Environmental Impact of Aerophilic Organisms on Bitumen Biodegradation

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Abstract- Biodegradation of Bitumen by aerophilic organisms was carried out by inoculating the bitumen sample on nutrient agar plate. The sample was divided into six as five parts were cultured aerobically while a part was cultured anaerobically using anaerobic jar. The isolation, characterization and identification of the microbial isolate revealed that five bacterial general: such as *Pseudomonas aeroginosa, Bacillus subtilis, Bacillus cereus, Aerobacter aerogenes and Aerobacter cecacac* are grown in each plate after 24 hours and recorded as positive (+). Conversely, characteristic of isolate was also investigated such as colour, shape, cell shape, spore shape and gram reaction, biochemical test was also carried out on each bacterial isolate such as motility test, coagulase test, indole and catalase test. However, result shows that sample A has (brown), B (white), C (white to yellow), D (thick white) and E (white spreading) colour, other morphological characteristics like shape and gram staining reaction are indicated as positive(+) and negative (-) respectively. The ability of isolated organisms to degrade bitumen was investigated, and the pH was also determined. It was established that aerophilic organisms were capable of degrading bitumen.

Index Terms- Biodegradation, bitumen, aerophilic organisms, isolate

I. INTRODUCTION

Petroleum is a complex mixture of hydrocarbons derived from the geologic transformation and decomposition of plants and animals that lived hundreds of millions of years ago. As a technical term, *petroleum* encompasses the liquid (crude oil), gaseous (natural gas), and viscous or solid (bitumen asphalt) forms of hydrocarbons that occur in the Earth, but the meaning is often restricted to the liquid oil form. Heavy oil and tar sand oil (bitumen) are petroleum hydrocarbons found in sedimentary rocks. Bitumen was discovered in Nigeria over a hundred years ago, preceding the discovery of oil by over 50 years. The Nigerian bitumen belt lies on the onshore areas of Eastern Dahomey (Benin) basin. Nigeria has a proven reserve of 42.47 billion metric tonnes, the second largest in the world, covering about 120 × 4.3 km (Oboh *et al.*, 2006). This is spread along the bitumen belt stretching from Lagos, Ogun and to Ondo and Edo states. Five distinct hydrocarbon types of occurrence have been identified within the tar sands belt: outcrop, rich sands, lean sands, shale and deep seated heavy crude. They are formed by the oxidation and biodegradation of crude oil, and occur in the liquid or semiliquid state in limestones, sandstones, or sands Bitumen is an organic material that surrounds tar sand. They are so highly viscous liquid and clings to the sand stone that is easy to remove from the mixture as a solid form of petroleum yet it is a mixture of high density liquids on its supporting solid (Speight, 1983). Bitumen mixed with mineral matter is defined as asphalt. Bitumen is black-brown in colour and almost completely soluble in solvents such as benzene, trichloroethylene and carbon disulphide. Bitumen is highly viscous liquid or sold at normal temperatures and exhibits thermoplastic behaviors softening when heated, becoming mobile liquids on further heating and returning to their original state on cooling. Bitumen is about 20% of the actual oil sands found in Nigeria. The remaining 76% is mineral matter including clay and sands and around 4% of water (Hobson, 1994).On average, bitumen is composed of carbon – 83.2%, hydrogen-10.4%, oxygen-0.94%, Nitrogen-0.36%, iron and vanadium-0.3% (Oguntimehin & Ipinmoroti, 2007). Concern about polycyclic aromatic hydrocarbons initially focused on their ability to cause cancer, but more recently concern has turned to their interference with hormone systems and their potential effects on reproduction, as well as their ability to depress immune function (Adewole, 2010). A particular concern is the effect of polycyclic aromatic hydrocarbons on egg production in fish, and their potential effects on the numerous early life stages that reside in the surface micro-layer of the oceans, where polycyclic aromatic hydrocarbons can become concentrated. Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous pollutants in the environment and are of major concern due to their well known carcinogenic and mutagenic properties (Kameda et al., 2005). Nutrient availability, especially of nitrogen and phosphorus seem to be the most limiting factor in petroleum biodegradation (Oloke *et al.*, 2009). It was confirmed that these nutrients enhance growth of microorganisms, which leads to more rapid decomposition of petroleum contaminants (Bramble et al., 1991; Chaineau et al., 2005). The microbial degradation of crude oil has become important as a result of massive oil pollution incidents. Numerous strains of bacteria, yeast, actinomyetes and filamentous fungi have been reported to degrade and mineralized crude oil (Atlas and Crawford 1981), and however, the impact of these organisms have not been established. This study is therefore designed to investigate the impact of aerophilic organisms on bitumen degradation.

II. MATERIALS AND METHODS

Source and collection of samples: Bitumen samples was collected from Agbabu village in the well at the back of Oba palace which refers to as closed sample and another one was

taken from the well of about 100m away which refers to as open sample. Agbabu village is within odigbo local government area of Ondo State Nigeria. Agbabu is a village of about 400 inhabitants at the south-western part of Nigeria in the coordinates of $E004^{0}48\text{-}49^{1}$ and $N06^{0}34\text{-}36^{1}$. This is where bitumen was first spotted in Nigeria in 1910 and the first bitumen well NBC-7 was drilled there.

Sterilization of glassware: The glass wares were washed with detergent and then rinsed with distilled water. They were then sterilized at 160^{0}C for 1 hour.

Preparation of media: All media were prepared from commercially available products (nutrient agar, nutrient broth and peptone water) and made according to manufacturer's instruction. They were sterilized by autoclaving at 121^{0}C for 15mins.

Characterization and identification of isolates: This was done based on morphological culture and biochemical characteristics. Gram reaction, catalase test, sugar fermentation test and motility test. Pure cultures were obtained by carrying out serial dilution, inoculation and incubation at 45^{0}C for 14 days and pure colonies were isolated after organisms were sub-cultured (Bergey et al., 1981).

Spore Staining: A smear is prepared on a slide; this was then flooded with malachite green stain and heated over a beaker of boiling water for 10 minutes. It was then washed off with water and flooded with safraline for 20 seconds and finally washed with water and blotted dry. This was observed under the oil immersion lens. The spores stained green while the vegetative cells stained pink to red.

Biochemical tests: The various biochemical tests were carried on each of the bacteria isolate to help in characterization by making sure that the drop of culture was within the ring. The slide with the cover slip was reinverted and examined under the microscope using X40 objective.

Sugar fermentation test: The sugars used were glucose, lactose, maltose, sucrose and arabinose. The sugars were sterilized by authoclaving at 110^{0}C for 15mins. The medium used was peptone water.

pH determination: This was carried out using pH meter which has been earlier standardized with buffers 4 and 9.

Catalase test: From the subcultures organism in a Mac Cartney bottle, a pinch of each organism was taken and placed on a slide and few drops of 8% hydrogen peroxide (H_2O_2) solution was added.

Indole test: The test organism was inoculated into sterile peptone water and incubated at 28^{0}C for 24hrs. After incubation 0.3-0.5ml of kovac's reagent was added to the culture and the tubes were shaken and allowed to stand for a minute or two. A dark red colour formation in the tube indicates a positive test, while an orange coloration showed a delayed positive test.

Coagulase test: This test was used to identify *staphylococcus aureus* which produces the enzymes coagulase that causes plasma to cloth by converting fibrinogen to fibrin.

Motility test: The hanging drop method was used; Vaseline was placed round the edge of the depression of a cavity slide. A loopful of the isolate was place on the centre of a clean dry cover slip. The slide was then inverted on the cover slip.

Determination of hydrocarbon degrading potential of microbial isolates: 100ml portion of sterile water was added to 3g of nutrient broth and sterilize. The nutrient serves as the control culture while the other one which was inoculated with microorganism and the original sample served as the degraded culture. Both culture was incubated for 24hrs, recorded as day 1 and studied weekly for 28days.

III. RESULTS AND DISCUSSION

Table 1: Growth of Bacteria on Nutrient Agar Plates

Samples	Growth of nutrient agar in plates
A_1	+
A_2	+
A_3	+
A_4	+
A_5	+
A_6	-

(+) = Positive (-) = Negative

A1 – Aerobic closed sample – (culture- incubated aerobically) A2 – Aerobic closed Sample – (incubated aerobically)
A3 – Aerobic open sample – (incubated Aerobically) A4 – Aerobic closed Sample – (incubated aerobically)
A5 – Anaerobic closed Sample – (incubated anaerobically) A6 – Anaerobic open Sample – (incubated anaerobically)

Table 1 shows the growth of bacterial in plates, all the samples were grown rapidly when cultured and it was indicated as (+) positive as obtained at 24hrs, only sample A6 – anaerobic open sample incubated anaerobically was (-) negative.

Table 2: Characteristics of Bacteria Isolates in Sugar Fermentation

Isolates Identity	Maltose	Lactose	Manitol	Arabinose	Sucrose	Fructose
Pseudomonas aeroginosa	-	-	-	-	-	-
Bacillus subtilis	+	-	+	+	+	+
Bacillus cereus	+	-	-	+	+	+
Aerobacter aerogenes Aerobacter cecacac	+	+	+	+	+	+
	+	+	+	+	+	+

(+) = Positive (-) = Negative

Table 2 reveals the results of sugar fermentation (arabinose, mannitol, sucrose, fructose, maltose and lactose) of each bacteria isolate in which sample A was negative in all sugars, isolate B are all positive except in lactose, isolate C lactose and mannitol are negative and the rest of sugar are positive, isolate D and E are all positive. The positive are indicated by colour change while negative are those that did not change.

The genetic ability of the isolate microorganisms identified in this study is in line with what was reported by Horowitz *et. al*, 1985 to be responsible for the different response to the degradation of crude oil in the culture medium.

Table 3: Characteristics of Bacteria Isolates Cellular Morphology and Biochemical Reactions

COLONIAL AND CELLULAR MORPHOLOGY						BIOCHEMICAL TEST			
Isolates	Colour on Agar Slant	Shape	Cell shape	Spore stain	Gram reaction	Motility	Coagulase	Indole	Catalase
A	Brown	Round	Short rods	-	-	+	+	+	+
B	White spreading	Round	Long rods	+	+	+	+	-	+
C	White to yellow	Round	Long rods	+	+	+	+	-	+
D	Thick white	Round	short rods	-	-	+	+	-	+
E	White spreading	Round	Short rods	-	-	+	+	-	+

A = Pseudomonas aeroginosa B = Bacillus subtilis C = Bacillus cereus D = Aerobacter aerogenes E = *Aerobacter cecacac*

The results of characteristics of isolate in each sample such as colour, shape, cell shape, spire shape and gram reaction are shown in table 3, it also contained the results of biochemical test of each bacteria isolate such as motility test, coagulase test, indole and catalase test. The samples colour are A (brown) B (white) C (white to yellow) D (thick white) and E (white spreading) and all the cells has rounded shape, other cellular morphology was the cell shape where sample A, D and E are short rod while B and C are long rod. There spore and gram staining reactions are the same in that sample A, D and E which shows pink colour indicate (-) gram negative while B and D shows purple colour indicated as (+) gram positive. In biochemical test, the results of each test carried on each sample was indicated by positive (+) and negative (-) for motility test all samples are positive as it was observed under electron microscope, coagulase test which was carried out by putting a drop of distilled water on each of the sample on a slide, it was observed that all organisms were clumped after 10 seconds, thus , indicate positive (+), on indole test, only sample A showed positive (+) which indicate by formation of dark red colour and the rest were negative (-) and lastly is the catalase test which was carried out by hydrogen peroxide method indicate that all samples are bubbles shows positive (+) reaction. This indicates that 60% of the isolates from samples were gram-negative and this was in line with the percentage reported by Benka-coker *et. al*, (1995) for gram-ve bacterial isolated from waste drilling fliud in a tropical mangrove swamp oil field location. Atlas, 1984, and Obire, 1988 also reported that most of the bacteria floras of hydrocarbon sources are gram-negative.

Table 4: Hydrocarbon Degrading Potential of Bacteria Isolates (cfu/g) in *28days*

Bacteria Isolates	***Pseudomonas aerogenosa***		***Bacillus sustilies***		***Bacillus cereus***		***Aerobacter aerogenes***		***Aerobacter cecacac***	
Samples / Days	Open	Close	Open	Close	Open	Close	Open	Close	Open	Close
1	3.5	2.5	3.8	3.4	1.2	1	3	2.7	3.1	2.8
7	7.5	4.8	6.5	5.6	2.7	1.5	5.5	4	6.4	5.6
14	13	9.2	11.3	10.0	5.8	3.6	8.5	7	13.5	10.2
21	18.5	13.5	13.2	12.1	7	5.3	12	10.5	14	12
28	19.7	17	18.5	15.5	9.2	8	16.1	14.0	15.5	13.7

Table 4 shows the biodegradation activities of each bacterial Isolate within the period of 28days. It shows the degradation profile of *Pseudomonas aerogenosa* in close and open samples which ranged from 3.5 – 19.7 in open and 2.5 – 17.0 (cfu/g) in closed sample whereas the pH decreased from 6.4 – 3.7 indicates that acidity increased, the profile of *Bacillus sustilies* where the total viable count ranged from 3.8 – 18.5 in open and closed 3.4 – 15.5 (cfu/g) with pH of 6.3 - 3.5, while profile of *Bacillus cereus* recorded total viable count range of 1.2 – 9.2 in open and 1.0 – 8.0 (cfu/g) in closed sample with pH of 6.5 – 2.8 conversely the table also show profile of *Aerobacter aerogenes* where total viable count ranges from 3.0 - 16.1 in open and 2.7 – 14.0 (cfu/g) in closed sample while pH range was 6.9 – 3.3 and lastly is the profile of *Aerobacter cecacac* where total viable count ranges from 3.1 – 15.5 in open and 2.8 – 13.7(cfu/g) in closed sample the pH range was 5.9 – 2.8 respectively. This implies that hydrocarbon degradation increases as the days increases while the pH decreases and that the activities was faster and higher in open samples than the closed samples which could be as a result of the presence of oxygen low molecular weight hydrocarbons. Hamme et al. (2003), Ghazali et al. (2004) observed that low molecular weight hydrocarbons are degraded most rapidly, or converted to some high molecular weight and more valuable compounds, when bacteria were made to grow on them. Some high molecular weight and more valuable of hydrocarbons compounds such as ethers, esters, lactones and silicon compound were formed after the biodegradation of bitumen. Hydrocarbon-degrading microorganisms act mainly at the oil-water interface and have been microscopically observed growing over the entire surface of an oil droplet Amund, 1984. The pH values obtained from the bitumen samples used in this study were within the normal range for the proliferation of bacteria and crude oil utilizing potential of the organisms was in line with what was demonstrated by (Ijah, *et. al*, 1988 and Amund, 1984).

IV. CONCLUSSION

The characterization of the bacteria isolates from Agbabu bitumen samples showed that two of the five genera were gram-positive while the remaining three general were gram-negative which indicate that 60% of the isolates from samples were gram-negative. The pH values obtained from the bitumen samples used in this study were within the normal range for the proliferation of bacteria. The genetic ability of the isolate microorganisms identified in this study may also be responsible for the different response to the degradation of crude oil in the culture medium. The number of viable cell increased enormously in 7 days of incubation upward indicating that the crude oil provided a good source of hydrocarbon for the growth of organisms and that toxic intermediate product were not found within the 7 days of incubation period. It was established from the study that aerophilic organisms are capable of degrading bitumen under aerobic and anaerobic conditions at low pH and this would enhance the remediation/reclamation of polluted land in bitumen exploitation areas. This suggest that some anthropogenic activities such as illegal felling of timber without regeneration should be avoided and method of bush burning as a form of farm practice should be discouraged in the bitumen deposit areas. Likewise, Government should be well informed about the impact of aerophillic organisms in bitumen degradation prior to the taping of bitumen in the study area.

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