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Optimization of cultural conditions for bitumen-degrading bacteria

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ABSTRACT

The biodegradability of bitumen was found to be highly influenced by incubation temperature, pH and inoculum size with five strains of bacteria (Pseudomonas fragii, Streptococcus zymogenes, P. aeruginosa, P. fluorescens and Bacillus macerans) isolated from water samples collected from Agbabu, a bitumen producing area of Ondo state, Nigeria. When grown on Thermophile Halophile Sulplur (THS) medium, the dispersion rate of bitumen was very high at $40^{O}C$ and $44^{O}C$ but bitu-oil was not produced, while dispersion rate was high at $37^{O}C$ and 0.33g/ litre of bitu-oil was produced. Very high bitumen dispersion rate and 0.43g/litre of bitu-oil were obtained at of pH 7.0. When two plates each of the isolated organism (each plate containing 1.0 x 10^{11} cfu/ml) in suspension was used as inoculum in two litre-fermenter, very high dispersions rate of bitumen and 0.35g/litre of bitu-oil were obtained. Results of this research shows that bitumen degradation was optimum at of pH 7.0, temperature of $37^{O}C$ and with two plates of each bacterium in suspension using two litre-fermenter.

Keywords; Bitumen, biodegradation, bitu-oil, Thermophile Halophile Sulphur bacteria consortium.

INTRODUCTION

Under certain conditions, living organisms primarily bacteria, yeast, molds, and filamentous fungi metabolize various classes of compounds present in oil. Biodegradation of oil alters subsurface oil accumulation (Winters and Williams, 1969) and it has been shown that Hydrocarbon degraders are found in almost all environments occurring in high numbers when oil is present (Atlas and Bartha, 1972). Tar sand or bitumen has been known to occur in Nigeria, and rank among the five largest deposits in the world. The first exploration of bitumen was by the defunct Nigerian Bituminous Corporation between 1908 and 1914. Environmental hazards of bitumen exploration include destruction of the ecosystem and pollution from bituminous toxic wastes (Aderemi, 2000).

The mineralization or complete biodegradation of an organic molecule in water and soil is almost always a consequence of microbial activity (Atlas, 1984). In general, the biodegradation of aliphatic pollutants is affected by biological and physicochemical factors. Biological factors include the enzymatic activity of the microorganisms on the alkanes and the transport limitation of the substrate across the membrane (Setti *et al.,* 1995). The rate of mineralization of the pollutant is a function of availability of the chemicals and quantity of the active microbes. These physicochemical factors also include the fermentation conditions and the substrate characteristics, such as its water solubility, viscosity, diffusivity, and surface tension (Setti *et al.* 1995). It is obvious that the degradation of petroleum and refined products proceed much faster in the presence of oxygen than under anoxic conditions. Other overriding limitations such as temperature, water, pH, and minerals nutrients have profound effect on biodegradation of petroleum (Verstraete *et al.,* 1976).

Some environmental constraints on degradation of petroleum hydrocarbons have been extensively investigated (Zobell, 1973). However, reports on the culture parameters on bitumen biodegradation have not been widespread. This is the report of the effect of temperature, pH and inoculums size on bitumen biodegradation and bitu-oil production by a bacteria consortium (*Pseudomonas fragii*, *Streptococcus zymogenes*, *P. aeruginosa, P. fluorescens* and *Bacillus macerans*), locally isolated from bitumen producing site.

MATERIALS AND METHODS

Isolation

Water samples (from abandoned borehole) and bitumen (flow or plain type) were collected from Agbabu area of

Ondo State, Nigeria. Pure cultures were obtained by carrying out serial dilution, inoculated and incubated at ambient temperature (30 $\pm$ 2^{o}C) for 14days (two weeks). Pure culture of each member of the consortium was made. Thermophile Halophile sulphur (THS) medium containing (g/l) K_2HPO_4 (0.5), $MgSO_4.7H_2O$ (0.1), $CaCl_2.2H_2O$ (0.1), $(NH_4)_2SO_4$ (0.2), NaCl (2.0), and Agar- agar (20) with 120ml/litre of solubilized bitumen (bitumen was solubilized by dissolving 0.9g of bitumen in 2ml of n-hexane) was used for isolation.

Determination Of An Optimum Inoculum Size

The optimum inoculum size was determined by evaluating three different levels of microbial concentration which are one plate of each organism, two plates each and three plates of each organism (each plate containing 1.0 x 10^{11} cfu/ml), using 2-litre fermenter. The medium was prepared, sterilized, inoculated and incubated at room temperature with aeration and agitation with aeration pump. In determining the optimum inoculum size, the rate of bitumen dispersion (by shaking the culture vigorously for two minutes, allowed the culture to stand and the time its take for dispersed bitumen to come together was recorded) and rate of medium disappearance, (by measuring the contents of medium used within three days interval) were determined and recorded. After 18days of incubation, the culture was harvested, centrifuged and extracted.

Determination Of An Optimum Temperature

Three different temperature values evaluated were 37^{o}C, 40^{o}C and 44^{o}C. The THS medium of two litres was prepared into reaction vessels, sterilized, inoculated and incubated. Also, rate of bitumen dispersion (bitumen biodegradation) and rate of medium disappearance were determined and recorded. The culture was harvested after 18 days, centrifuged and metabolite was extracted.

Determination Of An Optimum Ph Value

Three pH values evaluated were 5.5, 7.0 and 8.7. Hydrochloric acid (HCl) of 0.1ml and 0.1ml of sodium hydroxide (NaOH) were used as buffer. The medium was prepared, inoculated and incubated at 37^{o}C with aeration and agitation. Rate of bitumen dispersion and medium disappearance were determined and recorded. After 18 days of incubation, the culture was harvested, centrifuged and extracted. All experiments were carried out in triplicate.

EXTRACTION METHOD

After the incubation period, according to Hughes and McKenzie (1975), the culture was filtered (using Whatman paper 1) to separate the undegraded bitumen, the filtrate was centrifuged (at 2500rpm), supernatant was added to equal volume of n-hexane, shaking vigorously and allowed to settle showing 2 layers-the upper layer which was hexane layer with biodegraded bitumen, and lower layer which was medium layer. The upper layer was collected and n-hexane was removed (by exposing at room temperature for six hours) to obtain bitu-oil (oil produced from biodegradation of bitumen). The bitu-oil was weighed and recorded.

The Infrared Spectroscopy Analysis Of Bitumen And Bitu-Oil

The infrared spectroscopy (IR) analysis of bitumen and bitu-oil was carried out using Nicolet Avatar FT-IR330, by Thermo Electron Corporation to show the different peaks of absorbance wavelength in the two compounds.

RESULTS

Effect Of Inoculum Size On The Bitumen Biodegradation

The rate of biodegradation of bitumen when suspension of cell from one plate, two plates and three plates of the organism each were used as inoculum for between fourteen to eighteen days resulted in high dispersion rate in one plate and three plates of organism. However, very high dispersion rate was obtained for two plates of organism (Table 1). The rate of medium disappearance at one plate, two plates and three plates of the organism each, between ten to fourteen days of growth were moderate reduction, very high reduction and high reduction rate respectively (Table 2).

Tables 1: Effect of Inoculum size on bitumen degradation

Days	One plate of each organism	Two plates of each organism	Three plates of each organism
1-3 4-7 7-10 10-14 14-18	+ + + + + + + + +	+ ++ +++ +++ + + + +	+ ++ +++ +++ + + +

+ (1-2mins) $\Rightarrow$ Low dispersion, ++ (3-4mins) $\Rightarrow$ partial dispersion, +++ (5-7mins) $\Rightarrow$ high dispersion, + + + + (>8mins) $\Rightarrow$ very high dispersion

Table 2: The effect of inoculum size on rate of Medium disappearance

Days	One plate of each organism	Two plates of each organism	Three plates of each organism
1-3 4-7 7-10 10-14 14-18	Dried ++ ++ +++	++ Dried ++++ ++++ ++++	++ Dried +++ +++ +++

+ + $\Rightarrow$ Moderate Reduction, + + + $\Rightarrow$ high reduction, + + + + $\Rightarrow$ very high reduction

Effect Temperature On The Bitumen Biodegradation

At 40°C and 44°C, the rate of biodegradation of bitumen was very high, while it was low at 37°C using two plates each of organism between fourteen to eighteen days of growth (Table 3). The rate of medium disappearance at 37°C and 40°C was high, while very high reduction rate was obtained at 44°C, within eighteen days of growth (Table 4).

Table 3: Effect of temperature on biodegradation of bitumen

Days	37°C	40°C	44°C
1-3	+	++	++
4-7	++	+++	+++
7-10	+++	++++	++++
10-14	+++	++++	+ + + +
14-18	+ + +	+ + + +	+ + + +

+ (1-2mins) ⇒ Low dispersion, + + (3-4mins) ⇒ partial dispersion + + + (5-7mins) ⇒ high dispersion, + + + + (>8mins) ⇒ very high dispersion

Table 4: Effect of temperature on the rate of medium disappearance

Days	37°C	40°C	44°C
1-3	-	-	+
4-7	+	+	++
7-10	++	++	+++
10-14	+++	+++	++++
14-18	+ + +	+ + +	+ + ++

- ⇒ No reduction, + ⇒ Low reduction, + + ⇒ Moderate, reduction, + + + ⇒ high reduction, + + + + ⇒ very high reduction

Effect Of Ph On The Bitumen Biodegradation

The rate of biodegradation of bitumen between fourteen and eighteen days of growth at pH of 5.5, 7.0 and 8.7 were partial dispersion, very high dispersion and high dispersion respectively (Table 5) and there was no significant difference in medium disappearance at different pH levels.

Table 5: Influence of pH on bitumen degradation

Days	5.5	7.0	8.7
1-3	-	+	+
4-7	+	++	++
7-10	+	++	++
10-14	++	+++	++
14-18	++	++++	+++

- ⇒ No dispersion, + (1-2mins) ⇒ Low dispersion, + + (3-4mins) ⇒ partial dispersion,+ + + (5-7mins) ⇒ high dispersion, + + + + (>8mins) ⇒ very high dispersion

IR-Analysis of the Bitumen and Bitu-oil produced after biodegradation.

The different wave numbers (cm^{-1}) obtained with the spectroscopic analyses in the raw bitumen and bitu-oil obtained after bitumen biodegradation were (588.48, 723.39, 745.99, 812.90, 867.33, 1376.02, 1456.47, 1623.72, 1700.49, 2956.53, 3446.43 and 3749.17) (Fig 1) and (523.81, 745.14, 866.89, 927.47, 1013.89, 1109.51, 1296.17, 1343.90, 1373.42, 1455.35, 1643.05, 1728.82, 2015.10, 2868.78, 2969.88 and 3499.89) (Fig 2) respectively.

DISCUSSION

The utilization of bitumen as sole source of carbon and energy by *Pseudomonas fragii*, *Streptococcus zymogenes, P. aeruginosa, P. fluorescens* and *Bacillus macerans*, was influenced by some specific growth conditions, such as temperature, pH and inoculum size. From the results it can be seen that inoculum size plays a prominent role in biodegradation of bitumen. Table 1 show that the two plates each of the organism resulted in very high dispersion rate of bitumen, while high dispersion rate of bitumen was obtained in one plate and three plates of the organism each between fourteen to eighteen days of growth. This indicates that two plates each of the organism is an optimum inoculum size for bitumen biodegradation. Earlier report by Bartha and Atlas (1977), showed that an optimum concentration of the organism in a petroleum contaminated area enhances its biodegradation. The high microbial biomass, the great microbial diversity, and the abundant representation of bacterial and fungal genera capable of metabolizing hydrocarbons render soil a relatively favorable environment for petroleum hydrocarbon. By increasing the number of hydrocarbon- degrading microorganisms in the soil, the need for a long acclimatization period was avoided (Alexander, 1977). The rate of medium disappearance at one plate, two plates and three plates of each organism were as follows: high reduction for one and three plates of organism, while very high reduction was obtained at the two plates, between fourteen to eighteen days of growth (Table 2). According to previous work by Hughes and McKenzie (1975), the fast disappearances of substrate during crude oil degradation implies an effective metabolic activity.

At 40°C and 44°C, the rate of bitumen dispersion was very high (Table 3), but bitu-oil was not produced, while the dispersion rate was high at 37°C, and 0.33g/litre of bitu-oil was produced within eighteen days of growth. This agreed with Dubble and Bartha (1979), who observed that working with an oily sludge in a New Jersey soil, has the highest hydrocarbon

biodegradation rate occurring above 20°C, and with no further increase in rate at 37°C. The medium disappearance was very high at 44 °C, while it was high at 37°C and 40°C, between fourteen to eighteen days of growth (Table 4). Atlas and Bartha (1972), observed that at higher temperature, evaporation of hydrocarbon and lost of substrate usually occur.

The rate of bitumen dispersion, when two plates each of cell suspension were used as inoculum size at pH of 5.5, 7.0 and 8.5 are partial dispersion, very high dispersion and high dispersion rate respectively (Table 5) between fourteen to eighteen days of growth. This may imply that bitumen degradation is possibly more effective under neutral or slightly alkaline pH condition, which is in agreement with Vanlookce *et al.,* 1975. Dubble and Bartha (1979), reported that hydrocarbon biodegradation was minimal in a naturally acidic soil of pH 3.7 while stimulation of hydrocarbon biodegradation increased with rising soil pH in response to liming up to the highest value of pH 7.8 tested.

Prominent peaks in the spectra of bitumen include; 1700.49cm^{-1} (carbonyl group), 1623.72cm^{-1} (C=C stretch), 2956.53cm^{-1} ($-\overset{l}{\underset{l}{C}}-H$ stretch or Saturated C-H) and 3446.34cm^{-1} (Alkanol {OH}), while prominent peaks in spectra of the bitu-oil occurred at; 1109.51cm^{-1} ($-\overset{l}{\underset{l}{C}}-OH$ stretch), 1296.17cm^{-1} (Ethers), 1728.82cm^{-1} (Aryl and αβ unsaturated group), 3499.89cm^{-1} (OH-group), 1343.90cm^{-1} (C-O stretch) and 2868.78cm^{-1} ($-\overset{l}{\underset{l}{C}}-H$ stretch) (Fig. 1 and 2). The IR-analysis revealed the disappearance of low molecular hydrocarbon such as alkane ($-\overset{l}{\underset{l}{C}}-H$ stretch or Saturated C-H) and alkene (C=C stretch) after bitumen biodegradation. Hamme *et al.,* and Ghazali *et al.,* observed that low molecular weight hydrocarbons are degraded most rapidly, or converted to some high molecular weight and more valuable compounds, when bacteria were made to grow on them. Some high molecular weight and more valuable of hydrocarbons compounds such as ethers, esters, lactones and silicon compound were formed after the biodegradation of bitumen. In this study, it is shown that bacteria isolates that are able to grow on bitumen as carbon and energy sources, have oil-degrading properties when grown in an optimum conditions of cell suspension from two plates of each organism, pH of 7.0 and at 37°C resulting in high biodegradation of bitumen and high yield of bitu-oil.

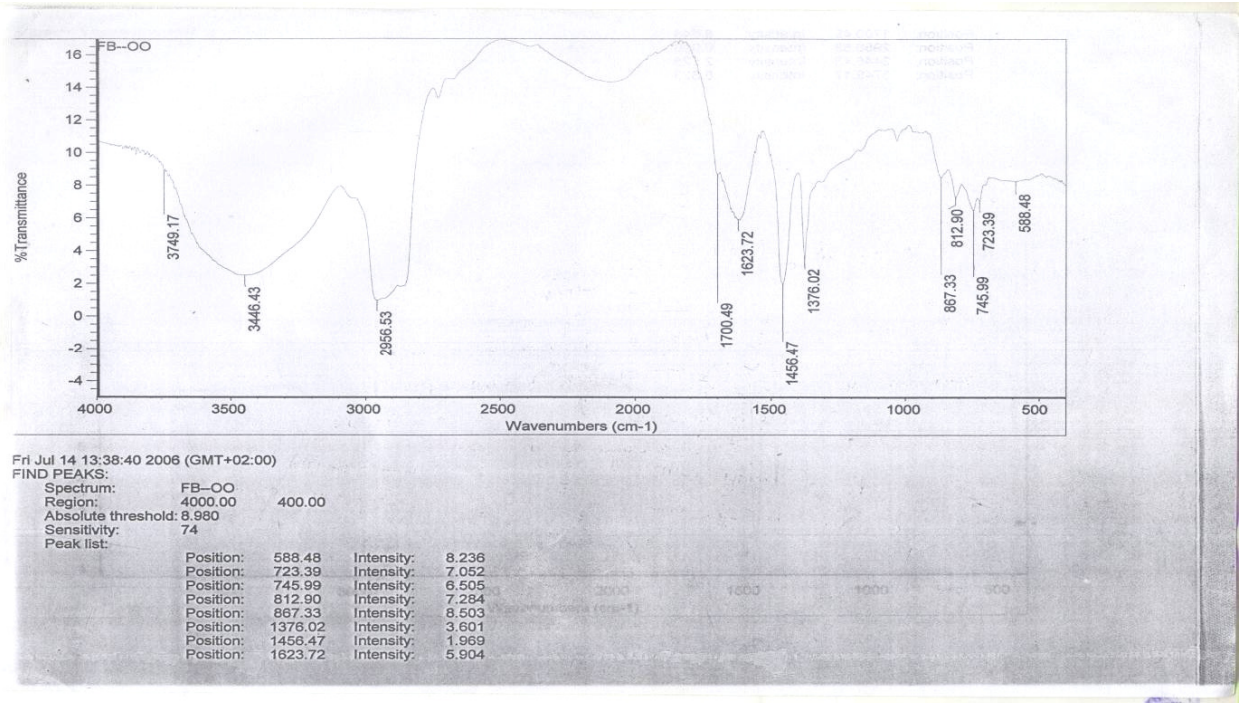

Fig.1: The IR analysis of bitumen before biodegradation.

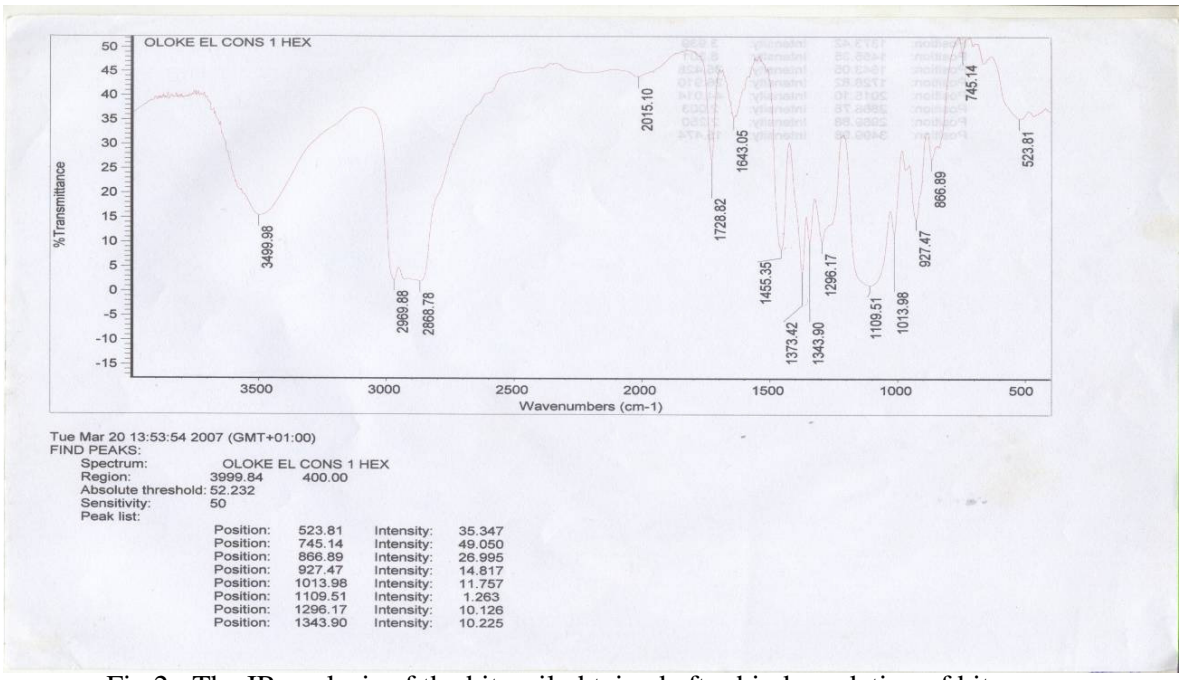

Fig.2: The IR analysis of the bitu-oil obtained after biodegradation of bitumen.

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