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## Preliminary Study on Biodegradation of Nigerian Natural Bitumen

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### ABSTRACT

One of the major problems facing the existence of man in this millennium is pollution. Thus, it is no surprise that much attention is given to pollution control and remediation of polluted environment. While remediation can be achieved by physicochemical and biological methods, application of the latter tends to be gaining upper hand due to some of its inherent advantages. This study investigated the biodegradability of bitumen from Agbabu, Southwestern Nigeria by hydrocarbon utilizing strains of *Pseudomonas putrefaciens*, *Pseudomonas nigrificans*, *Bacillus licheniformis*, *Pseudomonas fragi* and *Achromobacter aerogenes*. The organisms were used to inoculate Mineral Salts Medium (MSM) supplemented with 0.2 g L<sup>-1</sup> solution of Agbabu bitumen in dichloromethane. Cultures were inoculated at different temperature and pH (27°C/pH 3.5; 27°C/pH 5.6; 30°C/pH 7.0 and 40°C/pH 7.0) for two weeks. The quantity of bitumen degraded by each organism was determined gravimetrically at the end of first and second week. The rate of degradation was calculated for each organism and residual bitumen analyzed by infrared-Spectroscopy. In the first week of incubation, *Achromobacter aerogenes* exhibited the highest rate of degradation (1.750±0.027 mg h<sup>-1</sup>) at 30°C and pH 7 while *Bacillus licheniformis* showed the least degradation rate (0.300±0.018 mg h<sup>-1</sup>) at 40°C and pH 7. Structural indices such as aromaticity, sulphonation, aliphaticity and oxidation calculated from the infrared spectra of recovered bitumen from the inoculated samples were different from that of the control. This thus, confirms the degradation capability of the bacteria used in this work on the Agbabu bitumen and hence their potentials for use in bioremediation of bitumen-polluted environments.

**Key words:** Biodegradability, bacteria, bitumen, Agbabu, structural indices

### INTRODUCTION

Environmental pollution arising from processing of crude oil, use of petroleum products and oil spillage is of great global concern. Environment polluted by crude oil is injurious to animals and plants because crude oil contains many toxic compounds in relative high concentration (Franco *et al.*, 2004). The ecosystems of hydrocarbon contaminated soils are damaged due to accumulation of pollutants in animals and plants tissues. Such accumulation may lead to progeny's death or mutation (Alvarez *et al.*, 1991).

Physicochemical and biological methods are employed in the remediation of polluted soils (Okoh, 2006; Erdogan and Karaca, 2011). The physicochemical methods of remediation of oil polluted soils are associated with high cost, laborious and above all they often achieve incomplete removal of the contaminants from the soil (Eckenfelder and Noris, 1993). Biological method which is also known as bioremediation appears to be having upper hand over the physicochemical methods. This is due to its low cost and ability to halt the accumulation of contaminants (El-Nawawy *et al.*, 1987; April *et al.*, 2000; Jain *et al.*, 2011). Bioremediation refers to the decomposing of organic pollutants by soil microorganism with subsequent conversion of the decomposed materials to harmless end products such as carbon dioxide, water and methane (Walter *et al.*, 1997). Bioremediation is not only useful in the recovery of site contaminated with oil but also applicable to recovery of hazardous wastes contaminated sites or media (Chitra and Lakshmanaperumalsamy, 2010; Rezaee *et al.*, 2010; Thavasi *et al.*, 2006; Saber *et al.*, 2010; Akinyele *et al.*, 2011; Amini *et al.*, 2011; Ogwal *et al.*, 2011). The huge success recorded in the clean-up process of the oil tanker Exxon Valdez oil spill of 1989 attested to the efficacy of bioremediation process (Atlas and Bartha, 1998) in Prince William Sound and the Gulf of Alaska. Thus, there is growing interest in the use of biodegradation and bioremediation technology as a method of clean-up of environment contaminated by oil or any other contaminants.

Degradation of hydrocarbons through microbial activities has been detected for quite some time. As far back as 1895, the degradation of a thin layer of paraffin by the fungus *Bortrytis cineria* was reported (Higgins *et al.*, 1975). Since then, the quest by scientists to study biodegradation of petroleum, petroleum products, bitumen and allied materials in general has been on the increase. Recently Igwo-Ezikpe *et al.* (2010a) reported their investigations on the evaluation of potential of Tropical bacteria isolates and its consortium to biodegrade mixture of high molecular polycyclic aromatic hydrocarbons (PAHs). The same authors, Igwo-Ezikpe *et al.* (2010b), reported the biodegradative potentials of some bacteria isolated from various contaminated soils in Nigeria to degrade chrysene, fluorathene and pyrene. Lepo and Cripe (1999) in their own findings showed that biodegradation of PAHs was preferential to that of n-alkanes in crude oil when they used some bacteria (*Nocardia globerula*, *Rhodococcus fascians*, *Pseudomonas saccharophila*). These findings contradicted the earlier view that the n-alkanes were generally considered the most biodegradable compound class within crude oil (Leahy and Colwell, 1990; Atlas and Bartha, 1992; Prince, 1993). The possible application of biodegradation in Antarctic was reported by Vasileva-Tonkova and Gesheva (2004), who isolated 17 microbes from Antarctic soil and all of them showed a good biodegradative potential for hydrocarbons.

Nigeria is blessed with vast deposits of natural bitumen (Adegoke, 2000). Strausz *et al.* (2010) described bitumen as the heaviest form of petroleum which is viscous, black and sticky. It is a complex mixture of high boiling point range of hydrocarbon compounds and molecules with relatively low hydrogen to carbon ratio (Yoon *et al.*, 2009). The exploitation and exploration of the Nigerian natural bitumen deposits can best be described to be at the planning stage. Previous studies on the Nigerian bitumen were concentrated on geological surveying of the deposits and the composition and engineering properties of the bitumen (Adegoke *et al.*, 1980; Sonibare *et al.*, 2003; Olajire *et al.*, 2007; Adebisi and Asubiojo, 2008; Oloke *et al.*, 2009). Till date, there is little information regarding the biodegradability of Nigerian natural bitumen despite its potential as environmental pollutant. Hence, the present study was initiated to investigate the biodegradability of Agbabu natural bitumen by some locally isolated bacteria with a view to generate important data on the natural transformation of Nigerian natural bitumen and lay a solid background for the bioremediation of ecosystems polluted with this mineral and other hydrocarbon sources in Nigeria.

## MATERIALS AND METHODS

**Sampling site and enrichment cultures:** The bacterial strains used in this study were isolated from spent lubricant-contaminated soils collected from an Automobile Service Workshop in Ogbomoso, Nigeria. Soil samples were collected at subsurface level at five different points, pooled together and stored in closed containers at 4°C prior to use. The bitumen used for enrichment and biodegradation experiments were collected in September, 2007 from one of the observatory wells in Agbabu, Nigeria. Agbabu is one of the major towns located in the Nigerian natural bitumen belt and the place where bitumen was first discovered in Nigeria (Adegoke, 2000). All microbial enrichment and isolation were performed in the media prepared with the following composition (g L<sup>-1</sup>): NH<sub>4</sub>NO<sub>3</sub> (1); K<sub>2</sub>HPO<sub>4</sub> (0.5); MgSO<sub>4</sub>·7H<sub>2</sub>O (0.2); CaCl<sub>2</sub> (0.02); FeCl<sub>3</sub> (3 drops of 60% FeCl<sub>3</sub>). Solubilized bitumen sterilized by tyndallization was added as sole carbon source to the autoclaved medium. The pH of the culture media was adjusted appropriately with either HCl or NaOH.

**Isolation and purification of bitumen-degrading bacteria:** An enrichment culture technique was employed using the method of Palittapongarnpim *et al.* (1998) to isolate hydrocarbon-degrading bacteria. The Mineral Salts Medium (MSM) containing soil and sterilized bitumen was incubated at 27°C with orbital shaking (100 r min<sup>-1</sup>). Enrichment of microbial culture was carried out in 100 mL erlenmeyer flasks containing 50 mL of MSM. The pH was appropriately adjusted. The MSM was sterilized by autoclaving (121°C for 20 min). Nutrient Agar (NA) was used for isolation, numeration and maintenance of pure strains. One gram of the collected contaminated soil sample was added to 50 mL of MSM containing 0.5% v/v sterilized bitumen. At weekly intervals during the initial enrichment, transfers (1/100 v/v) were made to the same fresh medium. After six transfers, 1 mL was diluted and placed on agar plates and incubated for 72 h at 27°C in darkness. Distinct colonies were selected and sub-cultured repeatedly on NA plates until pure cultures were obtained. Five bacteria isolates obtained were then characterized morphologically, microscopically and biochemically at the soil laboratory, Institute of Agricultural Research and Training, Moore Plantation, Ibadan, Nigeria, using the scheme of Holt *et al.* (1994).

**Biodegradation of bitumen by the bacterial strains:** All the five strains isolated were used for the biodegradation of the bitumen. A series of 25 mL McCartney bottles (36 in number) were used for this experiment. Each flask contained 6.0 mL of MSM plus 4.0 mL of 0.2 g mL<sup>-1</sup> of bitumen and culture was inoculated by transferring 1 mL of pre-culture of strains, pH and temperature adjusted as shown on Table 1. Thirty six experiments were designed in a batch reactor under aerobic condition by shaking on orbital shaker (rotation speed of 160 rpm) for two weeks. Four treatments and one control were set up for this experiment varying the temperature of inoculation and pH of the MSM, as shown in Table 1. For accuracy, all treatments were performed in triplicate. Samples were removed at the end of first and second weeks to assess the concentration

Table 1: Temperature and pH conditions for the biodegradation experiment

Experiment	Condition
1	Inoculation temp. 30°C, pH = 7.0
2	Inoculation temp. 40°C, pH = 7.0
3	Inoculation temp. 27°C, pH = 3.5
4	Inoculation temp. 27°C, pH = 5.6

of the bitumen by gravimetric analysis while a change in the structure of bitumen was evaluated with infrared spectroscopy.

**Determination of residual bitumen:** The extent of utilization of the hydrocarbon in the bitumen was estimated gravimetrically at 7th and 14th day of incubation. This was achieved by harvesting the residual bitumen from the control and experimental set-ups, using the method of Sanni and Ajisebutu (2003). Five milliliter of Dichloromethane (DCM) was added to the culture solutions and shaken vigorously for 5 min to extract the residual bitumen. The extract was filtered and filtrate centrifuged (at 2500 rpm). Two different layers were formed; the upper layer which was DCM layer contained biodegraded bitumen and lower layer was medium layer. The DCM layer was collected into a pre-weighed beaker using separating funnel. The process was repeated thrice and the combined DCM layer was allowed to evaporate at room temperature and the beaker and its content was weighed. The amount of bitumen degraded was obtained using the equation:

$$X_0 - X_g \quad (1)$$

where,  $X_0$  is the amount in gram of residual bitumen recovered from the control test and  $X_g$  is the amount of residual bitumen recovered from the test experiments. The rate of degradation of bitumen was then calculated using the following equation:

$$R = \frac{X_0 - X_g}{t}$$

where,  $t$  is the period of incubation in h.

The infrared spectral of the residual bitumen and the control were analyzed using Fourier Transform Spectrophotometer (Nicolet Avatar 330FT-IR). The analysis was carried out by placing about 0.01 g of the withdrawn/residual bitumen in between two potassium chloride discs. The discs were gently pressed against each other to spread the sample across the diameter of the discs. The prepared disc was then mounted on the sample holder of the spectrophotometer and spectrum of the sample scanned between 4000-400  $\text{cm}^{-1}$ . Infrared absorption peaks were identified automatically by comparison with the standards in the library interfaced with the spectrophotometer.

**Statistical analysis:** Each of the results of Gravimetric Analysis is mean value of three Readings $\pm$ SD. Structural change indices are ratios of absorbance at the wavelengths indicated for each of them.

## RESULTS AND DISCUSSION

Five bacteria were isolated from the spent lubricant contaminated soil samples used in this study. The organisms were identified by morphological, microscopic and biochemical characterization tests (Holt *et al.*, 1994) as *Pseudomonas putrefaciens*, *Pseudomonas nigrificans*, *Bacillus licheniformis*, *Pseudomonas fragi* and *Achromobacter aerogenes*. These bacteria species were used in the biodegradation experiment.

The rate of degradation in the first week of incubation as evaluated gravimetrically is contained in Table 2. Results showed that at pH 7 and temperature 30°C, *Achromobacter aerogens* had the

Table 2: Comparative potential of the five selected bacteria to degrade Agbabu Bitumen in a week

Bacterium	Rate (mg h <sup>-1</sup> ) (Conditions of inoculation/incubation)			
	(Temp. 27°C, pH 3.5)	(Temp. 27°C, pH 5.6)	(Temp. 30°C, pH 7)	(Temp. 40°C, pH 7)
A	0.661±0.006	0.613±0.000	0.318±0.018	1.223±0.004
B	0.470±0.013	0.512±0.012	1.083±0.080	0.924±0.018
C	0.542±0.006	0.708±0.036	0.799±0.009	0.300±0.018
D	0.512±0.001	0.405±0.023	1.641±0.031	1.710±0.016
E	0.702±0.081	0.494±0.020	1.750±0.027	1.437±0.660

A: *Pseudomonas putrefaciens*, B: *Pseudomonas nigrificans*, C: *Bacillus licheniformis*, D: *Pseudomonas fragi*, E: *Achromobacter aerogenes*

greatest degradation rate (1.750±0.027 mg h<sup>-1</sup>) which agreed perfectly well with result of the infrared analysis (Table 4). Table 4 gives the infrared characterization factors of Agbabu bitumen degraded by the bacteria used in this study. The characterization factors otherwise called Structural Change Indices (SCI) were calculated from the infrared spectra of the control and test samples, Kovaleva and Golubev (2005). Difference in the values of SCI of control and that of test samples is an indication of structural change. There exist differences between the SCI of control and all the samples acted upon by the bacteria used in this study. For instance, the aromaticity of control (at pH 7 and temperature 40°C) was 1.067 while that of samples degraded by *Pseudomonas putrefaciens*, *Pseudomonas nigrificans*, *Bacillus licheniformis*, *Pseudomonas fragi* and *Achromobacter aerogenes* were 1.264, 1.290, 1.276, 1.252 and 0, respectively (Table 4).

When the conditions of incubation were changed, the degradation capability of all the bacteria used in this study also changed. Inoculation conditions such as temperature and pH have been observed by some workers (Dibble and Bartha, 1979; Thorn and Aiken, 1998) to influence the rate of biodegradation of crude oils. At pH 5.6 and temperature 27°C, *Bacillus licheniformis* had the highest degradation rate of 0.708 mg h<sup>-1</sup> while *Pseudomonas fragi* had the lowest value of 0.405 mg h<sup>-1</sup>. When the incubation conditions changed to pH 7 and temperature 30°C, *Achromobacter aerogenes* had the highest degradation rate of 1.75 mg h<sup>-1</sup> and *Pseudomonas putrefaciens* had the lowest of 0.318 mg h<sup>-1</sup> (Table 2).

The rate of biodegradation of the Agbabu bitumen by the five bacteria used in this study in the second week is as given in Table 3. Generally, a reduction in the rate of biodegradation of the bitumen was observed for each of the bacteria. The highest degradation rate observed in the second week was 0.970±0.001 mg h<sup>-1</sup>, at 40°C and pH 7 for *Pseudomonas putrefaciens* and the lowest was 0.271±0.240 mg h<sup>-1</sup> at 40°C for *Bacillus licheniformis*. At the same condition in the first week of incubation, *Pseudomonas fragi* exhibited highest rate (1.710±0.016 mg h<sup>-1</sup>) while *Bacillus licheniformis* showed the lowest rate (0.300±0.018 mg h<sup>-1</sup>). This observation (i.e., reduction in rate) might probably be due to reduction in efficiency of the bacteria arising from the accumulation of toxic degradation products. Some of the degradation products of crude oil and other hydrocarbons include alcohol, aldehyde and carboxylic acids (Taylor *et al.*, 1990). High concentrations of these compounds may be toxic to some bacteria.

The maximum level of degradation of the bitumen achieved by each of the bacteria at the first week was 25.68, 22.74, 16.78, 34.46 and 36.75% for *Pseudomonas putrefaciens*, *Pseudomonas nigrificans*, *Bacillus licheniformis*, *Pseudomonas fragi* and *Achromobacter aerogenes*, respectively. These were achieved at temperature 30°C and pH = 7.0 except for *Pseudomonas putrefaciens* which occurred at temperature 40°C and pH = 7.0. Relatively, the biodegradation potentials of the five bacteria used in this study were a little bit lower than previously reported by Silva *et al.* (2006),

Table 3: Comparative potential of the five selected bacteria to degrade Agbabu bitumen in two weeks

Bacterium	Rate (mg h <sup>-1</sup> ) (Conditions of inoculation/incubation)			
	(Temp. 27°C, pH 3.5)	(Temp. 27°C, pH 5.6)	(Temp. 30°C, pH 7)	(Temp. 40°C, pH 7)
A	0.643±0.006	0.396±0.027	0.315±0.008	0.970±0.001
B	0.634±0.001	0.521±0.014	0.592±0.030	0.540±0.095
C	0.560±0.005	0.589±0.009	0.577±0.004	0.271±0.240
D	0.643±0.008	0.545±0.018	0.920±0.011	0.893±0.003
E	0.577±0.006	0.661±0.013	0.875±0.009	0.740±0.016

A: *Pseudomonas putrefaciens*, B: *Pseudomonas nigrificans*, C: *Bacillus licheniformis*, D: *Pseudomonas fragi*, E: *Achromobacter aerogenes*

Table 4: Infrared characterization factors of Agbabu bitumen degraded by some selected bacteria

Infrared characterization factor	Bacterium					
	Control	<i>Pseudomonas putrefaciens</i>	<i>Pseudomonas nigrificans</i>	<i>Bacillus licheniformis</i>	<i>Pseudomonas fragi</i>	<i>Achromobacter aerogenes</i>
Aromaticity (A <sub>1600</sub> /A <sub>720</sub> )	1.067	1.264	1.290	1.276	1.252	-
Aliphaticity (A <sub>720</sub> A <sub>1380</sub> /A <sub>1600</sub> )	2.112	1.989	1.984	2.028	1.945	-
Oxidation (A <sub>1710</sub> /A <sub>1455</sub> )	0.623	0.710	0.706	0.680	0.776	1.074
Sulfurization (A <sub>1030</sub> /A <sub>1455</sub> )	0.562	0.672	0.675	0.635	0.714	-

A<sub>750</sub>: Absorbance at ~ 720 cm<sup>-1</sup>, A<sub>1600</sub>: Absorbance at ~ 1600 cm<sup>-1</sup>, A<sub>1380</sub>: Absorbance at ~ 1380cm<sup>-1</sup>, A<sub>1455</sub>: Absorbance at ~ 1455cm<sup>-1</sup>, A<sub>1030</sub>: Absorbance at ~ 1030cm<sup>-1</sup>, A<sub>1710</sub>: Absorbance at ~ 1710cm<sup>-1</sup>

Celik *et al.* (2008) and Salam *et al.* (2011). Improving the efficiency of these bacteria may require the use of enhancers such as biosurfactants as suggested by Abalos *et al.* (2004), Celik *et al.* (2008) and Qomarudin *et al.* (2010). Alternatively, the five bacteria can be used as bacterial consortium. Bacterial consortium has been reported to have greater ability to degrade hydrocarbons than individual bacterium (Lal and Khanna, 1996; Qomarudin *et al.*, 2010).

A typical Infrared spectrum of the bitumen sample after *Achromobacter aerogenes*, acted upon it for seven days is as shown in Fig. 2. Figure 1 shows the infrared spectrum of the bitumen prior to the biodegradation (i.e., control). From, Fig. 1 and 2, it can be seen clearly that the spectral features (absorbance and wavelength) of notable peaks of the spectrum of the bitumen were modified by the action of this bacterium. Similar changes were also observed in the spectra of the bitumen samples acted upon by the other bacteria used in this study. For instance, the broad peak due to O-H stretch in the infrared spectrum of the control sample was found to be flattened and shifted from 3446.43 to 3249.48 cm<sup>-1</sup> when *Pseudomonas putrefaciens* acted on the bitumen sample. Also, substituted benzene ring band (745, 810 and 860 cm<sup>-1</sup>) appeared relatively distinct in the spectrum of this sample compared to its appearance in the control sample. Furthermore, two new peaks (2340.23 and 2359.07 cm<sup>-1</sup>) due to C-H stretching vibration (Williams and Fleming, 1987) appeared in the spectrum of the bitumen sample degraded by *Pseudomonas putrefaciens*.

Arising from these observations, Structural Changes Indices as defined by Kovaleva and Golubev (2005), were calculated from the spectra and were presented in Table 4. Aromaticity, aliphaticity, oxidation and sulphurization measure changes in the aromatic, aliphatic, carbonyl and sulphony functional groups, respectively in a material (Lamontagne *et al.*, 2001; Kovaleva and Golubev, 2005; Mouillet *et al.*, 2008). Thus, changes in the values of these indices

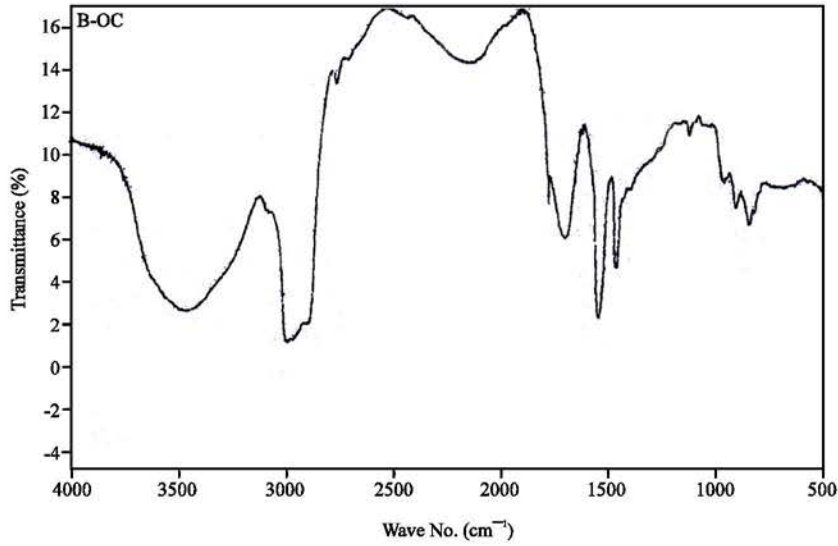


Fig. 1: Infrared spectrum of Agbabu bitumen (control)

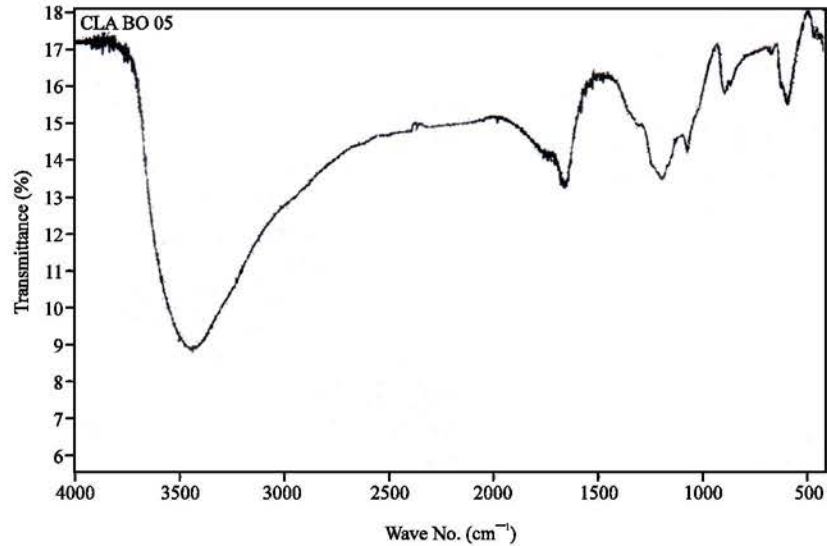


Fig. 2: Infrared spectrum of Agbabu bitumen degraded by *Achromobacter aerogenes*

as shown in Table 4 are evidences that bitumen samples recovered in this study were degraded products of the control sample. The degradation been brought about by the bacteria used in this study. The activity of *Achromobacter aerogenes* was exceptional because it led to the disappearance of almost all the notable peaks in the infrared spectrum of the undegraded bitumen (Fig. 2). Hence some of the structural changes indices could not be calculated for the bacterium. It therefore suggests that *Achromobacter aerogenes* has a higher tendency to degrade the Nigerian bitumen than the other four bacteria used in this study. The efficiency of *Achromobacter aerogenes* and *Pseudomonas fragi* to degrade bitumen was earlier reported by Oloke *et al.* (2009). Adelowo *et al.* (2006) in their earlier findings reported the ability of these bacteria to degrade about 80% of engine oil used in their study.



## CONCLUSION

In this study, biodegradation was monitored by gravimetric and infrared spectrophotometric analyses. The infrared spectra features of the Agbabu natural bitumen were altered when each of the five bacteria used in this study acted upon it. The changes in the infrared spectra and gravimetric analyses of the altered samples showed that the bacteria have potential to degrade the Agbabu bitumen. However, the rate at which they did this was different for each of the bacteria used and was found also to be a function of temperature, pH and period of incubation. Thus, the applicability of these bacteria in bioremediation of spillages from Nigerian natural bitumen is a possibility and should therefore be a subject of further studies.

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