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# Effect of Culture Parameters of a Bacterial Consortium on Biodegradation of Bitumen

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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 fragi, Streptococcus zymogenes, P. aeruginosa, P. fluorescens* and *Bacillus macerans*) isolated from water samples collected from bitumen producing area, Agbabu, ondo state, Nigeria. When grown on Themophile 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 P$^{H}$ of 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 litres 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 PH of 7.0, temperature of 37$^{O}$C and with two plates of each bacterium in suspension using two litres fermenter.

***Key words:*** Bitumen, biodegradation, bitu-oil,Thamophile Halophile Sulphur and 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 [22]. Hydrocarbon degraders are found in almost all environments [7] occurring in high numbers when oil is present [5, 18, 23, 21]. Tar sand or bitumen has been known to occur in Nigeria [1], and rank among the five largest deposits in the world. The first exploration of bitumen was by the defunct Nigerian bituminous corporation (1908-1914). There are grim hazards of the environment by bitumen exploration or degradation leading to destruction of socio-economic system, the ecosystem as well as pollution from bituminous toxic wastes [2]. However a wide variety of microbes are able to use long chain hydrocarbons as their sole source of carbon and energy [8].

The mineralization or complete biodegradation of an organic molecule in water and soil is almost always a consequence of microbial activity [4]. 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 [17]. The rate of mineralization of the pollutant is a function of availability of the chemicals and quantity of the active microbes [17]. The physicochemical factors include the fermentation conditions and the substrate characteristics, such as its water solubility, viscosity, diffusivity, and surface tension

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[17]. 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 [20]. Some environmental constraints on degradation of petroleum hydrocarbons have been extensively investigated [9, 7, 23 and 18]. However, reports on the culture parameters on bitumen biodegradation have not been widespread. Here we report the effect of temperature, pH and inoculum size on bitumen biodegradation and bitu-oil production by a bacteria consortium (*Pseudomonas fragi*, *Streptococcus zymogenes*, *P. aeruginosa, P. fluorescens* and *Bacillus macerans*) being 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 + 2$^{O}$C) for 14days (two weeks). The organism were sub-cultured, pure colonies were isolated. 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 dissolved 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 two litres fermenter. The medium was prepared, sterilized, inoculated and incubated at room temperature with aeration and agitation. 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 measured the content 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, 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. Two different layers were formed; 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 exposed at room temperature for six hour) 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 and discussion

### *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, very high dispersion rate 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).

*Effect temperature on the bitumen biodegradation*

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

*Effect ph on the bitumen biodegradation*

The rate of biodegradation of bitumen between fourteen to 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.

*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 fragi*, *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 shows that the two plates each of the organism resulted to very high dispersion rate of bitumen, while high dispersion rate of bitumen was obtained at 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. Previous studied [9] reported 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 general capable of metabolizing hydrocarbons render soil a relatively favourable environment for petroleum hydrocarbon. By increasing the number of hydrocarbon- degrading microorganism in the soil, the need for a long acclimatization period was avoided[3]. 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 [15] the fast disappearances of substrate during crude oil degradation implies an effective metabolic activity.

At 40ºC and 44ºC, the rate of bitumen dispersion was very high (Table 3), but bitu-oil was not produced, while the dispersion rate was high at 37ºC, and 0.33g/litre of bitu-oil was produced within eighteen days of growth. This agreed with Dubble and Bartha [10], observed that working with an oily sludge in a New Jersey soil, the highest hydrocarbon biodegradation rate occurred above 20ºC, and with no further increase in rate at 37ºC. The medium disappearance was very high at 44$^{0c}$, while it was high at 37ºC and 40ºC, between fourteen to eighteen days of growth (Table 4). Atlas [5] 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 (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,* [18]. Dubble and Bartha [10] reported that hydrocarbon biodegradation was minimal in a naturally acidic soil (pH 3.7). Stimulation of hydrocarbon biodegradation increased with rising soil pH in response to liming up to the highest value (pH 7.8) tested.

Prominent peaks in the spectra of bitumen include; 1700.49cm$^{-1}$ (carbonyl group), 1623.72cm$^{-1}$

**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) Low dispersion, ++ (3-4mins) partial dispersion,

+++ (5-7mins) high dispersion, + + + + (>8mins) 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 | Dried | Dried | Dried |
| 4-7 | ++ | ++ | ++ |
| 7-10 | ++ | ++++ | +++ |
| 10-14 | ++ | ++++ | +++ |
| 14-18 | +++ | ++++ | +++ |

+ + Moderate Reduction, + + + high reduction

+ + + + very high reduction

**Table 3:** Effect of temperature on biodegradation of bitumen.

| Days | 37$^{o}$C | 40$^{o}$C | 44$^{o}$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$^{o}$C | 40$^{o}$C | 44$^{o}$C |
|---|---|---|---|
| 1-3 | - | - | + |
| 4-7 | + | + | ++ |
| 7-10 | ++ | ++ | +++ |
| 10-14 | +++ | +++ | ++++ |
| 14-18 | +++ | +++ | ++++ |

- No reduction, + Low reduction

+ + Moderate, reduction,

+ + + high reduction

+ + + + very high reduction

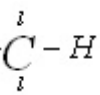
**Table 5:** Influence of pH on bitumen degradation.

| 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

(C=C stretch), 2956.53cm$^{-1}$ (  stretch or Saturated C-H) and 3446.34cm$^{-1}$ (Alkanol {OH}), while prominent peaks in spectra of the bitu-oil occurred at; 1109.51cm$^{-1}$ (  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$^{-1}$ (  stretch) (Fig. 1 and 2). The IR-analysis revealed the disappearance of low molecular hydrocarbon such as alkane

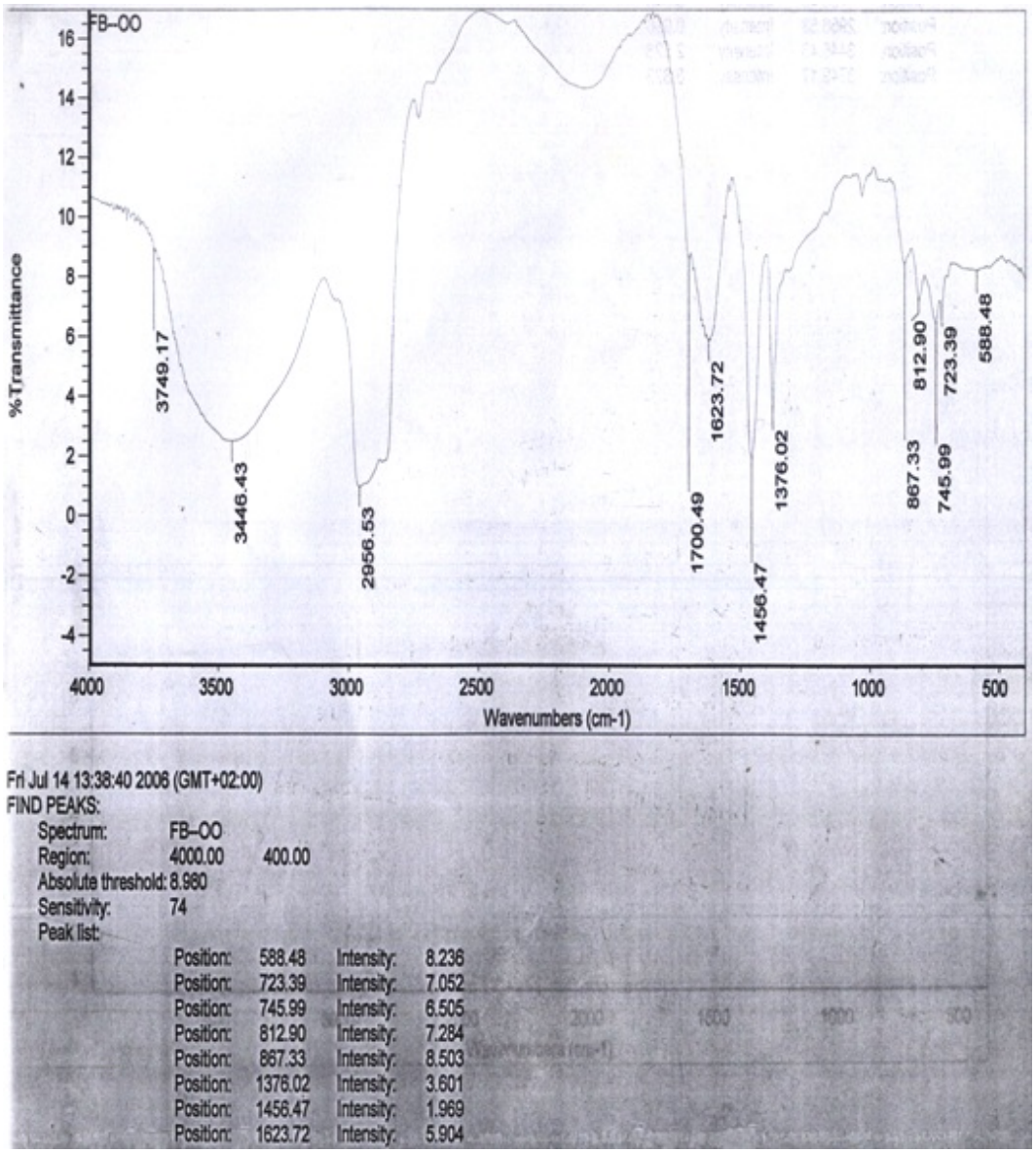



**Fig. 1:** The IR analysis of bitumen before biodegradation.

( $-\overset{|}{\underset{|}{C}}-H$ stretch or Saturated C-H) and alkene (C=C stretch) after bitumen biodegradation. Hamme *et al.* [13], Ghazali *et al.* [12] 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 at 37°C resulted in high biodegradation of bitumen and high yield of bitu-oil.

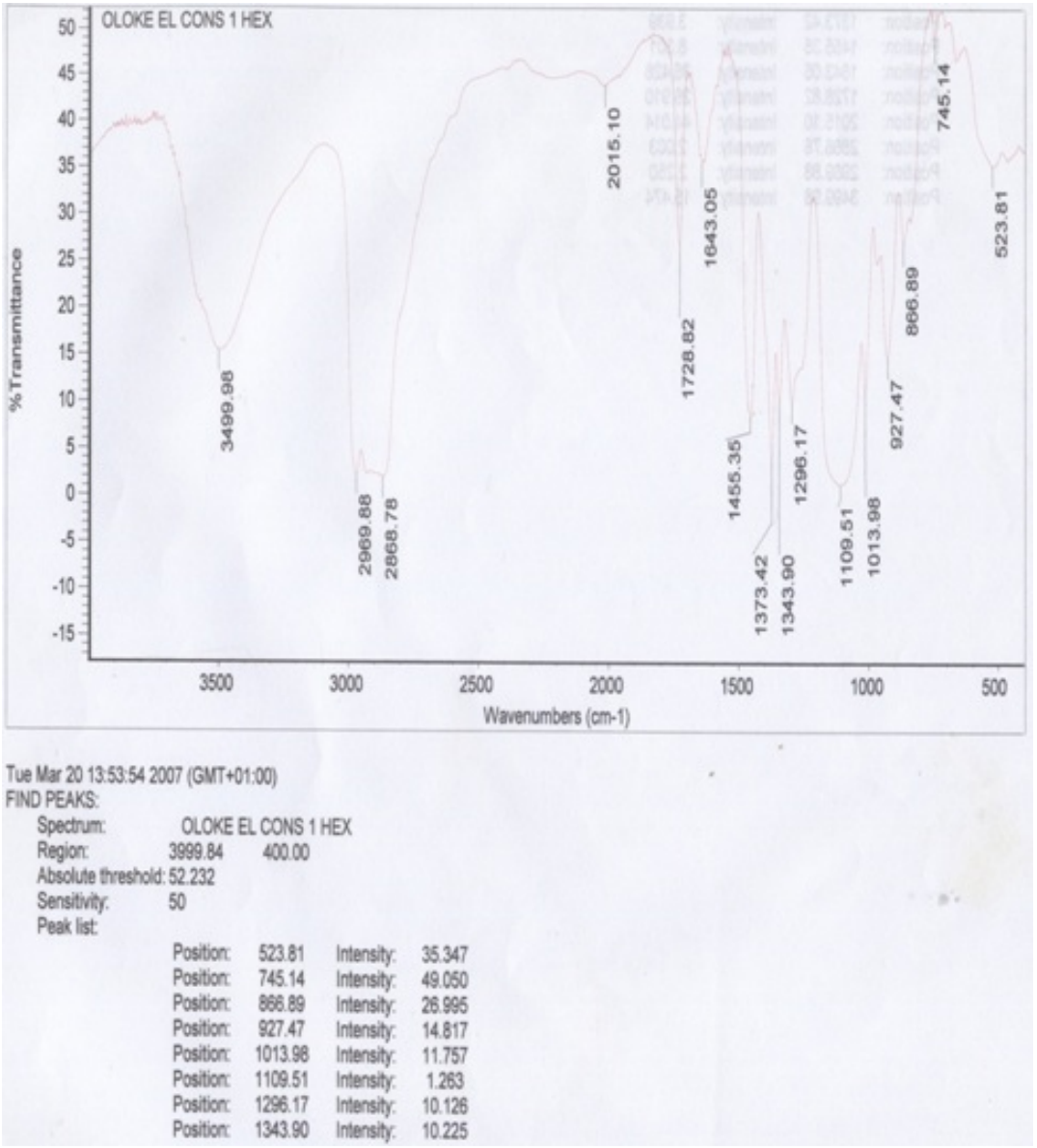



**Fig. 2:** The IR analysis of the bitu-oil obtained after biodegradation of bitumen.

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