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PETROLEUM HYDROCARBON DEGRADING CAPABILITY OF FRESHWATER AUTOCHTHONOUS FILAMENTOUS FUNGI

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ABSTRACT

Petroleum hydrocarbon degrading capability and growth profile of indigenous filamentous freshwater fungi from four (4) different streams were determined in vitro. The result indicated that the streams under investigation contained an average heterotrophic fungal count of 5.55 $\pm$0.25x10^5 cfu ml^{-1} while the mean count of biodegraders of Qua Iboe light crude oil after 15 days of incubation was 3.17$\pm$0.21 x 10^4 cfu ml^{-1} The best four utilizers of petroleum hydrocarbons for carbon and energy isolated from the streams were *Aspergillus-OUF7*, *Rhizopus-OUF8*, *Aspergillus-OUF11* and *Fusarium-OUF14*. The growth profile of the fungi on oil medium showed that the isolates have an average total viable count (TVC) of 16% after an incubation period of 35 days, hence their ability to utilize Qua Iboe light crude oil efficiently. The isolates equally demonstrated a high degree of crude oil degrading potentials with an average percentage weight loss of 92%, 78% and 55% after 35days of incubation on 1%, 5%, 10% pollution levels on Qua Iboe light crude oil respectively. The percentage weight loss among the test isolates showed a positive correlation between pollution levels and varied significantly ($p<0.05$) over incubation period. The results therefore suggest that systematic bioremediation involving these autochthonous freshwater fungi can be effectively utilized over a period in remediating considerable levels of crude oil pollution in a freshwater ecosystem within the Niger Delta region of Nigeria.

KEYWORDS: Freshwater, bioutilization, fungal isolate, crude oil, pollution levels.

INTRODUCTION

Oil exploration and oil related industrial activities have resulted in various degrees of oil pollution problems in many developing nations In the southern part of Nigeria, where most of the crude oil is exploited, there is a great deal of environmental deterioration due to spillage of oil and crude oil distillates. Petroleum hydrocarbon contamination of environments has aroused a tremendous interest of the public and stakeholders in the oil industry. Accidental oil spills on land are generally easier to control and cleanup than oil spills in water (Bartha, 1986, Atlas and Bartha, 1992). Here lies the problem.

Provision of freshwater, in terms of quantity and quality for domestic, agricultural and industrial purposes in Nigeria, is a hideous task. The contamination of freshwater sources (steams, lakes and ponds) with crude oil hydrocarbons may constitute public health hazards and socio-economic problems to their immediate communities (Kobayashi and Rittman, 1982).

Hydrocarbons in water may result in serious aquatic toxicity problems that affect microbial physiological processes, genetic machinery and population density (Atlas and Bartha, 1977). Very often, hydrocarbon pollutants inhibit important bio-geochemical cycles of the aquatic system and thus affects the productivity of the ecosystem (Atlas and Bartha, 1977). In Southern Nigeria, freshwater habitats contain a lot of debris and organic matter as sediments, and thus harbour diverse microbial populations including filamentous fungi capable of degrading various hydrocarbon pollutants such as petroleum hydrocarbons.

This study, therefore, evaluates the potential of freshwater fungal isolates from unpolluted streams to biodegrade the Qua Iboe light crude oil at different pollution levels in the Niger Delta region of Nigeria.

MATERIAL AND METHODS

Collection of samples

The Qua Iboe light crude oil sample used in this study was collected from the Qua Iboe Exxon Mobil Production Unlimited terminal, Akwa Ibom State, Nigeria The fungal isolates were obtained from perennial streams (Idim Nakanda, Idim Ebisa, Idim Ikot Eyo and Idim Esa Polycal) located in Akpabuyo and Calabar south local government areas in Cross River State, Nigeria. The freshwater samples were stored in an ice packed container (cooler, 4°C) prior to microbiological analysis, which was carried out within 24 hours.

Measurement of some physical properties of the water samples

The temperature of the water column was measured with a thermometer caliberated in degree centigrade (°C), the pH of the water was measured with an electronic (field) pH meter (Slop Labtech model, England). The turbidity of the water samples was measured as optical density (OD) at 540nm wavelength with a spectrophotometric colorimeter (model Spectronic 20 Genesy, Spectronic Inst. Inc. Rochester, NY).

Mycological analysis of water samples

The fungal count was determined by the spread plate method using potato dextrose agar supplemented with 50µg streptomycin/litre to inhibit bacterial contaminants. In this method, 0.1ml of prepared 10^{-4} dilution of the water samples A to D in sterile distilled water was inoculated in triplicate on the PDA. The plates were incubated at room temperature (28$\pm$2°C) for 96 hours in an inverted position. Pure colonies of the fungal isolates obtained from the agar were counted, and expressed as colony forming units (cfu ml^{-1}). The colonies also were characterised and identified as described by Mills *et al.* (1978) and Barnett and Hunter (1998).

Isolation and purification of oil utilizing fungi

Hydrocarbonoclastic fungi from the stream water samples were isolated using surface vapour phase respiratory transfer technique (SPRT). This involved surface spreading of 0.1ml of the 10^{-4} dilution of mineral salt medium. Then 50µg/ml concentration of filter sterilized streptomycin sulphate was added to the medium to inhibit the growth of bacterial contaminants Sterilized whatman No.1 filter paper was

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saturated with 2.0ml of sterile Qua Iboe light crude oil and aseptically placed on the inside cover of plate and sealed. The plates were inverted and incubated at room temperature (28±2°C) for seven (7) days from which discrete colonies that developed were aseptically picked and purified by repeated subculturing on malt extract agar slant.

Determination of the utilization of Qua Iboe light crude oil by freshwater fungal isolates

The gravimetric method described by Okpokwasili and Okorie, (1988) was used to screen the fungal isolates for their potential to utilize Qua Iboe light crude oil as their only source of carbon and energy. In this method, 9.9ml of mineral salt broth (MSB) of Bushnell and Haas, (1941) in test tubes were sterilized by autoclaving at 121psi for 15 minutes. Thereafter 0.1ml of filter sterilized (0.45 µm pore size) crude oil was added and aseptically inoculated with a 72-hour old malt broth culture of the test isolates. Three uninoculated test tubes containing mineral salt broth and 0.1ml of light crude oil only were prepared to serve as controls. Inoculated tubes were incubated at room temperature (28±2°C) undisturbed at a stationary condition for 10 days during which the tubes were compared with the controls and observed visually for turbidity. The ability of the fungal isolates to utilize the crude oil was graded as high (+++), moderate (++), weak (+) and no growth (-) as against the control and the optical density (OD) of the medium was measured at 540nm using a spectrophotometer (Model Spectronic 20 Genesys, Spectronic Institute, Inc., Rochester, N.Y.)

Determination of growth profile of fungal isolates in crude oil

To determine the ability of the fungal isolates to degrade Qua Iboe light crude oil, their growth profile in an oil medium was assayed using the method prescribed by Okpokwasili and Okorie, (1988) In this method 99.0ml of Bushnell and Haas. (1941) mineral salt medium (MSM) was measured into six 250ml capacity Erlenmeyer flasks and autoclaved at 121psi for 15 minutes. Then 0.1ml of filter sterilized Qua Iboe light crude oil was added to the medium. Four of the flasks were inoculated with the test organisms identified and coded as *Aspergillus-OUF7*, *Rhizopus-OUF8*, *Aspergillus-OUF11* and *Fusarium-OUF14*, while three un-inoculated flasks containing only the medium and crude oil served as controls. The flasks were incubated at room temperature (28±2°C) on a rotary shaker (Model Gallenhamp flask shaker, England) rotating at 110 rpm for 35 days. At interval of 7 days, 20.0ml of the samples were aseptically obtained from each flask and used for the following determinations. 1) Optical Density (OD) at 540nm wavelength determined with a spectrophotometer. 2) pH changes monitored with a digital electronic pH meter (Jenway 3510 model). 3) Total viable counts (TVC) determined by the spread plate technique.

Determination of the rate (percentage weight loss) of crude oil resulting from biodegradation by fungal isolates

In determining the percentage of crude oil biodegradability by the test fungi at three levels (1%, 5% and 10%) of oil pollution, mineral salt broth (Bushnell and Hass, 1941) was prepared and 9.90ml, 9.50ml and 9.0ml quantities were measured into nine 100ml capacity flasks. The flasks were then sterilized by autoclaving and on cooling 0.1ml, 0.5ml and 1.0ml (representing each level of pollution) of filter-sterilized Qua Iboe light crude oil were added into 9.9ml, 9.50ml and 9.0ml respectively. Then 0.1ml of a 72 hour broth culture of the test fungal isolates was then inoculated into the flasks and four unincoluated flasks remained as control. The flasks were incubated at room temperature (28±2°C) on a shaker for 35 days. At interval of 7 days a set of 4 flasks reflecting the levels of pollution per isolate were removed and the amount of crude oil left was determined by extracting the residual crude oil with 20.0ml of n-hexane and their spectrophotometric absorbance (optical density) read at 540nm using a Spectronic spectrophotometer (Model: Spectronic 20 Genesys Spectronic Institute, Inc., Rochester, NY). The weight of the extracted oil residue was obtained by reading off the respective absorbance from a previously prepared standard curve. Percentage biodegradation or weight loss of the added (polluted) crude oil was determined from the following relationship: *Weight of the crude oil (control) minus weight of crude oil (degrade) divided by weight of crude oil (control) divided by 100*

$$\text{Percentage degradation} = \frac{a - b}{a} \times \frac{100}{1}$$

Where a is the weight of crude oil (control), b the weight of crude oil (degraded).

RESULT

Physical properties of the representative streams considered in this study are shown in Table 1, which shows that the streams are perennial, slow running, and had an average temperature of 28°C. The mean pH recorded was 6.9±0.1 while the optical density (turbidity) ranged from 0.39±0.01 to 0.44±0.02. The mean total heterotrophic fungal count was 5.55 ±0.25x 10^5 cfu ml^{-1} Water sample B had the highest fungal count of 6.4 ±0.15 x 10^5 cfu ml^{-1} while sample D recorded the lowest count of 5.4 ±0.16 x 10^5 cfu ml^{-1} which was less than the mean count.

Among the isolates, *Aspergillus-OUF11*, *Fusarium-OUF14*, *Rhizopus-OUF8* and *Aspergillus-OUF12* exhibited abundant growth in mineral salt medium (MSM). Moderate growth and a fairly strong hydrocarbonoclastic potential was exhibited by *Candida-OUF 5*, *Penicillium-OUF 6*, *Turolopsis-OUF 9*. *Fusarium-OUF 10*, *Rhodoturula-OUF 12 and Mucor-OUF 13* while *Penicillium-OUF 1*, *Candida-OUF 2*, *Saccharomyces-OUF 3 and Mucor-OUF 4* exhibited relatively poor growth in (MSM) with low hydrocarbonoclastic potentials.

Growth profile results revealed the variable potentials of the fungal isolates to grow in crude oil mineral salt medium (Figures 1 – 3). The indices used to determine the growth profiles were; changes in pH, optical density (OD) of the oil medium and the total viable count expressed as colony forming units per milliliter (cfu. ml^{-1}). The results indicated that for each of the isolates, OD increases as the TVC increases after 7 – 21 days of incubation and thereafter dropped correspondingly

Figures 4 – 7 present percentage (%) weight loss for the three pollution (1%, 5%, 10%) levels. The best utilization rates or weight losses by all the isolates were recorded at 1% pollution level, followed by 5% and 10% being the least. The percentage weight loss among the test isolates showed a positive correlation between concentration levels of crude oil and varied significantly ($p<0.05$) over incubation period.

DISCUSSION

The study has shown that the streams under investigation are relatively rich in hydrocarbonoclastic filamentous fungi. A mean total heterotrophic fungal count obtained was 5.55 ±0.25 x 10^5 cfu ml^{-1} out of which only 33% of the isolates had little to high hydrocarbonoclastic potential. This finding agreed with the report that high fungal cells in freshwater are attributed to high organic contents generated by human activities of the streams, and are considered as

members of the ecosystem (Atlas and Bartha, 1992), and that aquatic ecosystems harbour a variety of microorganism most of which are hydrocarbonoclastic (Shekel and Ravid, 1977,

Atlas, 1981). Equally, economic activities generated through human activities within and around these streams with no recorded history of crude oil pollution could have been responsible for the increased level of nutrients in the streams, which could have enhanced the high fungal growth, population density and diversity (Roubal *et. al.,* 1979). The hydrocarbonoclastic fungi utilized the Qua Iboe light crude oil for growth and energy and proliferated as indicated by the level of turbidity produced in the mineral salt medium (MSM).

The low percentage (33%) of oil utilizing fungi is attributed to the fact that the streams investigated were not previously exposed to hydrocarbon pollution. It is clear from a number of studies that the distribution of hydrocarbon utilizing microorganisms reflects the historical exposure of the environment to hydrocarbons, and a number of laboratory studies have demonstrated sizeable increase in population when environmental samples are exposed to petroleum hydrocarbon (Atlas, 1981).

Table 1: Some physical properties of water samples from four (4) streams

Water sample	Temp. ^{O}C	PH	Turbidity OD 540nm	Total heterotrophic count (THC) c.f.u. ml^{-1}
A	28	6.90	0.42±0.01	4.6 ±0.15X 10^{-5}
B	27±0.5	6.9±0.06	0.39±0.01	6.4 ±0.15X 10^{5}
C	28±0.6	7.0±0.06	0.40±0 01	5.8 ±0.30X 10^{5}
D	28	7.0±0.06	0.44±0.02	5.4±0.06 X 10^{5}
Mean	27.5±0.5	6.95±0.1	0.413±0.02	5.55±0.25 X 10^{5}

Key: Water sample
A. Idim Nakanda
B. Idim Ebisa
C. Idim Ikot Eyo
D. Idim Esa Polycal

Profiles of the best four oil degrading fungal isolates in MSM are presented in figures 1-3. The growth profile or optical density (OD) is taken as an index of ability to utilize Qua Iboe light crude oil as the only source of carbon, energy and metabolism in MSM which is reflected by changes in pH of cultures at room temperature (28±2^{O}C) and total viable count (TVC), (Antai, 1990, Itah and Essien, 2001, 2005). *Aspergillus-OUF11* exhibited the highest OD of 0.296 followed by *Fusarium-OUF14, Aspergillus-OUF7* and *Rhizopus-OUF8* after 35 days of incubation (Figure 1). The pH of the MSM was affected by the bioutilization process (Figure 2). The utilization of crude oil hydrocarbons by microorganisms results in their growth and concomitant production of acidic metabolites (Ijah and Antai, 1988). Also, aerobic biodegradation of aliphatic and aromatic hydrocarbons leads to the production of organic acids (Nweke and Okpokwasili, 2003). The acidic metabolites are responsible for the decrease in pH of the growth medium as in the case of *Fusarium-OUF14* from pH 6.7 to a more acidic level pH 6.2 over time (Itah and Essien, 2005). The effect of pH varied with the fungal species and increases with time in each case. The acidity level of MSM were reasonably increased by the activities of *Fusarium-OUF14, Aspergillus-OUF7* and *Rhizopus-OUF8*. The total viable count was highly affected by the bioutilization process (Figure 3). The total cell count on MSM varied among the species tested. All the test organisms were at their peak after 21 days of incubation and relatively dropped after 35 days of incubation. *Aspergillus-OUF7* and *Rhizopus-OUF8* exhibited very little changes and differences in their growth pattern. *Aspergillus-OUF11* with the highest OD of 0.296 produced the highest cell count (Figure 2). The least number of cells was produced by *Rhizopus-OUF8* with a fluctuating OD between the incubation period of 14 – 28 days. These findings demonstrated that *Aspergillus-OUF11* and *Fusarium-OUF14* are better oil utilizers than *Aspergillus-OUF7* and *Rhizopus-OUF 8*.

The degree of weight loss for the three levels (1%, 5% and 10%) of Qua Iboe light crude oil pollution on MSM are presented in figures 4 – 7. The degree of weight loss was observed to increase with increase in incubation period; decrease with pollution levels and varied among the different fungal species tested. The average percentage weight loss for (1%) pollution level was 92%, 5% level was 78% and 10% level was 55% after 35 days of incubation. However, at 1% pollution level *Aspergillus-OUF7* (Figure 4) left little or no trace of oil after 28 days of incubation while at 10% pollution level *Aspergillus-OUF11* (Figure 6) bioutilized 60% of the oil better than the other tested isolates after 35 days of incubation. *Rhizopus-OUF8* performed poorly in all concentrations as compared with other tested species thereby making it not suitable for any bioremediation exercise.

The poor utilization degree by the isolates at 10% pollution level is related to the concentration level of the crude oil. At this concentration, the oil became toxic and lethal to the fungal isolates causing a reduction in their utilization rate. Secondly, at 10% concentration, the oil might have been in excess of its solubility limit and resulted in spreading at 28±2^{O}C. Atlas (1981) reported that the degree of oil spreading determined, in part, the surface area of oil available for microbial colonization by hydrocarbon degrading microorganism in aquatic systems. Therefore reduction in spreading of the oil reduced the available nutrients and oxygen. These negative conditions stressed the fungi and reduced their ability to breakdown the oil efficiently.

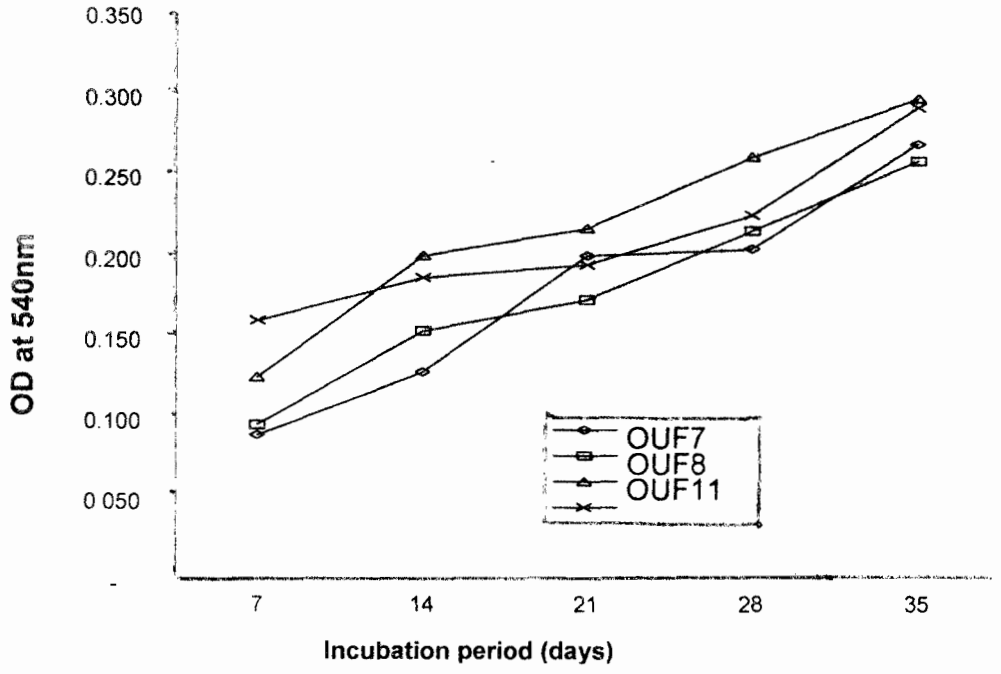

Figure 1: Growth profiles (OD) of the four isolates *Aspergillus-OUF7, Rhizopus-OUF8, Aspergillus-OUF11* and *Fusarium-OUF14* in crude oil mineral salt medium (MSM)

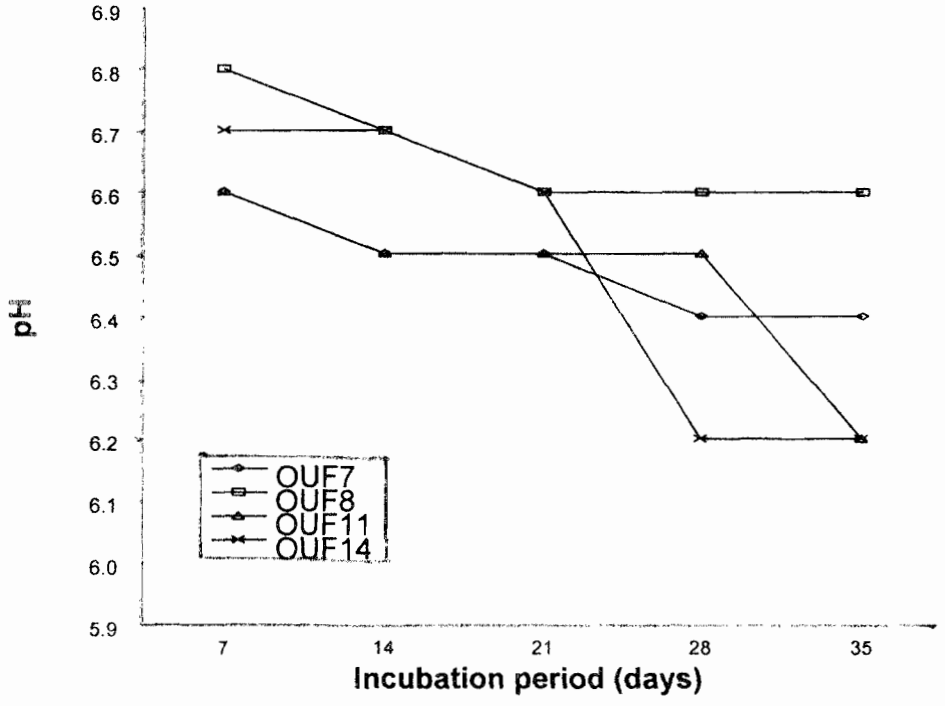

Figure 2: Growth profiles (pH) of the four isolates *Aspergillus-OUF7, Rhizopus-OUF8, Aspergillus-OUF11* and *Fusarium-OUF14* in crude oil.

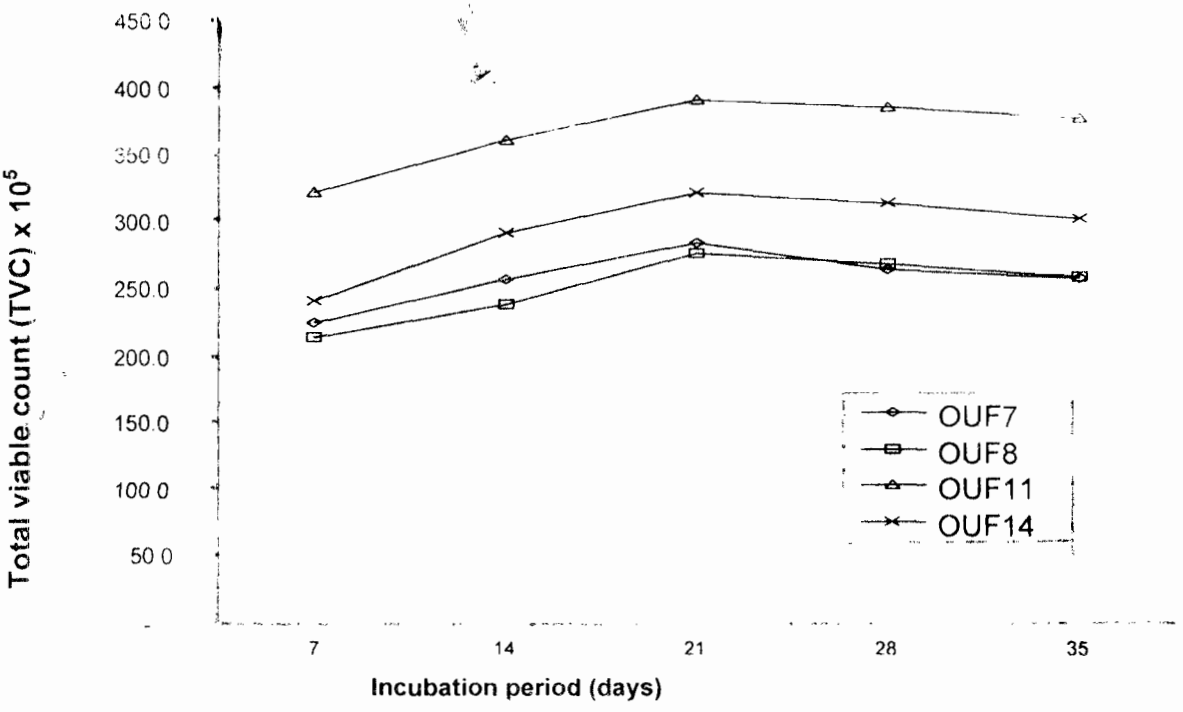

Figure 3: Growth profiles of total viable count, (TVC) of *Aspergillus-OUF7, Rhizopus-OUF8, Aspergillus-OUF11* and *Fusarium OUF14* in crude oil

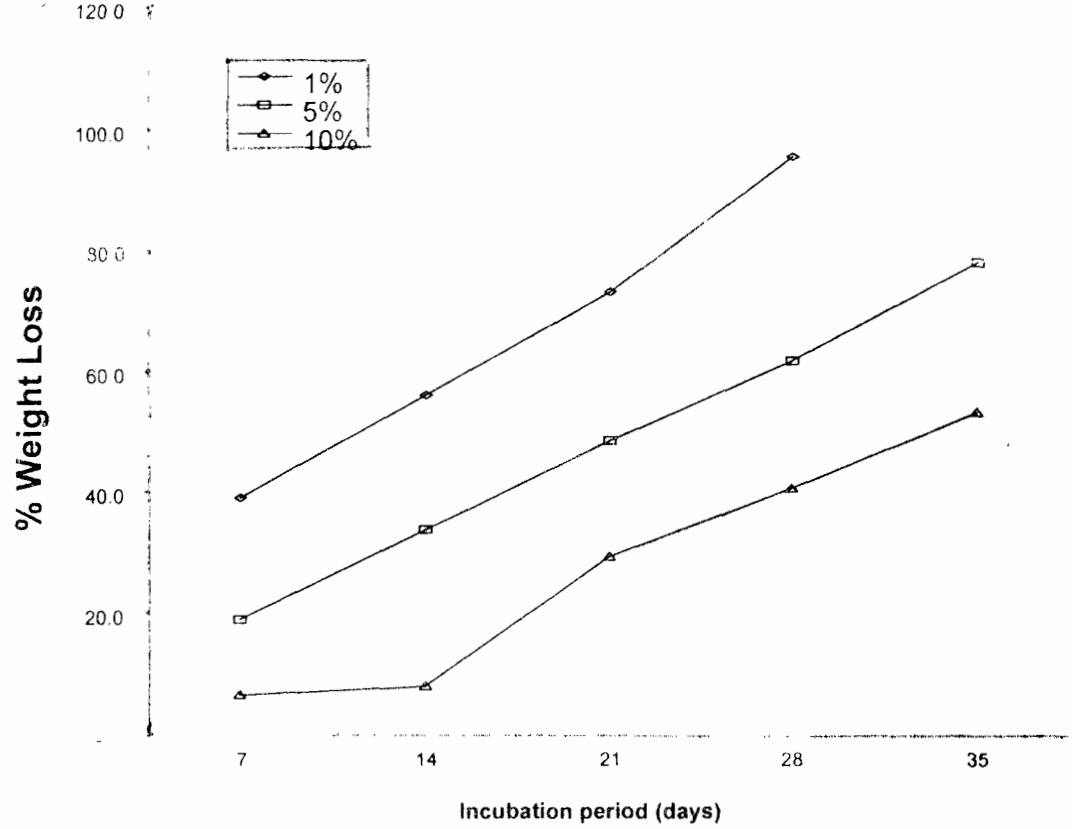

Figure 4: The percentage weight loss/bioutilization by *Aspergillus-OUF7* at 1%, 5% and 10% pollution levels of Qua Iboe light crude oil

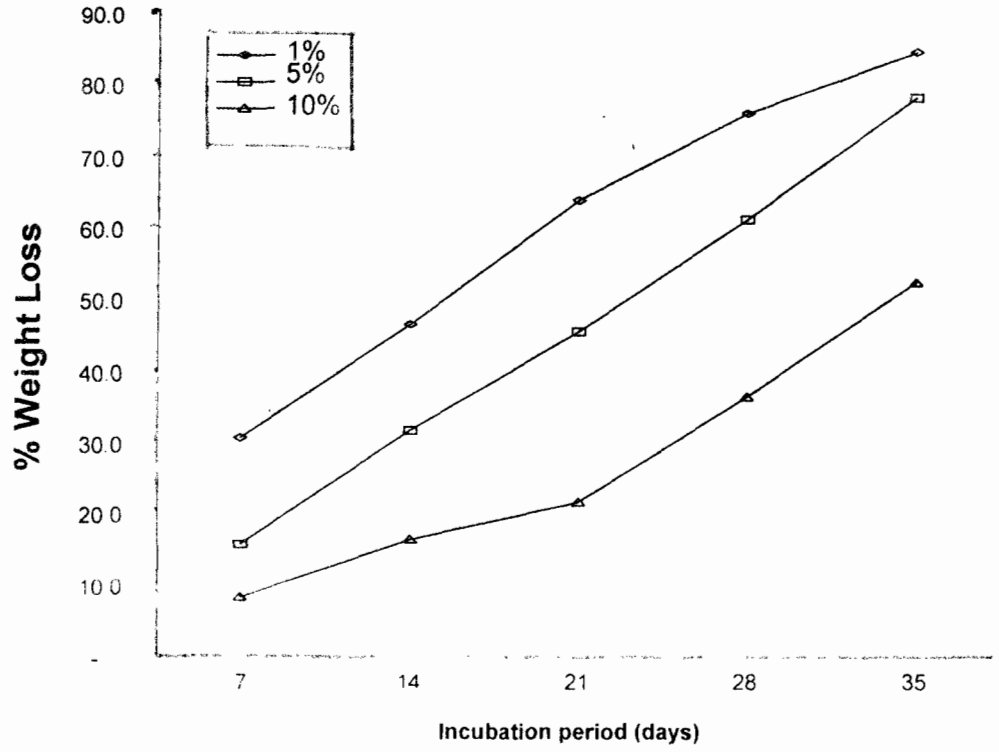

Figure 5: The percentage weight loss/bioutilization by *Rhizopus-OUF8* at 1%, 5% and 10% pollution levels of Qua Iboe light crude oil.

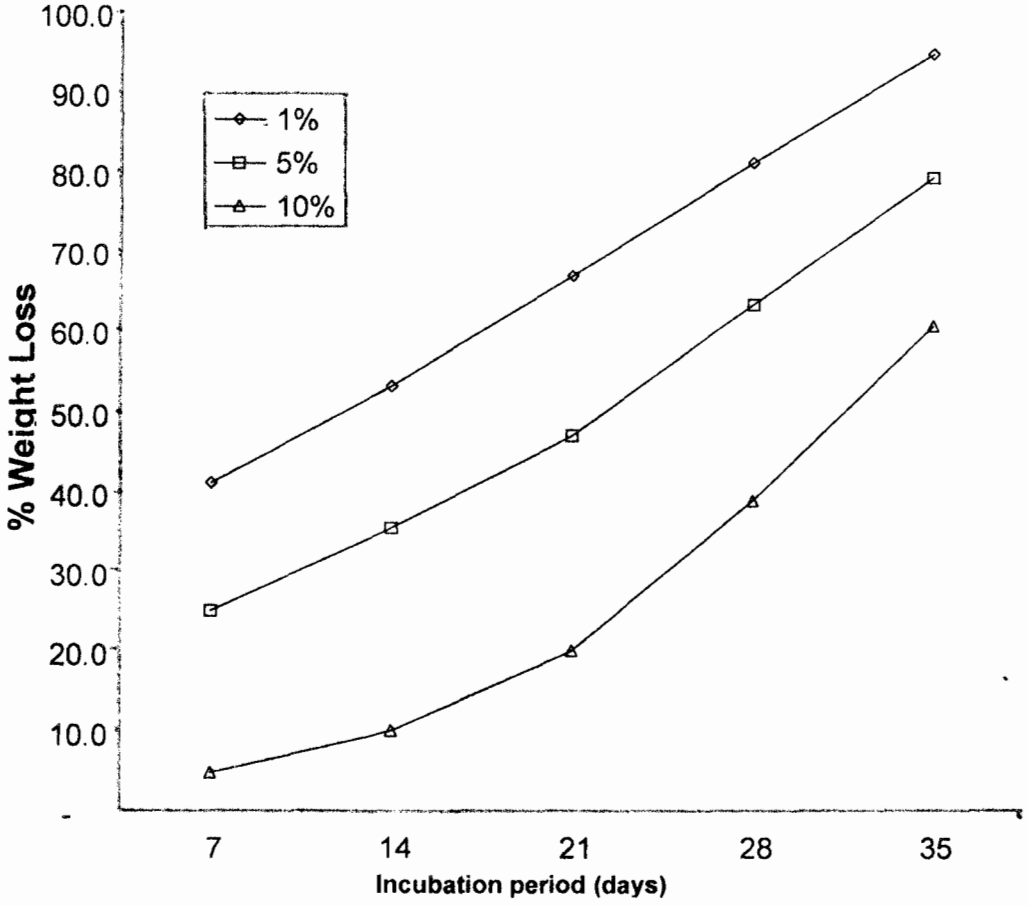

Figure 6: The percentage weight loss/bioutilization by *Aspergillus-OUF8* at 1%, 5% and 10% pollution levels of Qua Iboe light crude

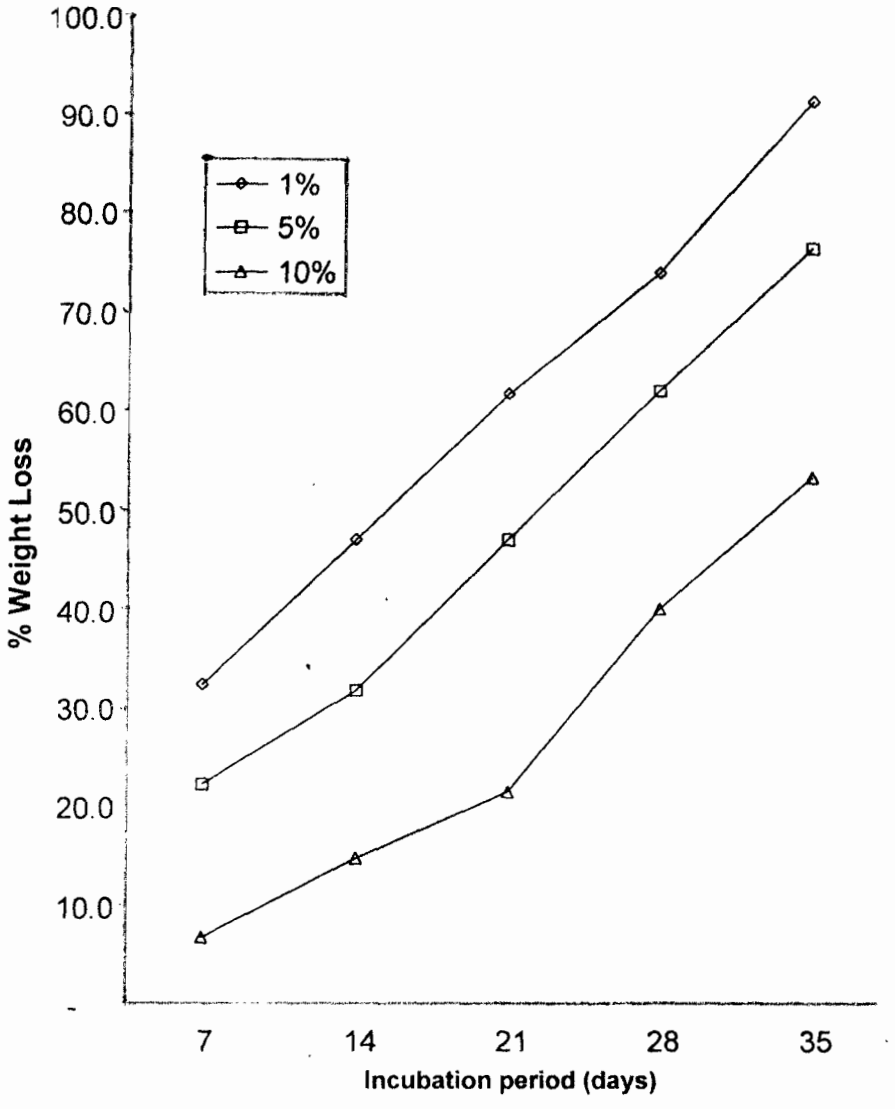

Figure 7: The percentage weight loss/bioutilization by *Fusarium-OUF 14* at 1%, 5% and 10% pollution levels of Qua Iboe light crude oil

CONCLUSION

The present study has revealed a remarkable relationship between growth profile and Qua Iboe light crude oil degradation. The direct increase in acidity (pH), optical density (OD) and total viable count (TVC) reflected the crude oil degrading potential of most hydrocarbonoclastic fungi (Itah and Essien, 2005). From the study, it is observed that the higher the OD of cultures, the more acidic the medium and the higher the cell count (TVC) and the capability of the fungi to degrade the crude oil. These observations are strongly demonstrated by *Aspergillus-OUF11* and *Fusarium-OUF14*. The study equally revealed that crude oil pollution level is a significant factor in biodegradation process involving filamentous fungi in freshwater ecosystem. The result confirmed that higher levels of crude oil (10%) pollution inhibited bioutilization compared to the low pollution levels of 1% and 5%. It therefore means that the more quantity of crude oil added as a pollutant to water, the more toxic and resistant the compounds become to microbial degradation (Ijah and Antai, 1988) and that bioremediation technology involving the use of these test organisms *Aspergillus-OUF11, Fusarium-OUF14* and *Aspergillus-OUF7* in freshwater at 1 – 5% pollution will be effective in the Niger Delta region of Nigeria

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