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Biodegradation of hydrocarbon compounds in Agbabu natural bitumen

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The recovery of an environment polluted by petroleum and allied hydrocarbons through bioremediation is being embraced globally as the best technology of removing hydrocarbon pollutants from environment. Infrared spectral changes and gravimetric analysis from the preliminary biodegradability study carried out on Agbabu Natural Bitumen showed the vulnerability of the bitumen to some bacteria: *Pseudomonas putrefaciens*, *Pseudomonas nigrificans, Bacillus licheniformis, Pseudomonas fragi* and *Achromobacter aerogenes*. This study investigates the ability of *P. putrefaciens*, *P. nigrificans, B. licheniformis, P. fragi* and *A. aerogenes* to degrade the aliphatic and polycyclic aromatic hydrocarbon fractions of Agbabu natural bitumen. Samples of the bitumen were separately inoculated with each of the bacteria for 14 days and the hydrocarbon profiles before and after inoculation were quantified using gas chromatography technique. The total aliphatic hydrocarbon compounds (C_{11} - C_{29}) in the bitumen degraded by *P. putrefaciens* and *P. nigrificans* was slightly higher than that in the undegraded bitumen, while the concentration of compounds (C_{11} - C_{29}) found in samples of the bitumen degraded by *B. licheniformis, P. fragi* and *A. aerogenes* was less than what was contained in the undegraded bitumen. Also the even-odd carbon-ratios of the degraded bitumen were higher than unity while these were less than unity in the undegraded bitumen. The polycyclic aromatic hydrocarbons (PAHs) profile in the bitumen degraded samples also differed from that of undegraded bitumen. A substantial reduction in the concentration of some PAHs was found in the bitumen samples following their degradation by the bacteria strains, typically from 55.98 to 30.79%, thus suggesting the possibility of using the bacteria strains for bioremediation process.

Key words: Agbabu, bitumen, bacteria, biodegradability, hydrocarbons.

INTRODUCTION

Petroleum hydrocarbon compounds are the most widespread pollutants in water and soil. Some PAHs have been implicated for having the capacity to damage genetic materials and consequently leads to development of different type of cancers. High molecular weight PAHs such as benzo(a)pyrene and dibenzo(a,h)anthracene

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are the most culprits of cancer inducers (Schneider et al., 2000). Thus, the global attention is focused on how to reduce the concentrations of these in the environments. Although, there is variety of techniques to achieving this, biodegradation appears to be a promising method of recovering petroleum polluted environment. Biodegradation refers to reduction in complexity of chemical compounds with aid of biological catalysts produced by microorganisms (Wilson and Jones, 1992). Biodegradation has some advantages such as complete removal of the pollutants at low cost from the petroleum environment compared to other methods of remediation which are based on physical or chemical principle (April et al., 2000).

Many components of petroleum hydrocarbons have been reported to be biodegradable (Jain et al., 2011, Margesin and Schneider 1999b, Braddock et al., 1997). As far back as early 70s, a wide range of microbial species have been identified to have capacity to degrade hydrocarbons. Among these are soil moulds, such as *P. glaucum,* some yeast (for example *Candida utilis*) and some species of bacterium (for example, *Pseudomonas norcardia and Mycobacteria*) (Wang et al., 1998).

In biodegradation of crude oil, bacteria are the major microbes involved. The popular view on biodegradation of crude oil is that only aerobic bacteria are involved, but recent studies have shown that anaerobic bacteria too can degrade crude oil (Higgins and Burns, 1975).

When petroleum is attacked and degraded by microbes, certain fundamental changes in the composition and properties of the crude oil resulted. Among these changes are sequential and systematic removal of various hydrocarbons and other compounds, selective degradation of specific isomers within individual compound classes and the production of acidic compounds (Reneter, 1994).

It has been established that biodegradation leads to oxidation of some components of crude oil. Six carbon atoms and above are oxidized; leading to a decrease in aromatic hydrocarbon content of the oil. (Widdel and Rabus, 2001, Ian et al., 2003, Volkmen et al., 1984). Generally, in biodegradation of crude oil, the hydrocarbons are preferentially destroyed in decreasing order of: n-alkanes>branched alkanes>aromatic hydrocarbon>alicyclic hydrocarbon (Wang et al., 1998; Meredith et al., 2000).

This is followed by sulpur-oxygen- and nitrogen-containing compounds (Wenger et al., 2001; Miller et al., 1987). Products of crude oil biodegradation include acyclic, and cyclic, saturated and aromatic carboxylic acids and phenols (Ian et al., 2003; Fedorak and Lake 1984; Huang et al., 2003). A complex variety of acidic non-hydrocarbons are also produced from biode-graded aromatic heterocyclics found in oil (Taylor et al., 1990; Mackenize et al., 1983; Thorn and Alken, 1998).

Nigeria has a large deposit of natural bitumen, which is ranked to be one of the five largest deposits of natural bitumen in the World (Adegoke, 2000).The bitumen deposit is located in the bitumen belt which spans across three states in Nigeria, but very often it is referred to as Agbabu Natural Bitumen (ANB), being the town (Agbabu) where the Nigerian natural bitumen was first discovered in 1900 (Adedimila, 2000). The full exploitation of this important engineering material has not commenced, however, intensive scientific research investigations on the material are currently being carried out by experts with a view to providing useful information for prospective investors.

Infrared data and gravimetric analysis from our previous study showed that, *P. putrefaciens*, *P. nigrificans, B. licheniformis, P. fragi* and *A. aerogenes* impacted qualitative and quantitative changes on ANB (Olabemiwo et al., 2011). The present study investigated the ability of these bacteria to degrade the aliphatic and polycyclic aromatic hydrocarbons. The objective is to provide information that would serve as template for the development of appropriate bioremediation scheme for the Agbabu bitumen spills in particular and petroleum hydrocarbons in general

MATERIALS AND METHOD

Biodegradation

The biodegradation experiment was as described in our earlier study (Olabemiwo et al., 2011). Summarily, this involved the isolation, purification and characterization of the bitumen degrading bacteria strains. (*P. putrefaciens*, *P. nigrificans, B. licheniformis, P. fragi* and *A. aerogenes*). Sample of bitumen was then inoculated with each of the bacteria strains for 14 d. The biodegraded/residual bitumen was subsequently harvested and kept at 4°C, for further analysis.

Fractionation of the biodegraded bitumen

Samples of fresh and degraded bitumen isolated from biodegradation experiment were separately dissolved in dichloromethane (DCM). The resulting solution was then fractionated into aliphatic, aromatic and polar fractions. The fractionation was achieved through column chromatography. Activated Silica gel (70-230 mesh) was packed into 100 mL chromatographic column. Added to the top of the silica in the column was 1 g of anhydrous sodium sulfate (Na_2SO_4). This was followed by the conditioning of the system using 20 ml hexane. Thereafter, the concentrated solution of dissolved degraded bitumen was introduced into the column. The sample was eluted with 3×25 ml of *n*-hexane followed by 3×25 ml DCM: *n*-hexane (95:5) to obtain the aliphatic and aromatic fractions, respectively. Eluates from each fraction were pooled together and rotary evaporated and kept in labeled sample vials at 4°C till analysis.

Gas chromatography

Analysis of aliphatic and aromatic fractions of residual oil were separately carried out on a 5890 series II Hewlett Packard gas chromatograph equipped with flame ionization detector (FID). A fused silica capillary column (30 m × 0.25 µm) coated with 0.25 µm

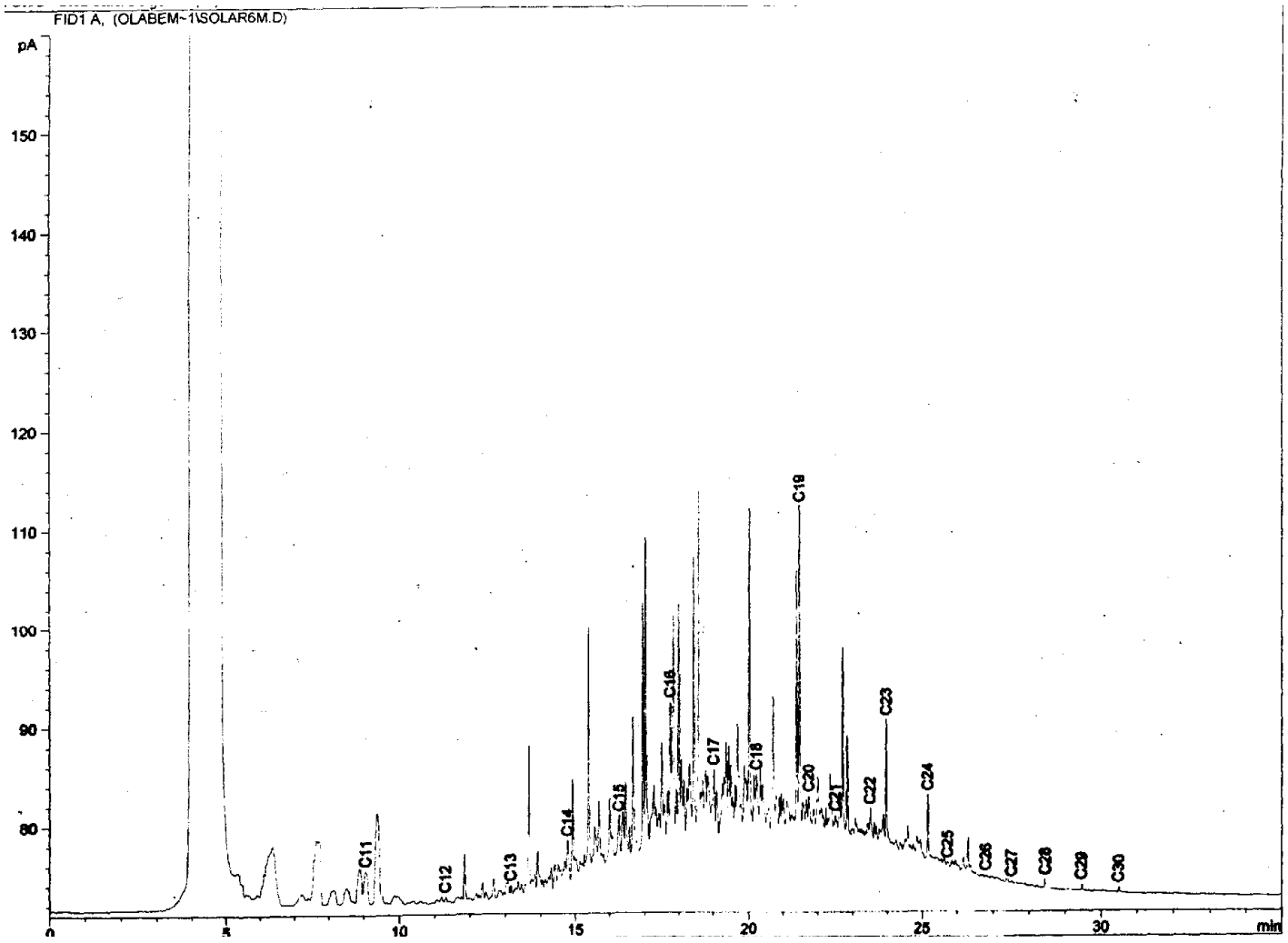

Figure 1. A typical chromatogram of degraded sample of ANB.

film of HP-5 was used. The chromatograph was powered with HPCHEM software. The instrumentation for quantification of aliphatic hydrocarbons was as follows: Nitrogen was used as carrier gas with a pressure of 30 psi. The column temperature was programmed with initial temperature of 60°C, held isothermally for 2 min and then increased to 260°C at the heating rate of 10°C/min for 20 min. It was held at this temperature for 2 min, thereafter, increased to 320°C at heating rate of 12°C/min for 5 min and held at this temperature for 2 min. The injector and detector temperatures were maintained at 250°C and 350°C, respectively. For the aromatic components, the column temperature started at 68°C and was held at this temperature for 2 min, thereafter, the temperature increased to 260°C at heating rate of 12°C/min for 16 min. It was held isothermally at 260°C for 4 min and thereafter increased to 320°C at heating rate of 15°C/min for 4 min and held at 320°C for 8 min. The carrier gas was nitrogen at a pressure of 35 psi. Hydrogen and air were supplied at 25 and 30 psi, respectively. Injector and detector temperatures were 300 and 320°C respectively and the volume of sample injected was 2 µL. Calibration curves for the aliphatic and polycyclic aromatic hydrocarbons were prepared using their standard solutions which were supplied by the manufacturer of the equipment.

RESULTS AND DISSCUSSION

A typical chromatogram of aliphatic hydrocarbons of ANB sample degraded by one of the bacteria used in this study is presented in Figure 1. The total and the distribution of individual aliphatic hydrocarbons (C_{11} - C_{29}) in ANB degraded by *P. putrefaciens, P. nigrificans, B. licheniformis, P. fragi* and *A. aerogenes* are presented in Table 1 and Figure 2. It can be observed that the total and distribution of individual aliphatic hydrocarbons vary with the type of bacteria used. The total and distribution of individual aliphatic hydrocarbons also differed from what was contained in the control sample (undegraded ANB). This is a very good evidence of vulnerability of the ANB to biodegradation and is in agreement with our previous findings (Olabemiwo et al., 2011). The distribution of individual aliphatic hydrocarbons (C_{11} - C_{29}), found in the degraded ANB is presented in Figure 2.

The biodegradative activity of *P. putrefaciens* and *P.*

Table 1. Proportion of odd and even numbered carbon atoms in ANB

Parameter	O	A	B	C	D	E
TOCA (%)	37.44	83. 28	82.97	75.78	80.40	79.70
TECA (%)	62.56	16.72	17.03	24.22	19.60	20.30
TALPH (g)	484.73	520.95	502.99	421.53	378.48	334.29
ROENA: TOCA/TECA	0.60	4.98	4.87	3.13	4.10	3.73

TOCA, Total Odd Numbered Carbon Atoms; TECA, Total Even Numbered Carbon Atoms; ALPH, Total Aliphatic Hydrocarbons; ROENA, Ratio of total Odd Numbered carbon Atoms to Total even numbered Carbon Atom; O, controls; A, ANB sample degraded by *Pseudomonas putrefaciens*; B, ANB sample degraded by *Pseudomonas nigrificans;* C, ANB sample degraded by *Bacillus licheniformis*; D, ANB sample degraded by *Pseudomonas fragi*; E, ANBB sample degraded by *Achromobacter aerogenes.*

nigrificans caused a relatively small increase in the total aliphatic hydrocarbons (TALPH) in the degraded ANB compared to the undegraded ANB (Table 1). The two bacteria probably converted higher aliphatic hydrocarbons (> C_{30}) to lower ones as evident by the increase in the abundance of some low molecular mass aliphatic hydrocarbons (C_{11} and C_{12}) (Figure 2). The degradative activity of the *B. licheniformis, P. fragi* and *A. aerogenes* caused a decrease in TALPH found in the ANB (Table 1). The lower molecular weight aliphatic hydrocarbons present were probably oxidized to carbon (IV) oxide and water (Obire and Nwaubeta, 2001) by these bacteria.

In the undegraded ANB, the even-odd numbered C-atoms was less than unity, while it was found to be greater than unity in all the biodegraded samples of ANB (Table 1). This implies that all the bacteria used in this work attacked mainly, the even-numbered carbon atoms in the ANB. The percentage of even-numbered carbon atoms are 17, 17, 24 and 20 in ANB samples degraded by *P. putrefaciens*, *P. nigrificans*, *B. licheniformis*, *P. fragi* and *A.* aerogenses, respectively, whereas it was about 63% in control sample. The degradative activity of all the bacteria used in this study, on the ANB led to the production of large quantity of lower molecular mass odd-numbered aliphatic hydrocarbons. This might account for the reversal of the proportion of odd to even carbon atom in the degraded samples (Figure 2). About ten-folds of aliphatic hydrocarbon with twenty-nine carbon atoms found in the undegraded ANB was found in ANB samples degraded by *P. putrefaciens* and *P. licheniformis*. Eight and five folds of this aliphatic hydrocarbon (C_{29}) were detected in ANB samples degraded by *B. licheniformis* and *P. fragi* respectively (Figure 2). On the other hand, the aliphatic hydrocarbon (C_{29}) was not detected in sample of ANB degraded by *A. aerogenes*.

The total PAHs and distribution of individual PAH in control and degraded samples of ANB are given in Figure 3. The total PAHs in each of the biodegraded samples was less than the total PAHs in the undegraded sample of ANB. Samples of ANB degraded by *P. putrefaciens* and *P. nigrificans* contained almost half the total PAHs in the undegraded ANB. In case of samples of ANB degraded by *B. licheniformis*, *P. fragi* and *A. aerogenes*, one third of the total PAHs contained in undegraded PFB was found in them. This implies that the three bacteria were able to degrade more than 60% of the total PAHs contained in undegraded ANB. The extent of degradation of PAHs achieved in this work is very close to the results of Gokcen et al. (2008), where degradation rates in the range of 42-59% were reported for some PAHs.

The distribution pattern of individual PAH in the biodegraded ANB samples also differed from that in the undegraded ANB (Figure 4). Naphthalene was found in undegraded ANB and samples of ANB degraded by *P. fragi* and *A. aerogenes*. Samples of ANB degraded by *P. putrefaciens*, *P. nigrificans* and *B. licheniformis* had no detectable quantity of naphthalene.

There were 2-, 3- and 4- fused ring PAHs in the ANB (Figure 4), prior to the degradation experiment. However, in the ANB samples degraded by *P. putrefaciens*, *P. nigrificans* and *B. licheniformis*, only 3- and 4- fused rings PAHs were detected. The ANB samples degraded by *P. fragi* and *A. aerogenes* had 2-, 3-, 4- and 5- fused rings PAHs. The 5- fused rings PAHs found in degraded samples may be due to polymerization or recombination of 2-, and 3- fused rings PAHs (Stern and Stern, 1971; Okerentugba and Ezeronye, 2003).

It can therefore be said that, all the bacteria used in this work displayed the capability of degrading some of the PAHs found in ANB. However, *B. licheniformis*, *P. fragi* and *A. aerogenes* appeared to be more efficient in degrading the PAHs in the ANB than the other two bacteria. As reported above for aliphatic hydrocarbons, the bacteria used in this work had also affected the total and distribution of individual PAHs detected in the ANB. These findings quite agree with our earlier preliminary study on biodegradation of ANB using infrared spectroscopy (Olabemiwo et al., 2011). The changes in the infrared spectrum and chromatographic analysis of the degraded ANB corroborate each other to show that the composition and structure of the ANB were altered by the bacteria used in this work.

The biodegradability of petroleum hydrocarbons by some bacteria strains have also been established by some other workers (Bushnell and Haas, 1941, Pahthapingam et al., 1998, Obire, 1990). The findings of

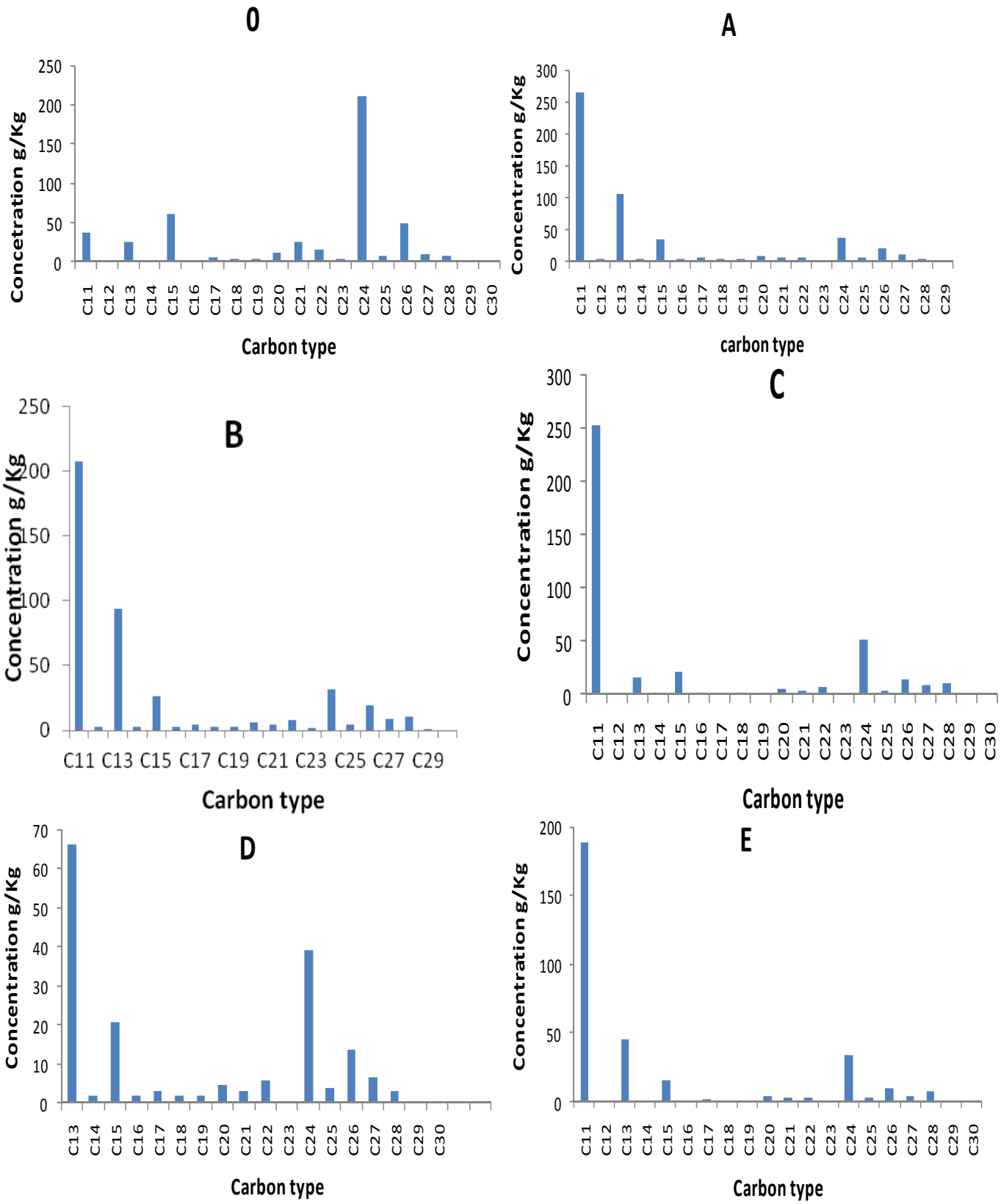

Figure 2. Distribution of aliphatic hydrocarbons in ANB degraded by some Bacteria. O = controls,A = PFB sample degraded by *Pseudomonas putrefaciens* B = PFB sample degraded by *Pseudomonas nigrificans* C = PFB sample degraded by *Bacillus licheniformis* D = PFB sample degraded by *Pseudomonas fragi* E = PFB sample degraded by *Achromobacter aerogenes.*

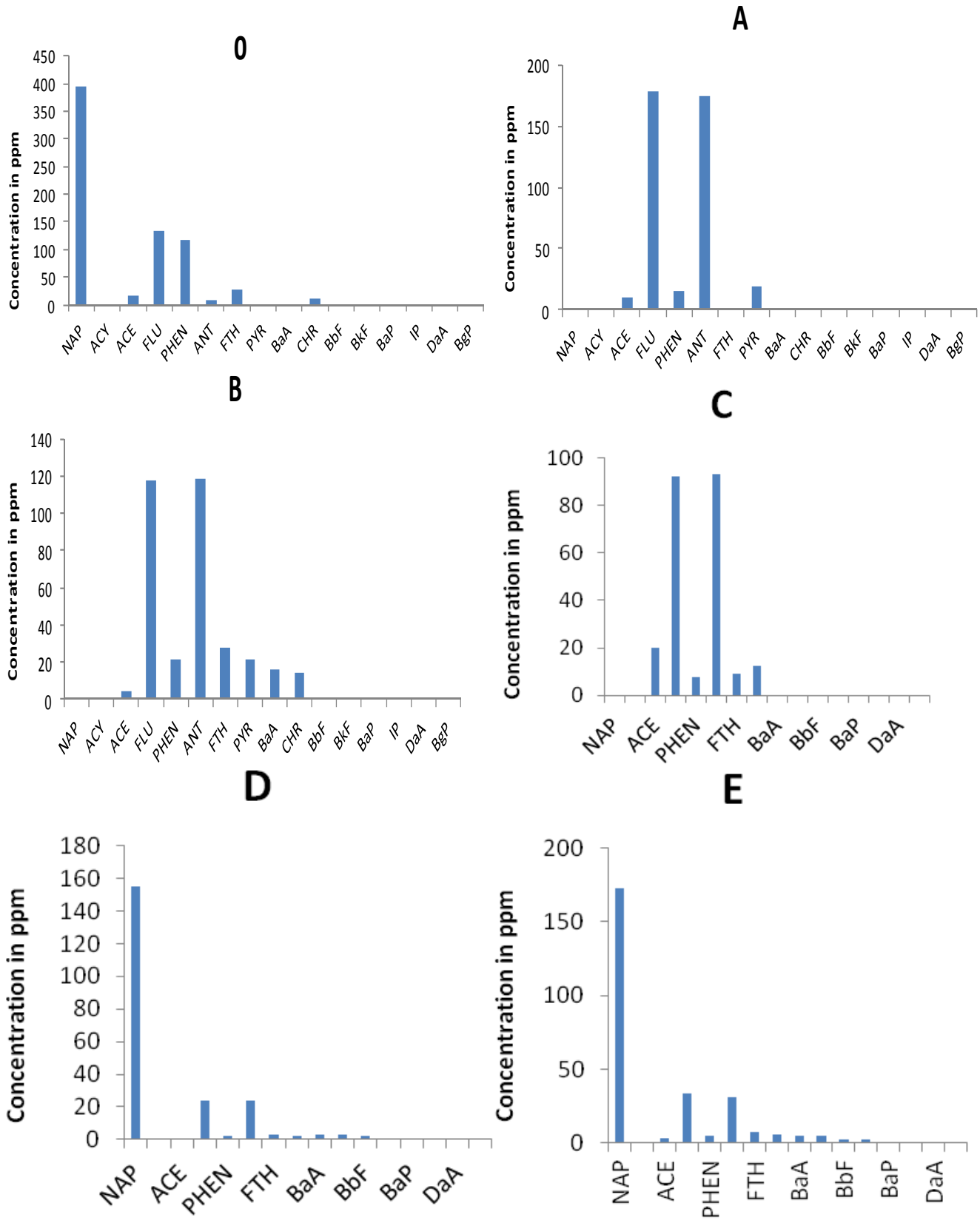

Figure 3. Polycyclic Aromatic Hydrocarbon (PAH) Profile of Biodegraded PFB. O = control,A = ANB sample degraded by *Pseudomonas putrefaciens*,B = ANB sample degraded by *Pseudomonas nigrificans*,C = ANB sample degraded by *Bacillus licheniformis*,D = ANB sample degraded by *Pseudomonas fragi*,E = ANB sample degraded by *Achromobacter aerogenes*

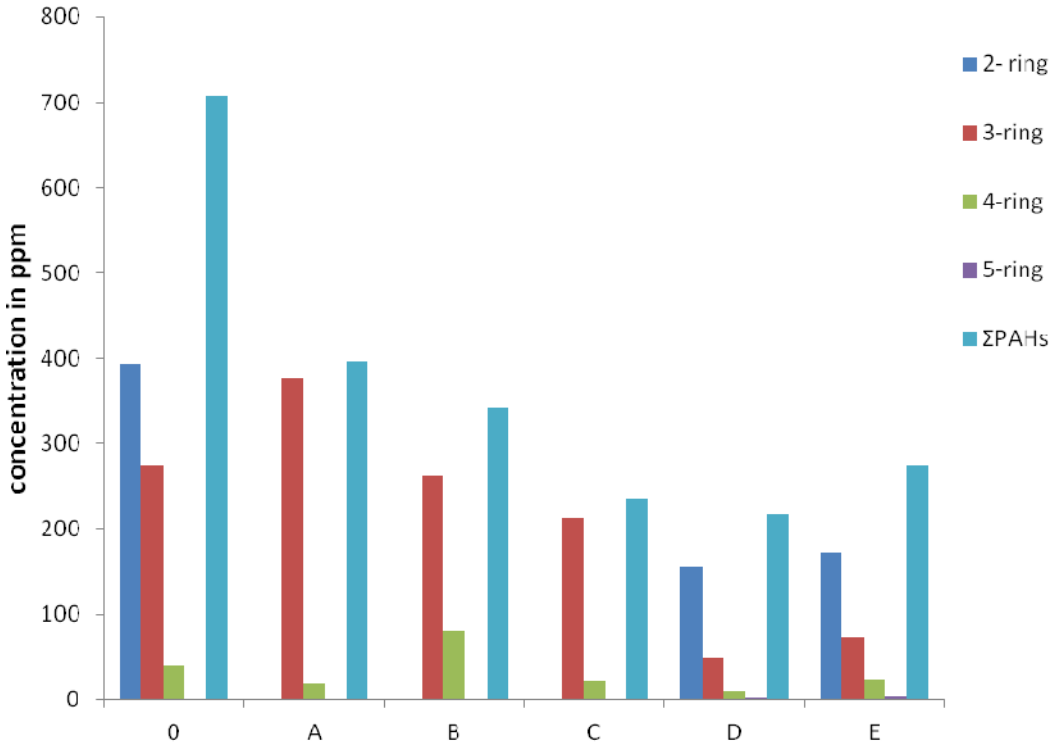

Figure 4. Group distribution of PAHs in biodegraded PFB. O = control,A = ANB sample degraded by *Pseudomonas putrefaciens*,B = ANB sample degraded by *Pseudomonas nigrificans*,C = ANB sample degraded by *Bacillus licheniformis*,D = ANB sample degraded by *Pseudomonas fragi*,E = ANB sample degraded by *Achromobacter aerogenes*.

this study show that *B. licheniformis*, *P. fragi* and *A. aerogenes* had potential of being useful in bioremediation of hydrocarbons in bitumen spill.

Conclusion

All the five bacteria strains used in this study altered the hydrocarbon profiles in ANB. However, three of these bacteria strains (*B. licheniformis*, *P. fragi* and *A. aerogenes)* reduced the total aliphatic and polycyclic aromatic hydrocarbons contents of the bitumen. Thus, these bacteria strains can be employed for bioremediation of bitumen spill in Agbabu area and its environs.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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