts which run for about 1hour, and then the separated gel was viewed at the documentation chamber under an ultra violet light.

2.8.3. DNA Quantity and Quality Analysis by Nanodrop2000 Spectrophotometer

The upper and lower optical surfaces of the micro volume spectrophotometer sample retention system was cleaned, by pipetting 2-3µl of clean deionized water unto the lower optical surface. The lever arm was then closed to ensure that the upper pedestal comes in contact with the deionized water before lifting the lever arm and wiping off both optical surfaces with a clean dry free lab wipe. The nano-drop software was then opened to select the nucleic acid application, after which a small volume calibrated pipette was used to perform a blank measurement by dispensing 1µl of buffer unto the lower optical surface then the lever arm was lowered to carry out a

blank measurement. Once the blank measurement was completed, cleaning of both optical surfaces with a laboratory wipe was done as before. Then 1µl of nucleic acid sample was transferred unto the lower optical pedestal and lever arm was closed, this was followed by selecting the measure icon in the application software, this software then automatically calculates the nucleic acid concentration and purity ratio following sample measurement.

2.8.4. DNA Amplification – Eppendorf Thermocycler Was Used

PCR reaction carried out by placing the required components in a tube on ice, the components added were; 10× PCR buffer 2.5µl, mgcl21.0µl, 5pmol forward primer 1.0µl, 5pmol reverse primer 1.0µl, DMSO 1.0µl, DNTPs 2.0µl, taq polymerase 0.1µl, DNA 3.0µl, H2O 13.4µl. The six DNA samples for amplification was then transferred to Eppendorf thermocycler for DNA amplification with a pre-programmed temperature cycle of 94°C to denature the DNA double strands, 56°C to enable the forward and reverse primer to anneal to the DNA single strands, and a final temperature of 72°C to allow for the extension of the individual DNA strands, a total number of 36 circles was carried out. The amplicon from the reaction was loaded on 1.5% agarose gel and the gel picture is called PCR product. The ladder used is 1kb plus ladder from Invitrogen. The expected base pair of the amplicon is around 1500bp.

2.8.5. PCR Products Purification and DNA Sequencing of 16s Rdnagene

This was forwarded outside Nigeria.

2.9. Statistical Analysis

The major statistical technique used in this study for the analysis of the collected data is Analysis of Variance (ANOVA). In this study, only one-way was used to present the data.

3. Results

3.1. Crude Oil Contaminated Soil

The physico-chemical analysis of the contaminated soil sample showing the percentage of total organic matter, pH, moisture content, water holding capacity of the crude oil soil samples are presented in Table 1: below.

Table 1. *Physico-chemical analysis/properties of the soil.*

Sample	pH	moisture content (%)	water holding capacity (%)	TOC (%)
A	3.7	3.0	28.7	3.4
B	5.9	1.6	22.4	4.6
C	6.6	2.0	32.0	3.6
D	6.1	4.4	28.0	7.1

TOC:Total organic carbon
A:soil sample from Abia State.
B:soil sample from Anambra State.
C:soil sample from River State.
D:Uncontaminated soil.

3.2. Isolation and Enumeration of Bacteria

The results of the total heterotrophic bacteria (THB) using plate count and hydrocarbon utilizing bacteria (HUB) using spectrophotometer were presented in Table 2: and Table 3: respectively. Results shows that the population of total heterotrophic bacteria (THB).

Table 2. *Microbial counts of the total heterotrophs in CFU/ml.*

Sample	dilution factor	No of colonies	Cfu/ml
A1	10^4	82	8.2×10^6
A2	10^6	48	4.8×10^8
A3	10^8	33	3.3×10^{10}
B1	10^4	71	7.1×10^6
B2	10^6	54	5.4×10^8
B3	10^8	39	3.9×10^{10}
C1	10^4	124	1.24×10^7
C2	10^6	96	$9.6x\ 10^8$
C3	10^8	59	5.9×10^{10}
D1	10^4	87	$8.7x\ 10^6$
D2	10^6	70	7.0×10^8
D3	10^8	35	3.5×10^{10}
Control	No growth		

3.3. Determination of Bacterial Growth Population

Optical density of 600nm was used to determine the growth population of bacterial isolate during vapour-phase method on mineral salt agar.

Table 3. *Optical density measurement at 600nm.*

Sample	1st day	2nd day	3rd day	4th day	5th day
A1	0.173	0.286	0.312	0.442	0.585
A2	0.168	0.274	0.288	0.394	0.318
A3	0.182	0.322	0.484	0.512	0.664
B1	0.177	0.280	0.281	0.282	0.311
B2	0.166	0.268	0.277	0.380	0.415
B3	0.175	0.282	0.289	0.398	0.467
C1	0.188	0.394	0.400	0.411	0.540
C2	0.180	0.382	0.494	0.598	0.614
C3	0.164	0.265	0.265	0.270	0.373
D1	0.168	0.269	0.270	0.374	0.375
D2	0.166	0.267	0.268	0.268	0.369
D3	0.167	0.267	0.268	0.369	0.370
Control	0.065	0.064	0.069	0.068	0.068
Standard	0.122	0.122	0.121	0.121	0.122

Table 4. *Strain improvement test at 600nm.*

Sample	% of crude oil	1st day	2nd day	3rd day	4th day	5th day
A1	1ml	0.114	0.287	0.418	0.426	0.522
A1	2ml	0.124	0.246	0.318	0.324	0.428
A2	1ml	0.116	0.218	0.223	0.326	0.429
A2	2ml	0.126	0.220	0.224	0.328	0.332
A3	1ml	0.112	0.212	0.321	0.423	0.543
A3	2ml	0.101	0.232	0.314	0.412	0.412
B1	1ml	0.112	0.114	0.245	0.248	0.255
B1	2ml	0.112	0.113	0.113	0.214	0.244
B2	1ml	0.113	0.217	0.224	0.328	0.336
B2	2ml	0.115	0.120	0.220	0.225	0.330
B3	1ml	0.112	0.216	0.318	0.419	0.520

Sample	% of crude oil	1st day	2nd day	3rd day	4th day	5th day
B3	2ml	0.110	0.212	0.227	0.317	0.418
C1	1ml	0.115	0.220	0.225	0.329	0.438
C1	2ml	0.114	0.221	0.232	0.326	0.332
C2	1ml	0.116	0.240	0.242	0.324	0.324
C2	2ml	0.113	0.118	0.219	0.220	0.322
C3	1ml	0.112	0.114	0.142	0.262	0.313
C3	2ml	0.110	0.110	0.110	0.221	0.241
Control	0.010	0.109	0.203	0.205	0.212	

Table 5. *Absorbance and pH value using spectrophotometer (600nm) showing growth of isolates during biodegradation.*

Isolate	Day 1	Day 7	Day 14
Hub-A1, A3	0.128	0.236	0.445
pH	7.22	7.43	7.96
Hub-B2, B3	0.127	0.330	0.538
pH	7.31	7.39	7.44
Hub-C1, C2	0.130	0.239	0.594
pH	7.32	7.38	7.46
Control	0.118	0.119	0.122
pH	7.23	7.20	7.12

Table 6. *Biochemical characterization of isolates.*

Isolate	Morphological Characteristics	Gram stain	Spore stain	Catalase	Oxidase	indole	Citrate
A1	Rod	+	+	+	-	-	+
A2	Rod	-	-	+	+	-	+
A3	Rod	-	-	+	-	-	+
B1	Cocci	+	-	+	-	-	+
B2	Rod	+	+	+	+	-	+
B3	Rod	+	+	+	+	-	+
C1	Rod	-	-	+	+	-	+
C2	Rod	+	+	+	+	-	+
C3	Rod	+	-	+	-	-	+
D1	Rod	+	+	+	+	-	+
D2	Rod	+	-	+	-	-	+
D3	Rod	-	-	+	-	-	-

Table 6. *Continued.*

Isolate	H_2S	Motility	Glucose	Lactose	Gas prod	TSI	Organism
A1	-	+	-	-	-	K/NC	*Bacillus spp.*
A2	-	+	-	-	-	K/NC	*Pseudomonas spp.*
A3	+	+	+	-	+	A/A	*Serratia spp.*
B1	-	+	+	-	-	A/A	*Micrococcus spp.*
B2	+	-	+	+	+	A/A	*Bacillus spp.*
B3	+	-	+	+	+	A/A	*Bacillusspp.*
C1	+	+	+	+	+	A/A	*Proteus spp.*
C2	+	-	+	-	-	K/A	*Bacillus spp.*
C3	+	-	+	+	+	A/A	*Arthrobacter spp.*
D1	-	+	+	-	-	K/A	*Bacillus spp.*
D2	+	+	+	+	+	A/A	*Arthrobacter spp.*
D3	-	-	+	-	-	K/A	*Shigella spp.*

keys: + = positive, – = negative,
K/A = only glucose fermented., A/A = glucose and lactose / sucrose fermented.
K/NC = no sugar fermented.

Table 7. *TPH concentration of the mineral salt medium with the organisms analyzed.*

Sample	Day 0(mg/kg)	Day 7(mg/kg)	Day 14 (mg/kg)
A	582.668	454.543	123.666
B	682.647	531.891	203.846
C	753.320	411.577	324.769
D	812.325	792.345	781.338

TPH: Total petroleum hydrocarbon
A:Abia state
B:Anambra state
C:River state
D:Control

Table 8. *Reaction mixture and volume pipetted.*

PCR Mixtures	Volume (µL)
10× PCR buffer	2.5
25mM Mgcl2	1.0
5pMol forward primer	1.0
5pMol reverse primer	1.0
DMSO	1.0
2.5mM DNTPs	2.0
Taq 5u/ul	0.1
10ng/µl DNA	3.0
H2O	13.4
Total/Rxn Volume	25µL

Table 9. *Steps involved in the polymerase chain reaction.*

Initial den.	Den.	Ann. tempt	Extension	No. of circles	Final extension	Hold tempt
94˚c	94˚c	56˚c	72˚c	36	72˚c	10˚c
5min	30sec	30sec	45sec		7min	∞

Table 10. *DNA Quantification of the six samples with Nanodrop-2000 spectrophotometer.*

Sample ID	Nucleic Acid (µg/µl)	260/280 (quality)
sample 1	80.1	1.81
sample 2	161.6	1.8
sample 3	149.2	1.85
sample 4	152	1.85
sample 5	125.7	1.86
sample 6	145.8	1.84

Table 11. *Result and Inferences from the molecular characterization and identification sequences.*

Isolate	Max Score	Total Score	Query Cover	E. value	Identity	Accession number	Description
A1	924 bits	924	100%	0.0	100%	KY705015.1	*Lysinibacillusspp*M2c.
A3	1286 bits	700	100%	0.0	99%	D10387	*Serratia spp* Mb4.
B2	1437 bits	1437	100%	0.0	100%	KX834865.1	*Bacillus aerius* TPM-23
B3	582 bits	582	100%	2e-16[2]	95%	KY777602.1	Unknown isolate
C1	998 bits	540	100%	0.0	100%	KX417275	*Proteus mirabilis* LS-3
C2	237 bits	237	67%	2e-58	79%	EF153309.1	New isolate

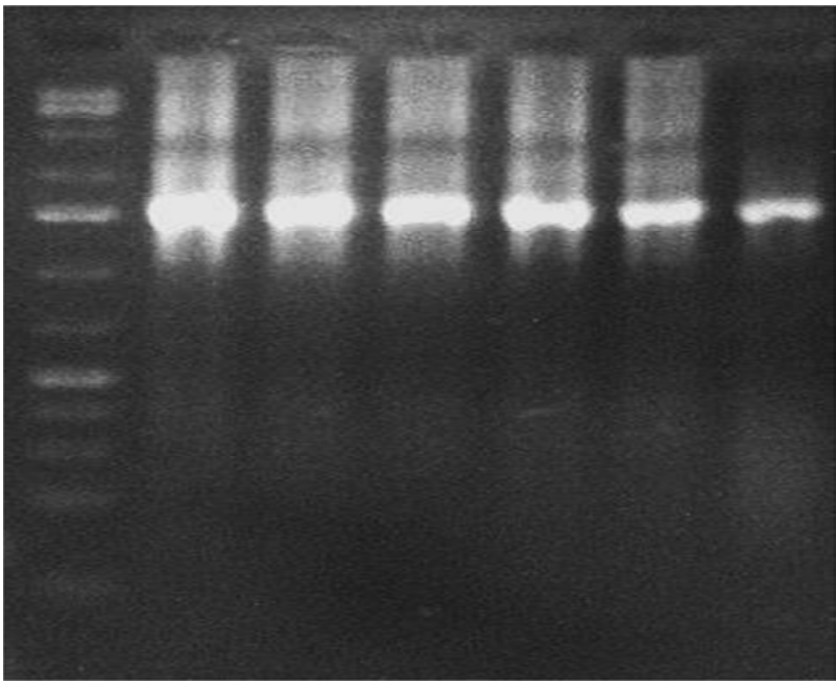

Figure 1. *Gel electrophoresis showing PCR product for the 6 samples on 1.5%Agarose gel where L = Ladder, samples 1–6, showing; Lysinibacillus spp. M2c, Serratiamarcescens Mb4., Bacillus aeriusTPM-23, Unknown isolate, Proteus mirabilis LS-3, a new isolate.*

TPH concentration Day 0 (Abia).

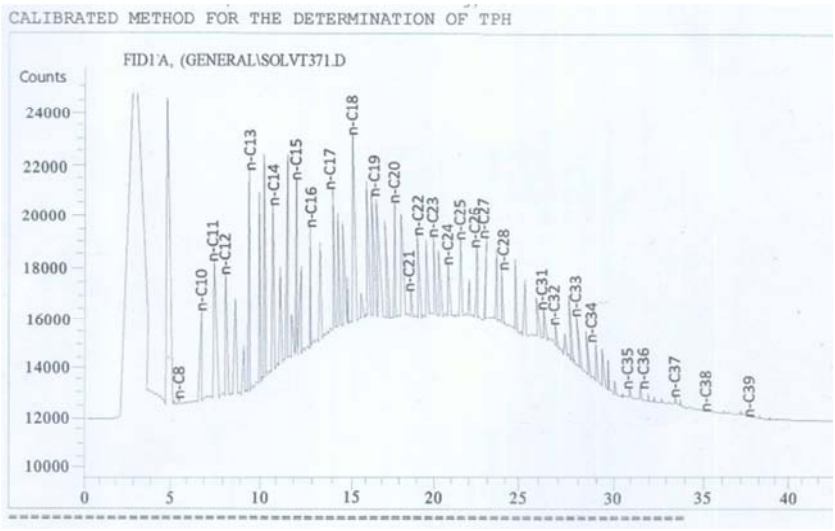

Figure 2. *TPH concentration Day 7 (Abia).*

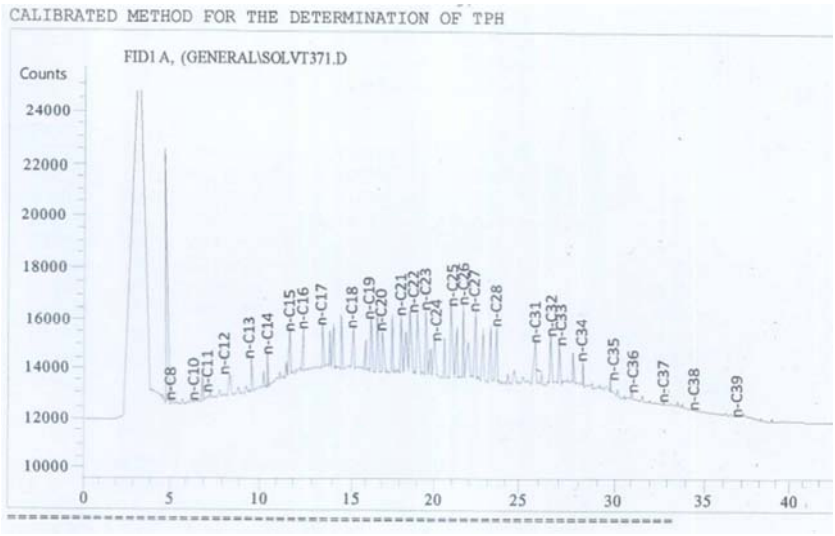

Figure 3. *TPH concentration Day 14 (Abia).*

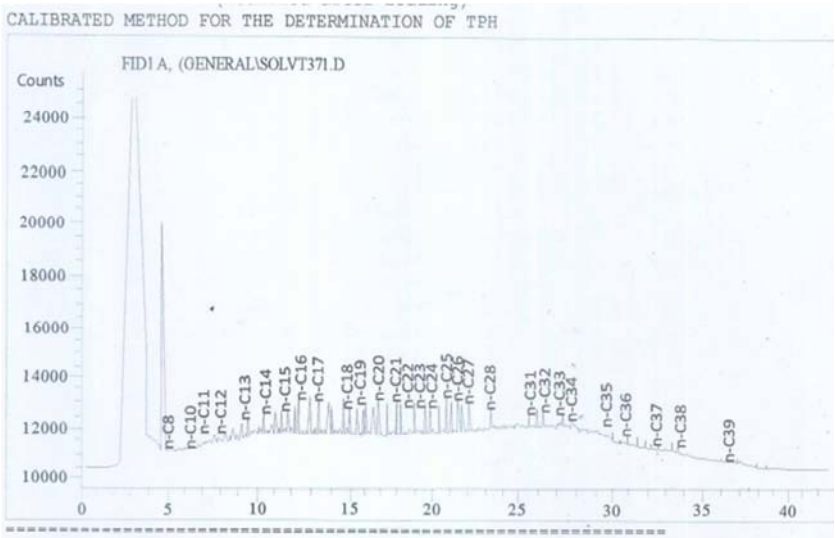

Figure 4. *TPH concentration Day 0(Anambra).*

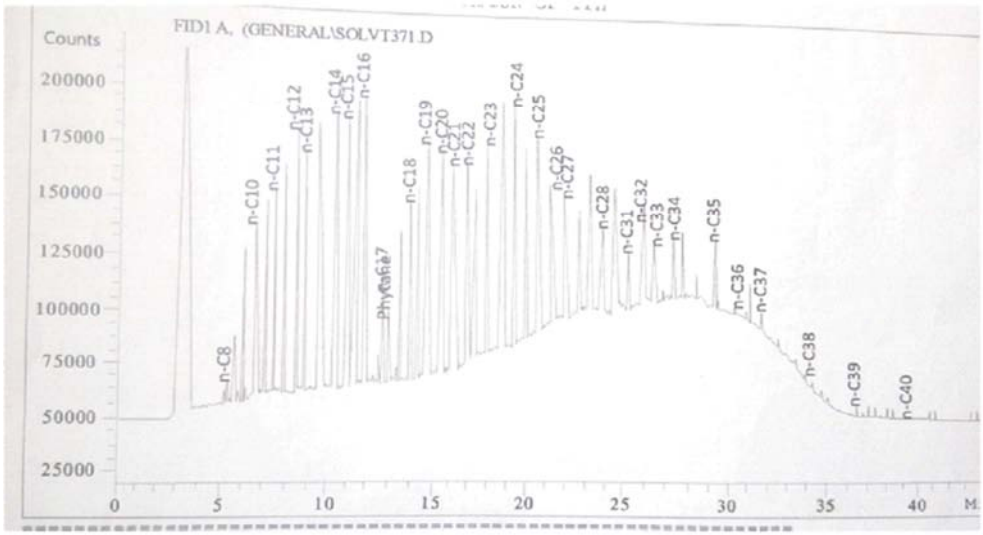

Figure 5. *TPH concentration Day 7 (Anambra).*

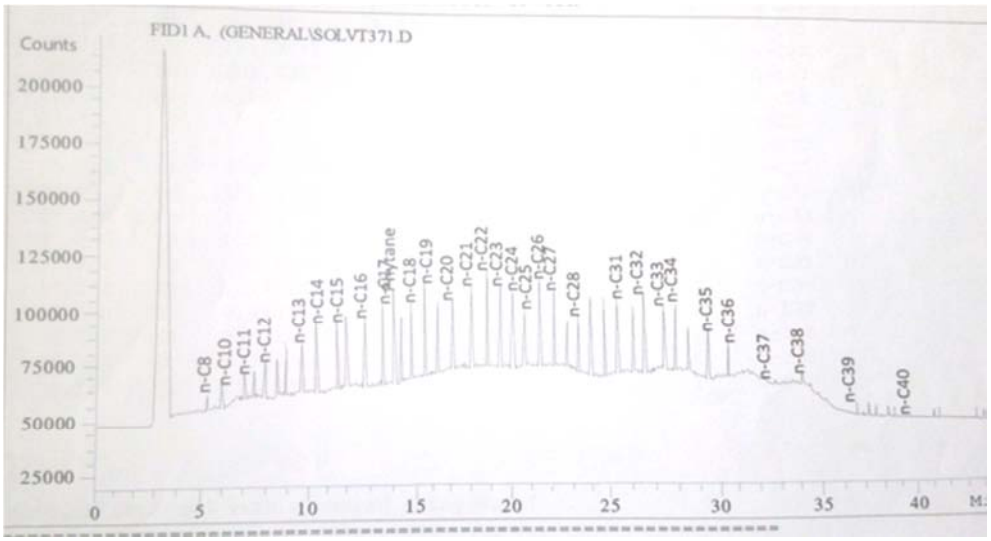

Figure 6. *TPH concentration Day 14 (Anambra).*

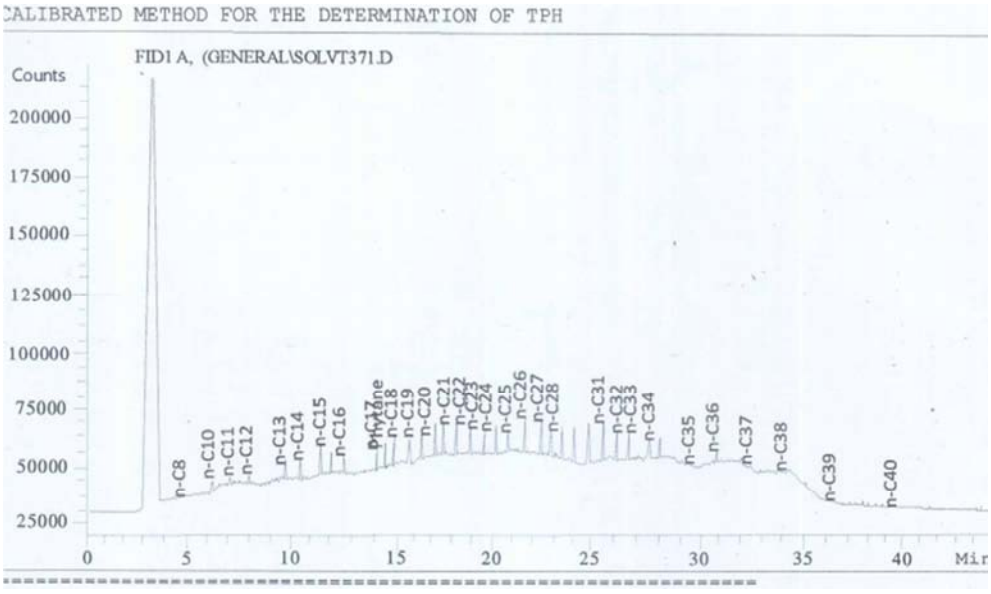

Figure 7. *TPH concentration Day 0 (Rivers).*

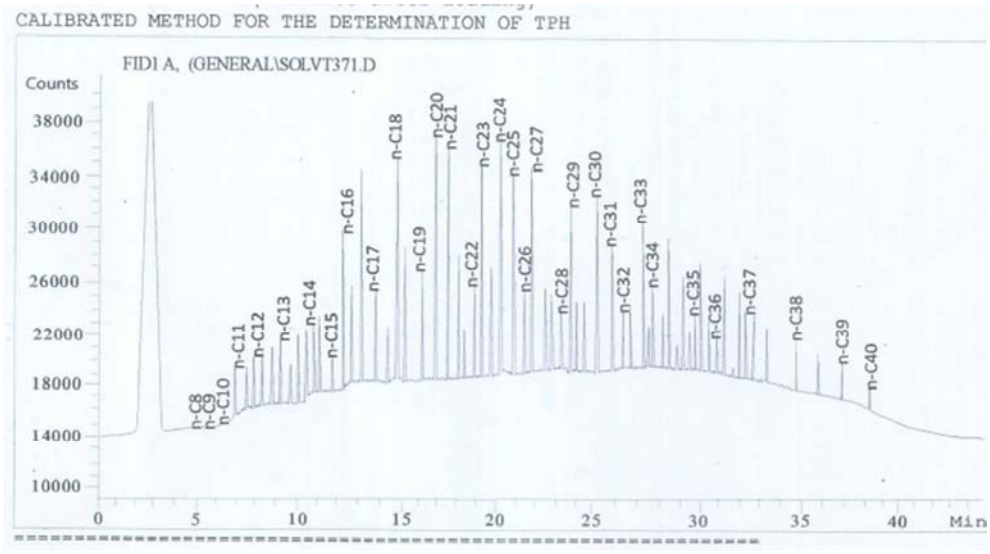

Figure 8. *TPH concentration Day 7 (Rivers).*

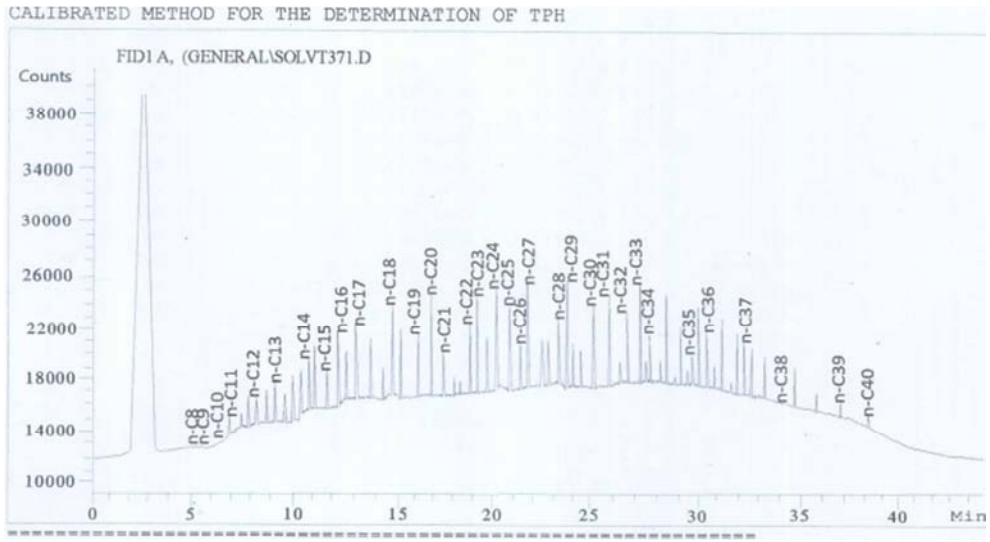

Figure 9. *TPH concentration Day 14 (Rivers).*

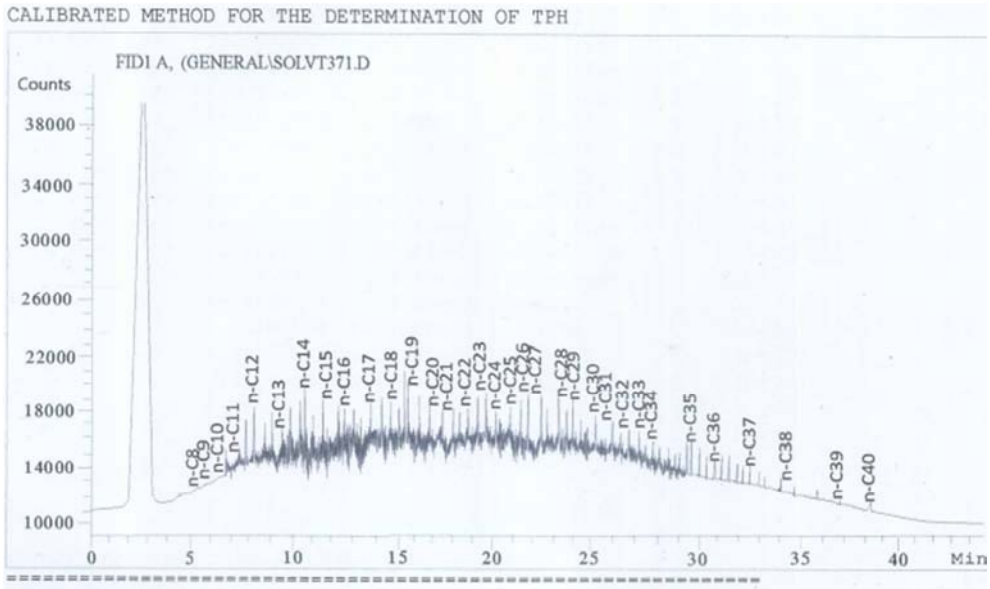

Figure 10. *TPH concentration Day 0 (control).*

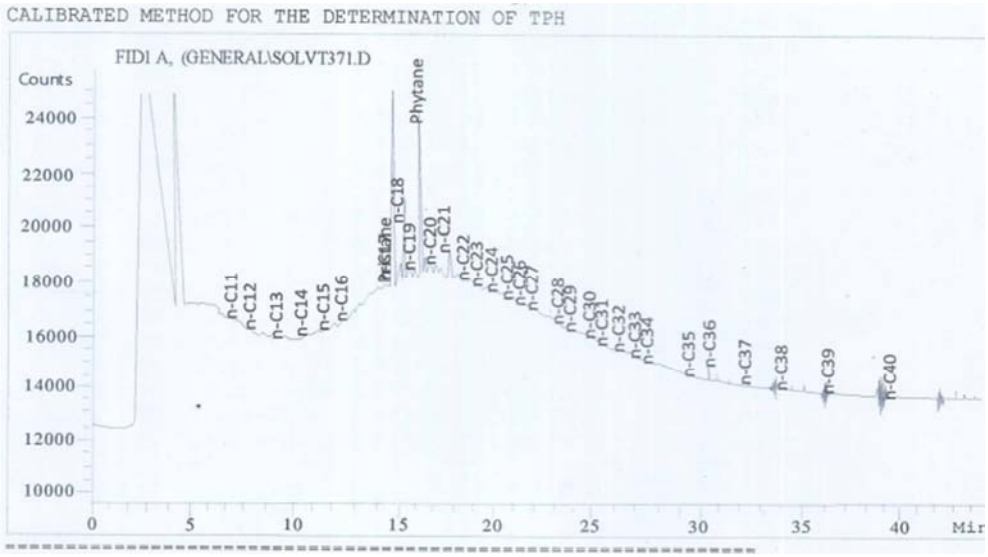

Figure 11. *TPH concentration Day 7 (control).*

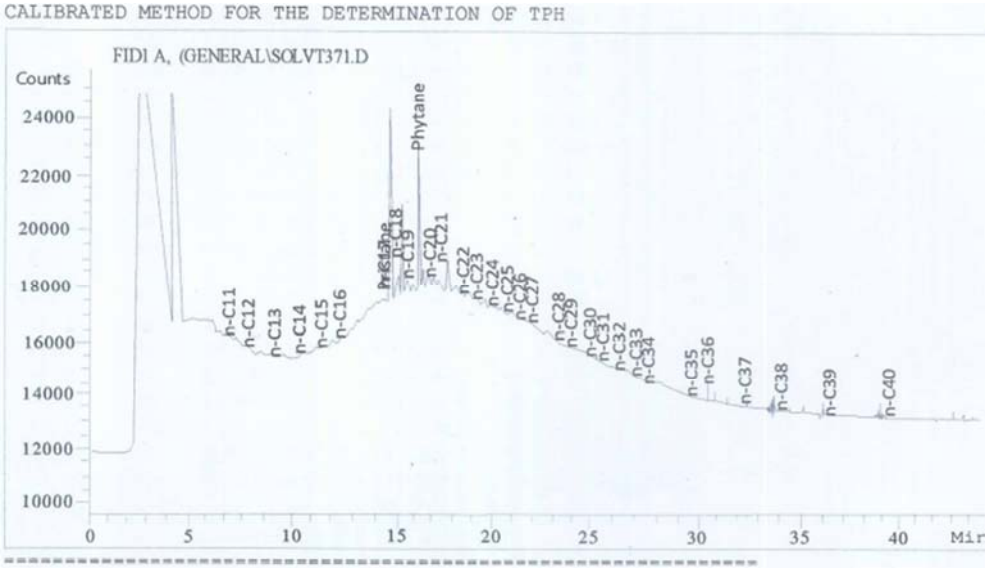

Figure 12. *TPH concentration Day 14 (control).*

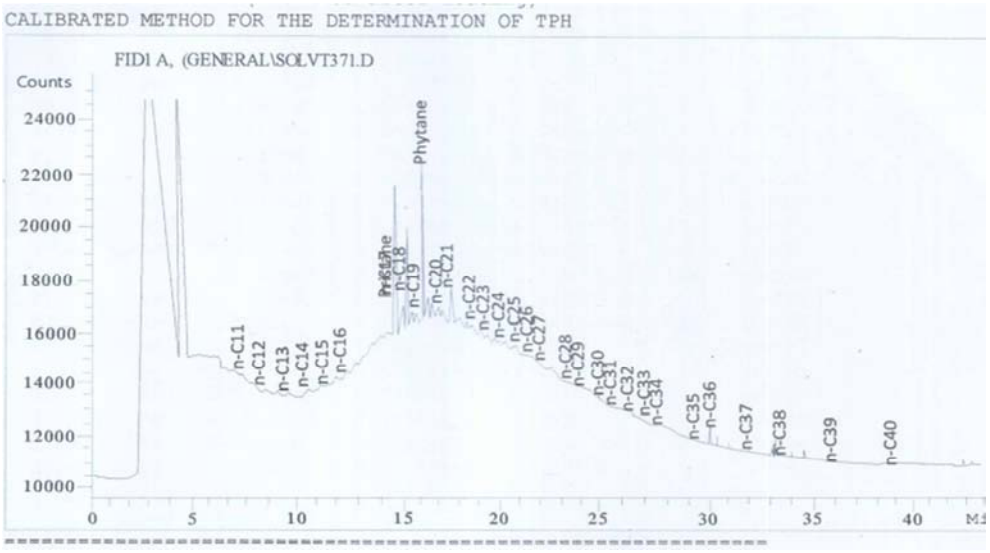

Figure 13. *TPH concentration Day 21.*

3.4. Statistical Analysis

In testing for the significance of the growth of the micro-organisms, bacteria, a One-Way ANOVA was conducted. This One-way ANOVA was conducted to test for the difference in growth across the types of bacteria and across the days for the 1ml and 2ml sample respectively.

4. Discussion

This study compared the ability of bacterial isolates from crude oil contaminated soil from three different soil locations; the isolated organism which included *Serratia marcescens* Mb4, *Lysinibacillus*spp. M2c, *Bacillus cereus* TPM-23, *Proteus mirabilis* LS-3 and new unidentified bacterium were subjected to hydrocarbon degradation test. Soil samples were collected from Abia, Anambra, and Rivers state.

The soil pH ranged from 3.65 – 6.12 (Table: 1), the decrease in pH value was as a result of the accumulation of acid metabolites previous studies the accumulations which has also shown to increases the ability of the heterotrophs microbes present in a crude oil contaminated area, to actively degrade the hydrocarbon present in the soil. This was also reported by Ekhaise and Nkwelle[9]. However, moisture content ranged from 1.6 – 3.0 and 4.4 for the control sample. The crude oil contaminated soil sample had lower moisture content when compared to the crude oil free sample, in the research work of Onifade and Abubakar[28]. They suggested that it may be due to the fact that crude oil coated the soil and consequently preventing the penetration of water this was shown in (Table: 1). Total organic carbon TOC ranged from 3.4 – 4.6 and 7.1% for the control sample, the TOC influences many soil characteristics including odour, nutrient holding capacity. The high TOC level obsevered was also seen in the research of Onifade and Abubakar. [28], which proved that the existence of microorganism is also dependent of the level of TOC as one of the major source of nutrient. Table: 2 showed that the bacterial load was high for all samples, this may be because the crude oil contaminated soil favored the growth of the organisms present in the soil sample, similar results were seen in the research conducted by Omotayo *et al*. [27]. The water holding capacity ranged from 22.4 – 32.0% the high value obtained was because of the high organic matter content, which is considered integral in the capacity of a soil to maximize water storage through its effect on creating and stabilizing soil pores and its absorption capacity. Bacterial growth curve which was monitored for 5 days on mineral salt agar at 600nm (table: 3), the absorbance result ranged from 0.17 - 0.70. similarly, strain improvement test was carried out to identify the bacterial isolates that adapted to 1ml and 2ml% of crude oil from the result obtained isolate; A1, A3, B2, B3, C1, C2 adapted very well to 1ml and 2ml% of crude oil with their optical density at 600nm ranging from 0.111 – 0.543 (Table4). The active isolates were subjected to biodegradation, which was monitored for 14 days in a mineral salt broth, pH value and absorbance value ranged from 7.2 – 7.9 and 0.12 – 0.60 respectively. The slight rise in pH showed that the pH was at the best range for hydrocarbon degradation, similar result was seen by Onuoha. [29]. Table: 7 showed result for TPH concentration, sample A showed a sharp decrease in TPH concentration which was observed from day 7 (454.54 mg/l) and day 14 (123.67mg/l) it showed that *Serratia marcescens* Mb4 and *Lysinibacillus* M2c were able to mineralize the hydrocarbon content to an appreciable level. Ichor, *et al*. [12], observed similar result, by using bacterial consortium. (Table: 6) showed result of the biochemical characterizations carried out on each isolates, result showed that the isolates were catalase positive and rod shaped and that the gram positive organisms dominated from the result shown. Molecular study using the phylogenetic tree with distance and without distance showed the evolutionary relationship between the isolates. SPSS was used in testing for the significance of growth for the microorganism here one way ANOVA was used to compare growth significance, across the types of bacterial which showed there was no significance, but across the days it showed that there was a significant growth. Since the values obtained were less than the α- value (0.01) for both the vapour phase method and adaptation test.

5. Conclusion

This study has been able to establish the ability of *Lysinibacillus* spp. M2c*, Serratia marcescens*Mb4*, Proteus mirabilis* LS-3*, Bacillus aerius*TPM-23, a new unidentified bacterium and unknown isolate to utilize and detoxify crude oil-contaminated soils of Owazangboko in Abia, Aguleriotu in Anambra and Obi-igbo River State, Nigeria. The study showed that a consortium of these organisms in a biodegradation process can increase the rate of hydrocarbon mineralization. The physicochemical characteristics of the soil proved that the soil condition was also conducive for the hydrocarbon degraders.

The study recorded the mineralization of some straight chain hydrocarbons, the bacterial isolates mentioned above were able to mineralize the following hydrocarbons; nonane, octane, decane, nonacosane, triacontane, tetracontane.

5.1. Contribution to Knowledge

A new organism was isolated in the course of this study effort will be made to ensure further identification and naming of the isolate.

Studies on biosurfactant production during a biodegradation process can also ensure a more comprehensive understanding on hydrocarbon degradation by microbial consortium to determine a more effective strategy.

This study has experimentally shown that the isolated microbial consortium be used for bioremediation of a crude oil polluted site. This can be made possible by the use of bioaugumentation.

5.2. Recommendation

Efforts should be made, to ensure the conservation of our natural resources. By reducing and monitoring the amount and level of oil spills taking place.

Government should set up Environmental Health Agencies, which will assist in a cleanup exercise in an event of crude oil spill.

Microbial isolates which has proven to have a more and adequate hydrocarbon degradative capabilities should be stored up for future reference and use in a well-designed culture collection center funded by the government.

Researchers should be given access to funds, and scholarship. To enable the advancement of researches in the future.

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