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Full Length Research Paper

Degradation of weathered crude oil (Escravos Light) by bacterial strains from hydrocarbons-polluted site

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Two hydrocarbon degrading Gram-negative bacteria isolated from hydrocarbon polluted power plant soil in Lagos, Nigeria, were studied for their degradation potentials on weathered crude oil (Escravos Blend). These bacteria isolates were identified as *Enterobacter cloacae* strain LG1 and *Burkholderia cepacia* strain LG2. The highest population density for LG1 was 2.07 x 10^7 cfu/ml, while for LG2 it was 1.63 x 10^7 cfu/ml. In the two cases slight but consistent pH drops to < 6.51 were observed. LG1 had a growth rate of 0.38 d^{-1} and doubling time 1.83 d. The corresponding values for LG2 during the first log phase were 0.58 d^{-1} and 1.19 d, respectively, while in the second log phase the values were 0.14 d^{-1} and 5.13 d Gas chromatographic analysis showed that chrysene was degraded by LG and LG2 by 92.91 and 80.25%, respectively, anthracene by 92.21 and 91.53%, respectively, and Benzo (b) fluoranthene was degraded by 77.19 and 98.41%, respectively. LG2 had a higher degradative capability of the polyaromatic fractions with 80.15% while LG I was lower at 52.33%. Statistical analysis showed that there was a significant difference ($p < 0.05$) in the degradation of various aromatic components of the oil by LG1 and LG2.

Key words: Biodegradation, crude oil, hydrocarbons, *Enterobacter cloacae*, *Burkholderia cepacia.*

INTRODUCTION

The dramatic increase in production, refining and distribution of crude oil and its products has brought with it an ever increasing problem of environmental pollution. Nigeria apart from being a major petroleum producer, invariably fated to experience the fallouts of such operations (Adebusoye et al., 2007), the Nigerian environment is characterised by nonchalant, indiscriminate and highly unregulated disposal of petroleum products including diesel and engine oil (Odjegba and Sadiq, 2000).

Spence et al. (2005) noted that even small releases of petroleum hydrocarbons into aquifers can lead to concentrations of dissolved hydrocarbons far in excess of regulatory limits. Research findings have shown statistically significant impact of such reckless disposal on plants, including height reduction, chlorophyll loss and protein level reduction (Oluwole et al., 2005; Umechuruba, 2005).

However, studies have shown that hydrocarbon degrading bacteria are naturally present in these environments and play a very important role in the removal of the pollutants (Abed et al., 2001). Species of *Bacillus*, *Micrococcus*, *Nocardia, Corynebacterium*, *Pseudomonas*, *Flavobacterium*, *Achromobacter*, *Alcaligenes* and *Proteus* are some of the commonly isolated degraders (Atlas, 1992; Okoh and Trejo-Hernandez, 2006). Also found to be important are numbers of fungal and actinomycetes strains.

Petroleum is a complex mixture composed primarily of aliphathic, alicyclic and aromatic hydrocarbons. There are thousands of individual compounds in every crude oil, the composition of each crude oil varying with its origin (Atlas 1995a). Whereas the aliphatic and low molecular weight (LMW) aromatic hydrocarbon components of crude oil and its products can be easily biodegraded, the high molecular weight fractions particularly the recalcitrant polycyclic aromatic hydrocarbons (PAHs) such as

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pyrene, benzo(a) pyrene and the likes often persist in soils (Heitkamp and Cerniglia, 1987 ; Leahy and Colwell, 1990; Cerniglia, 1992; Johnsen et al., 2005).

Furthermore, it is difficult to find organisms with degradative capability for a broad array of aliphatics and aromatics. In this paper, we report the isolation and characterisation of two Gram-negative strains with remarkable ability to degrade weathered Nigerian Escravos Light crude oil.

MATERIALS AND METHODS

Sample site

Soil samples were collected from a power generating plant soil site known to be chronically contaminated with diesel, engine oil, exhaust soot and other hydrocarbons for more than 20 years. The samples were collected at a depth of 10 to 12 cm with sterile trowel after clearing debris from the soil surface. Samples for physicochemical analysis were collected in polyethylene bags, while those for microbiological studies were kept in sterile sample bottles. Samples were transported to the laboratory and treated within 4 h.

Physico - chemical analysis of soil samples

The pH of the soil samples was determined with a pH meter (Jenway 3051) in 1:1 soil solution in distilled water. The moisture level, organic content, total nitrogen content, potassium content and available phosphorous were determined as described previously (Bray and Kurtz, 1945; Black, 1965; Chopra and Kanwar, 1998). The total hydrocarbon was extracted from the soil using n-hexane: dichloromethane solvent systems (1:1).

Microbiological analysis of soil sample

The total heterotrophic bacterial and fungal counts were enumerated by plating aliquots (0.1 ml) of appropriate diluted soil samples on nutrient agar and acidified potato dextrose agar containing streptomycin (1 mg/100 ml), respectively. Similarly, the population of hydrocarbon-utilisers was estimated on mineral salts medium (MSM) formulated by Kästner et al. (1994). The medium contained (in g/l) Na_2HPO_4, 2.13 g; KH_2PO_4, 1.30 g; NH_4Cl, 0.50 g and $MgSO_4.7H_2O$, 0.20 g. Sterile trace element solution (1.0 ml/l) of Bauchop and Elsden (1960) was aseptically added to the medium after sterilization. The pH of the medium was adjusted to 7.2 and 5.6, respectively for bacterial and fungal estimations. The MSM was also fortified with nystatin (50 µg/ml) for bacteria and 1 mg/100 ml of streptomycin for fungi. Sterile crude petroleum served as the sole carbon and energy source and was made available to the cultures through vapour-phase transfer (Raymond et al., 1976). Plates were counted after incubation at room temperature for 5 to7 days.

Enrichment and isolation of hydrocarbon-degrading bacteria

Bacteria able to degrade crude oil were isolated on mineral salt medium (MSM) containing crude oil as sole carbon and energy source by continual enrichment method. The medium described by Kästner et al. (1994) was used. The medium contained (in g/L) Na_2HPO_4, 2.13 g; KH_2PO_4, 1.30 g; NH_4Cl 0.50 g and $MgSO_4 .7H_2O$, 0.20 g. After the pH was adjusted to 7.2 it was fortified with nystatin (50 ppm) to suppress fungal growth. Trace elements solution (1 ml per liter) described by Bauchop and Elsden (1960) was sterilized separately and added aseptically to the medium.

Contaminated soil sample (0.4 g) was added to 40 ml MSM containing 1% crude oil. Enrichment was carried out with shaking at room temperature (29 ± 2.0°C) for 3 weeks in the dark until there was growth (turbidity). After 3 consecutive transfers at shorter intervals (7 to 15 days), hydrocarbon degraders were isolated by plating out the final flask on Luria-Bertani (LB) agar. Several colonies that appeared were further purified by subculturing once on LB agar. The ability to degrade hydrocarbons was confirmed by inoculating LB broth-grown pure cultures (18 h) into fresh MSM flask containing crude oil (1 % v/v) as sole carbon sources and incubated in the dark with shaking at room temperature for two weeks. However, utilization of crude oil was rated by turbidity observation.

Maintenance and identification of Isolates

Organisms were maintained in glycerol nutrient broth (1:1). Colonies growing on LB agar with very low percentage of PAH (0.005%) were harvested with sterile inoculating loop, pooled and transferred to the medium. The mixture was shaken well to homogenize it and kept at -20°C.

Isolates were identified on the basis of their colonial morphology, cellular morphology and biochemical characteristics according Cowan and Steel (Barrow and Feltham, 1995). This was complemented by using the API 20E rapid test kit phenotypic typing (BioMerieux, Durham, N.C.). API 20E kits were used according to the manufacturer's instruction.

Time course of isolates on crude oil

Replicate flasks containing 50 ml of MSM with 0.5 ml (1% v/v) of weathered crude oil (Escravos Blend) were prepared. Isolates were inoculated to achieve an initial concentration of the total viable count (TVC) of about 2.0×10^6 cfu/ml. Control flasks were inoculated with heat inactivated cells. Total viable counts were determined at 3 days interval (at each time point, entire replicate flasks were sacrificed) by plating out appropriate dilutions of the cultures onto nutrient agar. Residual oil concentration and pH were also determined at the same interval.

Extraction of residual oil

Residual oil was extracted by adding 20 ml of dichloromethane to broth culture in flask and shaking thoroughly as recently described by Adebusoye et al. (2007). After removing the aqueous phase with separating funnel, the extract was collected and concentrated in a rotary evaporator. The concentrated extract was separated into the aliphatic and aromatic fractions using a column packed with treated alumina. The polyaromatic fractions were later concentrated before injecting 1 µl into the gas chromatograph. Purification of the extract was carried out on activated silica gel in open column. The residual oil concentration was determined by gas chromatography. Control flasks were also extracted similarly. Results were expressed as percentages of respective controls.

Analytical method

Dichloromethane extracts (1.0 µl) were analyzed with Hewlett Packard 5890 series II gas chromatograph equipped with flame ionization detector (FID) and 30 m long HP - 5 column (internal diameter, 0.25 mm; film thickness, 0.25 µm). The carrier gas was nitrogen. The injector and detector temperatures were maintained at 300°C and 320°C, respectively. The column was programmed at

Table 1. Physico-chemical and microbiological characteristics of soil sample.

Parameter	Value
pH	7
Moisture (%)	5.49 ± 1.72
TOC (%)	7.07 ± 0.09
THC (mg/kg)	34211 ± 127.28
Nitrogen (%)	0.47 ± 0.07
Phosphorus (mg/kg)	0.36 ± 0.07
Potassium (mg/kg)	306.50 ± 7.78
Sulphate (mg/kg)	14.14 ± 0.20
TB (cfu/g)	9.50×10^7
THB (cfu/g)	7.10×10^6
TF (cfu/g)	6.00×10^5
THF (cfu/g)	1.64×10^4

TOC – total organic carbon; THC- total hydrocarbon; TB- total bacteria; THB - total hydrocarbon degrading bacteria; TF- total fungi; THF- total hydrocarbon degrading fungi.

an initial temperature of 60°C for 2 min, then ramped at 12°C/min to 320°C and held for 10 min. Nitrogen pressure was 35 psi, hydrogen pressure 25 psi and compressed air pressure 30 psi.

Statistical analysis

Mean generation times and specific growth rates were calculated using nonlinear regression of growth curves for the period when growth rates were maximal. Regression and variance analyses were performed using prism version 5.0 (Graphpad software, San Diego, CA, USA).

RESULTS

Physico-chemistry of soil sample

Table 1 shows the physico-chemical characteristic of the soil sample. The soil is rich in hydrocarbon (34211 ± 127.28 mg/kg) but poor in inorganic nutrients and moisture. The result shows that the concentration of PAHs in the soil was very low with pyrene and naphthalene being the highest (7.54×10^{-3} gm/l) and the lowest (8.07×10^{-4} gm/l), respectively. Other PAHs detected in the soil include acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene and benzo (a) pyrene. However, chrysene, benzo(b)flouranthene, benzo(k)flouranthene, benzo(a)pyrene, indeno(1, 2 ,3 - cd) pyrene, dibenzo (a, h) anthracene and benzo(g, h, i) perylene were not detected in the soil.

Isolation and identification of bacterial strains

Continuous enrichment resulted in the isolation of four morphologically distinct isolates. The isolates were Gram-negative motile rods. The two with the highest degradative ability on crude oil, as evidenced by turbidity increase, were selected for further study. The first strain, LG1, was a mucoid yellow-pigmented lactose fermenter, positive for arginine dihydrolase, ornithine decarboxylase, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, mygdalin, arabinose and nitrate reduction but negative for citrate, indole, voges preskaeur, gelatine and oxidase. It was identified as *Enterobacter cloacae*. The second organism utilized fewer sugars, namely glucose, rhamnose and sucrose. It was oxidase positive, liquefied gelatin, grew on citrate and was equally positive for arginine dehydrolase, lysine decarboxylase, ornithine decarboxylase. Additionally, it grew at 42°C but had no pigment. It was identified as *Burkholderia cepacia* .

Growth profile on crude oil

The growth profile of LG1 and LG2 are shown in Figure 1. From an initial value of 1.6×10^6 cfu/ml, LG1 grew rapidly to peak at 22.0×10^6 cfu/ml on day 6. LG2, on the other hand, described a biphasic growth curve. There was an initial growth from 2×10^6 cfu/ml within 3 days followed by a slight drop on day 6. Population rose again and peaked at 16×10^6 cfu/ml on day 9. In the two cases, slight but consistent pH drops at < 6.51 were observed. Kinetics of growth for the two isolates is shown in Table 2. LG1 had a growth rate of 0.38 d^{-1} and doubling time 1.83 d. The corresponding values for LG2 during the first log phase were 0.58 d^{-1} and 1.19 d, respectively, while in the second log phase the values were 0.14 d^{-1} and 5.13 d. Percentage degradation for the two organisms were 52.33 and 80.15%, respectively.

The degradation of selected aromatic hydrocarbon components of weathered crude oil (Escravos Light) is shown in Figure 2. Acenaphthene was degraded to undetectable level by both isolates. LG1 also completely depleted phenanthrene. There was over 90% removal of

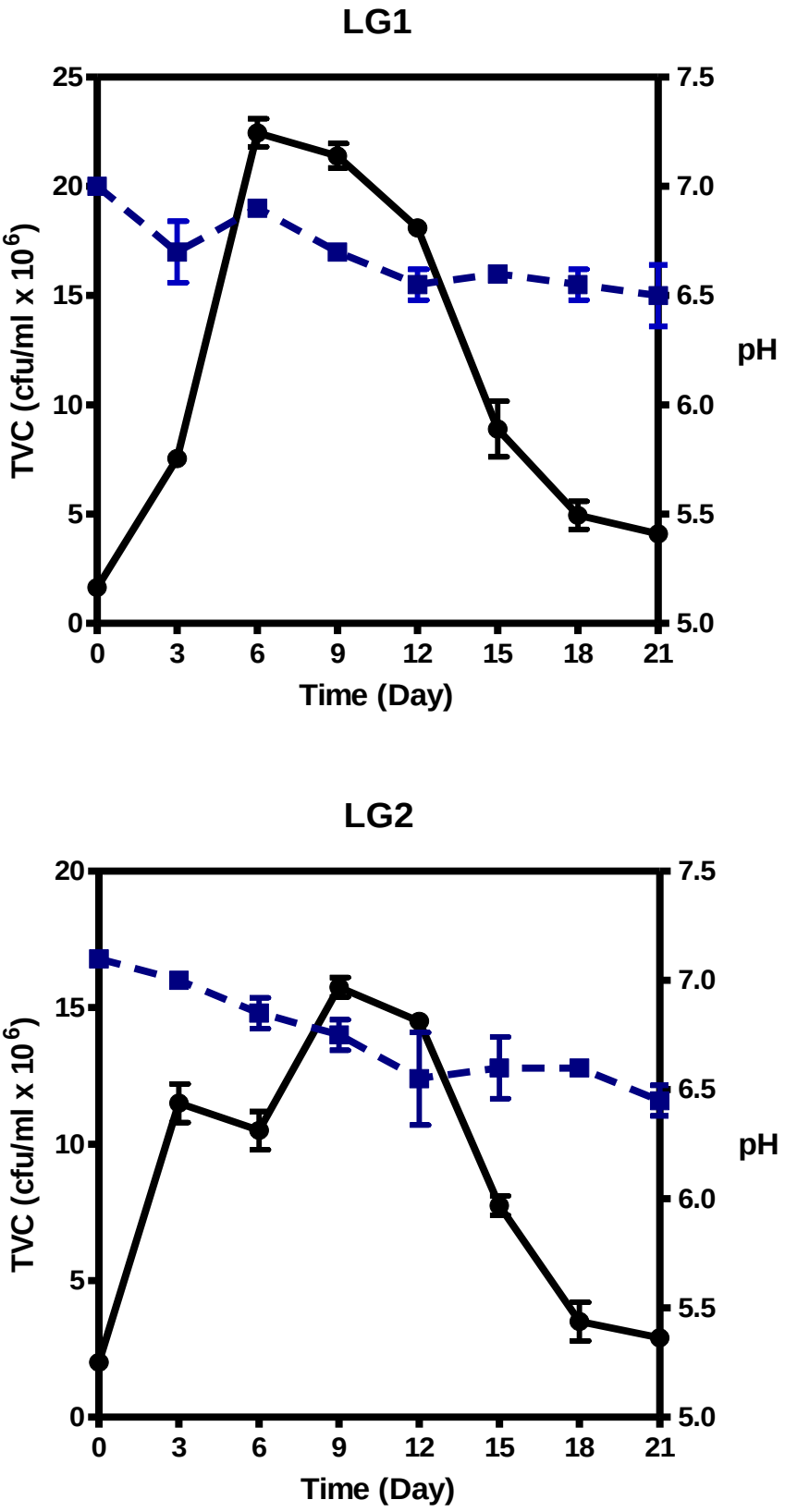

Figure 1. Growth dynamics of *E. cloacae* strain LG1 and *B. cepacia* strain LG2 in mineral medium supplemented with weathered crude oil showing total viable counts, TVC (●) and pH (■) changes. Data points represent the mean of three replicate flasks.

Table 2. Growth kinetics of isolates on crude oil.

Isolate	Growth rate (d^{-1})		Generation time (d)		Percentage degradation
	K_1	K_2	T_{g1}	T_{g2}	
LG 1	0.38	NA	1.83	NA	52.33
LG 2	0.5 8	0.14	1.19	5.13	80.15

K_1 and K_2 growth rates during first and second log phases respectively; T_{g1} and T_{g2} generation times during first and second log phases respectively.

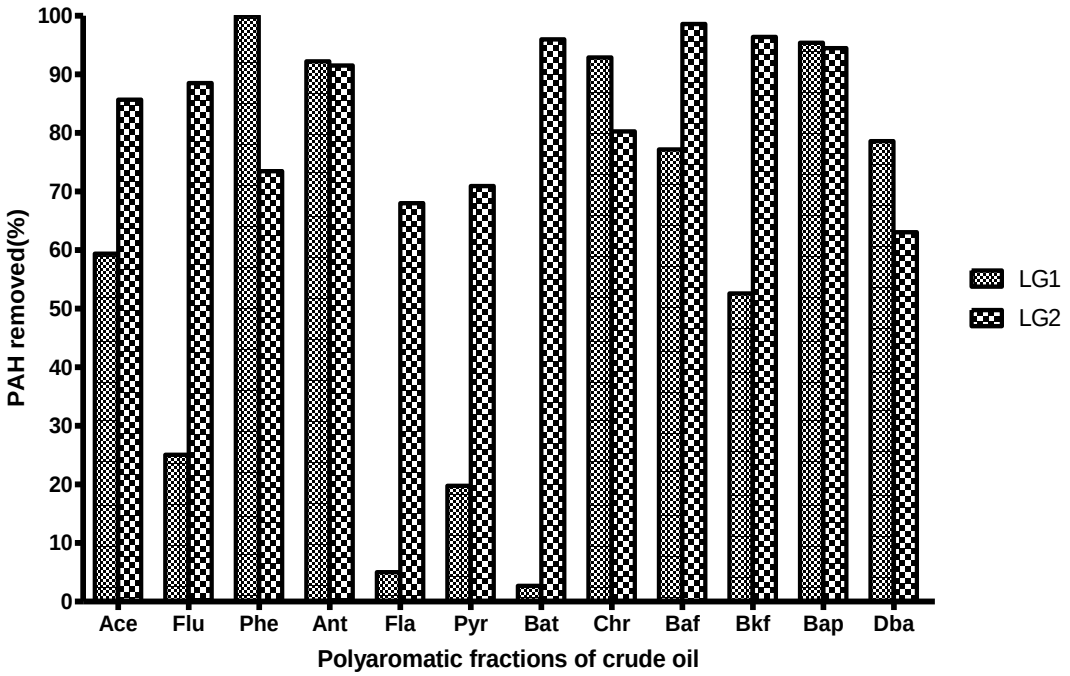

Figure 2. Percentage degradation of some aromatic components of weathered crude oil after 21 day incubation period with *E. cloacae* strain LG1 and *B. cepacia* strain LG2. Ace – acenaphthene, Flu – fluorene, Phe – phenanthrene, Fla – fluoranthene, Pyr – pyrene, Bat – benzo(a)anthracene, Chr – chrysene, Baf – benzo(a)fluoranthene, Bkf – benzo(k) fluoranthene, Bap – benzo(a)pyrene , Dba – dibenzo(b)anthracene.

both anthracene and benzo (a) pyrene by both LG1 and LG2. LG2 degraded most PAHs better than LG1, with 85.67% degradation of acenaphthylene, 88.51% of fluorene, 95.96% of benzo(a)anthracene, 98.61% of benzo(a)fluoranthene, and 96.41% of benzo(k)fluoranthene. The corresponding values for LG1 were 59.40, 25.06, 2.70, 77.19, and 52.61%, respectively. Statistical analysis showed that there was a significant difference ($P < 0.05$) between the percentage degradation of the various PAHs by the two isolates

DISCUSSION

Biodegradation is the major mechanism for the removal of petroleum pollutants from environmental compartments. In this regard, bacteria and fungi play by far the most important role. Whereas detection of the organisms involved in degradation can be achieved by non-cultural means, enrichment and isolation of degraders in pure culture remains an important technique for proper characterization of these organisms, correlation of biochemical properties to specific genes and, more importantly, obtaining seeds for bioremediation purposes (Hilyard et al., 2008).

The organisms isolated in this study are members of groups that have been well reported in the literature as hydrocarbon degraders. Although *E. cloacae* is an enteric organism, there are many reports of the ability of strains of this species to degrade environmental pollutants (Binks et al., 1996; Saadoun et al., 1999). Saadoun (2002) also isolated from a crude oil polluted site a strain

of *E. cloacae* capable of growing on diesel (0.1%).

Burkholderia c was formerly known as *Pseudomonas cepacia* and only recently reclassified on the basis of 16s rRNA analysis (Wackett and Hershberger, 2001), and since then there have been an avalanche of reports of strains of this species capable of growth on hydrocarbons. Okoh et al. (2001) reported the degradation of heavy crude oil (Maya) by *B. cepacia* Strain RQ1. There are other reports of *burkholderia* spp. capable of remarkable growth on aromatic fractions of crude oil (Kim et al., 2003; Nnamchi et al., 2006; Somtrakoon et al., 2008).

Microbial degradation of crude oil is often shown to occur by attack on alkanes or light aromatic fractions, while higher molecular weight aromatics, resins and asphaltenes are considered recalcitrant (Lal and Khana, 1996). Usually there is a positive correlation between increase in size of PAH molecule, and its angularity, and its hydrophobicity and stability. However, majority of the components of crude oil are ultimately biodegradable (Prince et al., 1994).

E. cloacae LG1 exhibited very low growth rates and higher generation times than Pseudomonas strain P11 which we recently reported (0.304 d^{-1} and 2.28 d) (Obayori et al., 2009b). But it is equally noteworthy that lower growth rates and higher doubling times than those reported here were observed for four Pseudomonas strains from the same environment (Obayori et al., 2009a). These organisms exhibited growth rates of 0.08 – 0.21 d^{-1} and generation times 3.27- 8.55 d. The lower order of magnitude of population increase observed in LG1 and LG2 may be attributed to the inability of the organisms to fully process the degraded components for biomass production. It could also mean that the organism did more of mineralisation than biomass production.

The biphasic growth exhibited by LG2 may be due to sequential utilisation of the more readily degradable components and the more recalcitrant fractions.

The degradation of oil in this study was concomitant with decrease in pH from 7.01 to 6.51. This may not be unconnected with the fact that degradation of hydrocarbon compounds, usually leads to production of organic acids, which invariably leads to lowering of pH (Adebusoye et al., 2007). However, the slight decrease in pH may be accounted for by the fact that the medium is buffered.

Percentage degradation for LG1 and LG2 were 52.33 and 80.15% respectively. The value for LG2 is remarkable as single organisms are generally only able to degrade limited range of hydrocarbons (Marin et al., 1996). During degradation of crude oil by *Acinetobacter lwoffi* and *P. aeruginosa*, more than 60% of the oil was depleted by the pure strain during 20 day incubation period (Adebusoye et al., 2007). The result obtained for LG2 is similar to what we previously reported for pseudomonads (Obayori et al., 2009a; Obayori et al., 2009b). Okoh et al. (2001) had also reported the degradation of the non-asphaltic fraction of heavy crude oil (Maya) by *B. cepacia* strain RQ1by about 89%.

Katsivela et al. (2003) reported the ability of *Enterobacter* sp. strain EK 3.1 to grow on mixture of aliphatic as well as monoaromatic and polyaromatic hydrocarbons, including toluene, acenaphthene and acenaphthalene. Juhazs et al. (1997) isolated three strains of *B. cepacia* capable of growing on pyrene as sole source of carbon and energy. These organism degraded fluorene, phenanthrene, and pyrene (100 mg/l) to undetectable levels within 7 to 10 d. They also cometabolised benzo(a)anthracene, benzo(a)pyrene and dibenzo(a,h)anthracene in the presence of phenanthrene.

The higher the growth rate, the higher the percentage degradation of oil and the ability to considerably reduce the aromatic components of weathered crude oil by *B. cepacia* LG2 makes it a candidate for consideration for bioaugmentation.

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