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CRUDE OIL DEGRADING POTENTIAL OF FRESHWATER BACTERIAL ISOLATES FROM SLOW RUNNING FRESHWATER SYSTEM LOCATED IN CROSS RIVER STATE, NIGERIA

L. B. ETIM, S. P. ANTAI AND G. IWAT

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ABSTRACT

The potential of freshwater bacterial isolates obtained from drinking water sources to utilize crude oil was studied. The result obtained from the study revealed that the average total heterotrophic count (THC) was $8.73 \pm 0.3 \times 10^{9}$ colony forming units per milliliter (cfu ml^{-1}) and the best hydrocarbonoclastic isolates were *Pseudomonas - OUB6, Micrococcus - OUB7, Bacillus - OUB8, Pseudomonas - OUB13 and Pseudomonas OUB14*. The growth profile as an index of the ability to utilize crude oil for carbon and energy revealed that the bacterial isolates had an average cell count of $6.33 \pm 0.26 \times 10^{8}$ cfu ml^{-1} at pH 5.9 after incubation period of 25 days. Furthermore, the isolates (*Micrococcus-OUB7, Pseudomonas - OUB13 and Pseudomonas - OUB14)* exhibited a high crude oil biodegradability of >90% at ten percent (10%) crude oil pollution level, 82% at (5%) pollution level and 80% at 1% pollution level respectively. The study therefore, indicated that freshwater ecosystem (streams, rivers, lakes etc.) can recover with time from crude oil pollution level of as high as 10% with the deployment of an integrated bioremediation technologies (bioaugmentation) involving the test bacterial isolates obtained from freshwater ecosystem.

KEYWORDS: Freshwater, bacterial isolates, biodegradation, crude oil pollution levels.

INTRODUCTION

Freshwater oil spillage and contamination is a frequent event in the Niger Delta region of Nigeria. Deliberate or accidental spills through the activities of the oil companies and pipeline vandals have exposed streams and other freshwater bodies to a high level of potentially toxic hydrocarbon pollutants. At any level the pollutants are hazardous to plants, animal and microbial populations within the aquatic and terrestrial environments of the region (Atlas, 1981, 1984, Leahy and Colwell, 1990). The options of chemical and mechanical treatments are considered ineffective and expensive and require special technological skills (Cox and Cowell, 1979). Biodegradation by microorganisms is considered the most effective means of remedy. Microorganisms are many and widespread in aquatic environments, and their effectiveness appears to be related to their activity rather than their number (Odu, 1972, Lee and Levy, 1989). Hydrocarbonoclastic microorganisms in aquatic environment use crude oil as their sole source of carbon and energy thereby cleansing the environment (Ijah and Antai, 1988, Antai and Mgbomo 1989, Ijah and Ukpe 1992). Such a potential is usually limited in freshwater systems not previously exposed to crude oil pollution (Roubal *et al.*, 1979).

In this study, the biodegradative potentials of the freshwater bacterial isolates from slow running streams located at Akpabuyo and Calabar South Local Government Areas of Cross River State, Nigeria are investigated. The study sites are located in Cross River State of Nigeria but the streams in study have no recorded incidence of oil pollution.

MATERIALS AND METHODS

Collection of Samples

The Qua Iboe light crude oil sample used in this study was collected from the Qua Iboe terminal of Exxon Mobil Production Nigeria Unlimited, Ibeno, Akwa Ibom State, Nigeria, while the water samples for physical/bacteriological analysis were obtained from four perennial streams (Idim Nakanda, Idim Ebisa, Idim Ikot Eyo and Idim Esa Polycal) located in Akpabuyo and Calabar South local government areas of Cross River State, Nigeria. The freshwater samples were stored in an ice packed container (4°C) prior to isolation of crude oil utilizing bacteria within 24 hours

Measurement of some physical properties of the water samples

The temperature of the water sample was measured with a thermometer calibrated in degree Celsius (°C), the pH of the water was measured with an electronic (field) pH meter (slop Laptech model, England). The turbidity of the water samples was measured as optical density (OD) at 540nm wavelength with a spectrophotometric colorimeter (model Spectronic 20 Genesys, Spectronic Inst Inc Rochester, NY)

Determination of heterotrophic bacterial load in freshwater samples

The heterotrophic bacterial load was determined by the pour plate method cultured in tryptone soya agar (TSA) (Atlas and Bartha, 1982). Ten-fold serial dilutions of the water samples (A to D) were prepared using sterile distilled water in the range of 10^{-1} to 10^{-8}. Then 1.0ml of 10^{-6} and 10^{-8} aliquot was seeded in (triplicate) sterile petri dishes and total heterotrophic count (THC) was determined after incubated at 30°C for forty-eight hours. The colonies were counted using Quebec counter and multiplied by the reciprocal of the dilution factor and recorded as colony forming units per millitre of water (cfu ml^{-1}).

Isolation and purification of oil utilizing bacteria

Crude oil-utilizing bacteria in the stream water samples were isolated by surface phase respiratory technique (SPRT). This involved spreading 0.1 ml of 10^{-6} dilution of water sample on mineral salt medium (MSM). Filter-sterilized 50.0μg/ml nystatin was added to the medium to inhibit the growth of fungai. Sterile No. 1 Whatman filter papers were impregnated to saturation with 2.0ml sterile Qua Iboe light crude oil and aseptically placed onto the inside of the covers of inverted plates and tightly taped around with a paper tape. The plates were incubated at room temperature (28±2°C) for 7 days from which discrete colonies that developed were

L. B. Etim, Department of Biological Sciences, Cross River University of Technology, Calabar, Nigeria
S. P. Antai, Department of Microbiology, University of Calabar, Calabar Nigeria
G. Iwat, Department of Microbiology, University of Calabar, Calabar Nigeria

aseptically picked and purified by repeated subculturing on nutrient agar (NA) slant and stored in a refrigerator at 4^{0}C for further microbiological studies.

Characterization and identification of oil utilizing isolates

The bacterial isolates obtained were characterized based on their cultural, morphological and biochemical characteristics described by Buchanan and Gibbons (1974), MacFaddin (1980) and Cowan, (1985).

Utilization of Qua Iboe light crude oil by freshwater bacterial isolates

The gravimetric method described by Venosa *et al.* (1993), was used to screen the isolates for their ability to utilize Qua Iboe light crude oil as the only source of carbon and energy for growth. In this method, sterile 9.8ml of mineral salt broth (Zajic and Supplisson, 1972) in test tubes were treated with 0.1ml of filter (0.45μm pore size) sterilized crude oil and inoculated with 0.1ml of 24 hour old nutrient broth culture of individual test bacterial isolates. Three uninoculated tubes containing the mineral salt broth and 0.1ml of Qua Iboe light crude oil were prepared to serve as controls. The Inoculated tubes were incubated at room temperature (28±2^{0}C) undisturbed during which the tubes were monitored on the seventh and tenth days. The degree of turbidity as gravimetric index of oil utilization by the isolates was observed visually and graded as maximum (+++), moderate (++), minimal (+) and inability to grow was recorded as no growth (-) as against the control. The optical density (OD) of the medium was measured at 540nm using a spectrophotometer (Spectronic 20 Genesys, Spectronic Inst, Inc. Rochester, N. Y).

Growth potential of six (6) most efficient Qua Iboe light crude oil utilizing bacterial isolates

To further investigate the ability of the bacterial isolates to degrade the crude oil, the growth profile in an oil mineral salt medium of six (6) most efficient isolates were determined as described by Okpokwasili and Naubia (1995) and recently adopted by Itah and Essien (2005). In this method, 99.0ml mineral salt broth (MSB) of Zajic and Supplisson (1972) were dispensed into seven 250ml capacity Erlenmeyer flasks, autoclaved and, on cooling, were supplemented with 1.0ml of filter-sterilized Qua Iboe light crude oil after which 0.1ml of 24hour nutrient broth culture of the test organisms was inoculated, while the seventh flask served as control. The flasks were incubated at room temperature (28±2^{0}C) with periodic gentle swirling agitation each day for 25 days. During incubation, at 5 days interval, 20.0ml of the representative samples were taken from each flask for determination of (a) Optical density at 540nm wavelength with spectrophotometer, (b) pH changes using digital electrical pH meter, (c) total viable count (TVC) determined by plating on a nutrient agar 0.1 ml of 10^{-6} dilution and incubated at room temperature (28±2^{0}C) for 24 hours after which the colonies were counted and expressed as colony forming units per milliliters (cfu ml^{-1}).

Assessment of percentage bioutilization of Qua Iboe light crude oil by the three (3) hydrocarbonoclastic freshwater bacterial isolates

In assessing the percentage bioutilization of Qua Iboe light crude oil by three (3) most efficient oil utilizing freshwater bacterial isolates (based on screen test), Zajic and Supplisson (1972) mineral salt broth (MSB) medium was prepared and 9.9ml, 9.5ml and 9.0ml respectively were measured into 3 sets of fifteen (15) 100ml capacity Erlenmeyer flasks and sterilized by autoclaving at 121^{0}C for 15 minutes. On cooling, 0.1ml, 0.5ml and 1.0ml of a 24-hour nutrient broth cultures of the bacterial isolates were respectively inoculated into the 3 sets of five (5) test tubes per isolate. Then 0.1ml, 0.5ml, 1.0ml of sterilized crude oil representing 1%, 5% and 10% pollution levels were dispensed into each tube plus three (3) uninoculated tubes to serve as controls. The flasks were incubated at room temperature (28±2^{0}C) on a shaker (Gallenkamp Flask Shaker, England) at 110 rpm for 25 days.

At every 5 days, a tube from a set of each isolate was removed and the amount of the crude oil left was determined by extracting the left over crude oil with 20ml of n-hexane and their absorbance reading taken at 540nm using a spectrophotometer (Spectronic 20 Genesys, Spectronic Inst. Inc. Rochester, NY)

The weights of the extracted oil residue were obtained by reading off the respective absorbances from a previously prepared standard curve. Percentage utilization or weight loss of the incorporated crude oil was calculated as weight of crude oil (control) minus weight of crude oil (degraded) divided by weight of crude oil (control) multiplied by 100.

RESULTS

The physical properties/the total heterotrophic count of the stream water under study are presented in table 1. The slow running streams had a mean temperature of 28°C, optical density (turbidity) of 0.41±0.02, pH 6.9 and a total aerobic heterotrophic count (THC) of 8.73±0.3 x 10^9 cfu ml^{-1}. Water sample A had the highest bacterial count of 10.6±0.41 x 10^9 cfu ml^{-1} while water sample D recorded 5.3±0.25 x 10^9 cfu ml^{-1} which was less than the average total heterotrophic count.

Figure 1 shows the ability to utilize Qua Iboe light crude oil as source of carbon by the isolates. The best crude oil utilizers identified and coded were *Pseudomonas-OUB6, Micrococcus-OUB7, and Bacillus-OUB8. Pseudomonas-OUB13* and *Pseudomonas-OUB14*. The result also shows that hydrocarbonoclastic isolates constituted a total of 33% of the total isolates.

Growth potential of the best crude oil utilizers revealed their variable potentials to grow in crude oil mineral salt medium (Figures 1 – 3) The growth profile as an index of ability to utilize Qua Iboe light crude oil as the only source of carbon and energy was based on changes in the optical density (OD) measured at 540nm wavelength, pH of the culture medium and total viable count (TVC) expressed as cfu ml^{-1}. The results show that for each of the bacterial isolates, optical density (OD) increased as viable cell count increased after 5-20 days of incubation and dropped correspondingly as incubation period extended beyond 25 days. The utilization process effected the shift in pH from 6.9 to 5.8 (Figure 2).

Percentage weight loss or bioutilization rate for the three-oil pollution (1%, 5% and 10%) levels is presented in Figures 4-6. The highest bioutilization rate among the test isolates was observed at 10% oil pollution level seconded by 5% level and lastly by 1% level. Comparison between days of incubation and mean bioutilization rate for each pollution level showed significant positive linear relationship ($p<0.05$) within the test isolates.

DISCUSSION

The present study has shown that freshwater aerobic bacterial isolates obtained from the four sampled streams had an average heterotrophic bacterial load of 8.73±0.3 x 10^9 with a great diversity of microorganisms at pH 6.9 and temperature of 28^{0}C (Table 1). The high level of bacterial load under the present conditions is believed to be as a result of the numerous anthropogenic economic activities around the streams that promote high nutrient level for the organisms (Cooney and Shiaris, 1982). The high nutrient level therefore encouraged high bacterial growth and diversity (Atlas and Bartha (1992), Roubal et al., 1979). The screen test revealed that the number of hydrocarbonoclastic bacterial isolates

represented a total of 33% of the total bacterial isolates. These crude oil-utilizers utilized the Qua Iboe light crude oil for growth, energy and metabolism as depicted by the levels of turbidity produced in the crude oil mineral salt medium (MSM). The turbidimetric measurement indicated that the isolates *Pseudomonas-OUB6*, *Micrococcus-OUB7*, *Bacillus-OUB8*, *Mycobacterium-OUB12*, *Pseudomonas-OUB13* and *Pseudomonas-OUB14* had strong potentials to utilize and degrade the Qua Iboe light crude oil. *Micrococcus-OUB1*, *Bacillus-OUB4* and *Micrococcus-OUB5* exhibited moderate growth while *Mycobacterium-OUB2*, *Nocardia-OUB3* *Vibrio-OUB9*, *Nocardia-OUB10*, *Alcaligenes-OUB11* and *Vibrio-OUB15* exhibited maximal growth with a low potential to utilize Qua Iboe light crude oil in MSM.

Figures 1 - 3 shows the growth profile of the six best crude oil-utilizers. The growth profile as an index of potential to utilize Qua Iboe light crude oil as the only source of carbon and energy was based on changes in the optical density (OD at 540nm wavelength) of the growth medium, pH of the culture at room temperature (28±2^{0}C) and total viable count (TVC) (Antai, 1990; Itah and Essien, 2001; 2005). *Pseudomonas-OUB14* exhibited the highest optical density level of 0.919 after 20 days of incubation (Figure 1). It demonstrated a steady rise of OD after day 5 to day 20 of incubation. This was followed by *Pseudomonas-OUB6*, *Pseudomonas-OUB13* and *Mycobacterium-OUB12*. These organisms recorded an average optical density of 0.596 after 10 days of incubation while *Micrococcus-OUB7* exhibited an OD of 0.583 after 5 days of incubation and remained slightly unchanged thereafter for the 10 to 20 days of incubation. The crude oil utilization process marginally affected the pH of the MSM (Figure 2). Bioutilization of crude oil by bacteria results in growth and production of acidic metabolites (Itah and Essien (2005), Ijah and Antai (1988), Madigan *et al* (1997) and Cernigali, (1992)). These metabolites are responsible for the decrease in pH of 6.85 to a more acidic level of 5.9 after 10 days of incubation by *Mycobacterium-OUB12*, and relatively no change by *Pseudomonas-OUB14*. The effect of pH varied with the test isolates and incubation time (Figure 2). No change in pH was recorded after the incubation period of 15 - 25 days for any of the bacterial isolates.

Table 1: Some physical properties and total heterotrophic bacterial count (THC) in the freshwater samples

S/No.	Water Sample	Temp ^{0}C	pH	Turbidity OD 540nm	Total Heterotrophic bacterial count (THC) cfu ml^{-1}
1	A	28	6.90	0.420±0.01	10.6±0.41 x 10^9
2	B	27±0.5	6.90	0.390±0.01	9.3±0.20 x 10^9
3	C	28±0.6	7.00	0.400±0.01	9.7±0.25 x 10^9
4	D	28	7.00	0.440±0.02	5.3±0.25 x 10^9
5	Mean	28	6.95	0.413±0.01	8.73±0.30 x 10^9

Key: Water sample
Idim Nakanda
Idim Ebisa
Idim Ikot Eyo
Idim Esa Polycal

Figure 3 presents the total viable count (TVC) of the bacterial isolates. The isolates exhibited different growth pattern over time (Antai and Mbgomo, 1993). These patterns reflect a typical Monod kinetic (Okerentuga and Ezeronye 2003). The prolonged log phase by *Pseudomonas-OUB13* and *Pseudomonas-OUB14* indicated that these organisms utilized the crude oil for carbon and energy at a rather slow rate. The utilization of the crude oil therefore gave an average cell count of 6.33±0.26 x 10^8 cfu ml^{-1} as against the total heterotrophic count of 8.73±0.3 x 10^9 cfu ml^{-1}. This result reflected the adaptability potential of the test isolates from unpolluted to a crude oil polluted freshwater environment (Okerentuga and Ezeronye, 2003) hence their ability to utilize Qua Iboe light crude oil.

Figures 4-6 show the percentage bioutilization rates of the 3 most efficient Qua Iboe light crude oil-utilizers. The bacterial species *Micrococcus-OUB7*, *Pseudomonas-OUB13* and *Pseudomonas-OUB14* were tested on three different concentration of crude oil (1%, 5% and 10%). The result revealed that the hydrocarbonoclastic bacteria had caused substantial reduction in weight loss in all concentration of the crude oil. The degree of weight loss is observed to increase with increase in crude oil concentration and incubation days. A percentage weight loss above 90% was recorded at 10% concentration while 82% and 80% were recorded at 5% and 1% respectively. In comparison, as the incubation days prolonged, the mean percentage weight loss (10%>5%>1%) showed significant linear relationship (p>0.05). The low total petroleum hydrocarbon (TPH) concentration limits bioutilization because the carbon supply may be low to support bacterial growth (Leahy and Colwell, 1990). This low concentration of TPH (1% and 5% pollution level) created a vaccum between the oil-water interface thereby reducing its metabolism and inhibited bacterial growth.

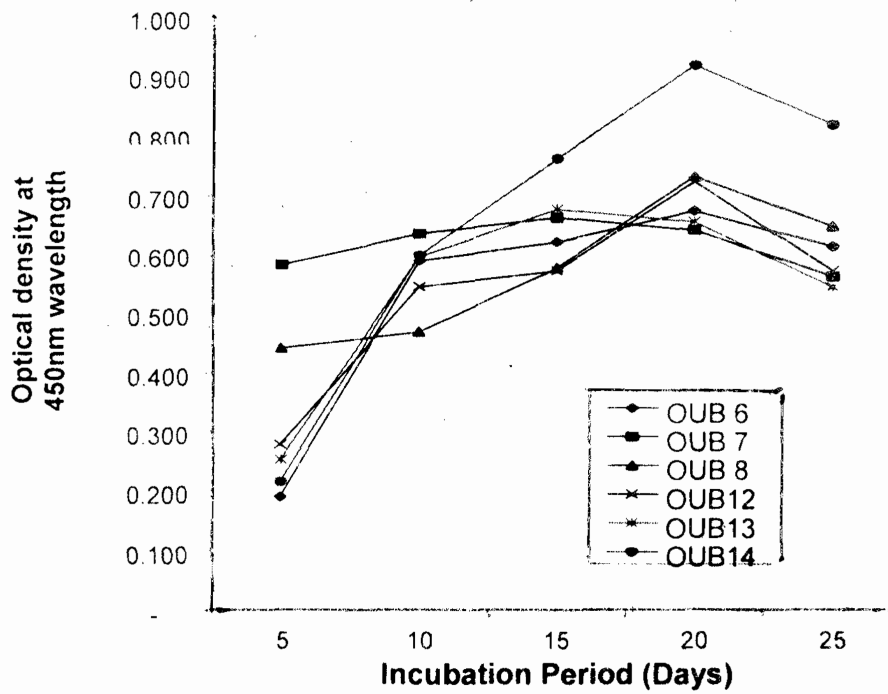

Figure 1: Growth profile (Optical density) of the 6 isolate as index of growth and utilization of Qua Iboe light crude oil.

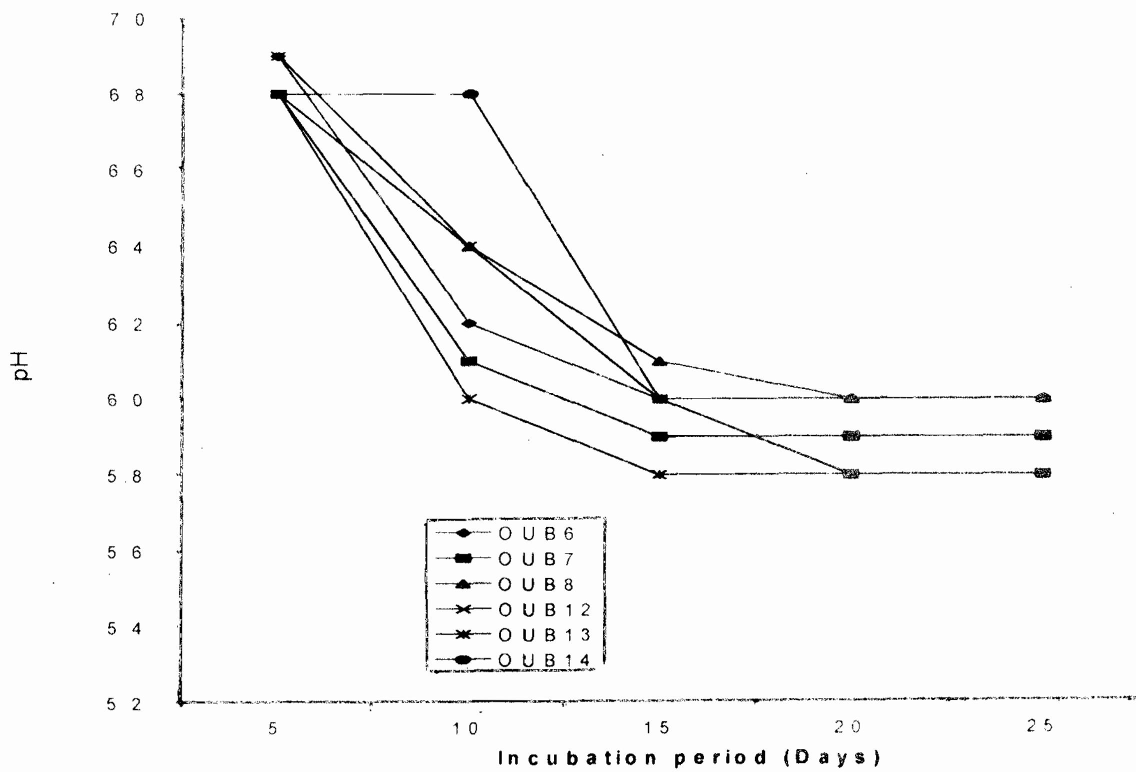

Figure 2: pH changes resulting from the growth of 6 bacterial isolates in crude oil.

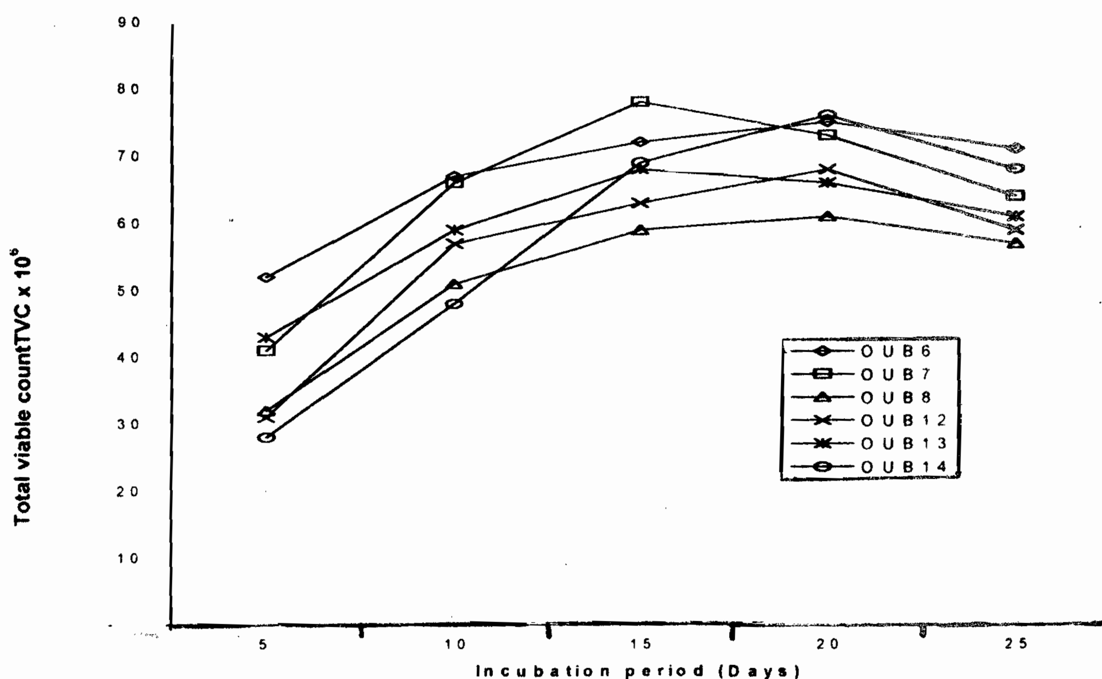

Figure 3: Total viable count (TVC) as a measure of growth of six bacterial isolates on Qua Iboe light crude oil.

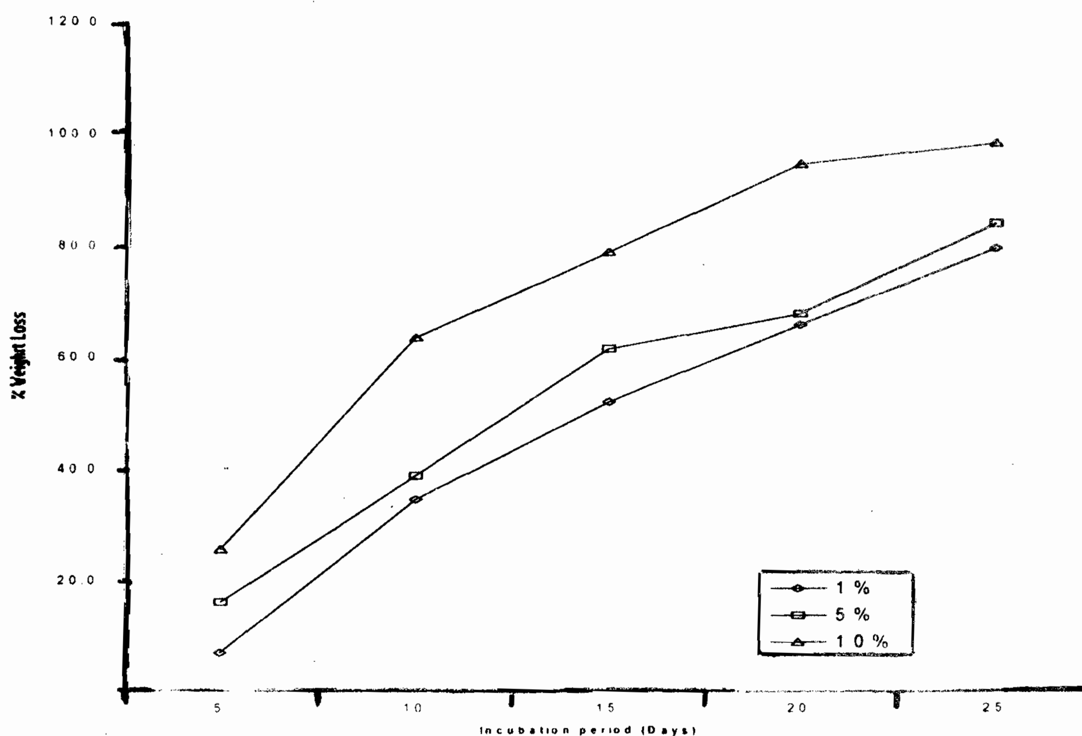

Figure 4 : Bioutilization of different concentration of Qua Iboe light crude oil by *Micrococcus*-OUB7.

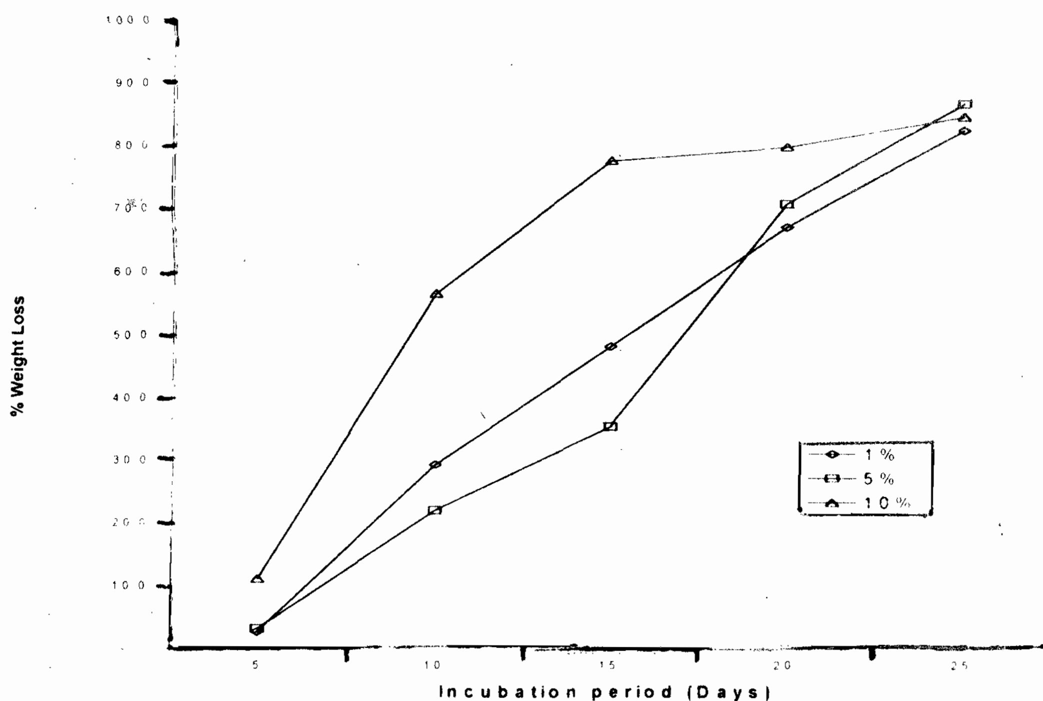

Figure 5: Bioutilization of different concentration of Qua Iboe light crude oil by *Pseudomonas*-OUB13.

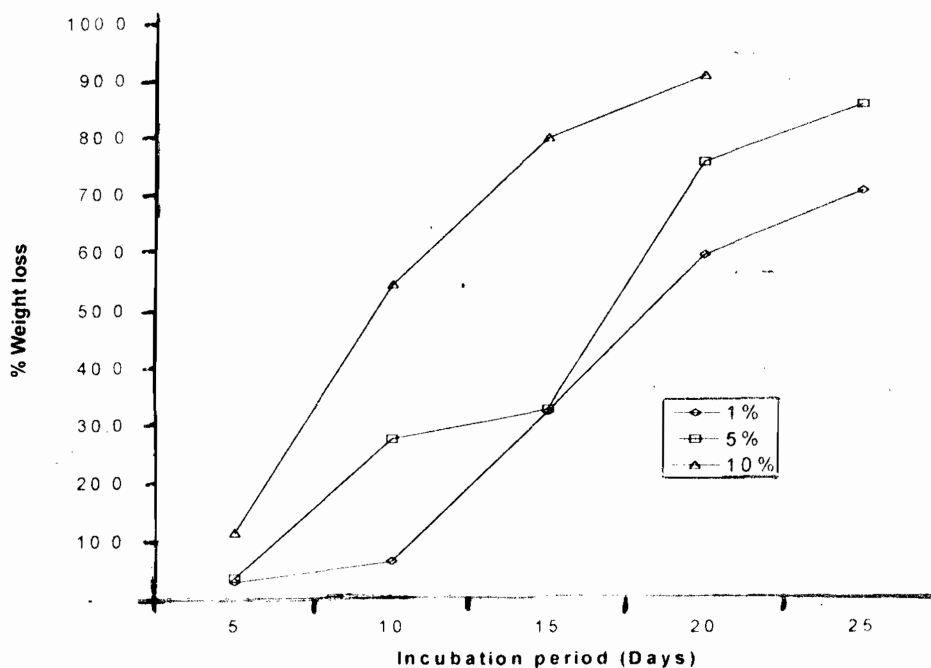

Figure 6. Bioutilization of different concentration of Qua Iboe light crude oil by *Pseudomonas*-OUB14.

SUMMARY

This study has revealed that there is a strong positive correlation (r = 0.878) between the increase in turbidity (OD), cell growth (TVC), pH and degradation (rate) of Qua Iboe light crude oil by the test bacterial isolates with increasing incubation days. This relationship is supported by the excellent hydrocarbonoclastic potential of *Micrococcus-OUB7*, *Pseudomonas-OUB13*, *Bacillus-OUB8*, *Mycobacterium-OUB12*,

Pseudomonas-OUB14 and *Pseudomonas-OUB6*. The study has further revealed that there is a positive correlation between the level of crude oil concentration and bioutilization by the test isolates. The test isolates *Micrococcus-OUB7 Pseudomonas-OUB13* and *Pseudomonas-OUB14* caused an average weight loss of approximately 90%, 82% and 80% of 10%, 5% and 1% crude oil pollution levels respectively after 25 days of incubation These freshwater isolates may be good candidates for bioaugmentation to bioremediate crude oil pollution in freshwater ecosystem in Cross River State of Nigeria.

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