

Hydrocarbon Degrading Potentials of Bacteria Isolated from a Nigerian Bitumen (Tarsand) Deposit

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Abstract: In an effort at developing active microbial strains that could be of relevance in bioremediation of petroleum contaminated systems in Nigeria, fifteen hydrocarbon degrading bacteria and fungal species were isolated from three bitumen deposits. The predominant species belonged to the genera *Pseudomonas* and *Aspergillus*. The ability of *Pseudomonas stutzeri*, *Pseudomonas mullei* and *Alcaligenes* sp. to degrade naphthalene, kerosene and diesel was studied. The results show maximal increase in optical densities and total viable counts concomitant with decrease in pH of the culture media. Typical generation times vary between 0.64 and 1.09 d, 0.97 and 3.03 d, 0.88 and 2.97 d respectively for kerosene, diesel and naphthalene. All the isolates utilized the hydrocarbons as sole carbon and energy sources equally well; there was no statistical difference ($P > 0.05$) in the utilization rates, thus suggesting close genetic similarities in respect of oil degradation capabilities. The study revealed for the first time, the types of microorganisms that are associated with Nigerian bitumen deposit and their potential to degrade oil. The knowledge of the potentials of these isolates to degrade hydrocarbons will increase the possibilities of developing models and strategies for removing hydrocarbon pollutants from the environment. [Nature and Science. 2006;4(3):51-57].

Keywords: bacteria; bitumen; degrade; Nigerian; hydrocarbon

Introduction

Tarsand is composed of sand, heavy oil and clays that are rich in minerals and water. The heavy oil in tarsand is called bitumen. Nigeria is richly endowed with many natural resources; the most important are crude oil, gas and bitumen. Of all the three natural resources, only the crude oil has been well developed and it contributes about 90 % of the country's foreign earnings (Adegoke et al., 1991). The Nigerian bitumen belt lies on the onshore areas of the Eastern Dahomey (Benin) Basin. The probable reserve of bitumen and heavy oil in the entire Nigerian belt is about 120 x 4.3 Km (Adegoke and Ibe, 1982).

The clay content of the Nigerian deposit is very low averaging about 5 % and heavy oil extracted from the deposits has an API gravity ranging between 5.00° and 14.6°. Physical properties reported include softening point (44 -52 °C), ductility (0.1 – 1.3 mm), penetration (80 – 100 mm), hydrocarbon content (7.2 % by wt - 18.2 % by wt), resins (32.12 % by wt - 34.0 % by wt) and sulphur (5.00 ppm to 10.00 ppm). Furthermore, the Nigerian bitumen possesses relatively large quantity of naphthenes, aromatics and asphaltenes that are similar to the conventional oil. This makes the Nigerian bitumen a very useful alternative source of petroleum hydrocarbon and a potential feedstock for petrochemical industries (Adegoke et al., 1991).

The high demand for petroleum and associated products during the last ten decades has made petroleum spills inevitable consequences of oil production and refining. Despite fluctuations in its prices, oil will remain a major source of energy in the next several decades because a reliable alternative has not yet been found. As a result, the problem of pollution during production and transportation of oil would remain a major issue. Microbial degradation appears to be the most environmentally friendly method of removal of oil pollutant since other methods such as surfactant washing and incineration lead to introduction of more toxic compounds to the environment. Hydrocarbon-degrading microorganisms are widely distributed in marine, freshwater, and soil ecosystems (Atlas and Bartha, 1973). The ability to isolate high numbers of certain oil-degrading microorganisms from an environment is commonly taken as an evidence that those organisms are the active degraders of that environment (Okerentugba and Ezeronye, 2003). Although, hydrocarbon degraders may be expected to be readily isolated from a petroleum-polluted environment, the same degree of expectation may be anticipated for microorganisms isolated from a total unrelated environment. There is an extensive body of knowledge on mineralization or degradation of hydrocarbons by microorganisms (Ojomu et al., 2005; Adenipekun and Fasidi, 2005). Most of these organisms, majority of

which are bacteria have been characterized and classified using cultural, biochemical and molecular techniques. Bacteria have evolved millions of years ago regulatory systems that ensure the synthesis of enzymes so that the initial attack on these compounds is induced only when required.

In a recent study, Ojo (2006) reported the capability of native bacterial population to mineralize petroleum hydrocarbons in wastewater. Similarly, Okoh (2003) demonstrated degradation rates of different strains of *Pseudomonas aeruginosa* on crude oil with evidence of significant reduction of major peak components of the oil. The organisms also showed multiple antibiotic resistance thus, the authors concluded that this could be an important factor to consider in their eventual use in bioremediation program. Virtually all of these studies utilized organisms isolated from petroleum contaminated sites and degradation competencies were only tested on crude oil. In this report however, we chose to isolate hydrocarbon degraders from bitumen deposits in Nigeria. Degradation potentials of the obtained isolates were not only demonstrated on crude oil, and petroleum feedstocks including kerosene, and diesel but naphthalene which is usually less degradable compared to the petroleum fractions. The results from this investigation would be useful for the prediction of the bioremediation mechanisms of these microbial isolates.

Materials and Methods

Chemicals

Escaros blend crude oil, petroleum feedstocks including kerosene and diesel obtained were all obtained from Chevron (Nigeria) Limited. Naphthalene was provided by the Department of Botany and Microbiology, University of Lagos.

Collection of bitumen and soil samples

The bitumen used was obtained from three different communities namely, Agbabu, Ilubirin and Mile 2, all in Odigbo Local Government Area of Ondo State, Nigeria. Three sets of samples were collected from these locations i) "core bitumen" represented bitumen samples seeping out directly from installation pipes; ii) "surface bitumen" represented bitumen samples collected from surfaces of installed pipes and; iii) "control samples" represented relatively undisturbed soils in the vicinities of these sampling stations. All samples were collected in two replicates and treated immediately.

Isolation and characterization of hydrocarbon-degrading microorganisms

The bacterial species indigenous to the bitumen and soil samples were isolated by spread plate technique using 0.1 ml aliquots of appropriate dilution into

nutrient agar slants. Individual cultures were identified by morphological and biochemical techniques using the taxonomic scheme of Bergey's Manual of Determinative Bacteriology (Holt et al., 1994).

The fungi species were also isolated from the samples in similar manner to bacteria but using potato dextrose agar (PDA) into which streptomycin (50 mg/ml) has been added to suppress bacterial growth. The microscopic and macroscopic features of the hyphal mass, morphology of cells and spores, nature of the fruiting bodies among other criteria were used for identification.

The obtained cultures of bacteria and fungi were screened for hydrocarbon utilization using crude oil as model substrate.

Growth of bacterial isolates on hydrocarbon substrate

Time-course degradation of kerosene, diesel and naphthalene was performed in mineral salts medium previously described by Mills et al. (1978). The medium was dispensed in 99 ml quantities into 250 ml Erlenmeyer flasks. Each flask was supplemented with 1 ml or 1 g as the case may be of selected carbon source and seeded with axenic cultures of isolates. The flasks were cultivated in a gyratory shaker incubator programmed at 120 rpm and 30 °C. The optical density (OD_{600nm}), total viable count (TVC) and pH of the culture fluids were monitored at determined time intervals as biodegradation indices.

Results and Discussion

The morphological and biochemical characterization of the bacterial isolates obtained from the different sites revealed the following genera: *Pseudomonas*, *Bacillus*, *Alcaligenes* and *Citrobacter*. All the isolates were motile, rod-shaped and Gram-negative with the exception of *Bacillus subtilis*. Most were indole-negative, urea and citrate utilizers. Majority of the organisms were isolated from the core bitumen while the predominant species were mainly *Pseudomonas* (Table 1). The fungi isolates were mainly *Aspergillus* species. Others were *Trichodema*, *Penicillium*, *Rhizopus* and *Rhodotorula* species (Table 1). All of these isolates were able to grow on crude petroleum as the sole source of carbon and energy when screened for hydrocarbon utilization. Interestingly, this same group of organisms have been implicated in hydrocarbon degradation, particularly *Pseudomonas* and *Alcaligenes* by several workers (Amund and Adebiji, 1991; Atlas, 1992; Nwachukwu and Ugoji, 1995; Nwachukwu, 2001).

Figures 1-3 show the degradation potentials of *Pseudomonas stutzeri*, *P. mullei* and *Alcaligenes* sp. on petroleum cuts and naphthalene under aerobic batch conditions. The growth dynamics of the organisms was

determined by the optical densities, total viable counts and the pH of the culture media. Our results showed that the isolates grew maximally on the four hydrocarbon substrates when supplied as the sole source of carbon and energy. The growth profiles showed that none of the bacterial isolates exhibited lag phases. This observation has been reported previously (Amund, 1984; Okerentugba and Ezeronye, 2003), and can be attributed to genetic make up due to the constitutive expression of hydrocarbon catalysing enzymes or physiological owing to previous exposure to exogenous hydrocarbons present in the bitumen deposits. Typical doubling times and specific growth rates on diesel ranged from 0.97 – 3.05 d and 0.23 – 0.71 d⁻¹ respectively (Table 2).

The utilization of the hydrocarbons resulted in increase in cell densities with a concomitant visual gradual reduction in the oil layer and complete disappearance of the oil with prolonged incubation. The reduction in pH of the culture fluids in the experimental flasks within the 8-day incubation period further confirmed chemical changes of the hydrocarbon substrates which must have been precipitated by microbial enzymes (Atlas and Bartha, 1972). Microbial degradation of hydrocarbons often leads to production of organic acids and other metabolic products (Nwachukwu and Ugoji, 1995; Okpokwasili and James, 1995). Thus, the acids probably produced account for the reduction in pH levels.

Of particular significance was the growth data obtained on naphthalene, an aromatic hydrocarbon that is relatively recalcitrant to aerobic degradation (Table 2). These values are comparable to those obtained for kerosene and diesel. It would appear that these organisms have aromatic degradation capabilities and could be relied upon for the destruction of both alkane and aromatic components inherent in crude petroleum.

On the basis of the growth data, we could infer that kerosene was slightly preferred by the isolates contrary to the reports of Amanchukwu et al. (1989) and Okpokwasili and James (1995). However, statistical analysis showed no significant difference ($P > 0.05$) in the degradation competence of the isolates on the four hydrocarbon substrates, thus suggesting possession of similar catabolic properties. This observation also confirmed the close genetic similarities of isolates in respect of oil degradation capabilities particularly of the *Pseudomonas* strains.

It is evident from this investigation that oil degrading microorganisms could readily be isolated from bitumen deposits without the need for time consuming traditional enrichment protocols. Further understanding of the metabolic process of these organisms on the hydrocarbons will increase possibilities of developing models and strategies for removing hydrocarbon pollutants from oil impacted environment.

Table 1. Hydrocarbon-utilizing microbial species isolated from various samples

ISOLATES	Source
<i>Pseudomonas stutzeri</i>	Core
<i>Pseudomonas putida</i>	Core
<i>Pseudomonas mellei</i>	Surface, core
<i>Bacillus subtilis</i>	Surface, core
<i>Alcaligene sp.</i>	Surface, core
<i>Aspergillus fumigatus</i>	Surface, soil
<i>Aspergillus oryzae</i>	Surface
<i>Aspergillus wentii</i>	Soil
<i>Aspergillus flavus</i>	Soil
<i>Aspergillus niger</i>	Core, surface
<i>Trichodema sp.</i>	Core
<i>Penicillium notatum</i>	Core, surface, soil
<i>Rhizopus stolonifer</i>	Surface
<i>Rhodotorula sp.</i>	Core

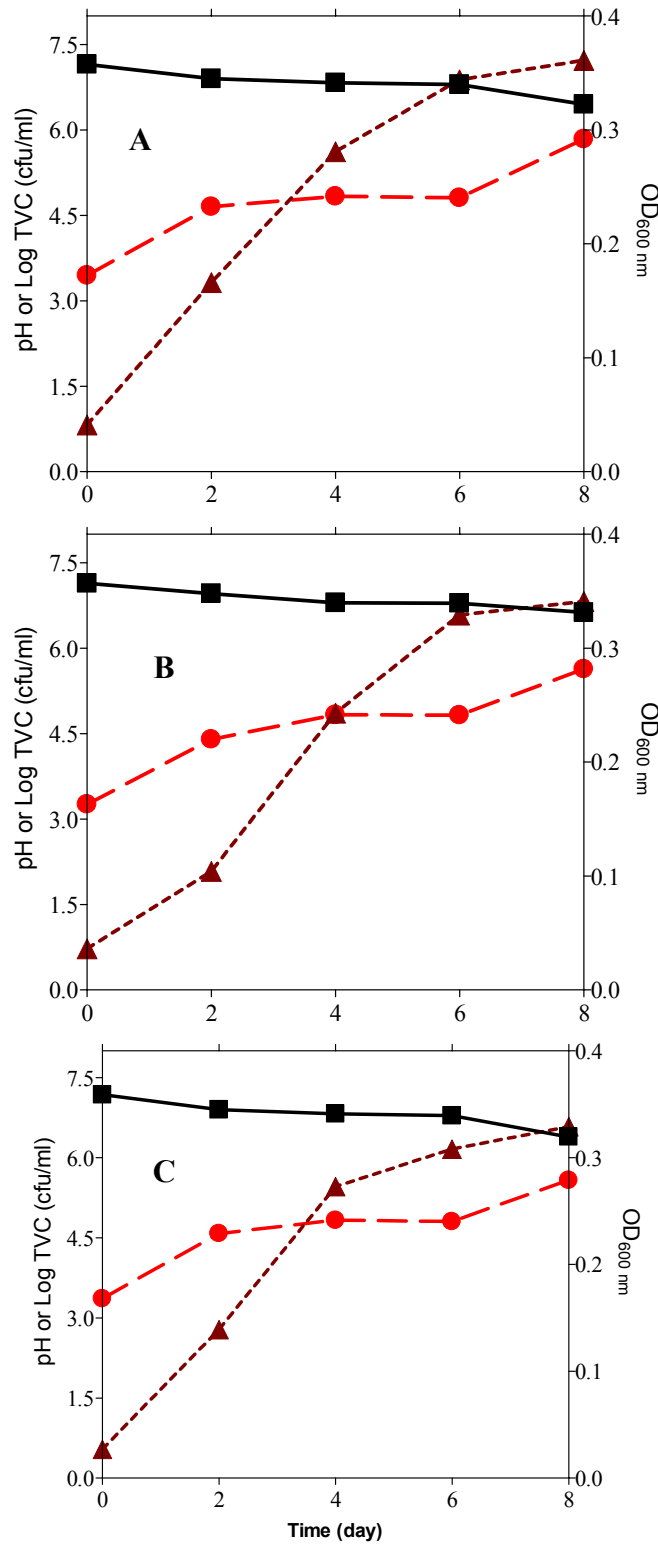


Figure 1. Growth Profiles of *Pseudomonas stutzeri* (A), *Pseudomonas mellei* (B) and *Alcaligenes* sp. on Kerosene. ●, Log TVC (cfu/ml); ▲, OD_{600nm}; ■, pH of culture fluids.

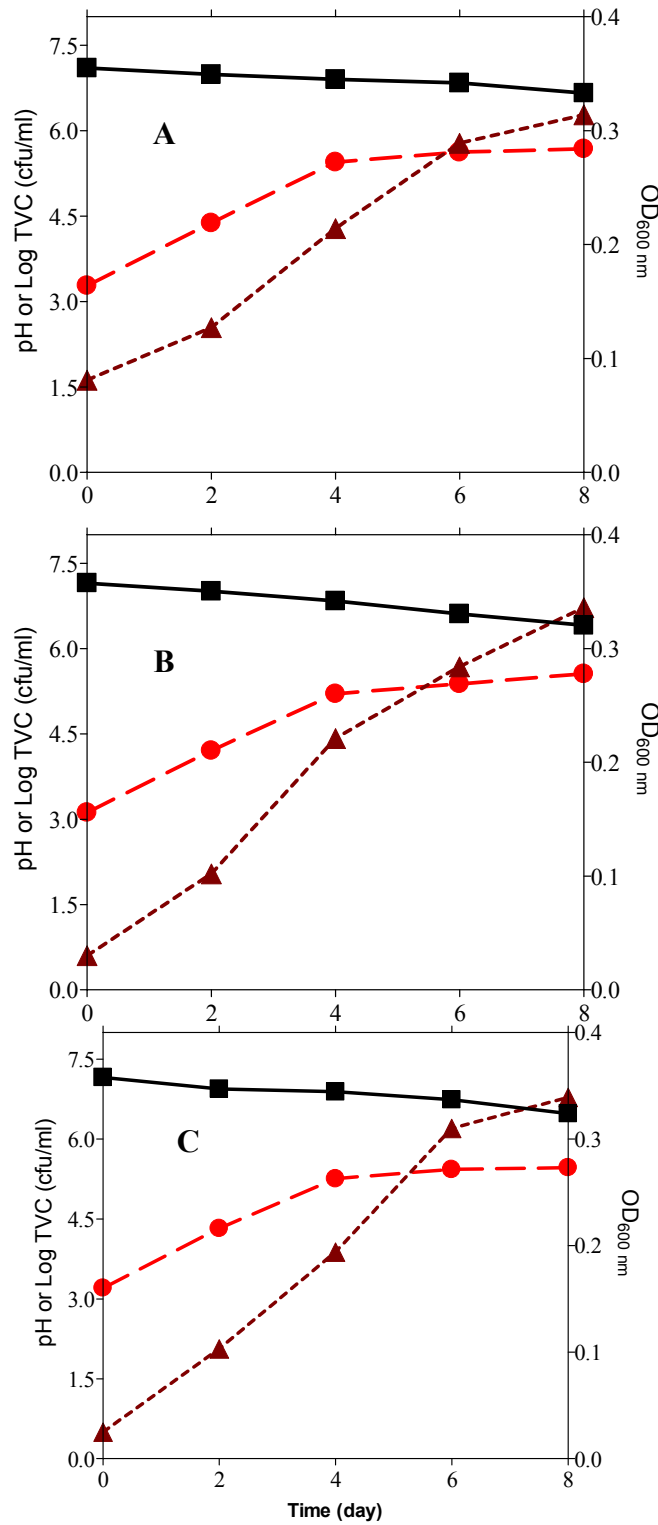


Figure 2. Growth Profiles of *Pseudomonas stutzeri* (A), *Pseudomonas mellei* (B) and *Alcaligenes* sp. on Diesel. ●, Log TVC (cfu/ml); ▲, OD_{600nm}; ■, pH of culture fluids.

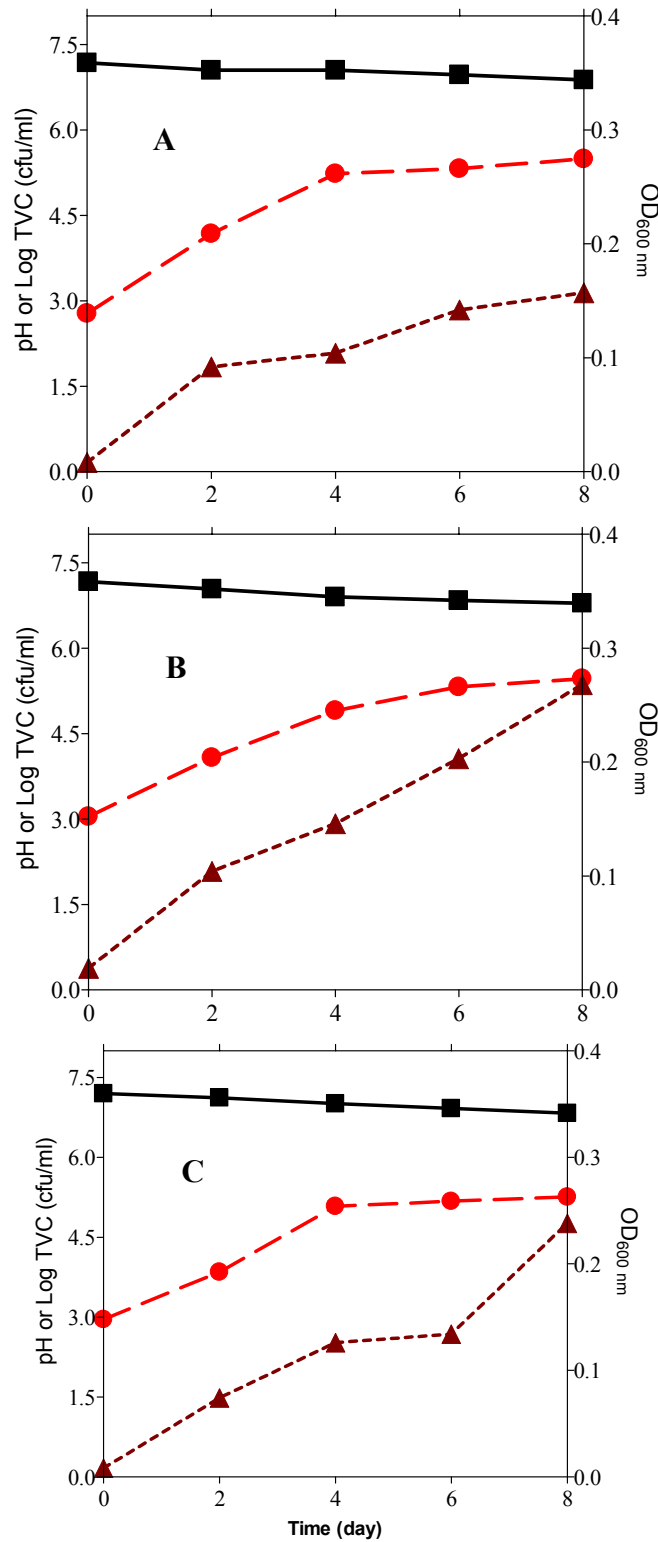


Figure 3. Growth Profiles of *Pseudomonas stutzeri* (A), *Pseudomonas mellei* (B) and *Alcaligenes sp.* on Naphthalene. ●, Log TVC (cfu/ml); ▲, OD_{600nm}; ■, pH of culture fluids.

Table 2. Growth potentials of hydrocarbon-utilizing bacteria

Isolate	Kerosene		Diesel		Naphthalene	
	μ (d ⁻¹) ^a	Tg (d) ^b	μ (d ⁻¹) ^a	Tg (d) ^b	μ (d ⁻¹) ^a	Tg (d) ^b
<i>Pseudomonas stutzeri</i>	1.08	0.64	0.24	2.91	0.79	0.88
<i>Pseudomonas putida</i>	0.79	0.88	0.3	2.34	0.25	2.76
<i>Pseudomonas mullei</i>	0.68	1.02	0.71	0.97	0.71	0.98
<i>Bacillus subtilis</i>	0.67	1.03	0.23	3.05	0.23	2.96
<i>Alcaligenes</i> sp.	0.64	1.09	0.23	3.03	0.23	2.97

^aspecific growth rate, ^bmean generation time

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