Nigerian Journal of Microbiology.2009, Vol 23(1); 1945 - 1952

Growth Profile, Sodium Chloride Tolerance and Substrate Specificity of a Hydrocarbon – utilizing *Mucor species*

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Abstract

The growth profile, sodium chloride tolerance and substrate specificity of a hydrocarbon –utilizing *Mucor* species was investigated using standard microbiological methods. The fungus caused a weight loss of 47.7% and 54.5% on crude oil and kerosene respectively after a 21 – day incubation at 28°C although growth was still at log phase, the pH decreased and dissolved oxygen concentrations fluctuated. The organism could only tolerate 1.0% NaCl. Above, this, total viable counts decreased from 8.0 x 10^3 cfu/ml at 1.0% to 7.2 x 10^3 cfu/ml at 2.0%. Optimum growth of the fungus was observed at the following substrate concentrations: Crude oil, 5%, Kerosine, 8%, Hexadecane, 5%, Naphthalene, 3% and Cyclohexane, 3%. This work therefore suggests that growth conditions must be greatly enhanced in order to optimize bioremediation of of impacted ecosystems.

Keywords: Sodium chloride, crude oil, tolerance, fungus utilization
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Introduction

The high demand for petroleum as a source of revenue and energy and as a raw material for chemical and petrochemical industries in Nigeria has resulted in corresponding increase in the rate of petroleum exploration and production activities. These activities have led to frequent oil spills, especially through well blow-outs, tanker accident and accidental rupture of oil pipelines. Bioremediation of oil contaminated environments is known to be the principal natural process for the removal of non-volatile fractions of oil from the environment, since it is safer and cheaper than the mechanical or chemical methods. Most studies cited in literature have investigated the ability of microorganisms to degrade crude oil (Antai and Mgbomo 1993). Attempts have also been made by various researchers to elucidate the pattern of hydrocarbon degradation and possible effects on micro flora and microfauna of the affected ecosystems. Much work has also been done on the effects of pH and temperature on biodegradation (Odu, 1981).

However, little has been done on the influence of sodium chloride and hydrocarbon concentrations on the growth and survival of hydrocarbon degrading fungi.. This work therefore was designed to achieve this since the rate of biodegradation generally depends on the the composition of the impacting compound and the prevailing growth conditions.

Materials and methods

Source of water samples

Water samples were collected from Ibeno beach in Akwa Ibom State, Calabar River, Great Kwa River and University of Uyo fish pond.

Source of Crude Oil and Kerosine

Nigerian light crude oil was collected from NNPC's Port-Harcourt Refinery, Alesa-Elema, Rivers State. Kerosene was obtained from NNPC Megastation, Uyo, Akwa Ibom State Nigeria.

Source of pure hydrocarbon substrates

Cyclohexane, naphthalene, hexane, hexadecane, toluene and benzene were obtained from the Chemistry Department, University of Lagos. All the hydrocarbon substrates were Analytical grade.

Collection of water samples

Water samples were collected aseptically in sterile sampling bottles and transported to the laboratory for analysis.

Enumeration of aerobic heterotrophic fungi

The total number of fungi was enumerated using surface spreading technique. Zero-point- one milliliter (0.1ml) of serially diluted water sample was plated on Malt Extract Agar supplemented with 100mg of Streptomycin and 15mg of Penicillin G per liter of medium (Fedorak *et al.*, 1984). Triplicate plates from 10^{-2} dilution of each sample was prepared and incubated at room temperature (28 +2°C) for 3 days. Counts were expressed as colony forming units (cfu) per ml of water sample.

Enumeration of Crude oil utilizing fungi

Serially diluted water sample was plated on modified Mineral salts medium solidified with 2% agar using the vapour phase techniqueas described by Okpokwasili and Okorie (1988). After inoculation, sterile Whatman No. 1 filter papers saturated with sterile crude oil were aseptically placed on the covers of inverted Petri dishes. Triplicate plates of 10^{-1} dilution were prepared, taped and incubated at 28°C for 7 days. Colony counts were expressed as cfu/ml.

Isolation of Kerosene- utilizing fungi

The fungi were isolated using modified mineral salt medium Okpokwasili and Okorie (1988) fortified with 100mg of Streptomycin and 15mg penicillin G per litre to inhibit bacterial growth. The medium was solidified with 2% agar. Aliquots of serially diluted water samples were spread, plated on mineral salt medium plates, sterile filter papers (Whatman No. 1) soaked with kerosene were placed aseptically unto covers of inverted Petri dishes. The plates were taped round with masking tape before incubating at 28°C for 7 days. Colonies that developed were transferred into fresh malt extract agar plates.

Determination of substrate specificity of *Mucor Species*

Ability of the isolate to utilize crude oil, kerosene, hexadecane, naphthalene and cyclohexane was tested using the method of Amadi and Antai (1991). Different amounts of each of the substrates were added to mineral salts medium to obtain 1.0%, 3.0%, 5.0%, 7.0%, 10.0%, 12.0% and 15.0% concentrations. The tubes were autoclaved at 121°C and 15 P.S.I for 15 mins. Each tube was inoculated with active inoculum of *Mucor species*. The tubes were incubated in a shaker-incubator at 120 rpm for 21 days at 28°C and total viable counts were determined.

Sodium chloride tolerance of *Mucor species*

Different quantities of NaCl were added to the mineral salts medium to obtain 0.05%, 0.1%, 1%, 2%, 3% 3.5%, 4.5%, 5.0% and 5.5% (w/v) concentrations. The tubes were autoclaved, cooled and inoculated with 0.1ml (2.3×10^3 cfu/ml) of active inoculum of *Mucor species.* Tubes were incubated at 28°C in a shaker-incubator at 120 rpm for 21 days and total viable counts determined.

Determination of degradation rate and growth profile in crude oil and kerosine

Two sets of bottles were set up as follows: one set containing 4.85ml of mineral salt medium and 0.05ml of

crude oil was inoculated with 0.1ml (3.7 x 10^3 cfu/ml) of active inoculum. The other set was 4.85ml mineral salt medium and 0.05ml of kerosene inoculated with 0.1 ml (2.8 x 10^3 cfu/ml) active inoculum. Control tubes were not inoculated. The bottles were incubated at 28 ± 2°C in a shaker incubator operated at 120rpm for 20 days N-hexane (20ml) was used to extract the residual crude oil or kerosine. The absorbance values of the extracts were read at 310nm for kerosine and 350nm for crude oil. Using previously prepared standard curves, the amounts of crude oil or kerosine degraded were obtained by the formula:

% weight loss of crude oil kerosine =

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

From the growth profile, samples were taken at four-day's interval for the determination of total viable count, pH change (using pH electronic meter (WTWG. MBH, Germany) and changes in dissolved oxygen concentration (using Oxygen meter, OxyGuard Handy MK II electronic meter).

Data analysis.

Results obtained were subjected to statistical analysis(ANOVA) to assess their significance.

Results and discussion

The abundance of aerobic heterotrophic and hydrocarbon utilizing fungi from the four water samples is shown on Table 1. The highest mean total fungal count of 3.4 ± 2.0 x 10^2 cfu/ml was obtained from Ibeno beach while the lowest count of 1.3 ± 1.0 x 10^2 cfu/ml was obtained from Great Kwa River. Hydrocarbon utilizing fungal counts ranged from 1.0 ± 1.7 x 10^1 cfu/ml to 2.3 ± 2.6 x 10^1 cfu / ml. The highest count was obtained from Ibeno while the lowest was from Great Kwa River. Analysis of variance revealed that there was a significant difference ($p < 0.05$) between counts obtained from samples previously exposed to oil pollution than those in non-polluted areas. This result agrees with the finding of Amadi and Antia (1991). It is likely that in polluted areas the spilled oil had enriched the native microflora with an inherent petroleum hydrocarbon assimilating capacity.

The substrate specificity of *Mucor* species is illustrated on Table 2. Total viable counts increased with crude oil and hexadecane concentrations up to 5%. Above this, the counts decreased. On the other hands, the fungus increased in counts with 3% naphthalene and cyclohexane. Further increase in concentration of the hydrocarbon substrates resulted in a decrease in total viable counts.

The results indicate that aliphatics (e.g hexadecane) or mixtures of aliphatic hydrocarbons (e.g kerosine) supported growth better than the aromatics (e.g naphthalene). Growth was least supported by the alicyclics (e.g cyclohexane). This result agrees with the report of Atlas (1981) showing that the n-alkanes are the most widely and readily utilized hydrocarbons. Aromatic hydrocarbons especially the polycyclic aromatics are quite toxic and recalcitrant to microbial degradation. The cycloalkanes are highly toxic and serve as growths substrates only in isolated exceptional cases.

The growth profile of the isolate is presented on Figure 1. As the total viable count increased, there was a corresponding decrease in the pH of the medium. At the end of 20 days of

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incubation, the growth of *Mucor* species was still at the log phase This indicated that growth could still continue after 30

days of incubation as the stationary growth phase had not been reached.

Table 3 shows the NaCl tolerance of hydrocarbon utilizing *Mucor* species. The organism grew well in the absence of NaCl (0.%). The total viable counts increased with increase in concentration of NaCl up to 1%. Concentrations of 2.0% and above caused a decrease in the total viable counts of the organism. This is because Nacl affects the physiology of petroleum hydrocarbon degraders. This is in consonance with Zobell (1973) and Rabus and Widdel (1995).

Figure 2 shows the biodegradation rate of *Mucor* on crude oil and kerosine. The organism caused a weight loss of 47.7% and 54.5% on kerosine and crude oil respectively. Many investigators have studied the biodegradation of crude oil by microorganisms (Antai and Mgbomo 1993, Ijah & Ukpe 1992) although majority of the studies were on bacteria.

This study therefore suggests that in order to greatly enhance the bioremediation of impacted ecosystems, key environmental factors governing the growth of the microfauna must be optimized

Table 1: Aerobic heterotrophic and hydrocarbon – utilizing microorganisms.

Sampling Site	Sampling code	Bacterial			Fungi		
		THB (x 10^5 cuf/ml)	HUB (x10^3 cuf/ml	% HUB	THF (X10^2 cfu/ml)	HUF (X 10^1 CFU/ML	% HUF
Ibeno beach	IB	8.3±0.5*	8.4±1.2	1.02	3.4±2.0	2.3±2.6	6.8
Calabar river	CR	6.4±1.5	6.2±2.0	0.96	2.0±2.0	1.3±1.7	6.5
Great kwa River	GK	3.1±1.7	1.6±2.6	0.52	1.3±1.0	1.1±1.0	8.5
University of Calabar fish pond	UP	3.8±1.0	1.9±1.7	0.5	1.5±1.0	1.0±0.5	6.7
Range	-	3.1-8.3	1.6-8.4	0.5-1.02	1.3-3.4	1.0-2.3	6.5-8.5

Key; THB=Total heterotrophic bacteria, HUB=Hydrocarbon utilizing bacteria
THF=Total heterotrophic fungi; HUF=Hydrocarbon utilizing fungi

Table 2: Growth of *Mucor* species in mineral salts medium supplemented with varying concentrations of crude oil, hexadecane, naphthalene or cyclohexane.

Hydrocarbon Concentration (%)	Total Viable count (TVC) (x 10^3 cfu ml^{-1}) on			
	Crude oil	Hexadecane	Naphthalene	Cyclohexane
1	8.2 ± 2.8*	7.8 ± 1.4	4.0 ± 2.8	3.0 ± 1.4
3	8.8 ± 1.4	8.2 ± 1.4	4.5 ± 1.4	3.2 ± 2.8
5	9.4 ± 1.4	8.9 ± 1.4	4.2 ± 2.8	2.6 ± 2.8
7	8.0 ± 1.4	8.5 ± 2.8	4.0 ± 1.4	2.0 ± 2.8
10	7.2 ± 2.8	8.2 ± 2.8	3.2 ± 2.8	1.8 ± 1.4
12	6.6 ± 2.1	7.6 ± 1.2	2.8 ± 2.1	1.6 ± 1.2
15	4.8 ± 1.2	5.9 ± 1.4	2.2 ± 1.2	1.0 ± 1.4

*Standard deviations based on values obtained from two replications

Table 3: Growth of Mucor Species in mineral salts medium supplemented with crude oil and varying concentration of sodium chloride

Sodium chloride concentration (% w/v)	Total viable counts (TVC) (x10^3 CFU ml^{-1})
0	7.0 ± 3.2*
0.05	7.4 ± 2.8
0.1	7.6 ± 1.4
1.0	8.0 ± 1.4
2.0	7.2 ± 2.1
3.0	5.4 ± 3.2
3.5	4.6 ± 2.1
4.5	3.4 ± 1.4
5.0	2.8 ± 1.2
5.5	1.4 ± 0.4

*Standard deviations based on values obtained from two replications

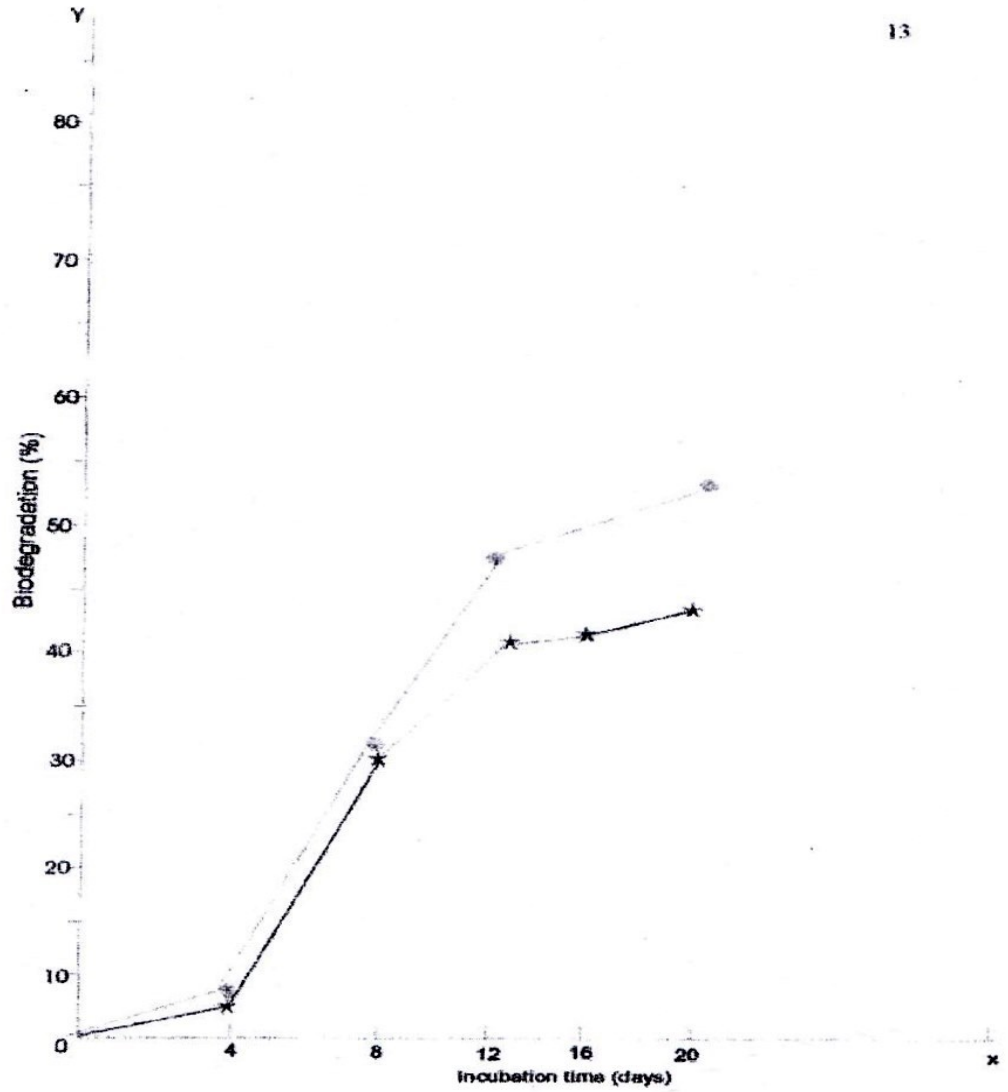

FIG. 2. Biodegradation of crude oil and kerosine by Mucor species during 20 days of incubation at 28°c.

Pink indicate kerosine
while black indicate crude oil

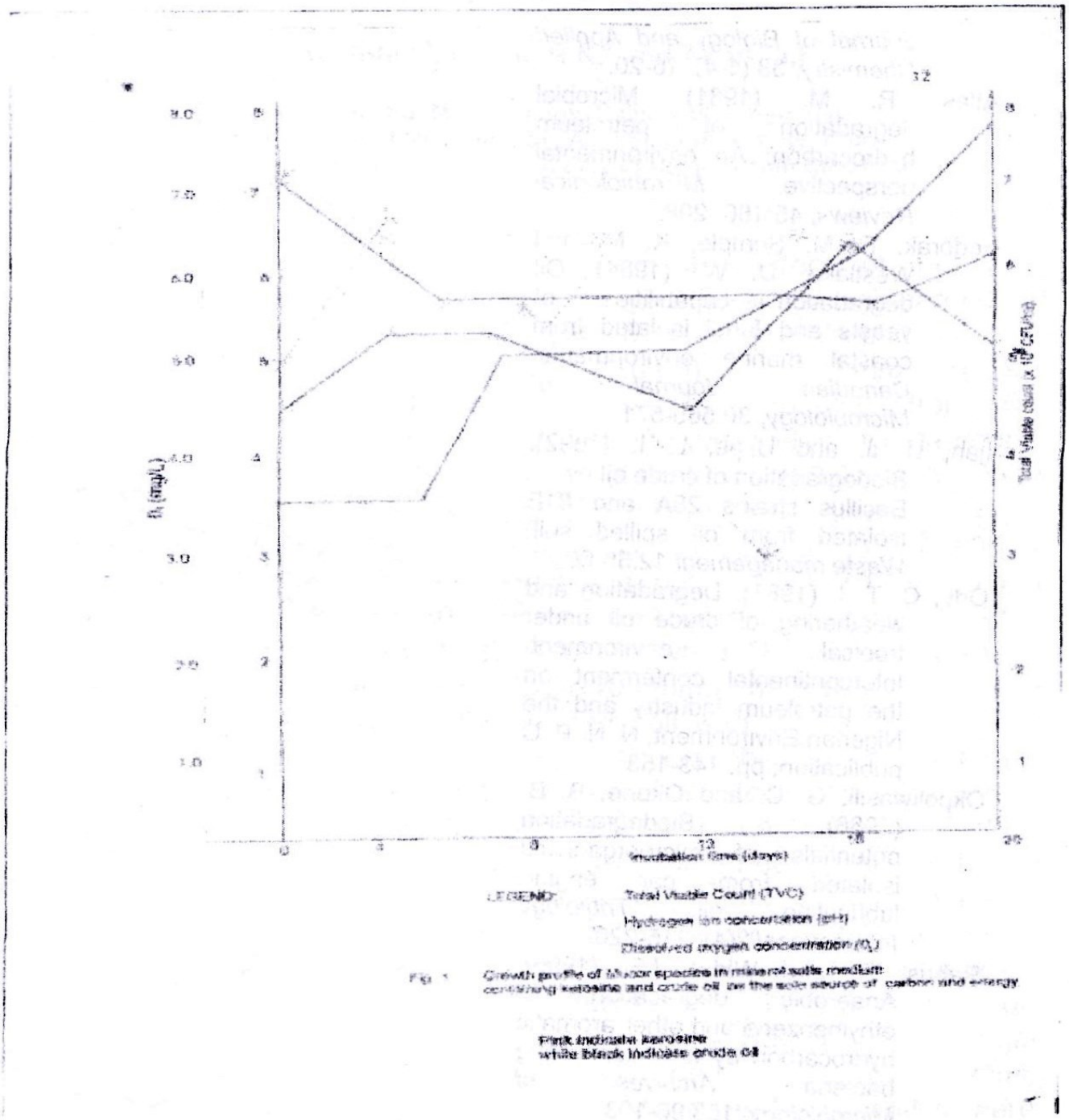

Fig. 1 Growth profile of Mixed species in mineral salts medium containing kerosene and crude oil as the sole source of carbon and energy

Pink indicate kerosene
while black indicate crude oil

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