CHARACTERIZATION OF INDIGENOUS BACTERIAL COMMUNITIES IN CRUDE-OIL IMPACTED SITES AT OBAGI TOWN, ONELGA, RIVERS STATE, NIGERIA.

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ABSTRACT

Hydrocarbon utilizers are expected to be indigenous in crude-oil polluted environments. The isolation and characterization of hydrocarbon utilizers is often a key strategy in bioremediation of hydrocarbon-polluted environments. In this study, crude-oil polluted soil samples from Obagi town, Onelga, Rivers state were enumerated and characterized for putative hydrocarbon utilizing bacterial populations. Biochemical characterization identified five bacterial species representative of five genera: *Bacillus, Pseudomonas, Acinetobacter, Micrococcus* and *Staphylococcus*. Amongst the genera of bacteria isolated, *Bacillus* had the highest frequency of occurrence (40%). The mean count of total heterotrophic bacteria was 1.7 X 10^7 cfu/g, while hydrocarbon utilizing bacteria (HUB) count mean density was 1.0 X 10^7 cfu/g for the three soil samples. Statistical analyses revealed no significant difference at $p>0.05$ between Total Heterotrophic Bacterial (THB) and Hydrocarbon Utilizing Bacterial (HUB) counts, suggesting that most of the bacteria present in the sampled sites were hydrocarbon utilizers. Findings from this study suggest the presence of indigenous putative hydrocarbon utilizing bacteria in the crude-oil polluted soil of Obagi town. Hence, a promising potential exists for future bioremediation studies on the site.

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KEYWORDS

- Hydrocarbon-utilizers
- Indigenous
- Bacteria
- Crude-oil
- Bioremediation

INTRODUCTION

One of the major environmental problems today is hydrocarbon pollution by the petrochemical industry (1), and widespread release of aromatic hydrocarbons through spillages and leakage from underground tanks and steamers, causing extensive contamination of surface soils, ground water, seas and oceans (2). Mechanical and chemical methods for remediation of hydrocarbon polluted environments are often expensive, technologically complex and lack public acceptance (3).

Biodegradation by microorganisms is fundamental in the removal of hydrocarbons and xenobiotic substances (4). Irrespective of the wealth of research relating to microbial degradation of hydrocarbons, knowledge pertaining to which organisms are the key players in hydrocarbon degradation in the environment is limited (5).

In order to combat this challenge, it is pertinent to first assess the hydrocarbon degrading potential of the microorganisms before any bioremediation intervention rather than just focus on the removal of individual hydrocarbon compounds *via* mechanical and chemical methods for remediation (6). This approach will provide new insights for improving the management of such environments.

It has been observed that low molecular weight hydrocarbons like anthracene and naphthalene are usually readily degraded by bacteria in soil and under laboratory conditions (7). Other studies have also shown that petroleum hydrocarbons can be degraded by microorganisms such as bacteria, fungi, yeast, and microalgae. Hydrocarbon degrading bacteria and fungi are widely distributed in marine, freshwater and soil habitats (16). Typical bacterial groups already known for their capacity to degrade hydrocarbons include *Pseudomonas*, *Marinobacter*, *Alcanivorax*, *Microbulbifer*, *Sphingomonas*, *Micrococcus*, *Cellulomonas*, *Dietzia* and *Gordonia* groups (8).

The aim of this study was to evaluate the microbial heterogeneity of crude-oil polluted soils at Obagi town, Onelga, Rivers state to be able to predict their inherent potential for hydrocarbon utilization.

MATERIALS AND METHODS

DESCRIPTION OF SAMPLE SITES

The study site, Obagi town, is a mangrove environment whose center lies at a latitude of 5.25114 and longitude of 6.61298. Surface soil from the three sample sites in Obagi town were collected to enumerate and characterize bacterial isolates that have the potential for utilizing hydrocarbons. The samples were placed in sterile polyethylene bags and transported to the laboratory for analysis.

ENUMERATION OF TOTAL HETEROTROPHS AND HYDROCARBON UTILIZING BACTERIA

One gram (1g) each of the soil samples were serially diluted (10^{-1} to 10^{-6}) in 9ml normal saline. Aliquots (0.1ml) from dilutions of 10^{-4}, 10^{-5} and 10^{-6} of soil samples were plated in duplicate on sterile Plate Count Agar (Merck, Germany) and incubated at 37°C for 24 hours for total culturable heterotrophic bacteria counts. For hydrocarbon utilizing bacterial counts, enumeration was performed as described by Hamamura *et al.* (15) where appropriate dilutions of soil sample suspensions were plated on Busnell-Haas Agar (Sigma-Aldrich, USA), and hydrocarbons were supplied through the vapour phase to putative hydrocarbon utilizers by placing sterile filter papers impregnated with 5ml Okono crude oil on the lids of the inverted plates and incubated for 7 days at 37°C.

PURIFICATION AND IDENTIFICATION OF PUTATIVE HYDROCARBON UTILIZING BACTERIAL ISOLATES

Discrete colonies of different putative hydrocarbon utilizing bacteria (HUB) were randomly picked using a sterile wire loop and subcultured for purification by streaking on nutrient agar plates and incubated at 37°C for 24 hours. Individual bacterial colonies were presumptively identified using morphological and biochemical tests as described in Bergy's

Manual for Determinative Bacteriology (Gram stain, motility test, catalase test, oxidase test, citrate utilization test, indole test, hydrogen sulphide test, urease test, triple sugar iron test, methyl red, and Voges–Proskauer test)(17).

STATISTICAL ANALYSIS

Data obtained from the study were subjected to statistical analysis using T-test and one way analysis of variance (ANOVA) at 0.05 confidence level ($p<0.05$).

RESULTS

Total heterotrophic bacterial counts for each soil sample were (1.9×10^7cfu/g, 2.0×10^6cfu/g, and 3.0×10^7cfu/g) (Fig. 1) and hydrocarbon utilizing bacterial counts were (2.1×10^6cfu/g, 1.4×10^6cfu/g and 2.7×10^7cfu/g) respectively (Fig. 2).

We observed that there was a significant difference between THB and HUB in soil sample A ($p=0.001$), which suggests that the hydrocarbon utilizers (HUB) present in soil sample A are not a majority proportion of the bacterial community (THB). However, in samples B and C (i.e, THB and HUB in sample B, and THB and HUB in sample C) there was no significant difference observed ($p=0.084$ and 0.441, respectively), which suggests that most of the culturable bacterial population (THB) have become putative hydrocarbon utilizers (HUBs).

A total of 28 bacterial species were isolated as THB, while 19 bacterial species were isolated on mineral salt medium (Bushnell Haas Agar) and identified morphologically and *via* biochemical tests.

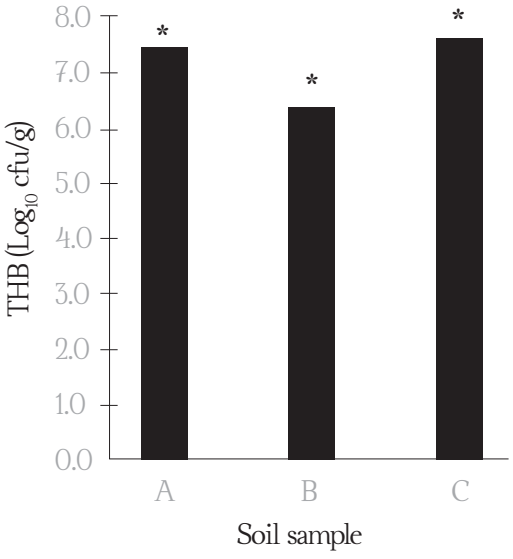

Figure 1. Log cfu/g of Total Heterotrophic Bacteria of the various oil impacted soil samples
*= significant difference exists at $p=0.05$

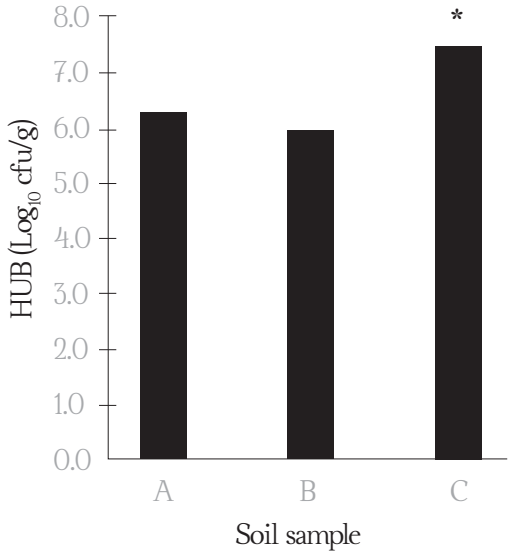

Figure 2. Log cfu/g of Hydrocarbon Utilizing Bacteria of the various oil impacted soil samples
*= significant difference exists at $p=0.05$

Table 1: Characterization of bacterial isolates.

Legends:
R: Rod; C: Cocci; +: Positive; –: Negative

Isolates	Gram reaction	Tentative identity
B2SA1	– R	*Klebsiella sp.*
B2SA2	+ R	*Corynebacterium sp.*
B2SA3	+ C	*Micrococcus sp.*
B2SA4	– R	*Enterobacter sp.*
B2SA5	– R	*Flavobacterium sp.*
B2SA6	– R	*Azotobacter sp.*
B4SA1	– R	*Escherichia coli*
B6SA1	+ C	*Proteus sp.*
B2SB1	+ C	*Staphylococcus sp.*
B2SB2	– R	*Proteus sp.*
B6SB1	– R	*Serratia sp.*
B8SB1	– R	*Pseudomonas sp.*
B8SC1	– R	*Pseudomonas sp.*
1A1	+ R	*Bacillus sp.*
1A2	+ R	*Bacillus sp.*
2A1	+ C	*Staphylococcus sp.*
2A2	– C	*Acinetobacter sp.*
2A3	+ C	*Micrococcus sp.*
1B1	– C	*Acinetobacter sp.*
1B2	– R	*Pseudomonas sp.*
2B1	+ C	*Micrococcus sp.*
2B2	– R	*Pseudomonas sp.*
2B3	+ R	*Bacillus sp.*
1C1	+ R	*Bacillus sp.*
1C2	+ C	*Staphylococcus sp.*
1C3	+ R	*Bacillus sp.*
2C1	+ R	*Bacillus sp.*
2C2	– R	*Pseudomonas sp.*

Table 2: Frequency of occurrence of hydrocarbon utilizing bacteria genera isolated from oil impacted soil samples.

Genus	Frequency of occurrence (%)
Bacillus spp.	31.6
Pseudomonas spp.	26.3
Acinetobacter spp.	10.5
Micrococcus spp.	15.8
Staphylococcus spp.	15.8

The characteristics of the Total Hydrocarbon Bacteria are presented in Table 1. The isolates were Gram positive and negative rods and cocci, and were tentatively identified to be representatives of the genera *Staphylococcus, Bacillus, Micrococcus, Pseudomonas Acinetobacter, Klebsiella,* and *Enterobacter*.

Nineteen putative HUB species were isolated with the dominance (frequency of occurrence) of representatives related to the genera *Bacillus and Pseudomonas* (Table 2). *Bacillus* spp. was observed to be the most isolated bacterial genera from the soil samples.

DISCUSSION

The study site, Obagi town, is a mangrove environment challenged by crude oil pollution. In this study, a culture dependent technique was used to isolate and characterize a putative hydrocarbon utilizing bacterial population indigenous in crude–oil impacted soil. Culture dependent techniques have been used by several researchers to isolate bacteria involved in petroleum hydrocarbon utilization (2,3,6,7,10,13,21,22–26).

Results showed that the mean values of total culturable heterotrophic and hydrocarbon utilizing bacterial counts from each soil sample (Soil A, B and C) were all moderate-to-high. These high counts may be attributed to the presence of organic matter content (nutrients) and favorable ecological factors that underpin the survival of these bacterial species. Consequently, it indicates a viable population with the potential to initiate and maintain hydrocarbon degradation. A similar finding was made by Abu and Dike (1) and Chikere and Ekwuabu (11).

Results revealed that most of the microorganisms present in the various sample sites were hydrocarbon degraders. A similar observation was reported by Chikere and Ekwuabu (11).

Furthermore, a one-way analysis of variance (ANOVA) showed that significant difference existed in the THB (Log10 cfu/g) counts in soil samples A, B and C with *p*-values of <0.0001 and HUB (Log10 cfu/g) counts in soil samples A, B and C with *p*-values of <0.0001.

It was also observed that HUB counts in Soil C were significantly higher compared to Soil A and B. This is likely due to the hydrocarbon concentration in Soil C. Soil C may have been chronically impacted by hydrocarbons compared to the other soils and the indigenous microbes as part of a survival strategy, and may have adapted to degrading/utilizing these hydrocarbons as carbon sources, thus increasing their counts in the area of concern. When environmental changes occur as a result of the presence of a pollutant, there will be the development of new metabolic pathways and acquisition of new functions by mutations. This increases diversity with one population by the formation of a new niche (hydrocarbon utilizers) such that under *in vitro* isolation processes, microorganisms with beneficial mutations have growth advantages in comparison to unadapted organisms (24).

A total of 28 bacterial isolates were obtained as THB while a total of 19 bacterial isolates were confirmed on mineral salt medium (Bushnell Haas Agar) with crude oil as the carbon source. These bacterial species were Gram positive and mostly Gram negative representative of the *Gammaproteobacteria* group. This correlates with earlier studies (11,20). The putative hydrocarbon utilizing bacterial isolates were observed to be representative of the genera *Bacillus*, *Staphylococcus*, *Acinetobacter*, *Micrococcus*, and *Pseudomonas*. These genera of bacteria have been reported as hydrocarbon utilizing bacteria by several researchers (9,12,18,27,28), and this suggests that these species are key players in biodegradation of hydrocarbons in these sites.

Bacillus spp. was recorded to be the dominant bacterial genus from the soil samples having 6 out of 15 isolates, followed closely by *Pseudomonas* spp. having 5 as shown in Table 1. This corroborates results observed by Chikere *et al.* and Kadali *et al.* (9,19). *Bacillus* spp. have been reported to be more tolerant to high concentrations of hydrocarbons in soils due to their resistant endospores (14,19,29). The capability of *Pseudomonas* species to grow and degrade different hydrocarbon content has also been reported. They have been isolated from worldwide polluted sites (5).

CONCLUSION

Mangroves are intertidal ecosystems along coastlines of tropical and subtropical regions, thus they are prone and subjected to urban and industrial effluent discharges and accidental oil spills. Results from this study revealed the utilization of hydrocarbons by the indigenous bacterial community in crude-oil polluted mangrove soil of Obagi town, evidenced by appreciable total heterotrophic bacterial and hydrocarbon utilizing bacterial counts from a mineral salt medium (Bushnell Haas) containing crude-oil as the only carbon source. The isolated bacterial species such as *Bacillus* and *Pseudomonas* amongst others have also been isolated and implicated as hydrocarbon utilizers in a number of studies. This study therefore provides a database of the indigenous hydrocarbon utilizing bacterial community present in Obagi town for application in bioremediation studies, although these data are based on culturable microbial populations present in the sampled locations. Hence, molecular methods that characterize functional genes would be a more powerful approach to study hydrocarbon utilizers in a mixed microbial community (8,30,31).

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