UNIVERSITY OF NAIROBI

SCREENING, ISOLATION AND CHARACTERIZATION OF HYDROCARBONOCLASTIC BACTERIA FROM OIL CONTAMINATED SOILS

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DECLARATION

This is my original work and it has not been presented for award of a degree in any other University.

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DEDICATION

I dedicate this work to the science fraternity and my family.

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LIST OF ABBREVIATIONS

AlkB	Alkane hydroxylase/ monooxygenase
ANOVA	Analysis of variance
ASS/MAS	Alkylsuccinate Synthase/ Methyl-alkyl Succinate Synthase
BHM/BHA	Bushnell Haas Media/ Bushnell Haas Agar
BLAST	Basic Local Alignment Search Tool
BTEX	Benzene, Toluene, Ethylbenzene and Xylene
CoA	Coenzyme A
CYP	Cytochrome P
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
EPA	Environmental Protection Agency
EtBr	Ethidium Bromide
EXDO	Extradiol dioxygenases
FPM	Flavoprotein monooxygenase
GC-MS	Gas Chromatography–Mass Spectrometry
KPA	Kenya Ports Authority
KPC	Kenya Pipeline Company
LB	Luria Bertani
MCMC	Markov Chain Monte Carlo
NCBI	National Center for Biotechnology Information
NCP	National Contingency Plan
NEMA	National Environmental Management Authority
NOPC	National Oil Pollution Committee
N-P-K	Nitrogen-Phosphorus-Potassium
PAH	Polycyclic Aromatic Hydrocarbon
PCR	Polymerase Chain Reaction
POPs	Persistent Organic Pollutants
RNHO	Rieske Non-heme Iron Oxygenase
SDM	Soluble Diiron multicomponent Monooxygenase

SDS	Sodium Dodecyl Sulfate
SPSS	Statistical Package for Social Sciences
TAE	Tris-Acetate-EDTA
TE	Tris EDTA
TPH	Total Petroleum Hydrocarbon

ABSTRACT

Petroleum and its products continue to serve as a principle source of energy for industries and daily life. However, their release into the environment is a worldwide concern since some products are acutely toxic or possess mutagenic, teratogenic and carcinogenic properties. Several oil disposal methods have been applied over time with bioremediation emerging as the most promising technology. It takes advantage of the versatility of soil microbes to degrade hydrocarbon contaminants. Unlike conventional disposal methods, bioremediation is an environmentally friendly and cost effective method that simulates natural processes for complete degradation of hydrocarbons into innocuous compounds. This study focused on isolation, morphological and biochemical characterization as well as molecular identification of bacteria possessing hydrocarbon-degrading properties. The study also aimed at optimizing appropriate culture conditions for the isolates as well as screening for alkane hydroxylase enzyme. Isolation of hydrocarbon degrading microbes from soils polluted with used motor oil around Ngara, Nairobi-Kenya was carried out using Bushnell Haas media supplemented with used engine oil. The isolates were screened for ability to utilize heating oil, hexane, octane, toluene and diesel oil hydrocarbons. Characterization of the isolates was carried out by performing Gram's iodine and potassium hydroxide as well as starch, catalase and carbohydrate fermentation tests. The isolates were also identified through PCR amplification and sequencing of 16S rDNA gene and comparison of obtained sequences with those retrieved from Genbank database. Optimization of culture conditions of three efficient degraders was performed using diesel oil and cellular growth monitored through biomass determination. Hydrocarbon analysis was performed using GC-MS following culturing in diesel oil. Alkane hydroxylase (alkB) gene was amplified using alk-3F and alk-3R primer pair. Among 21 microbes isolated, nine were selected based on their ability to utilize the hydrocarbons and characterized. The isolates were observed to mineralize heating oil, hexane, octane and toluene as well as diesel oil. PCR amplification of 16S rDNA gene revealed that the nine isolates belong to six different genera; *Pseudomonas, Acinetobacter, Klebsiella, Enterobacter, Salmonella* and *Ochrobactrum*. Based on their ability to degrade the hydrocarbons, three isolates were selected and their growth conditions optimized. Optimum degradation of diesel oil was recorded at <1 % substrate concentration, pH 7, temperature of 37 ^{0}C and using yeast extract as a nitrogen source. GC-MS analysis of diesel oil degradation demonstrated that the isolates were capable of readily degrading linear, branched, cyclic and isoprenoid alkanes as well as aromatic hydrocarbons with fatty acids, aldehydes and alcohols produced as intermediate metabolites. Isolate 1C was identified as the most efficient hydrocarbon degrader based on utilization of the different hydrocarbons tested. Its alkane hydroxylase gene was successfully amplified indicating the isolate's potential catabolic capability in degrading alkanes. Overall, the characterized bacterial isolates may constitute potential candidates for biotechnological application in environmental cleanup of petroleum contaminants.

CHAPTER ONE

1.0 INTRODUCTION

Petroleum-based products are a principle source of energy for industries and daily life, making them a vital commodity central to the global economy (Jahangeer & Kumar, 2013). These products include; petrol, gasoline, kerosene, diesel oil, lubricating oil among others. They originate from crude oil whose main constituents are hydrocarbon compounds (Harayama *et al.,* 1999) derived from ancient algae and plant remains found in reservoirs under the earth's surface. Petroleum products are divided into four classes: saturates, aromatics, resins and asphaltenes (Tebyanian *et al.,* 2013).

Accidental release of petroleum products occur regularly during exploration, production, refining, transportation, utilization and storage (Das & Chandran, 2010). For instance, it is estimated that globally, approximately 1.7-8.8 million metric tons of petroleum hydrocarbons are released into marine ecosystems annually (Zhu *et al.*, 2001). Water and soil pollution is a worldwide environmental problem that is of particular concern since it leads to uptake and accumulation of toxic substances including petroleum products in food chains consequently harming the flora and fauna (Rockne & Reddy, 2003).

Current conventional disposal methods of petroleum products include physicochemical techniques such as photo-oxidation, burying, dispersion, washing, incineration, thermal conversion and other pyrolysis techniques (Zhu *et al.,* 2001; Lam & Chase, 2012). Many of these methods are expensive and can result in incomplete decomposition of oil products. In addition, physicochemical methods such as volatization, photo-oxidation and chemical oxidation are rarely successful in rapid removal of hydrocarbon contaminants especially the aromatics (Hu *et al.,* 2013).

Bioremediation is emerging as one of the most promising technology for environmental removal of petroleum contaminants (Jahangeer & Kumar, 2013). It is a process through which microorganisms metabolize contaminants through oxidative/reductive processes. Bioremediation can be performed either through addition of oil degrading microbes into the soil in a process referred to as bio-augmentation or through provision of appropriate conditions and/or amendments (e.g. supplying oxygen, moisture and nutrients) for growth of the microorganisms, a process known as bio-stimulation (Das & Chandran, 2010).

Studies have shown that petroleum-based products can primarily be eliminated from the environment by hydrocarbonoclastic microbes such as bacteria, yeast, fungi and microalgae (Jahangeer & Kumar, 2013). Bacteria however play a major role in biodegradation of these hydrocarbon compounds. Some important microbial species with this potential are of the genera *Bacillus, Arthrobacter, Halononas, Pseudomonas, Klebsiella, Proteus* among others (Uzoamaka *et al.,* 2009). These microbes completely degrade or mineralize petroleum compounds into non-toxic end products that include carbon dioxide, water or organic acids and methane (Rockne & Reddy, 2003). Bioremediation is an effective technique that takes advantage of the versatility of microbes to completely degrade petroleum compounds into innocuous end products. Apart from being environmentally friendly, the method is also cost effective for treatment of oil pollution compared to physicochemical methods (Geetha *et al.,* 2013).

Although numerous studies have been conducted on microbial species capable of cleaning up petroleum contaminants around the world, there is no published work relating to potential oil degrading microbes in Kenya. Therefore, the objective of this study was to isolate, screen, characterize using morphological, biochemical and molecular methods and optimize appropriate culture conditions for oil degrading microbes that would be best suited to degrade petroleum-based contaminants in Kenya.

1.1 Statement of the research problem

As Kenya ventures into petroleum mining, efficient disposal methods must be thought out. The country's initial oil production is expected to be 2,000 barrels per day with Turkana oil reserves currently standing at 750 million barrels. With the ongoing oil exploration, accidental spills are likely to occur during drilling or transportation. In the recent years, tankers meant to transport petroleum oil within the country and to neighboring land-locked countries have been reported to cause major oil spills with subsequent fire accidents. Bursting of oil pipelines, leaking storage tanks and the consequential release of oil into soil, drainages and underground water is also a major environmental concern. Additionally, owing to the unregulated disposal of petroleum wastes, automobile garages often dispose waste oil indiscriminately on open grounds and this constitutes potential risk to human and animal health as well as soil and vegetation.

Current conventional disposal methods such as incineration, thermal conversion, landfilling and pyrolysis techniques (Lam & Chase, 2012) are expensive and can result in incomplete decomposition of the contaminants. With bioremediation, complete degradation of contaminants can be achieved via bio-stimulation or bio-augmentation processes (Das & Chandran, 2010). However, Kenya has not adopted this emerging technology and no data exists on its potential application in environmental conservation.

1.2 Justification

Despite the numerous studies conducted on bioremediation around the world, there is no published work relating to oil degrading microbes in Kenya. With the recent successful oil exploration in the country (Tullow oil plc, 2013), Kenya is likely to experience challenges associated with oil exploitation such as handling of accidental oil spillages during drilling, refining, transportation, as well as storage accidents such as bursting of storage tanks and pipelines. Of interest is the inevitable water and soil pollution by petroleum compounds. The country has no set mechanisms/technologies to avert this anticipated environmental challenge.

The present study aimed at providing an insight to an effective strategy to an environmentally friendly and cost effective means of environmental bioremediation of accidentally released petroleum oil and hence provide a possible effective oil spill response management strategy to oil prospecting, refining and transporting companies (Kenya Pipeline Company Ltd-KPC, Kenya Ports Authority-KPA), environmental protection agencies (National Environmental Management Authority-NEMA, National Oil Pollution Committee-NOPC) and the general public (automobile garages and petrol stations). Successful bioremediation techniques require the right combination of microbes and environmental conditions (Boopathy, 2000). Therefore there is need to screen for oil degrading microbes that would be best suited to degrade petroleum-based contaminants in Kenya.

1.3 Objectives

1.3.1 General Objective

To isolate, screen and characterize hydrocarbon degrading bacteria from oil contaminated soils.

1.3.2 Specific objectives

1. To isolate, screen and characterize bacteria with hydrocarbon degrading properties using morphological, biochemical and molecular methods
2. To determine optimum conditions of pH, temperature, substrate concentration and nitrogen source for the bacterial isolates' degradation of hydrocarbons
3. To determine the ability of bacterial isolates to degrade aliphatic hydrocarbons by screening for the catabolic alkB gene

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1.0 Petroleum compounds

Petroleum is made up of a hydrocarbon mixture, which can be divided into four classes: saturates (alkanes), aromatics, resins and asphaltenes (Widdel & Rabus, 2001). Saturated hydrocarbons lack double bonds in their structure and constitute alkanes and cycloalkanes. Alkanes are highly hydrophobic and at physiological temperatures, exist in either gaseous (C1-C4), liquid (C5-C17) or solid (C18-C38) states depending on their molecular weights (Nyyssönen, 2009). Alkanes are the major components of crude oil and natural gas. Aromatic hydrocarbons are made up of one or more aromatic rings usually substituted with dissimilar alkyl groups (Figure 1). Resins and asphaltenes on the other hand contain non-hydrocarbon polar compounds with additional nitrogen, sulfur or oxygen atoms and occur in trace amounts (Harayama *et al.,* 1999).

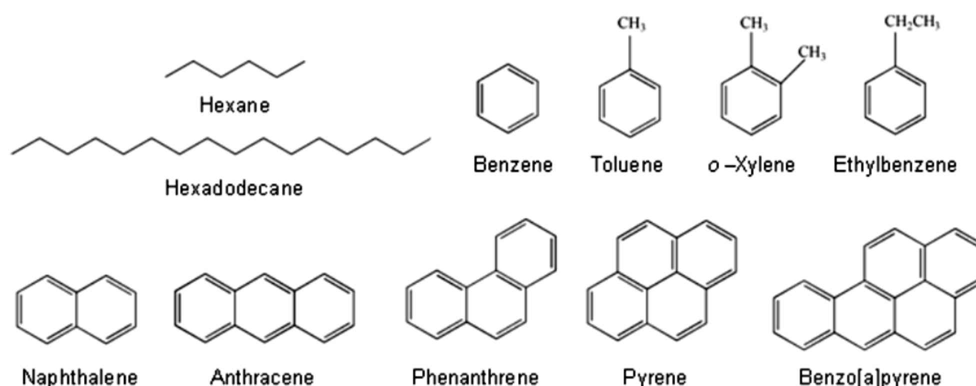


Figure 1: Chemical structures of some aliphatic and aromatic compounds (Adapted from Nyyssönen, 2009).

Petroleum can be fractionated by silica gel chromatography into aliphatic, aromatic, asphaltic (phenols and porphyrins) and resin (pyridines, quinolines, carbazoles, sulfoxides and amides) fractions (da Cruz *et al.,* 2008). The mono aromatic fraction comprises compounds referred to as BTEX (benzene, toluene, ethylbenzene and *ortho*, *para* & *meta* xylene). Polycyclic aromatic hydrocarbons (PAH) constitute a

wide and diverse group of recalcitrant, high molecular weight organic compounds made up of fused benzene rings in various structural configurations as shown in Figure 1 (Ivey, 2006).

Petroleum compounds are of immense environmental as well as human health concern owing to their potential trophic bio-magnifications (Malkawi *et al.,* 2009). Particularly, presence of low molecular weight compounds such as naphthalene and phenanthrene, among others, that are acutely toxic (Hamamura *et al.,* 2006) and high molecular weight compounds that have mutagenic, teratogenic and potential carcinogenic effects pose a serious threat (Selvakumar *et al.,* 2014). Presence of hydrocarbons in soil and water is a major problem since most of them are recalcitrant in nature (Lee *et al.,* 2010).

Oil spills have the largest immediate and economic effects as they harm not only the isolated location but to a larger extent, the ecosystem (Uzoamaka *et al.,* 2009). Many of these spills involve tankers or offshore oil wells some of which catch fire and consequently their combustion leads to emission of large amounts of toxic ash that is detrimental to human health. Seepage of used engine oil has been reported to cause loss of soil fertility, permeabilty, water holding and binding capacity (Khan & Rizvi, 2011). In addition, discharge of hydrocarbons into the environment has been shown to cause adverse effects such as mortality of marine mammals, seabirds, and large fishes as was observed during the Exxon Valdez oil spill of 1989 in Prince William Sound, Alaska. This led to the formation of oil film on shorelines leading to land degradation and water pollution (Peterson *et al.,* 2003). PAHs pose a potential risk to marine flora and fauna as well as to human health as many of them are carcinogenic and mutagenic in nature (Deziel *et al.,* 1996) with dibenz[a,h]anthracene, benzo[a]pyrene, chrysene and benz[a]anthracene being listed among priority harzadous contamintants by the US Environmental Protection Agency (US EPA) (Gan *et al.,* 2009).

2.2.0 Conventional disposal methods for petroleum products

A wide variety of physicochemical processes are used in cleaning up petroleum contaminants from the environment. Physicochemical methods such as washing, booming and skimming, mechanical removal, dispersion and dissolution are common for marine remediation (Zhu *et al.,* 2001), while landfilling, incineration, chemical oxidation and thermal conversion are used in soil clean up of these contaminants (Lam & Chase, 2012).

2.2.1 Incineration

This is a technique that is widely used in large refineries for waste oil treatment. The process involves complete combustion of oil wastes in presence of excess air and auxiliary fuels using incinerators such as rotary kiln and fluidized bed incinerator (Hu *et al.,* 2013). Combustion temperatures in the range of 980–1200 ^{0}C for 30 minutes using rotary kiln incinerator and 730–760 ^{0}C for several days in a fluidized bed incinerator are required. The process however suffers a number of limitations among them atmospheric pollution arising from fugitive emission of pollutants from incineration and incomplete combustion (Li *et al.,* 1995). In addition, ash residue and scrubber water and sludge generated are hazardous and require further treatment.

2.2.2 Oxidation treatment

The technique has been used to eliminate a wide range of organic contaminants using chemical or other enhanced oxidation processes (Ferrarese *et al.,* 2008). Chemical oxidation entails oxidation of organic compounds to carbon dioxide and water, or other non-hazardous compounds using reactive chemicals (Ferrarese *et al.,* 2008). Such chemicals include; Fenton's reagent, hypochlorite, ultrasonic irradiation, ozone, persulfate and permanganate which act by generating radicals such as hydroxyl radicals (Rivas, 2006).

Sonolysis using free radicals results in breakdown of complex and high molecular weight long chain alkanes or aromatic hydrocarbons into simple hydrocarbons

possessing higher solubility and bioavailabilty (Mason, 2007). Other advanced techniques for waste oil treatment include supercritical water oxidation, wet air oxidation and photocatalytic oxidation (Hu *et al.,* 2013).

2.2.3 Thermal conversion technique

Thermal conversion covers a wide range of thermal decomposition processes including gasification and pyrolysis in which the waste materials are heated and cracked in the absence of oxygen. The process results into smaller compounds that can be used as energy inputs for synthesis of new materials (Lam & Chase, 2012). For instance, hydrocarbon wastes are decomposed to produce syngas ($H_2 + CO$) which can be used directly as fuel or converted into liquid fuel through the Fischer-Tropsch process (Dry, 2001). The process is however energy intensive and usually entails large scale operation combined with a capital intensive plant.

Pyrolysis also involves thermal decomposition of the waste materials in an oxygen limited environmental. The process can be used as a thermal conversion technique for hydrocarbon wastes in which the materials are cracked to produce hydrocarbon gases, oils and char (Hu *et al.,* 2013).

2.2.4 Stabilization/solidification

This is a waste treatment technique designed to immobilize pollutants by transforming them into a less soluble or toxic form in a process called stabilization and then encapsulating them by creating a durable matrix with high structural integrity in a process known as solidification (Malviya & Chaudhary, 2006). The method is however considered less compatible with organic wastes since such wastes inhibit cement-based binder hydration and are normally not chemically bound in the binder hydration products (Leonard & Stegemann, 2010).

2.3.0 Degradation of hydrocarbons by microbes

A diverse group of micro-organisms (bacteria, fungi and algae) present in soil and aquatic environments possess enzymatic capabilities for complete mineralization of hydrocarbons (Jahangeer & Kumar, 2013). The process of bioremediation may be aimed at achieving: (a) mineralization, that is, complete oxidation of organic pollutants (b) biotransformation of organic contaminants into small, less toxic intermediates, or (c) reduction of compounds possessing highly electrophilic nitro- and halo- groups into less toxic forms by transfer of electrons from an electron donor usually a sugar or fatty acid to the contaminant (Rockne & Reddy, 2003).

Rising amount of microbiological research has been devoted to bioremediation of petroleum and petroleum products contaminated sites using microorganisms. Over 200 bacteria, 103 fungi and 14 algal genera, encompassing over 500 species, have been identified as potential degraders of hydrocarbon contaminants (Head *et al.,* 2006; Yakimov *et al.,* 2007). Notable among these are the bacterial species *Arthrobacter, Flavobacterium, Pseudomonas* sp. (most predominant), *Sphingomonas* (a novel *Pseudomonas* sp.) and *Acinetobacter* (Uzoamaka *et al.,* 2009). Others include *Micrococcus, Alcaligenes, Bacillus, Marcaxella and Comomanas*. Bacteria of the subphyla α-, β- and ω-proteobacteria are well established for their hydrocarbon degrading capabilities (Mahjoubi *et al.,* 2013).

Susceptibility of hydrocarbons to microbial attack differs with the nature of the hydrocarbon and are normally ranked in the following order of decreasing susceptibility: n-alkanes > branched alkanes > low-molecular-weight aromatics > cyclic alkanes (Atlas, 1981). Saturates have been shown to have the highest rates of biodegradation, followed by light aromatics, while high molecular weight aromatics and polar compounds show low biodegradation rates (Fusey & Oudot, 1984). However, this pattern is not universal as indicated by some studies in which for instance, naphthalene was observed to have high degradation rate compared to hexadecane in water sediment bacterial mixtures (Cooney *et al.,* 1985).

2.3.1 Hydrocarbon degrading bacteria

Mandal and co-workers (2012) isolated 324 bacteria belonging to 110 different species from oil contaminated soils and crude oily sludge and these were found to efficiently degrade different fractions of total petroleum hydrocarbons. In a study conducted by Mahjoubi and co-workers, bacteria of the genera *Pseudomonas, Ochrabactrum, Bacilllus, Agrobacterium, Stenotrophomonas, Brevundimonas, Gordonia, Acinetobacter, Achromobacter, Microbacterium, Sphingobium, Rhodococcus, Luteibacter, Kocuria* and *Novosphingobium* were isolated from oil contaminated environments (Mahjoubi *et al.,* 2013). In another study, bacterial genera *Gordonia*, *Burkholderia, Aeromicrobium, Mycobacterium, Dietzia,* and *Brevibacterium* were isolated from petroleum contaminated soil (Chaillan *et al.,* 2004).

Pseudomonas sp. has been indicated as the most predominant class of microbes that degrade xenobiotic compounds (Sharma *et al.,* 2015). In a study conducted by Sharma and co-workers, *P. aeruginosa* DSVP20 was shown to degrade 97 % eicosane, 75 % pristine and 47% of fluoranthene in the presence of purified biosurfactant following one week incubation (Sharma *et al.,* 2015). In a different study, *P. aeruginosa* was found to efficiently reduce hydrocarbon components ranging from C12 to C30 (Hamza *et al.,* 2010). The strain was found to degrade 48 % of total petroleum hydrocarbons after 24 hours and 77 % after 48 hours of incubation. Degradation of 88.5 % of 2 % petroleum by a *Pseudomonas* strain after 21 days of incubation was also reported by Linda and co-workers (2012). *Acinetobacter* species was found to be the most dominant strain capable of utilizing hydrocarbons in marine sediments in a study carried out by Mahjoubi and co-workers (2013). In a different study, *Acinetobacter* sp. strain DSM 17874 was found to mineralize alkanes with carbon chain lengths ranging from $C_{10}H_{22}$ to $C_{40}H_{82}$ (Throne-Holst *et al.,* 2007).

Ochrobactrum sp. is also a common bacterial species that has been reported to efficiently degrade polyaromatic hydrocarbons (Arulazhagan *et al.,* 2010; Mahjoubi *et*

al., 2013). In a study carried out by Katsivela and co-workers (2003), *Ochrobactrum* sp. EK6 was reported to co-metabolically assimilate significant amounts of a mixture of substrates consisting of many petroleum hydrocarbons in a complex LB media. In this study, *Ochrobactrum* sp. EK6 and two other strains of *Enterobacter* sp were found to notably degrade 97% of 2,2,4,4,6,8,8-heptamethylnonane, 72% of acenaphthene, 71% of acenaphthylene and 55% of toluene among other mono-aromatic and polycyclic aromatic hydrocarbons after 9 days of growth. Here, *Ochrobactrum* sp. EK6 was reported to give the best degradation rates. In a similar study, *Ochrobactrum* sp. strain PWTJD isolated from municipal waste contaminated soil was found to utilize 99% of phenanthrene within 7 days of culturing (Ghosal *et al.,* 2010).

Enterobacter species have also been found to mineralize a wide variety of hydrocarbon compounds. For instance, *Enterobacter cloacae* was observed to degrade benzene, hexane, xylene, paraffin, kerosene, wax, and different cooking oils by producing the biosurfactant exopolysaccharide EPS 71A (Iyer *et al.,* 2006). Two strains of *Enterobacter sp*, *Enterobacter* sp. EK3.1 and *Enterobacter* sp. EK4 were shown to utilize a mixture of hydrocarbons when used as the sole energy substrates or as co-metabolic substrates. The bacterial strains were reported to metabolize a hydrocarbon mixture of branched alkane 2,6,10,14-tetramethylpentadecane, toluene and the PAHs acenaphthylene and acenaphthene as carbon sources for growth and energy in mineral salts media (Katsivela *et al.,* 2003).

2.3.2 Hydrocarbon degrading fungi

Fungal genera namely, *Amorphoteca, Fusarium, Graphium, Aspergillus, Talaromyces Neosartorya, Paecilomyces and Penicillium* and yeast genera namely *Yarrowia, Candida* and *Pichia* were discovered in petroleum contaminated soil and proved to be potential degraders of hydrocarbons (Chaillan *et al.,* 2004). Uzoamaka and co-workers showed that eight out of twelve fungal isolates recovered from oil contaminated soils had potential for crude oil biodegradation and the fastest onset and

highest extent of biodegradation greater than 98% biodegradation efficiency was exhibited by *Aspergillus versicolor* and *Aspergillus niger* (Uzoamaka *et al.,* 2009).

2.4.0 Mechanisms of hydrocarbon biodegradation

Hydrocarbons are carbon and hydrogen containing compounds, are largely non-polar and at room temperature, exhibit little chemical reactivity due to lack of functional groups (Sierra-Garcia *et al.,* 2013). Based on their bonding nature, hydrocarbons can be classified into two groups; an aliphatic group made up of straight chain (n-alkanes), branched chain and cyclic alkanes as well as an aromatic group consisting of mono- or polycyclic hydrocarbons. Occurrence, type and arrangement of unsaturated bonds determine differences in hydrocarbon reactivity's (Sierra-Garcia *et al.,* 2013).

Numerous studies on biodegradation process have been carried out and results have revealed that many microbes can completely degrade most classes of hydrocarbons including alkanes, alkynes, alkenes and aromatic compounds. The process can take place in presence of molecular oxygen, *i.e.* aerobically or anaerobically using sulfate, ferric, nitrate or other oxidizing agents (Widdel & Musat, 2010b).

2.4.1 Activation of hydrocarbons for biodegradation

During initiation of biodegradation, the hydrocarbon must first be functionalized and currently it has been recognized that microbes have evolved an astonishing diverse range of activation (functionalizing) reactions (Sierra-Garcia *et al.,* 2013). These hydrocarbon activation mechanisms are different in aerobic and anaerobic micro-organisms (Widdel & Musat, 2010b). Under aerobic conditions, oxygen is used as a co-substrate in both mono and dioxygenase reactions that facilitate the terminal or sub-terminal hydroxylation of alkanes as well as the mono- and di- hydroxylation of the aromatic hydrocarbons (Boll & Heider, 2010).

Under anaerobic conditions, some proposed reactions comprise; methylation of unsubstituted aromatics, addition of fumarate by glycyl-radical enzymes, water-

mediated hydroxylation using molybdenum bound enzymes of an alkyl substituent via dehydrogenase as well as carboxylation catalyzed by uncharacterized enzymes which may represent a combination of the methylation reaction followed by the fumarate addition reaction (Foght, 2008; Boll & Heider, 2010).

2.4.2 Aerobic degradation of hydrocarbons

2.4.2.1 Aerobic degradation of aliphatic (alkane) hydrocarbons

Degradation of aliphatic hydrocarbons is essential as alkanes are quantitatively the most important components of petroleum with some being acutely toxic and difficult to remediate (Sierra-Garcia *et al.,* 2013). Under aerobic conditions, the methyl group of *n*-alkane is oxidized to the corresponding primary alcohol by substrate specific terminal hydroxylases (mono-oxygenases) (Rojo, 2009). The alcohol produced is then converted to an aldehyde that is finally oxidized to a fatty acid. The carboxylic acid formed is then conjugated to CoA and consequently converted to acetyl CoA molecules through the β-oxidation reaction (Figure 2).

Sub-terminal oxidation has also been observed for both short and long chain alkanes and it is also possible for both terminal and sub-terminal oxidation to co-exist in some microbes (Throne-Holst *et al.,* 2007). For sub-terminal oxidation, the alkane is oxidized to a secondary alcohol, which is converted to a corresponding ketone. This is then oxidized to an ester via a Baeyer-Villinger monooxygenase. The ester formed is hydrolyzed by an esterase generating an alcohol and a fatty acid (Rojo, 2009).

Among bacteria, hydroxylation of the terminal methyl group of aliphatics can be initiated by different classes of enzymes namely; propane monooxygenase (C3), different classes of butane monooxygenase (C3-C9), CYP 153 monooxygenases (C5-C12), AlkB-related non-heme iron monooxygenase (C3-C10 or C10-C20), Flavin binding monooxygenase AlmA (C20-C36), Flavin dependent monooxygenase ladA (C10-C30) and Copper flavin dependent dioxygenase (C10-C30) (Rojo, 2010). Among these enzymes, the integral membrane non-heme iron monooxygenase, alkane

hydroxylase (alkB) enzyme, is extensively studied and characterized (Salminen *et al.,* 2008).

Alkane monooxygenases are key enzymes in degradation of alkanes hence alkB gene is widely used as a functional biomarker for characterization of medium (C5-C11) and long (>C12) chain alkane degrading bacteria (Kuhn *et al.,* 2009). Alkane hydroxylase of one particular *Pseudomonas* strain, *Pseudomonas putida* (*Pseudomonas oleovorans*) GPo1, is well characterized with respect to its enzymology, genetics, and also potential applications (Van Beilen & Funhoff, 2007). A number of alkane oxidizing enzymes have been detected in *Acinetobacter* sp. M1, which are able to utilize alkanes with carbon range of C13–C44. For instance, alkMa and alkMb are membrane bound proteins related to *P. putida* GPo1 alk B (Rojo, 2010).

Most alkane hydroxylases possess a wide substrate range which translates to a number of products that find applications in synthesis of carboxylic acids, aldehydes, alcohols and epoxides. For instance, cytochrome P450 enzymes are used in production of drugs, fine chemicals and fragrances, as well as in bioremediation (Van Beilen & Funhoff, 2007).

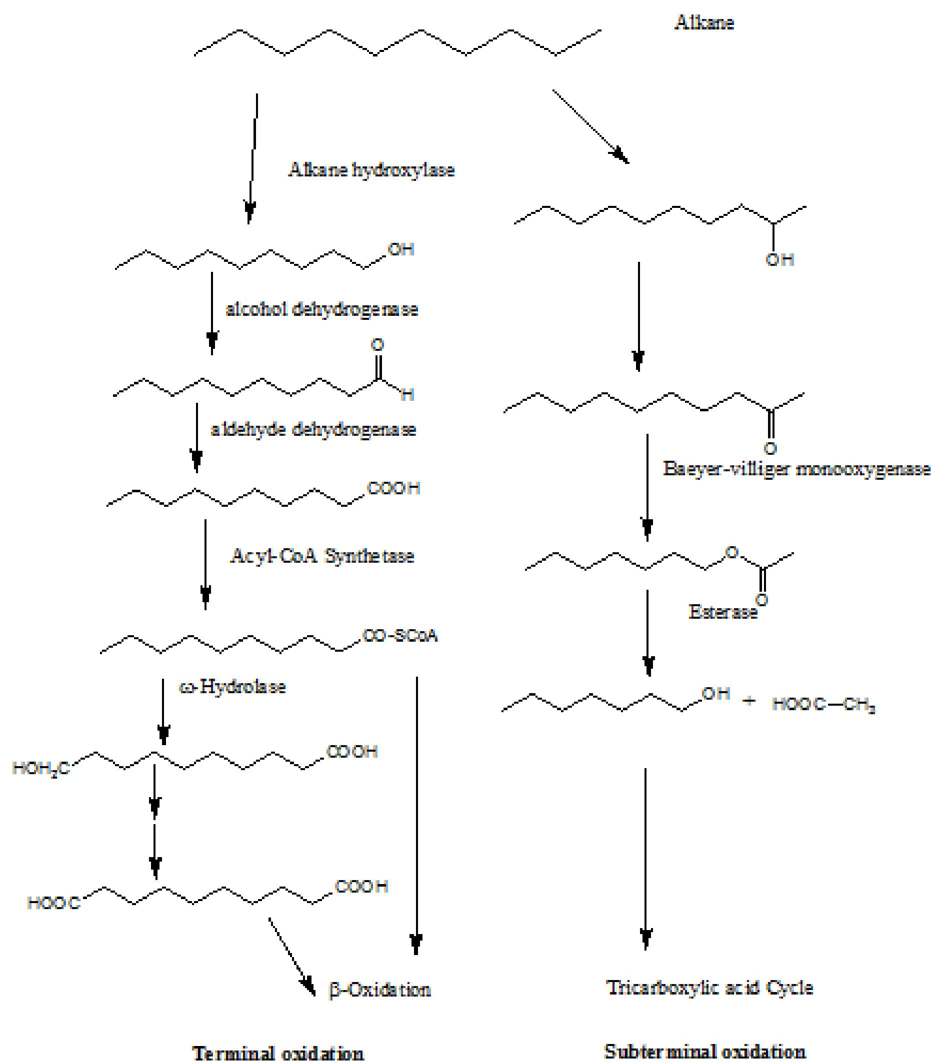


Figure 2: Aerobic pathway for degradation of alkanes by terminal and sub-terminal oxidation (Adapted from Rojo, 2009).

2.4.2.2 Aerobic degradation of aromatic hydrocarbons

A wide range of peripheral pathways are involved in aerobic catabolism of aromatic hydrocarbons. These pathways result in structurally diverse intermediates that are transformed into a number of common precursors that are further broken down and processed by few central reactions and the central metabolism of the cell (Carmona *et al.,* 2009). Biodegradation of aromatic hydrocarbons is typically initiated by enzymes belonging to either of three super families; Soluble Diiron multicomponent

Monooxygenase (SDM), Flavoprotein Monooxygenases (FPM) and Rieske Non-heme Iron Oxygenases (RNHO) (Sierra-Garcia *et al.,* 2013). In an alternative pathway, activation process can be initiated by CoA ligase enzymes and the resulting CoA derivatives processed through selective hydroxylation (Vilchez-Vargas *et al.,* 2010).

Aerobic aromatic degradation is normally initiated by oxygenation reactions catalyzed by RNHO enzymes which activate the aromatic ring for hydrophobic contaminants such as toluene, benzene (Figure 3), naphthalene, biphenyl and polycyclic aromatics, or through SDM enzymes (Sierra-Garcia *et al.,* 2013). Further breakdown of these compounds is attained via di- or trihydroxylated aromatic intermediates. There are two classes of enzymes which are phylogenetically unrelated and which are involved in further catalysis of di- or trihydroxylated aromatic intermediates; intradiol and extradiol dioxygenases (EXDO). These enzymes are vital in metabolism of aromatic compounds and a number of them and their encoding sequences have been recognized, purified and characterized (Brennerova *et al.,* 2009). All intradiol dioxygenases are included in the same superfamily while EXDO include at least three members of different families. The first group of EXDO (e.g. Catechol 1-2 dioxygenases and Catechol 2-3 dioxygenases) falls under the vicinal oxygen chelate superfamily. Type II EXDO (e.g. procatechuate 4, 5 dioxygenases) belong to LigB superfamily while type III EXDO (e.g. gentisate dioxygenases) are related to the cupin superfamily (Vilchez-Vargas *et al.,* 2010).

Monitoring of the capability of microbes to metabolize aromatic compounds in soils has been aided by knowledge on metabolic properties of various bacterial isolates (Pieper & Junca, 2004). Such studies have been carried out using primers designed based on conserved gene regions focusing on RNHO or SDM enzymes as targets for initiating biodegradation, or on aromatic ring cleaving EXDO (Sierra-Garcia *et al.,* 2013).

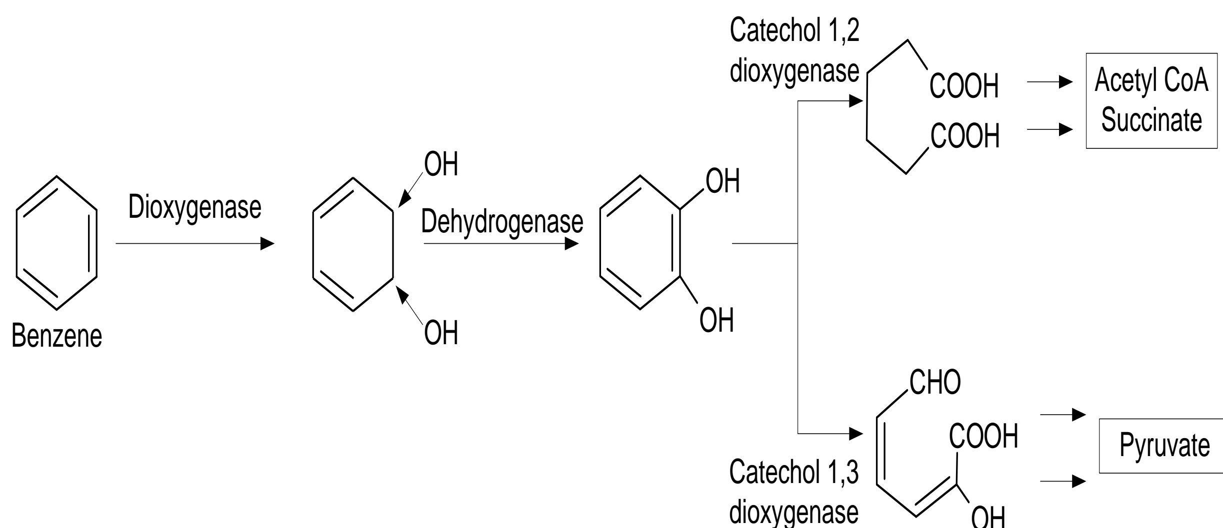


Figure 3: Biodegradation of a simple aromatic hydrocarbon, benzene (Adapted from Ivey, 2006).

2.4.3 Anaerobic degradation of hydrocarbons

2.4.3.1 Anaerobic degradation of aliphatic hydrocarbons

Two main mechanisms for anaerobic degradation of n-alkanes have been described. The first mechanism involves radical-catalyzed activation of the alkane through addition of fumarate which results in formation of succinate derivatives (Widdel & Grundmann, 2010a). Normally, the sub-terminal carbon of the hydrocarbon undergoes activation to form (1-methylalkyl)-succinates. Further breakdown of (1-methylalkyl) succinyl-CoA proceeds via rearrangement of the carbon skeleton as well as decarboxylation resulting in formation of 4-methyl-branched fatty acid thioesters which are eventually processed through the β-oxidation process (Widdel & Grundmann, 2010a).

Alkyl succinate synthase (ASS) also known as 1-Methyl-alkyl succinate synthase (MAS) is strictly anaerobic glycyl radical enzyme which catalyzes production of Alkyl succinates. Genes encoding this enzyme have been identified in bacteria that belong to the phylum proteobacteria for instance the sulfidogenic *Desulfococcus alkenivoras* AK-01 (Callaghan *et al.,* 2008). In a study conducted using propane and

paraffin degraders maintained under sulfate-reducing conditions, AssA genes were detected and suggested to be probable biomarkers for anaerobic alkane degradation (Callaghan *et al.,* 2010).

The second mechanism is proposed to involve a carboxylation reaction. This process was developed from the growth pattern of *Desulfococcus oleovoras*, which is a sulfate-reducing strain. Unlike other alkane degrading bacteria, this strain differs in conversion of carbon-even alkanes into carbon-odd cellular fatty acids and in conversion of carbon-odd alkanes into carbon-even cellular fatty acids (Widdel & Grundmann, 2010a).

2.4.3.2 Anaerobic degradation of aromatic hydrocarbons

The most widely understood anaerobic aromatic hydrocarbon degradation mechanism is one involving addition of fumarate to the compound resulting in substituted succinate derivatives. The process, which is a radical-catalyzed reaction, has been observed in activation of several alkyl-substituted benzenes and n-alkanes (Kube *et al.,* 2004). For toluene, benzylsuccinate synthase is the key enzyme involved in fumarate-dependent activation of the hydrocarbon (Boll *et al.,* 2002). The enzyme is widely used as a biomarker for aromatic hydrocarbon degradation (Carmona *et al.,* 2009).

Subsequent breakdown of toluene occurs through reductive de-aromatization and hydrolytic ring cleavage of benzoyl-CoA intermediate, β-oxidation of acetyl-CoA units and terminal oxidation to carbon dioxide (Boll *et al.,* 2002). For Ethylbenzene and probably other alkylbenzenes with carbon chain of at least 2, the process involves direct oxidation of the methylene carbon via S-1-Phenylethanol to acetophenone (Carmona *et al.,* 2009). This is then carboxylated and converted to benzoylCoA which is a common precursor for toluene and ethylbenzene degradation pathways (Boll *et al.,* 2002).

Complete genetic sequencing of six bacterial strains that belong to different taxonomic groups of bacteria and that are capable of anaerobic aromatic degradation using different electron acceptors has been carried out. These are; *Rhodopseudomonas palustris* strain CGA009 and the denitrifying *Magnetospirillum magneticum* strain AMB-1 (α-proteobacteria), *Thauera aromatica* and *Azoarcus species* EbN1 (denitrifying β-proteobacteria), and two obligate anaerobic δ-proteobacteria, the iron reducer *Geobacillus metallireducens* GS-15 and the fermenter *Syntrophus aciditrophicus* strain SB (Carmona *et al.,* 2009).

2.5.0 Biosurfactants

To overcome the challenge of low water solubility of petroleum hydrocarbons, microorganisms produce surfactants that facilitate emulsification (Banat *et al.,* 2000). Biosurfactants are a heterogeneous group of surface-active chemicals that enhance solubilization of the hydrocarbon through reduction of surface tension of the environment around the bacteria. Biosurfactants also act via reduction of interfacial tension between the bacterial cell wall and the petroleum compounds (Desai & Banat, 1997) and/or through various membrane modifications that increase the hydrophobicity of the cell wall.

Biosurfactants thus increase bioavailability of these contaminants hence speeding up uptake and biodegradation process (Deziel *et al.,* 1996). Pseudomonads, especially *P. aeruginosa* are best known for their ability to produce glycolipid type biosurfactants (Sharma *et al.,* 2015) which have been applied in bioremediation of oil sludge contaminated soils (Cameotra & Singh, 2008). Similarly, *P. putida* and *P. chlororaphis* have also been shown to possess such capability (Jahangeer & Kumar, 2013).

2.6.0 Factors affecting the rates of biodegradation

Biodegradation rate greatly depends on the physical state, chemical composition and concentration of the petroleum hydrocarbons (Jahangeer & Kumar, 2013). There exist

differences between the rate of biodegradation of petroleum in soil and in aquatic ecosystems following an oil spill (Margesin & Schinner, 1999). Key among these differences are those which affect the physicochemical nature of the oil and hence its movement and distribution. Terrestrial oil spillages are associated with vertical infiltration rather than horizontal movement of the oil into the soil and this prevents evaporative losses of volatile hydrocarbons which turn out to be toxic to micro-organisms (Leahy & Colwell, 1990).

Presence of particulate matter also affects the physico-chemical nature of oil in soil. Particulate matter can lower the effective toxicity of petroleum components through absorption although absorption and adsorption of hydrocarbons to humid substances could lead to formation of persistent oil film and silks (Weissenfels *et al.,* 1992). In addition, biodegradability of contaminants also depends on population size and activity level of the degrading bacteria (Nyyssönen, 2009).

The key factors that affect the rate of hydrocarbon degradation are temperature, oxygen and nutrient concentrations (Leahy & Colwell, 1990). In some aquatic environments, salinity and pressure may affect breakdown of the hydrocarbons, while in soils, moisture and pH may limit the degradation process (Nyyssönen, 2009). Temperature is vital in controlling the nature and extent of microbial hydrocarbon degradation (Leahy & Colwell, 1990). It directly affects the physico-chemical nature of hydrocarbons such as diffusion, viscosity and volatization which in turn alter the hydrocarbon composition and bioavailability leading to a net effect on the rate of biodegradation (Whyte *et al.,* 1998). Thus at high temperatures, higher degradation rates are expected. However, increased solubility and volatization observed at elevated temperatures may also increase membrane toxicity (Whyte *et al.,* 1998) and delay onset of the degradation process (Leahy & Colwell, 1990). Biodegradation of hydrocarbons occur over a wide range of temperature, for instance, Whyte and co-workers (1998) reported degradation of n-alkanes and diesel oil by *Rhodococcus* sp. strain Q15 at an optimal temperature of 0 °C and 5 °C while Holmes and co-workers

(2011) observed biodegradation at temperatures as high as 85 °C for the hyperthermophillic bacteria, *Ferroglobus placidus*.

Oxygen concentration has been identified as the rate-limiting variable in biodegradation of petroleum in soil and groundwater (Leahy & Colwell, 1990). The rates of microbial oxygen consumption, soil type, presence of utilizable oxygen depleting substrates (Bossert *et al.,* 1984) and whether the soil is waterlogged or not all determine oxygen availability in soils (Leahy & Colwell, 1990).

Carbon, nitrogen and phosphorus concentrations also affect the rate of microbial biodegradation. The biochemical oxygen demand of the contaminated site determines the actual required quantity of these nutrients. In a study conducted by Manilal & Alexander (1991), the effect of addition of phosphate, nitrogen and a combination of the two was investigated. Addition of phosphorus showed pronounced effects while nitrate was found to inhibit the biodegradation process. Additionally, combining the two minerals enhanced the rate of mineralization though the rate was lower than that observed when only phosphate was added. Nitrogen and Phosphorus may thus be limiting in soils and several studies have demonstrated accelerated rates of biodegradation following addition of urea-phosphate, N-P-K fertilizers, and ammonium and phosphate salts (Margesin & Schinner, 2001).

Typically, most heterotrophic bacteria and fungi favor a neutral pH with fungi being more tolerant to acidic conditions (Rockne & Reddy, 2003). Extremes in pH observed in some soils would therefore be expected to have a negative effect on the ability of microbes to metabolize hydrocarbons. Kästner and co-workers (1998) observed low pyrene biodegradation by *Sphingomonas paucimobilis* BA 2 at soil pH of 5.2 whereas a 10-fold increase was observed when the pH was raised to 7. Pyrene degradation by another strain BP 9 was however not affected by change in soil pH (Kästner *et al.,* 1998).

2.7.0 Bioremediation techniques

Numerous bioremediation agents have been proposed and these are classified based on the bioremediation approach as either bio-augmentation or bio-stimulation agents. These commercially available agents include microbial cultures, enzyme and nutrient additives (Das & Chandran, 2010). A list of bioremediation agents which may be used in response to oil spills on land and on/near waters in the United States was compiled by the U.S. EPA as a part of the National oil and hazardous substances pollution Contingency Plan (NCP) product schedule (Das & Chandran, 2010). These products are presented in Table 1.

Table 1: Bioremediation agents in NCP (National Contingency Plan) product schedule (Adapted from Das & Chandran, 2010).

Name or Trademark	Product Type	Manufacture
BET BIOPETRO	MC	BioEnviro Tech, Tomball, TX
BILGEPRO	NA	International Environmental Products,LLC,Conshohocken, PA.
INIPOL EAP 22	NA	Societe, CECA S.A., France
LAND AND SEA	NA	Land and Sea Restoration LLC, San Antonio, TX
RESTORATION MICRO-BLAZE	MC	Verde Environmental, Inc., Houston, TX
OIL SPILL EATER II	NA/EA	Oil Spill Eater International, Corporation, Dallas, TX
OPPENHEIMER FORMULA	MC	Oppenheimer Biotechnology, Inc., Austin, TX
PRISTINE SEA II	MC	Marine Systems, Baton Rouge, LA
STEP ONE	MC	B & S Research, Inc., Embarrass, MN
SYSTEM E.T. 20.	MC	Quantum Environmental Technologies, Inc (QET), La Jolla, CA
VB591TMWATER, VB997TMSOIL, AND BINUTRIX	NA	BioNutraTech, Inc., Houston,TX
WMI-2000	MC	WMI International, Inc

Abbreviations of product type:
MC: Microbial Culture
EA: Enzyme Additive
NA: Nutrient Additive.

A number of field studies have demonstrated the efficiency of bioremediation technique in cleanup of oil-polluted soils. For instance, in a field case study conducted on different oil refineries in India, a consortium consisting of four different uncharacterized species of bacteria was found to successfully degrade different fractions of total petroleum hydrocarbons (TPH) (Mandal *et al.,* 2012).About 48,914 tons of different types of oily wastes were treated in batches using the consortium which was previously isolated from oil-contaminated soils and later produced in 1500-litre bioreactors. The microbial consortium was applied together with a nutrient formulation consisting of nitrogen, phosphorous and potassium mineral salts to stimulate the population of the microbial consortium as well as to mitigate the initial toxic shock due to oil contamination. Initial TPH content, which varied from between 83.50 to 531.30 gm/kg of oily waste, was degraded to less than 10 gm/kg of oily waste in most cases within 2-12 months in 44 field case studies. Moreover, bio-remediated soil was found to be non-toxic to seed germination and natural vegetation was observed to grow on these sites following bioremediation (Mandal *et al.,* 2012).

The success of bioremediation approach in the clean-up of oil pollution after the Exxon Valdez oil spill of 1989 in Prince William Sound and the Gulf of Alaska generated remarkable interest in the potential of bioremediation technique (Zhu *et al.,* 2001). About 37,000 metric tons (11 million gallons) of crude oil was released into the environment following the oil spill which resulted in mortality of thousands of marine mammals and seabirds (Das & Chandran, 2010). Bio-stimulation technique was applied during this incident in which two fertilizers were used in large-scale to enhance growth of hydrocarbon degrading microbes. These were an oleophilic organic liquid compound designed to attach to oil (Inipol EAP22), as well as a slow-release granular inorganic fertilizer (Customblen) (Prince *et al.,* 2003). A monitoring program designed to examine biodegradation potential following application of the two fertilizers on shoreline sediments reported an increase in degradation of especially hexadecane and phenanthrene (Lindstrom *et al.,* 1991).

CHAPTER THREE

3.0 METHODOLOGY

3.1.0 Sample collection

Soil sample collection was randomly carried out at six sites in garages around Ngara area in Nairobi-Kenya. Area coordinates for this location are shown in appendix 1. The sampling sites were denoted as site 1 to site 6. Soil was collected from three depths; upto 1cm, 5cm and 15 cm. The collected samples were kept in sterile falcon tubes prior to transportation to the laboratory for further analysis and stored at 4 °C. Photograph of contaminated site showing waste engine oil flowing into a pool of stagnant water is shown in the Figure 4.

Figure 4: Photograph of contaminated site at one of the auto garages in Ngara area, Nairobi Kenya

3.2.0 Bacteria isolation

Isolation of hydrocarbon degrading bacteria was carried out using enrichment technique (Afuwale & Modi, 2012). Soil sample (1 g) from each site and soil depth was transferred into 250 ml Erlenmeyer flasks containing 100 ml sterile Bushnell Haas media (BHM) supplemented with 1% used engine oil as sole carbon source and incubated at 37 °C for 7 days in a rotary shaker (Gallenkamp, London, England) operating at 120 revolutions per minute (rpm). BHM composed of in (g/L): $MgSO_4$ (0.2), $CaCl_2$ (0.02), KH_2PO_4 (1.0), K_2HPO_4 (1.0), NH_4NO_3 (1.0), $FeCl_3$ (0.05) final pH 7 (Borah & Yadav, 2014). The media was sterilized using an autoclave (Tuttnauer,

USA) at 121 °C for 15 minutes. After one week, 1 ml of this suspension was transferred into freshly prepared BHM supplemented with 1% used engine oil and incubated at the same conditions as mentioned earlier. This was then followed by another enrichment process under the similar conditions.

An inoculum was then picked from each BHM flask and streaked on to Luria Bertani (LB) agar plates and incubated overnight at 37 °C. The LB media composed of in (g/L): Tryptone (10.0), Yeast extract (5.0), NaCl (10.0), Agar (15.0), final pH 7.2. Colonies were picked from each plate, transferred into test tubes containing LB broth and incubated in a rotary shaker at 150 rpm for 24 hours at 37 °C. Serial dilutions of up to 10^{-7} from each test tube was carried out and an aliquot of 100 µl plated on LB agar plates. Discrete colonies from 10^{-7} and 10^{-6} dilutions plates were then picked using sterile toothpicks and purified by plating on fresh LB agar plates and later LB broth before storage at 4 °C.

3.2.1 Preparation of glycerol stocks

Glycerol stocks of pure bacterial isolates were prepared by mixing 500 µl of an overnight culture with 500 µl of 80% sterile glycerol (Sigma, St. Louis, USA) in 2 ml eppendorf tubes and mixed briefly before storage at -80 °C.

3.3.0 Screening for hydrocarbon degraders

3.3.1 Degradation of mixed hydrocarbons

Pure isolates were screened for ability to metabolize heating oil using the procedure described by Afuwale & Modi (2012). Mineral salt media supplemented with heating oil as the sole carbon source was used. Bacterial cells cultured overnight in LB media were washed twice with 0.85 % NaCl solution before suspending in the same solution. An aliquot (100 µl) of bacterial culture was then transferred to test tubes containing 5 ml mineral salt media following autoclaving for 15 minutes at 121 °C. Heating oil (0.5 ml) was then added and incubation carried out at 30 °C in a shaker with a speed of 120 rpm. Mineral salt media composed of in (g/L): $(NH_4)_2SO_4$ (1.0),

$MgSO_4.7H_2O$ (0.1), KH_2PO_4 (0.5), K_2HPO_4 (0.76). Composition of 10X 1ml/L trace elements solution (mg/L): $ZnSO_4$ (100), H_3BO_3 (300), $CaCl_2.2H_2O$ (134.2), $FeSO_4.7H_2O$ (2000), $CuCl_2.2H_2O$ (10), $NaMoO_4.2H_2O$ (30), $NiCl_2.6H_2O$ (20), $MnCl_2.4H_2O$ (30). The pH was adjusted to 7 using 1 M NaOH. The experiment was carried out in triplicate and two controls prepared by excluding the substrate in one and inoculum in the other and the two kept under the same conditions. Change in turbidity was taken as the measure of growth. Optical density readings at 600nm were therefore taken for 7 days using cell density meter (WPA BioChrom, USA).

3.3.2 Degradation of individual hydrocarbons

Bacterial isolates were also screened for their ability to degrade toluene, octane and hexane. Pure bacterial cultures were streaked on BHA plates that were kept in a desiccator containing 10% toluene and 90% hexadecane (does not evaporate) in a 25 ml beaker and incubation carried out at 25 °C for 14 days. Replica control plates not exposed to hydrocarbons were also kept under similar conditions to eliminate autotrophs and agar-utilizing bacteria (modified Hassanshahian *et al.,* 2012). Isolates were also separately exposed to 20% hexane in 80% hexadecane and 20% octane in 80% hexadecane and treated as described for toluene.

3.4.0 Morphological characterization

All bacterial isolates were identified by their morphological characteristics based on colony morphology on LB agar plates.

3.4.1 Gram's staining test

Bacterial isolates were examined by Gram's staining test to differentiate between Gram positive and Gram negative bacteria. The procedure described by Hucker & Conn (1923) was applied. A thin bacterial smear was prepared on a clean microscopic glass slide. This was heat-fixed by passing the slide over a Bunsen burner flame. The slide was then flooded with crystal violet solution for 1 minute and briefly rinsed with running tap water. This was repeated by replacing crystal violet with Gram's iodine

solution. Decolourization was performed using 95% ethanol followed by rinsing with tap water. The slide was then counter-stained with safranin solution for 1 minute, rinsed with running tap water and allowed to air dry. The dry slides were then viewed under a microscope (63X/0.75) (LEICA DM 750, USA).

3.4.2 Potassium hydroxide (KOH) test

This was carried out as a confirmatory test for Gram's iodine staining using the procedure described by Buck (1982). Two drops of 3% Potassium hydroxide solution was placed on a glass slide. A loopful of pure bacterial cells was then mixed into the KOH drops. Observations were made on formation of a viscous string with the inoculating loop indicating positive results (Gram's negative) or lack of formation of the viscous string indicating negative results (Gram's positive).

3.5.0 Biochemical characterization

3.5.1 Starch hydrolysis test

The procedure described by Alariya and co-workers (2013) was applied with some modifications. A pure bacterial colony from each isolate was streaked in a straight line on mineral salt media agar plate containing 2% soluble starch and incubated at 37 °C for 48 hours. Mineral salt media composed of in (g/L): $(NH_4)_2SO_4$ (1.0), $MgSO_4.7H_2O$ (0.1), KH_2PO_4 (0.5), K_2HPO_4 (0.76), agar (15.0) supplemented with 1 ml trace element solution (mg/L): $ZnSO_4$ (100), H_3BO_3 (300), $CaCl_2.2H_2O$ (134.2), $FeSO_4.7H_2O$ (2000), $CuCl_2.2H_2O$ (10), $NaMoO_4.2H_2O$ (30), $NiCl_2.6H_2O$ (20), $MnCl_2.4H_2O$ (30). The plates were then flooded with Gram's iodine solution to produce a dark blue colored starch-iodine complex. Gram's iodine composed of: 250 mg iodine crystals, 2.5 g potassium iodide and 125 ml water. Observations were made on formation of a clear zone around the bacterial streaks indicating starch hydrolysis as a result of amylase enzyme activity. Negative results were indicated by lack of a clear zone around the bacterial streaks signifying absence of amylase enzyme activity.

3.5.2 Catalase test

A few drops of 5% hydrogen peroxide were placed on a glass slide and a loopful of pure bacterial cells added. Catalase enzyme activity was indicated by formation of air bubbles (Kumari *et al.,* 2013).

3.5.3 Carbohydrate fermentation test

Glucose, fructose, maltose, and sucrose fermentation tests were carried out using phenol red carbohydrate broth (Merck, 2007). The broth was sterilized separately from the carbohydrate solution to avoid hydrolysis of carbohydrates caused by excessive heating. Phenol red broth composed of in (g/L): protease peptone no. 3 (10.0), NaCl (5.0) and phenol red (0.0189). These ingredients were dissolved in 800 ml distilled water to prepare phenol red base broth and 4.5 ml of this transferred to capped test tubes. Durham tubes were then inserted inside the test tubes to detect gas production during fermentation. The broth was sterilized using an autoclave at 121 °C for 15 minutes. Carbohydrate solution was prepared by dissolving 10.0 g of individual carbohydrate in 200 ml of distilled water. The solution was then sterilized by filtering through a bacteria retaining membrane filter with a 0.22 µm pore size. An aliquot of 0.5 ml filtrate was then added to the phenol red base broth and mixed. Broth media had a red color with a final pH of 7.1.

Each test tube was aseptically inoculated with test bacteria using sterile toothpicks and the tubes incubated at 37 °C for 24 hours at 150 rpm. Observations were then made on color changes with yellow color indicating fermentation of the sugar hence positive results while pink-red/red color implied no fermentation hence negative reaction. Gas production was indicated by presence of bubbles trapped within the durham tubes.

3.6.0 Molecular characterization

3.6.1 Genomic DNA extraction

Isolates that showed bacterial growth with the different hydrocarbons tested were selected for identification through PCR amplification of 16S rDNA gene. DNA extraction was carried out using organic solvents (Miller *et al.,* 1999). Selected isolates were grown overnight in LB broth. Broth culture of 2 ml was centrifuged for 5 min at 10,000 x g in a 2 ml eppendorf tube using a Neofuge 13R centrifuge (heal Force Bio-Meditech, Shanghai China) to obtain cell pellet. An aliquot of 400 µl of lysis buffer composed of in (g/100ml): 2% Triton X-100 (2), 1% SDS (1), 100 mM NaCl (0.58), 1 mM EDTA (0.0288) and 10 mM Tris HCl (1 ml) (pH 8.2) was added followed by 1 small spoonful of ceramic beads. An aliquot of 400 µl Phenol/Chloroform/Isoamylalcohol in the ratio of 25:24:1 was then added and the mixture vortexed for 2 min. An aliquot of 300 µl of TE buffer composed of: 10 mM Tris, 1 mM EDTA, pH 7.5 was added and centrifuged for 15 min at 25 °C and 8,000 x g. The supernatant obtained was transferred to a new sterile eppendorf tube and 1 ml absolute ethanol at -20 °C added and gently mixed before centrifugation for 4 min at 10,000 x g at 4 °C. The supernatant was discarded and the pellet obtained dissolved in 400 µl TE buffer and 3 µl RNase A (10 mg/ml) added and incubated for 5 min at 37 °C. An aliquot of 10 µl of 4 M ammonium acetate was then added followed by 1 ml of cold absolute ethanol at -20 °C. Each tube was gently inverted several times before centrifugation at 16,000 x g for 2 min at 4 °C. The supernatant was discarded and the pellet left to air dry for 20 min. The pellet was finally dissolved in 100 µl of 10 mM Tris-HCl pH 8.

3.6.2 DNA analysis by gel electrophoresis

The quality of genomic DNA was determined on 1% (w/v) agarose gel in 1X TAE buffer. Composition of 1X TAE buffer was: 40 mM Tris (pH 7.6), 20 mM acetic acid and 1 mM EDTA. A 1% TAE /agarose/EtBr gel mix solution was prepared by boiling 1.0 g agarose in 100 ml of 1X TAE. The solution was allowed to cool down to 60 °C before addition of ethidium bromide to a final concentration of 0.5 µg/ml (Shahaby *et*

al., 2015). The solution was then poured into the gel casting chamber and a comb placed in to make the loading wells. After polymerization, the gel was transfered into an electrophoresis chamber covered with 1X TAE running buffer. An aliquot of 1 µl of DNA loading dye (6X purple) (Fermentas, Pittsburgh, USA) was premixed with 5 µl of each DNA sample prior to loading them onto the wells alongside GeneRuler 1 kb plus DNA ladder (ThermoFisher Scientific, USA). The DNA samples were then electrophoresed at 100V for 45 min and the DNA bands visualised under a UV transilluminator (Herolab, Wiesloch, Germany).

3.6.3 PCR amplification of 16S rDNA gene

An aliquot of genomic DNA extracted from each isolate was used as a template to amplify 16S rDNA gene. The gene was amplified using two universal primers (Eurofins genomics, Ebersberg Germany): 16S F27, forward 5'…AGA GTT TGA TCC TGG CTC AG…3' and 1492, reverse 5'…GGT TAC CTT GTT ACG ACT T …3' (Dasgupta *et al.,* 2013). The PCR reaction was performed in PCR reaction tubes of 25 µl using 12.5 µl One*Taq*® Quick-Load® 2X master mix with standard buffer (New England Biolabs, Massachusetts, United States), nuclease free water (9.5 µl), 10 µM forward primer (0.5 µl) , 10 µM reverse primer (0.5 µl) and genomic DNA template (2 µl). One*Taq*® Quick-Load® 2X master mix contains 20 mM Tris-HCl (pH 8.9 at 25 °C), 1.8 mM $MgCl_2$, 22 mM NH_4Cl, 22 mM KCl, 0.2 mM dNTPs, 5% glycerol, 0.06% IGEPAL® CA-630, 0.05% Tween® 20, Xylene Cyanol FF, Tartrazine and 25 units/ml One Taq DNA polymerase. Thermocycler (MJ research - PTC 200, Minnesota, USA) conditions for the PCR were as follows: An initial denaturation step at 95 °C for 3 min followed by 30 cycles of 95 °C for 30 sec, 52 °C for 45 sec and 68 °C for 45 sec and a final extension step at 68 °C for 5 min followed by a final hold at 4 °C.

Amplified PCR products were electrophoresed on a 1% agarose gel in 1X TAE buffer and visualized under UV light. A positive control (*E. coli* 16S rDNA), negative control (water) and GeneRuler 1 kb plus DNA ladder were also included in the gel

electrophoresis. The PCR products were then purified using Qiaquick PCR purification kit (Qiagen, Valencia, CA, USA) and sent to Eurofins Genomics Ebersberg, Germany for sequencing. Obtained sequences were analyzed and deposited in Genbank.

3.6.4 Phylogenetic analysis of 16S rDNA sequences

Obtained 16S rDNA sequences were compared with already known 16S rDNA sequences at National Center for Biotechnology Information (NCBI) database using Basic Local Alignment Search Tool (BLAST) algorithm obtained from; http://www.ncbi.nlm.nih.gov/BLAST. All the sequences were then aligned using CLUSTAL W algorithm in Geneoius 9.1.4® and Phylogenetic trees constructed based on the nucleotide sequences with the Bayesian phylogenetic method in MrBayes software obtained at http://mrbayes.net. The trees were then visualized using Fig tree version 1.3.1 software obtained at http://tree.bio.ed.ac.uk/.

3.7.0 Optimization of growth conditions for diesel oil degrading bacteria

Optimization of growth conditions for three selected bacterial isolates was conducted using diesel oil according to the procedure described by Dongfeng and co-workers (2011) with some modifications.

3.7.1 Effect of pH on bacterial growth during biodegradation of diesel oil

The effect of pH on growth of three bacterial strains was determined using 100 ml Bushnell Haas media supplemented with 1% diesel oil as the sole carbon source. Bacterial inoculums of 100 µl previously cultured overnight in LB media were inoculated in autoclaved BHM with pH values equating to 3, 5, 7, 9 and 11 following washing with physiological saline (0.85%). The pH values were adjusted appropriately using 1 M NaOH and 1 M HCl. The test was conducted in triplicate using 1% substrate concentration at a temperature of 37 °C for 7 days in a shaker with a rotational speed of 150 rpm. Control tests containing no bacterial inoculum were also included. Bacterial growth was then monitored daily using a spectrophotometer

at 600 nm and net dry biomass (g/L) determined simultaneously. An aliquot of 5 ml of culture media was poured into a pre-weighed centrifuge tube and spun in a centrifuge (Hanil Science Industrial, Korea) at 16,000 x g for 10 min. This was then washed twice with distilled water and dried overnight at 90 °C before reweighing and the difference in weight and the volume used considered to obtain dry biomass.

3.7.2 Effect of temperature on bacterial growth during biodegradation of diesel oil

The effect of temperature on growth of the three selected bacterial isolates was studied using BHM at pH 7 supplemented with 1% diesel oil. An aliquot of 100 µl of bacterial inoculum previously cultured overnight in LB media was separately inoculated in 100 ml sterile BHM following washing with physiological saline (0.85%). The test was conducted using 1% substrate concentration at varying temperatures as follows; 25 °C, 30 °C, 37 °C, 45 °C and 55 °C alongside control tests. Bacterial growth was determined after culturing for 7 days, in a shaker with a rotational speed of 150 rpm, using a spectrophotometer at 600 nm and the dry biomass (g/L) also determined simultaneously as described in section 3.7.1.

3.7.3 Effect of various concentrations of diesel oil on bacterial growth during biodegradation

The influence of substrate concentration on growth of the three bacterial isolates was determined using 100 ml sterile BHM at pH 7 supplemented with various concentrations of diesel oil as follows; 0.5, 1, 3, and 5% at 37 °C. Bacterial inoculum of 100 µl used was previously cultured overnight in LB culture media before washing with physiological saline. Bacterial growth was determined after culturing for 7 days in a shaker with a rotational speed of 150 rpm. Growth was determined using a spectrophotometer at 600 nm and the dry biomass (g/L) determined simultaneously as described in section 3.7.1.

3.7.4 Effect of nitrogen source on bacterial growth during diesel oil biodegradation

The influence of nitrogen source on growth of the three bacterial isolates was determined using autoclaved nitrogen- limited mineral salts media (100 ml) supplemented with 1% diesel oil at pH 7 and 37 °C. A 0.1% (w/v) of Ammonium nitrate (NH_4NO_3), yeast extract and tryptone were separately used as nitrogen sources. Bacterial inoculums of 100 µl used were previously cultured overnight in LB culture media before washing with physiological saline. Bacterial growth was determined after culturing for 7 days in a shaker with a rotational speed of 150 rpm using a spectrophotometer at 600 nm and the dry biomass (g/L) determined simultaneously as described in section 3.7.1.

3.8.0 Hydrocarbon analysis using GC-MS

Diesel oil hydrocarbons and intermediate metabolites in BHM inoculated with isolates 1C, 2C, 3A and 4A2 were analyzed after 21 days of incubation with some modifications (Hassanshahian *et al.,* 2012). Inoculums of these isolates were previously cultured overnight in LB media and afterwards washed twice with physiological saline. An aliquot of 100 µl of bacterial cells obtained was then transferred to a 250 ml volumetric flask containing 100 ml sterile BHM supplemented with 1% diesel oil. Un-inoculated BHM flask was kept as control. The hydrocarbons were extracted from 30 ml BHM using an equal volume of dichloromethane with aid of a separating funnel. This was repeated twice to ensure complete recovery of the hydrocarbons and the dichloromethane phases combined and treated with anhydrous Na_2SO_4 to remove emulsions and residual water. The resultant extracts were concentrated by evaporation under a stream of Nitrogen using a heidolph rotary evaporator (Goel Scientific, India). The residue obtained from each sample was then dissolved in dichloromethane. The hydrocarbon composition was analyzed by GC-MS using SHIMADZU QP2010SE series GC-MS (Shimadzu, Kyoto, Japan) equipped with Zebron GC column (ZB-1MS) 30.0 m by 0.25 mm inner diameter with a thickness of 0.50 µm. Helium was used as the carrier gas. A temperature program

consisting of an initial oven temperature of 55 °C for 3 minutes increased to 245 °C for 5 min at a rate of 4 °C /min was applied. An aliquot of 10 µl was injected as the sample. The injector and detector temperatures were maintained at 250 °C and 260 °C, respectively. Split ratio of 10.1 injection mode was applied. Hydrocarbon and intermediate metabolite peaks were identified through comparison of retention times with mass spectrometer database using Autochro-3000 software.

3.9.0 Alkane hydroxylase gene amplification

An aliquot of 2 µl of genomic DNA extracted from each isolate was used as a template to amplify alkB gene. The gene was amplified using two primers (Eurofins genomics, Ebersberg Germany): alk-3Foward 5'…TCG AGC ACA TCC GCG GCC ACC A…3' and alk-3Reverse 5'…CCG TAG TGC TCG ACG TAG TT…3' characterized from *Pseudomonas oleovorans* GPo1 (Tebyanian *et al.,* 2013). The expected PCR product was 330 bp. The PCR process was performed in 25 µl PCR reaction tubes by adding 12.5 µl One*Taq*® Quick-Load® 2X master mix with standard buffer, 9.5 µl nuclease free water, 0.5 µl 10 µM forward primer, 0.5 µl 10 µM reverse primer and 2 µl genomic DNA template. Thermocycler (MJ research - PTC 200, Minnesota, USA) conditions were as follows: An initial denaturation step at 94 °C for 2 min followed by 30 cycles of 94 °C for 1 min, 54 °C for 30 sec and 72 °C for 30 sec and a final primer extension step at 72 °C for 5 min and a final hold at 4 °C.

PCR products obtained were electrophoresed alongside GeneRuler 1kb DNA ladder (ThermoFisher Scientific, USA) on a 1% agarose gel with 1X TAE buffer and visualized under a UV light transilluminator before purification using Qiaquick PCR purification kit and sent to Eurofins Genomics Ebersberg, Germany for sequencing. Phylogenetic analysis was then carried out as previously described for 16S rDNA gene in section 3.6.4.

3.10.0 Data analysis

All analyses were carried out in triplicates and the experimental data analyzed using one way analysis of variance (ANOVA) using Statistical Package for Social Sciences (SPSS), version 19.0 (Shahaby *et al.,* 2015). Differences among mean values for treatments at $P<0.05$ were evaluated using Post hoc test (Tukey's test) ("SPSS," 2010). The data is presented as mean ± standard error.

CHAPTER FOUR

4.0 RESULTS

4.1.0 Bacteria isolation and purification

Photographs showing difference in turbidity of both control BHM and BHM inoculated with soil sample collected from site 1 after 7 days of incubation (Figure 5). The change in turbidity indicates growth of oil degrading bacteria.

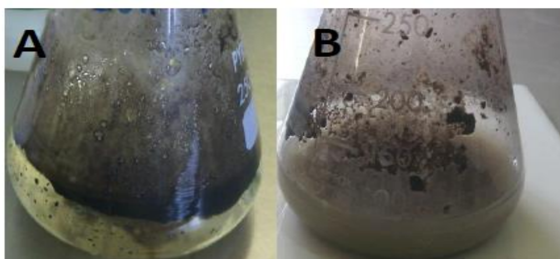


Figure 5: Bacteria isolation using BHM supplemented with 1 % used engine oil. (A) BH control media without inoculum and (B) inoculated BHM after 7 days of incubation.

Twenty bacterial isolates and 1 fungal isolate were obtained following isolation and purification procedure. These were denoted as 1A, 2A, 3A, 3AF (fungi), 4A, 4A2, 5A, 6A, 1B, 2B, 3B, 4B, 5B, 6B, 1C, 2C, 3C, 4C, 5C, 5CB and 6C as shown in Table 2 based on the sampling location.

Six sites denoted 1–6 were sampled at three different soil depths as; surface, sub-surface 1 and sub-surface 2 denoted as C, B & A respectively. The numbers 1–6 before each isolate in Table 2 corresponds to the sampling site.

Table 2: Microbial isolates as obtained from six sampling sites

Sampling Site	Soil Depth	Isolate
1	Surface	1C
	Sub-surface 1	1B
	Sub-surface 2	1A
2	Surface	2C
	Sub-surface 1	2B
	Sub-surface 2	2A
3	Surface	3C
	Sub-surface 1	3B
	Sub-surface 2	3A, 3AF
4	Surface	4C
	Sub-surface 1	4B
	Sub-surface 2	4A, 4A2
5	Surface	5C, 5CB
	Sub-surface 1	5B
	Sub-surface 2	5A
6	Surface	6C
	Sub-surface 1	6B
	Sub-surface 2	6A

Key: Surface=up to 1 cm, Sub-surface 1 = 5 cm, Sub-surface 2 = 15 cm

Figure 6 shows the sequence of isolation of isolates 5A, 5B and 5C from soil sampled from site 5. Discrete colonies were obtained through sequential culturing in LB agar and broth.

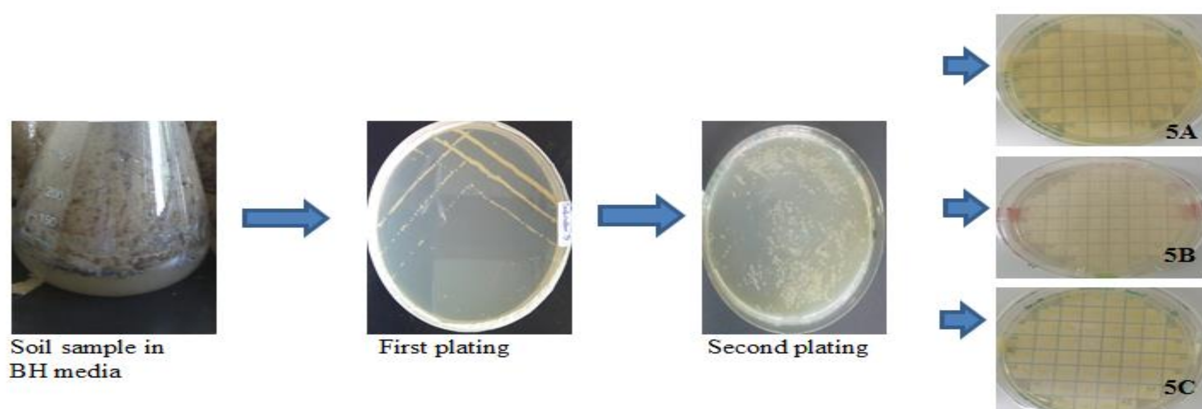


Figure 6: Photographs of soil in BH media and LB agar plates of mixed bacterial cultures and pure single cultures.

4.2.0 Screening for biodegradation potential

4.2.1 Degradation of mixed hydrocarbons

Following isolation, selection of efficient hydrocarbon degraders was carried out based on ability to grow in mineral salt media supplemented with heating oil which is a mixture of hydrocarbons. It was observed that all the isolates grew as shown in Appendix 2 and 3. Nine isolates; 3A, 4A2, 5A, 6A, 1B, 1C, 2C, 5C and 6C were selected for further studies since they showed a steady increase in cell density compared to the rest of the isolates as shown in Figure 7.

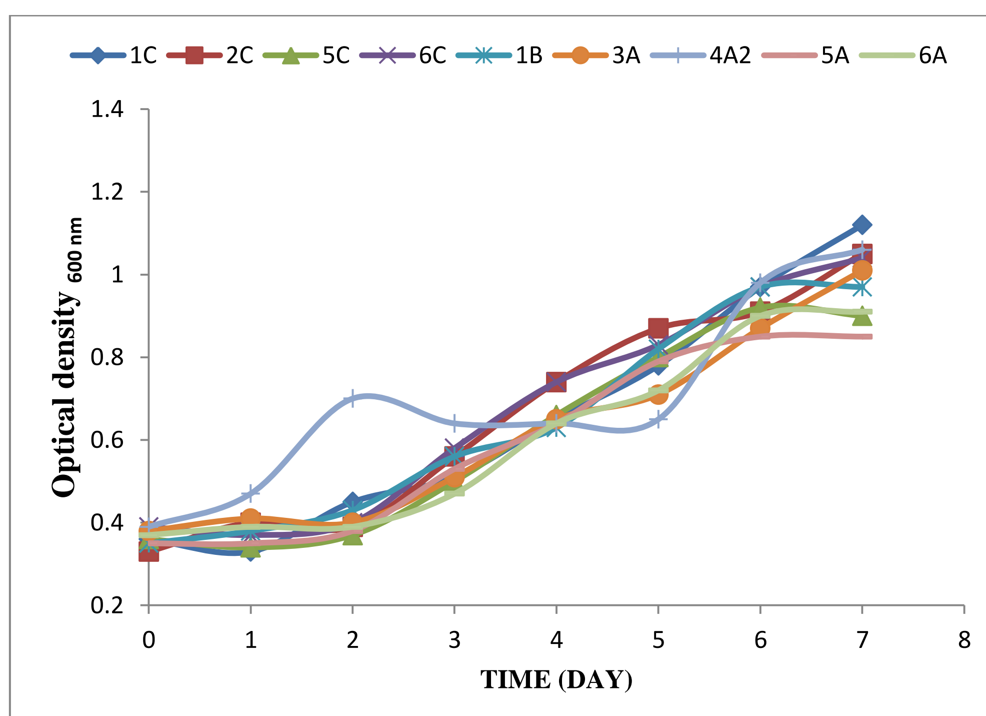


Figure 7: Time course of growth for bacterial isolates cultured in mineral salt media supplemented with 1 % heating oil for 7 days.

4.2.2 Degradation of individual hydrocarbons

It was found that isolates 1C, 3A, 2C, 6C and 4A2 utilized hexane vapor as indicated by growth on BHA plates while isolates 1C, 3A and 4A2 could utilize octane as the sole carbon source (Table 3). Only one isolate; 4A2, was able to utilize toluene, an aromatic hydrocarbon categorized under BTEX compounds as shown in Table 3.

Table 3: Growth of isolates cultured in BHA plates exposed to toluene, hexane and octane hydrocarbons after fourteen days of incubation at 25 °C

Isolate	10 % Toluene + 90 % Hexadecane	20 % Hexane + 80 % Hexadecane	20 % Octane + 80 % Hexadecane
1C	-	+	+
2C	-	+	-
5C	-	-	-
6C	-	+	-
1B	-	-	-
3A	-	+	+
4A2	+	+	+
5A	-	-	-
6A	-	-	-

Key: + denotes growth, - denotes no growth

4.3.0 Characterization

4.3.1 Morphological characterization

4.3.1.1 Colony characteristics

Variation in morphological characteristics of the colonies suggests that the selected isolates were different from each other. Plates of four of the nine selected isolates are shown in Figure 8. The rest of the selected isolates had diverse colony characteristics as summarized in Table 4.

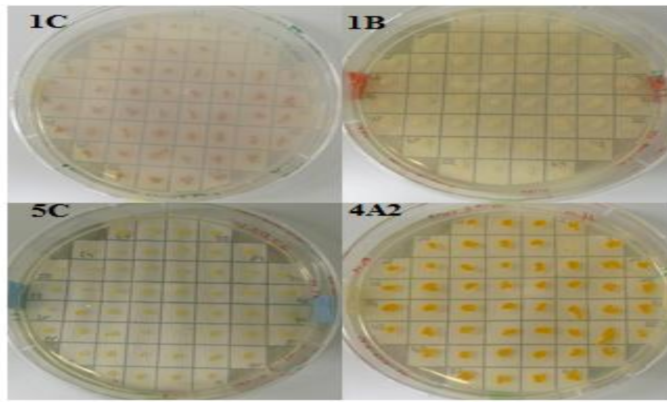


Figure 8: Plates of pure bacterial cultures of isolates 1C, 1B, 5C and 4A2 indicating different colors of colonies on LB agar plates.

4.3.1.2 Gram's iodine staining and Potassium hydroxide (KOH) test

All isolates were examined by Gram's staining reaction to differentiate between Gram positive and Gram negative bacteria. Table 4 shows Gram's stain and KOH test results of some of the selected isolates. Only isolate 1A (not included in the table) was found to be Gram positive (KOH negative) while the rest were Gram negative (KOH positive). Isolates 1C and 2C were rod-shaped while the rest were cocci-shaped.

Gram-positive bacteria have a thick mesh-like cell wall made up of 50-90% peptidoglycan while Gram-negative bacteria have a thinner cell wall with an additional lipid-rich outer membrane. Gram-positive bacteria are as a result able to retain the bluish-purple crystal violet dye during staining while Gram-negative bacteria retain the reddish-pink safranin counter stain.

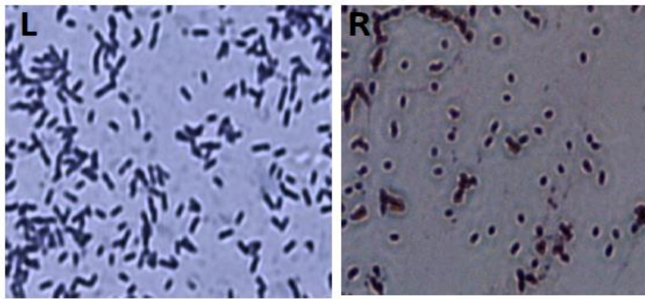


Figure 9: Gram's iodine test images. (L) Gram's positive rod-shaped bacteria (R) Gram's negative cocci-shaped bacteria.

Table 4: Morphological characteristics of selected oil degrading bacteria cultured on LB agar plates

	COLONY CHARACTERISTICS			GRAM'S STAINING AND KOH TEST		
ISOLATE	COLONY FORMS	COLOUR	MARGIN	GRAM'S IODINE STAINING	KOH TEST	SHAPE OF CELLS
1C	Irregular	Pale brown	Undulate	-	+	Rods
2C	Circular	Cream white	Entire	-	+	Rods
5C	Irregular	Cream yellow	Lobate	-	+	Cocci
6C	Circular	White	Curled	-	+	Cocci
1B	Irregular	Cream white	Undulate	-	+	Cocci
3A	Circular	Cream white	Undulate	-	+	Cocci
4A2	Irregular	Yellow	Lobate	-	+	Cocci
5A	Irregular	Cream	Undulate	-	+	Cocci
6A	Irregular	Cream	Curled	-	+	Cocci

4.3.2 Biochemical tests

4.3.2.1 Starch hydrolysis test

Isolate 4A2 was positive for starch hydrolysis test after 48 hours of incubation on starch agar plates. Starch hydrolysis was indicated by formation of a clear halo around the bacterial streaks as a result of amylase enzyme activity (Figure 10). The rest of the isolates gave negative results indicating their inability to hydrolyze starch (Table 5).

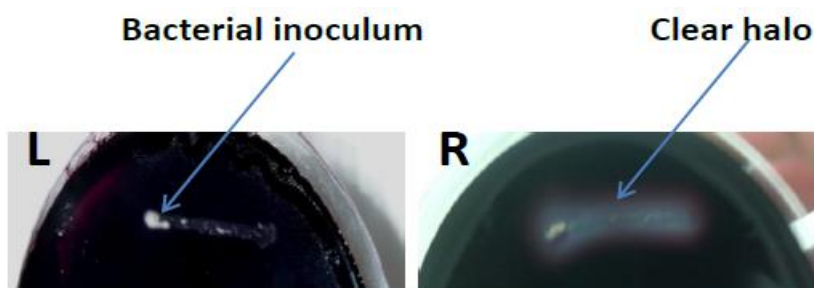


Figure 10: Starch hydrolysis test**.** (L) Starch agar plate for isolate 2C indicating absence of amylase enzyme activity, (R) starch agar plate for isolate 4A2 showing a clear zone round the bacterial streaks indicating amylase enzyme activity.

4.3.2.2 Catalase test

Gas bubbles were observed when all the bacterial cultures were separately mixed with hydrogen peroxide implying positive catalase enzyme activity (Table 5).

Table 5: Catalase test and starch utilization test for selected isolates

Isolate	1C	2C	5C	6C	1B	3A	4A2	5A	6A
Catalase Test	+	+	+	+	+	+	+	+	+
Starch Test	-	-	-	-	-	-	+	-	-

+ denotes positive test, - denotes negative test

4.3.2.3 Carbohydrate fermentation test

Ability of selected isolates to ferment carbohydrates was determined using phenol red carbohydrate broth. Phenol red dye in phenol red carbohydrate broth acts as an indicator to detect change in pH of the media during fermentation process. The dye is red at neutral pH and yellow at acidic pH indicating fermentation. Figure 11 shows color changes in phenol red with fermenting and non-fermenting isolates. Turbidity observed in the tubes with negative results indicates utilization of alternative substrates. Some isolates were able to ferment particular carbohydrates leading to

change in color of the broth to yellow as a result of formation of organic acids, and in some cases this was accompanied by gas production as shown in Figure 11. A summary of the carbohydrate fermentation test results is given in Table 6.

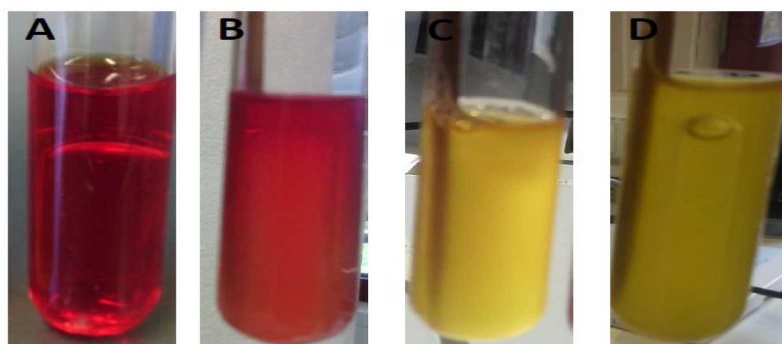


Figure 11: Phenol red fermentation test. (A) phenol red broth without inoculum (control), (B) Inoculated phenol red broth indicating negative fermentation results, (C) Inoculated phenol red broth indicating positive fermentation reaction, (D) Positive fermentation accompanied by gas production.

Table 6: Carbohydrate fermentation tests for selected isolates

ISOLATE	GLUCOSE FERMENTATION	FRUCTOSE FERMENTATION	MALTOSE FERMENTATION	SUCROSE FERMENTATION	GAS PRODUCTION
1C	-	-	-	-	-
2C	+	+	+	+	+
5C	+	+	+	+	+
6C	+	+	+	+	-
1B	+	+	+	+	-
3A	-	-	-	-	-
4A2	-	-	-	-	-
5A	+	+	+	+	+
6A	+	+	+	+	-

+ denotes positive reaction, - denotes negative reaction

4.3.3 Molecular identification

4.3.3.1 Genomic DNA extraction and PCR amplification of 16S rDNA gene

Molecular identification of the isolates was carried out using respective sequenced 16S rDNA gene fragments. Sequenced 16S rDNA gene amplicons were compared with those in the NCBI database as shown in Table 7. Genomic DNA extraction was carried out for selected candidates; 3A, 4A2, 5A, 6A, 1B, 1C, 2C, 5C and 6C. The DNA concentrations obtained were between 80 and 150 ng/µl. Figure 12 depicts gel photographs of extracted genomic DNA of high molecular weight.

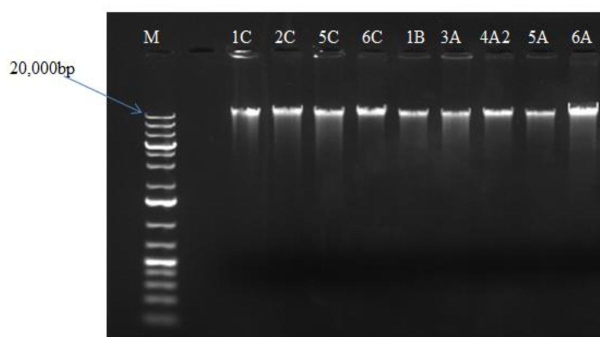


Figure 12: Agarose gel analysis of genomic DNA for isolates 1C, 2C, 5C, 6C, 1B, 3A, 4A2, 5A and 6A. M is the molecular marker [GeneRuler 1 kb plus, (ThermoFisher Scientific, USA)].

The gel image of 16S rDNA PCR amplicons (≈ 1500 bp DNA fragments) of selected bacterial isolates is shown in Figure 13.

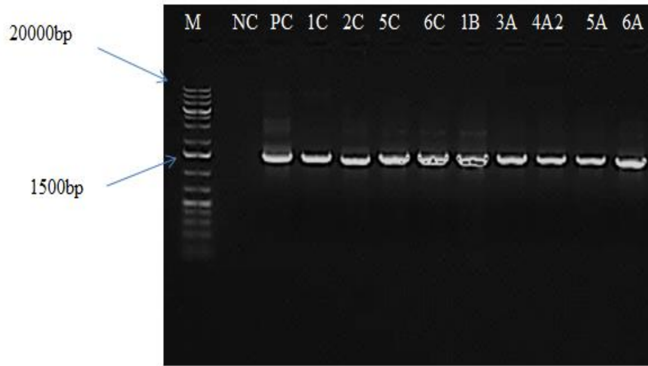
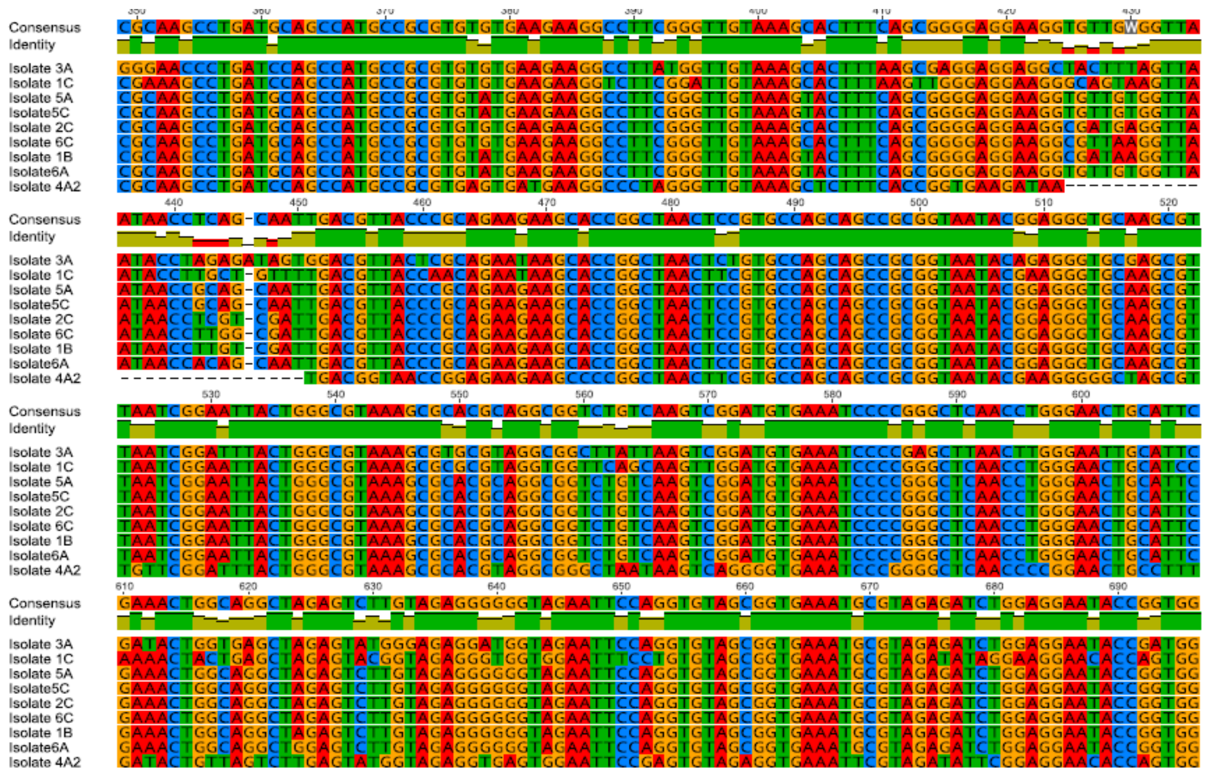


Figure 13: Gel image of 16S rDNA PCR amplicons for isolates 1C, 2C, 5C, 6C, 1B, 3A, 4A2, 5A and 6A. M is the molecular marker [GeneRuler 1 kb plus, (ThermoFisher Scientific, USA), NC is the negative control while PC is the positive control (*E. coli* 16S rDNA).

4.3.3.2 Alignment of 16S rDNA sequences

Multiple sequence alignment of the 16S rDNA sequences for the nine isolates using Clustal W alignment in Geneious® 9.1.4 software was carried out and a section of it is as shown in Figure 14. The full multiple sequence alignment is shown in appendix 14. From the multiple sequence alignment, nucleotides in positions 101, 153, 517, 729 and 1226 among others were noted to vary in the aligned sequences. For some sections, e.g. positions 26, 45, 65, 125, 442 and 1272, a less than 30 % similarity in nucleotides was observed in the alignment a strong indication that the isolates are different from each other.



Consensus graph: Solid green color: 100% identity, Green-brown: at least 30% and under 100% identity, Red: below 30% identity

Figure 14: A multiple sequence alignment (from nucleotide position 349 to 696) showing variation in nucleotide bases of the 16S rDNA gene fragments for the nine isolates. Sections highlighted with the same color show similarity in bases for that position.

4.3.3.3 Phylogenetic analysis of 16S rDNA gene sequences

A phylogenetic tree based on the BLAST search was constructed and the topological robustness of the tree evaluated using percentages of posterior probabilities. Figure 15 shows the phylogenetic tree for 16S rDNA gene sequences. The tree was constructed using Mr Bayes, a program for the Bayesian inference of phylogeny that is based on the Markov Chain Monte Carlo (MCMC) model. Numbers at the nodes show posterior probabilities as percentages.

The nine bacterial isolates from this study clustered with members of the following genera; *Pseudomonas, Acinetobacter, Ochrobactrum, Salmonella, Enterobacter and Klebsiella* (Figure 15). Table 7 shows the closest relatives to these isolates together with their percentage similarity as obtained from the search BLAST at the NCBI database. The sequences were deposited in NCBI Genbank and awarded accession numbers as indicated in the table 7.

Table 7: Closest relatives of selected bacteria based on 16S rDNA gene sequences

Isolate	Closest hit	Phylum: Proteobacteria	Accession number	Identity (%)
1C	*Pseudomonas aeruginosa*	Gammaproteobacteria	KX036860	99
2C	*Klebsiella variicola*	Gammaproteobacteria	KX036863	99
5C	*Enterobacter cloacae*	Gammaproteobacteria	KX036856	99
6C	*Klebsiella pneumoniae*	Gammaproteobacteria	KX036858	99
1B	*Enterobacter cloacae*	Gammaproteobacteria	KX036855	99
3A	*Acinetobacter baumannii*	Gammaproteobacteria	KX036861	99
4A2	*Ochrobactrum anthropi*	Alphaproteobacteria	KX036859	99
5A	*Enterobacter cloacae*	Gammaproteobacteria	KX036862	99
6A	*Salmonella enterica*	Gammaproteobacteria	KX036857	99

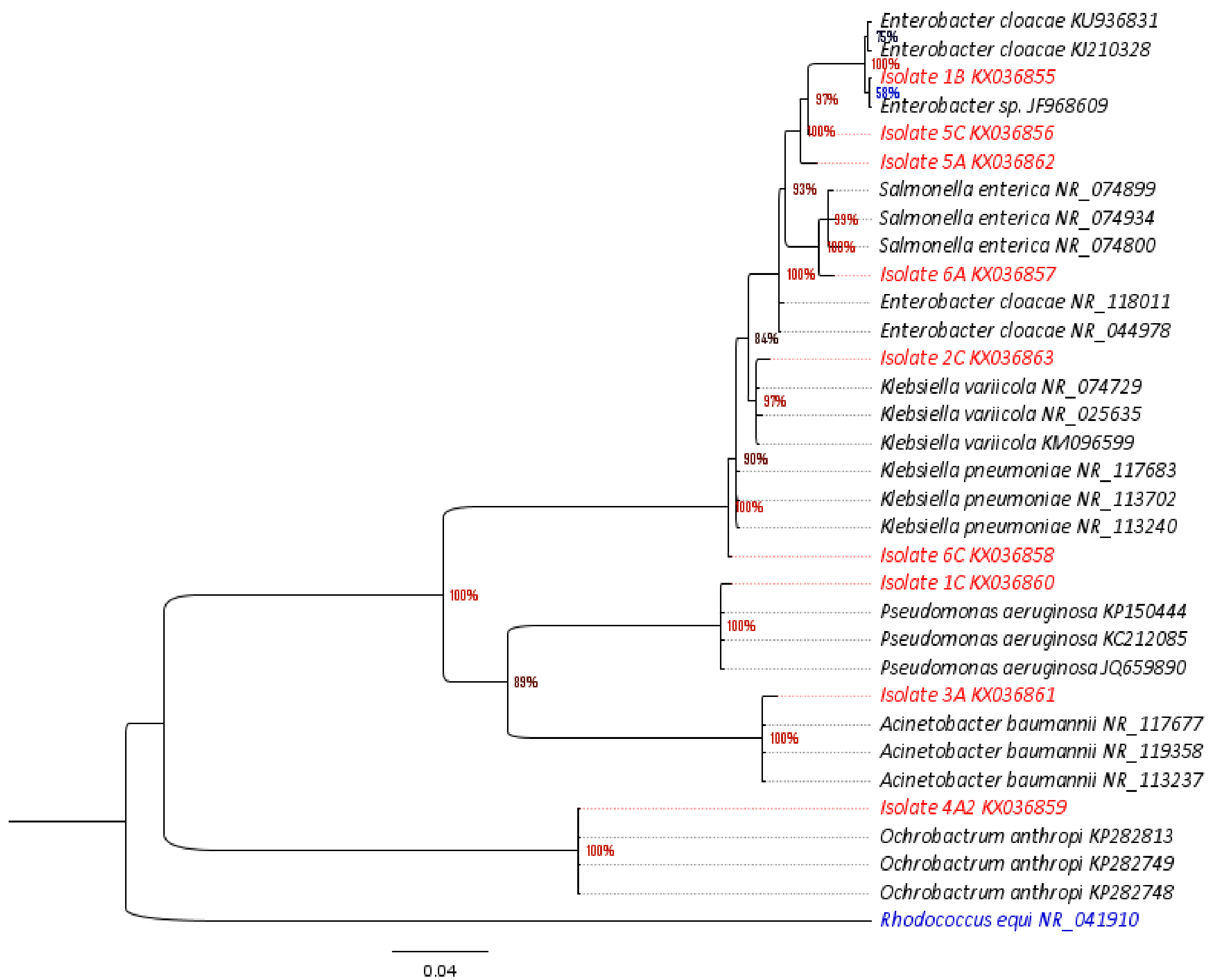


Figure 15: Phylogenetic tree based on 16S rDNA gene sequences. The tree was constructed using comparable 16S rDNA gene sequences of isolates from this study and those retrieved from the NCBI database. The numbers at the node indicate bootstrap values as percentages obtained with 1000 resampling analyses. Branch length unit is the number of substitutions per nucleotide position. Isolates from this study are shown in red. The tree is rooted using 16S rDNA sequence from *Rhodococcus equi* (shown in blue).

4.4.0 Optimization of growth conditions for diesel oil degrading bacteria

4.4.1 Effect of pH on bacterial growth during biodegradation of diesel oil

There was significant difference in diesel oil degradation depicted by microbial growth for the different pH values ($p<0.05$). Post hoc test was carried out using Tukey's test at a significance level of 0.05 as shown in Appendix 7. The optimum pH for microbial growth was 7 as shown in Figure 16. At this pH, maximum biomass obtained was 0.594 g/L, 0.742 g/L and 0.609 g/L for isolate 3A, 1C and 2C, respectively. Below and above pH 7, growth of the three isolates was reduced. Isolate 3A however, showed slight tolerance to alkaline pH compared to isolate 2C during the growth period.

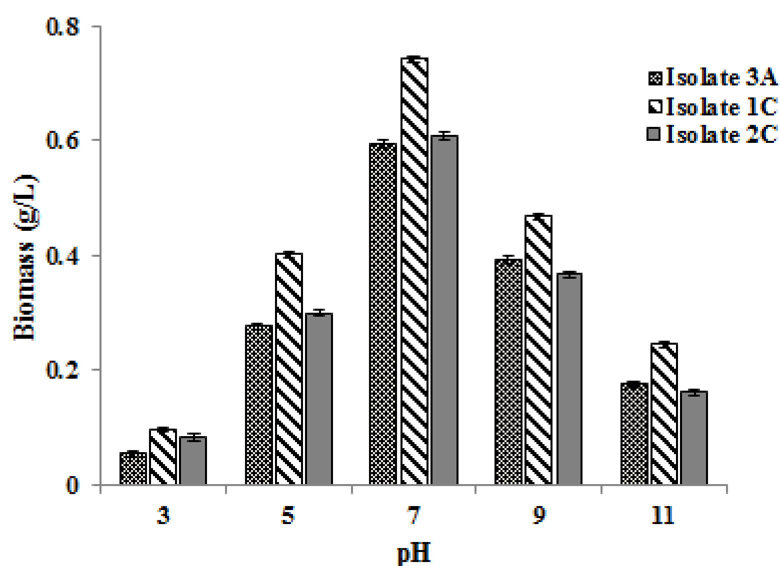


Figure 16: Effect of pH on growth of the three selected isolates. Bacterial growth was expressed in terms of biomass (g/L) after 7 days of incubation using BHM supplemented with diesel oil. Error bars have been displayed using standard error of the means. Microbial growth was highest at pH 7.

4.4.2 Effect of temperature on bacterial growth during biodegradation of diesel oil

To determine the influence of temperature on diesel oil degradation by the three selected isolates, growth was carried out at temperatures ranging from 25 °C to 55 °C and at the predetermined optimum pH. There was a significant variation in microbial growth at 25 °C, 37 °C and 55 °C with $p<0.05$. However, there was no significant difference in growth at 25 °C and 45 °C with $P = 0.153$ and at 30 °C and 37 °C with $P = 0.515$. Post hoc analysis for multiple comparisons was carried out using Tukey's test at a significance level of 0.05 as shown in Appendix 9. From these results, it is clear that a temperature range of between 30 °C and 37 °C is suitable for growth of the three selected bacterial isolates; 1C, 2C and 3A because this is where maximum biomass was obtained.

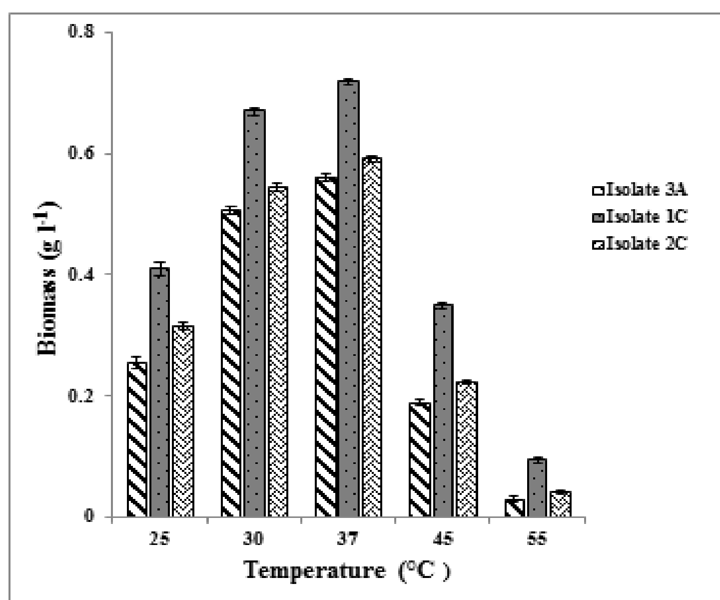


Figure 17: Effect of temperature on diesel oil degradation expressed in terms of biomass (g/L) after 7 days of incubation at pH 7. Error bars have been displayed using the standard error of the means. Optimum temperature for diesel oil biodegradation indicated by microbial growth was recorded at 37 °C for all the three isolates.

4.4.3 Effect of concentration of diesel oil on bacterial growth during biodegradation

Microbial growth decreased with increase in diesel oil concentration as shown in Figure 18. There was a significant difference in bacterial growth at diesel oil concentrations of 0.5%, 1%, 3% and 5% with P of 0.000 as shown in Appendix 10. Post hoc test was carried out using Tukey's test at a significance level of 0.05 as shown in Appendix 11. Notably, maximum biomass at 0.5% diesel oil concentration was obtained earlier, that is, on the 4th day during the culturing period compared to the rest of the increased diesel oil concentrations for all the three isolates. At 0.5% diesel oil concentration, maximum biomass obtained for isolate 3A, 1C and 2C was 0.757 g/L, 0.885 g/L and 0.7843 g/L, respectively.

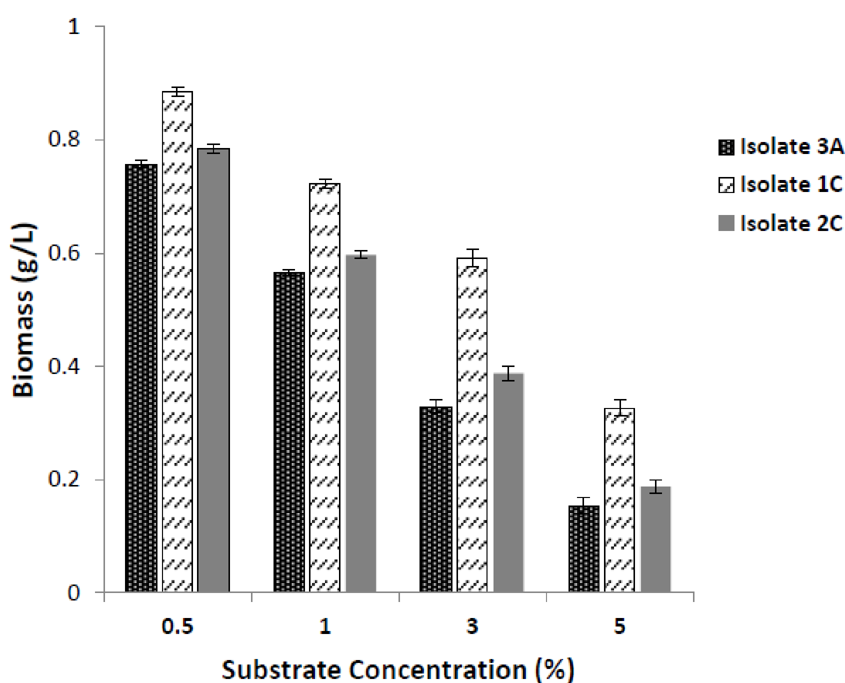


Figure 18: Effect of substrate concentration (diesel oil) on microbial growth in terms of biomass (g/L) after 7 days of incubation at 37 °C. Error bars have been displayed using standard error of the means. Optimum growth was recorded at diesel oil concentration of 0.5%.

4.4.4 Effect of nitrogen source on bacterial growth during diesel oil biodegradation

The effect of nitrogen source on diesel oil degradation by isolates 1C, 2C and 3A is presented in Figure 19. The test was carried out using ammonium nitrate, yeast extract and tryptone as nitrogen sources at predetermined optimum pH and temperature. Significant variation in microbial growth was observed for the three nitrogen sources with $p<0.05$ as shown in Appendix 12. Post hoc analysis was carried out using Tukey's test at a significance level of 0.05 as shown in Appendix 13. From these results, microbial growth was highest in presence of yeast extract. With yeast extract as a nitrogen source, maximum biomass obtained was 1.344 g/L, 1.163 g/L and 0.972g/L for isolate 3A, 1C and 2C, respectively.

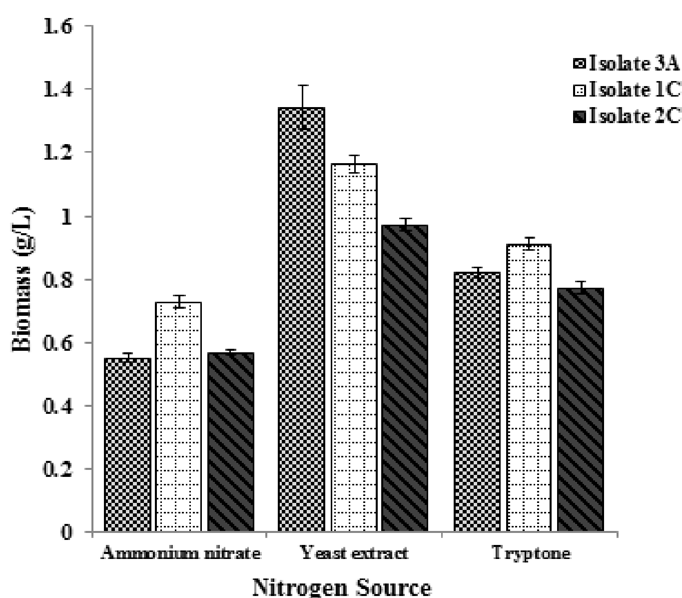
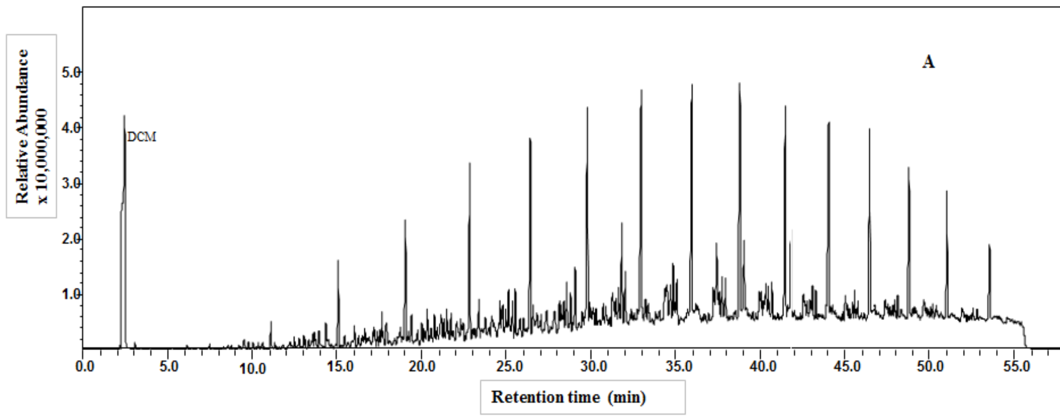


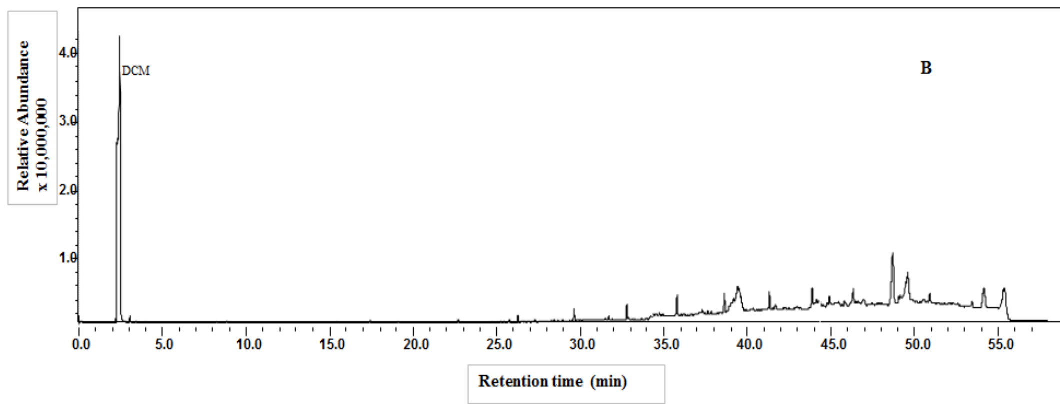
Figure 19: Effect of nitrogen source on microbial growth during diesel oil degradation. Growth expressed in terms of biomass (g/L) after 7 days of incubation at pH 7 and 37 °C. Error bars have been displayed using standard error of the means. For all the three isolates, microbial growth was highest using yeast extract as the nitrogen source followed by tryptone and then ammonium nitrate.

4.5.0 Analysis of diesel oil degradation by Gas Chromatography-Mass Spectrometry

4.5.1 GC-MS profiles of diesel oil biodegradation

The ability of isolates 1C, 2C, 3A and 4A2 to utilize diesel oil was determined through quantitative comparison of diesel oil extracted from inoculated BHM with a control obtained from un-inoculated media. Gas chromatogram profiles are shown in Figure 20. The data obtained shows that the isolates were capable of readily mineralizing most of the hydrocarbons present in diesel oil. This is indicated by decrease in relative abundance shown by the size of hydrocarbon peaks. Comparison of chromatogram profile of the control and the different isolates indicate that most of the branched chain and cyclic alkanes as well as aromatic hydrocarbons were completely degraded as is indicated by disappeared peaks. Peaks representing linear alkanes were still present though in reduced size. New peaks indicating formation of metabolic intermediates were also observed. Appendices 15 to 19 shows the identity and size of the different peaks as identified by comparison of their retention times and mass spectra in the mass spectrometer database.


A
DCM
Relative Abundance x 10,000,000
5.0
4.0
3.0
2.0
1.0
0.0
5.0
10.0
15.0
20.0
25.0
30.0
35.0
40.0
45.0
50.0
55.0
Retention time (min)


B
DCM
Relative Abundance x 10,000,000
4.0
3.0
2.0
1.0
0.0
5.0
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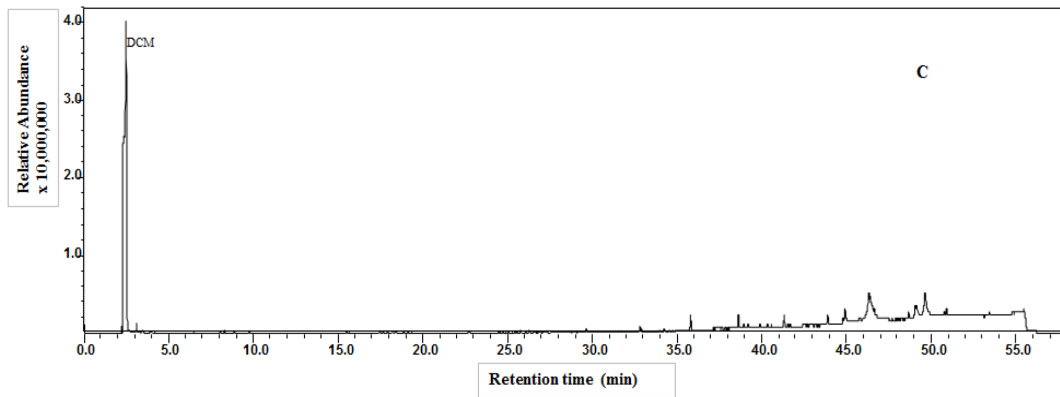
25.0
30.0
35.0
40.0
45.0
50.0
55.0
Retention time (min)


C
DCM
Relative Abundance x 10,000,000
4.0
3.0
2.0
1.0
0.0
5.0
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30.0
35.0
40.0
45.0
50.0
55.0
Retention time (min)

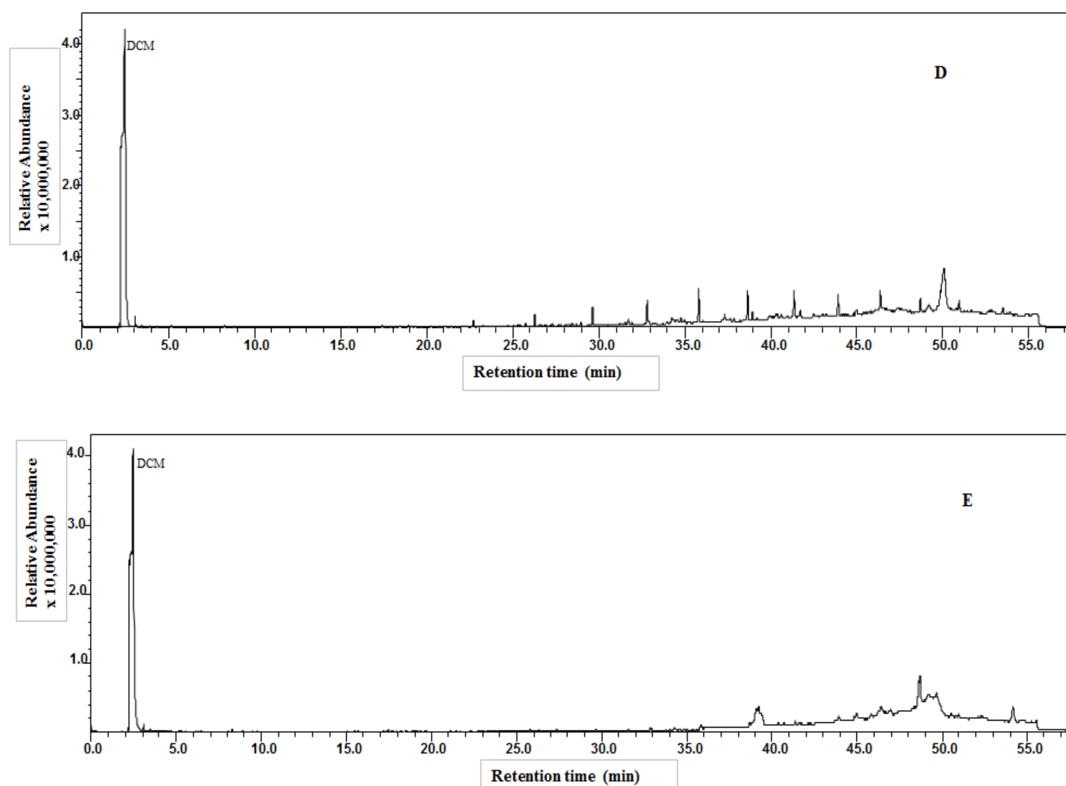


Figure 20: GC-MS profiles of diesel oil extracted from BHM after 21 days of incubation at pH 7.0 and 37° C with and without inoculation. (A) Control (uninoculated); (B) Isolate 1C; (C) Isolate 2C; (D) Isolate 3A and (E) Isolate 4A2. Diesel oil extraction was performed using dichloromethane (DCM) as the solvent. For identification of the peaks and peak size for GC-MS profiles A through E, see Appendices 15 to 19.

It was observed that most of the hydrocarbons present in control media are absent in inoculated media implying bacterial degradation activity. A summary of hydrocarbons identified in control media and those identified in media inoculated with the different isolates is given in Table 8. Mono-aromatics such as Benzene, 1,2,3-trimethyl- and 5-Ethyl-m-xylene and polycyclic aromatic hydrocarbons such as Naphthalene, 2-ethyldecahydro- and Naphthalene, 1,2,3,4-tetrahydro-2,5,8-trimethyl- were completely degraded by all the four isolates. Most branched chain alkanes such as Decane, 2-methyl- and Eicosane, 2,4-dimethyl- were also completely depleted. With

the exception of Cyclohexane, (4-methylpentyl)-, the isolates were also able to completely degrade cycloalkanes present in diesel oil. Conversely, almost all linear alkanes were still present at the end of the incubation period though in low levels. Isoprenoid hydrocarbons, phytane (hexadecane, 2,6,10,14-tetramethyl) and pristine (pentadecane, 2,6,10,14-tetramethyl) were also not completely degraded.

Table 8: Comparison list of hydrocarbons identified in un-inoculated (control) media and media inoculated with isolates 1C, 2C, 3A and 4A2

Ret. Time (min)	Compound Name	Chemical formula	Control	Isolate			
				1C	2C	3A	4A2
11.075	Nonane	C_9H_{20}	+	−	−	−	−
13.32	Benzene, 1,2,3-trimethyl-	C_9H_{12}	+	−	−	−	−
15.048	Undecane	$C_{11}H_{24}$	+	−	−	+	−
16.002	Decane, 4-methyl-	$C_{11}H_{24}$	+	−	−	−	−
16.61	Benzene, 1-methyl-2-propyl-	$C_{10}H_{14}$	+	−	−	−	−
17.613	Decane, 2-methyl-	$C_{11}H_{24}$	+	−	−	−	−
17.863	Decane, 3-methyl-	$C_{11}H_{24}$	+	−	−	−	−
19.366	trans-Decalin, 2-methyl-	$C_{11}H_{20}$	+	−	−	−	−
20.505	5-Ethyl-m-xylene	$C_{10}H_{14}$	+	−	−	−	−
20.714	Hexane, 2-phenyl-3-propyl-	$C_{15}H_{24}$	+	−	−	−	−
21.281	Dodecane, 4-methyl-	$C_{13}H_{28}$	+	−	−	−	−
21.446	Undecane, 2-methyl-	$C_{12}H_{26}$	+	−	−	−	−
21.69	Undecane, 3-methyl-	$C_{12}H_{26}$	+	−	−	−	−
22.788	Dodecane	$C_{12}H_{26}$	+	−	−	+	+
23.335	Undecane, 3,6-dimethyl-	$C_{13}H_{28}$	+	−	−	−	−
23.46	7-Ethylidenebicyclo[4.2.1]nona-2,4-diene	$C_{11}H_{14}$	+	−	−	−	−
23.702	Naphthalene, 2-ethyldecahydro-	$C_{12}H_{22}$	+	−	−	−	−
24.09	Benzene, (3-methyl-2-butenyl)-	$C_{11}H_{14}$	+	−	−	−	−
24.59	Naphthalene, 1,2,3,4-tetrahydro-5-methyl-	$C_{11}H_{14}$	+	−	−	−	−
24.68	Dodecane, 6-methyl-	$C_{13}H_{28}$	+	−	−	−	−
24.746	Nonane, 5-(2-methylpropyl)-	$C_{13}H_{28}$	+	−	+	+	+
25.083	Octadecane	$C_{18}H_{38}$	+	−	−	−	−

25.315	Dodecane, 3-methyl-	$C_{13}H_{28}$	+	−	−	+	−
25.475	Octane, 3,6-dimethyl-	$C_{10}H_{22}$	+	−	−	−	−
25.961	Cyclopentane, 1-methyl-3-(2-methylpropyl)-	$C_{10}H_{20}$	+	−	−	−	−
26.37	Pentadecane	$C_{15}H_{32}$	+	+	+	+	+
26.538	Naphthalene, 1,2,3,4-tetrahydro-2,6-dimethyl-	$C_{12}H_{16}$	+	−	−	−	−
27.336	1,7,7-Trimethyl-2-vinylbicyclo[2.2.1]hept-2-ene	$C_{12}H_{18}$	+	−	−	−	−
27.795	Cyclohexane, (cyclopentylmethyl)-	$C_{12}H_{22}$	+	−	−	−	−
28.081	Tridecane, 6-methyl-	$C_{14}H_{30}$	+	−	−	+	−
28.189	Tridecane, 5-methyl-	$C_{14}H_{30}$	+	−	−	−	−
28.35	Tridecane, 4-methyl-	$C_{14}H_{30}$	+	−	−	+	−
28.518	Eicosane, 10-methyl-	$C_{21}H_{44}$	+	−	−	−	−
28.741	Tetradecane, 3-methyl-	$C_{15}H_{32}$	+	+	−	+	−
29.02	Nonane, 3-methyl-5-propyl-	$C_{13}H_{28}$	+	−	−	−	−
29.744	Tetradecane	$C_{14}H_{30}$	+	+	+	+	+
30.07	Hexadecane	$C_{16}H_{34}$	+	+	+	+	+
30.256	Nonadecane	$C_{19}H_{40}$	+	+	+	+	+
30.832	Naphthalene, 1,2,3,4-tetrahydro-2,5,8-trimethyl-	$C_{13}H_{18}$	+	−	−	−	−
31.224	Cyclohexane, (4-methylpentyl)-	$C_{12}H_{24}$	+	+	+	+	+
31.32	Undecane, 6-methyl-	$C_{12}H_{26}$	+	−	−	−	−
31.415	Tetradecane, 5-methyl-	$C_{15}H_{32}$	+	−	−	+	−
31.592	Tetradecane, 4-methyl-	$C_{15}H_{32}$	+	−	−	+	−
32.925	Heptadecane	$C_{17}H_{36}$	+	+	+	+	−
33.185	Hexadecane, 7,9-dimethyl-	$C_{18}H_{38}$	+	−	−	−	−
34.309	Decane, 5-propyl-	$C_{13}H_{28}$	+	−	−	−	−
34.424	n-Nonyl cyclohexane	$C_{15}H_{30}$	+	−	−	−	−
34.644	Pentadecane, 4-methyl-	$C_{16}H_{34}$	+	−	−	+	−
34.81	Tetradecane, 2,6,10-trimethyl-	$C_{17}H_{36}$	+	−	−	+	−
34.94	Eicosane	$C_{20}H_{42}$	+	+	+	+	+
35.026	Pentadecane, 3-methyl-	$C_{16}H_{34}$	+	−	−	+	−
37.194	Heptadecane, 8-methyl-	$C_{18}H_{38}$	+	−	−	−	−
37.46	Cyclohexane, 1,1'-(1,3-propanediyl)bis-	$C_{15}H_{28}$	+	−	−	−	−
37.7	Hexadecane, 2-methyl-	$C_{17}H_{36}$	+	−	+	+	−
39.004	Pentadecane, 2,6,10,14-tetramethyl	$C_{19}H_{40}$	+	+	+	+	−

40.019	Pentadecane, 6-methyl-	$C_{16}H_{34}$	+	−	−	−	−
40.438	Heptadecane, 2-methyl-	$C_{18}H_{38}$	+	−	−	−	−
40.653	Heptadecane, 3-methyl-	$C_{18}H_{38}$	+	−	−	+	−
41.448	Heneicosane	$C_{21}H_{44}$	+	+	+	+	+
41.775	Hexadecane, 2,6,10,14-tetramethyl	$C_{20}H_{42}$	+	+	+	+	+
42.515	Nonadecane, 9-methyl-	$C_{20}H_{42}$	+	−	−	−	−
42.635	Decane, 2,5-dimethyl-	$C_{12}H_{26}$	+	−	−	−	−
42.744	Eicosane, 2,4-dimethyl-	$C_{22}H_{46}$	+	−	−	−	−
43.054	Octadecane, 2-methyl-	$C_{19}H_{40}$	+	−	+	−	−
43.254	Octadecane, 3-methyl-	$C_{19}H_{40}$	+	−	−	−	−
45.396	Heptadecane, 9-octyl-	$C_{25}H_{52}$	+	−	−	−	−
45.532	Octacosane	$C_{28}H_{58}$	+	−	−	+	−
48.103	2-methyloctacosane	$C_{29}H_{60}$	+	+	−	+	+
53.53	Hentriacontane	$C_{29}H_{60}$	+	−	−	−	−

Key: + Denotes compound present, −Denotes compound absent

4.5.2 GC-MS analysis of possible intermediates of diesel oil degradation

A list of fatty acids, alcohols and aldehydes identified in inoculated media using GC-MS is shown in Table 9. These compounds are similarly absent in extract obtained from control media indicating possible intermediate metabolites. Fatty acids, fatty esters, aldehydes and alcohols are intermediates in alkane and aromatic aerobic degradation pathways. Very long n-alkanes (C>30) possibly resulting from polymerization of medium and short chain alkanes and short branched chains alkanes arising from disintegration of long chain alkanes were also detected. The retention times of these compounds are shown in Appendices 16 to 19.

Table 9: Possible intermediate metabolites identified in media inoculated with 1C, 2C, 3A and 4A2 bacterial isolates

Isolate 1C	Isolate 2C	Isolate 3A	Isolate 4A2
2-Isopropyl-5-methyl-1-heptanol$^{\theta}$	Phenylethyl alcohol$^{\theta}$	1-Butanol, 3-methyl-$^{\theta}$	1-Butanol, 3-methyl-$^{\theta}$
11-Methyldodecanol$^{\theta}$	2-Isopropyl-5-methyl-1-heptanol$^{\theta}$	1-Octanol, 2,7-dimethyl-$^{\theta}$	1-Octanol, 3,7-dimethyl-$^{\theta}$
1-Tetradecanol$^{\theta}$	11-Methyldodecanol$^{\theta}$	Phenylethyl alcohol$^{\theta}$	Phenylethyl alcohol$^{\theta}$
1-Decanol, 2-hexyl-$^{\theta}$	n-Hexadecanoic acid*	2-Isopropyl-5-methyl-1-heptanol$^{\theta}$	2-Isopropyl-5-methyl-1-heptanol$^{\theta}$
1-Dodecanol, 2-hexyl-$^{\theta}$	1-Dodecanol, 2-hexyl-$^{\theta}$	n-Pentadecanol$^{\theta}$	11-Methyldodecanol$^{\theta}$
2-Isopropyl-5-methylhex-2-enal $^{\gamma}$	Oleic Acid*	11-Methyldodecanol$^{\theta}$	n-Hexadecanoic acid*
1-Dodecanol, 2-octyl-$^{\theta}$		1-Decanol, 2-hexyl-$^{\theta}$	Dodecanoic acid, ethenyl ester*
4-Cyclohexyl-1-butanol$^{\theta}$		1-Hexacosanol$^{\theta}$	Oleic acid*
Valtrate*		1-Heptacosanol$^{\theta}$	
n-Hexadecanoic acid*		Octacosanol$^{\theta}$	
cis-Vaccenic acid*		n-Hexadecanoic acid*	
cis-13-Eicosenoic acid*		7-Hexadecenal, (Z)- $^{\gamma}$	
Oleic Acid*		Octadecanoic acid*	

Key: * Fatty acid $^{\theta}$ Alcohol $^{\gamma}$ Aldehyde

4.6.0 AlkB gene PCR amplification

Alkane hydroxylase (AlkB) gene from genomic DNA of isolate 1C was amplified and sequenced. PCR amplification of the gene was however unsuccessful in isolates 2C and 3A. The quality of PCR products obtained was analyzed on a 1% agarose gel stained with ethidium bromide. An electrophoresis gel of the PCR amplicon of 313 bp is shown in Figure 21. The obtained sequence was compared with already known alkB gene sequences at the NCBI database using BLAST algorithm. The sequence was deposited at the NCBI database under the accession number, KX036864.

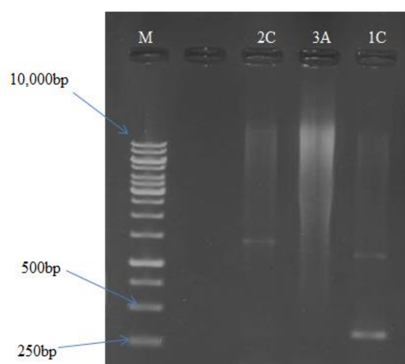
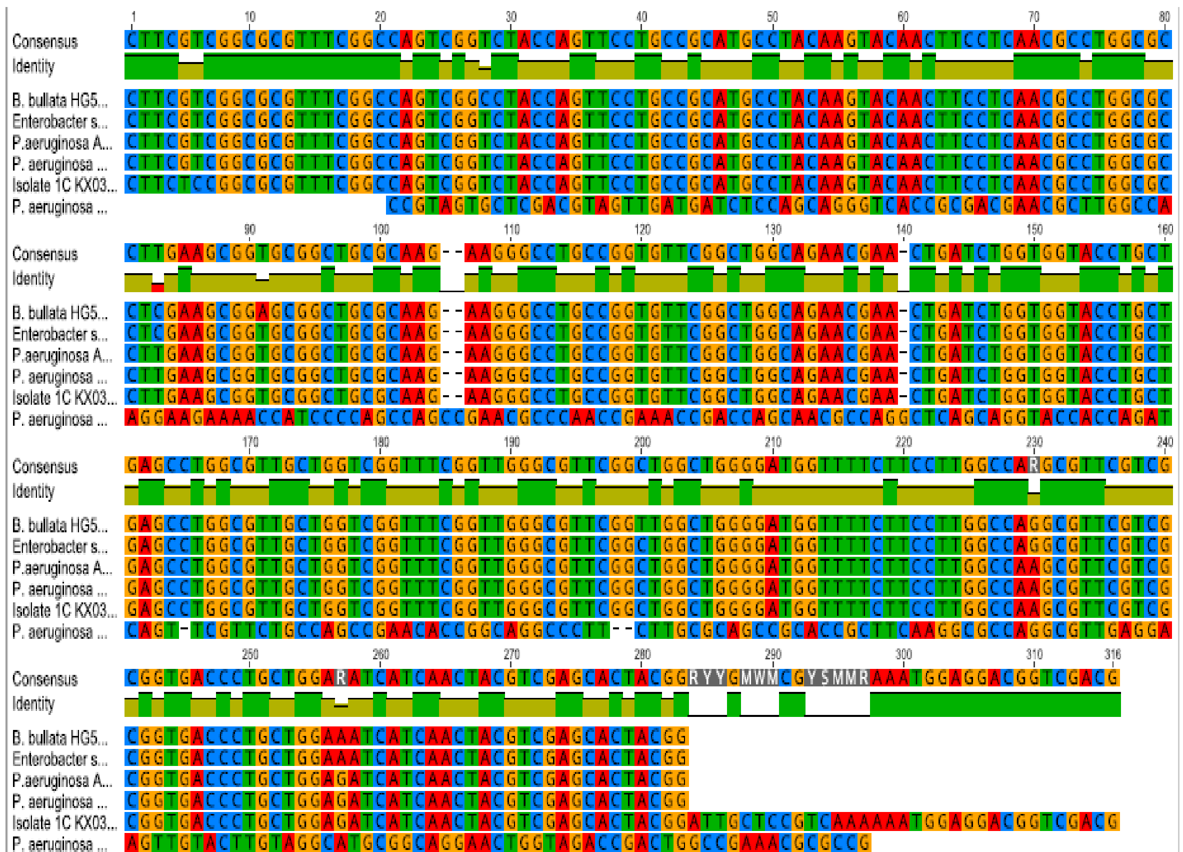


Figure 21: An electrophoresis gel image of alkB gene PCR amplicons for isolates 2C, 3A and 1C. M is the molecular marker {GeneRuler 1 kb (ThermoFisher Scientific, USA)}. Isolate 2C did not give the expected PCR product size while for isolate 3A, no PCR product was detected.

4.6.1 Alignment of alkB gene sequences

Multiple sequence alignment of the alkB gene sequences using Clustal W alignment in Geneious® 9.1.4 software indicates that nucleotides in positions 28, 79, 83, 91, 230 and 257 among others were noted to vary in the sequences as shown in figure 22. This indicates that the alkane hydroxylase coding sequences are different in the selected bacterial strains.



Consensus graph: Solid green color: 100% identity, Green-brown: at least 30% and under 100% identity, Red: below 30% identity

Figure 22: Multiple sequence alignment of alkB gene from isolate 1C and alkB gene sequences of five different bacterial strains obtained from the NCBI database.

4.6.2 Phylogenetic analysis of alkB gene sequences

A phylogenetic tree of the alkB gene obtained was constructed using sequences from BLAST search and the isolate 1C alkB sequence. The topological robustness of the tree was evaluated using percentages of posterior probabilities. Figure 23 shows the phylogenetic tree for alkB gene sequences constructed using Bayesian phylogenetic method in MrBayes.

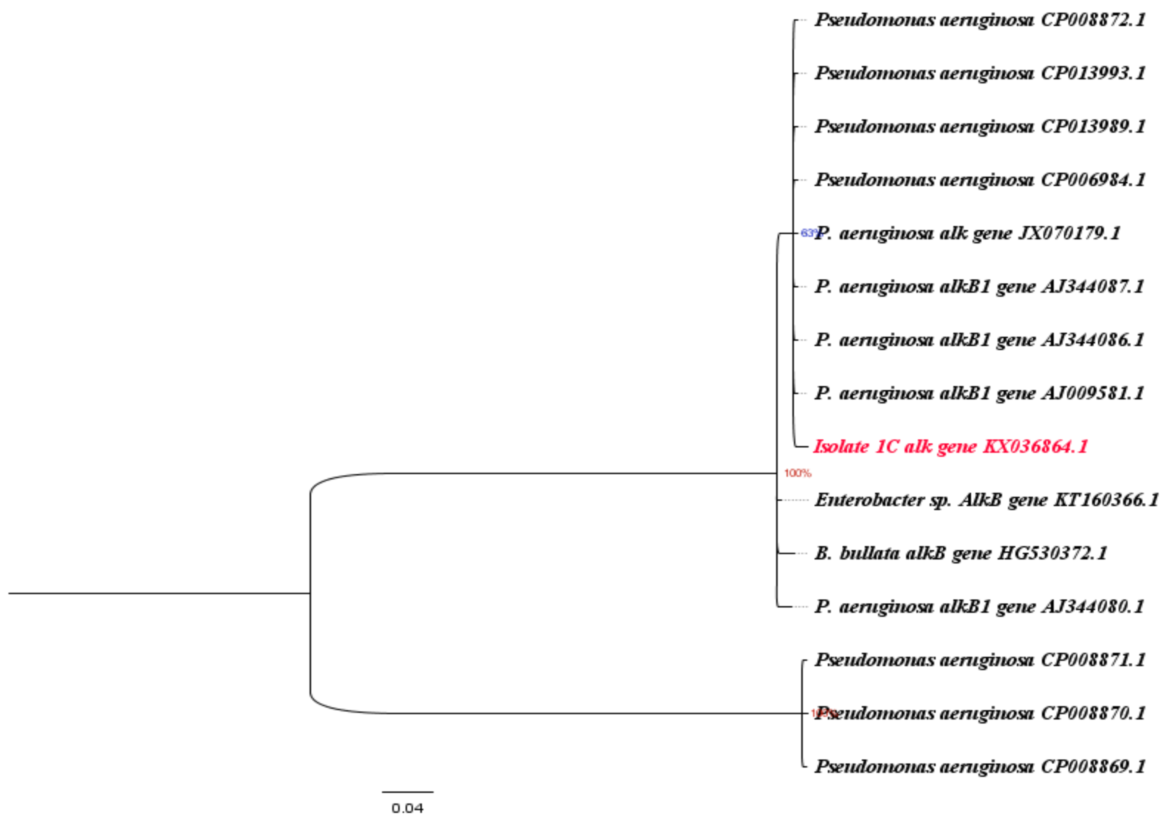


Figure 23: Phylogenetic relationship of isolate 1C alkB gene (shown in red) with alkB gene of 14 closely related bacterial strains from NCBI database. The tree is rooted at mid-point. The numbers at the node show bootstrap values as percentages obtained with 1000 resampling analyses. Branch length unit (0.04) represents the number of substitutions per nucleotide site.

CHAPTER FIVE

5.0 DISCUSSION

5.1.0 Discussion

Petroleum products are a major source of energy for industries and daily life. Increased oil explorations in the East African region and the anticipated oil spillages that occur during routine operations and transportation have raised concerns of environmental pollution (Gagandeep & Malik, 2013). As the utilization of petroleum oil products increases, soil, surface and ground water contamination with these products is becoming a major environmental concern (Hamza *et al.,* 2010). In particular, contamination with spilled diesel (Hamza *et al.,* 2010) and engine oil (Mandri & Lin, 2007) is a major problem as these products end up in soil and eventually in water bodies (Afuwale & Modi, 2012).

The use of bioremediation as opposed to physicochemical methods to counter this problem is emerging as a more efficient, economical and effective strategy (Mandri & Lin, 2007). Bacteria in particular have been identified as the most active agents in mitigation of petroleum oil pollution (Gagandeep & Malik, 2013). Thus, attempts to isolate hydrocarbon degrading microorganisms from different environments have led to isolation of a wide variety of potential bacterial candidates amenable to multiple biotechnological applications including bioremediation (Margesin & Schinner, 2001; Yakimov *et al.,* 2007; Mahjoubi *et al.,* 2013). Hydrocarbonoclastic bacteria tolerate oil-contaminated environments since they utilize hydrocarbons in the oil contaminants as energy sources. They are thus ideal for bioremediation as non-degrading bacteria are gradually eliminated (Mahjoubi *et al.,* 2013).

5.1.1 Isolation of hydrocarbon degrading bacteria

In this study, hydrocarbon degraders were isolated from auto garage contaminated soils from Ngara region in Nairobi-Kenya. Bushnell Haas media (BHM) supplemented with used engine oil was used as the sole carbon source for energy and subsequent purification carried out using LB media. As shown in Table 2, a total of

20 bacterial and 1 fungal isolate was obtained. Similar to this study, used engine oil has also been applied in other isolation studies. For instance, Mandri & Lin (2007) isolated three bacterial strains, *P. aeruginosa, Flavobacterium sp.,* and *A. calcoaceticum* using used engine oil while Gagandeep & Malik (2013), isolated nineteen different bacteria cultures using minimal media supplemented with 0.5% 2T engine oil as the sole carbon source.

5.1.2.0 Screening for microbial degradation of various hydrocarbons

5.1.2.1 Degradation of mixed hydrocarbons

Petroleum is typically found as a complex mixture consisting of a variety of hydrocarbons which include; aliphatics, heterocyclic alkanes and mono-aromatics as well as polyaromatics hence biodegradation studies using a mixture of hydrocarbon substrates is essential in biological treatment of petroleum contaminated sites (Katsivela *et al.,* 2003; Tebyanian *et al.,* 2013).

In this study, selection of efficient hydrocarbon degraders was based on ability to grow in mineral salt media supplemented with heating oil. Nine isolates, 3A, 4A2, 5A, 6A, 1B, 1C, 2C, 5C and 6C demonstrated high growth as indicated by increase in optical density readings at 600 nm. Post hoc analysis as shown in Appendix 5 showed that there was no significant difference in microbial growth of all isolates up to day 2 with $p > 0.05$ as was also evident from the growth curve (Figure 7). This was probably due to unavailability of enzymes (before induction) involved in mineralization of the complex hydrocarbons and/or minimal production of secondary metabolites important for microbial growth (Tebyanian *et al.,* 2013). Significant growth of $p < 0.05$ was later noted on day 3 to 7 after which growth remained fairly constant after the 7th day. This stationary phase may be attributed to the need by the cell to adapt continuously to more complex hydrocarbons, depletion of degradable substrates and/or accumulation of waste products.

For isolates 4A2 and 2A (included in appendix 3 only) the curves indicate a diauxic growth which is a common feature of microbes that utilize two or more sources of carbon at a time (Silva *et al.*, 2006). For isolate 4A2, the first stage of growth was observed between the first and second day of incubation in which simple hydrocarbons such as n-alkanes and some alkylic chains are possibly degraded. A stationary phase was then observed between the 3rd and 5th day and later a second exponential phase noted after the 5th day. More complex hydrocarbon molecules such as mono- and poly-aromatics are thought to be degraded during this phase.

5.1.2.2 Degradation of individual hydrocarbons

The structure of hydrocarbons is key in their biodegradability (Ivey, 2006). Among various classes of hydrocarbons, alkanes and n-alkyl-aromatics with medium length chain (C10-C22) are favorable substrates for microbes hence are rapidly biodegraded (Tebyanian *et al.*, 2013). Short-chain alkanes (C5-C9) on the other hand possess high membrane toxicity while long-chain alkanes (>C22) have low water solubility and sorption into surfaces hence reduced bioavailability and consequently biodegradation (Ivey, 2006; Tebyanian *et al.,* 2013).

The ability of the bacterial isolates to utilize individual hydrocarbons such as hexane (C6), octane (C8) and toluene (methylbenzene) as carbon sources (see Table 3), showed that isolate 4A2 was able to utilze the three hydrocarbons. Bacterial strains 1C, 2C, 6C, 3A and 4A2 were able to utilize hexane while only 1C, 3A and 4A2 could grow on octane. Only isolate 4A2 could grow on plates exposed to toluene indicating its ability to wishstand toluene toxicity. Toluene is an aromatic hydrocarbon of the BTEX compounds (Benzine, Toluene, Ethylbenzene & Xylene), which are classified as priority pollutants.

5.1.3.0 Morphological, biochemical and molecular characterization

5.1.3.1 Morphological characterization

Gram's staining and potassium hydroxide tests revealed that majority of the isolates in this study were Gram negative cocci-shaped with a few Gram negative rods, a finding that is in agreement with other studies. Geetha and co-workers (2013) reported that majority of hydrocarbon degrading bacteria isolated from soils obtained from oil production sites were Gram negative cocco-bacilli with only a few being Gram positive. Mahjoubi and co-workers (2013) also reported dominance of the Gram-negative bacteria represented by three subclasses; the *gammaproteobacteria* (most abundant), *alphaproteobacteria* and the *betaproteobacteria* isolated from oil-contaminated environments. Dominance of Gram negative bacteria in oil contaminated sites has been attributed to their lipopolysaccharide membrane which can play the role of a biosurfactant accelerating the biodegradation process (Mahjoubi *et al.,* 2013).

5.1.3.2.0 Biochemical characterization

5.1.3.2.1 Catalase and starch hydrolysis tests

Aerobic and facultative anaerobic microorganisms synthesize enzymes capable of detoxifying toxic oxygen metabolites such as superoxide radicals, which can be converted to hydrogen peroxide by superoxide dismutase. Catalase-positive bacteria are then able to convert the hydrogen peroxide to water and gaseous oxygen. As expected, all the isolates in this study were found to possess catalase enzyme activity since they were isolated from oxygenated soil where they are required to neutralize toxic oxygen compounds. This implies that the isolates are either aerobic or facultatively anaerobic (Taylor & Achanzar, 1972). From the starch hydrolysis test, only isolate 4A2 was shown to possess amylase enzyme activity as shown in Table 5. The test is used to differentiate bacteria that possess the α-amylase or oligo- 1, 6-glucosidase enzymes which enable them to utilize starch.

5.1.3.2.2 Carbohydrate fermentation tests

During the process of fermentation, an organic substrate may serve as the final electron acceptor resulting in a variety of end products depending on the substrate undergoing fermentation, specific organism, enzymes involved and environmental conditions such as temperature. Depending on the specific reaction, a number of end products may result from fermentation including acids such as lactic acid, acetic acid and butyric acid as well as ethanol, carbon dioxide gas, hydrogen gas and other organic compounds (Hemraj *et al.,* 2013). Isolates 2C, 5C, and 5A were able to ferment glucose, fructose, maltose and sucrose accompanied by gas production. Isolates 6A, 6C and 1B could ferment glucose, fructose, maltose and sucrose with no accompanying gas production. The rest of the isolates were found to be non-fermenters. Carbohydrate fermenting microbes are essential in petroleum industry as some have been reported to produce biosurfactants which find applications in biodegradation of petroleum contaminants as well as in oil recovery (Asfora-Sarubbo *et al.,* 2006).

5.1.3.3 Molecular identification

Phylogenetic analysis of 16S rDNA gene

Based on their biodegradation ability, nine bacterial isolates were selected and identified using molecular tools. The use of morphological and/or biochemical tests for taxonomical classification of microorganisms has been widely used in microbiological studies (Silva *et al.,* 2006; Geetha e*t al.*, 2013; Tebyanian *et al.,* 2013). However, the large diversity and abundance of microbes that share similar features is a limitation to these tests. Currently, phylogenetic trees are being used to infer relatedness between organisms based on measurement of sequence diversity of chronometers such as 16S ribosomal DNA (16S rDNA), 23S ribosomal DNA (23S rDNA), and elongation factor TU (Rudi, 2008). 16S rDNA is a mosaic of hypervariable and conserved regions. Hypervariable regions have evolved over time while the conserved regions which flank the hypervarible regions are used as targets

to which primers are designed enabling their amplification through polymerase chain reaction (Jonasson *et al.,* 2007).

From the tree topology shown in Figure 15, isolates 5A, 1B and 5C clustered with members of the genus *Enterobacter* with a posterior probability of 100%, 100% and 97% respectively implying that these isolates are members of the genus *Enterobacter.* Isolates 2C and 6C clustered with members of the genus *Klebsiella* with a posterior probability of 97% and 100% respectively while isolate 1C clustered with members of the genus *Pseudomonas* with a posterior probability of 100% implying that the isolate is a *Pseudomonas.* Isolates 3A, 4A2 and 6A clustered with members of the genus *Acinetobacter, Ochrobactrum and Salmonella* each *w*ith a posterior probability of 100% implying that these isolates are *Acinetobacter, Ochrobactrum and Salmonella* respectively.

All these strains fall under the phylum proteobacteria with *Ochrobactrum* being an alpha proteobacteria while the rest are gamma proteobacteria as depicted also by clustering in the phylogenetic tree. Bacterial strains of subphyla α-, β- and ω proteobacteria are well established for their ability to degrade a wide variety of hydrocarbons (Dasgupta *et al.,* 2013; Mahjoubi *et al.,* 2013). Mahjoubi and co-workers (2013) for instance reported that proteobacteria were the most predominant group (91.01%) among 125 bacterial strains isolated from contaminated sediments and seawater from a refinery harbor of the Bizerte coast, North of Tunisia. Similar to the present study, it was also observed that among the proteobacteria, the gamma group was the most abundant constituting 65% of proteobacteria.

5.1.4.0 Selected hydrocarbon degrading bacteria

5.1.4.1 Hydrocarbon degradation by *Pseudomonas sp***.**

Isolate 1C, identified as *Pseudomonas aeruginosa* was capable of utilizing heating oil, hexane, octane as well as diesel oil as the sole carbon source. Members of this genus have been identified as the most predominant group in metabolism of hydrocarbons

(Sharma *et al.,* 2015). This class of bacteria possesses a broad array of physiological and metabolic properties as well as a complex enzymatic system that enable them to utilize a wide range of aliphatic and aromatic compounds as their sole carbon source (Das & Chandran, 2010; Sharma *et al.,* 2015).

Ability of *Pseudomonas* to efficiently take up alkanes has been linked to production of rhamnolipid biosurfactants as was demonstrated in a study carried out by Sharma and co-workers (2015) using *P. aeruginosa* DSVP20. The metabolic versatility of *Pseudomonas* has been linked to presence of degradative plasmids such as OCT (octane), ALK (alkanes), TOL (toluene), XYL (xylene) and NAH (naphthalene) (Silva *et al.,* 2006). The efficiency of *P. aeruginosa* in hydrocarbon degradation has also been attributed to passive diffusion of the hydrocarbon across the cell membrane (Rojo, 2010).

5.1.4.2 Hydrocarbon degradation by Klebsiella sp.

Two of the isolates, 2C and 6C that demonstrated growth when cultured in heating oil belonged to the genus *Klebsiella*. The two were also capable of utilizing hexane vapour as well as diesel oil as the sole carbon source. *Klebsiella* species are well established in degradation of petroleum compounds. Among 45 hydrocarbon degrading isolates obtained from estuary sediments, Rodrigues and co-workers (2009) reported that bacteria of the genus *Klebsiella* were the most frequently encountered making 46.7% with some of them recording over 90% degradation of toluene, xylene, nonane and naphthalene. In a different study, two strains of bacteria *K. pneumoniae SS12* and *K. pneumoniae SS26* isolated from soils near petroleum pumps were also found to degrade toluene, benzene, octane and heptane (Survery *et al.,* 2004).

5.1.4.3 Hydrocarbon degradation by *Acinetobacter sp*.

Studies on alkane oxidation by members of the genus *Acinetobacter* have indicated that alkane utilization is widespread among this group (Ratajczak *et al.,* 1998). In this study, isolate 3A, identified as *A. baumannii* was found to utilize heating oil, diesel

oil, as well as hexane and octane vapors as the sole carbon source. In a study conducted by Mahjoubi and co-workers (2013), *Acinetobacte*r species was found to be the most abundant group. Similarly, Chaıneau and co-workers (1999) reported that *A. baumannii* was able to greatly assimilate saturated and aromatic hydrocarbons. Efficiency of *Acinetobacter sp.* in utilization of hydrocarbons could be attributed to their ability to produce biosurfactants as was observed in a study conducted by Barkay and co-workers (1999). In this study, *A. radioresistens* KA53 was reported to produce alasan which is a high-molecular-weight bioemulsifier complex observed to accelerate mineralization of recalcitrant PAHs (Barkay *et al.,* 1999).

5.1.5.0 Optimization of growth conditions for diesel oil degrading bacteria

In order to stimulate microbial growth, optimization of environmental conditions is very vital (Dongfeng *et al.,* 2011). Out of the nine bacterial isolates identified, three bacterial isolates that showed high potential for hydrocarbon degradation were selected and their growth conditions optimized. These were isolates 3A, 1C and 2C.

5.1.5.1 Determination of optimum pH for microbial growth in diesel oil

In the present study, an optimum pH of 7 was observed for all the three isolates. Isolate 1C however, displayed a higher biomass production of 0.742 g/L compared to the other two isolates as shown in Figure 16. A number of studies have also indicated optimal growth at or near pH 7 (Hamza *et al.,* 2010; Dongfeng *et al.,* 2011; Mahalingam & Sampath, 2014). In a similar study, the optimum pH for growth of *P. aeruginosa* was found to be 6.5 in a study carried out by Hamza and co-workers (2010) using crude oil and minimal salt media. Maintenance of an optimal pH condition is very vital as variation in pH of the culture media caused by accumulation of metabolic waste products affects microbial growth (Mahalingam & Sampath, 2014). Notably, Isolate 3A was slightly tolerant to alkaline pH compared to isolate 2C. This could be linked to soil pH where the isolate was sampled. Isolate 2C was obtained from the surface compared to isolate 3A from the sub surface 2, much

deeper than the former. Hamza and co-workers (2010) noted that for oil contaminated sites, as soil depth increases, the pH value also increases.

5.1.5.2 Determination of optimum temperature for microbial growth in diesel oil

The three bacterial strains were observed to grow well at 30 °C and 37 °C with $p<0.05$. At 37 °C however, a higher microbial biomass was noted for all the three strains with isolate 1C yet again recording the highest biomass of 0.718 g/L indicating higher diesel oil utilization as shown in Figure 17. Above 37 °C, growth was reduced. Though the optimum temperature was found to be 37 °C*, P. aeruginosa* AT18 strain was found to efficiently assimilate n-alkanes, naphthalene, toluene and crude oil at 41 °C in a study conducted by Silva and co-workers (2006). In a different study however, a low temperature *Pseudomonas* strain ST41 isolated from Antarctic soils was shown to degrade a wide range of aliphatic and aromatic hydrocarbons at 4 °C (Stallwood *et al.,* 2005).

5.1.5.3 Determination of optimum substrate concentration

In this study, optimization of diesel oil concentration revealed that 0.5% substrate concentration provided excellent growth for the three isolates. A suitable range of diesel oil concentration was observed between 0.5 to 1% although isolate 1C was able to tolerate up to 3% diesel oil concentration (Figure 18). The study findings concur with Mahalingam & Sampath (2014) study, in which *Pseudomonas sp.* and another strain of *Bacillus sp* were observed to tolerate increased diesel oil concentration. Tebyanian and co-workers (2013) also reported that when hexadecane concentration was increased from 1-7%, microbial growth decreased. Thus an optimum range of substrate concentration is very vital since biodegradation is not easily stimulated below the oil concentration range while above the range, growth inhibition may occur due to oxygen limitations as well as solvent toxic effect (Zhu *et al.,* 2001; Mahalingam & Sampath, 2014).

5.1.5.4 Determination of optimum nitrogen source for microbial growth in diesel oil

Isolates 3A, 1C and 2C were grown in a nitrogen-limited mineral salt media separately supplemented with 0.1% ammonium nitrate, yeast extract and tryptone as nitrogen sources. Significant difference in microbial growth was observed for the three nitrogen sources with addition of yeast extract giving the highest growth for the three isolates (Figure 19). With yeast extract, isolate 3A was observed to give the highest biomass of 1.344 g/L compared to the rest of the isolates. In a study performed by Hamza and co-workers (2010), *P. aeruginosa*, *P. putida, A. hydrophila* and *A. lwoffii* were observed to grow optimally using yeast extract and tryptone as nitrogen sources. In the same study however, it was observed that addition of ammonium nitrate and glycine as nitrogen sources did not give a significant increase in microbial growth. Although yeast extract was observed to provide excellent microbial growth as a nitrogen source in this study, growth was also observed for ammonium nitrate as nitrate possess high oxidation potential for elimination of hydrocarbon contaminants which normally exist in a reduced state (Borah & Yadav, 2014).

5.1.6 Analysis of microbial diesel oil degradation by GC-MS

Compared to straight chain alkanes, most branched-chain and cyclic alkanes were totally degraded as indicated by the GC-MS analyses in Figure 20 and Table 8. This is contrary to some studies which have reported slower degradation of branched chain alkanes compared to linear alkanes. Katsivela and co-workers (2003) for instance reported higher preference for straight chain alkanes compared to branched chain alkanes. In this study however, most linear alkanes were still present at the end of the incubation period possibly due to their higher concentration in diesel oil. Comparison of GC-MS profiles of control media with that inoculated with different isolates also revealed complete microbial degradation of mono- and polycyclic aromatic hydrocarbons in spite of their persistent and recalcitrant nature. PAHs show greater

resistance to degradation and are classified as persistent organic pollutants (POPs) (Ferrarese *et al.,* 2008).

Additionally, the ability of the isolates to also reduce acyclic isoprenoid hydrocarbons, phytane and pristine normally used as internal biomarkers in environmental hydrocarbon analyses, suggest that the microbes possess multiple degradative genes which facilitate a diverse catabolic ability. Similar to the present study, numerous studies have also reported that phytane and pristine degradation remains low until most alkanes and aromatics are removed mainly due to their persistent nature (Mills *et al.,* 2003; Salam, 2016).

GC-MS analysis of possible microbial degradation intermediates

GC-MS analyses revealed new peaks which were identified as possible intermediate metabolites that included alcohols, aldehydes and fatty acids as shown in Table 9. Alcohols, aldehydes, ketones and fatty acids are intermediates in terminal and sub-terminal aerobic degradation pathway for alkanes (Whyte *et al.,* 1998). The pathway is initiated by oxidation of the terminal or sub-terminal methyl group of the alkane to a corresponding alkanol which is first converted to an aldehyde or ketone and later to a carboxylic acid that is then completely mineralized to CO_2 and water via the β-oxidation pathway (Nyyssönen, 2009). Accumulation of these acids and the subsequent change in media pH has been reported to hamper biodegradation as they become toxic (Chaillan *et al.,* 2004). For aromatic hydrocarbon degradation, phenylethyl alcohol was the only metabolite identified in media inoculated with isolates 2C, 3A and 4A2. 1- Phenylethyl alcohol is a metabolic intermediate formed through oxidation of ethylbenzene by naphthalene dioxygenase enzyme (Choi *et al.,* 2013).

The isolates were thus capable of readily degrading diesel oil notwithstanding its higher toxicity compared to heavier oils such as crude oil. Diesel oil has been reported to possess high microbial toxicity due to presence of low molecular weight

hydrocarbons which increase the ease of uptake and hence bioavailability (Coulon *et al.,* 2005).

5.1.7 AlkB gene amplification

Alkane hydroxylase gene of isolate 1C was successfully amplified and sequenced indicating the isolate's potential catabolic capability in degrading alkane fraction of petroleum oils. Isolate 2C did not give the expected PCR product size probably due to nonspecific priming while 3A showed no PCR product for the gene. Lack of expected PCR products for this catabolic gene could be due to existence of completely different gene sequences from those characterized from the bacteria (*Pseudomonas oleovorans*). Alternatively, regions used to develop primer pairs may not have been well conserved due to existence of gene homologues in this bacteria (Katsivela *et al.,* 2005). Phylogenetic analysis indicated that the gene sequence for 1C isolate clustered with alkB gene from other *P. aeruginosa* strains with a posterior probability of 63% further supporting that isolate 1C is a *Pseudomonas*. AlkB gene catalyzes the first step in aerobic degradation of medium and long chain alkanes in which oxygen atom originating from molecular oxygen is introduced into the alkane substrate to form an alcohol.

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

A total of twenty bacteria and one fungus were isolated from oil-contaminated soils sampled from garages around Ngara area in Nairobi. From these, nine efficient bacterial isolates were identified based on morphological and biochemical tests as well as 16S rDNA sequence analyses. The isolates were observed to utilize heating oil, hexane, octane and toluene as well as diesel oil as the sole carbon source. Optimization of culture conditions using three of the most efficient degraders revealed that optimal degradation of diesel oil was recorded with <1% substrate concentration at pH 7 and temperature of 37 °C. Additionally, yeast extract was selected as the best nitrogen source for diesel oil biodegradation. GC-MS analyses demonstrated that the isolated bacterial strains were capable of readily degrading different alkane and aromatic hydrocarbons present in diesel oil thus exhibiting a broad range of catabolic activities. Alkane hydroxylase gene (AlkB) of isolate 1C was successfully amplified indicating the isolate's potential catabolic ability in alkane degradation. These findings clearly indicate the prospect to develop an environmentally friendly mitigation strategy against petroleum hydrocarbon pollution using the obtained bacterial isolates.

6.2 Recommendations

I. Though this study was carried out using pure single strains, several studies have shown that a consortium of several bacterial strains is required for complete mineralization of hydrocarbon contaminants given the complexity of oil products. This is due to the fact that single strains may not possess all the enzymatic machinery required for the degradation process. In addition, screening for hydrocarbon degrading fungi and yeast will lead to discovery of more efficient degraders amenable to biotechnological applications.

II. Numerous bioremediation studies have reported effectiveness of biodegradation in the laboratory but less so in pilot scale and field trials. This is due to the fact that laboratory studies rarely simulate complicated real world situations such as climatic effects, biological interactions, and spatial heterogeneity among others. Thus there is need to carry out field studies and applications which are the ultimate tests for demonstrating the effectiveness of a bioremediation technique.

III. Recently, much attention has been directed towards structural analysis of biosurfactants based on their broad range functional properties and potential commercial applications in the oil industry. Screening for biosurfactant production by the isolated microbes is thus essential.

IV. Finally, the use of genetic engineering to improve biodegradation agents (microbial cultures, biosurfactant, enzymes additives) may open potential prospects of obtaining highly effective and less costly agents for use in the cleanup of petroleum pollution.

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8.0 APPENDICES

Appendix 1: Soil sampling area coordinates

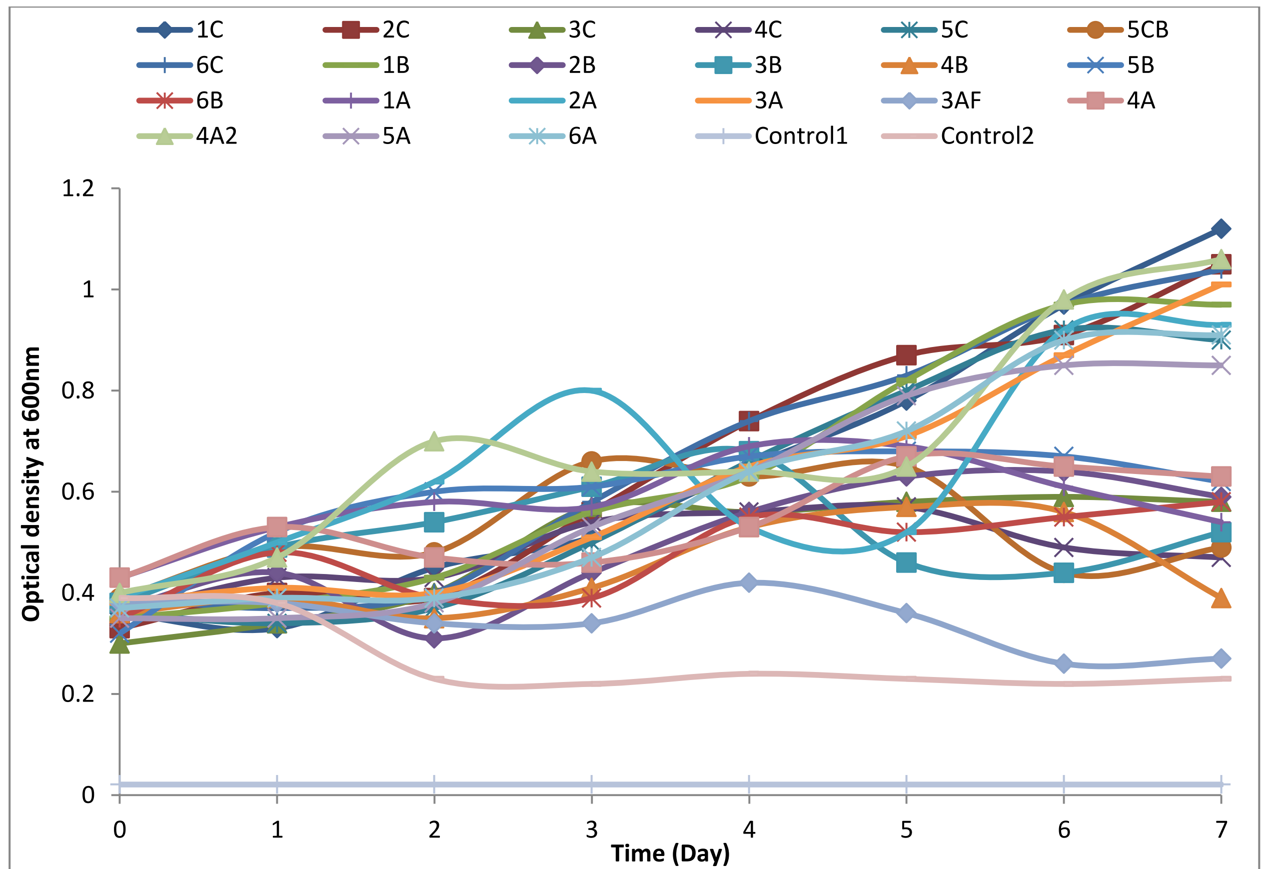
Isolation site (Ngara, Nairobi Kenya)	Geographical location (Latitude & Longitude)
Site 3 and 4	" 1°16'21.12""S" " 36°49'7.46""E"
Site 1	" 1°16'34.86""S" " 36°49'9.70""E"
Site 2 and 6	" 1°16'20.02""S" "36°49'50.20""E"
Site 5	" 1°16'41.45""S" " 36°49'53.73""E"

Appendix 2: Optical density readings obtained during bacterial culturing in mineral salt media supplemented with 1% heating oil

	Optical density readings (600nm)							
Isolate	**Day 0**	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**
1C	0.36	0.33	0.45	0.51	0.65	0.78	0.97	1.12
2C	0.33	0.40	0.39	0.56	0.74	0.87	0.91	1.05
3C	0.30	0.34	0.40	0.57	0.56	0.58	0.59	0.58
4C	0.36	0.43	0.43	0.54	0.56	0.57	0.49	0.47
5C	0.36	0.34	0.37	0.50	0.66	0.80	0.92	0.90
5CB	0.39	0.49	0.48	0.66	0.63	0.65	0.44	0.49
6C	0.39	0.37	0.40	0.58	0.74	0.83	0.97	1.04
1B	0.35	0.38	0.43	0.56	0.63	0.82	0.97	0.97

2B	0.37	0.44	0.31	0.44	0.56	0.63	0.64	0.59
3B	0.38	0.49	0.54	0.61	0.68	0.46	0.44	0.52
4B	0.36	0.39	0.35	0.41	0.53	0.57	0.56	0.39
5B	0.32	0.52	0.60	0.61	0.67	0.68	0.67	0.62
6B	0.35	0.48	0.39	0.39	0.55	0.52	0.55	0.58
1A	0.43	0.53	0.58	0.57	0.69	0.69	0.61	0.54
2A	0.39	0.50	0.62	0.80	0.53	0.52	0.92	0.93
3A	0.38	0.41	0.40	0.51	0.65	0.71	0.87	1.01
3AF	0.38	0.38	0.34	0.34	0.42	0.36	0.26	0.27
4A	0.43	0.53	0.47	0.46	0.53	0.67	0.65	0.63
4A2	0.40	0.47	0.70	0.64	0.64	0.65	0.98	1.06
5A	0.35	0.35	0.38	0.53	0.64	0.79	0.85	0.85
6A	0.37	0.39	0.39	0.47	0.64	0.72	0.90	0.91
Control1	0.021	0.021	0.021	0.021	0.021	0.021	0.021	0.021
Control2	0.39	0.38	0.23	0.22	0.24	0.23	0.22	0.23

Appendix 3: Time course of growth for bacterial and fungal (3AF) isolates cultured in mineral salt media supplemented with 1 % heating oil for 7 days



Appendix 4: One way ANOVA results on time course of growth of selected bacterial isolates during culturing in BHM supplemented with 1% heating oil

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	10.286	7	1.469	407.156	.000
Within Groups	.664	184	.004		
Total	10.950	191			

Appendix 5: Post hoc statistical analysis indicating multiple comparisons of results on time course of growth of selected bacterial isolates during culturing in BHM supplemented with 1% heating oil for 7 days

	(I) Day	(J) Day	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	0	1	-.01875	.01734	.960	-.0719	.0344
		2	-.07417*	.01734	.001	-.1273	-.0210
		3	-.17375*	.01734	.000	-.2269	-.1206
		4	-.30125*	.01734	.000	-.3544	-.2481
		5	-.40458*	.01734	.000	-.4578	-.3514
		6	-.56833*	.01734	.000	-.6215	-.5152
		7	-.64000*	.01734	.000	-.6932	-.5868
	1	0	.01875	.01734	.960	-.0344	.0719
		2	-.05542*	.01734	.034	-.1086	-.0022
		3	-.15500*	.01734	.000	-.2082	-.1018
		4	-.28250*	.01734	.000	-.3357	-.2293
		5	-.38583*	.01734	.000	-.4390	-.3327
		6	-.54958*	.01734	.000	-.6028	-.4964
		7	-.62125*	.01734	.000	-.6744	-.5681
	2	0	.07417*	.01734	.001	.0210	.1273
		1	.05542*	.01734	.034	.0022	.1086
		3	-.09958*	.01734	.000	-.1528	-.0464
		4	-.22708*	.01734	.000	-.2803	-.1739
		5	-.33042*	.01734	.000	-.3836	-.2772
		6	-.49417*	.01734	.000	-.5473	-.4410
		7	-.56583*	.01734	.000	-.6190	-.5127
	3	0	.17375*	.01734	.000	.1206	.2269
		1	.15500*	.01734	.000	.1018	.2082
		2	.09958*	.01734	.000	.0464	.1528
		4	-.12750*	.01734	.000	-.1807	-.0743
		5	-.23083*	.01734	.000	-.2840	-.1777
		6	-.39458*	.01734	.000	-.4478	-.3414
		7	-.46625*	.01734	.000	-.5194	-.4131
	4	0	.30125*	.01734	.000	.2481	.3544
		1	.28250*	.01734	.000	.2293	.3357
		2	.22708*	.01734	.000	.1739	.2803
		3	.12750*	.01734	.000	.0743	.1807

	5	-.10333*	.01734	.000	-.1565	-.0502
	6	-.26708*	.01734	.000	-.3203	-.2139
	7	-.33875*	.01734	.000	-.3919	-.2856
5	0	.40458*	.01734	.000	.3514	.4578
	1	.38583*	.01734	.000	.3327	.4390
	2	.33042*	.01734	.000	.2772	.3836
	3	.23083*	.01734	.000	.1777	.2840
	4	.10333*	.01734	.000	.0502	.1565
	6	-.16375*	.01734	.000	-.2169	-.1106
	7	-.23542*	.01734	.000	-.2886	-.1822
6	0	.56833*	.01734	.000	.5152	.6215
	1	.54958*	.01734	.000	.4964	.6028
	2	.49417*	.01734	.000	.4410	.5473
	3	.39458*	.01734	.000	.3414	.4478
	4	.26708*	.01734	.000	.2139	.3203
	5	.16375*	.01734	.000	.1106	.2169
	7	-.07167*	.01734	.001	-.1248	-.0185
7	0	.64000*	.01734	.000	.5868	.6932
	1	.62125*	.01734	.000	.5681	.6744
	2	.56583*	.01734	.000	.5127	.6190
	3	.46625*	.01734	.000	.4131	.5194
	4	.33875*	.01734	.000	.2856	.3919
	5	.23542*	.01734	.000	.1822	.2886
	6	.07167*	.01734	.001	.0185	.1248

* The mean difference is significant at the 0.05 level

Appendix 6: One way ANOVA results on effect of pH on bacterial growth during biodegradation of petroleum diesel oil

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.712	4	.428	171.945	.000
Within Groups	.100	40	.002		
Total	1.811	44			

Appendix 7: Post hoc statistical analysis on effect of pH on bacterial growth during biodegradation of diesel oil indicating multiple comparisons

	(I) pH	(J) pH	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	3	5	-.24789*	.02352	.000	-.3151	-.1807
		7	-.57122*	.02352	.000	-.6384	-.5041
		3	-.33189*	.02352	.000	-.3991	-.2647
		11	-.11633*	.02352	.000	-.1835	-.0492
	5	3	.24789*	.02352	.000	.1807	.3151
		7	-.32333*	.02352	.000	-.3905	-.2562
		9	-.08400*	.02352	.008	-.1512	-.0168
		11	.13156*	.02352	.000	.0644	.1987
	7	3	.57122*	.02352	.000	.5041	.6384
		5	.32333*	.02352	.000	.2562	.3905
		9	.23933*	.02352	.000	.1722	.3065
		11	.45489*	.02352	.000	.3877	.5221
	9	3	.33189*	.02352	.000	.2647	.3991
		5	.08400*	.02352	.008	.0168	.1512
		7	-.23933*	.02352	.000	-.3065	-.1722
		11	.21556*	.02352	.000	.1484	.2827
	11	3	.11633*	.02352	.000	.0492	.1835
		5	-.13156*	.02352	.000	-.1987	-.0644
		7	-.45489*	.02352	.000	-.5221	-.3877
		9	-.21556*	.02352	.000	-.2827	-.1484

* The mean difference is significant at the 0.05 level

Appendix 8: One way ANOVA results on effect of temperature on bacterial growth during diesel oil biodegradation

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.989	4	.497	113.030	.000
Within Groups	.176	40	.004		
Total	2.165	44			

Appendix 9: Post hoc statistical analysis on effect of temperature on bacterial growth during biodegradation of diesel oil showing multiple comparisons

	(I) Temperature (^{0}C)	(J) Temperature (^{0}C)	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	25	30	-.24789*	.03127	.000	-.3372	-.1586
		37	-.29744*	.03127	.000	-.3867	-.2081
		45	.07322	.03127	.153	-.0161	.1625
		55	.27156*	.03127	.000	.1823	.3609
	30	25	.24789*	.03127	.000	.1586	.3372
		37	-.04956	.03127	.515	-.1389	.0397
		45	.32111*	.03127	.000	.2318	.4104
		55	.51944*	.03127	.000	.4301	.6087
	37	25	.29744*	.03127	.000	.2081	.3867
		30	.04956	.03127	.515	-.0397	.1389
		45	.37067*	.03127	.000	.2814	.4600
		55	.56900*	.03127	.000	.4797	.6583
	45	25	-.07322	.03127	.153	-.1625	.0161
		30	-.32111*	.03127	.000	-.4104	-.2318
		37	-.37067*	.03127	.000	-.4600	-.2814
		55	.19833*	.03127	.000	.1090	.2876
	55	25	-.27156*	.03127	.000	-.3609	-.1823
		30	-.51944*	.03127	.000	-.6087	-.4301
		37	-.56900*	.03127	.000	-.6583	-.4797
		45	-.19833*	.03127	.000	-.2876	-.1090

* The mean difference is significant at the 0.05 level

Appendix 10: One way ANOVA results on effect of substrate concentration on bacterial growth during biodegradation of diesel oil

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.712	3	.571	73.541	.000
Within Groups	.248	32	.008		
Total	1.960	35			

Appendix 11: Post hoc statistical analysis indicating multiple comparisons on effect of substrate concentration on bacterial growth during biodegradation of diesel oil

	(I) Substrate Concentration	(J) Substrate Concentration	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	0.5	1	.18089*	.04152	.001	.0684	.2934
		3	.37056*	.04152	.000	.2581	.4831
		5	.58633*	.04152	.000	.4738	.6988
	1	0.5	-.18089*	.04152	.001	-.2934	-.0684
		3	.18967*	.04152	.000	.0772	.3022
		5	.40544*	.04152	.000	.2929	.5179
	3	0.5	-.37056*	.04152	.000	-.4831	-.2581
		1	-.18967*	.04152	.000	-.3022	-.0772
		5	.21578*	.04152	.000	.1033	.3283
	5	0.5	-.58633*	.04152	.000	-.6988	-.4738
		1	-.40544*	.04152	.000	-.5179	-.2929
		3	-.21578*	.04152	.000	-.3283	-.1033

* The mean difference is significant at the 0.05 level

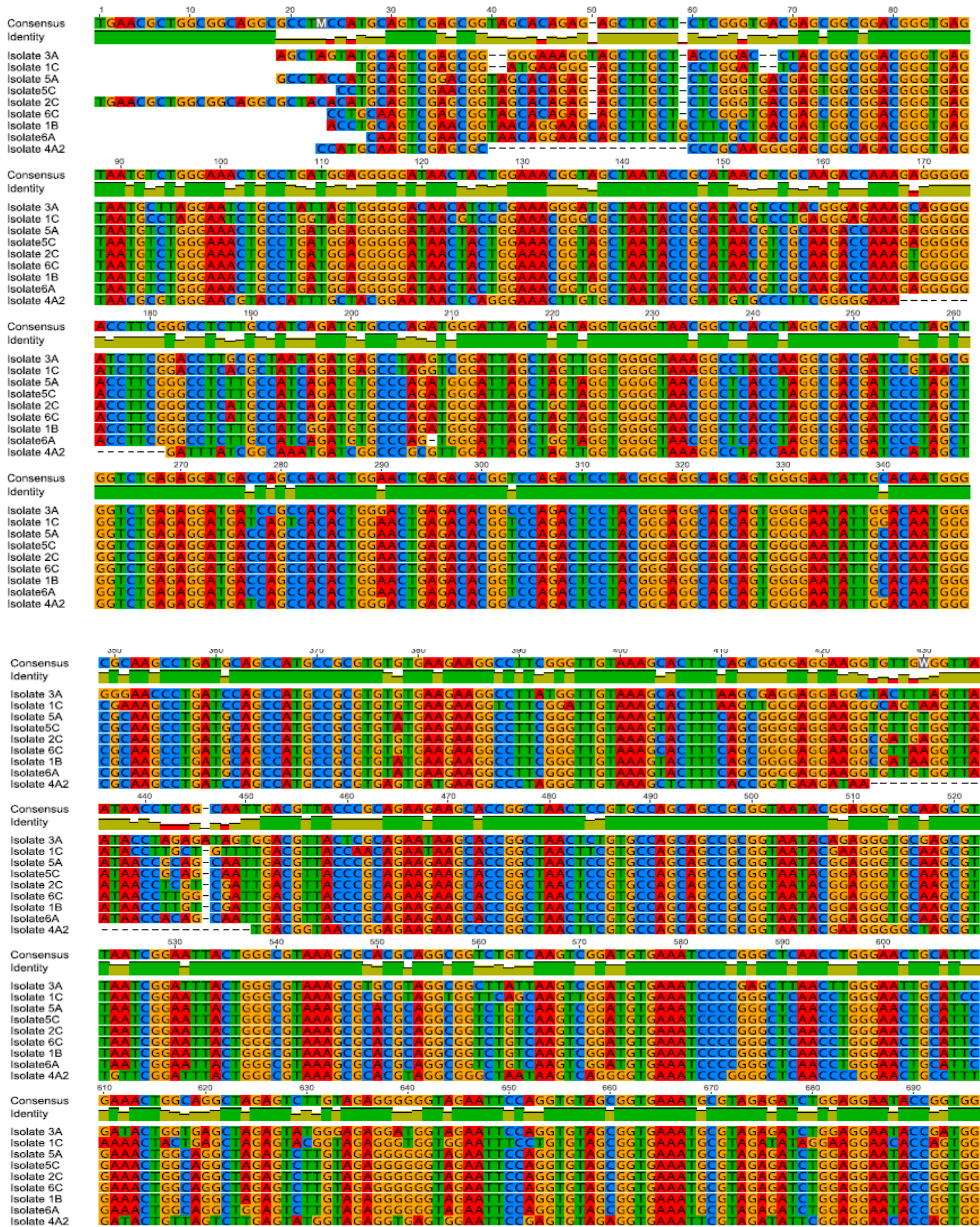
Appendix 12: One way ANOVA results on effect of nitrogen source on bacterial growth during diesel oil biodegradation

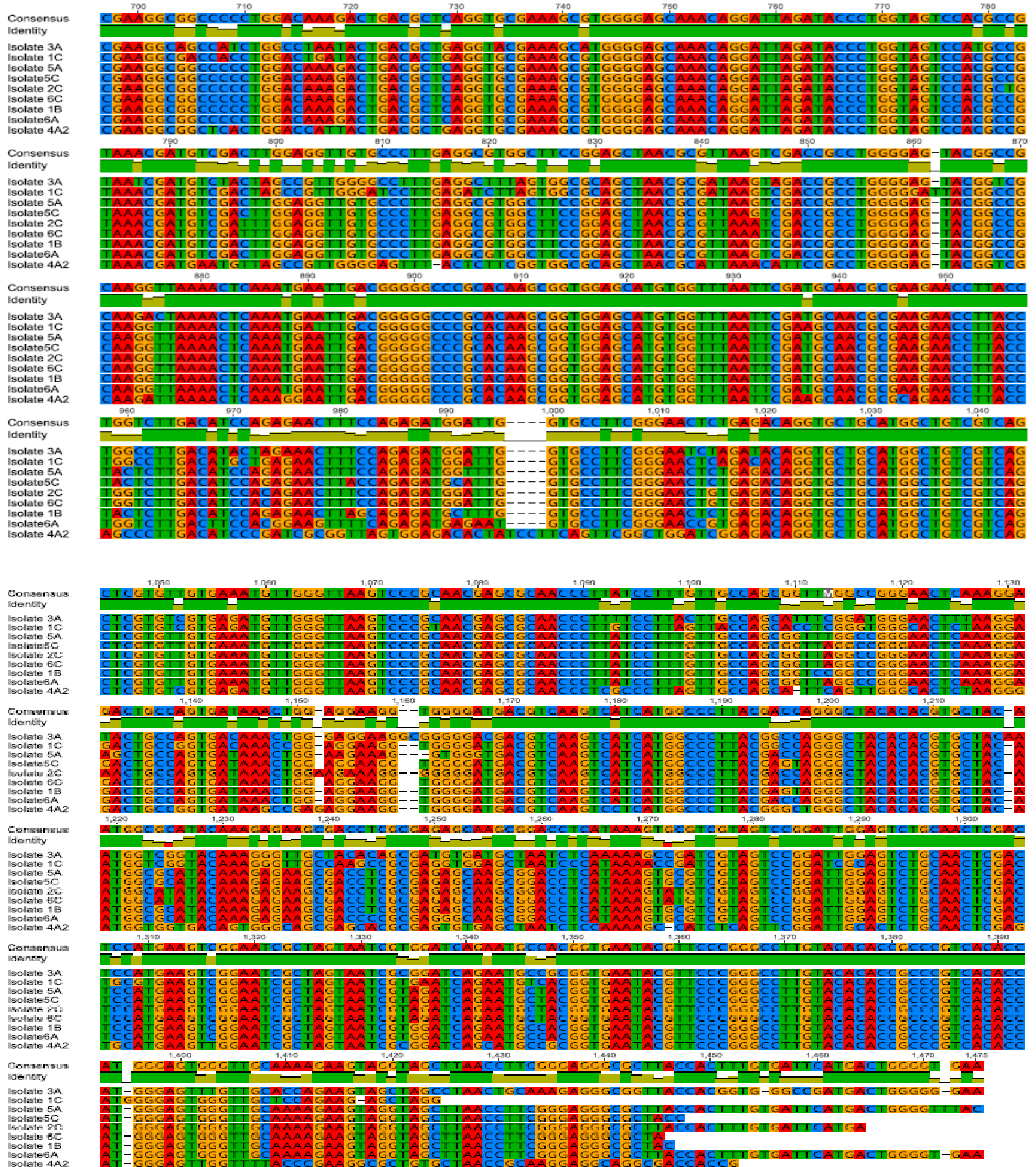
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.341	2	.671	47.478	.000
Within Groups	.339	24	.014		
Total	1.680	26			

Appendix 13: Post hoc statistical analysis indicating multiple comparisons on effect of nitrogen source on bacterial growth during diesel oil biodegradation

	(I) Nitrogen Source	(J) Nitrogen Source	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	Ammonium nitrate	Yeast extract	-.54256*	.05603	.000	-.6825	-.4026
		Tryptone	-.21856*	.05603	.002	-.3585	-.0786
	Yeast extract	Ammonium nitrate	.54256*	.05603	.000	.4026	.6825
		Tryptone	.32400*	.05603	.000	.1841	.4639
	Tryptone	Ammonium nitrate	.21856*	.05603	.002	.0786	.3585
		Yeast extract	-.32400*	.05603	.000	-.4639	-.1841

* The mean difference is significant at the 0.05 level





Appendix 14: A multiple sequence alignment showing variation in bases of the 16S rDNA gene of the nine isolates and a consensus sequence.

Appendix 15: Retention time in minutes and peak size of hydrocarbon compounds identified in un-inoculated (control) BH media

Ret. Time	Start time	End time	Area	Area %	Height	Height %	A/H	Compound
11.075	11.015	11.135	13881108	0.32	4584500	0.5	3.03	Nonane
13.32	13.255	13.385	12509430	0.29	3469390	0.38	3.61	Benzene, 1,2,3-trimethyl-
15.048	14.96	15.12	50821817	1.19	14482209	1.59	3.51	Undecane
16.002	15.945	16.07	10319744	0.24	3166669	0.35	3.26	Decane, 4-methyl-
16.61	16.54	16.69	10317541	0.24	2442150	0.27	4.22	Benzene, 1-methyl-2-propyl-
17.613	17.525	17.66	22109143	0.52	5773175	0.63	3.83	Decane, 2-methyl-
17.863	17.78	17.915	15063304	0.35	3594018	0.39	4.19	Decane, 3-methyl-
19.005	18.9	19.07	75274911	1.76	20168618	2.21	3.73	Undecane
19.366	19.24	19.44	22018431	0.51	4545821	0.5	4.84	trans-Decalin, 2-methyl-
20.505	20.465	20.64	10929344	0.26	2106801	0.23	5.19	5-Ethyl-m-xylene
20.714	20.64	20.81	18701076	0.44	4237089	0.46	4.41	Hexane, 2-phenyl-3-propyl-
21.281	21.2	21.34	9752768	0.23	3087142	0.34	3.16	Dodecane, 4-methyl-
21.446	21.345	21.495	13762589	0.32	4334147	0.48	3.18	Undecane, 2-methyl-
21.69	21.62	21.755	14256641	0.33	4092834	0.45	3.48	Undecane, 3-methyl-
22.788	22.69	22.87	118327935	2.76	29645443	3.25	3.99	Dodecane
23.335	23.275	23.395	24967984	0.58	6426936	0.71	3.88	Undecane, 3,6-dimethyl-
23.46	23.405	23.59	10009224	0.23	1421092	0.16	7.04	Nona-2,4-diene
23.702	23.66	23.775	11509640	0.27	2912718	0.32	3.95	Naphthalene, 2-ethyl decahydro-
24.09	24.005	24.165	13665312	0.32	2626349	0.29	5.2	Benzene, (3-methyl-2-butenyl)-
24.59	24.54	24.64	21455963	0.5	5768760	0.63	3.72	Naphthalene, 1,2,3,4-

								tetrahydro-5-methyl-
24.68	24.64	24.715	13044578	0.3	3384960	0.37	3.85	Dodecane, 6-methyl-
24.746	24.715	24.815	18018487	0.42	5104939	0.56	3.53	Nonane, 5-(2-methylpropyl)-
24.918	24.815	24.965	20190145	0.47	4518566	0.5	4.47	Dodecane, 4-methyl-
25.083	24.965	25.18	40171951	0.94	7870446	0.86	5.1	Octadecane
25.315	25.18	25.38	30591980	0.71	6643720	0.73	4.6	Dodecane, 3-methyl-
25.475	25.395	25.56	31013045	0.72	8287502	0.91	3.74	Octane, 3,6-dimethyl-
25.961	25.89	26.04	11397287	0.27	2743135	0.3	4.15	Cyclopentane, 1-methyl-3-(2-methyl propyl)-
26.37	26.25	26.425	141217663	3.3	33856816	3.71	4.17	Pentadecane
26.538	26.435	26.63	25745996	0.6	4878267	0.54	5.28	Naphthalene, 1,2,3,4-tetrahydro-2,6-dimethyl-
27.336	27.21	27.43	25409465	0.59	3764416	0.41	6.75	1,7,7-Trimethyl-2-vinyl bicyclo[2.2.1] hept-2-ene
27.795	27.705	27.93	21996965	0.51	2962052	0.32	7.43	Cyclohexane, (cyclo pentylmethyl)-
28.081	28.01	28.14	19903545	0.46	4902763	0.54	4.06	Tridecane, 6-methyl-
28.189	28.14	28.235	17253375	0.4	4442784	0.49	3.88	Tridecane, 5-methyl-
28.35	28.235	28.4	25741792	0.6	5096762	0.56	5.05	Tridecane, 4-methyl-
28.518	28.4	28.63	42668530	1	8619265	0.95	4.95	Eicosane, 10-methyl-
28.741	28.63	28.805	27384976	0.64	6906209	0.76	3.97	Tetradecane, 3-methyl-
29.02	28.935	29.11	48076623	1.12	11048129	1.21	4.35	Nonane, 3-methyl-5-propyl-
29.744	29.63	29.81	188203539	4.4	38254359	4.2	4.92	Tetradecane
29.89	29.81	29.96	21855780	0.51	4170154	0.46	5.24	Pentadecane
30.07	29.96	30.19	24688004	0.58	3354897	0.37	7.36	Hexadecane

30.256	30.19	30.33	12121317	0.28	3053840	0.34	3.97	Nonadecane
30.832	30.755	30.895	11351875	0.27	2760507	0.3	4.11	Naphthalene, 1,2,3,4-tetrahydro-2,5,8-trimethyl-
31.224	31.135	31.285	31256828	0.73	5589874	0.61	5.59	Cyclohexane, (4-methylpentyl)-
31.32	31.285	31.355	13186863	0.31	3778248	0.41	3.49	Undecane, 6-methyl-
31.415	31.355	31.465	19119723	0.45	4674908	0.51	4.09	Tetradecane, 5-methyl-
31.592	31.52	31.675	28375106	0.66	6439957	0.71	4.41	Tetradecane, 4-methyl-
31.782	31.675	31.875	81609944	1.91	17548895	1.93	4.65	Hexadecane
31.981	31.875	32.075	33376889	0.78	8955471	0.98	3.73	Tetradecane, 3-methyl-
32.925	32.74	33.055	227069160	5.31	40061747	4.39	5.67	Heptadecane
33.185	33.145	33.27	16655158	0.39	3767732	0.41	4.42	Hexadecane, 7,9-dimethyl-
33.332	33.27	33.39	14929006	0.35	3162725	0.35	4.72	Hexadecane, 7,9-dimethyl-
34.309	34.215	34.345	25203412	0.59	5591901	0.61	4.51	Decane, 5-propyl-
34.424	34.345	34.535	51805808	1.21	6260561	0.69	8.27	n-Nonylcyclohexane
34.644	34.535	34.735	38217496	0.89	6480404	0.71	5.9	Pentadecane, 4-methyl-
34.81	34.735	34.9	49554431	1.16	10395298	1.14	4.77	Tetradecane, 2,6,10-trimethyl-
34.94	34.9	34.97	12477747	0.29	3734977	0.41	3.34	Eicosane
35.026	34.97	35.14	32969405	0.77	7799615	0.86	4.23	Pentadecane, 3-methyl-
35.923	35.76	36.005	204362519	4.77	39673574	4.35	5.15	Heptadecane
37.194	37.115	37.24	22296400	0.52	5295396	0.58	4.21	Heptadecane, 8-methyl-
37.38	37.24	37.45	75148977	1.76	13127907	1.44	5.72	Heptadecane
37.46	37.45	37.505	9706073	0.23	3737669	0.41	2.6	Cyclohexane, 1,1'-(1,3-propanediyl)bis-
37.553	37.505	37.63	17711966	0.41	4757783	0.52	3.72	Tetradecane, 4-methyl-
37.7	37.63	37.8	28510319	0.67	7137976	0.78	3.99	Hexadecane, 2-methyl-
37.918	37.8	37.975	26277544	0.61	6904853	0.76	3.81	Tetradecane, 3-methyl-

38.76	38.615	38.82	196925531	4.6	39831235	4.37	4.94	Heptadecane
39.004	38.88	39.07	52058586	1.22	12149546	1.33	4.28	Pentadecane, 2,6,10,14-tetramethyl-
39.915	39.83	39.985	23337115	0.55	4189502	0.46	5.57	Heptadecane, 8-methyl-
40.019	39.985	40.08	13055461	0.3	4146252	0.45	3.15	Pentadecane, 6-methyl-
40.438	40.385	40.505	15643641	0.37	4817732	0.53	3.25	Heptadecane, 2-methyl-
40.653	40.575	40.73	20646398	0.48	6165425	0.68	3.35	Heptadecane, 3-methyl-
41.448	41.31	41.525	175300419	4.1	36418601	4	4.81	Heneicosane
41.775	41.685	41.895	66103402	1.54	15209218	1.67	4.35	Hexadecane, 2,6,10,14-tetramethyl-
42.515	42.46	42.595	25622996	0.6	4430857	0.49	5.78	Nonadecane, 9-methyl-
42.635	42.595	42.685	11347268	0.27	3097679	0.34	3.66	Decane, 2,5-dimethyl-
42.744	42.685	42.83	13567935	0.32	3232367	0.35	4.2	Eicosane, 2,4-dimethyl-
42.906	42.83	42.94	13273368	0.31	3488104	0.38	3.81	Tetradecane, 4-methyl-
43.054	42.94	43.125	30021217	0.7	5886560	0.65	5.1	Octadecane, 2-methyl-
43.254	43.125	43.325	18419687	0.43	5016464	0.55	3.67	Octadecane, 3-methyl-
44.005	43.88	44.065	155007346	3.62	33440453	3.67	4.64	Heneicosane
44.986	44.905	45.08	26148195	0.61	4234543	0.46	6.17	Eicosane
45.135	45.08	45.195	11748571	0.27	2743653	0.3	4.28	Pentadecane, 6-methyl-
45.396	45.305	45.455	13489823	0.32	3213358	0.35	4.2	Heptadecane, 9-octyl-
45.532	45.455	45.595	18013037	0.42	5099968	0.56	3.53	Octacosane
45.738	45.7	45.795	10215939	0.24	3325636	0.36	3.07	Heptadecane, 3-methyl-
46.441	46.31	46.495	125225887	2.93	31900744	3.5	3.93	Heneicosane
47.905	47.84	47.95	11748108	0.27	3213823	0.35	3.66	Octadecane
48.103	48.04	48.165	13710065	0.32	3910246	0.43	3.51	2-methyloctacosane
48.766	48.645	48.84	103849904	2.43	25218287	2.77	4.12	Heneicosane
51.004	50.895	51.085	83761457	1.96	21524145	2.36	3.89	Heneicosane

53.53	53.405	53.625	62011382	1.45	12712540	1.39	4.88	Hentriacontane

Appendix 16: Retention time in minutes and peak size of hydrocarbon compounds and possible intermediates identified in BH media inoculated with isolate 1C

Ret. Time	Start time	End time	Area	Area %	Height	Height %	A/H	Compound
8.267	8.225	8.325	737466	0.09	256679	0.17	2.87	Heptane, 2,4-dimethyl-
17.436	17.395	17.495	835727	0.1	294609	0.2	2.84	Octane, 5-ethyl-2-methyl-
22.724	22.675	22.785	994757	0.12	329552	0.22	3.02	Tridecane
25.764	25.72	25.83	867645	0.11	278435	0.19	3.12	Dodecane, 4,6-dimethyl-
26.285	26.22	26.355	3220087	0.4	942092	0.63	3.42	Tetradecane
26.584	26.525	26.66	475261	0.04	133562	0.07	3.56	2-Isopropyl-5-methyl-1-heptanol
26.88	26.82	26.935	480869	0.04	132560	0.07	3.63	11-Methyldodecanol
27.177	27.12	27.23	414006	0.04	112178	0.06	3.69	11-Methyldodecanol
29.38	29.335	29.52	224244	0.02	63870	0.03	3.51	1-Tetradecanol
29.642	29.57	29.72	5520539	0.68	1643611	1.11	3.36	Tetradecane
31.718	31.655	31.795	1982379	0.25	528943	0.36	3.75	Hexadecane
31.922	31.88	31.975	622656	0.08	224133	0.15	2.78	Tetradecane, 3-methyl-
32.808	32.74	32.87	7739684	0.96	2215566	1.49	3.49	Pentadecane
32.9	32.87	32.98	1033360	0.13	305943	0.21	3.38	Heptadecane
33.983	33.865	34.03	743354	0.09	141149	0.08	5.27	1-Decanol, 2-hexyl-
34.248	34.18	34.295	1081841	0.13	295922	0.2	3.66	Hexadecane
34.885	34.825	34.91	606299	0.05	137140	0.07	4.42	1-Dodecanol, 2-hexyl-
35.17	35.155	35.185	69120	0.01	44449	0.02	1.56	2-Isopropyl-5-methylhex-2-enal

35.206	35.185	35.25	139282	0.01	60109	0.03	2.32	1-Dodecanol, 2-hexyl-
35.804	35.725	35.88	8434891	1.04	2565841	1.73	3.29	Hexadecane
37.299	37.245	37.37	1395598	0.17	369792	0.25	3.77	Pentadecane, 2,6,10-trimethyl-
37.852	37.805	37.905	778628	0.1	259154	0.17	3	Heptadecane, 2,6,10,15-tetramethyl-
38.641	38.565	38.715	8407962	1.04	2444713	1.65	3.44	Nonadecane
38.925	38.855	38.97	3010786	0.37	695012	0.47	4.33	Pentadecane, 2,6,10,14-tetramethyl-
39.035	38.97	39.125	8581060	1.06	1038737	0.7	8.26	Heneicosane
39.196	39.125	39.255	10262056	1.27	1619292	1.09	6.34	Eicosane
39.33	39.255	39.36	9422369	1.17	1711227	1.15	5.51	Dodecane, 2,7,10-trimethyl-
39.425	39.36	39.49	20383646	2.52	2894709	1.95	7.04	Heneicosane
39.515	39.49	39.61	16303940	2.02	2661074	1.79	6.13	Tetratetracontane
39.635	39.61	39.805	9222438	1.14	1629170	1.1	5.66	2-methyloctacosane
40.229	40.165	40.245	814373	0.1	235144	0.12	3.46	1-Dodecanol, 2-octyl-
40.27	40.245	40.28	372273	0.03	186179	0.1	2	4-Cyclohexyl-1-butanol
41.332	41.265	41.405	7184731	0.89	2085716	1.4	3.44	Nonadecane
41.704	41.64	41.79	2114045	0.26	530516	0.36	3.98	Hexadecane, 2,6,10,14-tetramethyl-
43.904	43.785	43.975	8114365	1	2111423	1.42	3.84	Heneicosane
44.17	44.085	44.2	1746735	0.22	401158	0.27	4.35	Valtrate
44.809	44.76	44.84	787578	0.1	290925	0.2	2.71	Tetratetracontane
44.923	44.845	45.075	9404354	1.15	1288662	0.71	7.3	n-Hexadecanoic acid
45.815	45.75	45.85	1796319	0.22	537529	0.36	3.34	Eicosane
46.25	46.225	46.29	1291983	0.16	360586	0.24	3.58	2-methylhexacosane
46.352	46.29	46.415	7758657	0.96	1913408	1.29	4.05	Heneicosane
46.974	46.83	46.995	3233414	0.4	593919	0.4	5.44	Tetrapentacontane, 1,54-dibromo-

47.02	46.995	47.055	1370765	0.17	500793	0.34	2.74	Tetrapentacontane, 1,54-dibromo-
48.703	48.495	48.855	51369390	6.35	6210742	4.18	8.27	Heneicosane
49.072	48.99	49.12	2780268	0.34	632664	0.43	4.39	cis-Vaccenic acid
49.075	48.995	49.115	7604921	0.95	1353992	0.75	5.62	cis-13-Eicosenoic acid
49.179	49.115	49.285	12470326	1.06	1484943	0.76	8.4	Oleic Acid
49.5	49.365	49.535	10797166	1.34	1754201	1.18	6.16	Heneicosane
49.611	49.535	49.8	24657723	3.05	3086372	2.08	7.99	Tetratetracontane
50.939	50.86	51.02	4145426	0.51	1137353	0.7	3.64	Heneicosane
53.463	53.385	53.55	2753302	0.34	679530	0.46	4.05	Heneicosane
54.165	53.975	54.37	24954944	3.09	2425947	1.63	10.29	Heneicosane
55.413	55.05	55.69	49523777	6.13	3276002	2.21	15.12	Benzene, 1,4-bis(3-formylcyclo hexan-2-one-1-yl)-

Appendix 17: Retention time in minutes and peak size of hydrocarbon compounds and possible intermediates identified in BH media inoculated with isolate 2C

Ret. Time	Start time	End time	Area	Area %	Height	Height %	A/H	Compound
3.447	3.395	3.515	638849	0.12	225919	0.22	2.83	Cyclopentane, methyl-
8.267	8.21	8.335	853927	0.16	293212	0.28	2.91	Heptane, 2,4-dimethyl-
8.859	8.81	8.93	515079	0.1	177330	0.17	2.9	2,4-Dimethyl-1-heptene
17.435	17.37	17.505	1052658	0.2	340648	0.32	3.09	Octane, 5-ethyl-2-methyl-
18.411	18.385	18.5	559717	0.11	167213	0.16	3.35	Phenylethyl Alcohol
19.13	19.075	19.195	486088	0.09	160990	0.15	3.02	Undecane, 5-methyl-

25.762	25.695	25.845	1176804	0.22	350377	0.33	3.36	Nonane, 5-(2-methylpropyl)-
26.279	26.215	26.36	1215934	0.23	357351	0.34	3.4	Tetradecane
26.577	26.5	26.66	702231	0.13	164885	0.16	4.26	2-Isopropyl-5-methyl-1-heptanol
26.88	26.81	26.95	589537	0.11	162540	0.15	3.63	1-Undecene, 7-methyl-
27.171	27.125	27.235	313344	0.06	107324	0.1	2.92	11-Methyldodecanol
27.306	27.24	27.39	814419	0.15	222443	0.21	3.66	Dodecane, 4,6-dimethyl-
29.639	29.57	29.715	1648784	0.31	485608	0.46	3.4	Tetradecane
31.513	31.45	31.595	564769	0.11	144032	0.14	3.92	Hexadecane, 1,1-bis(dodecyloxy)-
31.709	31.63	31.785	665029	0.13	173171	0.16	3.84	Hexadecane
32.809	32.735	32.86	2480853	0.47	731323	0.7	3.39	Pentadecane
32.907	32.86	32.995	1108983	0.21	318705	0.3	3.48	Heptadecane
33.978	33.92	34.045	377950	0.07	114331	0.1	3.31	2-Isopropyl-5-methyl-1-heptanol
34.238	34.165	34.315	1011198	0.19	264721	0.25	3.82	Heneicosane
35.799	35.725	35.88	2686113	0.51	802642	0.76	3.35	Nonadecane
37.302	37.24	37.365	486862	0.09	129240	0.12	3.77	Pentadecane, 2,6,10-trimethyl-
38.641	38.565	38.72	2734566	0.52	780504	0.74	3.5	Heptadecane
38.925	38.85	39	694459	0.13	178911	0.17	3.88	Pentadecane, 2,6,10,14-tetramethyl-
39.189	39.115	39.245	882922	0.17	265141	0.25	3.33	Eicosane
40.321	40.265	40.46	976411	0.19	215229	0.2	4.54	Eicosane
41.331	41.26	41.4	2186360	0.42	657262	0.63	3.33	Nonadecane
41.697	41.63	41.775	834182	0.16	196437	0.19	4.25	Hexadecane, 2,6,10,14-tetramethyl-
42.991	42.88	43.05	448428	0.09	86674	0.08	5.17	Octadecane, 2-methyl-
43.897	43.845	43.97	2173802	0.41	626617	0.6	3.47	Nonadecane
44.805	44.735	44.85	797002	0.15	271109	0.26	2.94	Eicosane
44.92	44.85	44.99	3178746	0.6	758680	0.72	4.19	n-Hexadecanoic acid

45.485	45.43	45.535	336145	0.06	106749	0.1	3.15	1-Dodecanol, 2-hexyl-
45.802	45.745	45.86	763464	0.15	219204	0.21	3.48	Eicosane
46.035	46.025	46.115	547216	0.1	95591	0.09	5.72	Hexadecane, 2-methyl-
46.351	46.115	46.39	12651892	2.4	1545373	1.47	8.19	Heneicosane
46.66	46.6	46.845	3932465	0.75	511764	0.49	7.68	Pentatriacontane
48.545	48.5	48.62	487717	0.09	89146	0.08	5.47	2-methylhexacosane
48.684	48.62	48.76	1726508	0.33	461138	0.44	3.74	Heneicosane
49.067	48.975	49.115	3418812	0.62	736651	0.69	4.64	Oleic Acid
49.169	49.115	49.31	3634900	0.66	673682	0.64	5.4	Oleic Acid
49.653	49.42	49.83	12903417	2.45	1222397	0.61	10.56	2-methylhexacosane
49.88	49.83	49.96	1230696	0.23	262820	0.61	4.68	Eicosane
50.78	50.71	50.85	766722	0.15	188287	0.61	4.07	2-methylhexacosane
50.937	50.85	51.02	1616785	0.31	404873	0.61	3.99	Heneicosane
53.458	53.35	53.54	970876	0.18	200835	0.61	4.83	Heneicosane
55.489	55.39	55.67	4238150	0.81	427319	0.61	9.92	2-methyltetracosane

Appendix 18: Retention time in minutes and peak size of hydrocarbon compounds and possible intermediates identified in BH media inoculated with isolate 3A

Ret. Time	Start time	End Time	Area	Area %	Height	Height %	A/H	Name
3.448	3.4	3.51	700676	0.31	237576	0.48	2.95	Cyclopentane, methyl-
3.969	3.93	4.025	351832	0.15	132016	0.26	2.67	Cyclohexane
5.174	5.125	5.24	1036740	0.47	381474	0.77	2.72	1-Butanol, 3-methyl-
8.266	8.21	8.34	976028	0.43	309736	0.62	3.15	Heptane, 2,4-dimethyl-
8.863	8.815	8.925	519806	0.23	178985	0.36	2.9	2,4-Dimethyl-1-heptene

17.442	17.385	17.52	1201407	0.52	344306	0.69	3.49	Octane, 5-ethyl-2-methyl-
17.667	17.63	17.74	364994	0.16	115123	0.23	3.17	Octane, 5-ethyl-2-methyl-
18.36	18.28	18.395	446626	0.19	121616	0.24	3.67	1-Octanol, 2,7-dimethyl-
18.445	18.395	18.585	1051525	0.47	182597	0.37	5.76	Phenylethyl Alcohol
18.963	18.91	19.035	912820	0.4	279080	0.56	3.27	Undecane
19.143	19.095	19.21	505884	0.22	158767	0.32	3.19	Decane, 3,7-dimethyl-
22.74	22.665	22.85	3369332	1.47	859272	1.72	3.92	Dodecane
23.313	23.26	23.375	596782	0.26	180762	0.36	3.3	Undecane, 2,5-dimethyl-
25.064	25.005	25.17	810241	0.35	208586	0.42	3.88	Dodecane, 2-methyl-
25.293	25.24	25.365	684995	0.3	198691	0.4	3.45	Dodecane, 3-methyl-
25.45	25.39	25.53	928488	0.41	236927	0.48	3.92	Octane, 2,3,7-trimethyl-
25.777	25.705	25.865	1536275	0.67	401715	0.81	3.82	Hexadecane
26.308	26.22	26.4	6316372	2.76	1632985	3.28	3.87	Tetradecane
26.586	26.465	26.675	1176665	0.51	181146	0.36	6.5	2-Isopropyl-5-methyl-1-heptanol
27.325	27.26	27.42	1235156	0.54	318954	0.64	3.87	Nonane, 5-(2-methylpropyl)-
28.054	28.005	28.11	402217	0.18	130982	0.26	3.07	Tridecane, 6-methyl-
28.318	28.275	28.38	423314	0.18	154792	0.31	2.73	Tridecane, 4-methyl-
28.482	28.42	28.565	1236321	0.54	316953	0.64	3.9	Tridecane, 2-methyl-
28.984	28.91	29.095	1941568	0.85	442043	0.89	4.39	Dodecane, 2,6,10-trimethyl-
29.402	29.345	29.455	266985	0.1	82318	0.16	3.24	n-Pentadecanol
29.663	29.585	29.76	9094980	3.97	2417626	4.85	3.76	Tetradecane
31.203	31.135	31.325	1530435	0.67	218932	0.44	6.99	1-Tricosene
31.381	31.325	31.44	646795	0.28	167775	0.34	3.86	Tetradecane, 5-methyl-
31.545	31.44	31.615	1519449	0.66	375036	0.75	4.05	Tetradecane, 4-methyl-
31.735	31.615	31.85	3821440	1.67	844413	1.69	4.53	Hexadecane

31.942	31.88	32.01	1151793	0.5	317181	0.64	3.63	Tetradecane, 3-methyl-
32.833	32.75	32.9	12113081	5.29	3134392	6.29	3.86	Pentadecane
32.93	32.9	33.015	1219986	0.53	383175	0.77	3.18	Heneicosane
33.728	33.67	33.785	236570	0.25	70939	0.14	3.33	11-Methyldodecanol
33.986	33.92	34.065	654552	0.29	163766	0.33	4	1-Decanol, 2-hexyl-
34.26	34.19	34.325	1588652	0.69	430397	0.86	3.69	Heptadecane
34.597	34.5	34.695	1781767	0.78	272799	0.55	6.53	Pentadecane, 4-methyl-
34.777	34.71	34.845	2055727	0.9	476237	0.96	4.32	Tetradecane, 2,6,10-trimethyl-
34.99	34.935	35.07	1236223	0.54	322405	0.65	3.83	Pentadecane, 3-methyl-
35.826	35.74	35.925	12332005	5.38	3333638	6.69	3.7	Hexadecane
37.154	37.1	37.21	836334	0.37	212892	0.43	3.93	Hexacosane
37.339	37.21	37.405	2478781	1.08	572171	1.15	4.33	Heptadecane
37.512	37.475	37.575	345445	0.15	117084	0.23	2.95	Tetradecane, 4-methyl-
37.662	37.605	37.76	1206414	0.53	314488	0.63	3.84	Hexadecane, 2-methyl-
37.874	37.81	37.945	1197657	0.52	323740	0.65	3.7	Heptadecane, 2,6,10,15-tetramethyl-
38.445	38.44	38.45	33582	0.01	59649	0.12	0.56	1-Hexacosanol
38.67	38.58	38.765	11851304	5.17	3009478	6.04	3.94	Nonadecane
38.947	38.87	39.03	2767144	1.21	711875	1.43	3.89	Pentadecane, 2,6,10,14-tetramethyl-
39.218	39.17	39.28	762695	0.33	244891	0.49	3.11	Eicosane
39.935	39.815	40.035	2221445	0.97	284713	0.57	7.8	Eicosane
40.265	40.225	40.305	604590	0.26	151191	0.3	4	Heptadecane, 4-methyl-
40.335	40.305	40.5	2327944	1.02	300739	0.6	7.74	Hexadecane, 2,6,10,14-tetramethyl-
40.621	40.56	40.685	1060972	0.46	265145	0.53	4	Heptadecane, 3-methyl-
40.735	40.685	40.78	318868	0.14	92283	0.18	3.46	1-Heptacosanol

41.26	41.215	41.29	477646	0.21	137767	0.28	3.47	Cyclohexane, 1,2,3,5-tetraisopropyl-
41.368	41.29	41.475	10545389	4.6	2634252	5.29	4	Nonadecane
41.57	41.5	41.64	1012130	0.44	160133	0.32	6.32	Octacosanol
41.723	41.66	41.805	3383250	1.48	791758	1.59	4.27	Hexadecane, 2,6,10,14-tetramethyl-
42.509	42.44	42.545	901677	0.39	228670	0.46	3.94	2-methylhexacosane
43.03	42.97	43.09	924883	0.4	295952	0.59	3.13	Octacosane
43.229	43.18	43.28	700219	0.31	239070	0.48	2.93	2-methyloctacosane
43.925	43.84	44.015	8008584	3.5	2136127	4.29	3.75	Heneicosane
44.834	44.765	44.885	1115339	0.49	329163	0.66	3.39	Eicosane
44.986	44.88	45.19	7304707	3.21	688803	1.38	10.6	n-Hexadecanoic acid
45.385	45.31	45.48	1134560	0.5	198608	0.4	5.71	Tetradecane, 4-methyl-
45.509	45.48	45.565	724644	0.32	235530	0.47	3.08	2-methyltetracosane
45.82	45.77	45.87	892263	0.39	269099	0.54	3.32	2-methylhexacosane
46.221	46.18	46.24	382973	0.17	161447	0.32	2.37	2-methylhexacosane
46.376	46.31	46.47	7724475	3.37	1915008	3.84	4.03	Heneicosane
46.711	46.68	46.75	630614	0.28	204255	0.41	3.09	17-Pentatriacontene
47.351	47.295	47.37	330431	0.14	117374	0.24	2.82	Pentatriacontane
47.608	47.55	47.65	494457	0.22	162758	0.33	3.04	2-methylhexacosane
47.8	47.79	47.83	2134276	0.95	928971	1.9	2.3	7-Hexadecenal, (Z)-
48.199	48.18	48.345	516831	0.23	98489	0.2	5.25	17-Pentatriacontene
48.714	48.625	48.805	5469932	2.39	1341160	2.69	4.08	Heneicosane
49.71	49.52	49.74	16438308	1.16	1540532	0.82	10.67	Octadecanoic acid
49.87	49.755	49.895	10453490	4.56	1891158	3.8	5.53	Tetratetracontane
50.02	49.895	50.035	23945647	10.45	4022573	8.07	5.95	Tetracontane
50.084	50.035	50.515	36737717	15.99	4030478	8.1	9.11	Tetratetracontane

50.78	50.71	50.79	663900	0.29	231269	0.46	2.87	17-Pentatriacontene
50.962	50.895	51.075	4517369	1.97	1078638	2.16	4.19	Heneicosane
51.654	51.635	51.755	839470	0.37	191693	0.38	4.38	17-Pentatriacontene
52.752	52.705	52.875	1307703	0.57	187956	0.38	6.96	2-methylhexacosane
53.496	53.375	53.605	3059503	1.34	579650	1.16	5.28	Heneicosane
53.76	53.745	53.865	470388	0.21	74614	0.15	6.3	2-Methyl-E-7-octadecene

Appendix 19: Retention time in minutes and peak size of hydrocarbon compounds and possible intermediates identified in BH media inoculated with isolate 4A2

Ret. Time	Start time	End time	Area	Area %	Height	Height %	A/H	Compound
3.45	3.395	3.52	726873	0.09	250255	0.22	2.9	Cyclopentane, methyl-
5.183	5.135	5.245	512409	0.07	192410	0.17	2.66	1-Butanol, 3-methyl-
8.279	8.22	8.35	856097	0.11	285867	0.25	2.99	Heptane, 2,4-dimethyl-
8.869	8.815	8.94	506885	0.07	174403	0.15	2.91	2,4-Dimethyl-1-heptene
17.451	17.39	17.535	993098	0.13	291414	0.26	3.41	Octane, 5-ethyl-2-methyl-
17.678	17.625	17.745	387650	0.05	117434	0.1	3.3	Octane, 5-ethyl-2-methyl-
18.206	18.15	18.275	369571	0.05	108357	0.09	3.41	1-Decene, 2,4-dimethyl-
18.36	18.3	18.415	401027	0.05	93758	0.08	4.28	1-Octanol, 3,7-dimethyl-
18.45	18.415	18.55	472646	0.06	115603	0.1	4.09	Phenylethyl Alcohol
19.142	19.09	19.21	434669	0.06	136140	0.12	3.19	Decane, 3,7-dimethyl-
19.357	19.315	19.425	211561	0.03	71151	0.06	2.97	Decane, 3,7-dimethyl-
21.261	21.22	21.325	187515	0.02	64349	0.05	2.91	Decane, 3,7-dimethyl-
21.427	21.38	21.485	273219	0.04	91707	0.08	2.98	Dodecane

22.736	22.68	22.815	447805	0.06	122452	0.11	3.66	Dodecane
25.78	25.705	25.86	1070987	0.14	305011	0.27	3.51	Dodecane, 4,6-dimethyl-
26.295	26.23	26.38	894504	0.12	239385	0.21	3.74	Tetradecane
26.603	26.52	26.68	660326	0.08	147854	0.13	4.47	2-Isopropyl-5-methyl-1-heptanol
26.9	26.83	26.975	523751	0.07	132731	0.12	3.95	1-Undecene, 7-methyl-
27.198	27.155	27.255	226184	0.03	82285	0.07	2.75	11-Methyldodecanol
27.324	27.27	27.415	682752	0.09	189974	0.17	3.59	Nonane, 5-(2-methylpropyl)-
29.66	29.59	29.75	998226	0.13	270397	0.24	3.69	Tetradecane
31.724	31.695	31.795	164714	0.02	53656	0.05	3.07	Hexadecane
32.824	32.755	32.875	1256844	0.16	335008	0.29	3.75	Pentadecane
32.924	32.875	33.015	982697	0.13	270016	0.24	3.64	Dodecane, 4,6-dimethyl-
34.255	34.19	34.345	904461	0.12	231551	0.2	3.91	Eicosane
34.547	34.485	34.605	371972	0.05	98708	0.09	3.77	Eicosane
35.822	35.75	35.915	1349631	0.17	357062	0.31	3.78	Hexadecane
38.658	38.585	38.715	1775136	0.23	402839	0.35	4.41	Nonadecane
38.8	38.715	38.875	2470686	0.32	285616	0.25	8.65	Hexacosane
39.061	38.875	39.11	13688210	1.77	1413577	1.24	9.68	Heneicosane
39.209	39.11	39.26	11901775	1.54	1529988	1.34	7.78	Eicosane
39.27	39.26	39.28	1251420	0.16	1071048	0.94	1.17	Nonadecane
39.319	39.28	39.625	11125569	1.44	1052611	0.92	10.57	2-methyloctacosane
40.352	40.275	40.495	925969	0.12	184665	0.16	5.01	Eicosane
41.351	41.28	41.415	1055070	0.14	291533	0.26	3.62	Nonadecane
41.712	41.66	41.81	405816	0.05	103615	0.09	3.92	Hexadecane, 2,6,10,14-tetramethyl-
43.695	43.655	43.78	645131	0.08	94565	0.08	6.82	Eicosane
43.917	43.78	44.025	2698281	0.35	427938	0.38	6.31	Heneicosane
44.825	44.735	44.87	995800	0.13	245868	0.22	4.05	Eicosane
44.936	44.87	45.075	2908366	0.37	456200	0.39	6.38	n-Hexadecanoic acid

45.268	45.18	45.335	850920	0.11	138453	0.12	6.15	2-methylhexacosane
45.82	45.75	45.88	1121854	0.14	322163	0.28	3.48	Eicosane
46.265	46.06	46.315	3547607	0.46	421702	0.37	8.41	Tetracosane
46.38	46.315	46.555	8085880	1.04	765248	0.67	10.57	Hexacosane
46.595	46.555	46.625	1298305	0.17	342975	0.3	3.79	17-Pentatriacontene
46.695	46.625	46.745	1934666	0.25	307219	0.27	6.3	17-Pentatriacontene
46.953	46.745	47.155	6076857	0.79	425356	0.37	14.29	Pentatriacontane
47.478	47.365	47.555	1747170	0.23	212787	0.19	8.21	Cyclohexane, eicosyl-
48.105	48.015	48.165	583147	0.07	101668	0.09	5.74	Dodecanoic acid, ethenyl ester
48.7	48.465	48.835	36030250	4.66	2965350	2.6	12.15	Heneicosane
49.206	48.835	49.485	49334618	6.3	1590769	1.37	31.01	Oleic Acid
50.546	50.41	50.76	1978041	0.26	220451	0.19	8.97	Tetracontane
50.81	50.76	50.91	997508	0.13	175011	0.15	5.7	2-methylhexacosane
50.96	50.91	51.06	1059982	0.14	227258	0.2	4.66	Heneicosane
51.833	51.585	51.96	1422264	0.18	125983	0.11	11.29	2-methylhexacosane
52.11	51.96	52.21	2129809	0.28	210341	0.18	10.13	Heneicosane
52.61	52.485	53.11	3244609	0.42	232765	0.2	13.94	2-methylhexacosane
53.264	53.135	53.435	425478	0.05	56834	0.05	7.49	2-Cyclohexylnonadecane
54.178	53.935	54.435	11624741	1.5	1167375	1.03	9.96	Heneicosane