# ISOLATION, CHARACTERISATION AND BIODEGRADATION ABILITY OF BACTERIA ISOLATED FROM SOIL CONTAMINATED WITH HYDROCARBONS

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## ABSTRACT

Contamination of land and water bodies by crude oil and refined petroleum products is a major challenge worldwide. Indiscriminate disposal of crankcase oil into the environment has increased hydrocarbon pollution in Nigeria. Microorganisms have been identified as major contributors in fighting pollution. The remediation ability of bacteria isolated from hydrocarbon contaminated organic rich soil has not been fully investigated. This research was designed to study in-situ genera and hydrocarbon degrading ability of bacteria isolated from an organic rich tropical soil deliberately contaminated with a Nigerian crude oil and crankcase used oil

Bacterial enrichment for hydrocarbon degradation was carried out by deliberately contaminating garden soil samples collected from the Nursery of the Department of Microbiology, University of Ibadan. Top soil were collected and mixed with Forcados Blend crude and used crankcase oil at a mixed ratio of 5:1. Hydrocarbon degrading bacteria counts were obtained at two week intervals for ten weeks by sub-culturing on mineral salts oil agar supplemented with the hydrocarbons. Isolation was done by randomly selecting colonies of bacteria based on morphological and growth characteristics. Isolated bacteria were screened on sterile Hydrocarbon agar plates and were identified by classical methods. The DNA extraction and amplification of ten selected strains were carried out using molecular technique. Amplified DNA was digested by HaeIII and Rsal restriction enzyme and subjected to Restriction Fragment Length Polymorphism analysis and sequencing of the 16SrRNA.The BLAST search for the obtained sequences were made and phylogenetic tree of

amplicons constructed using MEGA4.1. Plasmid presence, sizes and numbers in the isolates were determined. Hydrocarbon degradation rate by the bacteria isolates was determined by gravimetry and Gas Chromatography analysis using Flame Ionisation Detector.

Hydrocarbon-utilising bacteria increased from $35 \times 10^4$ to $265 \times 10^4$ cfu/mL, while total bacteria count decreased from $245 \times 10^4$ to $123 \times 10^4$ cfu/mL between the second to tenth week. Ten out of forty-two hydrocarbon-utilizing bacteria detected showed high crude and crankcase oil degrading ability. Phylogenetic analyses of the isolates showed high sequence identities (75-100%) in amplified genes when compared to those in the GenBank. The isolates belonged to four genera; *Bacillus* (5), *Providencia* (3), *Proteus* (1) and *Alcaligenes* (*1*). Utilization of complex hydrocarbons present in crude and crankcase oil by these isolates ranged between 51.9-77.0% and 42.4-75.8% respectively. Four out of the ten bacterial isolates contained plasmids of varying sizes. *Bacillus* OUE3 and *Providencia* OCR1 contained two plasmids each of sizes 2.57 kb and 2.0 kb, and 1.3 kb and 1.9 kb respectively, while *Bacillus* OUE6 and *Providencia* OCR2 contained a plasmid each. The percentage total degradation for polycyclic aromatic hydrocarbon ranged from 29.64 to 98.45% for crude and crankcase oil. About 25.2 to 91.7% and 98.2 to 99.6% of aliphatic groups were utilised by the isolates in crude and crankcase oil respectively within 20 days.

Ten of the isolated bacteria could remediate hydrocarbon pollution from soil environment. *Providencia* sp. had the highest degradative ability.

**Keywords**: Oil degradation, Soil contamination, Oil pollution, *Providencia* sp.

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Abiodun Ayanfemi, Ayandele.

## CERTIFICATION

I certify that this work was carried out by Miss. A. A. Ayandele in the Department of Microbiology, University of Ibadan, Ibadan, Nigeria.

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## DEDICATION

I dedicate this thesis to:

Mrs. Ayandele, A. I.

Mother, I appreciate you for all your help, words of encouragement, care and prayers. You shall eat the fruit of your labour in Jesus name (amen).

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A sister that is just like a mother, I really thank you for all you have been doing for me since my secondary school days and till date.

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## Abbreviations

AD - Alcohol Dehydrogenase

ACS - Acyl-CoA Synthetase; ω-H, ω -hydroxylase

AH - Alkane Hydroxylase

AHc - Aliphatics

ALD - Aldehyde Dehydrogenase

BLAST - Basic Local Alignment Search Tool

BTEX - Benzene, Toluene, Ethybenzene, Xylene

BVM - Baeyer-Vlliger Monooxygenase

CoA - Co enzyme A

DNA - Deoxyribonucleic Acid

E - Esterase

EDTA - Ethlenediaminetetraacetic Acid

FID - Flame Ionization Detector

FLD - Formaldehyde Dehydrogenase

FMD - Formate Dehydrogenase

GC-MS - Gas Chromatography-Mass Spectrophotometer

MD - Methanol dehydrogenase

MMO - Methane monooxygenase

MSM - Minimum Salt Medium

PAH - Polycyclic Aromatic Hydrocarbon

PCR - Polymerase Chain Reaction

RFLP - Restriction Fragment Length Polymorphism

rRNA - ribosomal Ribonucleic Acid

STE buffer - Saline Tris EDTA buffer

TCA - Tricarboxylic Acid Cycle

TE buffer - Tris EDTA buffer

TOL - Aryl Toluyl functional group

Tris-HCl - Hydroxymethy amino methane

# TABLE OF CONTENTS

.

## List of Figures

## List of Tables

## List of Plates

# CHAPTER ONE

# INTRODUCTION

## 1.1 Oil Pollution and Biodegradation of Polluted Soil by Microorganisms

The quality of life on earth is indirectly linked to the over all quality of the environment. In early time, it was believed that we had an unlimited abundance of land and resources. But in the last years, a large number of ecosystems have been changed by the growing influence of human activity, most especially contamination of the ecosystem due to explorative activity of crude oil (Ekpo and Udofia, 2008). The world today is very much dependent on oil either to fuel the vast majority of its mechanized transportation equipment or as the primary feedstock for many of the petrol-chemical industries. Crude oil production volume has increased to over 82.3million barrels per day in 2003, and this volume is estimated to increase to 94.3 barrels per day in 2010 and up to 101.6 barrels per day by the year 2015 (US DOE / EIA, 2006).

Researches have shown that over five million tons of crude oil and refined oil enter the environment each year as a result of anthropogenic sources and since most of it travels by water, at some instances, certain amount of oil will inevitably spills from tankers and pipeline (Rob *et al*., 2009). Accidental and deliberate crude oil spills have been, and will still continue to be, a significant source of environmental pollution and this poses a serious environmental problem to living organisms as a result of air, water and soil contamination (Trindade *et al*., 2005 ).

Over 17,000 organic compounds have been identified in crude oil, and subdivided into four main classes; saturates, aromatics, asphaltenes and resins (Marshall and Rodger, 2004). Aliphatic and Aromatic Hydrocarbons which are part of the constituents of crude oil and its refined products are widespread in the environment since they form part of the natural components of the soil. They are formed naturally in geochemical processes from buried biomass as well as in microbial and plant metabolism (Tissot and Welte, 1984; Jüttner and Henatsch, 1986). Petroleum-hydrocarbon composition varies greatly in its

complex mixture of hydrocarbon and other organic and inorganic compounds, which contribute to its diversity and physical properties (Van Hamme *et al*., 2003). The Polycyclic Aromatic Hydrocarbon (PAH) which consists of aromatic and asphaltenes are very recalcitrant under normal conditions because of their strong molecular bounds. Microbial degradation studied has shown that susceptibility of hydrocarbons ranged from linear alkanes> branched alkanes > small aromatics > cyclic alkanes (Leahy and Colwell, 1990). Major products of petroleum hydrocarbons include, liquefied petroleum gas, gasoline or petrol, naphtha, kerosene, diesel oil, heavy fuel oil, lubricating oils that include engine oil, paraffin wax, asphalt and tar and petroleum coke (Leffler, 1985).

Used engine oil or used crankcase oil is defined as refined oil from crude oil or any synthetic oil that has been used and as a result is contaminated by chemical impurities which contribute to its chronic hazards which include mutagenicity and carcinogenicity as well as environmental hazard with global ramifications (Blodgette, 2001). Used crankcase oil is a mixture of different chemicals including low and high molecular weight (C15-C20), aliphatic hydrocarbon, polychlorinated biphenyls, chlorodibenzofurans, lubricate additives and decomposition of products (Onwuka and Igwe, 2010). Used crankcase oil has higher concentrations of polycyclic aromatic hydrocarbons than new motor oil (Jahir and Syed, 2011).

Used crankcase oil is released into the environments by the automobile mechanic, generators, and discharge from exhaust system during use and engine leaks (Anoliefo and Edegbai, 2000; Osubor and Anoliefo, 2003).and most of the time, used crankcase oil is discharged into open farms, vacant plots, water drains gutter and stream. One of the significant impact associated with seepage of used crankcase oil include loss of soil fertility, water holding capacity, permeability and binding capacity (Udeani *et al*., 2009).

Soil is the key component of natural ecosystem because environmental stabilities depends largely on sustainable soil ecosystem (Adedokun and Ataga, 2007; Adenipekun, 2008) and since soil is the habitat of many living organisms, any change in the number or forms of living organisms may upset or cause a total collapse of the ecosystem (Akoachere *et al*., 2008). So, when the soil is polluted the ecosystem is altered and agricultural activities are affected. It has been reported that oil is the major pollutant of

the soil and when the hydrocarbon or oil concentration exceed 3% in the soil, it becomes much deleterious to soil biota and crop growth (Achuba and Peretiemo-Clark, 2008).

Nigeria is a major producer of crude oil in the world, and therefore environmental impact of petroleum exploration is increasing daily. Pollution of environment due to accidental oil spillage, seepage, and ruptured pipeline is a common occurrence in Nigeria and has become a major concern to government, individuals, environment activists and the communities in the immediate environment. This problem is further compounded by sabotage and pipeline vandalization in many communities and in the Niger Delta area alone, there have been many reported cases of crude oil spillage into the environment since 1970 and researches have shown that about 2.8 million barrels of crude oil has been released into their environment (Nwaogu *et al*., 2008). Oil spills are destructive to the ecosystems and it affects both the vegetation and animals present in them, it is toxic and also reduces the oxygen tension when present in the soil, thereby increases anaerobiosis which is harmful to plant root (Bossert and Bartha, 1989).

Improper management and disposal of wastes arising from crude oil and its refined products may cause environmental pollution particularly to the soil and groundwater systems, due to their low volatile and aqueous solubility. These pollutants are not only carcinogenic and mutagenic, but they are also potent immunotoxicants (Mishra *et al*., 2001; Bach *et al*., 2005).

Many methods, including physical, chemical and biological means have been used in the treatment of contaminated sites but among the several clean up techniques available, biological method known as bioremediation is gaining ground (Alexander, 1994; Miller *et al.*, 1988; Mulligan and Yong, 2004). Microbial biodegradation is an effective and inexpensive approach to degrade and remove hydrocarbon compounds from contaminated soil, provided the correct population of microorganisms is employed and the wastes are conducive to biodegradation by these organisms (Phillips *et al*., 2000).

Bioremediation is a modern method in which the natural ability of microorganism is employed for the reduction of the concentration and /or toxicity of various chemical substances, such as petroleum derivatives, aliphatic and aromatic hydrocarbons, industrial solvent and metals (Jelena *et al*., 2009). Bioremediation is an attractive alternative method because it transforms environmental contaminants into less harmful products and

removes contaminants from the polluted environment as well as preserving and /or restoring soil back to its original state (Mrozik *et al*., 2003). This technology accelerate the naturally occurring biodegradation under optimized conditions such as oxygen supply, temperature, pH, the presence or addition of suitable microbial population (bioaugmentation) and nutrients (biostimulation), water content and mixing (Trindade *et al*., 2005; Andreoni and Gianfreda, 2007). The type of soil in which the process occurs also influenced the degree and rate of biodegradation (Jelena *et al*., 2009). All these factors discussed above must be fulfilled before a successful bioremediation process can be implemented. Bioremediation program according to Sarkkila *et al.,* (2004) is divided into four steps which include investigation of the site and the extent of contamination, design and development of a treatment method and implementation of bioremediation measures and monitoring of the effectiveness of the bioremediation. Finally, for an effective bioremediation process, there is need to have a good understanding of the presence and activities of these hydrocarbon degrading microorganisms at every stage.

Some microorganisms can decompose or transform the chemical substances present in petroleum and petroleum derivates. Hydrocarbon from crude oil represent substrates for microorganism, hence the numbers of hydrocarbon degrading microorganisms in the ecosystem continue to increase. Different species of *Pseudomonas* strains capable of degrading polycyclic aromatic hydrocarbons have been isolated from soil (Kiyohara *et al*., 1992; Johnson *et al*., 1996). Other petroleum hydrocarbon degraders found in the soil include *Alcaligenes* sp, *Acinetobater* sp, *Stenotrophomonas* sp, *Flavobacterium* sp, *Moraxella* sp *Bacillus* sp (Antai, 1990; Bhattacharya *et al*., 2002). Other microorganisms such as fungi, yeast and micro algae (Riser- Roberts, 1992; Bundy *et al*., 2004) are also capable of degrading hydrocarbon and it derivates, although they take longer period of time to grow compared to bacteria (Prenafeta- Boldu *et al*., 2001).

Microorganisms have shown high degree of success on their abilities to completely mineralize crude petroleum and petroleum products most especially bacteria under laboratory conditions (Obayori *et al*., 2008). The use of microbes therefore in pollution abatement either through natural selection or recombinant DNA technology is receiving interest as this is cheap and most effective (Deni and Pennick, 1999; Daane *et al*., 2001; Lalithakumari, 2001). It is very uncommon to find organism that could degrade

effectively both aliphatics and aromatics possibly due to differences in metabolic routes and pathway for the degradation of two classes of hydrocarbons (Salam *et al*., 2011) and the ability of an organism to degrade a specific substrate is clear evidence that its genome harbours the relevant degrading gene (Cowan and Strafford, 2007). But some researchers have suggested the possibility of bacterial species with abilities to degrade both aliphatic and aromatic hydrocarbon simultaneously (Amund *et al*., 1987; Obayori *et al*., 2009).

However, lack of appropriate data on the polluted site such as microbial flora, environmental conditions and other factors like nitrogen sources e.t.c necessary for optimization of the degradation of hydrocarbon polluted sites is also affecting the process of bioremediation of polluted sites in Nigeria.

This research work was therefore designed to investigate the degradation abilities of bacteria strains isolated from soil samples deliberately contaminated with crude and used crankcase oil, and also to determine the ability of isolated bacteria in degrading both aliphatic and polycyclic aromatic hydrocarbon compounds present in the crude and used crankcase oil. Pollution of the environment by crude oil and used crankcase oil is a common phenomenon in Nigeria and much data is not available on the bacterial strains that can be used for field experiment on bioremediation of the polluted sites.

Thus, the aim and objective of this research work are;

- To isolate hydrocarbon-degrading bacteria in soil samples deliberately contaminated with crude and used crankcase oil respectively
- Identification of the isolated bacteria using classical methods and molecular techniques of 16SrRNA analysis
- Determination of the biodegradation abilities of the bacterial isolates in laboratory Experiments
- Determination of the effect of two nitrogen salts on the biodegradation abilities of these bacterial strains.
- Determination of the amount and types of aliphatic and aromatic compounds degraded from the crude and used crankcase oil by using GC analysis.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 **Cases of Oil Spills and Methods of Remediating Polluted Sites**

The adverse effect of oil exploration on the environment has been of a public concern several years ago and many people have become aware of the need to protect ecosystems as well as to evaluate the damage caused by the contamination. Environmental pollution with petroleum and petroleum products (complex mixture of hydrocarbons) has been recognized as one of the most serious current problems especially when associated with accidental spill on large scale (Udeani *et al*., 2009). The toxic effect of crude oil and refined petroleum oils on plant, animals, humans and the environment are devastating (Elliot, 1997), because the hydrocarbon may also reach the water table before it become immobilized in the soil.

Past analysis of reported oil spills in the marine environments indicated that most of the oil comes from tankers, barges and other vessels as from land pipeline spills. Extensive changes in marine, as well as terrestrial ecosystems resulting from the grounding of the Exxon Valdez (1989), the Nahodka oil spill, the Erica spill (1999) and the prestige spill (2002) have recently increases the attention of Environmentalists, Chemists, Biologist, Biotechnologists and Engineers (Braddock *et al*., 1995; Tazaki *et al*., 2004) to this problem. Another reported case of oil spill occurred in Gulf of Mexico on April 20, 2010 (Boboye *et al*., 2010). Oil pollution persistence and its transport in water, subsoil and ground water aquifers should be monitored in order to predict its impacts, assess the impacts and find a solution to such impacts (Isola-Kayode *et al*., 2008). Environmental monitoring of petroleum hydrocarbons pollution include the use of radioactive labelled compounds to general methods that include quantifying of gross contamination and evaluating the extent of changes caused in the environment by the

presence of that pollutant. The conventional techniques used for remediation of the polluted sites are the digging up of a contaminated soil and remove it to a landfill, or capping of the contaminated areas or a site. But this method has some drawback because the first method simply moves the contamination elsewhere and may create significant risks in the excavation, handling, and transport of hazardous material (Vidali, 2001). Most of the physico-chemical methods that are used for remediation use chemical agents and the emulsion formed with the oil during remediation process causes toxicity to aquatic organisms. They may also produce another source of pollution and thereby increase the oil recovery cost. Additionally, abiotic losses due to evaporation of low molecular hydrocarbons, dispersion and photooxidation play a major role in decontamination of the oil spill environments (Mills *et al*., 2003)

Microbial biodegradation known, as bioremediation is an effective and inexpensive approach to degrade and remove hydrocarbon compounds from contaminated soils, as long as the correct population of microorganisms is employed and the wastes are conducive to the biodegrading of the contaminants (Phillips *et al*., 2000). The recent developments and application of state of the art molecular techniques has made the process of hydrocarbon catabolism to advance substantially. This technique is considered an effective technology for the treatment of oil pollution because it offers the possibility of destroying or renders harmless various contaminants using natural biological activity (Vidali, 2001).

## 2.2 **Crude Oil Composition**

Petroleum products are used as fuels, solvents and feedstock in the textile, pharmaceutical and plastics industries. Petroleum is a complex mixture of natural gas, condensate and crude oil (Okoh, 2006). It is also a heterogeneous consisting of the mixture of hydrocarbons which consists of hydrogen and carbon in ratio 2:1. Crude oil or hydrocarbons also contains elements such as nitrogen, sulphur and oxygen, all of which constitutes less than 3% (v/v). Organometallo – constituents, like phosphorus and heavy metals such as vanadium and nickel are also present (van Hammer *et al*., 2003). Crude oil could be classified according to their respective distillation residues as paraffin, naphthenes or aromatics and based on the relative proportions of the heavy molecular

weight constituents as light, medium or heavy (Okoh, 2003). Crude oil can be separated into four fractions namely, the saturated, aromatics, resin and asphaltene fractions by using absorption chromatography (Karlsen and Larder, 1991). Saturates are further classified according to their chemical structures into alkenes (paraffin's) and cycloalkanes (naphthenes). Aromatics have one or more aromatic ring with or without an alkyl substituted, while both the resin and asphaltene fractions contain non-hydrocarbon polar compounds (Harayama *et al*., 1999). Also, the composition of crude oil may vary with the location and age of an oil field, and may even be depth dependent within an individual well. Hydrocarbon composition affects their physicochemical properties and it differs in their solubility, from polar compounds, such as methanol, to very low solubility non-polar compounds, such as high molecular weight polynuclear aromatic hydrocarbons. These polynuclear aromatic hydrocarbons are fused- ring compounds that are structurally complex and they are highly recalcitrant under normal conditions because of their strong bonds (Bach *et al*., 2005).

### 2.3 **Composition of Engine oil and Used Crankcase oil**

Engine oil is one of the components of crude oil containing up to 20-70 carbon atoms in its chain and more than 75% C-alkanes and most of the C-alkanes in the base oil have long alkyl side chains (Koma *et al*., 2003; Bagherzadeh- Namazi *et al*., 2008). They are made from a heavier and thicker petroleum hydrocarbon base stock derived from crude oil with additives that help in maintaining a lubricating film between moving parts of a car or machine engines.

Spent engine oil which is also known as used crankcase oil is a brown to black liquid produced when new Crankcase oil is subjected to high temperature and mechanical process (Achuba and Peretiemo-Clark, 2008; Onwuka and Igwe, 2010) and it contains more metals like lead, zinc, chromium, barium and arsenic and heavy polycyclic aromatic hydrocarbons (PAHs) that come from engine parts as they wear down (Wang *et al*., 2000) and this contribute to chronic hazard, which include mutagenicity and carcinogenicity (Keith and Telliard, 1979; Hagwell et al., 1992; Bonchan *et al*., 2000). Crankcase oil had a density of 0.828g/ml and contained 14% aromatics and 65.4% aliphatics by weight. The illegal and indiscriminate dumping of used engine oil is an environmental hazard with

global ramification (Blodgette, 2001). Table 1 showed the different chemicals present inside the engine oil and crude oil composition (Koma *et al.*, 2001; Ganguli, 2010).

Table 1. Chemical composition of car engine base Oil and Crude Oil Composition

| Components of engine oil | % | Elements of crude oil | % weight |
|---|---|---|---|
| Saturated Fraction | 90.9 | Carbon | 83.0 – 87.0 |
| Normal Paraffin | 15.5 | Hydrogen | 10.0 – 14.0 |
| Cyclic Paraffin | 75.4 | Sulphur | 0.05 – 6.0 |
| Aromatic Fraction | 9.1 | Nitrogen | 0.1 – 2.0 |
| Naphthalene | 1.7 | Oxygen | 0.05 - 1.5 |
| Fluorene | 1.2 | Metals (Fe,Cu, Ni, | 0.00 - 0.14 |
| Benzene | 1.1 | V, Mg, Al) | |
| Dibenzofuran | 1.0 | | |
| Dinaphthenebenzene | 0.8 | | |
| Dibenzanthracene | 0.6 | | |
| Naphthobenzothiophene | 0.3 | | |
| Perylene | 0.2 | | |
| Benzothiophene | 0.2 | | |
| Chrysene | 0.1 | | |
| Unknown | 1.9 | | |

(Adapted from Koma *et al*., 2001; Ganguli, 2010)

Analyses of these hydrocarbons, i.e. the crude oil and used engine oil showed that they contain aliphatic and aromatic hydrocarbons.

## 2.4 **Aliphatic Hydrocarbon Compounds**

Aliphatic or alkanes are saturated hydrocarbons that are formed exclusively by carbon and hydrogen atom. They can be linear n-alkanes, cyclo-alkanes or branched Iso-alkanes (Fernando, 2009). Alkanes are highly hydrophobic and depending on their

molecular weight; exist as either gases ($C_1$- $C_4$), liquids ($C_5$- $C_{17}$) or solids ($C_{18}$- $C_{38}$) at physiological temperatures. Aliphatic hydrocarbons (AHc) are complex mixture in both composition and molecular structure, they originated mostly from crude oil. AHc are found in a wide range of chemical products such as gasoline, kerosene, fuel oil, jet oil, heavy oil and lubrication oil. Alkanes are also produced by many living organisms such as plants, green algae bacteria or animals (Fernando, 2009). Aliphatic hydrocarbons can be environmental polluting agents of risk for ecosystems and human health (Guo *et al*., 2010).

The solubility values of alkanes are well below the micromolar range ($1.4 \times 10^{-4}$ M for hexane and $2 \times 10^{-10}$ M for hexadecane). Their low solubility hampers their uptake by microorganisms and it is still unclear, how the alkanes are able to enter microorganisms' cells. Though the mechanism of the uptake of alkane differs among microorganisms, but it depends on the molecular weight of the alkane and the physico-chemical characteristics of the environment (Wentzel *et al*., 2007).

Uptake of the low molecular alkanes is still possible because they are soluble enough in water and there is a sufficient mass-transfer to the cell. Microorganisms may gain access to medium- and long-chain length n-alkanes, either by adhering to hydrocarbons droplets or by a surfactant- facilitated process (Fernando, 2009).

### 2.4.1 **Aerobic Degradation of Aliphatic Compounds**

Aerobic alkane degraders use $O_2$ as a reactant for the activation of the alkane molecule by using the enzyme, monooxygenases which overcome the low chemical reactivity of the hydrocarbons. Methane is oxidized to methanol, which is subsequently transformed to formaldehyde and then to formic acid (fig 1). The formic acid can be converted to $CO_2$ or assimilated for biosynthesis of multicarbon compound either by the ribulose monophosphate pathway, or by the serine pathway, depending on the microorganisms involved in the degradation process (Lieberman and Rosenzweigh, 2004).

Aerobic degradation usually starts by the oxidation of a terminal methyl group to give a primary alcohol in n-alkanes containing two or more carbon atoms, this is further oxidized to aldehydes and finally converted to a fatty acid. The fatty acids are conjugated

to CoA, which are further processed by β-oxidation to generate acetyl-CoA (Watkinson and Morgan 1990; Van Hamme *et al*., 2003; Wentzel *et al*., 2007). But in some cases, both ends of the alkane molecules are oxidized through ω-hydroxylation or fatty acids at the terminal methyl group (the ω position), rendering an ω-hydroxyl fatty acid that is further converted into a dicarboxylic acid and processed by β-oxidation (Watkinson and Morgan, 1990; Coon, 2005).

Subterminal oxidation of n-alkanes has also been reported (Kotani *et al*., 2006; 2007). The product generated a secondary alcohol which is converted to corresponding ketone (fig. 2.1) and then oxidized by a Baeyer-Villiger monooxygenases to produce an ester. Esterase hydrolysed ester into alcohol and a fatty acid. Both terminal and subterminal oxidation can coexist in some microorganisms.

Short-chain-length alkanes ($C_2$-$C_4$) are acted upon by the enzyme, methane monooxygenases (Hamamura *et al*., 1999; Dubbels *et al*., 2007). While medium-chain-length alkanes ($C_6$-$C_{11}$), or long-chain-length alkanes (>$C_{12}$) are degraded by bacterial strains that contain integral membrane non-haem iron monooxygenases related to the well-identified *Pseudomonas putida* GPO1 AlkB alkane hydrolase. However, some bacteria contain enzymes that belong to a family of soluble cytochrome P450 that hydroxylate $C_5$-$C_{11}$ alkanes, while those strains that can assimilate alkanes of more than 18 carbons contain alkane hydroxylases which is unrelated to those enzymes found in *Pseudomonas putida* (Van Beilen *et al*., 2003; Van Beilen and Funhoff, 2007). Several bacterial strains like *P. putida* GPo1 AlkB or *Acinetobacter* sp. EB104 cytochrome P450 can degrade alkane of $C_{10}$-$C_{22}$ but some strains that can oxidize alkanes larger than $C_{13}$-$C_{44}$ like *Acinetobacter* sp. M1 contain a soluble, $Cu^{2+}$- dependent alkane hydroxylase that is active on $C_{10}$-$C_{30}$ alkanes and the enzyme responsible is known as dioxygenase that generate n-alkyl hydroperoxides to render the corresponding aldehydes (Maeng *et al*., 1996; Tani *et al*., 2001). While *Acinetobacter* strain, DSM 17874 contain a flaving-binding monooxygenases, named Alm A, which can oxidizes $C_{20}$ to > $C_{32}$ (Throne-Holst *et al*., 2007), while *Geobacillus thermodenitrificans* NG80-2, contain alkane hydroxylase named Lad A, which can oxidizes $C_{15}$-$C_{36}$ to primary alcohols (Feng *et al*., 2007), the crystal structure of the primary alcohol showed that it is a two-component flaving-dependent oxygenase that belong to the bacterial luciferase family of proteins (Li *et al*.,

2008). Branched-chain alkanes are more difficult to degrade than linear n-alkanes (Pirnik *et al*., 1974). But some strains of bacteria have been found that can degrade branched-chain alkanes like isooctane (Solano-Serene *et al*., 2004) or pristine (Britton. 1984; Watkinson and Morgan, 1990). Pristane and phytane can also be degraded by *Alcanivorax* sp. (Hara *et al*., 2003).

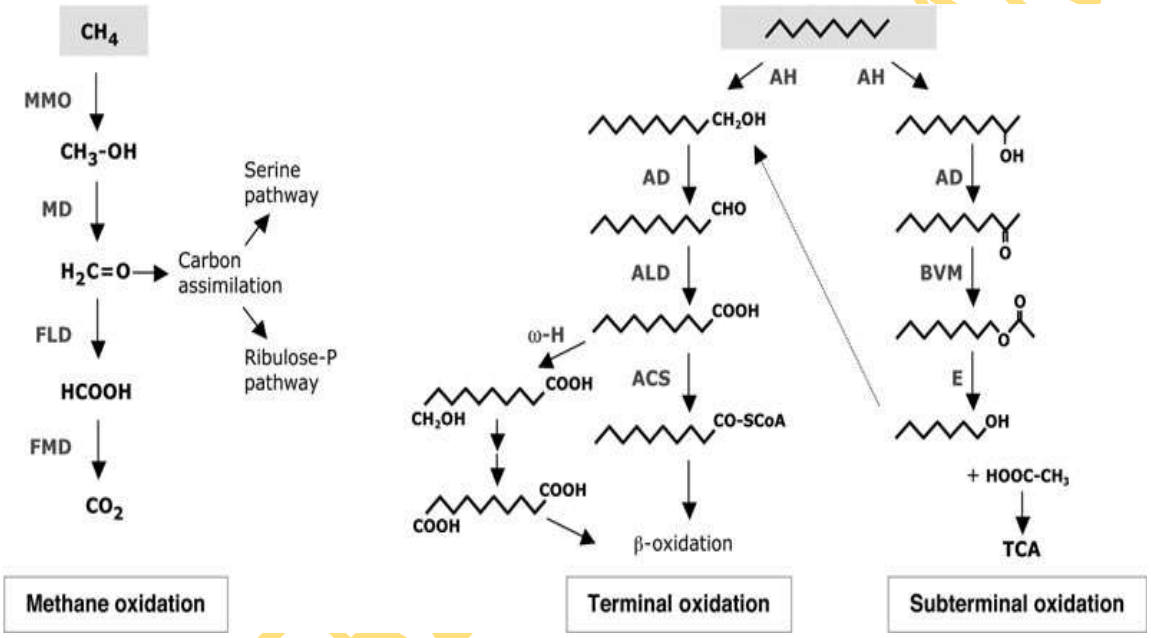



Fig 2.1. Aerobic Pathways for the degradation of methane (left), and larger n-alkanes by terminal and subterminal oxidation (right). MMO, methane monooxygenase; MD, methanol dehydrogenase; FLD, formaldehyde dehydrogenase; FMD, formate dehydrogenase; AH, alkane hydroxylase; AD, alcohol dehydrogenase; ALD, aldehyde dehydrogenase; ACS, acyl-CoA synthetase; ω-H, ω-hydroxylase; BVM, Baeyer-Villiger monooxygenase; E, esterase; TCA, tricarboxylic acids cycle (Kotani *et al*., 2006).

### 2.4.2 **Anaerobic Degradation of Alkanes**

Some microorganisms can degrade alkane under strictly anaerobic conditions by

using nitrate or sulfate as electron acceptor (Widdel and Rabus, 2001), though their growth is slower than that of aerobic alkane degraders. Those strains that can use this mechanism can only use a narrow range of alkanes as substrate for example, strain BuS5, a sulfate reducing bacterial that belongs to the *Desulfosarcina, Desulfococcus* cluster, can assimilate only propane and butane (Kniemeyer *et al*., 2007), *Azoarcus* sp. HxN1, a denitrifying bacteria metabolizes $C_{12}$-$C_{20}$ alkanes (Widdel and Rabus, 2001). The metabolic pathway used by some strains have been investigated to be two, one involves activation of the alkane at a subterminal position by addition of a fumarate molecule to the alkane, that yield an alkyl-succinate derivates, the reaction occur through a generation of an organic radical intermediate, which is believed to be a glycyl radical (Rabus *et al*., 2001), the reaction product is linked to CoA and converted into an acyl-CoA that is further metabolized by β-oxidation. While the second reaction has only been studied in propane in which fumarate molecule is added to one of the terminal carbon atoms of the alkane (Kniemeyer *et al*., 2007). However, several multispecies consortia have been identified that cycle methane and single-carbon compounds under sulfate-reducing or nitrate-reducing conditions (Caldwell *et al*., 2008).

### 2.5 **Polycyclic Aromatic Hydrocarbons**

Polycyclic aromatic hydrocarbons (PAHs) are composed of two or more condensed aromatic rings of carbons and hydrogen atoms, the rings are linked together in linear and angular arrangements. PAHs are chemically stable at ambient temperature because of their large negative resonance energy and because of their hydrophobic structures, they are nearly or totally insoluble in water and highly lipophilic (Hughes *et al*., 1997). PAHs are ubiquitous environmental pollutants that are generated from both natural and anthropogenic processes which pose a serious concern to the health of aquatic life and human through bioaccumulation (Hughes *et al*., 1997; Okafor and Opuene, 2007; Fagbote and Olanipekun, 2010; Lee and Byeon, 2010.)

The low molecular weight PAHs include naphthalene, with two six-membered rings; biphenylene, acenaphthylene, acenaphthene and fluorine, with two six membered rings and a four-or five- membered rings; and phenanthrene and anthracene with three six-membered rings. The high molecular weight PAHs include fluoranthene, pyrene,

benz[a]anthracene, and chrysene, with four rings; perylene, benzo[a]pyrene, benzo[e]pyrene, and dibenz [a, h] anthracene with five rings; and many others with six or more rings (Fig 2.2).

PAHs are emitted from a number of sources (fossil, fuel combustion, waste incineration, oil refinery, steel and iron manufacturing, coke and asphalt production, e.t.c.), the freely generated PAHs are emitted in the form of gases and ultra-fine particles (Richter and Howard, 2000). Although PAHs are found in coal and petroleum, the man modern way of introducing PAHs into the environment now is by the incomplete combustion of organic matter from sources such as motor vehicles, coal-fired plants, home heating furnaces and forest fires (Finlayson-Pitts and Pitts, 1997). PAHs are also released when coal, petroleum products, wood, urban solid wastes or old tires are burned (Mastral and Callén, 2000). Other sources of PAHs include oil refineries, coal gasification plants, steel mills, and aluminium plants. Crude oil spills from pipelines and supertankers deposit large amount of PAHs on the soil and in the ocean. In the atmosphere, PAHs are partitioned between the gaseous and the particulate phase with the carcinogenic 5- and 6-ring species being mostly associated with particles (Ravindra *et al*., 2008).

Polycyclic Aromatic Hydrocarbons are hydrophobic and readily adsorbed onto particulate matter and thus, coastal and marine sediments become the ultimate sinks for such compounds (Hughes *et al*., 1997; Yu *et al*., 2005; Osuji and Ezebuiro, 2006)

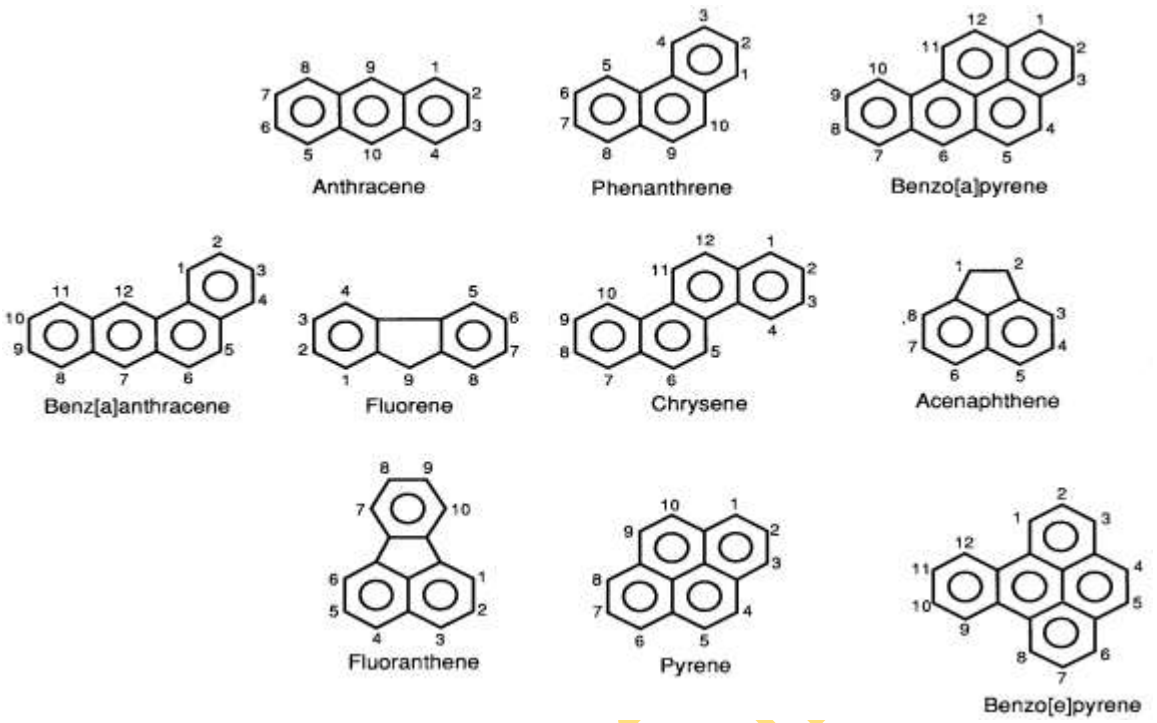



**Fig. 2.2.** Chemical structures of some selected polycyclic aromatic hydrocarbons (Mrozik *et al*., 2003)

### 2.5.1 **Occurrence and Toxicity of Polycyclic Aromatic Hydrocarbons**

PAHs are widespread pollutants, some of which have been found to be carcinogenic, genotoxic, cytotoxic or ecotoxic by studies performed in vitro and in vivo in humans, plants and aquatic microorganisms (Anyakona, 2007). Photo-chemical reactions may convert PAHs to carcinogenic nitro-PAHs (Finlayson-Pitts and Pitts, 1997) or to PAH endo-peroxides and radicals that are quickly transformed to quinines (Yu, 2002). Highway runoff water containing phenanthrene, fluoranthene, pyrene, and other PAHs arising from wear and tear on vehicles and the road; many of these PAHs are predominantly associated with particulate matter (Shinya *et al*., 2000).

Soils along highway margins are polluted by PAHs from motor fuels, exhaust, road tar, and street dust (Tuháčková *et al*., 2001). PAHs may bind to organic matter, silt or other fine-textured particles in the topsoil, or they may adsorb to dissolved organic matter and leach into the subsoil (Wilcke, 2000).

Some low molecular weight PAHs are acutely toxic to aquatic organisms, in addition some high molecular weight PAHs are mutagenic to bacteria and teratogenic or carcinogenic to mammals. Phenanthrene inhibits spore germination in some fungi (Lisowska *et al*., 2004. Light irradiation of PAHs that are in contact with DNA may induce single-strand cleavage of the DNA, oxidation of guanine to 8-hydroxyguanine, or formation of DNA-PAH adducts (Yu, 2002). When benzo[a]pyrene and other high molecular weight PAHs are adsorbed to particulate matter in soil, water, or sediments, they are biotransformed more slowly, although they can be oxidized, by cytochrome P450 monooxygenases in the mammalian liver (Miller and Ramos, 2001). Although the end products that are usually water-soluble are excreted, but some of these metabolites may be genotoxic compounds that may form adducts with DNA (Warshawsky, 1999). Researches have shown that benzo[a]pyrene and several other PAHs have the ability to cause cancer in experimental animals and also workers that are exposed to PAHs in their industries have a higher incidence of lung and bladder cancer (Mastrangelo *et al.*, 1996). While some PAHs have weak estrogenic or anti-estrogenic effects (Santodonato, 1997).

Researches over the past years have shown that much of the carcinogenesis associated with coal tar is due to benzo[a]pyrene (Philips, 1983). Though dibenz [a, h] anthracene was first found to cause skin tumour in mice, but benzo[a]pyrene was found to be even more carcinogenic.

### 2.5.2 **Aerobic Degradation of Polycyclic Aromatic Hydrocarbons**

The biological degradation of PAHs, as outlined by Johnsen *et al*., (2005), serves three different functions that include; assimilative biodegradation that yield carbon and energy for the microorganisms and mineralization of the compound, followed by intercellular mechanism that render the PAHs water-soluble and this and cometabolism, which is the degradation of PAHs without production of energy and carbon for the organism metabolism (Boonchan *et al*., 2000)

The biodegradation pathway for polycyclic aromatic hydrocarbons can be divided into three steps, in the first step; the aromatic ring is activated and transformed into hydroxylated aromatic metabolites including (alkyl- substituted) catechols, protocatechuic acid and gentisic acid by aromatic ring oxygenase and dehydrogenase, while in the

second stage, the aromatic ring is opened by ring-cleavage dioxygenases to yield unsaturated aliphatic acids and aldehydes which are used in central metabolism for energy and biomass production in the third stage (Smith, 1990; Peng *et al*., 2008).

In aerobic bacteria, the initial reaction is the incorporation of both atoms of oxygen molecules. The majority of the low molecular weight (LMW) PAHs-degrading isolates belong to the genus *Pseudomonas*, but degradation has also been demonstrated by strains of *Ralstonia*, *Sphingomonas* and *Burkholderia*. In Pseudomonads, the biodegradation of naphthalene, the smallest of the PAHs, is initiated by a naphthalene dioxygenase-catalysed activation reaction that produces naphthalene 1, 2-dihydrodiol (Resnick *et al*., 1996). The dihydroxylated intermediate is then rearomatized and ring-cleaved to produce salicylic acid that is further degraded to catechol. The catechol is either meta-cleaved by using catechol 2, 3-dioxygenase or ortho-cleaved by catechol 1,2-dioxygenase enzymes (Yen and Serdar, 1988; Habe and Omori, 2003). Phenanthrene, a three-ring PAH, is first converted to 3,4-dihydrodiol, which is further degraded to 1-hydroxy-2-naphtoic acid, phthalate and protocatechuic acid or dihydroxylated to 1,2-dihydroxynaphthalene, which is degraded via the naphthalene pathway to catechol. The ring-cleavage products are further metabolized to tricarboxylic acid cycle intermediates and eventually to $CO_2$ (Barnsley, 1983).

The metabolism of high molecular weight (HMW) PAHs containing four or more aromatic rings have been described in isolates of *Mycobacterium*, *Rhodococcus* and *Gordona*, like LMW PAHs, their biodegradation also starts with dioxygenation (Kanaly and Harayama, 2000). In *Mycobacterium* sp. PYR-1 pyrene is degraded via two dioxygenation reactions and ring-cleavage to 3,4-dihydroxyphenanthrene and further to O-phthalate in a similar way to the phenanthrene catabolic pathway (Kim *et al*., 2007). The PAHs which contain more than five rings, such as benzo[a]pyrene, are degraded by cometabolism (Kanaly and Harayama, 2000).

Generally, aerobic microorganisms degrade high molecular weight PAHs more slowly than low molecular weight PAHs (Kanaly and Harayama, 2000). The reasons may be due to;

Lower water solubility, slower uptake into the cells

Insufficient ability to induce degradative enzymes and lastly,

Lower energy yield for growth.

### 2.5.3 **Anaerobic Degradation of Polycyclic Aromatic Hydrocarbons**

The mechanism for anaerobic hydrocarbon biodegradation is not well understood. Under anaerobic degradation, nitrate, manganese (iv), iron (iii), sulphate or carbon (iv) oxide serve as terminal electron acceptors (Anderson and Lovely, 1997). The process is slower compared to aerobic process because the energy yield obtained is much slower (Madigan *et al*., 2003).

Aromatic hydrocarbon-degrading sometimes use the pathway of aerobic bacteria. The compounds are first degraded to the more common intermediates, after which the aromatic ring is cleaved and the noncyclic intermediates are converted to central metabolites. In naphthalene and phenanthrene metabolism, the initial activation occurs through carboxylation to form the central intermediates, 2-naphthoic or phenanthoic acid. The aromatic ring of the acid is then reduced to a hydroxylated intermediates followed by ring cleavage and central metabolism (Meckenstock *et al*., 2004). Sometimes the initial activation occurs by methylation (Safinowski *et al*., 2006).

## 2.6 **Oil Pollution and its Effect on Ecosystems**

The increase in demand for crude oil as a source of energy and as a primary raw material for industries has resulted in an increase in its production, transportation, and refining which in turn has resulted in gross contamination of the environment (Obire, 1988). Though petroleum is the principal source of energy worldwide but despite its importance, petroleum hydrocarbons also pose as a globally environmental pollutant (Plohl *et al*., 2002).

Accidental and deliberate crude oil spills continues to be a significant source of environmental pollution, and poses a serious environmental problem due to the possibility of air, water and soil contamination (Trindade *et al*., 2005), especially when it is associated with accidental spills on large-scale (Udeani *et al*., 2009). The impact of this spillage will also depend on the magnitude of the spill, the chemical composition and the nature of the contaminated ecosystems (Amund *et al*., 1993). The significance of any

given spill is dependent on the amount of oil spilled in terms of barrels measurement and on the impact of the environment.

According to Adekunle and Furster (1987), oil spills are classified into three main categories; minor oil spillage, medium oil spillage and major oil spillage. When this complex mixture of hydrocarbon enters into the aquatic and terrestrial environments, the composition of this hydrocarbon will change progressively due to physico-chemical changes such as dissolution, evaporation, absorption, degradation and photo- oxidation. Apart from physico – chemical changes, there are various effect on the biota depending on the substrate. The effect of oil pollution on the environment can both be biological and ecological, and this effect can either be a short or long term one (Mills *et al*., 2003).

The toxic effects of crude oil and refined petroleum oils on plants, animals, humans, and the environment are devastating (Elliot, 1997) and when oil also reach the soil, it can become immobilized and reach the water table (Udeani et al., 2009).

The effect of oil spill on both the vegetation and animals in the soil is not only because of their contact toxicity but also because hydrocarbons in the soil reduces oxygen tension and increases anaerobiosis which is harmful to plant roots (Bossert and Bartha, 1984). When hydrocarbon oil enters the water bodies, it induces essential changes in the functioning conditions of the biological systems, pH of the medium, aeration, living organisms are also exposed to the toxic effect, the stability of the communities is also violated and the species diversity gets impoverished (Ignatavicius *et al*., 2006).

The effect on the ecosystem may be a long term effect due to the release of toxic component of the oil over a prolonged period as the oil break up and the concentration of toxicants in the organisms toward the top of the food chain increases (Samanta *et al*., 2002).

The problem of oil pollution is worldwide and the estimated number of contaminated sites is significant (Cairney, 1993), and many cases of oil spills have been reported all over the world, for example, the Exxon Valdez in 1989, Prestige spill in 2002. Also, in South Africa, many cases of oil spills have been reported since 1983 till present time. In Nigeria, many cases of oil spills have been reported, with about 734 cases of spill within two years alone, i.e. from 1978 to 1980 (Ekpo and Udofia, 2008).

Polycyclic Aromatic Hydrocarbons are found in almost all the hydrocarbon oil, PAHs are chemical compounds consisting of atoms of C and H, arranged in the form of two or more aromatic rings, they are fused-ring compounds that are structurally complex (Jacques *et al*., 2007). PAHs are highly recalcitrant under normal conditions because of their strong molecular bonds. These groups of petro-chemicals are mainly found in the areas surrounding petroleum-refining plants, accidental oil spills and pipe leakages, and rainwater runoff from contaminated sites (Soriano and Pereira, 1998; Angelidaki *et al*., 2000; Bach *et al*., 2005). Researches have shown that the many constituents of PAHs are not only carcinogenic and mutagenic, but they are also potent immunotoxicants (Mishra *et al*., 2001; Bach *et al*., 2005). PAHs are lipid soluble and quickly absorbed by the mammals (Netto *et al*., 2000) and the prolonged exposure and high concentrations may cause development of liver or kidney disease, possible damage to the bone marrow and an increased risk of cancer (Propst *et al*., 1999; Lloyd and Cackette, 2001; Mishra *et al*., 2001). There have been reports of their impact on critical habitats such as the benthic ecosystems, which may ultimately get into the marine food chain (Bach *et al*., 2005). Aliphatic and aromatic hydrocarbons have detrimental effects on environmental quality and health. Many are considered toxic, and their toxicity is sometimes difficult to assess in the environment (Overton, 1997). These toxic effects are devastating on plants, animals, humans and the environment (Elliot, 1997; Kayode *et al*., 2008). Polycyclic aromatic compounds of four rings and above and cyclic alkanes are usually recalcitrant in the soil because of their low solubility in water (Cerniglia, 1992) and they can accumulate in the food chain (Nikunen *et al*., 2000).

The release of aliphatic and aromatic hydrocarbons in to the environment can also cause physically and aesthetically effects, such as film formation on shorelines or smells. All these effects of aliphatic and aromatic compounds in the environment can resulted to land degradation and water pollution limiting land use and damaging the ecosystem, which all forms of life are dependent on (Peterson *et al*., 2003).

Since petroleum hydrocarbons are among the most ubiquitous contaminants in the environment (Watanabe, 2001; Margesin *et al*., 2003), their vast range of substrates and metabolites provides an environment for the development of quite complex microbial

communities (Butler and Mason, 1997) and the ability to degrade them in the environment is widespread (MacNaughton *et al*., 1999; Rolling *et al*., 2004).

The concentrations of organic compounds in the environment also affect the level of tolerance. At low concentration, all fractions are likely to be attacked. However, at high concentrations, only those fractions most susceptible to degradation will be broken down. Also the concentration of contaminants will affect the number of organisms present. Dong and Wu (1995) reported that the higher concentrations of gasoline in contaminated water were related to higher counts of microorganisms.

## 2.7 **Ecology of Hydrocarbon Biodegradation**

The potential for hydrocarbon biodegradation is common in both the contaminated and uncontaminated environments (Margesin *et al*., 2003; Johnsen and Karlson, 2005; Saul *et al*., 2005). Hydrocarbon degrading bacteria always thrive as minor members in an uncontaminated environment but when contamination occurs, the hydrocarbon degrading bacteria population then can assimilate the available carbon to biomass increments in the microbial community (Mesarch *et al*., 2004; Katsivela *et al*., 2005; Hamamura *et al*., 2006). If the environment is contaminated by a mixture of contaminants, the microbial community changes in a new direction due to the sequential utilization of different compounds and other microbial populations that are responsible for their degradation become enriched (Kaplan and Kitts, 2004; Viňas *et al*., 2005; Powell *et al*., 2006). The abundance of the contaminants degrading microorganisms may also correlate with the abundance of bioavailable hydrocarbons in these contaminated environments (Sanseverino *et al*., 1993; Baldwin *et al*., 2008).

In environments that have been previously contaminated, the structure of those environments would have been changed as a result of the selection caused by contamination. Generally, overall loss of diversity (Saul *et al*., 2005), higher hydrocarbon biodegradation potentials (Johnsen and Karlson, 2005), diverse numbers of contaminant degrading bacteria (Sanseverino *et al*., 1993; Johnsen and Karlson, 2005; Baldwin *et al*., 2008) and faster response to any contaminant addition (Johnsen *et al*., 2007) are common occurrence in contaminated environments.

Multiple carbon sources are available for bacterial biodegradation at sites polluted with different contaminants but single bacteria can only metabolize a limited range of substrates, although some bacteria may carry multiple genes with different substrate specificities (Tani *et al*., 2001). Therefore mixed population with different degradation capabilities are required for the degradation of mixtures of hydrocarbons (Bouchez *et al*., 1995; Sei *et al*., 2003), while these different bacteria that are capable of degrading the same compound, may do so under different environmental conditions and substrate concentrations (Ghiorse *et al*., 1995; Cavalca *et al*., 2004). The order at which these different compounds are biodegraded depends on their bioavailability, the more available a compound is, the more rapidly it will be transformed (Hamamura *et al*., 2006).

Microorganisms also prefers the simple and more easily degraded hydrocarbons like short chain length n-alkenes and the low molecular weight (LMW) PAHs, over the more recalcitrant and complex carbon sources like the long chain length n-alkenes and high molecular weight (HMW) PAHs (Katsivela *et al*., 2005; Viňas *et al*., 2005; Hamamura *et al*., 2006). Also, different bacteria are found at different phases of degradation (Kaplan and Kitts, 2004; Katsivela *et al*., 2005; Viňas *et al*., 2005; Hamamura *et al*., 2006). Generally, the fast-growing bacteria known as r-strategists that belong to the genus *Pseudomonas* or *Sphingomonas* are found to metabolize the more easily available and biodegradable hydrocarbons, while the slow-growing more stable K-strategist, such as *Actinobacteria* degrade the less bioavailable compounds (Margesin *et al*., 2003; Kaplan and Kitts, 2004; Leys *et al*., 2005; Johnsen *et al*., 2007). However, the removal of one compound may also be inhibited by another compound in the mixture (Bouchez *et al*., 1995) and the preferential for the biodegradation of the more bioavailable fractions can leave the more recalcitrant hydrocarbons in the environment (Shuttleworth and Cerniglia, 1995).

Bacteria have different genetic and physiological mechanisms for adapting to the accumulation of hydrocarbons in the environment. Hydrocarbon-degradative genes often reside in mobile genetic elements, such as conjugative catabolic plasmids and catabolic transposons, which can be transferred to other bacteria via horizontal gene transfer (Tan, 1999). Novel biodegradation mechanisms are also constantly evolving through genetic rearrangements, recombination and transposition, or by point mutations (van der Meer *et*

*al*., 1992). Some bacteria can also form biofilms on solid hydrocarbons or can produce biosurfactants that can increase their access to substrates and make the hydrophobic contaminants more available by increasing the mass transfer rate of hydrocarbon into the bacterial cell wall (Johnsen *et al*., 2005), while the hydrophobic cell wall in some hydrocarbon degrading bacteria is also believed to aid in adhesion to poorly water soluble substrates (Watkinson and Morgan, 1990).

## 2.8 **Bioremediation**

As landfills is becoming scarcer and concomitantly more cost prohibitive. Interest in biological methods in organic wastes treatment has increased. The area that has received much attention is the biological treatment of petroleum-contaminated sites.

Contaminated sites can be treated using various means and applications, which include physical treatment, thermal or chemical processes (Piskonen and Itavaara, 2004). Although, they can be very effective at reducing the levels of a range of contaminants, they have several drawbacks, their technology is complex, the cost of small application is high, and they are also prone to prolong cycle time (Leah and Colwell, 1990; Ward *et al*., 2003) and the lack of public acceptance, especially for incineration that increases the exposure to contaminants for both the workers at the site and nearby residents (Vidali, 2001).

Bioremediation on the other hand can degrade contaminants, converting them to carbon dioxide, water, and new cells or convert the waste to non-toxic products, some of which may be useful to the ecosystem (Okoh and Trejo-Hernandez, 2006).

Bioremediation, which employs the biodegradative potentials of organisms or their attributes, is an effective technology that is being used to accomplish effective detoxification and volume reduction. It is useful in the recovery of sites contaminated with oil and hazardous wastes (Caplan, 1993). This method is not a new concept because it has been studied extensively in controlled conditions (Sugiura *et al*., 1997; Chaillan *et al*., 2004). And in open field experiments (Chaineau *et al*., 2003; Gogoi *et al*., 2003), but the method has acquired a new significance as an increasingly effective and potentially inexpensive clean-up technology. The process is complex , and its qualitative and quantitative aspect depend on the nature and amount of pollutants present , the ambient

and the seasonal environmental conditions and the constitution of the indigenous microbial community (Leahy and Colwell, 1990; Hinchee and Olfenbuttel. 1991).

The control and optimization of bioremediation processes is a complex system of many factors, which include, the microbial populations that are capable of degrading the pollutants, availability of contaminants to the microorganisms and the environmental factors. Understanding of these factors will help in manipulating the microbial activities. Other factors that need to be well understood for a successful bioremediation technology include;

Physical Characteristics of the Hydrocarbon constituents: - The fate of any hydrocarbon for bioremediation depends on the molecular size and topology of the compound, low molecular weight hydrocarbon are easily degraded than the high molecular weight (Kanaly and Haramaya, 2000). While the concentration of hydrocarbon present in the environment is also necessary in determining the rate of degradation (Ward *et al*., 2003). Maximum metabolic activities are observed in the upper soil layer of about 10 to 15cm deep.

Choice of Microbial Consortium:- Many microbial strains are capable of degrading only a specific hydrocarbons, but oily sludge wastes which contain many mixtures of PAH, alkanes, resin fractions (MacNaughton *et al*., 1999) are difficult to be degraded by only a single species of bacteria (Loser *et al*., 1998). Hence, there is need to employ a broad range of the indigenous microorganisms present in the environment, as they can degrade the constituents and have a higher tolerance to the toxicity of the pollutants (Mishra *et al*., 2001).

Factors affecting the biodegradation mechanisms: - Physical, chemical and biological factors will determine the effective uptake of hydrocarbons by microorganisms (van Hamme *et al*., 2003). These factors include; biosurfactants which are important agents in the effective uptake of hydrocarbon by microorganisms (Leahy and Colwell, 1990).

PH, many microorganisms will perform best at neutral pH. However, fungi are more tolerant to the acidic conditions (Al-Daher *et al*., 1998).

Nutrient; van Hamme et al., (2003) reported that nitrogen and phosphorus contents enhance the microbial degradation of hydrocarbons. Huesmann (1997), also reported that

application of fertilizers on the contaminated sites enhance the metabolic activities of the microbial community.

Oxygen; aerobic biodegradation is the most effective pathway for bioremediation. Presence and concentration of oxygen is the rate-limiting parameter in the aerobic biodegradation but anaerobic degradation of contaminants by microorganisms also occur but the rate is very low (Angelidaki *et al*., 2000).

Temperature; the best temperature for biodegradation process is between 30 to 40$^{o}$C, at above this temperature, enzymatic activities are inhibited as proteins denature (Leahy and Colwell. 1990), while low temperatures affect microbial growth and propagation.

Water activities; the level of moisture also determine the rate at which degradation will take place (Vinas *et al*., 2005), since water is needed for microbial growth and enzymatic biochemical activities (Leahy and Colwell, 1990).

Genetic mechanisms: - Researches have shown that genetic compatibility is one of the important factors in the success of microbial catabolism of hydrocarbons. The roles of plasmid in the environment have been well documented. It has been reported that the metabolic pathways for some compounds like naphthalene, toluene, xylene, salicylate have been shown to be encoded on plasmids, most especially in the *Pseudomonas* sp. Exposure of the indigenous microbial communities to pollutants may favour species harbouring the necessary survival plasmids (i.e. OCT, NAH, AND TOL) (Sayler, 1990). The ability to degrade more recalcitrant components of the hydrocarbons are plasmid mediated (Cerniglia, 1984).

### 2.8.1 **Bioremediation methods/ strategies**

Different techniques are employed in the bioremediation technology depending on the degree of saturation and aeration of an area. In Situ techniques, this is defined as those methods that are applied to soil and groundwater at the site with minimal disturbance. Ex Situ techniques on the other hand involves removal of the contaminant from the site via excavation or pumping (water) for Biotreatment.

In Situ bioremediation

This technique is the most desirable options because it is cost effective and less disturbance because there is no excavation of contaminants (Okoh and Trejo-Hernandez, 2006). Some of the methods used under In Situ bioremediation include;

Bioventing: - this is the supply of air and nutrients through wells to contaminated sites to stimulate the indigenous bacteria. It is the most common and is used when the contamination is deep under the surface.

Biosparging: - Biosparging involves the injection of air under pressure below the water table to increase ground water oxygen concentrations and enhance the rate of biological degradation of contaminants by naturally occurring microorganisms.

Biostimulation and bioaugmentation are the two best methods of microbial bioremediation, because they are cost effective, cause minimal environmental impact and very effective (Kaplan and Kitts, 2004).

Biostimulation: - This involves the modification of the environment to stimulate existing organisms capable of bioremediation. The growth of indigenous organisms present in the contaminated site by providing nutrients for their growth, the nutrient can be provided in form of fertilizer (Nester *et al*., 2004).

Bioaugmentation: - This is the addition or introduction of microorganisms indigenous or exogenous to the contaminated sites. Genetically engineered variant can also be used in treating the contaminated site.

Ex Situ Bioremediation: - these techniques involve the excavation or removal of contaminated sites.

Land farming: - It is a simple technique in which contaminated soil is excavated and spread over a prepared bed and periodically tilled until pollutants are degraded.

Composting: - In this method, contaminated soil is combined with non-hazardous organic ammendants, such as manure and agricultural wastes. These organic wastes will then stimulate the growth of microorganisms and increase the temperature, which is a characteristic of composting.

Biopiles: - These are the hybrid of land farming and composting. Engineered cells are constructed as aerated composted piles. The method is used for the treatment of surface contamination with petroleum hydrocarbons. Biopiles provide a favourable environment

for indigenous aerobic and anaerobic microorganisms and it also prevents physical losses of the contaminants by leaching and volatilization (van Fahnestock *et al*., 1998).

Bioreactors: - Slurry reactors or aqueous reactors are used for Ex Situ treatment of contaminated soil and water pumped up from a contaminated plume. Bioremediation in reactors involves the processing of contaminated solid material (soil, sediment, sludge) or water through an engineered containment system. The bioreactor may be defined as a containment vessel and apparatus used to create a three-phase (solid, liquid and gas) mixing condition to increase the bioremediation rate. The method is reliable because the contained environment is more manageable and hence more controllable and predictable but the disadvantage is that it needs pretreatment like excavation.

The following parameters can be used in determining the efficacy of bioremediation in the laboratory tests; counting or enumeration of microbial population using designed minimal medium, (Rice and Hemmingsen, 1997; Peressutti *et al*., 2003). Rate of hydrocarbon degradation or disappearance of the individual hydrocarbon and/or total hydrocarbon by microorganisms can also be determined (Okoro, 2008). It can also be determined by measuring the disappearance of hydrocarbon in an experiment set up by using, Gas Chromatography Mass spectrometer (GC-MS), Flame Ionization Detector (FID) (Angelidaki *et al*., 2000; Bach *et al*., 2005). Other methods that can be used include, determining the microbial respiratory activity ($CO_2$ production), (Zucchi *et al*., 2003), another method that can be used is Resting-cells Assay (Goris *et al*., 2004).

## 2.9 **Microorganism Associated with Biodegradation**

In recent years, many microbial ecologists have identified various microbial species that are effective degraders of hydrocarbons in natural environment. Many microbial consortia were isolated from heavily contaminated areas based on their ability to metabolize various carbon sources, such as aliphatic and aromatic compounds and their chlorinated derivatives.

Many microorganisms such as bacteria, fungi and micro algae can degrade petroleum hydrocarbons (Riser-Roberts, 1992; Bundy *et al*., 2004).

A large number of *Pseudomonas* strains capable of degrading PAHs have been isolated from soil and aquifers (Johnson *et al*., 1996). Other petroleum hydrocarbon

degrading organisms include *Acinetobacter*, *Alcaligenes*, *Bacillus*, *Arthrobacter*, *Flavobacterium*, *Nocardia*, *Serratia*, *Corynebacterium* (Bhattacharya *et al*., 2002; Chaillana *et al*., 2004). Several fungi and actinomycetes, which include *Penicillium*, *Phanerochaete*, *Nitrisomonas*, *Mucor*, *Aspergillus, and Fusarium,* have been confirmed to be important agents for bioremediation of hydrocarbon contaminated sites (April *et al*., 2000; Zhang *et al*., 2006). Fungi take longer periods of time to grow as compared to their bacterial counterparts that play central role in hydrocarbon degradation (Prenafeta-Boldu *et al*., 2001).

These microorganisms known as Petrophiles are unique because they can naturally degrade or utilize large hydrocarbons to satisfy their cell growth and energy needs (Harder, 2004). Microorganisms degrade these compounds by using enzymes in their metabolism to clean the contaminated sites (Alexander, 1999). A large number of microorganisms like *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Bacillus subtilis*, *Bacillus cereus*, *Bacillus licheniformis* and *Bacillus laterospor*, excrete emulsifiers that increase the surface area of the substrate. Some microorganisms can modify their cell surface to increase their affinity for hydrophobic substrates, and thus facilitate absorption (Cybulski *et al*., 2003; Carvalho and Fonseca, 2004). A large number of reports have shown that low molecular weight hydrocarbons are degraded more rapidly than the heavy weight one's like PAHs.

Many indigenous microbial communities capable of extensive oil biodegradation have been isolated from many contaminated sites when the conditions are favourable for oil-degrading metabolic activity (Capelli *et al*., 2001; Kim *et al*., 2004).

There are many advantages when relying on indigenous microorganisms rather than adding microorganisms to degrade hydrocarbons, because these indigenous microorganisms are adapted for survival and proliferation in that environment and secondly, the ability to utilize hydrocarbons is distributed among a diverse microbial population. This population occurs in natural ecosystem and can act either independently or in combination to metabolize various hydrocarbons. Mixed cultures carry out more extensive biodegradation of petroleum than pure cultures (Ghazali *et al*., 2004, Trindade *et al*., 2004; Sun *et al*., 2004; Oteyza *et al*., 2005).

Microorganisms are equipped with metabolic machinery to use petroleum products as a carbon and energy source. The metabolic pathways that hydrocarbon-degrading microorganisms use can either be aerobic (oxygen is used as the primary electron acceptor) or anaerobic (they utilize alternative electron acceptor such as nitrate or sulfate). Aerobic degradation usually proceeds more rapidly and is considered to be more effective than anaerobic degradation because they require less free energy for initiation and yield more energy per reaction (van Hamme *et al*., 2003).

## 2.10 **Metabolic Machinery of Degrading Hydrocarbons by Bacteria**

### 2.10.1 **Aerobic Degradation of Hydrocarbons by Microorganisms**

Aerobic biodegradation of hydrocarbons and crude oil is a long known and well-studied process. The hydrocarbons are broken down by a series of enzyme-mediated reactions. Oxygen serves as an external electron acceptor, while the organic components of the contaminating substances function as the electron donor or energy source.

The general degradation pathway for an alkane involves sequential formation of an alcohol, an aldehydes and a fatty acid. The fatty acid is first cleaved, releasing carbon dioxide and forming a new fatty acid that is two carbon units shorter than the parent molecule in a process known as beta-oxidation. The initial enzymatic attack involves a group of monooxygenases (van Hamme *et al*., 2003).

Extensive methyl branching interferes with the beta-oxidation process and necessitates diterminal attack or other bypass mechanisms. Therefore, n-alkanes are degraded more rapidly than Iso alkanes. Cycloalkanes are transformed by a not fully characterized oxidase system to a corresponding cyclic alcohol, which is dehydrated to ketone. The monooxygenases system lactonises the ring and this is subsequently opened by a lactones hydrolase but the two oxygenase systems that are needed for the opening are never found in the same organism (Okoh, 2006). However, synergistic action of microbial communities can degrade the various cycloalkanes effectively.

*Pseudomonas* is the most ubiquitous bacteria found in oil-contaminated sites and they are responsible for the degradation of most aromatics and aliphatic. Although the efficiency of degradation varies among the different strain but the most extensively characterized PAHs degradation pathway is encoded by the NAH7 plasmids from *P*.

*putida*. The first operon encodes the pathway for naphthalene conversion to salicylates, while the second codes for the conversion of salicylate via catechol meta-cleavage to acetaldehyde and pyruvate. Molecular oxygen is introduced into the aromatic nucleus via naphthalene dioxygenase (van Hamme *et al*., 2003).

The catabolic pathways for three- and four- ring PAHs in *P*. *putida* have been studied extensively. Reports have shown that phenanthrene was degraded by *Pseudomonas* sp strain PP2 via a dioxygenase- initiated pathway that converged with the naphthalene degradation pathway. Parales and Haddock (2004), postulated that the secretion of surfactant into the medium, increased the cell-surface hydrophobicity during their growth has led to increase in the uptake of the poorly soluble phenanthrene.

Some other *Pseudomonas* species were found to grow on aromatic constituents of gasoline as a sole source of carbon. Strains of *Bijerinckia* genus are also active in aerobic hydrocarbon degradation. The presence of biphenyl dioxygenase enables these microorganisms to oxidize benzo (a) pyrene, benzo (a) anthracene and the aromatic N-heterocyclic carbozole (Resnicek *et al*., 1993).

### 2.10.2 **Anaerobic Degradation of Hydrocarbons**

Oxygen is not available in some places where hydrocarbon contamination occurs like in the deep sediments, flooded soils, eutrophic lagoons, stagnant fresh and ocean waters and in oil reservoirs. The different roles of bacteria that can participate in these processes under anoxic/anaerobic conditions were not first understood until 1980. It was later discovered that these microorganisms can activate organic compounds by special biochemical mechanisms that differ completely from those employed in aerobic hydrocarbon metabolism (Riser-Roberts, 1992).

N-alkanes, branched alkanes, cycloalkanes, and some alkenes have been shown to be degraded under anaerobic conditions. For example, unsubstituted, methyl-substituted, and ethyl-substituted cyclopentenes, cyclopentanes and cyclohexanes were consumed without a substantial lag in the presence of sulfate but rather less effectively under methanogenic conditions. Dimethyl-substituted cyclopentanes and cyclohexanes were biodegraded only in the presence of sulfate (Widdel and Rabus, 2001).

PAHs are also metabolized under anaerobic conditions. Naphthalene degradation proceeds via carboxylation to form 2-naphtoate (the central intermediate in a pathway analogous to the benzoyl-CoA pathway for monoaromatic compounds) in sulfate reducing as well as denitrifying bacteria. The identification of other metabolites in a sulphate-reducing enrichment culture indicated further the metabolism of 2-naphthoate (presumably as activated acid) via subsequent reduction of the two rings to yield decalin-2-carboxylate. Alkylnaphthalenes appear to be activated by a mechanism similar to that of toluene (van Hamme *et al*., 2003). It was observed too that benzene, toluene, ethylbenzene, and xylene (BTEX) are degradable without oxygen in contaminated groundwater (Coates *et al*., 2002; Johnson *et al*., 2003).

# CHAPTER THREE

# Materials and Methods

## 3.1 Soil Samples Collection and Isolation of Bacteria

Agricultural soil sample was collected from the Nursery of the Department of Microbiology, University of Ibadan, Ibadan for this experiment. Soil sample was sieved to remove big stones from it. About 2kg of the soil sample was put in pots and labeled accordingly. Soil sample was mixed thoroughly with each of the hydrocarbon i.e. Crude oil (Forcados Blend) and Used Crankcase oil at the ratio of 5:1 in the labelled pots and the experiment was duplicated. Contaminated soil samples were turned every 5 days to provide aerobic conditions for the microorganisms present in the soil samples**.**

### 3.1.1 Isolation of Microorganisms

Microorganisms were then isolated after the $2^{nd}$ weeks, $4^{th}$ weeks,$6^{th}$ weeks, $8^{th}$ weeks and $10^{th}$ weeks respectively using Nutrient Agar (NA) and Minimal Salt Medium for total bacterial counts and total oil degraders counts respectively. About1g of the soil sample was taken from each of the soil samples contaminated with the different hydrocarbons and suspended in 10ml of sterile water. The suspension was serially diluted and 0.1ml of appropriate diluents was evenly spread on the surface of the already prepared Nutrient Agar. Duplicate plates were incubated for 24 hours at $37^{o}$C and morphologically distinct colonies were subcultured onto fresh plates. Pure colonies of each isolated bacteria strains were stored on Nutrient agar slants at $4^{o}$C for further study.

### 3.1.2 Screening for oil Degrading Bacteria

Isolated microorganisms were then streaked on Minimal Salt Medium (MSM) to determine which of them can utilize these hydrocarbons as their only source of carbon and energy.

The modified oil agar medium (Appendix I) according to Gogoi *et al*., (2003) consisted of basal medium (mineral salt) medium; 1.8 $K_2HPO_4$, 1.02 $KH_2PO_4$, 4.0 Urea, 2.0 $MgSO_4.7H_2O$, 0.1 NaCl, 0.1 Yeast Extract, 0.05 $FeCl_2$ and trace elements which are 0.1 $H_3BO_3$, 0.1 $ZnSO_4$ and 0.4 $MnSO_4.H_2O$ in 1liter of distilled water and 2% agar agar was added to the medium before sterilization, they were sterilized at 121$^oC$ for 15 minutes. The two hydrocarbons (Crude and Used crankcase oil) used were also sterilized by using tyndallization method. After cooling the sterilized MSM medium and the hydrocarbon were mixed together aseptically.

Molten sterile oil agar medium was aseptically poured into sterile Petri dish and allowed to solidify. Testing of each isolate was done by streaking a portion of the colony from previous cultures of 24 hours on the solidified oil agar medium. Plates were incubated at 25$^oC$ for 14 days. The bacteria isolates were selected on the basis of their counts and growth on the minimum salt medium supplemented with crude oil (Forcados Blend) and used crankcase oil. The bacteria isolates were further sub cultured on the surface of already prepared MSM before subculturing on Nutrient Agar. Ten bacterial isolates that were able to utilize both crude and used crankcase oil as their sole sources of carbon and energy were stored on slant bottles for biochemical characterization and 16SrRNA analysis. The ten bacterial isolates were then used for biodegradation experiment.

## 3.2 Characterization of Bacterial Isolates

Microscopic and biochemical tests were carried out on the bacterial isolates to determine their probable identities. The result of each test was recorded and the probable identity of the bacteria was determined by the use of Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994).H

### 3.2.1 Microscopy Test

#### 3.2.1.1 Gram's staining

A heat fixed smear from 24 hours old culture was stained with crystal violet solution for 1-2 minutes, the dye was washed off with clean water. The slide was flooded with Gram's Iodine to react for 1 minute and washed off with 95% alcohol until no more violet runs from the slide, the slide was then rinsed with clean water and counter stained with Safranin for 1-2 minutes. Wash the slide with water, air dried and observed at X1000 magnification using oil immersion under microscope. Gram positive bacteria stained purple while Gram negative bacteria stained red.

### 3.2.2 Biochemical Tests

The isolate bacteria were subjected to different biochemical test to determine their probable identities. The tests include, catalase test, coagulase test, Oxidase test, Urease test, Indole test, Methyl red-Voges-Proskauer test, Casein hydrolysis, Starch hydrolysis, Production of $H_2S$ and Fermentation of sugars including lactose, fructose, glucose, mannitol, xylose and dulcitol as described by Barrow and Feltham (1999). Details of the methods used for Biochemical tests in Appendix II.

## 3.3 Molecular Identification

Ten bacterial isolates were selected for the molecular characterization. They were made up of 5 strains each from the crude oil and used crankcase oil contaminated soil samples. The molecular characterizations were carried out using standard method at the Environmental Microbiology Laboratory, Patagonian National Research Center (CENPAT- CONICET), Puerto Madryn, Argentina.

### 3.3.1 Extraction of Total Genomic DNA

Single colony of each isolate on LB agar plates were selected and used to inoculate 1ml of LB broth in 10ml test tubes. The tubes were incubated aerobically at $37^oC$ overnight on a rotary shaker at 160 rpm after which the 1ml of the cultures were transferred to 1.5ml eppendoff tubes and centrifuged at 800 rpm for 1 min. The cells pellet were washed with 500µl STE buffer and centrifuged again at 800 rpm for 1 min,

and the cell pellets were resuspended in 557µl TE buffer. This was followed by the addition of 100µl of Tris- saturated phenol.

The mixture was then mixed thoroughly using a vortex mixer for 60s to lyse the cells and the lysate centrifuged at 12000 rpm for 5 min at 4$^{o}$C to separate the aqueous phase from the phenol. The aqueous supernatant was then transferred to a fresh microcentrifuge tube, and 0.6mL of isopropanol was added and the DNA precipitation was performed at room temperature after which the tubes were centrifuged for 10 min at 1600rpm to pellet DNA. The pellet was washed twice with 500 ul of 70% Ethanol to remove salts. The DNA were then dried at 25$^{o}$C for 20 minutes, and then resuspended in 50 µl of Tris-HCl 10 mM at pH 8.0. The DNA that was used were later diluted to 1:10 in sterile double distilled water and stored at 4ºC.

The diluted DNA was then quantified using a machine from Hoechst 33258 (Amersham Biosciences, Piscataway, Wj) and a Fluorometer Hoefer DyNA Guant 200 (Hoefer Scientific Instruments, San Francisco, CA). DNA samples were run on 0.8% Agarose gel with 200ng DNA per lane.

### 3.3.2 Polymerase Chain Reaction (PCR) Amplification of 16S rRNA genes

In order to determine the phylogenetic grouping of the ten bacteria isolates from the contaminated soil samples, their 16SrRNA was amplified by using standard PCR. About 2ng/µl solution of DNA was prepared for each bacteria strain. PCR analysis was performed on the isolates using 1 X buffer, 1.5mM $MgCl_2$, 0.2mMd NTPs, 0.2uM universal Primers (27F and 1492R) designed to target the conserved region of bacteria 16srRNA. The PCR mixture contained 0.04U/ul of the enzyme Taq polymerase and 1 or 10ng DNA template. The program used consisted in: 5 min at 94ºC, the PCR cycles consisted of 40 cycles of denaturation for 1 min at 94ºC, annealing at 55ºC for 1 min, elongation at 72ºC for 90 s, before cooling at 72ºC for 15 minutes. An Agarose gel (3.5 ul of the 25-ul reaction) indicated that 1 ng DNA was the optimal concentration for the amplification, with the exception of strain 9, in which the DNA amplification was visible at 10ng. The amplified products were analyzed by electrophoresis on 2% Agarose gel. The rest of the reaction was used to digest the HaeIII and RsaI restriction enzyme and

incubated overnight. Restriction Fragment Length Polymorphism (RFLP) was then carried out.

### 3.3.3 16S rRNA Sequencing and Phylogenetic Analysis of Isolates

PCR of 16S rRNA gene (using primers 27F and 1492R) was repeated for the bacterial strains, and the PCR products were purified using the kit from Promega SV Wizard for PCR. The PCR products were analyzed by electrophoresis on 0.8% agarose gels with DNA marker III as the molecular weight marker to confirm that the right sized fragments were inserted into the vector. Clones carrying the correct inserts were sequenced to obtain the 16s rRNA nucleotide sequences which were used for BLAST (Basic Local Alignment Search Tool) searches at http://blast.ncbi.nlm.nih.gov/Blast.cgi to confirm the identity of the isolates.

Sequences were analyzed phylogenetically using the MEGA 4 program (Tamura et al., 2007). Phylogenetic trees were constructed using the neighbour joining (NJ) algorithm. Stability among the class was assessed with 1000- replication bootstrap analysis.

## 3.4 Isolation of Plasmids

Pure culture of the bacteria strains were grown in Luria Bertani broth and incubated for 16-24 hours, after which the strains were harvested into a phosphate buffer. About 200 µL of buffer 1A was then added to the cells and the mixture was vortexed, after which 400 µL Lysing solutions is added to the cell pellets and the tubes are inverted at room temperature for 20 minutes. After 20 minutes, 300 µL ice cold buffer 2B (Appendix III) was added, vortexed again and kept on ice for 30 minutes. The mixture was then centrifuged at 300rpm for 15 minutes, 700 µL of chloroform was added to the supernatant and vortexed again while supernatant was then centrifuged again at 300rpm for 10 minutes. Two layers were then formed and 1ml of ice cold absolute ethanol was then added to the 500 µL aqueous layer and kept on ice for 1 hour and centrifuged again at 300rpm for 30 minutes. The pellets were washed with 70% ethanol, decanted and dried. 100 µL of buffer 3C was added to the dry pellet. The plasmid DNA was loaded along with the loading buffer into the wells. Electrophoresis was carried out on 0.8%

agarose gel and the DNA bands were viewed under UV trans-illuminator. Molecular weight of the isolated plasmids was determined by using online molecular weight calculator at www.insilico.ehu.es.

### 3.5. Inoculum Development and Biodegradation Experiment

Colonies of the bacterial isolates grown on agar plate's i.e. *Providencia* species, *Alcaligenes* sp., Bacillus sp., *B. cereus*, and *Proteus* sp. were washed separately into distilled water in 250 ml Erlenmeyer flasks and the absorbance at 600nm for each bacterial isolates in the distilled water was taken using GENESYS 10 UV scanning Spectrophotometer. The bacteria inoculum was standardized and then used for the biodegradation experiment.

MSM (8.5mL) was dispensed into labelled bottles and sterilized, after cooling 1.5mL of each sterilized hydrocarbon was added to the labeled bottles separately. About 0.5mL of the already prepared bacteria suspension was then used to inoculate each of the labelled bottles. A control devoid of the bacteria suspension was also prepared for each of the hydrocarbon.

All the experiments were carried out in duplicate. Two different nitrogen salts, namely ammonium nitrate and ammonium chloride ($NH_4NO_3$ and $NH_4Cl$) were added to the MSM preparation separately and labelled accordingly to determine the one that will favour the biodegradation process. All the experiments were carried out at room temperature for 20 days.

#### 3.5.1 Gravimetric Estimation of Hydrocarbon Oil Degradation

Estimation of the residual oil was carried out on every 5 days interval for 20 days. The mixture that contains the MSM, hydrocarbon oil and the bacterial isolate was centrifuged at 400 rpm for 15 minutes. Residual oil was then extracted by liquid-liquid extraction as described by Adebusoye et al. (2007). Equal volume of N-hexane (1.5mL) was added to the liquid culture in flask and shaken thoroughly. The mixture was then put in a separating funnel and mixed very well until the mixture is separated into two phases; aqueous and organic phases. The organic phase was then discharged into a pre-weighed Petri dish and left for 24hrs to allow the N-Hexane to evaporate.

The percentage of the residual oil that remained was then determined by using gravimetric method. In this method, the initial weight of the hydrocarbon oil is taken before the experiment and the final weight of the treated oil is also taken after the N-Hexane has evaporated. i.e.

$$\% \text{ of the degraded oil} = \frac{\text{Weight of the hydrocarbon oil (initial)} - \text{weight of oil after treated (final)} \times 100}{\text{Weight of hydrocarbon oil (initial)}}$$

The rate of degradation of crude oil and used crankcase oil was determined by statistics analysis using Ward method (Clifford and Stephenson, 1975).

### 3.5.2 Total Bacterial Count Determination

Growth pattern of each of the bacterial isolate was determined every 5 days interval by measuring the optical density at 600nm. Total viable counts (cfu/ml) of the isolates were also determined by using pour plate technique.

About 1ml of the aliquot was taken from the sampling bottle containing the mixture of MSM and bacterial suspension, introduced into cuvette and the absorbance was read at 600nm using the same spectrophotometer.

For total viable count, 1ml was also taken from the sampling bottle and serial dilution was carried out up to $10^{-5}$, 0.2ml was then taken from the appropriate diluents and introduced into the already prepared Nutrient Agar plate using spread plate method. Plates were then incubated at $37^{0}C$ for 24hrs. Colonies were counted and recorded as cfu/ml for each of the isolate. Absorbance of the bacterial isolate at 600nm using GENESYS 10 UV scanning spectrophotometer was also taken to confirm the viability of bacterial isolates used in this experiment.

### 3.5.3 Gas Chromatography Analysis for Aliphatic and Aromatic Hydrocarbons in Biodegraded Samples

The biodegraded and control oil were separated into the aliphatic profiles and aromatic hydrocarbons profiles by packing the glass column with activated alumina neutral and activity/grade.

About 10ml of the treated packed alumina was cleaned properly with redistilled hexane. The oil was introduced onto the alumina and allowed to run down with the aid of

the redistilled hexane to remove the aliphatic profiles into a pre-cleaned 20ml capacity glass container. The aromatic fraction was removed by allowing the mixture of hexane and dichloromethane into the pre-cleaned borosilicate beaker.

The mixture was concentrated to 1.0ml by passing it through a stream of the Nitrogen gas before chromatography analysis.

#### 3.5.3.1 Gas Chromatography Analysis for Poly Aromatic and Aliphatic Hydrocarbons in Biodegraded Samples

Analysis of the PAH was performed by Gas Chromatography. The GC model was HP 6890 II Hewlett Packard gas 1, equipped with a split injector and a Flame Ionization Detector (FID) both set at 250$^{o}$C-320$^{o}$C, using Nitrogen as carrier gas. The Nitrogen column pressure was 30psi, the column was fused silica capillary column (30m X 0.25µm, and the film thickness is 25µm). The initial temperature was 60$^{o}$C for 3 min, while the temperature programming was 15$^{o}$C min$^{-1}$ for 14 mins and maintained for 3 min and also 10$^{o}$C min$^{-1}$ for 5 min and maintained for 4 min and the injection volume was 1µl. The chromatograph was powered with HPCHEM software. The same procedure was used for aliphatic hydrocarbon analysis except that the temperature programming was 10$^{o}$C min$^{-1}$ for 20 mins and maintained for 4 min and also 15$^{o}$C min$^{-1}$ for 4 min and maintained for 10 min and the injection volume was also 1µl. The chromatographs were analysed using peak area ratio to determine extent of degradation of different carbon chains present with period of degradation.

# CHAPTER FOUR

# RESULTS

## 4.1 Sample Collection and Isolation of Bacterial Isolates

Total bacteria counts in crude oil and used crankcase oil soil samples were 220 cfu/ml and 245 cfu/ml respectively at the 2nd weeks of the experiment (Table 2), but as the week of the experiment progresses, the total bacteria counts were reducing in the two soil samples contaminated with crude oil and used crankcase oil, reaching the counts of 203 cfu/ ml and 215 cfu/ml respectively by the fourth week. Also the Total oil degrading bacteria counts in the soil samples contaminated with crude oil and used crankcase oil respectively at the 4th week were 62 cfu/ml and 57 cfu/ml, at the 6th week, the Total Bacteria Count had dropped to 195 Cfu/ml and 205 Cfu/ml in crude and used crankcase oil respectively, while the Total Oil Degraders increased to 108 and 103 cfu/ml in soil samples contaminated with crude and used crankcase oil respectively.

By the 10th week, the Total oil degrading bacteria counts increased to 205 cfu/ml and 245 cfu/ml in both soil samples (Table 2). The result obtained showed that the total oil degrading bacteria were increasing in the contaminated soil samples as they were metabolizing the hydrocarbons, while the Total Bacteria counts were decreasing in both soil samples contaminated with crude and used crankcase oil.

## Table 2: Total Bacteria counts and Total Oil Degraders in Contaminated Soil Samples

| HYDROCARBONS | DURATION | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2nd Weeks (Cfu/ml) | | | 4th Weeks (Cfu/ml) | | | 6th Weeks (Cfu/ml) | | | 8th Weeks (Cfu/ml) | | | 10th Weeks (Cfu/ml) | | |
| | TBC | TOD | $^{TOD}/_{TBC}$ | TBC | TOD | $^{TOD}/_{TBC}$ | TBC | TOD | $^{TOD}/_{TBC}$ | TBC | TOD | $^{TOD}/_{TBC}$ | TBC | TOD | $^{TOD}/_{TBC}$ |
| Crude oil | 220 | 45 | 1:5 | 203 | 62 | 1:3 | 195 | 108 | 1:2 | 162 | 178 | 1:1 | 145 | 205 | 2:1 |
| Used Crankcase Oil | 245 | 35 | 1:7 | 215 | 57 | 1:4 | 205 | 103 | 1:2 | 147 | 215 | 2:1 | 123 | 245 | 2:1 |

Key

TBC – Total Bacterial Count
TOD – Total Oil Degraders

## 4.2 Isolation and Identification of Bacteria

### 4.2.1 Morphology and Biochemical Characterisation of Bacterial Isolates

Forty –two bacteria isolates obtained from the two soil samples contaminated with crude oil and used crankcase oil. The ten (10) bacteria that were able to utilize these two hydrocarbons as their sole source of carbon and energy were then characterized. Results of the biochemical characterization tests (Table 4) revealed that the organisms belong to four different genera namely; *Providencia*, *Bacillus*, *Alcaligenes* and *Proteus* (Table 3). Among these bacterial isolates, members of the genus *Bacillus* had 50% of occurrence, followed by the genus *Providencia.*

*Bacillus* species stained Gram positively, while *Proteus* sp, *Alcaligenes* sp and *Providencia* species were Gram negative. All the bacteria strains were catalase positive and were all motile. Out of the five *Bacillus* species isolated from the soil samples, only one was identified to species level, *Bacillus cereus*. *Bacillus cereus* and *Bacillus* sp 3 were urease positive, while the remaining 3 were urease negative. Also, *Bacillus cereus* was producing both gas and acid during sugar fermentation. All the *Bacillus* species were able to hydrolyze gelatin and catalase, Indole and Methyl Red negative.

*Proteus* sp and the 3 *Providencia* species were Indole and Methyl Red positive. While *Proteus* species were Urease positive, can hydrolyze Gelatin and Starch but all the species of *Providencia* were Urease negative and cannot hydrolyze both Gelatin and starch. *Alcaligenes* was able to hydrolyze starch and cannot produce acid during the fermentation of Lactose, Fructose, Maltose and Arabinose sugars.

**Table 3: Results of Microscopy and Biochemical Characterisation Tests**

| Isolates | GR | SH | MO | CA | OX | UR | IN | MR | VP | CH | GH | SH | $H_2S$ | FERMENTATION OF SIGARS LA | FR | GL | MA | AR | RA | XY | DU | PROBABLE IDENTITY |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BD1 | - | R | + | + | - | - | + | + | - | - | - | - | - | - | A | A | A | - | - | - | - | *Providencia* sp 1 |
| BD2 | - | R | + | + | - | - | + | + | - | - | - | - | - | - | A | A | A | - | - | - | - | *Providencia* sp 2 |
| BD3 | - | R | + | + | - | + | + | + | - | - | + | + | + | - | A/G | A/G | - | - | - | AG | - | *Proteus* sp |
| BD5 | - | R | + | + | - | - | + | + | - | - | - | - | - | - | A | A | A | - | - | - | - | *Providencia* sp 3 |
| BD4 | + | R | + | + | + | - | - | - | - | + | + | + | - | A | A/G | A | A | A/G | A/G | A | A | *Bacillus* sp 1 |
| BD7 | + | R | + | + | + | + | - | - | - | + | + | + | - | A/G | A/G | A/G | A/G | A/G | A/G | A/G | A/G | *Bacillus cereus* |
| BD6 | + | R | + | + | + | - | - | - | - | + | + | + | - | A | A | A | A | A/G | A /G | A | A | *Bacillus* sp 2 |
| BD8 | + | R | + | + | + | + | - | - | - | + | + | + | - | A | A | A | A | A/G | A | A | A | *Bacillus* sp 3 |
| BD9 | - | R | + | + | + | - | - | - | - | - | - | + | - | - | - | A | - | - | A | A | A | *Alcaligenes* sp |
| BD10 | + | R | + | + | - | - | - | - | - | + | + | + | - | A | A/G | A | A | A/G | A | A | A | *Bacillus* sp 4 |

+: Positive reaction, -: negative reaction, R: Rod Shaped, SH: Shape, MO: Motility, CA: Catalase Test, OX: Oxidase Test, UR: Urease Test, IN: Indole Test, MR: Methyl Red Test, VP: Voges-Proskauer Test, CH: Casein Hydrolysis, GH: Gelatin Hydrolysis, SH: Starch Hydrolysis, $H_2S$: $H_2S$ Production, LA: Lactose, FR: Fructose, GL: Glucose, MA: Mannitol, AR: Arabinose, RA: Raffinose, XY: Xylose, DU: Ducitol.

### 4.3.2 Phylogenetic Characterization of Isolates

Analysis of the 16SrRNA sequencing of the ten bacterial isolates by PCR with Primers 27F and 1492F resulted in characteristics bands of about 50bp in 0.8% Agarose gel (Plate 1). The molecular weight ladder at the left represented the Lambda Hind III, while the right mass lanes represented each of the bacterial isolates. Lanes 1, 2 and 5 represented *Providencia* species, lane 3 was for *Proteus* sp, while lanes 4, 7. 8 and 10 were for *Bacillus* species, lane 6 was for *Bacillus cereus*, and lane 9 represented *Alcaligenes* sp. The result showed that the γ Hind of the bacteria strains had a molecular weight of 2.03 to 23.13. The amount of DNA in ng used for each of the bacterial isolates varies between 20-200 ng.

The Restriction Fragment Length Polymorphism (RFLP) analysis showed five unique patterns. Strains 1, 2, and 5 had the same RFLP pattern which are for *Providencia* species, strains 4, 7, 8, and 10 had the same RFLP pattern (*Bacillus* species), while strains 3, 6 and 9 showed unique pattern each for *Proteus*, *Bacillus cereus* and *Alcaligenes* respectively (Plate 2). A bacteria strain was chosen from each of the RFLP analysis that has the same pattern for the 16SrRNA sequencing. The obtained sequences (Appendix IV) were used for a BLAST search. Results of the BLAST search showed that the bacterial isolates can be classified into four distinct groups with high sequence identities (≥92%) with type strains. Five bacterial isolates were gram positive belonging to class Bacilli, while the gram negative isolates belong to three distinct classes of the Phylum Proteobacteria, the beta-Proteobacteria which consist of the *Alcaligenes* sp and the gamma-Proteobacteria consist of the *Providencia* species and the *Proteus* sp.

Some of the isolates had the same percentage similarity with type strains, indicating that they may belong to the same genera. The three strains of *Providencia* species (OCR1, OUEI, and OCR2), had 99% similarity with *Providencia rettgeri* CTB05 and 96% similarity with *Providencia stuartii* (Table 4). *Bacillus* species (OUE3, OCR3, OUE5 and OUE6) showed similarity to their phylogenetic descendants in their genus, while *Bacillus cereus* (OUE4) showed 92% sequence identity with *Bacillus cereus* ATCC14579. *Alcaligenes* sp. (OCR4) showed sequence identities with *Alcaligenes faecalis* MRb512 and *Alcaligenes* sp. JF3 while *Proteus* sp. (OUE2) also showed 95-99% similarities to its phylogenetic descendants in the genus (Table 4).

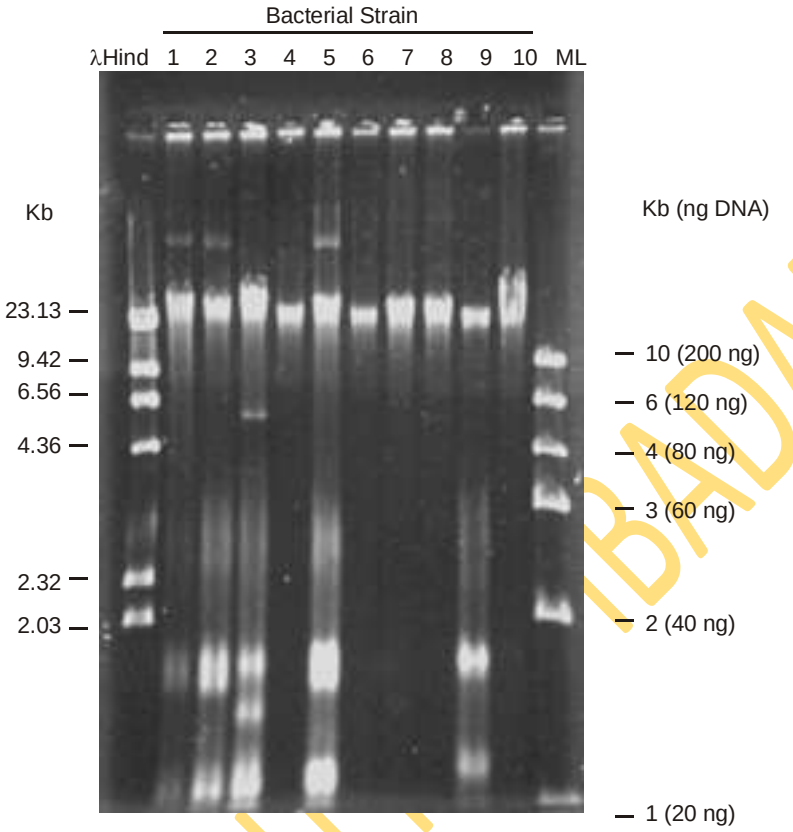



Plate1. Agarose gel with genomic DNA purified from strains 1 to 10. Molecular weight ladders: left, lambda HindIII; right, mass ladder. Lanes 1, 2, 5: *Providencia* spp., 3: *Proteus* sp., 4, 7, 8, 10: *Bacillus* sp., 6: *Bacillus cereus*, 9: *Alcaligenes* sp.

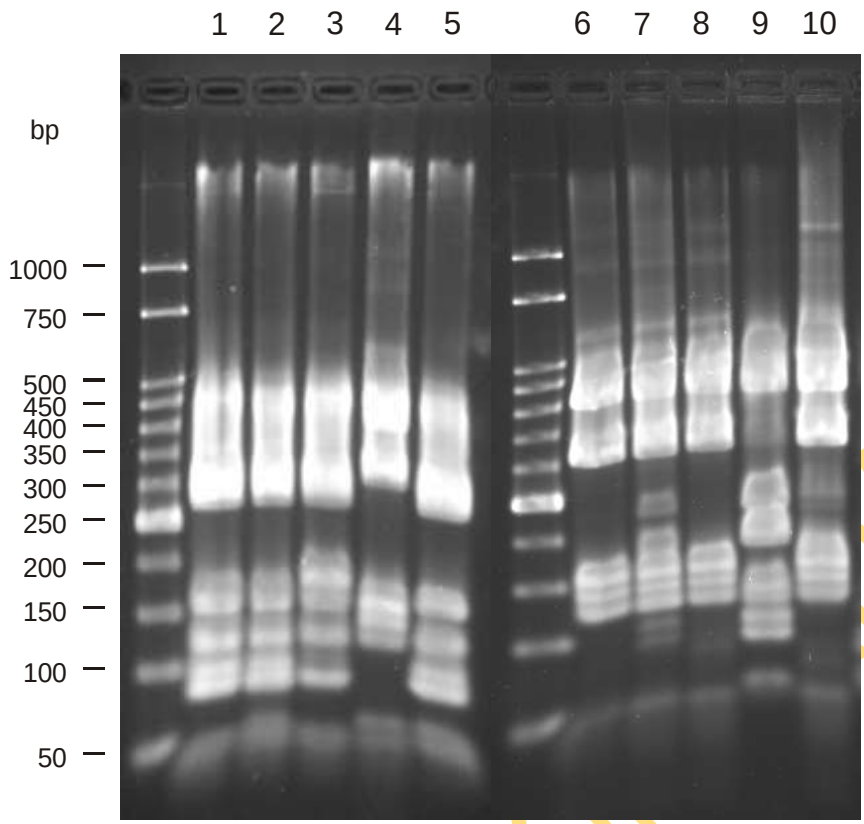



Plate 2. Agarose gel with 16S rRNA gene RFLP analysis of strains 1 to 10. Molecular weight ladder: 50 bp.

RFLP analysis showed five unique patterns and one strain per each pattern was chosen for 16s rRNA sequencing. Lanes 1, 2, 5: *Providencia* spp., 3: *Proteus* sp., 4, 6, 8, 10: *Bacillus* sp., 7: *Bacillus cereus*, 9: *Alcaligenes* sp.

**Table 4: Phylogenetic Identities of the Bacterial Isolates from the Contaminated Soil Samples**

| Bacterial Division | # Best Phylogenetic Match | % Identity | Number of Isolates |
|---|---|---|---|
| Firmicutes | *Bacillus cereus* | 92 | 1 |
| | *Bacillus* sp | 90 -100 | 4 |
| β- Proteobacteria | *Alcaligenes* sp | 75-100 | 1 |
| γ- Proteobacteria | *Providencia* sp | 94-96 | 3 |
| | *Proteus* sp | 95-99 | 1 |

### 4.3.3 Sequence and Phylogenetic Analysis

Percentage similarities of the sequence of 16S rRNA of the bacterial strains were compared with that of database. Database sequences and the sequences of the bacterial isolates that give the highest scores were retrieved to construct the phylogenetics trees. Separated neighbour-joining trees were used for the bacterial isolates. One phylogenetic tree was constructed for both *Providencia* species and *Proteus* sp. using *Escherichia coli* KI2 for the outgroup (Fig. 3). The sequence and phylogenetic analysis showed that the deduced nucleotides of the bacterial strains BD1, BD2 and BD5 formed a distinct cluster with the *Providencia rettgeri* CTB05, *Providencia stuartii* ATCC2991 and the other *Providencia* species (Fig. 3), while the bacteria strain BD3 formed another clusters with its evolutionary descendant like *Proteus myxofaciens* NCIMB1327, *Proteus mirabilis* HI4320, and *Proteus penneri* ENT229 (Fig. 3).

Similar analysis of BD4, BD6, BD8, and BD10, showed that it formed three distinct clusters (Fig. 4), while BD7 alone formed a distinct cluster with *Bacillus cereus* ATCC14579 with 92% similarity and *Bacillus anthracis* TC-3 with 46% similarity. While the remaining bacterial isolates formed three distinct clusters (Fig. 4). *Clostridium cellulolyticum* H10 was used as the outgroup bacteria strain for the *Bacillus* species.

The phylogenetic tree constructed for the bacterial strain BD9 showed only one distinct cluster (Fig. 5) with the closest evolutionary relationship to the *Alcaligenes faecalis* with subspecies *faecalis* AE1 and *faecalis persicum*, Alcaligenes sp. JF3 and *Alcaligenes faecalis* MRbS12 (Fig. 5). *Nitrosomonas eutropha* C91 was used as the outgroup bacterial strain. (Appendix IV)

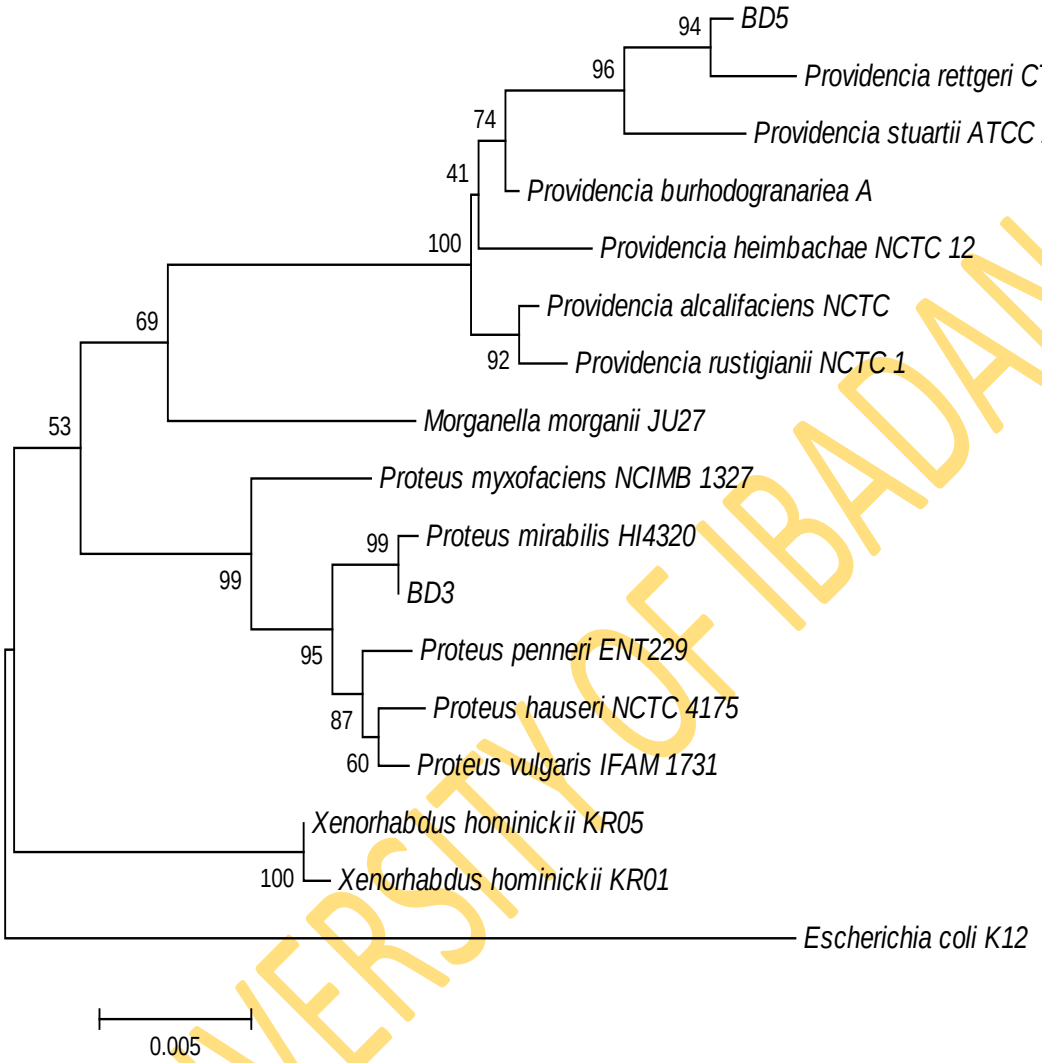



Fig. 3. Neighbour-joining phylogenetic tree based on partial 16S rRNA gene sequences of strains 3 and 5, as well as related genera. Numbers at nodes represent the percentage occurrence of nodes in 100 bootstrap trials. Bar represents 0.005 nucleotide substitutions per site. Outgroup: 16S rRNA gene from *Escherichia coli* K12. Phylogenetic analyses were conducted in MEGA4.1.

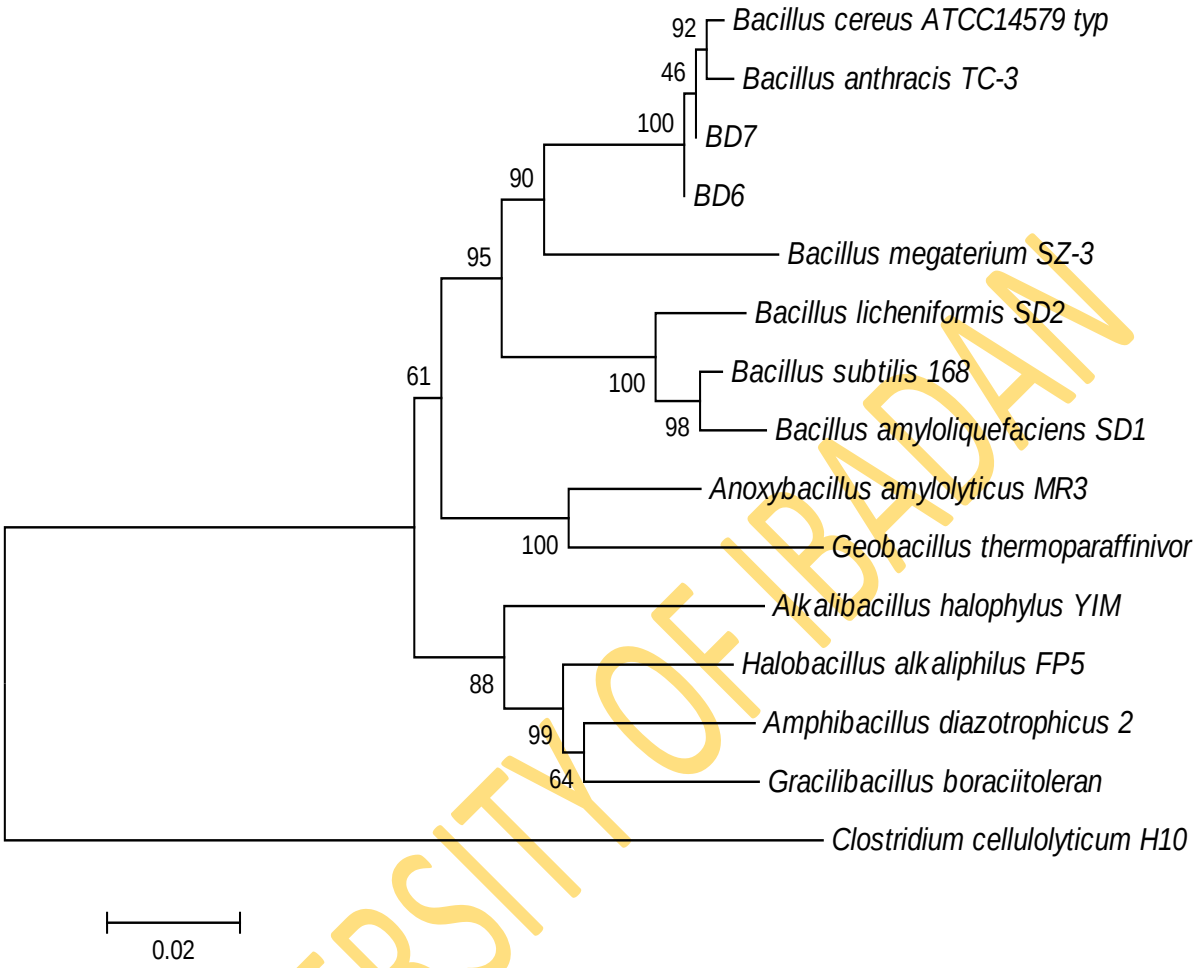



Fig. 4. Neighbour-joining phylogenetic tree based on partial 16S rRNA gene sequences of strains 6 and 7, as well as related genera. Numbers at nodes represent the percentage occurrence of nodes in 100 bootstrap trials. Bar represents 0.02 nucleotide substitutions per site. Outgroup: 16S rRNA gene from *Clostridium cellulolyticum* H10. Phylogenetic analyses were conducted in MEGA4.1.

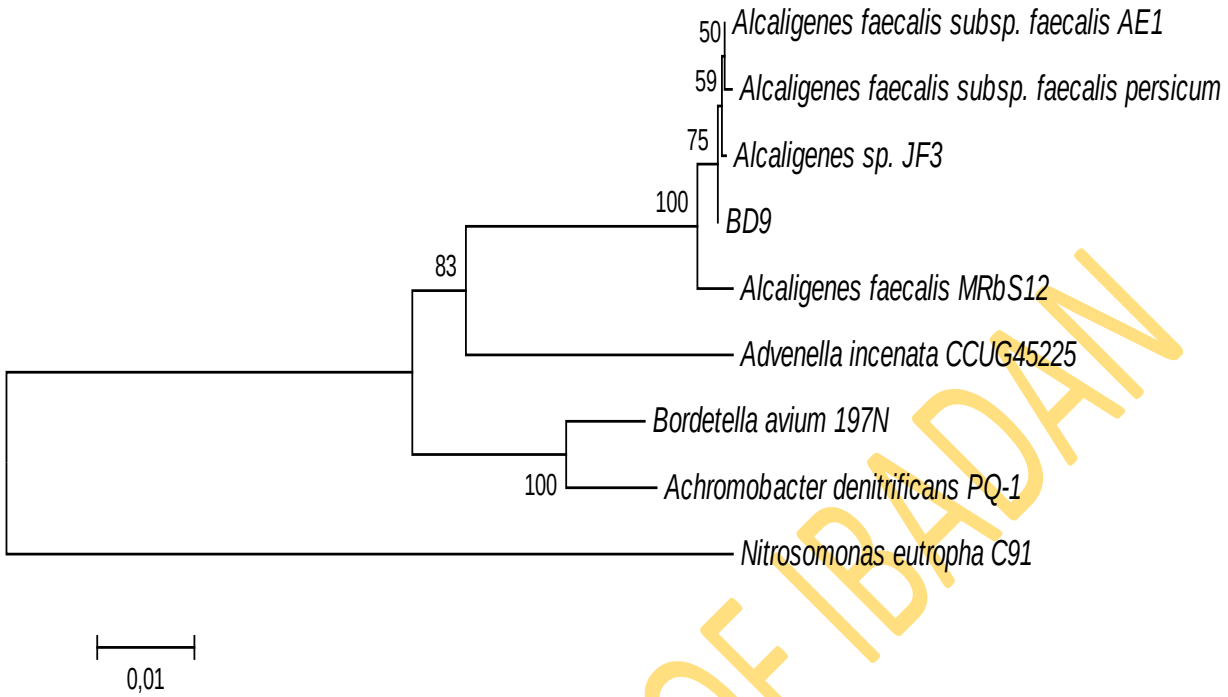



Fig. 5. Neighbour-joining Phylogenetic tree based on partial 16S rRNA gene sequences of strain 9 (*Alcaligenes*) and related genera. Numbers at nodes represent the percentage occurrence of nodes in 100 bootstrap trials. Bar represents 0.01 nucleotide substitutions per site. Outgroup: 16S rRNA gene from *Nitrosomonas eutropha* C91. Phylogenetic analyses were conducted in MEGA4.1.

### 4.4 Plasmid profile

Plate3. shows the plasmid profile of the bacterial strains, a total of 4 out of the bacterial isolates carried various sizes of plasmids. The percentage of the bacterial strains that carried plasmid corresponded to 40%, however two out of four isolates which were *Providencia* sp 1 (BD1) and *Bacillus* sp 1 (BD4) carried 2 plasmids in their genomes (Plate 3). The organisms and the plasmid sizes are as follows; *Providencia* sp 1 (1.88kb and 1.26kb), *Providencia* sp 3 (BD5) ((32.71kb), *Bacillus* sp 1 (2.58kb and 2.03kb) and *Bacillus* sp 4 (BD10) (1.37kb)

1 2 3 4 5 6 7 8 9 10 11 12

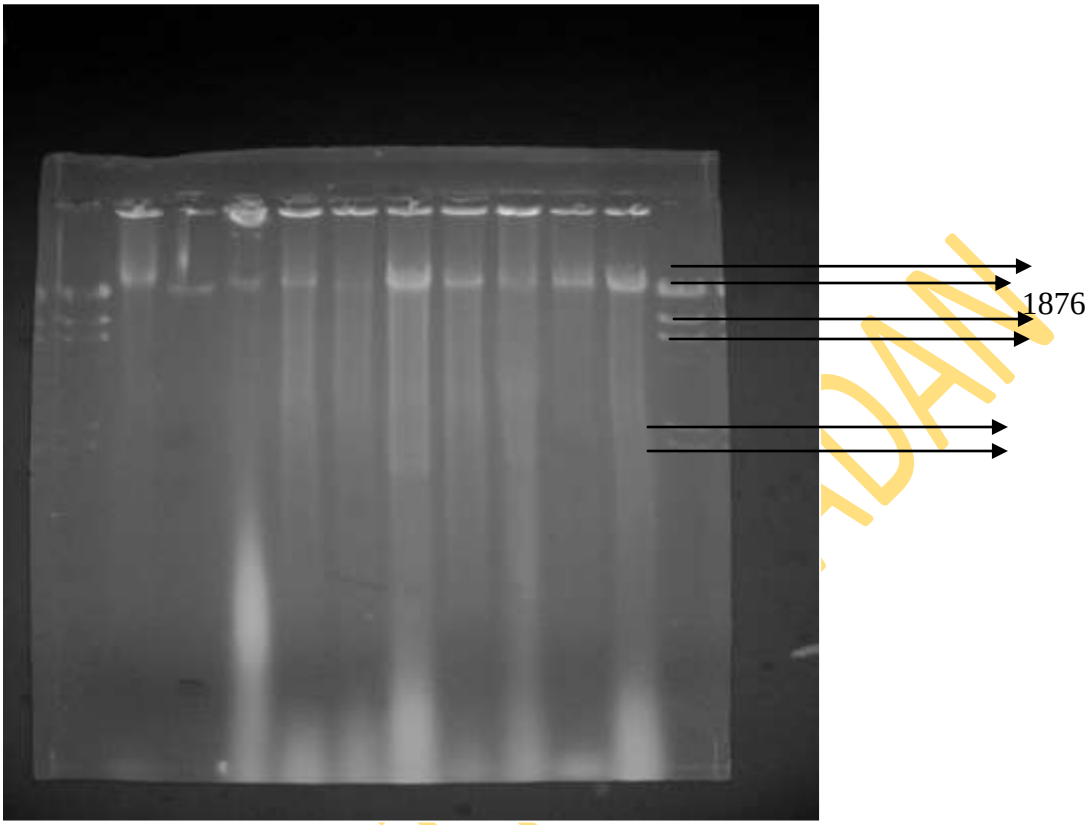



Plate3. Plasmid profiles of the bacterial isolates in 0.8% agarose gels. From the right (All molecular weights were calculated using on online molecular weight calculator at insilico.ehu.es)

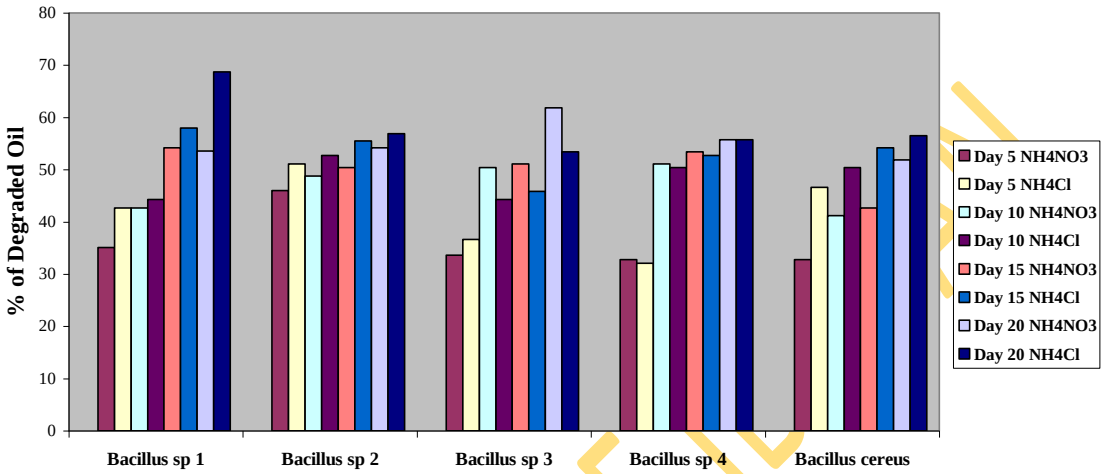
Lane 1 – DNA HIND III digest molecular weight marker
Lane 2 – *Bacillus cereus*
Lane 3 – *Bacillus* sp 1 had plasmids of 2577bp and 2031 bp
Lane 4 – *Bacillus* sp 4 had a plasmid of 1366bp
Lane 5 – *Bacillus* sp 2
Lane 6 – *Providencia* sp 1 had plasmids of 1876bp and 1261bp.
Lane 7 – *Alcaligenes* sp
Lane 8 – *Providencia* sp 2
Lane 9 – *Providencia* sp 3 had plasmid of 32707bp
Lane 10 – *Bacillus* sp 3
Lane 11 – *Proteus* sp
Lane 12 – DNA HIND III digest molecular weight marker.

### 4.5 Percentage of the Residual Oil

#### 4.5.1 Percentage of the Degradation Rate of Crude Oil by Bacterial Isolates

Fig. 6A shows the degradation of crude oil by *Bacillus* species. *Bacillus* sp 1 showed the highest degradation rate of 53.6 and 68.7% in the MSM supplemented with $NH_4NO_3$ and $NH_4Cl$ respectively, followed by *Bacillus* sp 2. *Bacillus cereus* showed the lowest degradation rate of 51.9 and 56.9% in the MSM supplemented with $NH_4NO_3$ and $NH_4Cl$ respectively (Fig. 6A).

Fig. 6B shows the degradation of crude oil by the bacterial strains. *Providencia* sp3 had the highest degradation of crude oil with the rate of 77.1 and 71.0% in the MSM supplemented with $NH_4NO_3$ and $NH_4Cl$ respectively followed by *Providencia* sp 1. While *Alcaligenes* sp had the lowest degradation rate of 53.4 and 55.2% in the MSM supplemented with $NH_4NO_3$ and $NH_4Cl$ respectively. (Appendix V)




**Bacterial Strains**

**Fig 6A: Rate of Degradation of Crude Oil by Bacillus species with NH4NO3 and NH4Cl as Nitrogen Sources**

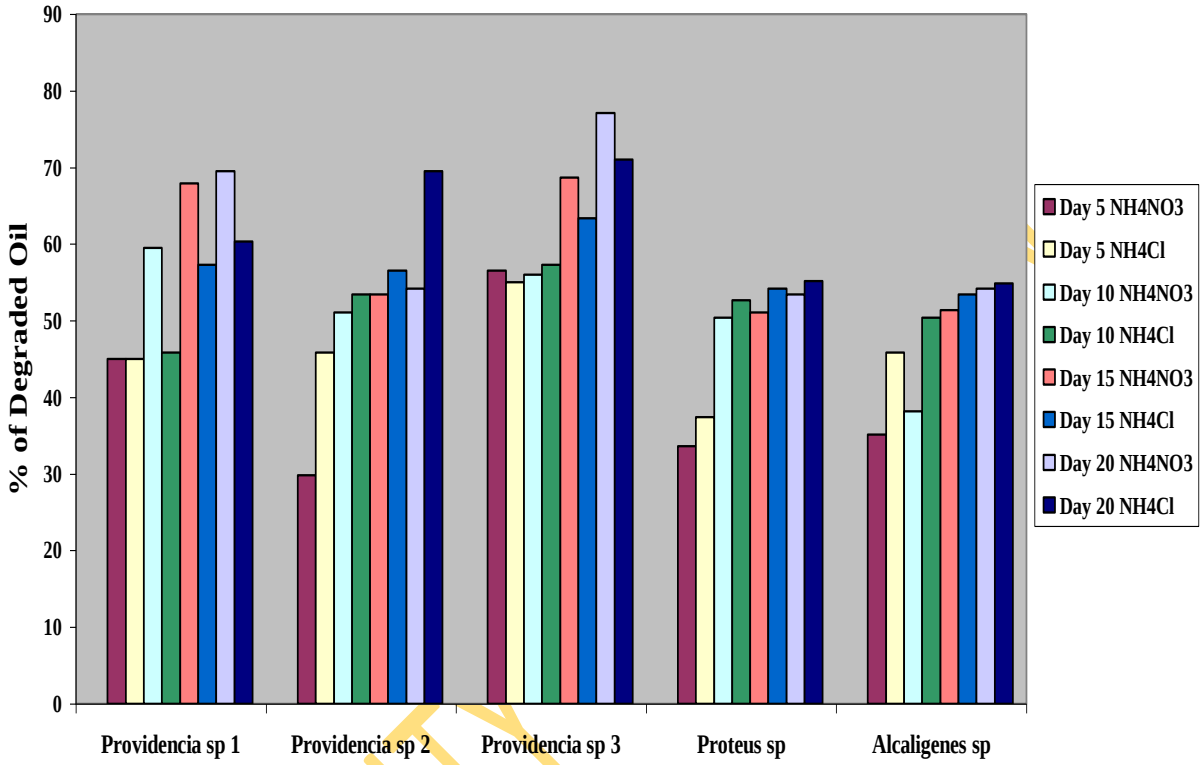



**Fig 6B: Rate of Biodegradation of Crude Oil by Bacterial Isolates with NH4NO3 and NH4Cl as Nitrogen Sources**

### 4.5.2 Percentage of the Degradation Rate of Used Crankcase Oil by Bacterial Isolates

Fig. 7A shows the degradation of used crankcase oil by *Bacillus* species. *Bacillus* sp 4 showed the highest degradation rate of 60.4 and 67.4% in the MSM supplemented with $NH_4NO_3$ and $NH_4Cl$ respectively, while Bacillus sp 2 followed. *Bacillus* sp 3 showed the lowest degradation rate of 47.7 and 42.4% in the MSM supplemented with $NH_4NO_3$ and $NH_4Cl$ respectively (Fig. 7A).

Fig. 7B shows the degradation of used crankcase oil by the bacterial strains. *Providencia* sp 1 had the highest degradation rate of 70.5 and 75.8% in the MSM supplemented with $NH_4NO_3$ and $NH_4Cl$ respectively followed by *Providencia* sp 3. While *Proteus* sp had the lowest degradation rate of 47.0 and 47.2% in the MSM supplemented with $NH_4NO_3$ and $NH_4Cl$ respectively (Appendix VII)

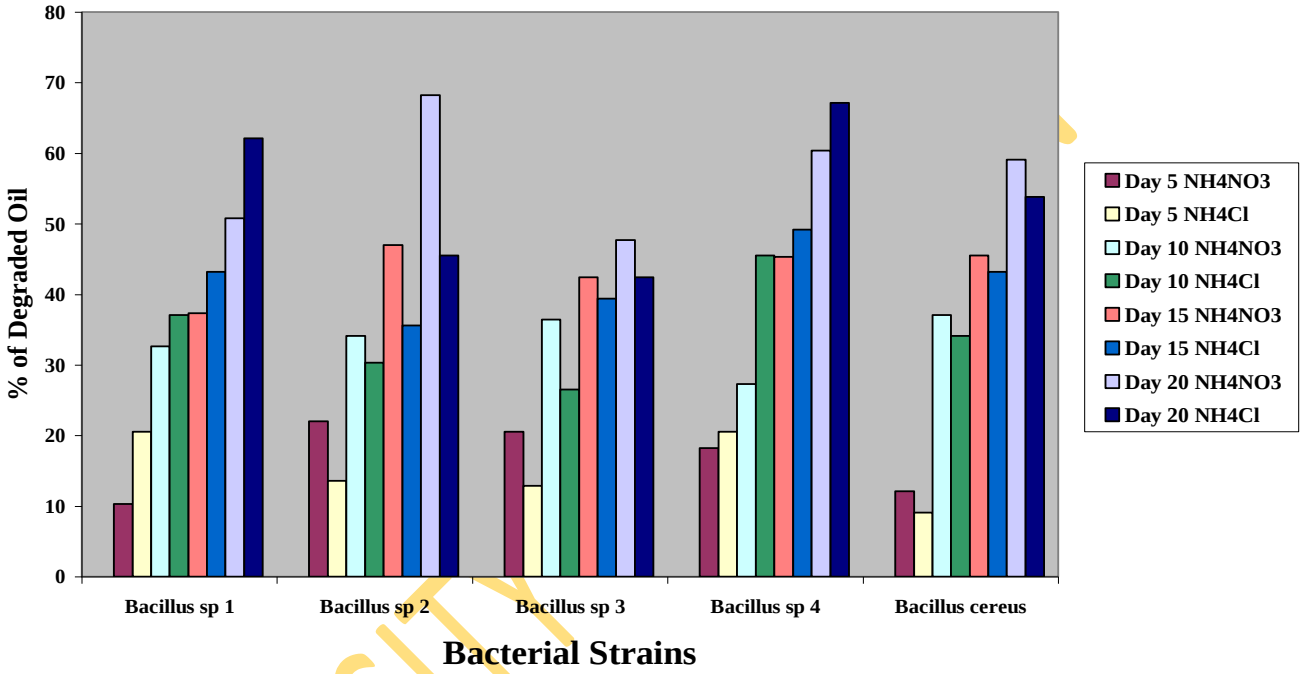



**Fig 7A: Rate of Degradation of Used Crankcase Oil by Bacillus species with NH4NO3 and NH4Cl as Nitrogen Sources**

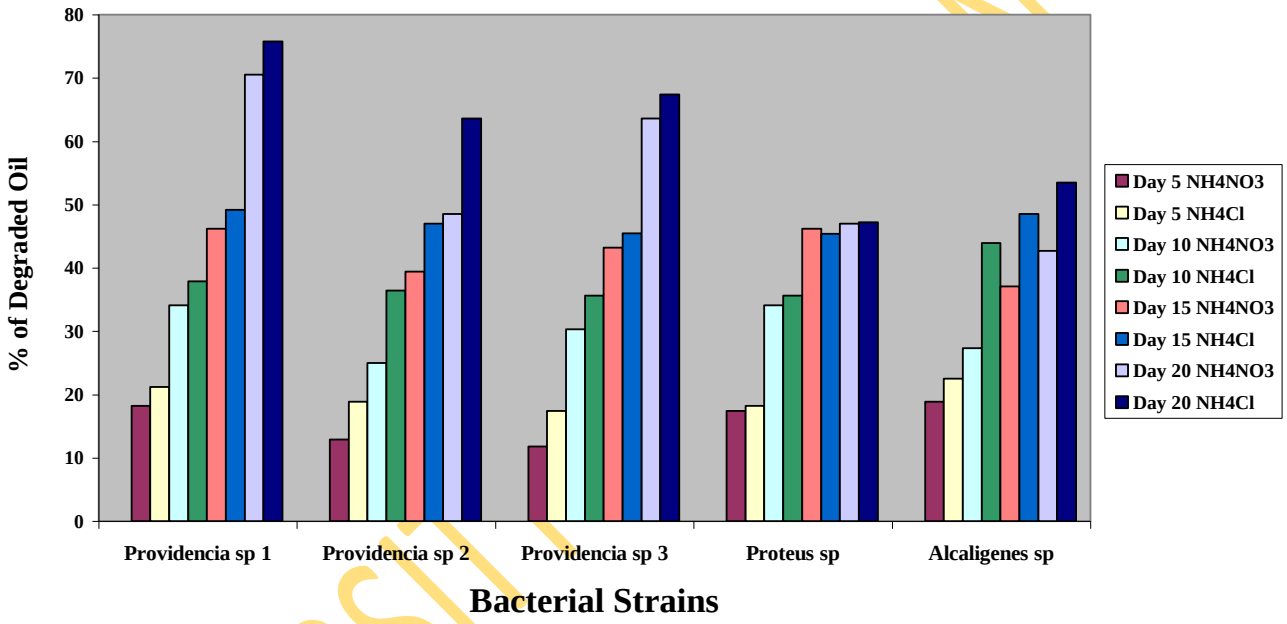



**Fig 7B: Rate of Biodegradation of Used Crankcase Oil by Bacterial Isolates with NH4NO3 and NH4Cl as Nitrogen Sources**

### 4.5.3 Statistical Analysis

Statistical analysis using the Ward method to determine the different groups of the bacterial isolates based on their degradation rate.

The degradation abilities of the 10 bacterial isolates were analyzed statistically using Ward method. The result showed two distinct Clusters (A and B). The genetic dissimilarity coefficients between Cluster A varied widely, and it is further divided into 3 subgroups (1, 2, 3) (Fig. 8). Subgroups 1 and 3 both consisted of sub-groups 'a' and 'b' respectively (Appendix VI). *Providencia* sp 1 and sp 3 that had the highest degradation rate for crude oil were found in Cluster b (Fig. 8). While *Bacillus cereus*, *Bacillus* sp 2 and *Alcaligenes* sp which had the lowest degradation rate were found in the subgroup 3 in Cluster A (Appendix VI).

The genetic dissimilarity coefficient based on the degradation abilities of the 10 bacterial isolates to utilize used crankcase oil as their carbon source was analyzed statistically using Ward method. Two distinct Clusters (A and B) were identified, Clusters A and B were further divided into subgroups 1 and 2 (Fig. 9). These two Subgroups both consisted of sub-groups 'a' and 'b' respectively (Appendix VIII). Subgroup 2 consists of three bacterial isolates that had better degradation abilities. *Providencia* sp 3 and *Bacillus* sp 4 belong to sub-group a, while *Providencia* sp 1 belong to sub-group b (Fig. 9) (Appendix VIII).

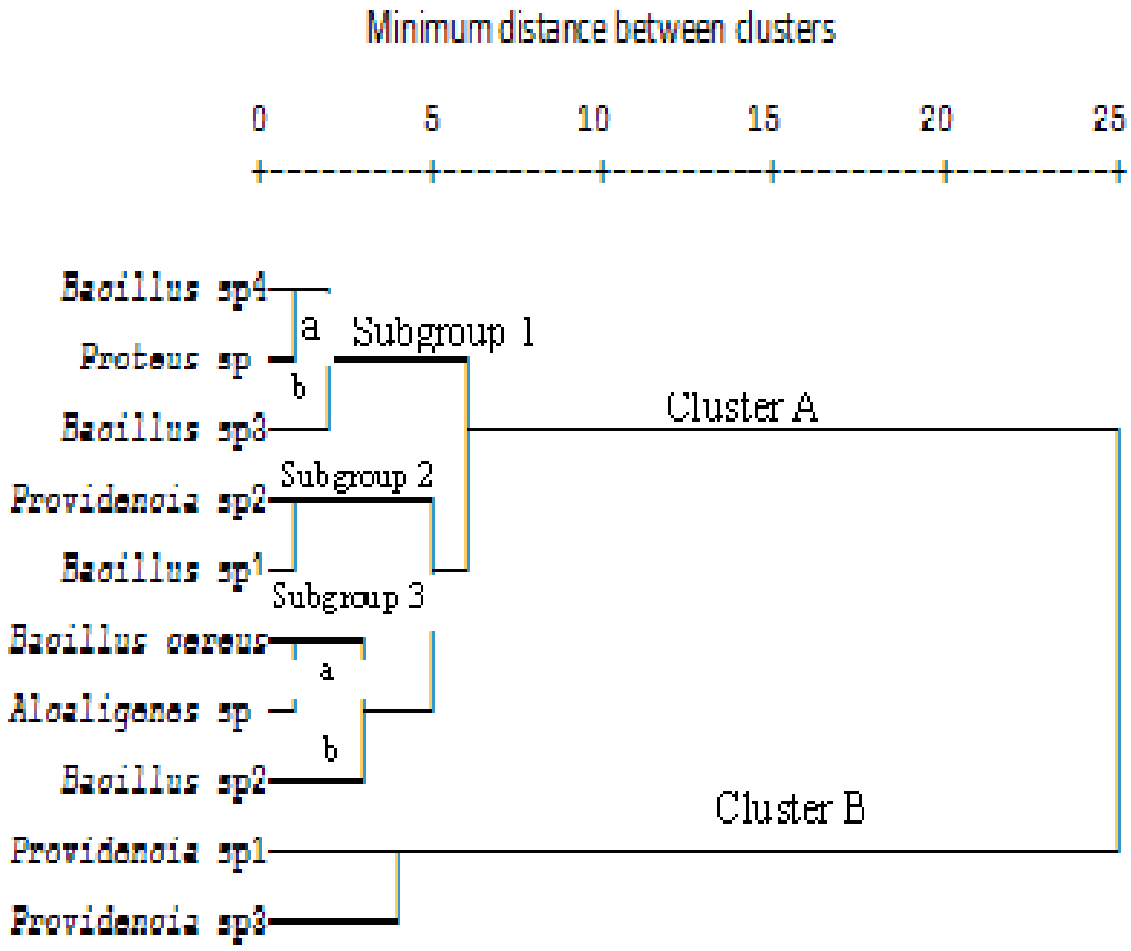



Fig. 8 Dendrogram generated using Ward clustering method depicting genetic relationship among ten bacterial isolates based on their degradation potential of using crude oil as the sole carbon source

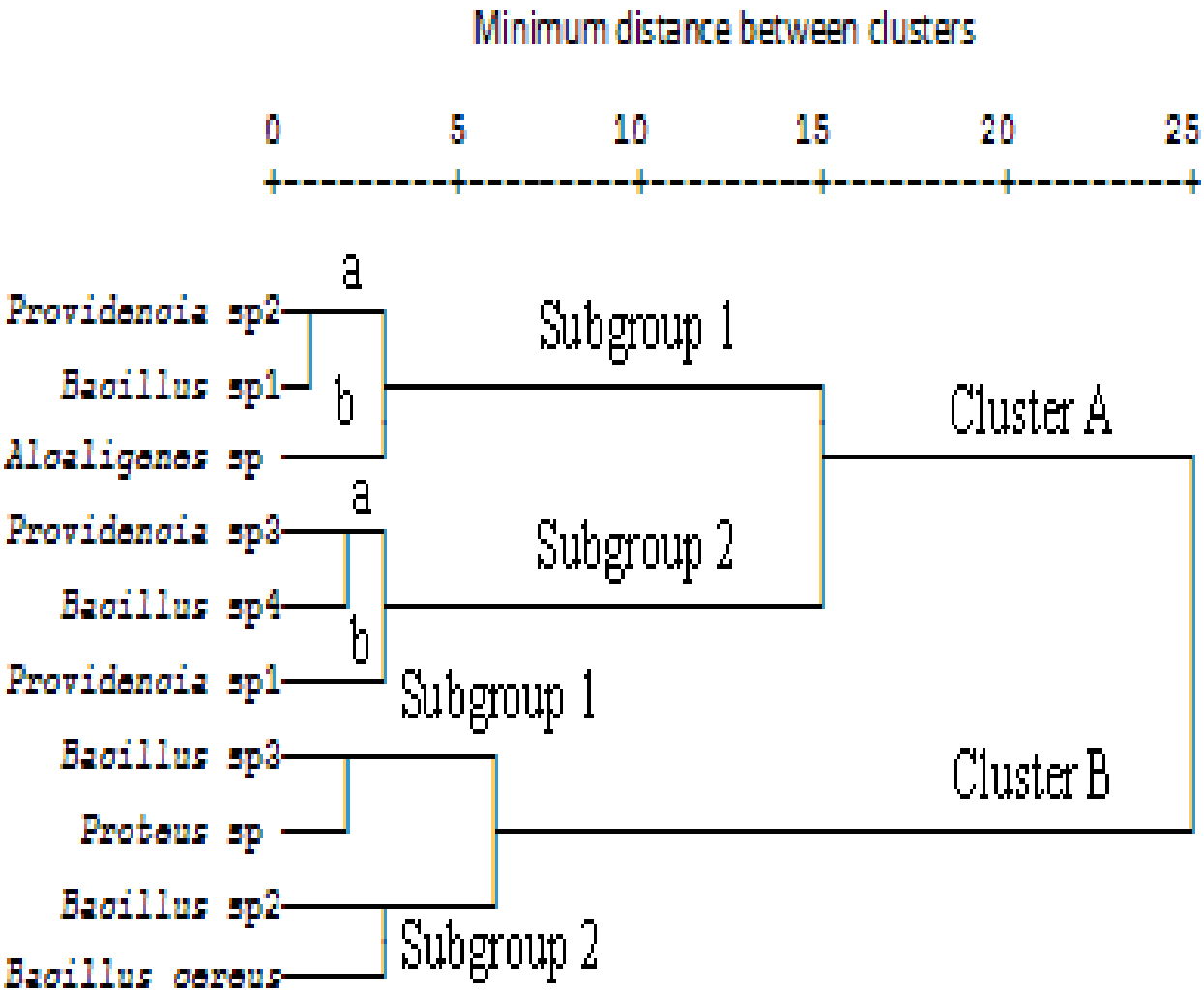



Fig. 9. Dendrogram generated using Ward clustering method depicting genetic relationship among ten bacterial isolates based on their degradation potential of using used crankcase oil as the sole carbon source

### 4.5.4 Bacteria Growth in Hydrocarbons

#### 4.5.4.1 Determination of Optical Density of Bacteria and Total Bacteria Count in MSM Supplemented With Crude Oil

The optical density of the bacterial isolates was determined at 600nm at days 5, 10, 15 and 20. The result showed that there was a gradual increase in the absorbance measurement from day 5 to day 10 in most of the bacterial isolates but it reduced at day 15 in *Bacillus* sp. strain 2, *Bacillus cereus* and *Bacillus* sp. strain 4 (Table 5). But by day 20, the absorbance measurement of *Providencia* sp. strain 2, *Providencia* sp. strain 3, *Alcaligenes* sp. and *Bacillus* sp. strain 4 had reduced by the $20^{th}$ day (Table 5).

Total bacterial count was done to determine whether the bacterial isolates were viable or not during the experimental days. The result obtained showed that the bacteria were growing and metabolizing the hydrocarbons during the experimental days (Table 6). Total bacteria count increased from day 5 till day 15 and the count ranged from $1.20 \times 10^4$ Cfu/ml to $9.10 \times 10^4$ Cfu/ml (Table 6). By day 20, there was a gradual decrease in the Total bacterial count for all the bacterial isolates.

**Table 5. $OD_{600}$ of the Bacterial Isolates during their Growth in MSM Supplemented with Crude Oil**

| ORGANISM | DAYS | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0 | 5 | | 10 | | 15 | | 20 | |
| | | $NH_4NO_3$ | $NH_4Cl$ | $NH_4NO_3$ | $NH_4Cl$ | $NH_4NO_3$ | $NH_4Cl$ | $NH_4NO_3$ | $NH_4Cl$ |
| *Providencia* sp (strain 1) | 0.800 | 1.694 | 1.595 | 1.940 | 1.865 | 2.456 | 2.127 | 1.912 | 2.308 |
| *Providencia* sp (strain 2) | 0.800 | 1.419 | 1.602 | 1.869 | 1.963 | 2.485 | 2.464 | 1.751 | 1.528 |
| *Proteus* sp | 0.800 | 1.705 | 1.636 | 1.647 | 2.138 | 2.642 | 1.984 | 2.187 | 2.118 |
| *Bacillus* sp (strain 1) | 0.800 | 1.432 | 1.765 | 1.713 | 1.938 | 2.187 | 1.977 | 2.476 | 1.924 |
| *Providencia* sp (strain 3) | 0.800 | 1.525 | 1.572 | 1.423 | 1.506 | 2.315 | 1.986 | 2.100 | 1.784 |
| *Bacillus* sp (strain 2) | 0.800 | 1.481 | 1.862 | 1.601 | 1.908 | 1.482 | 1.647 | 1.510 | 1.785 |
| *Bacillus cereus* | 0.800 | 1.469 | 1.746 | 1.755 | 2.099 | 1.683 | 1.935 | 2.035 | 1.915 |
| *Bacillus* sp (strain 3) | 0.800 | 1.529 | 1.834 | 1.883 | 2.048 | 1.422 | 1.861 | 1.784 | 1.757 |
| *Alcaligenes* sp | 0.800 | 1.736 | 1.766 | 1.829 | 1.974 | 2.198 | 2.235 | 1.873 | 1.841 |
| *Bacillus* sp (strain 4) | 0.800 | 1.681 | 1.999 | 1.975 | 2.270 | 2.174 | 2.109 | 1.936 | 1.844 |

**Table 6: Total Hydrocarbon Degrading Bacteria Count on MSM Supplemented with Crude oil**

| ORGANISM | DAYS | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0 | 5 | | 10 | | 15 | | 20 | |
| | | $NH_4NO_3$ | $NH_4Cl$ | $NH_4NO_3$ | $NH_4Cl$ | $NH_4NO_3$ | $NH_4Cl$ | $NH_4NO_3$ | $NH_4Cl$ |
| | (Cfu/ml X $10^4$) | (Cfu/ml X $10^4$) | | (Cfu/ml X $10^4$) | | (Cfu/ml X $10^4$) | | (Cfu/ml X $10^4$) | |
| *Providencia* sp (strain 1) | 1.50 | 2.70 | 2.30 | 4.60 | 4.40 | 7.70 | 7.30 | 7.40 | 6.20 |
| *Providencia* sp (strain 2) | 1.80 | 3.10 | 2.90 | 4.40 | 3.80 | 7.50 | 6.90 | 8.10 | 6.30 |
| *Proteus* sp | 2.20 | 3.60 | 4.70 | 4.40 | 5.10 | 6.90 | 7.10 | 6.20 | 6.90 |
| *Bacillus* sp (strain 1) | 1.40 | 3.40 | 3.10 | 4.70 | 4.40 | 7.50 | 5.90 | 6.90 | 5.60 |
| *Providencia* sp (strain 3) | 1.20 | 2.80 | 2.30 | 4.60 | 4.20 | 8.30 | 5.90 | 7.90 | 5.30 |
| *Bacillus* sp (strain 2) | 1.30 | 3.20 | 2.10 | 4.80 | 3.60 | 7.40 | 7.70 | 6.70 | 7.30 |
| *Bacillus cereus* | 1.50 | 2.70 | 2.20 | 4.70 | 3.80 | 8.30 | 8.50 | 7.90 | 8.10 |
| *Bacillus* sp (strain 3) | 1.40 | 2.90 | 2.30 | 4.60 | 3.70 | 8.50 | 8.40 | 8.10 | 7.70 |
| *Alcaligenes* sp | 1.70 | 3.10 | 2.90 | 4.90 | 4.50 | 9.10 | 8.80 | 8.50 | 8.10 |
| *Bacillus* sp (strain 4) | 1.90 | 3.40 | 2.80 | 5.20 | 4.90 | 8.90 | 8.10 | 8.30 | 7.80 |

#### 4.5.4.2 Determination of Optical Density of Bacteria and Total Bacteria Count in MSM Supplemented With Used Crankcase Oil

The absorbance measurement of the bacterial isolates taken at 600nm showed an increase from day 0 to day 15 during the experimental days (Table 8). But there was a gradual decrease in the absorbance from day 15 till day 20, except in *Bacillus cereus* grown in MSM supplemented with NH4Cl, in which the absorbance measurement had increased from 1.594 to 2.113 (Table 7).

The total bacteria count ranged from $1.10 \times 10^4$ Cfu/ml to $9.70 \times 10^4$ Cfu/ml from day 0 to day 15 (Table 9). By day 20, the Total bacterial count has started reducing in all the bacterial isolates except in *Bacillus cereus* and *Bacillus* sp strain 3 in which the Total bacterial count has increased to $7.50 \times 10^4$ Cfu/ml and $7.20 \times 10^4$ Cfu/ml respectively in MSM supplemented with $NH_4Cl$ (Table 8).

**Table 7: $OD_{600}$ of the Bacterial Isolates during their Growth in MSM Supplemented with Used Crankcase oil**

| ORGANISM | DAYS | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0 | 5 | | 10 | | 15 | | 20 | |
| | | $NH_4NO_3$ | $NH_4Cl_2$ | $NH_4NO_3$ | $NH_4Cl$ | $NH_4NO_3$ | $NH_4Cl$ | $NH_4NO_3$ | $NH_4Cl$ |
| *Providencia* sp (strain 1) | 0.800 | 1.546 | 1.433 | 1.752 | 1.693 | 1.921 | 1.815 | 1.251 | 1.287 |
| *Providencia* sp (strain 2) | 0.800 | 1.222 | 1.625 | 1.472 | 1.755 | 1.698 | 1.921 | 1.005 | 1.798 |
| *Proteus* sp | 0.800 | 0.988 | 1.878 | 1.360 | 2.348 | 1.736 | 2.019 | 1.007 | 1.311 |
| *Bacillus* sp (strain 1) | 0.800 | 1.444 | 1.738 | 1.794 | 2.053 | 2.072 | 1.578 | 1.900 | 1.371 |
| *Providencia* sp (strain 3) | 0.800 | 1.294 | 1.591 | 1.344 | 1.604 | 1.628 | 1.737 | 1.580 | 1.420 |
| *Bacillus* sp (strain 2) | 0.800 | 1.470 | 1.620 | 1.819 | 1.968 | 2.297 | 1.740 | 1.339 | 1.725 |
| *Bacillus cereus* | 0.800 | 1.521 | 1.713 | 1.731 | 1.839 | 1.955 | 1.594 | 1.489 | 2.113 |
| *Bacillus* sp (strain 3) | 0.800 | 1.382 | 1.763 | 1.674 | 2.005 | 1.966 | 2.682 | 1.929 | 1.913 |
| *Alcaligenes* sp | 0.800 | 1.566 | 1.807 | 1.410 | 2.122 | 1.667 | 2.636 | 1.097 | 1.436 |
| *Bacillus* sp (strain 4) | 0.800 | 1.450 | 1.084 | 1.359 | 1.901 | 1.769 | 2.364 | 1.374 | 1.367 |

**Table 8: Total Hydrocarbon Degrading Bacteria Count on MSM Supplemented with Used Crankcase oil**

| ORGANISM | DAYS | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0 | 5 | | 10 | | 15 | | 20 | |
| | (Cfu/ml X $10^4$) | $NH_4NO_3$ (Cfu/ml X $10^4$) | $NH_4Cl$ | $NH_4NO_3$ (Cfu/ml X $10^4$) | $NH_4Cl$ | $NH_4NO_3$ (Cfu/ml X $10^4$) | $NH_4Cl$ | $NH_4NO_3$ (Cfu/ml X $10^4$) | $NH_4Cl$ |
| *Providencia* sp (strain 1) | 1.20 | 2.90 | 3.20 | 4.10 | 4.80 | 9.20 | 9.70 | 8.70 | 9.30 |
| *Providencia* sp (strain 2) | 1.30 | 2.40 | 3.00 | 2.80 | 3.50 | 6.90 | 7.10 | 6.40 | 6.90 |
| *Proteus* sp | 1.60 | 2.70 | 2.10 | 4.80 | 3.90 | 7.80 | 8.40 | 7.50 | 8.10 |
| *Bacillus* sp (strain 1) | 1.80 | 2.90 | 2.70 | 4.50 | 4.40 | 7.20 | 7.40 | 6.70 | 6.90 |
| *Providencia* sp (strain 3) | 1.90 | 3.20 | 3.00 | 4.20 | 7.40 | 7.70 | 8.70 | 7.30 | 7.50 |
| *Bacillus* sp (strain 2) | 2.10 | 4.10 | 3.80 | 5.20 | 4.60 | 7.90 | 8.60 | 7.60 | 8.10 |
| *Bacillus cereus* | 1.10 | 2.30 | 2.40 | 3.10 | 4.30 | 6.70 | 6.60 | 6.30 | 7.50 |
| *Bacillus* sp (strain 3) | 1.30 | 2.50 | 2.10 | 3.40 | 2.80 | 5.40 | 6.30 | 5.10 | 7.20 |
| *Alcaligenes* sp | 2.30 | 4.20 | 3.90 | 5.60 | 5.50 | 8.10 | 8.30 | 7.70 | 7.90 |
| *Bacillus* sp (strain 4) | 1.30 | 3.90 | 3.70 | 5.50 | 5.30 | 7.90 | 8.80 | 7.10 | 8.30 |

## 4.6 Gas Chromatographic Analysis of the Aliphatic and Polycyclic Aromatic Hydrocarbons Profiles

### 4.6.1 Gas Chromatography results of the Aliphatic hydrocarbon profile present in the Crude oil

Fig.10. shows the degradation of Aliphatic carbon chains present in the crude oil by *Providencia* sp 1. The result showed that there was an increase in the amount of C16, 20, 26, 34, 35, 36 and 37 present in the degraded oil at $10^{th}$ when compared with the control but by day 20, the amount had reduced.

The amount of C10-C20 chains present in the crude oil increased to 219.18g/kg at day 10 when compared to the control (day 0) which was 184.47g/kg, but by day 20, it had reduced to 208.96 g/kg (Fig. 10). The amount of C21-C30 chains present in the crude oil was 600.44 g/kg in the control (day 0), but by day 10 it reduced to 157.07 g/kg and finally to 11.54 g/kg by day 20 (Fig 10). Also the amount of C31-C40 chains present in the control (day 0) was 136.88 g/kg but by day 10, it had increased to 346,419.29 mg/kg, but by day 20 it had reduced to 253,259.92 mg/kg (Fig. 10).

The result obtained showed that about 47.15% of the aliphatic hydrocarbon compound present in the crude oil has been degraded by *Providencia* sp 1 by day 20 (Appendix X A and B).

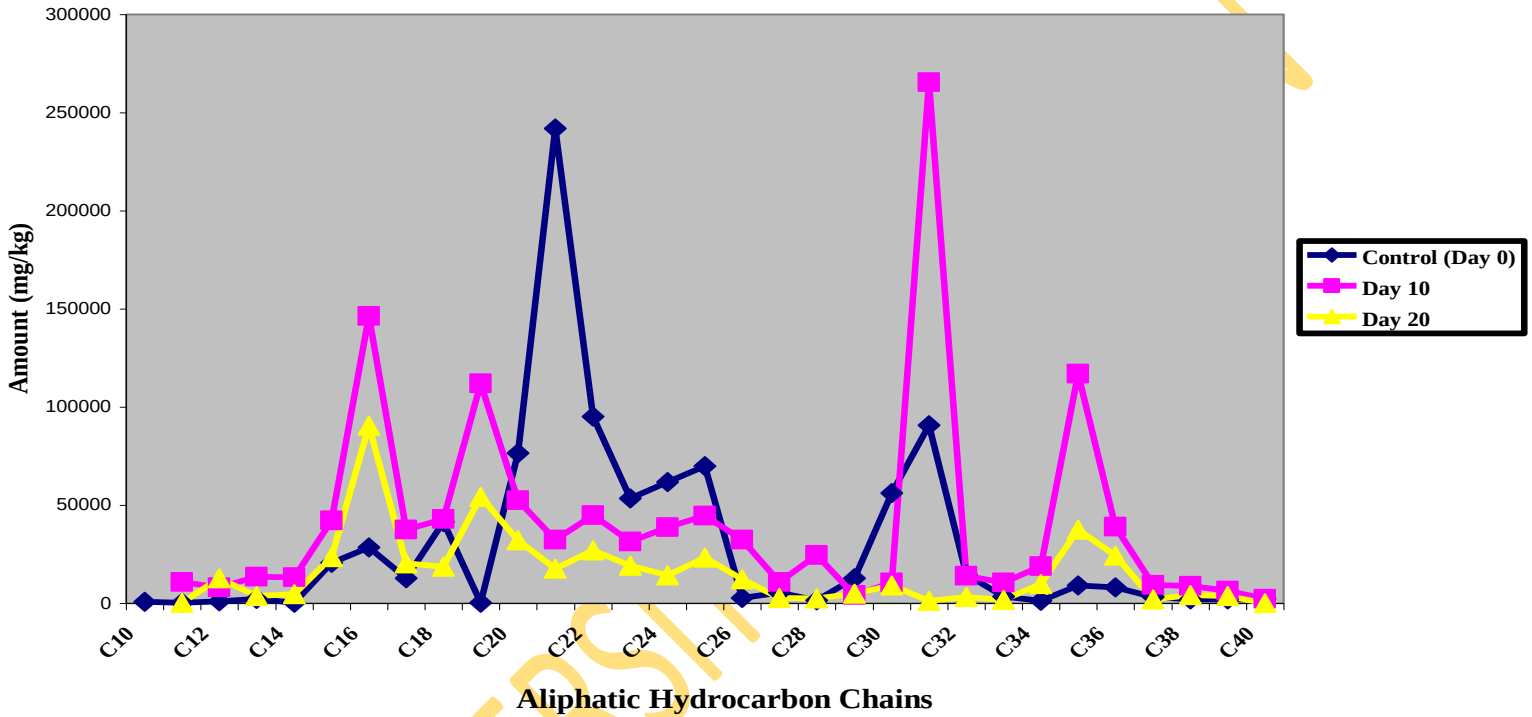



**Fig 10: Biodegradation of C- Chains present in Crude Oil by *Providencia* sp 1**

Fig. 11. shows the degradation of aliphatic carbon chains present in crude oil by *Providencia* sp 3. The GC analysis showed that Carbons 18, 20, 34, 35, 36 and 37 had increased in the degraded oil by day 10. But by day 20, these carbons chains have been degraded except in C18 which increased slightly at day 20.

The amount of C10-C20 chains present in the crude oil at day 0 (control) was 184.47 g/kg but by day 10 it has increased to 250.01 g/kg but by day 20, it has reduced to 173.57 g/kg. While the amount of C21-C30 present in the crude oil at day 10 had reduced to 145.09 g/kg as compared to 600.44 g/kg in the control (day 0) and by day 20, it has reduced further to 78.57 g/kg (Fig.11). C31-C40 chains present in the crude oil had increased to 253.25 g/kg as compared to 136.88 g/kg present on day 0 but by day 20, it has reduced considerably to 55.59 g/kg.

The amount of total aliphatic hydrocarbon compounds degraded from the crude was 65.62% at day 20 by *Providencia* sp 3 (Appendix XI A and B).

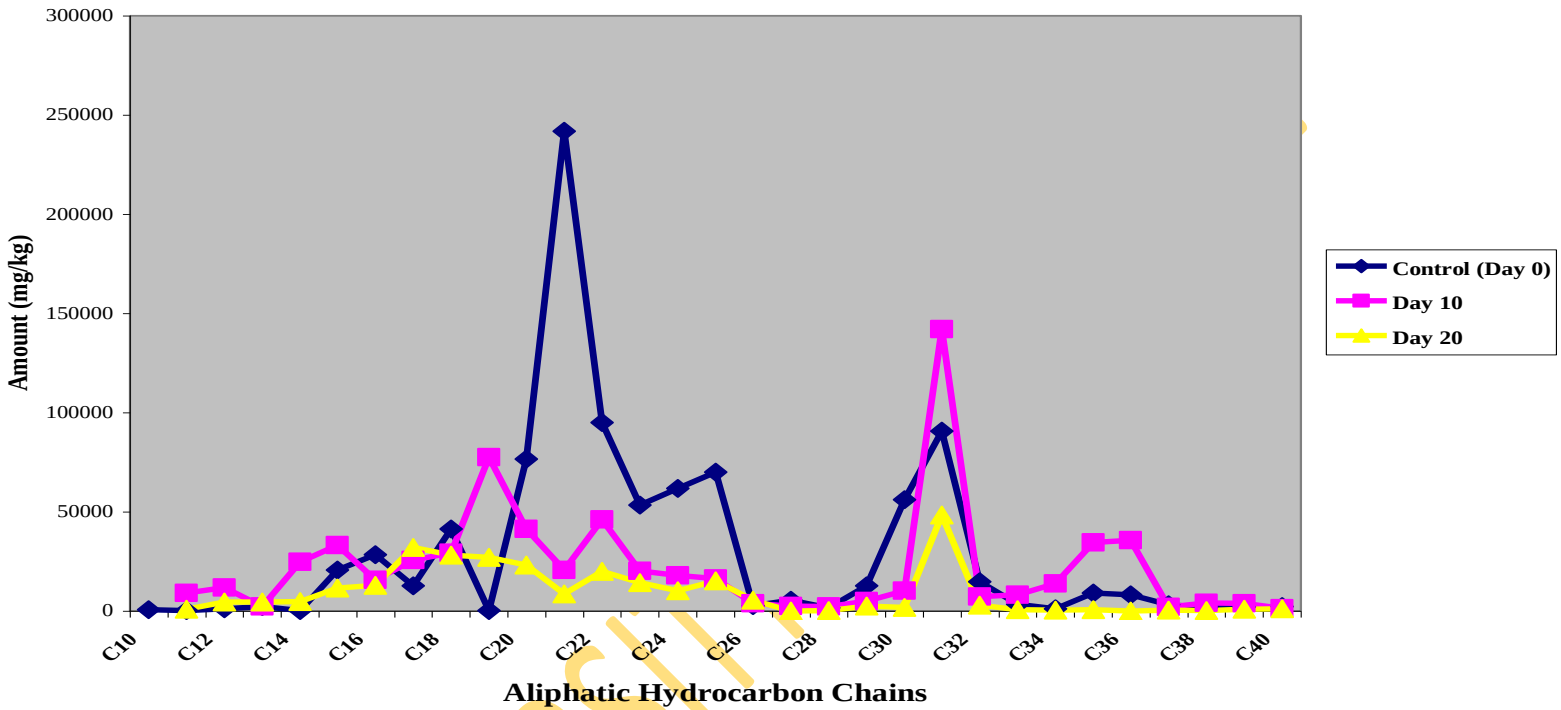



**Fig 11: Biodegradation of C- Chains present in Crude Oil by Providencia sp 3**

Fig. 12. shows the degradation of aliphatic carbon chains present in the crude oil by *Bacillus* sp 1. The results obtained showed that Carbons chain 12, 13, 14, 26, 27, 28 and 29 had increased in the degraded oil by day 10 as compared with the control, but by day 20 the amount has reduced considerably except Carbon 21 which had increased.

About 184.47 g/kg of C10-C20 was present in the control (day0) but by 20$^{th}$ day, it has reduced to 97.87 g/kg. For C21-C30 chains, the amount present in the crude oil at day 0 (control) was 600.44 g/kg but by day 20, it has reduced significantly to 7.43 g/kg (Fig. 12). At day 10, the amount of C31-C40 chains present in the crude oil was 121.97g/kg as compared to 136.88 g/kg present in the control (day 0) and by the 20$^{th}$ day it has reduced further to 76.79 g/kg.

Result obtained showed that about 77.20% of aliphatic compounds present in the crude oil had been degraded by *Bacillus* sp 1 (Appendix XII A and B).

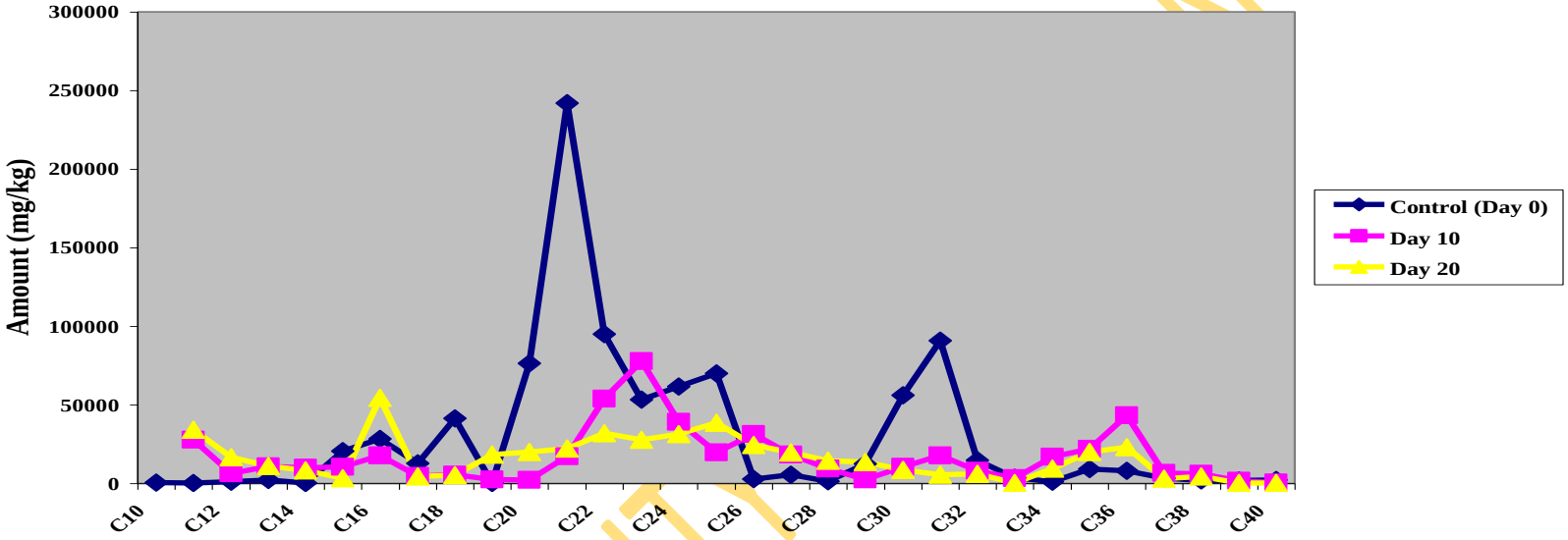



**Fig 12: Biodegradation of C-Chains present in Crude Oil by Bacillus sp 1**

### 4.6.2 Gas Chromatography Result of Aliphatic Hydrocarbon profile present in the Used Crankcase Oil as Degraded by *Providencia* sp 1

The GC analysis revealed that only few carbon chains were present in the control at day 0 (used crankcase oil). These were C15, C17, C18, C19, C20, C21, C23, C24, C25, C26, C28 and C33. The carbon chains are further grouped to C10-20, C21-30 and C31-40.

Fig. 13 shows the degradation of aliphatic carbon chains present in the used crankcase oil by *Providencia* sp 1. The amount of C10-C20 chains present in the control (day 0) was 464.44 g/kg but by day 20, only 2.47% of C10-C20 was degraded from the used crankcase oil. About 379.12 g/kg of C21-C30 chains of aliphatic hydrocarbon compounds was present in the control (day), but by day 20, 72.96% of C21-C30 chains had been degraded (Fig 13). About 8.44 g/kg of C31-C40 was present in the control (day 0) but by day 20, 99.47% of C31-C40 chains had been degraded by *Providencia* sp 1.

The total amount of aliphatic hydrocarbon compounds degraded by the *Providencia* sp 1 by day 20 was 34.85% (Appendix XIV A and B).

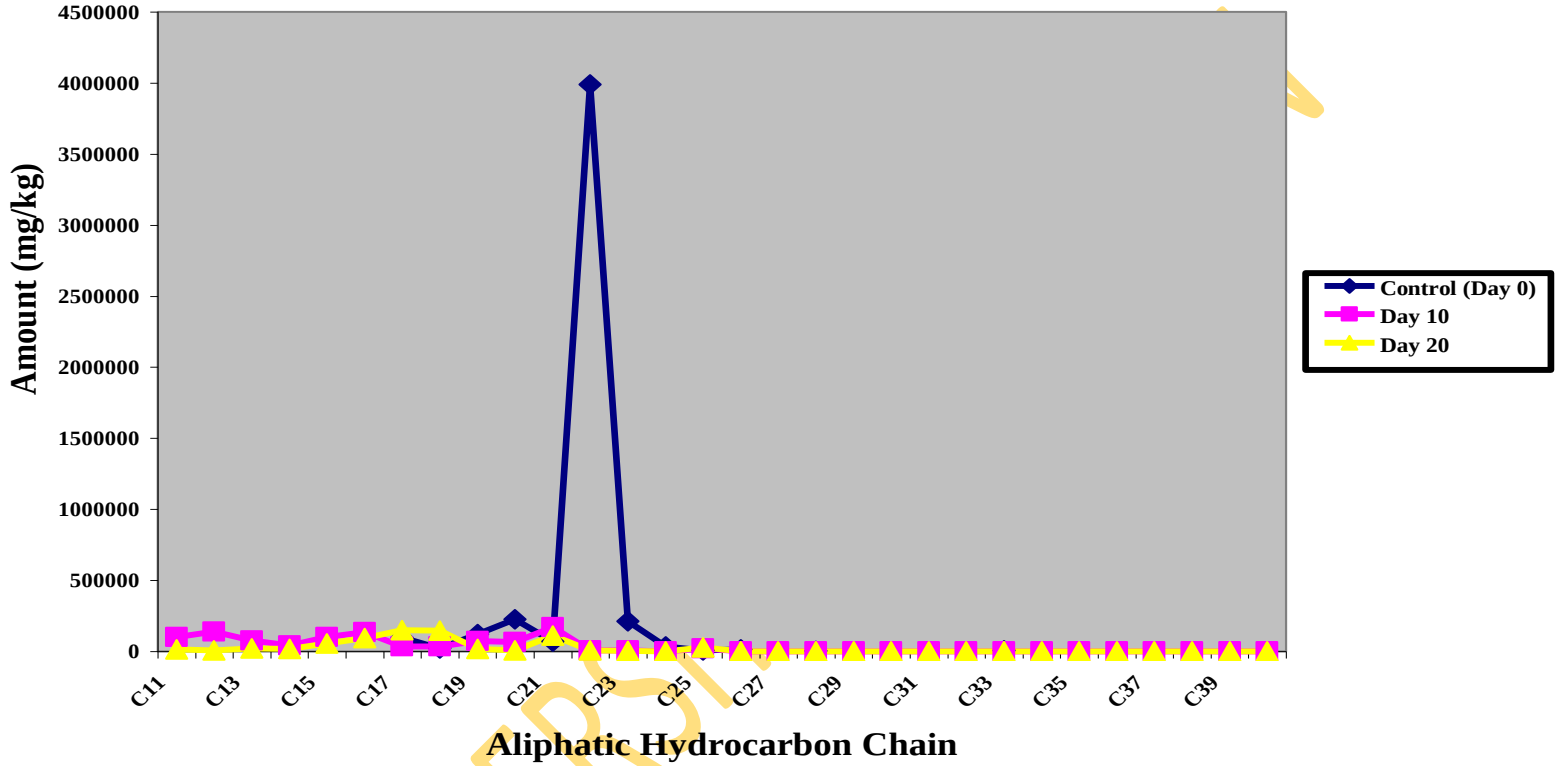



**Fig 13: Biodegradation of C-Chains present in Used Crankcase Oil by Providencia sp 1**

#### 4.6.2.2 Gas Chromatography Result of Aliphatic Hydrocarbon profile present in the Used Crankcase Oil as Degraded by *Providencia* sp 3

Fig. 14. shows the degradation of used crankcase oil by *Providencia* sp 3. About 469.44 g/kg of C10-C20 chains was present in the control (day 0), but by day 20, 28.32% had been degraded from this amount. At day 20, 92.85% of C21-C30 had been degraded from 379.12 g/kg preset in the C21-C30 chains of used crankcase oil at day 0 (control). There was a gradual decrease in the amount of C31-C40 chains present in the used crankcase oil after the experimental days. About 8443.54mg/kg of C33 was present in the control at day 0 and after 20 days, 99.52% had been degraded by *Providencia* sp 3 (Fig. 14).

About 54.32% of aliphatic hydrocarbon compound present in the used crankcase oil was degraded by *Providencia* sp 3 (Appendix XV A and B).

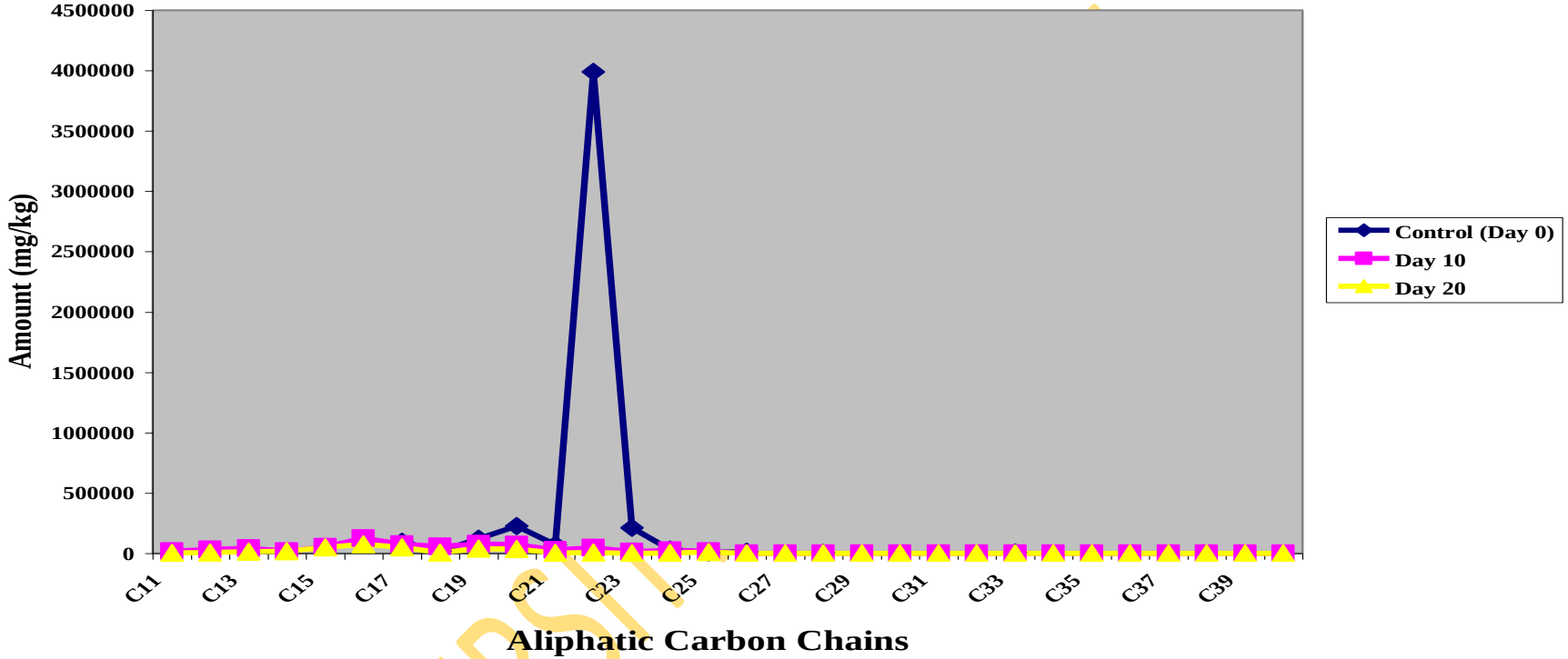



**Fig 14: Biodegradation of C-Chains present in Used Crankcase Oil by Providencia sp 3**

#### 4.6.2.3 Gas Chromatography Result of Aliphatic Hydrocarbon profile present in the Used Crankcase Oil as Degraded by *Bacillus* sp 4

Fig. 15 shows the degradation of aliphatic carbon chains present in used crankcase oil by *Bacillus* sp 4. There was a gradual increase in the amount of C10-C20 chains present in the used crankcase oil from 464.44 g/kg present in the control (day 0) to 1132.83 g/kg on the $10^{th}$ day . But by the $20^{th}$ day, it has reduced to 680.23 g/kg. The C21-C30 chains present in the used crankcase oil has been degraded by the *Bacillus* sp 4 from 379.12 g/kg to 74.52 g/kg on the $20^{th}$ day. About 93.59% of C31-C40 chains present in the used crankcase oil was degraded by the *Bacillus* sp 4 after 20 days. 8.44 g/kg of C31-40 was present in the control (Day 0), but by day 20, only 0.04 g/kg of C31-40 was present (Fig. 15).

About 31.68% of aliphatic hydrocarbon compounds present in the used crankcase oil was degraded by the *Bacillus* sp 4 after 20 days of degradation experiment (Appendix XVI A and B).

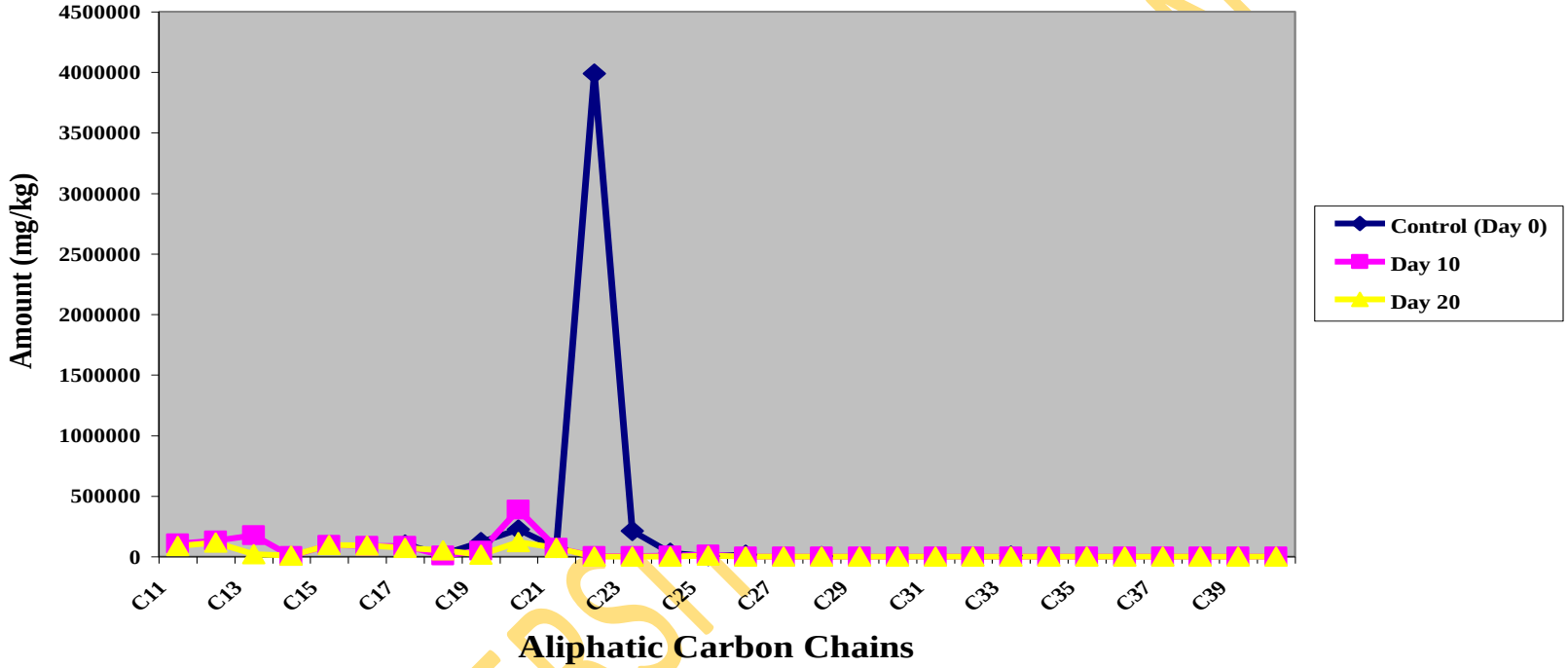



**Fig 15: Biodegradation of C-Chains present in Used Crankcase Oil by Bacillus sp 4**

### 4.6.3 Gas Chromatography Result of the Polycyclic Aromatic Hydrocarbon Profile present in the Crude oil

#### 4.6.3.1 Gas Chromatography Result of Polycyclic Aromatic Hydrocarbon profile present in Crude Oil as Degraded by *Providencia* sp 1

Fig. 16 shows the degradation of polycyclic aromatic hydrocarbons (PAH) present in the crude oil by *Providencia* sp 1. The amount of PAH present in the crude oil at day 10 was 12.73 g/kg as compared to 16.56 g/kg present in the control (day 0). But by day 20, it has reduced considerable to 0.57 g/kg. The rate of degradation of PAH present in the crude oil by *Providencia* sp 1 was 96.54% (Fig. 16) (Appendix XVIII A and B).

At day 10, the amount of polycyclic aromatic hydrocarbon (PAH) present in the crude oil was 13.75 g/kg as compared to 16.56 g/kg in the control (day 0) (Fig. 17). At day 20, the amount of PAH present in the crude oil has reduced further to 11.68 g/kg and this corresponded to 39.46% of PAH degraded at day 20 by the *Providencia* sp 3 (Fig. 17) (Appendix XIX A and B) .

Fig. 18 shows the degradation of polycyclic aromatic hydrocarbon (PAH) by the *Bacillus* sp 1. About 25.51% of PAH present in the crude has been degraded by *Bacillus* sp 1. The amount of PAH present in the crude oil was 15.23 g/kg at day 10 and reduced to 12.34 g/kg by day 20 as compared to 16.565 g/kg present in the control (day 0) (Fig. 18) (Appendix XX A and B) .

Indeno (1,2,3-cd) pyrene which was absent in the control (day 0) (Appendix XVII) was found in all the MSM media at day 10 but the amount has reduced considerably by day 20. The GC profile showed that the three bacterial isolates was able to degrade about 25.51% to 96.54% of PAH present in the crude oil.

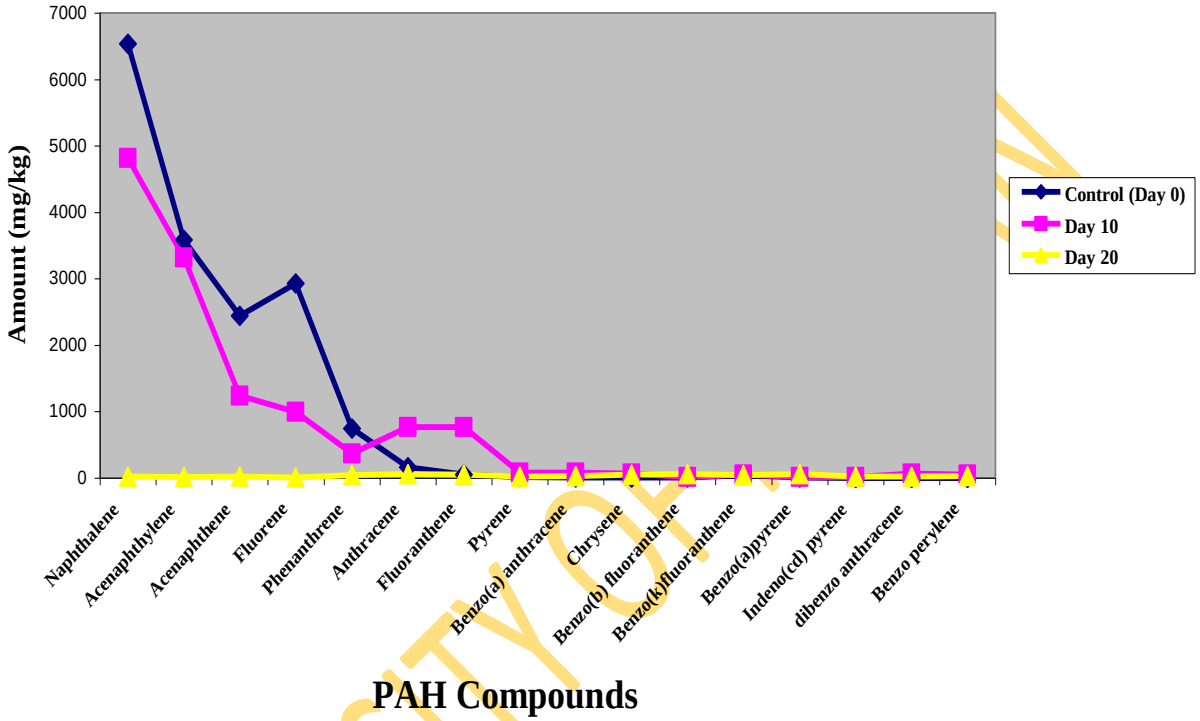



**Fig 16. Biodegradation of PAH Compounds present in Crude Oil by *Providencia* sp 1**

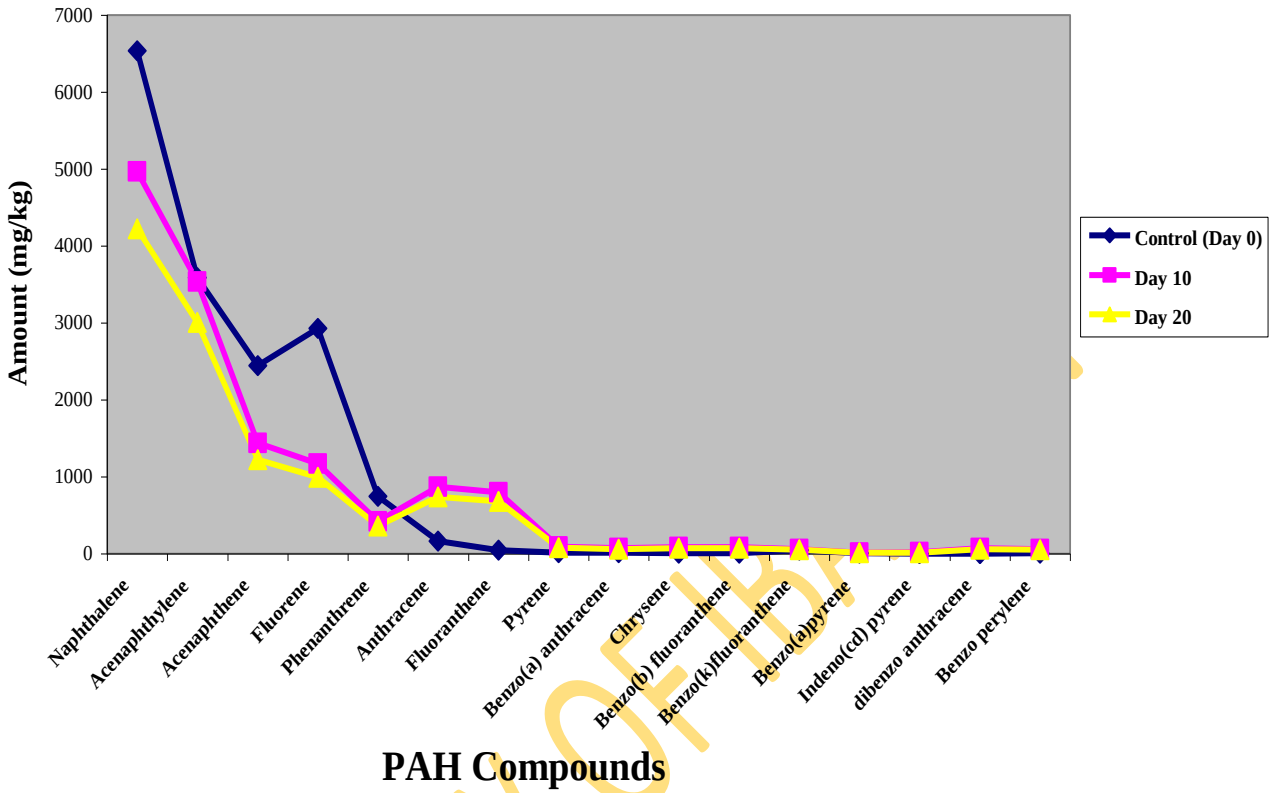



**Fig 17. Biodegradation of PAH Compounds present in Crude Oil by Providencia sp 3**

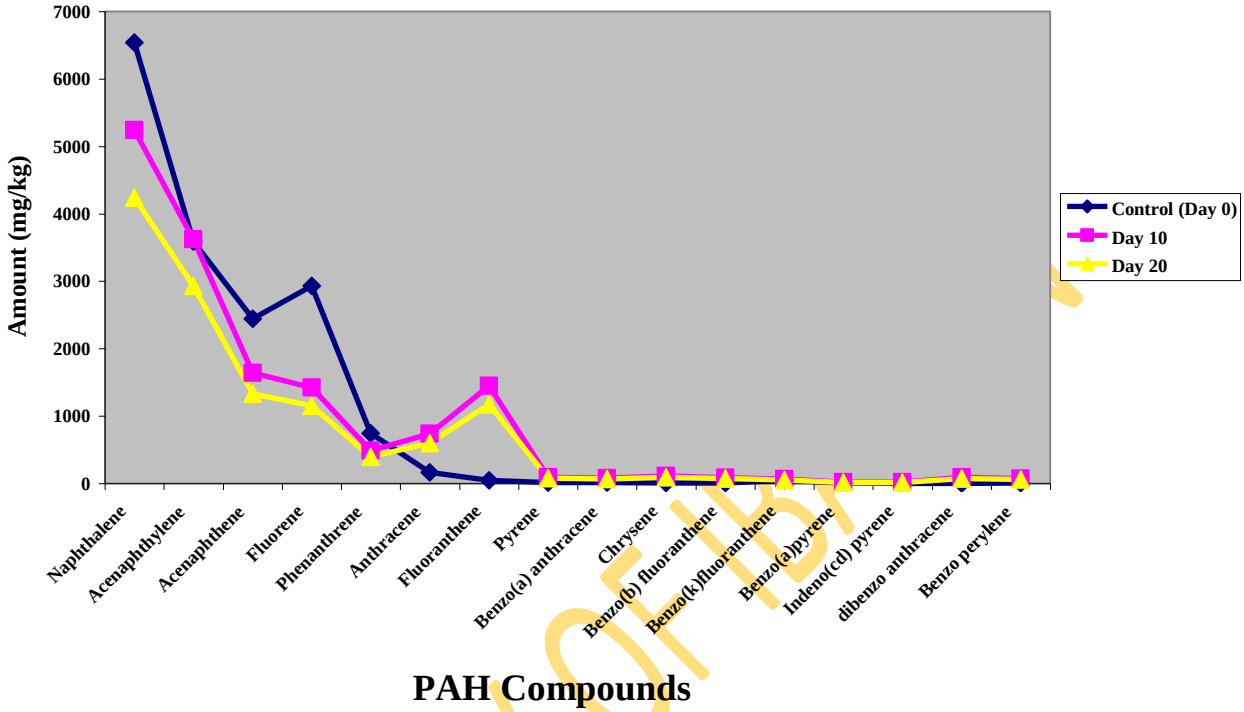



**Fig 18. Biodegradation of PAH Compounds present in Crude Oil by Bacillus sp 1**

#### 4.6.3.2 Gas Chromatography Result of Polycyclic Aromatic Hydrocarbon Profile present in Used Crankcase Oil

##### 4.6.3.2.1 Gas Chromatography Result of Polycyclic Aromatic Hydrocarbon profile present in Used Crankcase Oil as Degraded by *Providencia* sp 1

Fig. 19 shows the degradation of polycyclic aromatic hydrocarbon (PAH) present in the used crankcase oil by *Providencia* sp 1. At day 10, the amount of PAH present in the used crankcase oil was 0.78 g/kg as compared to 0.99 g/kg present in the control (day 0). And by day 20, the PAH has reduced to 0.62 g/kg, which showed that about 37.41% of PAH present in the used crankcase oil has been degraded by the *Providencia* sp 1 (Fig. 19) (Appendix XXII A and B).

Fig. 20 shows the degradation of polycyclic aromatic hydrocarbon (PAH) present in the used crankcase oil by *Providencia* sp3. At day 10, the amount of PAH present in the used crankcase oil was 0.88 g/kg as compared to 0.99 g/kg present in the control (day 0) but by day 20, it has reduced further to 0.69 g/kg. The rate of degradation of PAH present in the used crankcase oil was 29.97% as degraded by the *Providencia* sp 3 by the $20^{th}$ day (Fig. 20) (Appendix XXIII A and B).

The amount of polycyclic aromatic hydrocarbon (PAH) present in the control (day 0) of used crankcase oil was 0.99 g/kg but by day 10, it has reduced to 0.97 g/kg (Fig. 21). At day 20, the amount of PAH present in the used crankcase oil has reduced further to 0.81 g/kg which showed that about 19.34% of the PAH present in the used crankcase oil has been degraded by the *Bacillus* sp 4(Appendix XXIV A and B).

Acenaphthylene which is a two membered rings of polycyclic aromatic hydrocarbon was absent in the control (day 0) (Appendix XXI) but was found in the MSM medium containing the three bacterial isolates in varying amount at day 10. But by day 20, the amount has reduced further in all the MSM media.

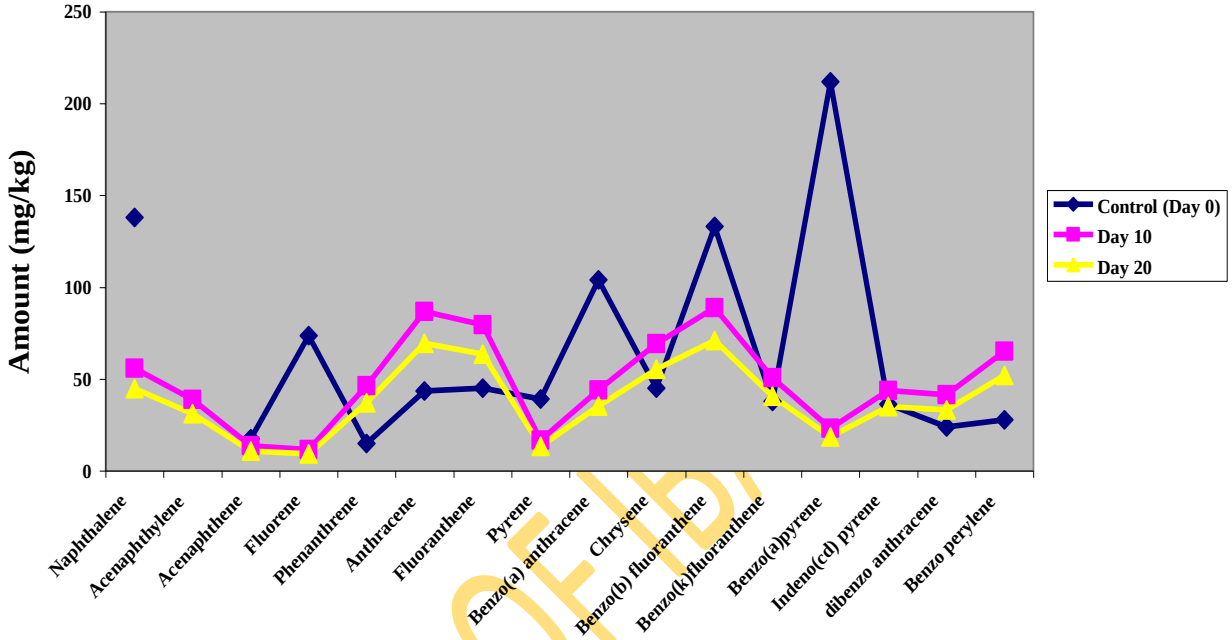



**Fig 19. Degradation of Polycyclic Aromatic Hydrocarbons present in Used Crankcase Oil by Providencia sp 1**

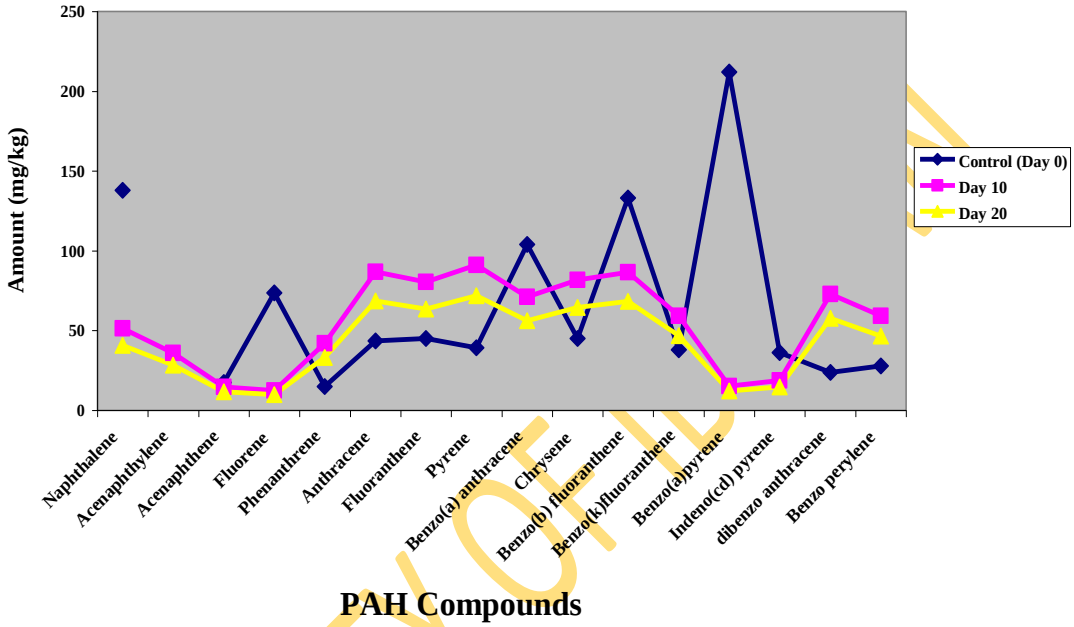



**Fig 20. Degradation of Polycyclic Aromatic Hydrocarbons present in Used Crankcase Oil by Providencia sp 3**

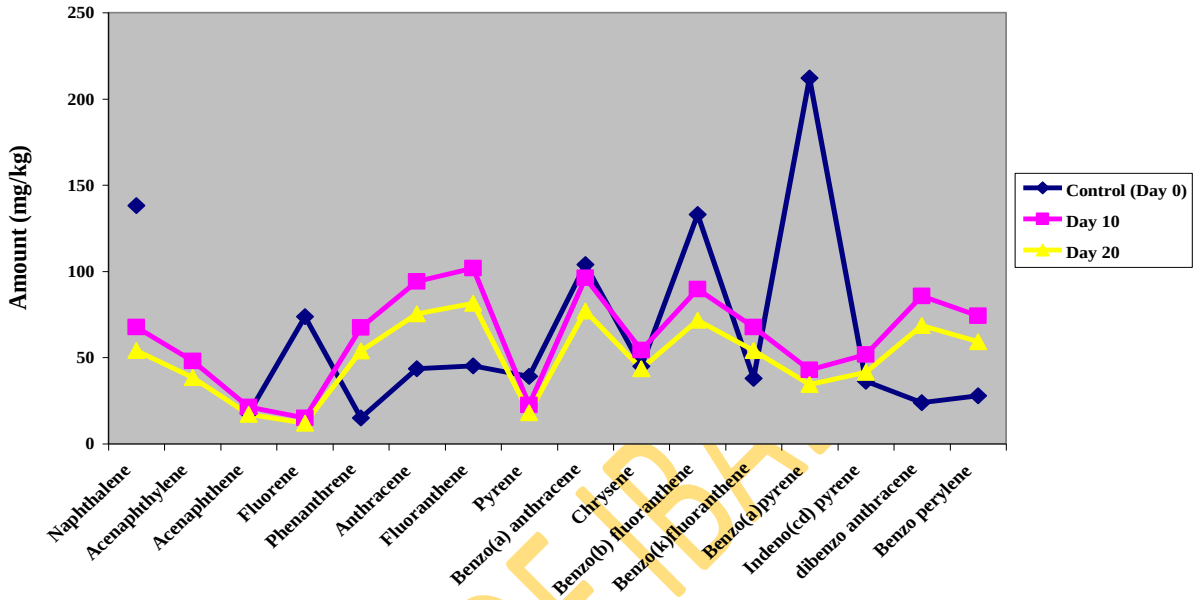



**Fig 21. Degradation of Polycyclic Aromatic Hydrocarbons present in Used Crankcase Oil by Bacillus sp 4**

## 4.7 Gas Chromatography Analysis Profiles showing the Effects of Nitrogen Salts on the Degradation of Polycyclic Aromatic Hydrocarbons present in the Crude Oil

### 4.7.1 Gas Chromatography Analysis showing the Effects of Nitrogen Salts on the Degradation of Polycyclic Aromatic Hydrocarbons present in the Crude Oil by *Providencia* sp 1

Fig. 22 shows the effects of two ammonium nitrogen salts ($NH_4NO_3$ and $NH_4Cl$) on the degradation of polycyclic aromatic hydrocarbon (PAH) present in the crude oil by *Providencia* sp 1. The amount of PAH present in the crude oil at day 10 was 12.73 g/kg and 14.66 g/kg in the MSM supplemented with $NH_4NO_3$ and $NH_4Cl$ respectively as compared to 16.57 g/kg present in the control (day 0) (Fig. 22). But by day 20, the amount had reduced further to 0.57 and 12.46 g/kg in the MSM supplemented with $NH_4NO_3$ and $NH_4Cl$ respectively by *Providencia* sp 1. The rates of degradation of PAH present in crude oil in the MSM supplemented with $NH_4NO_3$ and $NH_4Cl$ by Providencia sp 1 was 96.54 and 27.78% respectively.

The results obtained showed that $NH_4NO_3$ favoured the degradation of PAH present in the crude oil by *Providencia* sp 1(Appendix XVIII A, B, C and D).

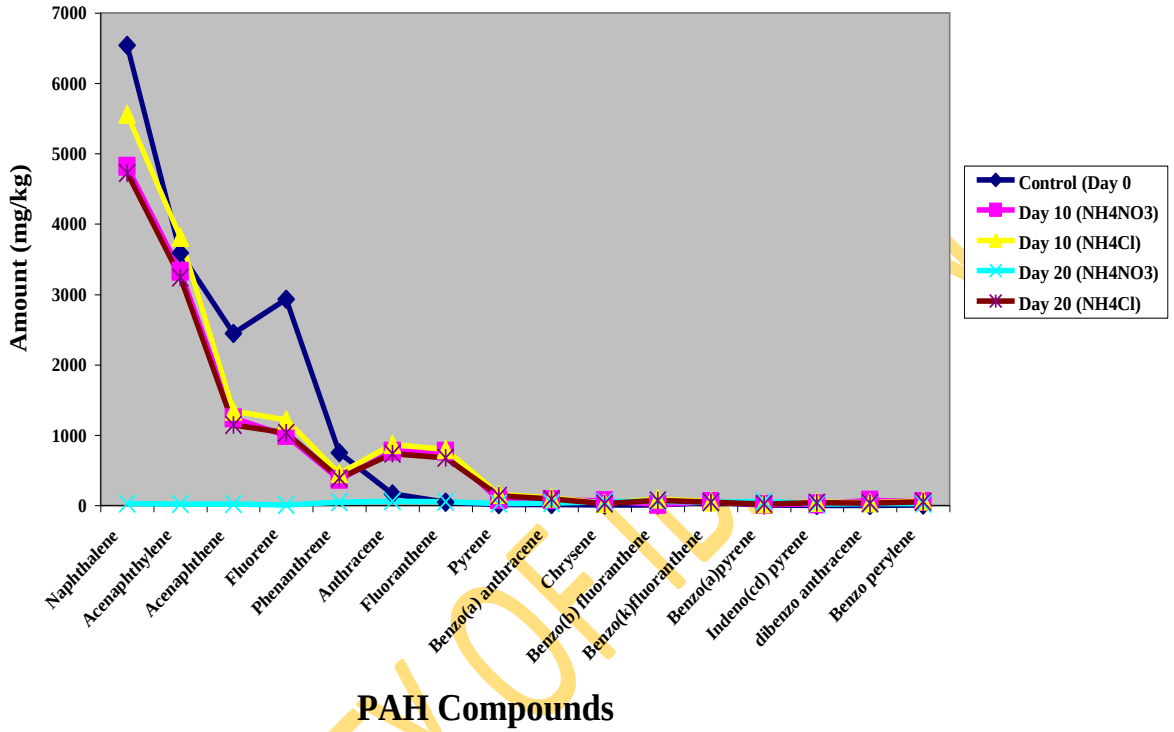



**Fig 22. Effects of Nitrogen Salts on Biodegradation of PAH Compounds present in Crude Oil by Providencia sp 1**

## 4.8 Gas Chromatography Analysis Profiles showing the Effects of Nitrogen Salts on the Degradation of Polycyclic Aromatic Hydrocarbons present in the Used Crankcase Oil

### 4.8.1 Gas Chromatography Analysis showing the Effects of Nitrogen Salts on the Degradation of Polycyclic Aromatic Hydrocarbons present in the Used Crankcase Oil by *Providencia* sp 3

Fig. 23 shows the effect of two Nitrogen salts ($NH_4NO_3$ and $NH_4Cl$) on the degradation of polycyclic aromatic hydrocarbons (PAH) present in the used crankcase oil by *Providencia* sp 1. At day 10, the amount of PAH present in the used crankcase oil were 0.88 and 1.43 g/kg in MSM supplemented with $NH_4NO_3$ and $NH_4Cl$ respectively as compared to 0.99 g/kg present in the control (day 0). But by day 20, the amount of PAH in the used crankcase oil had reduced to 0.69 and 0.71g/kg in the MSM supplemented with $NH_4NO_3$ and $NH_4Cl$ respectively. The rate of degradation of PAH present in the used crankcase oil at the $20^{th}$ day by *Providencia* sp 3 were 29.97 and 28.51% respectively in the MSM supplemented with $NH_4NO_3$ and $NH_4Cl$ respectively.

The results of the GC analysis showed that $NH_4NO_3$ favoured the degradation of PAH present in the used crankcase oil by *Providencia* sp 3(Appendix XXIII A, B, C and D).

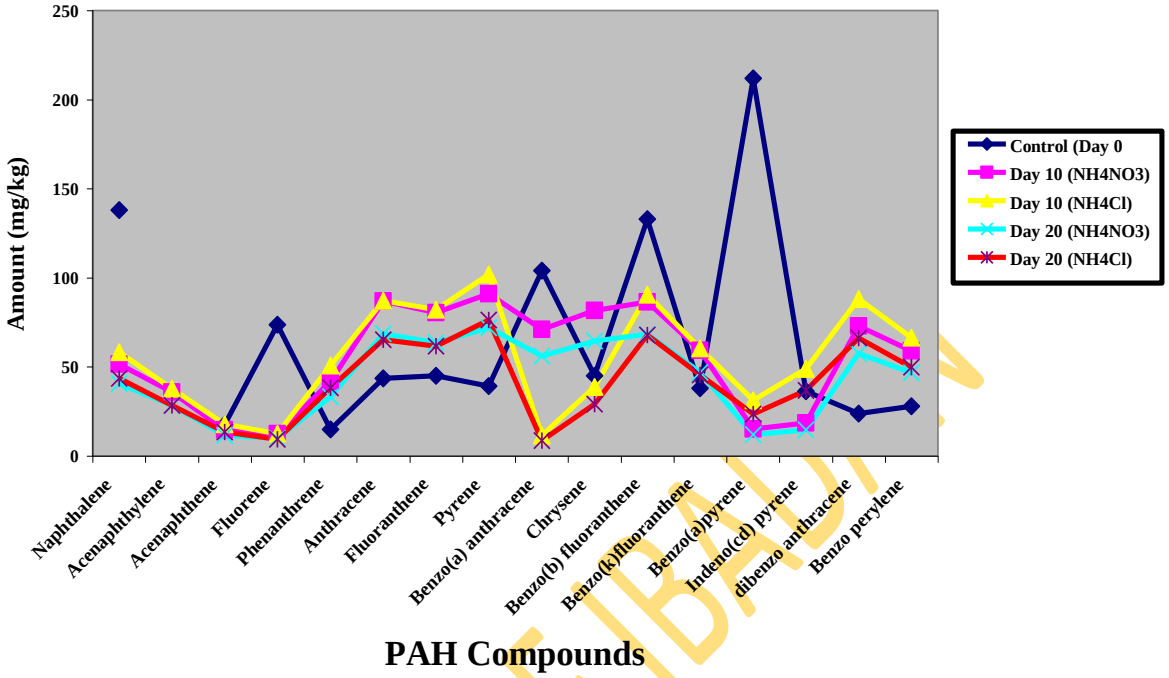



**Fig 23. Effects of Nitrogen Sailts on Biodegradation of PAH Compounds present in Used Crankcase oil by Providencia sp 3**

# CHAPTER FIVE

# DISCUSSION

## 5.1 Total Bacterial and Total Oil Degraders Enumeration

The results obtained from this research work showed that there was a gradual increase in the total oil degraders from the $6^{th}$ week till the $10^{th}$ of the experimental period, it increased from 108 to 205 cfu/ml in the soil contaminated with crude oil and from 103 to 245 cfu/ml in soil contaminated with used crankcase oil. This result showed that the application of the two hydrocarbons had increased the number of hydrocarbon degraders present in the contaminated soil samples.

Hydrocarbon degrading bacteria are widely spread in polluted soil, water, and the application of hydrocarbons increases the number of hydrocarbon utilizing bacteria (Leah and Colwell (1990), Chang *et al*. (2000), Barathi and Vasudevan (2001) and Zhuang *et al*. (2002). Total oil degraders were increased gradually during the experimental period in this work, which was similar to the work of Jane-Francis *et al*. (2008), who reported that oil-degrading bacteria counts ranged from $6 \times 10^4$ to $49\times 10^4$ cfu/ml in contaminated samples as against 0 to $14 \times 10^4$ cfu/ml in uncontaminated soil, the increase in the oil degrading bacteria counts might be due to the nutrient-induced desorption of hydrocarbons present in the soil sample. Desorption of hydrocarbons in the contaminated soil sample might lead to an increase in the microbial mineralization, either by increasing hydrocarbon solubility or by increasing the contact surface with hydrophobic compounds (Moran *et al*., 2000, Rahman *et al*., 2002, Ghulam *et al*., 2008). Microbial growth can also be enhanced by the addition of hydrocarbons to the soil samples in which the hydrocarbon served as a nutrient to the microorganisms present in the soil samples (Raza *et al*., 2007).

Atlas and Bartha (1972) observed that the application of crude oil to Arctic tundra soil caused overall increase in microbial numbers compared to un-oiled reference (control) soil, in which $7.5 \times 10^5$ cfu/g in the un-oiled soil, while $41 \times 10^7$ cfu/g was recorded for the soil sample contaminated with crude oil after 14 months. Ghulam *et al*. (2008) also reported that total bacterial count present in the soil contaminated with kerosene increased from $9 \times 10^8$ cfu/g at first week of the experiment to $9.6 \times 10^8$ by the 3rd week.

In this work, it was observed that total bacterial count decreased, while total oil degrader counts increased and this observation was also reported by Ramsay *et al*. (2000) who observed decrease in the heterotrophic bacteria count and increase in the hydrocarbon-degrading bacteria from the soil samples from oiled mangrove and untreated sediments. Olivera *et al*. (2003), also reported an increase of $2.0 \times 10^6$ to $1.3 \times 10^8$ cfu/ml during the first 24hrs in the soil contaminated with bilge waste till 17 days when the population had increased to $8.8 \times 10^8$ Cfu/ml. Rahman *et al*. (2003) reported an increase in the bacterial population from all the soil samples amended with hydrocarbons, especially the soil amended with 10% petroleum after 56 days of incubation. Increase in the hydrocarbon-degrader bacteria population from 1 × 105 to 1 × 107 cfu/g, between 4 and 7 days of incubation was observed by Kirsten *et al*. (2005), while the total heterotrophic population of the soil remained relatively unchanged during the incubation period.

Abioye *et al*. (2009) reported an increase in the hydrocarbon utilizing bacteria in a soil contaminated with used lubricating oil, which changed from $10.2 \times 10^6$ Cfu/g to $80.5 \times 10^6$ cfu/g, Hanan *et al*. (2009) also reported increase in number of microbes in a consortium used in the biodegradation of petroleum hydrocarbons that ranged from $6.14 \times 10^7$ to $3.5 \times 10^8$ and Udeani *et al*. (2009) also reported an increase from $1.25 \times 10^4$ to $6.25 \times 10^5$ in the hydrocarbon degraders present in the soil sample collected from mechanic workshop.

## 5.2 Bacterial Identification

### 5.2.1 Microscopy and Biochemical Identification of Bacterial Isolates

High prevalence of *Bacillus* species in hydrocarbon contaminated sites had been reported by many workers. Toledo *et al*. (2006) reported high percentage of *Bacillus* strain (66%) in their work. Ijah and Antai (2003) also reported *Bacillus* spp. as being the predominant isolate of all the crude oil utilizing bacteria that were isolated from highly polluted soil samples that contain 30 and 40% crude oil. Nwaogu *et al*. (2008) also reported the ability of *Bacillus subtilis* in the degradation of diesel oil in a polluted soil. *Bacillus* sp had also been reported to be involved in the degradation of aliphatic (Cybulski *et al.*, 2003) and polycyclic aromatic (Kazunga and Aitken, 2000).

The ability of the *Bacillus* spp. to grow in the hydrocarbon contaminated sites has been ascribed to their possession of resistant endospores, their ability to tolerate high levels of hydrocarbons in soil and also their abilities to survive in extreme environments (Ijah and Antai, 2003). *Bacillus* spp. can also colonize many environments (Shimura *et al*., 1999, Zhuang *et al*., 2002). The role of *Bacillus* spp in the degradation of complex hydrocarbons has been characterized as that of secondary degraders using metabolites produced by the primary hydrocarbon degraders (Chailan *et al*., 2004). Therefore, there is growing evidence that *Bacillus* species could be used effectively in clearing oil spills (Ghazali *et al*., 2004).

Not much literature is available on the degradation of hydrocarbon by *Providencia* species. In this work, *Providencia* spp. showed the highest degradation ability in the degradation of both crude oil and used engine oil. This genus *Providencia* spp. has not been previously reported to degrade PAHs but the degradation of hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine (RDX) and nitroso-RDX by this genus has been reported (Kiits *et al*. (1994)). *Providencia stuartii* has been identified as a bacterial isolate that is capable of utilizing chlorpyrifos as a source of carbon and energy (Rani *et al*., 2008).

*Proteus* sp is another bacterial isolate that was used in this work and the ability of this genus to degrade crude oil and its refined products has been reported by many workers. Olajide and Ogbeifun (2010) reported that the isolate, *Proteus vulgaris* SR-1 was able to grow on crude petroleum as the sole source of carbon and energy, when the

isolate was screened for hydrocarbon utilization. Kayode-Isola *et al*. (2008) also reported the ability of *Proteus* spp. in the biodegradation of hydrocarbon used in their work.

*Alcaligenes* sp has been known to be excellent hydrocarbon degraders (Krooneman *et al*., 1996) and some members of this genus that include, *A. dinifricans, A. odorans* and *A. eutrophus* have been reported to degrade hydrocarbons which include the polycyclic aromatic hydrocarbons (Weissenfeis *et al*., 1990, Harayama *et al*., 1991), polychlorinated biphenyls in mixed culture (Clark, 1979). Okoro and Amund (2010) also reported the biodegradation of produce water effluents from Chevron Escravos tank by the *Alcaligenes* sp.

### 5.2.2 Phylogenetic Identification of Bacterial Isolates

The results from the Gen Bank showed that the bacterial isolates were divided into two major groups, phylum Firmicutes, which consist of *Bacillus* species, while the second phylum is Proteobacteria which is further divided into β Proteobacteria which consist of *Alcaligenes* sp. and γ Proteobacteria that consist of *Providencia* species and *Proteus* sp.

Only *Bacillus cereus* was identified to its specific level by the 16S rRNA sequencing, while the remaining nine bacterial isolates were only identified to their generic level. The inability of the 16S rRNA sequencing to resolve isolates to their species names have been reported by many workers (Woo *et al*., 2003).

Researches have shown that cumulative results from a limited number of studies till date suggest that 16S rRNA gene sequencing can only provide generic identification in most cases (> 90%) but less with regard to species identification (65 to 83 %), with 1 to 14% of the isolates remaining unidentified after testing (Drancourt *et al*., 2000; Woo *et al*., 2003; Mignard and Flandrois, 2006). Difficulties encountered in obtaining a genus and species identification in the isolates include the recognition of novel taxa, too few sequences deposited in nucleotide databases, species sharing similar and / or identical 16S rRNA sequences, or nomenclature problems arising from multiple genomovars assigned to single species or complexes (Janda and Abbott, 2007).

Although 16S rRNA gene sequencing is highly useful in bacterial classification, it has low phylogenetic power at the species level and poor discriminating power for some genera (Bosshard *et al*., 2006; Mignard and Flandrois, 2006) and DNA relatedness studies

are necessary to provide absolute resolution to these taxonomic problems. Researches have shown that some species of the genus *Bacillus* are sometimes difficult to assign specific names, example of this are the Type strains of *B. globisporus* and *B. psychrophilus* which shared > 99.5% sequence similarity with regard to their 16S rRNA genes, but at the DNA level, they only exhibit 23 to 50% relatedness in reciprocal hybridization reaction (Fox *et al*., 1992). Janda and Abbott (2007) also reported that *Edwardsiella* species isolated from their laboratory exhibited 99.35 to 99.8% similarities to each other, and yet these three species were clearly distinguishable biochemically and by DNA homology (28 to 50% relatedness). Many workers had also reported resolution problem at the genus / and / or species level with regard to 16S rRNA gene sequencing data for some bacterial group that include family Enterobacteriaceae, rapid growing Mycobacteria, the *Acinetobacter baumannii*-A. *calcoaceticus* complex, *Achromobacter stenotrophomonas* and *Actinomyces.* Some of these problems are related to bacterial nomenclature and taxonomy, while others are related to sequence identity or very high similarity scores (Clayton *et al*., 1993; Stackebrandt and Goebel, 1994; Tang *et al*., 1998). Palleroni (1992) also reported difficulties in resolving bacterial taxonomy when a combination of RNA homology and phenotypic characteristics were only used for identification.Vandamme *et al*. (1996) concluded that ideal identification of any taxon is based upon a polyphasic approach that includes a combination of phenotypic testing methods (e.g. biochemical testing, cellular fatty acid analysis and numerical analysis) and genotypic testing methods (e.g. DNA-DNA hybridization, analysis of G + C content (in moles percent) and 16S rRNA gene sequencing).

**5.3 Plasmid Profile**

Result obtained from plasmid analysis showed that four bacterial isolates out of the ten isolates harbour plasmids with different molecular weight (Plate 3). *Providencia* sp 1 and *Bacillus* sp 1 harboured two plasmids each with molecular weight of 1876bp and 1261 bp, and 2577 bp and 2031 bp respectively, while *Bacillus* sp 4 and *Providencia* sp 3 harbour just a single plasmid each of molecular weight of 1366 bp and 32707 bp respectively.

Researches have shown that genetic factors play important roles in conferring biodegradation potentials on microorganisms and plasmids found in these organisms play a leading role in this aspect. Cerniglia (1984) reported that the ability of microorganisms to degrade more recalcitrant component of petroleum products like polycyclic aromatic hydrocarbons are sometimes plasmid mediated.

Plasmids that have been found to harbour genes encoding for the transformation of environmental pollutants are known as catabolic plasmids. The incidence of plasmids in oil degrading bacteria had been reported by many workers, Devereux and Sizemore (1982) reported the incidence of plasmids in 21% of the strains isolated on crude oil and 17% on polynuclear aromatic hydrocarbons, multiple plasmids in 50% of the plasmids containing strains were also similar to what was obtained in the multiple plasmids obtained in *Providencia* sp 1 and *Bacillus* sp 1, Thavasi *et al*. (2007) also reported the presence of multiple plasmid in *P. aeruginosa* isolated from their work.

Small plasmid was obtained from *Pseudomonas* strain with a molecular weight of 3.2 MDa in sediments from Campeche Bank (Leahy *et al*., 1990), Thavasi *et al*. (2007), also reported molecular weight of 3.8 to 4.2 kb in oil degrading bacteria, which also agreed with the results obtained in this work in which *Providencia* sp 3 and *Bacillus* sp. 4 also harboured a single plasmid each of molecular weight of 3.3kb and 1.4kb respectively.

Bacteria isolated from oil polluted environments have been shown to be more effective in degrading hydrocarbons than bacteria from unpolluted environments (Colwell *et al*., 1973) because exposures of a microbial community to hydrocarbons have been shown to increase the incidence of different types of plasmids in isolated bacteria (Hada and Sizemore, 1981; Burton *et al*., 1996; Ogunseitan *et al*., 1987; Day *et al*., 1988; Schutt, 1989; Leahy *et al*., 1996).

Researches have shown that plasmid is very important from single step reaction to multi step pathways in degradation pathway, and they appear to be a versatile means by which microorganisms can gain metabolic capacities in the exploitation of otherwise unavailable resources (Anthony *et al*., 2000). Presence of catabolic genes responsible for the degradation of naphthalene in plasmid found in *Pseudomonas putida* was reported by Park *et al*. (2003). Results obtained from this work showed that the four bacterial isolates

that harboured different sizes of plasmids were able to degrade both crude and used crankcase oil better than the remaining isolates that have no plasmids.

## 5.4 Effects of Nitrogen Sources on the Degradation of Hydrocarbons by Bacterial Isolates

Effect of two nitrogen salts; ammonium nitrate ($NH_4NO_3$) and ammonium chloride ($NH_4Cl$) was determined on the degradation of crude oil and used crankcase oil by the bacterial isolates.

### 5.4.1 Effects of $NH_4NO_3$ and $NH_4Cl$ on the Degradation of Crude oil by Bacterial Isolates

Results obtained from this research work showed that *Providencia* sp 3 had the highest degradation rate of 77.1% and 71.0% in the MSM supplemented with $NH_4NO_3$ and $NH_4Cl$ respectively for crude oil. $NH_4Cl$ enhanced the degradation of crude oil by five of the bacterial isolates after $20^{th}$ day. $NH_4NO_3$ and $NH_4Cl$ had no effect on the degradation of crude oil by the Bacillus sp 1 because 55.7% degradation rate was observed for both nitrogen sources at the $20^{th}$ day. $NH_4NO_3$ favoured the degradation of crude oil by *Providencia* sp 1, *Providencia* sp 3 and *Bacillus* sp 3.

The result showed that *Providencia* sp 3 performed better in the degradation of the crude oil than the two other *Providencia* species, while *Providencia* sp 2 showed the least degradation rate of 54.2 and 69.5% in the MSM supplemented with $NH_4NO_3$ and $NH_4Cl$. *Bacillus* sp 1 showed better degradation ability among the five *Bacillus* species used, while *Bacillus cereus* showed the least degradation rate.

The GC results obtained showed that $NH_4NO_3$ enhanced the degradation of polycyclic aromatic hydrocarbons (PAH) present in the crude oil than the medium containing the $NH_4Cl$. At day 20, the amount of PAH present in the crude oil has reduced to 0.57 and 12.46 g/kg as compared to 16.57 g/kg present in the control (day 0) in the MSM medium containing $NH_4NO_3$ and $NH_4Cl$ respectively (Fig 22). *Providencia* sp 1 was able to degrade 96.54 and 27.78% of PAH present in the crude oil at day 20 in the medium containing $NH_4NO_3$ and $NH_4Cl$ respectively.

### 5.4.2 Effects of $NH_4NO_3$ and $NH_4Cl$ on the Degradation of Used Crankcase oil by Bacterial Isolates

*Providencia* sp 1 showed the highest degradation rate of 70.5% and 75.8% in the MSM supplemented with $NH_4NO_3$ and $NH_4Cl$ respectively (Fig 7A and 7B). $NH_4Cl$ favoured the degradation rate of six bacterial isolates, while the two nitrogen sources had no significant effect on the *Proteus* sp since the degradation rate was 47.0% and 47.2% in the MSM supplemented with $NH_4NO_3$ and $NH_4Cl$ respectively. $NH_4NO_3$ favoured the degradation of used engine oil by *Bacillus* sp 2, *Bacillus cereus* and *Bacillus* sp 3 (Fig 7A and 7B).

*Providencia* sp 1 showed the highest degradation rate among the three *Providencia* species used in this work, while *Providencia* sp 2 showed the least degradation rate of 48.5 and 63.6% in the MSM supplemented with $NH_4NO_3$ and $NH_4Cl$ respectively. Among the five *Bacillus* species used, *Bacillus* sp 4 showed the highest degradation rate, while *Bacillus* sp 3 had the least degradation rate of 47.7 and 42.4% in the MSM supplemented with $NH_4NO_3$ and $NH_4Cl$ respectively.

The two nitrogen salts used did not have much effect on the degradation of polycyclic aromatic hydrocarbon (PAH) present in the used crankcase oil. At day 10, 0.88 and 1.43 g/kg of PAH was present in the medium containing $NH_4NO_3$ and $NH_4Cl$ respectively as compared to the control (day 0) which had 0.99 g/kg of PAH (Fig 36). But by day 20, it has reduced to 0.69 and 0.71 g/kg in the medium containing $NH_4NO_3$ and $NH_4Cl$ respectively. *Providencia* sp 3 was able to degrade about 29.97 and 28.51% of PAH present in the used crankcase oil by day 20 in the MSM supplemented with $NH_4NO_3$ and $NH_4Cl$ respectively (Fig 23).

Many workers have reported that the addition of nutrients in organic or inorganic forms into contaminated environments enhanced the breakdown of hydrocarbons and also that biodegradation conditions in contaminated environments can be limited by the availability of nutrients which include nitrogen (Atlas and Bartha, 1992; Mukred *et al*., 2008 a, b). Rahman *et al*. (2002) also reported that optimal rates of growth of microorganisms and biodegradation of hydrocarbon can be sustained when adequate concentrations of nutrients are present. Nitrogen sources that can be used in different concentrations to enhance the biodegradation rate include urea, ammonium nitrate,

ammonium chloride, ammonium or nitrate ions and phosphorus from orthophosphate, yeast extract, peptones (Atlas, 1981) but researches have shown that ammonium nitrogen is the preferred form for microbial metabolism as it required less energy to be assimilated (Walworth and Reynolds, 1995; Jorio *et al*., 2000). While further research by Lieberg and Cutright (1999) showed that the application of ammonium nitrogen stimulated hydrocarbon degradation rates to a greater extent than the application of nitrate-nitrogen at the same level, Brook *et al*. (2001) also observed that ammonium-nitrogen enhanced diesel fuel degradation to a greater extent.

Two different ammonium salts containing $NO_3^+$ and $Cl^-$ ions were used as the nitrogen sources for this study. The result obtained showed that ammonium chloride favoured the degradation rate of both the crude oil and used crankcase oil by the bacterial isolates, this was in agreement with the results obtained by Ciawi and Santi (2000) that glucose and $NH_4Cl$ enhanced oil degradation when they were added to Minimum salt Medium (MSM). But these results were in contrast to the works of Wrenn *et al*. (1994), Al-Awadhi *et al*. (1996), Braddock *et al*. (1997), Foght *et al*. (1999) and Aislabie *et al*. (2001) that reported that addition of ammonium-nitrate applied to contaminate soil resulted in acidification of the soil because of the microbial metabolism and not due to nitrification or toxicity of the ammonium nitrate and also that ammonium nitrate is considered as the most effective degradation enhancer but it had to be applied along with a buffer to be successful. While Graham *et al*. (1999) also reported that nitrate is the preferred form of nitrogen because it is more water soluble and does not depend on pH for speciation.

Brook *et al*. (2001) reported that the addition of excess nitrate-nitrogen can be inhibitory in some cases, while Kirsten *et al*. (2005) summarized the effect of ammonium chloride and nitrate as followed; ammonium nitrogen ($NH_4^+$-N) have short lag time before degradation and that degradation rates is enhanced with or without the addition of buffer, but nitrate-nitrogen ($NO_3^-$-N) do not need pH adjustment or buffer and saturated degradation is enhanced but more concentration of the salt is required to enhance degradation rate and longer lag time is observed. But Hazel and Lewis (1981) observed that different nitrogen ($NH_4^-$N and $NO_3^-$N) sources during naphthalene oxidation resulted in difference in cellular morphology, salicylic acid accumulation, Carbon-dioxide

evolution and the production of yellow colouration in the medium. While Brock *et al*. (1989) observed that oil biodegradation started more quickly in the ammonia cultures than it did in the cultures containing nitrate.

But when Bayoumi and Abul-Hamd (2010) compared the effects of different nitrogen sources, sodium nitrate, ammonium chloride, ammonium monohydrogen phosphate, ammonium sulphate and potassium nitrate on the growth of two bacterial isolates on toluene and phenol MSM media, they concluded that ammonium chloride was the best nitrogen source. Hamzah *et al*. (2010) reported that $NH_4NO_3$ and glycine has no significant effect on the growth of bacteria used in the degradation of Sumandak oil, while some workers reported that addition of nitrogen has no effect on biodegradation rates (Johnson and Scow, 1999). Mona *et al*. (2007) reported that the use of different nitrogen sources on kerosene showed different or varied degradation capabilities, and they concluded that the degradation rate varied with different nitrogen sources and bacterial isolates used.

### 5.5 Growth of Bacterial Isolates on the Hydrocarbons

Total viable counts and absorbance reading of the bacterial isolates in the hydrocarbons were taken to determine whether they were still growing and utilizing the crude oil and used engine oil. The absorbance reading was taken at 600nm using spectrophotometer.

#### 5.5.1 Growth of Bacterial Isolates in the MSM supplemented with Crude Oil

The absorbance reading of the bacterial isolates inside the Minimum Salt Medium (MSM) containing crude oil at 600nm, showed that the absorbance reading continue to increase from day 5 to day 10, but at day 15 and day 20, there was an increase in the absorbance reading of some bacterial isolates, while it decreased for some.

At day 15, absorbance reading at 600nm for *Bacillus* species 2 and 3, and *Bacillus cereus* decreased, but for *Proteus* sp, only the absorbance reading in the MSM supplemented with $NH_4Cl$ decreased (Table 5). While the absorbance reading for other bacterial isolates continue to increase till day 15. At day 20, absorbance reading of *Bacillus* sp 2 has increased, but for *Bacillus* sp 3, *Bacillus cereus*, and *Bacillus* sp 1, only

the absorbance reading in MSM supplemented with $NH_4NO_3$ had increased. But for *Providencia* sp1 and *Proteus* sp, the absorbance reading in MSM supplemented with $NH_4Cl$ only increased at day 20, while the absorbance reading for the remaining bacterial isolates at day 20 had decreased.

Total viable Counts of the bacterial isolates were also determined to confirm whether all the isolates can utilize crude oil as carbon and energy source. The viable count at Cfu/ml was increasing till day 15, which ranged from $1.40 \times 10^4$ Cfu/ml to $9.10 \times 10^4$ (Table 6). But at day 20, the viable counts for all the bacterial isolates had decreased.

### 5.5.2 Growth of Bacterial Isolates in the MSM Supplemented with Used Crankcase Oil

The absorbance measurement of bacterial isolates growing in used engine oil was determined at 600nm. The absorbance measurement of bacterial isolates continued to increase till day 15 except in *Proteus* sp, *Bacillus* sp 1, *Bacillus* sp 2 and *Bacillus cereus* in which the absorbance reading at 600nm decreased only in MSM supplemented with $NH_4Cl$ (Table 7). At day 20, all the absorbance reading of the bacterial isolates had decreased, except in *Bacillus cereus*, in which the absorbance measurement had increased in the MSM supplemented with $NH_4Cl$.

Total Viable counts at Cfu/ml were determined for bacterial isolates growing in the used crankcase oil. Total viable counts ranged from $1.10 \times 10^4$ Cfu/ml to $9.70 \times 10^4$ at day 0 till day 15. But at day 20, Total viable count was decreasing gradually in al the bacterial isolates except in *Bacillus cereus* and *Bacillus* sp 3 that increased in MSM supplemented with $NH_4Cl$ to $7.50 \times 10^4$ Cfu/ml and 7.20 × 104 Cfu/ml respectively (Table 8).

The ability to utilize and degrade hydrocarbon substrates is exhibited by a wide variety of bacterial genera (Leahy and Colwell, 1990; Dally *et al*., 1997; Bogan *et al*., 2003) that are widely distributed in oil polluted as well as pristine soils (Smith *et al*., 1999; Bogan *et al*., 2003; Van Beilen and Funhoff, 2005; Cappello *et al*., 2007).

The results showed that all the bacterial isolates used in this work can utilize both crude oil and used crankcase oil as their sole sources of carbon and energy and similar

results had been observed by many workers (Dally *et al*., 1997; Bogan *et al*., 2003; Cappello *et al*., 2007). Different growth rate was observed for each of the bacterial isolates used in this work using the different hydrocarbon substrates which collaborated with the work of Stanbury and Whitaker (1989) that reported that different organisms have different incubation periods, which may range from minutes to several hours.

Optical densities and Total viable counts have been used in several studies to show the potential of different types of bacteria in utilizing crude oil or hydrocarbons as a source of energy and carbon (Rahman *et al*., 2002; Emtiazi and Sharami, 2004). The results showed that all the bacterial isolates introduced into the culture media did not exhibit any lag phase (Tables 5-8). These results can be attributed to genetic make-up of the organisms, which may be due to the constitutive expression of hydrocarbon catalyzing enzymes present in them (Okerentugba and Ezeronye, 2003) and that microorganisms growing on crude oil did not exhibit any lag phase.

The survival of most microorganisms in petroleum hydrocarbon medium after their inoculation is a key factor which can be used to decide the rate of biodegradation of hydrocarbons either in soil or in liquid phases (Ramos *et al*. 1991). The ability of the bacterial isolates used in this work to utilize crude oil and used crankcase oil might be due to the fact that they were isolated from contaminated soil samples (Suguira *et al*. (1997), Rahman *et al*. (2003) and Kishore and Mukherjee (2006). Bacteria that were isolated from crude oil contaminated soil samples degrade or mineralize crude oil hydrocarbons because they also have the capability of native bacterial populations (Kasai, 2002; Okerentugba and Ezeronye, 2003; Emtiazi and Sharami, 2004) . Jacques *et al*. (2007) and Mandri and Lin (2007) had also reported an increase in cell population of each bacterial isolate used in their work, while Mukred *et al*. (2008) reported an increase in the cell numbers of bacteria consortia used in crude oil degradation from $243 \times 10^{22}$ to $178 \times 10^{23}$ Cfu/ml within 5 and 10 days of experiment.

The absorbance reading in this work was increasing from day 0 till day 10, while it increased and decreased at day 15 and day 20 but this was against the report of Nwaogu *et al*. (2008) that reported increment in the absorbance reading at 540nm throughout the experimental days of 12 days for *Bacillus subtilis* used in their work.

**5.6 Degradation of Aliphatic and Polycyclic Aromatic Hydrocarbons Present in the Hydrocarbons by the Bacterial Isolates** Degradation of the aliphatic i.e. different Carbons present in the crude oil and used crankcase oil was determined using the selected bacterial strains. The Carbon present in both the crude oil and used crankcase oil ranged from C10 to C40.

Degradation of polycyclic aromatic hydrocarbon present in the crude oil and used crankcase oil by the bacterial isolates was determined by GC analysis.

Sixteen different polycyclic aromatic hydrocarbons consisting of Naphthalene, Acenaphthylene, Acenaphthene, Fluorene, Phenanthrene, Anthracene, Fluoranthene, Pyrene, Benzo(a)anthracene, Chrysene, Benzo(b)fluoranthene, Benzo(k)fluoranthene, Benzo(a)pyrene, Dibenzo(a,h)anthracene, Indeno(1,2,3,-cd)pyrene and Benzo(g,h,l)perylene were all present in both the crude oil and used crankcase oil while Indeno(1,2,3,-cd) is not present in the crude oil (control). The polycyclic aromatic hydrocarbons (PAH) can be grouped into Low Molecular Weight (LMW) PAH and High Molecular Weight (HMW) PAH which consist of 2 and 3 rings and > 3 rings respectively or the PAH can be grouped into their different rings, 2, 3, 4, 5, and 6.

#### 5.6.1.1 Degradation of Aliphatic Present in Crude Oil

Degradation of the C10-C20 present in the crude oil ranged from 5.3 to 77.20%% at day 20. *Bacillus* sp 1 had the highest degradation rate of 77.2%, while Providencia sp 3 had least degradation rate of 5.3% (Figs 10. 11, 12)

The selected bacterial strains were able to degrade C21-C30 effectively more than the other C groups. The degradation rate ranged from 81.42 to 93.79% (Figs 10. 11.12). The highest degradation rate of 93.79% was found in Bacillus sp 1 and least degradation 81.42% was observed with *Providencia* sp 1. *Providencia* sp 3 was able to degrade 85.9% of C21-C30 present in the crude oil.

The degradation rate of C31-C40 present in the crude oil ranged from 43.90 to 53.38%. *Providencia* sp 3 had the highest degradation rate of 53.38% (Fig 11) while C31-C40 present in the crude oil containing Providencia sp 1 increased from 136,885.27mg/kg present in the control (day 0) to 253,259.98mg/kg at day 20. *Bacillus* sp 1 was able to degrade about 43.90% of C31-C40 present in the crude oil (Fig 12).

The three bacteria strains selected for GC analysis was able to degrade about 47.15%, 65.62% and 77.20% of aliphatic hydrocarbon compounds present in the crude oil by *Providencia* sp 1, *Providencia* sp 3 and *Bacillus* sp 1 respectively (Appendices IX to XIII).

#### 5.6.1.2 Degradation of Aliphatic Present in Used Crankcase Oil

The GC results showed that the selected three bacterial strains were not able to degrade the C10-C20 present in the used crankcase oil effectively. *Providencia* sp 1 was able to degrade about 2.57% of C10-C20 present in the crude oil (Fig 13), while 23.04% was degraded by *Providencia* sp 3 (Fig 14). But the amount of C10-C20 present in the use crankcase oil containing *Bacillus* sp 4 had increased from 46.44 g/kg present in the control (day 0) to 507.58 g/kg at day 20 (Fig15).

But the bacterial strains were able to degrade C21-C30 present in the crankcase oil effectively. *Providencia* sp 1 was able to degrade about 73.96% of C21-C30 present in the used crankcase oil, while *Providencia* sp 3 degraded 93.85% and about 80.34% of C21-C30 was degraded by the *Bacillus* sp 4.

Also the bacterial strains were able to degrade the C31-C40 present in the used crankcase oil effectively. The degradation rate ranged from 93.46 to 93.59%%, *Bacillus* sp. 4 had the highest rate of degradation of 93.59%, followed by Providencia sp 3 with 93.52% degraded. *Providencia* sp 1 showed the least degradation (93.465%) of C31-C40 present in the used crankcase oil.

The GC results showed that *Providencia* sp 1, *Providencia* sp 3 and *Bacillus* sp 4 were able to degrade 34.85, 54.32 and 312.68% of aliphatic hydrocarbon compounds present in the used crankcase oil respectively (Figs 13, 14 and 15).

The GC results showed that *Providencia* sp 1, *Providencia* sp 3 and *Bacillus* sp 4 were able to degrade 34.85, 54.32 and 312.68% of aliphatic hydrocarbon compounds present in the used crankcase oil respectively.

The results obtained in this study corresponded with the reports of many researchers that bacteria strains can degrade aliphatic hydrocarbon compounds present in different compounds. Degradation of aliphatic fractions of crude oil and petroleum refined products had been reported by many workers. Radwan *et al*. (1999) reported the

degradation of medium and long-chain n-alkanes with up to C40 Carbon atoms present in Arabian Gulf oil by *Acinetobacter calcoaceticus*; Sharma and Pant (2000), also reported the degradation of aliphatic fraction of crude oil present in the chronically polluted marine site by *Rhodococcus* sp. Zinjarde and Pant (2000) and El-Rafie and Helmy (2001) had also reported the degradation of aliphatic fraction of Bombay high crude oil and N-alkanes of an Egyptian crude oil respectively by different bacterial isolates.

Stroud *et al*. (2007) and Throne-Holst *et al*. (2007) had reported that long –chain hydrocarbons can contaminate soils for a long period of time and Matsumiya and Kubo (2007) had also reported that higher C-alkanes chains are especially difficult for microorganisms to degrade in nature, but this is in contrast to what was obtained in this work, in which 43.90 to 53.38%% of C31-C40 present in crude oil and 93.45 to 93.56% of C31-C40 present in the used crankcase oil were degraded within 20 days compared to the low and medium – chain aliphatic hydrocarbons.

Hamzah *et al*. (2010) reported different degradation rate of C8-20 with the degradation ranging from 10-77% within 48 hours of incubation, they reported that short chain are degraded less, which is similar to what was obtained in this result, where degradation rate of C10-C20 ranged from 2.52 to 23.04% for used crankcase oil.

Biodegradation of short-chain, medium-chain and long-chain aliphatic hydrocarbon present in used engine oil ranged from 2.52 to 23.04%, 73.96 to 93.85% and 93.46 to 93.59.6% respectively within 20 days. While Rahman *et al*. (2003) reported degradation rate of 100%, 83-98%, 80-85% and 57-73% for nC8-nC11, nC12-nC21, nC22-nC31 and nC32-nC40 respectively in their own work. Similar work by Mukred *et al*. (2008a) reported different biodegradation abilities by the bacterial isolates and consortium used in the degradation of crude oil, in which about 100% short- chain and medium- chain aliphatic were degraded by the consortium, while 97.12% and 98.35% of C28 to C33 was degraded singly within 15 days.

Sorkhoh *et al*. (1993), Ijah (1998), Verma *et al*. (2006) and Mukred *et al*. (2008b) had reported maximum removal of short-chain and medium-chain aliphatic compounds compared to longer-chain aliphatic in their work which was similar to what was observed in this work, in which 43.90 to 53.38% of long chain (C31-C40) was degraded in crude oil.

#### 5.6.2.1 Degradation of Polycyclic Aromatic Hydrocarbons Present in Crude oil

The degradation of PAH present in the crude oil was observed using selected bacterial isolates (*Providencia* sp 1, *Providencia* sp 3, and *Bacillus* sp 1) for 20 days.

*Providencia* sp 1 was able to degrade about 96.54% of polycyclic aromatic hydrocarbons present in the crude oil after 20 days (Fig 16), while just 39.46% of PAH present in the crude oil was degraded by *Providencia* sp 3 (Fig 17). Also, *Bacillus* sp 1 showed the least degradation of 25.51% by the 20$^{th}$ day (Fig 18)

#### 5.6.2.2 Degradation of Polycyclic Aromatic Hydrocarbons Present in the Used crankcase Oil.

The GC result showed that *Providencia* sp 1 had the highest degradation rate of 37.41% (Fig 19), followed by *Providencia* sp 3 (29.97%), at day 20 in the medium containing used crankcase oil as substrate (Fig 20). *Bacillus* sp 4 showed the least degradation of PAHs with 19.34% at day 20 (Fig 21).

The GC results obtained for crude oil and used crankcase oil showed that *Providencia* species performed better than the *Bacillus* species used in this research work, many researchers had reported that bacterial strains, especially gram-negative bacteria have the ability to degrade polyaromatic hydrocarbons (PAHs) compounds at various concentrations (Kiyohara *et al*., 1982; Cerniglia, 1992; Sutherland *et al*., 1995). The subclass gamma Proteobacteria is known to harbour most aerobic hydrocarbon degrading bacteria (HUB) (Van Hamme *et al*., 2003; Berthe-Corti and Hopner, 2005; Tapilatu *et al*., 2009).

The GC results showed that the PAHs content of the crude oil were degraded better by the selected bacterial isolates than that of the used crankcase oil, which confirmed the report of Atlas (1984) that crude oil were degraded better by microorganisms because apart from the carbon and energy supplied by the refined petroleum to the resident microorganisms, crude oil also supply mineral nutrients such as nitrogen, sulphur, and heavy metals in addition to carbon and energy.

The result obtained in this work for degradation of polycyclic aromatic hydrocarbon present in used crankcase oil also agreed with the report of Jacques *et al*.

(2007) in which the highest rate of degradation is recorded for the phenanthrene in their work, they concluded that this may be due to the higher solubility exhibited by phenanthrene than the other PAHs. But this is in contrast to what was observed in the degradation of PAHs present in the crude oil, except in *Providencia* sp 1 in which the phenanthrene has reduced to 25mg/kg compared to control.

The removal of different rings of PAHs present in the crude oil and used crankcase oil ranged from 19.34 to 96.54%, different degradation rate of PAHs in crude oil and petroleum refined products have been reported by many workers. Olivera *et al*. (2003) reported degradation rate of 100%, 95.2%, 93.6%, 70.3% and 71.6% for acenaphthylene, fluorene, phenanthrene, anthracene, and pyrene respectively. Zhang *et al*. (2004) reported rates of degradation of mineral medium of 0.500, 0.333, and 0.083mgL$^{-}$ day$^{-}$, for anthracene, phenanthrene, and pyrene respectively, while Hanan *et al*. (2009) reported degradation of toluene, benzene, naphthalene, anthracene, pentane and heptane at different concentrations. Sutiknowati (2007) also reported the degradation of Naphthalene, Phenanthrene, and Benzo (a) pyrene at different concentrations. While Udotong *et a*l. (2008) reported the degradation of different concentration of PAH present in the Mangrove environment by different hydrocarbon utilizing microorganisms. Toledo *et al*. (2006) in their work reported different degradation rate of Naphthalene, Phenanthrene, Fluoranthene and pyrene by *B. pumilus*, *B. subtilis*, *M. luteus*, *A. faecalis* and *Enterobacter* sp., while Hassan *et al*. (2009) reported degradation rate of 28.57, 30.19, 26.58 and 32.1% for anthracene by *Escherichia coli* (EF105548), Soil bacterium (EF105549), *Alcaligenes* sp. (EF105546), and *Thiobacter subterraneus* (EF105547) respectively, while those of Phenanthrene were 42.45, 48.44, 34.35 and 40.45% for these strains respectively.

Unfortunately, Indeno (1, 2, 3-cd) pyrene which was absent in the crude oil (control) at day 0, was found in all the MSM media containing all the selected bacterial strains at day 10. In *Providencia* sp1, it was 0.016 g/kg, for *Providencia* sp. 3, it was 0.019 g/kg and 0.02 g/kg for *Bacillus* sp. 1. There was also an increment in the amount of Benzo (g, h, l) perylene present in the MSM media at days 10 and 20 compared with the control of 0.004 g/kg at day 0. Naphthalene had also increased from 0.14 g/kg present in the used crankcase oil (control) at day 0, to 3.95 g/kg in the MSM containing *Providencia*

sp 1 at day 10 but by day 20, it had reduced to 0.031 g/kg. Although little increment was also observed in the Benzo (g, h, l) perylene in the liquid media supplemented with at day 10 for *Providencia* sp. 3 and *Bacillus* sp. 4 but by day 20, it had reduced considerably. Results observed in this work was in agreement with the work of Bayoumi (2009), who also observed increment in the concentrations of naphthalene and dibenzo (a, h) anthracene from 0.321 and 1.339 ppm to 0.60363 and 8.58807 ppm respectively after biodegradation of heavy oil by *Burkholderia cepacia*- DAFS11. Fluoranthene, chrysene and benzo (k) fluoranthene were also formed in liquid culture inoculated with this organism but these PAHs were not present in the control. Increment was also observed in some other PAHs and two other PAHs which are not found in the control in liquid media were observed in the media containing the bacterial strains used in his work. This might be due to the fusion of the lower rings PAH present in the control and also researches have shown that some bacteria can produce hydrocarbons depending on the type of substrates used (Park *et al*., 2001).

Generally, reduction in total PAHs was observed at day 20 for all the selected bacterial strains used in this work, which was in agreement with the work of Bayoumi, (2009).

# CHAPTER SIX

# CONCLUSION AND RECOMMENDATION

This study investigated the biodegradation abilities of ten bacterial isolates from crude oil and used crankcase oil contaminated soil samples. Gravimetric method was used to determine the biodegradation rate of the ten bacterial isolates using crude oil and used engine oil as carbon and energy sources. GC analysis was also used to determine the degradation rate of Polycyclic Aromatic Hydrocarbons (PAHs) and aliphatic compounds present in the crude oil and used crankcase oil by the selected bacterial isolates. At the end of the study, the results revealed that;

Ten bacterial isolates belonging to 4 genera were used in this work, in which most of them are common hydrocarbon degrading bacteria which have been reported by many workers.

There was an increase in the Hydrocarbon Utilizing Bacteria (HUB) from the contaminated soil samples during the 10 weeks of isolation.

Phylogenetic characterization of the 10 bacteria isolated from the contaminated soil samples and sequencing of their 16S rRNA genes revealed that the nucleotides sequence of their 16S rRNA genes from the test organisms have high nucleotide sequence identity with type strains from Gen Bank.

*Providencia* species had the highest degradation rate when both crude oil and used crankcase oil were used as the carbon and energy sources in the biodegradation experiment.

Optical density determination and Total bacterial count showed that the bacterial isolates were growing and utilizing both crude oil and used crankcase oil during the 20 days of biodegradation experiment.

The presence of mobile genetic elements (plasmids) present in four bacterial isolates, (*Providencia* sp 1, *Providencia* sp 3, *Bacillus* sp 1 and *Bacillus* sp 4) out of the 10 bacterial isolates showed that the presence of plasmids in bacteria enhanced their degradation abilities.

The two nitrogen salts ($NH_4NO_3$ and $NH_4Cl$) used in this work had varied effects on the degradation abilities of these bacterial isolates.

The GC results showed that aliphatic and polycyclic aromatic hydrocarbons present in the crude oil and used crankcase oil were degraded by the selected bacterial isolates.

Future Perspective and Recommendation

One can study different environmental conditions that will favour or enhanced the degradation ability of these bacterial strains

The catabolic gene present in the *Providencia* species can also be studied, since much scientific reports are not available on their hydrocarbon degradation abilities. Also, there is need to study the multi stage procedures used by Providencia species in the degradation of different hydrocarbons.

One can also extend the experimental days in order to study in detail the degradation of aliphatic and PAHs by these bacterial isolates.

One can also increase and use varying concentrations of different nitrogen sources, in order to know the appropriate nitrogen source to be used and also the exact quantity or amount to be used, since a successful bioremediation process also depend much on supplying an appropriate nitrogen source.

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## APPENDIX I

MEDIA

NUTRIENT AGAR

Formula in g/l

| | |
|---|---|
| Peptone | 5.0 |
| Meat extracts | 3.0 |
| Agar | 15.0 |

Final pH 7.0+. 0.2

MINIMUM SALT MEDIUM (MSM)

| | |
|---|---|
| $K_2HPO_4$ | 1.8 |
| $KH_2PO_4$ | 1.02 |
| Urea | 4.0 |
| $MgSO_4.7H_2O$ | 2.0 |
| NaCl | 0.1 |
| Yeast Extract | 0.1 |
| $FeCl_2$ | 0.05 |

Trace Elements

| | |
|---|---|
| $H_3BO_3$ | 0.1 |
| $ZnSO_4$ | 0.1 |
| $MnSO_4. H_2O$ | 0.4 |

## Appendix II

### Biochemical Tests

#### Catalase Test

An emulsion of each isolates (24 hours old) was made on a clean slide. A drop or two of freshly prepared 3% hydrogen peroxide ($H_2O_2$) was added. Positive result was indicated by the production of gas bubbles while the absence of bubbles indicated a negative result.

#### Oxidase Test

Two to three drops of 1% tetramethyl-paraphenylene diamine hydrochloride were applied unto Whatman No 1 filter paper placed on a glass slide moisten it. A smear of each bacterial isolate was then placed on the moistened filter paper by means of sterile platinum inoculating wire loop. A positive result was recorded if the smeared moistened filter paper turns purple within seconds. A delayed reaction was regarded as negative.

#### Indole Production

5ml of Tryptone water was dispensed into test tubes and sterilized by autoclaving for 15 minutes at 121$^{o}$C for 5-7 days. At the end of the incubation period, 0.5ml Kovac's indole reagent was added to each tube and mixed properly by rotating the tube between the palms. A deep red colour develops in the presence of indole which separate out in the alcohol layer.

#### V. P. Test

The medium used was glucose phosphate broth. This is a test for the production of acetyl methyl carbinol from glucose. Bacterial isolate was inoculated into the sterile medium in the test tube and incubated at 37$^{o}$C for 18hrs, an uninoculated tube served as control. At the end of the incubation period, 0.5ml of 40% potassium hydroxide and 0.5ml of 5% solution of α-Anaphthnol in absolute ethanol were added to the test tubes.

A positive reaction was indicated by the development of pink colour between 1-5 minutes.

#### Citrate Utilization Test.

The medium used was Koser citrate medium. The medium was prepared and 1- ml was dispensed in to each test tube. These were sterilized and allowed to solidify in

slanting position. Slant was inoculated with a loopful of the individual bacteria isolate and incubated at 37$^{o}$C for 48 hours. Changes in the colour of the medium from green to blue indicate positive result. An uninoculated citrate medium served as control.

**Urease Production**

Urea (20g) was dissolved in 100ml of distilled water and sterilized by filtration. A basal medium was prepared and dissolved by steaming with 12g of phenol red crystal and agar was added at 20g/l. The medium was sterilized and allowed to cool to 60$^{o}$C. The sterile urea solution was warmed and poured into the basal medium. The medium was distributed aseptically into sterile test tubes and allowed to set in a slanting position. The bacterial isolates were then inoculated and incubated at 30$^{o}$C for 7 days with daily observation. A change in the colour of the phenol red indicator from yellow to pink show that urea has been hydrolysed with liberation of ammonia.

**Fermentation of Sugars**

The bacteria isolates were tested for their ability to ferment Glucose, Lactose, Maltose fructose and Mannitol. This test was carried out using 1% peptone water and 1% fermentable sugar with phenol red (0.01%) as indicator. The medium was mixed thoroughly and 5-10ml of it was discharged into a clean test tubes and Durham tube was put in each of the test tube for accumulation of gas if produced. Sterilization was done at 121oC for 10 minutes. The tubes were inoculated with each of the bacterial isolate except the control tubes, incubation was at 37oC for up to 7 days. Acid production was shown by a change in the colour of the indicator from red to yellow

## Appendix III

PLASMID BUFFERS

Buffer 1A

400mM Tris

200mM Na EDTA

Acetic acid to pH 8.0

Buffer 2B

3M Na acetate

Acetic acid to pH 5.5

Buffer 3C

10mM Tris

2mM Na2 EDTA

Acetic acid to pH 8.0

Lysing Solution

4% SDS

100mM Tris

## Appendix IV

Nucleotide Sequence

16S rRNA

*Proteus* sp

GGCGGAcGGGTGAGTAAtGTATGGGGATCTGCCCGATAGAGGGGGATAACTACTGGAAACGGTGGCTAATAC
CGCATAATGTCTACGGACCAAAGCAGGGGCTCTTCGGACCTTGCACTATCGGATGAACCCATATGGGATTAG
CTAGTAGGTGGGGTAAAGGCTCACCTAGGCGACGATCTCTAGCTGGTCTGAGAGGATGATCAGCCACACTGG
GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATG
CAGCCATGCCGCGTGTATGAAGAAGGCCTTAGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGTGATAAGGT
TAATACCCTTATCAATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATA
CGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCAATTAAGTCAGATGTGA
AAGCCCCGAGCTTAACTTGGGAATTGCATCTGAAACTGGTTGGCTAGAGTCTTGTAGAGGGGGGTAGAATTC
CATGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACT
GACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTC
GATTTAgAGGTTGTGGTCTTGAACCGTGgCTTCTGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGG
CCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGC
AACGCGAAGAACCTTACCTACTCTTGACATCCAGCGAATCCTTTAGAGATAGAGGAGTGCCTTCGGGAACGC
TGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAA
CCCTTATCCTTTGTTGCCAGCACGTNATGGTGGGAACTCAAAGGAGACTGCCGGTGATAAACCGGAGGAAGG
TGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCAGATACAAAGA
GAAGCGACCTCGCGAGAGCAAGCGGAACTCATAAAGTCTGTCGTAGTCCGGATTGGAGTCTGCAACTCGACT
CCaTGAAGTCGGAATCGCTAGTAATCGTAgATCAgAATGCTACGGTGAATACGTTCCCGGGCCTTGTACACA
CCGCCCGTCACACCATGGGAGTGGGTT

*Providencia* spp

CGCTGacgAGCGGCGGaCGGGTGAGTAAtGTATGGGGATCTGCCCGATAGAGGGGGATAACCACTGGAAACG
GTGGCTAATACCGCATAATCTCTTAGGAGCAAAGCAGGGGAACTTCGGTCCTTGCGCTATCGGATGAACCCA
TATGGGATTAGCTAGTAGGtGNgGtAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATC
AGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCG
CAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGcCNTAGGGTTGTAAAGTACTTTCAGTCGGGAGGAA
GGCGTTGATGCTAATATCATCAACGATTGACGTTACCGACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAG
CCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTGATTAA
GTTAGATGTGAAATCCCCGGGCTTAACCTGGGAATGGCATCTAAGACTGGTCAGCTAGAGTCTTGTAGAGGG
GGGTAGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAATACCGGTGGCGAAgGCGGCCCCCT
GGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTG
TaaACGATGTCGATTTGAAGGTTGTTCCCTTGAGGAGTGGCTTTCGGAGCTAACGCGTTAAATCGACCGCCT
GGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTT
TAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTAGCAGAGATGCTTTGGTGCC
TTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGC
AACGAGCGCAACCCTTATCCTTTGTTGCCAGCGATTCGGTCGGGAACTCAAAGGAGACTGCCGGTGATAAAC
CGGAgGAAgGTGGGGATGACGTCAaGTCATCATGGCCCTTACg

*Bacillus* spp

GCGGCGGacGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAA
TACCGGATAACATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCC
GCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGAT
CGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGAC
GAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGA
ACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGC
AGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTT
AAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAG
GAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTT
CTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGC
CGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCG
CCTGGGGAGTACGGcCGCAAGGCTGAAACTCaAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAgCATGTG
GTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTC
TCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCC
CGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTTAGTTGGGCACTCTAAGGTGACTGCCGGTGACAA
ACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGG
ACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCTAATCTCATAAAACCGTTCTCAGTTCGGATTGTAGGC
TGCAACTCGCCTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAgCATGCCGCGgtGA

*Bacillus cereus*

TGAGTTaGCGGcGGacGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCG
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ACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAgCTAATCTCATAAAACCGTTCTCAGTTCGGAT
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TTCC

*Alcaligenes* sp

GacGGGTGAGTAATaTaTCGGAACGTGCCCAGTAGCGGGGGATAACTACTCGAAAGAGTGGCTAATACCGCA
TACGCCCTACGGGGGAAAGGGGGGGATCGCAAGACCTCTCACTATTGGAGCGGCCGATATCGGATTAGCTAG
TTGGTGGGGTAAAGGCTCACCAAGGCAACGATCCGTAGCTGGTTTGAGAGGACGACCAGCCACACTGGGACT
GAGACACGGCCCAGACTCCTACGGGAGGcNGcAGTGGGGAATTTTGGACAATGGGGGAAACCCTGATCCAGC
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GGGTGCAAGCGTTAATCGGAATTACTGgGCGTaAAGCGTGTGTAGGCGGTTCGGAAAGAAAGatGTGAAATC
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TTGTCATTAGTTGCTACGCAAGAGCACTCTAATGAGACTGCCGGTGACAAACCGGAgGAAGGTGGGGATGAC
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CGCGAGGGGGAGCCAATCTCAgAAACCCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTC
GGAATCGCTAGTAATCGCGGATCAgAATGtCGCGGTGA

## Appendix V

Table 9: Rate of Biodegradation of Crude Oil by Bacterial Strains Isolated in this Study with $NH_4NO_3$ and $NH_4Cl$ as Nitrogen Sources

| ORGANISM | DAYS | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 5 | | 10 | | 15 | | 20 | |
| | $NH_4NO_3$ | $NH_4Cl$ | $NH_4NO_3$ | $NH_4Cl$ | $NH_4NO_3$ | $NH_4Cl$ | $NH_4NO_3$ | $NH_4Cl$ |
| *Providencia* sp (strain 1) | 45.0 | 45.0 | 59.5 | 45.8 | 67.9 | 57.3 | 69.5 | 60.3 |
| *Providencia* sp (strain 2) | 29.8 | 45.8 | 51.1 | 53.4 | 53.4 | 56.5 | 54.2 | 69.5 |
| *Proteus* sp | 33.6 | 37.4 | 50.4 | 52.7 | 51.1 | 54.2 | 53.4 | 55.2 |
| *Bacillus* sp (strain 1) | 35.1 | 42.7 | 42.7 | 44.3 | 54.2 | 58.0 | 53.6 | 68.7 |
| *Providencia* sp (strain 3) | 56.5 | 55.0 | 56.0 | 57.3 | 68.7 | 63.4 | 77.1 | 71.0 |
| *Bacillus* sp (strain 2) | 46.0 | 51.1 | 48.8 | 52.7 | 50.4 | 55.5 | 54.2 | 56.9 |
| *Bacillus cereus* | 32.8 | 46.6 | 41.2 | 50.4 | 42.7 | 54.2 | 51.9 | 56.5 |
| *Bacillus* sp (strain 3) | 33.6 | 36.6 | 50.4 | 44.3 | 51.1 | 45.8 | 61.8 | 53.4 |
| *Alcaligenes* sp | 35.1 | 45.8 | 38.2 | 50.4 | 51.4 | 53.4 | 54.2 | 54.9 |
| *Bacillus* sp (strain 4) | 32.8 | 32.1 | 51.1 | 50.4 | 53.4 | 52.7 | 55.7 | 55.7 |

## Appendix VI

**Proximity Matrix**

| Case | Squared Euclidean Distance | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 1 | .000 | 889.620 | 899.340 | 586.850 | 755.450 | 1476.300 | 806.970 | 849.980 | 1124.210 | 895.210 |
| 2 | 889.620 | .000 | 194.960 | 1645.110 | 465.070 | 410.720 | 619.130 | 412.820 | 430.270 | 301.690 |
| 3 | 899.340 | 194.960 | .000 | 1752.090 | 457.430 | 358.380 | 558.530 | 427.560 | 286.870 | 366.530 |
| 4 | 586.850 | 1645.110 | 1752.090 | .000 | 1318.980 | 2504.830 | 2226.700 | 2198.350 | 2089.960 | 2092.420 |
| 5 | 755.450 | 465.070 | 457.430 | 1318.980 | .000 | 323.970 | 601.720 | 566.350 | 273.960 | 349.720 |
| 6 | 1476.300 | 410.720 | 358.380 | 2504.830 | 323.970 | .000 | 471.230 | 440.080 | 99.110 | 249.710 |
| 7 | 806.970 | 619.130 | 558.530 | 2226.700 | 601.720 | 471.230 | .000 | 153.990 | 390.800 | 215.560 |
| 8 | 849.980 | 412.820 | 427.560 | 2198.350 | 566.350 | 440.080 | 153.990 | .000 | 366.770 | 47.590 |
| 9 | 1124.210 | 430.270 | 286.870 | 2089.960 | 273.960 | 99.110 | 390.800 | 366.770 | .000 | 228.400 |
| 10 | 895.210 | 301.690 | 366.530 | 2092.420 | 349.720 | 249.710 | 215.560 | 47.590 | 228.400 | .000 |

This is a dissimilarity matrix

A Dissimilarity Matrix for Degradation of Crude Oil by Bacterial Isolates

## Appendix VII

Table 10: Rate of Biodegradation of Used Crankcase Oil by Bacterial Strains Isolated in this Study with $NH_4NO_3$ and $NH_4Cl$ as Nitrogen Sources

| ORGANISM | DAYS | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 5 | | 10 | | 15 | | 20 | |
| | $NH_4NO_3$ | $NH_4Cl$ | $NH_4NO_3$ | $NH_4Cl$ | $NH_4NO_3$ | $NH_4Cl$ | $NH_4NO_3$ | $NH_4Cl$ |
| *Providencia* sp (strain 1) | 18.2 | 21.2 | 34.1 | 37.9 | 46.2 | 49.2 | 70.5 | 75.8 |
| *Providencia* sp (strain 2) | 12.9 | 18.9 | 25.0 | 36.4 | 39.4 | 47.0 | 48.5 | 63.6 |
| *Proteus* sp | 17.4 | 18.2 | 34.1 | 35.6 | 46.2 | 45.4 | 47.0 | 47.7 |
| *Bacillus* sp (strain 1) | 17.3 | 20.5 | 32.6 | 37.1 | 37.3 | 43.2 | 50.8 | 62.1 |
| *Providencia* sp (strain 3) | 16.8 | 17.4 | 30.3 | 35.6 | 43.2 | 45.5 | 63.6 | 67.4 |
| *Bacillus* sp (strain 2) | 22.0 | 13.6 | 34.1 | 30.3 | 47.0 | 35.6 | 68.2 | 45.5 |
| *Bacillus cereus* | 12.1 | 09.1 | 37.1 | 34.1 | 45.5 | 43.2 | 59.1 | 53.8 |
| *Bacillus* sp (strain 3) | 20.5 | 12.9 | 36.4 | 26.5 | 42.4 | 39.4 | 47.7 | 42.4 |
| *Alcaligenes* sp | 18.9 | 22.5 | 27.3 | 43.9 | 37.1 | 48.5 | 42.7 | 53.5 |
| *Bacillus* sp (strain 4) | 18.2 | 20.5 | 27.3 | 45.5 | 45.3 | 49.2 | 60.4 | 67.4 |

## Appendix VIII

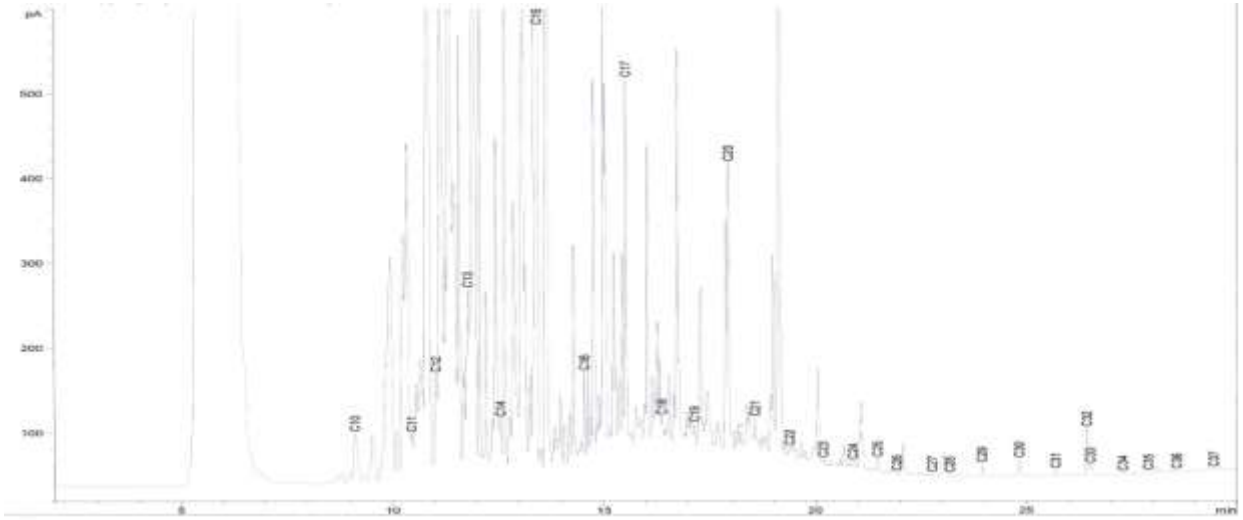
**Proximity Matrix**

| Case | Squared Euclidean Distance | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 1 | .000 | 802.360 | 695.180 | 304.990 | 1238.940 | 857.510 | 1955.310 | 277.870 | 1437.850 | 1371.230 |
| 2 | 802.360 | .000 | 106.560 | 327.330 | 1134.340 | 508.430 | 838.570 | 314.450 | 253.690 | 408.050 |
| 3 | 695.180 | 106.560 | .000 | 359.430 | 848.360 | 391.270 | 632.950 | 319.710 | 248.590 | 315.650 |
| 4 | 304.990 | 327.330 | 359.430 | .000 | 901.230 | 361.260 | 1243.620 | 274.920 | 926.540 | 800.100 |
| 5 | 1238.940 | 1134.340 | 848.360 | 901.230 | .000 | 353.410 | 487.930 | 1067.630 | 1298.690 | 621.370 |
| 6 | 857.510 | 508.430 | 391.270 | 361.260 | 353.410 | .000 | 427.220 | 615.860 | 785.580 | 311.100 |
| 7 | 1955.310 | 838.570 | 632.950 | 1243.620 | 487.930 | 427.220 | .000 | 1397.600 | 739.400 | 204.820 |
| 8 | 277.870 | 314.450 | 319.710 | 274.920 | 1067.630 | 615.860 | 1397.600 | .000 | 581.280 | 733.080 |
| 9 | 1437.850 | 253.690 | 248.590 | 926.540 | 1298.690 | 785.580 | 739.400 | 581.280 | .000 | 280.420 |
| 10 | 1371.230 | 408.050 | 315.650 | 800.100 | 621.370 | 311.100 | 204.820 | 733.080 | 280.420 | .000 |

This is a dissimilarity matrix

A Dissimilarity Matrix for Degradation of Used Crankcase Oil by Bacterial Isolates

## Appendix IX



Gas Chromatograph of Crude oil at day 0 (Control)

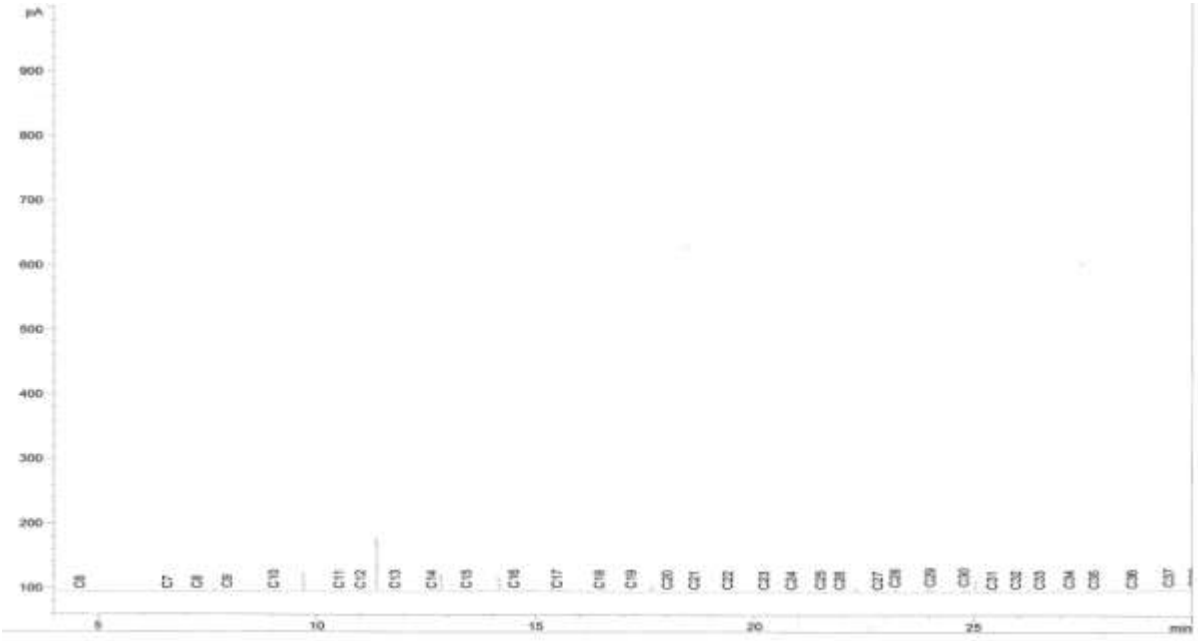
| Sorted By | : | Signal |
|---|---|---|
| Calib. Data Modified | : | 12/15/2008 11:18:03 AM |
| Multiplier | : | 1.0000 |
| Dilution | : | 1.0000 |

Signal 1: FID1 A,

| RetTime [min] | Type | Area [pA*s] | Amt/Area | Amount [mg/Kg] | Grp. | Name |
|---|---|---|---|---|---|---|
| 4.560 | | - | - | - | | C6 |
| 6.600 | | - | - | - | | C7 |
| 7.250 | | - | - | - | | C8 |
| 8.000 | | - | - | - | | C9 |
| 9.119 | VV T | 238.33218 | 2.85714 | 680.94810 | | C10 |
| 10.449 | VV T | 118.89053 | 2.85714 | 339.68722 | | C11 |
| 11.028 | VV T | 379.55246 | 2.85714 | 1084.43560 | | C12 |
| 11.785 | VV T | 758.70129 | 2.85714 | 2167.71790 | | C13 |
| 12.555 | VV T | 104.53365 | 2.85714 | 298.66758 | | C14 |
| 13.431 | VV T | 8270.44043 | 2.50000 | 2.06761e4 | | C15 |
| 14.541 | VV T | 142.06659 | 200.00000 | 2.84133e4 | | C16 |
| 15.533 | VV T | 1014.05642 | 12.50000 | 1.26757e4 | | C17 |
| 16.379 | VV T | 39.26150 | 1052.63158 | 4.13279e4 | | C18 |
| 17.146 | VV T | 20.71967 | 14.28571 | 295.99538 | | C19 |
| 17.949 | VB T | 765.14471 | 100.00000 | 7.65145e4 | | C20 |
| 18.580 | VV E | 169.35336 | 1428.57143 | 2.41933e5 | | C21 |
| 19.395 | VV E | 56.98819 | 1666.66667 | 9.49770e4 | | C22 |
| 20.188 | VV E | 31.90860 | 1666.66667 | 5.33160e4 | | C23 |
| 20.893 | VV E | 37.05124 | 1666.66667 | 6.17521e4 | | C24 |
| 21.477 | VV E | 41.95883 | 1666.66667 | 6.99314e4 | | C25 |
| 21.937 | VV X | 2.01400 | 1333.33333 | 2685.33548 | | C26 |
| 22.766 | VV T | 4.11212 | 1333.33333 | 5482.83195 | | C27 |
| 23.162 | VV T | 1.13473 | 1333.33333 | 1512.97283 | | C28 |
| 23.954 | VV T | 25.26761 | 500.00000 | 1.26338e4 | | C29 |
| 24.837 | VV T | 56.22549 | 1000.00000 | 5.62255e4 | | C30 |
| 25.700 | VV T | 9.07605 | 1.00000e4 | 9.07605e4 | | C31 |
| 26.443 | VV T | 146.82168 | 100.00000 | 1.46922e4 | | C32 |
| 26.519 | VB T | 35.19200 | 100.00000 | 3519.20013 | | C33 |
| 27.284 | VV T | 1.23515 | 1000.00000 | 1235.14557 | | C34 |
| 27.887 | VV T | 9.14677e-1 | 1.00000e4 | 9146.76607 | | C35 |
| 28.577 | VV T | 8.15167e-1 | 1.00000e4 | 8151.67427 | | C36 |
| 29.454 | VV T | 3.22989 | 1000.00000 | 3229.89440 | | C37 |
| 30.122 | VV T | 1.91543 | 1000.00000 | 1915.42876 | | C38 |
| 30.862 | VV T | 20.31062 | 100.00000 | 2031.06194 | | C39 |
| 31.172 | VV T | 22.03399 | 100.00000 | 2203.39947 | | C40 |
| Totals : | | | | 9.21810e5 | | |

Results obtained with enhanced integrator!

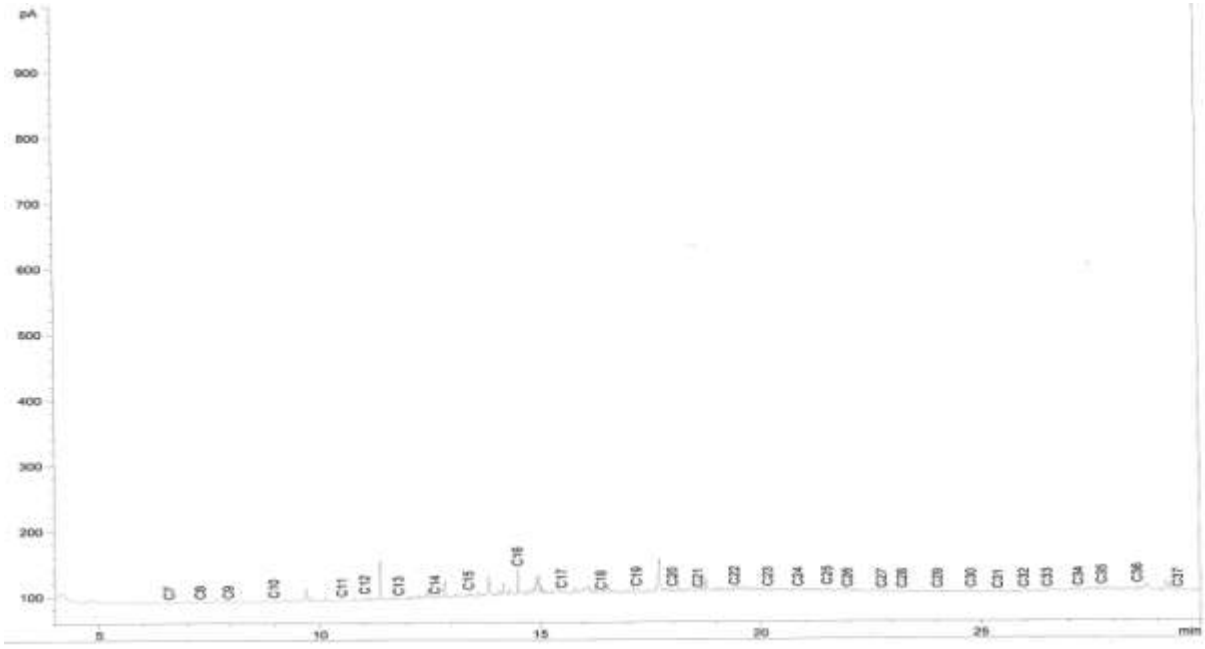
## Appendix X A




Gas Chromatograph of Crude Oil (Residual oil) as degraded by *Providencia* sp 1 10th day

| Time | Type | Area [pA*s] | Amt/Area | Amount [mg/Kg] | Grp | Name |
|---|---|---|---|---|---|---|
| 4.500 | | - | - | - | | C6 |
| 6.588 | VV T | 8.91070 | 0.00000 | 0.00000 | | C7 |
| 7.286 | VV T | 13.31381 | 0.00000 | 0.00000 | | C8 |
| 7.950 | VV T | 9.93204 | 0.00000 | 0.00000 | | C9 |
| 8.966 | VV T | 15.87074 | 0.00000 | 0.00000 | | C10 |
| 10.515 | VV T | 11.29511 | 5.00000e-5 | 9036.08627 | | C11 |
| 11.030 | VV T | 14.75290 | 5.00000e-5 | 1.18023e4 | | C12 |
| 11.804 | VV T | 2.77133 | 5.00000e-5 | 2217.06657 | | C13 |
| 12.622 | VV T | 5.83615 | 5.00000e-5 | 4668.92090 | | C14. |
| 13.393 | VV T | 41.35609 | 5.00000e-5 | 3.30849e4 | | C15 |
| 14.509 | VV T | 161.35486 | 5.00000e-5 | 1.29084e5 | | C16 |
| 15.506 | VV T | 32.00352 | 5.00000e-5 | 2.56028e4 | | C17 |
| 16.393 | VV T | 35.14053 | 5.00000e-5 | 2.81124e4 | | C18 |
| 17.211 | VV T | 96.76241 | 5.00000e-5 | 7.74099e4 | | C19 |
| 18.019 | VV T | 51.57598 | 5.00000e-5 | 4.12608e4 | | C20 |
| 18.590 | VV T | 27.57800 | 5.00000e-5 | 2.20624e4 | | C21 |
| 19.424 | VV T | 43.13705 | 5.00000e-5 | 3.45096e4 | | C22 |
| 20.184 | VV T | 29.45934 | 5.00000e-5 | 2.35675e4 | | C23 |
| 20.868 | VV T | 30.82500 | 5.00000e-5 | 2.46600e4 | | C24 |
| 21.530 | VV T | 33.79352 | 5.00000e-5 | 2.70348e4 | | C25 |
| 21.987 | VV T | 16.08513 | 5.00000e-5 | 1.28681e4 | | C26 |
| 22.758 | VV T | 1.32907 | 5.00000e-5 | 1063.25350 | | C27 |
| 23.189 | VV X | 8.11430e-1 | 5.00000e-5 | 649.14408 | | C28 |
| 24.035 | VV T | 5.69592 | 5.00000e-5 | 4556.73256 | | C29 |
| 24.784 | VV T | 7.62782 | 5.00000e-5 | 6102.25220 | | C30 |
| 25.378 | VV T | 1.77416 | 5.00000e-3 | 1.41933e5 | | C31 |
| 25.991 | VV T | 9.00498 | 5.00000e-5 | 7203.98254 | | C32 |
| 26.525 | VV X | 10.33173 | 5.00000e-5 | 8265.38162 | | C33 |
| 27.229 | VV X | 17.42724 | 5.00000e-5 | 1.39418e4 | | C34 |
| 27.764 | VV X | 43.27594 | 5.00000e-5 | 3.46208e4 | | C35 |
| 28.573 | VV X | 44.62811 | 5.00000e-5 | 3.57025e4 | | C36 |
| 29.496 | VV X | 2.60572 | 5.00000e-5 | 2084.57909 | | C37 |
| 30.213 | VV X | 5.11655 | 5.00000e-5 | 4093.24379 | | C38 |
| 30.815 | VV X | 4.94411 | 5.00000e-5 | 3955.29022 | | C39 |
| 31.255 | VV T | 1.82427 | 5.00000e-5 | 1459.41324 | | C40 |
| Totals : | | | | 7.72612e5 | | |

## Appendix X B

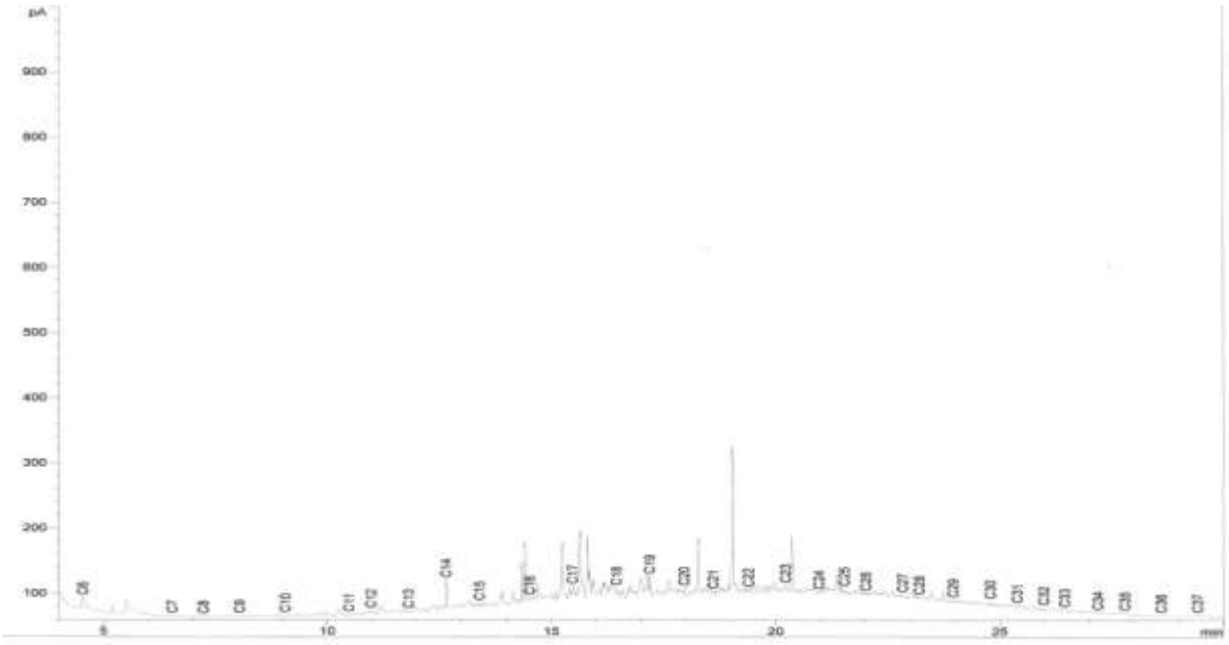



Gas Chromatograph of Crude Oil (Residual oil) as degraded by *Providencia* sp 1 20th day

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 4.500 | | | | | | | |
| 6.593 | VV | T | 8.94920e-1 | 0.00000 | 0.00000 | C6 |
| 7.294 | VV | T | 9.35990e-1 | 0.00000 | 0.00000 | C7 |
| 7.932 | VB | T | 2.01416e-1 | 0.00000 | 0.00000 | C8 |
| 8.966 | VV | T | 1.22743 | 0.00000 | 0.00000 | C9 |
| 10.511 | VV | T | 2.83985e-1 | 5.00000e-5 | 227.18799 | C10 |
| 11.027 | VV | T | 13.33047 | 5.00000e-5 | 1.06644e4 | C11 |
| 11.797 | VV | T | 3.81509 | 5.00000e-5 | 3052.07176 | C12 |
| 12.617 | VV | T | 4.79918 | 5.00000e-5 | 3839.34097 | C13 |
| 13.387 | VV | T | 24.79769 | 5.00000e-5 | 1.98382e4 | C14 |
| 14.502 | VV | T | 95.08427 | 5.00000e-5 | 7.60674e4 | C15 |
| 15.497 | VV | T | 21.43193 | 5.00000e-5 | 1.71455e4 | C16 |
| 16.392 | VV | T | 19.73493 | 5.00000e-5 | 1.57879e4 | C17 |
| 17.200 | VV | T | 56.90509 | 5.00000e-5 | 4.55241e4 | C18 |
| 18.011 | VV | T | 33.79787 | 5.00000e-5 | 2.70383e4 | C19 |
| 18.583 | VV | T | 18.66969 | 5.00000e-5 | 1.49357e4 | C20 |
| 19.417 | VV | T | 28.46371 | 5.00000e-5 | 2.27710e4 | C21 |
| 20.175 | VV | T | 20.07249 | 5.00000e-5 | 1.60580e4 | C22 |
| 20.857 | VV | T | 14.92138 | 5.00000e-5 | 1.19371e4 | C23 |
| 21.524 | VV | T | 24.41051 | 5.00000e-5 | 1.95284e4 | C24 |
| 21.983 | VV | T | 12.91786 | 5.00000e-5 | 1.03343e4 | C25 |
| 22.755 | VV | T | 2.72287 | 5.00000e-5 | 2178.29647 | C26 |
| 23.218 | VV | X | 2.57514 | 5.00000e-5 | 2060.11276 | C27 |
| 24.027 | VV | X | 5.14279 | 5.00000e-5 | 4114.22806 | C28 |
| 24.783 | VV | X | 9.53603 | 5.00000e-5 | 7628.82233 | C29 |
| 25.386 | VV | X | 2.79338 | 5.00000e-3 | 2.23470e5 | C30 |
| 25.980 | VV | X | 14.59071 | 5.00000e-5 | 1.16726e4 | C31 |
| 26.516 | VV | X | 10.97364 | 5.00000e-5 | 8778.91312 | C32 |
| 27.219 | VV | X | 19.74021 | 5.00000e-5 | 1.57922e4 | C33 |
| 27.754 | VV | X | 39.35261 | 5.00000e-5 | 3.14821e4 | C34 |
| 28.564 | VV | X | 41.07481 | 5.00000e-5 | 3.28598e4 | C35 |
| 29.485 | VV | X | 9.68034 | 5.00000e-5 | 7744.26880 | C36 |
| 30.205 | VV | X | 9.33755 | 5.00000e-5 | 7470.04242 | C37 |
| 30.798 | VV | X | 6.77138 | 5.00000e-5 | 5417.10663 | C38 |
| 31.241 | VV | X | 2.26532 | 5.00000e-5 | 1812.25910 | C39 |
| | | | | | | C40 |
| Totals : | | | | | 6.77230e5 | |

Results obtained with enhanced integrator

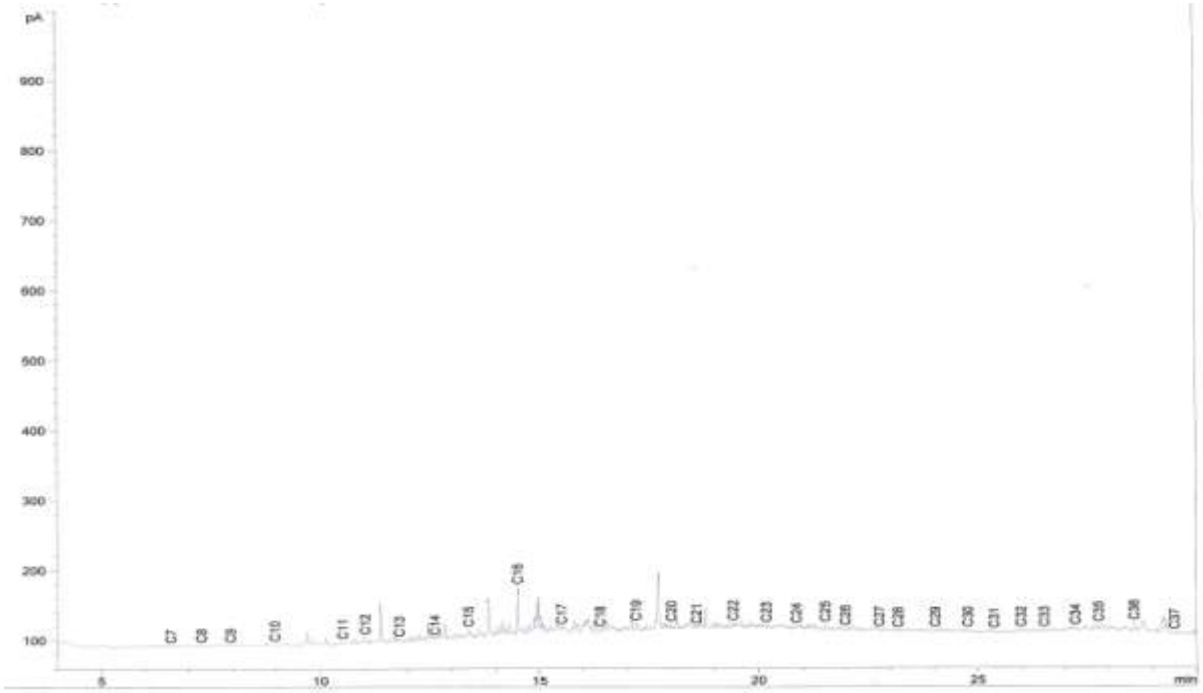
## Appendix XI A




Gas Chromatograph of Crude Oil (Residual oil) as degraded by *Providencia* sp3 at day 10

| [min] | | | [pA*s] | | [mg/Kg] | | |
|---|---|---|---|---|---|---|---|
| 4.537 | BB | X | 122.45176 | 0.00000 | 0.00000 | | C6 |
| 6.536 | VV | X | 4.51758 | 0.00000 | 0.00000 | | C7 |
| 7.230 | VV | T | 4.71414e-1 | 0.00000 | 0.00000 | | C8 |
| 8.039 | VV | T | 3.96150 | 0.00000 | 0.00000 | | C9 |
| 9.060 | VV | T | 8.59019 | 0.00000 | 0.00000 | | C10 |
| 10.486 | VV | T | 6.99374 | 5.00000e-5 | 979.12348 | | C11 |
| 10.981 | VV | T | 33.25709 | 5.00000e-5 | 4655.99228 | | C12 |
| 11.813 | VV | T | 31.93569 | 5.00000e-5 | 4470.99632 | | C13 |
| 12.652 | VV | T | 177.32233 | 5.00000e-5 | 2.48251e4 | | C14 |
| 13.393 | VV | T | 82.69505 | 5.00000e-5 | 1.15773e4 | | C15 |
| 14.516 | VV | T | 111.87270 | 5.00000e-5 | 1.56622e4 | | C16 |
| 15.474 | VV | T | 228.89275 | 5.00000e-5 | 3.20450e4 | | C17 |
| 16.456 | VV | T | 209.07533 | 5.00000e-5 | 2.92705e4 | | C18 |
| 17.196 | VV | T | 192.49004 | 5.00000e-5 | 2.69486e4 | | C19 |
| 17.967 | VV | T | 165.29655 | 5.00000e-5 | 2.31415e4 | | C20 |
| 18.609 | VV | T | 110.10258 | 5.00000e-5 | 1.54144e4 | | C21 |
| 19.398 | VV | T | 100.09388 | 5.00000e-5 | 1.40131e4 | | C22 |
| 20.226 | VV | T | 319.97269 | 5.00000e-5 | 4.47962e4 | | C23 |
| 20.971 | VV | T | 110.49709 | 5.00000e-5 | 1.54696e4 | | C24 |
| 21.536 | VV | T | 174.90193 | 5.00000e-5 | 2.44863e4 | | C25 |
| 22.020 | VV | T | 53.26179 | 5.00000e-5 | 7456.65024 | | C26 |
| 22.842 | VV | T | 169.06552 | 5.00000e-5 | 2.36692e4 | | C27 |
| 23.208 | VV | T | 61.45157 | 5.00000e-5 | 8603.21960 | | C28 |
| 23.964 | VV | T | 113.86894 | 5.00000e-5 | 1.59417e4 | | C29 |
| 24.796 | VV | T | 53.56254 | 5.00000e-5 | 7498.75534 | | C30 |
| 25.391 | VV | T | 34.51935 | 5.00000e-3 | 4.83271e5 | | C31 |
| 25.989 | VV | T | 21.01003 | 5.00000e-5 | 2941.40430 | | C32 |
| 26.456 | VB | T | 4.68647 | 5.00000e-5 | 656.10547 | | C33 |
| 27.194 | VV | X | 2.67469 | 5.00000e-5 | 374.45714 | | C34 |
| 27.801 | VV | X | 4.90074 | 5.00000e-5 | 686.10315 | | C35 |
| 28.613 | VV | X | 6.47459e-2 | 5.00000e-5 | 9.06443 | | C36 |
| 29.426 | VV | X | 3.43185 | 5.00000e-5 | 480.45946 | | C37 |
| 30.194 | VV | X | 1.62855 | 5.00000e-5 | 227.99727 | | C38 |
| 30.802 | VV | X | 6.16346 | 5.00000e-5 | 862.88450 | | C39 |
| 31.236 | VV | X | 7.38012 | 5.00000e-5 | 1033.21711 | | C40 |
| Totals : | | | | | 8.41468e5 | | |

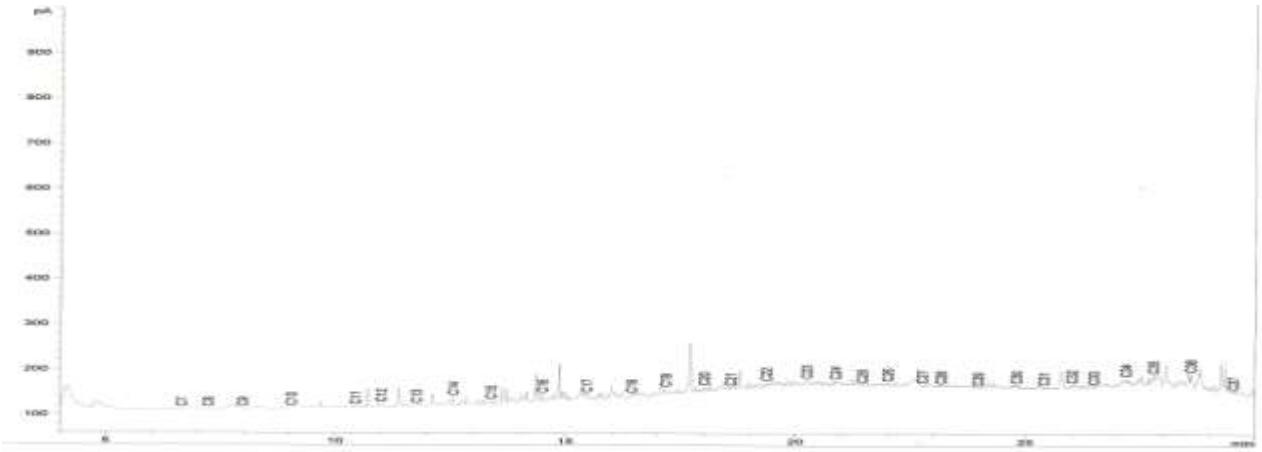
## Appendix XI B




Gas Chromatograph of Crude Oil (Residual oil) as degraded by *Providencia* sp3 20th day

| | | | | | | |
|---|---|---|---|---|---|---|
| 4.500 | | | | | | C6 |
| 6.588 | VV | T | 8.91070 | 0.00000 | 0.00000 | C7 |
| 7.286 | VV | T | 13.31381 | 0.00000 | 0.00000 | C8 |
| 7.950 | VV | T | 9.93204 | 0.00000 | 0.00000 | C9 |
| 8.966 | VV | T | 15.87074 | 0.00000 | 0.00000 | C10 |
| 10.515 | VV | T | 11.29511 | 5.00000e-5 | 9036.08627 | C11 |
| 11.030 | VV | T | 14.75290 | 5.00000e-5 | 1.18023e4 | C12 |
| 11.804 | VV | T | 2.77133 | 5.00000e-5 | 2217.06657 | C13 |
| 12.622 | VV | T | 5.83615 | 5.00000e-5 | 4668.92090 | C14 |
| 13.393 | VV | T | 41.35609 | 5.00000e-5 | 3.30849e4 | C15 |
| 14.509 | VV | T | 161.35486 | 5.00000e-5 | 1.29084e5 | C16 |
| 15.506 | VV | T | 32.00352 | 5.00000e-5 | 2.56028e4 | C17 |
| 16.393 | VV | T | 35.14053 | 5.00000e-5 | 2.81124e4 | C18 |
| 17.211 | VV | T | 96.76241 | 5.00000e-5 | 7.74099e4 | C19 |
| 18.019 | VV | T | 51.57598 | 5.00000e-5 | 4.12608e4 | C20 |
| 18.590 | VV | T | 27.57800 | 5.00000e-5 | 2.20624e4 | C21 |
| 19.424 | VV | T | 43.13705 | 5.00000e-5 | 3.45096e4 | C22 |
| 20.184 | VV | T | 29.45934 | 5.00000e-5 | 2.35675e4 | C23 |
| 20.868 | VV | T | 30.82500 | 5.00000e-5 | 2.46600e4 | C24 |
| 21.530 | VV | T | 33.79352 | 5.00000e-5 | 2.70348e4 | C25 |
| 21.987 | VV | T | 16.08513 | 5.00000e-5 | 1.28681e4 | C26 |
| 22.758 | VV | T | 1.32907 | 5.00000e-5 | 1063.25350 | C27 |
| 23.189 | VV | X | 8.11430e-1 | 5.00000e-5 | 649.14408 | C28 |
| 24.035 | VV | T | 5.69592 | 5.00000e-5 | 4556.73256 | C29 |
| 24.784 | VV | T | 7.62782 | 5.00000e-5 | 6102.25220 | C30 |
| 25.378 | VV | T | 1.77416 | 5.00000e-3 | 1.41933e5 | C31 |
| 25.991 | VV | T | 9.00498 | 5.00000e-5 | 7203.98254 | C32 |
| 26.525 | VV | X | 10.33173 | 5.00000e-5 | 8265.38162 | C33 |
| 27.229 | VV | X | 17.42724 | 5.00000e-5 | 1.39418e4 | C34 |
| 27.764 | VV | X | 43.27594 | 5.00000e-5 | 3.46208e4 | C35 |
| 28.573 | VV | X | 44.62811 | 5.00000e-5 | 3.57025e4 | C36 |
| 29.496 | VV | X | 2.60572 | 5.00000e-5 | 2084.57909 | C37 |
| 30.213 | VV | X | 5.11655 | 5.00000e-5 | 4093.24379 | C38 |
| 30.815 | VV | X | 4.94411 | 5.00000e-5 | 3955.29022 | C39 |
| 31.255 | VV | T | 1.82427 | 5.00000e-5 | 1459.41324 | C40 |
| Totals : | | | | | 7.72612e5 | |

## Appendix XII A



Gas Chromatograph of Crude Oil (Residual oil) as degraded by *Bacillus* sp 1 10th day

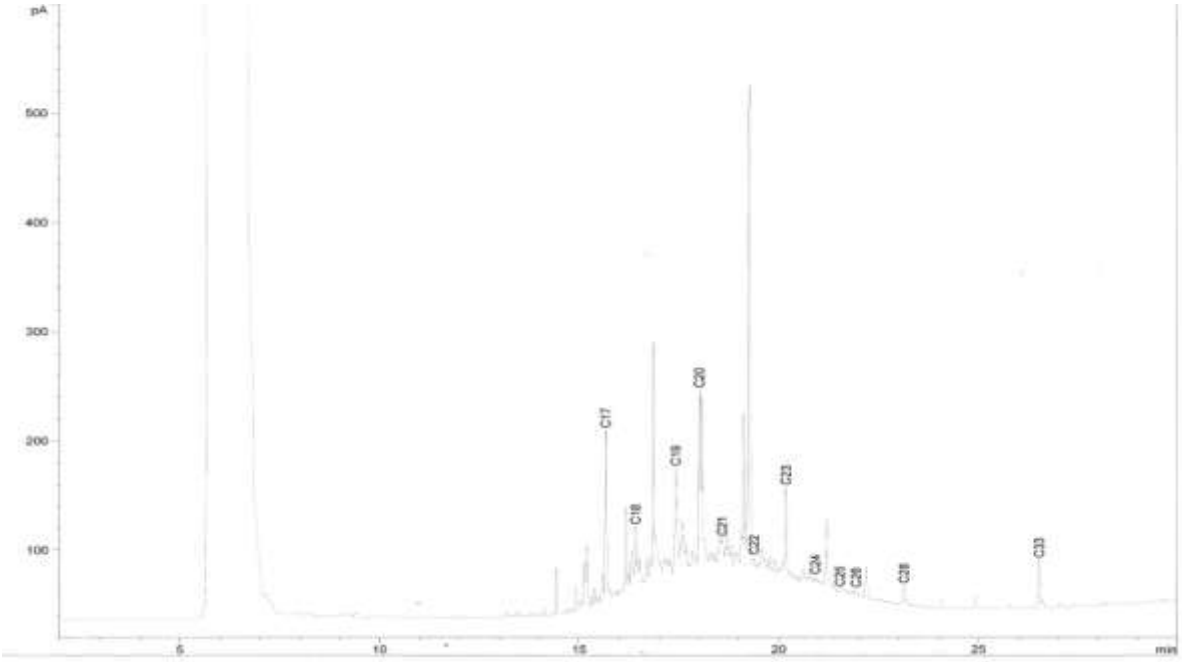
| | | | | | | |
|---|---|---|---|---|---|---|
| 4.500 | | | | | | C6 |
| 6.662 | VV | T | 7.75618e-1 | 0.00000 | 0.00000 | C7 |
| 7.238 | VV | T | 9.43288e-1 | 0.00000 | 0.00000 | C8 |
| 7.989 | VV | T | 7.92285 | 0.00000 | 0.00000 | C9 |
| 9.049 | VV | T | 26.52982 | 0.00000 | 0.00000 | C10 |
| 10.445 | VV | T | 27.61328 | 5.00000e-5 | 1.13214e4 | C11 |
| 11.003 | VV | T | 26.40612 | 5.00000e-5 | 1.08265e4 | C12 |
| 11.774 | VV | T | 13.62038 | 5.00000e-5 | 5584.35596 | C13 |
| 12.567 | VV | T | 6.69613 | 5.00000e-5 | 2745.41244 | C14 |
| 13.391 | VV | T | 1.74632 | 5.00000e-5 | 715.99174 | C15 |
| 14.513 | VV | T | 22.37705 | 5.00000e-5 | 9174.58910 | C16 |
| 15.474 | VV | T | 33.60405 | 5.00000e-5 | 1.37777e4 | C17 |
| 16.443 | VV | T | 36.17227 | 5.00000e-5 | 1.48306e4 | C18 |
| 17.194 | VV | T | 62.79039 | 5.00000e-5 | 2.57441e4 | C19 |
| 18.025 | VV | T | 76.07710 | 5.00000e-5 | 3.11916e4 | C20 |
| 18.601 | VV | T | 41.82484 | 5.00000e-5 | 1.71482e4 | C21 |
| 19.370 | VV | T | 131.30925 | 5.00000e-5 | 5.38368e4 | C22 |
| 20.252 | VV | T | 189.61656 | 5.00000e-5 | 7.77428e4 | C23 |
| 20.880 | VV | T | 95.51603 | 5.00000e-5 | 3.91616e4 | C24 |
| 21.461 | VV | T | 48.37814 | 5.00000e-5 | 1.98350e4 | C25 |
| 22.017 | VV | T | 76.77691 | 5.00000e-5 | 3.14785e4 | C26 |
| 22.768 | VV | T | 44.87670 | 5.00000e-5 | 1.83994e4 | C27 |
| 23.169 | VV | T | 23.11263 | 5.00000e-5 | 9476.18021 | C28 |
| 23.979 | VV | T | 6.29215 | 5.00000e-5 | 2579.78170 | C29 |
| 24.812 | VV | T | 25.68465 | 5.00000e-5 | 1.05307e4 | C30 |
| 25.407 | VV | T | 4.36957 | 5.00000e-3 | 1.79152e5 | C31 |
| 26.015 | VV | T | 19.85170 | 5.00000e-5 | 8139.19537 | C32 |
| 26.488 | VV | T | 5.20533e-1 | 5.00000e-5 | 213.41854 | C33 |
| 27.189 | VV | T | 41.39936 | 5.00000e-5 | 1.69737e4 | C34 |
| 27.789 | VV | T | 53.34585 | 5.00000e-5 | 2.18718e4 | C35 |
| 28.606 | VV | X | 105.84746 | 5.00000e-5 | 4.33975e4 | C36 |
| 29.527 | VV | X | 15.93920 | 5.00000e-5 | 6535.07256 | C37 |
| 30.169 | VV | X | 14.80622 | 5.00000e-5 | 6070.55022 | C38 |
| 30.775 | BV | X | 8.02774e-1 | 5.00000e-5 | 329.13720 | C39 |
| 31.200 | VV | X | 1.28526 | 5.00000e-5 | 526.95551 | C40 |
| Totals : | | | | | 6.89311e5 | |

## Appendix XII B

Gas Chromatograph of Crude Oil (Residual oil) as degraded by *Bacillus* sp 1 20th day

| | | | | | |
|---|---|---|---|---|---|
| 4.500 | | - | - | - | C6 |
| 6.674 | VV T | 1.63993 | 0.00000 | 0.00000 | C7 |
| 7.298 | VV T | 1.57184 | 0.00000 | 0.00000 | C8 |
| 7.968 | VV T | 7.21582e-1 | 0.00000 | 0.00000 | C9 |
| 8.965 | VV T | 1.02129 | 0.00000 | 0.00000 | C10 |
| 10.514 | VV T | 8.98924e-1 | 5.00000e-5 | 1707.95559 | C11 |
| 11.026 | VV T | 10.46445 | 5.00000e-5 | 1.98824e4 | C12 |
| 11.801 | VV T | 3.34595 | 5.00000e-5 | 6357.31294 | C13 |
| 12.618 | VV T | 4.65310 | 5.00000e-5 | 8840.88550 | C14 |
| 13.388 | VV T | 17.49683 | 5.00000e-5 | 3.32440e4 | C15 |
| 14.503 | VV T | 60.09430 | 5.00000e-5 | 1.14179e5 | C16 |
| 15.495 | VV T | 16.62737 | 5.00000e-5 | 3.15920e4 | C17 |
| 16.390 | VV T | 17.35717 | 5.00000e-5 | 3.29786e4 | C18 |
| 17.195 | VV T | 27.89467 | 5.00000e-5 | 5.29999e4 | C19 |
| 18.011 | VV T | 21.03042 | 5.00000e-5 | 3.99578e4 | C20 |
| 18.582 | VV T | 11.70123 | 5.00000e-5 | 2.22323e4 | C21 |
| 19.415 | VV T | 16.87922 | 5.00000e-5 | 3.20705e4 | C22 |
| 20.174 | VV T | 14.67912 | 5.00000e-5 | 2.78903e4 | C23 |
| 20.859 | VV T | 16.49481 | 5.00000e-5 | 3.13401e4 | C24 |
| 21.521 | VV T | 20.37197 | 5.00000e-5 | 3.87067e4 | C25 |
| 21.977 | VV T | 12.81740 | 5.00000e-5 | 2.43531e4 | C26 |
| 22.849 | VV T | 10.31307 | 5.00000e-5 | 1.95948e4 | C27 |
| 23.218 | VV T | 7.62481 | 5.00000e-5 | 1.44871e4 | C28 |
| 24.021 | VV T | 7.22010 | 5.00000e-5 | 1.37182e4 | C29 |
| 24.778 | VV T | 4.57821 | 5.00000e-5 | 8698.60330 | C30 |
| 25.390 | VV T | 2.87355e-2 | 5.00000e-3 | 5459.75311 | C31 |
| 25.986 | VV T | 3.19485 | 5.00000e-5 | 6070.21811 | C32 |
| 26.520 | VV T | 1.71781 | 5.00000e-5 | 3263.83476 | C33 |
| 27.216 | VV T | 4.99322 | 5.00000e-5 | 9487.11863 | C34 |
| 27.757 | VV T | 10.55244 | 5.00000e-5 | 2.00496e4 | C35 |
| 28.567 | VV T | 12.11037 | 5.00000e-5 | 2.30097e4 | C36 |
| 29.488 | VV T | 1.57984 | 5.00000e-5 | 3001.69499 | C37 |
| 30.197 | VV T | 2.37975 | 5.00000e-5 | 4521.52684 | C38 |
| 30.810 | VV X | 9.06966e-1 | 5.00000e-5 | 1723.23568 | C39 |
| 31.159 | VV X | 1.09414e-1 | 5.00000e-5 | 207.88689 | C40 |
| Totals : | | | | 6.51627e5 | |

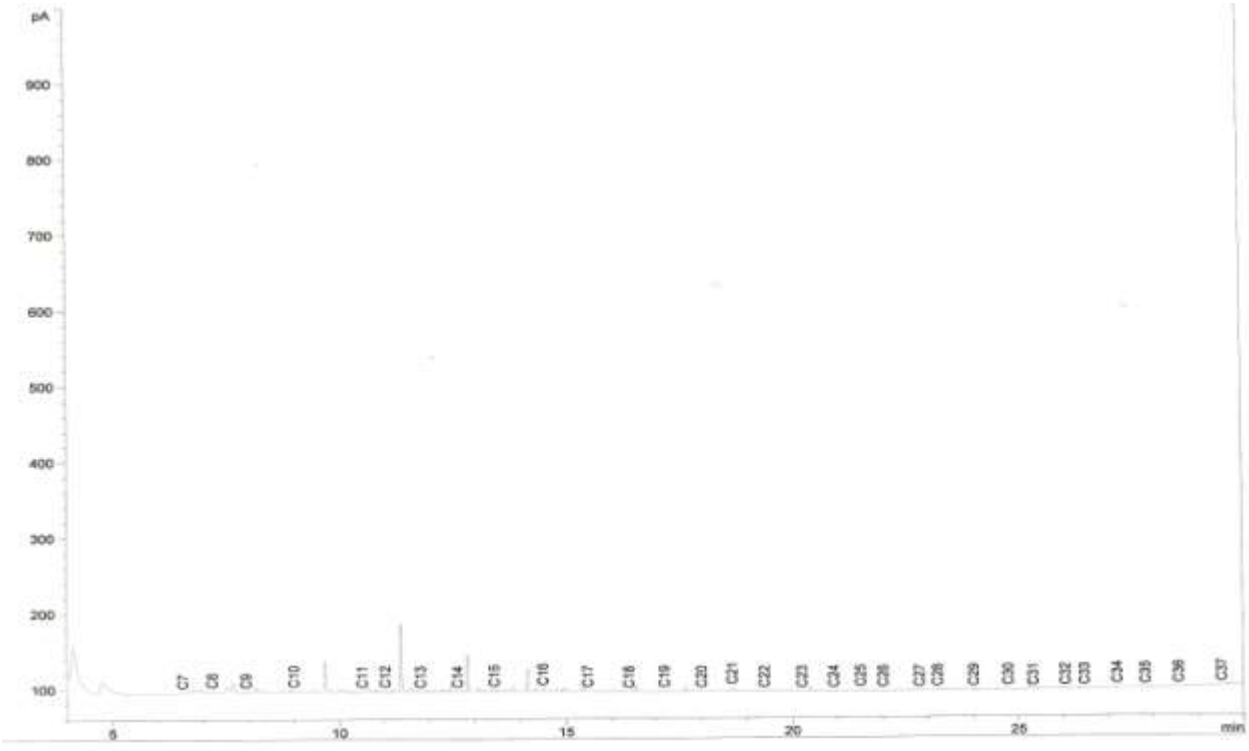
**Appendix XIII**




Gas Chromatograph of Control at day 0 (Used Crankcase Oil)

| RetTime [min] | Type | Area [pA*s] | Amt/Area | Amount [mg/Kg] | Grp | Name |
|---|---|---|---|---|---|---|
| 4.596 | BP | 1.78443e-1 | 0.00000 | 0.00000 | | C6 |
| 6.504 | VB S | 3.33293e4 | 0.00000 | 0.00000 | | C7 |
| 7.153 | BB X | 74.62476 | 0.00000 | 0.00000 | | C8 |
| 7.935 | VV X | 35.42345 | 0.00000 | 0.00000 | | C9 |
| 8.993 | VV X | 11.27350 | 0.00000 | 0.00000 | | C10 |
| 10.500 | | - | - | - | | C11 |
| 11.000 | | - | - | - | | C12 |
| 11.800 | | - | - | - | | C13 |
| 12.600 | | - | - | - | | C14 |
| 13.400 | | - | - | - | | C15 |
| 14.500 | | - | - | - | | C16 |
| 15.676 | VV T | 123.13949 | 800.00000 | 9.85116e4 | | C17 |
| 16.422 | VV T | 24.23392 | 800.00000 | 1.93871e4 | | C18 |
| 17.430 | VV T | 151.82550 | 800.00000 | 1.21460e5 | | C19 |
| 18.024 | VV T | 281.35226 | 800.00000 | 2.25082e5 | | C20 |
| 18.586 | VV X | 88.55001 | 800.00000 | 7.08400e4 | | C21 |
| 19.381 | VV X | 49.85208 | 800.00000 | 3.98817e4 | | C22 |
| 20.186 | VV X | 264.05435 | 800.00000 | 2.11243e5 | | C23 |
| 20.920 | VV X | 41.65187 | 800.00000 | 3.33215e4 | | C24 |
| 21.538 | VV X | 7.25956 | 800.00000 | 5807.65190 | | C25 |
| 21.937 | VB X | 15.17893 | 1000.00000 | 1.51789e4 | | C26 |
| 22.800 | | - | - | - | | C27 |
| 23.138 | VV T | 2.84848 | 1000.00000 | 2848.47593 | | C28 |
| 24.000 | | - | - | - | | C29 |
| 24.800 | | - | - | - | | C30 |
| 25.400 | | - | - | - | | C31 |
| 26.000 | | - | - | - | | C32 |
| 26.534 | VV T | 8.44354 | 1000.00000 | 8443.54439 | | C33 |
| 27.200 | | - | - | - | | C34 |
| 27.800 | | - | - | - | | C35 |
| 28.600 | | - | - | - | | C36 |
| 29.500 | | - | - | - | | C37 |
| 30.200 | | - | - | - | | C38 |
| 30.800 | | - | - | - | | C39 |
| 31.200 | | - | - | - | | C40 |
| Totals : | | | | | | |

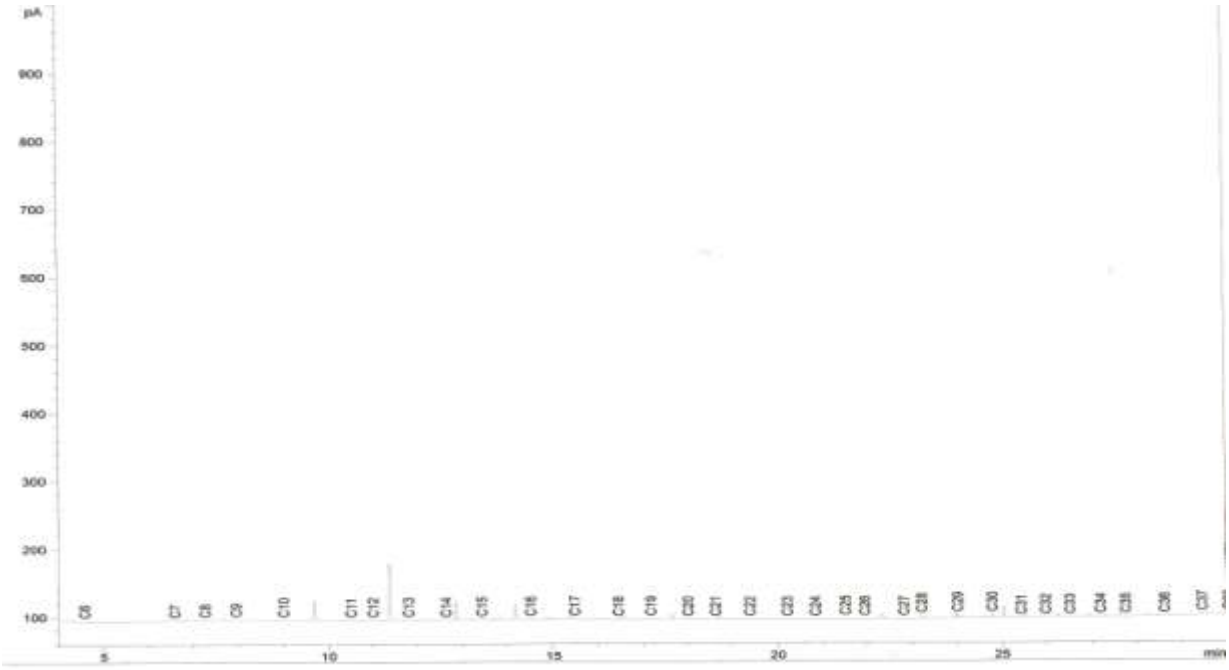
## Appendix XIVA




Gas Chromatograph of Used Crankcase Oil (Residual oil) as degraded by *Providencia* sp1 10th day

| | | | [mg/Kg] | | |
|---|---|---|---|---|---|
| 4.580 | BV x | 2.71243e-1 | 0.00000 | 0.00000 | C6 |
| 6.586 | VV | 1.30504 | 0.00000 | 0.00000 | C7 |
| 7.258 | VV | 3.36927 | 0.00000 | 0.00000 | C8 |
| 7.949 | VV | 8.16344 | 0.00000 | 0.00000 | C9 |
| 9.007 | VV | 1.19563 | 0.00000 | 0.00000 | C10 |
| 10.510 | VV | 5.65794e-1 | 5.00000e-5 | 1.41448e4 | C11 |
| 11.001 | VP | 3.10904e-1 | 5.00000e-5 | 7772.58798 | C12 |
| 11.785 | VV | 8.96393e-1 | 5.00000e-5 | 2.24098e4 | C13 |
| 12.622 | VV | 6.41831e-1 | 5.00000e-5 | 1.60458e4 | C14 |
| 13.419 | VV | 2.24958 | 5.00000e-5 | 5.62394e4 | C15 |
| 14.500 | VV | 5.37636 | 5.00000e-5 | 1.34409e5 | C16 |
| 15.488 | VV | 6.01163 | 5.00000e-5 | 1.50291e5 | C17 |
| 16.455 | VV | 5.82468 | 5.00000e-5 | 1.45617e5 | C18 |
| 17.178 | VV | 2.94713 | 5.00000e-5 | 7.36782e4 | C19 |
| 18.008 | VV | 2.57889 | 5.00000e-5 | 6.44721e4 | C20 |
| 18.616 | VV | 1.36470 | 5.00000e-5 | 3.41176e4 | C21 |
| 19.387 | VV | 1.41211 | 5.00000e-5 | 3.53027e4 | C22 |
| 20.216 | VV | 5.83540e-1 | 5.00000e-5 | 1.45885e4 | C23 |
| 20.843 | VV | 2.68959e-1 | 5.00000e-5 | 6723.97241 | C24 |
| 21.510 | VV | 1.92569 | 5.00000e-5 | 4.81423e4 | C25 |
| 21.942 | VV | 9.14902e-1 | 5.00000e-9 | 2.28726 | C26 |
| 22.813 | VV | 1.12570e-1 | 5.00000e-9 | 2.81426e-1 | C27 |
| 23.210 | VV | 8.22166 | 5.00000e-9 | 20.55416 | C28 |
| 24.009 | VV | 10.38613 | 5.00000e-9 | 25.96532 | C29 |
| 24.777 | VV | 10.21759 | 5.00000e-9 | 25.54398 | C30 |
| 25.422 | VV | 7.09439e-1 | 5.00000e-9 | 1.77360 | C31 |
| 25.968 | VV | 2.18402 | 5.00000e-9 | 5.46005 | C32 |
| 26.509 | VV | 3.78490e-1 | 5.00000e-9 | 9.46224e-1 | C33 |
| 27.189 | VV | 6.04637 | 5.00000e-9 | 15.11593 | C34 |
| 27.750 | VV | 7.97071e-1 | 5.00000e-9 | 1.99268 | C35 |
| 28.622 | VV | 2.94114 | 5.00000e-9 | 7.35285 | C36 |
| 29.466 | VV | 18.02285 | 5.00000e-9 | 45.05712 | C37 |
| 30.053 | VV | 23.66722 | 5.00000e-9 | 59.16804 | C38 |
| 30.805 | VV | 2.84365 | 5.00000e-9 | 7.10912 | C39 |
| 31.193 | VV | 6.26395 | 5.00000e-9 | 15.65987 | C40 |
| Totals : | | | | 8.24189e5 | |

## Appendix XIV B

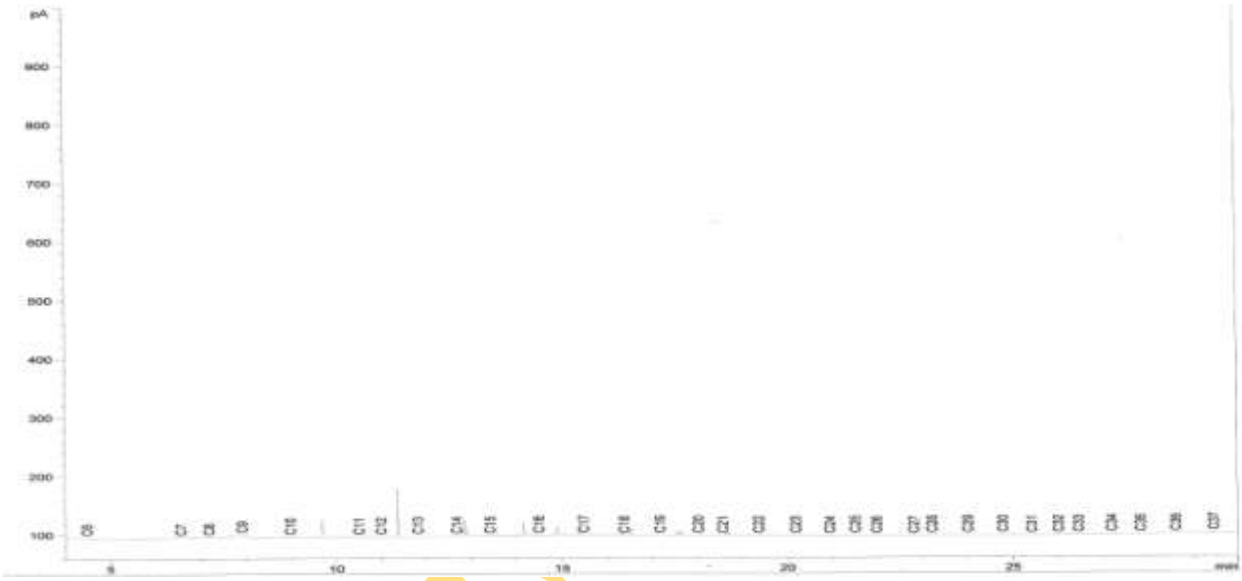



Gas Chromatograph of Used Crankcase Oil (Residual oil) as degraded by *Providencia* sp 1 20th day

Signal 1: FID1 A,

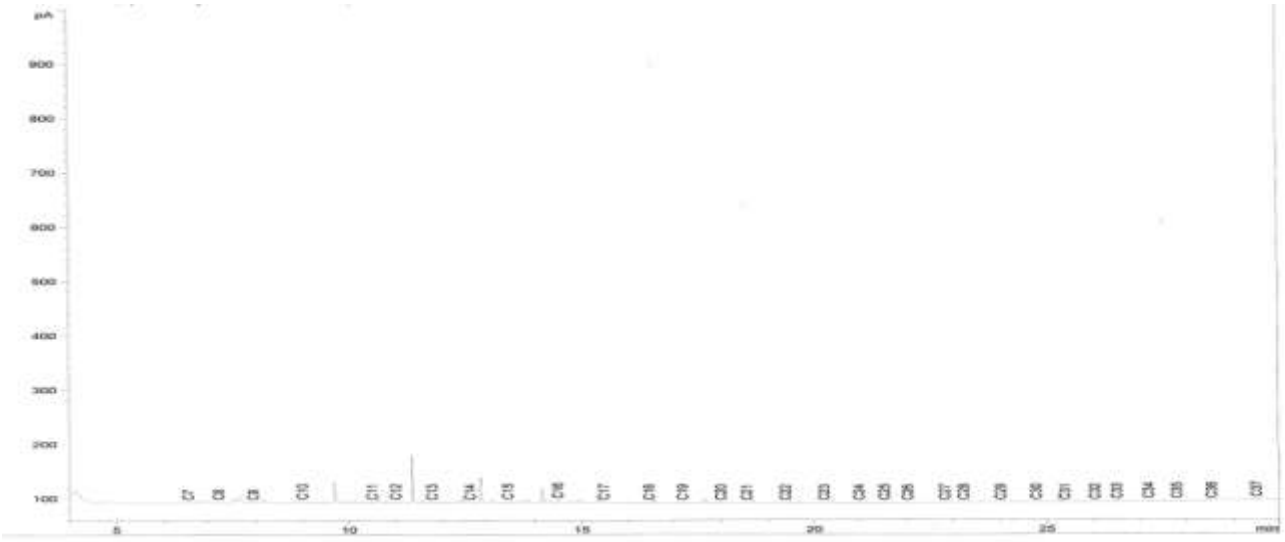
| RetTime [min] | Type | Area [pA*s] | Amt/Area | Amount [mg/Kg] | Grp | Name |
|---|---|---|---|---|---|---|
| 4.500 | | - | - | - | | C6 |
| 6.545 | VV T | 13.43958 | 0.00000 | 0.00000 | | C7 |
| 7.204 | VV T | 29.79893 | 0.00000 | 0.00000 | | C8 |
| 7.951 | VV T | 14.18145 | 0.00000 | 0.00000 | | C9 |
| 9.003 | VV T | 11.97800 | 0.00000 | 0.00000 | | C10 |
| 10.511 | VV T | 12.16189 | 5.00000e-5 | 9.94235e4 | | C11 |
| 11.016 | VV T | 17.06508 | 5.00000e-5 | 1.39507e5 | | C12 |
| 11.794 | VV T | 9.15095 | 5.00000e-5 | 7.48091e4 | | C13 |
| 12.613 | VV T | 5.02894 | 5.00000e-5 | 4.11116e4 | | C14 |
| 13.423 | VV T | 12.18761 | 5.00000e-5 | 9.96337e4 | | C15 |
| 14.497 | VV T | 11.67239 | 5.00000e-5 | 9.54218e4 | | C16 |
| 15.488 | VV T | 4.28811 | 5.00000e-5 | 3.50553e4 | | C17 |
| 16.458 | VV T | 4.28103 | 5.00000e-5 | 3.49975e4 | | C18 |
| 17.179 | VV T | 1.99542 | 5.00000e-5 | 1.63125e4 | | C19 |
| 18.004 | VV T | 9.46727e-1 | 5.00000e-5 | 7739.49304 | | C20 |
| 18.673 | VV T | 9.96194 | 5.00000e-5 | 8.14388e4 | | C21 |
| 19.384 | VV T | 1.06692e-1 | 5.00000e-5 | 872.20869 | | C22 |
| 20.218 | VV T | 3.24055e-1 | 5.00000e-5 | 2649.14854 | | C23 |
| 20.912 | VV | 1.23489e-1 | 5.00000e-5 | 1009.52245 | | C24 |
| 21.512 | VV | 2.02537 | 5.00000e-5 | 1.65574e4 | | C25 |
| 21.993 | VV | 1.39481e-1 | 5.00000e-5 | 1.14026e-1 | | C26 |
| 22.796 | VV | 2.67170e-1 | 5.00000e-5 | 2.18411e-1 | | C27 |
| 23.211 | VV | 2.33581 | 5.00000e-5 | 1.90953 | | C28 |
| 24.010 | VV | 1.45659 | 5.00000e-5 | 1.19076 | | C29 |
| 24.777 | VV | 2.05906 | 5.00000e-5 | 1.68328 | | C30 |
| 25.387 | VV | 1.48712e-1 | 5.00000e-5 | 1.21572e-1 | | C31 |
| 25.980 | VV | 7.14879e-1 | 5.00000e-5 | 5.84414e-1 | | C32 |
| 26.462 | VV | 1.40137 | 5.00000e-5 | 1.14562 | | C33 |
| 27.192 | VV | 5.01833 | 5.00000e-5 | 4.10248 | | C34 |
| 27.796 | VV | 8.55641e-1 | 5.00000e-5 | 6.99487e-1 | | C35 |
| 28.595 | VV | 6.96959e-1 | 5.00000e-5 | 5.69764e-1 | | C36 |
| 29.471 | VV | 9.58558 | 5.00000e-5 | 7.83621 | | C37 |
| 30.246 | VV | 15.30632 | 5.00000e-5 | 12.51291 | | C38 |
| 30.795 | VV | 14.07312 | 5.00000e-5 | 11.50478 | | C39 |
| 31.239 | VV | 8.42496 | 5.00000e-5 | 6.88741 | | C40 |
| Totals : | | | | 7.46590e5 | | |

## Appendix XV A




Gas Chromatograph of Used Crankcase Oil (Residual oil) as degraded by *Providencia* sp 3 10th

## Appendix XV B

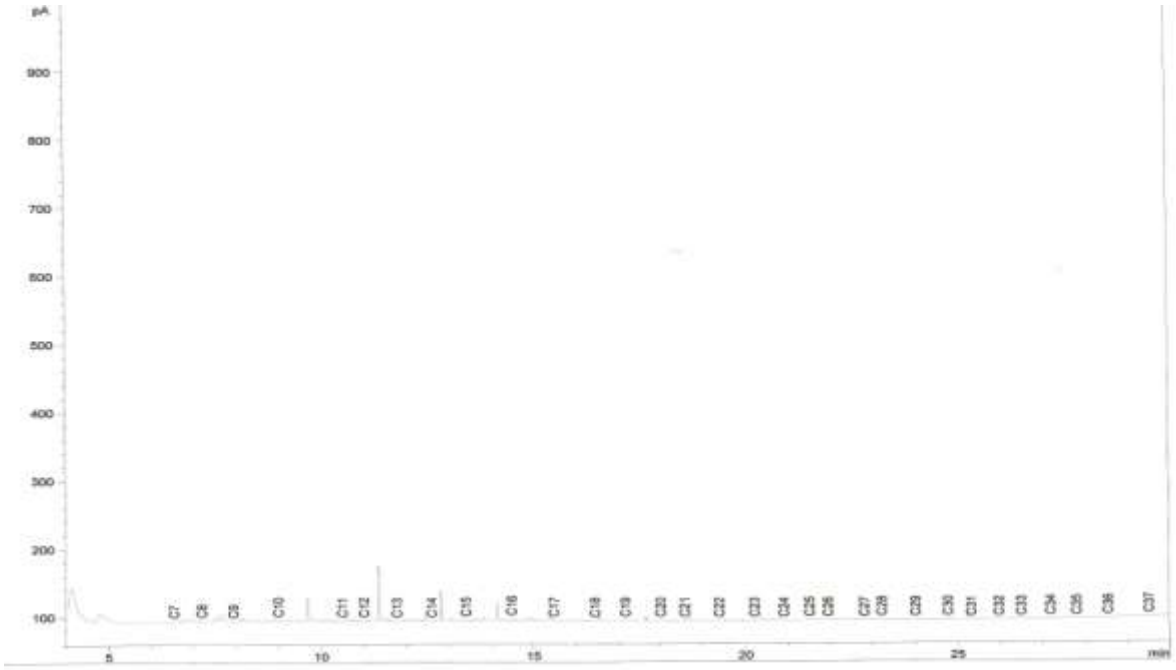


Gas Chromatograph of Used Crankcase Oil (Residual oil) as degraded by *Providencia* sp 3 20th day

| Time [Min] | Type | Area [pA*s] | Amt/Area | Amount [mg/Kg] | Grp | Name |
|---|---|---|---|---|---|---|
| 4.500 | | - | - | - | | |
| 6.547 | VV T | 6.52833 | 0.00000 | 0.00000 | | C6 |
| 7.199 | VV T | 10.99609 | 0.00000 | 0.00000 | | C7 |
| 7.945 | VV T | 7.66393e-1 | 0.00000 | 0.00000 | | C8 |
| 9.002 | VV T | 2.93248 | 0.00000 | 0.00000 | | C9 |
| 10.508 | VV X | 6.45004 | 5.00000e-5 | 9.14293e4 | | C10 |
| 11.014 | VV X | 9.60403 | 5.00000e-5 | 1.36137e5 | | C11 |
| 11.793 | VV X | 5.31460 | 5.00000e-5 | 7.53344e4 | | C12 |
| 12.609 | VV X | 3.17001 | 5.00000e-5 | 4.49349e4 | | C13 |
| 13.420 | VV X | 8.86524 | 5.00000e-5 | 1.25665e5 | | C14 |
| 14.494 | VV X | 8.62446 | 5.00000e-5 | 1.22252e5 | | C15 |
| 15.484 | VV X | 3.33871 | 5.00000e-5 | 4.73262e4 | | C16 |
| 16.451 | VV X | 2.15286 | 5.00000e-5 | 3.05168e4 | | C17 |
| 17.176 | VV X | 1.13846 | 5.00000e-5 | 1.61376e4 | | C18 |
| 18.000 | VV X | 8.18654e-1 | 5.00000e-5 | 1.16044e4 | | C19 |
| 18.549 | VV X | 2.71914e-1 | 5.00000e-5 | 3854.38609 | | C20 |
| 19.381 | VV X | 5.14927e-1 | 5.00000e-5 | 7299.08895 | | C21 |
| 20.215 | VV T | 9.95764e-2 | 5.00000e-5 | 1411.49587 | | C22 |
| 20.961 | VV T | 3.41962e-1 | 5.00000e-5 | 4847.30811 | | C23 |
| 21.509 | VV T | 6.82938e-1 | 5.00000e-5 | 9680.64840 | | C24 |
| 22.011 | VV T | 3.69638e-1 | 5.00000e-9 | 5.23961e-1 | | C25 |
| 22.806 | VV T | 4.26978e-1 | 5.00000e-9 | 6.05241e-1 | | C26 |
| 23.208 | VV T | 1.14794 | 5.00000e-9 | 1.62720 | | C27 |
| 24.004 | VV | 1.11664 | 5.00000e-9 | 1.58284 | | C28 |
| 24.774 | VV | 1.40387 | 5.00000e-9 | 1.98999 | | C29 |
| 25.388 | VV | 1.86985e-1 | 5.00000e-9 | 2.65051e-1 | | C30 |
| 26.031 | VV | 1.47077e-1 | 5.00000e-9 | 2.08481e-1 | | C31 |
| 26.502 | VV | 4.60742e-1 | 5.00000e-9 | 6.53101e-1 | | C32 |
| 27.187 | VV | 6.45570 | 5.00000e-9 | 9.15095 | | C33 |
| 27.795 | VV | 1.33279 | 5.00000e-9 | 1.88922 | | C34 |
| 28.541 | VV | 3.64054 | 5.00000e-9 | 5.16047 | | C35 |
| 29.508 | VV | 1.91311 | 5.00000e-9 | 2.71183 | | C36 |
| 30.222 | VV | 21.93631 | 5.00000e-9 | 31.09472 | | C37 |
| 30.789 | VV | 28.28961 | 5.00000e-9 | 40.10053 | | C38 |
| 31.223 | VV | 13.70686 | 5.00000e-9 | 19.42947 | | C39 |
| | | | | | | C40 |
| Totals : | | | | 7.28547e5 | | |

Results obtained with

## Appendix XVI A

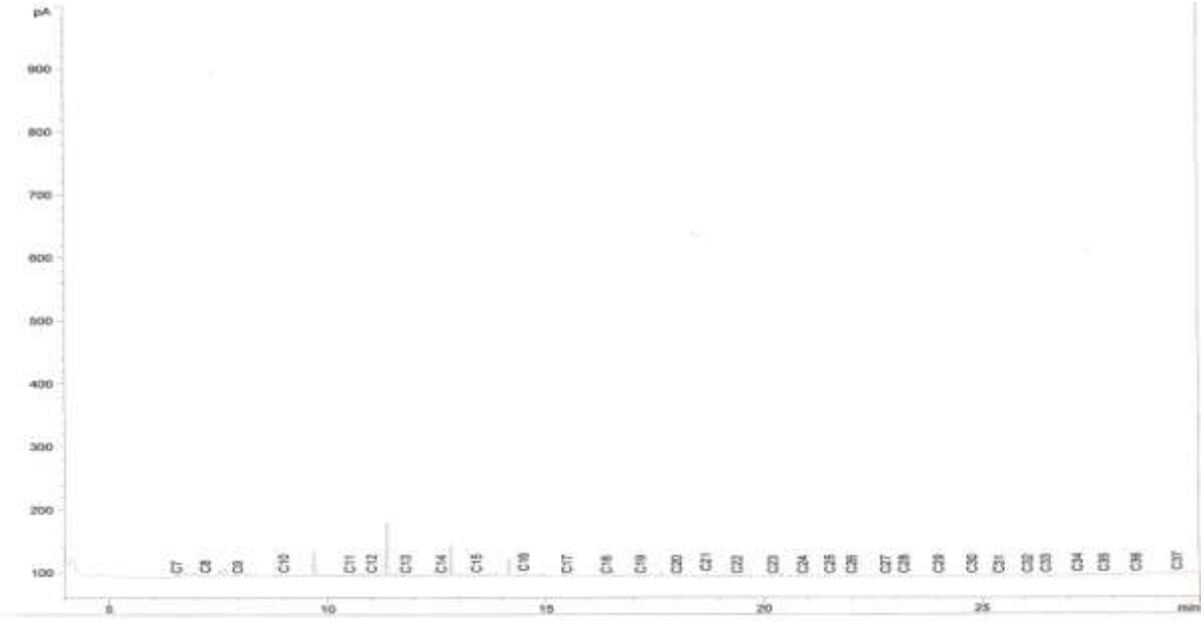



Gas Chromatograph of Used Crankcase Oil (Residual oil) as degraded by *Bacillus* sp 4 10th day

| Time [min] | Type | Area [pA*s] | Amt/Area | Amount [mg/Kg] | Grp | Name |
|---|---|---|---|---|---|---|
| 4.500 | | - | | - | | C6 |
| 6.543 | VV T | 19.17635 | 0.00000 | 0.00000 | | C7 |
| 7.202 | VV T | 33.83142 | 0.00000 | 0.00000 | | C8 |
| 7.948 | VV T | 16.24557 | 0.00000 | 0.00000 | | C9 |
| 9.003 | VV T | 12.41432 | 0.00000 | 0.00000 | | C10 |
| 10.510 | VV T | 31.71941 | 5.00000e-5 | 8.40867e4 | | C11 |
| 11.015 | VV T | 16.53834 | 5.00000e-5 | 1.18663e5 | | C12 |
| 11.797 | VV T | 10.07683 | 5.00000e-5 | 7.23012e4 | | C13 |
| 12.614 | VV T | 5.20453 | 5.00000e-5 | 3.73425e4 | | C14 |
| 13.422 | VV T | 12.64304 | 5.00000e-5 | 9.07138e4 | | C15 |
| 14.497 | VV T | 11.42992 | 5.00000e-5 | 8.20097e4 | | C16 |
| 15.488 | VV T | 5.10887 | 5.00000e-5 | 3.66561e4 | | C17 |
| 16.458 | VV T | 5.17602 | 5.00000e-5 | 3.71379e4 | | C18 |
| 17.179 | VV T | 2.13768 | 5.00000e-5 | 1.53378e4 | | C19 |
| 18.004 | VV T | 1.61235 | 5.00000e-5 | 1.15686e4 | | C20 |
| 18.673 | VV T | 10.23846 | 5.00000e-5 | 7.34609e4 | | C21 |
| 19.387 | VV T | 1.14225e-1 | 5.00000e-5 | 819.56710 | | C22 |
| 20.215 | VV T | 6.06523e-1 | 5.00000e-5 | 4351.80536 | | C23 |
| 20.915 | VV T | 1.31498 | 5.00000e-5 | 9434.98856 | | C24 |
| 21.512 | VV T | 9.22477e-1 | 5.00000e-5 | 6618.77039 | | C25 |
| 21.957 | VV T | 6.00428 | 5.00000e-9 | 4.30807 | | C26 |
| 22.801 | VV T | 4.07957 | 5.00000e-9 | 2.92709 | | C27 |
| 23.215 | VV T | 5.42587 | 5.00000e-9 | 3.89306 | | C28 |
| 24.009 | VV T | 4.36689 | 5.00000e-9 | 3.13324 | | C29 |
| 24.776 | VV T | 5.63091 | 5.00000e-9 | 4.04018 | | C30 |
| 25.395 | VV T | 3.36463 | 5.00000e-9 | 2.41412 | | C31 |
| 25.970 | VV T | 3.23869 | 5.00000e-9 | 2.32376 | | C32 |
| 26.497 | VV T | 3.52843 | 5.00000e-9 | 2.53165 | | C33 |
| 27.189 | VV T | 8.16132 | 5.00000e-9 | 5.85575 | | C34 |
| 27.852 | VV T | 5.21609 | 5.00000e-9 | 3.74254 | | C35 |
| 28.552 | VV T | 3.00391 | 5.00000e-9 | 2.15531 | | C36 |
| 29.465 | VV T | 6.25784 | 5.00000e-9 | 4.49000 | | C37 |
| 30.233 | VV T | 10.22303 | 5.00000e-9 | 7.33503 | | C38 |
| 30.797 | VV T | 18.89508 | 5.00000e-9 | 13.55722 | | C39 |
| 31.223 | VV T | 7.80235 | 5.00000e-9 | 5.59818 | | C40 |
| Totals : | | | | 6.80571e5 | | |

Results obtained with enhanced integrator!

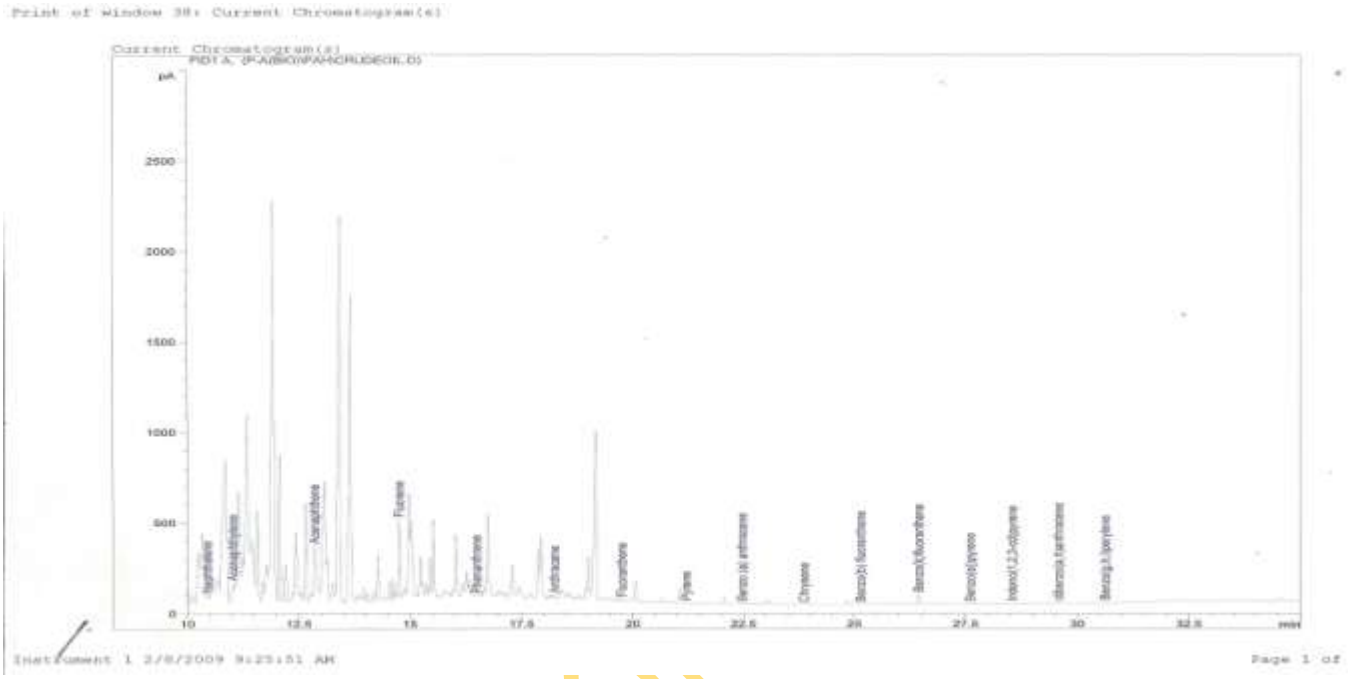
Appendix XVI B




Gas Chromatograph of Used Crankcase Oil (Residual oil) as degraded by *Bacillus* sp 4 20th day

| Time [n] | Type | Area [pA*s] | Amt/Area | Amount [mg/Kg] | Grp | Name |
|---|---|---|---|---|---|---|
| 4.500 | | | | | | C6 |
| 6.549 | VV T | 17.29001 | 0.00000 | 0.00000 | | C7 |
| 7.207 | VV T | 32.65989 | 0.00000 | 0.00000 | | C8 |
| 7.948 | VV T | 13.15596 | 0.00000 | 0.00000 | | C9 |
| 9.002 | VV T | 11.04802 | 0.00000 | 0.00000 | | C10 |
| 10.509 | VV T | 10.95402 | 5.00000e-5 | 8.95491e4 | | C11 |
| 11.016 | VV T | 14.24525 | 5.00000e-5 | 1.16455e5 | | C12 |
| 11.796 | VV T | 7.79625 | 5.00000e-5 | 6.37344e4 | | C13 |
| 12.612 | VV T | 3.95321 | 5.00000e-5 | 3.23175e4 | | C14 |
| 13.422 | VV T | 10.60496 | 5.00000e-5 | 8.66956e4 | | C15 |
| 14.496 | VV T | 9.62150 | 5.00000e-5 | 7.86558e4 | | C16 |
| 15.487 | VV T | 2.80561 | 5.00000e-5 | 2.29359e4 | | C17 |
| 16.391 | VV T | 2.84614e-1 | 5.00000e-5 | 2326.71699 | | C18 |
| 17.178 | VV T | 1.07181 | 5.00000e-5 | 8762.03609 | | C19 |
| 18.003 | VV X | 7.52653e-1 | 5.00000e-5 | 6152.93976 | | C20 |
| 18.673 | VV X | 8.84165 | 5.00000e-5 | 7.22805e4 | | C21 |
| 19.385 | VV X | 5.84882e-1 | 5.00000e-5 | 4781.41150 | | C22 |
| 20.210 | VV | 2.02572e-1 | 5.00000e-5 | 1656.02223 | | C23 |
| 20.891 | VV | 1.11650e-1 | 5.00000e-5 | 912.73952 | | C24 |
| 21.513 | VV | 1.88786 | 5.00000e-5 | 1.54333e4 | | C25 |
| 22.013 | VV | 1.75927e-1 | 5.00000e-9 | 1.43820e-1 | | C26 |
| 22.791 | VV | 2.81474e-1 | 5.00000e-9 | 2.30105e-1 | | C27 |
| 23.210 | VV | 2.20587 | 5.00000e-9 | 1.80330 | | C28 |
| 24.010 | VV | 1.29949 | 5.00000e-9 | 1.06233 | | C29 |
| 24.777 | VV | 1.50574 | 5.00000e-9 | 1.23094 | | C30 |
| 25.395 | VV | 2.17673e-1 | 5.00000e-9 | 1.77948e-1 | | C31 |
| 26.043 | VV | 4.59824e-1 | 5.00000e-9 | 3.75906e-1 | | C32 |
| 26.467 | VV | 1.73155 | 5.00000e-9 | 1.41555 | | C33 |
| 27.191 | VV | 4.13213 | 5.00000e-9 | 3.37802 | | C34 |
| 27.800 | VV | 6.04411e-1 | 5.00000e-9 | 4.94106e-1 | | C35 |
| 28.540 | VV | 1.61974 | 5.00000e-9 | 1.32414 | | C36 |
| 29.478 | VV | 8.46712 | 5.00000e-9 | 6.92187 | | C37 |
| 30.231 | VV | 12.70624 | 5.00000e-9 | 10.38735 | | C38 |
| 30.800 | VV | 9.81145 | 5.00000e-9 | 8.02086 | | C39 |
| 31.191 | VV | 2.90450 | 5.00000e-9 | 2.37443 | | C40 |
| Totals : | | | | 6.02688e5 | | |

## Appendix XVII




Gas Chromatograph of Crude oil (PAH) at day 0 (Control)

s Method : C:\HPCHEM\1\METHODS\PAH-AYAN.M
anged : 2/8/2009 9:39:56 AM
(modified after loading)
aromatic hydrocarbons

External Standard Report

Sorted By : Retention Time
Calib. Data Modified : 2/8/2009 9:39:13 AM
Multiplier : 3.7000
Dilution : 1.0000

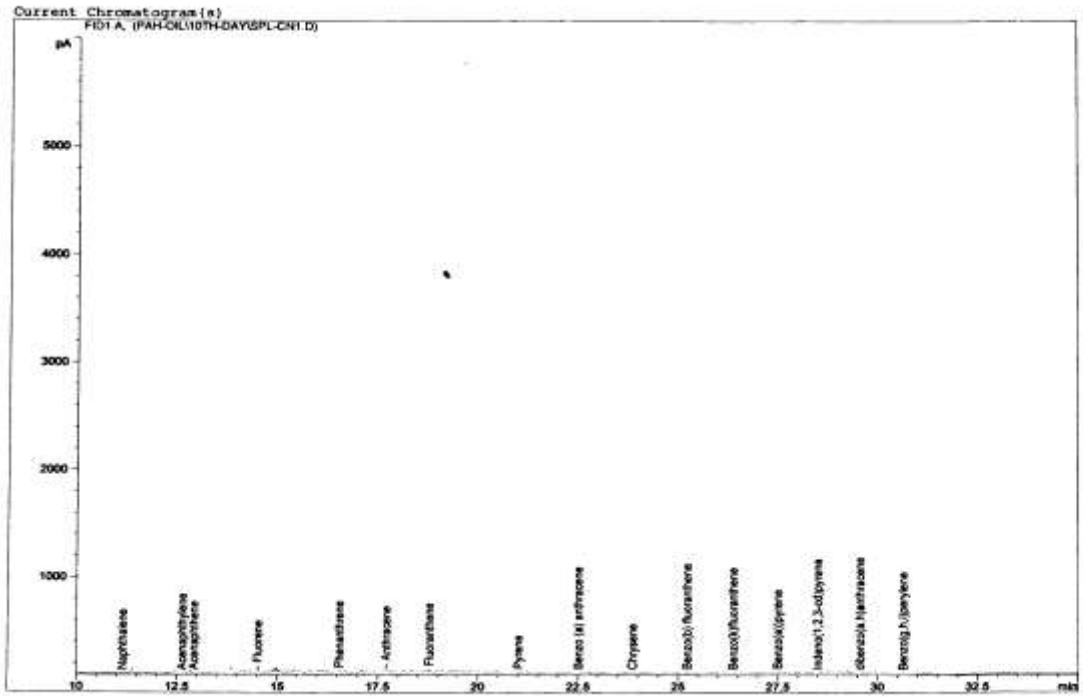
Signal 1: FID1 A,

| RetTime [min] | Sig | Type | Area [pA*s] | Amt/Area | Amount [mg/Kg] | Grp | Name |
|---|---|---|---|---|---|---|---|
| 10.449 | 1 | VV | 197.13710 | 8.96294 | 6537.63146 | 1 | Naphthalene |
| 11.028 | 1 | VV | 525.84741 | 1.84308 | 3585.96149 | 1 | Acenaphthylene |
| 12.861 | 1 | VV | 1754.13135 | 3.76606e-1 | 2444.28004 | 1 | Acenaphthene |
| 14.768 | 1 | VV | 1299.11438 | 6.13535e-1 | 2949.09116 | 1 | Fluorene |
| 16.488 | 1 | VV | 87.94422 | 2.29661 | 747.30235 | 1 | Phenanthrene |
| 18.241 | 1 | VV | 183.71959 | 2.41254e-1 | 163.99572 | 1 | Anthracene |
| 19.753 | 1 | VV | 189.77417 | 6.92109e-2 | 48.59746 | 1 | Fluoranthene |
| 21.211 | 1 | VV | 33.45435 | 8.96954e-2 | 11.10260 | 1 | Pyrene |
| 22.476 | 1 | VV | 32.39096 | 8.26508e-2 | 9.90541 | 1 | Benzo (a) anthracene |
| 23.861 | 1 | VV | 6.70775 | 2.46938e-1 | 6.12866 | 1 | Chrysene |
| 25.146 | 1 | VV | 8.60535 | 2.06315e-1 | 6.56902 | 1 | Benzo(b) fluoranthene |
| 26.443 | 1 | VV | 153.54095 | 7.80379e-2 | 44.33348 | 1 | Benzo(k)fluoranthene |
| 27.602 | 1 | VV | 6.67795 | 1.96591e-1 | 4.85745 | 1 | Benzo(a))pyrene |
| 28.541 | 1 | VV | 5.20329 | 0.00000 | 0.00000 | 1 | Indeno(1,2,3-cd)pyrene |
| 29.583 | 1 | VV | 19.13095 | 2.54674e-2 | 1.80269 | 1 | dibenzo(a,h)anthracene |
| 30.628 | 1 | VV | 4.19277 | 2.37368e-1 | 3.68235 | 1 | Benzo(g,h,i)perylene |
| Totals : | | | | | 1.65652e4 | | |

Results obtained with enhanced integrator!

Appendix XVIII A

of Window 38: Current Chromatogram(s)




went 1 8/10/2010 4:07:36 PM Page 1 of 1

Gas Chromatograph of Crude Oil (PAH) as degraded by *Providencia* sp 1 10th day ($NH_4NO_3$)

nalysis Method : C:\HPCHEM\1\METHODS\PAH-CN1.M
Last changed : 8/10/2010 5:39:26 PM
Polyaromatic hydrocarbons

External Standard Report

Sorted By : Retention Time
Calib. Data Modified : 8/10/2010 5:33:36 PM
Multiplier : 1.0000
Dilution : 1.0000

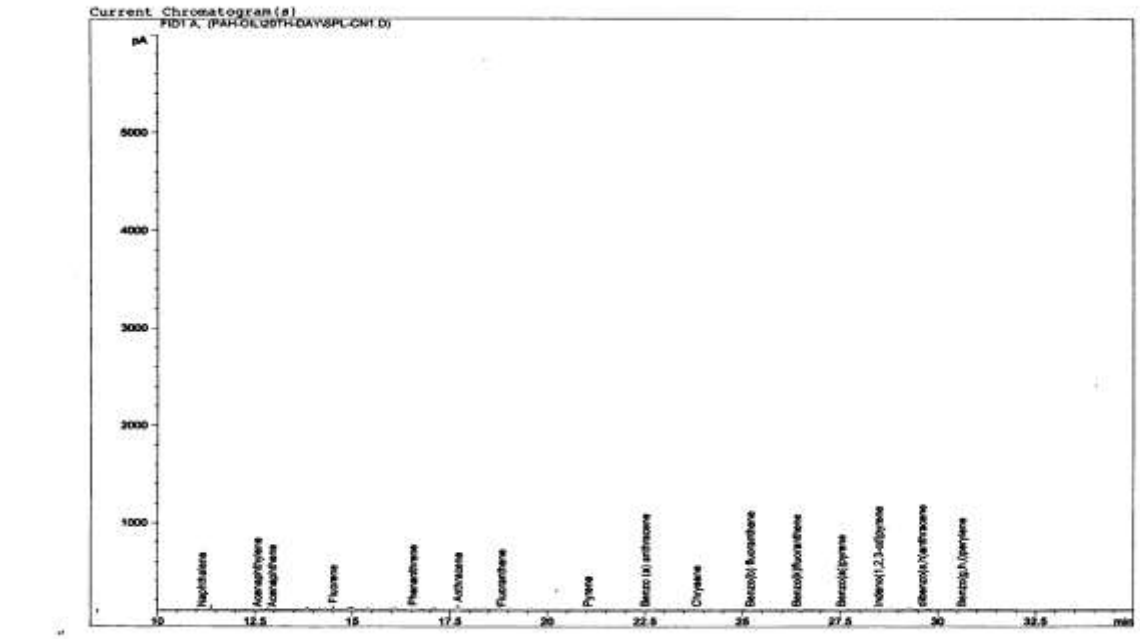
Signal 1: FID1 A,

| RetTime [min] | Sig | Type | Area [pA*s] | Amt/Area | Amount [mg/Kg] | Grp | Name |
|---|---|---|---|---|---|---|---|
| 11.140 | 1 | VV | 39.54055 | 121.86828 | 4818.73829 | 1 | Naphthalene |
| 12.568 | 1 | VV | 24.68416 | 134.61222 | 3322.78911 | 1 | Acenaphthylene |
| 12.913 | 1 | VV | 40.53079 | 30.63287 | 1241.57418 | 1 | Acenaphthene |
| 14.509 | 1 | VV | 221.15024 | 4.49175 | 993.35070 | 1 | Fluorene |
| 16.506 | 1 | VV | 9.26577 | 39.76777 | 368.47885 | 1 | Phenanthrene |
| 17.708 | 1 | VV | 320.08145 | 2.39783 | 767.50028 | 1 | Anthracene |
| 18.783 | 1 | VV | 186.10780 | 4.13859 | 770.22319 | 1 | Fluoranthene |
| 21.026 | 1 | VV | 12.09833 | 6.51197 | 78.78394 | 1 | Pyrene |
| 22.504 | 1 | VV | 12.33571 | 6.43464 | 79.37577 | 1 | Benzo (a) anthracene |
| 23.818 | 1 | VV | 9.10280 | 7.39687 | 67.33220 | 1 | Chrysene |
| 25.183 | 1 | VV | 18.95400 | 7.94514e-1 | 15.05923 | 1 | Benzo(b) fluoranthene |
| 26.380 | 1 | VV | 73.29287 | 7.49865e-1 | 54.95972 | 1 | Benzo(k)fluoranthene |
| 27.517 | 1 | VV | 19.68209 | 7.54610e-1 | 14.85230 | 1 | Benzo(a))pyrene |
| 28.472 | 1 | VV | 6.57804 | 2.49967 | 16.44291 | 1 | Indeno(1,2,3-cd)pyrene |
| 29.580 | 1 | VV | 16.55700 | 4.19987 | 69.53726 | 1 | dibenzo(a,h)anthracene |
| 30.604 | 1 | VV | 6.84056 | 8.08564 | 55.31033 | 1 | Benzo(g,h,i)perylene |
| Totals : | | | | | 1.27343e4 | | |

Results obtained with enhanced integrator!
Group summary :

## Appendix XVIII B

Print of window 38: Current Chromatogram(s)




Instrument 1 8/10/2010 4:28:35 PM

Page 1 of 1

Gas Chromatograph of Crude Oil (PAH) as degraded by *Providencia* sp 1 $20^{th}$ day ($NH_4NO_3$)

Method : C:\HPCHEM\1\METHODS\PAH-EN1.M
anged : 8/12/2010 12:56:54 PM
(modified after loading)
aromatic hydrocarbons

External Standard Report

Sorted By : Retention Time
Calib. Data Modified : 8/12/2010 10:53:50 AM
Multiplier : 0.7800
Dilution : 1.0000

Signal 1: FID1 A,

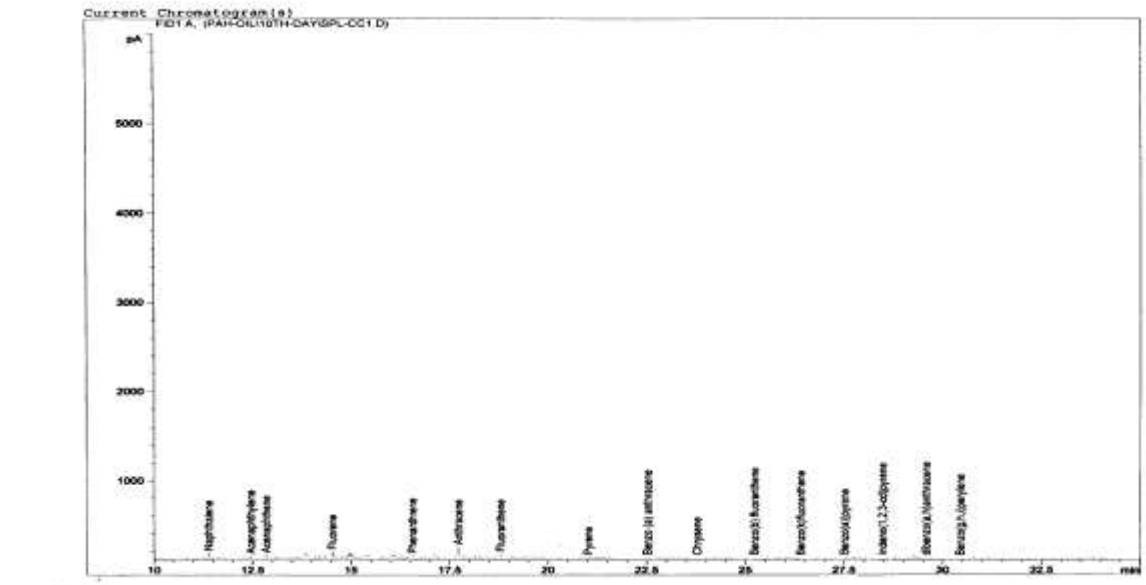
| RetTime [min] | Sig | Type | Area [pA*s] | Amt/Area | Amount [mg/Kg] | Grp | Name |
|---|---|---|---|---|---|---|---|
| 11.140 | 1 | VV | 39.54055 | 8.22925e-1 | 25.38035 | 1 | Naphthalene |
| 12.568 | 1 | VV | 24.68416 | 8.72496e-1 | 16.79872 | 1 | Acenaphthylene |
| 12.942 | 1 | VV | 9.46793 | 3.00865 | 22.21884 | 1 | Acenaphthene |
| 14.509 | 1 | VV | 221.15024 | 4.96509e-2 | 8.56463 | 1 | Fluorene |
| 16.539 | 1 | VV | 138.90569 | 3.96580e-1 | 42.96808 | 1 | Phenanthrene |
| 17.708 | 1 | VV | 320.08145 | 2.39783e-1 | 59.86503 | 1 | Anthracene |
| 18.913 | 1 | VV | 9.18592 | 7.07786 | 50.71297 | 1 | Fluoranthene |
| 21.091 | 1 | VV | 93.22626 | 3.38301e-1 | 24.60005 | 1 | Pyrene |
| 22.521 | 1 | VV | 6.20616 | 6.48337 | 31.38474 | 1 | Benzo (a) anthracene |
| 23.827 | 1 | VV | 9.53225 | 7.39109 | 54.95389 | 1 | Chrysene |
| 25.198 | 1 | VV | 10.08203 | 7.45551 | 58.62997 | 1 | Benzo(b) fluoranthene |
| 26.380 | 1 | VV | 73.29287 | 7.49865e-1 | 42.86858 | 1 | Benzo(k)fluoranthene |
| 27.532 | 1 | VV | 10.84838 | 7.19811 | 60.90856 | 1 | Benzo(a))pyrene |
| 28.467 | 1 | VV | 10.23357 | 2.47589 | 19.76298 | 1 | Indeno(1,2,3-cd)pyrene |
| 29.598 | 1 | VV | 7.84477 | 4.17817 | 25.56591 | 1 | dibenzo(a,h)anthracene |
| 30.627 | 1 | VV | 4.44658 | 8.13760 | 28.22392 | 1 | Benzo(g,h,i)perylene |
| Totals : | | | | | 573.40723 | | |

Results obtained with enhanced integrator!
Group summary :

| Group ID | Use | Area [pA*s] | Amount [mg/Kg] | Group Name |
|---|---|---|---|---|

## Appendix XVIII C

Print of window 38: Current Chromatogram(s)




Instrument 1 8/10/2010 4:06:48 PM Page 1 of 1

Gas Chromatograph of Crude Oil (Residual oil) as degraded by *Providencia* sp1 10th day ($NH_4Cl$)

is Method : C:\HPCHEM\1\METHODS\PAH-CC1.M
changed : 8/11/2010 9:31:07 AM
(modified after loading)
yaromatic hydrocarbons

External Standard Report

Sorted By : Retention Time
Calib. Data Modified : 8/11/2010 9:29:29 AM
Multiplier : 1.0000
Dilution : 1.0000

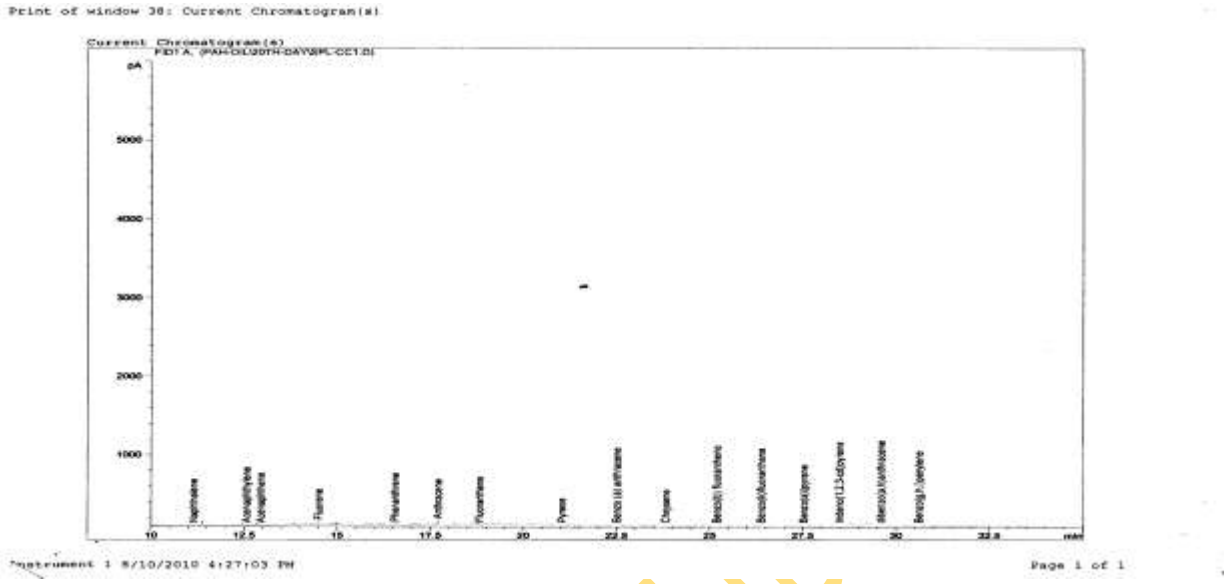
Signal 1: FID1 A,

| RetTime [min] | Sig | Type | Area [pA*s] | Amt/Area | Amount [mg/Kg] | Grp | Name |
|---|---|---|---|---|---|---|---|
| 11.148 | 1 | VV | 34.50134 | 161.14382 | 5559.67695 | 1 | Naphthalene |
| 12.573 | 1 | VV | 20.69040 | 184.19915 | 3811.15358 | 1 | Acenaphthylene |
| 12.921 | 1 | VV | 30.24895 | 44.43278 | 1344.04474 | 1 | Acenaphthene |
| 14.517 | 1 | VV | 198.36563 | 6.13691 | 1217.35125 | 1 | Fluorene |
| 16.506 | 1 | VV | 9.29664 | 48.77243 | 453.41973 | 1 | Phenanthrene |
| 17.715 | 1 | VV | 361.73413 | 2.39772 | 867.33892 | 1 | Anthracene |
| 18.788 | 1 | VV | 191.53635 | 4.13857 | 792.68675 | 1 | Fluoranthene |
| 21.023 | 1 | VV | 24.70873 | 6.47629 | 160.02082 | 1 | Pyrene |
| 22.510 | 1 | VV | 16.78744 | 6.42155 | 107.80138 | 1 | Benzo (a) anthracene |
| 23.818 | 1 | VV | 4.29380 | 7.54064 | 32.37799 | 1 | Chrysene |
| 25.186 | 1 | VV | 11.94565 | 7.43789 | 88.85037 | 1 | Benzo(b) fluoranthene |
| 26.362 | 1 | VV | 6.75505 | 7.50555 | 50.70040 | 1 | Benzo(k)fluoranthene |
| 27.510 | 1 | VV | 31.31542 | 7.38818e-1 | 23.13638 | 1 | Benzo(a))pyrene |
| 28.468 | 1 | VV | 17.76516 | 2.45774 | 43.66218 | 1 | Indeno(1,2,3-cd)pyrene |
| 29.596 | 1 | VV | 9.93081 | 4.18684 | 41.57867 | 1 | dibenzo(a,h)anthracene |
| 30.611 | 1 | VV | 8.07257 | 8.07091 | 65.15299 | 1 | Benzo(g,h,i)perylene |
| Totals : | | | | | 1.46590e4 | | |

Results obtained with enhanced integrator!
Group summary :

| Group ID | Use | Area [pA*s] | Amount [mg/Kg] | Group Name |
|---|---|---|---|---|

## Appendix XVIII D




Gas Chromatograph of Crude Oil (PAH) as degraded by

*Providencia* sp 1 20$^{th}$ day ($NH_4Cl$)

ysis Method : C:\HPCHEM\1\METHODS\PAH-CC1.M
st changed : 8/12/2010 12:00:51 PM
(modified after loading)
Polyaromatic hydrocarbons

External Standard Report

Sorted By : Retention Time
Calib. Data Modified : 8/11/2010 9:29:29 AM
Multiplier : 0.8500
Dilution : 1.0000

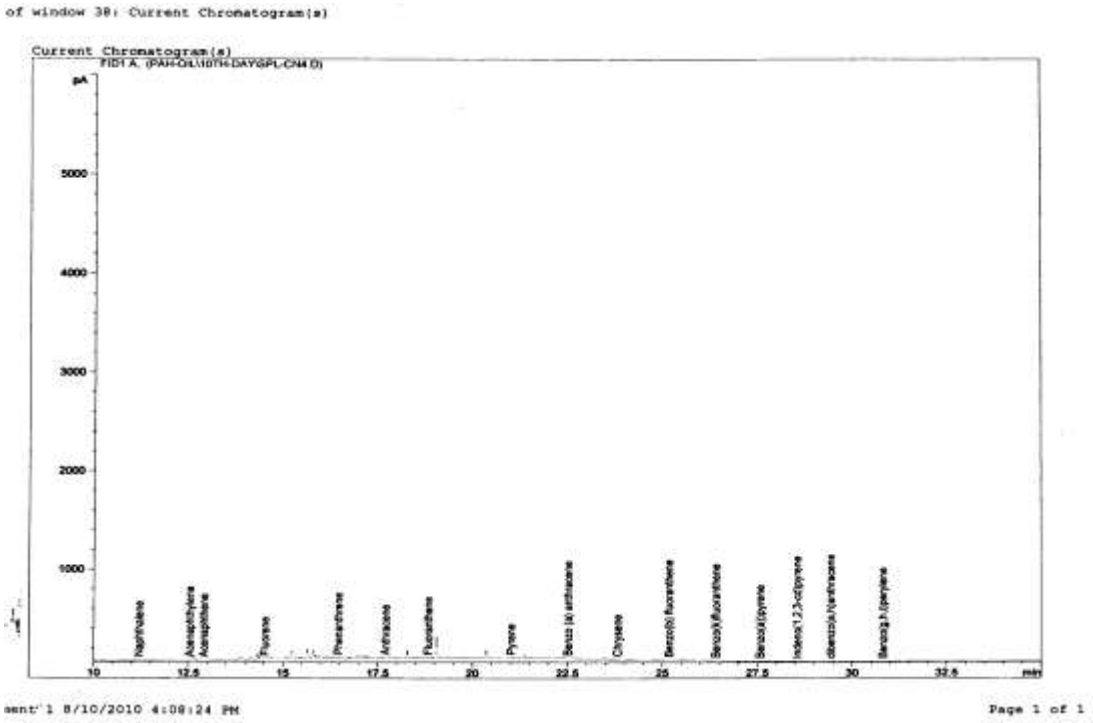
Signal 1: FID1 A,

| RetTime [min] | Sig | Type | Area [pA*s] | Amt/Area | Amount [mg/Kg] | Grp | Name |
|---|---|---|---|---|---|---|---|
| 11.148 | 1 | VV | 34.50134 | 161.14382 | 4725.72541 | 1 | Naphthalene |
| 12.573 | 1 | VV | 20.69040 | 184.19915 | 3239.48054 | 1 | Acenaphthylene |
| 12.921 | 1 | VV | 30.24895 | 44.43278 | 1142.43803 | 1 | Acenaphthene |
| 14.517 | 1 | VV | 198.36563 | 6.13691 | 1034.74856 | 1 | Fluorene |
| 16.506 | 1 | VV | 9.29664 | 48.77243 | 385.40677 | 1 | Phenanthrene |
| 17.715 | 1 | VV | 361.73413 | 2.39772 | 737.23808 | 1 | Anthracene |
| 18.788 | 1 | VV | 191.53635 | 4.13857 | 673.78374 | 1 | Fluoranthene |
| 21.023 | 1 | VV | 24.70873 | 6.47629 | 136.01770 | 1 | Pyrene |
| 22.510 | 1 | VV | 16.78744 | 6.42155 | 91.63118 | 1 | Benzo (a) anthracene |
| 23.818 | 1 | VV | 4.29380 | 7.54064 | 27.52129 | 1 | Chrysene |
| 25.186 | 1 | VV | 11.94565 | 7.43788 | 75.52282 | 1 | Benzo(b) fluoranthene |
| 26.362 | 1 | VV | 6.75505 | 7.50555 | 43.09534 | 1 | Benzo(k)fluoranthene |
| 27.510 | 1 | VV | 31.31542 | 7.38818e-1 | 19.66593 | 1 | Benzo(a))pyrene |
| 28.468 | 1 | VV | 17.76516 | 2.45774 | 37.11286 | 1 | Indeno(1,2,3-cd)pyrene |
| 29.596 | 1 | VV | 9.93081 | 4.18684 | 35.34187 | 1 | dibenzo(a,h)anthracene |
| 30.611 | 1 | VV | 8.07257 | 8.07091 | 55.38005 | 1 | Benzo(g,h,i)perylene |

Totals : 1.24601e4

Results obtained with enhanced integrator!
Group summary :

## Appendix XIX A

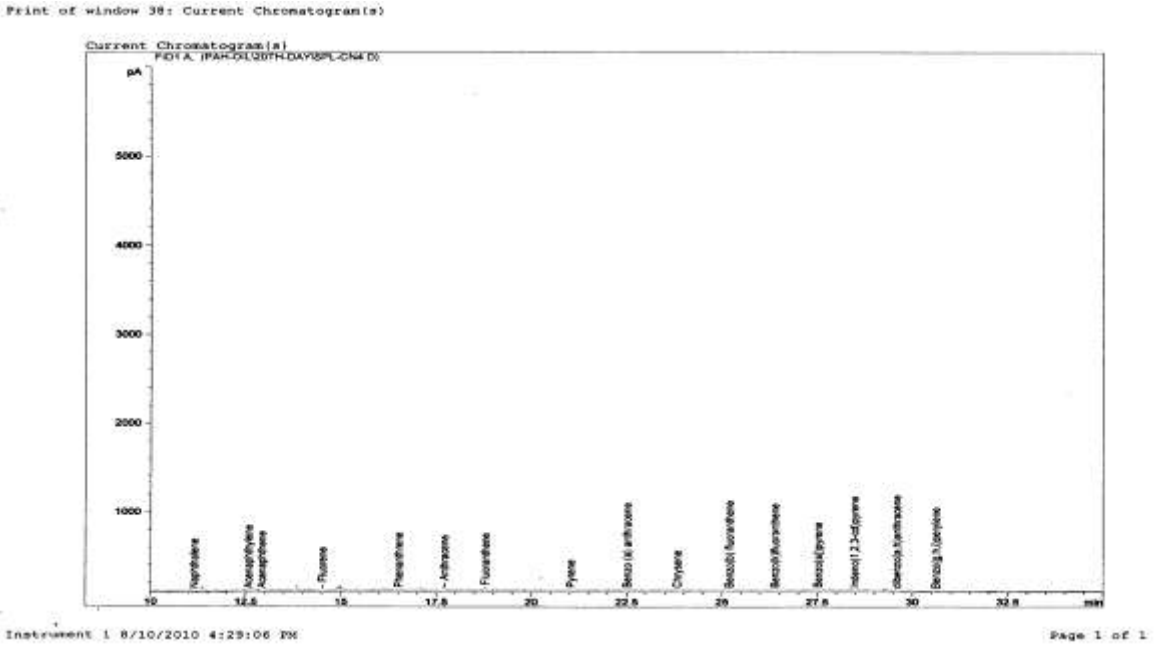



Gas Chromatograph of Crude Oil (PAH) as degraded by *Providencia* sp3 10th day

| RetTime [min] | Sig | Type | Area [pA*s] | Amt/Area | Amount [mg/Kg] | Grp | Name |
|---|---|---|---|---|---|---|---|
| 11.194 | 1 | VV T | 56.22594 | 88.37157 | 4968.77484 | 1 | Naphthalene |
| 12.547 | 1 | VV T | 15.21623 | 232.42731 | 3536.66798 | 1 | Acenaphthylene |
| 12.905 | 1 | VV T | 1.19848 | 1197.74614 | 1435.47109 | 1 | Acenaphthene |
| 14.516 | 1 | VV T | 8.41617 | 138.92534 | 1169.21906 | 1 | Fluorene |
| 16.459 | 1 | VV T | 10.26153 | 40.84107 | 419.09196 | 1 | Phenanthrene |
| 17.715 | 1 | VV T | 3.61386 | 239.77256 | 866.50428 | 1 | Anthracene |
| 18.821 | 1 | VV X | 5.08821 | 156.45370 | 796.06959 | 1 | Fluoranthene |
| 21.010 | 1 | VV T | 10.35688 | 8.78820 | 91.01836 | 1 | Pyrene |
| 22.537 | 1 | VV T | 1.10289 | 64.40386 | 71.03012 | 1 | Benzo (a) anthracene |
| 23.879 | 1 | VV X | 1.10855 | 73.73843 | 81.74297 | 1 | Chrysene |
| 25.188 | 1 | VB T | 1.16208 | 74.40496 | 86.46446 | 1 | Benzo(b) fluoranthene |
| 26.455 | 1 | VV T | 1.42237 | 41.70168 | 59.31541 | 1 | Benzo(k)fluoranthene |
| 27.330 | 1 | VB X | 2.33105 | 6.54336 | 15.25286 | 1 | Benzo(a))pyrene |
| 28.544 | 1 | VV X | 1.12638 | 16.55578 | 18.64806 | 1 | Indeno(1,2,3-cd)pyrene |
| 29.425 | 1 | VV | 3.11066 | 23.43026 | 72.88364 | 1 | dibenzo(a,h)anthracene |
| 30.700 | 1 | VV | 1.29214 | 45.72668 | 59.08550 | 1 | Benzo(g,h,i)perylene |
| Totals : | | | | | 1.37472e4 | | |

Results obtained with

## Appendix XIX B

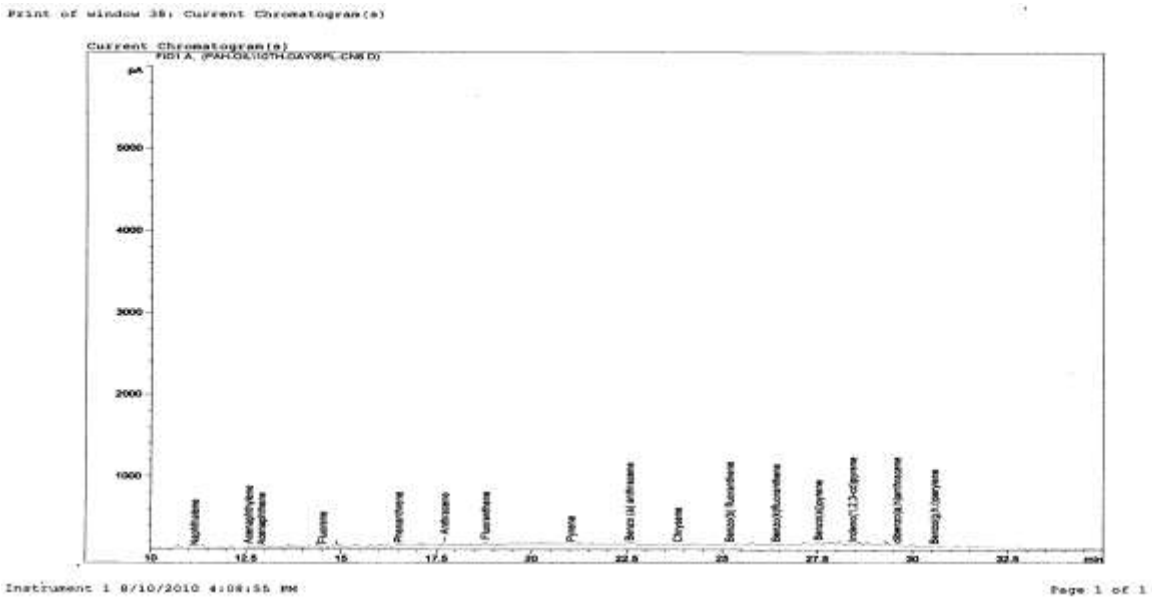



Gas Chromatograph of Crude Oil (PAH) as degraded by *Providencia* sp 3 20th day

| RetTime [min] | Sig | Type | Area [pA*s] | Amt/Area | Amount [mg/Kg] | Grp | Name |
|---|---|---|---|---|---|---|---|
| 11.194 | 1 | VV T | 56.22594 | 88.37157 | 4223.45861 | 1 | Naphthalene |
| 12.547 | 1 | VV T | 15.21623 | 232.42731 | 3006.16778 | 1 | Acenaphthylene |
| 12.905 | 1 | VV T | 1.19848 | 1197.74614 | 1220.15043 | 1 | Acenaphthene |
| 14.516 | 1 | VV T | 8.41617 | 138.92534 | 993.83620 | 1 | Fluorene |
| 16.459 | 1 | VV T | 10.26153 | 40.84107 | 356.22817 | 1 | Phenanthrene |
| 17.715 | 1 | VV T | 3.61386 | 239.77256 | 736.52864 | 1 | Anthracene |
| 18.821 | 1 | VV X | 5.08821 | 156.45370 | 676.65915 | 1 | Fluoranthene |
| 21.010 | 1 | VV T | 10.35688 | 8.78820 | 77.36561 | 1 | Pyrene |
| 22.537 | 1 | VV T | 1.10289 | 64.40386 | 60.37560 | 1 | Benzo (a) anthracene |
| 23.879 | 1 | VV X | 1.10855 | 73.73843 | 69.48153 | 1 | Chrysene |
| 25.188 | 1 | VB T | 1.16208 | 74.40496 | 73.49479 | 1 | Benzo(b) fluoranthene |
| 26.455 | 1 | VV T | 1.42237 | 41.70168 | 50.41810 | 1 | Benzo(k)fluoranthene |
| 27.330 | 1 | VB X | 2.33105 | 6.54336 | 12.96493 | 1 | Benzo(a))pyrene |
| 28.544 | 1 | VV X | 1.12638 | 16.55578 | 15.85085 | 1 | Indeno(1,2,3-cd)pyrene |
| 29.425 | 1 | VV | 3.11066 | 23.43026 | 61.95109 | 1 | dibenzo(a,h)anthracene |
| 30.700 | 1 | VV | 1.29214 | 45.72668 | 50.22267 | 1 | Benzo(g,h,i)perylene |
| Totals : | | | | | 1.16852e4 | | |

Results obtained with

## Appendix XX A

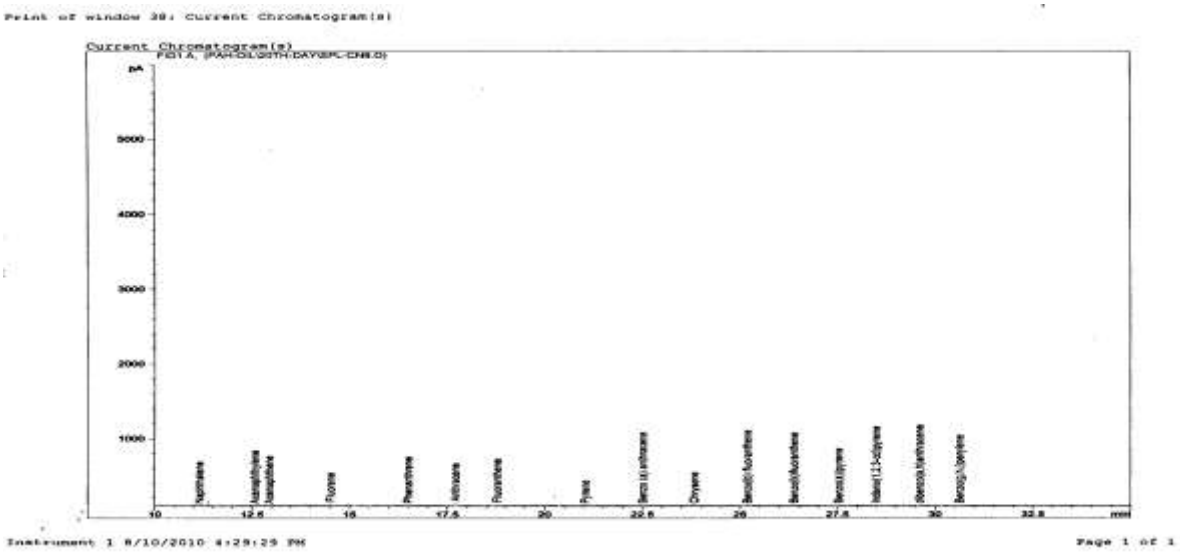



Gas Chromatograph of Crude Oil (PAH) as degraded by *Bacillus* sp 1 10$^{th}$ day

Signal 1: FID1 A,

| RetTime [min] | Sig | Type | Area [pA*s] | Amt/Area | Amount [mg/Kg] | Grp | Name |
|---|---|---|---|---|---|---|---|
| 11.146 | 1 | VV | 8.33748 | 628.42228 | 5239.46097 | 1 | Naphthalene |
| 12.566 | 1 | VV | 86.62402 | 41.80262 | 3621.11116 | 1 | Acenaphthylene |
| 12.931 | 1 | VV | 5.27191 | 311.39722 | 1641.65950 | 1 | Acenaphthene |
| 14.512 | 1 | VV | 128.01324 | 11.08842 | 1419.46456 | 1 | Fluorene |
| 16.504 | 1 | VV | 94.46140 | 5.13540 | 485.09710 | 1 | Phenanthrene |
| 17.709 | 1 | VV | 421.59790 | 1.74985 | 737.73275 | 1 | Anthracene |
| 18.790 | 1 | VV | 338.52213 | 4.27645 | 1447.67148 | 1 | Fluoranthene |
| 21.027 | 1 | VV | 29.54856 | 3.22223 | 95.21235 | 1 | Pyrene |
| 22.509 | 1 | VV | 48.93847 | 1.63426 | 79.97824 | 1 | Benzo (a) anthracene |
| 23.824 | 1 | VV | 29.28207 | 3.75571 | 109.97503 | 1 | Chrysene |
| 25.186 | 1 | VV | 44.95369 | 1.95782 | 88.01131 | 1 | Benzo(b) fluoranthene |
| 26.367 | 1 | VV | 36.20217 | 1.88379 | 68.19719 | 1 | Benzo(k)fluoranthene |
| 27.516 | 1 | VV | 140.61249 | 1.36329e-1 | 19.16963 | 1 | Benzo(a))pyrene |
| 28.471 | 1 | VV | 9.75345 | 2.05919 | 20.08420 | 1 | Indeno(1,2,3-cd)pyrene |
| 29.581 | 1 | VV | 36.52466 | 2.43390 | 88.89748 | 1 | dibenzo(a,h)anthracene |
| 30.603 | 1 | VV | 16.31768 | 4.44259 | 72.49274 | 1 | Benzo(g,h,i)perylene |
| Totals : | | | | | 1.52342e4 | | |

## Appendix XX B

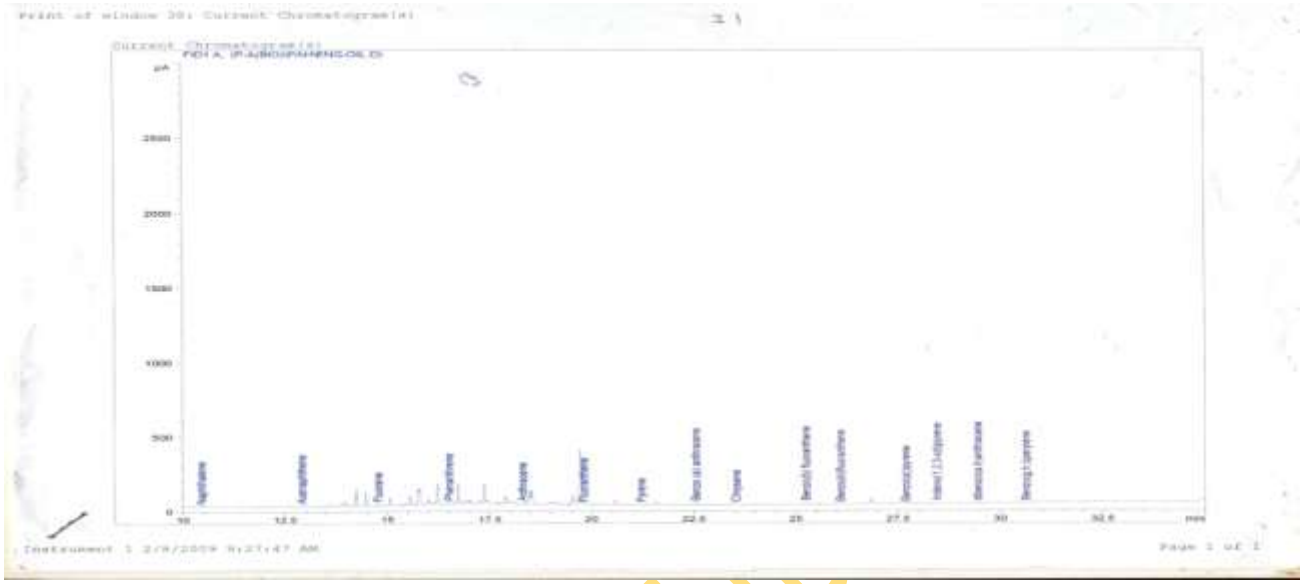



Gas Chromatograph of Crude Oil (PAH) as degraded by *Bacillus* sp 1 20$^{th}$ day

Signal 1: FID1 A,

| RetTime [min] | Sig | Type | Area [pA*s] | Amt/Area | Amount [mg/Kg] | Grp | Name |
|---|---|---|---|---|---|---|---|
| 11.146 | 1 | VV | 8.33748 | 628.42228 | 4243.96338 | 1 | Naphthalene |
| 12.566 | 1 | VV | 86.62402 | 41.80262 | 2933.10004 | 1 | Acenaphthylene |
| 12.931 | 1 | VV | 5.27191 | 311.39722 | 1329.74420 | 1 | Acenaphthene |
| 14.512 | 1 | VV | 128.01324 | 11.08842 | 1149.76629 | 1 | Fluorene |
| 16.504 | 1 | VV | 94.46140 | 5.13540 | 392.92865 | 1 | Phenanthrene |
| 17.709 | 1 | VV | 421.59790 | 1.74985 | 597.56353 | 1 | Anthracene |
| 18.790 | 1 | VV | 338.52213 | 4.27645 | 1172.61390 | 1 | Fluoranthene |
| 21.027 | 1 | VV | 29.54856 | 3.22223 | 77.12201 | 1 | Pyrene |
| 22.509 | 1 | VV | 48.93847 | 1.63426 | 64.78237 | 1 | Benzo (a) anthracene |
| 23.824 | 1 | VV | 29.28207 | 3.75571 | 89.07978 | 1 | Chrysene |
| 25.186 | 1 | VV | 44.95369 | 1.95782 | 71.28916 | 1 | Benzo(b) fluoranthene |
| 26.367 | 1 | VV | 36.20217 | 1.88379 | 55.23973 | 1 | Benzo(k)fluoranthene |
| 27.516 | 1 | VV | 140.61249 | 1.36329e-1 | 15.52740 | 1 | Benzo(a))pyrene |
| 28.471 | 1 | VV | 9.75345 | 2.05919 | 16.26820 | 1 | Indeno(1,2,3-cd)pyrene |
| 29.581 | 1 | VV | 36.52466 | 2.43390 | 72.00696 | 1 | dibenzo(a,h)anthracene |
| 30.603 | 1 | VV | 16.31768 | 4.44259 | 58.71912 | 1 | Benzo(g,h,i)perylene |
| Totals : | | | | | 1.23397e4 | | |

## Appendix XXI



Gas Chromatograph of Control at day 0 (Used Crankcase Oil)

1.0000

Signal 1: FID1 A,

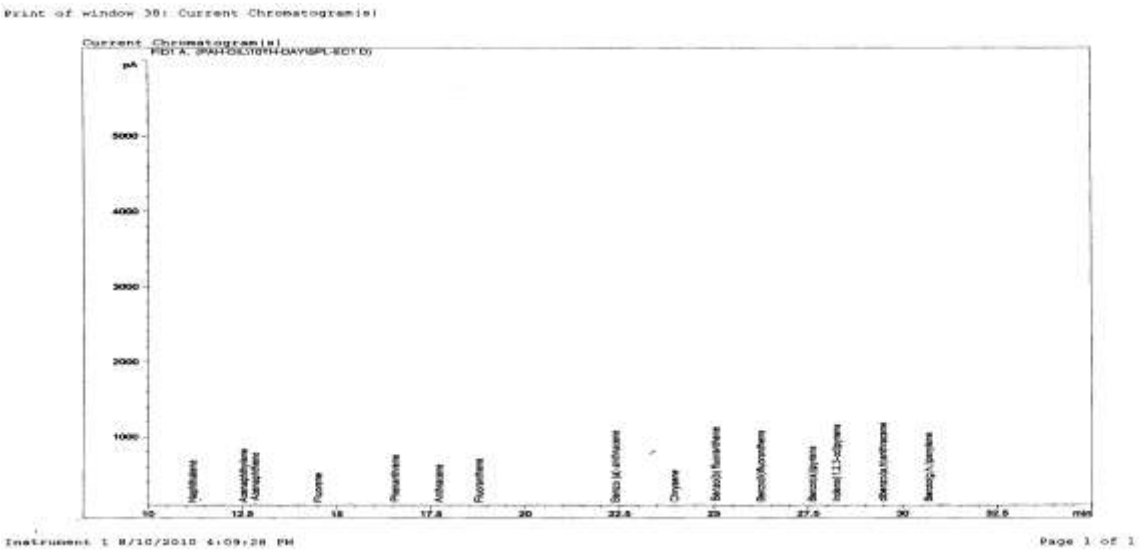
| RetTime [min] | Sig | Type | Area [pA*s] | Amt/Area | Amount [mg/Kg] | Grp | Name |
|---|---|---|---|---|---|---|---|
| 10.452 | 1 | VP | 10.16333 | 3.67072 | 138.03499 | 1 | Naphthalene |
| 11.085 | 1 | | - | - | - | 1 | Acenaphthylene |
| 12.908 | 1 | VV | 15.32259 | 3.08971e-1 | 17.51665 | 1 | Acenaphthene |
| 14.775 | 1 | VV | 33.38937 | 5.96401e-1 | 73.67978 | 1 | Fluorene |
| 16.504 | 1 | VV | 119.94686 | 3.37134e-2 | 14.96214 | 1 | Phenanthrene |
| 18.308 | 1 | VV | 115.56732 | 1.01905e-1 | 43.57432 | 1 | Anthracene |
| 19.812 | 1 | VV | 38.57516 | 3.16223e-1 | 45.13384 | 1 | Fluoranthene |
| 21.214 | 1 | VV | 34.14906 | 3.09866e-1 | 39.15201 | 1 | Pyrene |
| 22.550 | 1 | VV | 43.01077 | 6.53523e-1 | 104.00160 | 1 | Benzo (a) anthracene |
| 23.541 | 1 | VV | 15.03747 | 8.09820e-1 | 45.05731 | 1 | Chrysene |
| 25.241 | 1 | VV | 47.32387 | 7.59792e-1 | 133.03822 | 1 | Benzo(b) fluoranthene |
| 26.092 | 1 | VV | 13.09636 | 7.82913e-1 | 37.93726 | 1 | Benzo(k)fluoranthene |
| 27.685 | 1 | VP | 8.10298 | 7.07068 | 211.98631 | 1 | Benzo(a))pyrene |
| 28.449 | 1 | VV | 16.13564 | 6.07787e-1 | 36.28604 | 1 | Indeno(1,2,3-cd)pyrene |
| 29.449 | 1 | VV | 6.97884 | 9.23963e-1 | 23.85829 | 1 | dibenzo(a,h)anthracene |
| 30.622 | 1 | VV | 85.85564 | 8.75814e-2 | 27.82163 | 1 | Benzo(g,h,i)perylene |

Totals : 992.04040

Results obtained with enhanced integrator!

Group summary :

## Appendix XXII A

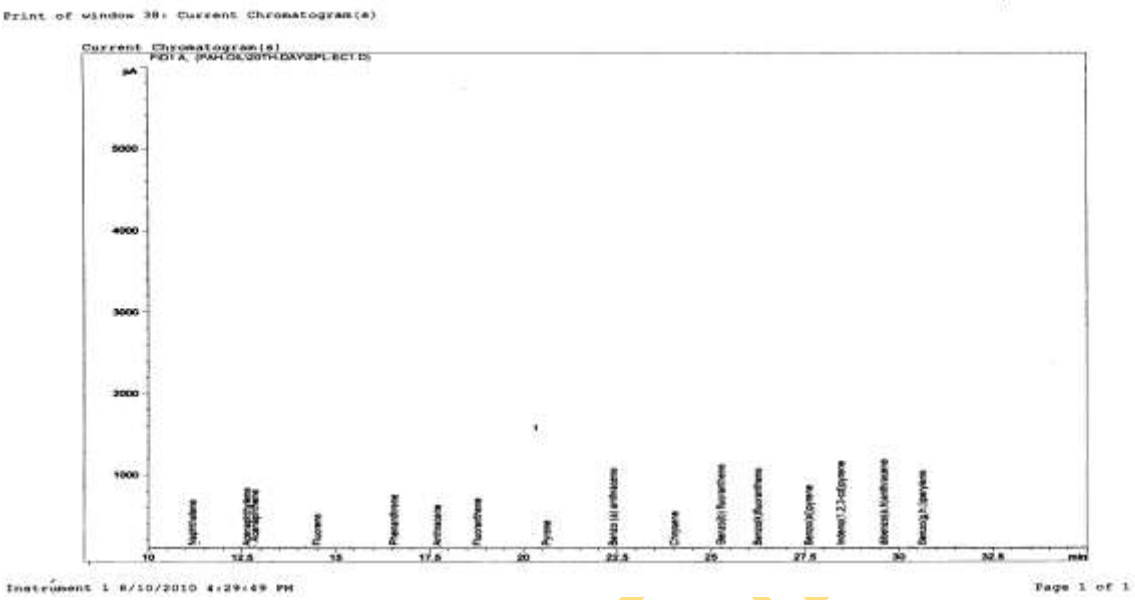


Gas Chromatograph of Used Crankcase Oil (PAH) as degraded by *Providencia* sp 1 10th day

Signal 1: FID1 A,

| RetTime [min] | Sig | Type | Area [pA*s] | Amt/Area | Amount [mg/Kg] | Grp | Name |
|---|---|---|---|---|---|---|---|
| 11.148 | 1 | VV | 34.50134 | 1.62078 | 55.91901 | 1 | Naphthalene |
| 12.573 | 1 | VV | 20.69040 | 1.87871 | 38.87120 | 1 | Acenaphthylene |
| 12.921 | 1 | VV | 30.24895 | 4.51368e-1 | 13.65341 | 1 | Acenaphthene |
| 14.517 | 1 | VV | 198.36563 | 5.84076e-2 | 11.58606 | 1 | Fluorene |
| 16.506 | 1 | VV | 9.29664 | 4.99115 | 46.40089 | 1 | Phenanthrene |
| 17.715 | 1 | VV | 361.73413 | 2.40136e-1 | 86.86532 | 1 | Anthracene |
| 18.788 | 1 | VV | 191.53635 | 4.15066e-1 | 79.50016 | 1 | Fluoranthene |
| 21.023 | 1 | VV | 24.70873 | 6.81102e-1 | 16.82917 | 1 | Pyrene |
| 22.510 | 1 | VV | 16.78744 | 2.62697 | 44.10012 | 1 | Benzo (a) anthracene |
| 23.818 | 1 | VV | 4.29380 | 16.15180 | 69.35253 | 1 | Chrysene |
| 25.186 | 1 | VV | 11.94565 | 7.43788 | 88.85037 | 1 | Benzo(b) fluoranthene |
| 26.362 | 1 | VV | 6.75505 | 7.50555 | 50.70040 | 1 | Benzo(k)fluoranthene |
| 27.510 | 1 | VV | 31.31542 | 7.38818e-1 | 23.13638 | 1 | Benzo(a))pyrene |
| 28.468 | 1 | VV | 17.76516 | 2.45774 | 43.66218 | 1 | Indeno(1,2,3-cd)pyrene |
| 29.596 | 1 | VV | 9.93081 | 4.18684 | 41.57867 | 1 | dibenzo(a,h)anthracene |
| 30.611 | 1 | VV | 8.07257 | 8.07091 | 65.15299 | 1 | Benzo(g,h,i)perylene |
| Totals : | | | | | 776.15887 | | |

## Appendix XXII B

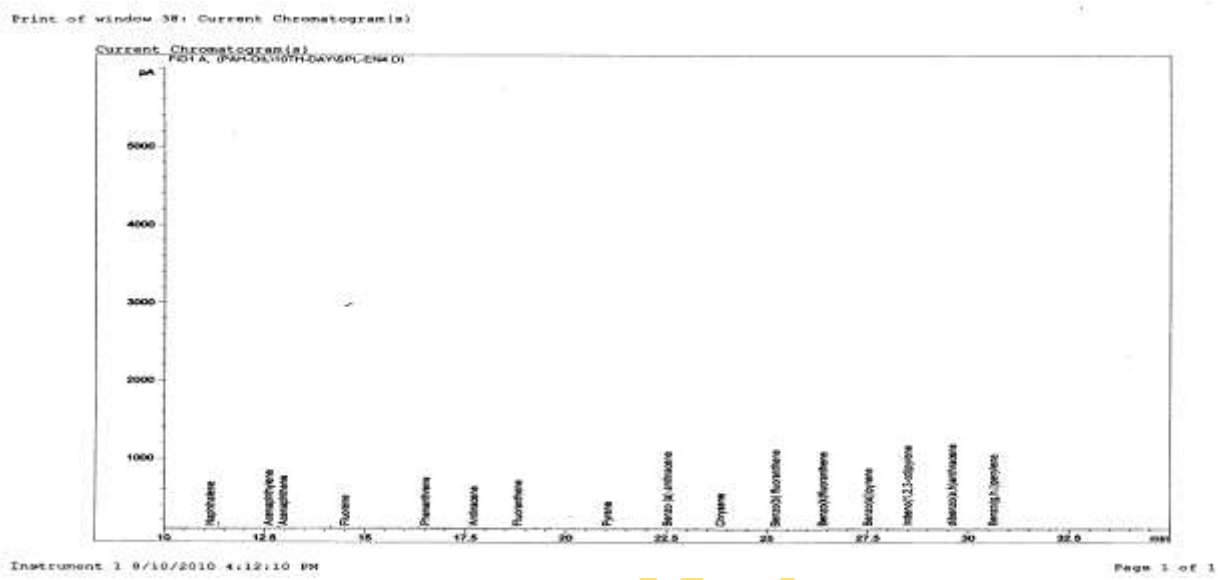



Gas Chromatograph of Used Crankcase Oil (PAH) as degraded by *Providencia* sp 1 20th day

Signal 1: FID1 A,

| RetTime [min] | Sig | Type | Area [pA*s] | Amt/Area | Amount [mg/Kg] | Grp | Name |
|---|---|---|---|---|---|---|---|
| 11.148 | 1 | VV | 34.50134 | 1.62078 | 44.73520 | 1 | Naphthalene |
| 12.573 | 1 | VV | 20.69040 | 1.87871 | 31.09696 | 1 | Acenaphthylene |
| 12.921 | 1 | VV | 30.24895 | 4.51368e-1 | 10.92273 | 1 | Acenaphthene |
| 14.517 | 1 | VV | 198.36563 | 5.84076e-2 | 9.26885 | 1 | Fluorene |
| 16.506 | 1 | VV | 9.29664 | 4.99115 | 37.12071 | 1 | Phenanthrene |
| 17.715 | 1 | VV | 361.73413 | 2.40136e-1 | 69.49225 | 1 | Anthracene |
| 18.788 | 1 | VV | 191.53635 | 4.15066e-1 | 63.60012 | 1 | Fluoranthene |
| 21.023 | 1 | VV | 24.70873 | 6.81102e-1 | 13.46334 | 1 | Pyrene |
| 22.510 | 1 | VV | 16.78744 | 2.62697 | 35.28010 | 1 | Benzo (a) anthracene |
| 23.818 | 1 | VV | 4.29380 | 16.15180 | 55.48203 | 1 | Chrysene |
| 25.186 | 1 | VV | 11.94565 | 7.43788 | 71.08030 | 1 | Benzo(b) fluoranthene |
| 26.362 | 1 | VV | 6.75505 | 7.50555 | 40.56032 | 1 | Benzo(k)fluoranthene |
| 27.510 | 1 | VV | 31.31542 | 7.38818e-1 | 18.50911 | 1 | Benzo(a))pyrene |
| 28.468 | 1 | VV | 17.76516 | 2.45774 | 34.92975 | 1 | Indeno(1,2,3-cd)pyrene |
| 29.596 | 1 | VV | 9.93081 | 4.18684 | 33.26294 | 1 | dibenzo(a,h)anthracene |
| 30.611 | 1 | VV | 8.07257 | 8.07091 | 52.12240 | 1 | Benzo(g,h,i)perylene |
| Totals : | | | | | 620.92710 | | |

## Appendix XXIII A




Gas Chromatograph of Used Crankcase Oil (PAH) as degraded by *Providencia* sp 3 10th day ($NH_4NO_3$)

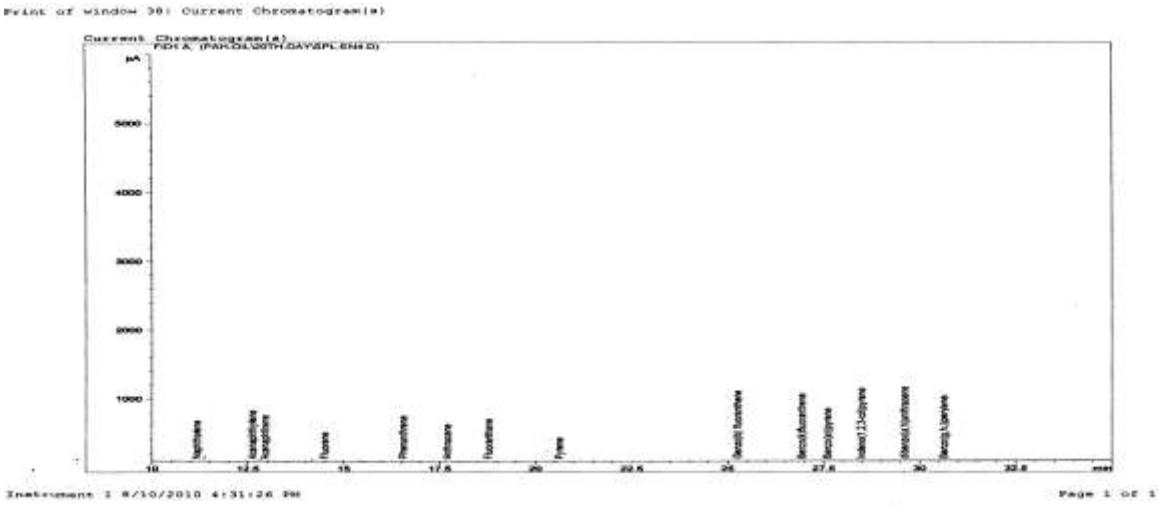
Signal 1: FID1 A,

| RetTime [min] | Sig | Type | Area [pA*s] | Amt/Area | Amount [mg/Kg] | Grp | Name |
|---|---|---|---|---|---|---|---|
| 11.194 | 1 | VV T | 56.22594 | 9.15737e-1 | 51.48817 | 1 | Naphthalene |
| 12.547 | 1 | VV T | 15.21623 | 2.35527 | 35.83833 | 1 | Acenaphthylene |
| 12.905 | 1 | VV T | 1.19848 | 12.25937 | 14.69257 | 1 | Acenaphthene |
| 14.516 | 1 | VV T | 8.41617 | 1.48261 | 12.47790 | 1 | Fluorene |
| 16.459 | 1 | VV T | 10.26153 | 4.09330 | 42.00354 | 1 | Phenanthrene |
| 17.715 | 1 | VV T | 3.61386 | 24.04869 | 86.90858 | 1 | Anthracene |
| 18.821 | 1 | VV X | 5.08821 | 15.82870 | 80.53977 | 1 | Fluoranthene |
| 21.010 | 1 | VV T | 10.35688 | 8.78820 | 91.01836 | 1 | Pyrene |
| 22.537 | 1 | VV T | 1.10289 | 64.40386 | 71.03012 | 1 | Benzo (a) anthracene |
| 23.879 | 1 | VV X | 1.10855 | 73.73843 | 81.74297 | 1 | Chrysene |
| 25.188 | 1 | VB T | 1.16208 | 74.40496 | 86.46446 | 1 | Benzo(b) fluoranthene |
| 26.455 | 1 | VV T | 1.42237 | 41.70168 | 59.31541 | 1 | Benzo(k)fluoranthene |
| 27.330 | 1 | VB X | 2.33105 | 6.54336 | 15.25286 | 1 | Benzo(a))pyrene |
| 28.544 | 1 | VV X | 1.12638 | 16.55578 | 18.64806 | 1 | Indeno(1,2,3-cd)pyrene |
| 29.425 | 1 | VV | 3.11066 | 23.43026 | 72.88364 | 1 | dibenzo(a,h)anthracene |
| 30.700 | 1 | VV | 1.29214 | 45.72668 | 59.08550 | 1 | Benzo(g,h,i)perylene |

Totals : 879.39025

Results obtained with enhanced integrator!
Group summary :

## Appendix XXIII B



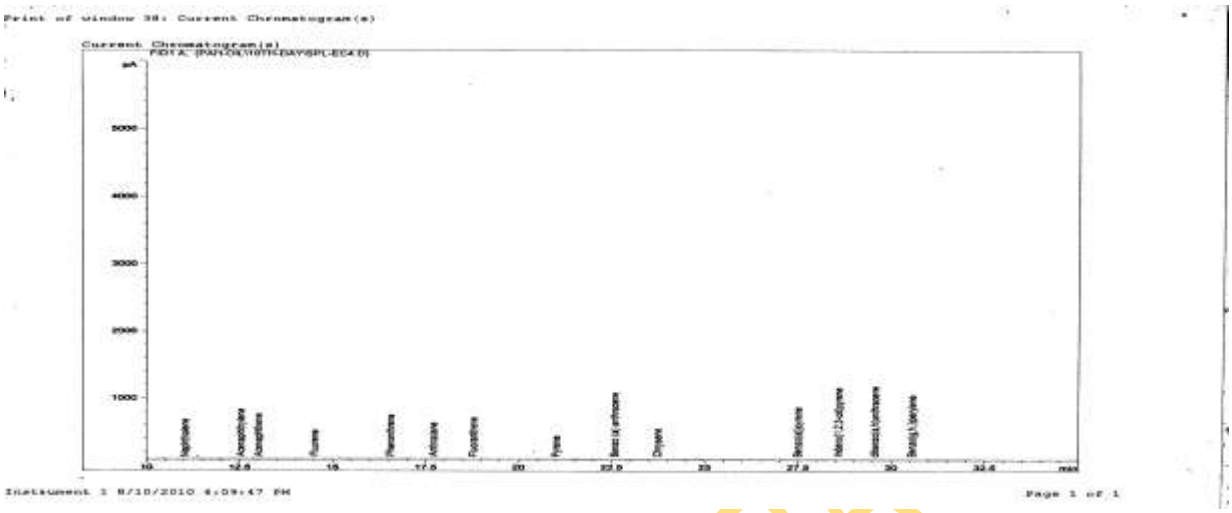
Gas Chromatograph of Used Crankcase Oil (PAH) as degraded by *Providencia* sp 3 20th day ($NH_4NO_3$)

Signal 1: FID1 A,

| RetTime [min] | Sig | Type | Area [pA*s] | Amt/Area | Amount [mg/Kg] | Grp | Name |
|---|---|---|---|---|---|---|---|
| 11.194 | 1 | VV T | 56.22594 | 9.15737e-1 | 40.67566 | 1 | Naphthalene |
| 12.547 | 1 | VV T | 15.21623 | 2.35527 | 28.31228 | 1 | Acenaphthylene |
| 12.905 | 1 | VV T | 1.19848 | 12.25937 | 11.60713 | 1 | Acenaphthene |
| 14.516 | 1 | VV T | 8.41617 | 1.48261 | 9.85754 | 1 | Fluorene |
| 16.459 | 1 | VV T | 10.26153 | 4.09330 | 33.18280 | 1 | Phenanthrene |
| 17.715 | 1 | VV T | 3.61386 | 24.04869 | 68.65778 | 1 | Anthracene |
| 18.821 | 1 | VV X | 5.08821 | 15.82870 | 63.62642 | 1 | Fluoranthene |
| 21.010 | 1 | VV T | 10.35688 | 8.78820 | 71.90451 | 1 | Pyrene |
| 22.537 | 1 | VV T | 1.10289 | 64.40386 | 56.11380 | 1 | Benzo (a) anthracene |
| 23.879 | 1 | VV X | 1.10855 | 73.73843 | 64.57695 | 1 | Chrysene |
| 25.188 | 1 | VB T | 1.16208 | 74.40496 | 68.30692 | 1 | Benzo(b) fluoranthene |
| 26.455 | 1 | VV T | 1.42237 | 41.70168 | 46.85918 | 1 | Benzo(k)fluoranthene |
| 27.330 | 1 | VB X | 2.33105 | 6.54336 | 12.04976 | 1 | Benzo(a))pyrene |
| 28.544 | 1 | VV X | 1.12638 | 16.55578 | 14.73197 | 1 | Indeno(1,2,3-cd)pyrene |
| 29.425 | 1 | VV | 3.11066 | 23.43026 | 57.57808 | 1 | dibenzo(a,h)anthracene |
| 30.700 | 1 | VV | 1.29214 | 45.72668 | 46.67754 | 1 | Benzo(g,h,i)perylene |
| Totals : | | | | | 694.71830 | | |

Results obtained with enhanced integrator!
Group summary :

## Appendix XXIII C



Gas Chromatograph of Used Crankcase Oil (PAH) as degraded by *Providencia* sp 3 10th day ($NH_4Cl$)

Sorted By : Retention Time
Calib. Data Modified : 8/12/2010 11:26:56 AM
Multiplier : 1.0000
Dilution : 1.0000

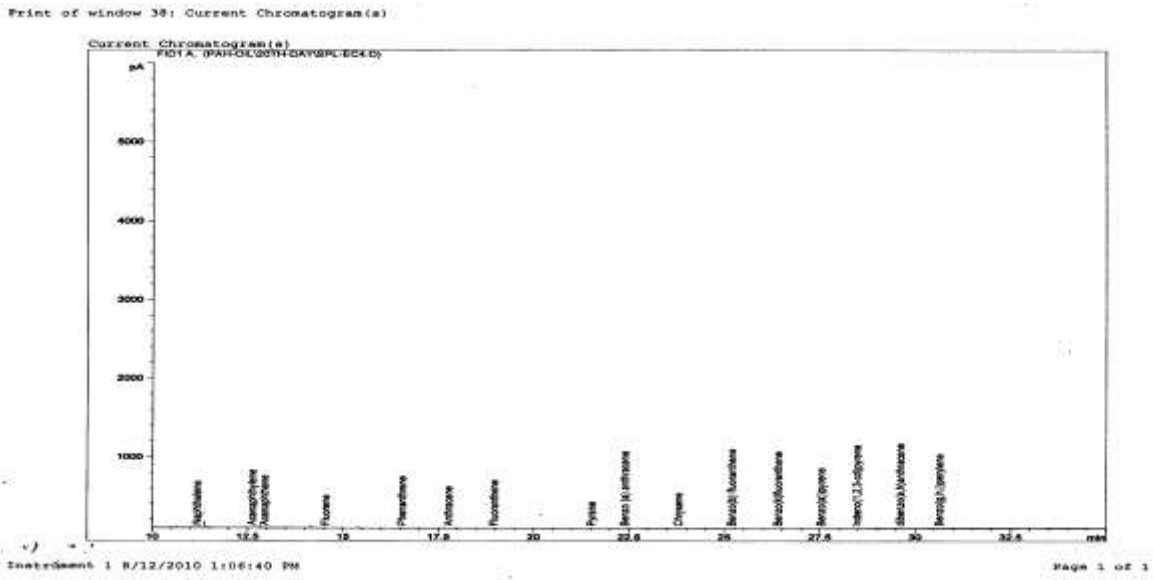
Signal 1: FID1 A,

| RetTime [min] | Sig | Type | Area [pA*s] | Amt/Area | Amount [mg/Kg] | Grp | Name |
|---|---|---|---|---|---|---|---|
| 11.142 | 1 | VV T | 34.24001 | 1.69976 | 58.19975 | 1 | Naphthalene |
| 12.570 | 1 | VV T | 12.54994 | 3.01574 | 37.84734 | 1 | Acenaphthylene |
| 12.918 | 1 | VV T | 14.36796 | 1.24766 | 17.92633 | 1 | Acenaphthene |
| 14.519 | 1 | VV T | 333.92368 | 3.73900e-2 | 12.48540 | 1 | Fluorene |
| 16.544 | 1 | VV T | 38.24645 | 1.33145 | 50.92306 | 1 | Phenanthrene |
| 17.725 | 1 | VV T | 580.02148 | 1.50186e-1 | 87.11092 | 1 | Anthracene |
| 18.792 | 1 | VV T | 190.05005 | 4.32590e-1 | 82.21370 | 1 | Fluoranthene |
| 21.025 | 1 | VB T | 2.25042 | 45.24006 | 101.80906 | 1 | Pyrene |
| 22.495 | 1 | VV T | 1.39185 | 8.32443 | 11.58636 | 1 | Benzo (a) anthracene |
| 23.778 | 1 | VB T | 1.90731 | 20.35575 | 38.82481 | 1 | Chrysene |
| 24.792 | 1 | VV T | 1.13686 | 79.62730 | 90.52511 | 1 | Benzo(b) fluoranthene |
| 26.385 | 1 | VV T | 3.83192 | 15.80644 | 60.56898 | 1 | Benzo(k)fluoranthene |
| 27.502 | 1 | VV T | 23.03803 | 1.36233 | 31.38549 | 1 | Benzo(a))pyrene |
| 28.473 | 1 | VV X | 3.01026 | 16.27246 | 48.98434 | 1 | Indeno(1,2,3-cd)pyrene |
| 29.566 | 1 | VV X | 9.77367 | 9.02159 | 88.17406 | 1 | dibenzo(a,h)anthracene |
| 30.615 | 1 | VV X | 1.45845 | 45.57665 | 66.47106 | 1 | Benzo(g,h,i)perylene |

Totals : 885.03578

Results obtained with enhanced integrator!
Group summary :

## Appendix XXIII D




Gas Chromatograph of Used Crankcase Oil (PAH) as degraded by *Providencia* sp3 20th day ($NH_4Cl$)

Signal 1: FID1 A,

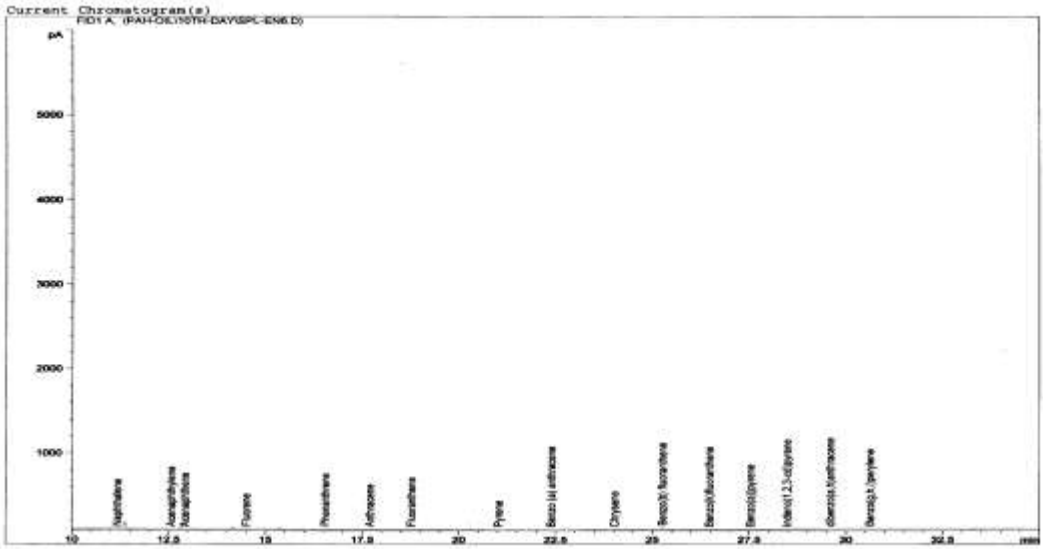
| RetTime [min] | Sig | Type | Area [pA*s] | Amt/Area | Amount [mg/Kg] | Grp | Name |
|---|---|---|---|---|---|---|---|
| 11.142 | 1 | VV T | 34.24001 | 1.69976 | 43.64981 | 1 | Naphthalene |
| 12.570 | 1 | VV T | 12.54994 | 3.01574 | 28.38551 | 1 | Acenaphthylene |
| 12.918 | 1 | VV T | 14.36796 | 1.24766 | 13.44475 | 1 | Acenaphthene |
| 14.519 | 1 | VV T | 333.92368 | 3.73900e-2 | 9.36405 | 1 | Fluorene |
| 16.544 | 1 | VV T | 38.24645 | 1.33145 | 38.19229 | 1 | Phenanthrene |
| 17.725 | 1 | VV T | 580.02148 | 1.50186e-1 | 65.33319 | 1 | Anthracene |
| 18.792 | 1 | VV T | 190.05005 | 4.32590e-1 | 61.66027 | 1 | Fluoranthene |
| 21.025 | 1 | VB T | 2.25042 | 45.24006 | 76.35679 | 1 | Pyrene |
| 22.495 | 1 | VV T | 1.39185 | 8.32443 | 8.68977 | 1 | Benzo (a) anthracene |
| 23.778 | 1 | VB T | 1.90731 | 20.35575 | 29.11861 | 1 | Chrysene |
| 24.792 | 1 | VV T | 1.13686 | 79.62730 | 67.89383 | 1 | Benzo(b) fluoranthene |
| 26.385 | 1 | VV T | 3.83192 | 15.80644 | 45.42673 | 1 | Benzo(k)fluoranthene |
| 27.502 | 1 | VV T | 23.03803 | 1.36233 | 23.53912 | 1 | Benzo(a))pyrene |
| 28.473 | 1 | VV X | 3.01026 | 16.27246 | 36.73825 | 1 | Indeno(1,2,3-cd)pyrene |
| 29.566 | 1 | VV X | 9.77367 | 9.02159 | 66.13055 | 1 | dibenzo(a,h)anthracene |
| 30.615 | 1 | VV X | 1.45845 | 45.57665 | 49.85330 | 1 | Benzo(g,h,i)perylene |
| Totals : | | | | | 663.77683 | | |

Results obtained with enhanced integrator!

Group summary :

## Appendix XXIV A

Print of window 38: Current Chromatogram(s)




Instrument 1 8/10/2010 4:12:33 PM

Page 1 of 1

Gas Chromatograph of Used Crankcase Oil (PAH) as degraded by *Bacillus* sp 4 10th day

Signal 1: FID1 A,

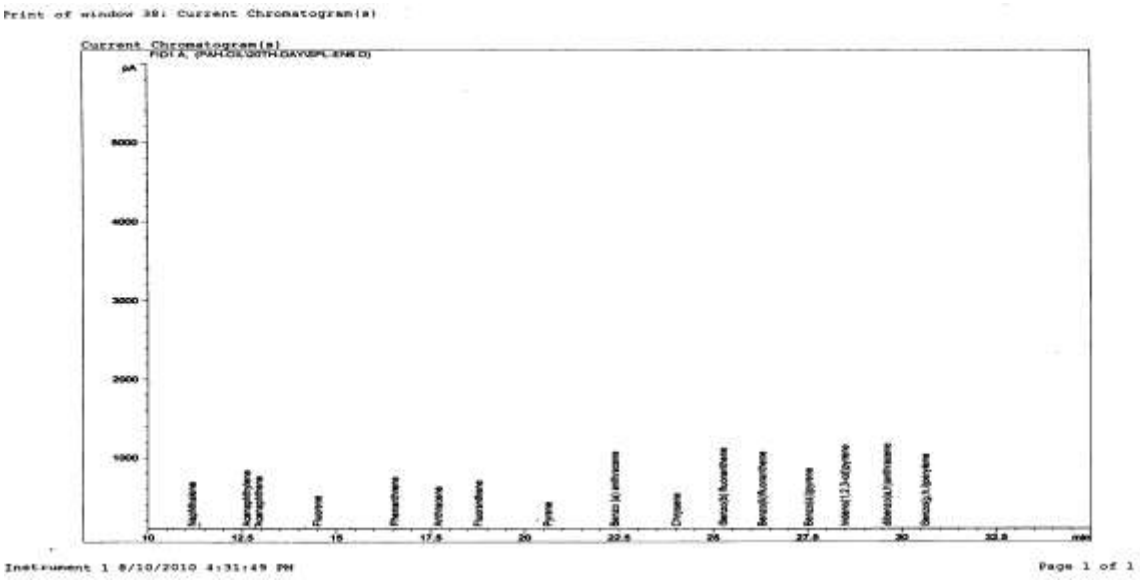
| RetTime [min] | Sig | Type | Area [pA*s] | Amt/Area | Amount [mg/Kg] | Grp | Name |
|---|---|---|---|---|---|---|---|
| 11.146 | 1 | VV | 8.33748 | 7.77889 | 64.85640 | 1 | Naphthalene |
| 12.566 | 1 | VV | 86.62402 | 4.83703e-1 | 41.90033 | 1 | Acenaphthylene |
| 12.931 | 1 | VV | 5.27191 | 3.82079 | 20.14289 | 1 | Acenaphthene |
| 14.512 | 1 | VV | 128.01324 | 1.13174e-1 | 14.48780 | 1 | Fluorene |
| 16.504 | 1 | VV | 94.46140 | 5.30642e-1 | 50.12521 | 1 | Phenanthrene |
| 17.709 | 1 | VV | 421.59790 | 1.75652e-1 | 74.05453 | 1 | Anthracene |
| 18.790 | 1 | VV | 338.52213 | 3.52379e-1 | 119.28814 | 1 | Fluoranthene |
| 21.027 | 1 | VV | 29.54856 | 3.22233 | 95.21525 | 1 | Pyrene |
| 22.509 | 1 | VV | 48.93847 | 1.63426 | 79.97824 | 1 | Benzo (a) anthracene |
| 23.824 | 1 | VV | 29.28207 | 1.91113 | 55.96183 | 1 | Chrysene |
| 25.186 | 1 | VV | 44.95369 | 1.95782 | 88.01131 | 1 | Benzo(b) fluoranthene |
| 26.367 | 1 | VV | 36.20217 | 1.88379 | 68.19719 | 1 | Benzo(k)fluoranthene |
| 27.516 | 1 | VV | 140.61249 | 1.36329e-1 | 19.16963 | 1 | Benzo(a))pyrene |
| 28.471 | 1 | VV | 9.75345 | 2.05919 | 20.08420 | 1 | Indeno(1,2,3-cd)pyrene |
| 29.581 | 1 | VV | 36.52466 | 2.43390 | 88.89748 | 1 | dibenzo(a,h)anthracene |
| 30.603 | 1 | VV | 16.31768 | 4.44259 | 72.49274 | 1 | Benzo(g,h,i)perylene |
| Totals : | | | | | 972.86316 | | |

Results obtained with enhanced integrator!

Group summary :

| Group ID | Use | Area [pA*s] | Amount [mg/Kg] | Group Name |
|---|---|---|---|---|

## Appendix XXIV B




Gas Chromatograph of Used Crankcase Oil (PAH) as degraded by *Bacillus* sp 4 20th day

Signal 1: FID1 A,

| RetTime [min] | Sig | Type | Area [pA*s] | Amt/Area | Amount [mg/Kg] | Grp | Name |
|---|---|---|---|---|---|---|---|
| 11.146 | 1 | VV | 8.33748 | 7.77889 | 53.83081 | 1 | Naphthalene |
| 12.566 | 1 | VV | 86.62402 | 4.83703e-1 | 34.77727 | 1 | Acenaphthylene |
| 12.931 | 1 | VV | 5.27191 | 3.82079 | 16.71860 | 1 | Acenaphthene |
| 14.512 | 1 | VV | 128.01324 | 1.13174e-1 | 12.02487 | 1 | Fluorene |
| 16.504 | 1 | VV | 94.46140 | 5.30642e-1 | 41.60393 | 1 | Phenanthrene |
| 17.709 | 1 | VV | 421.59790 | 1.75652e-1 | 61.46526 | 1 | Anthracene |
| 18.790 | 1 | VV | 338.52213 | 3.52379e-1 | 99.00915 | 1 | Fluoranthene |
| 21.027 | 1 | VV | 29.54856 | 3.22233 | 79.02866 | 1 | Pyrene |
| 22.509 | 1 | VV | 48.93847 | 1.63426 | 66.38194 | 1 | Benzo (a) anthracene |
| 23.824 | 1 | VV | 29.28207 | 1.91113 | 46.44832 | 1 | Chrysene |
| 25.186 | 1 | VV | 44.95369 | 1.95782 | 73.04938 | 1 | Benzo(b) fluoranthene |
| 26.367 | 1 | VV | 36.20217 | 1.88379 | 56.60367 | 1 | Benzo(k)fluoranthene |
| 27.516 | 1 | VV | 140.61249 | 1.36329e-1 | 15.91079 | 1 | Benzo(a))pyrene |
| 28.471 | 1 | VV | 9.75345 | 2.05919 | 16.66989 | 1 | Indeno(1,2,3-cd)pyrene |
| 29.581 | 1 | VV | 36.52466 | 2.43390 | 73.78491 | 1 | dibenzo(a,h)anthracene |
| 30.603 | 1 | VV | 16.31768 | 4.44259 | 60.16897 | 1 | Benzo(g,h,i)perylene |
| Totals : | | | | | 807.47643 | | |