

PETROLEUM HYDROCARBON DEGRADING CAPABILITIES AND GROWTH PROFILE OF BACTERIA FROM CRUDE OIL POLLUTED ULTISOL AND BRACKISH WATER

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ABSTRACT

Hydrocarbon degrading capabilities and growth profile of bacteria from Qua Iboe Light crude oil contaminated ultisols and brackish water were determined *in vitro*. The results revealed variations in crude oil utilization ability between the hydrocarbonoclastic bacteria isolated. The isolates were *Bacillus subtilis* IBU-1, *B. cereus* IBU-2, *B. licheniformis* IBU-3, *Micrococcus varians* IBU-5, *M. roseus* IBU-6, *Pseudomonas aeruginosa* IBU-7 and *Sarcina* species IU-8. *Bacillus subtilis* IBU-1, *Micrococcus varians* IBU-5, *Micrococcus roseus* IBU-6 and *Pseudomonas aeruginosa* IBU-7, were the most prevalent hydrocarbonoclastic bacteria encountered in the polluted environment. *Bacillus subtilis* IBU-1, exhibited the highest capability to degrade Qua Iboe Light crude oil with a 52% reduction in weight of crude oil after 10 days of incubation while *Sarcina* species IU-8 demonstrated the lowest (16% reduction in weight of crude oil after 10 days) degrading capability. The growth profiles of the bacteria on oil medium showed a positive relationship between oil degradability and turbidity development in all the isolates tested except *Sarcina* species IU-8. The results also showed that the higher the optical density, the more acidic the medium, a pointer to a high oil degrading capability of the bacteria. These characteristics confirmed the superior hydrocarbonoclastic potential of *B. subtilis* IBU-1, *B. licheniformis* IBU-3, and *M. varians* IBU-5 in environment contaminated with Qua Iboe light Nigerian crude oil.

Key Words: Hydrocarbonoclastic, Bacteria, Polluted, Ultisol, Brackishwater

INTRODUCTION

The immediate risk induced by oil when it first enters the environment is that of fire hazard, followed by possible damage to plants, animals and microbiota (Odu 1972). Atlas (1977) reported that the complex nature of petroleum pollutants may influence microbial activities with respect to the ratio of available nutrients.

However, microorganisms in soil and aquatic ecosystems have the capability of degrading hydrocarbon pollutants and utilizing them as the sole source of carbon and energy thereby decontaminating the environment (Ijah and Antai 1988, Antai and Mgbomo 1989, Ijah and Ukpe 1992). Common among the hydrocarbonoclastic bacterial genera that have been isolated from oil spilled soils are *Micrococcus*, *Alcaligenes*, *Flavobacterium*, *Pseudomonas* and *Arthrobacter* species (Odu 1980, Antai and Mgbomo 1989, Ijah and Ukpe 1992). These microorganisms vary in their oil-degrading ability. Some can utilize aliphatic hydrocarbon (hexane) or mixtures of aliphatic hydrocarbons (kerosine and diesel) than the aromatic hydrocarbon such as benzene and toluene (Ijah and Ukpe 1992), while others cannot degrade aromatic hydrocarbons. The resistance of aromatic hydrocarbons to microbial attack is ascribed to its condensed polycyclic nature and the presence of carbon-12 Olefins which are recalcitrant to biodegradation (Atlas and

Bartha 1973).

Considerable attention has been given to bioremediation of petroleum contaminated soils and waters, but with little interest on the specificity and rate of hydrocarbon utilization by microorganisms. This report highlights the crude oil degrading ability of bacteria from petroleum contaminated ultisols and brackish water and their growth profile on Qua Iboe light crude oil.

MATERIALS AND METHOD COLLECTION OF SAMPLES:

Oil contaminated water and soil samples analysed were obtained respectively from Qua Iboe River estuary and the effluent discharge point of Mobil Producing Nigeria Unlimited (MPN ULTD) both situated in Ibeno Local Government Area, Akwa Ibom State, Nigeria.

The Qua Iboe River estuary characterized by its considerably high salinity (about 25ppt) is surrounded by an acidic sandy loam ultisol found in the Niger Delta region of Nigeria (D'Hoore 1964). The water and soil of the area have been under persistent contamination by hydrocarbons released as a result of intense petroleum exploration and refining activities of the oil companies located within the area. The most recent case of pollution in the area is the Idoho-QIT pipeline rupture which occurred in January 1998, resulting in

the release of about 40,000 billion barrels (BBLs) of the Nigeria Qua Iboe light crude oil into the marine environment.

Qua Iboe light (QIL) crude oil used in this work was collected from Qua Iboe Terminal of Mobil Producing Nigeria Unlimited, Ibeno, Akwa Ibom State, Nigeria. The samples contained in sterile glass bottles were immediately transported to the laboratory for microbiological analysis.

MICROBIOLOGICAL ANALYSIS:

ANALYTICAL MEDIA: The media used for the microbiological studies were

- (a) Mineral salt medium, MSM (Zajic and Supplisson 1972): The mineral salt broth contained KH_2PO_4 , 0.11g; K_2HPO_4 , 0.65g; NH_4NO_3 , 0.5g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.29g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.025g; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.25mg; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 1mg and $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 4mg., distilled H_2O 100ml.
- (b) Nutrient agar (Oxoid); Yeast extract; 2g, Peptone, 5g; NaCl 0.1g; agar, 15g; distilled H_2O 1000ml.
- (c) Oil agar: This medium contained all the components of Zajic and Supplisson (1972) medium plus 15g agar and 0.2% Qua Iboe light crude oil.

ISOLATION AND PURIFICATION OF BACTERIA:

Bacteria in soil and water samples were isolated by the enrichment technique earlier described by Fedorak and Westlake (1981). Briefly, 2ml of the crude oil were added to 200ml of MSM and the mixture sterilized by autoclaving at 121°C for 15 mins under a pressure of 15 pounds per square inch (Psi). The supplemented medium was allowed to cool after which 2g (of soil) or 2ml (of water) portion of the samples were added to separate tubes. Inoculated tubes were incubated at $28 \pm 2^\circ\text{C}$ using the orbital shaker (SGM-300, Gallenkamp, England), at 120rpm for 7 days. 0.1ml amount of the enrichment culture was then directly inoculated onto nutrient agar plates and incubated at $28 \pm 2^\circ\text{C}$ for 48 hours.

CHARACTERIZATION AND IDENTIFICATION OF ISOLATES:

The isolates were characterized and identified using the taxonomic schemes of Buchanan and Gibbons (1974); Cowan and Steel (1985)

DETECTION OF HYDROCARBONOCLASTIC BACTERIA:

The hydrocarbonoclastic bacteria among the isolates obtained from the soil and water samples were ascertained by growing the isolates on oil agar plates at 30°C (Antai and Mgbomo (1989). Non-oil degrading

Table 1: Occurrence and hydrocarbonoclastic potential of bacteria isolated from oil contaminated environment in Ibeno, Nigeria.

Isolates	Ultisol (U)	Brackish water (B)	% Frequency of Occurrence	Ratio of Occurrence (U:B)	Growth on Oil agar
<i>Bacillus subtilis</i> IBU-1	+ (10)	+ (7)	85	10:7	+
<i>B. cereus</i> IBU-2	+ (7)	+ (1)	40	7:1	+
<i>B. licheniformis</i> IBU-3	+ (7)	+ (5)	60	7:5	+
<i>Escherichia coli</i> IBU-4	+ (10)	+ (10)	100	10:10	-
<i>Micrococcus varians</i> IBU-5	+ (6)	+ (10)	80	6:10	+
<i>M. roseus</i> IBU-6	+ (8)	+ (4)	60	8:4	+
<i>Pseudomonas aeruginosa</i> IBU-7	+ (7)	+ (7)	70	7:7	+
<i>Sarcina</i> sp IU-8	+ (10)	-	50	-	+
<i>Staphylococcus aureus</i> IB-9	- (7)	+ (10)	50	-	-
<i>Enterococcus faecalis</i> IBU-10	+ (5)	+ (7)	60	5:7	-
<i>Enterococcus faecium</i> IB-11	-	+ (8)	40	-	-
Total	9	10			7

+ = present/growth on oil agar
 - = absent/inability to grow on oil agar
 * = isolate code.

The % frequency of occurrence was calculated jointly from 20 samples (10 soil + 10 water samples). Values in brackets represent the frequencies of distribution of the bacterial isolates per 10 samples in each habitat.

Table 2. Percentage weight loss from QIL crude oil resulting from growth of hydrocarbonoclastic bacteria.

Isolates	Incubation period (days)				
	2	4	6	8	10
<i>Bacillus subtilis</i> IBU-1	4±2.2	18±1.4	32±1.3	38±2.2	52±1.4
<i>B. cereus</i> IBU-2	2±0.8	8±0.9	14±1.2	18±1.2	34±2.1
<i>B. licheniformis</i> IBU-3	5±1.2	12±2.2	20±2.1	28±2.1	40±2.2
<i>M. varians</i> IBU-5	7±1.5	20±2.1	24±1.51	28±2.1	42±2.2
<i>M. roseus</i> IBU-6	5±2.0	14±1.4	18±1.2	20±1.4	22±2.3
<i>Pseudomonas aeruginosa</i> IBU-7	4±0.9	10±1.4	23±2.1	27±2.1	40±1.1
<i>Sarcina</i> sp IU-8	3±1.2	2±2.1	4±2.1	6±1.8	7±2.2

* Standard deviation is based on the mean of three determinations

Table 3. Screen test for utilization of Qua Iboe Light crude oil by the bacterial isolates.

Isolate / code	level of turbidity
<i>B. subtilis</i> IBU -1	++
<i>B. cereus</i> IBU -2	+
<i>B. licheniformis</i> IBU -3	+
<i>E. coli</i> IBU -4	+
<i>M. varians</i> IBU -5	+++
<i>M. roseus</i> IBU -6	+
<i>P. aeruginosa</i> IBU -7	++
<i>Sarcina sp</i> IBU -8	+++
<i>Staph. aureus</i> IBU -9	-
<i>Ent. faecalis</i> IBU -10	-
<i>Ent. faecium</i> IBU -11	-

No growth -
 Heavy growth +++
 Moderate growth ++
 Little growth +

bacteria are not expected to grow on oil agar.
DETERMINATION OF CRUDE OIL DEGRADING CAPABILITIES OF THE HYDROCARBONOCLASTIC BACTERIA:

The capability of the bacterial isolates to degrade hydrocarbon was determined by the method of Okpokwasili and Okorie (1988) using mineral salt broth (Zajic and Supplison 1972). 0.1ml of filter sterilized (0.45 um pore size filter, Millipore Corp. England) crude oil was incorporated into tubes containing 5ml of sterile mineral salt broth. The crude oil supplemented broth was then inoculated with 0.1ml amount of active inocula from 24 hr old nutrient broth culture of each isolate. Uninoculated tubes containing only the mineral salt broth and 0.1ml of Qua Iboe Light crude oil were also prepared to serve as control.

All the tubes were incubated undisturbed at 28°C for 10 days. The tubes were monitored every two days and the amount of oil left in the tubes were measured using the method described by Odu (1972).

$$\frac{\text{Weight of crude oil (control)} - \text{Weight of crude oil (degraded)}}{\text{Weight of crude oil (control)}} \times 100$$

The degree of turbidity in tubes was also observed and used as an index of oil utilization by microorganisms after 10 days of incubation.

DETERMINATION OF GROWTH PROFILE OF HYDROCARBONOCLASTIC BACTERIA:

To further assess the ability of the bacterial isolates to degrade the crude oil, their growth profile in oil medium were determined as earlier described by Okpokwasili and Okorie (1988). Sterile mineral salt broth in 200ml amounts were dispensed into 250ml conical flasks. To each flask 2ml of crude oil and 0.1ml (about 4.5×10^7 cells) of the test organisms was incorporated. An uninoculated flask served as control for each of the bacterial cultures tested. The flasks were incubated on an orbital shaker operated at 120 rpm for 20 days. After incubation, 30ml of samples were obtained from each flask for the following determinations;

1. Changes in the pH of culture medium was measured by means of a pH meter (EIL 7020 Kent Industrial Measurements Ltd).
2. While the optical density (OD) was determined at 560nm wavelength using a spectrophotometer (Spectronic 20 Milton Roy Company, New York).

RESULTS AND DISCUSSION

The aerobic bacterial isolates from the crude oil contaminated ultisol and brackish water are presented in Table 1. Very little variation in species diversity was obtained between the aquatic (brackish water) and terrestrial (ultisol) microcosms. More species of bacteria were encountered in the water than the soil of the polluted environment. *Sarcina* species IU-8 was isolated only from the ultisol while *Staphylococcus aureus* IB-9, and *Enterococcus faecium* IB-11 were found only in brackish water samples. Their presence in the water samples may be ascribed to the persistent human activities in the river estuary under investigation. *Staphylococcus* and *Enterococcus* species are common human skin and intestinal microflora (Jawetz *et al* 1989) and may have been introduced into the water body through human activities. On the other hand *Bacillus subtilis* IBU-1, *B. cereus* IBU-2, *B. licheniformis* IBU-3, *Escherichia coli* IBU-4, *Micrococcus varians* IBU-5, *M. roseus* IBU-6, *Pseudomonas aeruginosa* IBU-7, and *Enterococcus faecalis* IBU-10, were encountered in the soil and water samples but in variable proportions (Table 1).

Escherichia coli IBU-4 with a 100% frequency of occurrence was the most predominant bacteria isolated from the contaminated environments followed by *Bacillus subtilis* IBU-1 (85%) and *Pseudomonas*

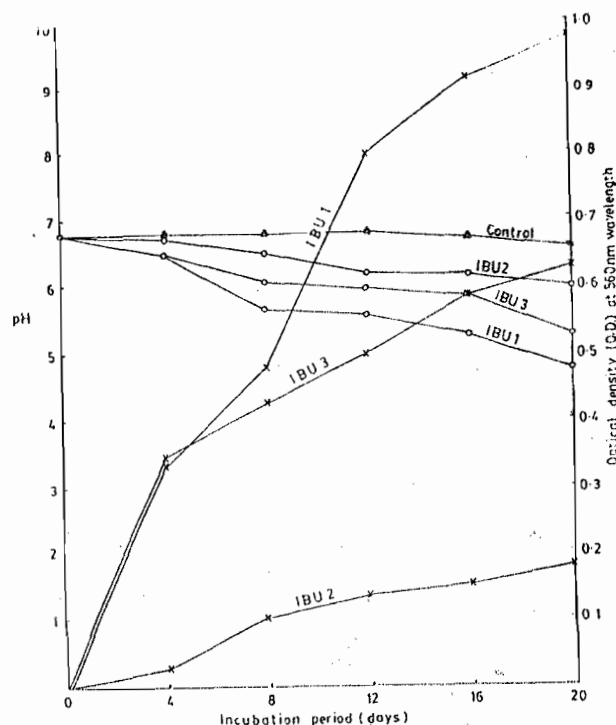


Fig. 1: Growth profile (pH, O and O D; x) of *Bacillus subtilis* IBU-1, *B. cereus* IBU-2 and *B. licheniformis* IBU-3 on crude oil medium. Uninoculated crude oil medium served as control (▲).

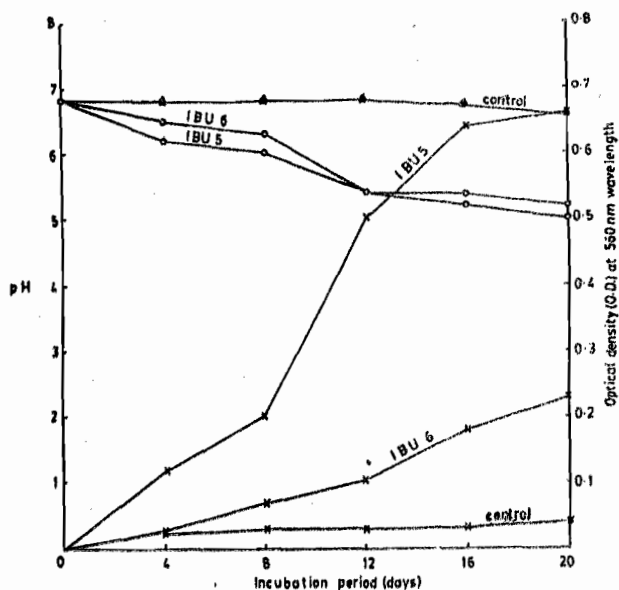


Fig. 2: Growth profile (pH, \circ and O.D., X) of *Micrococcus varians* IBU-5 and *M. roseus* IBU-6 on crude oil medium. Uninoculated crude oil medium served as control (A)

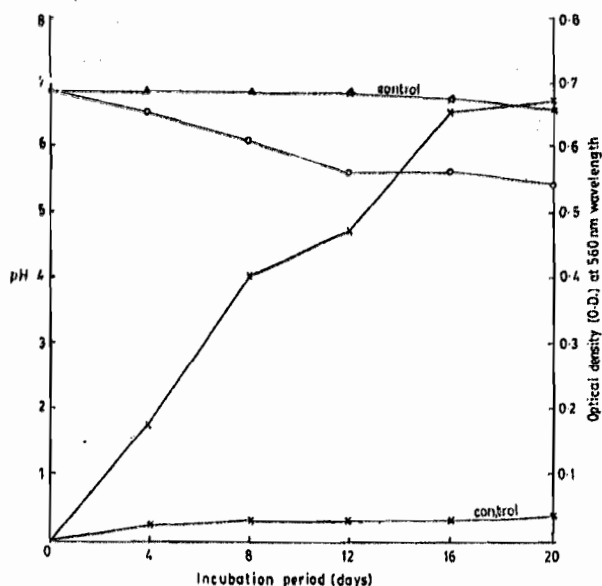


Fig. 3: Growth profile (pH, \circ and O.D., X) of *Pseudomonas aeruginosa* IBU-7 on crude oil medium. Uninoculated crude oil medium served as control (A)

aeruginosa IBU-7 (70%) *Bacillus licheniformis* IBU-3 *Micrococcus roseus* IBU-6 and *Enterococcus faecalis* IBU-9 were present only in 60% of the samples analysed. The frequency of occurrence of other isolates was less than 50% of the total samples studied.

Variation in the level of occurrence of the bacterial species in the oil-contaminated environment may be ascribed to the differences in the genetically endowed ability of individual micro organisms to resist environment stresses. *Escherichia coli* and *Pseudomonas* species are known to easily develop resistance to anti growth factors (Jawetz et al 1989). *Micrococcus* species are commonly associated with oil contaminated soils and water bodies. Their presence in such stressed conditions have been attributed to their high osmophilic properties (Atlas and Bartha 1972,

Walker et al 1976. Antai and Mgbomo 1989, Ijah and Okang 1993). The ability of *Bacillus subtilis* IBU-1, *B. cereus* IBU-2, *B. licheniformis* IBU-3, *Micrococcus varians* IBU-5, *M. roseus* IBU-6, *Pseudomonas aeruginosa* IBU-7, and *Sarcina* species IU-8 to utilize hydrocarbon for growth (Table 1) contributed to their presence in the crude oil contaminated brackish water and uftisol. Their capabilities to utilize the Qua Iboe Light crude oil are shown in Table 2. The hydrocarbonoclastic bacteria caused a reduction in weight of Qua Iboe light crude oil at 28°C. The degree of weight loss was observed to increase with increase in incubation period, but varied with the different species tested. *Bacillus subtilis* IBU-1 exhibited the highest capability to degrade the oil and was able to cause 52% reduction in weight of crude oil after 10 days of incubation. The oil-degrading capabilities of *M. varians* IBU-5 (42%), *B. licheniformis* IBU-3 (40%) and *P. aeruginosa* IBU-7 (40%) were also remarkably high over the same period of incubation. *Sarcina* species IU-8 demonstrated the lowest (6%) capability to utilize hydrocarbons for growth.

The hydrocarbonoclastic bacteria utilize the crude oil for growth and proliferation as depicted by the level of turbidity produced in the oil medium (Table 3).

Contrastingly *Sarcina* species IU8 produced a high level of turbidity in the oil medium despite its low oil degradability (Table 2). This suggests that the ability of a bacterium to produce turbidity in oil medium might not necessarily be an indication of the organisms oil degrading capability. Such capability is greatly determined by the ability of the microorganisms to elaborate the vital enzymes required for oil decomposition rather than being nutritionally fastidious or able to grow profusely.

The growth profiles of the oil-degrading bacteria on the oil medium are presented in Figures 1 to 4. The profiles are based on changes in the optical density (O.D. at 560nm wavelength) and pH of cultures at 28°C. *Bacillus subtilis* IBU-1 recorded the highest O.D. of 0.98 in crude oil after 20 days of incubation (Fig.1). This was followed by *Pseudomonas aeruginosa* IBU 7(0.67) and *Micrococcus varians* IBU-5 (0.66) presented in Figures 2 and 3 respectively. *Sarcina* IU-8 (Fig.4), *Micrococcus roseus* IBU- 6 (Fig.2) and *Bacillus cereus* IBU 2 (Fig.1) recorded very low O.D at 560 nm over 20 days of incubation.

The pH of the oil medium was affected by the biodegradation process. The utilization of crude oil by microorganisms resulted in their growth and concomitant production of acidic metabolic products. The acidic metabolites are responsible for the decrease in pH of the growth medium from pH 6.8 to more acidic levels. The effect on pH increases with increase in incubation period but however varied with the species of bacteria involved. The levels of acidity attained at the end of the 20 days incubation period were as follows: 4.8 for *B. subtilis* IBU-1, 5.3 for *B. licheniformis* IBU-3, and 6 for *B. cereus* IBU-2 (Fig.1.) Other levels attained were 5 and 5.2 for *M. varians* IBU-5 and *M. roseus* IBU-6 respectively (Fig. 2). *Pseudomonas aeruginosa* IBU-7 recorded a pH of 5.4 over 20 days (Fig.3), while *Sarcina* IU8 induced a pH of 5.5 under

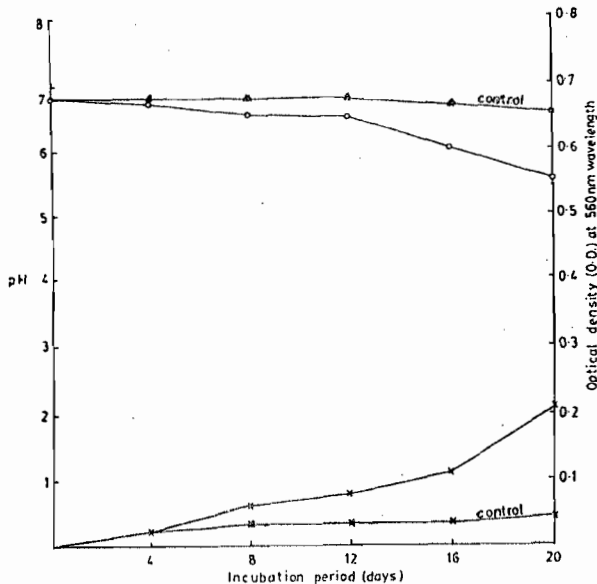


Fig. 4: Growth profile (pH; O and O.D, x) of *Sarcina* IU8 on crude oil medium. Uninoculated crude oil medium served as control (A)

the same period of incubation (Fig.4).

The growth profiles of the hydrocarbonoclastic bacteria revealed a relationship between growth profile and crude oil degradation. The level of O. D. and extent of pH recorded by the bacteria reflected their oil-degrading capabilities. The higher the O.D. of the cultures the more acidic the medium and probably the more capable the organism to utilize crude oil for growth. This relationship is confirmed by the superior hydrocarbonoclastic potential of *B. subtilis* IBU-1, *B. licheniformis* IBU-3 and *M. varians* IBU-5 revealed by this investigation. These organisms are recommended for bioremediation of soils and rivers contaminated with Qua Iboe Light crude oil.

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