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Isolation and Characterization of Crude Oil Degrading Bacteria in Association with Microalgae in Saver Pit from Egbaoma Flow Station, Niger Delta, Nigeria

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Abstract

The capability of indigenous bacteria and microalgae in crude oil effluents to grow in and utilize crude oil as their sole source of carbon and energy provides an environmentally friendly and economical process for dealing with crude oil pollution and its inherent hazards. In view of the toxicity of crude oil spillages to indwellers of the affected ecosystems and the entire affected environment, the isolation of pure bacterial and microalgae cultures from crude effluents is a step in the right direction, particularly for bio-augmentation or bioremediation purposes. The total heterotrophic bacteria count and hydrocarbon utilizing bacteria count, as well as the microalgae count, were determined with the pour plate technique. The physicochemical properties of the effluent samples were also analyzed. Identification of the hydrocarbon utilizing bacteria was performed with phenotypic techniques. The result shows a mean total heterotrophic bacterium count of 5.91 log CFU/ml and a mean microalga count of 4.77 log cells/ml. When crude oil and polycyclic aromatic hydrocarbon (PAH) were used as sole carbon sources, total hydrocarbon utilizing bacteria counts were respectively estimated at 3.89 and 2.89 log CFU/ml. Phenotypic identification of hydrocarbon utilizing bacteria in the crude oil effluents revealed the presence of two main bacterial genera: *Streptococcus* and *Pseudomonas*. Data obtained from this study confirmed the biodegradative abilities of indigenous bacterial species, thus, ultimately resulting in the amelioration of the toxicity associated with the crude oil effluents.

1. Introduction

Petroleum hydrocarbons have undoubtedly emerged as significant environmental contaminants (Eriyamremu *et al.*, 2007; Liu *et al.*, 2010). Environmental pollution especially oil spills are major threats predominantly in the Niger Delta region of Nigeria, due to intensive exploration and other petroleum related legal and illegal activities resulting in several environmental hazards ranging from soil infertility, erosion of soil microbial diversity and total damage to both flora and fauna in the environment (Nweke and Okpokwasili, 2004; Head *et al.*, 2006; Emtiazi *et al.*, 2009; Mittal and Singh, 2009). Despite the immense economic benefit of crude oil, oil spills in soils have been traceable to various hazards in the environment and could cause several diseases like Kidney disease, probable destruction of the bone marrow and a risk factor in cancer due to the presence of harmful carcinogenic and mutagenic substances (Lichtfouse *et al.*, 1997; Lloyd *et al.*, 2001; Mishra *et al.*, 2001; Sing and Lin, 2008), and even death in lower animals (Phyllis *et al.*, 1989; Eriyamremu *et al.*, 2007). The high demand for petroleum products contributes to the increase in crude oil extraction, processing and large amounts of oily waste through various means. Crude oil is a complex mixture of

chemicals varying widely in their composition of hydrocarbon and hydrocarbon-like (polycyclic aromatic hydrocarbons; PAH) chemicals and may persist in the environment for prolonged periods, posing a major threat to ecosystems. Biodegradation by intrinsic microbial populations is one of the reliable tools through which a great deal of xenobiotic contaminants, comprising crude oil spill and effluents are gradually degraded and subsequently eradicated from the environment (Cappello *et al.*, 2007). The catabolic abilities of microorganisms such as fungi, bacteria and algae to degrade petroleum hydrocarbons had been previously reported (Wang *et al.*, 2011); these microorganisms possess specific enzyme systems that enable them to degrade and utilize hydrocarbons as their carbon and energy sources. The most important means of aerobic PAH biodegradation is the primary oxidation of the aromatic benzene ring through which molecular oxygen is incorporated by the dioxygenase enzymes to form *cis*-dihydrodiols. The intermediates of dihydrodiol dehydrogenation are metabolized to carbon dioxide and water through the catechols by the actions of catechol dioxygenases and other enzymes (Chikere *et al.*, 2011). However, one of the major limitations of (PAHs)

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biodegradation is the low bioavailability of the pollutants to the degrading microorganisms. The efficient disposal of crude oil waste has thus become a source of concern and has resulted in environmental pollution in the areas where crude oil exploration and exportation is predominant. Bioremediation has been used as a biological approach that involves the use of microorganisms' metabolic potential to degrade pollutants into innocuous compounds in the environment (**Hara et al., 2013; Singh and Chandra, 2014**). Most constituents of crude oil sludge are biodegradable, and the use of bioremediation techniques has proven to be economical, environmentally friendly and flexible (**Niti et al., 2013**). The present study deals with the isolation and characterization of bacteria that is capable of growing in and utilizing crude oil as their sole source of carbon and energy during compost bioremediation of crude oil effluents.

2. Material and methods

2.1 Sample Size and Collection

The sample site for this research was the Egbaoma flow station (Formerly Asuokpu/Umutu) located in the Northern Niger Delta of Delta State with coordinates 50 SS ON and 614' OE. Oil effluent samples were collected from the saver pit (crude oil effluent collection basin) into sterile plastic screw-capped bottles by dipping the bottles into the pit after which the bottle was corked and swabbed with cotton wool saturated with 95% ethanol. The samples were transported to the Microbiology Laboratory, Igbinedion University Okada, Edo State for analysis.

2.2 Enumeration of Total Heterotrophic Bacteria

The total heterotrophic bacteria count (HPC) were conducted according to protocols described in standard methods for the examination of water and waste water (**Baird et al., 2017**). The HPC was determined with the pour plate technique. One milliliter of the crude oil effluent sample was aseptically mixed with 9 ml of sterile distilled water and serially diluted up to 10^{-3} dilution. One milliliter of each of the dilutions was subsequently poured into sterile Petri dishes containing 20 ml sterile molten nutrient agar, swirled gently and allowed to solidify. The plates were incubated at room temperature for 72 hours and colonies on each plate enumerated.

2.3 Enumeration of Total Hydrocarbon Utilizing Bacteria

The total hydrocarbon utilizing bacteria was counted on minimal salts agar with the pour plate method according to the method of **Mills et al., 1978** as modified by **Okpokwasili and Odokuma (1990)**. Ten-fold serial dilutions were performed on the crude oil effluent. To isolate the hydrocarbon utilizing bacteria in the crude oil effluent, 1 ml of each dilution was mixed with 20 ml of sterile molten oil-agar medium (sterile minimal salts agar plus either sterile crude oil or PAH carbon sources) in a Petri dish. The Petri dish was incubated at 30 °C for 5 – 7 days. Colonies on the respective oil-agar plates were then counted and subsequently expressed as hydrocarbon utilizing bacteria per ml of the effluent. The bacterial colonies which developed on the plates were randomly picked and streaked onto sterile nutrient agar slants. The pure isolates in the slants were then kept in the refrigerator until characterization/identification of the isolates.

2.4 Characterization of Hydrocarbon Utilizing Bacteria

The isolates were identified using various morphological and biochemical tests as described by **Holt (1995)** Bergey's Manual of Determinative Bacteriology.

2.5 Enumeration of Total Heterotrophic Micro Algae

The crude oil effluent samples from the saver pit were filtered using a sterile muslin cloth so as to recover a concentrated amount of the micro algae. A surface sterilized spatula sterilized with 95 % ethanol was then used to transfer the algae from the cloth to 2.5 ml of sterile Allen medium and the mouth of the flask was plugged with sterile cotton wool (**Stein, 1980**). The flask was shaken vigorously for 30 seconds to allow the dispersal of algae in the medium as well as to discourage algae from setting at the base of the flask. The flask was then incubated for 7-14 days under an illumination provided by two (2) 2 ft. fluorescent tubes at room temperature (**Ajao and Fagade, 1990**). The cell was counted using a haemocytometer under $\times 40$ objectives, and calculated using the standard formula.

2.6 Purification of Micro Algae Culture

The algae culture was plated out on Allen medium using the pour plate method and incubated for 24 hours after which distinct colonies were picked using a sterile inoculating needle and transferred into Allen medium. Incubation was for 7-14 days under an illumination provided by two (2) 2 ft. fluorescent tubes at room temperature after which the cells were viewed under $\times 40$ objectives to ascertain purity (**Omoni and Abu, 2014**).

2.7 Characterization of Micro Algae

Pure algal isolates sub-cultured from the heterotrophic algal onto broth medium were examined based on their morphology and possible extracellular structures. A wet mount preparation was carried out and view under $\times 40$ objectives. Characterization was done by comparison with those documented in the identification guide of fresh-water and terrestrial algae (**John et al., 2003**).

2.8 Physicochemical Properties of the Effluent Sample

The physicochemical parameters assessed for the effluent samples include temperature, pH, and electrical conductivity, dissolved oxygen, turbidity, Polycyclic Aromatic Hydrocarbons (PAHs)-assessment, salinity and alkalinity and were determined using standard methods (**Baird et al., 2017**).

3. Results

The pH of the effluent was estimated at 7.2, while the pH of the PAH and crude oil carbon sources ranged from 7.3 to 7.5. Hence there was no significant difference ($P < 0.05$) in the pH of the effluent and the carbon sources examined in this study. While the nitrogen content of the effluent was significantly higher than the values reported in the PAH and crude oil carbon sources; the phosphorus content in the PAH and crude oil were much higher than the value reported in the crude oil effluent. PAH was the least turbid while the crude oil effluent was found to be most highly turbid fluid. The lead content of the crude oil effluent was significantly lower than those reported in the PAH and crude oil carbon sources. Other heavy metals such as zirconium and copper were found at lower levels in the effluent when compared to PAH and crude oil carbon sources.

Total heterotrophic bacteria and microalgae isolated from the saver pit in Egbaoma flow station in Delta State is presented in Table 2. The result shows a total mean heterotrophic bacterium count of 5.91 log CFU/ml and a mean microalga count of 4.77 log cells/ml.

Table 1. Some physicochemical parameters of the crude oil effluent, polycyclic aromatic hydrocarbon (PAH) and crude oil carbon sources

Parameter	Crude Oil Effluent	PAH	Crude Oil
pH	7.2±0.1	7.5±0.1	7.3±0.1
Moisture %	20.1±0.1	14.1±0.7	13.4±0.7
Organic matter %	2.19	1.95	2.25
N %	5.3±0.1	0.1±0.0	0.17±0.0
P %	0.01±0.00	1.24±0.03	1.16±0.0
Turbidity µS/cm	367.3	95.0	248.0
Pb (mg/L sample)	57.8	337.8	347.8
Zr (mg/L sample)	247	556	504
Cu (mg/L sample)	111.8	143.8	164.8

Legend: N- Nitrogen; P- Phosphorus; PAH-Polycyclic Aromatic Hydrocarbons; Pb-Lead; Zr-Zirconium; Cu-Copper; some values are presented as Mean ± Standard Deviation

Table 2. Total microbial counts of Heterotrophic bacteria and Microalgae from the saver pit in Egbaoma flow station Delta State

Sample	Heterotrophic bacteria count		Micro algae	
	Rep. Bacteria (log CFU/ml)	Mean count (log CFU/ml)	Rep. microalgae count (log cells/ml)	Mean count (log cells/ml)
1	5.90	5.91	4.76	4.77
2	5.94		4.87	
3	5.90		4.66	

Table 3. Hydrocarbon utilizing bacteria isolated from the saver pit using different hydrocarbon materials as sole carbon sources

Hydrocarbon used as sole carbon source	Hydrocarbon utilizing bacteria counts	
	Rep bacteria count (log CFU/ml)	Mean count (log CFU/ml)
Polycyclic aromatic hydrocarbon (PAH)	1. 2.90	2.89
	2. 2.87	
	3. 2.91	
Crude oil	1. 3.89	3.89
	2. 3.92	
	3. 3.85	

Table 4. Phenotypic characterization of hydrocarbon utilizing bacteria

Carbon Source	Isolates	Cultural Examinations	Gram Staining	Biochemical Examinations								Probable Organism
				Co	Ca	Ox	Ur	In	Mr	Vp	Ci	
Crude oil	1	Mucoid colony with entire margin	Gram positive cocci in chains	-	-	-	-	-	+	-	-	<i>Streptococcus</i> species
Crude oil	2	Greenish colony with entire margin	Gram negative rods	-	+	+	-	-	-	-	+	<i>Pseudomonas</i> species
PAH	3	Mucoid colony with entire margin	Gram positive cocci in chains	-	-	-	-	-	+	-	-	<i>Streptococcus</i> species

Legend: Co-coagulase test, Ca-catalase test, Ox-oxidase test, Ur-urease test, In-indole test, Mr-methyl red test, Vp-voges Proskauer test, Ci-citrate test

Table 3 represents the hydrocarbon utilizing bacteria fraction of the total heterotrophic bacteria isolated from the saver pit in Egbaoma flow station in Delta state. When crude oil and PAH were used as sole carbon sources, hydrocarbon utilizing bacteria counts were 3.89 and 2.89 log CFU/ml for crude oil and PAH respectively.

Table 4 represents the use of phenotypic methods to identify isolated hydrocarbon utilizing bacteria. Upon colonial examination, three bacterial isolates were examined. Two of these isolates were indicated as Gram positive cocci occurring in chains, while the other isolate was observed as Gram-negative rods. The three isolates were subjected to an array of biochemical tests. The results indicated that the two isolates belong to the *Streptococcus* genus, while the other isolate was identified as *Pseudomonas* species. In essence, two main hydrocarbon utilizing bacteria were isolated from the saver pit in Egbaoma flow station, Delta state.

4. Discussion

Hydrocarbon utilizing bacteria exhibit extreme diversity in nature and can readily adapt to survive in unsuitable environments (Sohal and Srivastava, 1994). Previous studies (Survery et al., 2004; Chaillan et al., 2004; Li et al., 2005; Sathishkumar et al., 2008) have implicated some microbes such as *Achromobacter*, *Bacillus*, *Corynebacterium*, *Escherichia*, *Micrococcus*, *Vibrio* and *Pseudomonas* species as hydrocarbon utilizing bacteria. In this research, indigenous hydrocarbon utilizing bacteria were isolated from crude oil effluents collected from the saver pit in Egbaoma flow station in Delta state. Mean total heterotrophic bacteria count was estimated at 5.91 log CFU/ml (Table 2). From the pool of the total heterotrophic bacteria count, mean hydrocarbon utilizing bacteria counts of 3.89 and 2.89 log CFU/ml were respectively reported for crude oil and PAH carbon sources (Table 3). The hydrocarbon utilizing

bacteria that were isolated belong to the *Streptococcus* and *Pseudomonas* genera (Table 4). *Pseudomonas* species have been frequently detected by several authors in various hydrocarbon-contaminated environments (Li *et al.*, 2005; Sathishkumar *et al.*, 2008). Mean microalgae count was estimated at 4.77 g cells/ml (Table 2). This result was in line with the findings of Tan and Ji (2010) that these classes of bacteria and microalgae possess the ability to use the nitrogen-sulphur-oxygen (NSO) fractions of crude oil effluents as their sources of nitrogen, carbon and energy. This result is comparable to the findings of some studies (Hara *et al.* 2013; Molina *et al.* 2009; Mishra *et al.* 2014) that confirmed some organism including the ones isolated in this study to have the abilities to use crude oil effluents for their carbon and energy requirements.

5. Conclusion

The data from this study supports past research findings that indigenous bacteria and microalgae grow in and utilize crude oil effluents as their carbon source for energy. Our findings have indicated that the toxicity of crude oil effluents to the environment could be ameliorated by the biodegradative activities of indigenous microbes in the crude oil effluents. Data obtained from this study specifically confirmed the biodegradative abilities of *Streptococcus* and *Pseudomonas* species, their ubiquity in hydrocarbon polluted environments and their potential for bioremediation of hydrocarbon-polluted sites. Further work to determine the optimum environmental conditions favorable for their application in bioremediation is hereby suggested.

Declaration of interest

The authors report no conflicts of interest.

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