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Full Length Research Paper

Effect of nitrogen and phosphate limitation on utilization of bitumen and production of bitu-oil and gas by a bacterial consortium

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Five strains of bacteria; *Pseudomonas fragi*, *Streptococcus zymogenes*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens* and *Bacillus macerans*, capable of utilizing bitumen as source of carbon and energy were isolated from water sample obtained from bitumen producing area, Agbabu, Ondo state, Nigeria. The degradation of bitumen was found to be associated with the production of carbon (IV) oxide, natural gas and oil. As a result of using nitrogen limited and phosphate limited media, 1750 and 1250 cm^3 of gas and 0.95 and 0.85 g/l of oil were obtained respectively. Nitrogen and phosphate limitation have profound effect on bitu-oil and gas production.

Key words: Bitumen, biodegradation, phosphate limited, nitrogen, bitu-oil.

INTRODUCTION

Petroleum entering an aquatic ecosystem provides a source of carbon and energy for microbial growth. But growth may be limited by the amount of available nitrogen and phosphorus, with a consequent limitation on degradation of the petroleum (Atlas, 1984). Nutrient availability, especially of nitrogen and phosphorus seem to be the most limiting factor in petroleum biodegradation. It was confirmed that these nutrients enhance growth of microorganisms, which leads to more rapid decomposition of petroleum contaminants (Bramble et al., 1991; Chaineau et al., 2005).

Nitrogen and phosphorus are the usual nutrients, which have been used in biostimulation process in order to support microbial growth (Liebeg and Cutright (1999). These nutrients have been considered as limiting factors for the bioremediation of contaminated soils, but when used properly, they may stimulate soil biodegradation (Zhou and Crawford, 1995). The effect of nitrogen and phosphate availability has been widely investigated (Chaineau et al., 2005; Rosenberg et al., 1980; VerstraeSte et al., 1976; Ward and Brock, 1976).

However, reports on nitrogen and phosphate limitation on bitumen biodegradation and metabolites production have not been exploited. This study was designed to investigate the effect of nitrogen and phosphate limitation in the medium composition which have on bitumen biodegradation and metabolites produced using isolated bacteria consortium from bitumen producing area.

MATERIALS AND METHODS

Isolation

The bacterial consortium (*Pseudomonas fragi*, *Streptococcus zymogenes*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens* and *Bacillus macerans)*, used were isolated from water samples collected in bitumen producing site from Agbabu area of Ondo state, Nigeria. Pure cultures were obtained by carrying out serial dilution, inoculation and incubation at 44°C for 14 days (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 120 ml/L of solubilized bitumen (bitumen was solubilized by dissolved 0.9 g of bitumen in 2 ml of n-hexane) was used for isolation.

Media composition

Two optimized media profiles were used. Medium one contains (g/l)

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(NH4)$_2$SO$_4$ (0.2), $CaCl_2.2H_2O$ (0.1), K_2HPO_4 (0.6), $MgSO_4.7H_2O$ and NaCl (2.5), while medium two contains (g/l) $(NH_4)_2SO_4$ (0.4), $CaCl_2.2H_2O$ (0.1), k_2HPO_4 (0.5), $MgSO_4.7H_2O$ (1.0) and NaCl (2.0).

Effect of complete medium composition on bitumen biodegradation and metabolites produced

The two culture media were prepared into separate reaction vessel, sterilized, inoculated and incubated at 37°C with aeration and agitation for five days. After the incubation, the culture was extracted; the wet weight, dry weight of cell, and weight of bitu-oil (oil produced from bitumen) were taken.

Effect of nitrogen limitation on bitumen biodegradation and metabolites produced

To investigate the effect of nitrogen limitation on growth, $(NH_4)_2SO_4$ at 0.002 g/l was the sole source of Nitrogen. The medium was prepared, sterilized, inoculated and incubated at 37°C for five days with aeration and agitation. After five days, the culture was harvested, extracted and the products were quantified.

Effect of phosphate limitation on bitumen biodegradation and metabolites produced

To investigate the effect of phosphate limitation on growth, K_2HPO_4 at 0.005 g/l was the sole source of phosphate. The medium was prepared, sterilized, inoculated and incubated at 37°C for five days with aeration and agitation. After five days, the culture was harvested, extracted and the products were quantified.

Extraction method

The culture was filtered (using Whatman paper 1) after incubation period to separate the undegraded bitumen. The filtrate was centrifuged (at 2500 rpm), supernatant was added to equal volume of n-hexane, shaking vigorously and allowed to settle. Two different layers were formed; the upper layer which is hexane layer with biodegraded bitumen, and lower layer which is medium layer. The upper layer was collected and n–hexane was removed (by exposed at room temperature for 6 h) to obtain bitu–oil. The bitu–oil was weighed and recorded.

Wet weight of cell

The wet weight was obtained by using the method of Rosenberg et al. (1972).

Dry weight of cell

The cell was dried at 60°C for 20 min in an oven and weighed. The process was repeated until constant weight of cell was achieved, which is final weight recorded.

Determination of volume of gas evolved and preliminary qualitative test of the gas

The volume of gas evolved were taken by inverted the burret filled with water into a bowl brim filled with water and a hole from reaction vessel was passed to the burret, which shows the volume of water displaced as corresponding volume of gas evolved. The preliminary qualitative test of gas evolved was carried out by passing gas over some chemicals; Lime water, bromine solution, acidified potassium tetraoxomanganate (vii) and silver trioxonitrate (V). Also, the combustive property of the gas was tested for, by passing through Bunsen burner.

The infrared spectroscopy analysis of bitumen and bitu-oil

The infrared spectroscopy (IR) analysis of bitumen and bitu-oil obtained from nitrogen and phosphate limited culture 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 complete medium composition on gas and bitu-oil production

The highest amount of gas (1750 cm^3) was obtained from medium with less concentration of $(NH_4)_2SO_4$ (0.2 g/l) (Figure 1), while medium with higher concentration of $(NH_4)_2SO_4$ (0.4 g/l) gave the least volume of 1500 cm^3 (Figure 2). The highest biomass yield (12.75 g/l) was obtained from medium containing 0.2 g/l of $(NH_4)_2SO_4$, while the lowest yield (10.05 g/l) was obtained at medium with 0.4 g/l of $(NH_4)_2SO_4$. The highest yield (0.56 g/l) of bitu-oil and least yield (0.05 g/l) of bitu-oil were obtained from medium with 0.2 and 0.4 g/l of $(NH_4)_2SO_4$, respectively (Table 1).

Effect of nitrogen and phosphate limitation on gas and bitu-oil production for medium with 0.2 g/l of $(NH_4)_2SO_4$

Nitrogen–limited growth culture gave the highest bitu-oil yield of 0.85 g/l and highest yield of biomass (7.5 g/l), while the phosphate–limited growth culture gave the lowest bitu–oil yield of 0.8 g/l and lowest yield of biomass (6.15 g/l) (Table 2). The highest volume (1750 cm^3) gas evolved was obtained at nitrogen–limited growth culture (Figure 3), while the lowest volume (1500 cm^3) of gas evolved was obtained at phosphate–limited growth culture (Figure 4).

Effect of nitrogen and phosphate limitation on gas and bitu-oil production for medium with 0.4 g/l of $(NH_4)_2SO_4$

Nitrogen–limited growth culture gave the highest bitu–oil yield of 0.95 g/l and lowest biomass yield (5.55 g/l), while the phosphate– limited growth culture gave the lowest bitu–oil yield of 0.50 g/l and highest biomass yield (6.45 g/l) (Table 3). The highest volume of (1750 cm^3) of gas evolved was obtained from nitrogen–limited growth culture (Figure 5), while the lowest volume (1500 cm^3) of

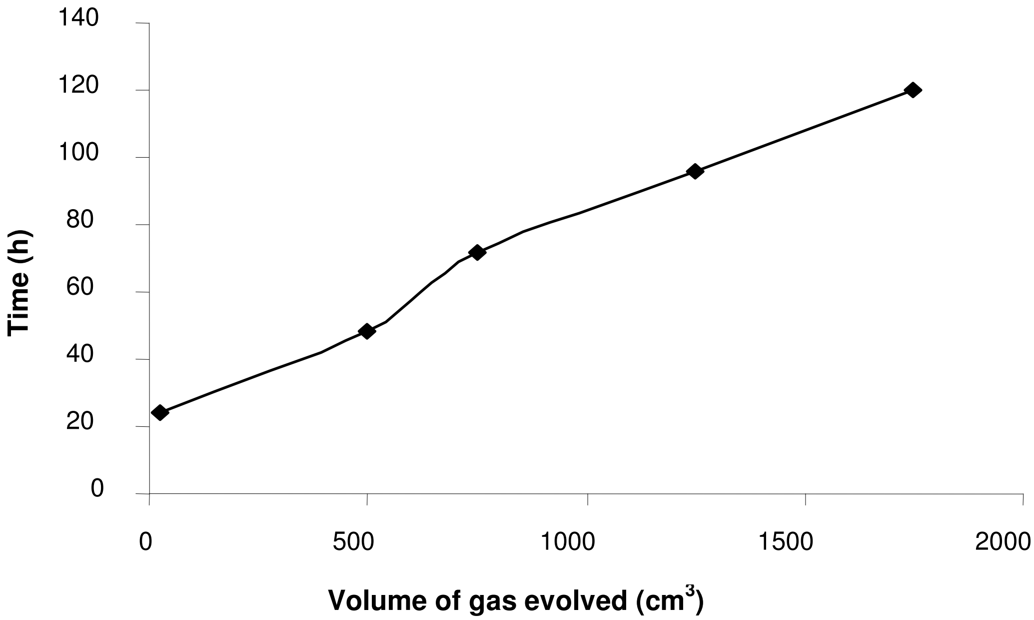

Figure 1. Volume of gas evolved in complete medium composition for medium with 0.2 g/l of $(NH_4)_2SO_4$.

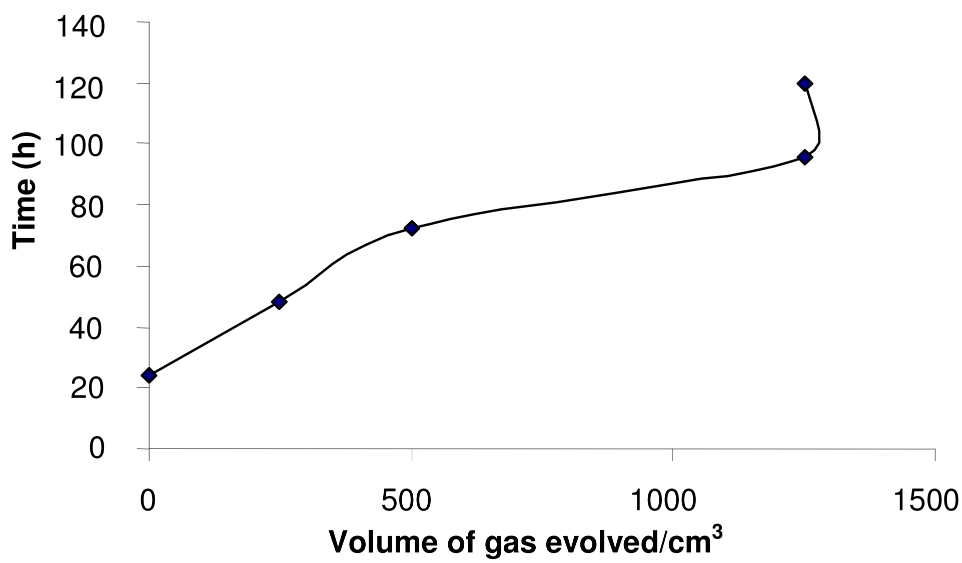

Figure 2. Volume of gas evolved in complete medium composition for medium with 0.4 g/l of $(NH_4)_2SO_4$.

Table 1. The wet weight (W/W), Dry weight (D/W) and bitu–oil weight in complete medium composition.

Medium	Cell (g/l)	DW of cell (g/l)	Bitu-oil weight (g/l)
M_1	12.75	4.1	0. 56
M_2	10.05	4.25	0.5

M_1 = Medium one; M_2 = Medium two.

Table 2. The effect of nitrogen and phosphate limitation on bitu-oil and biomass production in medium with 0.2 g/l of $(NH_4)_2SO_4$.

Limiting factors	Cell (g/l)	DW of cell (g/l)	Bitu- oil weight (g/l)
N_2A	7.0	3.6	0.85
N_2B	7.5	4.1	0.80
PO_4A	6.15	3.55	0.75
PO_4B	5.5	3.25	0.80

N_2A = Nitrogen limitation, vessel A; N_2B = nitrogen limitation, vessel B; PO_4A = phosphate limitation, vessel A; PO_4B = Phosphate limitation, vessel B.

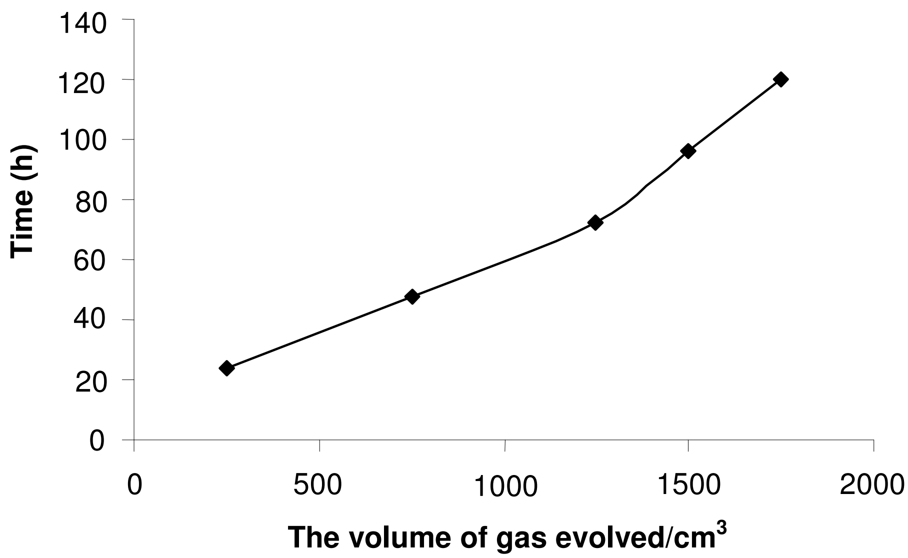

Figure 3. Volume of gas evolved in nitrogen-limited culture for medium with 0.2 g/l of $(NH_4)_2SO_4$.

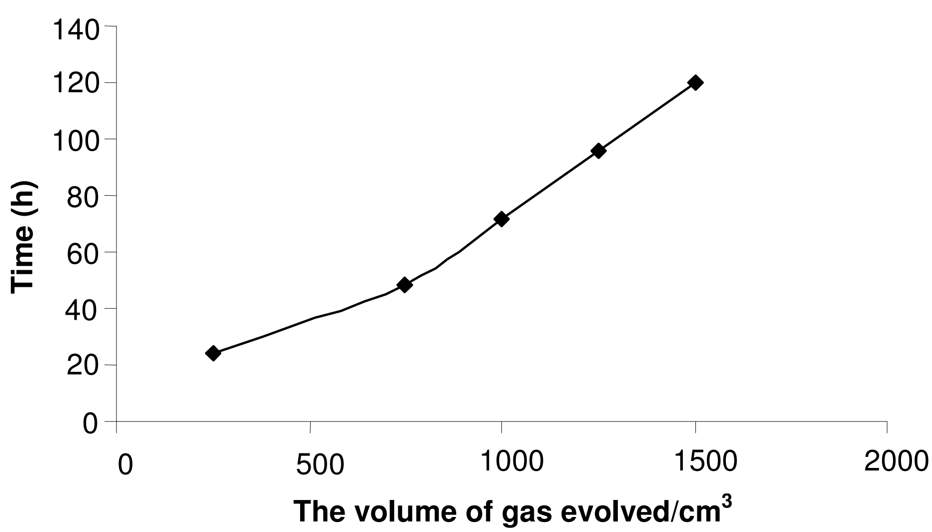

Figure 4. Volume of gas evolved in phosphate-limited culture for medium with 0.2 g/l of $(NH_4)_2SO_4$.

Table 3. The effect of nitrogen and phosphate limitation on bitu-oil and biomass production in medium with 0.4 g/l of $(NH_4)_2SO_4$.

Limiting factors	Cell (g/l)	DW of cell (g/l)	Bitu-oil weight (g/l)
N_2A	6.05	3.10	0.85
N_2B	5.55	2.95	0.95
PO_4A	5.50	2.80	0.50
PO_4B	6.45	3.55	0.85

N_2A = Nitrogen limitation, vessel A; N_2B = nitrogen limitation, vessel B; PO_4A = phosphate limitation, vessel A; PO_4B = Phosphate limitation, vessel B.

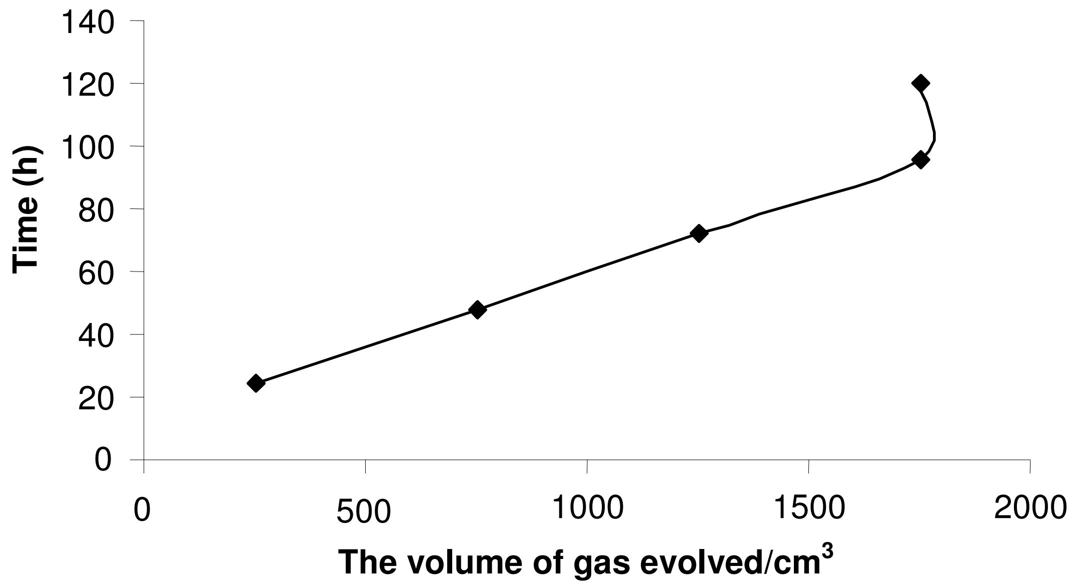

Figure 5. Volume of gas evolved in nitrogen-limited culture for medium with 0.4 g/l of $(NH_4)_2SO_4$.

gas evolved was obtained at phosphate–limited growth culture (Figure 6). The results of preliminary test for gas produced were shown in the Table 4. The addition of gas and lime water formed milky solution, while reaction of gas with bromine water, and cold acidified potassium tetraoxomanganaate (vii) turned colourless and addition of gas with silver trioxonitrate (v) gave no visible reaction. Different wave numbers (cm^{-1}) obtained with the spectroscopic analyses in the raw bitumen and bitu-oil from nitrogen and phosphate limited cultures 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 (Figure 7), 743.31, 929.21, 967.47, 1015.21, 113.17, 1273.22, 1343.62, 1374, 1460.88, 1729.88, 1641.43, 2855.94, 2925.22, 2962.02 and 3450.79 (Figure 8) and 651.70, 45704.98, 743.91, 867.27, 928.30, 967.25, 1014.52, 1112.42, 1273.53, 1343.52, 1373.91, 1461.14, 1729.73, 1641.61, 2857.70, 2925.69, 2966.03 and 3466.21 (Figure 9).

DISCUSSION

As expected the variation in media composition gave significantly different yield of metabolites (bitu-oil and gas) direct on bitumen biodegradation. The highest gas of 1750 cm^3 was obtained from medium with less concentration of $(NH_4)_2SO_4$ (0.2 g/l) (Figure 1), while medium with higher concentration of $(NH_4)_2SO_4$ (0.4 g/l) gave the least volume of 1500 cm^3 (Figure 2). The highest biomass yield (12.75 g/l) was obtained from medium containing 0.2 g/l of $(NH_4)_2SO_4$, while the lowest yield (10.05 g/l) obtained from medium with 0.4 g/l of $(NH_4)_2SO_4$. The maximum yield of 0.56 g/l and least yield (0.5 g/l) of bitu-oil were obtained from media with 0.2 and 0.4 g/l of $(NH_4)_2SO_4$, respectively (Table 1). Thomson et al. (2005) using sixteen different growth conditions containing different concentrations of lactic acid and benzoic acid found out that the media differently affected the fermentative capacity of *Saccharomyces cerevisiae*.

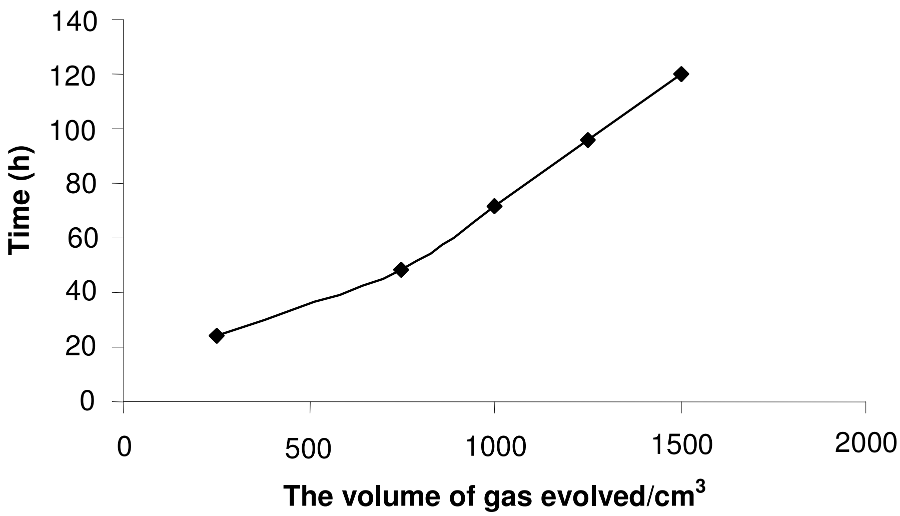

Figure 6. Volume of gas evolved in phosphate–limited culture for medium with 0.4 g/l of $(NH_4)_2SO_4$.

Table 4. Preliminary test on the gas evolved.

Test	Reaction	Inference
Lime water + gas	Milky solution	Carbon (iv) oxide
Bromine + gas	Decolourization	Alkene and alkyne suspect
Cold acidified potassium tetraoxomanganate(vii) + gas	Decolourzation	Alkene and alkyne suspect
Silver trioxonitrate (v) + gas	No reaction	Alkene confirmed

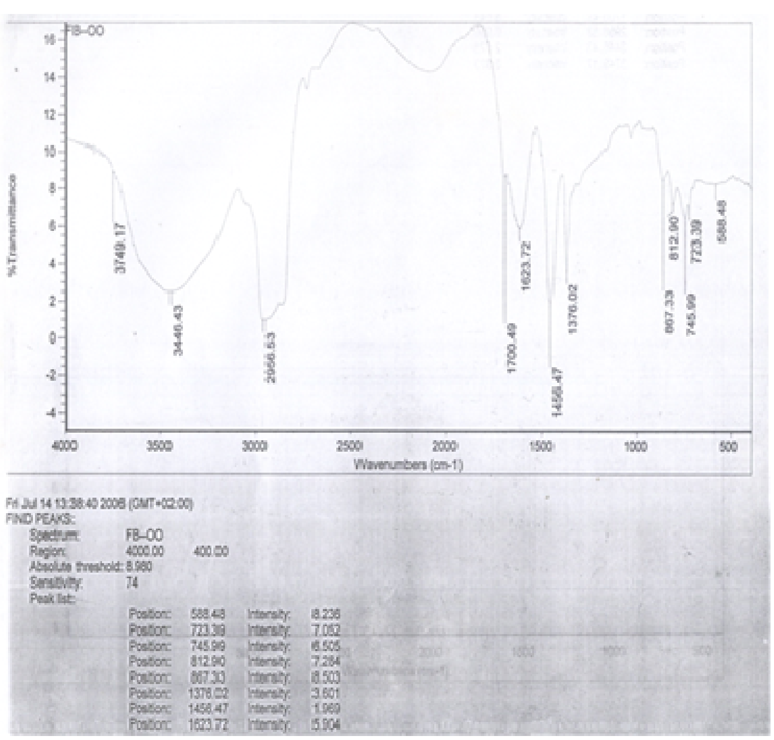

Figure 7. The IR analysis of bitumen before biodegradation.

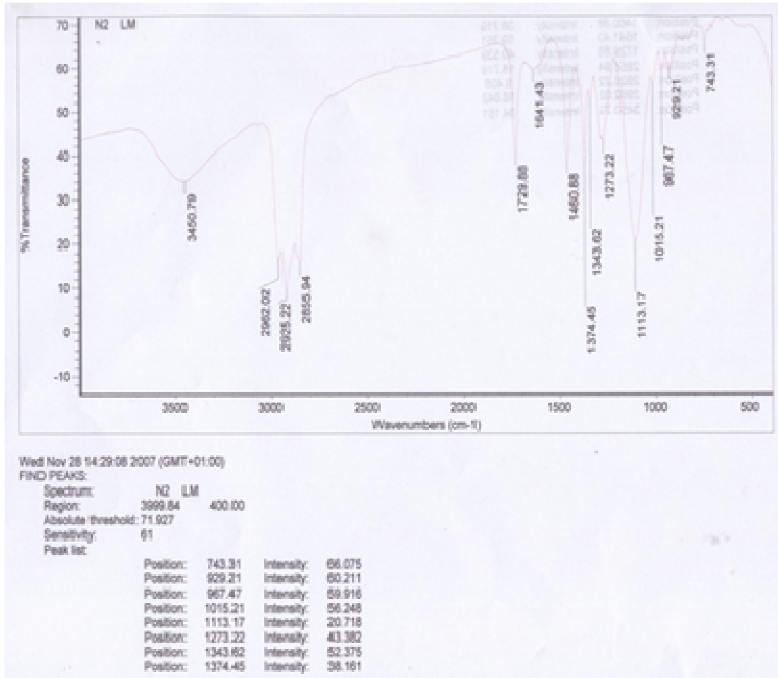

Figure 8. The IR analysis of the oil obtained from the nitrogen limited culture after biodegradation.

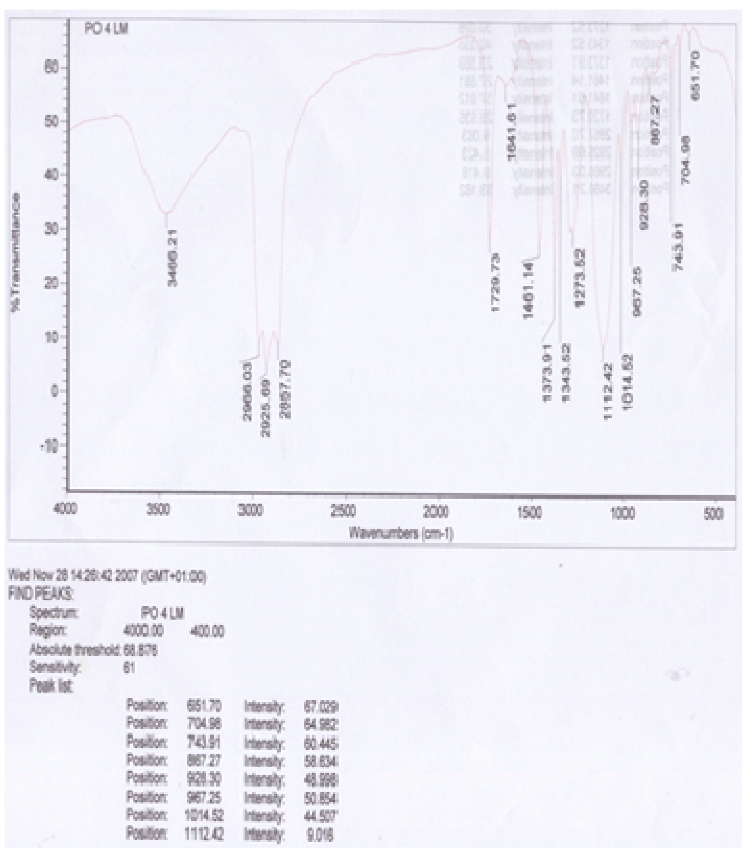

Figure 9. The IR analysis of the oil obtained from the phosphate limited culture after biodegradation.

Previous works have shown that availability of required substrate constituents in required concentrations strongly stimulated oil biodegradation in contaminated soils (Kincannon, 1972; VerstraeSte et al., 1976; Dibble and Bartha, 1979).

In medium containing 0.2 g/l of $(NH_4)_2SO_4$, nitrogen–limited growth culture gave the highest yield of bitu–oil (0.85 g/l) and biomass (7.5 g/l), while the phosphate–limited growth culture gave the least yield of bitu–oil (0.8 g/l) and biomass (6.15 g/l) (Table 2). Figure 3 shows the highest volume of gas (1750 cm^3) obtained from nitrogen–limited growth culture and the least volume of gas (1500 cm^3) obtained from phosphate–limited growth culture (Figure 4). The medium with higher concentration of $(NH_4)_2SO_4$ (0.4 g/l), nitrogen-limited growth culture gave highest bitu-oil yield of 0.95 g/l and lowest biomass yield (5.55 g/l), while the phosphate–limited growth culture gave least bitu-oil yield of 0.50 g/l and highest biomass yield (6.45 g/l) (Table 3). The highest volume (1750 cm^3) of gas evolved was obtained from nitrogen–limited growth culture (Figure 5), while the least volume (1500 cm^3) of gas evolved from phosphate-limited growth culture (Figure 6). Thomson et al. (2005) working on starvation response of *S. cerevisiae* grown in anaerobic nitrogen or carbon-limited chemostat cultures showed that nitrogen limited cells that were starved for carbon lost almost all their fermentatives capacity. The carbon limited cells managed to preserve a large portion of their fermentative capacity during carbon starvation. They concluded that in industrial setting, carbon starvation in anaerobic fermentation should be avoided to maintain productive yeast. Zenk et al. (1977) reported that decreased in phosphate concentration correlated with an increased in alkaloids, anthocyanins and phenolics production in *catharanthus.* Stimulated caffeine production has been reported in *coffea arabica* cell suspension and that of cinnanoyl patrescine in those of *Nicotiana tabaccum* under phosphate–deficient conditions (Bramble et al., 1991; Knobloch et al., 1981). Ward and Brock (1976) observed that nitrogen and phosphate limitation have profound effect on oil biodegradation and metabolite production. When the phosphate or nitrogen concentration in the culture medium decreases below a threshold level, bacteria increase their production of a variety of metabolites (Viining, 1992).

The results obtained from this work shows that limiting the growth factors in the growth substrate of a certain microorganism enhances the overproduction of its metabolites. Nitrogen and phosphate limitation increased the rate of bitumen biodegradation with direct effect on the bitu-oil and gas produced. The addition of gas with lime water produced milky solution which suggested the presence of carbon (IV) oxide, also decolourization of bromine water and potassium tetraoxomanganase (VII) when reacted with gas, confirmed the presence of alkene group of hydrocarbon (ethylene gas) (Table 4). The non-combustive property confirmed that the gas produced is an ethylene gas. The test confirmed that the mixture of gases evolved comprises carbon (IV) oxide, ethylene gas and other unidentified gases.

Prominent peaks in the spectra of bitumen (Figure 7) include; 1700.49 cm^{-1} (carbonyl group), 1623.72 cm^{-1} (C=C stretch), 2956.53 cm^{-1} ($-\overset{|}{\underset{|}{C}}-H$ stretch or saturated C-H) and 3446.34 cm^{-1} (Alkanol {OH}), while prominent peaks in spectra of the bitu-oil (Figures 8 and 9) occurred at; 1113.17 cm^{-1} ($-\overset{|}{\underset{|}{C}}-OH$ stretch), 1273.22 cm^{-1} (Ethers), 1729.88 cm^{-1} (Aryl and αβ unsaturated group), 3450.76 cm^{-1} (OH-group), 1343.62 cm^{-1} (C-O stretch) and 2855.94 cm^{-1} ($-\overset{|}{\underset{|}{C}}-H$ stretch). The IR-analysis revealed the disappearance of low molecular hydrocarbon such as alkane ($-\overset{|}{\underset{|}{C}}-H$ stretch or saturated C-H) and alkene (C=C stretch) after bitumen biodegradation. Hamme et al. (2003), Ghazali et al. (2004) 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.

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