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Effect of pH, Temperature, and Nutrient Supplement on the Growth of Micrococcus Luteus and on its Biodegradation of Crude Oil Hydrocarbons

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Abstract:
The effect of pH, temperature and nutrient supplement on the growth of Micrococcus luteus and its rate of biodegradation of crude oil hydrocarbons obtained from a crude oil contaminated farmland in Ekpe East Local Government Area of Rivers State was studied following laboratory simulation of the stated factors and monitoring the growth and the residual oil. This was done under five different pH values, five different temperature values and five different nutrient concentrations. The bacterium was isolated from crude oil contaminated farmland by enrichment culture method. The growth of the organism was found to be highest at a temperature of 43.7^{o}C and biodegradation of the crude oil was 78%. The highest growth under pH was at pH 8.0 while 71% hydrocarbon was removed. The result for the nutrient supplementation gave 65% crude oil degradation with the highest growth at the nutrient concentration of 0.434g/ml oil. This nutrient concentration corresponds to carbon to nitrogen (C: N) ratio of 10:2.

Keywords: *Micrococcus luteus, biodegradation, crude oil, nutrient, temperature and pH*

1. Introduction

The environmental contamination as a result of oil spill incidents into the environment, especially farmlands has tremendous negative effects and implications for the sustainability of the environment with particular reference to agricultural products. The frequency and size of the spill resulting from complex activities inherent in oil exploration, exploitation and distribution is of great concern to the world and to the Nigerian Nation in particular whose spill management exercises are still undeveloped. The increasing global demand for petroleum hydrocarbons and its products has led to the frequent contamination of the environment as a result of increased activity in order to meet up with the demand. Pollution of the soil with crude oil or its hydrocarbon products affect soil micro fauna and micro flora, it also affects plants and ground water. The severity of this on the environment depends on the magnitude of the spilled oil. The ecological problems observed as a result of oil pollution include a brownish vegetation and soil erosion, diminishing resources of the natural ecosystem, lands that were originally fertile were turned barren and it also brought with it adverse effects on the life, health and negatively affects the economy of the people of the area (Roberts, 1977). Oil pollution damages vegetation, it causes premature defoliation and loss of plants productive cycles and may even result in outright death of the affected plants. Essien and John, (2010) reported that plants germinate, develop and grow in soil medium where air, water and other nutrients are available for good healthy growth and also for productive and profitable agriculture. They also reported that the spillage of the crude oil on agricultural soils has fouling effect on all forms of life, that it renders the soil especially the biologically active surface layer toxic and unproductive. Abii and Nwosu, (2009), observed in their work that crude oil reduces the soil's fertility such that most of the essential nutrients are no longer available for plant and crop utilization. Brain, (1979), reported that crude oil reduces and restricts permeability, that organic hydrocarbons fill the pores of the soil, prevent water and air from reaching the soil thus preventing or depriving the plant roots the much-needed water and air.

As the contamination of the soil by crude oil affects the fertility of the soil, it also has an adverse effect on the microbial biomass. Microbial presence in the soil is of great importance in maintaining soil fertility as reported by Torstenssen et al., (1998), that soils which maintain a high level of microbial biomass are capable of not only storing more nutrients, but also of cycling more nutrients through the system. Akpoveta et al., (2011) reports that the sustainability of soil fertility, quality and productivity is of immense interest to and concern to man because of the attendant detriments of hydrocarbon contamination on soil, since there is direct reliance and dependence of man's existence on the soil. The effects of crude oil on natural microbial populations will depend on the composition of oil spilled, oil temperature, viscosity of the oil, the level or concentration of the oil spilled and some other environmental factors such as pH, nutrient concentration, soil moisture, soil texture etc. It is believed that petroleum pollution causes microbial population changes, such as increases or decreases in

microbial numbers. Ekpo and Ebeagwu (2009), reported that the initial reaction of the microorganism as it gets contact with the oil both in the soil and river is a reduction of activity due to reduced air availability and that this initial suppression of the microbial count is attributed to selective destruction of the microorganisms by the crude oil. In the soil the crude oil produces an anaerobic condition as it is introduced into the soil and this automatically eliminates most of the aerobic organisms. This agrees with the report of Odu (1972) that crude oil introduced into the soil causes initial damage to the soil biota.

This frequent crude oil spillage on agricultural soils affects the microbial population and the vegetation negatively and as a result, the farming activities of the community of the area in question are badly affected. The natural removal of the crude oil hydrocarbons by the indigenous microbial population is a very slow process that can last for over ten years. Biodegradation of the hydrocarbons is further delayed by the initial reduction of the microbial biomass when the spill first occurred. The process of biodegrading the hydrocarbon pollutants can be optimized by ensuring a favorable environmental condition that will enhance the rate of microbial transformation of the oil. The right favorable environmental conditions such as temperature, pH, nutrients etc. will increase the rate of both growth of the organism and the breakdown of the hydrocarbons. This work is aimed at studying the effects of these environmental conditions (temperature, pH and nutrient concentration) on the growth of Micrococcus luteus and the rate at which it transforms or removes the crude oil hydrocarbons. The Micrococcus luteus was isolated from the contaminated Ekpe soil; it is indigenous to the Ekpe soil.

2. Materials and Method

2.1. Sample Collection

The soil samples used were collected from oil impacted farmland at Ekpe in Ahoda East Local Government Area of Rivers State. The samples (oil polluted soil and an unpolluted) were collected with a soil augur at surface not below 15cm. The unpolluted (Pristine soil) was collected from a forest also in Ekpe that has no oil pollution history.

2.1.1. Isolation and Identification of the Bacterium

Mineral salts media were prepared and properly labelled 1 and 2. These were used for the isolation of the hydrocarbon degrading bacterium. Medium 1 in addition was also used for biodegradation studies. Medium 2 is mineral salt agar.Medium 1 was formulated by mixing the following reagents:
$(NH_4)_2SO_4$ (1.25g); $(NH_4)_2HPO_4$, (1.95g); KH_2PO_4, (0.85g); $MgSO_4.7H_20$, (0.09g); $CaCL_2$, (0.001g); and distilled water 1000ml.
Medium 2 was gotten by mixing the following: K_2HPO_4, (0.05g); NH_4CL (1.0g); Na_2SO_4 (2.0g); KNO_4 (2.0g); $MgSO_4$ (1.0g); Agar (15.0g); distilled water (1000ml) and $CaCl_2$ (0.001g). The prepared media pH was adjusted to 7.0.

2.1.2. Procedure

Two 50ml volumes of medium 1 were measured out and dispensed into two 250ml Erlenmeyer Flasks and sterilized for fifteen minutes at 121^oC. A 250ml flask containing 200ml of medium 2 was also sterilized at 121^oC for fifteen minutes and dispensed into three petri-dishes. Plates were allowed to solidify, dried and kept for 24 hours to test for sterility.
The contaminated soil sample was taken and mixed thoroughly; 2g of it was weighed and used to inoculate each flask of medium 1. Thereafter, 1.0ml of kerosene filtered through a 25mm Whatmann syringe filter was introduced into each flask and the flasks incubated on an orbital shaker (Gallenkamp, England) at 30^oC for 24 hours. After 24 hours incubation, a loopful of the content of each flask 1 was used to streak triplicate petri-dishes of medium 2 using a sterile wire loop and incubated at room temperature (25-30^oC) for 48 hours. Kerosene was introduced as carbon source to each petri-dish by the vapour phase transfer method. This was done by impregnating sterile filter paper with sterile kerosene and placing it in each petri-dish cover and incubating in an inverted position. All the resulting colonies were repeatedly sub cultured in the same manner twice and pure isolates were gotten and stored in nutrient agar slants for use in the experiment.

2.2. Identification of Crude Oil Degrading Strain

Identification of the isolates were based on the cultural, morphological and biochemical characteristics. This was carried out by conventional, microbiological and biochemical procedures and reference to the Bergey's Manual of Determinative Bacteriology. The following identification test were carried out in addition to cultural and morphological properties: Gram reaction, spore stain, motility, catalase, oxidase, and starch hydrolysis, nitrate reduction, Growth on 7% Nacl, Arginine hydrolysis, Voges Proskauer test. Fermentation tests of sucrose, dextrose, xylose, maltose, mannitol, mannose, and lactose were also done.

2.3. Biodegradation Studies in Soil

The effects of temperature, pH and nutrient supplement on the growth of one of the isolates (Micrococcus luteus) and on its crude oil utilization were investigated. The growth and the degree of crude oil depletion were evaluated using bacteria number (measured in colony forming units (cfu) per gram soil) and quantity of residual oil. Each of the three factors tested (pH, temperature and nutrient supplements) was applied at five different levels to the organism (Micrococuss luteus). Urea fertilizer was the nutrient supplement used in carbon to nitrogen ratio (C: N) of 10:5, 10:4 10:3, 10:2, 10:1.

The sandy loamy soil (Pristine soil) obtained from the forest with no history of oil pollution in Ekpe in Ahoda East Local Government Area of Rivers State was air dried, sieved and dispensed in 200g weights into thirty-two 500ml Erlenmeyer flasks. The soil samples measured were sterilized by autoclaving three times at 121°C for fifteen minutes. The thirty-two flasks were divided into three and placed into three cells, each cell containing ten flasks which were duplicates of the variables (pH, temperature and nutrient) being tested while two flasks served as controls for the nutrient supplement. Each flask was contaminated with filter-sterilized Bonny Light Crude Oil at 20% (v/w) level of pollution. Also, each of the thirty-two flasks was inoculated with 5ml of inoculum of the isolate (Micrococus luteus) suspended in normal saline to a level of 0.5 McFarland standard. The flasks containing the nutrient supplement also received sterilized solution of urea fertilizer. The experiment lasted for one month (28 days) and samples were collected every seven days for analysis to determine the bacterial number and residual oil.

2.4. Determination of Hydrocarbon Degrading Bacteria (Cfu/Ml)

Ten-fold serial dilution was carried out. A gram of the oil impacted soil sample was measured out from each of the sample flasks and introduced into the first test tube containing 9ml of sterile distilled water for serial dilution. The tube was shaken vigorously for thorough mixing of the mixture. 1.0ml was then pipetted from this first test tube and transferred into another test tube containing 9ml of sterile distilled water to give a 10^{-1} dilution. The sample was diluted serially up to 10^{-10}. This same procedure was performed for the thirty-two flasks, (Nwosu et al., 2018). The enumeration proper was carried out by inoculating 0.1ml aliquot from an appropriately serially diluted sample onto mineral salts agar medium 2. Kerosene supplied carbon by the vapour phase transfer method. The plates inoculated were incubated for 48 hours at room temperature (25-30°C) after which the resulting colonies were counted and the bacterial number estimated as colony forming units (cfu) using the following expression:

$$\text{cfu}) = \frac{\text{average number of colonies} \times \text{original dilution}}{\text{volume of inoculum} \times \text{dilution factor}} = \frac{Y \times 1}{V \times 10^{-x}}$$

V = Volume of inoculum
10^{-x} = Dilution factor
Y = Average number of colonies
$10^{o} = 1$ = Original dilution

2.5. Extraction of Residual Oil

Twenty milli- litre of n-hexane was divided into two equal parts and poured into the bottle containing 5g crude oil polluted soil with the microorganism and centrifuged at 1000rpm for 5 minutes and decanted into a sterile test-tube. The extraction was performed twice with 10ml of the n-hexane stated earlier. The combined extract which contains the residual crude oil was kept overnight for the n-hexane to evaporate and leaving the residual oil in the test tube. The oil left was weighed and the volume calculated based on the fractional recovery rate (FRR). Two extractions were done and the average value recorded.

2.6. Fractional Recovery Rate (FRR)

Preliminary extraction was done to help in this work in determining the actual percentage of the crude oil biodegraded by the organism used. 20g of soil was mixed with ten milli-litre of crude oil. Using the method of Toogood and McGill (1977), the crude oil was extracted from the soil using n-hexane. It was discovered that the recovery rate of the oil was consistently 80% (i.e. from the 10ml introduced into the soil, 8.0ml was consistently recovered with the remaining adsorbed to the soil particles). There was a consistent 20% crude oil loss which amounts to 2ml of the 10ml of crude oil introduced. With this it was possible then to calculate the fractional lost to the soil.

Quantity of oil used = 10ml
Quantity of oil recovered = 8.0ml
Recovered oil per ml = 8/10 = 0.8ml
The fractional recovery rate = 0.8ml
Percentage recovery rate = 80%
The fractional lost per ml of oil = 0.2ml per ml of oil

The calculated amount of oil lost was added to the residual oil obtained from the extraction of residual oil. The actual amount of oil biodegraded will now be the amount used minus the total recovery residue and the fractional lost calculated

3. Results and Discussion

3.1. Microbial Isolate

The bacterial strain used in this study was isolated from the crude oil polluted soil obtained from Ekpe in Ahoda East Local Government Area of Rivers State, Nigeria. The isolate was a gram-positive coccus, yellow in color and was identified as

Micrococcus luteus based on its morphological and biochemical properties, Table1. It exhibited positive results for catalase, oxidase, starch hydrolysis, nitrate reduction, growth on 7% Nacl, Voges Proskauer tests, sucrose fermentation, dextrose, mannitol, maltose and xylose fermentation. Negative results were recorded for sporulation, Arginine hydrolysis tests and lactose and mannose fermentation. The effects of temperature, pH and nutrient supplementation, on the growth of this isolate and its ability to biodegrade crude oil and use it as a source of carbon and energy were investigated.

Tests	Results
Color of colony	Yellow
Shape	Cocci
Gram stain	+
Sporulation	_
Oxidase	+
Catalase	+
Starch hydrolysis	+
Growth on 7% Nacl	+
Arginine hydrolysis	_
Nitrate reduction	+
Voges Proskauer	+
Dextrose fermentation	+
Mannitol fermentation	+
Lactose fermentation	_
Sucrose fermentation	+
Maltose fermentation	+
Mannose fermentation	_
Xylose fermentation	+
Implicated organism	Micrococcus Luteus

Table 1: Biochemical Test Results Conducted for the Identification of the Isolate Micrococcus Luteus
+ = positive reaction to the test
= negative reaction to the test

The growth of Micrococcus luteus at five different temperatures of 30.27^{o}C, 33°C, 37°C, 41°C and 43.7^{o}C and at a time ranging from 0- 672 hours and the rate at which it degrades the crude oil are shown in figures 1, 2 and 3.

3.2. Effect of Time and Temperature on the Growth of Micrococcus Luteus

In this work, the highest growth of this organism was at the temperatures of 43.7^{o}C and 41°C giving bacteria population of 2.74 x 10^{12} cfu/g soil and 2.30 x 10^{12} cfu/g soil respectively. This organism showed no lag phase at the temperature of 43.7^{o}C. In the third week of the experiment, the rate of growth was slow between the temperatures 30.27 C and 33°C and picked up at the temperature above 33°C. The same can be said in the second week of the experiment, but the rate of growth picked here at the temperature of 37°C. At the start of the experiment and in the first week, the organism was in complete dormant condition. The growth in the first week started slowly at the temperature of 37°C. This is shown in the plot of bacteria number against time, Figure 1.

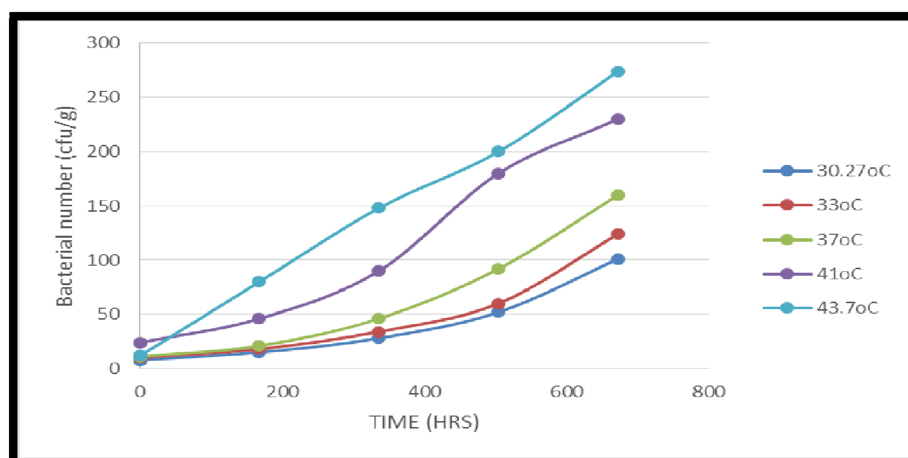

Figure 1: Effect of Temperature on Bacterial Growth

In Figure 2 which is the plot of bacteria number against temperature, the growth again was highest at the temperature of 43.7C. There was no lag phase at this temperature. This was followed by the growth at 41°C, which showed a little lag phase. The growth started immediately at the temperature of 43.7^{o}C but the rate was slow initially at 41°C until after 33°C in the third week. At the other three temperatures, the lag phase shown in each week was prominent and growth started at the temperature of 37°C.

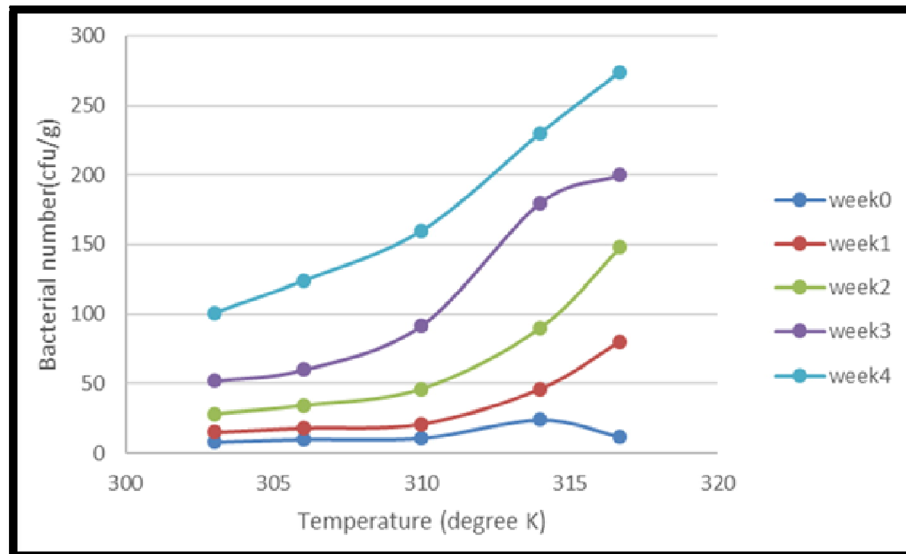

Figure 2: Variation of Bacterial Growth with Temperature and Time

3.3. Effect of Time and pH on Growth

Figure 3 is the plot of bacterial number against time at different pH values. The highest growth was observed at pH 8.0 followed by that at pH 8.68 with viable bacterial numbers of 85 x 10^{10} cfu/g soil and 56 x 10^{10} cfu/g soil respectively. Except at pH 5.32, where it showed delay before growth started, there was no lag phase in all the other pH values of 6.0, 7.0, 8.0 and 8.68. There was no growth from initial zero-hour time to 168 hours at pH 5.32.

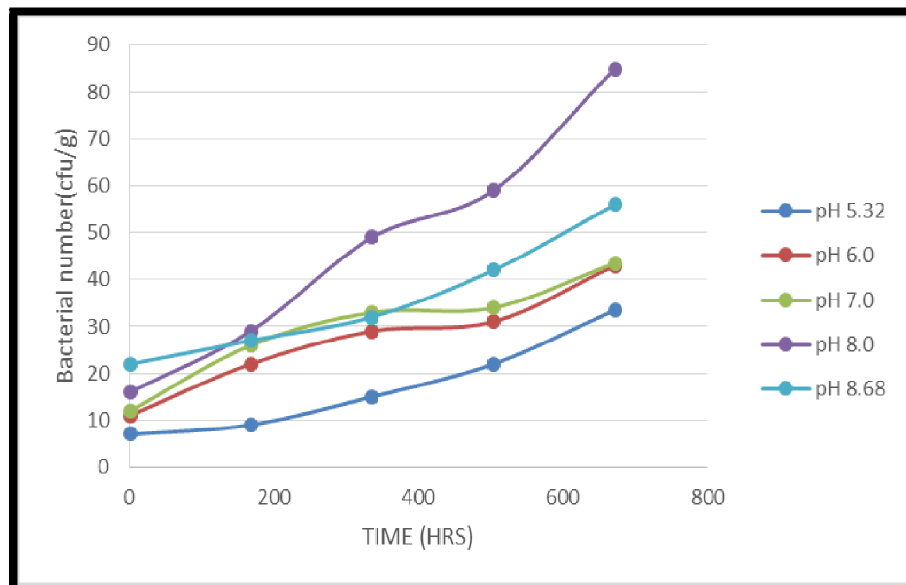

Figure 3: Effect of pH on the Growth of M. Luteus

Growth commenced after 168 hours at this pH. This result is in agreement with the report of Dibble and Bartha, (1979), that the growth and degradation of oil sludge was greatest at pH of 7.5 and 8.0.

The plot of bacterial number against pH showed clearly how pH affected the growth of Micrococcus luteus. In all the weeks, pH 8.0 gave the highest growth. In weeks 1, 2 and 3, the growth at pH 6 showed decrease and increased again at pH 7 and had the highest growth at pH 8.0 and sharply decreased, though giving the second highest growth at pH 8.68, Figure 4.

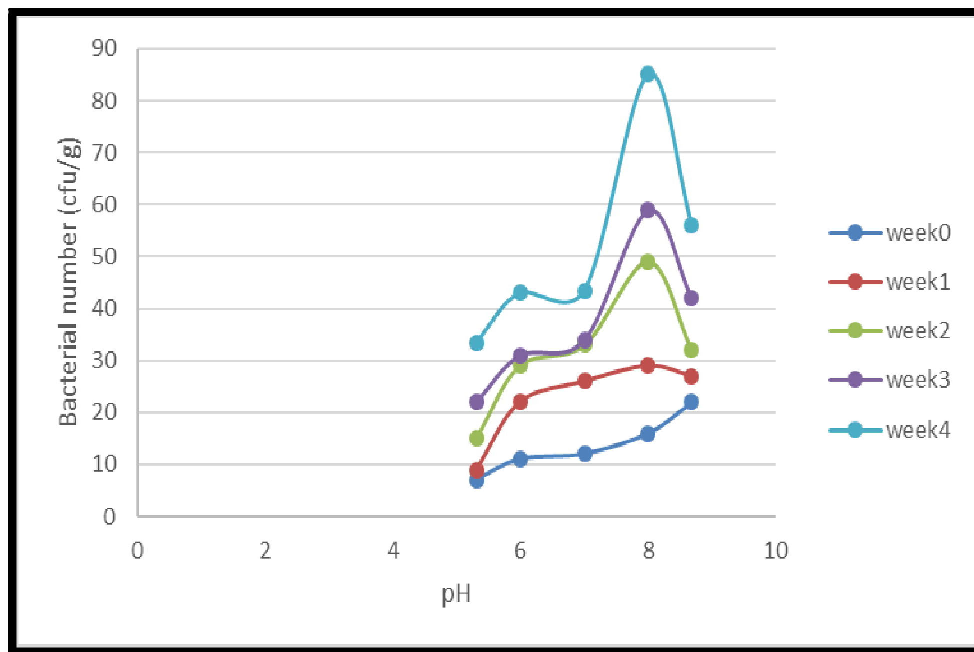

Figure 4: Variation of Micrococcus Luteus Population with pH and Time

3.4. Effect of Time and Nutrient on Growth

Nitrogen as a nutrient is necessary for microbial growth and plays a vital role in the utilization of crude oil and other organic pollutants that leads to proper growth in their natural environments. It is a basic nutrient for growth and maintenance of metabolic functions. Urea was used in this work in different concentrations containing calculated amounts of nitrogen and its effect on the growth of Micrococcus luteus measured. The nutrient urea was applied at a carbon to nitrogen ratio (C:N ratio) of 10:1, 10:2, 10:3, 10:4, and 10:5, which gives nitrogen concentrations of 0.217, 0.434, 0.651, 0.868 and 1.085g/ml oil respectively. The results obtained showed that 0.434g/ml oil gave the highest bacterial growth. The viable bacterial number at this nutrient value is 96 x 10^{10} cfu/g soil from the initial value of 9.0x 10^{10} cfu/g soil. The contour plot of bacterial number against time Figure 5 showed the pattern of growth of Micrococcus luteus with time.

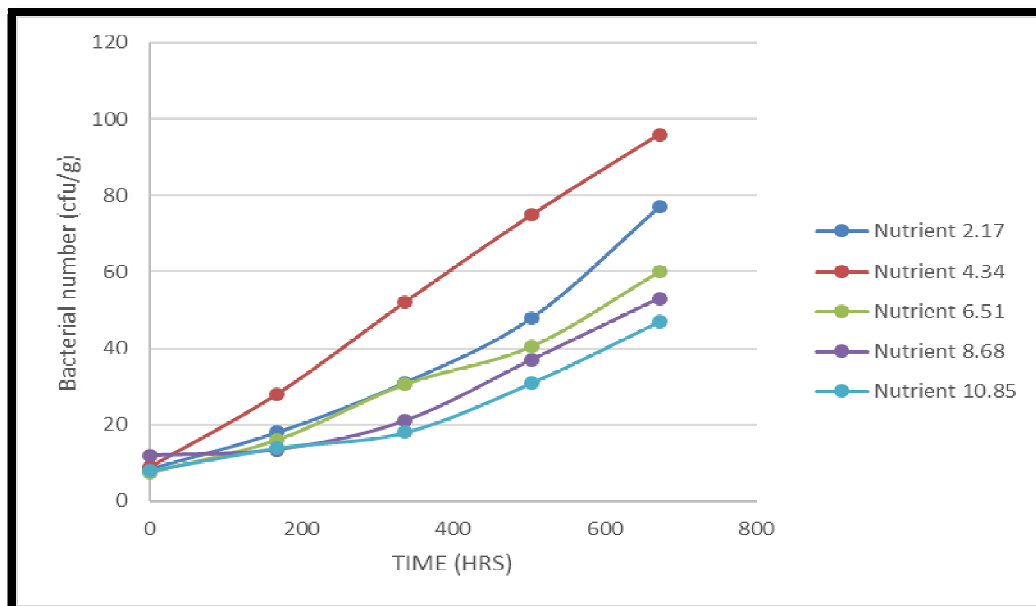

Figure 5: Effect of Nutrient on Growth of Micrococcus Luteus

The second highest growth of 77 x 10^{10} cfu/g oil was obtained at the nutrient value of 0.217g/ml oil. At the nutrient value of 0.434g/ml oil, the growth was instant, there was no delay (lag phase), unlike what happened at nutrient concentrations of 0.868g/ml oil and 1.085g/ml oil where the growth started after about 168 hours have elapsed. It showed little lag phase at nutrient concentrations of 0.217g/ml oil and 0.651g/ml oil.

The plot of bacterial number against nutrient concentration showed the variation of bacterial population with nutrient concentration and time Figure 6. The highest growth through the four weeks the work lasted was at the nutrient concentration of 0.434g/ml oil. The trend of growth at different nutrient concentrations as shown in this plot is the same. Apart from week zero that showed no growth at nutrient concentrations of 0.217g/ml and 0.434g/ml. The growth started from nutrient concentration of 0.217g/ml oil from week one to week four and showed the highest growth at 0.434g/ml oil throughout the period of the experiment as a shown in Figure 6.

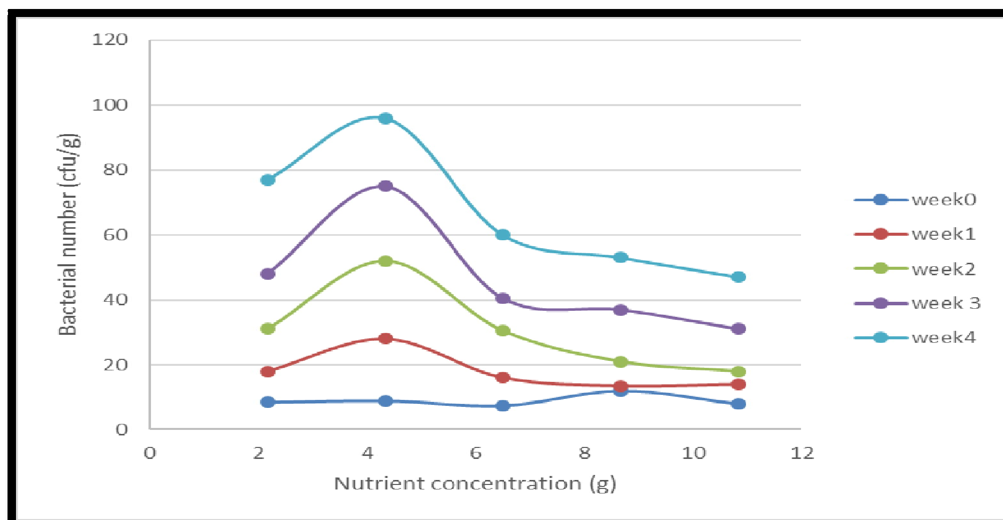

Figure 6: Variation of Bacterial Population with Nutrient Concentration and Time

3.5. Effect of Time and Temperature on Utilization of Crude Oil

The trend of degradation and utilization of the crude oil by Micrococcus luteus in this work followed the growth pattern. The highest crude oil degradation was recorded at the temperature with the highest bacterial number, resulting in the least residual oil at this temperature. The residual oil at this temperature (43.7^{o}C) was observed to be 0.2g from the initial crude oil value of 0.9g. At the temperature of 41°C with the second highest bacterial number the residual oil was 0.25g.
The crude oil decreased with time as shown in Figure 7 following the increasing number of the Micrococcus luteus since the organism was breaking down the hydrocarbons and utilizing it as food for growth and energy.

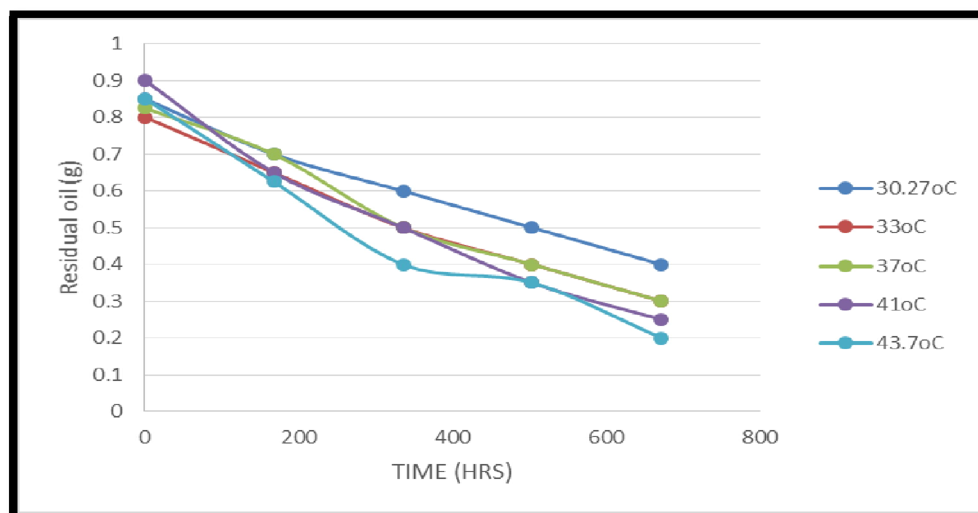

Figure 7: Effect of Temperature on Degradation of Crude Oil by M. Luleus

This observation is in agreement with the report of Mohamed and Amnah, (2012). In their report, they observed that Micrococcus luteus was more efficient in degrading hydrocarbons at temperatures of 40-43°C.They also reported that result revealed that temperature increased the rate of degradation when compared with the degradation at 37°C. The percentage degradation of the crude oil at 43.7^{o}C was 78% and 72% at the temperature of 41°C as against 64% at 37°C. This temperature at which the organism had the highest growth and the highest degradation of the crude oil is the optimum growth temperature for Micrococcus luteus and the optimum for its enzymatic activity since the least residual oil was recorded at this temperature.

3.6. Effect of Time and pH on the Utilization of Crude Oil by Micrococcus Luteus

The effect of pH and time on the breakdown and utilization of crude oil by Micrococcus luteus also followed the growth trend. This is shown in Figure 8. The high viable bacterial number was reflected in the degradation of the crude oil. The crude oil reduced from its initial value of 0.85g to 0.25g at the pH value of 8.0. This is about 71% crude oil removal in four weeks.

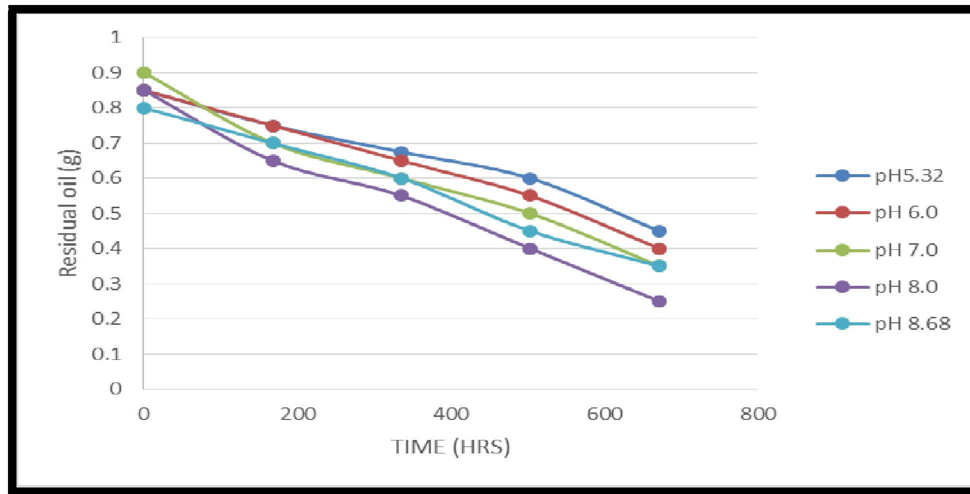

Figure 8: Effect of pH on Degradation of Crude Oil by M. Luteus

This result is in agreement with that of Akpoveta et al., (2011). They reported in their work that the rate of microbial degradation was dependent on availability of nutrient source and pH and that high, biodegradation rate occasioned by an increase in microbial population was favored between pH 6.7 to 9.6. The high microbial count observed in this work between pH of 8.0 and 8.68 is also in agreement with the report of Dibble and Bartha (1979) where they recorded oil sludge biodegradation to be optimal at pH 7.5 to 8.0. Ritter and Scarborough, (1995) reported in their work that the optimum pH for the degradation of crude oil by Micrococcus luteus to be between 6.5 and 8.5. In the work of Olabisi et al., (2009), they reported that pH of the polluted soil amended with Mellon shell was raised from 7.6 to 8.03 and argued that this could be one of the conditions that increased the rate of biodegradation of crude oil in amended soil since crude oil degrading bacteria grow and utilize hydrocarbon better at slightly alkaline pH.

3.7. Effect of Time and Nutrient on Crude Oil Utilization by Micrococcus Lutues

The effect of nutrient on the Micrococcus luteus utilization of the crude oil follows the growth pattern discussed above. The highest hydrocarbon removal was recorded at the nutrient concentration of 0.434g/ml oil. The least residual oil was observed at this nutrient value of 0.434g/ml oil followed by that at nutrient value of 0.217g/ml oil. The crude oil decreased from the initial value of 0.85g to 0.3g in the fourth week at the nutrient concentration of 0.434g/ml and from 0.9g to 0.35g at the nutrient concentration of 0.217g/ml. This represents 65% and 61% hydrocarbon removal at the two nutrient concentration values respectively. This is shown in the contour plot of residual oil against time, Figure 9. This nutrient concentration of 0.434g/ml corresponds to carbon to nitrogen ratio (C: N) of 10: 2.

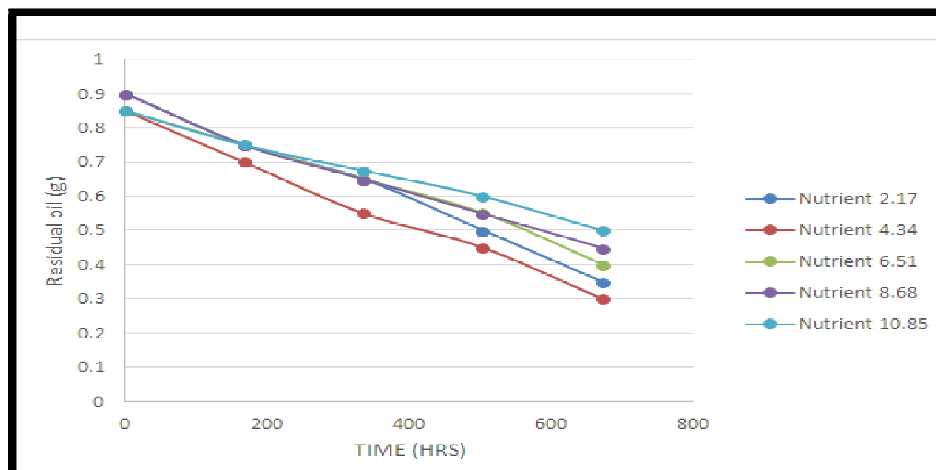

Figure 9: Effect of Nutrient on Degradation of Crude Oil by M. Luteus

4. Conclusion

The result of this work showed that Micrococcus luteus has the capacity to breakdown and utilize petroleum hydrocarbon when the environmental conditions are favorable. The result also showed that the optimum growth temperature at which the growth rate was most rapid was the optimum for the enzymatic activity. This is evident in the observed direct relationship of the rate of biodegradation and the microbial biomass. In the three conditions (temperature, pH and nutrient supplementation), studied, the trend was the same, the highest bacterial growth resulting in the highest crude oil removal. At the temperature of 43.7^{O}C, the bacterial number was 27.4 x10^{12}cfu/g soil giving crude oil removal of 78%. At the pH 8.0, the viable bacterial number was highest and the percentage crude oil removal at this pH was 71%, while at the nutrient concentration of 0.434g/ml, the highest bacterial number was 96 x 10^{10}cfu/g soil giving the highest percentage crude oil removal of 65%.

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