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PETROLEUM HYDROCARBON DEGRADING POTENTIALS OF INDIGENOUS BACTERIA STRAINS FROM "BLACKWATER" ECOSYSTEM OF ENIONG RIVER – NIGERIA

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ABSTRACT

Crude oil degradation by bacteria from sediments of blackwater ecosystem of Enoing River, Itu, Akwa Ibom State, Nigeria was investigated using standard microbiological and analytical protocols. Enrichment of sediment sample in mineral salt medium spiked within 2% and 1% percent crude oil resulted in Darwinian selection of 2.0 x 10^3 cfu/ml and 1.4 x 10^4 cfu/ml bacteria respectively. Crude oil utilizing rating studies revealed that isolates $EHSC_1$ and $EHSC_2$ could multiply using test oil as only source of carbon and energy as they exhibited 18mm and 17mm zone of inhibition on oil-mineral salt agar. These bacteria where further identified as *Azotobacter* $EHSC_2$ and *Bacillus* $EHSC_1$ species. Results of total viable count (TVC) revealed *Azotobacter* $EHSC_2$ increased by 2-fold magnitude (10^3 to 10^5 bacteria/ml) within 6 days while showing early decline. On the other hand, *Bacillus* $EHSC_1$ was observed to utilize crude oil more rapidly and increased by 5-fold (10^3 to 10^8 cells/ml) peaking on day 9 while maintaining profuse growth till the day 18. Chromatographic profiling revealed some crude oil fractions including complex carbon or long chain components (C_4, C_{15}, C_{25}, C_{26} and C_{30}-C_{34}) showed high level of degradation; these bacteria may be adapted for possible application in bioremediation strategies.

INTRODUCTION

Anthropogenic activities involved in exploration, refining, transportation, storage and use of crude oil and its products are responsible for the steady introduction of petroleum hydrocarbons into the environment (Osu *et al.*, 2021). When present in soil, sediment and water, crude oil as pollutant exhibits phenomenal toxicity that is related to the type of organism, nature of the system, concentration of crude oil and exposure route. In soils, for example, crude oil adversely affects it fertility including nutrient status and soil enzymes (Udofia, 2018), other biophysical properties (Wang *et al*., 2013). Crops and vegetation (Ezeji *et al.*, 2007; Doesch *et al*., 2006).

In the marine environment, crude oil has disastrous effect on sea birds, corals, mammals and fishes (Teal and Howard. 1990). Formation of oil slick after spillage reduces levels of dissolved oxygen and in turn affect aerobic entities in the sea and also reduces photosynthesis as the oil slick hampers light penetration into the water (Dave and Ghaly, 2011). In recent years, spillage of crude oil and inputs of its refined products into the environment have been a source of concern as available matrices are gradually getting contaminated (Adekunle, 2011; Ani and Chukwuma, 2020).

There have been lots of researches in the area of remediation due to increasing need to scale down effect of crude petroleum spill on public and environmental health. A number of technologies have been used for conventional remediation of hydrocarbon contamination including biopiling, soil flushing, solvent extraction, electro kinetic remediation and photo-catalytic degradation (Gan *et al.*, 2009; USEPA, 2001). These conventional technologies are associated with high capital, energy and technological inputs. The methods are ecologically unfriendly and do not offer viable alternatives. However, microbial degradation presents better option and is gaining wider acceptance due to its low cost and simplicity in operation. Microbial genetic plasticity enables them to develop catabolic pathways that efficiently utilizes hydrocarbon. During microbial metabolism, enzymes that could oxidize and subsequently transform hydrocarbon contaminants into less toxic compounds are elaborated (Siciliano *et al.*, 2002). In search of active strains that could be of relevance in bioremediation of petroleum and its complex hydrocarbons, studies were conducted on petroleum hydrocarbon degradation by bacteria from black water ecosystem of Eniong River.

MATERIALS AND METHODS

Study Area

The study area is a humic freshwater or blackwater ecosystem of Eniong River, a tributary of the lower Cross River traversing Itu through Okopedi. The area is located between the coordinates 05^{o}12^I 0.54^{II} N and 007^o 58^I 48.6^{II} E (downstream); 05^o 16^I 0.54^{II} N and 007^o 57^I 28.7^{II} E (midstream) and 05^o 22^I 56^{II} N and 007^o 54^I 59.1^{II} E (upstream) (Figure 1). The ecosystem is unique and the river is characterized by intense coloration due to the presence of humic substances and possibly soluble iron. The ecosystem supports the remaining populations of an endangered aquatic mammal the manatee (plate 1).

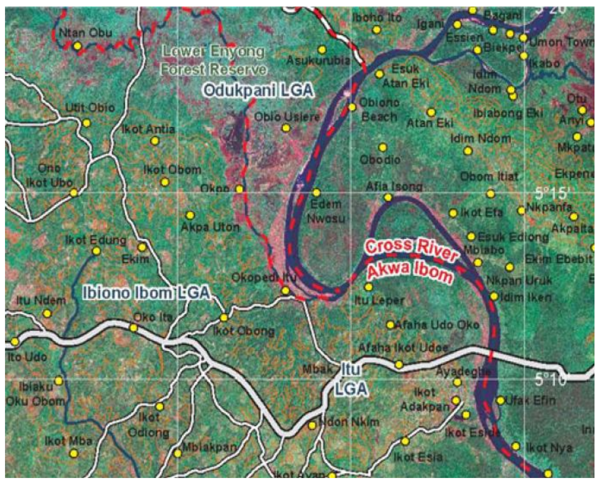

Figure 1: Map of humic freshwater Eniong River, Itu, Nigeria

Plate 1: The confluence point of the humic and non-humic Eniong river

Sample and Test Chemical Collection

Sediment samples were collected from the benthic zone of the river with the aid of a grab sampler and stored separately in sterile amber bottles to avoid photo-oxidation. Sediment sample containers were placed in an ice-packed chest and transported to the Postgraduate Laboratory of the Department of Microbiology, University of Uyo within 6 hours of collection for subsequent analyses. Bonny light crude (specific gravity $0.818g/cm^3$) was sourced from an oil servicing company that deals with Exxon Mobil located in Eket, Akwa-Ibom State.

Determination of Physicochemical Properties of sediment

Sediment samples were analysed for pH, total organic carbon (TOC), available phosphorous, total nitrogen (TON), total salinity, nutritive salts, particle sizes and exchangeable cations (IITA, 1979; Jacobsen, 1992; Essien, *et al*., 2011; APHA, 1998; AOAC, 1975).

Determination of Total petroleum Hydrocarbon (TPH).

Before and after treatment with test isolates, one hundred (100) ml of sample was measured into a separating funnel and 10 ml of Dichloromethane: Hexane (1:1) was added into it. The mixture was shaken gently and vented for 5mins. The aqueous layer was allowed to separate and was decanted. The extract was concentrated by rotary evaporator into 1ml. precisely 1.0µL of the extracts was injected into a pre-programmed Hewlett-Packard HP 5890 GC-FID. The concentration of TPH was calculated from the peak area of the calibration standards. The GC operational conductions for TPH were as follows: 50^oC-320^oC at 10^oC/min. initial holding temperature 2.0 minutes. Injector temperature was 250^oC while detector temperature was 340^oC. Helium was used as the carrier gas (at 1.5ml/min) with hydrogen and air as the ignition gas (Aceves, *et al*, 1988).

Microbiological Analyses

Determination of Total heterotrophic bacteria count in sediments

Twenty (20) grams of sediment was weighed and added to 180 ml of sterile deionized water and vigorously agitated for a minute using a vortex shaker. The classical ten-fold decimal dilutions of the sediment were carried out (Oboh *et al*., 2006). Bacterial species indigenous to the sediment samples were enumerated after spread plating of 0.1ml aliquots of appropriate dilutions unto Bacto Nutrient Agar (NA) plates and incubating at 28 ± 2^oC for 48 hours.

Enrichment for Isolation of test bacteria

Enrichment for bacteria capable of utilizing crude oil as sole sources of carbon was performed and involved the use of liquid crude oil within a shaking batch liquid system according to Chadhain, *et al*., (2006). Bacteria with the capability of utilizing crude oil were obtained by inoculating the sediment (20g) into enrichment medium made up of 180ml sterilized minimal medium in a 250ml flasks supplemented with crude oil as the sole source of carbon. The medium contained: K_2HPO_4 (1.8g/L); NH_4CL (4.0g/L); $MgSO_4.7H_2O$ (0.2g/L); NaCl (0.1g/L); $Na_2SO_4.7H_2O$ (0.01g/L); KH_2PO_4 (1.2g/L); $FeSO_4.7H_2O$ (3.5g/L); carbon source (4% crude oil) and distilled water (IL) and pH adjusted to 7.2 using aqueous HCl and NaOH. The flasks were inoculated with 1ml aliquot from an overnight broth cultures of the bacteria. Culture flasks were incubated on a shaker (80rpm) at 28 ± 2^oC for 15 days.

Isolation and Identification of crude oil utilizing bacteria.

Hydrocarbonoclastic bacteria were isolated on mineral salt medium (Same composition as enrichment medium) to which 15g per litre of agar agar was added and overlaid with 1% (v/v) sterile crude oil. Inoculum (0.1ml) was aseptically removed from enrichment flasks and inoculated by spread plating onto the above medium and incubated at room temperature for 10 days. Colonies that developed on plates on plates were picked and based on the zones of halo created, were rated +, ++ or +++ to indicate the magnitude of potentials.

Discrete colonies exhibiting widest zone of clearing in all instances were picked with platinium wire loop and subcultured repeatedly on fresh NA. Pure Bacterial culture plates were incubated at 28 ± 2^oC for 24. Conventional microbiological procedures were used to identify bacteria.

Time-course degradation of crude oil by test Bacteria isolates.

This was carried out in sterile 200ml minimal medium (as previously constituted) contained in 250ml capacity Erlenmeyer flasks to which 1 ml of a 24 hours old broth culture of the test bacteria were added. The flasks were supplemented with filter sterilized crude oil (2% v/v) to provide carbon and energy. A second set devoid of microbial cells but containing appropriate concentration of crude oil pollutants served as control. Flasks were incubated on a mechanical shaker at 120rpm for 5 days and then at 80rpm for the remaining 15 days in the dark at room temperature. Pollutant degradation and utilization for growth purposes was monitored indirectly by measuring Total Viable Count (TVC) at 3 days intervals. The residual pollutants after degradation were determined by Gas Chromatographic analyses.

RESULTS AND DISCUSSIONS

The heterotrophic bacteria load in sediments from humic and non-humic freshwater ecosystem of Eniong River is presented in Table 1. The results revealed a bacterial count of $2.7 \pm 1.02 \times 10^3$ cfu/g for station A; $4.0 \pm 0.52 \times 10^4$ cfu/g for station B; $1.2 \pm 1.57 \times 10^4$ cfu/g (station C) and $2.5 \pm 1.49 \times 10^6$ cfu/g for non-humic sediments. The results revealed further the bacterial counts of cultures enriched with crude oil at two levels of pollution (Table 2). The result of the enrichment showed that the resultant population were affected by the concentration of the pollutant, confirming the point that toxicity of the pollutants is amongst other factors dependent on concentration. This agrees with the work of Gans *et al* (2009) whose findings indicated that higher concentrations of chemical pollutants depressed populations of species.

On Table 3 is presented pollutant utilizing ratings and other characteristics of test bacterial isolates. Results on the table clearly show that the isolates $EHSC_1$ and $EHSC_2$ could multiply in the presence of crude oil following the wide zones of inhibition (18mm and 17mm respectively) exhibited. The identity of the bacteria species used in this study is presented in Table 4. The results have revealed the catabolic diversity of microbial assemblage from the humic freshwater Eniong River incriminated in the degradation of crude oil. The potentials of the bacteria *Bacillus* $EHSC_1$, and *Azotabcter* $EHSC_2$ as oil degraders have been established.

Table 1: Heterotrophic bacteria load in sediments from humic and non-humic freshwater Eniong River (cfu/g)

Sediment sample code	Mean count ± SD
HAS	$2.7 \pm 1.02 \times 10^3$
HSB	$4.0 \pm 0.52 \times 10^4$
EHSC	$1.2 \pm 1.57 \times 10^4$
NHS	$2.5 \pm 1.49 \times 10^6$

Table 2: bacterial counts from cultures enriched with test pollutants (cfu/ml)

Percent crude oil (%)	Counts
2%	2.0×10^3
1%	1.4×10^4

Table 3: Pollutants utilization ratings and characteristics of test bacteria isolates

Bacterial codes	Pollutant type	Gram reaction	Cell forms	Inhibition zones (mm)	Utilization rating
$EHSC_1$	Crude oil	+	Chains	18	+++
$EHSC_2$	Crude oil	+	Single	17	+++
$EHSC_3$	Crude oil	+	Chains	9	++
$EHSC_4$	Crude oil	+	Single	4	+

Table 4: Pollutants used and the identity of the utilizing bacteria

Isolate code	**Pollutant utilized**	**Probable organism**
$EHSC_1$	Crude oil	*Bacillus* sp
$EHSC_2$	Crude oil	*Azotobacter* sp

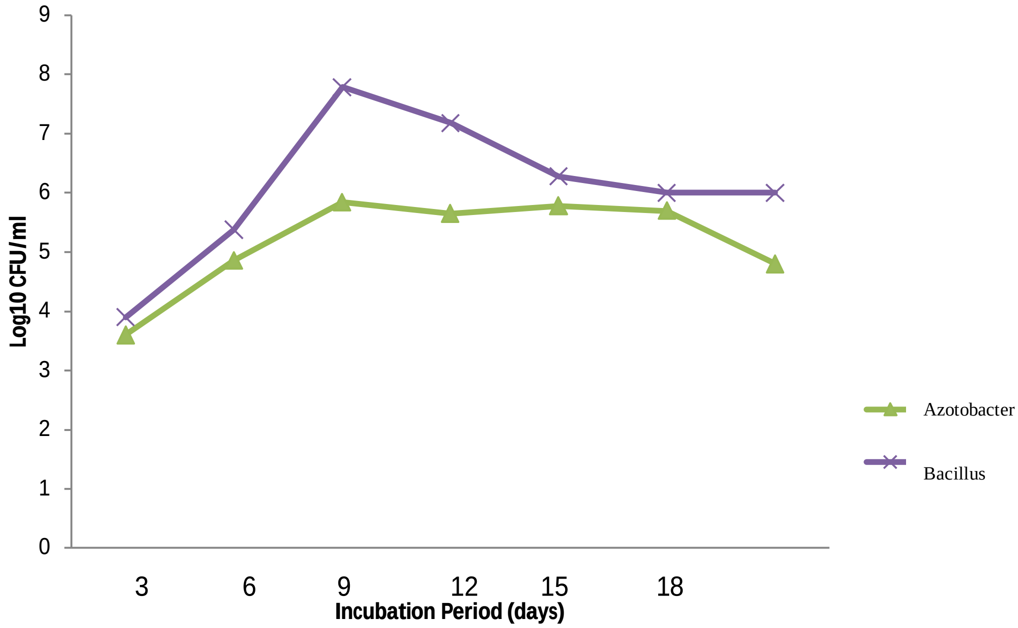

Figure 2: Population Dynamics of Bacteria Exposed to Test Hydrocarbons for 21 Days

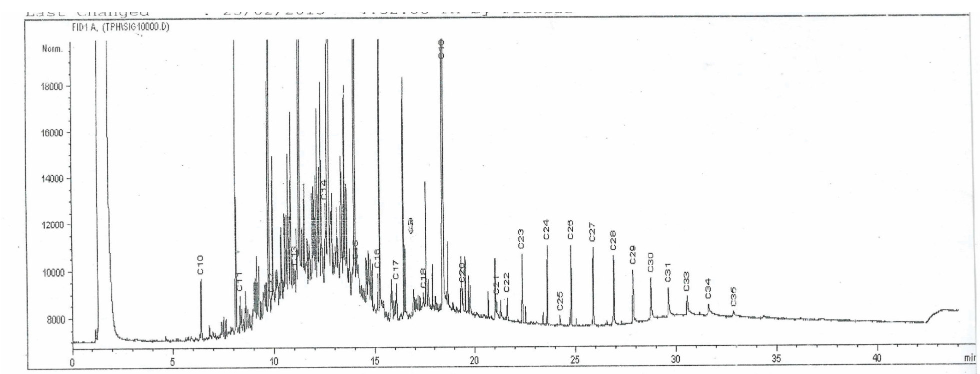

Figure 3: Profile of un-inoculated crude oil after 21 days.

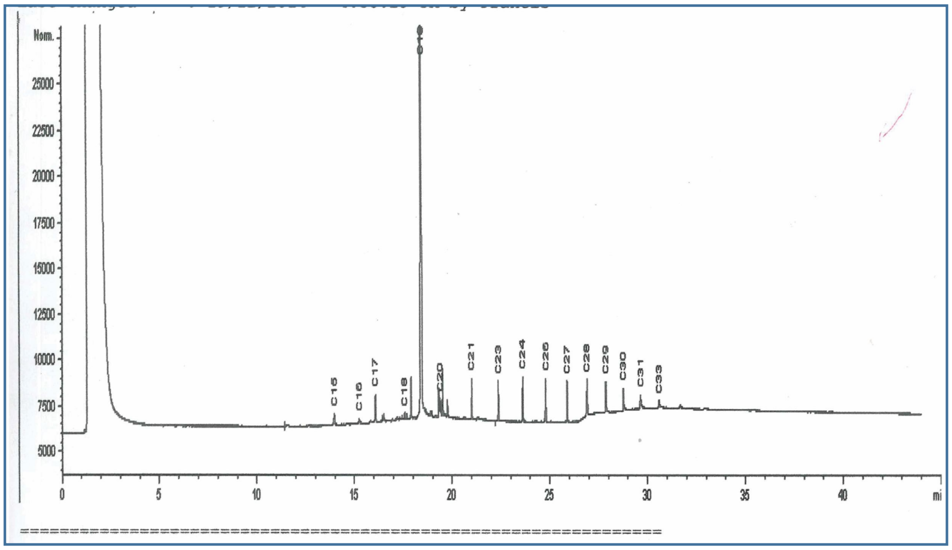

Figure 4: Profile of crude oil degradation by *Azotobacter* sp $EHSC_2$ after 21 days.

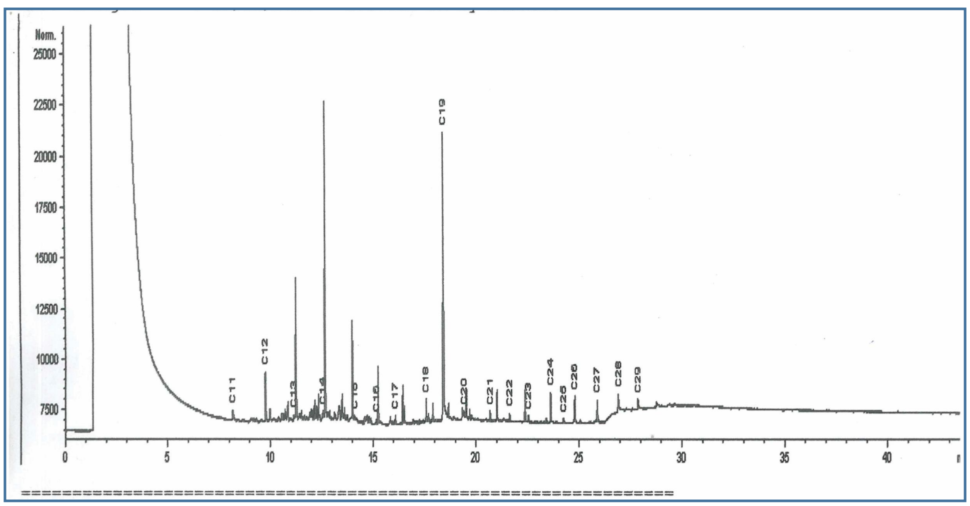

Figure 5: Profile of crude oil degradation by *Bacillus* sp $EHSC_1$ after 21 days

Crude oil was utilized by *Azotobacter* $EHSC_2$ and increased by two-fold magnitude (10^3 to 10^5 bacteria/ml) within 6 days to reach a population of 10^7 cells/ml between the 9th and 12th day of incubation. On the other hand, *Bacillus* $EHSC_1$ was observed to utilize crude oil more rapidly and increased by five-folds (10^3 to 10^8 cells/ml) peaking on the 9th day, maintaining profuse growth till the 18th day before noticeable reduction (figure 2.)

The result of gas chromatographic profile of saturated fraction in crude oil (control) and persistent residue after degradation by *Azotobacter* $EHSC_2$ and *Bacillus* $EHSC_1$ are presented in figures 3, 4 and 5 respectively. Chromatogram peaks corresponds to different fractions in oil, peak heights depict concentrations while values on ordinate signify signal retention time of fraction in column of chromatography. The results revealed that while some fractions were poorly degraded, others including the complex carbon or long chain components (C_4, C_{15}, C_{25}, C_{26} and C_{30}-C_{34}) showed high rate of degradation. The spectral pattern exhibited sixteen peaks and twenty three peaks for *Bacillus* $EHSC_1$ and *Azotobacter* $EHSC_2$. Result revealed that fractions from C_{10} to C_{34} were effectively eluted and detected and the eluted fractions include Octadecane, Tetracosane, Pentacosane, Hexacosane, n-Docosane and n-Elcosane.

The trophodynamic roles of sediment humid acid is poorly understood. However, evidence is increasing that humic acid catalysis both oxidative and reductive reactions among chemical species and interact with aquatic organisms to provoke a variety of specific reactions in organisms including potential to influence physiologic properties and probably trigger micro evolution. It is hypothesized that microbes from the black water ecosystem may adapt to interact with heterogenous materials that serve as primary environmental sorbent for hydrophobic hydrocarbon in ways that facilitate uptake of crude oil pollutant and subsequent metabolism. Sequential enrichment technique activated the catabolic potentials and selected for crude oil utilizing *Bacillus* $EHSC_1$ and *Azotobacter* $EHSC_2$ from humic sediment.

CONCLUSION

The present study shows that certain indigenous bacteria including (*Azotobacter* and *Bacillus*) from sediment of Eniong River ecosystem have high capacity to degrade petroleum hydrocarbon. This capacity appears to be enhanced by prior exposure of isolates to sub concentrations of either stressors or chemical pollutants. These organisms may be adapted for possible application in bioremediation of contaminated site. However, more detailed information is required in order to optimize conditions for maximum utilization.

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