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BACTERIZATION OF SPENT ENGINE OIL IMPACTED TROPICAL GARDEN SOIL HYDROCARBON FOR DEGRADATION AND BIOREMEDIATON

NKANTION, N. U*. AND ITAH, A.Y.
Department of Microbiology, University of Uyo, Nigeria.
***CORRESPONDENCE**: *nkantionnseobong@gmail.com*

ABSTRACT

Spent engine oil has significant negative impact on microbial communities in soil and water; this disruption can have cascading effects on various ecosystem processes, such as nutrient cycling, nitrogen fixation, carbon sequestration, and the degradation of organic matter. This study investigates the hydrocarbon degradation potentials of *Alcaligenes aquatilis* isolated from adjoining farmland to the Mechanic Village, Uyo, Akwa Ibom State, Nigeria. Bioremediation was monitored using the percentage ratio of total petroleum hydrocarbons (TPH) at day 0 to Total Residual Hydrocarbons (TRH) after 56 days treatment by gas chromatography- mass spectrometry (GC-MS) techniques. Over 97.77 %, 98.08 %, 95.91 %, and 98.47 %, of the total petroleum hydrocarbons fractions of the spent engine oil contamination at 5 %, 10 %, 15 % and 20 % concentration were respectively degraded in 4 kg of garden soil augmented with *Alcaligenes aquatilis*. Statistical analysis (ANOVA) showed highly significant difference ($p<0.001$) in TPH content between the mean initial and residual TPH. Monitored natural attenuation showed the highest percentage degradation (marginal increase) of about 98.79 % at 5 % contamination with spent engine oil. The molecular characteristics of the best degrading bacterial strains by PCR, sequencing and blasting suggest the isolates as, *Alcaligenes aquatilis* strain 4p171492R. Based on the results of this work *A. aquatilis* strain 4p171492R showed strong degradative activity and is strongly recommended for the clean- up and bioremediation of spent engine oil impacted soil in contaminated sites.

KEYWORDS: Spent engine oil, bioaugmentation, monitored natural attenuation, gas chromatography

INTRODUCTION

Spent engine oil is a term used to describe engine oil that has been used for lubrication of automobile engines and is no longer suitable for use due to contaminants and breakdown of additives. Due to global increase in the utilization of petroleum products, soil pollution by lubricant is speedily increasing. In the developing country like Nigeria, hydrocarbon contamination of terrestrial and aquatic environment has been a problem since oil was discovered as fuel for servicing of engines (Itah and Essien, 2005). Lubricating oil usage has increased as a result of the variety of machinery and automobile. According to Ekanem *et al*., (2019) attention is attracted due to hydrocarbon contamination of air, soil and fresh water particularly polycyclic aromatic hydrocarbons because it is noxious, mutagenic and carcinogenic and constitute public health hazard. Complete clean up cannot be achieved by physical and mechanical mechanisms which are always the first response; Bioremediation technologies harness this process by promoting the growth of competent population of microorganisms that can biodegrade contaminants. Some microbes with the capability of converting and mineralizing aimed pollutants already exist at hazardous waste site; although not necessarily in the number required in remediating the site (Pilon, 2005), hence the need for this investigation. Therefore, techniques have been designed to stimulate the growth and biodegradative activities of the existing autochthonous microbial communities. Such techniques include bioaugmentation which ensures the acceleration of microbiological processes through the addition of known active or pre- grown microorganisms to the soil based on their metabolic capabilities. Whereas monitored natural attenuation is a remediation strategy that involves monitoring the natural processes that occur in the environment to break down and eliminate contaminants. *Alcaligenes aquatilis* was the organism of choice in this study based on its hydrocarbon utilizing capability.

MATERIALS AND METHODS

Sources of Materials

The soil samples were collected from adjoining farmlands to the automobile workshops in mechanic village, Uyo, Akwa Ibom State where there are traces of hydrocarbons for isolation of hydrocarbonoclastic microorganisms and University of Uyo garden soil. Spent engine oil was obtained from the motor vehicle (automobile) workshops at the same mechanic village, Uyo, Akwa Ibom State

Isolation of Spent Engine Oil Degrading Bacteria from Spent Engine Oil Impacted Soil Enrichment and Isolation of Bacterial Isolates

The enrichment culture technique was employed. Precisely 1 g of spent engine oil impacted soil sample from mechanic village was inoculated into eight different sets of conical flask containing 50 mL of sterile mineral salt medium [K_2HPO_4- 1.8 g, NaCl- 0.1 g, KH_2PO_4- 1.2 g, NH_4Cl- 4.0 g, $MgSO_4$- 0.2 g, $FeSO_4$- 0.001 g, per 1 liter (pH 7.0± 0.2)] enriched with 1 % of spent engine oil as carbon source. The medium was incubated at 28^oC in shaker incubator (100 rpm) for 7 days. The samples were thereafter serially diluted using sterile water as diluents and plated on Nutrient Agar (NA) to obtain viable bacterial cells. Discrete colonies obtained were sub-cultured onto fresh NA plate using streak method as described by Cheesbrough (2006) to obtain pure cultures. The best spent engine oil utilizing bacterial isolates were characterized based on their cultural and morphological attributes as well as their responses to standard biochemical test as described by Holt *et al*., (1994), Barrow and Feltham (2003).

Identification of the more promising Spent Engine oil Degrading Bacterial Isolate using New Generation techniques

According to Tamura *et al*. (2013) Genomic DNA from the isolates was extracted using ZR fungal/bacterial DNA Miniprep (Manufactured by Zymo Research). The extraction was done according to the manufacturer's instructions. Two millilitres (2 mL) of broth culture of bacterial cell were added to a ZR Bashing TM Lysis tube, 750 µl Lysis solution was also added to the tube. It was then secured in a bead fitted with 2 mL tube holder assembly and processed at maximum speed for 5 minutes. The ZR Bashing Bead TM Lysis tube was centrifuged in a micro-centrifuge at > 10,000 xg for 1 min. 400 µl supernatant was transferred to a Zymo-Spin TM IV Spin Filter (orange top) in a Collection Tube and centrifuged at 7,000 xg for 1minute after which 1200 µl of Fungal/Bacterial DNA Binding Buffer was added to the filtrate in the Collection tube. 800 µl of the mixture was then transferred to a Zymo-Spin TM IIC Column in a Collection Tube and centrifuged at 10,000 xg for 1 minute. The flow through from the Collection Tube was discarded and the last step was repeated. 200 µl of DNA Pre-Wash Buffer was added to the Zymo-Spin TM IIC Column in new Collection Tube and centrifuged at 10,000 xg for 1min, after which 500 µl of Fungal/Bacterial DNA Wash Buffer was added to the Zymo-Spin TM IIC Column and centrifuged at 10,000 xg for 1min. The Zymo-Spin TM IIC Column was transferred to a clean 1.5 mL micro-centrifuge tube and 100 µl DNA Elution Buffer was added directly to the column matrix and centrifuged at 10,000 xg for 30 seconds to elute the DNA.

DNA Sequencing

Utilizing the Big Dye terminator volume 3.1 cycles sequencing kit, MEGA X, Bio-Edit software, and an Applied Bio-systems Genetic Analyzer 3130 xl sequencer, the amplified fragments DNA were sequenced in accordance with the manufacturer's instructions.

Screening for the Hydrocarbon Utilizing ability of Bacterial Isolates

Spent engine Oil utilizing potential of the bacterial isolates was carried out using hydrocarbon overlay method. Precisely 15 g of agar- agar was added to mineral salt medium, sterilized and allowed to set. The solidified plates were overlaid with 1mL of spent engine oil, allowed for 15 to 30 minutes then the test isolates were streaked on the surface of the plate. All inoculated plates were incubated at room temperature for 10 to 15 days for periodic observation. Colonies that eventually developed showing area of clearing were selected and rated. Utilization was rated based on diameter and luxurious nature of the developed colonies, ie. +, ++ or +++ indicating the magnitude of the oil degrading potentials as described by Ekundayo and Obire (1987).

Ex- situ Examination of the Impact of Bioaugmentation on the Isolates' Degradation of Spent Engine Oil-Related Soil

Before being sieved with a 2 mm sieve, garden soil from the University of Uyo agricultural farm was meticulously and physically sorted to remove debris. Using a 20 kg table measuring scale, 4 kg of soil was weighed and placed into nine wooden boxes with perforations. Following that, 4 kg of soil in nine perforated 30 by 30 cm wooden boxes were polluted with graded percentage of spent engine oil (5 %, 10 %, 15 %, and 20 %), and were augmented with 20 mL broth culture of *Alcaligenes aquatilis* (2.0×10^6) broth cultures. The control soil was polluted but not augmented. The rate of spent engine oil biodegradation was monitored for a period of two months at seven-day intervals (0, 7th, 14th, 21st, 28th, 35th, 42nd, 49th and 56th days after augmentation). The impact of the bioaugmentation on the physico-chemical parameters of the treated and untreated soil was evaluated at the end of the degradation course (2 months after treatment).

Determination of Residual Petroleum Hydrocarbons after Degradation by Gas Chromatography-Mass Spectrometry Technique

Soil Sample Extraction for TPHs TRHs Analysis

Fifty milligram of the sample extract was weighed with an electronic weighing balance (XY3000MB/XY5000MB) into a beaker and 10 mL of the solvent mix (1:1 - Hexane: Dichloromethane) was measured and poured into the beaker to dissolve and homogenize the extract. The homogenized extract was cleaned up with 100 -200 mm mesh silica gel and 3 g of anhydrous sodium sulfate in a well-packed column, conditioned with hexane to form a slurry. 1 microlitre of the sample was injected through the injection port into the Gas Chromatography Flame Ionization Detection **(**GC-FID).

Gas Chromatography-Mass Spectrometry (GC-MS)

An Agilent 6890N gas chromatography equipped with an auto sampler connected to an Agilent Mass Spectrophotometric Detector was used. One microlitre of the sample was injected in the pulsed split less mode onto a 30 m x 0.25 mm ID DB 5 MS coated fused silica column with a film thickness of 0.15 micrometer. Helium gas was used as a carrier gas, and the column head pressure was maintained at 20 psi to produce a constant of 1ml/min. Other operating conditions were preset. The column temperature was initially held at 55 ºC for 0.4 min, increased to 200 ºC at a rate of 25 ºC/mins, then to 280 ºC at a rate of 8 ºC/mins and to a final temperature of 300 ºC at a rate of 25 ºC/mins, held for 2 mins. The identification time was based on retention time, and components with lower retention time elute first before those with higher retention time as described by Olajire *et al*. (2005).

Determination of Total Residual Petroleum Hydrocarbons

The samples were injected into the inlet where it volatilized and an appreciable portion was taken onto the column aided by the carrier gas (N_2 or He). Differential partitioning separated the sample components into stationary and mobile phases. The separated sample components were removed from the column into the detector where their physicochemical property was detected and a signal generated. The signal was then amplified and sent to the data system where the chromatograph was electronically

constructed. The percentage degradation rate was calculated using the formula below:

$$\frac{\text{TPH- TRH}}{\text{TPH}} \times 100$$

Statistical Analysis of Data

One-way ANOVA (Analysis of Variance) was used to compare the initial TPH and Residual TPH using Statistical Package for Social Sciences (SPSS) version 22 by IBM Corporation, USA. Significance was determined at $p<0.001$.

RESULTS

Molecular Identification of the Best Degrader

The PCR amplified I6S rDNA analysis showed that isolates with codes NA3 has 100.0 % Pair wise Identity to *Alcaligenes aquatilis* strain (4p17_1492R) with NCBI accession number MK139482.1, zero E- value providing evidence that this species is present in the environment and may be contributing to the high degradation rate of petroleum hydrocarbons by producing enzymes such as alkanes hydroxylases.

Table 1 shows the potentials of autochthonous bacterial taxon isolated from mechanic village automobile workshops in Uyo to utilized spent engine. The results divulged the existence of bacterial isolates with strong spent engine utilization capabilities. *Psedomonas aeuuginosa*, *Pseudomonas putida*, *Alcaligenes aquatilis* and *Bacillus subtilis* were the efficacious degraders; *Corynebacterium* sp. and *Micrococcus* sp. were the restrained degraders.

Residual Petroleum Hydrocarbons

Figures 1 shows the concentration (ppm) of different residual petroleum hydrocarbons and Aliphatic Hydrocarbons present in the spent engine oil contaminated soil (at 5 %, 10 %, 15 % and 10 %) at the end of the biodegradati on studies. The ex-situ degradation study carried out for 8 weeks showed that, the degradation of spent engine oil and its components was faster when enhanced by augmentation with *Alcaligenes aquatilis* as well as under natural attenuation

For contaminated soils without augmentation the degradation rate was marginally high (98.78 %) within 8weeks while the contaminated soil augmented with *Alcaligenes aquatilis* showed the percentage rate of biodegradation of 97.77 %, 98.04 %, 95.91 % and 98.47 % respectively (Figure 3).

Figure 4 to 13 show the chromatogram of total petroleum hydrocarbons and residual hydrocarbons of 4 kg garden soil contaminated with different percentage of spent engine oil and augmented with *Alcaligenes aquatilis*

For clarity, The Y- Axis represent Abundance signal intensity (counts in milivolts) of each molecule in the mixture. The X- Axis represent retention time (minutes) which is the amount of time a molecule spends in the column. Each peak of Gas chromatography and mass spectrophotometer (GC/MS) chromatogram represents an individual compound separated from the mixture. The area under the graph represents the relative amount of each component. The concentration of molecular weight of hydrocarbons was relatively high (9000 counts) in TPHs but at the end of treatment with *Alcaligenes aquatilis* the residual petroleum hydrocarbons concentration of each compounds was reduced or broken down to 80 mvolts at most.

Table 1: Spent engine oil utilization potential of hydrocarbonoclastic bacteria

Isolates after 7 days	Growth on spent engine after 7 days	Growth on spent engine after 14 days
A. aquatilis	+++	+++
B. subtilis	+++	+++
P. putida	+++	+++
P.aeruginosa	++	++
Corynebacterium sp.	+	++
Micrococcus sp	++	++

Keys: - = Lack of growth, + = 1-5 mm (debile growth), + = 6-10 mm, (restrained growth), ++ = 11-15 mm (strong growth),
+++ = 16- 20 mm (strongest growth)

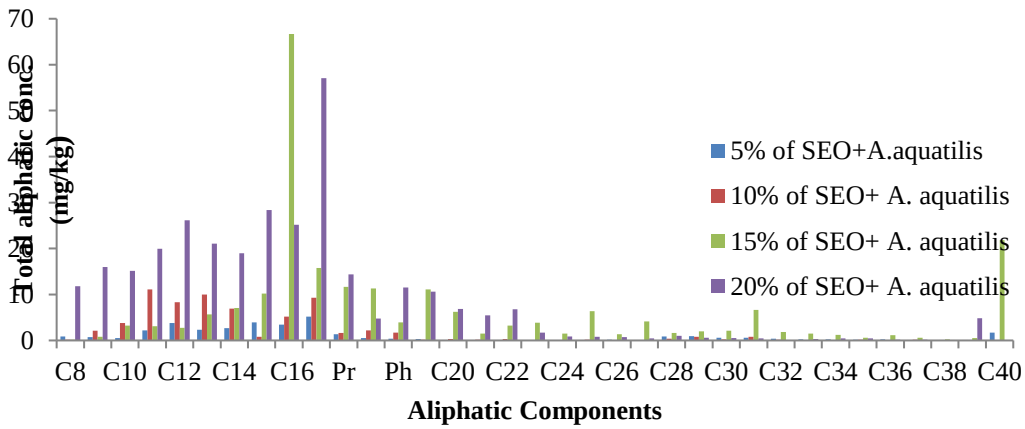

Fig. 1: Total residual concentration of hydrocarbons detected for 8 weeks bioremediation studies in different percentage of contamination of garden soil with spent engine oil augmented with *Alcaligenes aquatilis*

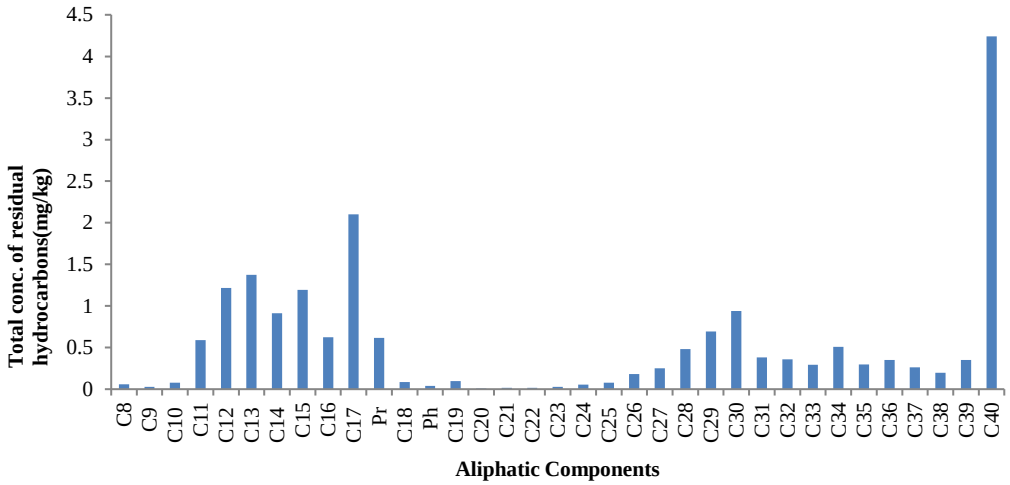

Fig. 2: Total concentration of residual hydrocarbons detected for 8 weeks bioremediation studies in 5 % contamination of garden soil with spent engine oil without augmentation

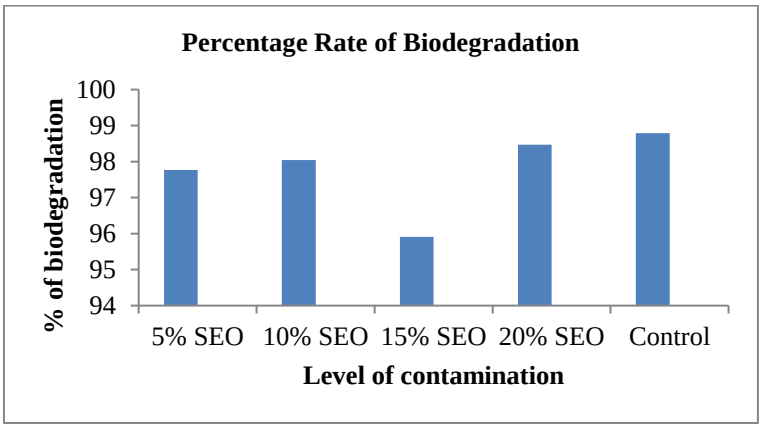

Fig. 3: The rate of degradation obtained from 5 %, 10 %, 15 % and 20 % contamination of spent engine oil, augmented with *Alcaligenes aquatilis* and without augmentation (control) during bioremediation

Chromatograms of TPHs and TRHs of Spent Engine oil

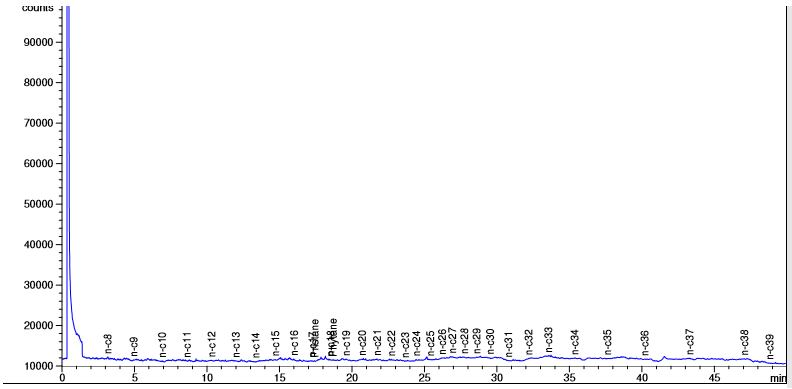

Fig. 4: Chromatogram of total petroleum hydrocarbon in 5 % of spent engine oil contaminated soil before treatment

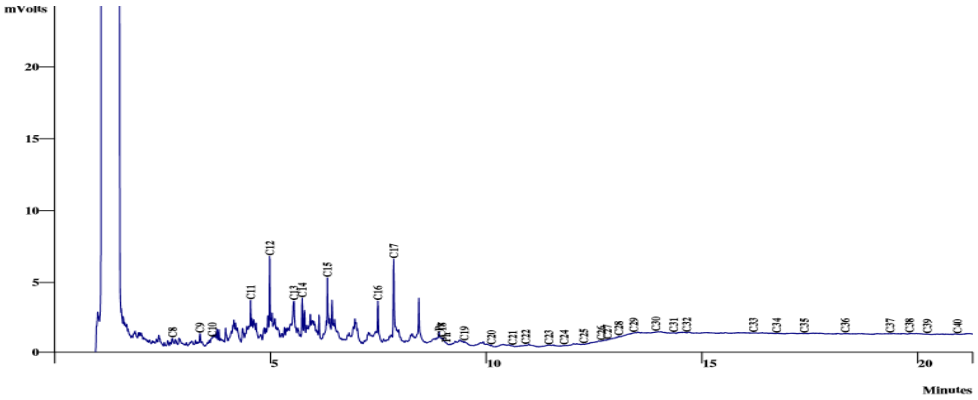

Fig. 5: Chromatogram of total residual petroleum hydrocarbon in 5 % of spent engine oil contaminated soil augmented with *Alcaligenes aquatilis* for 56 days

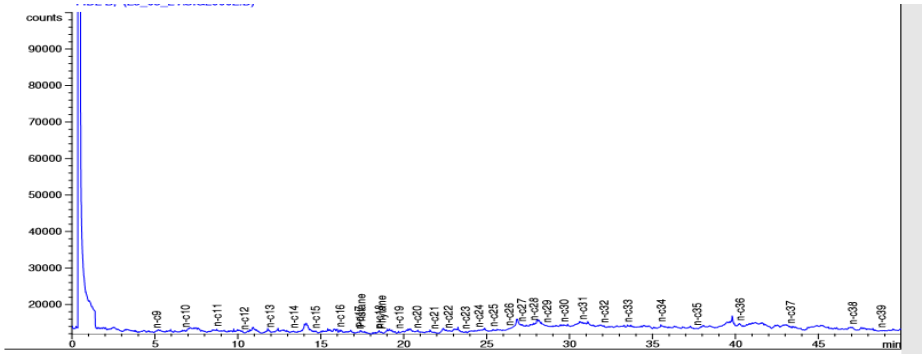

Fig. 6: Chromatogram of total petroleum hydrocarbon in 10 % of spent engine oil contaminated soil before treatment

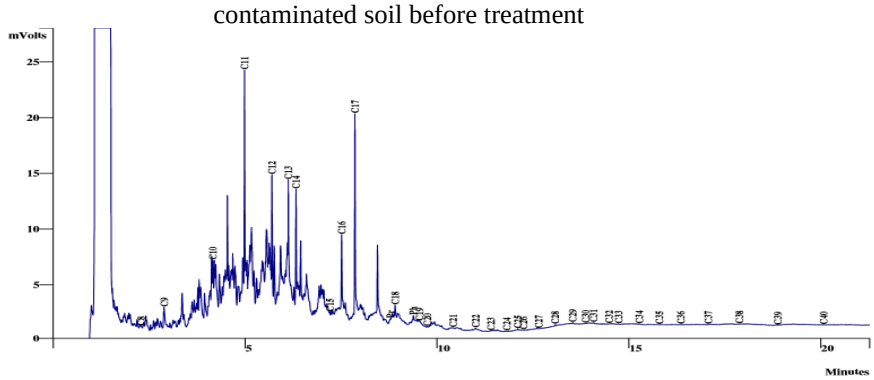

Fig. 7: Chromatogram of total residual petroleum hydrocarbon in 10 % of spent engine oil contaminated soil augmented with *Alcaligenes aquatilis* for 56 days

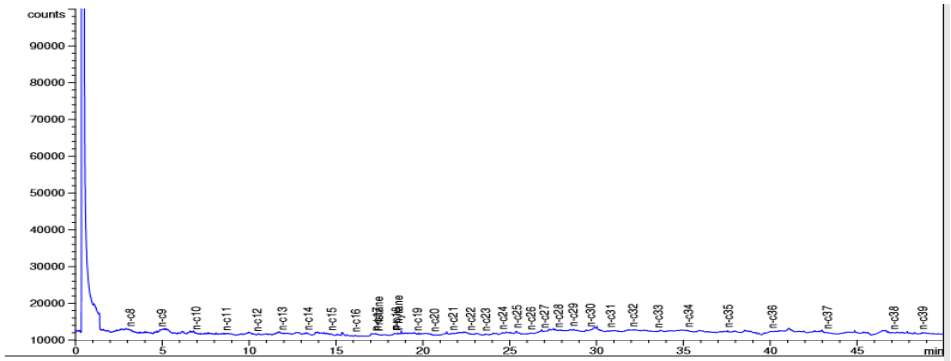

Fig. 8: Chromatogram of total petroleum hydrocarbon in 15 % of spent engine oil contaminated soil before treatment

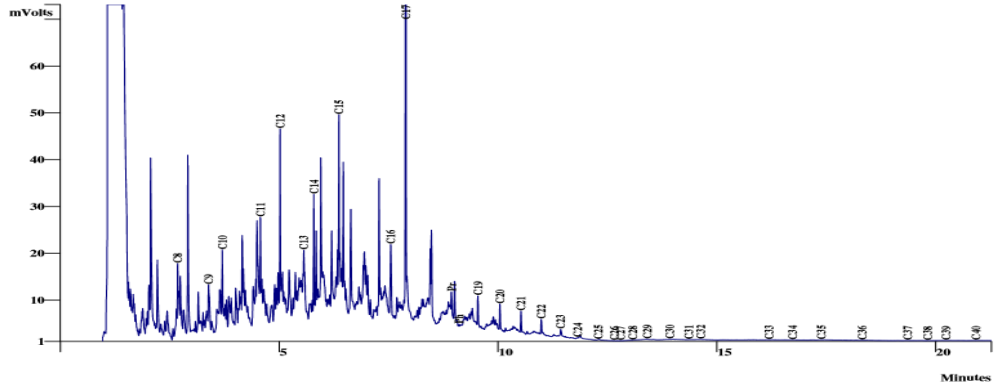

Fig. 9: Chromatogram of total residual petroleum hydrocarbon in 15 % of spent engine oil contaminated soil augmented with *Alcaligenes aquatilis* for 56 days

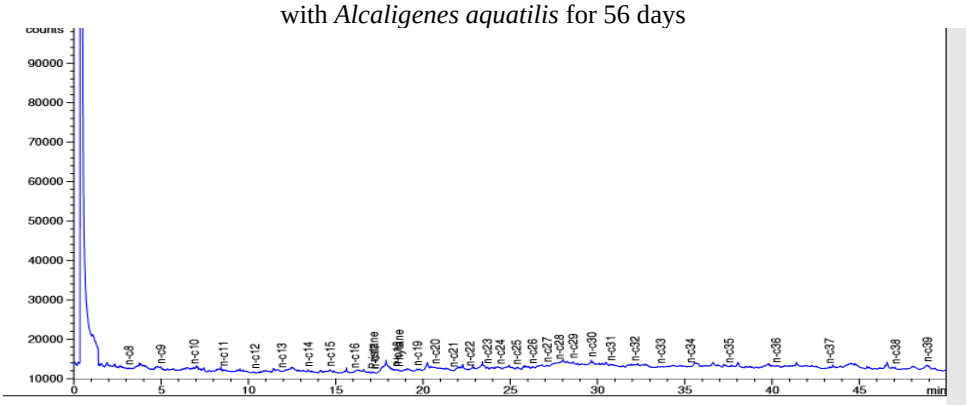

Fig. 10: Chromatogram of total petroleum hydrocarbon in 20 % of spent engine oil contaminated soil before treatment

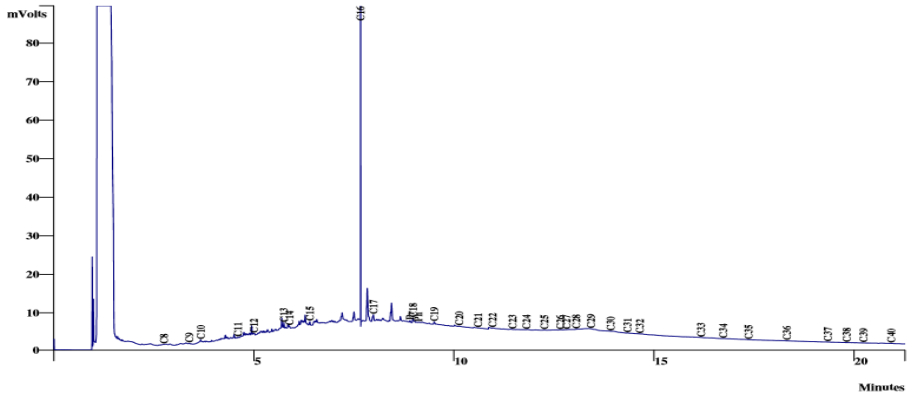

Fig. 11: Chromatogram of total residual petroleum hydrocarbon in 20 % of spent engine oil contaminated soil augmented with *Alcaligenes aquatilis* for 56 days

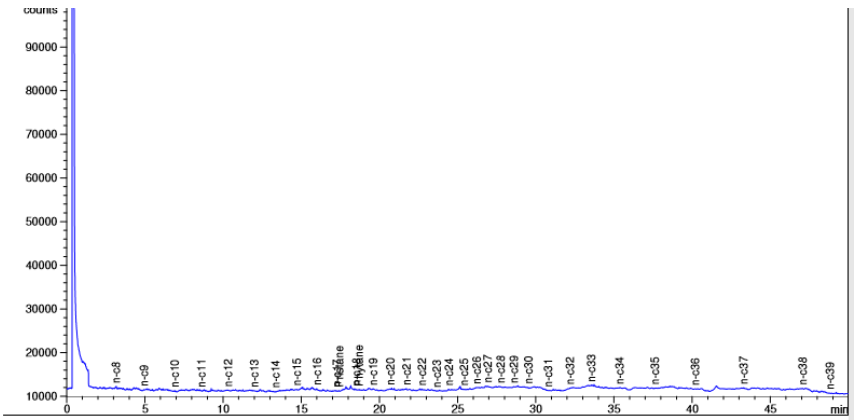

Fig. 12: Chromatogram of total petroleum hydrocarbon in 5 % of spent engine oil contaminated soil before treatment

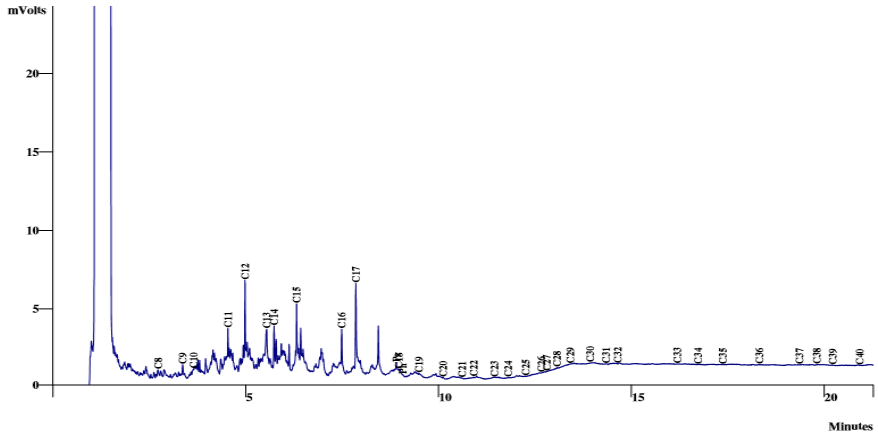

Fig. 13: Chromatogram of total residual petroleum hydrocarbon in 5 % of spent engine oil contaminated soil without augmentation during 56 days

DISCUSSION

With bacteria predominating in the microhabitats, soil-based microbes play a significant role in microbial food webs, biogeochemical cycles, and energy transport (Unimke *et al.*, 2017). Their biodiversity can serve as a reflection of local environmental conditions because it is influenced and determined by the temporal and geographical variation of physicochemical and biological components (Antai *et al.*, 2014). The initial hydrocarbon content (THC) of the 4 kg of garden soil contaminated with 5 %, 10 %, 15 % and 20 % used for this study were 1570.95904 mg/kg, 3450.18405 mg/kg, 5466.01408 mg/kg and 20607.4 mg/kg respectively.

Alcaligenes aquatilis used in augmenting the growth of some indigenous microorganisms is a type of bacterium that produces cationic biosurfactants in response to the presence of hydrocarbons which induces the expression genes in biosurfactant production to solubilized spent engine oil, enhancing high degradation rate. The GC analysis shows that both monitored and enhanced natural attenuation degraded the total petroleum hydrocarbon (TPH) content above 95 %. Ekanem *et al*. (2019) had also reported the reduction of engine oil hydrocarbons above 95 % by *Pseudomonas* sp. Spent engine oil degradation rates were found to increase with increase in the percentage of spent engine oil added in the microcosm except at 15 % (95.91 %) level of contamination which might be due to saturation effects or inhibition, where the reaction rate is limited by reactants or presence of inhibitors.

There was a marginal increase in soil remediated using monitored natural attenuation (98.79 %) which may be due to the absence of any inhibitory effect from contaminant, the treatment supported more hydrocarbonoclastic activities because it greater the number of oil degraders it's faster the rate of degradations. This is conformed to the findings of Pilon (2005) who stated that, natural attenuation can be used to restore areas with low contamination levels. Approximately 25 % of all petroleum contaminated land has been remediated with natural attenuation (Stroud *et al.*, 2007). Guarino *et al*. (2017) reported that Hydrocarbons contaminated soil reached a 57 % reduction of the Total Petroleum Hydrocarbons (TPH). In the present study 5 % contamination of 4 kg garden with spent engine oil was not toxin to microbial communities even without augmentation rather it serves as sole source of carbon and they worked in synergy or consortium to ensure effective bioremediation. Bartha and Atlas (2009) reported that when natural environment are contaminated with pollutants, the indigenous microbial communities are likely to contain microbial pollution of different taxonomic characteristics which are capable of degrading the pollutant.

The ability to isolate high numbers of hydrocarbonoclastic microorganisms from oil polluted environment is commonly taken as evidence that these microorganisms are active degraders in the environment. On the other hand, contaminated soil augmented with *Alcaligenes aquatilis* showed the percentage rate of biodegradation of 97.77 %, 98.04 %, 95.91 % and 98.47 % respectively. These values suggest that the degradation process is highly efficient and consistent, with most values exceeding 95 %. The ANOVA result confirms that the remediation process has a significant impact on the TPH content of the soil.

CONCLUSION

The autochthonous bacteria (*Alcaligenes aquatilis*) with the capabilities of metabolizing spent engine oil were encountered in this research. The biological decomposition of petroleum and other hydrocarbons in a polluted site is a complicated approach with quantitative and qualitative facet based on the concentration of contaminants, nutrients availability, optimum environmental matrix and variety of bacterial genera. Adsorption, volatilization and biodegradation of total petroleum hydrocarbon in spent engine were taking place via cationic interaction that is why high rate of remediation was achieved.

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