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# Modeling the Effects of Bile and Nutrients on Microbes and the Evolution of Temperature, Electrical Conductivity, Surface Tension and pH during Biodegradation of Used Engine Oil Contaminated Water

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**Abstract**

At five levels of Bile (No bile, 1.2 ml, 1.4 ml, 1.6 ml and 1.8 ml) and two nutrient levels (Nutrient medium and No Nutrients) the effect of Bile and Nutrients on microbes and the evolution of temperature, electrical conductivity, surface tension and pH were studied and modeled during the bioremediation of a petroleum hydrocarbon contaminated water. Analysis of variance (ANOVA) for $\alpha$=0.05 was used to show the variations in parameter values according to the levels of Bile and Nutrients. Microbial colony numbers and pH generally did not show a uniform evolution trend as the level of Bile increased but showed an increasing trend with increment in Nutrients level. Temperature increased with increasing Bile and Nutrients levels only when either Bile or Nutrients Medium was the lone supplement in the experiment. Electrical conductivity increased with increasing levels of Bile whiles surface tension decreased with increasing levels of Bile and Nutrients.

**Keywords:** Bile, Nutrients, microbes, temperature, electrical conductivity, surface tension, pH Biodegradation, Bioremediation

## 1. Introduction

Petroleum-based products are the major source of energy for industry and our daily life. Leaks and accidental spills occur regularly during the exploration, production, refining, transport, and storage of petroleum and petroleum products (Nilanjana and Preethy, 2010). One of the most widely used petroleum-based products is engine oil especially because of the proliferation of vehicle manufacturing industry. Engine oil is a complex mixture of hydrocarbons and other organic compounds, including some organometallic constituents (Butler and Mason, 1997) that is used to lubricate the parts of an automobiles' engine, in order to keep everything running smoothly (Hagwell et al., 1992).

Unused engine oil poses relatively less toxicity to organisms (more volatile and water soluble) as compared to used ones. Used motor oil contains more metals and heavy Polycyclic Aromatic Hydrocarbons (PAHs) that would contribute to chronic hazards including mutagenicity and carcinogenicity (Boonchan et al., 2000) and therefore, its disposal is of great concern to public health. Unfortunately, in Ghana used engine oil is carelessly disposed off into the environment (open areas and into drains) and this is particularly evident in automobile garages throughout the country. Except for containment in large barrels and concrete pits by some garages little has been done to curb this menace or remediate these contaminated systems by city authorities. Bioremediation is an evolving method for the removal and degradation of many environmental pollutants including the products of petroleum industry (Medina-Bellver et. al, 2005). The process of bioremediation is defined as the use of microorganisms to detoxify or remove pollutants owing to their diverse metabolic capabilities (Medina-Bellver et. al, 2005).

The rationale for this study was to provide some information as to how surface tension reducing substances influences the dynamics of temperature, electrical conductivity, surface tension and pH evolutions during biodegradation of hydrocarbon contaminated water. This is particularly important for process control in biodegradation processes involving the use of biosurfactants. Bile was used in this study because of its ability to reduce surface tension due to its micelle-forming properties and has been used by Lingxiang *et al.* (2008) to achieve vesicle-to-micelle transition (VMT) in catanionic surfactant systems by the addition of two kinds of bile salts, sodium cholate (SC) and sodium deoxycholate (SDC) (Vethamuthu *et al.*, 1992c).

 

## 2. Materials and Methods

### 2.1Used Engine Oil and Bile

Used engine oil was obtained from a mechanic workshop at Suame 'magazine' light industrial area, Kumasi and fresh cattle bile from the Kumasi abattoir.

### 2.2 Nutrient Source

Nutrients to support microbial growth was prepared based on the recommendation of Fei-Baffoe (2003). This comprised of 2.0 g of $Na_2HPO_4.2H_20$, 0.8 g of $KH_2PO_4$, 0.2 g of $MgSO_4$ and 1.8 g of $(NH_4)_2SO_4$ dissolved in 1liter of distilled water.

### 2.3 Microbial Support Material

Sterilised bamboo pieces of lengths 1-2 cm and 0.3-0.5 cm diameters were provided in the reactors as microbial support material.

### 2.4 Equipments for Measurements

Temperature, pH, and Electrical Conductivity, were measured using a multi-parameter probe meter (YSI 550A). Surface tension readings were taking with Krüss tensiometer and Microbial Colony Numbers with a Stuarts' automated scientific colony counter. Residual TPH was determined with a Varian CP-3800 GC – FID after solvent extraction in methylene chloride (GC grade).

### 2.5 Bioreactor Setup

The bioreactor setup used (fig 1) in the study comprised of eight reaction containers constructed with six inch Polyvinyl Chloride (PVC) pipes. Each reactor was designed to have a height of 50cm and volume 0.009 $m^3$. 1/3 of each reactor's volume was occupied by the microbial support material and held in place by rubber mesh.

The eight bioreactors were arranged as $R_{1A}$, $R_{2A}$, $R_{3A}$, and $R_{4A}$ and $R_{1B}$, $R_{2B}$, $R_{3B}$ and $R_{4B}$. In each set, four reactors were connected in series to one another. The two sets of serially connected reactors were arranged parallel to each other with bioreactor set B serving as a replicate of bioreactor set A as shown in Fig. 1.

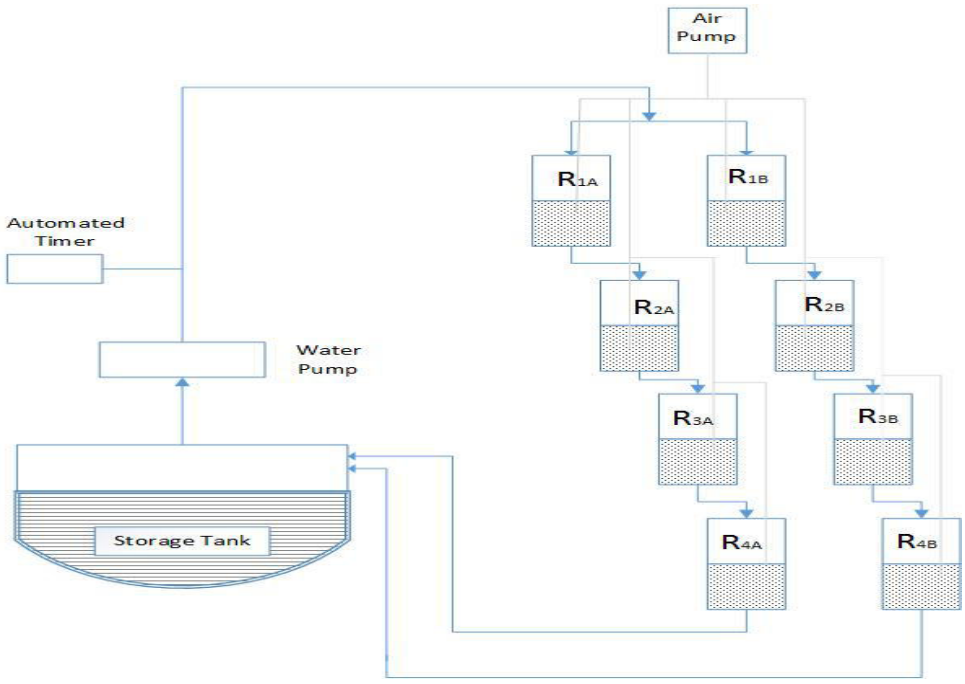


**Figure 1 Bioreactor setup**

### 2.6 Operation of Bioreactor

A 0.5 hp (horse power) water pump was used in driving samples (water (15L) + oil + bile/nutrients) through the bioreactors and eventually back to the storage tank (Fig. 1.0). The flow regimes through the bioreactors were

regulated using an automated timer. A total of eight (8) regimes per day were employed for each experimental run with each pumping period lasting for 30 minutes at a flow rate of 1 L/min.

### 2.7 Isolation of Heterogeneous Hydrocarbon Degrading Microorganisms

Soil samples collected from oil contaminated sites (Suame Magazine) were homogenized and 1g of it introduced to 500 ml of distilled water and shaken for 5 minutes. Serial dilutions from the third enrichment process were plated onto Nutrient Agar (NA), which was covered with 100 µL of used engine oil and incubated at 37 °C for 24 hours. Single colonies developed were again streaked on NA and incubated at 37 °C overnight.

Two loops full of grown bacteria from inoculated plates were introduced to 1 L of distilled water containing 1ml of used engine oil and 50 ml of nutrient medium and then incubated for 72 hours. 1000 ml of the resulting liquid inoculum was dispensed aseptically into each reactor as microbial biomass.

### 2.8 Characterization of Isolates

The oil degrading isolates were characterized by gram stain and biochemical tests.

- **Gram Staining**

**Staining Protocol**

A small drop of sterile distilled water was placed on a sterilized grease free slide. A small quantity of bacteria transferred from NA and placed on the slide with the aid of a sterilized loop. The preparation was allowed to dry and then fixed by passing 3 times through a Bunsen flame.

A 0.5% crystal violet stain was placed on the bacteria smear for 2 minutes. Then washed off with water and stained with dilute iodine for another 2 minutes. Absolute alcohol was carefully dripped on smear and allowed to run off. This process was repeated two more times and then washed off with water. A 1% safranin was used to counter-stain the smear for 2 minutes, and then smear washed, drained and blot dried (Standards Unit, Evaluations and Standards Laboratory, UK (2007)). This procedure was carried out on 2 more slides and the prepared smears presented for microscopy.

- **Microscopy**

Stained smears were observed initially using low power objective lenses of X10 and X20. Afterwards immersion oil was carefully dripped on the smears and viewed under a high power lens (X100) to characterize isolates.

- **Biochemical Test**

**Oxidase Test (Filter paper method)**

Three pieces of filter papers were soaked in reagent solution (0.5ml of 1% N, N, N', N'-tetra-methyl-p-phenylenediamine dihydrochloride) after which a loop full each of freshly grown bacteria rubbed on them and the resultant reaction observed after 10 seconds for colour change (Standards Unit, Evaluations and Standards Laboratory, UK ,2007).

- **Catalase Test**

Three millilitres (3 ml) of 3% $H_2O_2$ was poured in a test tube. Using a sterile glass rod, several colonies of isolates removed and immersed in the hydrogen peroxide solution contained in a test tube. The test tube was immediately investigated for a bubbling reaction (Cheesbrough, 2006).

### 2.9 Acclimatization of Hydrocarbon-Degrading Microbes in the Bioreactors

This was done by running contaminated water containing 6000 mg of used motor oil for a week to expose the microbes to the reactor conditions.

## 3. Biodegradation Experiments

Three sets of experiments were carried out as follows;

1. Effect of nutrients only on microbes and the evolution of temperature, electrical conductivity, surface tension and pH during biodegradation
2. Effects of bile only on microbes and the evolution of temperature, electrical conductivity, surface tension and pH during biodegradation
3. Effect of combinations of nutrient and bile on microbes and the evolution of temperature, electrical conductivity, surface tension and pH during biodegradation

### 3.1 Effects of Nutrients on Microbes (MCN) and the Evolution of Temperature (TP), Electrical Conductivity (EC), Surface Tension (ST) and pH during Biodegradation

This was determined by introducing 50 ml of nutrient medium into contaminated water (6000 mg/L of used oil in water). With a constant flow rate of 1 L/min and cycling period of 30 minutes for every 3 hours, biodegradation

 

of contaminated water was carried out for a week and parameters of interest (MCN, TP, EC, ST and pH) duly measured.

### 3.2 Effects of Bile on MCN and the Evolution of TP, EC, ST and pH during Biodegradation

1.2, 1.4, 1.6 and 1.8 ml of bile were added to separately prepared contaminated water (6000 mg/L of used oil in water). Each was run for a period of 7 days at a constant flow rate of 1 L/min and cycling period of 30 minutes for every 3 hours. Parameters of interest (MCN, TP, EC, ST and pH) were duly monitored and measured.

### 3.3 Effect of Combination of Bile and Nutrients on MCN and the Evolution of TP, EC, ST and pH during Biodegradation

This was determined by combining 50 ml of nutrient medium and bile additions of 1.2, 1.4, 1.6 and 1.8 ml separately to four different preparations of contaminated water. Each combination of nutrient and bile was run for a week also at a constant flow rate of 1 L/min and cycling period of 30 minutes for every 3 hours. Parameters of interest (MCN, TP, EC, ST and pH) were measured as with the other two experiments.

### 3.4 Enumeration of Hydrocarbon Degrading Bacteria

Water samples were collected in tightly corked sterile bottles and immediately transported to the laboratory. Samples were serially diluted in sterile distilled water and the $10^{-7}$ to$10^{-10}$ dilution plated on PCA. Inoculated agar plates were labeled and incubated at 37 $^{o}$C for 24 hours. Microbial colony numbers on plates were obtained with the aid of Stuarts automated scientific colony counter.

## 4. Results and Discussions

### 4.1 Characterized Isolates

*Pseudomonas* sp. and *Bacillus* sp. are able to degrade hydrocarbons in order to produce energy and biomass (Van Hamme *et al*., 2003) and they do so because of their effective enzyme (oxygenases, dioxygenase, dehydrogenases) systems (www.wiley-vch.de/books/biotech/pdf). Therefore the identification of these bacteria (Table 1) in the study implied that, they were able to utilize hydrocarbon contaminants as carbon and energy sources for growth. The isolation of two different species of bacteria from contaminated water also implied that, these bacteria were possibly co-metabolizing hydrocarbon contaminants. In co-metabolism it is possible that one species can remove the toxic metabolites of the other species, or degrade some compounds better than others (Alexander, 1999). Thus it was possible that, one of the species was mineralizing the by-products of the other or was effectively degrading the hydrocarbons more than the other.

Table 1 Characterized Bacteria Isolates

| Bacteria Group | Gram Stain | Shape | Oxidase Test | Catalase Test |
|---|---|---|---|---|
| ***Bacillus* Sp.** | + | rod | - | + |
| ***Pseudomonas* Sp** | - | rod | + | - |

### 4.2 Effects of Nutrients only on Measured Parameters (MCN, TP, EC, ST and pH)

Mean values and standard errors (standard errors in parenthesis) for MCN and TP, EC, ST, pH at various nutrient levels are given in Table 2. Across the two levels of nutrients MCN ranged from 66.03CFU/ml (No nutrients) to 75.42 CFU/ml (Nutrients Medium) and TP from 27.946 $^{o}$C (No nutrient) to 28.117 $^{o}$C (Nutrients Medium). This indicates that nutrients medium resulted in increasing MCN and TP. Also, whiles mean values for EC and ST decreased with increasing nutrient levels, mean values for pH increased with increasing nutrient levels.

T-test conducted revealed that Mean values for MCN, TP, EC, ST and pH differ significantly across the two nutrient levels ($p<0.05$).

**Table 1: Means (Standard Errors) for MCN, TP, EC, ST and pH at various Nutrient levels**

| Nutrients | MCN x$10^{10}$ CFU/ml | TP ($^{o}$C) | EC (µS/cm) | ST (mN/m) | pH |
|---|---|---|---|---|---|
| **No Nutrient** | 66.03a | 27.946a | 3458.835a | 59.747a | 6.758a |
| | (5.609) | (0.089) | (52.909) | (0.618) | (0.061) |
| **Nutrients Medium** | 75.42b | 28.117b | 2729.427b | 52.521b | 7.038b |
| | (3.163) | (0.089) | (52.909) | (0.618) | (0.061) |

*Means in the same column that do not share a common alphabet are significantly different at 0.05 level of*

 

*significance*

### 4.3 Effects of Bile only on Measured Parameters (MCN, TP, EC, ST and pH)

Mean values and standard errors (standard errors in parenthesis) for MCN, TP, EC, ST, and pH at various levels of Bile are given in Table 3. Across the different levels of Bile, mean values for pH and MCN did not show any uniform changes with increasing levels of Bile. Thus increasing Bile levels did not reflect increments in MC and pH. Mean TP ranged from 27.515 $^{o}$C (No Bile) to 28.389 $^{o}$C (Bile at 1.6ml). This indicated that increasing Bile levels resulted in increasing TP. Also, whiles mean values for EC increased with increasing Bile levels (1342.217 µS/cm for No Bile to 4985.617 µS/cm for Bile at 1.6ml), mean values for ST decreased with increasing Bile levels (64.188 mN/m for No Bile to 47.217 mN/m for Bile at 1.8ml) (Table 3).

Analysis of variance conducted revealed that Mean values for MCN were significantly different ($p<0.05$) of each other except for Bile at 1.2 ml and 1.8 ml. In the case of EC and ST mean values differed significantly across the various levels of Bile. For TP, mean value without Bile was significantly different from mean values with Bile. Also for pH, mean value for Bile at 1.4ml was significantly different from the other mean values.

**Table 3: Means (Standard Errors) for MCN, TP, EC, ST and pH at various levels of Bile**

| Levels of Bile | MCN x$10^{10}$ CFU/ml | TP ($^{o}$C) | EC (µS/cm) | ST (mN/m) | pH |
|---|---|---|---|---|---|
| **No Bile** | 41.08a | 27.515a | 1342.217a | 64.188a | 7.030a |
| | (8.853) | (0.141) | (83.656) | (0.976) | (0.096) |
| **Bile at 1.2ml** | 79.42b | 28.017b | 3416.373b | 53.993b | 7.090a |
| | (4.398) | (0.141) | (83.656) | (0.976) | (0.096) |
| **Bile at 1.4ml** | 68.29c | 28.070b | 2443.076c | 49.948c | 6.449b |
| | (6.484) | (0.141) | (83.656) | (0.976) | (0.096) |
| **Bile at 1.6ml** | 85.04d | 28.389b | 4985.617d | 48.325d | 7.098a |
| | (7.283) | (0.141) | (83.656) | (0.976) | (0.096) |
| **Bile at 1.8ml** | 79.79b | 28.167b | 3283.372e | 47.217e | 6.823a |
| | (4.872) | (0.141) | (83.656) | (0.976) | (0.096) |

*Means in the same column that do not share a common alphabet are significantly different at 0.05 level of significance*

### 4.4 Effects of Combination of Bile and Nutrients on Measured Parameters (MCN, TP, EC, ST and pH)

Mean values and standard errors (standard errors in parenthesis) for MCN, TP, EC, ST and pH at various levels of Bile and Nutrient combinations are given in Table 4. In the absence of Nutrients, mean values for MCN did not show any uniform changes with increasing levels of Bile. Implying increasing levels of Bile did not reflect an increment in MCN. TP and EC generally increased from 28.010 to 28.355 $^{o}$C and 3846.493 to 4532.000 µS/cm respectively as level of bile increased whiles surface tension and pH generally decreased from 57.275 to 53.360 mN/m and 6.797 to 6.353 respectively at increasing levels of Bile.

In the presence of Nutrients, mean values for MCN again did not show any uniform pattern as level of bile increased. Mean values for TP were generally not significantly different from each other for the various levels of bile investigated. Whiles EC generally increased with increasing levels of bile (1833.000 to 5439.234 µS/cm) ST on the other hand, generally decreased as levels of bile increased (64.175 to 41.075 mN/m). pH too did not show any uniformity in pattern with increasing levels of Bile. Thus increasing levels of Bile did not reflect increments in pH

Analysis of variance conducted revealed that, mean values for MCN were significantly different across the various levels of Bile except for Bile at 1.2 and 1.8 ml. The mean values for EC differed significantly across the various levels of Bile ($p<0.05$). With regards to TP, mean value for bile at 1.4 ml was significantly different from the other means whiles for ST the mean values of Bile at 1.2 ml, 1.4 ml and 1.8 ml were significantly different of each other and across the various levels of bile. At the various levels of bile, mean pH values were significantly different across the levels of Bile but not of each other. pH values at Bile level 1.2 and 1.6 ml were not significantly different of each other. Also pH values at Bile level 1.4 and 1.8 ml were not significantly different of each other too.

 

**Table 4: Means (Standard Errors) for MCN, TP, EC, ST and pH at various levels of Bile and Nutrients**

| Nutrients | Bile Content | MCN x$10^{10}$ (CFU/ml) | TP ($^{o}$C) | EC (µS/cm) | ST (mN/m) | pH |
|---|---|---|---|---|---|---|
| **No Nutrient** | No Bile | 1.50a | 27.002a | 851.434a | 64.850a | 7.020a |
| | | (1.567) | (0.199) | (118.308) | (1.381) | (0.136) |
| | Bile at 1.2ml | 80.92b | 28.010b | 4035.750b | 57.275b | 6.797b |
| | | (1.567) | (0.199) | (118.308) | (1.381) | (0.136) |
| | Bile at 1.4ml | 80.42b | 28.185b | 3846.493c | 56.775c | 6.353b |
| | | (1.567) | (0.199) | (118.308) | (1.381) | (0.136) |
| | Bile at 1.6ml | 84.42c | 28.355b | 4532.000d | 66.475d | 6.875b |
| | | (1.567) | (0.199) | (118.308) | (1.381) | (0.136) |
| | Bile at 1.8ml | 82.92c | 28.180b | 4028.500b | 53.360e | 6.743b |
| | | (1.567) | (0.199) | (118.308) | (1.381) | (0.136) |
| **Medium Nutrients** | No Bile | 80.67b | 28.027b | 1833.000e | 63.527a | 7.041a |
| | | (1.567) | (0.199) | (118.308) | (1.381) | (0.136) |
| | Bile at 1.2ml | 77.92d | 28.024b | 2796.997f | 50.710f | 7.383c |
| | | (1.567) | (0.199) | (118.308) | (1.381) | (0.136) |
| | Bile at 1.4ml | 56.17e | 27.955a | 1039.660g | 43.120g | 6.545b |
| | | (1.567) | (0.199) | (118.308) | (1.381) | (0.136) |
| | Bile at 1.6ml | 85.67c | 28.422b | 5439.234h | 64.175a | 7.320c |
| | | (1.567) | (0.199) | (118.308) | (1.381) | (0.136) |
| | Bile at 1.8ml | 76.67d | 28.153b | 2538.245i | 41.075h | 6.903b |
| | | (1.567) | (0.199) | (118.308) | (1.381) | (0.136) |

*Means in the same column that do not share a common alphabet are significantly different at 0.05 level of significance*

### 5. Total Petroleum Hydrocarbon Degraded (TPH) Degraded across the Various Levels of Bile

Although the interest of this work was on the effects of bile on MCN, TP, EC, ST and pH evolutions, there was the need to determine residual TPH at the end of each biodegradation period since this work was based on bioremediation technique. Residual TPH was determined with a Varian CP-3800 GC – FID after solvent extraction in methylene chloride (GC grade). Percentage TPH degraded for all experiments in the study are presented in table 5 below.

**Table 5 Percentage TPH degraded in Experiments**

| Experiment | Nutrient Medium (NM) | Bile at 1.2 ml | Bile at 1.4 ml | Bile at 1.6 ml | Bile at 1.8 ml | Bile at 1.2 ml + NM | Bile at 1.4 ml + NM | Bile at 1.6 ml + NM | Bile at 1.8 ml + NM |
|---|---|---|---|---|---|---|---|---|---|
| **MCN x$10^{10}$ (CFU/ml)** | 80.67 | 80.92 | 80.42 | 84.42 | 82.92 | 77.92 | 56.17 | 85.67 | 76.67 |
| **Percentage (%)TPH degraded** | 95.03 | 95.38 | 94.52 | 97.33 | 96.21 | 87.63 | 51.11 | 97.34 | 86.36 |

Percentage TPH degraded did not correspond to volume of bile supplied as show in table 5.Thus percentage TPH degraded was possibly influenced by some other factors (Possibly experimental conditions (oxygen, carbon

 

dioxide, by-products of degradation)) aside the effects of Bile and nutrients. However, it was observed that percentage TPH degraded depended on microbial colony numbers. The larger the microbial colony numbers, the higher the percentage TPH degraded

## 6. Conclusion

It was established that microbial colony numbers and pH showed no uniform pattern with increasing levels of Bile. It was also observed that, increasing the volume of bile generally resulted in an increase in electrical conductivity but a reduction in surface tension. This observation was possibly due to the ability of bile to reduce surface tension. Reduction in surface tension reduces viscosity thus increases the dissolution of ions in solution. Temperature increased with increasing levels of bile for experiments involving Bile only but remained roughly unchanged when modeled against No Nutrient level and Nutrient Medium experimental results, thus there was a weak relationship between increasing bile level and temperature evolution.

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