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Biodegradation of Bonny Light Crude Oil by Bacteria Isolated from Contaminated Soil

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ABSTRACT

Two hydrocarbon degraders isolated from an abandoned coal power plant soil in Lagos, Nigeria, were tested. The isolates were identified as belonging to the genus *Pseudomonas*. *P. putida* G1 exhibited strong degradative ability on crude oil, kerosene, fair ability on engine oil, brake oil and petrol and a weak ability on pyrene, while *P. aeruginosa* K1 had corresponding values of 0.21days-1 and 3.31 days. The amount of crude oil degraded by strain G1 and K1 after a 21 day incubation period were 92.05% and 90.89% respectively. Data obtained from gas chromatographic analysis of oil recovered from culture fluids of G1 and K1 confirmed a near-disappearance of aliphatic fractions and a significant reduction of aromatic fractions in the hydrocarbon mixture. © 2010 Friends Science Publishers

Key Words: biodegradation; Crude oil; Hydrocarbons; Pollution; *Pseudomonas* strains

INTRODUCTION

Crude oil is a complex mixture of hydrocarbons, composed of aliphatic, aromatic and asphaltene fractions along with nitrogen, sulfur and oxygen-containing compounds (Jain *et al*., 2005). The constituents of these hydrocarbon compounds are present in varied proportions resulting in high variability in crude oil from different sources (Speight, 1999). Contamination of soils and aquifers by oil spills is a persistent and widespread pollution problem ravaging almost all compartments of the environment and imposing serious health implication and ecological disturbances (Bundy *et al*., 2002; Okoh, 2006). Nigeria, a major producer of petroleum, is enmeshed in serious environmental pollution problems arising from petroleum exploration, exploitation, transportation and consumption coupled with inadequate maintenance of oil pipelines resulting in seepages and ruptures (Okerentugba & Ezeronye, 2003). This problem has become compounded in recent years by increasing sabotage and vandalization of pipelines by restive oil communities particularly in the Niger Delta area of the country. Petroleum pollutants are not only toxic to biological component of the environment, but some hydrocarbon components are indeed carcinogenic and mutagenic, with immunomodulatory effects on humans, animals and plant life (Miller & Miller, 1981; Obayori *et al*., 2009b). Mechanical and chemical methods for remediation of hydrocarbon-polluted environment are often expensive, technologically complex and lack public acceptance (Vidali, 2001). Thus, bioremediation remains the method of choice for effective removal of hydrocarbon pollutant in the environment (Okoh & Trejo-Hernandez, 2006).

Hydrocarbon degrading bacteria and fungi are mainly responsible for the mineralization of oil pollutants and are distributed in diverse ecosystems (Leahy & Colwell, 1990). Gram negative bacteria such as species of *Pseudomonas*, *Sphingomonas*, *Acinetobacter*, *Flavobacterium*; Gram positive bacteria with low G+C such as *Bacillus*, *Micrococcus* and the high G+C particularly the Actinomycetes such as species of *Arthrobacter*, *Nocardia*, *Rhodococcus* and *Streptomyces* have been implicated in the mineralization of hydrocarbon pollutants (Leahy & Colwell, 1990; Amund, 2000; Okoh, 2006).

It is uncommon to find organisms that could effectively degrade both aliphatics and aromatics possibly due to differences in metabolic routes and pathways for the degradation of the two classes of hydrocarbons. However, some reports have suggested the possibility of bacterial species with propensities for degradation of both aliphatic and aromatic hydrocarbons simultaneously (Amund *et al*., 1987; Obayori *et al*., 2009b). This rare ability may be as a result of long exposure of the organisms to different hydrocarbon pollutants resulting in acquisition of appropriate degradative genes. In this study, we report the degradative potentials of two *Pseudomonas* species isolated from an abandoned coal power plant soil on Bonny Light crude oil.

MATERIALS AND METHODS

Sampling: Soil samples for this study were collected from an abandoned coal power plant soil in the premises of

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Power Holding Company (PHC) formerly National Electric Power Authority (NEPA) yard at Ijora-Olopa, Lagos. The site has a long history of contamination due to the use of coal plant for electric power generation in the 1950s. Soil samples were collected at a depth of 10–12 cm using sterile trowel after clearing debris from the soil surface. Samples for physico-chemical analyses were collected in polyethylene bags, while those for microbiological analysis were collected in sterile screw-capped bottles. Analysis commenced immediately upon arrival in the laboratory. Leftover samples were refrigerated at 4°C.

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 content, organic content, total nitrogen content, potassium content and available phosphorous were determined as described previously (Bray & Kurtz, 1945; Black, 1965; Chopra & Kanwar, 1998). Conductivity was determined using a conductivity cell (PW 9504 Philips) with a cell constant of 1.2, while the heavy metal content of the soils was determined using atomic absorption spectrophotometer (Alpha 4, AAS) following mixed acid digestion and extraction of the soil samples. The total hydrocarbon content of the soils was extracted using n-hexane: dichloromethane solvent systems (1:1).

Microbiological analysis of soil samples: The total heterotrophic bacterial and fungal counts were enumerated by plating aliquots (100 µL) of appropriate diluted soil samples on nutrient agar and acidified potato dextrose agar containing streptomycin (1 mg/100 mL), respectively. All plates were incubated aerobically at room temperature (29±2°C) and counted after 24 h and 48 h for bacteria and fungi, respectively. Similarly, the population of hydrocarbon-utilizers was estimated on mineral salts medium (MSM) formulated by Kästner *et al.* (1994). The medium contained per litre 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 oil served as the sole carbon and energy source and made available to the cultures through vapour-phase transfer (Raymond *et al*., 1976). Plates were counted after incubation at room temperature for 5-7 days. The percentage of hydrocarbon-utilizers relative to the heterotrophic population for each time point was subsequently determined.

Enrichment and isolation of hydrocarbon degrading bacteria: Bacteria able to degrade crude oil were isolated on crude oil amended mineral salts medium (MSM) by continual enrichment method. The MSM of Kästner *et al*. (1994) described above was used. Contaminated soil sample (5.0 g) was added to 45 mL of MSM containing crude oil (Bonny light) at 1% (v/v) as the sole source of carbon and energy. Enrichment was carried out with shaking at room temperature for 4 weeks until there was turbidity. After 3 consecutive transfers, hydrocarbon degraders were isolated by plating out dilutions from the final flask on Luria-Bertani (LB) agar. The colonies that appeared were further purified on LB agar and screened for crude oil utilization. Pure isolated were maintained at -20°C in glycerol: LB broth medium (50:50).

Identification and characterization of isolates: Pure cultures of bacterial isolates were identified on the basis of their colonial morphology, cellular morphology and biochemical characteristics according to the taxonomic scheme of Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994).

Substrate specificity of isolates: Abilities of the isolates to grow on varieties of hydrocarbon substrates were evaluated in liquid media supplemented with 50 ppm of respective hydrocarbons as a sole carbon and energy source. Incubation was carried out under similar conditions as described above. Degradation was monitored by cell increases and visual observation for turbidity coupled with disappearance of the oil slick in case of petroleum cuts. The hydrocarbons tested include 2-naphtol, anthracene, pyrene, biphenyl, crude oil, petrol, engine oil, brake oil and kerosene. Liquid hydrocarbons were autoclaved and separately added to sterile MSM at 0.1% (v/v). Inocula were 24-h LB-grown cultures and inoculation was carried out to achieve initial optical density of 0.05 (OD_{500}). Incubation was carried out for 14 days.

Growth kinetics: Mean generation times (T_d) and specific growth rates (µ) of the two isolates on crude oil were calculated using non-linear regression of growth curves for the period when growth rates were maximal using prism version 5.0 (Graphpad software, San Diego, CA).

Extraction of residual oil: Residual oil was extracted by liquid-liquid extraction as described by Adebusoye *et al*. (2007). Briefly, equal volume of hexane (20 mL) was added to broth culture in flask and shaken thoroughly. After removing the aqueous phase with separating funnel, the organic fraction was reconstituted to 5 mL and the residual oil concentration was determined by gas chromatography. Control flasks were also extracted similarly.

Analytical method: Hexane extracts (1.0 µL) of residual oil 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 250°C and 350°C, respectively. The column temperature was programmed at an initial temperature of 70°C; this was held for 2 min, and then ramped at 10°C/min to 320°C and held for 10 min.

RESULTS

Physicochemical properties of soil: The polluted soil had a

pH that was slightly acidic (5.4) with a moisture content of about 11%. The total hydrocarbon content (THC) of the soil was high with a value of 13382.1 mg/kg, while the detection of lead in the soil further confirmed the extent of pollution of the site (Table I).

Microbial characterization of the study area: The microbial load of the polluted soil is presented in Table I. The result shows a total heterotrophic bacterial (THB) and fungal counts (THF) of 6.18 x 10^7 cfu/g and 6.09 x 10^7 cfu/g, respectively in the polluted soil. Additionally, it was observed that the proportions of hydrocarbon utilizing bacteria (HUB) and fungi (HUF) within the heterotrophic communities of the polluted soil were generally less than 1.0% (0.78%, 0.74%).

Identification of bacterial strains: The enrichment of the polluted soil with crude oil led to the isolation of several hydrocarbon degraders. Two bacterial strains with the best degradative abilities on crude oil based on visual observation for turbidity were used for this study. The two isolates were Gram negative, motile rods, which were positive for oxidase, catalase, nitrate reduction and citrate utilization. They failed to ferment most sugars tested. Strain G1 was indole negative and was able to ferment fructose and xylose. It exhibited positive reaction to methyl red and Voges-Proskauer and could not grow at 42°C and failed to produce any pigment. It was thus identified as *Pseudomonas putida*. Strain K1 was positive for gelatin hydrolysis and was able to ferment cellobiose. It did not ferment xylose and was methyl red and Voges-Proskauer negative It produced pyocyanin and grew at 42°C. It was thus identified as *Pseudomonas aeruginosa*.

Substrate susceptibility of isolates: Strain K1 showed luxuriant growth on crude oil, petrol, kerosene, brake oil and engine oil. It grew moderately on anthracene but failed to grow on 2-naphtol, biphenyl and pyrene. Strain G1 degraded petrol, engine oil and brake oil moderately, but exhibited luxuriant growth on crude oil and kerosene. However, G1 fail to degrade all the PAHs tested, except pyrene on which moderate growth was observed (Table II).

Growth kinetics of isolates on crude oil: Following a 21 day incubation of the two isolates on crude oil as sole carbon and energy source, significant increases in cell densities were observed with concomitant decreases in different components of the crude oil (Table III, Figs. 1 & 2). Strain G1 exhibited specific growth rate and mean generation time of 0.12 $days^{-1}$ and 5.78 days, respectively. However, higher specific growth rate and lower mean generation time were observed in strain K1 with values of 0.21 $days^{-1}$ and generation time of 3.31 days. Interestingly, strain G1 with the least growth rate displayed higher degradative ability with 72% of the oil degraded in 12 days as compared to 69% obtained with K1 during the same period. At the end of the 21 days cultivation period, more than 92% of the oil was consumed by G1, a value slightly higher than ~ 91% obtained with K1.

Table I: Physicochemical and microbiological properties of the polluted soil

Parameter	Value
Moisture content (%)	11.1
pH	5.4
Conductivity (µS/m)	67.4
Chloride (mg/kg)	68.0
Total organic carbon (mg/kg)	3.1
Total hydrocarbon content (mg/kg)	13382
Potassium (mg/kg)	18.4
Total nitrogen (%)	0.18
Available phosphorus (mg/kg)	363.4
Sodium (mg/kg)	542
Lead (mg/kg)	4.7
Total heterotrophic bacteria (cfu/g)	6.18 x 10^7
Total heterotrophic fungi (cfu/g)	6.09 x 10^7
Hydrocarbon utilizing bacteria (cfu/g)	4.85 x 10^5
Hydrocarbon utilizing fungi (cfu/g)	4.48 x 10^5

Table II: Substrate susceptibility of isolates on various components of crude oil

Substrate	Isolates	
	***P. putida* (G1)**	***P. aeruginosa* (K1)**
Crude oil	+++	+++
Petrol	++	+++
Kerosene	+++	+++
Engine Oil	++	+++
Brake Oil	++	+++
2-naphtol	-	-
Anthracene	-	+
Pyrene	+	-
Biphenyl	-	-

+++: luxuriant growth; ++: very good growth; +: poor growth; -: no growth
Cultures were incubated for 14 days
All substrates were supplied at a concentration of 50 mg/L

Table III: Growth kinetics of bacterial strains on crude oil

Isolate	Specific growth Rate (day^{-1})	Mean generation Time(day)	% degradation*	Degradation Rate (%/day)
P. putida (G1)	0.12	5.78	92.05	4.38
P. aeruginosa (K1)	0.21	3.31	90.89	4.32

*Percent degradation values represent the net decrease (FID area counts) calculated with reference to the amount recovered from heat killed control tubes

DISCUSSION

The use of autochthonous microorganisms inhabiting hydrocarbon-polluted niches for biodegradation and bioremediation has been widely accepted as a formidable approach due to avalanche of successes recorded by various researchers. The mechanisms of adaptation employed by the autochthonous microorganisms to achieve this feat includes synthesis of inducible enzymes, mutations such as single nucleotide change or DNA re-arrangement that results in degradation of the compound and acquisition of genetic information from closely related or phylogenetically distinct population within the hydrocarbon-challenged community

Fig. 1: Gas chromatographic fingerprints of Bonny Light crude oil recovered from flasks inoculated with heat-inactivated cells of *Pseudomonas* strain G1 (A), experimental flasks containing G1 after incubation for 12 days (B), and experimental flasks containing G1 after incubation for 21 days (C)

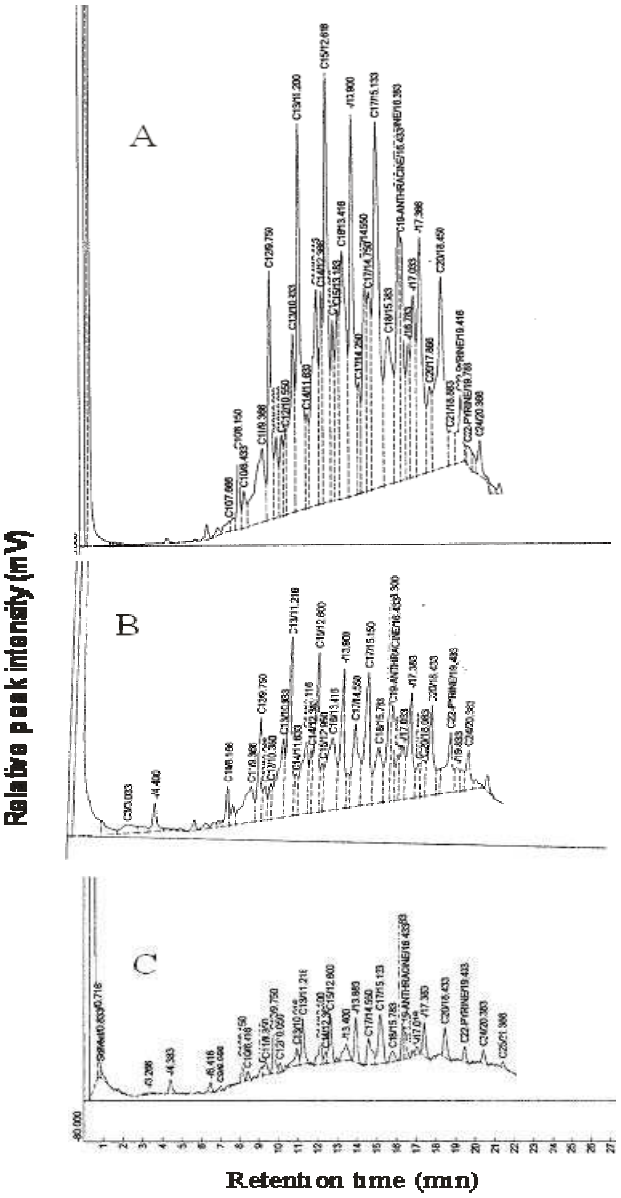
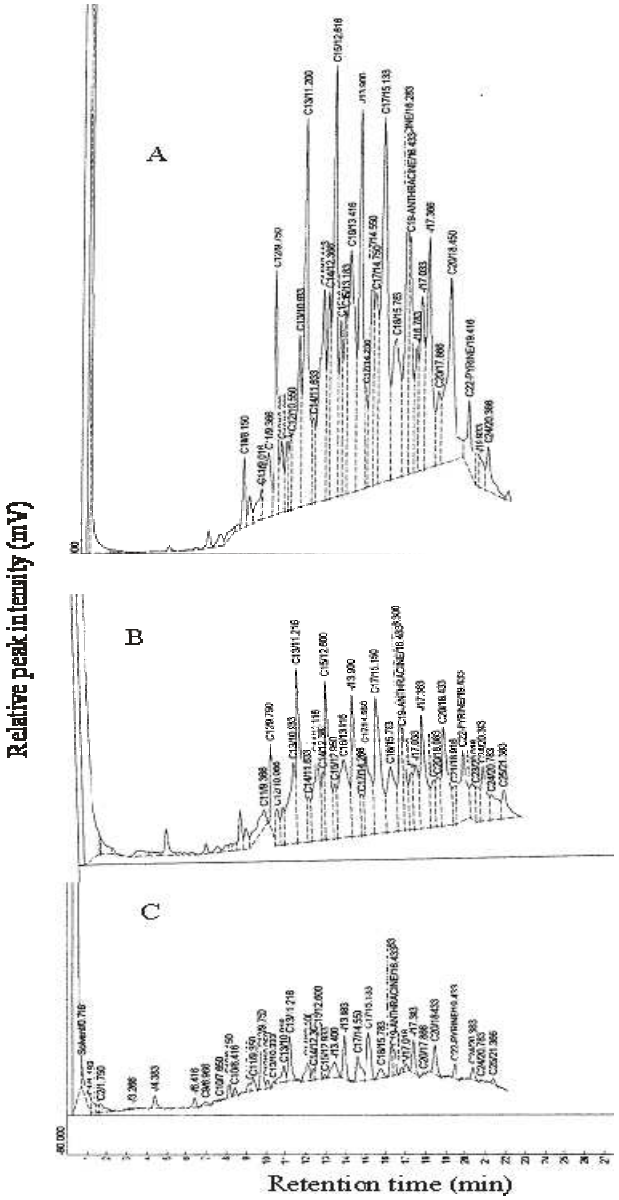


Fig. 2: Gas chromatographic fingerprints of Bonny Light crude oil recovered from flasks inoculated with heat-inactivated cells of *Pseudomonas* strain K1 (A), experimental flasks containing K1 after incubation for 12 days (B), and experimental flasks containing K1 after incubation for 21 days (C)



through horizontal gene transfer (HGT) (Top & Springael, 2003).

The present study examined the biodegradative ability of two bacterial strains isolated from an abandoned coal power plant soil on bonny light crude oil. The two isolates were identified as species of *Pseudomonas* and exhibit the ability to grow on both the aliphatic and aromatic fractions of petroleum. Earliest reports have indicated the catabolic versatility and genetic plasticity of *Pseudomonas* species and their ubiquity in hydrocarbon polluted environments (Chakrabarty, 1972; Dunn & Gunsalus, 1973; Atlas, 1981). The genus *Pseudomonas* is reputed to possess broad substrate affinity not only for different classes of hydrocarbons such as alkanes, alicyclics, heterocyclics and aromatics (Vankateswaran *et al*., 1995; Nojiri *et al*., 1999; Obayori *et al*., 2008) but also for a plethora of xenobiotic compounds (Amund & Adebiyi, 1991; Habe *et al*., 2001; Wackett & Hershberger, 2001).

The aliphatic fraction, consisting of straight chain, branched chain and cyclic chain carbon moieties, is the

major constituent of crude oil (Jain *et al*., 2005) and is the most readily degraded hydrocarbon compounds with species of *Pseudomonas*, *Acinetobacter*, *Arthrobacter*, *Burkholderia* etc., as common culprits (Mishra *et al*., 2001; Bhattacharya *et al*., 2003). As indicated in the GC fingerprints of the two isolates on crude oil for 21 days, the C_{10}-C_{14} fractions of the crude oil was nearly completely utilized by the *Pseudomonas* species with a drastic reduction in the major peaks C_{15}, C_{17}, C_{19} and C_{20} (as shown in panel A-C of Figs. 1 & 2). This observation give credence to the fact that aromatic components of hydrocarbons (especially higher aromatic hydrocarbons) exhibit high degree of recalcitrance due to their stability, hydrophobicity and high resonance energy informed by their benzene backbone. Significant reductions in concentration of naphthenes and aromatics components of the crude oil as shown in the GC fingerprints indicates the catabolic versatility of the two *Pseudomonas* isolates. This observation may not be unconnected to the fact that acquisition of intrinsic abilities to degrade crude oil components by the *Pseudomonas* species must have been, because of long exposure to the pollutants resulting in induction of appropriate degradative genes.

It is widely believed that individual organisms could only metabolize limited range of hydrocarbon substrates (Britton, 1984; Adebusoye *et al*., 2007). This has led to the assertion that mixed culture exhibited superior degradative competence than pure culture strains (Leahy & Colwell, 1990; Adebusoye *et al*., 2007). However, Obayori *et al.* (2009a) reported the isolation of a *Pseudomonas* strain MVL1 from a mechanic workshop in Lagos that degraded the aliphatic and aromatic fractions of Bonny Light crude oil after 18 days of incubation with the GC fingerprints showing a near total disappearance of these fractions. In the present study, we report more than 90% degradation of Bonny Light crude oil by each of the two *Pseudomonas* isolates at 1 g/L concentration over a period of 21 days incubation with a near total disappearance of the aliphatic fractions and significant reductions in aromatic fractions. This result is similar to that of Obayori *et al.* (2009b), which showed that *Pseudomonas* strain LP1 a biosurfactant producer degraded 92.34% of crude oil in 21 days. It is also noteworthy that comparatively Escravos crude oil degradation rates of only 60 to 66% were reported by Adebusoye *et al*. (2007) for pure culture strains isolated from polluted tropical streams after 20 days of incubation.

The substrate spectrum analysis of the two *Pseudomonas* isolates on various hydrocarbon substrates used in this study showed different utilization patterns. This may be attributed to the varied composition of the substrates and the diverse nature of hydrocarbon products present at the site from which the isolates were recovered (Leahy & Colwell, 1990). Crude oil, a complex mixture of different chemical composition may favorably support growth of microorganisms better than refined petroleum products such as brake oil and engine oil due to diverse of nutrients options available in crude oil as source of carbon and energy. Another reason may be that at the abandoned coal power plant soil, where these organisms were isolated, different types of oil products may have been used for lubrication and fuelling of the coal plant, which inevitably found their way into the soil along with products of coal combustion. This often results in adaptation of the autochthonous organisms to the pollutants due to selective pressure and acquisition of degradative abilities (Wackett & Hershberger, 2001).

In conclusion, this study has confirmed the biodegradative abilities of *Pseudomonas* species, their ubiquity in hydrocarbon polluted environments and their potential for bioremediation of hydrocarbon-polluted sites. Further works to determine the optimum environmental conditions favorable for their application in bioremediation will be the focus of our future research.

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