Bacterial Utilization of Petroleum in Liquid and Simulated Soil Environments

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Abstract

Ten bacterial isolates designated S1 to S10 were obtained from hydrocarbon. Polluted soils in Nsukka and studied for their hydrocarbonoclastic potentials. After screening for hydrocarbon utilizing capability, isolates S2 and S8, identified as Bacillus subtilis and Pseudomonas aeruginosa respectively, were selected for the study owing to their impressive utilization of crude oil as sole carbon source. Hydrocarbon utilization in liquid medium as assessed by the parameters of optical density (OD_{600}), surface tension and pH showed engine oil as the worst substrate for the growth of the two isolates. S2 grew best on kerosene (maximum OD_{600} = 1.56). Optical density increased progressively with a concomitant fall in medium pH and surface tension for the two isolates during the 96h-experiment. A simulated bioremediation study was conducted using soil samples dispensed in 50.0g weights into 250ml conical flasks and contaminated with different levels (2.5%, 5.0%, 7.5% or 10.0% $^{v}/_{w}$) of Bonny light crude oil. A saturated solution of NPK (15:15:15) compound fertilizer was added at the ratio of crude oil to NPK of 10.0:1.0 and each flask inoculated with 1.0ml of S8 isolate containing $3x10^{8}$ cells. Microbial utilization of the oil in the soil as assessed by the weight of extracted residual oil indicated that the extents of oil removal in the stimulated and/or bioaugmented soils were significantly high in relation to unsupplemented and uninoculated controls.

Keywords: Bacterial utilization, Petroleum in liquid, Petroleum in soil, Environments

Introduction

The petroleum industry has been a major source of ecological problems due to the release of petroleum hydrocarbons into the environment. Crude oil is a complex mixture of thousands of hydrocarbon and non-hydrocarbon compounds. Non-hydrocarbons in petroleum are compounds containing oxygen, sulphur, nitrogen or metals, in addition to hydrogen and carbon.

Petroleum can adversely affect living organisms by smothering, decreased light, oxygen and food availability, and toxic actions (Neff, 1985). Accounts of the effects of oil spills on plants contain reports of the death of mangroves, sea grasses and algae (Baker et al., 1980; Floc'h and Diouris, 1980).

Recovery from the effects of oil spills of most local plant population can require from a few weeks to five years depending on the type of soil, circumstances of the spill and species affected (Baca et al., 1987). Mechanical removal of petroleum from wetlands can increase the recovery time 25 to 50% (Baca et al., 1987).

Oil spill reports also often contain accounts of the death or debilitation of aquatic animals (Harrel, 1990; Crunkilton and Duchrow, 1990; Garrity and Levings, 1990). Petroleum affects animals ranging from invertebrates to mammals (Eisler, 1987; McOrist and Lenghaus, 1992). Bioremedation can be carried out in two ways, viz: bioaugmentation and biostimulation. In bioaugmentation microbial oil degraders are added to the contaminated site to dissimilate the oil pollutant. Biostimulation is carried out by introducing inorganic nutrients to the polluted site to help indigenous microorganisms clean up the area. The only shortcoming associated with bioremediation is the prolonged reclamation time – it takes from a few months to many years to clean up a contaminated site (Oppenheimer, 1999). This reclamation time is dependent on many factors, prominent among which is the degradative capability of the microbes used. The release of crude oil and its products into the environment is also a threat to agricultural land. Petroleum hydrocarbons 'sterilize' the soil and prevent crop growth and yield for varying periods of time (Plice, 1948). Restoration of the fertility of arable lands previously contaminated by spilled petroleum is of overriding importance, and to this end attempts are being made by many nations to reclaim crude oil – polluted agricultural lands.

Methods employed to clean up oil polluted environments are either physical or biological. Physical oil clean – up strategies includes burning, use of detergents and sorbent materials, sinking and mechanical removal. Most of these physical methods have undesirable ecological side-effects. Those that do not impact on the ecosystem have the shortcoming of being capital – intensive. Bioremediation, which is the use of biological agents or their products to reclaim contaminated soils and waters ranks best among other oil clean-up methods because it is both ecofriendly and cost-effective (USEPA, 2001; Adenipekun and Fasidi, 2005).

This work was undertaken to isolate and characterize hydrocarbon-utilizing bacteria from Nsukka soils and study their potentials for hydrocarbon metabolism in both liquid and soil environments with the aim that bacteria with high hydrocarbon-degrading capabilities could be obtained, mass-produced and used as inoculants for the clean-up of spilled oils in the environment.

Materials and Methods

Source of crude oil and crude oil products: The crude oil and crude oil products used were obtained

from the Shell Petroleum Development Company (SPDC), Port-Harcourt River State, Nigeria.

Source of test organism: The source of the organism used as inoculants for the study was soil sample with a long history of petroleum – hydrocarbon pollution collected from automobile servicing workshop.

Isolation of test organism: An enrichment medium was prepared; it consist in g/l of the following: $(NH_4)_2$ SO_4, 1.25; $(NH_4)_2$ HPO_4, 1.95; KH_2 PO_4, 0.85; $MgSO_4$. $7H_2O$, 0.09; $CaCl_2$, 0.001; distilled water, 1.00L. Ten grams of the petroleum hydrocarbon-polluted soil sample was inoculated into the sterile medium and 1.0ml of filter-sterilized kerosene was added and the mixture shake-incubated (Gallenkamp, England) at 100 rpm and 30^0C for seven days.

Thereafter the viable organisms in the enrichment medium (described above) were sub-cultured onto sterile mineral salts agar plates prepared with 2.0% agar. Sterile filter papers impregnated with kerosene, sterilized using a 25mm Whatman syringe filter, were placed in the Petri dish covers and the plates incubated upside down. The kerosene in the filter papers supplied the organisms with the hydrocarbon through the vapour phase transfer as described by Okpokwasili et al., (1988). Incubation was for 48h at room temperature (25-30^0C). Each of the resulting colonies was repeatedly sub-cultured in this manner twice and ten isolates were obtained and designated S1-S10. After screening for hydrocarbon-utilizing capability using Bonny light crude oil, isolates S2 and S8 owing to their superior growth on crude oil as sole carbon source were selected for the study. Upon morphological ad biochemical characterization they were presumptively identified as strains of *Bacillus subtilis* (S2) and *Pseudomonas aeruginosa* (S8).

Determination of the rate of hydrocarbon utilization by the Isolates: The mineral salts medium (previously described) was prepared and dispended in 50.0ml volumes into eight 250ml Erlenmeyer flasks. Each of the isolates (S2 and S8) was used to inoculate four flasks, each flask containing one of the following hydrocarbons: kerosene, crude oil, diesel and engine oil. The inoculation was done using 0.5ml containing $6x10^6$ cfu/ml of each organism per flask. The oils were introduced in 1.0ml volume each into the sterile medium by filtering through a 25mm Whatman syringe filter. Thereafter the flasks were plugged with cotton wool and incubated on an orbital shaker (previously described) at 30^0C and 100rpm for 96h. A 5.0ml volume was withdrawn from each flask at twelve-hourly intervals for the determination of optical density, pH and surface tension.

Determination of optical density, surface tension and pH: The optical density (OD) was determined using a spectrophotometer (Spectronic 20, Mitton Roy Company, USA) at 600nm. Interfacial tension was determined by means of a Tensiometer (Kruss 3412, Hamburg), and the pH was measured with an electronic pH meter (H198127, Hanna industries, Mauritius).

Biodegradation experiment in simulated soil environment: Pristine soil sample (Sandy loam) was sun-dried, sieved and dispensed in 50.0g weights into sixty-four 250ml conical flasks. Filter-sterilized Bonny light crude oil was added to the soil samples in the following varying levels: 2.5, 5.0, 7.5 or 10.0% (v/w). A saturated solution of compound fertilizer (NPK, 15:15:15) was added to each flask at a ratio of crude oil to NPK of 10.0:1.0. One millilitre of bacterial inoculant containing $3x10^8$ cells/ml was added to each flask. Finally, 15.0ml of tap water was introduced into each flask and all flasks including the control were plugged with cotton wool and incubated by keeping them in a well ventilated room at room temperature for eight weeks. A 10g sample was taken from each flask at four weekly intervals and used for the determination of both the utilized and residual crude oil.

Determination of residual and utilized crude oil: Residual oil was determined by putting 10g of each soil sample into an extraction tube containing 20ml of n-hexane. The mixture was shaken (in a shaker) for 1h, and allowed to settle. Ten millilitre of the supernatant was removed by means of a syringe and put into pre-weighted evaporation dish. After evaporation of the solvent, the weight of residual oil was determined in the following manner: weight of residual oil = weight of bottle containing oil (W2) – weight of empty bottle (W1) → W2 – W1. Approximate weight of utilized oil = weight of initial quantity of oil – weight of residual oil.

Results

Identification of Isolates: Two isolates, S2 and S8, used in this study were identified as *Bacillus subtilis* and *Pseudomonas aeruginosa* respectively. The morphological and biochemical characteristics of the isolates are presented in Table 1.

Table 1: The morphological and biochemical characteristics of S2 and S8 isolates

Test	S2	S8
Colour of Colonies	White	Greenish
Shape	Short rods	Long rods
Gram reaction	+	-
Spore Stain	+	-
Motility	+	-
Catalase	+	+
Oxidase	-	+
Starch hydrolysis	+	-
Nitrate reduction	-	-
Growth on 7% NaCl	+	-
Arginine	-	-
Voges Proskauer	+	+
Sucrose fermentation	A/G	-
Dextrose fermentation	A/G	A
Mannitol fermentation	A/G	-
Lactose fermentation	-	-
Maltose fermentation	A	-
Mannose fermentation	A	-
Xylose fermentation	-	-

Key: + = Positive reaction; - = Negative reaction; A = Acid production; A/G = Acid and gas production

Biodegradation studies in liquid medium using crude oil and crude oil products as substrates: There was a marked increase in the optical density (OD) of the medium during the biodegradation experiment in liquid medium using the isolates, *B. subtilis and P. aeruginosa* as inoculants and crude oil and its products as substrates. This is presented in Figure 1. The mean maximum OD values of both isolates are shown in Table 2. *Bacillus subtilis* grew best on kerosene (maximum OD_{600} = 1.42) while *Pseudomonas aeruginosa* grew best on crude oil (maximum OD_{600} = 1.56).

Table 2: The mean maximum optical density (OD_{600}) values for isolates S2 and S8 during their 96h- growth on crude oil and crude oil products in shake flask cultures. Optical Density (λ = 600nm)

Isolate	Kerosene	Crude oil	Diesel oil	Engine oil
S2	1.42	1.29	1.33	0.25
S8	1.17	1.56	1.39	0.24

The surface tension of the medium decreased remarkably during the growth of both isolates as shown in Figure 2. Similarly the pH of the medium decreased with each organism (Fig. 3).

Biodegradation studies in simulated soil environment: The effects of biostimulation and bioaugmentation on the rate of oil metabolism in the soil are presented in Table 3. There were marked increases in the percentage of degraded oil in biostimulated and bioaugmented samples when compared with uninoculated and unsupplemented samples (controls).

Discussion

The two isolates, *Bacillus subtilis* (S2) and *Pseudomonas aeruginosa* (S8) involved in the degradation of petroleum in this study are members of the genera of bacteria implicated in petroleum hydrocarbon biodegradation (Ekundayo and Omokaro, 1987).

The observed increase in optical density ($OD6_{00}$) during the 96h-degradation of crude oil and crude oil products in liquid medium by the two isolates confirmed previous reports (Okpokwasili and Okorie, 1991). The *P. aeruginosa* grew best on crude oil as indicated by its highest mean maximum $OD6_{00}$ value during its growth on the substrate (Table 2). The ability of the crude oil to support good microbial growth could be due to its complex composition which in addition to hydrocarbons may also contain other compounds and elements which may serve as nutrients to the organism. In fact according to Okpokwasili and James (1995) some of the compounds in crude oil have been shown to be sources of readily available nitrogen and mineral nutrients. The OD of *P. aeruginosa* was higher with diesel oil than with kerosene, indicating better growth with diesel oil. This further confirms previous report that oil degrading microorganisms show a preference for higher molecular weight hydrocarbons (Wilkinson, 1971). On the contrary, *B. subtilis* grew best on kerosene as shown by its highest OD (OD_{600}) = 1.42) with the substrate. This best growth of *B. subtilis* on kerosene markedly deviates from previous findings and is indicative of the presence of novel degradative system in the organism. Growth of the two isolates on engine oil was poor, and this could be attributed to the high viscosity of the oil which could reduce its bioavailability.

The surface tension of the medium decreased remarkably during the growth of both isolates on the oils (Fig. 2). The fall in surface tension could be due to the elaboration of biosurfactants in the medium, because microorganisms are known to produce biosurfactants when they grow on carbohydrates, hydrocarbons and oils (Mercade et al., 1993). Production of biosurfactants makes it possible for microorganisms to render such hydrophobic substrates as hydrocarbons and oils amenable to biodegradation. A decrease in pH due to medium acidification was also observed during the degradation study (Fig.3). A fall in pH under similar conditions has been reported (Okpokwasili and Okorie, 1991), and this may be caused by the production of carboxylic acids during the degradation process (Atlas, 1984).

The extracted weights of residual oils (Table 3) from the simulated soil experiment show that biostimulation (nutrient addition) and bioaugmentation (inoculum addition) increased the rate of oil metabolism in the soil by the microbes. This is in agreement with the report of Atlas (1984), that addition of nitrogen fertilizer and oil – degrading microbes increases the rate of microbial metabolism of petroleum in the soil. Generally, rates and extents of crude oil removal in nutrient-supplemented or inoculated soils were significantly ($p < 0.01$) high in relation to unsupplemented and uninoculated samples (controls).

Conclusion: The present study brings to the limelight the high hydrocarbonclastic qualities of the isolates used, projecting them as good candidates for the clean-up of environments contaminated by crude oil and crude oil products. The study also reveals the presence of novel degradative systems in the strain of *B. subtilis* (S2) used, as evidenced by its ability to utilize kerosene more than the other petroleum substrates. This deviates remarkably from previous findings that microbes have a preference for crude oil and diesel than kerosene, petrol and engine oil as growth substrates.

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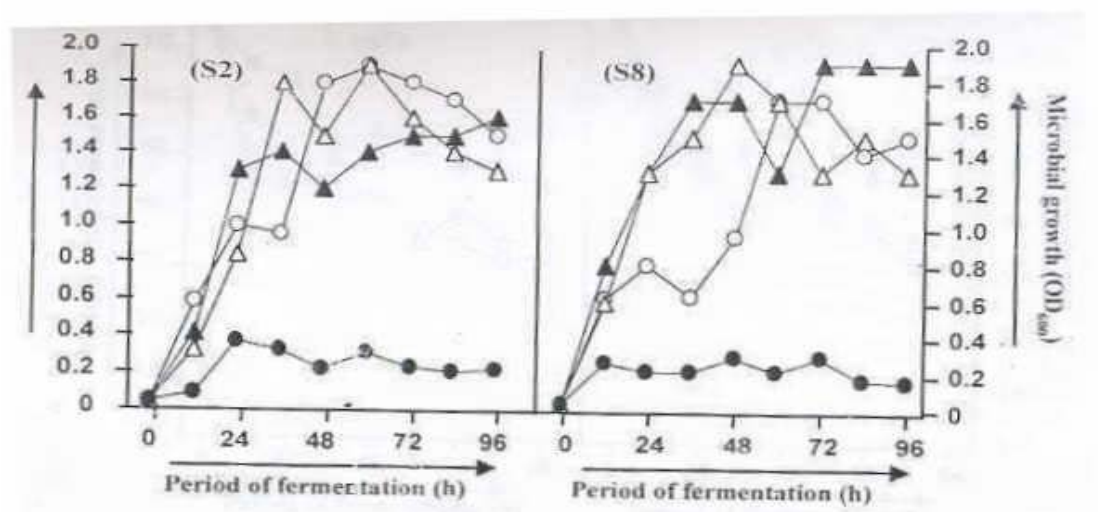

Fig. 1: Time – course of bacterial growth on petroleum hydrocarbons ○, kerosene; △ diesel; ●, engine oil; ▲, crude oil

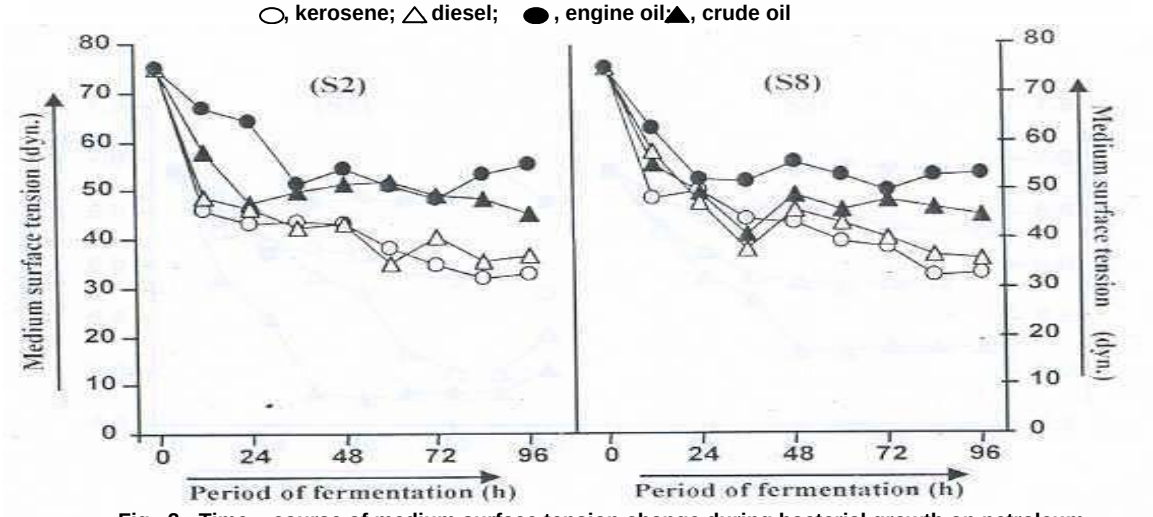

Fig. 2: Time – course of medium surface tension change during bacterial growth on petroleum hydrocarbons ○ kerosene; △ diesel; ● engine oil; ▲ crude oil

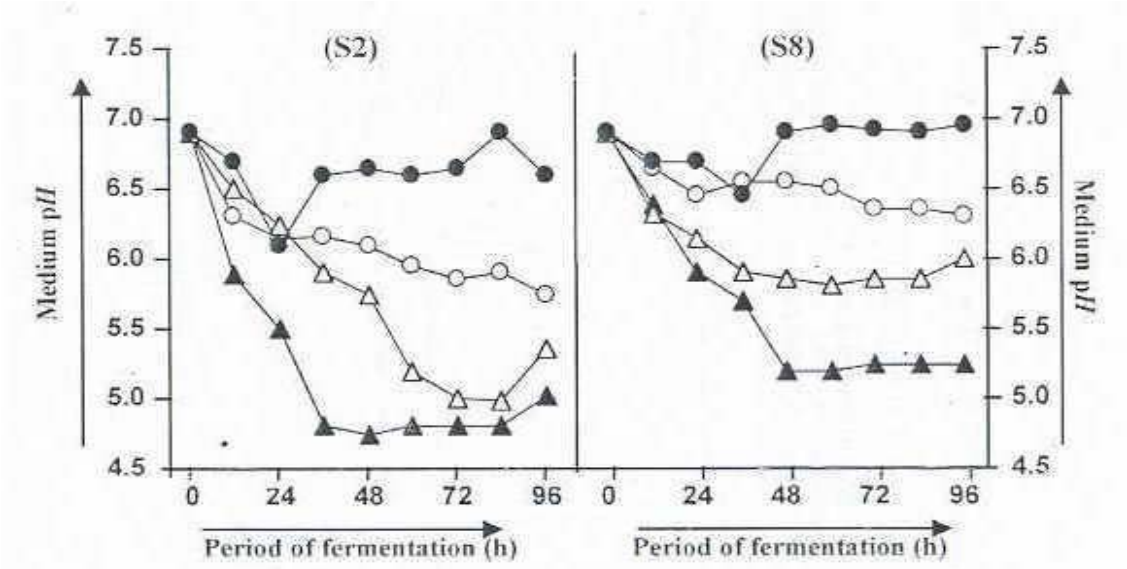

Fig. 3: Time – course of medium pH change during bacterial growth on petroleum hydrocarbons ○ kerosene; △ diesel; ● engine oil; ▲ crude oil

Table 3: The effects of biostimulation and bioaugmentation on biodegradation rate as assessed by the percentage of degraded oil in the samples

Crude oil levels	A	B	C	D	E	F	G	H	I
2.5% crude oil pollutant	1.05g	0.60g	42.85%	0.50g	52.38%	0.50g	52.38%	0.45g	57.14%
5.0% crude oil pollutant	2..35g	1..50g	36.17%	1.15g	51.07%	1.45g	38.29%	0.90g	61.70%
7.5% crude oil pollutant	3.40g	2.25g	35.71%	1.75g	50.00%	1.55g	55.71%	1.10g	68..57%
10.0% crude oil pollutant	4.80g	2..90g	39.58%	2.30g	52.08%	1.55g	67.70%	1.65g	65.62%

A = Initial weight of oil in soil sample; B = Weight of residual oil in uninoculated and unsupplemented soil (control); C = % of degraded oil in uninoculated and unsupplemented soil sample; D = weight of residual oil in nutrient supplemented soil sample E = % of degraded oil in inoculated soil sample; F = Weight of residual oil in inoculated soil sample; G = % of degraded oil in inoculated soil sample; H = Weight of residual oil in inoculated and supplemented soil sample; I = % of degraded oil in inoculated and supplemented soil sample.

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