DIVERSITY OF POLYAROMATIC HYDROCARBON UTILIZING MICROBES IN AN ABANDONED CRUDE OIL POLLUTED FARMLAND

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Abstract: Polvcyclic aromatic hydrocarbons (PAHs) are hydrophobic organic compounds known to he highly toxic and recalcitrant in the environment and are present in hydrocarbon contaminated soil and sediment. This study was investigated to selectively ascertain the prevalence of anthracence utilizing microbes in a chronically polluted site. Samples were collected from crude oil contaminated sites in Obagi Town Omoku, Rivers State, Nigeria. Five samples were collected from 3 sites (Obi 27, Obi 22 and Obi 10, all in Obagi Town, Omoku), and designated A, B and C respectively. Soil and sediments samples were collected from sites A and B, while only soil sample was collected from site C. These samples were analyzed for the presence of anthracene utilizing microbes. A total of 12 bacterial species which fall under 7 genera were isolated, characterized and identified from 5 petroleum polluted sample using anthracene as the sole carbon source. The isolates obtained were *Acinectobacter, Alcaligenes, Bacillus, Enterobacter, Flavobacterium, Psendomonas* and *Staphylococcus. A* total of 5 fungal species which fall under 3 genera were also isolated and identified from the 5 petroleum polluted samples using Rose Bengal Chloramphenicol agar. *Penicillium* sp., *Microsporium* sp., and *Aspergillus* sp., were the dominant fungi genera isolated from all the samples using Rose Bengal Chloramphenicol agar. None of the isolated organisms tested positive for catechol- 2, 3-dioxygenase after catechol solution was sprayed on the isolates and incubated for 10 minutes. These findings demonstrate the selective enrichment of anthracene utilizing microbes following crude oil pollution in the environment.

Keywords: Anthracene, Crude oil, Hydrocarbon, Poly cyclic aromatic hydrocarbon, Toxic.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are hydrophobic organic compounds consisting of two or more fused benzene rings arranged in linear, angular, or cluster forms and have a low water solubility which is responsible for its persistence in the environment (Juhasz and Naidu, 2000).

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PAHs susceptible to biodegradation include naphthalene, phenanthrene, anthracene, pyrene, fluorine, and catechol. Anthracene is a low molecular weight solid polycyclic aromatic hydrocarbon consisting of three fused benzene rings. It is found in high amounts in PAH –contaminated environments, and a variety of bacterial species have the ability to utilize anthracene as their sole source of carbon and energy (Peng *et al.*, 2008). The contamination of soil and water with crude oil is among the most prevalent

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problem in Nigeria and the world at large (Claudia *et al.*, 2005). The presence of PAHs in the hydrocarbon contaminated soil and sediment poses a significant risk to the soil microbes, because many of these PAH compounds are known or suspected to be toxic, mutagenic and, in some cases carcinogenic (Peng *et al.*, 2008). The implications of crude oil pollution include: damage to the DNA of living organisms, de-vegetation, and fall in reproduction of both animal and plant, contamination of drinking water (Chikere and Azubuike, 2013; Macaulay and Rees, 2014).

Microorganisms have been found to possess the metabolic potential to degrade a variety of polycyclic aromatic hydrocarbons (PAHs) and they are widely distributed in nature (Thenmozhi *et al.*, 2012). The degradation of PAH by bacteria under aerobic conditions starts with oxidation of the aromatic ring, which is mostly catalyzed by dioxygenases. In this reaction, two atoms of molecular oxygen are incorporated into the PAH to form cis-dihydrodiol metabolites (Brezna *et al.*, 2003). Some features also enable fungi to play great role in bioremediation of polluted environment and there are: secretion of extracellular enzymes, utilization of cytochrome P450 system, ability to grow under stressed environmental conditions (low nutrient, pH, and water activity), extension in biomass location through hyphal growth, easy and rapid growth on agricultural or forest waste, and other enzyme systems (Chikere and Azubuike, 2014). Hydrocarbons in the environment are most times biodegraded primarily by bacteria, yeast and fungi (Das and Chandran, 2010). Examples of some of the bacteria that have shown this PAH degradative ability according to Adebusoye *et al.* (2007) include *Acinetobacter*, *Achromobacter*, *Athrobacter*, *Alcaligenes*, *Staphylococcus*, *Flavobacterium*, *Micrococcus*, and the fungi are *Penicillium*, *Microsporium*, *Aspergillus*, and *Fusarium*. Mixed populations with broad enzymatic capacities are required to degrade complex mixtures of hydrocarbons such as crude oil in soil, fresh water and marine environments (Das and Chandran, 2010).

Therefore, this study was designed to selectively consider the diversity of PAH utilizing microbes from abandoned crude oil polluted farmland.

MATERIALS AND METHODS

Description of sampling site

Crude oil polluted samples were collected from Obagi town Omoku, Rivers State, which is located in the Niger Delta Region of Nigeria. This site has been contaminated with crude oil arising from spillage resulting from pipeline vandalisation.

Sampling

Samples were collected from crude oil polluted soils at a depth of 15 cm using a soil auger. Five samples were collected from 3 sites (Obi 27, Obi 22 and Obi 10, all in Obagi Town, Omoku), the sites were designated A, B and C respectively. Soil and sediments samples were collected from sites A and B, while only soil sample was collected from site C.

Selective enrichment of anthracene utilizing microbes

A portion of 10 g each of soil and sediment samples were transferred into 250 ml conical flasks each containing 90

ml of diluents (normal saline). The conical flasks were immediately shaken for 2 minutes, after which an aliquot of 10 ml supernatants were transferred into new sets of 250 ml conical flasks each containing 90 ml sterile Bushnell Haas broth (mineral salt), following this 2% v/v anthracene was added at a final concentration of 200 mgl^{-1}. The flasks were then incubated in the dark at room temperature at 1000 rpm.

After incubation for 14 days, an aliquot of 10 ml enriched culture was transferred from each of the 250 ml conical flasks to new sets of 250 ml conical flasks containing 90 ml of Bushnell Haas broth to which 2% v/v anthracene was added at a final concentration of 200 mgl^{-1}. The flasks were then incubated in the dark at room temperature at 1000 rpm for another 14 days.

The enrichment culture technique was used to isolate pure microbial strain capable of utilizing anthracene as sole source of carbon and energy.

Isolation of Anthracene utilizing bacteria and fungi

Isolation of hydrocarbon utilizing bacteria and fungi by spray method using anthracene as carbon source. From the 250 ml conical flask containing dilution 1:10, 1 ml each of the samples were transferred into 9 ml diluents in test tubes, these also gave a dilution of 1:10, then the solutions were serial diluted by ten-fold. Following this, 2 g of anthracene was carefully dissolved in 200 ml of absolute organic solvent, 0.2 ml of the suspension was sprayed on each of the Bushnell Haas agar plates, allowed for 10 minutes until a thin film developed, following vapourization of the solvent, then 0.1 ml aliquots were spread over the Bushnell Hass agar plates coated with a thin layer of anthracene and incubated at 30°C for 12 days. This process was also repeated using Rose Bengal Chloramphenicol agar and incubated at 25°C for 7 days.

According to Kiyohara *et al.* (1982), the bacterial colony surrounded by a clear zone was scored positive. The bacteria colony was then picked up from the plates, and purified by repetitive streaking on nutrient agar plates. The morphology of the bacterial colony on the nutrient agar was observed. Gram staining was conducted and observed under the microscope at x100 magnification. The purified strains were stocked on nutrient agar slants and stored at 4°C.

Also, after incubation, Rose Bengal Chloramphenicol agar plates were examined for growth, and purification. Identification of the colonies was done using wet mount using wet mount method, after which stock cultures were preserved on Rose Bengal Chloramphenicol agar slants and stored at 4°C.

Enumeration of Total Hetrotrophic bacteria and Fungi

From the 250 ml conical flask containing dilution 1:10, 1 ml each of the samples were transferred into 9 ml diluents in test tubes, these also gave a dilution of 1:10, then the solutions were serial diluted by ten-fold and then 0.1 ml aliquots were spread over the plate count agar plates and incubated at 30°C for 24 hours. This process was also repeated using Rose Bengal Chloramphenicol agar and incubated at 25°C for 7 days.

After the incubation, the nutrient agar and Rose Bengal Chloramphenicol

plates were examined for bacterial and fungal growth respectively, purification and identification of the colonies were also done, after which stock cultures were preserved and stored at 4°C.

Detection of bacterial species with catechol-2, 3- dioxygenase

To assay for the presence of catechol-2, 3- dioxygenase, a 100 mM catechol solution was spread on the colonies previously identified and purified. The presence of the enzyme was supposed to be indicated by yellow formation by the colonies following conversion of the substrate, catechol to 2-hydroxymuconic semialdehyde, a yellow compound (Sahar, 2006).

RESULTS

Bacterial counts

The culturable bacterial count (THB and AUB) is presented in Table 1. The presence of microbial activity was determined by the enumeration of THB and AUB. Soil A recorded $1.65x10^5$cfu/g, soil B was $68x10^5$cfu/g, soil C was too numerous to count, sediment A was too few to count and sediment B was.$5.0x10^4$cfu/g. All samples for AUB were too few to count.

Table 1: Total heterotrophic and Anthracene utilizing bacterial (AUB) counts

Samples	Mean values of THB	Means values of AUB
Soil A	$1.65x10^5$ cfu/g	Too few to count
Soil B	$2.68x10^5$ cfu/g	–
Soil C	Too numerous to count	–
Sediment A	Too few to count	–
Sediment B	$5.0x10^4$ cfu/g	–

Legend: cfu/g-colony forming unit per gram

3.2 Phenotypic characterization of bacterial isolates

Phenotypic characteristics of probable identities of anthracene utilizing bacterial are shown in Table 2, and 3. Bacterial genera identified are *Pseudomonas, Alcaligenes, Flavobacterium, Acinetobacter, Enterobacter, Bacillus,* and *Staphylococcus.* The isolates were mainly Gram negative bacteria and the Gram positive bacteria such as *Staphylococcus*, and *Bacillus* were also present. Soil has the highest number of isolates followed by sediments.

Table 2: Probable identities of anthracene utilizing bacteria isolated from sediment samples.

S/N	Isolate code	Gram reaction	Shape	C23DO	Tentative identity
1	DAA1	-	R	nd	*Pseudomonas* sp
2	DAA2	-	R	nd	*Alcaligenes* sp
3	DAA3	-	R	nd	*Pseudomonas* sp
4	DBA1	-	R	nd	*Flavobacterium* sp
5	DBA2	-	R	nd	*Pseudomonas* sp

Legends: R: rod; -: negative; C23DO: catechol-2,3-dioxygenase; nd: not determined

Table 3: Probable identities of anthracene utilizing bacteria isolated from soil samples

S/N	Isolate code	Gram reaction	Shape	C23DO	Tentative identity
1	SAA1	-	C	nd	*Acinetobacter* sp.
2	SAA2	-	R	nd	*Enterobacter* sp.
3	SAA3	-	R	nd	*Alcaligenes* sp.
4	SBA1	+	R	nd	*Bacillus* sp.
5	SBA2	+	R	nd	*Bacillus* sp.
6	SCA1	+	C	nd	*Staphylococcus* sp.
7	SCA2	+	R	nd	*Bacillus* sp.

Legends: R: rod; C: cocci; +: positive; -: negative; C23DO: catechol-2,3-dioxygenase; nd: not determined

3.3 Phenotypic characterization of Fungal isolates

The phenotypic characteristics and tentative identities of anthracene utilizing Fungi are presented in Table 4 and 5. A total of 3 fungal genera were isolated from the study sites. The isolates obtained include: *Aspergillus, Penicillium,* and *Aspergillus*

Table 6: Fungal morphology, and tentative identities of anthracene utilizing Fungi isolated from soil samples

Isolate codes	Microscopic observation	Morphological characteristics	Tentative identity
SAA1	Branched cell, smooth conidia in long chain	Green velvet colony. Reverse is cream	*Penicillium* sp.
SBA1	Unbranched conidiophores with multiseptate	Web-like colony with a raised and foamy center. Reverse is cream	*Microsporum* sp.
SCA1	Unbranched conidiophores with multiseptate	Web-like colony with a raised and foamy center. Reverse is cream	*Microsporum* sp.

Table 7: Fungal morphology, and tentative identities of anthracene utilizing Fungi isolated from sediment samples

Isolate codes	Microscopic observation	Morphological characteristics	Tentative identity
DBA1	Unbranched conidiophores, swollen apex with aseptate hyphae	Dark brown colony. Reverse in tan	*Aspergillus* sp.
DBA2	Unbranched conidiophores, swollen apex with aseptate hyphae	Dark brown colony. Reverse in tan	*Aspergillus* sp.

3.4 Frequency of occurrence of bacterial isolates

Twelve bacterial genera were isolated and they include: *Acinetobacter* sp., *Alcaligenes* sp., *Bacillus* sp., *Enterobacter* sp., *Flavobacterium* sp., *Pseudomonas* sp. and *Staphylococcus* sp. It could be seen based on the frequency of occurrence that *Pseudomonas* sp. was the most abundant among the Gram negative bacteria and *Bacillus* sp. for the Gram positive bacteria.

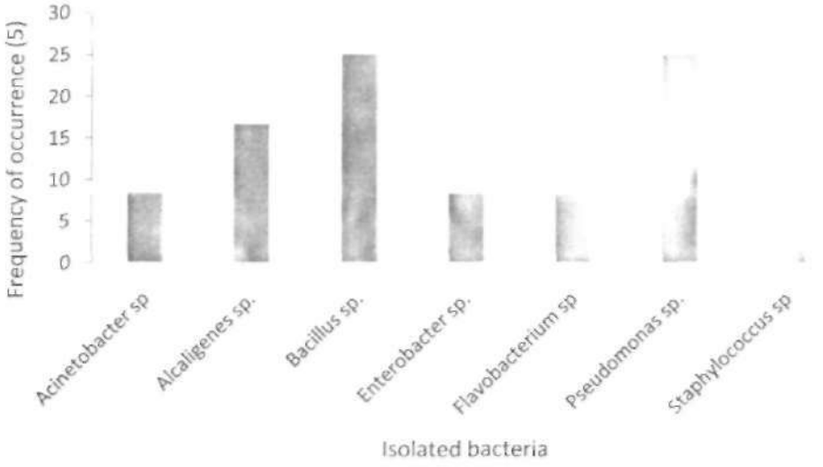

Fig.3. Frequency of occurrence of anthracene utilizing bacterial genera isolated from soil and sediment samples

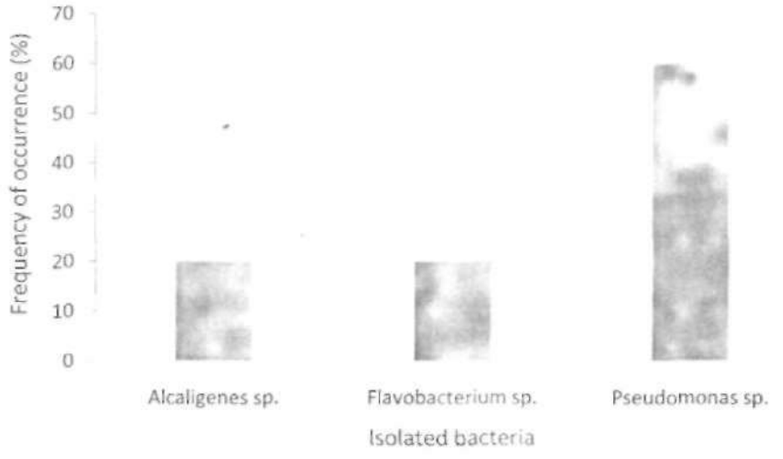

Fig.4. Frequency of occurrence of anthracene utilizing bacteria genera isolated from sediment samples

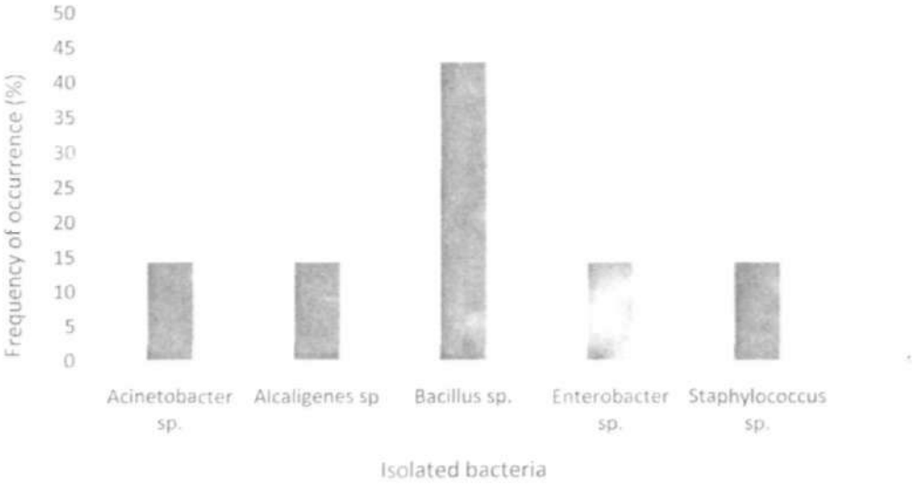

Fig.5. Frequency of occurrence of anthracene utilizing bacteria genera isolated from soil samples

3.5 Frequency of occurrence of fungal isolates

Five fungal isolates obtained covered three genera namely: *Aspergillus* (40%), *Penicillium* (20%) and *Microsporum* (40%). It was observed that *Aspergillus* and *Microsporum* were most abundant.

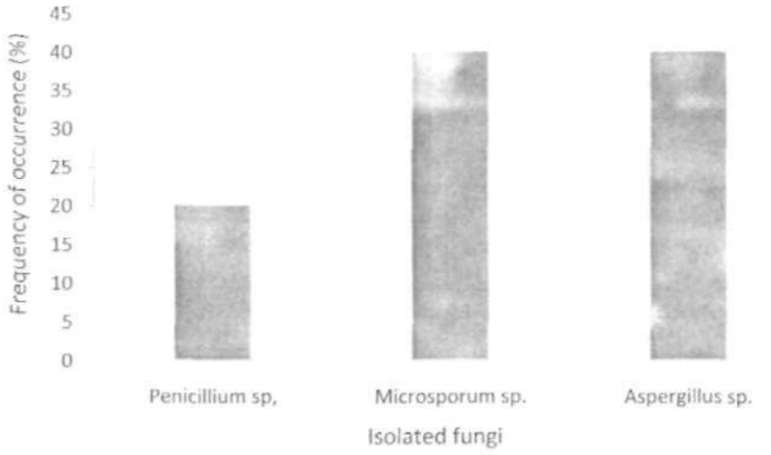

Fig.8. Frequency of occurrence of anthracene utilizing fungal genera isolated from soil and sediment samples.

DISCUSSION

The presence of microbial activity was determined by the enumeration of total heterotrophic bacteria and total anthracene utilizing bacteria as presented in Table 1 and soil samples recorded higher cfu/g soil for THB than sediment samples. A similar observation was reported by Eze and Okpokwasili (2010). The high counts recorded in soil samples could be attributed to the myriad of nutrients, high organic matter concentration and other ecological factors that influence the survival of soil microorganisms that play important roles in the decomposition and recycling of nutrients.

A total of 12 bacterial pure cultures were able to grow on mineral salt medium (Bushnell Haas Agar) with anthracene as carbon source and were identified using phenotypic and biochemical tests. The population of culturable anthracene utilizers from soil, and sediment samples investigated showed that majority of the bacteria were Gram negative belonging to *Gamma proteobacteria* gr oup, this agrees with the findings of Kaplan and Kitts, (2004), although some Gram positive isolates were also observed. The 12 isolates belonged to the genera *Bacillus, Pseudomonas, Enterobacter, Flavobacterium, Alcaligenes, Acinetobacter,* and *Staphylococcus* as presented in Table 2 and 3.

Majority of these organisms isolated were *Pseudomonas* and *Bacillus*. Hydrocarbon polluted sites have been shown to contain predominantly a high number of anthracene degrading species and this suggests that these species are probably the active hydrocarbon utilizers in this environment. These isolates have also been demonstrated by other researchers to be hydrocarbon utilizer (Sarma and Sarma, 2010; Ebrahimi *et al.*, 2012). Watanabe (2001) isolated *Micrococcus, Pseudomonas* and *Bacillus* from sediments as marine petroleum hydrocarbon utilizers. Members of the above mentioned genera have been reported by various authors (Chikere and Azubuike, 2013) as organisms which utilize hydrocarbon as their sources of carbon and energy. Chikere and Azubuike, (2013) reported that bacterial species isolated from hydrocarbon polluted sites such as *Acinetobacter, Achromobacter, Athrobacter, Alcaligenes, Staphylococcus, Flavobacterium* and *Micrococcus,* have all been confirmed by other researchers as having hydrocarbon degradative capabilities. Obire and Nwanbeta, (2002) also reported the isolation of *Serratia, Pseudomonas, Proteus, Klebsiella, Micrococcus* and *Staphylococcus* species from samples collected from petroleum hydrocarbon contaminated soil in Port Harcourt while Eze and Okpokwasili, (2010) isolated *Flavobacterium, Proteus, Bacillus, Klebsiella, Lactobacillus* among other bacteria from Okpoka-Woji river sediment serving as a sink for industrial effluents. The Catechol color assay employed for the detection of C23DO activity did not yield positive results for the isolates screened. The negative result for Catechol 2, 3- dioxygenase could be as a result of the experimental design employed, showing that microorganisms could possess other enzyme involved in PAHs degradation. Furthermore, a total of 5 fungal species which fall under 3 genera were also isolated and identified from the 5 petroleum polluted samples using Rose Bengal Chloramphenicol agar. The fungal genera isolated from this study have been implicated in the degradation of hydrocarbon such as in crude oil,

anthracene and refined petroleum products. The co-existence of different fungal isolates belonging to different genera was attributed to the concept of co-metabolism, a form of microbial interaction involving simultaneous degradation of two compounds (Chikere and Azubuike, 2014). The fungal isolates include: *Penicillium* sp., *Microsporium* sp., and *Aspergillus* sp. It was discovered that the *Aspergillus* and *Microsporium* sp. were more predominant.

CONCLUSION

These findings have revealed that through selective enrichment that PAH utilizing microbes can be isolated and there is an appreciable population of indigenous anthracene utilizing bacteria in crude oil-polluted sites in Obagi Town Omoku. These microbes through the process of bioremediation remove toxic, mutagenic, carcinogenic and recalcitrant polyaromatic hydrocarbons in the environment. Furthermore, Catechol 2, 3- dioxygenase which is an essential enzyme involved in PAH degradation was not detected using the catechol test method. Therefore, it is possible that these organisms possess other enzymes such as catechol 1, 2- dioxygenase and Cytochrome P450 which they use in PAH degradation.

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