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In Vitro Degradation of Bitumen from Tar Sand by Microorganisms around the Bitumen Deposit

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ABSTRACT

In vitro degradation of bitumen by microorganisms isolated around bitumen deposit at Agbabu was investigated. The microorganisms were isolated from soil sample collected around bitumen deposit and bitumen itself. The ability of individual isolates to utilize bitumen as sole carbon source in mineral salt medium was investigated. The results showed a decrease in the pH of the medium with an increase in the bacteria cell densities within the period of incubation, thereby confirming activities of the isolates in the medium. *Bacillus subtilis* (29.15%) caused highest weight loss from the bitumen, while *Pseudomonas aeruginosa* (12.03%) caused the least among the bacteria isolate. Other bacteria isolated were *Acinetobacter* sp. and *Staphylococcus aureus.* All the isolates caused weight loss from the bitumen. For moulds, *Arthrobotrys oligospora* caused higher percentage weight loss (19.13%), than *Aspergillus niger* in 28 days of incubation. By the 56 days of incubation, *Bacillus subtilis* was responsible for highest weight loss (49.50%), while *Pseudomonas aeruginosa* exhibited the lowest percentage (23.17%). For the moulds, *Arthrobotrys oligospora* caused the higher weight loss (42.83%) while *Aspergillus niger* had 37.33%. Thus, this established that the microorganisms isolated from the soil around the Agbabu bitumen and the bitumen itself could degrade bitumen from tar sand, hence their potential usefulness in remediation of bitumen polluted environment when the exploitation of the resource commenced.

Keywords: Bitumen; Bacteria; Mould; Hydrocarbon

INTRODUCTION

Bitumen is a viscous liquid, or a solid consisting essentially of hydrocarbons and their derivatives, and is soluble in carbon disulphide, substantially non-volatile and softens gradually when heated. It occurs naturally on its own or as a by-product of vacuum distillation of crude oil. It is blackish or brownish in colour and possesses water proofing and adhesive properties (Olutoye, 2005). Elemental analysis showed that bitumen is composed principally of carbon and hydrogen, with traces of nitrogen, sulphur and oxygen (Meyer and Witt, 1990; Guma *et al.,* 2012).

Nigeria has a considerable large deposit of bitumen which is found in the Western part of the country (Oboh *et al.,* 2006; Fagbote and Olanipekun, 2012; Ayoade *et al.,* 2014). It has been reported that Nigeria has a reserve of 42.47 billion metric tonnes of bitumen, covering about 120 × 4.3km (Oboh *et al.,* 2006; Ayoade *et al.,* 2014). Although exploration of the Nigerian tar sand oil deposit has not yet commenced, the environment is already battling with the problem of pollution arising from seepages of the natural resource into the surface water and soil in the communities where the resource is found (Adesanya *et al.,* 2014).

During dry season, the tar sand oil is free flowing contaminating the surrounding environment, water surface and under aquifer (Olabemiwo *et al.,* 2011b). The presence of this hydrocarbon in the environment has resulted in the infertility of the land, reduction of water quality, shortage of oxygen and reduction in aquatic population (Abii and Nwosu, 2009; Akpor *et al.,* 2014; Ajayi, 2015). The dominance of polyaromatic hydrocarbon in soil and water polluted with tar sand oil has been linked to health issues such as

damage to body organs, carcinogenicity and mutagenicity (Fagbote and Olanipekun, 2010; Olabemiwo *et al.,* 2011b).

Biodegradation of hydrocarbon contaminated sites, which exploit the ability of microorganisms to degrade and detoxify organic contamination, has been established as efficient, economic, versatile and environmental friendly (Okoh and Trejo-Hernandez, 2006; Adesanya *et al.,* 2014), and it has also proved that bitumen is not recalcitrant. Oboh *et al.* (2006) reported the hydrocarbon degrading potential of bacteria strains, namely *Pseudomonas stutzeri, Pseudomonas mulli* and *Alcaligenes* sp. isolated from bitumen sample and undisturbed soil of Agbabu, Ilubirin and Mile 2, all in Odigbo local government area of Ondo state, to degrade kerosene, diesel and naphthalene. Olabemiwo *et al.* (2011a) reported the ability of *Pseudomonas putrefaciens*, *Pseudomonas nigrificans*, *Bacillus licheniformis*, *Pseudomonas fragi* and *Achromobacter aerogenes* isolated from spent oil contaminated soil in Ogbomoso, Nigeria to degrade Agbabu bitumen and his study showed that bitumen is biodegradable. In this present study, isolates from the soil around the bitumen deposit site and bitumen itself were investigated for their ability to degrade natural tar sand bitumen.

METHODOLOGY

Collection of samples

The site of collection was Agbabu village in Odigbo Local Government Area of Ondo state, Nigeria, within the geographical grids of latitude 6^0 35' 16.3''N and 6^0 37' 13.9''N and longitude 4^0 29' 29.0E and 4^0 50' 20.7''E (Amigun *et al.,* 2012).

Soil samples were collected around the bitumen deposit site at two different locations. The distance was about 2-5 metres and 5-10 metres to the bitumen borehole well for the first and second sample point respectively. The soil was collected at 0-5cm depth and the other at 10-15cm depth from the surface.

The bitumen sample was collected from the bitumen well at mile 2 where the resource seeps to the surface of the land. Sterile metallic spoon was used to fetch the bitumen sample and kept in a sterile reagent bottle.

Isolation and identification microorganisms

Isolations were carried out from the soil and bitumen sample using pour plate method under aseptic conditions. Nutrient agar and acidified Malt extract agar were employed for bacteria and mould isolations respectively. Sub-culturing was carried out until pure cultures of each organism were obtained.

Bacteria isolates were identified by cultural, morphological and biochemical characteristics using the taxonomical scheme of Bergey's manual of Determinative Bacteriology (Bergey et al., 1994).

The macroscopic and microscopic characteristics of colour on the plate, hyphal diameter, spore shape and size among other criteria were used for the mould identification as reported by Barnatte and Hunter (1998) and Watanabe (2002).

Preparation of inoculum

The microbial inoculum for *in vitro* study was prepared as a suspension. Bacteria inoculum was prepared by adding 10ml of sterile water to the Petri dish containing 24 hour old culture of bacterium. The dish was gently shaken thoroughly to have the bacterium cell separated from the agar in which is grown. For the determination of the number of cells, haemocytometer was used. The inoculum density was adjusted by diluting appropriately with sterile water. Then it was covered with a coverslip and mounted on light microscope. The counting was done using x40 objective.

The mould inoculum was prepared by cutting the mould on the malt extract agar from the Petri dish, put into a sterile beaker, and 100ml of sterile water was then added to the beaker and

was shaken thoroughly to have the spores dislodge. The suspension formed was filtered using sterile funnel in which sterile cotton wool was inserted. Using haemacytometer, spore counting was done. Further dilution of the suspension was done where necessary. The number of cells (bacteria) and spores (moulds) used for inoculation was standardised by dilution.

Testing for microbial growth on bitumen

Basal mineral salt medium described by Oboh *et al*. (2006) was used. The medium composition was 0.7g K_2HPO_4, 0.7g KH_2PO_4, 0.7g $MgSO_4$, 1.0g NH_4NO_3, 0.005g NaCl, 0.002g $FeSO_4.7H_2O$, 0.002g $ZnSO_4.7H_2O$, 0.001g $MnSO_4.7H_2O$ per litre of distilled water. It was dispensed in 99ml portions in 250ml Erlenmeyer flask. Two gram of bitumen sample was then introduced into the flask and then sterilized at 121^0C for 15minutes. Two ml of the bacteria inoculum was later added to the flask containing the cooled medium and the bitumen sample. Same procedure was followed for inoculation of the mould inoculum. Before the introduction of the test microbes, the pH of the medium was adjusted to be 8.60 using the Hanna pH meter model HI96107. The controls contained only the medium and bitumen sample without any organism. The flask was plugged with cotton wool and wrapped with aluminium foil and then incubated at 30^0C for 28days and then the other set extended to 56days.

Assessment of microbial growth on bitumen sample

The growth of each microbe in culture was assessed by determining the pH and total cell count after incubation for bacteria.

Determination of weight loss

The extent of the utilization of hydrocarbon in the bitumen was estimated gravimetrically on the 28th and 56th days of incubation. This was achieved by harvesting the residual bitumen from both the control and experimental set-ups, using modified method of Olabemiwo *et al.,* (2011a). Ten milliliter of dichloromethane (DCM) was added to the culture and shaken vigorously for 5 min to extract the residual bitumen. The extract was filtered and the residue on the filter paper aired for five to six hours in order for the DCM to evaporate. The filter paper with its content was weighed.

Weight loss of the bitumen was calculated using the equation;

$X_3 = X_1 - X_2$.

Where X_1 is the weight of the bitumen before inoculation, X_2 is the weight of the residual bitumen recovered after incubation and X_3 is the weight loss.

Percentage weight loss = $(X_3 \div X_1) \times 100$.

The data was analysed using one way analysis of variance (ANOVA) followed by Tukey's test for mean separation.

RESULTS and DISCUSSION

Figure 1 and 2 shows the change in the pH of the medium at the end of incubation. There was much decrease in the pH of the inoculated culture media and slight change in that of the control. *Acinetobacter* sp. caused the most acidification of medium. pH decrease caused by moulds was not as much as that of bacteria. The change in pH observed in the experimental set-up and the control agree with the findings of Omotayo *et al.* (2011), Moneke and Nwangwu (2011) who stated that the utilization and introduction of petroleum hydrocarbons in medium changes the pH of the medium. Okerentugba and Ezeronye (2003) noted that the utilization of petroleum hydrocarbon as carbon source by microorganisms resulted in the production of acidic metabolic products which might account for the decrease in the pH of culture medium. Oboh *et al*. (2006) stated that the reduction of pH in experimental flasks confirmed chemical changes in hydrocarbon substrate which must have been precipitated by microbial enzymes.

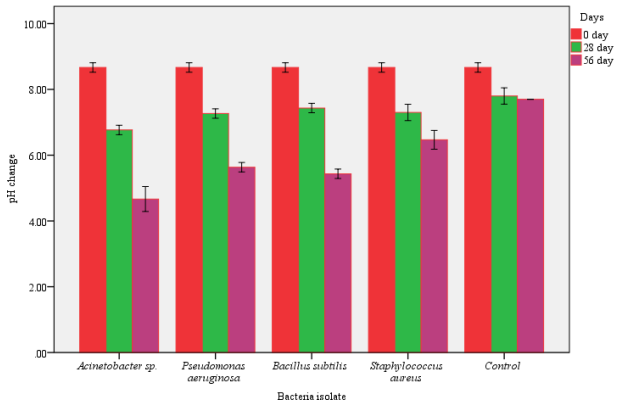

Figure 1: pH of the mineral salt medium containing bitumen inoculated with bacteria and incubated at 30^{0}C.

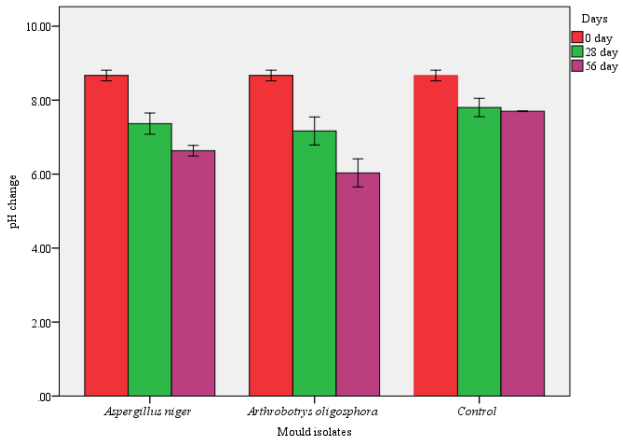

Figure 2: pH of the mineral salt medium containing bitumen inoculated with mould spores and incubated at 30^{0}C.

The cultural and morphological characteristics of each bacteria isolates such as colony shape, elevation, pigmentation, colour, cell shape, Gram reaction, motility and endospore test are indicated on Table 1. The table also has the results of biochemical test for the bacteria isolates such as catalase test, oxidative fermentative test, sulphate reduction test and starch hydrolysis test, and also the sugar fermentation test. The features revealed the following probable bacteria: *Acinetobacter* sp., *Pseudomonas aeruginosa, Bacillus subtilis,* and *Staphylococcus aureus.* The probable mould isolates were *Aspergillus niger* and *Arthrobotrys oligospora.* Most of these organisms have been isolated from soil and hydrocarbon polluted environments by other researchers (Ogunjobi and Fagade, 2000; Oboh *et al.,* 2006; Boboye *et al.,* 2010; Olabemiwo *et al.,* 2011a; Omotayo *et al.,* 2011; Al-Jawhari, 2014). The study revealed for the first time the association of *Arthrobotrys oligospora* with natural tar sand bitumen.

On the basis of growth profile, it could be observed that all the bacteria isolates were able to utilize bitumen as growth substrate which resulted in the increase in cell density, though there was disparity in the rate of growth as shown in Figure 3. The ability of the isolates to utilize bitumen as growth substrate might be attributed to either the constitutive nature of bitumen assimilating capacities of the microorganisms or reflects the adaptation of the strains as a result of previous exposure to bitumen which may be followed by concurrent development of the ability to use bitumen as carbon source (Adebusoye *et al.,* 2006; Omotayo *et al.,* 2011). Interestingly most of the isolates used in this study had been reported by other researchers to utilize other hydrocarbon with similar chemical composition to that bitumen. Oboh *et al.* (2006) in their earlier study revealed the ability of microorganisms associated with Nigeria bitumen deposit to utilize kerosene, diesel and naphthalene as growth substrate. Olabemiwo *et al*. (2011a) reported the ability of *Pseudomonas putrefaciens*, *Pseudomonas nigrificans*, *Bacillus lincheniformis*, *Pseudomonas fragi* and *Achromobacter aerogenes* from spent oil contaminated soil to utilize tar sand bitumen as growth substrate. The utilization of aviation fuel as growth substrate by *Pseudomonas aeruginosa*, *Micrococcus luteus*, *Corynebacterium* sp., *Flavobacterium rigense*, *Bacillus subtilis*, *Aspergillus niger*, *Penicillium* sp., *Rhodotorula* sp., *Candida tropicalis* and *Saccharomyces* sp., had also been demonstrated by Omotayo *et al*. (2011).

Table 1: Cultural, Morphological and Biochemical characteristics of bacteria isolates from soil around Agbabu bitumen site and bitumen.

Cultural Characteristics					Morphological Characteristics			Biochemical Characteristics				Sugar Fermentation						Probable Organism
Colony Shape	Elevation	Pigmentation	Colour	Cell Shape	Gram Reaction	Motility	Endospore	Oxidative Fermentation Test	Starch Hydrolysis	Sulphate Reduction	Catalase Test	Glucose	Fructose	Sucrose	Maltose	Lactose	Raffinose	
Regular	Raised	_	White	Short rod	_	_	_	O/F	_	_	+	+	_	_	_	+	_	*Acinetobacter* sp
Regular	Raised	Greenish	White	Short rod	_	+	_	O/F	+	_	+	+	+	_	_	_	_	*P.aeruginosa*
Irregular	Raised	_	Cream	Short rod	+	+	+	O/F	_	_	+	+	+	+	+	_	_	*B. subtilis*
Regular	Raised	_	White	Cocci	+	_	_	O/F	+	_	+	+	+	+	+	+	_	*S. aureus*

Key: - = Negative Test, + = Positive Test, O/F = both oxidative and fermentative positive.

Adesanya *et al.* (2014) noted the utilization of diesel as growth substrate by *Bacillus subtilis, Micrococcus* sp., *Pseudomonas aeruginosa, Bacillus licheniformis, Bacillus sphaericus, Citobacter freundii, Flavobacterium, Acinetobacter* sp., *Staphylococcus aureus, Bacillus thuringiensis, Bacillus cereus*, *Penicillium* sp. and *Bacillus megaterium* isolated from bitumen contaminated water and sediment. The utilization of Forcados blend crude oil, benzene, toluene, phenol and catechol by *Acinetobacter* (AI), *Pseudomonas* (A5), *Bacillus subtilis* (A3), *Pseudomonas aeruginosa* (strain K2) and *Acinetobacter calcoaceticus Iwoffi* (strain K4) had been reported by Ogunjobi and Fagade (2000). Al-Ghamdi (2011) reported the ability of *Aspergillus niger*, *Aspergillus terreus*, *Aspergillus fumigatus*, *Trichoderma viride* to utilize gasoline as growth substrate. Al-Jawhari (2014) also found that *Aspergillus niger, Aspergillus fumigatus, Aspergillus flavus, Fusarium solani* and *Penicillium funiculosum* isolates were able to utilize crude oil as growth substrate.

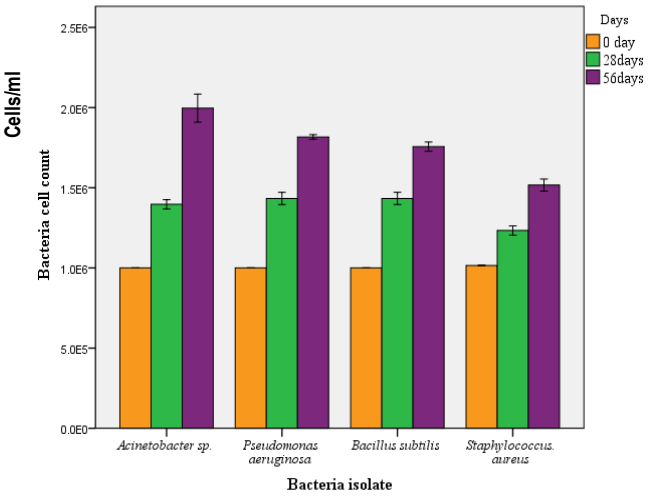

Figure 3: Bacteria cell count in mineral salt medium and bitumen after incubation at 30^{0}C.

The weight loss in the bitumen harnessed after the period of incubation is shown in Figure 4 and 5, indicates that all microbial isolates degraded the bitumen. This confirms the report of Olabemiwo *et al.* (2011a) who noted that bitumen is biodegradable. The result showed that *Bacillus subtilis* degraded bitumen most, followed by *Acinetobacter sp*. and the least degradation was found with *Pseudomonas aeruginosa*.

For the mould isolates, *Arthrobotrys oligospora* exhibited higher degradation ability while *Aspergillus niger* had lower capacity. *Aspergillus niger* had been found to be more effective in the degradation of crude oil and gasoline (Al-Ghamdi, 2011; Al-Jawhari, 2014). This observation could be as a result of genetic adaptation that had taken place with regard to its previous association with natural tar sand.

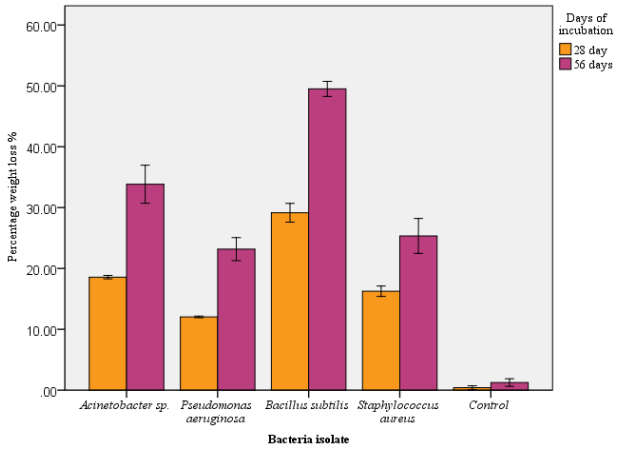

Figure 4: Graph of percentage weight loss in bitumen samples inoculated with bacteria cells after the period of incubation.

CONCLUSION

In this study, *in vitro* degradation of bitumen at 30^{0}C by natural microorganisms isolated from bitumen and soil sample collected around the bitumen deposit occurred. Therefore, the fact that the isolated microorganisms were able to grow and degrade bitumen showed their potential in the remediation of bitumen polluted environment. The efficacy and effectiveness of the microorganisms in the degradation of bitumen could be improved by incorporating other remediation strategies. The change in chemical structure of the bitumen can be ascertained using Gas chromatography or Mass chromatography.

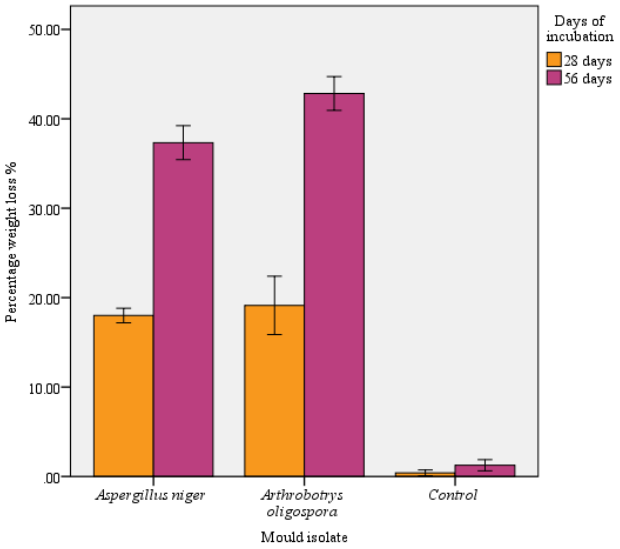

Figure 5: Graph of percentage weight loss in bitumen samples inoculated with mould spores after the period of incubation.

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