Effect of Nitrogen Sources on Crude Oil Utilization by Mangrove Bacterial Strains

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Abstract: Batch experiment was conducted to investigate the effect of nitrogen source on biodegradation of crude oil by hydrocarbon utilizing bacteria isolated from Eagle Island mangrove ecosystem of Niger Delta, Nigeria. The organisms were identified as *Pseudomonas* sp. MW3 and *Bacillus* sp. SD4. The ability of the organisms to solubilize crude oil was indicated by having emulsification index values of 62.1, 58.7, 60.6 and 61.2 % for crude oil, diesel, petrol and kerosene by *Pseudomonas* sp. MW3 and 61.4, 58.8, 59.4 and 60.3 % for crude oil, diesel, petrol and kerosene by *Bacillus* sp. SD4) respectively. After 15 days of incubation, the maximum total petroleum hydrocarbon (TPH) removal observed was 90.3 % regardless of treatments applied. Bushnell-Haas (BH) medium amended with urea showed 88.1 and 90.3 % TPH removal in both *Pseudomonas* sp. MW3 and *Bacillus* sp. SD4 cultures respectively. The lowest TPH removal (43.1 %) was observed in culture medium of *Bacillus* sp. SD4 supplemented with potassium nitrate. The high TPH removal in medium supplemented with implies that urea is an appropriate nitrogen source during bioremediation of crude oil contaminated sites.

Keywords: Bacteria, Mangrove, Nitrogen sources, Crude oil, Utilization

Introduction:

One of the growing threats to the good health of our environments is contamination by crude oil and its products. This is mainly due to oil spills that occur during discharge from the refineries, accidents of ships/tankers, their grounding, rupture on seabed and on shore pipelines, offshore oil production and exploration platforms (Subathra *et al.*, 2013). Environmental contamination with crude oil and crude oil products has caused critical environmental and health defects. This is not just because of their contact toxicity but because the amount of crude oil introduced into the environment is on the increase and this influences the oxygen concentration in the water and sediment resulting in an anaerobic condition which is highly detrimental to aquatic lives.

In Nigeria, oil activities in terms of exploration, exploitation and mining are known to take in the coastal zones of the country which is referred to as the Niger Delta. The Niger Delta is mainly associated with mangrove ecosystem. The mangrove ecosystems differ in terms of their physical, chemical, and biological characteristics (Pinto *et al.*, 2015). Mangrove swamps are particularly unique as they serve as ecotones between land and sea environments along tropical and subtropical latitudes. The mangrove plants are salt tolerant species that grow on sheltered shores in the tropics and sub-tropical estuaries where they provide ecosystem functions and several human utility benefits especially for coastal communities where it exist. Their halophytic nature and ability to compensate for low oxygen in the soil allows them to flourish in the environment (Chindah *et al.*, 2007). Despite their great ecological and economic importance, mangroves are often situated in areas of high anthropogenic influence, being exposed to pollutants, such as those released by oil spills (dos Santos *et al.*, 2011). The mangrove ecosystems of the Niger Delta are polluted not only by crude oil exploration but also by different human activities which include untreated or improperly purified organic effluents, as well as industrial pollutants such as hydrocarbons and heavy metals, all of which negatively impact the environment and its local biota. The function of mangrove root system is primarily affected by crude oil pollution as the surface of the plant's organ functioning in the exchange of carbon dioxide (CO_2) and oxygen is covered by oil residue hence lowered the oxygen levels by 1-2% within 48 hours (Gofar. 2011).

Crude oil is a complex mixture of many petroleum hydrocarbons such as alkanes, aromatics, resins and asphaltenes associated with other organic compounds containing sulfur, nitrogen and oxygen. Among petroleum hydrocarbons, aromatic compounds constitute a major fraction of the hydrocarbon containing 30 polyaromatic hydrocarbons (PAHs) (Kumaria *et al.*, 2012).

Microorganisms being ubiquitous are responsible for the maintenance of productivity, conservation and recovery of mangroves. They are directly involved in the transformation of nutrients, photosynthesis, nitrogen fixation, methanogenesis, phosphate solubility, sulfate reduction and production of other substances, including antibiotics and enzymes and are reservoirs of products of biotechnological interest as, for example, bacteria that produce bioemulsifiers. However, these microbial processes are affected by crude oil toxicity as a result of pollution of mangrove ecosystem by hydrocarbons (dos Santos *et al.*, 2011). Despite the effects of crude oil toxicity on the microbial processes, there are some of these microbial species such as *Bacillus*, *Pseudomonas*, *Corynebacterium*, *Rhodococcus* as well as *Aspergillus*, *Fusarium*, *Penicillium*, *Articulosporium* species (Snape *et al.*, 2001; Al-Jawhari, 2014) that utilize crude oil as sole sources of carbon and energy. The rate of

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utilization of crude oil by a microbial population is a complex process which depends on its nature and concentration present as well as other number of environmental factors which include pH, temperature, oxygen and nutrients availability (Sathishkumar *et al.*, 2008).

The utilization of crude oil by microbial cells is a slow process but is a means of restoring polluted sites to their state. The growth and proliferation of microbial cells during crude oil utilization is greatly influenced by the availability of nutrients (Santos *et al.*, 2011) especially nitrogen which may become limiting factor thus affecting crude oil degradation. The type and nature of nitrogen sources may greatly influence the utilization of crude oil by the organisms. Nitrogen is very essential in microbial metabolism of organic carbon containing compounds and it is necessary in biosynthesis of protein and nucleic acids. Media rich in nitrogen has good metabolic activity and good microbial biomass (Acuna *et al.*, 2012)

The aim of the present study was to assess the effect of different nitrogen sources on crude oil utilization by bacterial strains isolated from Eagle Island mangrove ecosystem in Port Harcourt, Rivers State Nigeria.

Materials and Methods

Sample collection:

Composite samples of Eagle Island mangrove water and sediments were collected in polyethylene containers and were transported in icebox to the laboratory for microbiological analyses within 6 h of sampling.

Isolation and identification of organism

The heterotrophic and crude oil utilizing bacterial species of the samples were isolated by spread plate technique by transferring one tenth (0.1 ml) of decimally diluted sample water onto nutrient agar (NA) and mineral salt agar respectively (Mills *et al.*, 1978). For crude oil utilizing bacteria, the crude oil source was supplied through the vapour phase by placing filter papers (Whatman No.1) impregnated with 3 ml of filter-sterilized Bonny-Light crude oil on the lids of the plates. The plates were incubated at 30^{O}C for 48 and 168 h respectively. Isolates that developed on the mineral salt medium were screened by subculturing them on nutrient agar (NA) and Bushnell-Haas agar (BH) media overlaid with 0.1 ml of crude oil. The culture media were incubated at 30^{0}C for 5 and 14 days respectively (Subathra *et al.*, 2013). Any isolate that grew on NA-crude oil plates but failed to grow on BHA-crude oil plate were confirmed as non-degraders. The isolates which grew on both the agar-crude oil plates were confirmed as hydrocarbon degraders. The diameter of zone of clearance around the colonies of the isolates grown on BHA plate was measured. Two isolates with largest zone of clearance were selected and tentatively characterized using a battery of biochemical tests. The isolates were identified to the generic level following Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994) and stored at 4^{0}C on nutrient agar slants for further studies.

Preparation of Inoculum

The inoculum of crude utilizing bacterial strains were prepared by growing the isolates in 100ml of sterile nutrient broth media (HIMEDIA) contained in 250 ml Erlenmeyer flasks. The flasks were incubated on a rotary shaker incubator operating at 150 rpm for 48 h at room temperature (28 ± 2^{O}C). Cells were harvested by centrifugation at 6000 rpm for 10 minutes. Harvested cells were washed twice in sterile phosphate buffered saline (PBS, 0.02M). The washed cells were resuspended in the same medium and the turbidity adjusted spectrophotometrically to give an optical density of 0.5 at 540nm. The inoculum was used throughout unless otherwise stated.

Screening for utilization of hydrocarbon substrates

The screening assay for petroleum products utilization by the test isolates was done as described by Resnick and Chapman (1994) with little modification. Petroleum substrate utilization by the test organisms was determined in sterile Bushnell-Haas (BH) broth medium (9.9 ml in 20 ml culture test tubes) supplemented with various petroleum products [0.1 % (v/v)] such as crude oil, Lubricating oil, Diesel, petrol, kerosene and hexane. Others included Hexane, Toluene and phenol. This was followed by inoculation of the medium with 0.1 ml of bacterial cell suspensions (OD_{540nm} 0.1). Controls consisted of inoculated media devoid of petroleum products (positive) as well as with petroleum products but without inoculum addition (negative). The tubes were incubated at 28^{O}C for a period of 14 days. Development of turbid culture was indicative of utilization of the petroleum hydrocarbons as carbon source. Cultures without increase in turbidity over positive and negative control were visually scored.

Emulsification measurement

Emulsifier activity was measured as described by Cooper and Goldenberg (1987) with little modification. This was carried out by adding 4 ml of hydrocarbon compounds of crude oil, diesel, petrol and kerosene to equal volume of aqueous cell-free broth sample contained in 15 ml screw capped culture glass test tube and vortexing at high speed for 120 s. The mixture was left undisturbed for 24 h at room temperature (28 ± 2)^{0}C to allow for the formation of biphasic layers. Thereafter, measurements of the heights of total of the mixtures and emulsified layers were made. The emulsion index (E_{24}) was estimated using the expression:

$$Emulsion\ index,\ E_{24} = \frac{Height\ of\ emulsion\ layer}{Total\ height\ of\ Mixture} \times 100$$

Culture medium and biodegradation assay

This was done by adopting the method of Fathabadi *et al.*, (2011). Mineral salt medium supplemented with 1% (w/v) of crude oil was prepared as per the formula of Bushnell and Haas (BH) (1941) with a substitution of Ammonium nitrate (NH_4NO_3) with NH_4Cl, KNO_3 or Urea as nitrogen sources. The media was sterilized for 15mins at 121°C by autoclaving and on cooling at ambient temperature were inoculated with the standardized cultures. The cultures were incubated at 30°C and were agitated daily throughout the duration of incubation (Regina *et al.*, 2006; Omotayo *et al.*, 2012). Medium containing 1% (w/v) of the crude oil and was given same treatment but without test organisms served as control. Cell biomass of the culture was estimated by spectrometric technique at 540 nm while uninoculated medium sample served as blank. The amount of crude oil utilized by the isolates and residual oil in the control were estimated at every 5 days time interval using cell free cultures sample prepared by removal of cells via centrifugation (700 rpm). 3.0 ml of the cell free culture was mixed with equal volume of hexane and thereafter vortexed thoroughly for 60 sec and the extracted crude oil detected spectrophotometrically at 340 nm. This was then compared with standard crude oil calibration curve and the percentage (%) degradation was calculated using the expression (John and Okpokwasili, 2012).

$$\%\ Degradation = \left[\frac{(a-b)}{a}\right] \times 100$$

Where, a and b are the residual oil in the control and test sample respectively.

Results and discussion

The total microbial load of the water and sediment samples collected from Eagle Island mangrove ecosystem of Niger Delta of Nigeria are as shown in Table 1. The total aerobic heterotrophic bacterial densities for both water and sediment samples are 2.65 x 10^6 CFU/ml and 2.83 x 10^6 CFU/g. Also, the total hydrocarbon utilizing bacterial count in both samples is 1.62 x 10^6 CFU/ml and 1.88 x 10^6 CFU/g respectively. Lakshmipriya and Sivakumar (2012) had reported a total aerobic heterotrophic bacteria count of 2.84 x 10^9 CFU/g from rhizosphere sediment of Pitchavaram mangrove environment of India while Sousa *et al.*, (2006) reported aerobic heterotrophic bacterial count in the Log CFU of 4.04 (1.09 x 10[illegible] CFU/ml) obtained in the mangrove water of Ceara in Brazil. These values of aerobic heterotrophic bacterial count are in the same range for the result obtained in this study. Also, similar total hydrocarbon utilization bacterial count (1.52 x 10^6 CFU/g) was obtained by Antai *et al.*, (2014) in their study of heterotrophic and crude oil utilizing microorganisms of Imo River estuary of the Niger Delta mangrove ecosystem. The organisms that exhibited the largest diameter of zone of clearance around their colonies were identified as *Pseudomonas* sp. MW3 and *Bacillus* sp. SD4 respectively. These organisms have been reported to be the most predominant bacterial species in the mangrove environments and known to be hydrocarbon compounds degraders (Antai *et al.*, 2014; Bharathkumar *et al.*, 2008).

Table 1: Total microbial load of water and sediment samples of Eagle Island mangrove ecosystem

Sample/unit	Total aerobic heterotrophic bacterial count (x 10^6)	Total hydrocarbon-utilizing bacterial count (x 10^6)
Water (CFU/ml)	2.34	1.62
Sediment (CFU/g)	2.83	1.88

Different petroleum hydrocarbon substrates utilization by the test organisms are as depicted in Table 2. The results obtained showed that the isolates could utilize crude oil and its petrochemical products, such as lubricating oil, kerosene and phenol for growth. This is in corroboration with the reports of Ezekoye *et al.* (2015) and John and Okpokwasili (2012) that mangrove bacteria was an excellent degrader of crude oil and had ability to utilize crude oil and its products. Though, both organisms could not show any growth in Hexane and Toluene. This showed the devastating effects of these two products on the organisms. *Bacillus* sp. SD4 showed better growth in most of the hydrocarbon substrates than Pseudomonas sp. MW3.

Table 2: Screening test for the utilization of hydrocarbon substrates by the bacterial isolates

	Organism	
Hydrocarbon substrate	*Pseudomonas* sp. MW3	*Bacillus* sp. SD4
Crude oil	++	++
Lubricating oil	+	+
Diesel	-	++
Petrol	-	+
Kerosene	+	+
Hexane	-	-
Toluene	-	-
Phenol	+	+

Key: +++ = Heavy growth, ++ = Moderate growth, + = growth, - = No growth

The diameter of zone of clearance around the bacterial colonies (3.4 and 3.1 mm for *Pseudomonas* sp. MW3 and *Bacillus* sp. SD4) and emulsification activities on various hydrocarbons (62.1, 58.7, 60.6 and 61.2 % for crude oil, diesel, petrol and kerosene by *Pseudomonas* sp. MW3 and 61.4, 58.8, 59.4 and 60.3 % for crude oil, diesel, petrol and kerosene by *Bacillus* sp. SD4) respectively as shown in Table 3. The results obtained in the study indicated that the cells have strong affinity for the crude oil and its other products hence able to produce extracellullar biosurfactant capable of emulsifying these products. These are the mechanisms used by microorgnisms to take up substrates with low water solubility (Nweke and Okpokwasili, 2003).

Table 3: Diameter of zone of clearance on BHA and emulsification activity of the isolates

	Zone of clearance	Emulsification index (%)			
		Hydrocarbon compound			
Organism	(mm)	Crude oil	Diesel	Petrol	Kerosene
Pseudomonas sp. MW3	3.4	62.1	58.7	60.6	61.2
Bacillus sp. SD4	3.1	61.4	58.8	59.4	60.3

Figure 1 showed the crude oil reduction and growth profile of the test organisms in response to BH medium supplemented with different nitrogen sources in 15 days of incubation. Though, similar incubation period was employed by Dibble and Barth (1976) to assess the effect iron on the biodegradation of petroleum in sea water. There was progressive increase in the percentage of biodegradation of crude oil in all the nitrogen sources amended medium of the isolate during the incubation period except in the control that showed no increase in percentage of biodegradation of crude oil. The percentage of crude oil degradation was high (88.1 and 90.3 %) in *Pseudomonas* sp. MW3 and *Bacillus* sp. SD4 culture BH medium supplemented with urea as nitrogen sources respectively. The least of percentage of crude oil degraded was obtained in both culture media amended with potassium nitrate after the incubation period. The percentage of biodegradation of crude oil in medium amended with various nitrogen sources were in the following order: Urea (88.1 %) > ammonium chloride (74.5 %) > potassium nitrate (43.1 %) for *Pseudomonas* sp. MW3 and Urea (90.3 %) > ammonium chloride (80.1 %) > potassium nitrate (46.1 %) for *Bacillus* sp. SD4 after 15 days of incubation respectively. The results obtained in this study is in corroboration with results obtained by Chekroud *et al.*, (2011) who reported that 60 % of crude oil in marine medium was degraded by *Pseudomonas* species and *Rhodococcus* species in the presence of urea employed as sources of nitrogen after 15 days of incubation. Also, Sharma and Pant (2000) obtained similar result of 15 % reduction of crude oil after 15 days of incubation in the presence of urea. The growth profiles with time for *Pseudomonas* sp. MW3 and *Bacillus* sp. SD4 during crude oil reduction in BH medium supplemented with different nitrogen sources are as depicted in Figure 1.

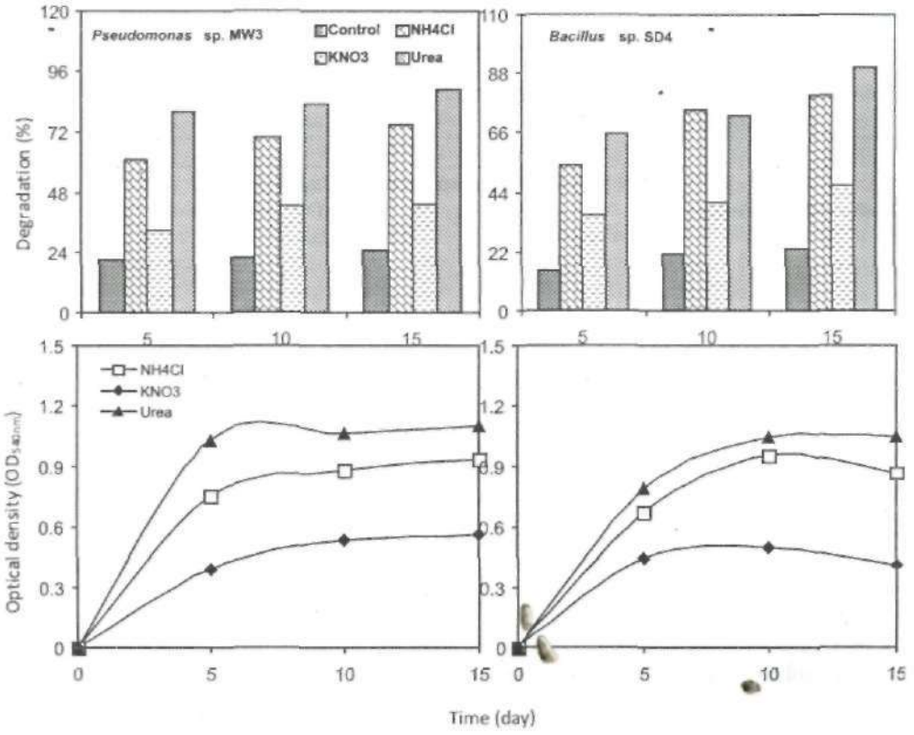

Figure 1: Crude oil reduction by hydrocarbon utilizing bacterial strains and their growth profile in biodegradation of crude oil in BH medium

The growth profile of the test organisms expressed as optical density (OD_{540nm}) in medium amended with respective sources followed same pattern of results obtained in crude oil reduction of respective nitrogen sources. This indicated that there was maximal utilization of the crude oil hence growth rate of the organisms. The highest and lowest growth rate of the organisms measured as optical density was observed in the media supplemented with urea and potassium nitrate in both cultures.

In conclusion, this study demonstrated that urea as organic nutrient would stimulate more rate and extent of biodegradation of crude oil in any media hence a contributing factor in removal of total petroleum hydrocarbon in polluted sites.

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