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Monitoring the degradation of total petroleum hydrocarbon in hydrocarbon impacted site using a consortium of hydrocarbon utilizing bacteria

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Abstract
Bacteria (Hydrocarbon utilizing Bacteria) are ever present in natural history, and they make use of these blends as the origin of carbon and energy, however bacteria with such potential are frequently utilized for bioremediation of fossil fuel polluted earth, and have develop corrective measures against petroleum contaminants, by improving the bonding capacity of their cells changing their exterior mechanism and secreting surface active biomolecule materials to augment contact to objective hydrocarbon substrates. The essence of the study is to examine the degradation of total petroleum hydrocarbon in fossil oil polluted soil matrix using consortium of bacteria in three concentrations. (10%, 1.0% 0.1%. for 16 weeks). Consortium of bacteria used is *Bacillus thuringiensis*, *Bacillus velezensis* and *Stenotrophomonas species.* However result from the Gas chromatography flame induction detector (GC-FID) analysis showed the extent of degradation 10% (948.46±0.07 mg/kg and 2.97±0.01 mg/kg), 1.0% (110.07±0.10 mg/kg and 21.95±0.08 mg/kg) and 0.1% (99.99±0.01 mg/kg and 13.88±0.04), at the initial and final stage of exposure respectively. The study specifies that, augmenting biodegradation prospective via the application of bacterial consortia with several catabolic genes, is practical and possible approach for facilitating the elimination competence of petroleum hydrocarbons from contaminated environments.

Keywords: Consortium, hydrocarbon utilizing bacteria, degradation, genbank

Introduction
Bioremediation practice is economical, cost effective and has no secondary pollutants identified with its end product, even in ecological security. (Dvořák *et al*., 2017; Wanapaisan *et al*., 2018) [7, 28].

Furthermore, petroleum hydrocarbons are totally petrified into carbon dioxide and water under the act of diverse microbes, though bioremediation is laborious. Numerous usual and great bacterial species have been isolated and utilized as bio-degraders for treating petroleum hydrocarbons. The degradation trail of a diversity of petroleum hydrocarbons (e.g., aliphatics and polyaromatics) are known to utilize oxidizing reactions. However, these route differ deeply due to the specific oxygenase's identified with different bacterial species, occasionally some bacteria can absorb a certain alkanes, whereas others split down aromatic or resin fractions of hydrocarbons. A number of these bacteria such as *Achromobacter*, *Acinetobacter*, *Alkanindiges*, *Alteromonas*, *Arthrobacter*, *Burkholderia*, *Dietzia*, *Enterobacter*, *Kocuria*, *Marinobacter*, *Mycobacterium*, *Pandoraea*, *Pseudomonas*, *Staphylococcus*, *Streptobacillus*, *Streptococcus* and *Rhodococcus* function better in cleaning these fossil fuel petroleum (Jin *et al*., 2012; Sarkar *et al*., 2017; Varjani, 2017) [12, 17, 18]. However a number of bacteria have been said to posses a wide range of petroleum hydrocarbon degradative ability, *Dietzia* sp. DQ12-45-1b make use of s *n*-alkanes (C6+C40) and other elements as the only carbon sources and *Achromobacter xylosoxidans* DN002 performs better on a diversity of monoaromatic and polyaromatic hydrocarbons, approximately no single bacteria can solely degrade the entire portion of petroleum hydrocarbon certainly, some array of bacteria can only efficiently degrade certain portion of petroleum hydrocarbon components, as others are entirely unavailable (Varjani, 2017) [18]. Generally, these work point out that improving the biodegradation potential by the application of bacterial consortia with multiple catabolic genes is a practical and possible approach to speed up the removal competence of petroleum hydrocarbons from contaminated environments.

A number of work have as well stated that some catabolic and anabolic intermediates with comparatively elevated dispersible formed from the degradation of petroleum hydrocarbons by bacteria and it might contain elevated cytotoxicity than the primary molecules as it can engender damage to the microbe (Hou *et al*., 2018) [10]. On the other hand, indigenous bacteria configure large mass and cluster, each of the specie performs its own role accordingly, as some bacteria that are sensitive to petroleum hydrocarbons are deeply reserved upon contact to petroleum hydrocarbons, the main factors depriving the biodegradation competence of petroleum hydrocarbons are as follows:

1. Inadequate solubility of petroleum hydrocarbons to the organisms (bacteria).
2. The fact that bacterial cell contact with hydrocarbon substrates is a requirement before introduction of molecular oxygen into molecules by the functional oxygenases. Nonetheless, bacteria improves the bonding capacity of cells by changing their exterior mechanism and secreting bio emulsifier to improve their contact to target hydrocarbon substrates bacteria with such functions are often screened and used to remediate the environment, (Kaczorek *et al*., 2012; Krasowska & Sigler, 2014; Wang *et al*., 2018; Guerra, 2018) [13, 14, 21, 27].

Materials and Method

Collection of Soil Sample

The earth used in this work was sourced through the Integrated Institute of Environment and Development (IIED), in Federal University of Petroleum Resources Effurun, (FUPRE) Hydrocarbon Pollution Research/ Training site located in Obi-Ayagha, Ughelli South, Delta State, Nigeria.

Sample location

Composite soil samples from the surface horizon (0-15m) were collected from the impacted site with the history of Fossil-oil contamination using a soil auger. Composite sample of unpolluted soil was also collected (0-15m) and used as the control. It was collected from the College of Science premises in Federal University of Petroleum Resources Effurun, Delta State.

Fossil oil was sourced from Bonny (Light crude).

Table 1: Soil sample collection locations

Sample Collection Site	Type of Sample	Latitude	Longitude
Obi-Ayagha Artisanal refinery abandoned site	Fossil oil Contaminated Soil	5.3674330	5.8499400
FUPRE Garden	Pristine soil	5.570334.5	5.840970

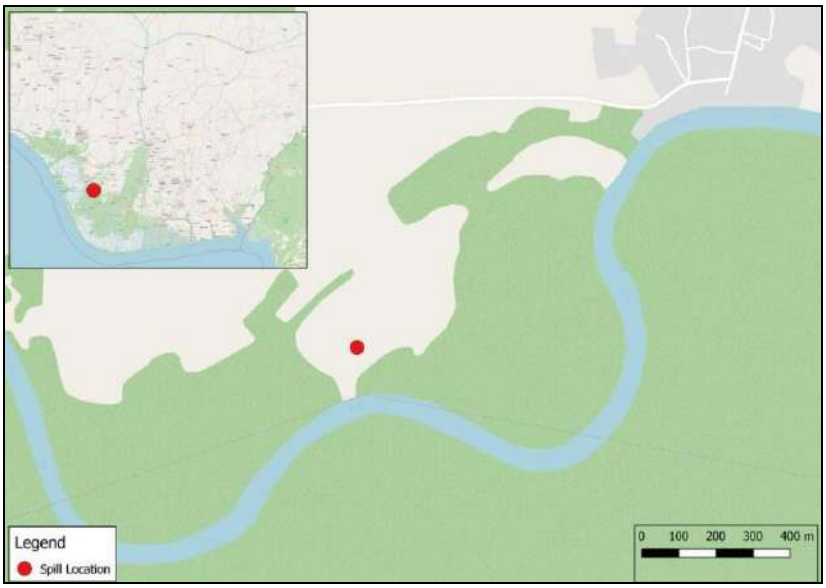

Fig 1: GPS map of the Study Area

Plate 1: Fossil-oil Abandoned Artisanal refinery site

Microbiological analysis

Isolation of hydrocarbon utilizing bacteria (using spread plate method)

The total heterotrophic bacterial (THB) and hydrocarbon utilizing bacterial (HUB) counts were concluded using the method described i ten-fold serial dilution with normal saline was used, in which one gram of the impacted soil was weighed into a test tube containing 10ml of normal saline. 1ml of the diluent was then transferred from the stock into another test tube containing 9ml normal saline giving 10^{-1} dilution. This procedure was repeated up to 10^{-3}. Aliquots of the dilutions (0.1ml) were inoculated onto nutrient agar (NA) plates in triplicates using spread plate method. The plates were incubated at 30 ± 2 ºC for 24hours. The HUB count was accomplished in triplicates on mineral salt agar (MSA) of as modified by Okerentugba *et al*. (2016) [16].

Screening and Identification of Bacterial Isolates

High through put screening using Turbidometric and spectrophotometric approach was used to quantify the growth of the isolates and the quantity of hydrocarbon present, following the method of (Habib, *et al.*, 2017) [9].

Molecular Identification of Isolates

Using pure bacterial isolates, Genomic deoxyribonucleic acid (DNA) extraction, sequencing and bioinformatics were done in International Institute of Tropical agriculture (IITA) a federal research institute located at Ibadan, in Nigeria, to identify the bacteria isolates with high hydrocarbon utilizing capability.

DNA extraction and molecular identification of isolate were described in the work of (Tudararo *et al*., 2023).

PCR Product Purification

The PCR conditions were as follows: 2 vol (20ul) of absolute ethanol was added to the PCR product, the tube was then incubated at room temperature for 15 minutes and Spinned down at 10,000rpm for 15minutes. The Decant supernatant was Spin down as well at 10,000rpm for 15 minutes, then add 2vol (40ul) of 70% ethanol Decant supernatant Air dry Add about 10ul of ultrapure water Check for amplicon on 1.5% agarose then the PCR product is ready for sequence reaction. As displayed below:

Primer: 27F: AGAGTTTGATCCTGGCTCAG
1492R: GGTTACCTTGTTACGACTT

Total Petroleum Hydrocarbon (TPH)

Gas chromatograph-flame ionization detector (GC-FID), was used to achieve the analysis was carried out in thermo steel laboratory situated in Warri, Delta State, Nigeria. Ten grams (10g) of soil samples (polluted sample and control sample) was transferred into an extraction bottle, spiked with known amount of the internal standard (0.1ml of squalene) and dried with anhydrous sodium sulphate, then the dried sample was extracted with a known volume of a mixture of n-hexane and dichloromethane in the ratio of 3:1 by shaking with a so nicator. The extract was cleaned in a silica gel column and the final volume of extract is taken, 1.0µl of the final volume of the extract was injected into an already calibrated Gas Chromatograph equipped with capillary column and identification with data processing software (DATA APEX CLARITY). Total petroleum, hydrocarbon (TPH) was extracted and quantified using the gas chromatography flame ionization detection (GC-FID) Agilent 7890. ASTM-D2862, (2016).

Results and Discussion

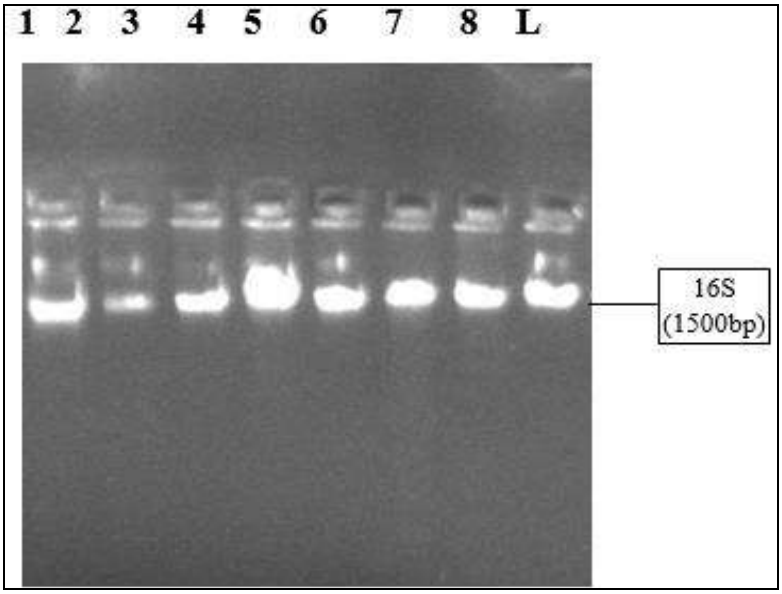

Fig 2: Agarose gel electrophoresis showing the DNA gel L: represents the 1kb ladder, 1-6 represents 16 S gene bands of the isolates

Figure 2: Shows the phylogenetic analysis of the 16S rRNA of the isolates showing their percentage similarities to other species at 98.30% beingclosely related Bacillus thuringiensis, Bacillus velezensis and Stenotrophomonas species.

Table 2: Molecular Identification of Bacterial Isolates

S/N	Sample Identity	Percentage	GenBank Accession Number
1.	Bacillus thuringiensis	97.18%	CP044978.1
2.	Bacillus velezensis	93.06%	Cp1016121
3.	Stenotrophomonas species	98.30%	JNOOO347.1

Table 3: Experimental Design

Treatment	10% Amendments	1% Amendments	0.1% Amendments
Bioaugmentation using Composite microbial culture (CMC)	12g (Consortium of organisms)	1.2g (Consortium of organisms)	0.12g (Consortium of organisms)
CS (Fossil-oil Contaminated soil sample)	8.0g (polluted sample)	0.8g (polluted sample)	0.08g (polluted sample)
NS (Natural sample) Control	180g (unpolluted sample)	198g (unpolluted sample)	199.8g (unpolluted sample)
Total	200g	200g	200g

Key: 10%
1.0% =
0.1% =

Table 2: Mean Concentration of residual total petroleum hydrocarbon in natural attenuation microcosms

Exposure Period (Days)	A (10%)	B (1.0%)	C (0.1%)
0	12504.50±0.71	1071.62±0.54	1026.52±0.01
28	859.97±0.04	756.39±0.19	423.76±0.03
56	267.41±0.24	182.38±0.11	119.79±0.26
84	200.08±0.16	53.88±0.00	27.84±0.01

Table 3: Mean TPH Concentration in bio augmentation with composite microbial culture microcosms

Exposure Period (Days)	A (10%)	B (1.0%)	C (0.1%)
0	948.46±0.07	99.99±0.01	110.07±0.10
28	600.13±0.91	60.25±0.01	84.90±0.01
56	54.73±0.01	25.55±0.01	47.76±0.01
84	2.97±0.01	13.88±0.04	21.95±0.08

Key: A =12:8:180= (200 g)
B= 1.2: 0.8:198 = (200 g)
C= 0.12:0.08:199.8 = (200 g)

The residual TPH concentrations for the control sample (Natural attenuation) at the end of the test duration (84 days), were 200.08±0.16 mg/kg, 53.88±0.00 mg/kg and 27.84±0.01 for the 10%, 1.0% and 0.1% treatment respectively. For the contaminated samples exposed to the bacteria consortium (bioaugmentation), residual TPH concentrations at the test termination (84 days), were 13.88±0.04 mg/kg, 21.95 ±0.08 and 2.97±0.01 mg/kg for the 10%, 1.0% and 0.1% treatments respectively.The residual TPH concentrations of the control samples(Natural attenuation microcosms)were higher than the samples exposed to the consortium of bacteria because TPH degradation and removal was slower and could be attributed to mainly volatilization and reduction of the microbial population due to toxicity of the oil on the microbes.

The consortium of bacteria that was used were *Bacillus thurigiensis, Bacillus velezensis* and *Stenotrophomonas species.* The result from the GC-FID analysis revealed that the bacteria consortium (bioaugmentation treatment) degraded the polluted samples in the 10%, 1% and 0.1% treatments, leading to drastic reduction of the TPH concentrations from the first day of exposure to the last period as displayed in Table 2 above.This also imply that the remediation of petroleum hydrocarbon contamination requires the combined achievement of numerous useful bacteria to accomplish the most excellent environmental decontamination outcome (Dombrowski *et al*., 2016) [5]. This is accredited to the reality that diverse indigenous bacteria posses different enzyme capability, their function in fossil fuel contaminated sites also vary widely. Nevertheless, microbial remediation practice plays a unique function in biological safety when dealing with petroleum hydrocarbon polluted environments due to its low cost, positive effect, little environmental influence and lack of secondary pollution (Dvořák *et al*., 2017) [7]. No single species of bacteria can effectively breakdown hydrocarbon element whereas others are totally engaged (Varjani, 2017) [18]. Their activities in fossil fuel contaminated areas differ widely.

Though some bacteria have been said to fossilize petroleum hydrocarbons totally in a short time under culture setting, the degradative potential of these microbes makes it practically difficult to convene normal effects. (Chen *et al*., 2017) [4]. The compound mixture of diverse organic and abiotic factors restricts the role of petroleum hydrocarbon-degrading bacteria in diverse facet. (Zhao, *et al*., 2017; Wang Y, *et al*., 2018) [25, 21]. The return of microbial communities is accessible since there are a diversity of catabolic genes in a bacterial consortium, and the synergistic property of these genes are helpful to achieving the decontamination of pollutants. A bacterial consortium of five culturable bacteria has been constructed by (Wanapaisan, *et al*. 2018) [28]. However these multiple catabolic genes present in the fossil fuel is practical and realistic approach for speeding up the removal competence of petroleum hydrocarbons from contaminated areas.

Conclusion

Bioremediation using consortium of organisms tends to have a higher ecological significance and that depend on the indigenous microorganisms available to mineralize the organic contaminants. The application of this technology in degrading hydrocarbon polluted soil can also be looked at as it is more promising, eco-friendly and cost effective. Hence this biological method is more promising than other methods such as mechanical, burying, evaporation dispersion and washing because these technologies are expensive and can lead to incomplete decomposition of contaminants.

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