KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY

DEPARTMENT OF BIOCHEMISTRY AND BIOTECHNOLOGY

COLLEGE OF SCIENCE

ISOLATION AND CHARACTERIZATION OF HYDROCARBON-DEGRADING BACTERIA FROM PETROLEUM STORAGE FACILITIES IN THE KUMASI METROPOLITAN AREA.

A THESIS SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE MASTER OF SCIENCE DEGREE IN BIOTECHNOLOGY.

BY

ADANE GORDON.

MARCH 2013.

Declaration

I Adane Gordon, hereby declare that this piece of work (thesis) herein now submitted as dissertation for a Masters Degree in this University is the result of my own findings and that no previous submission of this work for a Masters degree has been made elsewhere except those references which have been duly acknowledged in the text.

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Dedication

I devote this work to devote Jehovah Jireh, whose provision and grace has brought me this far. To the memory of my late mother Christiana Abisa (Adwoa Boadiwaa), my daughter Jacqueline Adom Boadiwah, and the entire family. It also goes to the 'Biotek Klan' 2008. I have enjoyed your company over the years; your influence just like salt has seasoned my life. During my stay in this Institution and in writing this thesis many encouraged. Many people have contributed to the success of this thesis some will be on the pages of this book but all are on the pages of my heart.

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Looking back over the period of this thesis writing, the knowledge, insight and challenges that were encountered made it altogether provoking and enjoyable.

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Abstract

Hydrocarbon degrading microorganisms isolated from five different petroleum storage facilities in the Kumasi Metropolitan area were investigated. A total of five bacterial isolates were obtained. Culture-based techniques i.e., isolation, enumeration, purification, biochemical tests were carried out to obtain the aforementioned organisms. The isolates (*Pseudomonas cepacia, Pseudomonas aeruginosa, Enterobacter, Proteus and Bacillus*), were from petroleum sludge of gasoline, diesel and kerosene. The research also focused on screening for the potentials of the isolates in utilizing the following hydrocarbon substrates (gasoline, diesel and kerosene) in Bergs Mineral Salt Medium (BMSM). The effects of different nutrients (soy and pito waste) supplementation on biodegradation indices were also assessed. The biodegradation indices that were evaluated include Optical Density ($OD_{600\ nm}$), floating abilities of the isolates, emulsification (E_{24}) indices and Gas Chromatography (GC) profiles. The isolates were able to reduce the hydrocarbon substrates considerably as revealed in the percent area report values over the time frame of two weeks compared to the control (abiotic). The results from the GC profiles showed that all the isolates had well above 80% reduction in the hydrocarbon substrates. *Enterobacter cloacae* showed the greatest potential with respect to gasoline degradation efficiency under soy supplement thus (99.66%) followed closely by consortium (soy added), *Proteus mirabilis* (soy added) had 99.4%, followed lastly by *Bacillus firmus* (97.2%). For diesel degradation, *Pseudomonas cepacia* (soy added) and the consortium (no nutrients added) were the same with respect to the degradative efficiency of 99.80% followed by *Enterobacter cloacae* (soy added) with 99.6%, *Pseudomonas aeruginosa* and consortium each gave an efficiency of 99.55% with soy added. *P. Aeruginosa* gave the least efficiency of 93.0% under pito waste inclusion. For kerosene degradation studies, *Enterobacter cloacae* (soy added) gave 96.0%;

consortium (no nutrient added) gave 95.60%; *Enterobacter cloaeca* (no nutrient added) recorded 95.5% with the least efficient being *Bacillus* (soy added) recording 83.4%. Compared to the controls these degradation efficiencies were relatively high.

Key words: hydrocarbon contamination, bacterial isolates, biostimulation, gas chromatography.

TABLE OF CONTENTS

Content Page

Chapter One

Chapter Two

Content Page

Chapter Four

Content Page

Chapter Five

Chapter Six

Content page

List of Acronyms

(BMSM) = Bergs Mineral Salt Medium

(BOST) = Bulk Oil and Storage Transportation

(BP) = British Petroleum

(BTEX)= Benzene, Toluene, Ethylbenzene, Xylenes

(CFU) = Colony forming units

(E_{24})= Emulsification index

(EPs)=Endopolysaccharides

(FID)=Flame Ionization Detector

(GC)= Gas Chromatography

(LPG)= Liquefied Petroleum Gas

(MSM)=Mineral Salt Medium

(NA)= Nutrient Agar

(NGOs)=Non Governmental Organizations.

(NPK)=Nitrogen, Phosphorous, Potassium

(NYMEX)= New York Mercantile Exchange

(OD)= Optical Density

(OPEC)=Organization of Petroleum Exporting Countries

(PAHs)= Polycyclic Aromatic Hydrocarbons

(THB)= Total Heterotrophic Bacteria

(TOR)=Tema Oil Refinery

(USTs)=Underground Storage Tanks.

List of Tables

Content Page

Content Page

Content Page

List of figures

Content Page

List of plates

Chapter One

1.0 INTRODUCTION

Bacteria are the most abundant microorganisms in the soil. They are equipped with the ability to make use of diverse substances including organic substances, such as petroleum, and inorganic substances for energy and growth (Marquez-Rocha *et al*., 2001). The metabolic pathways that hydrocarbon-degrading heterotrophs use can be either aerobic (i.e. they utilize oxygen as the primary electron acceptor) or anaerobic (i.e. they utilize an alternative electron acceptor such as nitrate or sulfate) (Braddock *et al*., 1997). Aerobic degradation usually proceeds more rapidly and is considered to be more efficient than anaerobic degradation. The rational is that, aerobic reactions require less free energy for initiation and yield more energy per reaction. Throughout the course of

evolution, microorganisms that have been constantly exposed to petroleum have been found to tolerate these compounds as a result of the genetic adaptations that have taken place (Atlas 1981, van der Meer, 1994). Rabus *et al*., (2005) has stated that, this has culminated in the emergence of specific population of bacterial species (ecotypes) that thrive in petroleum environment. Alvares and Illman, (2006) have noted that key component of the adaptation process among microbial communities is the role of genes which induce enzyme formation responsible for petroleum hydrocarbon-degrading are borne on plasmids or transposons that may be exchanged between species. New catabolic pathways may ultimately be assembled and adapted for efficient regulation. Another feature is cell adaptation leading to new ecotypes where cell envelope may be modified to tolerate solvents (Ramos *et al.*, 2002) and also development of community level interactions which may aid cooperation within consortia. The enzymes that are produced act on the components of crude petroleum breaking them down into simpler forms. In recent years, many microbial ecologists have identified various microbial species that are effective degraders of hydrocarbons in natural environments. Petroleum hydrocarbons are degraded not only by bacteria but by fungi, yeast and microalgae as well (Bundy *et al.*, 2004). However, bacteria play an essential role in hydrocarbon degradation. Petroleum biodegradation revolves around the ability of microorganisms to utilize hydrocarbons for growth and energy needs. In many ecosystems there is already an adequate indigenous microbial community capable of extensive oil biodegradation, provided that environmental conditions are favourable for oil-degrading metabolic activity (Kim *et al*., 2005). Oteyza *et al*., (in press) among other researchers have stated that mixed cultures carry out more extensive biodegradation of petroleum than pure cultures. The metabolic diversity of microorganisms in the natural environments is an important factor in the biodegradation of hydrocarbons. These microorganisms are adapted for survival and

proliferation in that environment. Secondly, the ability to utilize hydrocarbons is distributed among a diverse microbial population. A number of bacterial genere are commonly known to grow on petroleum as substrate when sample of the contaminant are taken for analysis. Some of them are *Arthrobactor* sp., *Alcaligenes piechaudii* sp., *Acinetobacter* sp., *Bacillus* sp., *Brevibacillus* sp., *Corynebacterium* sp., *Pseudomonas* sp., *Rhodococcus* sp., *Flavobacterium*sp., Micrococcus sp., *Staphylococcus* sp., *Serratia* sp., and *Mycobacterium* sp. (Munoz-Castellanos *et al.*, 2006, Arvanitis *et al.*, 2008, Abdulsalam *et al.*, 2011)

1.1 **Petroleum industry past and present**

Petroleum exploration activities in Ghana started in 1896 with wells being drilled in the surrounding area of Half-Asini (Western Region) as a result of oil seeps found in the onshore Tano Basin in the Western Region of Ghana (www.tullowoil.com/ghana/index.asp?pageid=27). Some of the giants oil companies which have been in the forefront of oil exploration activities in Ghana over past eight years, include; Tullow, Kosmos, Hess Corporation, Hunt Oil, Afren and Norsk Hydro Oil and Gas. These companies have struck petroleum in commercial quantities, making Ghana to join the Organisation of Petroleum Exporting Countries (OPEC) (www.ghanaweb.com). Industries the world over depend heavily on the use of petroleum and its products. In time past, this precious resource has been exploited for use in mortar, for coating walls and boat hulls, and as a fire weapon in defensive warfare dating back to historical times as reported by historians. Petroleum is a complex mixture of varying molecular weight hydrocarbons and other organic compounds found beneath the earth's surface. It is formed from pyrolysis of hydrocarbon, in a variety of reactions, mostly endothermic at high temperature and pressure (Kumar *et al*., 2011).

As a technical term, petroleum encompasses the liquid (crude oil), natural gas, and viscous or solid (asphalt and bitumen) forms of hydrocarbons that occur in the Earth, but the meaning is often restricted to the liquid oil form. Petroleum is recovered from drilled wells, transported by pipeline or tanker ship to refineries, and then converted to fuels and petrochemicals (http://ww.britannica.com/EBchecked/topic/454269/petroleum Encyclopedia Britannica). What has increased its usage over the past decades is the development of the automobile which has given petroleum the impetus as primary source of energy (Rahman *et al*., 2002). Today the world is heavily dependent on petroleum not for only motive power but also for lubrication, fuel, dyes, drugs, and many synthetics. Chief among the producers of crude oil and natural gas are Iran, Saudi Arabia, the U.S. and Russia, accounting for more than 60% of world energy consumption; the U.S. is by far the largest consumer. Petroleum production in the United States peaked during the 1960s, however, Saudi Arabia and Russia have surpassed the U.S. (Akiner and Aldis, 2004). According to Lambertson (2008), about 90% of vehicular fuel needs are met by oil; thus making it important or of critical concern to many nations. Petroleum's worth as a convenient, dense energy source powering vast majority of vehicles and as the base of many industrial chemicals makes it one of the world's most important traded items. The worldwide consumption is about 30 billion barrels (4.8 km^3) of oil per year, and the top oil consumers largely consist of developed nations. In fact, 24% of the oil consumed in 2004 was by the U.S. alone, though by 2007 this dropped to 21% of world oil consumed (New York Mercantile Exchange (NYMEX) 2006; Mabro, 2006). The table below shows the quota of petroleum with respect to the world's energy consumption pattern according to geographic regions.

Table 1: **World energy consumption patterns according to geographic regions.**

Continent	percentages
Asia	32%

Europe	32%
North America	40%
Africa	41%
South and Central America	44%
Middle East	53%

(Courtesy: New York Mercantile Exchange (NYMEX) 2006, Mabro 2006)

1.2 Petroleum in the environment and its impact.

Oil will continue to be used as a source of energy for powering our engines and for other processes until cheaper and more abundant clean technologies are found (Oboh *et al.*, 2006). An extensive rate of industrialization is driving the quest for more energy and this has culminated in an increased petroleum exploration activities worldwide with its attendant negative consequence being the pollution of the environment (Okoh and Trejo-Hernandez, 2006). Amongst the numerous contaminants polluting the environment, hydrocarbons play a special role, which is related to their wide-scale distribution and hazardous physicochemical and biological properties (Lisovitskaya and Mozharova. 2008). Their presences in the environment need not only be the result of anthropogenic activities such exploration, drilling, extraction, refining and combustion, but seepages as well (Kenovolden and Cooper, 2003). The discharge of petroleum products in large quantities into the environment has impacted negatively on various ecosystems (sea, lands, wetlands and underground water). Their undesirable effects endanger plants and animals lives (Atlas and Philp. 2005). Regardless of the source of contamination, petroleum's effects on the environment are similar. Some components of petroleum such as benzene, toluene, xylenes and ethylbenzene (BTEX) have high water solubility and poses serious health threat as these contaminants are linked with mutation, cancer and susceptibility to immuno-toxicants. This problem is most serious in areas which rely on groundwater and rivers as major sources of drinking water. The quality of water is also dented in terms of taste and smell even at very low

level of concentration of these contaminants (Adebusoye *et al*., 2006, Margesin and Schinner 2001, Rahman *et al*., 2002).

Plate 1. A badly damaged ecosystem as a result of petroleum discharge in Nigeria (Niger Delta Region).

Soil contaminations are from cars and trucks, leaky containers thus underground storage tanks (usts), industrial accidents, and poorly disposed of wastes (hydrocarbons) on land as a result of field operations involving fuel storage, refueling of vehicle, industrial plants among others. Technical faults and equipment failure, sometimes due to sheer negligence on the part of Trans–national oil corporations cause environmental degradation/pollution in the soil and fragile wetlands (Mishra *et al*., 2001; Amunwa, 2006).

1.3 Premise for degradation of hydrocarbons

Hydrocarbons are considered to be of biological source and processes (Prenafeta-Boldu *et al.*, 2006). Drawing inference from Surridge, (2007), it could be said that a hydrocarbon compound

called hopanoid associated with petroleum are commonly found in bacterial cell walls and that the 'biological evidence' within these hydrocarbons could be a possible reason for the adaptation of microorganisms to degrade them so readily. The genetic potential and certain environmental factors such as temperature, pH, available nitrogen and phosphorus sources determine the rate and the extent of degradation. Efforts targeted at using microorganisms and optimizing the conditions of the degradative process to ameliorate soil and groundwater bodies are increasingly becoming a popular alternative (Singh and Lin, 2008).

1.4 Proposed interventions or strategies.

In dealing with the above problem, the catch phrase in use today is bioremediation. This technology has been adopted by many companies in South Africa and other developed countries for dealing with municipal waste. It has been classified as either *ex situ* or *in situ*. *Ex situ* bioremediation involves the physical removal of the contaminated media to another location for treatment. In situ involves the treatment of the contaminated media in place. It is envisaged that regardless of the method chosen, coupled with environmental conditions, the identification of key organism(s) is pertinent to achieving a higher level of degradation (Watanabe, 2002).

1.5 Problem statement

Rojo, (2009) has reported that with the development of the economy and industries (petroleum exploration), contamination of soil with petroleum compounds is of concern worldwide. Hydrocarbon discharges are not only a problem in countries that produce oil but also in countries that purchase, process and use them. Oil tanker accidents and similar occurrences elsewhere have long drawn attention to the problem of petroleum hydrocarbon in the environment. Though oil tanker accidents attract great media attention, they are not the most common contamination

problem. Environmental problems caused by oil from cars and trucks, leaky containers, industrial accidents, and poorly disposed of wastes are much more common cause for concern. This often results in cleanup delays while the contaminated soil continues to pollute groundwater resources if on land, and death of aquatic life if on waterways. Kumasi the secondary largest city in Ghana is sprawling with the establishment of filling stations and sooner or later the city will experience petroleum contamination. This problem of hydrocarbon contamination will be most serious in areas which rely on groundwater and river as major source of drinking water (Samanta *et al*., 2002).

In Ghana there is little or no data on sites that has been contaminated by the storage or operations of hydrocarbons. Wherever the source of contamination may be from, petroleum products may reach groundwater reserves, lakes or water courses providing water for domestic and industrial use. Apart from the obvious altered taste and smell of water when contaminated with minute quantities of petroleum hydrocarbons Onosode, (2001) has intimated that oil spills have destroyed farmlands, polluted surface and groundwater caused drawbacks in fishing and killed many rural Nigerians through fire outbreaks and explosions in the region. Diesel constituents (PAHs) are known to be carcinogenic, mutagenic and a potent immuno-toxicant, thus posing a serious threat to human, animal health and the ecosystems over a prolonged period of release. These pollutants could also hinder some microbial communities that are important in some biogeochemical cycles of that ecosystem and this affects the productivity of such ecosystem.

1.6 Justification

Bioremediation, using biological processes to ameliorate hydrocarbons from the environment, is already a successful technology for cleaning up soil and marine sediments (van Herwijnen *et al.*,

2006; Das and Mukherjee, 2007). When the oil spill by British Petroleum (BP) in the Gulf of Mexico occurred in April 2010, stakeholders in fishing communities, environmentalist, and tourism agencies doted along Miami beach and elsewhere got tensed up because the problem had gotten out of hand. It was expected that the spill would reach the shorelines and dwindle the fortunes of the communities doted along the coastline but that never occurred. How was the problem solved, and who were the unsung heroes?. Some microbes (bacteria) appeared and consumed the contaminant which had spread over a long distance in nautical miles for several weeks. The Alaskan experience has shown that bioremediation is doable. Several reviews and research publications have shown that degradation of hydrocarbons is not uncommon and that the degradative process is not restricted to only a few microorganisms but widely distributed among numerous genera or taxa (Yakubu, 2007). Biodegradation of hydrocarbon-contaminated soils, which exploits the ability of microorganisms to degrade and/or detoxify organic contaminants, has been established as an efficient, economic and versatile that suits local conditions and sites (Mehrashi *et al*., 2003, Taki *et al*., 2007, Obayori *et al*., 2008). A need exists to develop methods or techniques to accelerate the removal of these contaminants from the environments. Identifying and using microbes that have the ability to degrade both soil and groundwater with aromatic hydrocarbons is possible whereas conventional technologies have failed to give the desired impact. A research in South Africa, by Atangana, (2008) has shown that the concentration of most of the selected hydrocarbon components used in a field experiment were reduced by up to 100% and that microbial activities were shown to correlate with the reduction in hydrocarbon contents of the soil. In fact there are several companies including Bio-systems and MRO Product Management (Pty) Ltd both South African companies; who have specialized in bioremediation of hydrocarbons in soil, chlorinated organic compounds as well as

refinery and petro-chemical waste using non-pathogenic prepared microorganism to treat these hazardous xenobiotics.

1.7 The main objective

Microbial activity alone is not sufficient evidence of natural attenuation; it can be used in conjunction with other evidence such as physical and chemical treatment. The aim of this present work is to isolation and characterise hydrocarbon-degrading bacteria from petroleum storage facilities in the Kumasi metropolitan area.

1.8 Specific objectives

- To determine hydrocarbon-degrading microorganisms indigenous to some storage facilities within the Kumasi metropolitan area.
- Identify the organism by morphology; Gram staining, catalase test and other biochemical tests (API 20 E and API CH 50).
- To determine the effect of soy residue and pito waste on degradation of the various petroleum hydrocarbons by individual and mix cultures.
- Investigate the biodegradation potential of each strain by determining the emulsifying stability test (E_{24}) floating test and Optical Density (OD_{600nm}) and the Gas Chromatography profile of individual species and mix cultures on various petroleum hydrocarbon substrates.

Chapter Two

2.00 LITERATURE REVIEW

2.10 Role of microorganisms in bioremediation

Bioremediation is the use of biological systems or agents to destroy or reduce the concentrations of hazardous wastes from contaminated site. The bottom line of information pouring in from all sides indicate that one does not need to search far to find microorganisms with the potential to degrade petroleum compounds (Odokuma and Dickson, 2003). The findings of several studies have been published on the response of the bacterial community to bioremediation treatment of soils polluted with crude oil or specific hydrocarbon classes. Indigenous microorganisms abound in sites contaminated with petroleum are suitable for inocula during bioremediation.

A field experiment in the Niger Delta dry and wetlands was carried out in which the indigenous microorganisms were identified and used to demonstrate their ability to metabolize Bonny light crude oil (Odokuma and Dickson, 2003). In Egypt an isolated strain from water treatment plant identified as *Pseudomonas* sp. (H12) was found to be highly efficient in degrading hydrocarbon mixture of benzene, toluene, xylene, etc. (Amer *et al*., 2008). Reports and publications of native soil microorganisms including those mentioned earlier cannot be overlooked. These microbes act as agents in transforming complex organic compounds into simple constituent elements. This process is termed mineralization. The end-products of petroleum hydrocarbon degradation are carbon-dioxide and water which are also measures of microbial respiration and activity in soils, (Obire and Nwaubeta, 2001). Below is a plate describing the mineralization process.

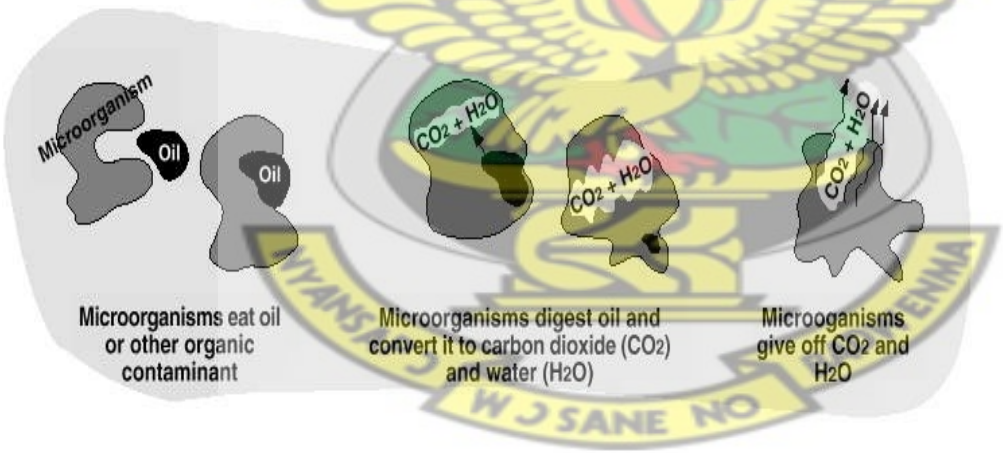

Plate 2: A diagram showing mineralization of organic contaminant

(Courtesy: Response, EPA 2001, 542-F-01-001)

Several research and reviews indicate that microorganisms (bacteria) can take the contaminants for their own growth and build up new cells. However, in soils, some of the hydrocarbons

especially polycyclic aromatic hydrocarbons (PAHs) disappearance does not necessarily involve complete conversion to carbon dioxide and water (Obire and Nwaubeta, 2001). Bossert and Bartha (1984) stated that partially oxidized PAH compounds may be incorporated into soil humus.

2.11 Bioremediation: approaches and adaptation

Bioremediation being a natural attenuation relies on the capabilities of naturally occurring microbial communities to degrade environmental pollutants, a classic example is the case–study where it was used in a very large scale application in cleaning up shoreline in Alaska, after the "Exxon Valdez" oil tanker oil spill in 1989. Bioremediation systems have potentially broad-spectrum site applications including groundwater, soils, lagoons, and sludge and process waste-streams (Shell Petroleum Development Company, 2002). Aichberger *et al*, (2005) and several reviews suggest that at sites where there is petroleum products contamination, a spectrum of necessary professional expertise is greatly expanded. However, three important aspects are necessary in bioremediation studies, and these include microbial composition, contaminant type, geology of polluted site and chemical conditions at the contaminated site (Hamdi *et al*., 2007). The goal of bioremediation completely destroy the pollutants if possible, or at least to transform them to innocuous substances (Sheetal, 2012). This technology quickens the naturally occurring biodegradation under enhanced conditions such as oxygen supply, temperature, pH, the presence or addition of suitable microbial population (bioaugmentation) and nutrients (biostimulation), water content and mixing (Margesin and Schinner, 1999). Studies have shown that a wide range of compounds that may be subjected to biological decontamination by one or other bioremediation system is long.

2.12 Biostimulation

This process involves the stimulation of indigenous microorganisms to degrade the contaminant. The microbial degradation of many pollutants in aquatic and soil environments is hindered primarily by the availability of nutrients, such as nitrogen, phosphorus, and oxygen supply. The addition of nitrogen, phosphorus and provision of additional oxygen or aeration has been shown to stimulate the indigenous microbial populations to be able to perform better (Trindade *et al*., 2005). Without ample supplies of oxygen and nutrients, the microbes will eventually deplete the existing supplies, thus limiting the amount of contamination removal that can occur. Margesin and Schinner, (2001); Roland and Atlas (2011), and Van Hamme *et al*., (2003) have documented that the slow nature of biodegradation and the biodegradative ability of microbes could be addressed by the addition of fertilizers. Another course of action is the addition of a second carbon source to stimulate the synergy of the consortia.

2.13 Nutritional content of soy residue and pito waste.

The high cost along with unavailability of inorganic fertilizers in Ghana has promoted a renewed interest in the use of agro-waste such as pito waste and soy residue as nutrient sources for the many process such as biostimulation. Sorghum is a rich source of potassium, phosphorus and nitrogen; it has a good amount of calcium, iron and sodium. In addition it has some amount of thiamine, niacin and riboflavin; these are identified to promote the growth of living things (www.organicfacts.net/nutrition, Nishidha Patil). Soy on the other hand, is well known for its high protein contents and contains all the essential amino acids necessary for growth and

development. Foremost in its mineral components are potassium, sodium, calcium, magnesium, sulfur and phosphorus. The water-soluble vitamins in soybeans are thiamine, riboflavin, niacin, pantothenic acid, biotin, folic acid, inositol and choline. Fat-soluble vitamins present in the soybean are vitamins A and E (www.soytech.com/soy)

2.14 Co–metabolism

Synergistic activities or co–metabolism occurs when an organism is using one compound for growth and gratuitously oxidizes a second compound that is resistant to being utilized as a nutrient and energy source by the primary organism, but the oxidation products are available for use by other microbial populations. This synergistic action facilitates the degradation of many recalcitrant pollutants by introducing analogous compounds to the target pollutant thereby creating positive conditions for metabolism and growth (Igwo-Ezikpe *et al*., 2010). A study by Burback and Perry (1993) described this phenomenon when *Mycobacterium vaccae* co–metabolized cyclohexane while growing on propane. The cyclohexane was oxidized to cyclohexanol, which other bacterial populations (*Pseudomonas*) then utilized. Co–metabolism transformation ensures that there is recycling of relatively recalcitrant compounds that do not support the growth of any microbial culture (Atlas and Bartha, 1993). The genus *Pseudomonas* is known for their immense ability to grow on various organic compounds. Phenol biodegradation studies with the bacterial species have resulted in bringing out the possible mechanism and also the enzyme involved in the process (Annadurai *et al*., 2000; Nair *et al*., 2008).

2.15 Biosurfactants

Bacteria and fungi are predominant in degrading hydrocarbons; these microbes have developed different adaptations for exploiting poorly soluble substrates. Majority of them produce biosurfactants, amphiphilic molecules of diverse chemical nature and molecular size with effective surface active and biological properties (Desai and Banat 1997). They help to disperse the hydrocarbons, increase the surface area of hydrophobic water-insoluble substrates and increase their bioavailability, thereby encouraging the growth of bacteria and the rate of bioremediation (Rosenberg and Ron, 2002). There has been quite an extensive research on some microbes that have the capacity to degrade many pollutants and produce biosurfactants or emulsifiers with beneficial applications (Bell *et al.*, 1998). A wide variety of petroleum degrading microorganisms has been found to bring about the formation of oil–in–water emulsions while growing on hydrocarbons. Employing biosurfactants have unique advantages which have been briefly outlined.

- Structural diversity that have unique properties. The option of cost effective production and their biodegradability. These characteristics make them a promising choice for applications in enhancing hydrocarbon bioremediation (Whang *et al.*, 2009).
- Ability to retain their properties even under extreme conditions of pH, temperature, salinity (Rosenberg and Ron, 2002).
- Low irritancy and compatibility with human skin (Pornsunthorntawee *et al*., 2009).
- Bioavailability activity under diverse conditions, ecological acceptability, low toxicity. They could also be altered by biotechnology and genetic engineering by this means increasing the bioavailability of poorly soluble organic compounds, decreasing surface tension and increasing the displacement of oily substance from soil (Banat *et al.*, 2000; Tugrul and Cansunar, 2005).

According to Yakubu (2007), biodegradation research has two spin-offs: many of the enzymes used in the pathways for degrading unusual substrates catalyze novel reactions. They are often non-specifically and have potential use as biocatalysts in industry for the production of novel fine chemicals which are otherwise difficult to synthesize and secondly an understanding of the pathways and their genes can lead to the use of recombinant strains carrying pathways that has been enhanced by genetic engineering to degrade compounds that are to resistant to microbial attack.

2.16 Bioaugmentation

Bioaugmentation is the introduction of cultured microorganisms into a contaminated environment for the purpose of facilitating biodegradation process (Mrozik andPiotrawska-Peget, 2009). Information about the bacteria's presence and concentration might then be used to assess the progress of efforts to remove pollutant from the contaminated soil. Individual isolates have lower ability than microbial consortia in biodegradation, especially for complex mixtures of compounds such as diesel oil and crude oil (Wang *et al*., 2008). Whiles some publications (Prince, 1997; Swannell *et al*., 1996) claim that bioaugmentation has usually proved ineffective in stimulating degradation of petroleum hydrocarbon contamination; a study by Ruberto *et al*., (2003) on the bioremediation of a hydrocarbon contaminated Antarctic soil demonstrated a 75% removal of the hydrocarbon when the contaminated soil was bioaugmented with a psychrotolerant strain (B-2-2) and that bioaugmentation improved the bioremediation efficiency. A publication by Vecchiolli *et al*., (1990) stated that in addition to the natural presence of hydrocarbon degrading bacteria in soils, exogenous microbial inoculation is able to accelerate the biodegradation whenever conditions are appropriate. Watanabe, (2002) described the former

statement as a direct biochemical method that takes the attendance of the bacterial organisms in the soil. Recalcitrant components in crude oil such as PAHs has been broken down and rendered harmless by microbes that have been engineered (Nilanjana, and Preethy, 2011**)**.Catabolic pathways have been proposed for various organisms since the 1960's and gene clusters involved in their degradation characterized (Frantz and Chakrabarty1986). Reports on manipulating the genes of bacteria to enhance the degradation of some hydrocarbons has been stated by Ramos *et al.,* (2002) explaining some roles played by plasmids in encoding the enzymes of biodegradative pathways. However, some degradative pathways can produce intermediates, which are trapped in dead end pathways, or transform the pollutants into toxic compounds. Such situation can be improved by the addition of a seed culture (bioaugementation) of selected or genetically engineered microorganisms.

2.17 Composition and characteristics of petroleum hydrocarbon.

Petroleum is a liquid mixture of hydrocarbons (oil) obtained from natural underground reservoirs (Hynes 2001, Kumar *et al*., 2011). Petroleum hydrocarbons can be categorized for simplicity into four fractions: Saturates, Aromatics, Resins (N, O, S,) and Asphaltene. Saturates are are characterized by the absence of (naphthenes). Aromatic hydrocarbons have one or several aromatic rings and are usually substituted with different alkyl groups. The Aromatic fraction contains volatile mono–aromatic hydrocarbons (benzene, toluene, xylenes etc.) and polycyclic aromatic hydrocarbons (PAHs) such as naphtheno aromatics and aromatic sulphur compounds such as thiophenes and dibenzothiophenes. It is noteworthy that PAHs fractions which is associated with oil contamination, includes both suspected and known carcinogens, the most toxic being benzo(a)pyrene. Resins are amorphous solids which are truly dissolved in oil, whereas asphaltenes are large molecules colloidally dispersed in oil (Alloway and Ayres 1993; Sheetal, 2012). The relative proportions of these fractions are dependent on many factors such as the source, geological history, age, migration and alteration of crude oil (Gallego *et al*., 2001, Harayama

et al., 1992). The most common petroleum hydrocarbons contaminating environment are the gasoline, diesel and fuel oils. Petroleum hydrocarbons are between C_6 and C_{25}. Gasoline is a light fraction in the range from C_6 to C_{10} with a boiling temperature ranging from 23^OC to 204^OC. Diesel fuel is in the middle distillate group (C_6 to C_{24}) with boiling temperature between 202^OC and 320^OC. Most diesel hydrocarbons are between the C_{10} and C_{18} other literature by Speight (1991**)** stated that molecules in the gasoline range have 4-12 carbon atoms, kerosene has 12-16, diesel or fuel oil has 14-20, and heavier oils have 20 or more carbon atoms. Molecules with the same number of carbon atoms can vary in their number of hydrogen atoms. Fuel oil (kerosene) and lubricants are heavier cuts in petroleum products and similar in composition and characteristics to middle distillates. These types of fuels are relatively viscous and insoluble in water and are relatively immobile in the subsurface. Below are some structural components of petroleum.

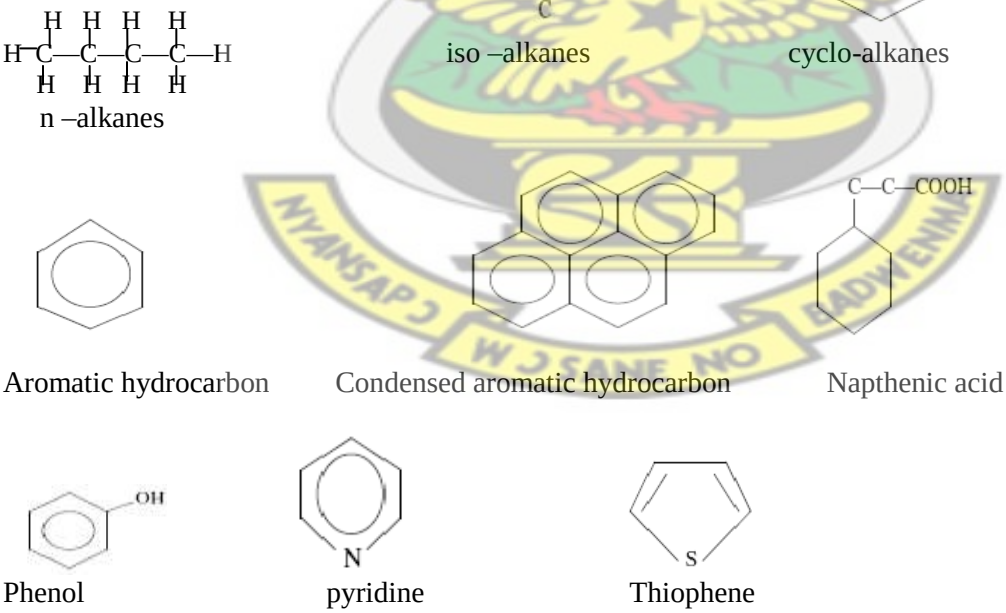

Figure 1. Structural categories of some crude petroleum components (Alloway and Ayres 1993).

Petroleum products have basically similar chemical and physical properties. For the purpose of remediation of contaminants the most important physical properties are volatility, solubility in water and viscosity. The viscosity of spilled oils determines the spreading and dispersion of the hydrocarbon mixture and also the surface area available for microbial attack. The most common distillates are gasoline (petrol), diesel, kerosene and liquefied petroleum gas (LPG).

Table 2. **Composition by percentage of the components of petroleum**

Elements	Percentage range (%)
Carbon	83-87
Hydrogen	10-14
Nitrogen	0.1-2
Oxygen	0.1-1.5
Sulfur	0.5-1.6

Source: Speight (1999).

2.18 Toxicity of petroleum hydrocarbons

The use of petroleum as a primary source of energy is convenient in terms of its availability; but the detrimental effects it leaves on various ecosystems have far reaching consequences, as reiterated by several environmentalists. Media reports indicate that large amount of these toxins are released into both populated areas and ecosystem globally. The most common soil contaminants are petroleum-based. These chemicals tend to spread through soil by diffusion. Hydrocarbons from diesel fuel and gasoline are widespread problems, because of PAHs. Many PAHs are known carcinogens, and others are suspected deleterious chemicals that need to be kept from contaminating drinking water (van Grevenynghe *et al.*, 2005; Samanta *et al.*, 2002). It is believed that shorter carbon molecules facilitate microorganism degradation (Montagnolli *et al*., 2009). There are many views as to what may constitute toxicity. Many compounds contribute to high toxicity, but the PAHs formed from fuel combustion and lubricant decomposition in high

concentration are found lethal to soil microorganisms (Henry, 1998). Generally oil products with low boiling points appear to be more toxic than the heavier fuel oils while crude oils are intermediate with respect to toxicity. Small hydrophobic molecules are highly toxic for microorganisms due to their partition into the cytoplasmic membrane (Sikkema *et al*., 1995). High concentrations of petroleum components like BTEX might be toxic to microbial populations and therefore delay degradation (Alagappan and Cowan, 2003). These petroleum fractions disrupt the protein-lipid and lipid-lipid connections in the membrane, cause functional disturbances, increase membrane fluidity and passive diffusion of the hydrophobic compounds into the cell (Sikkema *et al.*, 1995).

Toxicity of crude oil on humans includes liver necrosis, congestion of the liver, fat degeneration and dissociation of hepatocytes. Animals and birds in oil-contaminated area are found to have black emulsion in the digestive tract with a petroleum odour. This reduces the absorption of nutrients and finally leads to the death of these birds (Khan and Ryan, 1991).

2.19 The nature of petroleum sludge

A mixture of wax, oil, sand, and water is referred to as slop or sludge, in the petroleum industry. The percentage of sludge oil varies according to the type of crude and the conditions under which it has been transported. Usually the amount of sludge oil ranges from a low value of 0.5% to a high value of 10%; normally, it is in the range of 2% to 5% (Goldman and Gordon, 2006; www.worldintellectualpropertyorganisation). The presences of sludge tend to trap the moisture and increase corrosion and rusting in tanks. One of the major economic impacts of asphaltene and paraffin sludge on the petroleum market occurs when paraffins and asphaltenes separate from crude oils during ocean transport. If a pipeline becomes plugged up or clogged because of paraffin wax precipitating out during the pumping operation, a crisis can arise. Numerous

pipelines worldwide are clogged daily or monthly due to wax precipitating out of the crude oil (Goldman and Gordon 2006, www.worldintellectualpropertyorganisation).

2.20 The influence of soil and water in relation to degradation by hydrocarbon degraders.

According to (Manilal and Alexander, 1991) petroleum hydrocarbons tends to adhere onto soil with high organic content like humic substances and clay minerals. The ease with which hydrocarbon-degrading microbial biomass move is affected by both texture and structure of soil. Coarse materials offer less resistance to microorganisms than fine textured soil. In fine textured soil the hydrocarbons become less available for biodegradation. The fine particles and significant amounts of organic matter in the sediment also adsorb the pollutants to the matrix. Generally, petroleum hydrocarbon compound bind to soil components and are difficult to remove or degrade (Barathi and Vasuden, 2001). A high molecular weight compound with very low solubility in water prevents natural biodegradation process from working efficiently in hydrocarbon contaminated soils. These compounds also penetrate macro-and micro-pores in soil and thus limit water and air transport that would be necessary for organic matter conversion (Caravaca and Roldan, 2003).

2.21 Biochemistry of microbial degradation of petroleum

Evolutionary trends show that microorganisms that have been constantly exposed to a wide range of petroleum have evolved enzymes required to degrade them. Many microorganisms, such as *Pseudomonas aeruginosa, Pseudomonas putida* and *Bacillus subtilis, Bacillus cereus, Bacillus licheniformis* and *Bacillus laterospor* excrete emulsifiers that increase the surface area

of the substrate. These microorganisms modify their cell surface to increase its affinity for hydrophobic substrates and thus facilitate their absorption (Cybulski *et al*., 2003; Carvalho and Fonseca, 2005). Petroleum compounds are degraded by microorganisms to satisfy nutritional, energy requirements and also to detoxify the immediate environment. Sometimes the mineralization reactions occur fortuitously such that the organism receives no nutritional or energy benefit. Normally it takes the synergy (co–metabolism) of two or more microbes (consortium). There are a variety of bacteria and fungi, which are capable of co–metabolizing polycyclic aromatic hydrocarbons. Once the petroleum compound enters into the soil or groundwater, natural biodegradation will take place by the indigenous microbes that seem to be ever-present even in cold marine ecosystem **(**Christofi and Ivshina, 2002). The formation of enzymes for breaking down those compounds is induced only when required by bacteria. Some of these organisms have developed an additional and highly effective system for responding to a variety of potential growth substrate. Elucidating the fate of hydrocarbon contamination by microorganisms is quite complex as it borders on enzymatic reaction of the indigenous microbial population. Metabolic pathways within microbial cells are concisely regulated (expressed constitutively) and extremely sensitive to the cell's need. *Pseudomonas* sp, among other species are versatile in surviving and metabolize the hydrocarbon contaminations. Vidali, (2001) has explained that for these biological entities to carry out effective biodegradation, the presence of an inducer is imperative for the synthesis of specific enzymes for the target compound(s). The key features in their metabolic versatility include:

1) The possession of hydrocarbon-group-specific oxygenases and mechanisms for optimizing contact with the hydrocarbon.

2) Producing extracellular emulsifying agents by microbes that are induced by growth on hydrocarbons.

Microbial degradation pathways start with the action of multi component dioxygenases (Mason and Cammack, 1992) or through successive monooxygenations of the aromatic ring to produce diols (Harayama, 1992) followed by dehydrogenation of the two adjacent hydroxylated carbon atoms.

The enzymes (bioemulsifiers) produced by the hydrocarbon degraders facilitate their abilities to degrade hydrocarbons. Emulsification helps the dissolution of hydrocarbons in water and provides an enlarged surface area for direct contact of microorganisms with liquid hydrocarbon droplets Rosenberg and Ron, (1999). Parales and Haddock, (2004) have stated that microorganisms can efficiently transport into their cells (poor soluble) dissolved liquid hydrocarbons and use it for growth. A hydrocarbons' chemical structure affects its biodegradation in two ways. First, the molecule may contain groups or substituents that cannot react with available or inducible enzymes. Second, the structure may determine the compound to be in a physical state where microbial degradation does not easily occur. The solubilisation is not the only factor that influences the degradation of hydrocarbons (Cybulski *et al*., 2003). These enzymes play an important role in biodegradation since they define the substrate range of each bacterium. The aromatic compounds are transformed into a limited number of central metabolites that are further degraded to Krebs cycle intermediates. Nevertheless, genes for some of these enzymes are evolutionarily related to each other even though they oxidize different pollutants. Microorganisms decompose most organic compounds into carbon dioxide, water and mineral matter, such as sulfate, nitrate and other inorganic compounds. The aerobic pathway

proceeds most rapidly and most efficiently, because aerobic reactions require less free energy for initiation and yield more energy per reaction (http://home.eng.iastate.edu, Todd Brubaker).

The hydrocarbons are broken down by a series of enzyme-mediated reactions oxygen serves as an external electron acceptor, while an organic component of the contaminating substance functions as the electron donor or energy source. In absence of O_2, NO^-_3, SO_4^{2-} are used as electron acceptors (Braddock *et al*., 1997). The general degradation pathway for an alkane involves sequential formation of an alcohol, an aldehyde and a fatty acid. The fatty acid is 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 mono–oxygenases (Atlas and Bartha, 1998;http://home.eng.iastate.edu)

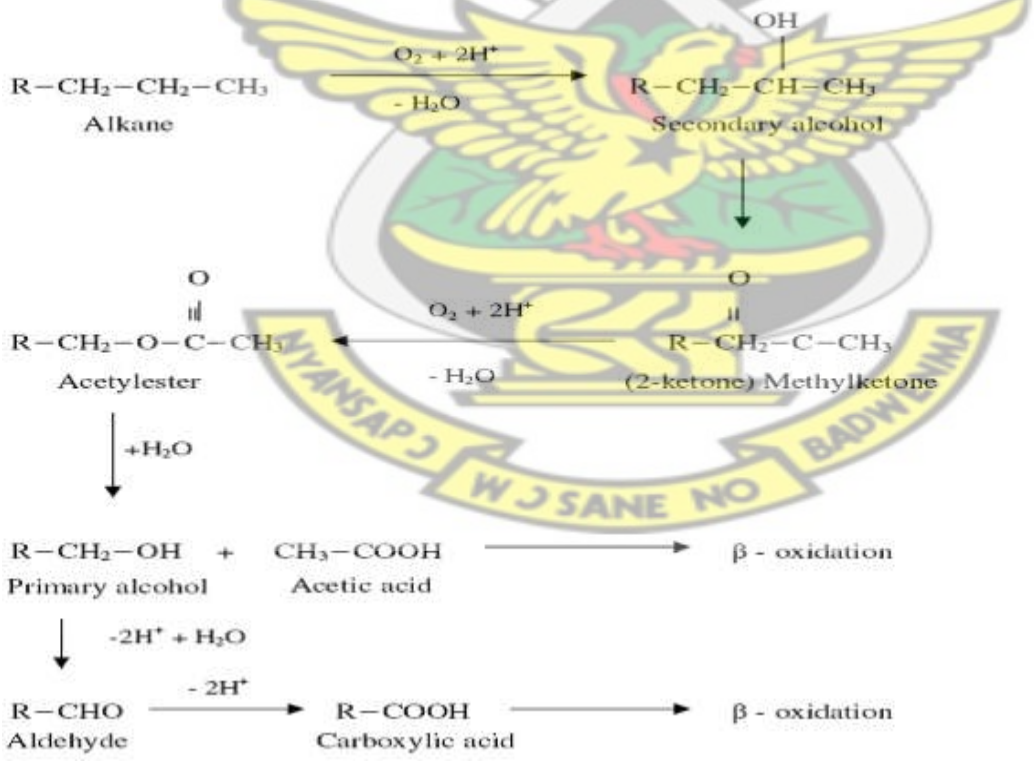

Figure 2. Pathway, through which sub–terminal oxidation of alkanes yield two fatty acid moieties, which are metabolized further by beta-oxidation (Atlas and Bartha, 1998).

The energy gained from these electron transfers is "invested" along with some electrons and carbon from the contaminant to produce more cells. These two materials, the electron donor and acceptor, are essential for cell growth and are called the primary substrates (Atlas, 1981). The general pathway for aromatic hydrocarbons involves *cis*-hydroxylation of the ring structure forming a diol (e.g. catechol) using dioxygenase. The ring is oxidatively cleaved by dioxygenases, forming a dicarboxylic acid (e.g. muconic acid). Oxidation of substituted aromatics generally proceeds by initial beta-oxidation of the side chain, followed by cleavage of the ring structure (Atlas and Bartha, 1998).

The above reactions in Figure 2 can be summarized as follows:

Organic substances + microbes + O_2 →biomass + CO_2 + H_2O + other inorganic.

Thus, the organic substance is oxidized (addition of oxygen), and the O_2 is reduced (addition of electrons and hydrogen to water (H_2O). In this case, the organic substance serves as sources of energy (electrons) and the source of cell carbon used to build microbial cell (biomass).

Different microbial species have different biodegradation capabilities. Due to this fact, the biodegradation profiles of different bacteria are important task for selecting microorganisms in bioremediation processes.

Chapter Three

3.00 Materials and methods

3.10 Sampling

Sludge samples were collected from five (5) petroleum storage facilities (locations) within the Kumasi Metropolis using the systematic area sampling technique. The areas sampled include North (Suame–Maakro) area–Goil filling station, South (Kwamo)-Goil filling station, East (Tafo) Goil filing station, West (Kaase)- Bulk Oil and Storage Transportation (BOST) and (Kotei)-from local retailer. The plate below indicates (in spiked circle) the geographic locations where sludge samples were taken from.

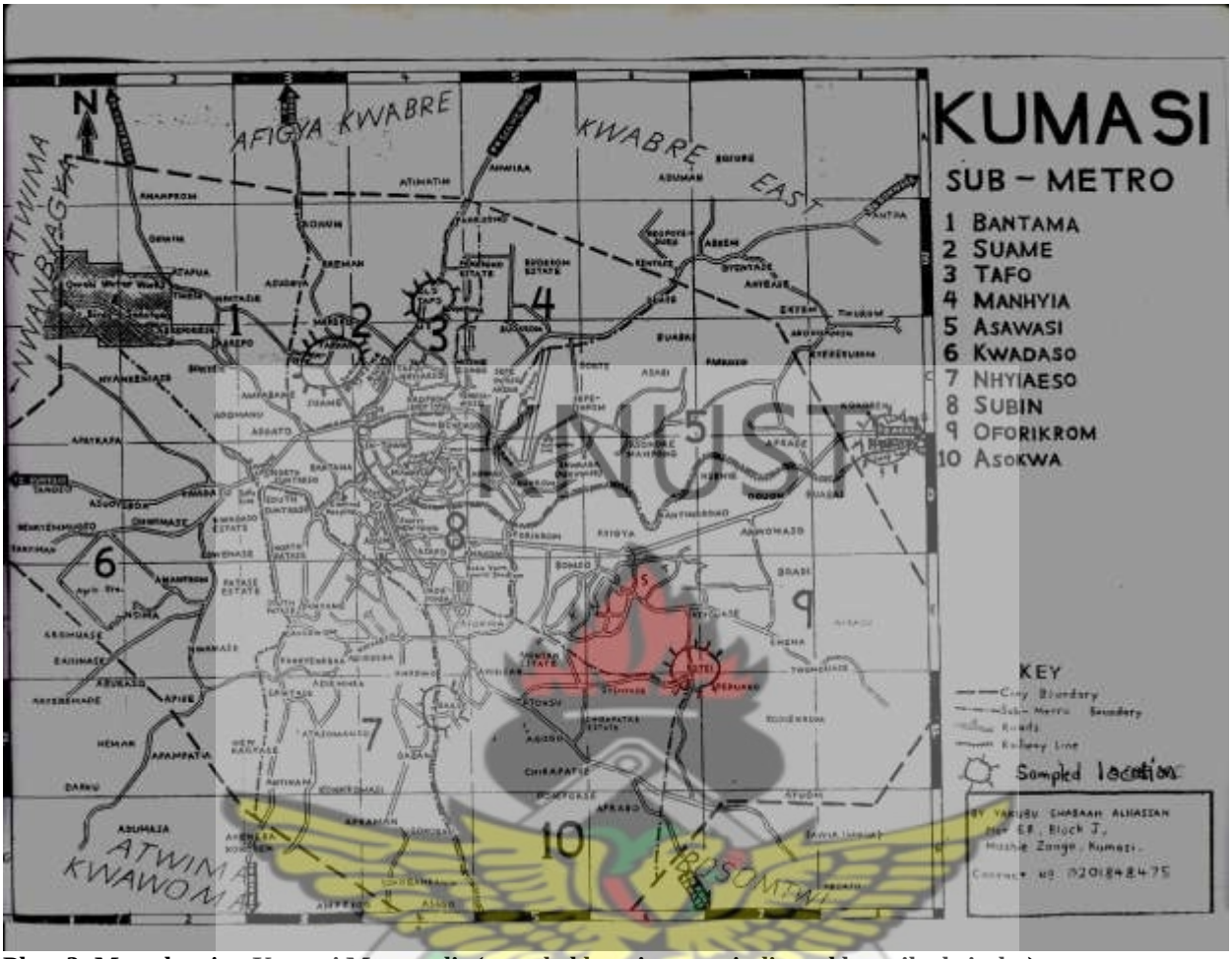

Plate 3: Map showing Kumasi Metropolis (sampled locations are indicated by spiked circles).

A total of fifteen samples were collected, three from each location. The three samples from each location were gasoline (1), diesel (2) and kerosene (3). Prefix (1), (2) and (3) represent gasoline, diesel and kerosene sludge samples respectively.

The samples were collected with spatula and sterile plastic bags. Commercial (petroleum) products of gasoline, diesel and kerosene that were used for the experiment were purchased from Kentinkrono Mobil filling station while the kerosene was obtained from a local retailer (Gawo).

Plate 4. Sludge samples from Tafo

3.11 Isolation and enumeration of total heterotrophic bacteria (THB) cultures

One (1) g of oil sludge each from petrol, diesel and kerosene samples from each of the five locations were weighed into test tubes containing 9 ml of sterile distilled water. The contents were shaken vigorously to obtain a homogenous mixture. Ten-fold serial dilutions in the range of $10^{-6} – 10^{-9}$ were prepared using sterile distilled water. Pour plate technique was used to isolate total heterotrophic bacteria (THB) (Oxid). Aliquots (1ml) of sample dilutions of $10^{-6} – 10^{-9}$ were each plated. The above experiment was performed in duplicates. The mixture was allowed to solidify and then incubated at 37°C for 24 hrs. Total Heterotrophic bacteria (THB) and colony forming units /g (CFU/g) of the sludge which is index of microbial contamination of the waste and site is calculated from each of the bacterial colonies on plates and multiplied by the reciprocal of the appropriate dilution.

i.e. No of CFU/g of sludge = $\frac{\text{average number of colony x Initial weight of soil}}{\text{Dilution factor}}$

Plate 5. A representative petri dish showing bacterial isolates.

3.12 Purification of culture isolates

Discrete colonies from each of the isolated microorganisms were picked and sub-cultured onto nutrients agar by streaking to obtain pure cultures. Thus colonies that were single and non-crossed were picked and streaked onto nutrient agar plates, incubated at 37°C for 24 hrs, and stored at 4°C until further use.

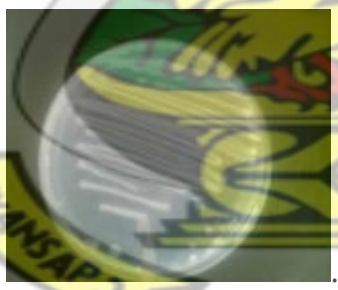

Plate 6. A representative petri dish showing purified bacteria culture.

The purified strains were maintained on nutrients agar slants and stored at 4^{o}C with successive subcultures every two weeks.

3.13 Bacterial identification

The oil-degrading isolates (bacteria) from the representative sampled areas were identified by their morphological characteristics based on the shape, size colony morphology on nutrient agar (NA) Oxid plates and catalase test. All isolates were examined by Gram's staining reaction to differentiate between Gram–positive and Gram–negative bacteria. Physiological characteristics were determined based on battery of biochemical tests set within the API Test kits i.e. (API 20E and API CH 50).

3.14 Identification of colonies by Gram stain technique

Bacterial cells are difficult to observe because they are nearly transparent. However, most bacteria can be stained by dyes to increase the contrast between the cells and the background.

The Gram Stain technique consists of the following steps

- A smear of each colony was prepared on a glass slide and fixed.
- Smear was stained with crystal violet solution for one minute. Then washed off with iodine solution.
- Iodine solution was left on the smear for one minute, then washed with water and drained.
- Smear was decolorized with alcohol (75%) (approximately 30 seconds), and slide was washed with water and drained.
- Smear was flooded with Safranine, then washed and bloated dry by placing the slide between two pieces of Whiteman's paper. The shapes of the cells were then observed through a microscope.

Plate 7. (A) **Gram–positive (N2) Microscopic view (1000x)**

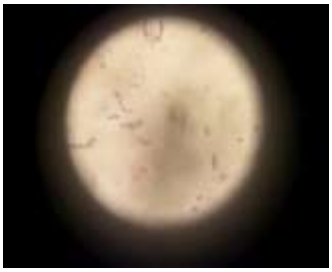

Plate 8. (B) Gram–negative (N1) Microscopic view (1000x)

3.15 Catalase test [Method]

A loopful of cells from each agar culture was mixed on a slide with a drop of 30% hydrogen peroxide. The appearance of bubbles within a time range of 5 sec to 3 mins is indicative of the presence of catalase. The bubbles are O_2 produced by the reaction.

$H_2O_2 + H_2O_2 \rightarrow 2\ H_2O + O_2$.

3.16 Bacterial growth

3.17 Preparation of degradative media

Nitrogen sources such as soy and malted sorghum (pito waste) were used in this study. Each of these nitrogen sources was added to 33 ml of Bergs Mineral Salt Medium (BMSM) (Bergs *et al*., 1990) at a concentration of 0.5% (w/v) at the pH of 10± 0.5. Two percent (2% v/v) of gasoline, diesel and kerosene were each distributed in 100 ml sterilized bottles containing the above BMSM under aseptic conditions. The same experiment was repeated but with no nutrient supplement. One loopful of each isolate (pure culture) was aseptically introduced into each of the medium. A mix culture (consortium) was also introduced; a control was also set up without any microorganism. The set ups (treatments) were kept at room temperature on a rotary shaker (Lab line No 3590), with shaking at 110 rpm for fourteen days. The indices of growth thus the Optical Density (OD_{600nm}) and the floating test were monitored. The (OD) of the culture media was measured spectrophotometrically as described by Rahman *et al*., (2002) at an interval of two days to allow appreciable monitoring of bacteria growth under experimental conditions alongside that of sterile control. The pH values of various culture media were also measured. Uninoculated

control was used to monitor abiotic loss of the petroleum products. Below is the outline of the set up or treatments.

MSM +Gasoline + pure culture

MSM +Gasoline + pito waste +pure culture

MSM +Gasoline + soy residue + pure culture

MSM +Gasoline + soy residue +consortium

Control: MSM +Gasoline (no culture added)

MSM +Diesel + pure culture
MSM + Diesel + pito waste +pure culture
MSM + Diesel + soy residue +pure culture
MSM +Diesel + soy residue +consortium
Control: MSM + Diesel (no culture added)

MSM +Kerosene + culture
MSM + Kerosene + pito waste + culture
MSM + Kerosene + soy residue culture
MSM +Kerosene + soy residue +consortium
Control (without microbes): MSM + Kerosene (no culture added)

3.18 Floating test

8 ml of BMSM were placed in test tubes and autoclaved. The media were supplemented with 2 ml of a mixture of the three petroleum products under aseptic conditions. These were seeded with single and mixed culture. The set up was plugged with cotton wool and placed on a rotary shaker at a speed of 108 rpm for 14 days. The set up was performed in duplicate. The floating ability of each of the microorganisms(s) in the test tube was determined by visual observation of each tube. The set up revealed different floating pattern of the microbes in the test tubes.

3.19 Hydrocarbon biodegradation measurements.

The biodegradation potential of individual isolates were measured. Each isolated microbial strain was subjected to treatment for detailed investigation of hydrocarbons utilization. The growths of microorganisms were monitored regularly by measuring $OD_{(600nm)}$ of the culture media. Additionally, emulsification index (E_{24}) was also determined as a second parameter for evaluating the biodegradation potential of isolated bacteria. They are rapid and easy to be carried out, and do not require specialized equipment. Emulsification is expressed as a percentage of the height of emulsified layer over the total height of liquid column after 24 hrs of growth.

3.20 Quantitative analysis

3.21 Recovery of residual hydrocarbon substrates/losses.

The hydrocarbon substrates (undegraded oil / residual oil) in the BMSM were extracted using the liquid–liquid extraction procedure, using hexane as the extractant. Initially 15ml of hexane was added to the culture media in a 250 ml separating funnel, capped and shaken thoroughly for about 2 minutes to partition the contaminants into the solvent phase. After settling, the mixture in the funnel separated into two phases thus the solvent phase and the aqueous phase. The aqueous phase was drained off into a bottle, while the solvent phase was kept in a sterile bottle capped and stored in a refrigerator until analysis. These steps were repeated for all the samples including the controls. The aqueous phase was used for the emulsification (E_{24}) index.

Plate 9: A diagram illustrating liquid–liquid separation.

3.22 Screening for emulsification stability

3.23 Emulsification (E_{24}) indices.

The E_{24} index of the culture sample was determined as described by (Desai and Banat., 1997). Two percent (v/v) of gasoline, diesel and kerosene mixture were added to same volume of the culture medium from the various treatments respectively in a 15 ml centrifuge tube. The mixture was vortexed for 2 minutes and left to stand for 48 hours. The E_{24} index is determined as the percentage of the emulsified layer (mm) divided by the total height of the liquid column (mm).

$$\text{i.e. } E_{24} \text{ index} = \frac{\text{height of emulsified layer (mm)}}{\text{Total height of the liquid column (mm)}} \times 100$$

3.24 Analysis of extracted hydrocarbon substrates.

The hydrocarbon analysis was performed at the Tema oil Refinery (TOR) quality assurance laboratory. Gas chromatographic device Agilent 6890 gas chromatography (GC), equipped with a flame ionization detector (FID) using an enhance integrator software. The oven temperature was initially set to 30^{o}C and increased at a rate of 6^{o}C per minute to 300^{o}C. The carrier gas, helium was allowed to flow at a constant rate of 1.0 ml/min. Three microliter of the extractable substrates was analyzed on a 30 m polydimethylsiloxane capillary column.

The extracted petroleum products with the hexane solvent are assumed to be 100% according to the percent area report (see appendix). The hexane being lighter and pure comes out first and shows the highest peak. The percent area report values of hexane are subtracted from total petroleum mixture to obtain the percent area report values of the residual hydrocarbons. This is compared with the area percent report values of authentic standards determined initially. The degradation efficiency (D%) of the isolates was calculated using the formula stated below.

$$\mathbf{D\% = \frac{\sum TPHs\ sd - \sum TPHs\ trd}{\sum TPHs\ sd} x\ 100}$$

D%= (sum of area peak values for TPHs of kerosene before (sd)–sum of total area peak values for TPHs of kerosene treated after (trd) / sum of area peak values for TPHs before (sd) X 100

Where sd = standard, trd= treated.

Chapter Four

4.00 RESULTS

4.10 Colony characteristics

Table 3.Colony characteristics

Sample spread	size of colony	Colour of colony	Average colony forming units (cfu/g)	Log_{10} of cfu/g	Shape	G_S	C_t
N1	medium	Cream/white	$1.40x10^7$	7.1	Circular short rods	+	++
E1	medium	White/cream	$7.4x10^6$	6.9	Short rods	+	++
W1	medium	White/cream	$1.20x10^9$	9.1	Rods in chains	_	_
S1	medium	White/cream	$1.80x10^9$	9.3	Long rods	_	_
K1	medium	Cream/white	$7.2x10^8$	8.9	Rods	_	_
N2	medium	Cream/white	$1.25x10^9$	9.18	Short rods in chains	_	_
E2	medium	White/cream	$1.00x10^9$	9.00	Small coliforms (spores)	_	_
W2	medium	White/cream	$1.5x10^9$	9.10	Rods	_	_
S2	medium	White/cream	$1.40x10^9$	9.15	Rods	_	_
K2	medium	Cream/white	$1.00x10^9$	9.00	Short rods	+	+
N3	medium	Yellow	$8.0x10^6$	6.9	Rods in chains	+	++
E3	medium	White/cream	$1.00x10^9$	9.00	Rods	_	_
W3	medium	White/cream	$1.80x10^9$	9.26	Short rods	+	+
S3	medium	Cream/white	$1.28x10^9$	9.11	short rods	_	_
K3	medium	Cream/white	$1.36x10^9$	9.13	Short rods	+	+

Gs= Gram staining and Ct =catalase test. N = Suame–Markro, S= Kwamo, Tafo =E, W= Kaase, K=Kotei
1= gasoline sludge sample, 2= diesel sludge sample and 3=kerosene sludge samples.

4.12 Graphs showing microbial load expressed in Log_{10} of cfu/g (figure 4-6).

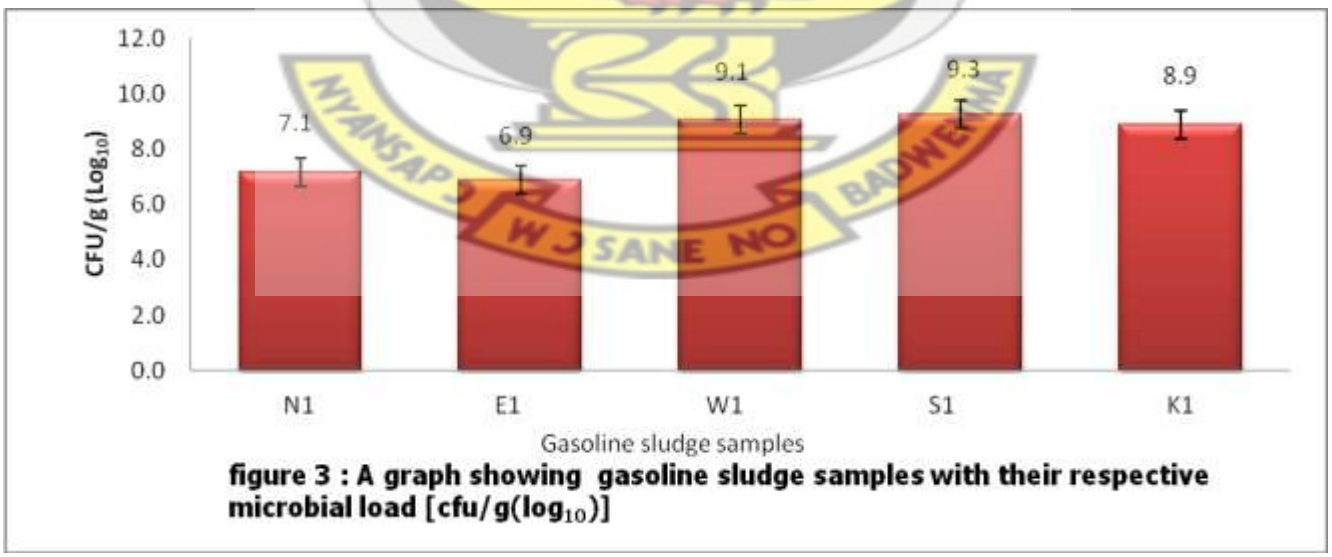

figure 3 : A graph showing gasoline sludge samples with their respective microbial load [cfu/g(log_{10})]

Figure 4: A graph showing diesel sludge samples with their respective microbial load [cfu/g(log_{10})]

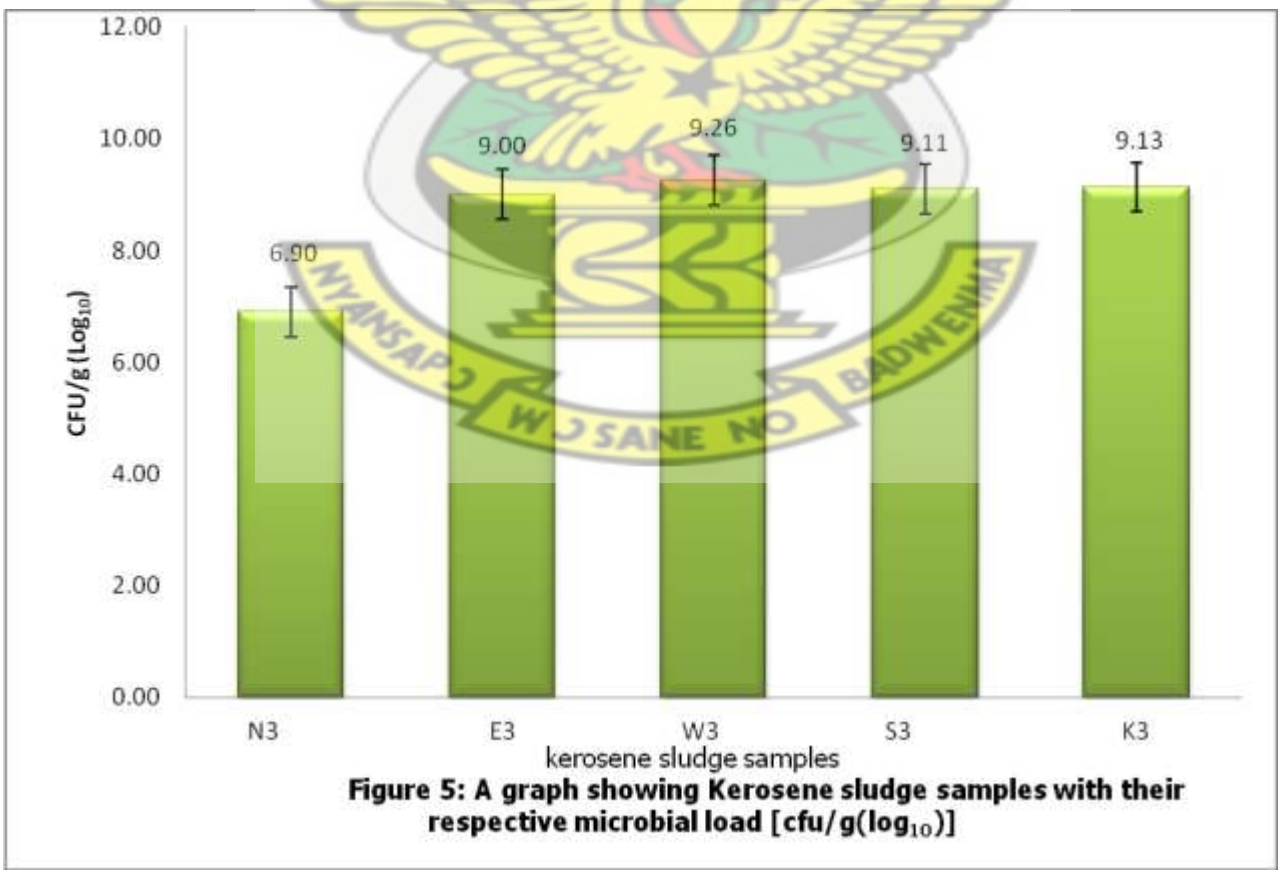

Figure 5: A graph showing Kerosene sludge samples with their respective microbial load [cfu/g(log_{10})]

The average microbial load recorded for sludge samples of gasoline varied as shown figure 3; it was worth noting that statistically there was no significant ($p > 0.05$) differences between samples W1, S1, and K1 however they were significantly different ($p>0.05$) from samples N1[7.1 cfu/g (log_{10})] and E1[6.9cfu/g (log_{10})]. It was observed that petroleum storage facilities and sites which have for a long time and continuously been receiving petroleum products had the highest microbial load which reflected in the microbial load for Kaase (W1) where the Bulk Oil and Storage Transport Company has its depot.

The microbial load in the diesel sludge samples was generally high. Samples N2, W2 and S2, were not significantly different from each other, though the highest value was recorded for N2 (figure 4), whereas E2 and K2 were statistically different even though the same value were recorded at $p >0.05$ as depicted in figure 4. The above difference could be attributed to the fact that the floor of the container where samples E2 and K3 were taken from had been concreted and receives regular flushing of the container.

For kerosene sludge samples there were no significant difference among samples E3, W3, S3 and K3; notwithstanding the fact that W3 recorded the highest microbial load of 9.62 cfu/g (log_{10}) at $p < 0.05$. N3 however was statistically different at p value < 0.05. The above reason could also be assigned to the above results why a lower microbial load was recorded as seen in figure 5.

4.13 Results of microorganism identified using API CH 50 test kit

Table 4. Results of microorganism identified using API CH 50 test kit.

API CH 50 test		
N1	positive rods	*Bacillus firmus*
N3	positive rods	*Bacillus firmus*
E1	positive rods	*Bacillus firmus*
K2	positive rods	*Bacillus firmus*
K3	positive rods	*Bacillus firmus*

4.14 Results of microorganism identified using API 20E test kit

Table 5. Results of microorganism identified using API 20E test kit.

Sample code	API code	Identified organism
N2	2 202 004	*Pseudomonas aeruginosa*
S1	2 213 004	*Pseudomonas aeruginosa*
S2	4 302 004	*Pseudomonas cepacia*
S3	4 302 004	*Pseudomonas cepacia*
E2	2 202 004	*Pseudomonas aeruginosa*
E3	2 212 004	*Pseudomonas aeruginosa*
W1	3 304 573	*Enterobacter cloacae*
W2	3 304 573	*Enterobacter cloacae*
W3	3 305 573	*Enterobacter cloacae*
K1	0 636 000	*Proteus mirabilis*

4.15 Result of microbial distribution in terms of percentage

Table 6. **The percentage of bacteria in gasoline, diesel and kerosene sludge samples from five locations within the Kumasi Metropolitan area.**

ORGANISMS	(1)	(2)	(3)	over all percentage (%)
Bacillus firmus	2	1	2	33%*
Pseudomonas sp.	3	2	_	40%
Enterobacter cloacae	1	1	1	20%
Proteus mirabilis	1	0	0	6.7%*
Total number of sites	5	5	5	100%

(1) = gasoline sludge sample (2) diesel sludge sample (3) kerosene sludge sample

* approximate value(s).

From the sampled locations and the various tests carried out, it came to light that *Bacillus firmus* was associated more with gasoline sludge samples from Suame-Maakro, Tafo and Kotei. *Bacilli* were also found in Kerosene sludge samples from Suame and Kotei. Diesel sludge sample from Kotei had *Bacilli* also present as seen from Table 6.

4.16 Distribution of bacteria in sludge samples from various locations.

From the five sampled locations two out of the five sites for gasoline sludge samples indicated *Bacillus firmus* to be present; accounting for a percentage of 33.3%, while *Pseudomonas* sp. were more associated with diesel and kerosene sludge samples. For diesel three out of the five (60%) sampled sites were found to contain *Pseudomonas* sp. For kerosene sludge sampled sites, *Pseudomonas* and *Bacillus* seemed to co-dominate the sites as each were present in two of the sampled sites. The results of the distribution are presented in Table 6. The highest microbial population was *Pseudomonas* (40%) followed by *Bacillus* (33%), *Enterobacter* (20%) and *Proteus* (6%). These isolates have established themselves as being able to live in various hydrocarbon substrates; however, it was not conclusive that these were the only bacterial isolates that could be present.

4.17 Results of measurement of growth potential: Optical Density (OD_{600mn}) viz –a –viz their pH (Tables 7-15)

Table 7. **Measurement of OD against pH of culture media containing gasoline (2%) supplemented with pito waste over a period of 14 days**

	Days							
	0	2nd	4th	6th	8th	10th	12th	14th
	OD/ pH							
Bacillus firmus	0.00	0.05	0.28	0.45	0.48	0.45	0.40	0.40
	10.0	8.50	8.30	8.10	7.60	7.40	7.00	6.70
Enterobacter cloacae	0.00	0.18	0.40	0.79	0.80	0.80	0.74	0.70
	10.0	8.75	8.60	8.40	7.80	7.60	7.40	6.9 0
Psuedomonas aeruginosa	0.00	0.12	0.30	0.45	0.50	0.80	0.60	0.54
	10.0	8.77	8.40	8.00	7.70	7.30	7.00	6.90
Proteus mirabilis	0.00	0.48	0.38	0.81	0.90	0.84	0.83	0.70
	10.0	8.74	8.30	8.10	7.80	7.20	7.10	6.50
Consortium	0.00	0.2	0.57	0.6	0.75	0.83	0.87	0.86
	10.0	8.84	8.40	7.90	7.30	7.40	7.10	6.85
Control	0.00	0.00	0.00	0.02	0.02	0.04	0.05	0.05
	10.00	10.0	10.0	9.50	9.40	9.40	9.30	9.00

Table 8. **Measurement of OD against pH of culture media containing Diesel (2%) supplemented with pito waste over a period of 14 days**

	Days							
	0	2nd	4th	6th	8th	10th	12th	14th
	OD/ pH							
Psuedomonas aeruginosa	0.00	0.75	0.69	0.64	0.90	0.42	0.24	0.27
	9.80	8.70	8.40	7.90	7.70	7.40	7.00	6.80
Enterobacter cloacae	0.00	0.65	0.75	0.69	0.95	0.42	0.29	0.52
	10.00	8.80	8.20	7.60	7.50	7.20	6.80	6.30
Psuedomonas cepacia	0.00	0.85	0.43	0.75	0.45	0.30	0.15	0.60
	10.00	8.50	8.20	7.90	7.40	7.00	6.70	6.40
Bacillus firmus	0.00	0.90	0.75	0.77	0.93	0.45	0.14	0.07
	9.80	8.75	8.30	8.00	7.80	7.20	7.00	6.80
Consortium	0.00	0.85	0.50	0.60	0.60	0.48	0.28	0.28
	10.0	8.50	8.10	7.85	7.50	7.25	6.95	6.70
Control	0.00	0.00	0.00	0.01	0.00	0.02	0.18	0.20
	0.00	0.00	9.80	9.80	9.80	9.40	9.50	9.00

Table 9. Measurement of OD against pH of culture media containing kerosene (2%) supplemented with pito residue over a period of 14 days.

	Days							
	0	2nd	4th	6th	8th	10th	12th	14th
	OD/ pH							
Psuedomonas aeruginosa	0.20	0.80	0.55	0.55	0.65	0.41	0.41	0.75
	10.00	8.90	8.60	8.10	7.60	7.20	6.75	6.50
Enterobacter cloacae	0.00	0.37	0.12	0.12	0.38	0.37	0.37	0.40
	10.0	8.60	8.20	7.80	7.40	7.00	6.85	6.45
Psuedomonas cepacia	0.02	0.69	0.66	0.66	0.74	0.06	0.06	0.30
	9.90	8.50	8.10	7.60	7.10	6.90	6.70	6.35
Bacillus firmus	0.00	0.44	0.70	0.70	0.88	0.50	0.56	0.60
	10.0	8.70	8.25	7.85	7.50	6.95	6.60	6.30
Consortium	0.21	0.72	0.45	0.54	0.98	0.80	0.60	0.50
	10.0	8.60	8.40	7.90	7.40	7.10	7.00	6.80
Control	0.00	0.00	0.00	0.02	0.02	0.04	0.02	0.05
	10.20	10.00	10.00	10.00	9.85	9.70	9.50	9.50

Table 10.Measurement of OD against pH of culture media containing gasoline (2%) over a period of 14 days.

	Days							
	0	2nd	4th	6th	8th	10th	12th	14th
	OD/ pH							
Bacillus firmus	0.00	0.00	0.00	0.00	0.02	0.06	0.00	0.40
	10.00	8.84	8.60	7.80	7.20	7.00	6.50	6.50
Enterobacter cloacae	0.00	0.00	0.00	0.00	0.02	0.05	0.02	0.06
	10.00	8.42	7.90	7.50	7.20	7.10	6.80	6.50
Pseudomonas aeruginosa	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.20
	10.0	8.70	8.20	7.90	7.30	7.20	6.80	6.40
Proteus mirabilis	0.00	0.00	0.02	0.00	0.01	0.4	0.02	0.05
	10.0	8.85	8.40	8.00	7.80	7.30	6.50	6.50
Consortium	0.00	0.18	0.2 0	0.20	0.03	0.00	0.04	0.10
	10.0	8.85	8.3	8.0	7.70	7.20	6.70	6.40
(Abiotic control)	0.00	0.00	0.00	0.05	0.05	0.02	0.02	0.03
	10.0	10.00	10.00	10.0	9.80	9.80	9.80	9.80

Table 11.Measurement of OD against pH ofculture media containing diesel (2%) over a period of 14 days.

	Days							
	0	2nd	4th	6th	8th	10th	12th	14th
	OD/ pH							
Pseudomonas aeruginosa	0.09	0.40	0.60	0.30	0.30	0.19	0.04	0.02
	10.0	8.70	8.40	7.90	7.00	6.80	6.50	6.30
Enterobacter cloacae	0.05	0.10	0.12	0.14	0.37	0.28	0.20	0.03
	10.0	8.80	8.20	7.80	7.10	7.00	6.8 0	6.50
Pseudomonas cepacia	0.00	0.04	0.05	0.29	0.30	0.25	0.10	0.10
	10.0	8.70	8.00	7.60	6.90	6.80	6.05	6.20
Bacillus firmus	0.02	0.24	0.42	0.24	0.27	0.1	0.12	0.09
	10.0	8.50	8.10	7.80	7.40	6.80	6.90	6.20
Consortium	0.28	0.42	0.46	0.54	0.6	0.5	0.35	0.21
	10.00	8.90	8.20	7.50	7.20	7.00	6.90	6.50
Control	0.00	0.00	0.00	0.02	0.02	0.00	0.01	0.02
	10.00	10.00	10.00	9.80	9.90	9.80	9.80	9.53

Table 12. Measurement of OD against pH of culture media containing kerosene (2%) over a period of 14 days.

Days								
	0	2nd	4th	6th	8th	10th	12th	14th
	OD/ pH							
Pseudomonas aeruginosa	0.00	0.00	0.05	0.02	0.17	0.18	0.21	0.26
	9.80	8.95	8.30	7.80	7.40	6.80	6.50	6.20
Enterobacter cloacae	0.00	0.00	0.01	0.00	0.15	0.16	0.18	0.10
	10.0	8.72	8.20	7.50	7.00	7.00	6.70	6.40
Pseudomonas cepacia	0.00	0.00	0.02	0.20	0.05	0.07	0.12	0.20
	10.0	8.77	8.20	7.60	7.20	6.80	6.40	6.20
Bacillus firmus	0.00	0.00	0.00	0.08	0.11	0.15	0.18	0.28
	10.00	8.80	8.00	7.60	7.00	6.80	6.50	6.10
Consortium	0.00	0.20	0.21	0.16	0.20	0.30	0.36	0.26
	10.00	8.50	7.90	7.40	6.70	7.00	6.30	6.30
Control	0.00	00.00	0.00	0.01	0.01	0.00	0.01	0.00
	10.00	10.00	10.00	10.0	10.00	9.80	9.70	9.00

Table 13: Measurement of OD against pH of culture media containing gasoline (2%) supplemented with soy residue over a period of 14 days.

	Days							
	0	2nd	4th	6th	8th	10th	12th	14th
	OD/ pH							
Bacillus firmus	0.30	0.72	0.35	0.35	0.28	0.60	0.52	0.50
	9.85	8.54	8.20	7.80	7.20	6.30	6.50	6.30
Enterobacter cloacae	0.28	0.88	0.67	0.65	0.35	0.44	0.50	0.45
	10.0	8.50	8.30	7.90	7.20	6.20	6.20	6.20
Psuedomonas aeruginosa	0.02	0.70	0.60	0.54	0.27	0.50	0.57	0.54
	10.00	8.35	8.20	7.20	7.00	6.80	6.30	6.80
Proteus mirabilis	0.25	1.00	0.89	0.50	0.38	0.69	0.48	0.50
	9.95	8.60	8.00	7.40	7.20	6.70	6.50	6.50
Consortium	0.20	0.90	0.78	0.70	0.57	0.60	0.40	0.45
	10.20	8.70	8.20	7.80	7.30	6.90	6.70	6.50
Control	0.00	0.00	0.01	0.01	0.05	0.05	0.05	0.02
	10.20	10.00	10.00	9.95	9.90	9.80	9.80	9.00

Table 14. Measurement of OD against pH of Culture media containing diesel (2%) supplemented with soy residue over a period of 14 days.

	Days							
	0	2nd	4th	6th	8th	10th	12th	14th
	OD/ pH							
Psuedomonas aeruginosa	0.00	0.46	0.60	0.60	0.40	0.43	0.45	0.60
	10.05	8.90	8.10	7.50	6.90	6.70	6.50	6.50
Enterobacter cloacae	0.02	0.50	0.53	0.53	0.50	0.46	0.16	0.30
	10.00	8.50	8.00	7.70	6.80	7.00	6.80	6.60
Psuedomonas cepacia	0.04	0.75	0.77	0.72	0.43	0.3	0.175	0.50
	9.90	8.70	7.30	7.00	6.80	7.10	7.00	6.80
Bacillus firmus	0.10	0.70	0.72	0.68	0.4	0.16	0.2	0.27
	10.0	8.50	7.40	7.10	7.00	6.80	6.50	6.40
Consortium	0.33	1.00	0.80	0.70	0.65	0.35	0.51	0.70
	10.00	8.50	7.70	7.20	7.00	7.00	6.80	6.50
Control	0.00	0.00	0.02	0.00	0.01	0.02	0.02	0.00
	10.00	10.0	10.00	9.85	9.80	9.80	9.70	9.50

Table 15. Measurement of OD against pH of Culture media containing kerosene (2%) supplemented with soy residue over a period of 14 days.

	Days							
	0	2nd	4th	6th	8th	10th	12th	14th
	OD/ pH							
Bacillus firmus	0.05	0.40	0.55	0.60	0.63	0.60	0.54	0.50
	10.0	8.60	8.20	7.70	7.30	7.05	6.80	6.70
Psuedomonas aeruginosa	0.02	0.50	0.38	0.40	0.50	0.53	0.40	0.42
	10.20	8.55	8.00	7.60	7.00	7.00	6.70	6.70
Enterobacter cloacae	0.05	0.46	0.43	0.46	0.44	0.40	0.38	0.30
	10.20	8.50	8.00	7.40	7.10	6.80	6.40	6.30
Psuedomonas cepacia	0.35	0.52	0.51	0.65	0.64	0.58	0.55	0.44
	10.20	8.52	7.90	7.20	6.90	6.70	6.30	6.20
Consortium	0.03	0.20	0.60	0.70	0.64	0.63	0.58	0.54
	9.85	8.75	8.0	7.50	7.10	6.90	6.40	6.40
Control	0.00	0.00	0.01	0.00	0.02	0.02	0.00	0.00
	10.20	10.00	10.00	9.50	9.10	9.00	8.90	8.50

There was a general trend in the pH and OD values across the treatments (Tables 7-15) thus a gradual fall in the pH values with concomitant rise in the $OD_{(600nm)}$ values to some point where they either became constant, decrease or experience some increases. An exception to this trend was observed and recorded for the treatments where no microbes were introduced (control); there were marginal increases in the OD values and very little changes in pH values. This is not unusual according to Mills *et al*., (2003).

4.18 Floating test results

Table 16. Floating test/distribution in test tubes:

Pito waste + BMSM+ microorganism(s)	floating ability
Bacillus firmus	_
Enterobacter cloacae	+++
Psuedomonas aeruginosa	+
Proteus mirabilis	_
Soy + BMSM+ microorganism(s)	
Psuedomonas aeruginosa	_
Enterobacter cloacae	++
Psuedomonas cepacia	+++
Bacillus firmus	_
BMSM + microorganism(s) (no nutrients added)	
Bacillus firmus	_
Psuedomonas aeruginosa	_
Enterobacter cloacae	+ +
Psuedomonas cepacia	_

(-) suspension (+) poor (++) moderate (+++) very good

Enterobacter cloacae in all the test tubes were observed to float (coagulate at the top) in all the treatments. *Bacillus firmus* was generally found to be suspending in the media. The floating behavior of the other species did not follow any pattern as in some episodes they floated while in some treatments they suspended (Table 16).

4.19 Results for emulsification (E_{24}) indices

Table 17. Emulsification (E_{24}) indices

	Percentage of (E_{24})		
	Pito	Soy	no nutrients added
	Gasoline		
Bacillus firmus	1.5/4 x 100 = **37.5%**	1.5/4 x100= **37.5%**	N/S (0%)
Enterobacter cloacae	2.5/4 x 100 = **62.5%**	3/4 x 100 =**75.0%**	N/S (0%)
Psuedomonas aeruginosa	2/4 x 100 = **50.0%**	1.5/4 x 100= **37.5%**	N/S (0%)
Proteus mirabilis	1.5/4 x 100= **37.5%**	1.5/5 x 100=**37.5%**	N/S (0%)
Control	N/S (0%)	N/S(0%)	(0%) N/S
	Diesel		
Psuedomonas aeruginosa	3.5/4 x 100= **87.5%**	2.5/4 x 100 = **62.5%**	N/S (0%)
Enterobacter cloacae	1.5/4x100= **37.5%**	1.5/4 x 100=37.5%	N/S (0%)
Pseudomonas cepacia	1.75/4x100= **44.0%**	1.5/4 x 100=**37.5%**	1.5/4 x 100=**37.5%**
Bacillus firmus	N/S (0%)	1.7/4 x 100= **42.5%**	1.7/4 x 100= **42.5%**
Control	N/S (0%)	1.5/4 x 100 =**37.5**	N/S (0%)
	Kerosene		
Pseudomonas aeruginosa	2.7/4 x 100=**67.5%**	0.3/4 x 100=**75%**	1.7/4 x100=**42.5%**
Enterobacter cloacae	3/4 x 100=**75%**	N/S (0%)	2/4 x 100=**50%**
Pseudomonas cepacia	3.5/4 x 100=**87.5%**	N/S (0%)	2.5/4x100=**62.5%**
Bacillus firmus	2/4 x 100= **50%**	1.7/4 x 100=**42.5**	N/S (0%)
Control	N/S (0%)	N/S (0%)	N/S (0%)

Those less than 0.2/4 was considered as Not significant (N/S) given scored a percentage of zero (0%)

4.20 Gas chromatographic (GC) profiles (figure 3-figure 5)

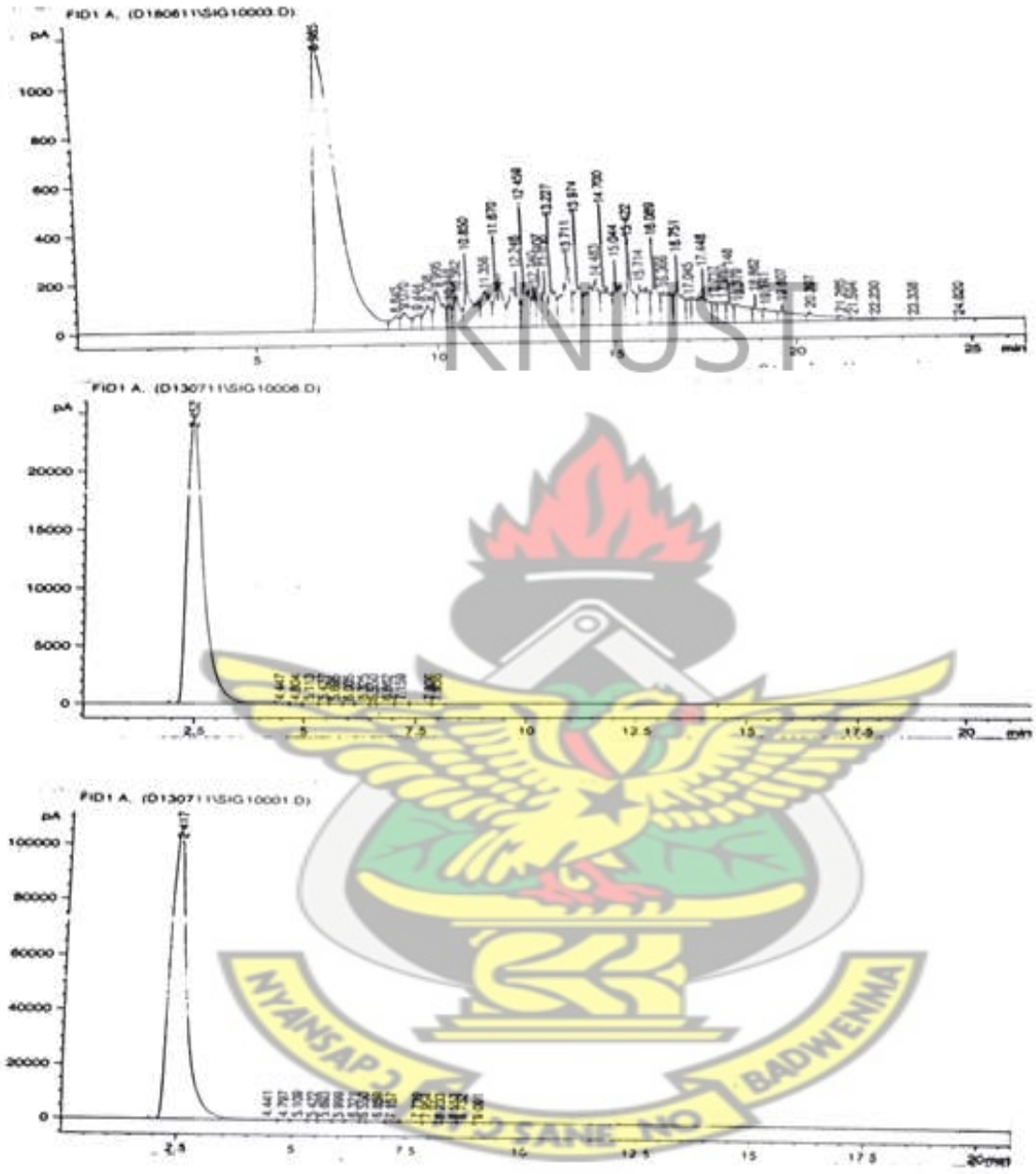

Figure 6. Diesel degradation. GC profile comparing standard (top most), consortium (middle) and *Pseudomonas cepacia (*bottom) with soy added after 14 days period a storage period of one month.

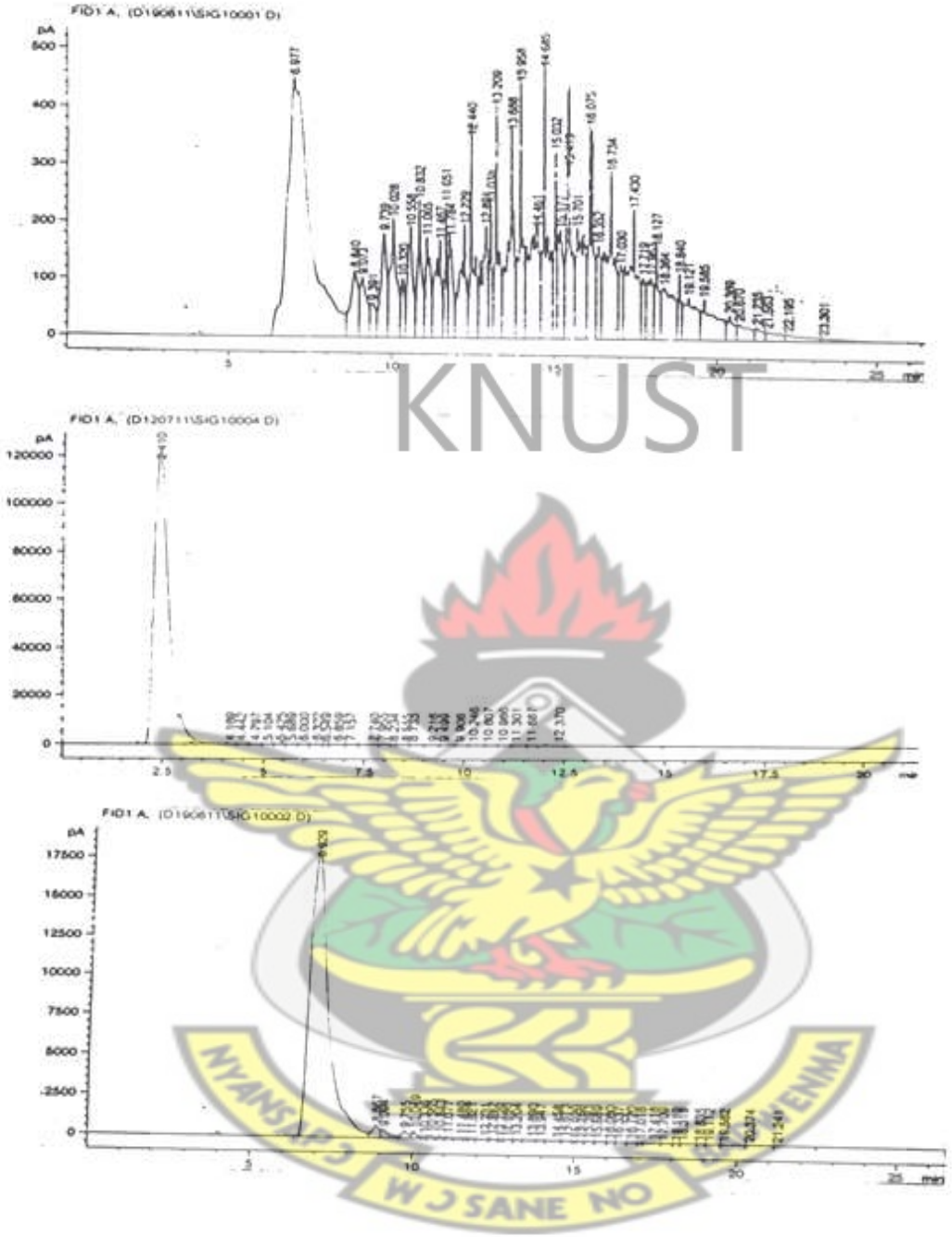

Figure 7. Gasoline degradation. GC profile comparing standard (top most) with *Enterobacter cloacae*–soy added (middle) and control (bottom) after 14 days period a storage period of one month.

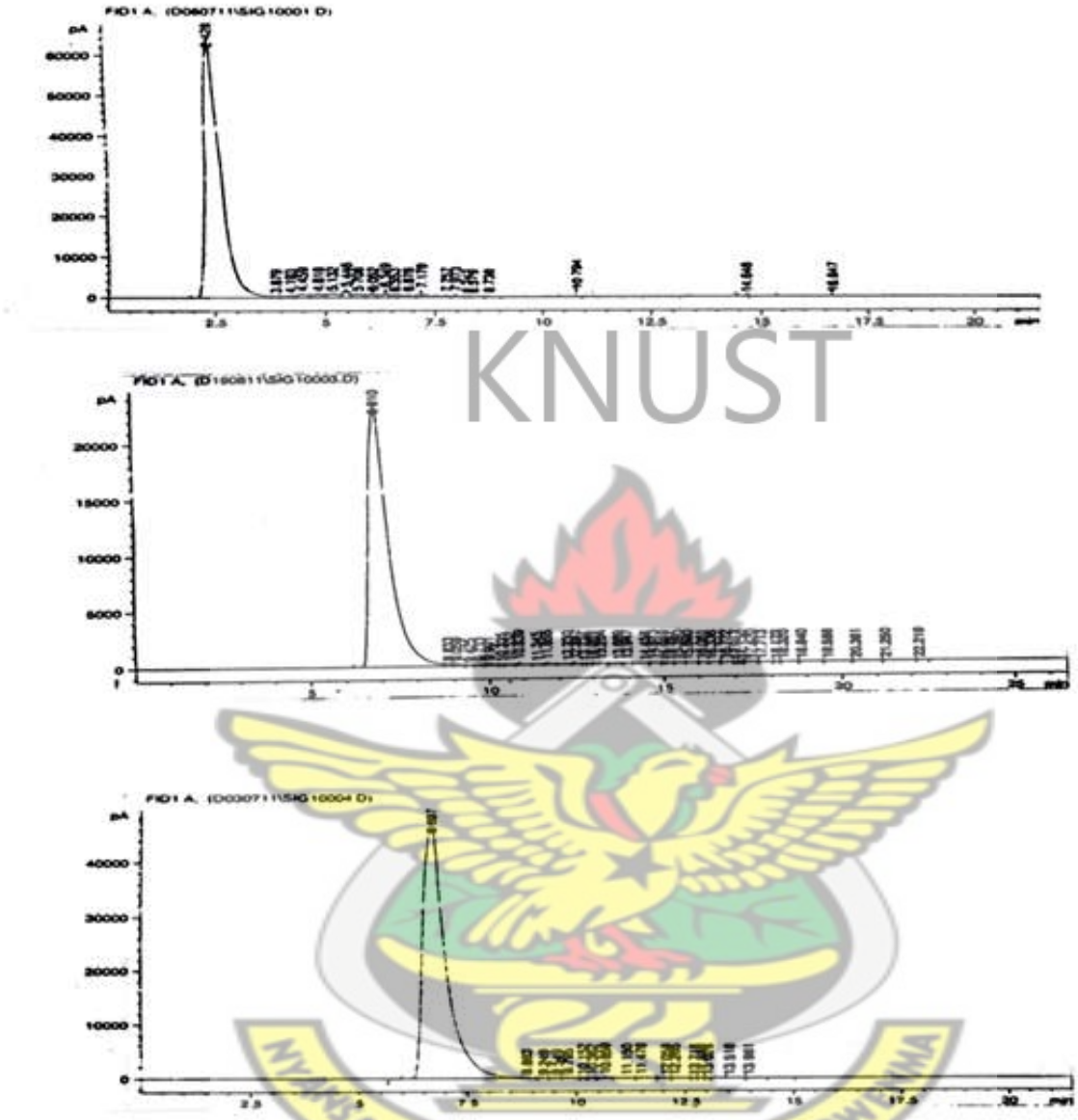

Figure 8. Kerosene degradation: GC profile comparing standard (top most), with control (middle) and *Enterobacter cloacae* (bottom) with soy added after 14 days period and a storage period of one month.

From the Gas chromatographic (Gc) profiles of residual oils left at the end of the 14 day period, reductions in area percent reports of all substrates and their components showed a marked effect of the isolates in utilizing the hydrocarbon substrates.

From the GC profiles, the growth of *Pseudomonas, Enterobacter, Bacillus* and *Proteus* as well as the mixed culture (consortium) in various substrates resulted in a substantial disappearance of the fraction within hydrocarbon substrates as shown (Figure 6-8). The Gram-negative bacteria (*Pseudomonas, Enterobacter* and *Proteus*) gave a relatively higher petroleum-degradation efficiency compared with *Bacillus firmus* (the only Gram-positive bacteria) and the control.

In the degradation of diesel, *Pseudomonas cepacia* (soy added) and consortium (without nutrient addition) achieved the same level of substrate removal of 99.8 % efficiency as seen from Table 19 below.

Gasoline degradation revealed that *Enterobacter cloacae* (soy added) and microbial consortium (*Bacillus firmus, Enterobacter cloacae, Pseudomonas aeruginosa, Proteus mirabilis*) with soy exhibited the highest degradation potential (99.9% and 99.6%, respectively) followed by *Bacillus firmus* (97.2%).

In the degradation of kerosene, *Enterobacter cloacae* exhibited the highest degradation efficiency of 96% (under soy treatment) over a period of 14 days which a little higher than microbial consortium degradation efficiency of (95.6%). The differences in the efficiency of the isolates are very close as seen from Table 20. Relatively, the least successful was the attempt to utilize kerosene with *Bacilli* (soy added) that is 83.3%.

4.21 Degradation efficiencies of bacteria isolates (Table 18-20)

Table 18. A comparison of gasoline degradation efficiencies (%) of bacterial isolates.

Treatment	Microorganisms					Abiotic
Gasoline substrates	*Bacillus firmus*	*Enterobacter cloacae*	*Pseudomonas aeruginosa*	*Proteus mirabilis*	Consortium *(Bacillus firmus, Enterobacter cloacae, Pseudomonas aeruginosa, Proteus mirabilis)*	Control
Raw	**97.2%**	**98.0%**	**97.9%**	**97.7%**	**98.0%**	**49.0%**
Pito	**98.7%**	**97.5%**	**99.2%**	**98.5%**	**99.3%**	
Soy	**98.4%**	**99.6%**	**99.6%**	**99.4%**	**99.8%**	

Table 19. A comparison of diesel degradation efficiencies (%) of bacterial isolates.

Treatment	Microorganisms					Abiotic
Diesel substrates	*Bacillus firmus*	*Enterobacter cloacae*	*Pseudomonas aeruginosa*	*Pseudomonas cepacia*	Consortium *(Bacillus firmus, Enterobacter cloacae, Pseudomonas aeruginosa, Proteus mirabilis)*	Control
Raw	**98.9%**	**99.2%**	**98.4%**	**97.7%**	**99.8%**	–
Pito	**98.8%**	**97.6%**	**93.6%**	–	**99.2%**	
Soy	**99.1%**	**99.6%**	**99.5%**	**99.8%**	**99.5%**	

–= not available (treatment had spilled in the cause of the 14 days period).

Table 20. A comparison of kerosene degradation efficiencies (%) of bacterial isolates.

Treatment	Microorganisms					Abiotic
Diesel substrates	*Bacillus firmus*	*Enterobacter cloacae*	*Pseudomonas aeruginosa*	*Pseudomonas cepacia*	Consortium *(Bacillus firmus, Enterobacter cloacae, Pseudomonas aeruginosa, Proteus mirabilis)*	Control
Raw	**91.0%**	**95.2%**	**95.0%**	**86.0%**	**95.6%**	**32%**
Pito	**95.3%**	**92.5%**	**90.0%**	**92.8**	**86.78%**	
Soy	**83.3%**	**96.0%**	**95.35%**	**92.1%**	**88.3%**	

[Please refer to appendices to see how degradation efficiencies were computed for tables 18, 19 and 20]

Chapter Five

5.0 DISCUSSION

5.1 Microbial enumeration and identification

There was a myriad of microorganisms that thrive in facilities and sites having oil spills as indicated in Table 3 and confirmed in Table 4 and5. Results of cfu/g counts demonstrate that the bacterial populations obtained (Table 3) were relatively high as compared with the minimum value of 10^5 that had been prescribed by Forsyth *et al*., (1995). In our estimation these observation correspond with the statement that these bacterial isolates multiply at an exponential rate and metabolize the hydrocarbon substrates there by reducing the levels dramatically hence the outcome of what were observed in Tables 18-20.

In confirming the biochemical identity of the above microorganisms, the API test kits systems (API CH 50 and API 20 E) sort the microorganisms into Gram-positive and Gram-negatives. Bacteria species identified in the test sludge were *Bacillus firmus* (Gram-postive), *Pseudomonas aeruginosa, Pseudomonas cepacia, Enterobacter cloacae and Proteus mirabilis* all Gram-negative. The identification of the species has been reported by researchers who have undertaken similar work. Ten of the isolates were found to be Gram-negative while five were Gram-positive. Of the ten species, *Pseudomonas aeruginosa, Pseudomonas cepacia, Enterobacter cloacae and Proteus mirabilis* were the members. While the remaining five belonged mainly to one species, *Bacillus firmus*. The dominance (more common) of Gram-negative bacteria especially *Pseudomonas*, at the locations comes as no surprise as it has been documented by several reporters and that Gram-positive bacteria if found in an oil spill environment are rarely dominant (Rahman *et al*., 2002; Oboh *et al*., 2006). This could be attributed to the fact that these bacteria

have a stronger cell envelope and are more tolerant to high levels of hydrocarbons due to their resistant spores than Gram positive bacteria (Zheng, and Obbard, 2002; Ramos *et al*., 2002).

5.2 Floating test

From the test results obtained (Table16), only *Enterobacter cloacae* was able to float in the hydrocarbon mixture under various treatments; exhibiting the chemostatic and aerotaxis response in accordance with what has been stated by Rosenberg and Ron (1996). *Bacilli* were found predominantly suspending in the hydrocarbon mixture. *Pseudomonas* sp. however showed varying response in their ability to float or suspend and thus grew as a mass of cells between the oil and aqueous interface in the test tubes. Interestingly the isolates that were able to float were *Enterobacter* and *Pseudomonas* both Gram-negative whereas those that were found to be suspending were the Gram positive contrary to the findings of Ta-Chen *et al*., (2005). It is not clear whether the Gram-status of a microbe has an influence on its ability to float. However it appears that microorganisms migrate towards where they would obtain nourishment and or favorable environmental condition(s). In principle cell with lower densities thus float as against those that have higher densities. Other possible reason could be that *Enterobacter* and *Pseudomonas* secretes a bioemulsifyer called an extracellular polymeric thus endopolysaccharides (EPs) biofilms enabling cells to float. By nature EPs biofilms are known to facilitate the contact between cells and oil substrates that is cells in proximity adhere to each other forming flocculates which easily enable them to float. (Wolfaardt *et al*., 1998; Fusconi and Godinho, 2002; Ta-Chen *et al*., 2005). EPs allow cells to stick to each other forming flocculates which enable them to float easily. Perhaps it is no surprise that *Enterobacter* and *Pseudomonas*

sp. were able to utilize the oil substrate which was found mostly at the top of the BMSM than *Bacillus firmus* which were found predominantly suspending.

5.3 Analysis of OD $_{(600\ nm)}$ and pH values.

The pH and OD values for the treatment over the 14days of investigation revealed that the top degraders(s) thus consortium and *Enterobacter* recorded optimum pH and OD values which were within a range of 8.0–5.0 and 0.5–0.9 respectively required for effective bioremediation (Atlas 1981; Song and Bartha 1990, Nilanjana and Preethy, 2011). Most heterotrophic bacteria thrive in a near pH. Exceptions to this were slight variations in pH and OD values over certain episodes of treatments (abiotic control). Therefore, pH and optical density of the microbial cultures were not limiting factors in this study as all bacterial isolates acquitted themselves as being able to grow (increase in OD_{600nm}) thus lowering the pH of the medium in which they grew. The utilization of the hydrocarbon substrates (gasoline, diesel and kerosene) by isolates was evident by the increase in cellular optical density of the culture. The results showed maximal increase in OD values with fall in pH values across all the results (Tables 7-15) except for the control which experienced marginal decreases. The pH and OD values for treatments that were supplemented with nutrients (soy and pito waste) were enhanced as a result of the nutrients that were provided which enabled them to grow and quickly utilize the carbon substrates with a resultant production of organic acids and other metabolites which lowed the pH of the media (Tables 7-15) (Nwachukwu and Ugoji, 1995; Okpokwasilis and James, 1995).

5.4 Emulsification (E_{24}) indices.

Incorporation of different hydrocarbon (gasoline, diesel and kerosene) culture extracts showed an appreciable emulsion after 24 hours except the uninoculated tubes. Those that were not amended with nutrients and some isolates did not also show any sign of emulsification. Varying percentages of (E_{24}) indices were achieved by various isolates but *Enterobacter cloacae* gave the highest E_{24} indices of 75% and 62.5% with addition of soy and pito waste respectively, for gasoline.

The (E_{24}) indices were 87.5%, and 62.5**%** for *Pseudomonas aeruginosa* with diesel under pito and soy residue amendment, respectively. The (E_{24}) indices of the isolates in MSM having kerosene as carbon source were 87.5% for *Pseudomonas cepacia* (pito amended), 75% for both *Enterobacter cloacae* and *Pseudomonas aeruginosa* under pito and soy amendment respectively. Emulsification indices or stability of emulsions were high and showed no patterns of relatedness except for the influence of the media from which the isolates grew i.e. whether it was nutrient amended or not. Isolates that were cultured with pito and soy supplements produced surfactants and it seem that the nutrients influenced (E_{24}) indices to an appreciable levels thus increased surfactant activities of the isolates as shown in Table 17. This result is similar to a study by Monteiro *et al.,* (2006) who recorded an emulsification index of 70% after 30 days of incubating *Psuedomonas aeruginosa* which produced emulsions that were stable and could be used in the control of environmental contamination Monteiro *et al*., (2006).

5.5 Hydrocarbon degradation analysis /GC analysis

Gas chromatography profiles of the inoculated and uninoculated hydrocarbons at the end of the degradation period revealed a reduction in the percent area report of the inoculated hydrocarbons as compared to the uninoculated control refer to (appendices). The incidence of disappearing peaks (percent area report values) may be due to the fact that isolates had an ample supply of their required energy sources, and nutrients. The experiments showed hydrocarbon degrading potential of the isolates similar to what has been made by Bento *et al*., (2005) that biodegradation of petroleum hydrocarbon depends on the specific microbial population present. All three hydrocarbons were degraded by microbes at a relatively faster rate, compared with Adebusoye *et al*., (2006), and Attia *et al.,* (2009). These results indicate that hydrocarbon biodegradation can proceed in the presence of these microbes.

In this study, all the isolate exhibited the ability to degrade hydrocarbon substrates to varying degrees with most of them having an efficiency of over 90%. It is worth noting as the figures suggest that *Pseudomonas* and *Enterobacter* were at par with respect to their efficiency at degrading hydrocarbons. The rate of degradation and or loss of the substrates in the media were relatively fast almost 100% as shown in Tables 18-20 above.

From Tables 18-20, the optimum degradation efficiency was that of gasoline, this was achieved by *Enterobacter cloacae* (99.66%), whereas the mix culture and other isolates achieved a rate quite close to the degradation efficiency of *Enterobacter* over the same period of time. All isolates showed high degrees of degradation under nutrient supplementations which were remarkable but the Gram-positive were relatively consistent in their efficiency in all the culture media supplemented with gasoline, diesel and kerosene. These bacteria have a stronger cell envelope and are more tolerant to high levels of hydrocarbons due to their resistant spores, than

Gram-negative bacteria which allow them to thrive in the highly variable hydrocarbon contaminated environment (Zheng and Obbard, 2002).

The observation that the gasoline and diesel were the most degraded hydrocarbon compounds indicated that these were probably the most preferred substrates by the microbial consortia and the individual microbe(s) that carried out the metabolic process (Figures 3-5). With the addition of the nutrients (agro-residues) i.e. soy and pito waste their metabolic ability were enhanced, but the pattern of relatedness was not very clear as some of the treatments indicated that pito waste addition was not better. There were also few episodes where those treatments that were not supplemented with nutrients did better than those supplemented with nutrients (Table 18-20). Generally, addition of pito and soy residues as nutrient sources had a significant impact on degradation abilities of the isolates. The combination of soy residue and consortium achieved the highest degradation efficiency of 99.8% for gasoline substrate followed closely by *Enterobacter cloacae* and *Pseudomonas aeruginosa* both having 99.6%, *Proteus mirabilis* 99.4%, and *Bacillus firmus* 98.4%.

For diesel degradation, microbial consortium achieved the highest degradation efficiency of 99.8% (no nutrients added) this value was achieved by *Pseudomonas cepacia* with the addition of soy residue (Table 19).

For kerosene degradation profile a combination of soy and *Enterobacter cloacae* gave the highest degradation efficiency of 96.0% followed by consortium (no nutrient addtion), *Enterobacter cloacae* (raw), and *Pseudomonas aeruginosa* (soy added).

The results in Table 18-20 also revealed that the consortia did not achieve the highest level of degradation for the gasoline, diesel and kerosene. In all cases degradation was consistent with what has been reported by Ausma *et al*., (2002) and this could be attributed to the assumption

that the different microorganism might have acted antagonistically as reported by Okpokwasili and James (1995) and also competition for nutrients or unfavorable change in pH. Relatively the least successful was the episode where consortium (soy added) utilized less amount of the kerosene (Table 20); this could also be ascribed to the reason stated above.

Recapping and summing up the above outcomes, the soy residue stimulated the hydrocarbon degradation abilities of all isolates better. The use of pure cultures in this study, alongside the mix culture (*Bacillus firmus, Enterobacter, Pseudomonas* sp. and *Proteus mirabilis*) provides practical advantages by eliminating the ambiguity associated with the treatments. From the study it was evident that *Enterobacter cloacae* and consortium were most effective in utilizing the substances. Many literature states that in a mixed culture system; the growth of the organism cannot be regulated because of nutrient stress and competition (Okpokwasilli and James 1995). This could be one of the many possible reasons why in some of the treatments the single culture tended to be superior to the consortium with respect to their degradative and mineralization profiles. From the research it seems that the pure culture alone or in a mix culture can make use of most hydrocarbon fuel substances as expressed tentatively by Venkateswaran *et al*., (1995). Although mixed cultures did not give the highest proportion of degradation efficiency in all the treatments evidence of the cooperation of mixed cultures in dealing with hydrocarbons contamination is still relevant as been reported elsewhere by Bounchan *et al*., (2000). Additionally, abiotic losses due to evaporation of low molecular hydrocarbons (aromatic compounds in the substrates) and photo-oxidation may have played a major role in reducing the levels of the oils in the culture media support as has been documented by Mills *et al*., (2003). Survival of microorganisms in petroleum hydrocarbon media during degradation period was a key factor in the rate of biodegradation of hydrocarbons in substrates Ramos *et al*., (1991). Since all the bacteria in the present study were isolated from petroleum sludge, they survived and adopted the

oil substrates easily as report elsewhere by Sugiura *et al*., (1997). This was evident from the significant increase in $OD_{(600nm)}$ values viz-a-viz a decline in pH values, floating test, and $E_{(24)}$ indices in all cultures as compared to control. The supplementation of soy and pito waste impacted on the degradation efficiency compared to the control (no microbes added).

Chapter Six

6.0 **CONCLUSION**

The results from this empirical work affirms the increasing awareness that bioremediation as a means of dealing with oil spill or contamination is real and practicable. The microbes identified and used in this study fall into two groups mainly Gram-positive (*Bacillus firmus*) and Gram-negative (*Enterobacter cloacae, Pseudomonas aeruginosa, Pseudomonas cepacia and Proteus mirabilis*). There was statistical differences ($p>0.05$) among the microbial load with respect to all the hydrocarbon sludges. The bacterial population ranged from higher value of 9.26 cfu/g (log_{10}) to a low value of 6.9 cfu/g (log_{10}) for *Enterobacter cloacae* (W3) and *Bacilli* (E1) respectively. It was reckoned that old petroleum storage facilities abound in greater proportion of the microbes vis-à-vis new petroleum storage facilities as reflected at Kaase (W3) and Tafo (E1) respectively. The GC percent area report indicates that all five species showed a remarkable degradation of the petroleum products used and more so the nutrients that were added enhanced the biodegradation. From the study there was no consensus on how best to optimize nutrient addition because in some episodes with no nutrients did better. The growth and survival of the microbes was an indication that wherever oil spill occurs that are native to the environment developed metabolic capabilities to be able to utilize the hydrocarbon substrates. The microbes move in the medium either by floating or suspending in the medium while making use of the contaminant. They make use of the pollutants by secreting bioemulsions that facilitates the dissolution of the hydrocarbons. Some bacteria are mobile and exhibit chemotactic response that is they sense the contaminant and moving towards it.

6.1 **Recommendations**:

Bioremediation processes are intriguing and many opportunities exist to further elucidate the application of our bacterial isolates on industrial scale. Characterization of bacteria nutrition and oxygen, pH among others are not fully understood as pattern of relatedness seem to somehow defy our understanding to some extent.

- There should be periodic sampling of water bodies and tap water in industrial areas to ensure that there are no xenobiotics in the water being consumed by people living close to those areas.
- Culture collections of these microbes having the potential to metabolize petroleum should be collected and by research institutions, universities and government agencies in Ghana since spills are inevitable.
- In the future studies; efforts should to be directed at factors affecting the ability and efficiency of petroleum hydrocarbon degradation, such as type of nutrients, their concentrations, oxygen content among others by process control and optimization.
- There should be more collaboration between universities, companies, non-governmental organizations (NGOs) and government agencies to ensure that research findings become more meaningful to the development of the country.

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Appendices (Table 21-23)

Calculation on degradation profiles of bacteria isolates.

Comparison of gasoline Degradation efficiency (D %) of bacteria isolates.

Control (abiotic) D%

$$\frac{(79.97-40.773)}{79.97} \times 100$$

$$=\frac{39.2}{79.97} \times 100$$

$$=\mathbf{49.0}\%$$

Unamended (no nutrients added)	pito waste added	soy amended
Bacillus firmus **D%** $\frac{(79.97-2.21)}{79.97} \times 100$ $=\frac{77.76}{79.97} \times 100$ **=97.2%**	*Bacillus firmus* **D%** $\frac{(79.97-0.965)}{79.97} \times 100$ $=\frac{79.0}{79.97} \times 100$ **=98.7%**	*Bacillus firmus* **D%** $\frac{(79.97-0.644)}{79.97} \times 100$ $=\frac{77.676}{79.97} \times 100$ **=98.4%**
Enterobacter cloacae D% $\frac{(79.97-1.53)}{79.97} \times 100$ $=\frac{78.44}{79.97} \times 100$ **=98.0%**	*Enterobacter cloacae* D% $\frac{(79.97-1.93)}{79.97} \times 100$ $=\frac{78.04}{79.97} \times 100$ **=97.5%**	*Enterobacter cloacae* D% $\frac{(79.97-0.27)}{79.97} \times 100$ $=\frac{79.7}{79.97} \times 100$ **=99.66%**
Pseudomonas aeruginosa D% $\frac{(79.97-1.94)}{79.97} \times 100$ $=\frac{78.3}{79.97} \times 100$ **=97.90%**	*Pseudomonas aeruginosa* D% $\frac{(79.97-0.566)}{79.97} \times 100$ $=\frac{79.404}{79.97} \times 100$ **=99.2%**	*Pseudomonas aeruginosa* D% $\frac{(79.97-0.503)}{79.97} \times 100$ $=\frac{79.467}{79.97} \times 100$ **=99.37%**
Proteus Mirabilis D% $\frac{(79.97-1.78)}{79.97} \times 100$ $=\frac{78.19}{79.97} \times 100$ **=97.7%**	*Proteus Mirabilis* **D%** $\frac{(79.97-1.17)}{79.97} \times 100$ $=\frac{78.8}{79.97} \times 100$ **=98.5%**	*Proteus Mirabilis* D% $\frac{(79.97-0.434)}{79.97} \times 100$ $=\frac{79.53}{79.97} \times 100$ **=99.4%**
Consortium (mixed culture) D% $\frac{(79.97-0.644)}{79.97} \times 100$ $=\frac{78.6}{79.97} \times 100$ **=98.4%**	Consortium (mixed culture) D% $\frac{(79.97-0.644)}{79.97} \times 100$ $=\frac{79.45}{79.97} \times 100$ **=99.3%**	Consortium (mixed culture) D% $\frac{(79.97-0.644)}{79.97} \times 100$ $=\frac{79.64}{79.97} \times 100$ **=99.6%**

Comparison of diesel degradation efficiency (D %)of bacteria isolates

Comparison of gasoline Degradation efficiency (D %) of bacteria isolates.

Control (abiotic) D% =not available due to loss

Unamended (nonutrients added)	pito waste added	soy amended
Bacillus firmus **D%** (61.8-0.7) X100 61.8 =61.1 X 100 61.8 **=98.9%**	*Bacillus firmus* **D%** (61.8-0.7) X100 61.8 =61.1 X 100 61.8 **=98.9%**	*Bacillus firmus* **D%** (61.8-0.7) X100 61.8 =61.1 X 100 61.8 **=98.9%**
Enterobacter cloacae D% (61.8-0.508) X100 61.8 =61.259 X 100 61.8 **=99.2%**	*Enterobacter cloacae* D% (61.8-1.444) X100 61.8 =60.356 X 100 61.8 **=97.6%**	*Enterobacter cloacae* D% (61.8-0.23) X100 61.8 =61.57 X 100 61.8 **=99.6%**
Pseudomonas aeruginosa D% (61.8-1.014) X100 61.8 =60.786 X 100 61.8 **=98.4%**	*Pseudomonas aeruginosa* D% (61.8-3.9) X100 61.8 =57.9 X 100 61.8 **=93.6%**	*Pseudomonas aeruginosa* D% (61.8-0.327) X100 61.47 =61.1 X 100 61.8 **=99.5%**
*Pseudomonas cepacia*D% (61.8-1.421) X100 61.8 =60.379 X 100 61.8 **=97.7%**	*Pseudomonas cepacia*D% Not available	*Pseudomonas cepacia*D% (61.8-0.112) X100 61.8 =61.689 X 100 61.8 **=99.8%**
Consortium (mixed culture) D% =(61.8-0.127) X100 61.8 =61.673 X 100 61.8 **=99.8%**	Consortium (mixed culture) D% =(61.8-0.5024) X100 61.8 =61.2977 X 100 61.8 **=99.2%**	Consortium (mixed culture) D% =(61.8-0.3379) X100 61.8 =61.46 X 100 61.8 **=99.5%**

Comparison of diesel degradation efficiency (D %)of bacteria isolates

Comparison of gasoline Degradation efficiency (D %) of bacteria isolates.

Control D%

$=\frac{(9.57-6.43)}{9.57} \times 100$

$=\frac{3.14}{9.57} \times 100$

=32.8%

Unamended (no nutrients added) pito waste added soy amended

Bacillus firmus **D%**	*Bacillus firmus* **D%**	*Bacillus firmus* **D%**
$=\frac{(9.57-0.85)}{9.57} \times 100$ $=\frac{8.72}{9.57} \times 100$ **=91.0%**	$=\frac{(9.57-0.453)}{9.57} \times 100$ $=\frac{9.117}{9.57} \times 100$ **= 95.3%**	$=\frac{(9.57-1.5899)}{9.57} \times 100$ $=\frac{7.98}{9.57} \times 100$ **=83.3%**
Enterobacter cloacae D% $=\frac{(9.57-0.43)}{9.57} \times 100$ $=\frac{9.14}{9.57} \times 100$ **= 95.5%**	*Enterobacter cloacae* D% $=\frac{(9.57-0.742)}{9.57} \times 100$ $=\frac{8.828}{9.57} \times 100$ **= 92.5%**	*Enterobacter cloacae* D% $=\frac{(9.57-0.37)}{9.57} \times 100$ $=\frac{9.20}{9.57} \times 100$ **= 96.0%**
Pseudomonas aeruginosa D% $=\frac{(9.57-0.46)}{9.57} \times 100$ $=\frac{9.11}{9.57} \times 100$ **= 95.0%**	*Pseudomonas aeruginosa* D% $=\frac{(9.57-0.9)}{9.57} \times 100$ $=\frac{8.67}{9.57} \times 100$ **= 90.6%**	*Pseudomonas aeruginosa* D% $=\frac{(9.57-0.445)}{9.57} \times 100$ $=\frac{9.125}{9.57} \times 100$ **= 95.35%**
Pseudomonas cepacia D% $=\frac{(9.57-1.35)}{9.57} \times 100$ $=\frac{8.22}{9.57} \times 100$ **= 86.0%**	*Pseudomonas cepacia* D% $=\frac{(9.57-0.6845)}{9.57} \times 100$ $=\frac{8.886}{9.57} \times 100$ **= 92.8%**	*Pseudomonas cepacia* D% $=\frac{(9.57-0.758)}{9.57} \times 100$ $=\frac{8.812}{9.57} \times 100$ **= 92.10%**
Consortium(mixed culture) D% $=\frac{(9.57-0.422)}{9.57} \times 100$ $=\frac{9.1418}{9.57} \times 100$ **= 95.6%**	Consortium(mixed culture) D% $=\frac{(9.57-1.265)}{9.57} \times 100$ $=\frac{8.305}{9.57} \times 100$ **= 86.78%**	Consortium (mixed culture) D% $=\frac{(9.57-1.12)}{9.57} \times 100$ $=\frac{8.45}{9.57} \times 100$ **= 88.3%**