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BIODEGRADABILITY OF THE MAJOR COMPONENTS OF BONNY LIGHT CRUDE OIL BY *Bacillus Subtilis*

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ABSTRACT

Of the known 92 potential hydrocarbon-utilizing bacteria and fungi isolated from crude oil polluted soil and water samples, only one soil bacterial isolate (SB_{17}), identified as *Bacillus subtilis,* was found to efficiently degrade 72.23% of the Bonny light crude oil sample after 25 days of incubation, and thus was used for further investigations. Biodegradability of the major components of Bonny light crude oil was determined by column chromatographic analysis, and asphaltene, saturated hydrocarbon fractions, aromatic fraction and the nitrogen – sulphur – oxygen (NSO) containing fractions were found to be the major components. The chromatographic analysis after 25 days of incubation at 28°C also revealed that during the degradation of Bonny light crude oil, there was a continuous decrease of the saturated fraction and a relative increase of the aromatic and NSO fractions. These results suggest that *Bacillus subtilis* is a good candidate for microbial seeding of Bonny light crude oil polluted terrestrial environment.

Key words: Biodegradability, *Bacillus subtilis,* Bonny light crude oil, saturated hydrocarbon fractions, aromatic and NSO fractions.

INTRODUCTION

Crude oil, a dark sticky, viscuous liquid found in huge underground deposits in many parts of the world is Nigeria's and the World's most important energy resource. Increasing demand for it has resulted in a high rate of activities such as sabotage, oil tanker accidents, oil-well blow outs and accidental rupture of oil pipelines, causing oil spills and pollution of the aquatic and terrestrial environments.

Although remediation of oil polluted environments by mechanical and chemical methods have been successful to some extent (Amadi and Antai, 1991), some limitations such as the difficult terrain in which most oil spillages occur, side effects (toxicity to organisms) and high expense, render the traditional clean up methods sometimes unsuitable. Since biodegradation (which is the breakdown of complex compounds to simpler forms by biological means) is relatively inexpensive, non-toxic and microorganisms with the ability to utilize hydrocarbons as sole sources of carbon and energy are widely distributed, Atlas (1981), Prince (1993) and Ijah and Antai (1995) all reported that microbial degradation is the most environmentally acceptable method for the elimination of spilled oil from the environments.

Although there are numerous reports on microbial degradation and utilization of petroleum products (Jobson *et al.*, 1972; Colwell and Walker, 1977; Atlas, 1981; Okpokwasili and Amanchukwu, 1988; Ijah and Antai, 1988; Antai, 1990) to date, no reports on the biodegradability of the major components of Bonny light crude oil in Nigeria have been published. Thus, this study was undertaken to determine the extent of microbial degradation of the major components of Bonny light crude oil.

MATERIALS AND METHODS

The soil and water samples used were collected from crude oil polluted sites and water bodies at Brass town in Bayelsa State, in sterile sampling bottles and transported in a cool box to the laboratory for isolation of crude oil utilizing bacteria and fungi.

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The Bonny light crude oil used was collected from the Nigerian National Petroleum Corporation (N.N.P.C.) refinery at Alesa-Eleme, Rivers State, Nigeria and stored at room temperature (28 ± 2°C) till when needed.

The mineral salts medium (MSM) of Zajic and Supplisson (1972) used had the following composition: K_2HPO_4, 1.8g; KH_2PO_4, 1.2g; NH_4CL, 4.0g; $MgSO_4.7H_2O$, NaCl, 0.1g; $FeSO_4.7H_2O$, 0.01g in 1000ml of distilled water (pH7.4).

Isolation of crude oil – utilizing bacteria and fungi

Crude oil-utilizing bacteria in the soil and water samples were isolated using the surface spreading technique. Serial ten-fold dilutions of the soil and water samples were prepared respectively. 0.1ml of the 10^{-3} dilution of each sample was plated in triplicates on mineral salts medium of Zajic and Supplisson (1972) which was solidified with 2% agar. Nystatin (50μg/ml) was added to inhibit fungal growth.

Sterile filter papers (Whatman No. 1) saturated with 2.0ml of sterile Bonny light crude oil were aseptically placed onto the inside of the covers of inverted petri-dishes and sealed around with a masking tape. The plates were incubated at room temperature for 5 to 7 days, from which discrete colonies which developed, were picked and purified by repeated subculturing and stored on nutrient agar slants at 4°C in a refrigerator for further studies.

The crude oil-utilizing fungi were isolated using the same procedure but mineral salts medium supplemented with filter-sterilized Streptomycin and Penicillin G, at a concentration of 50μg/ml each, incorporated to inhibit bacterial growth were used. A portion of each fungal colony which developed, was picked and purified by repeated subculturing and stored on fresh malt extract agar plates for further studies.

Screen test for the utilisation of bonny light crude oil by bacterial and fungal isolates

The bacterial and fungal isolates were screened for their ability to utilize Bonny light crude oil as their sole source of carbon and energy for growth by the method of Okpokwasili and Okorie (1988).

Mineral salt broth was dispensed in 9.9mls amounts into test tubes and sterilized by autoclaving. On cooling, 0.1ml of filter-sterilized Bonny light crude oil (using membrane filter with 0.45 μm pore size, SM 16510 Satorius, Germany) was added to the MSM tubes.

Then one (1) subset of the MSM + Bonny light crude oil test tubes were inoculated with 0.1ml of a 24 hour old nutrient broth culture of each bacterial isolate while the remaining subset of the test tubes were inoculated with 0.1ml of 72 hour old malt extract broth culture of each fungal isolate. Two control tubes remained uninoculated and all the test tubes were incubated at room temperature for 16 days under a stationary condition.

After every four (4) days, the tubes were compared with the controls and observed visually for turbidity as an index of utilization of the incorporated Bonny light crude oil. Maximum (+++), moderate (++) and minimal (+) ability to grow in crude oil media were recorded while inability to grow was recorded as no growth (-). At the end of the 16 days incubation period, the final turbidity of the medium in the tubes were read at 450nm using a spectrophotometer (model HACH DR 3000/USA).

Characterization and identification of the bonny light crude oil utilizing bacterial isolate

Characterisation and identification of the most efficient Bonny light crude oil-utilizing bacterial isolate followed the scheme of MacFaddin (1980).

Determination of growth profile of the most efficient bonny light crude oil utilizing bacterial isolate

To assess the ability of the bacterial isolate to degrade the Bonny light crude oil, its growth profile in the crude oil medium was monitored by the method of Okpokwasili and Okorie (1988). Mineral salts broth was autoclaved in 99mls amounts in four 250ml Erlenmeyer flasks. To each of the four flasks was added one millilitre of filter sterilized Bonny light crude oil. Then two of the flasks were inoculated with one millilitre of the test organism while the other two flask were uninoculated and served as controls. The flasks were continuously shaken on a rotary

shaker (SGM – 300, Gallenkamp, England) operating at 120rpm at room temperature for 25 days.

At intervals of 5 days, twenty millilitres of the samples were taken from each flask for determinations of (1) optical density (OD) i.e. turbidity at 450nm using HACH DR 300 spectrophotometer. (2) pH changes monitored with an electronic pH meter (Wisenschaftlich Technische – Werkstatten G. M. B. H., Germany), and (3) Total viable counts (TVC) determined by serially diluting the culture, plating on nutrient agar and incubating at 30°C for 24 – 48 hours after which counts were taken and expressed as colony forming units per millilitre (CFU ml^{-1}).

Assessment of biodegradability of bonny light crude oil by spectrophotometric method

To assess the biodegradability of Bonny light crude oil by the best hydrocarbon-utilizing bacterial isolate, mineral salts broth (Zajic and Suplisson, 1972) was prepared and transferred in 9.88 ml quantities into eighteen 100 ml capacity Erlenmeyer flasks. These flasks were then sterilized by autoclaving and on cooling, 0.12 ml of filter – sterilized Bonny light crude oil was added to each of the eighteen (18) flasks. Three uninoculated flasks were kept as control. 0.1ml (8.6 x 10^6 cells) of a 24 hour nutrient broth culture of the bacterial isolate was inoculated into the remaining fifteen Erlenmeyer flasks. The flasks were all incubated on a shaker incubator (Model BKS 300 – 010F, Gallenkamp, England) set at 30°C and 120 rpm for 25 days. Every 5 days interval, a set of 3 inoculated flasks were removed and the amount of the crude oil left was determined by extracting the residual crude oil with 20 mls of n-hexane and noting

Table 1:
Screen test for the utilization of Bonny Light Crude Oil by some bacteria and fungi isolated from soil and water samples.

S/N	SOIL SAMPLES				WATER SAMPLES			
	Bacterial Isolates	Absorbance (OD) at 450nm	Fungal Isolates	Absorbance at 450nm	Bacterial Isolates	Absorbance at 450nm	Fungal Isolates	Absorbance at 450nm
Control		0.015		0.015		0.015		0.015
1.	SB_1	0.072	SF_1	0.040	WB_1	0.612	WF_1	0.037
2.	SB_2	0.353	SF_2	0.030	WB_3	0.130	WF_2	0.048
3.	SB_3	0.137	SF_3	0.022	WB_5	0.683	WF_3	0.029
4.	SB_4	0.205	SF_4	0.031	WB_6	0.042	WF_5	0.041
5.	SB_5	0.097	SF_5	0.029	WB_7	0.029	WF_9	0.036
6.	SB_6	0.056	SF_6	0.042	WB_{10}	0.030	WF_{10}	0.074
7.	SB_7	0.024	SF_9	0.019	WB_{16}	0.154	WF_{11}	0.052
8.	SB_8	0.237	SF_{10}	0.047	WB_{22}	0.090		
9.	SB_9	0.215			WB_{26}	0.039		
10.	SB_{10}	0.271			WB_{30}	0.464		
11.	SB_{11}	0.028			WB_{36}	0.042		
12.	SB_{12}	0.046			WB_{41}	0.030		
13.	SB_{13}	0.101			WB_{45}	0.594		
14.	SB_{14}	0.018			WB_{51}	0.051		
15.	SB_{15}	0.029						
16.	SB_{16}	0.194						
17.*	SB_{17}	3.104						
18.	SB_{18}	0.422						
19.	SB_{19}	0.160						
20.	SB_{20}	0.254						

SB = Bacteria isolated from soil samples
WB = Bacteria isolated from water samples
SF = Fungi isolated from soil samples
WF = Fungi isolated from water samples

their absorbance readings at 390 nm respectively using the HACH DR 300 spectrophotometer.

The weights of the extracted residual crude oil 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.

Assessment of the biodegradability of the major components of bonny light crude oil by column chromatographic analyses

The same procedures and number of flasks (eighteen) already described in the measurement of crude oil degradation studies were repeated in parallel for column chromatography. The extraction and column chromatographic analyses were applied to the crude oil before (control) and after bacterial degradation.

At 5 days interval, three Erlenmeyer flasks were removed and the residual crude oil in the flasks were extracted with n-hexane (ALDRICH HPLC grade), separated by column chromatography and quantified in milligram.

The crude oil extracts were deasphaltenated by precipitation in dichloromethane (methylene chloride) and petroleum benzene (1:30 ml) in a centrifuge running at 3,000 rpm for 20 minutes. The asphaltene and maltene fractions were separated, evaporated to dryness and quantified in milligrams respectively.

The maltene extracts were separated on wet-packed (n-hexane) activated silica gel (Merck, Germany mesh size 0.063 – 0.200mm) and alumina columns.

The colourless saturated fractions were eluted with n-hexane, the yellowish aromatic fractions were eluted with dichloromethane while the dark brown NSO fractions were eluted with a mixture of dichloromethane and methanol in the ratio 1:2. After evaporation to dryness, the separated fractions were quantified in milligrams. The entire procedures were carried out in triplicates for the controls and the degraded samples at 5 days interval for 25 days.

RESULTS AND DISCUSSION

The result of the screen test for the utilization of Bonny light crude oil as sole carbon and energy source by some bacteria and fungi isolated from crude oil polluted soil and water samples is presented in Table 1. The table shows that of the 92 hydrocarbon-utilizing bacteria and fungi isolated from soil and water samples, only one soil bacterial isolate (SB_{17}) had the highest turbidimetric (optical density) reading of 3.104 resulting from the utilization of Bonny light crude oil against control of 0.015. Because of its efficient ability to utilize the Bonny light crude oil, SB_{17} was selected for detailed biodegradation studies and identified as *Bacillus subtilis* following the scheme of MacFaddin (1980).

Figure 1 shows the growth profile of *Bacillus subtilis* determined by monitoring the

Table 2: Components of degraded Bonny light crude oil as revealed by column chromatographic method

Incubation period (Days)	Weight of Estract (mg)	% weight Loss	Asphaltene fraction (mg)	Maltene fraction (mg)	% weight loss of maltene	SHC # fraction (mg)	% weight loss of SHC	Aromatic franction (mg)	% weight fain of Aromatics	NSO fraction (mg)	% weight gain of NSO	% of fractions left after degradation		
												% SHC	% AROM	% NSO
0**	91.80±0.8*	-	0.70±0.1	90.70±1.0	-	53.50±0.8	-	16.00±0.5	-	12.90±1.0	-	64.49	17.64	14.22
5	80.10±0.8	12.75	0.50±0.2	79.40±0.9	12.46	46.70±0.7	20.17	16.80±0.7	5.00	13.70±0.5	6.20	58.82	21.16	17.25
10	66.00±1.0	28.10	0.70±0.2	65.30±1.0	28.00	33.20±1.0	43.24	17.30±0.5	8.13	14.50±0.4	12.40	50.84	26.49	22.21
15	53.40±0.7	41.83	0.40±0.1	53.00±0.7	41.57	18.00±0.6	69.23	17.60±0.6	10.00	17.10±0.3	32.56	33.96	33.21	32.26
20	50.00±1.0	45.53	0.40±0.1	49.40±0.9	45.53	11.40±0.3	80.51	17.80±0.5	11.25	19.50±0.5	51.16	23.08	36.03	39.47
25	47.50±0.4	48.26	0.60±0.1	46.50±0.5	48.73	8.80±0.5	84.96	17.90±0.7	11.88	19.70±0.5	52.71	18.92	38.50	42.37

* Standard deviations based on values obtained from three replications

\# Saturated hydrocarbons

++ Control

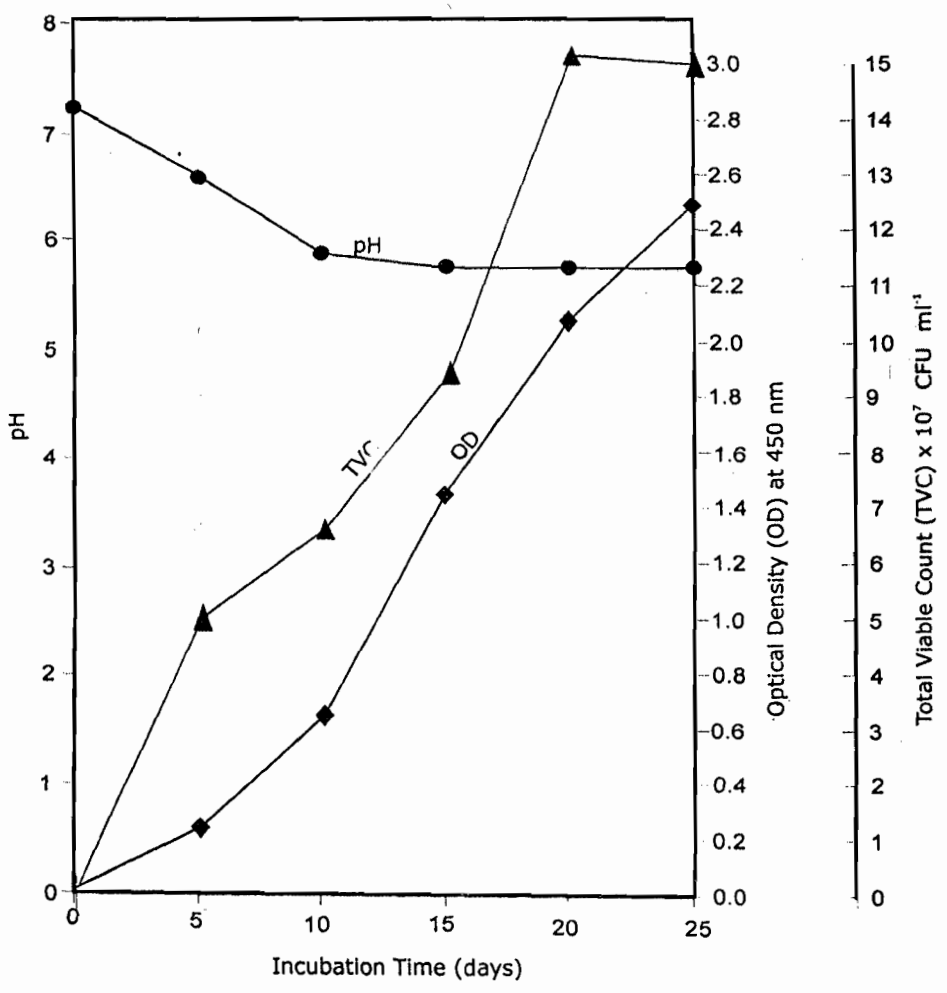

Fig. 1: Growth profile of *Bacillis subtilis* in mineral salts medium containing Bonny Light Crude Oil as the sole source of Carbon and energy.

pH, turbidity (optical density) and total viable count (TVC) of the culture as it utilized Bonny light oil as the sole source of carbon and energy. The figure shows that the total viable counts of the organism increased with increase in the optical density and decrease in pH of the culture as incubation progressed. The utilisation of Bonny light crude oil by *Bacillis subtilis* resulted in its growth probably with a concomitant production of acidic metabolic products which may have accounted for the decrease in pH of the culture. These observations confirm the screen test results (Table 1) that *B. subtilis* actually utilized the Bonny light crude oil. It was also observed at the end of the 25 days incubation period, that the growth of *B. subtilis* had reached the stationary phase. This may be as a result of depletion of the nutrients present in the medium and/or the accumulation of the toxic metabolites produced.

Results obtained from spectrophotometric evaluations of degradability of Bonny light crude oil by *Bacillus subtilis* is presented in figure 2. The figure reveals that there was continuous increase in the rate of degradation as incubation progressed. At the end of the 25 days degradation period, *Bacillus subtilis* was capable of efficiently degrading Bonny light crude oil by causing a weight loss of 72.23%. This suggests that the organism has a competent and active hydrocarbon degradation enzyme. The efficient ability of *B. subtilis* to degrade Bonny light crude oil is not surprising since the results of many investigators have shown the role *Bacillus* species play in biodegradation of crude oil

(Antai and Mgbomo, 1989; Okpokwasili and James, 1995). The differences in the results of studies on crude oil degradation by *Bacillus* species reported by different investigators might depend on the chemical composition of the crude oil, the genetic constitution of the strains and species of *Bacillus* involved, the incubation period, and the prevailing environmental and physiological conditions in the system. Westlake *et al.* (1974) reported that chemical compositions of a crude oil, has a marked effect on its biodegradability and determines the type of bacteria which will metabolise the oil.

Figure 3 presents the major components of Bonny light crude oil as revealed by column chromatographic analysis. The figure shows that the major components of Bonny light crude oil are the saturated hydrocarbons, the aromatic hydrocarbons and the NSO fractions. The figure clearly shows that bulk of the Bonny light crude oil sample was the saturated hydrocarbons (63.73%) with less quantities of aromatic (17.40%) and NSO fractions (14.0%). Figure 3 also reveals that with increasing days of incubation, from the fifth day to the twenty-fifth day, there was a continuous decrease of the saturated fractions with corresponding relative increase of the aromatic and NSO fractions. The 84.96% degradation of the saturated fraction indicates that, it is the most readily degraded fraction of all the components of oil. That the bulk of the Bonny light crude oil is mostly saturated hydrocarbons explains why 84.96% degradation of the saturated fractions resulted in 72.23% degradation of the entire crude oil. This shows that the saturated content is

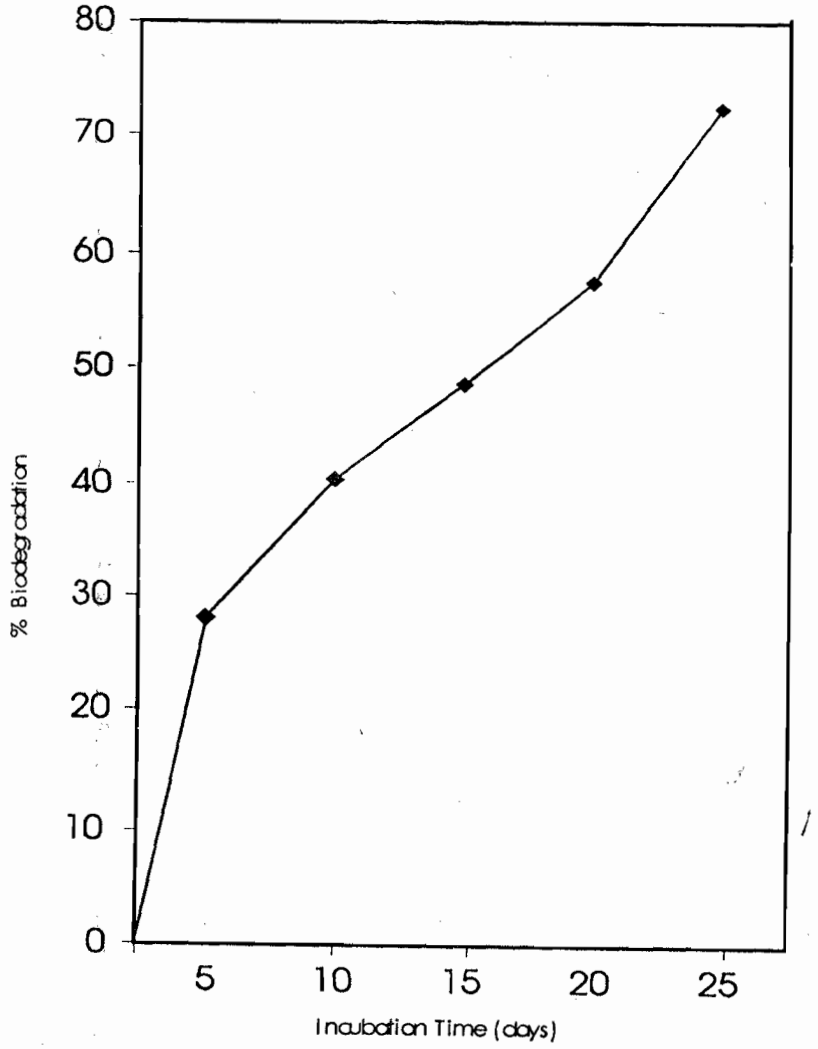

Fig. 3. Biodegradability of Bonny light Crude oil (BLCO) by *Bacillus subtilis* using Spectrophotometric method.

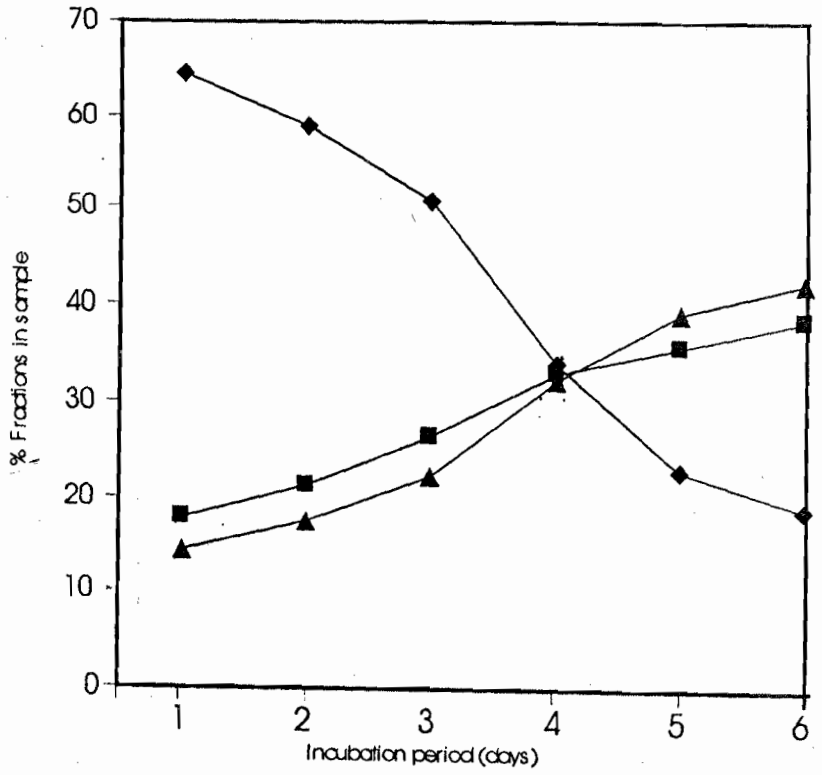

Fig. 3: Biodegradability of the major components of Bonny light Crude oil as revealed by column chromatographic analysis.

possibly the most important component with regards to the biodegradability of the oil. The reason for the increment of the aromatic fractions in the degraded Bonny light crude oil can be attributed to aromatization of the degraded products. The increase in the content of the polar NSO fractions as a result of microbial activity indicates that a considerable amount of transformation resulting from the accumulation of metabolites in these fractions has taken place. These results are in agreement with the findings of Rontani *et al.* (1986) who reported an increase in the aromatic and NSO fractions of degraded Asthart crude oil.

The difference in the biodegradability of the saturated, aromatic and NSO fractions may be attributed to the fact that the saturated fractions are more readily degraded due to their straight chain nature and the preference of the organism for the saturated hydrocarbon which might be associated with the enzymatic complement of the organism. The aromatics and NSO fractions have been reported to be recalcitrant to microbial degradation (Atlas, 1981).

Table 2 shows that there were inconsistent variations in the asphaltene fractions present in the degraded Bonny light crude oil. These changes could therefore not be interpreted.

In conclusion, the results presented in this study show that although the factor determining the biodegradability of Bonny light crude oil resides primarily in the composition of the n-saturate fractions, it is also affected by the contents of the other components (such as the aromatic and NSO fractions), the genetic capability of the hydrocarbon-utilizing bacterium involved and the prevailing environmental conditions.

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