DIOXYGENASE SCREENING IN INDIGENOUS HYDROCARBON UTILIZING BACTERIA FROM OIL-POLLUTED SITES IN NIGER DELTA, NIGERIA

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Abstract: Soil and sediment samples collected from crude oil impacted sites at Ogale-Eleme, Rivers State, and five water samples from effluent discharge points at five different flow-stations in Delta State, all in the Niger Delta area of Nigeria respectively were analyzed for the presence of hydrocarbon utilizing bacteria using Bushnell-Haas agar, followed by dioxygenase screening. A total of sixty bacterial species under eleven genera were isolated, characterized and identified as *Acinetobacter*, *Alcaligenes*, *Bacillus*, *Edwardsiella*, *Entervbacter*, *Flavobacterium*, *Klebsiella*, *Micrococcus*, *Proteus*, *Pseudomonas* and *Staphylococcus.* Out of the sixty bacteria characterized, only four isolates from the water sample obtained from Ahia flow station in Delta State and sediment sample E identified as *Pseudomonas* spp. were found to possess the enzyme dioxygenase. This was detected using indigo production by putative hydrocarbon utilizing bacteria. Indigo is produced as an intermediate in indole metabolism by dioxygenases via the meta-cleavage pathway. Bacteria possessing dioxygenases reduced indole to indigo while colonies of such isolates turned navy blue. The findings demonstrate that autochthonous hydrocarbon utilizing bacteria associated with crude oil polluted sites in Niger Delta may probably possess the natural propensity to degrade aromatic hydrocarbons in crude oil.

Key words: Dioxygenases, indole, meta-cleavage pathway, hydrocarbon utilizing bacteria, Niger Delta.

Running title: Detection of dioxygenases in PAH degraders.

INTRODUCTION

Petroleum (crude oil) is a complex mixture of hydrocarbons, mostly saturated or aromatic. The molecular sizes are separated into fractions based on boiling points. The components are dissolved natural gas, gasoline, benzene, xylenes, naphthalenes, octanes, camphor, kerosene, diesel, fuel, heating oil and tars (Dubey, 2009). Since the discovery of petroleum in 1956 in Nigeria, it is known to have much economic importance, producing at a rate of 818 million barrels in 2004 from more than 150 oil fields, mostly in the Niger Delta. It is a major source of Nigeria's export earnings. Virtually, 100% of export earnings and about four-fifths of government revenues are derived from petroleum. In fact, the benefits of crude oil cannot be overemphasized. It has been the major sources of revenue, energy and

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employment generation in Nigeria (UNEP, 2011).

The continuous exploration, production and processing of crude oil and their transportation exposes the environment to constant threat of oil pollution. Crude oil is among the most ubiquitous and persistent environmental contaminants (Onwuka, 2005) because it is capable of causing serious damage to human and the ecosystem. It has been reported that the greatest single environmental problem connected with crude oil exploration in Nigeria is oil spillage both on shore and off shore (Okpokwasili, 1996).

Oil spillage has detrimental effects on both plants and animals. It is reported that oil spillage has caused constant threat to farmlands, crop plants, forest tree species and other vegetation in oil producing areas in Nigeria (Ogri, 2001; Agbogidi, 2003). There have been over 4,000 oil spills in the Niger-Delta area of Nigeria since 1960 (UNEP, 2011). Toxicity of crude oil depends on its physical and chemical composition, the amount of the oil, the plant species and time of application as well as other environmental conditions (Agbogidi *et al.*, 2005). The most promising of many researches carried out to deal with large scale oil spillage is the use of microorganisms to provide an effective alternative (Singh *et al.*, 2001; Atlas and Philp, 2005). This is bioremediation, a process that uses microorganisms or their enzymes to return the environment altered by contaminants to its near-original state at least (Okon and Trejo-Hernandez, 2006). Dua *et al.* (2002) reported that microorganisms are capable of using organic substances, natural or synthetic, as sources of nutrients and energy hence, exhibiting remarkable range of degrading capabilities. For bioremediation to be considered as an applicable technology to the clean-up of a specific pollutant, it is necessary to show that a specific chemical or chemical mixture is biodegradable and that the process of bioremediation will not result in outward ecological side effect (Atlas and Bartha, 1998). The qualitative and quantitative aspects of bioremediation of pollutants are dependent on the composition of the indigenous microbial community, the ambient and seasonal environmental conditions and as such, the temperature, pH, adequate inorganic nutrients and relative humidity of the environment are factors that affect the growth of these microorganisms (Dubey, 2009). These microbes derive nutrients and energy for optimal growth and reproduction from the hydrocarbons so as to degrade them. Biodegradation, which is the breakdown of organic compounds by microorganisms, is carried out largely by diverse bacterial populations, mostly *Pseudomonas* species and other Gram negative bacteria. The hydrocarbon-degrading populations are widely distributed in terrestrial and aquatic ecosystems. In a research carried out by Ojo (2006), hydrocarbons utilizers commonly associated with hydrocarbon degradation included *Bacillus megaterium, Pseudomonas putida, Micrococcus luteus, Bacillus brevis, Bacillus pumilis and Enterobacter aerogenes.* When an environment is contaminated with petroleum, the proportion of hydrocarbon-degrading microorganisms increases rapidly, (Leahy and Colwell, 1990). In particular, there is an increase in the proportion of bacterial populations with plasmids containing genes for hydrocarbon utilization. It is reported that the proportion of hydrocarbon-degrading bacterial population in hydrocarbon-contaminated environments often exceeds 10% of the total bacterial population (Atlas, 1995; Coulon *et al.*, 2012).

There is the need to screen for the biodegrading capabilities of microorganisms associated with hydrocarbon contamination because not all microbes isolated from hydrocarbons are able to degrade/utilize them. The ability of an organism to degrade a specific substrate is clear evidence that its genome harbours the relevant degrading gene (Cowan and Strafford, 2007).

Bacteria have adaptively evolved by developing catabolic pathways to utilize a variety of compounds that are available in the natural environment. Many soil bacteria possess the metabolic potential to degrade a variety of aromatic compounds (Atlas and Philp, 2005). Although the metabolic pathways of these aromatic compounds are very diverse, they are channeled into a limited number of key intermediates, such as catechol and substituted catechols (Akiko *et al.*, 2003). The most common target used to detect PAH-degrading bacterial populations is the presence of dioxegenases that catalyze the initial step of aerobic aromatic hydrocarbon degradation pathways (Marcos *et al.*, 2012; Todorova *et al.*, 2014). The main objectives of the present study were to characterize the hydrocarbon utilizing bacteria community inhabiting the sediment, soil and water environments from Niger Delta, Nigeria, and to screen for the presence of dioxygenases in the isolates.

MATERIALS AND METHODS

Sampling

Four soil samples labeled A, B, C, and D and sediment samples labeled E, F, G, and H were collected from crude oil impacted sites at Ogale-Eleme, Rivers State Nigeria. Five water samples labeled J, K, L, M, and N were obtained from the discharge points of five different flow-stations in Delta State, Nigeria. The soil samples were collected using the composite method with sterile soil auger, from four different points at the contaminated site at a 15cm-depth, and kept in labeled sterile polythene bags. The sediment samples were collected using Eckman sediment grab and were stored in clean-labeled sterile polythene bags. The water samples were collected using water sampler and kept in sterile 500ml plastic cans, then taken to the laboratory for analyses.

Experimental design

One gram of each of the soil and sediment samples and 1ml of the water samples were suspended in 9ml of diluent (nutrient broth), the soil suspensions were serially diluted to 10^{-7} dilutions of which 0.1ml aliquot was spread on plate count agar and incubated for 24 hours at 30°C while 0.1ml aliquot of the same dilution was spread on Bushnell-Haas medium. Sterile Wattman filter papers were impregnated with Okono medium crude oil and aseptically placed on the lids of the Petri dishes, covered, inverted and incubated for 7days at 30°C, using the vapour phase transfer method. For the isolation of PAH utilizing bacteria, 0.1ml of 10^{-7} dilution was spread on the Bushnell-Haas medium after which 0.5g of anthracene crystals were gently spread on the Petri dishes, covered, inverted and incubated for 7 days at 30°C. For the isolation of PAH utilizers using the spray method, 0.5g anthracene was dissolved in 100ml of absolute ethanol, 0.2ml of the PAH-solvent mixture was spread on the Bushnell-Haas medium. This was allowed to stand for some 15 min for the alcohol to evaporate and for a thin anthracene film to develop after which 0.1ml of 10^{-7} dilutions of the samples were spread over the film and incubated for 7 days at 30°C. After incubation, the plates were examined for growth and the morphological characteristics of each colony were noted. The colonies on the plate count agar were subcultured onto nutrient agar, incubated for 24 hours at 30°C, while the colonies on the Bushnell-Haas medium were subcultured onto Bushnell-Haas medium, and were incubated for 7 days at 30°C, after sub-culturing on the primary medium, the cultures were aseptically transferred into nutrient agar slant, streaked aseptically using wire loop and incubated

for 24 h. The pure cultures were characterized and identified to genera level using Gram staining, motility test and presence of endospores. Biochemical tests such as catalase, Voges-Proskauer, methyl red, oxidase, indole production, citrate utilization, hydrogen sulphide production, lactose and sucrose utilization. They were preserved in a refrigerator at 4°C as pure cultures prior to dioxygenase enzyme screening.

Dioxygenase enzyme screening

The aromatic ring dioxygenase activity was examined using indole because the formation of indigo from indole is presumptive for the presence of aromatic ring dioxygenases (Ensley *et al.*, 1983). Dioxygenases cleave indole with the production of indigo (navy blue colour). Bacteria possessing dioxygenases reduce indole to indigo while colonies of such isolates turn navy blue (Fig.1). Briefly, bacterial colonies were pre-grown on Yeast Extract-Poly-peptone-Glucose (YEPG) agar (0.25g/L glucose, 0.5g/L polypeptone, 0.05g/L yeast extract, 0.05g/L ammonium nitrate, 15g/L agar, pH 7.0; Sanseverino *et al.*, 1993a) plates for 24 hours at room temperature, and then 5g of indole crystals were placed in the lids of the Petri dishes containing the isolates and incubated. After 24h incubation at 28°C, colonies producing blue colour were scored positive for dioxygenase enzyme activity.

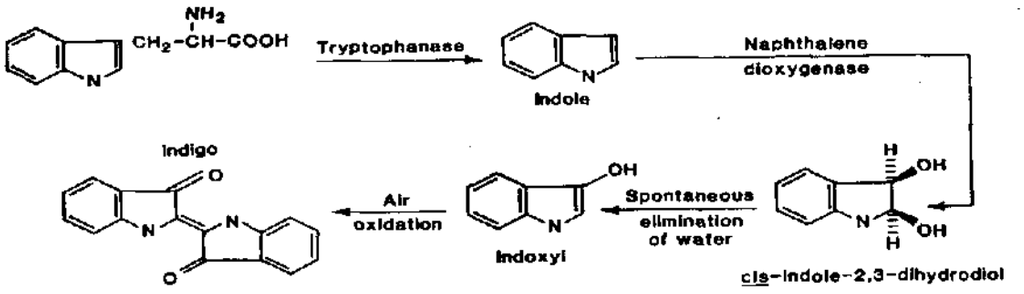

Fig 1. Biosynthesis of indigo from indole through the naphthalene dioxygenase pathway. (Ensley *et al.*, 1983).

RESULTS AND DISCUSSION

A total of sixty bacterial species which fell under eleven genera were isolated, characterized and identified from thirteen crude oil contaminated samples using Bushnell-Haas (Sigma-Aldrich) and plate count (Merck) media.

In all, 36.67% of the isolates were obtained from the Bushnell-Haas agar using the vapour phase transfer technique, 25% were isolated using the plate count agar, 23.33% and 15% of the isolates were obtained from anthracene spray and spread plate techniques respectively as presented in the table 1.below.

Table 1. Frequency of occurrence of Isolates

Isolates/Organisms	*Pseudomonas* sp.	*Flavobacterium* sp.	*Acinetobacter* sp.	*Alcaligenes* sp.	*Enterobacter* sp.	*Bacillus* sp.	*Micrococcus* sp.	*Klebsiella* sp.	*Proteus* sp.	*Staphylococcus* sp.	*Edwardsiella* sp.	Total no. of Isolates	% of Isolates
HUB (Using VPT)	9	2	4	1	1	2	1	1	-	1	-	**22**	**36.67%**
HUB (Anthracene Spray)	6	1	1	-	-	2	1	-	1	1	1	**14**	**23.33%**
HUB (Anthracene Spread)	3	2	-	-	-	1	1	1	1	-	-	**9**	**15%**
THB (Using PCA)	2	-	5	1	-	4	-	-	2	1	-	**15**	**25%**
Sum Total	**20**	**5**	**10**	**2**	**1**	**9**	**3**	**2**	**4**	**3**	**1**	**60**	**100%**

Key: HUB,Hydrocarbon utilizing bacteria, VPT, Vapour phase transfer, PCA, Plate count agar.

The bacteria isolated were *Acinetobacter* spp. (16.67%), *Alcaligenes* spp. (3.33%), *Bacillus* spp. (15%), *Edwardsiella* spp. (1.67%), *Enterobacter* spp. (1.67%), *Flavobacterium* spp. (8.33%), *Klebsiella* spp. (3.33%), *Micrococcus* spp. (5%), *Proteus* spp. (6.67%), *Pseudomonas* spp. (33.33%) and *Staphylococcus* spp. (5%). Members of these genera have been reported by various researchers (Floodgate, 1984; Okpokwasili and Nnubia, 1995; Ollivier and Magot, 2005) as bacteria that utilize petroleum hydrocarbons as their source of carbon and energy. Kasai *et al.* (2002) isolated *Flavobacterium* spp. from oil polluted marine environment capable of degrading aromatic hydrocarbons in crude oil. In a related study, Edlund and Janson (2006) discovered that members of the class *Gammaproteobacteria* (*Pseudomonas* spp. inclusive) and *Flavobacterium* spp. were the most dominant bacteria in a highly PAH- and polychlorinated biphenyl- polluted sediment before and after dredging. Said *et al.* (2008) isolated *Bacillus, Staphylococcus, Pseudomonas* and *Acinetobacter* spp. capable of degrading PAHs from a polluted sediment. Members of the *Enterobacteriaceae* family were isolated from hydrocarbon-impacted marine sediment by Chikere *et al.* (2009b). The presence of *Alcaligenes* spp. and *Micrococcus* spp. corroborates the findings of Chikere et al. (2009a) who isolated *Alcaligenes* and *Micrococcus* spp. as degraders from crude oil-polluted soil. The predominance of *Pseudomonas* spp. (33.33%) can be explained on the basis that *Pseudomonas* spp. are well known hydrocarbon degraders (Kostka *et al.*, 2011; Korenblum *et al.*, 2012; Kigigha and Odayibo, 2014).

More isolates were obtained from anthracene spray (23.33%) protocol than anthracene spread (15%) protocol. This could be attributed to the solvent used in dissolving the anthracene in the spread plate method, since the solvent alcohol that was used is a solvent that dissolves the membrane of bacteria making it permeable for intracellular materials to leak out of the cell, it could have possibly led to the lyses of some of the cells and hence the reduced number of isolates (Prescott *et al.*, 2008). Also the low percentage of growth obtained in both anthracene spray and spray methods when compared with other

methods could be attributed to the fact that anthracene is a polyaromatic hydrocarbon containing 3 fused benzene rings with known angularity making it less bioavailable. Before a microorganism can start growing and multiplying; it must first expend energy for maintenance of viability, called maintenance energy. This energy is derived from the oxidation of organic compound (Atlas and Philp, 2005).

The high percentage of bacterial growth from the water samples which were collected from flow station discharge points is a reflection of the degree of contamination of the sites with crude oil. Since the points are constantly contaminated with crude oil, it could be indicative of the fact that these microorganisms metabolize hydrocarbons for their growth and survival (Atlas, 1981), and hence are adapted to that environment.

For the detection of dioxygenase activity, four isolates from water sample J obtained from Ahia flow station in Delta State and sediment sample E characterized as *Pseudomonas* spp. were found to possess the enzyme dioxygenases, this was detected from the observation of indigo which is the intermediate of indole metabolism in the presence of the dioxygenase enzyme via the meta cleavage pathway. Members of this genus have been reported by various researchers to possess the enzyme dioxygenases (Obayori and Salam, 2010; Thavamani *et al.*, 2012). It could be that these isolates tentatively shown to possess the enzyme using the indole production method probably have different classes of dioxygenases encoded in their chromosomal or plasmid DNA molecules (Mesarch *et al.*, 2000; Nakamura *et al.*, 2007; Marcos *et al.*, 2012).

Hydrocarbons, especially polyaromatic hydrocarbons which consist of two or more fused benezene rings, are widespread in the environment and persist over a long period of time, the removal of such pollutant is of importance because some are toxic, mutagenic and carcinogenic and therefore are hazardous to human health, and both terrestrial and aquatic ecosystems (Obayori and Salam, 2010). The detection of dioxygenase enzyme activity in some of the hydrocarbon utilizing bacteria is very important because the enzyme converts polyaromatic hydrocarbons into metabolic intermediates that are channeled through the meta-cleavage pathway to the tricarboxylic acid cycle and finally yielding carbon dioxide, energy, biomass and water (Iwai *et al.*, 2011). Effluents, sludges and spilled oil are PAH-laden and are usually discharged into vulnerable ecosystems. They persist in the environment and hence the presence of aromatic degraders in these hydrocarbon polluted sites could help to mitigate environmental pollution and holds good promise for bioremediation (Atlas and Philp, 2005; Thavamani *et al.*, 2012).

CONCLUSION

The findings from this research show that aliphatic and aromatic hydrocarbon utilizing bacteria in hydrocarbon impacted sites may probably possess diverse dioxygenase and other hydrocarbon degradative enzymes as detected in four *Pseudomonas* spp. isolated from hydrocarbon inundated samples. It is recommended that the use of culture independent techniques like nucleic acid-based methods such as 16S rRNA-based polymerase chain reaction (PCR), denaturing gradient/temperature gradient gel electrophoresis (DGGE/TGGE) and other microbial community profiling techniques such as high throughput next generation sequencing platforms should be adopted (Alquati et al. 2005; Chikere, 2013; Salipante *et al.*, 2014) in order to effectively monitor bioremediation and also develop field evidence in support of bioattenuation of hydrocarbon pollutants. It has been estimated that only less than 1% of

environmental isolates are culturable (Chikere *et al.*, 2011).

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