BEHAVIORAL PROPERTIES OF LOCALLY ISOLATED *ACINETOBACTER* SPECIES IN DEGRADING HYDROCARBON CHAIN IN CRUDE OIL AND USED COOKING OIL

BY

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ABSTRACT

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Tan Yong Hui

Oil degrading bacteria were isolated from soil and pond water in Kuala Lumpur. Oil degradability of the bacterial isolates was tested in the minimal salt broth with 1% spent engine oil, crude oil and used cooking oil. Isolates that showed flocculation activity were selected for further studies. These isolates were confirmed by 16S rRNA sequencing tools. Based on BLAST database homology search results, the 16S rRNA amplification sequence revealed the individual isolates were closely related to *Acinetobacter radioresistens*, *Acinetobacter baumannii*, *Acinetobacter calcoaceticus*, *Stenotrophomonas maltophilia* and *Entrerobacter sakazakii*, respectively. Three strains that have the faster flocculation formation were selected for further tests. Their behavioral properties in terms of cell mass, pH change and hydrocarbon chain degradation pattern in minimal salt broth supplemented with different types of oil were monitored for 17 days. Based on the morphology in minimal salt broth supplemented with crude oil, the isolates demonstrated a unique mechanism in clumping oil and forming aggregates, suggesting these mechanisms are to facilitate the hydrocarbon uptake. The formation of the aggregate was clearly seen with nigrosin background staining and observed under the phase contrast microscope. Among *A. radioresisten, A. baummanii* and *A. calcoaceticus*, the

species *A. calcoaceticus* showed promising degrading ability in spent engine oil, crude oil or used cooking oil. The ability of the isolates in degrading petroleum hydrocarbon chains was monitored using gas chromatography mass spectrometry (GCMS). Gas chromatography mass spectrometry analysis revealed several metabolites, which include Bis [3-(3, 5-di-tert-butyl-4-hydroxyphenyl)propyl] maleate. Decanedioic acid detected at 39.81 min from the gas chromatography analysis was believed to be the byproduct of the longer carbon chain length degradation. On top of that, nutritional effects in terms of nitrogen source and metal ions in enhancing the bioremediation rate by *A. calcoaceticus* were studied. From the present study, *A. calcoaceticus* was found to be the best degrader of crude oil and used cooking oil. The best nitrogen source and metal ion that enhanced hydrocarbon chain degradation were polypeptone and magnesium.

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APPROVAL SHEET

This dissertation entitled "**BEHAVIORAL PROPERTIES OF LOCALLY ISOLATED *ACINETOBACTER* SPECIES IN DEGRADING HYDROCARBON CHAIN IN CRUDE OIL AND USED COOKING OIL**" was prepared by TAN YONG HUI and submitted as partial fulfillment of the requirements for the degree of Master of Science at Universiti Tunku Abdul Rahman.

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PERMISSION SHEET

It is hereby certified that **TAN YONG HUI** (ID No: **07UEM02856)** has completed this thesis entitled "BEHAVIORAL PROPERTIES OF LOCALLY ISOLATED *ACINETOBACTER* SPECIES IN DEGRADING HYDROCARBON CHAIN IN CRUDE OIL AND USED COOKING OIL" under supervision of DR WOO KWAN KIT (Superior) and DR HII SIEW LING (Co-supervisor) from the Department of Bioscience, Faculty of Engineering and Science.

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been dully acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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TABLE OF CONTENTS

	Page
ABSTRACT	**ii**
AKNOWLEDGEMENT	**iv**
APPROVAL SHEET	**v**
PERMISSION SHEET	**vi**
DECLARATION	**vii**
TABLE OF CONTENTS	**viii**
LIST OF TABLES	**xi**
LIST OF FIGURES	**xii**
LIST OF ABBREVIATIONS	**xiv**
CHAPTERS	
1.0 INTRODUCTION	**1**
2.0 LITERATURE REVIEW	**4**
2.1 Composition and Properties of Petroleum	4
2.2 Effect of Oil Spill to Living Organisms	8
2.3 Conventional Methods Involve in Oil Spill Cleaning Up	9
2.3.1 Chemical Methods	9
2.3.2 Mechanical Methods	11
2.3.2.1 After Treatments of Mechanical Methods	12
2.4 Biodegradation and Bioremediation	14
2.4.1 Bioaugmentation	18
2.4.2 Biostimulation	20
2.5 Oil Transformation in Marine Environment	22
2.6 General Family of Microbes Involved in Oil Degrading	26
2.7 Mode of Action for Microbes in Degrading Oil	30
2.8 Factors Affecting Cleanup Process by Microbes	34
2.9 New Trend in Oil Spill Management and Cleaning	37
3.0 MATERIAL AND METHODS	**38**
3.1 Material	38
3.1.1 Equipments and apparatus	38
3.1.2 Kits, Chemicals and reagents	39
3.2 Methods	39
3.2.1 Sample collection	39

3.2.2 Isolation and Screening of Hydrocarbon Degrading Bacteria 40
3.2.3 Isolate Identification 41
3.2.3.1 The Biolog Identification System 41
3.2.3.2 Molecular Sequencing and Phylogeny 41
3.2.4 Hydrocarbon Degradation Analysis 42
3.2.4.1 Measurement of Cell Optical Density 43
3.2.4.2 Determination of Hydrocarbon Chain Degradation Pattern using GCMS 43

3.2.5 Culture Medium Modification 44
3.2.5.1 Nitrogen Source Substitution 45
3.2.5.2 Metal Ion Substitution 46
3.2.6 Metabolite Analysis 46
3.2.6.1 Protein Analysis 47

4.0 RESULTS 48
4.1 Screening and Isolation 48
4.2 Speciation of Isolates 53
4.3 Hydrocarbon Degradation Analysis 56
4.3.1 Spent Engine Oil as Sole Carbon Source 56
4.3.2 Crude Oil as Sole Carbon Source 60
4.3.3 Used Cooking Oil as Sole Carbon Source 65
4.4 Culture Medium Modification 67
4.4.1 Nitrogen Source Substitution 67
4.4.1.1 Nitrogen Source Substitution in MSM with 1% v/v Crude Oil 69
4.4.1.2 Nitrogen Source Substitution in MSM with 1% Used Cooking Oil 73
4.4.2 Metal Ion Substitution 76
4.4.2.1 Metal Ion Substitution in MSM with 1% v/v Crude Oil 76
4.4.2.2 Metal Ion Substitution in MSM with 1% v/v Used Cooking Oil 80
4.5 Metabolite Analysis 83
4.5.1 SDS-PAGE 83

5.0 DISCUSSION 85
5.1 Isolation and Screening of Hydrocarbon Degrading Bacteria 85
5.2 Hydrocarbon Utilization Pattern of Bacterial Species 87
5.2.1 Degradation Pattern on Spent Engine Oil 88
5.2.2 Degradation Pattern on Crude Oil 90
5.2.3 Degradation Pattern on Used Cooking Oil 92
5.3 Culture Medium Modification 93
5.3.1 Nitrogen Source 93
5.3.2 Metal Ions 95
5.4 Lipase Activity 96
5.5 Metabolite Analysis 98

6.0 CONCLUSION AND FUTURE PERPECTIVES **100**
6.1 Conclusion 100
6.2 Future Perspectives 101

REFERENCES **102**

APPENDICES **111**

LIST OF TABLES

Table		Page
2.1	16 PAH priority pollutants defined by US-EPA	6
2.2	Chemical composition and the function of each component of Inopol EAP22	17
2.3	Genera of hydrocarbon degrading bacteria	28
4.1	Summary of the species identification test results	54

LIST OF FINGURES

Figure		Page
2.1	Chemical structure for alkanes, alkenes and aromatic hydrocarbons	7
2.2	Pathways by which spilled oil may enter the marine ecosystem	23
2.3	Microbial degradation network of oil	30
2.4	Basic metabolic pathway involved in the metabolism of n-alkanes	32
2.5	Metabolic pathways involved in the degradation of the isoprenoid alkane pristine	33
4.1	Comparison between isolates with and without floccules formation	50
4.2	Close up view of the floccules formation by *A.calcoaceticus* culturing in spent engine oil medium	51
4.3	Appearance of culture medium after 5 days of incubation	52
4.4	Phylogenetic tree, constructed by the neighbour joining method	55
4.5	Gas chromatography profile of the culture medium supplemented with 1% spent engine oil on day 17	58
4.6	Time course of (a) pH; (b) growth; for *Acinetobacter* bacteria strains cultured in minimal salt broth supplemented with 1 % of spent engine oil for 17 days	59
4.7	Gas chromatography profile of the culture medium supplemented with 1% crude oil on day 17	62
4.8	Time course of (a) pH and (b) growth for *Acinetobacter* bacteria strains cultured in minimal salt broth supplemented with 1 % of crude oil for 17 days	63
4.9	Phase contrast microscopic morphology of the isolates in minimal salt medium supplemented with crude oil on day 3	64
4.10	Gas chromatography profile of the culture medium supplemented with 1% used cooking oil on day 17	66

4.11 Time course of (a) pH; (b) growth; for *Acinetobacter* bacteria strains cultured in minimal salt broth supplemented with 1 % of used cooking oil for 17 days 67

4.12 Gas chromatography profile for culture medium of *Acinetobacter calcoaceticu*s in 1% crude oil with the substitution of different nitrogen sources on day 14 71

4.13 Time course of (a) pH; (b) growth; (c) lipase activity of *A. calcoaceticus* cultured in minimal salt broth supplemented with 1 % of crude oil and different nitrogen sources 72

4.14 Gas chromatography profile for the culture medium of *Acinetobacter calcoaceticu*s in 1% used cooking oil with different nitrogen source substitution on day 14 74

4.15 Time course of (a) pH, (b) growth, (c) lipase activity for *A. calcoaceticus* cultured in minimal salt broth supplemented with 1 % of used cooking oil and different nitrogen sources substitution respectively 75

4.16 Gas chromatography profile for culture medium of *Acinetobacter calcoaceticu*s in 1% crude oil with different metal ion substitution on day 14 78

4.17 Time course of (a) pH; (b) growth; (c) lipase activity for *A. calcoaceticus* cultured in minimal salt broth supplemented with 1 % of crude oil and different metal ion substitution respectively 79

4.18 Gas chromatography profile for culture medium of *Acinetobacter calcoaceticu*s in 1% used cooking oil with different metal ion substitution on day 14 81

4.19 Time course of (a) pH; (b) growth; (c) lipase activity for *A. calcoaceticus* cultured in minimal salt broth supplemented with 1 % of used cooking oil and different metal ion substitution respectively 82

4.20 SDS PAGE for metabolite of *A. baumannii* and *A. calcoaceticus* 84

LIST OF ABBREVATIONS

Ă	Angstrom
ABA	Autochthonous Bioaugmentation
β	Beta
BLASTn	Basic Local Alignment Search Tool (nucleotide)
CABI	CAB International
CFU	Colony Forming Unit
cm	centimeter
DNA	Deoxyribonucleic Acid
g	gram
GC	Gas Chromatography
GCMS	Gas Chromatography Mass Spectrometer
g/L	Gram per liter
IMO	International Maritime Organization
kDa	Kilo Dalton
KOH	Potassium Hydroxide
PCR	Polymerase Chain Reaction
mg/mL	milligram per milliliter
min	minute
mL	milliliter
NCBI	National Center for Biotechnology Information
nm	nanometer
NO_2	Nitrogen dioxide
PAGE	Polyacrylamide gel eletropheresis

PAH	Polycylic aromatic hydrocarbon
PFTBA	Perfluorotributylamine
pNPL	p-nitrophenil-laurate
rpm	Rotation per minute
SDS	Sodium dodecyl sulphate
SO_2	Sulfur dioxide
TCA	Tricarboxylic Acid
US EPA	United States Environment Protection Agency
UV	Ultra Violet
uL	micro liter
µmol	Micro Molar
w/v	Weight per volume
x g	times gravity
16S rRNA	16 svedberg ribosomal ribonucleic acid
°C	Degree Celsius

CHAPTER 1

INTRODUCTION

Petroleum hydrocarbons are the most widespread contaminants entering the aquatic environments (Cappello, Denaro, Genovese, Guiliano & Yakimov, 2006). These contaminants are exposed to our environment through industrial waste, urban runoffs, refineries/terminal, natural sources, tanker operations, tanker accidents, and leakage of oil from shipping or offshore production (Ventikos & Psaraftis, 2004). Pollution of petroleum hydrocarbon can cause detrimental effect to human and the ecosystem because many of the constituents of petroleum hydrocarbons or oily sludge are carcinogenic and potent immunotoxicants (Singh & Lin, 2008).

Efforts have been taken by scientists and researchers to combat oil spill and oil pollution. Among the common techniques to clean up oil spill, utilisation of microorganism to degrade the hydrocarbon is considered to be the most environmental friendly technique. According to Leahy & Cowell (1990), microorganisms are the major degraders of petroleum hydrocarbons in contaminated ecosystems. This is supported by Grifoll, Casellas, Bayona & Solanas (1992) that microorganisms play an important role in the degradation of aliphatic and aromatic hydrocarbons in terrestrial and aquatic ecosystems. Microorganisms use oil hydrocarbons as their exclusive source of carbon and

energy.

Hydrocarbon utilising bacteria are also known as hydrocarbonoclastic bacteria, they were first isolated almost a century ago. Review lists 79 bacterial genera that can use hydrocarbons as the sole source of carbon and energy; also 9 cyanobacterial genera, 103 fungal genera and 14 algal genera that are known to degrade or transform hydrocarbons (Head, Jones & Roling, 2006). Examples of oil degrading bacteria are *Acinetobacter, Pseudomonas, Phenylobacterium, Stenotrophomonas, Gluconobacter, Agrobacterium, Vibrio, Micrococcus, Aeromonas, Beijerinckia, Flavobavterium, Norcadia, Corynebacterium, Spingomonas, Microbacterium, Paracoccus, Burkholderia* and many more (Jacques, Okeke, Bento, Peralba & Camargo, 2007).

The natural process where microorganisms utilise the contaminant as food and energy source is known as biodegradation. Biodegradation is a slow process and one of the primary mechanisms to eliminate petroleum and other hydrocarbon pollutants from the environment. It is considered an environmentally acceptable way of eliminating oils and fuel because the majority of hydrocarbons in crude oils and refined products are biodegradable (Calvo, Manzanera, Silva-Castro, Uad & Gonzalec-Lopez, 2008). The technique of using indigenous microorganisms *in situ* to decontaminate the affected sites by nutrient addition or seeding of microorganisms to the contaminated area with the aim to speed up biodegradation is known as bioremediation. According to Venosa et al. (1996), bioremediation enhances

the removal of crude oil several times more than the intrinsic rate. This approach is able to reclaim contaminated land and therefore reduces the threat to groundwater and enhances the rate of biodegradation (Mishra, Jyot, Kuhad & Lai, 2001).

This project aims to establish information on oil and hydrocarbon chain degradability of the locally isolated bacterial strains, to investigate the effect of nutrient, different nitrogen and metal ion sources, towards the hydrocarbon chain degradation ability and to study the feature of metabolite produce by the selected strains.

CHAPTER 2

LITERATURE REVIEW

Oil is a type of hydrocarbon which is toxic, it harms the environment where it is spilled, affecting the wildlife there. Therefore, quick cleaning up of oil spills is important to limit damage to the environment. Oil spill cleaning up with the aid of oil degrading bacteria is fairly important in such conditions.

2.1 Composition and Properties of Petroleum

There are three basic classes of hydrocarbon compounds in petroleum oils: 1) alkanes which are also referred to as paraffins or saturates; 2) alkenes (oleofins) and 3) aromatic or arenes. Alkanes have a single bond between the carbon atoms and it can be formed in simple straight chain, branched chain or simple rings (naphthetic or cycloalkanes). Alkanes are non polar compounds and generally are soluble in non polar solvents such as carbon tetrachloride and insoluble in polar compounds such as water (Sackheim & Lehman, 1998). Low boiling point alkanes produce anaesthesia and narcosis at low concentrations, whilst exposure to high concentration can cause cell damage and death in low invertebrates (Boesch, Hershner & Milgram, 1974). Higher boiling point alkanes are not normally toxic; however they may affect chemical

communication and interfere with metabolic processes. On the other hand, alkenes have a double bond between two of the carbon atoms. Generally, alkenes are more reactive than alkanes due to the presence of double bonds.

Aromatics hydrocarbons consist of benzene rings that have multiple double bonds. The term polycyclic aromatic hydrocarbons (PAHs) refer to hydrocarbon with more than one benzene ring. PAHs are formed by incomplete combustion of organic substances. They are highly toxic to organism due to their carcinogenic and mutagenic potential (Pizzul, Castillo & Stentrom, 2006). PAHs have low water solubility and hydrophobicity, therefore they tend to adsorb and accumulate sediments. The degradation of PAHs with high molecular weights is particularly slow (Readman, Mantoura, Rhead & Brown, 1982). Sixteen individual PAH compounds are classified as priority pollutants by the United States Environment Protection Agency (US-EPA) as shown in Table 2.1.

PAHs occur as colourless, white or pale yellow solids with low solubility in water, high melting and boiling points, and low vapour pressure. With an increase in molecular weight, their water solubility and vapour pressure decreases; melting and boiling point increases (Haritash & Kaushik, 2009; Patnaik, 1999). The higher the number of carbon atoms in a molecule, the higher the boiling point and less volatile it is. This is due to the large surface area and increased intermolecular forces among atoms. Figure 2.1 shows chemical structure for alkanes, alkenes and aromatic hydrocarbons.

Table 2.1: 16 PAH Priority Pollutants Defined by US-EPA

Two-ring	Three-ring	Four-ring	Five-ring	Six-ring
Napthalene	Flouranthene	Crysene	Benzo[a]pyrene	Benzo[g,h,i]perylene
Fluorene	Phenanthrene	Pyrene	Indenol[1,2,3-c,d]pyrene	
Acenaphthene	Anthracene	Benzo[a]anthracene	Dibenzo[a,h]anthracene	
Acenaphthylene		Benzo[b]fluoranthene		
		Benzo[k] fluoranthene		

(Perelo, 2009)

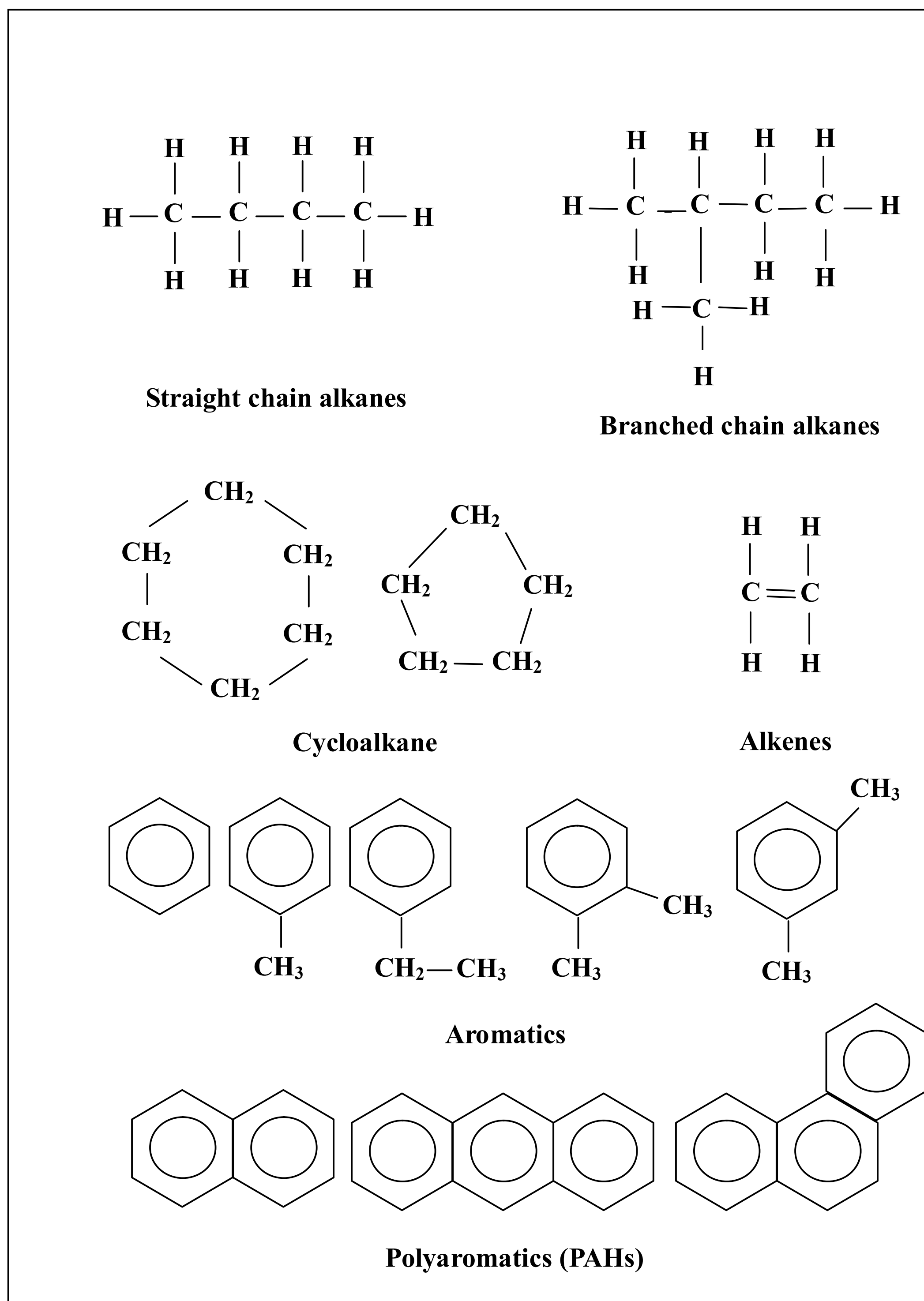

Figure 2.1: Chemical structure for alkanes, alkenes and aromatic hydrocarbons. (Source: Phang, 1990).

2.2 Effect of Oil Spill to Living Organisms

Most of the major oil spills occur in mid ocean. However the actual harmful effects are felt only in the coastal regions and estuaries. In 24 March 1989, the Exxon Valdez oil tanker ran aground on Bligh Reef in Prince William Sound, Alaska and spilled 10.9 million gallons of North Slope Crude Oil into the ocean. More than 88 species of birds were affected. They lost their insulating ability and suffered hypothermia when their feathers were soaked with oil (Pritchard, Mueller, Rogers, Kremer & Glaser, 1992). Some suffered anaemia when oil entered into the blood stream; causing the red blood cells to wrinkle.

Effects of the spill in Prince Sound William also caused seven thousand sea otters killed by variety of conditions resulted from oil getting into the blood stream. It caused nose bleeding due to blood thinning, that subsequently lead to infection. Hydrocarbon could also cause liver and kidney damage to sea otters when the organs attempt to clean the oil out of the system (Pritchard et al., 1992). Some of the sea otter suffered emphysema, which compromise their diving ability and eventually lead to death. Another cause was blindness when oil entered into otter's eyes which then lead to starvation (Pritchard et al., 1992).

Another such tragedy was on November 2004 when Greek tanker Athos I struck a submerged object in the Delaware River resulting spill of 264,000 gallons of heavy crude oil. Large number of migratory birds that feed on eggs layed by the

horseshoe crabs (*Limulus polyphemus*) in the intertidal sands of the Delaware and New Jersey shores suffered from shortage of food. The spill that occurred during spawning season was believed to affect the bird populations and the future generations of horseshoe crabs in that area (Venosa et al., 1996).

2.3 Conventional Methods Involve in Oil Spill Cleaning Up

Oil spill cleaning up can be carried out by several ways. Basically, the methods applied can be divided into chemical and mechanical methods. Chemical method disperses the oil into small droplets while mechanical method blocks the spread of oil, concentrate it into one area, then it could be removed from the water.

2.3.1 Chemical Method

The mechanism of oil removal chemically from the water surface is to disperse oil into small droplets. The types of chemical treatment agents used to remove or reduce oil slicks are dispersants, biodegradants, chemicals forming oil-water suspension, sinking agents, material put on the slick to assist ignition or enhance combustion of the oil spill for *in-situ* burning. Dispersants are chemicals containing surfactants that act to break up oil into tiny droplets. These tiny droplets provide more surface area for the oil to weather and break down under the natural

processes such as wind, waves and current. However, the effectiveness of a dispersant is determined by the composition of the oil being treated. Examples of dispersant are Corexit 9500 (Zahed, Aziz, Isa, & Mohajeri, 2010).

Dispersants are most effective when used within an hour or two of the initial spill. However, they are not appropriate for all oils and all locations. Successful dispersion of oil through the water column can affect marine organisms like deep-water corals and sea grass. They can also cause oil to be temporarily accumulated by subtidal seafood (University of Delaware Sea Grant Program, 2004).

Biodegradants are substances that promote oxidation of oil by microbial action. The other chemicals used are gelling agents that form semi-solid oil agglomerates to facilitate removal of oil spills. Herding agents are chemicals that concentrate the spilled oil in small area (Boesch et al., 1974). The chemical method particularly with the use of dispersant causes a big ecological concern. It is prohibited in many countries due to possible long-term negative environmental effects and toxicity concerns.

2.3.2 Mechanical Method

Mechanical method incorporates the use of boomer, sorbent and skimmer. Boomer is a device floated on the surface of the sea to prevent the passage of an oil slick from one side of the barrier to the other. It is able to clean up oil spill by diverting the oil into a collection device or channel it to other desired destination. However it is not suitable in cleaning spills in fast current or rough sea, where trapping of the oil layer is very difficult (Ernst, 1979).

Another type of mechanical oil removal method commonly applied is sorbents. Sorbents are materials that absorb oil to form a floating mass for later collection and removal (Adebajo, Frost, Kloprogge, Carmody & Kokot, 2003). A sorbent must be able to attract oil and repel water. Examples of sorbent are peat moss, saw dust, feathers, clay, sand, volcanic ash, polyethylene and nylon. A sorbent demonstrates promising results in oil removal; but it will sink if filled up with water.

Oleophilic skimmer on the other hand is applied with the different materials in removing oil. In this method belts, disks, or mop chains are used to attract oil, so that oil can be squeezed or scraped out.

In suction skimmers, oil will be sucked up from the water surface and stored in a tank. This method requires big area for the oil storage tanks and high

labour input to empty the tanks which subsequently leads to more cost and create potential danger to the service personnel.

Oil spills should be cleaned up as soon as possible before the oil emulsifies (Stevens, 2000). This is because in emulsification the oil mixes with water and the resulting mixture become a thick, pudding-like substance. This emulsified oil is very difficult to clean up using standard materials like sorbents, dispersants and skimmers. Despite the capabilities of boomers and skimmers as mentioned above, they have some limitations too. They are less effective when applied in high winds and rough seas.

Disadvantages of using mechanical methods in oil spill cleaning up is the involvement of high cost, being time consuming and requiring excavation of soil for treating the spill in separate areas or better treatment facilities. These treatments include incineration or burial in the secure landfills (ITOPF, 2006).

2.3.2.1 After Treatments of Mechanical Methods

Waste management is needed after the oil is contained by mechanical means. There are two methods to dispose the waste, landfill and incineration. In landfill, the waste is compacted into natural or man-made cavities in the landscape. Such disposals become eye-sore and encourage rats and other vermins. Besides,

microbiological action in the organic landfill-waste leads to the production of methane, which is explosive and categorised as the fire hazard. Methane is also a greenhouse gas and to avoid its release to the atmosphere, action has to be taken to collect from landfill sites and flamed it off or used it as fuel. Microbial action may also lead to toxic products such as hydrogen sulphide gas, which affect human nervous system. Liquid leaching from landfill sites and pollution of the ground water is yet another serious problem. However, this danger can be lessened by lining the site with impermeable material (Clugston & Flemming, 2000).

Landfill is an increasingly expensive way of disposing wastes, due to the shortage of suitable sites and the cost of meeting higher environment standards. Therefore incinerators are being used to burn the increasing amount of wastes (Yemashova et al., 2007). Incineration sterilises the material for the final disposal and reduces it in volume by about 90%. Also most new incinerators are designed to use the heat from burning rubbish to generate electricity.

The combustion product released from the incinerator to the atmosphere may harm the environment. A large proportion of domestic waste is organic in origin and produces CO_2 and H_2O on combustion and hence contributes to the greenhouse effect. Moreover, the incomplete combustion of the waste produces toxic gases such as CO (carbon monoxide) together with soot (Yemashova et al., 2007). Sulphur containing compounds produce SO_2 while nitrogen containing compounds NO_2. Both these gases can cause acid rain. Besides, insufficiently high

temperature during incineration may lead to chlorinated compounds producing dioxins. Dioxins are the name given to a whole range of highly toxic compounds that consist of two benzene rings connected via two oxygen atoms (Clugston & Flemming, 2000). In toxicity, dioxin is next to the radioactive waste.

Incineration and burial of collected oil in secure landfills are effective treatments but on burning, the soil loses most of its nutritional value and structure. These methods do not remove the contamination but only relocate the problem (Jain, Gupta, Pathak, Lowry, & Jaroli, 2010; Lageman, Clarke, & Pool, 2005).

2.4 Biodegradation and Bioremediation

Biodegradation is a process by which microorganisms such as bacteria, fungi, and yeast break down complex compounds into simple products to obtain energy and nutrients. Biodegradation of oil is a natural process that slowly over the course of weeks, months or years, removes the oil spilled from the environment (US EPA, 1999). However, rapid removal of spilled oil from shorelines and wetlands is necessary in order to minimize potential environmental damage to these sensitive habitats.

Bioremediation technologies are able to speed up the biodegradation processes. Bioremediation is referring to the act of adding biological agents to the

environment, such as nutrients/fertilizers, surfactant, enzyme or beneficial microorganisms (US EPA, 1999).

In the bioremediation process, indigenous microbes (biomass) and microbial inoculants degrade contaminants and detoxify the soil. The by-products resulting from this process are carbon dioxide and water (Keeler, 1991 cited in Adekunle & Adebambo, 2007). In short, bioremediation takes contaminated soil and converts the contaminants into non hazardous substances. Bioremediation is often used after all mechanical oil recovery methods have been used (US EPA, 1999). The goal of bioremediation is to degrade organic pollutants to concentrations that are either undetectable or the concentration is below the limits established as safe by regulatory agencies.

The bioremediation process can be performed *in situ* or *ex situ*. The *in situ* process is adopted where excavation is impractical and involves either biostimulation or bioaugmentation depending on environmental condition and microbial community structure. The guidelines of the United States Environment Protection Agency (US EPA) suggested that bioremediation is feasible when there is about 10^3 CFU/g soil of the microbial population (Lin, Pan & Cheng, 2009).

According to Mohajeri, Aziz, Isa & Zahed (2010), bioremediation is not recommended for crude oil concentrations of 2000 mg/L or higher, lower concentration of crude oil demonstrated more efficient hydrocarbon removal.

Besides, heavy crude oil is not dispersed as well as light and middle weight oils.

A successful case on application of bioremediation was during the shoreline clean up in Alaska after the accidental spill of 10.9 million gallons of crude oil from the tanker Exxon Valdez in 1989. The bioremediation process involved the use of Inopol EAP22, a nitrogen and phosphorus fertilizer mix. Inopol EAP22 was sprayed on oil that has been washed up on beach. It encouraged the growth of indigenous oil degrading bacteria. Although Inopol EAP22 demonstrated successful result, this method was not widely used in other spill incidents due to uncertainty of the possible side effects (Pritchard, et al., 1992). The chemical composition and the function of each component of Inopol EAP22 are showed in Table 2.2. Studies conducted after Exxon Valdez oil spill has shown that pristine and phytane can be biodegraded (Bragg, Prince, Harner & Atlas., 1994).

Table 2.2: Chemical composition and the function of each component of Inopol EAP22

Component	Chemical composition	Function
Oleic acid	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$	Emulsifying agent
Tris (laureth) phosphate	$C_{12}H_{25}PO_4$	Hydrophobic phase, phosphorus source and surfactants
2-Butoxy-1-ethanol	$HO-C_2H_4-O-C_4H_9$	Surfactant and emulsion stabiliser
Urea	$NH_2-CO-NH_2$	Nitrogen source
Water	H_2O	Solvent

(Swannell, Lee & McDonagh, 1996)

2.4.1 Biaugmentation

Bioaugmentation is the introduction of exogenous microorganisms to treatment facilities in order to enhance the degradation of organic pollutant (Watanabe, 2001). These pre-grown exogenous microorganisms have an affinity towards a specific contaminant. These microbes are suspended by a stabilizing agent and lie dormant in a spore until activated in solution and applied together with micronutrients and biostimulants.

The purpose of seeding (introduction of exogenous microorganisms) is to increase the population of microorganisms that can biodegrade the spilled oil. Bacterial species that do not naturally exist in an area is added to the native population together with added nutrients.

Bioaugmentation can serve many functions in municipal waste-water treatment. This process improves treatment efficiency and reduces sludge production. A sufficient quality and diversify of microorganisms will allow a better processing of wastewater. The benefits of bioaugmentation are low operating and maintenance cost, production of a more thoroughly processed effluent containing fewer organic pollutants and less sludge to be treated and disposed of by the treatment plant (Ueno, Ito & Yumoto, 2007).

Bioaugmentation is particularly effective if indigenous microorganisms in a particular contaminated area are not capable of degrading the contaminants.

However, the US EPA commented that bioaugmentation or seeding with native microorganisms do not result in faster biodegradation because hydrocarbon degrading bacteria exist almost everywhere and exogenous species are often unable to compete successfully with native microorganisms (Norman, Frontera-Suau & Morris, 2002).

In 1997, bioaugmentation using seed culture of petroleum-degrading bacteria, Terrazyme (Oppenheimer Biotechnology, Austin, Texas, USA) was attempted, on a trial basis on the coast of Japan facing the Japan Sea after a heavy oil spill from the Russian tanker Nakhodka. According to Tsutsumi et al. (2000), Terrazyme was able to enhance the biodegradation of oil on the shore however such exogenous microbial products have never been widely used in Japan probably due to the unclear guidelines for bioremediation. On top of that, bioaugmentation using exogenous microorganisms were not enacted in Japan until 2004 (Hosokawa, Nagai, Morikawa & Okuyama, 2009).

According to Hosokawa et al. (2009), the efficiency of bioaugmentation is only during the early stages of the process. Hence, bioaugmentation treatment must be adjusted intermittently with the addition of more microorganisms. As an alternative to the survival and the xenobiotic degrading ability of introduced microorganisms, autochthonous bioaugmentation (ABA) is proposed by Hosokawa et al. (2009) to overcome these difficulties.

ABA method is a bioaugmentation technology that contains the data of microorganisms indigenous to the contaminated site or predicted contamination site. Indigenous microorganisms of that particular contaminated site will be enriched under conditions where bioaugmentations will be conducted. For these reasons information in advance on the chemical and physical characteristics of potential oil spill sites and type of oils that might be spilled is needed. Hosokawa et al. (2009) also claimed that it is not impossible to predict location of oil spills. Areas close to oil fields are threatened with many types of oil spills, such as eruption of oil wells and spills from storage tanks and pipelines. The most recent oil wells leakage incident in 2010 was Deepwater Horizon Spill that contaminated Gulf of Mexico. The advantages of ABA method to decontaminate oil polluted lands, coasts and waters are shorter treatment time, greater potential efficiency, lower impact on the environment, and relative ease in obtaining public support.

2.4.2 Biostimulation

Biostimulation involves aeration and the application of selected micronutrients and biostimulants such as phosphorus, nitrogen or biosurfactant to a contaminated environment to stimulate the growth of the microorganisms that break down the contaminants (Kim, Choi, Sim & Oh, 2005). According to Neralla, Wright & Weaver (1995), inorganic nutrient supplementation may speed up the process, because the addition of large quantities of oil results in a high C: N ratio

that is unfavourable for microbial activity.

Biostimulation is effective if high indigenous microbial population is present in the contaminated site. When nutrients are added, the native microbial population can grow rapidly, potentially increasing the rate of biodegradation. The two nutrients most likely to limit microbial population in sea water are nitrogen and phosphorus (Capone & Bauer, 1992).

The advantage of biostimulation is that bioremediation has been undertaken with the native microorganisms that are well suited to the subsurface environment, and are well distributed specially within the sub surface. The primary disadvantage is that the delivery allows the additives to be readily available to the subsurface microorganisms which are based on the local geology of the sub surface. Tight clays or other fine-grained materials make spreading additives throughout the hydrocarbon polluted area difficult. Fractures in the subsurface create preferential pathways in the subsurface where additives will flow through, hence preventing the even distribution of additives. In a nutshell, bioavailability of a compound to the microbes is determined by the rate of mass transfer of the substrate to the microbial cells in relation to intrinsic catabolic activity (Johnsen, Wick & Harms, 2005).

Proof of the effectiveness of biostimulation as an oil spill cleanup technology was developed on the shoreline of Delaware Bay in 1994 (US EPA,

1999). This EPA-funded study, which involved an intentional release of light crude oil into small plots, demonstrated a several-fold increase in the biodegradation rate due to the addition of fertilizer as compared to the unfertilized control plots (US EPA, 1999).

Another successful case was during the Exxon Valdez oil spill cleanup and restoration activities, the Alaska Regional Response Team authorised the use of bioremediation products, including biostimulation and bioaugmentation (US EPA, 1999). Addition of nutrients was approved for 100 miles of Prince William Sound shoreline. Data collected through a monitoring protocol required by the State of Alaska indicated that nutrient addition accelerated the natural degradation of oil without observed eutrophication or toxicity (US EPA, 1999).

2.5 Oil Transformation in Marine Environment

Oil spilled to marine environment goes through a variety of physical, chemical and biological transformation during its transport by the wave. Advention and spreading begin immediately after the oil is spilled in the ocean and cause rapid increase in the exposure area of the oil. The subsequent process followed by the spreading will be weathering which include evaporation, photochemical oxidation, dissolution, dispersion, emulsification and sedimentation (National Academy Press, 1985). These processes are influenced by density,

viscosity, pour point and aqueous solubility of the oil being spilled. The pathway in which spilled oil may enter into the marine ecosystem is shown in Figure 2.2.

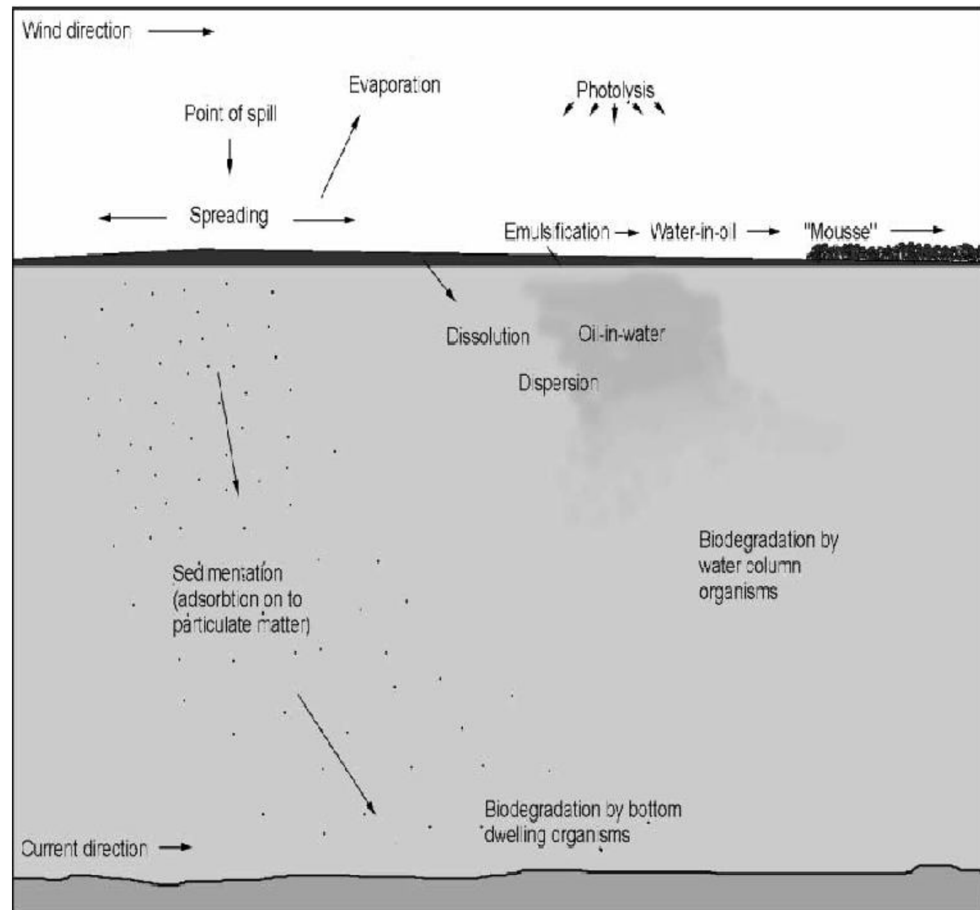

Figure 2.2: Pathways by which spilled oil may enter the marine ecosystem. (Source: Kingston, 2002).

When an oil spill occurs, oil spreads over the water surface and slick of a few millimeters thick will be formed and the volatile component of oil will rapidly evaporate (Kingston, 2002). This includes most of the toxic components.

Evaporation causes the loss of one to two-third of an oil spill mass in a period of few hours or a day (Jordan & Payne, 1980). It is estimated that at least 30% of the oil spilled by Exxon Valdez (35, 000 tones) and 40% of the Amoco Cadiz oil (240, 000 tones) disappeared by evaporation (Kingston, 2002). Light oil evaporates faster from the sea surface.

Some hydrocarbons are oxidized by photo oxidation or photolysis. Photolysis is the oxidation of oil components by UV radiation from the sun. The oxidation products include acidic and phenolic compounds, some of which may be more toxic than the original hydrocarbons. However, their concentrations are relatively low and have no significant ecological effect (Kingston, 2002).

Certain amounts of hydrocarbons undergo dissolution. Concentrations of hydrocarbon dissolved in water are particularly important due to their potential to exert toxic effect on the biological systems (National Academy Press, 1985). Most of the low molecular weight compounds which are relatively toxic dissolve in the sea water. Dissolution process takes up less than 1% of the spilled oil (Kingston, 2002).

Dispersion is probably responsible for the natural removal of most of the oil from the water surface. The oil is disseminating by the wave action into small droplet, 0.01-1 mm in diameter and is retained in the water column until biodegradation takes place (Kingston, 2002).

Oil emulsification in the marine environment depends on the composition of oil and the turbulent regime of the water mass. According to Patin (1999), the most stable emulsions such as water-in-oil contain 30-80% water. Emulsions usually appear after strong storm in the zones of spills of heavy oils with an increased content of nonvolatile fractions especially asphaltenes. Stability of the emulsions usually increases with decreasing temperatures.

Sedimentation mainly happens in the narrow coastal zone and shallow water where particulates are abundant and water is subjected to intense mixing. About 10-30% of oil adsorbed on the suspended material in the sea and deposited to the bottom (Patin, 1999). In deeper areas remote from the shore, sedimentation of light fraction of oil is an extremely slow process. Other than that, plankton filtrators and other organisms absorb the emulsified oil and sediment it to the bottom of sea with their metabolites and remainder. Under the anaerobic condition in the bottom of the sea, the oxidation process is slowed down and due to this the heavy oil fractions are preserved for many months and years.

2.6 General Family of Microbes Involved in Oil Degradation

Hydrocarbonocastic bacteria are ubiquitous in nature (Alexander, 1994). Table 2.3 shows the genera family of bacteria involved in the oil degrading process. According to Matsuoka, Miura & Hori (2009), *Burkholderia arboris* secretes lipase to aid biodegradation process while *Candida cylindracea* is responsible in glycerol assimilation. On the other hand, Koma et al. (2003) reported that *Rhodococcus* species and *Gordonia* species used n-alkane and c-alkane as sole carbon and energy source and the strains degraded more than 27% of car engine based oil (1% addition).

According to Sayavedra-soto, Chang, Lin, Ho & Liu (2006), *Rhodococcus* strain NTU-1 carried out the degradation of alkane via a hydroxylase, while *Bacillus fusiformis* L-1 and *Ochrobactrum* species would not degrade alkanes but aided flocculation by forming more rigid bacterial aggregates that enhanced the trapping of alkanes. They also reported that on batch culture of these three bacteria, transformation and removal of the linear and branched alkanes was achieved within 66 hours with more than 95% efficiency.

Kasai, Kishira & Harayama (2002) observed that polycyclic aromatic hydrocarbon (PAH) degradation proceeds parallel with the growth of *Cycloclasticus* cells on the surfaces of the oil polluted gravel. They reported that bacteria belonging to the genus *Cycloclasticus* play an important role in the

degradation of petroleum PAHs in the marine environment. Other marine bacteria that have previously been reported to be PAH degraders include members of the genera *Flavobacterium, Marinobacter, Moraxella, Pseudomonas, Sphingomonas* and V*ibrio*. However, the activities of these organisms in the natural environment remained unknown (Kasai et al., 2002).

Iyer, Mody & Jha (2006) reported that *Enterobacter cloacae* produced exopolysaccharides that can emulsify hexane, benzene, xylene, kerosene, paraffin wax, cotton seed oil, coconut oil, jojoba oil, castor oil, groundnut oil and sunflower oil.

Based on the study from Haritash & Kaushik (2009), enzymes involved in the degradation of PAHs are oxygenase, dehydrogenase and lignolytic enzymes. Fungal lignolytic enzymes are lignin peroxidase, laccase, and manganese peroxidase. They are extracellular and can catalyze radical formation by oxidation to destabilise bonds in a molecule. Lynolytic fungi that are able to degrade PAH are *Phanerochate chrysoosporium, Bjerkandera adusta* and *Pleurotus ostreatus.*

Table 2.3: Genera of Hydrocarbon Degrading Bacteria

Genus	**Function**	**Reference**
Acinetobacter	Bioemusifier producer, diesel degrader	Singh & Lin, 2008
Aeromonas	PAH degrader	Jacques et al., 2007
Alcanivorax	Alkane degrader	Kasai et al., 2002
Aspergillus	Engine oil degrader	Adekunle & Adebambo, 2007
Bacillus	Flocculation, diesel degrader	Sayavedra-soto et al., 2006; Singh & Lin, 2008
Beijerinckia	PAH degrader	Jacques et al., 2007
Burkholderia	PAH degrader, Lipase secretion	Jacques et al., 2007; Matsuoka et al., 2009
Candida	Glycerol assimilating	Matsuoka et al., 2009
Citrobacter	Diesel degrader	Singh & Lin, 2008
Corynebacterium	PAH degrader	Jacques et al., 2007
Cycloclasticus	Aromatic degrader	Kasai et al., 2002
Enterobacter	Exopolysaccharide producer	Iyer et al., 2006
Flavobacterium	PAH degrader	Jacques et al., 2007

Table 2.3 (Cont'): Genera of hydrocarbon degrading bacteria

Gordonia	N- and C-alkane degrader	Koma et al., 2003
Haemophilus	PAH degrader	Haritash & Kaushik, 2009
Lutibacterium	Phenenthrene, PAH degrader	Chung & King, 2001
Moraxella	PAH degrader	Kasai et al., 2002
Mycobacterium	PAH degrader	Jacques, 2007; Haritash & Kaushik, 2009
Nocardia	PAH degrader	Jacques, 2007
Paenibacillus	PAH degrader	Haritash & Kaushik, 2009
Paracoccus	PAH degrader	Jacques, 2007
Pseudomonas	PAH degrader, biosurfactant producer	Yateem, Balba , Al-Shayji & Al-Awadhi, 2002; Jacques, 2007; Bordoloi & Konwar, 2009; Haritash & Kaushik, 2009
Rhodococcus	Alkane degrader	Sayavedra-soto et al., 2006
Spingomonas	PAH degrader	Jacques, 2007
Stenotrophomonas	PAH degrader	Jacques, 2007
Vibrio	PAH degrader	Kasai et al., 2002

2.7 Mode of Action for Microbes in Degrading Oil

Oil degrading bacteria form part of an ecological network. The degrading process involves many direct and indirect interactions with other community members and the environment, hence it is greatly influenced by environmental variables such as nutrient availability or physiological parameters. According to Head et al. (2006), such interactions include competition for limiting nutrients, predation by protozoa, lysis by phage and cooperative interactions that increase hydrocarbon degradation (Figure 2.3).

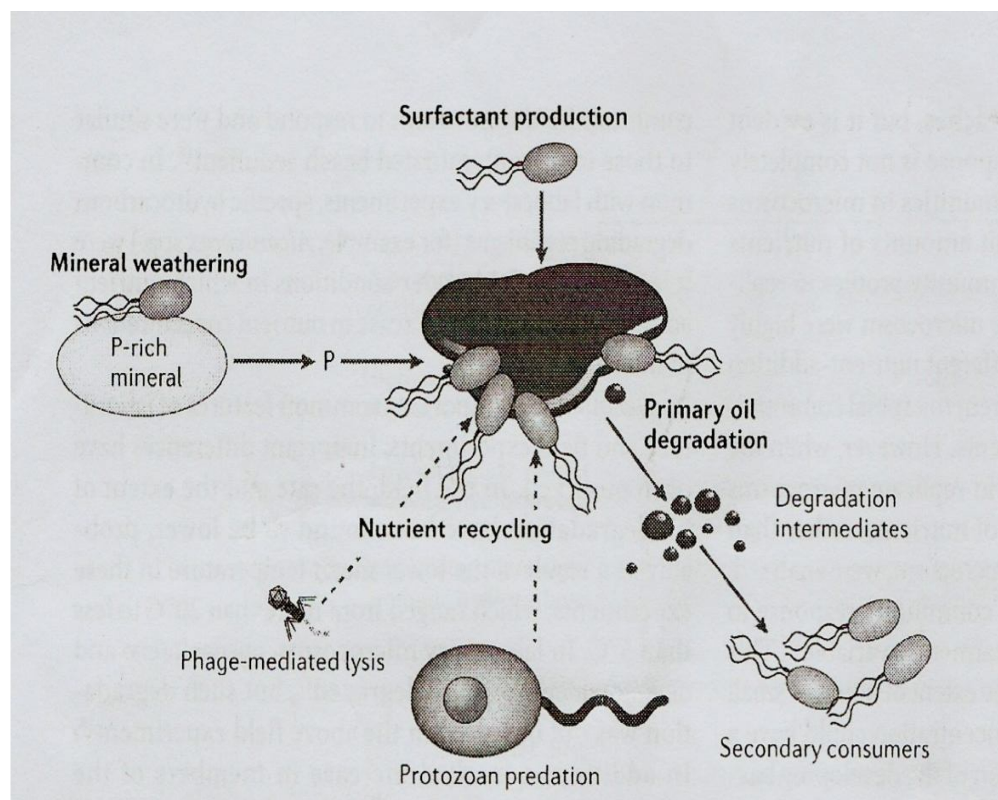

Figure 2.3: Microbial degradation network of oil. Solid arrows indicate material fluxes, and broken arows indicate direct interactions. (Source: Head et al., 2006)

The growth of bacteria on oil is thought to involve three specific processes; 1) the interaction of the cells and uptake of the insoluble substrate via adherence at the oil-water interface, also refer as pseudosolubilization of the carbon source or uptake of hydrocarbons dissolved in the bulk aqueous phase; 2) introduction of molecular oxygen into the hydrocarbons; 3) biotransformation to a form which can then enter the normal metabolic pathway of the microbial cell and the expression of specific regulatory signal governing these pathways (Runsansky, Avigad, Michaeli & Gutnick, 1987). The main products of microbial metabolism of hydrocarbon are carbon dioxide, water, small quantities of fatty acid and surfactants that participate in the stabilization of water-oil emulsion.

Microbial biodegradation of linear alkanes starts via a hydroxylase with terminal or subterminal oxidation (Kasai et al., 2002). The resulting primary and secondary alcohols are then metabolized to aldehydes, acid and gone through β-oxidation pathway, followed by TCA cycle (Figure 2.4). β-oxidation is the degradation of fatty acids that removes two carbon segments from the fatty acid at the oxidised β carbon (Timberlake, 2009).

Branched alkanes such as 2, 3-dimethylhexane, 3-ethylpentane and 4-ethyl-2-methylhexane are generally more recalcitrant to biodegradation than linear alkanes. Microbial degradation of branched isoprenoid alkanes (Figure 2.5), such as pristine, go on to form an acid but before proceeding toward β-oxidation, the methyl branching point esterifies with coenzyme A (Sayavedra-Soto et al., 2006).

Terminal oxidation

CH_3-CH_2-$(CH_2)_n$-CH_2-CH_3

Subterminal oxidation

CH_3-CH_2-$(CH_2)_n$-CH_2-CH_3OH

OH

CH_3-CH_2-$(CH_2)_n$-CH_2-CH_3

CH_3-CH_2-$(CH_2)_n$-CH_2-CHO

O

CH_3-CH_2-$(CH_2)_n$-CH_2-COOH

CH_3-CH_2-$(CH_2)_n$-C-CH_3

ω - oxidation

O

CH_3-CH_2-$(CH_2)_{n-1}$-CH_2-O-C-H_3

$HOCH_3$-CH_2-$(CH_2)_n$-CH_2-COOH

HOOC-CH_2-$(CH_2)_n$-CH_2-COOH

CH_3-CH_2-$(CH_2)_{n-1}$-CH_2OH + HOOC-CH_3

CH_3-CH_2-$(CH_2)_{n-1}$-COOH

TCA cycle

β- oxidation

Figure 2.4: Basic metabolic pathway involved in the metabolism of n-alkanes. (Source: Tzeng, 1994).

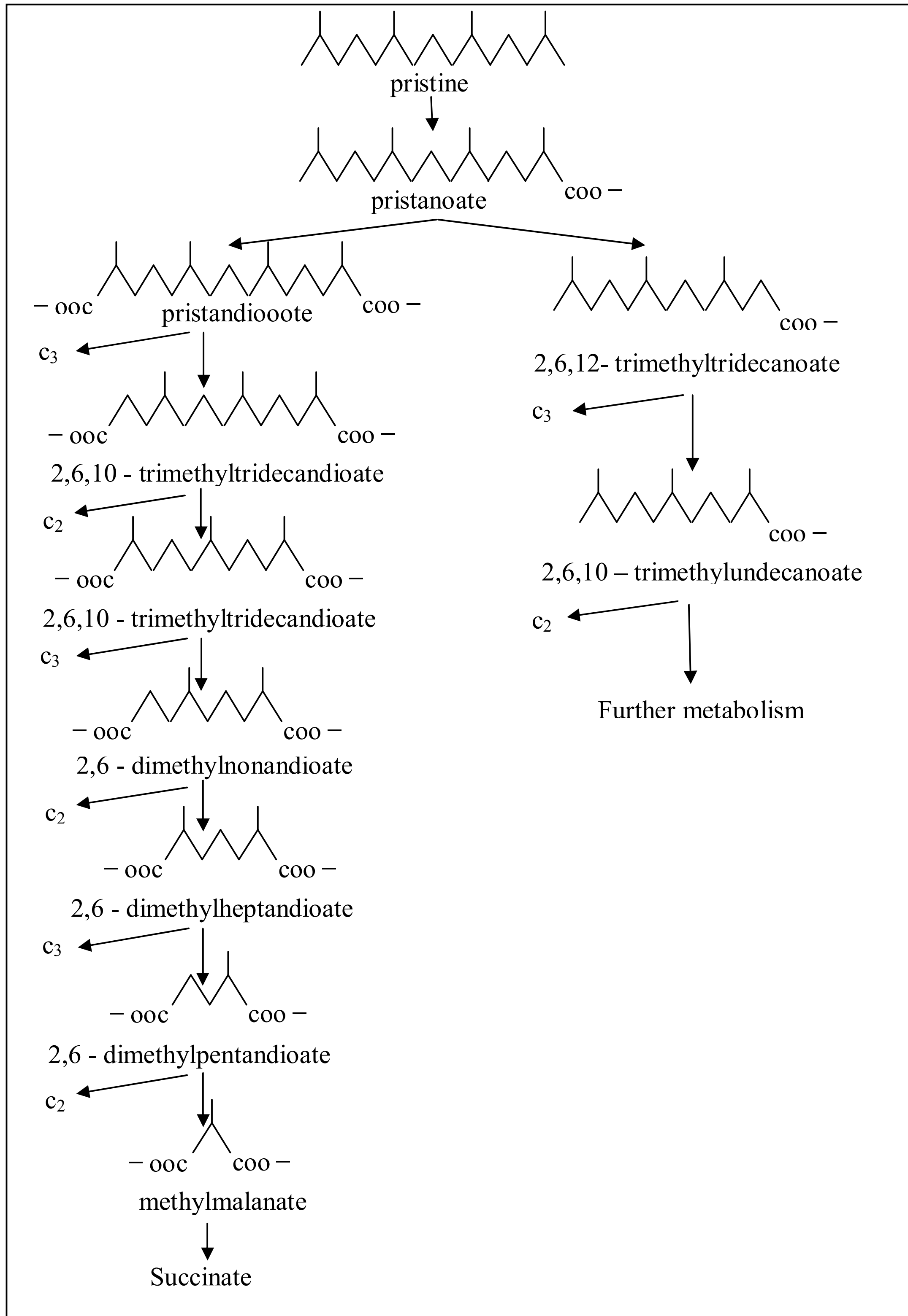

Figure 2.5: Metabolic pathways involved in the degradation of the isoprenoid alkane pristine. (Source: Tzeng, 1994).

2.8 Factors Affecting Cleanup Process by Microbes

Microbial degradation of petroleum hydrocarbons is affected by temperature, metabolic and oxygen limitations, nutrient status and biological factors such as microbial community. Temperature influences hydrocarbons, physical nature and chemical composition (Leahy & Colwell, 1990) by changing the composition of the soluble fraction and thus limiting the bioavailability of soluble compounds to the microorganisms (Northcott & Jone, 2000). Bioavailability is the tendency of individual oil components to be taken up by microorganisms. Besides, temperature also determines the structure and metabolism of microbial communities, rate of hydrocarbon degradation, and the composition of the microbial communities (Atlas & Neilson, 1997), as well as the mass transfer of substrate and electron acceptors in the contaminated site (Yang et al., 2009). Ground temperatures can remarkably affect the degradation rates. Hydrocarbon degradation is faster at 25°C than at 5°C (Atlas & Neilson, 1997). Biodegradation of heavy fuel by indigenous organisms in the North Sea was four times greater in summer (18°C) than in winter (4°C) (Barnes & Filler, 2003).

Biodegradation of hydrocarbon also relies on cellular enzymatic system within the bacterial cells. The microorganisms can be metabolically active only when mass transfer across the cell membrane occurs. In cold temperature area, cryogenic stresses resulting in closing the transport channels or freezing the cytoplasm. Cryogenic stresses are very common in extreme conditions for several

seasons and may restrict mass transport and limit contaminants to gain access into the cells (Yang et al., 2009). These will subsequently lead to low substrate bioavailability that hinders the hydrocarbons transferring into microbial cellulous enzymes and result in limited energy for maintaining degradation (Yang et al., 2009).

Metabolic limitations may result from the enzyme-substrate interaction and the energy needed to activate metabolism. If the proper enzyme already exists, the degradation rate will then be determined by specific interactions of the compound with the enzyme. Generally, the enzymatic limitations result from substrate recognition and steric hindrance of substrate because the recognition and approach are required for enzymatic catalysis. The larger the compound size, the bigger the steric hindrance and more difficult the compound interaction with the active center of the enzyme (Yang et al., 2009).

Oxygen limitation is one of the crucial reasons for bioremediation failures. Oxygen serves as the terminal electron acceptor in aerobic metabolism. The importance of oxygen comes from the participation of oxygenases and molecular oxygen involved in major degradation pathways for hydrocarbons. Aerobic processes mostly yield a considerably greater potential energy yield per unit of substrate and tend to occur considerably more rapidly. Theory suggests that the mass of oxygen necessary to remediate the hydrocarbon load is about 0.3 g oxygen for each gram of oil oxidized (Yang et al., 2009).

The nutrient status of a soil directly impacts microbial activity and biodegradation. A group of nutrient elements or organic compounds are required as a source of carbon or electron acceptor. Inorganic nutrients including exchangeable cations, nitrates and phosphates are important for bioremediation. Aerobic organisms utilize elemental oxygen as their ultimate electron acceptor. Anaerobes utilize nitrates, sulfates, CO_2 and ferrous metals as electron acceptors and often have unique reaction mechanisms in the deep surface, where the anaerobic pathway is likely to be meritorious to degrade some resistant compounds (Yang et al., 2009).

Biological factors such as the structure of the microbial community play an important role in hydrocarbon degradation (Arturo, Antoine, Pelletier, Delille & Ghiglione, 2009). Structure and diversity of microbial communities determined the metabolic capacity in a contaminated site (Galvao, Mohn & Lorenzo, 2005). Single species are capable of degrading only limited number of compounds found in crude oil while complex communities with broad enzymatic capacities can degrade a wide range of molecules in crude oil (Leahy & Colwell, 1990). This is supported by Jacques et al. (2007) that the advantage of using microbial consortia is the efficiency of the bioremediation process.

2.9 New Trend in Oil Spill Management and Cleaning

Prevention is better than cure. Prevention of oil spill is better in term of environmental conservation and cost effectiveness than the cleanup process. Preventative measures for oil spill have been taken by International Maritime Organization (IMO) of the United Nations and individual nations. Measures implemented are towing arrangements, enhanced inspection, equipment duplication on ships and double-hull requirements (IMO, 1996).

In order to reduce the risk and severity of oil spill caused by shipping collision, grounding pipelines employ computers, electromagnetic instruments, and ultrasonic devices to detect weak spots so that they can be repaired before a leak develops (IMO, 1996). Besides, marine terminals and vessels are being designed differently. Vessels are being built with double hulls and storage tanks are constructed of special materials to withstand corrosion.

On top of that, advances in remote sensing technologies help to identify agencies potentially responsible for pollution and to identify minor spills before they cause widespread damage (Jha, Levy & Gao, 2008). Plan that identifies the personnel, equipment and materials is needed to deal with the spill developed in the tanker facility, platform and pipeline. It includes information about the storage capacity, environmentally and economically sensitive areas, personnel training, practice drills in a worst case scenario.

CHAPTER 3

MATERIAL AND METHODS

3.1 Material

3.1.1 Equipments and Apparatus

Laboratory equipment used in this research include incubator shaker (Memmert, Germany), centrifuge (Sigma, USA), gel electrophoresis apparatus (Bio-Rad, USA), SDS-PAGE electrophoresis (Bio-Rad, USA), UV transilluminator (Promega, USA) and GCMS (Shimadzu, Japan). Milli-Q and RiOs (Millipore, USA) was used in the entire research work. Other common equipment used were autoclave (Hirayama,Japan), UV visible spectrophotometer (Thermo Scientific, USA), pH Meter (Mettler Toledo, USA), weighing machine (Sartorius, Germany), waterbath and oven (Memmert, Germany), microwave oven (Sharp Electronics, Japan), vortex mixer (Labnet, USA), Thermo Scientific (USA) micropipette range of 0.5-10 ul, 2-20 ul, 20-200 ul and 100-100 ul. Glasswares used in this research included measuring cylinder (Furex, Germany), Erlenmeyer flask, Schott Duran bottle (Boeco, Germany) and beaker (Bomex, USA).

3.1.2 Kits, Chemicals and Reagents

Chemicals used throughout this research were: hexane (GC grade), ferric chloride from Merck (USA), soy peptone, tryptone, yeast extract and beef extract from Pronadisa (Spain), polypeptone from Becton Dickison (USA), peptone from meat, ammonia sulphate, nickel chloride, lithium chloride, magnesium chloride, sodium chloride, potassium chloride, manganese chloride, ferrous chloride and pristine from Sigma Aldrich (USA), copper chloride from Systerm (Malaysia), cobalt chloride from HmbG Chemicals (Germany), cadmium chloride from Hopkins & Williams (UK), and lastly DNA extraction kits from iNtRON (Korean). The spent engine oil was obtained from a car mechanic in Kuala Lumpur, crude oil from Petronas Research Center at Bangi, Selangor, and cooking oil from a local restaurant.

3.2 Methods

3.2.1 Sample Collection

Sample collection was carried out at two locations: road side of Desa Setapak, Kuala Lumpur and pond water at Kepong. The collected samples were kept in sterile specimen bottles prior to transporting to the laboratory for further analysis.

3.2.2 Isolation and Screening of Hydrocarbon Degrading Bacteria

Isolation of hydrocarbon degrading bacteria was carried out using direct plating of dilutions of each sample on minimal salts agar (MSA) medium containing spent engine oil as sole source of carbon and energy. Each soil sample weighing 10 g was suspended in 100 ml sterile distilled water and vortexed for even distribution. Serial dilution was carried out up to 10^{-4} before plating. Aliquot of 100 ul was plated on MSA containing spent engine oil. Minimal salt medium (MSM) was prepared according to Appendix A. MSM cultures were incubated at 35°C for 5 days. Discrete colonies were picked and purified by streaking two times on nutrient agar before storage as the slant cultures at 4°C.

Individual pure isolates were further screened for their ability to degrade hydrocarbons by growing them on the minimal salts broth supplemented with spent engine oil under 35°C for 10 days. Changes in the culture medium were closely monitored. Isolates that could form floccules in the culture flasks were selected for further studies. Prior to further studies, the pure cultures of bacteria were identified on the basis of biochemical tests using the breath print pattern from the Biolog Identification system (USA), followed by the molecular sequencing method.

3.2.3 Isolate Identification

All bacterial isolates (as in Section 3.2.2) were subjected to Gram staining (Claus, 1992) prior to Biolog Test (Appendix B) and molecular sequencing with slight modification. Gram staining differentiation by KOH (Buck, 1982) was also carried out (Appendix C).

3.2.3.1 The Biolog Identification System

Biolog® commercial identification kits were utilised to identify the genus and species of the isolates. The procedures were conducted according to the instruction given by the supplier. Results were compared with the database (Biolog's MT MicroPlate™, version 3). The tests were performed at CABI, Serdang, Malaysia. Details of the test procedures were at Appendix D.

3.2.3.2 Molecular Sequencing and Phylogeny

Genus and species of the isolates were further confirmed with 16S rRNA molecular sequencing. DNA of the isolates were extracted with DNA isolation kit using i-genomic CTB DNA extraction mini kit (iNtRON, Korea) for Gram negative bacteria and i-genomic BYF DNA extraction mini kit ((iNtRON, Korea)

for Gram positive bacteria. 16S rRNA encoding genes were amplified by PCR using universal primer 27F AgAgTTTgATCMTGGCTCAg, 1492R TACggYTACCTTgTTACgACTT. PCR program were set at 35 amplification cycles at 94°C for 45 sec, 55°C for 60 sec, and 72°C for 60 sec. A positive control (*E.coli* genomic DNA) and a negative control (sterile deionized water) were included in the PCR. The 16S rRNA sequencing service was carried out by Macrogen, Korea.

The sequences were analyzed using the BLASTn program of National Center for Biotechnology Information (NCBI) and aligned with Clustal X software (version 2.0.11). Phylogenetic tree was constructed based on partial 16S rRNA gene sequences using Neighbor-joining method (Saitou & Nei, 1987).

3.2.4 Hydrocarbon Degradation Analysis

Interest was focused on the isolated *Acinetobacter* species that formed foccules in a shorter incubation period as compared to the other isolates. Three species were selected for further study.

The carbon utilization profile of these 3 species was carried out by inoculating each of the isolates in MSM supplemented with different carbon sources like spent engine oil, crude oil and cooking oil. The cultured media were

then incubated in shaker incubator under 180 rpm and temperature 35°C. Samples were collected daily; pH and optical density were monitored for a period of 17 days. GCMS analysis was done at the end of the study period.

3.2.4.1 Measurement of Cell Optical Density

Cell optical density was measured by pipetting 1 ml of the culture broth into a 15 ml centrifuge tube and centrifuged to obtain the cell pellet. The pellet was washed twice with 2 ml of distilled water and the spectrometric absorbance was measured at 600 nm. A standard curve was constructed based on the cell dry weight versus the optical density (Appendix E).

3.2.4.2 Determination of Hydrocarbon Chain Degradation Pattern by GCMS

An aliquot of 1 ml culture medium was pipetted into 3 ml of GC grade hexane to extract the hydrocarbons in the medium. The mixture was vortexed for 5 min followed by centrifugation to separate the aqueous and organic layer. Approximately 1 ml of organic layer was aliquot to the crimp-seal glass sample vial. Internal standard, the pristine (100 ppm) was included before GCMS analysis.

The extracted hydrocarbon chain samples were then analysed using a Shimadzu model 2010 Gas chromatography with a model QP2010 plus mass spectrometer detector. GC was operated in split mode with inlet temperature of 310°C. The oven initial temperature was 90°C for 1 min, ramp to 196°C in 5 min, ramp to 260°C in 4 min, hold 2 min, and ramp to 300°C in 3 min hold for 5 min with the total program time of 58.53 min. The column used was SGE BXP 5, length 30 m, diameter 0.25 mm, and thickness 0.25 um.

The mass spectrometer was operated under the condition of ion energy 70 eV and the mass range scanned 140-465 m/z. The MS was tuned to m/z 69, 219 and 502 with perfluorotributylamine (PFTBA) as a calibration standard. SCAN acquisition mode was used and solvent cut of time as 2.5 min.

3.2.5 Culture Medium Modification

The bacterial isolates demonstrated the best hydrocarbon chain degrading profile in section 3.2.4 was selected to further test the effect of nitrogen and metal ion sources toward the hydrocarbon chain degradation properties. The crude oil followed by cooking oil was found to be most drastically degraded among the oils tested, hence the crude oil was chosen as a carbon source for the subsequent procedures.

3.2.5.1 Nitrogen Source Substitution

Ammonium nitrate in minimal salt broth was substituted with six different organic nitrogen sources such as soy peptone, polypeptone, tryptone, yeast extract, meat peptone and beef extract. The amount of organic nitrogen substituted was determined by total nitrogen content calculation (Appendix F). Samples were collected daily; pH, lipase activity and optical density were monitored for a period of 14 days. GCMS analysis was done at day 14 as retrieved in section 3.2.4.2.

Lipase activity was determined by spectrophotometric assay using p-nitrophenil-laurate (pNPL) as substrate according to Castro-Ochoa, Rodriguez-Gomez, Valerio-Alfaro & Ros (2005). The reaction mixture consisted of 0.1 ml production medium, 0.8 ml 0.05 M phosphate buffer (pH 6.5) and 0.1 ml of 0.01 M pNPL in ethanol. The hydrolytic reaction was carried out at 37°C for 30 min, after which 0.25 ml of 0.1 M Na_2CO_3 was added. The mixture was centrifuged (16000 x *g*, 15 min, 25°C) and the absorbance at 410 nm was determined. One unit of lipase activity was defined as the amount of enzyme that caused the release of 1 μmol of p-nitrophenol (molar absorption coefficient, 4.6 mM^{-1} cm^{-1}) from p-nitrophenol-laurate in 30 min under the test condition. Lipase activity standard curve was prepared (Appendix G).

3.2.5.2 Metal Ion Substitution

The metal ion ferrous chloride (0.05 mg/l) used in the minimal salt broth was substituted with 0.05 mg/ml of any one of cuprum chloride, cobalt chloride, cadmium chloride, ferric chloride, nickel chloride, lithium chloride, magnesium chloride, sodium chloride, potassium chloride and manganese chloride. Hydrocarbon degradation rate by GCMS, pH changes, lipase activity and optical density of the medium were examined for two weeks. Analysis procedures were similar to those in section 3.2.4 for GCMS and cell optical density measurement, and section 3.2.5.1 for lipase activity test.

3.2.6 Metabolite Analysis

The cell free extract with crude oil as the hydrocarbon source was fractionated and concentrated by solid ammonium sulphate [$(NH_4)_2SO_4$] at 80%. The mixture was stirred gently at $4^{\circ}C$ overnight. Precipitated protein or endopeptide was collected by centrifugation at 15, 000 x g for 15 min and subsequently dialysed against distilled water at $4^{\circ}C$ for 24 hours. Dialyzed samples were freeze dried and tested further using SDS-PAGE.

3.2.6.1 Protein Analysis

The molecular mass of proteins were estimated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Samples were resolved in linear acrylamine/bis vertical slab gel according to Laemmli (1970), with a discontinuous buffer system, using Bio-Rad Mini-Protean II electrophoresis apparatus. Resolving gel (12% w/v) was prepared in 0.375 M Tris-HCl, pH 8.8 buffer whereas the stacking gel comprised of 4% (w/v) acrylamine/bis in 0.125 M Tris-HCl, pH 6.8 buffer. Both gels contained 0.1% SDS.

Samples for electrophoresis were diluted 1:4 with sample buffer containing 0.325 M Tris-HCl, pH 6.8, 2% (w/v) SDS, 20% (w/v) glycerol, 5% mercapthoethanol, 0.01% bromophenol blue as tracking dye. The sample mixture was boiled for 1 min and centrifuged for 5 min at 10 000 x g in a microcentrifuge. The gel was loaded in tank buffer (25 mM Tris-HCl, 0.5% (w/v) SDS, 200 mM glycine pH 8.3) and electrophoresed at 150 V constant voltage for 60 min in ice bath or until the tracking dye migrated to the base. Gel staining and destaining procedures are described in Appendix H.

CHAPTER 4

RESULTS

4.1 Screening and Isolation

From the serial dilution plate, a total of 12 bacteria were isolated and named as strain F1 to F12. Seven strains were isolated from the pond water of Kepong and 5 strains from the soil along the road side. These 12 strains were then cultured in minimal salt broth supplemented with 1% spent engine oil and incubated in a shaker at 35°C. Flocculates formation were observed in flask F1, F4 and F12 on the 3rd day of incubation followed by flask F2 and F10 on the 5th day. Cloudiness in the media was noticed in all five flasks, which suggested bacterial growth in the minimal salt medium supplemented with spent engine oil, though engine oil contained antimicrobial substances.

Floccules formed in the medium demonstrated round and discrete aggregates floating on the medium. Different isolates showed different floccule morphologies and differed among sizes. Floccules formed by F12, F4 and F2 were very discrete with the size of 1 mm whereas floccules formed by F1 and F10 were 2 mm in diameter. Figure 4.1 shows the comparison between isolates with and without floccule formation whereas Figure 4.2 is the close up of the flocculates.

Different appearance was observed in the culture flasks showing diverse morphology on oil dispersion and turbidity of culture medium (Figure 4.3). This further confirmed that the isolates were able to grow in hydrocarbon such as spent engine oil. In the contrary, the control flask contained a clearer medium, a distinct layer between the oil and water interfaces was observed. A bigger clump was observed in the control flask after 10 days. This was probably due to the weathering of the oil in the incubator shaker which was kept constantly at $35^{\circ}C$ and without the presence of any microbe.

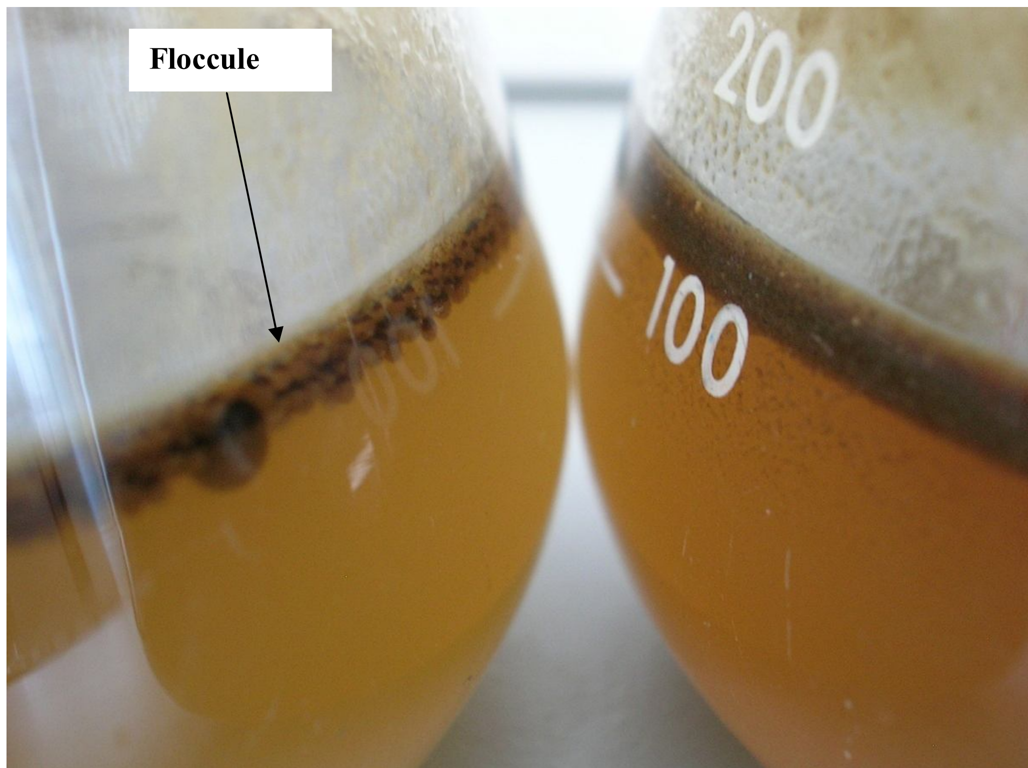

Figure 4.1: Comparison between isolates with and without floccule formation

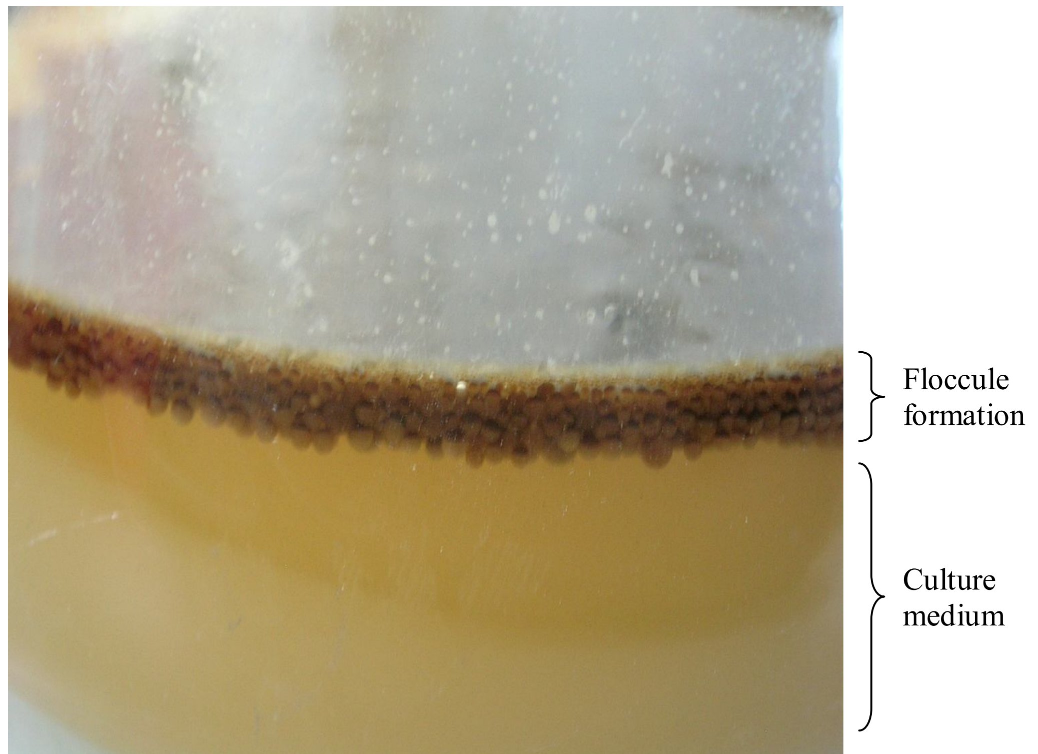

Figure 4.2: Close up view of the floccule formation by *A.calcoaceticus* culturing in spent engine oil medium

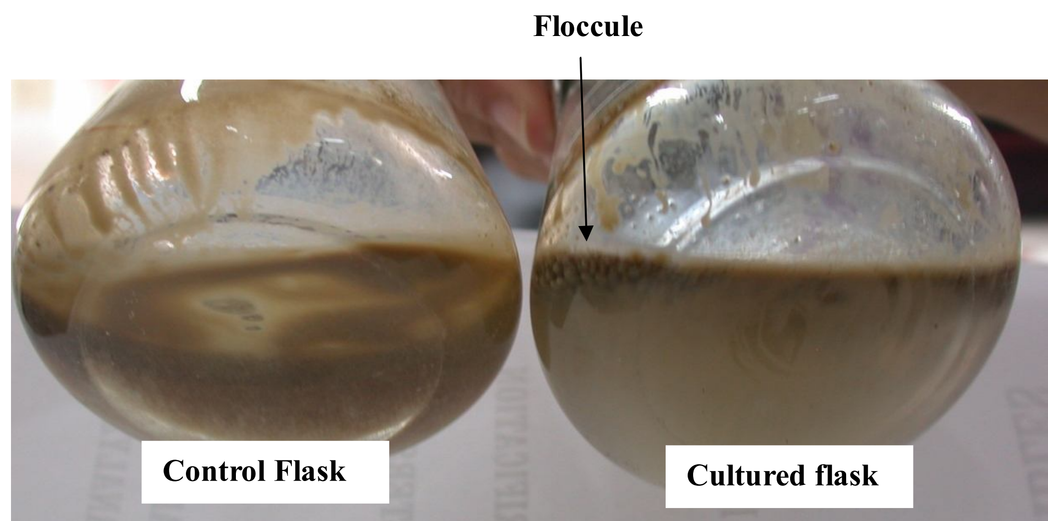

Figure 4.3: Appearance of culture medium after 5 days of incubation. Differences between oil dispersion pattern and floccule formation was observed

4.2 Speciation of Isolates

A total of 5 pure cultures with floccule formation were isolated and identified with 16S rRNA molecular sequencing. Both Gram staining and KOH method showed the same result in identifying whether the strain was Gram positive or Gram negative. Table 4.1 summarises the result of Biolog test and 16S rRNA molecular sequencing results for each of the isolates, respectively.

Five individual isolates F1, F2, F4, F10 and F12, showed high similarities to *Acinetobacter radioresistens* (100%), *Stenotrophomonas maltophilia* (100%), *Acinetobacter baumannii* (100%), *Entrerobacter sakazaki*i (100%) and *Acinetobacter calcoaceticus* (100%), respectively from the nucleotide Blast in NCBI. Sequences of the analysis are presented at Appendix I. Figure 4.4 shows the phylogenetic relationship among 5 strains. Phylogenetic tree showed the relationship of 16S rRNA gene sequences of the bacteria isolated. *A. radioresistens* and *A. baumannii* are from the same group and they possesed high sequence similarity with the distance 0.01125 and 0.01375, respectively. *A. radioresistens*, *A. baumannii* and *A. calcoaceticus* were chosen for subsequent experimental works as the floccule formation among these strains was observed within shorter incubation period as compared to other strains.

Table 4.1: Summary of the species identification test results

Isolate Labelling	Isolation Location	Gram Differentiation	Biolog Test	16S rRNA Sequencing Analysis
F1	Pond water	Gram negative	*Corynebacterium jeikeium*	*Acinetobacter radioresistens*
F2	Pond water	Gram negative	*Corynebacterium jeikeium*	*Stretomonas maltophilia*
F4	Pond water	Gram negative	*Acinetobacter baumannii*	*Acinetobacter baumannii*
F10	Soil	Gram negative	*Corynebacterium jeikeium*	*Enterobacter sakazakii*
F12	Soil	Gram negative	*Acinetobacter calcoaceticus*	*Acinetobacter calcoaceticus*

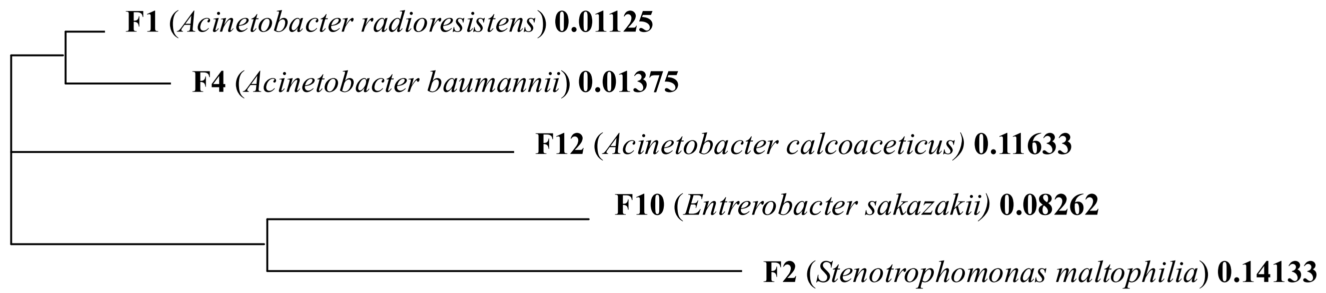

Figure 4.4: Phylogenetic tree constructed by neighbour joining method

4.3 Hydrocarbon Degradation Analysis

Hydrocarbon degradation ability of spent engine oil, crude oil and used cooking oil were carried out with the three strains of *Acinetobacter* species from section 4.2. Hydrocarbon degradation profile was analysed using GCMS. Medium pH and bacterial growth rate were monitored at the same time.

4.3.1 Spent Engine Oil as Sole Carbon Source

Three species of *Acinetobacter*, *A. radioresistens, A. baumannii,* and *A. calcoaceticus* were separately inoculated into minimal salt broth supplemented with 1% v/v spent engine oil respectively. Figure 4.5 shows GCMS results obtained from the continuous monitoring on the hydrocarbon degradation profile. The three isolates were able to grow on spent engine oil supplemented culture medium. A significant peak was observed at 51.96 min in the GCMS chromatogram (Figure 4.5), which was identified as Bis [3-(3, 5-di-tert-butyl-4-hydroxyphenyl)propyl] maleate (63% matches with the GCMS library). This metabolite was produced in the culture media of *A. calcoaceticus* and *A. baumannii*. On the other hand, an additional peak at 39.81 min was observed in the culture medium of *A. calcoaceticus*. The compound was suggested to be Decanedioic acid (88% matches with the GCMS library). The distinct peak at 34.75 min present in all 3 test samples and control was identified to be 79%

similar to 1, 2-Benzenedicarboxylic acid bis [(4-hydroxylmethyl cyclohexyl) methyl] ester from the mass spectra library similarity search.

The change of pH during the bacterial growth was monitored. Results from the observation indicated that the medium with *A. baumannii* possessed the lowest pH (pH 6.3) among the three strains on day 17 (Figure 4.6a). *A. radioresistents* did not show obvious pH changes as compared to the control flask (with oil but without microbe). The pH of the control medium was in between 6.9 throughout the length of the study. But, flasks with bacteria achieved slightly lower pH (pH 6.4 to 6.8) as compared to control. This observation might be due to the bacterial metabolism or production of secondary metabolites during the growth. Overall, *A. calcoaceticus* possesed the highest growth rate (0.91 to 0.94 g/L) as compared to *A. baumannii* and *A. radioresistents* (Figure 4.6b).

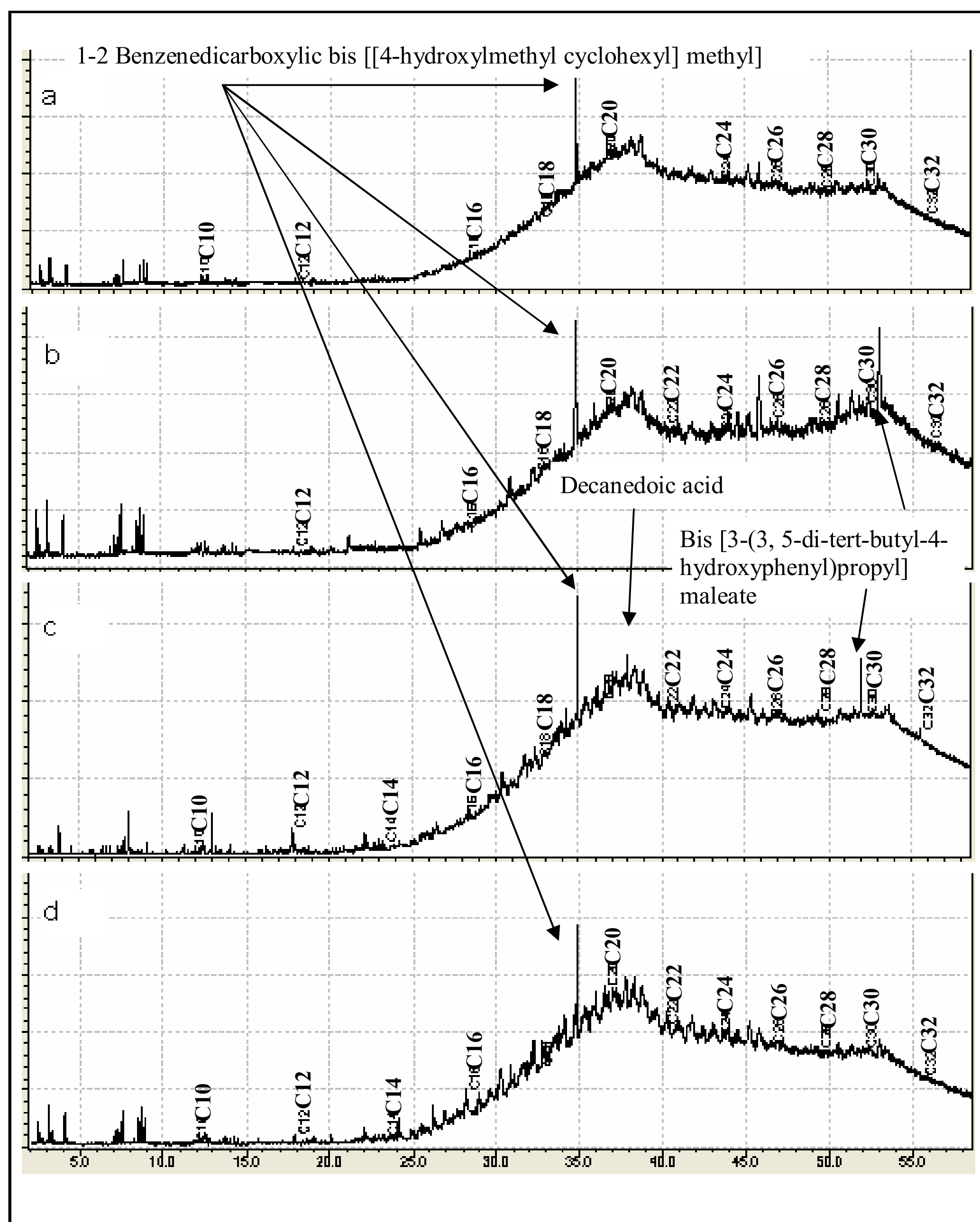

Figure 4.5: Gas chromatography profile of the culture medium supplemented with 1% spent engine oil on day 17: (a) *Acinetobacter radiaoresistens;* (b) *Acinetobacter calcoaceticus;* (c) *Acinetobacter baumannii* and (d) control

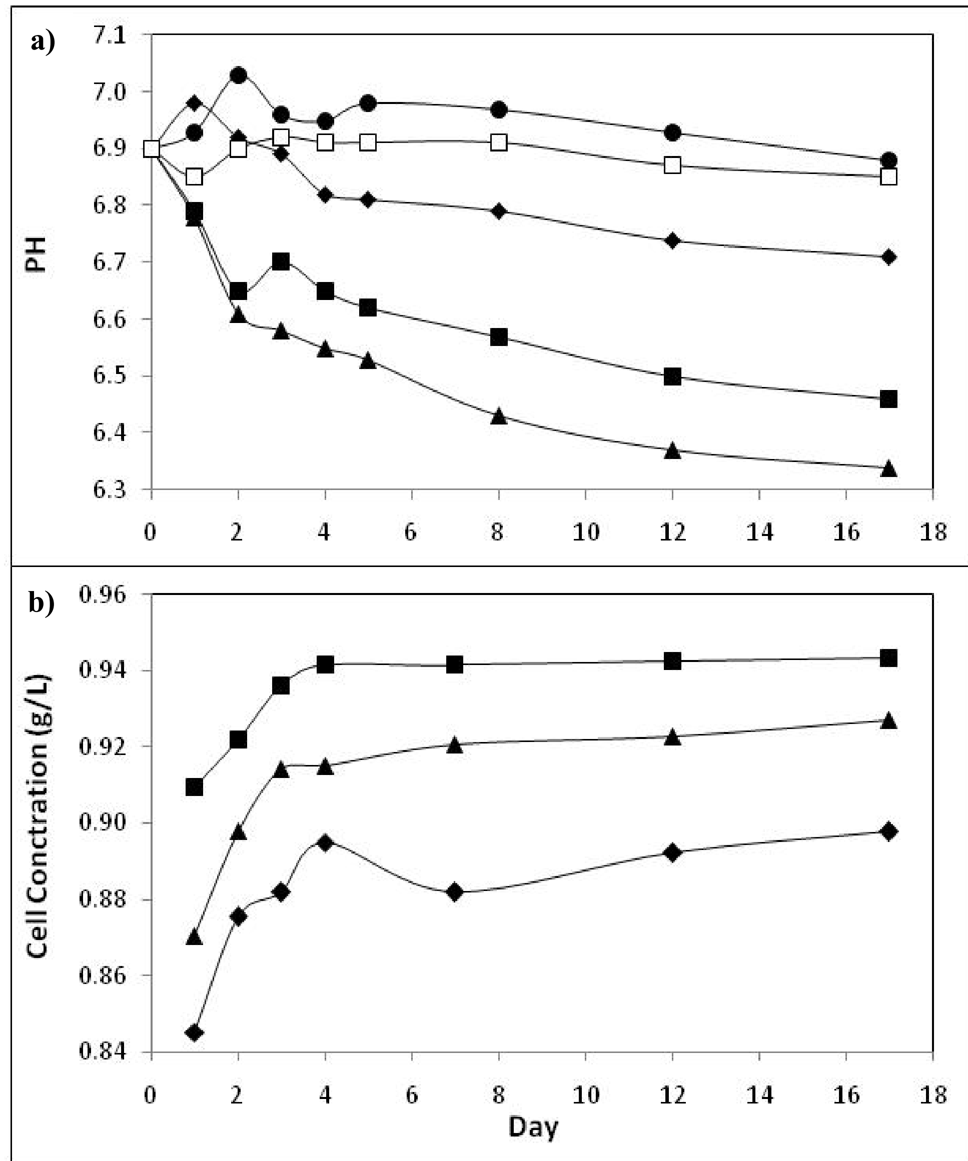

Figure 4.6: Time course of (a) pH and (b) growth for *Acinetobacter* bacteria strains cultured in minimal salt broth supplemented with 1 % of spent engine oil for 17 days

Symbols: (◆) *A. radioresistents*, (▲) *A. baumannii*, (■) *A.calcoaceticus*, (●) control and (□) medium

4.3.2 Crude Oil as Sole Carbon Source

The selected 3 *Acinetobacter* species were inoculated into minimal salt broth supplemented with crude oil for further tests. Hydrocarbon degradation pattern was monitored by GCMS for 17 days, at the same time pH and growth profiles were also observed. The series of chromatograms in Figure 4.7 show that *A. calcoaceticus* achieved enhancing degradation pattern as compared to the control or the other two strains as most of the peaks from carbon chain length C8 to C28 were degraded. GCMS analysis revealed Octadecanal compound at 34.8 min and 4-(2,2-Dimethyl-6-methylenecyclohexylidene)-3-methylbutane-2 at 51.5 min from *A. calcoaceticus* culture medium on day 17.

From Figure 4.8, changes in cell density and pH suggested that cells have entered the exponential phase after day 2. However, growth of *A. radioresistents* was not as promising as the other two strains because the growth rate was rather slow, no drastic change of pH was observed. *A. baumannii* achieved pH 4.5 on day 2. *A. calcoaceticus* had the fastest growth among the three species (1.6 to 2 g/L).

Nigrosin staining (negative staining) was carried out to the culture on day 3 and examined under the phase contrast microscope. Figure 4.9 represents the phase contrast microscopic observation of the isolates in crude oil medium on day 3. The clear zone represents the oil droplet. Obvious changes of the species under the microscopic view could be seen with background staining. The species *A.*

calcoaceticus clumped compactly around the discrete oil droplet, which clearly distinguished from the other two strains. This observation suggested that the bacteria surrounded the oil droplet for carbon source uptake.

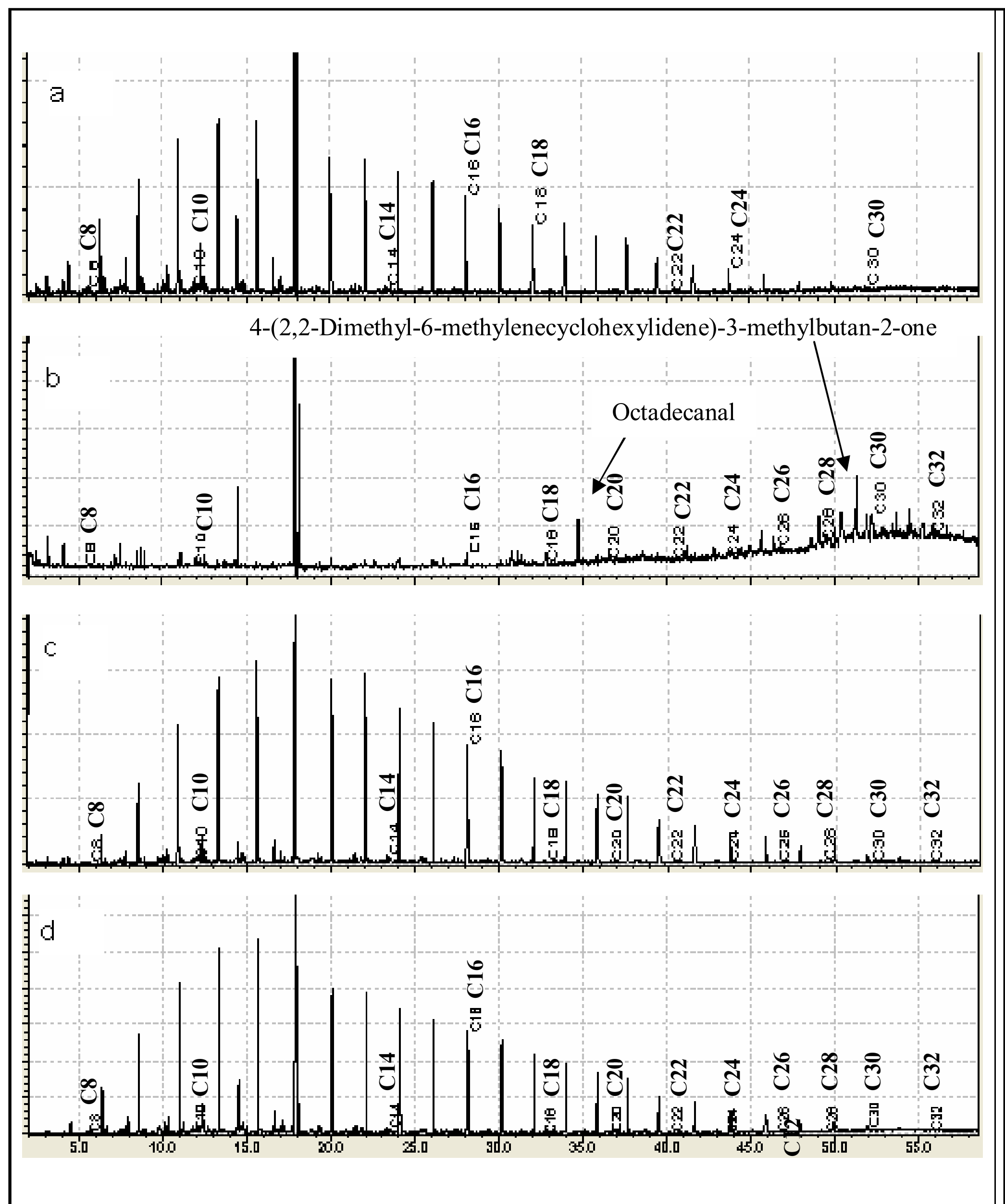

Figure 4.7: Gas chromatography profile of the culture medium supplemented with 1% crude oil on day 17: (a) *Acinetobacter radiaoresistens,* (b) *Acinetobacter calcoaceticus,* (c) *Acinetobacter baumannii* and (d) control

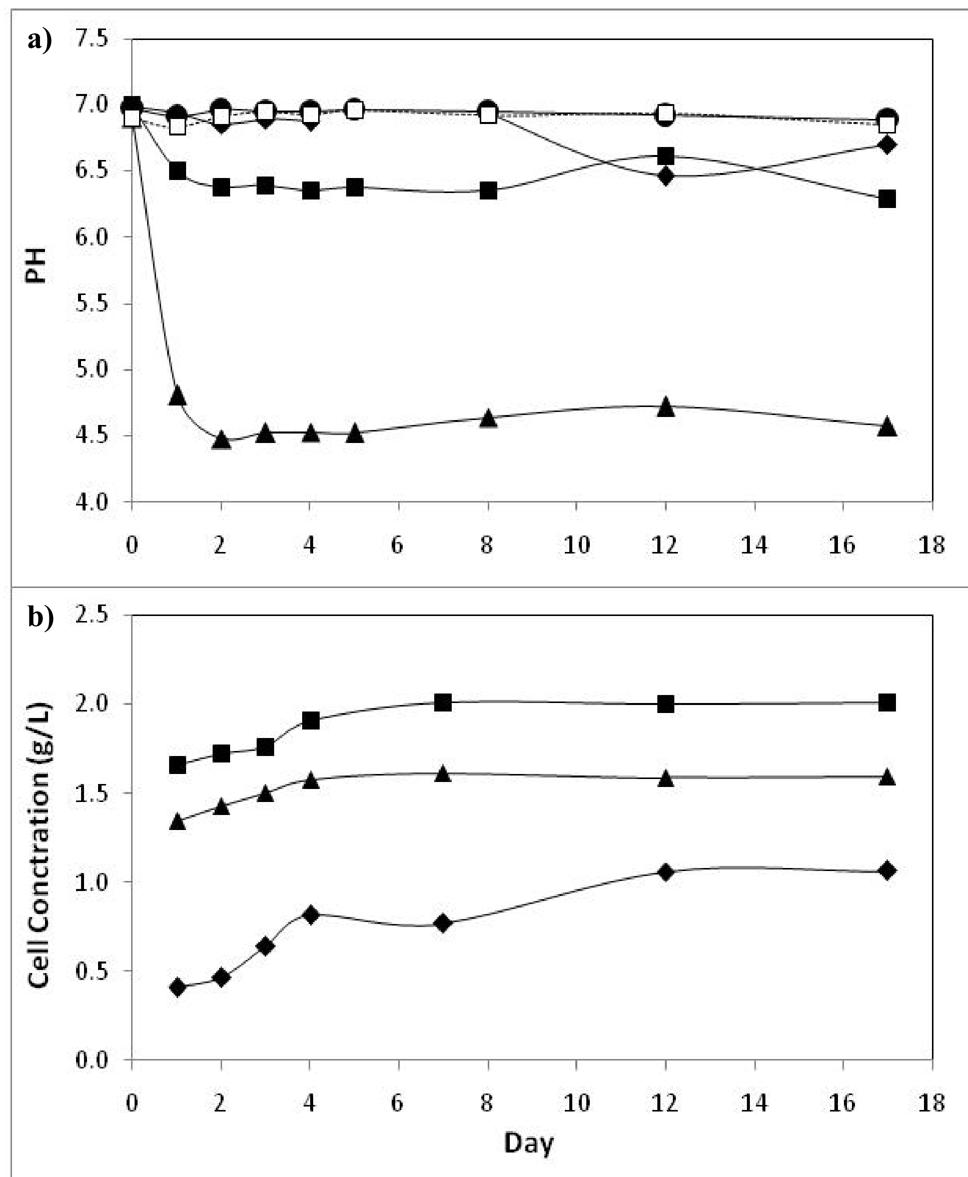

Figure 4.8: Time course of (a) pH and (b) growth for *Acinetobacter* bacteria strains cultured in minimal salt broth supplemented with 1 % of crude oil for 17 days

Symbols: (◆) *A. radioresistents*, (▲) *A. baumannii*, (■) *A.calcoaceticus*, (●) control and (□) medium

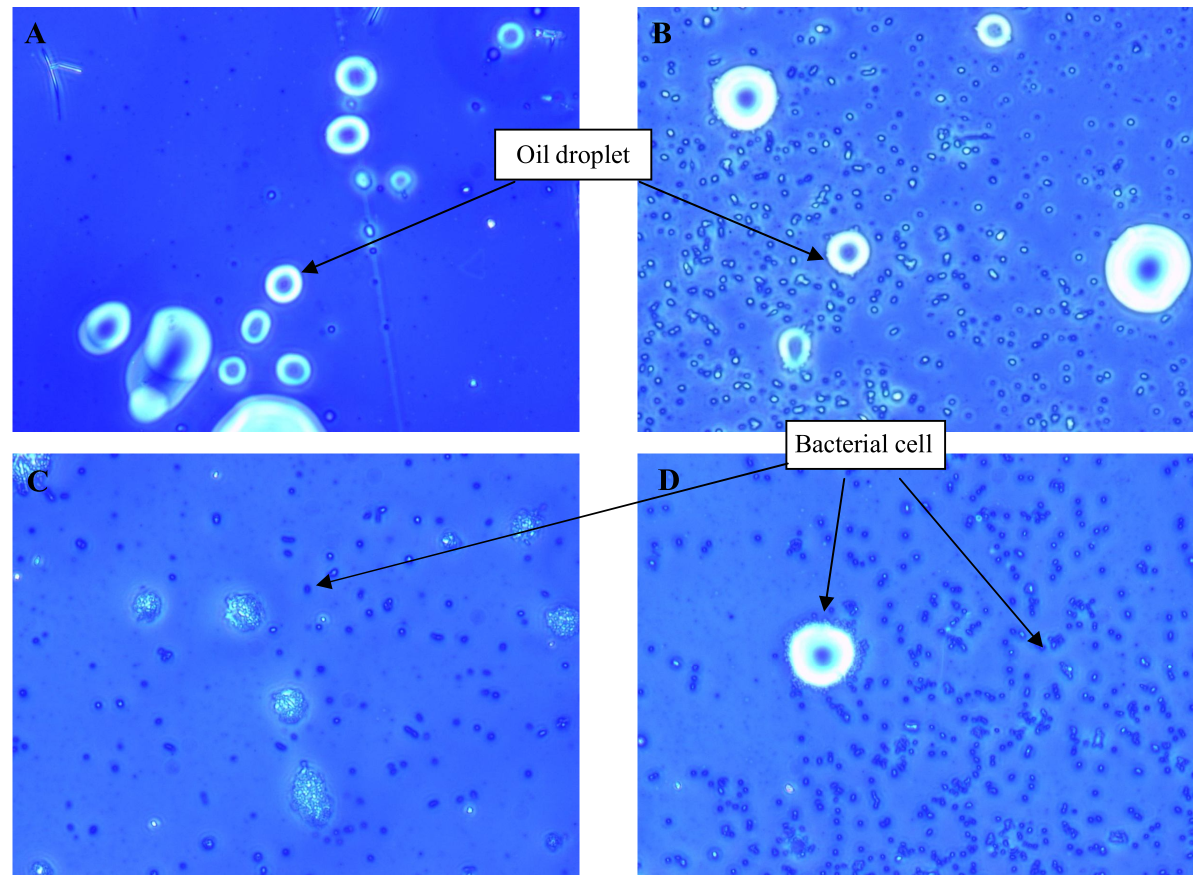

Figure 4.9: Phase contrast microscopic morphology of the isolates in minimal salt medium supplemented with crude oil on day 3: A) control, B) *A. radioresistens,* C) *A. calcoaceticus* and D) *A. baumannii*

4.3.3 Used Cooking Oil as Sole Carbon Source

Under the same test condition as described previously, crude oil was substituted with used cooking oil. The two peaks labelled as Peak I (PI) and Peak II (PII) (Figure 4.10) appeared in between 30 to 35 min in Figure 4.10 were the two hydrocarbons detected in used cooking oil. *A. radioresistents* was able to degrade Peak I when tested on day 17 whereas *A. calcoaceticus* degraded both peaks. *A. baumannii* was also able to degrade both peaks as compared to the control but in a slower rate than the other two species.

pH changes during incubation were monitored. Figure 4.11a shows the changes of pH in the culture medium. pH changed in between day 1 and day 8 for *A. radioresistents* and *A. baumannii*. A drastic pH decrease in the culture medium of *A. calcoaceticus* from pH 6.9 to 4.6 was observed at day 1 but increased gradually to pH 6.5 at day 6 and remained until day 17.

For growth profile, the cultures reached stationary phase at day 4 (Figure 4.11b). The highest cell concentration of 1.72 g/L was achieved by *A. calcoaceticus* at day 4.

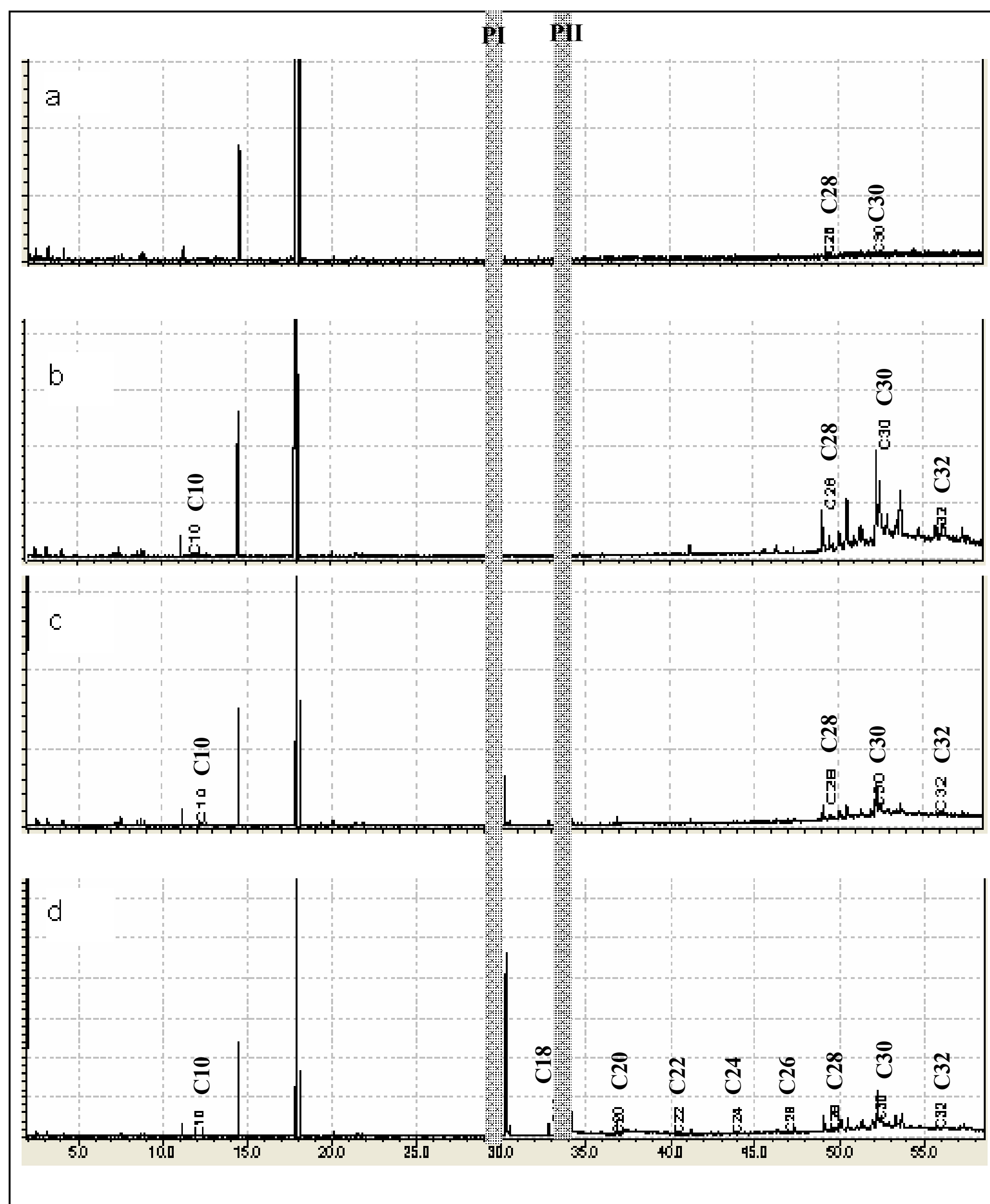

Figure 4.10: Gas chromatography profile of the culture medium supplemented with 1% used cooking oil on day 17: (a) *Acinetobacter radiaoresistens,* (b) *Acinetobacter calcoaceticus,* (c) *Acinetobacter baumannii* and (d) control

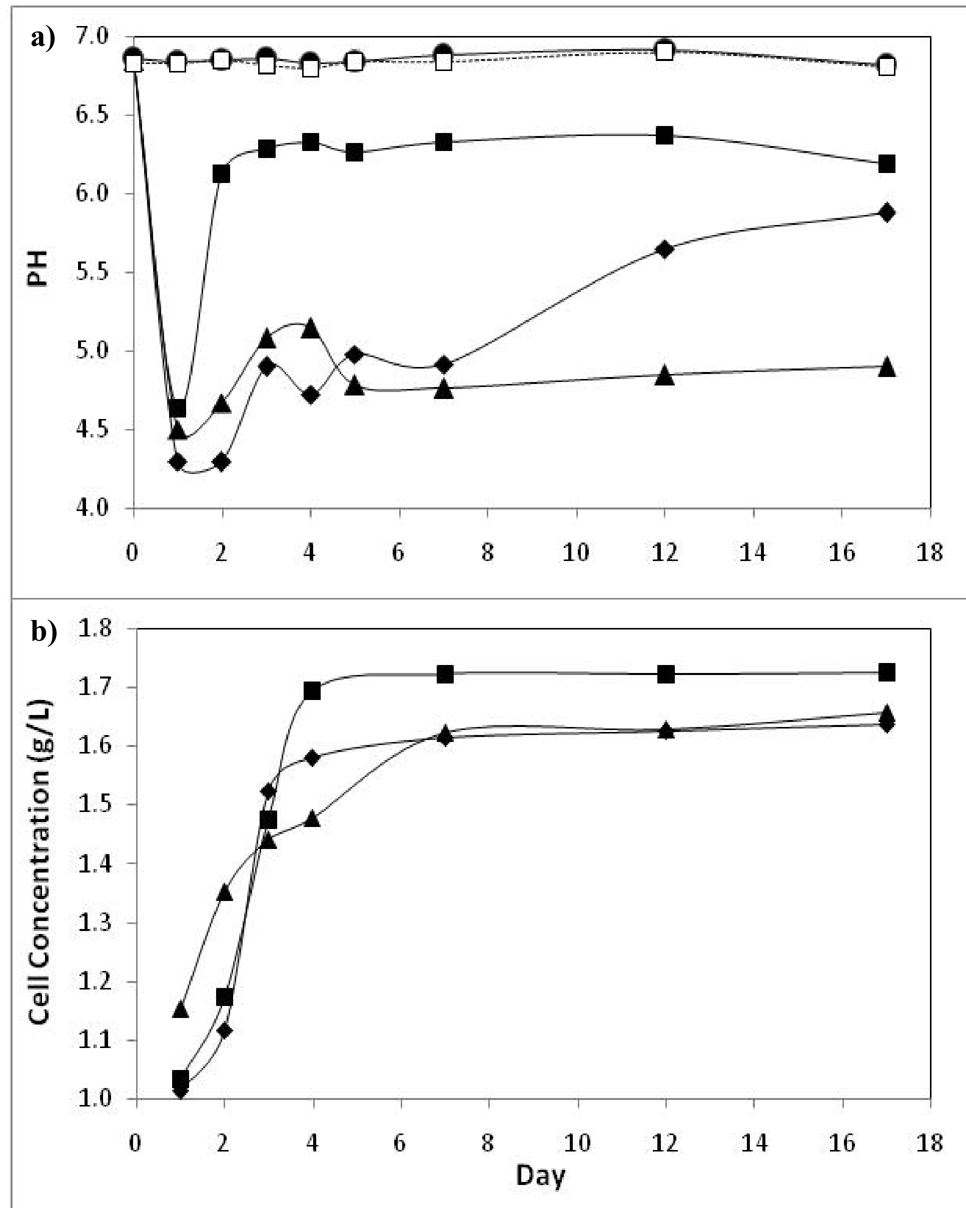

Figure 4.11: Time course of (a) pH and (b) growth for *Acinetobacter* bacteria strains cultured in minimal salt broth supplemented with 1 % of used cooking oil for 17 days
Symbols: (◆) *A. radioresistents,* (▲) *A. baumannii*, (■) *A.calcoaceticus,* (●) control, (□) medium

4.4 Culture Medium Modification

Optimisation of culture medium in term of nitrogen source and metal ion was carried out to facilitate the bacterial growth and promote bioremediation. From Section 4.3, *A. calcoaceticus* was found to be the best degrader of crude oil or used cooking oil as compared to *A. radiaoresistens* and *A. baumannii*.

4.4.1 Nitrogen Source Substitution

Six different organic nitrogen sources, namely, yeast extract, beef extract, tryptone, polypeptone, soy peptone and peptone from meat were used to replace 1 g/L of NH_4NO_3 in the original culture medium. The amount of organic nitrogen substituted was determined by the total nitrogen content in ensuring that each flask had the same nitrogen content. The modified culture medium was then supplemented separately with crude oil and cooking oil to monitor the degradation pattern of oils. Gas chromatography was carried out to examine the degraded oil product after 14 days. pH, growth and lipase activity were monitored at the same time.

4.4.1.1 Nitrogen Source Substitution in MSM with 1% v/v Crude Oil

Calculated amount of yeast extract, beef extract, tryptone, polypeptone, soy peptone and peptone from meat were added separately to each flask containing MSM with 1% v/v of crude oil. Parameters examined were oil degrading profile using GCMS, pH, growth and lipase activity of each flask for 14 days. Gas chromatogram in Figure 4.12 shows that nitrogen sources supplement with yeast extract, tryptone, polypeptone and soy peptone respectively exhibited better degradation of crude oil as compared to medium with beef extract and peptone from meat as the source of nitrogen.

The fluctuation of pH was not obvious for all the samples (Figure 4.13a). pH reduced slightly (pH 6.45 to 6.74) on day 2 but remained almost neutral after day 3. *A. calcoaceticus* in culture medium with tryptone, polypeptone, peptone from meat and soy peptone possessed a pH range of 6.6 to 6.7 starting from day 3 onward.

A. calcoaceticus showed the highest growth rate when cultured in medium amended with yeast extract (2 g/L) (Figure 4.13b). In the yeast extract medium, the pH tended to remain near neutral between pH 6.7 to 6.9.

The highest lipase activity was achieved by *A. calcoaceticus* in tryptone-culture medium (16.8 U/ml), followed by beef extract (10 U/ml) and polypeptone

(9.2 U/ml) as the nitrogen source on the first day (Figure 4.13c). The culture medium with soy peptone and peptone showed minimal or nearly no lipase activity. Lipase activity peaked at 10.1 U/ml on day 11 in the beef extract substituted flask.

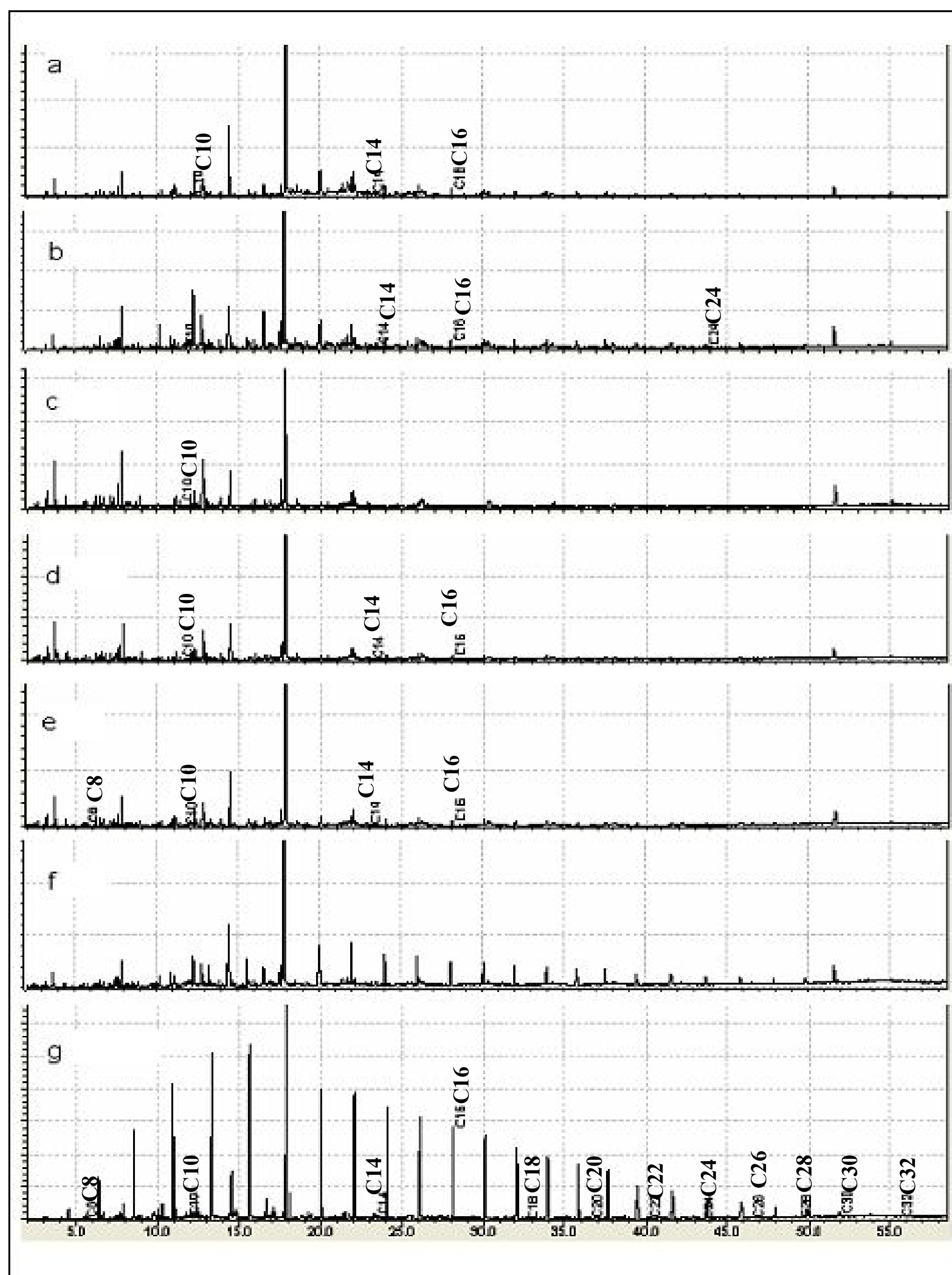

Figure 4.12: Gas chromatography profile for culture medium of *Acinetobacter calcoaceticus* in 1% crude oil with the substitution of different nitrogen sources on day 14: (a) yeast extract (b) beef extract (c) tryptone (d) polypeptone (e) soy peptone (f) peptone and (g) control flask

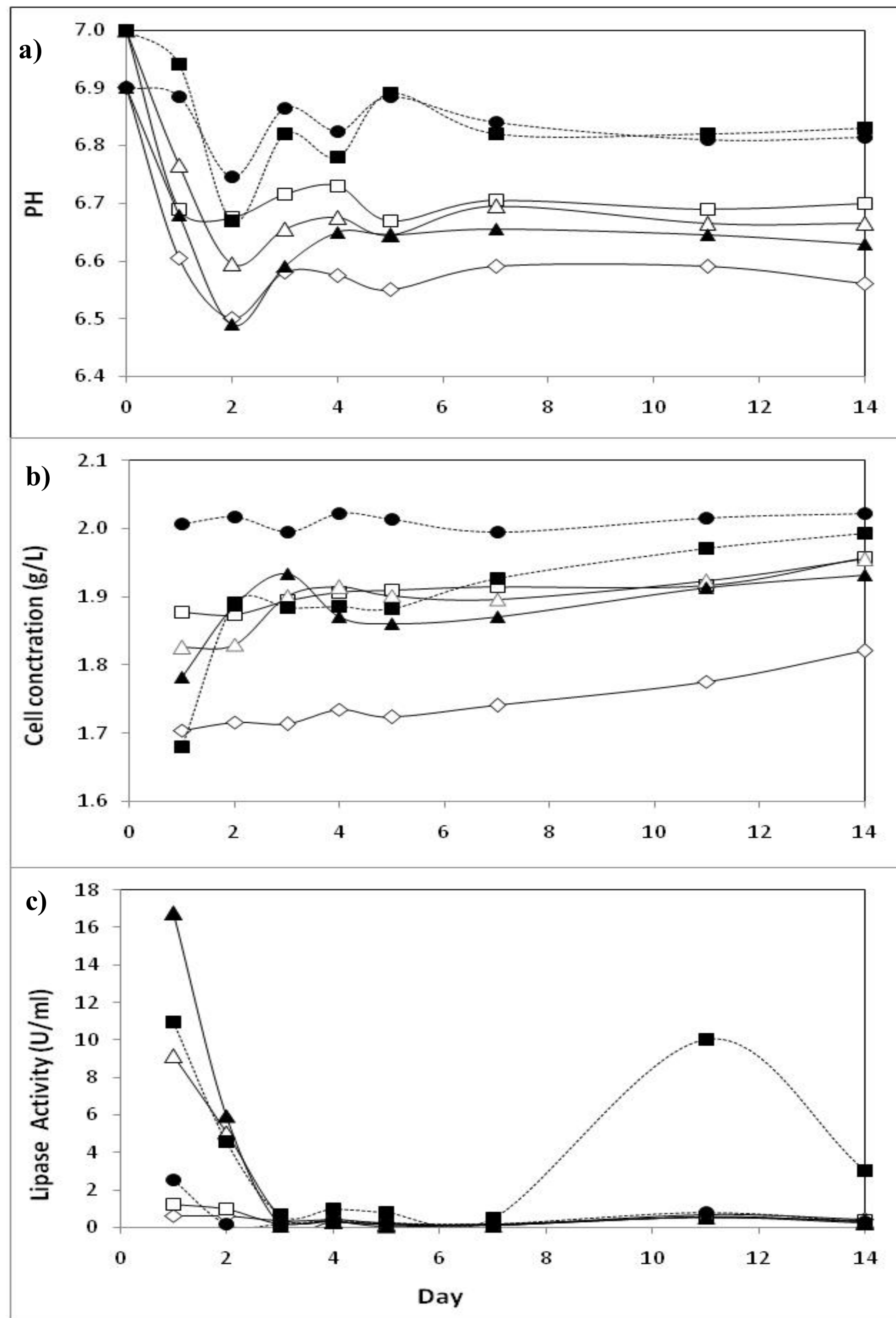

Figure 4.13: Time course of (a) pH; (b) growth; (c) lipase activity of *A. calcoaceticus* cultured in minimal salt broth supplemented with 1 % of crude oil and different nitrogen sources

Symbols: (◇) peptone from meat, (□) soypeptone, (△) polypeptone, (▲) tryptone, (■) beef extract, (●) yeast extract

4.4.1.2 Nitrogen Source Substitution in MSM with 1% v/v Used Cooking Oil

Culture of bacteria in different nitrogen sources was also carried out with the used cooking oil as the sole source of carbon as described in section 4.6.1. Based on the GC analysis results (Figure 4.14), culture medium supplemented with polypeptone exhibited the best degradation of used cooking oil in which peak I (PI) and peak II (PII) were completely degraded.

The pH of all the media dropped on day 2 and 3 from 6.6 to 4.2 (Figure 4.15). Culture flasks with soy peptone regained their pH to almost the original stage from day 4 onward. Peptone supplemented flask with 1% used cooking oil possessed acidic pH (4.0) on day 2 onward.

The yeast extract supplemented flask exhibited the highest growth rate (2.2 g/L) and the minimum lipase activity (0.3 to 1.8 U/ml) as depicted in Figure 4.15. For soy peptone and yeast extract supplemented culture medium, *A. calcoaceticus* lipase activity increased from 11.1 U/ml on day 7 to the maximum 4.8 U/ml on day 10.

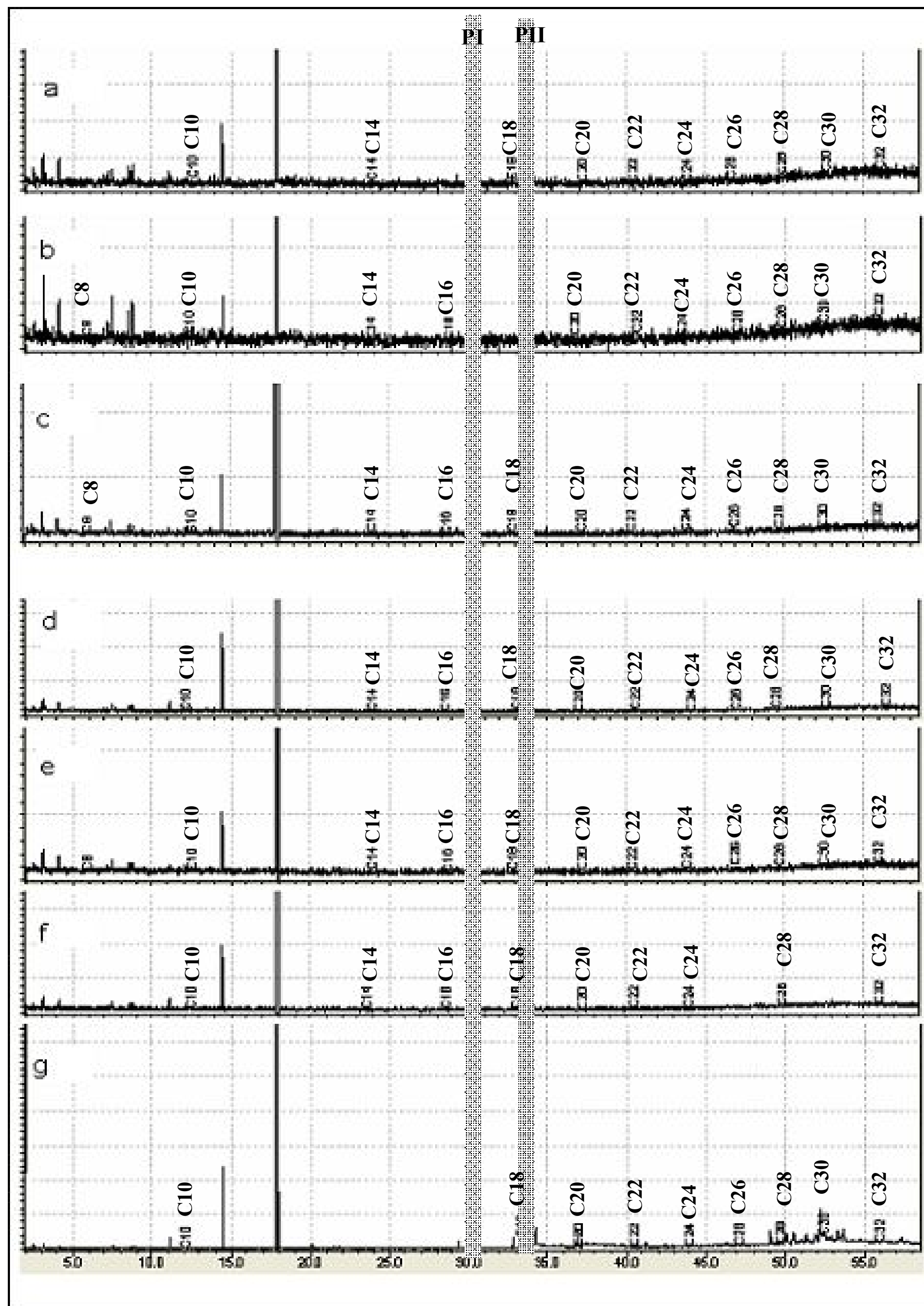

Figure 4.14: Gas chromatography profile for the culture medium of *Acinetobacter calcoaceticus* in 1% used cooking oil with different nitrogen source substitution on day 14: (a) Yeast extract (b) beef extract (c) tryptone (d) polypeptone (e) soy peptone (f) peptone and (g) control flask

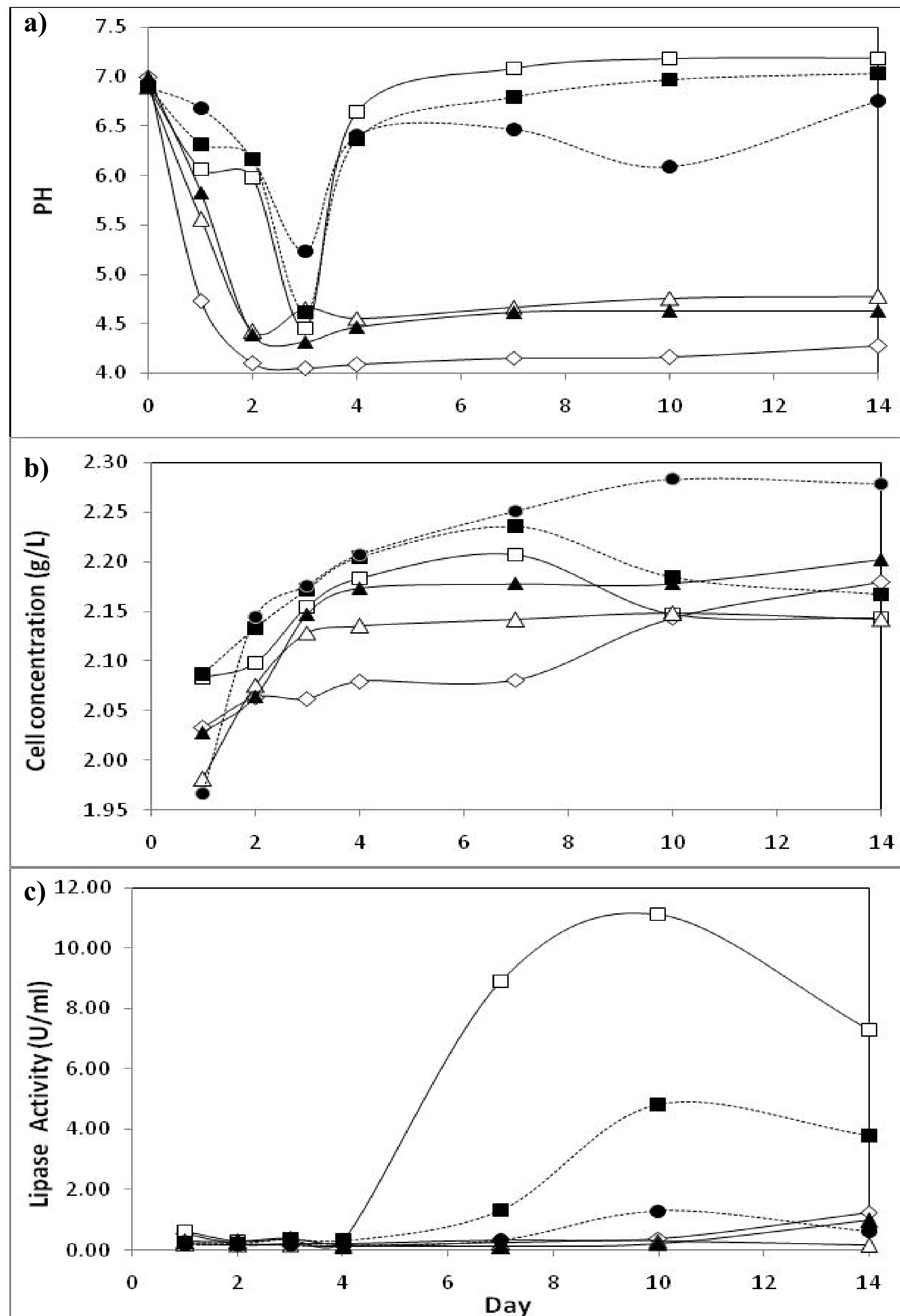

Figure 4.15: Time course of (a) pH, (b) growth, (c) lipase activity for *A. calcoaceticus* cultured in minimal salt broth supplemented with 1 % of used cooking oil and different nitrogen sources substitution respectively

Symbols: (◇) peptone from meat, (□) soypeptone, (△) polypeptone, (▲) tryptone, (■) beef extract, (●) yeast extract

4.4.2 Metal Ion Substitution

Incorporation of metal ions to the standard culture medium was carried out using 0.05 mg/ml cuprum chloride, cobalt chloride, cadmium chloride, nickel chloride, ferric chloride, lithium chloride, magnesium chloride, sodium chloride, potassium chloride and manganese chloride respectively to substitute ferrous chloride in the original formulation. Either crude oil or cooking oil was used as the carbon source. Among the metal ions tested, cuprum chloride, cobalt chloride, cadmium chloride and nickel chloride failed to cause growth of the bacterial cultures. The observation suggested that these ions might have intoxicated the cells and inhibited growth.

4.4.2.1 Metal Ion Substitution in MSM with 1% v/v Crude Oil

Six different metal ion sources namely ferric chloride, lithium chloride, manganese chloride, potassium chloride, sodium chloride and magnesium chloride were substituted to each flask singly with 1% v/v of crude oil. Parameters examined were oil degradation profile, pH profile, growth profile and lipase activity of each flask for 14 days. *A. calcoaceticus* tended to degrade shorter carbon chain length (C8 to C12) hydrocarbons in the crude oil supplemented with potassium chloride (Figure 4.16d). Magnesium supplementation showed the best degradability in 1% crude oil with the most carbon chains C8 to C32 being

degraded with the highest growth rate of 1.5 g/L as depicted in Figure 4.17.

Culture medium with ferric chloride showed the lowest pH (4.0) in day 1 onward among all test samples. The pH decreased gradually 7 to 5.5 in potassium supplemented medium (Figure 4.17a).

A. calcoaceticus in potassium chloride and ferric chloride supplemented media possessed a slightly slower growth rate (0.6 to 1.2 g/L). The highest growth rate (1.3 to 1.6 g/L) was achieved by *A. calcoaceticus* in magnesium or potassium chloride supplementation (Figure 4.17b).

According to Figure 4.17c, the entire test samples showed elevated lipase activity on day 11. The highest lipase activity (0.8 U/ml) was achieved in potassium chloride supplemented medium.

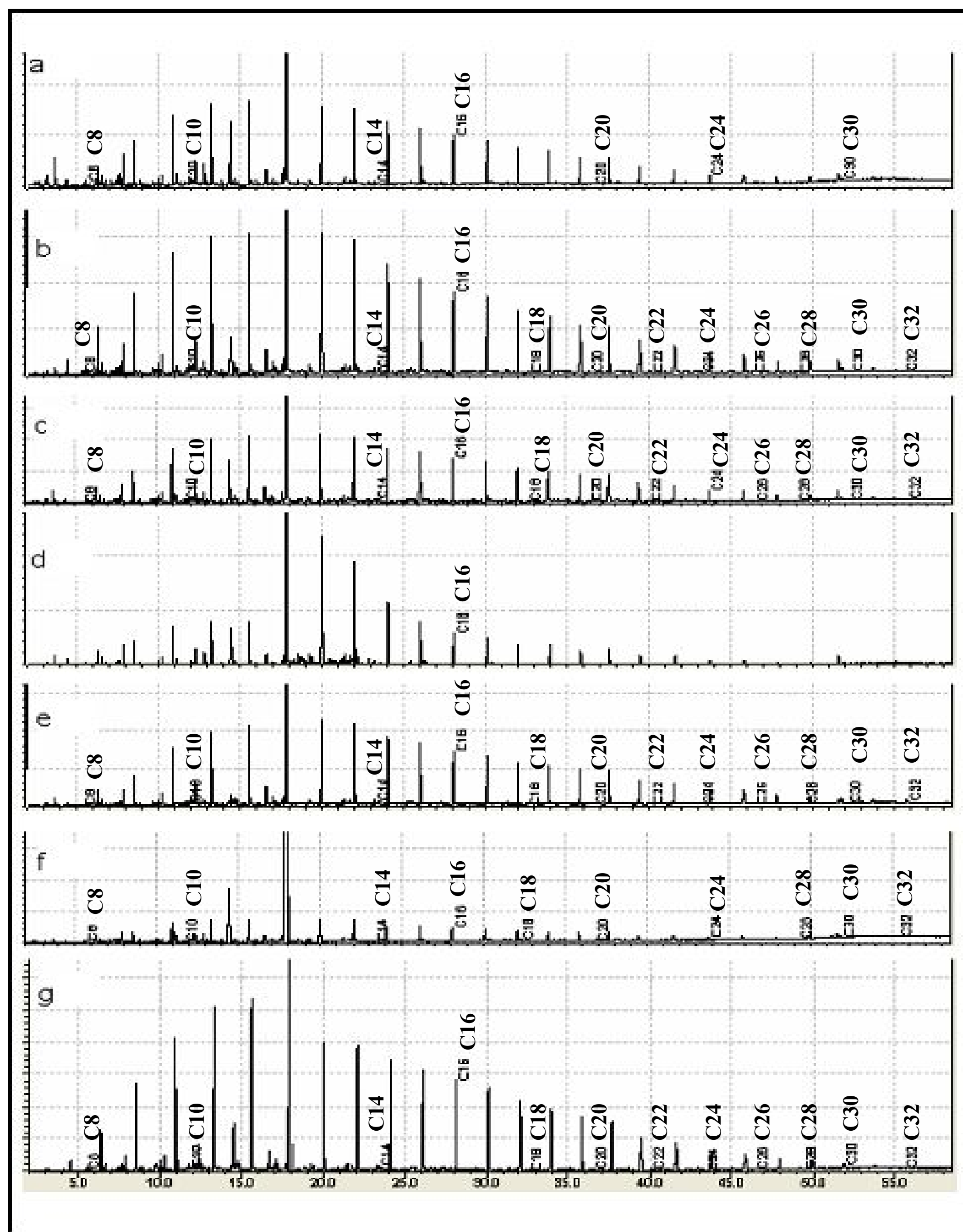

Figure 4.16: Gas chromatography profile for culture medium of *Acinetobacter calcoaceticus* in 1% crude oil with different metal ion substitution on day 14: (a) ferric chloride (b) lithium chloride (c) manganese chloride (d) potassium chloride (e) sodium chloride (f) magnesium chloride and (g) control flask

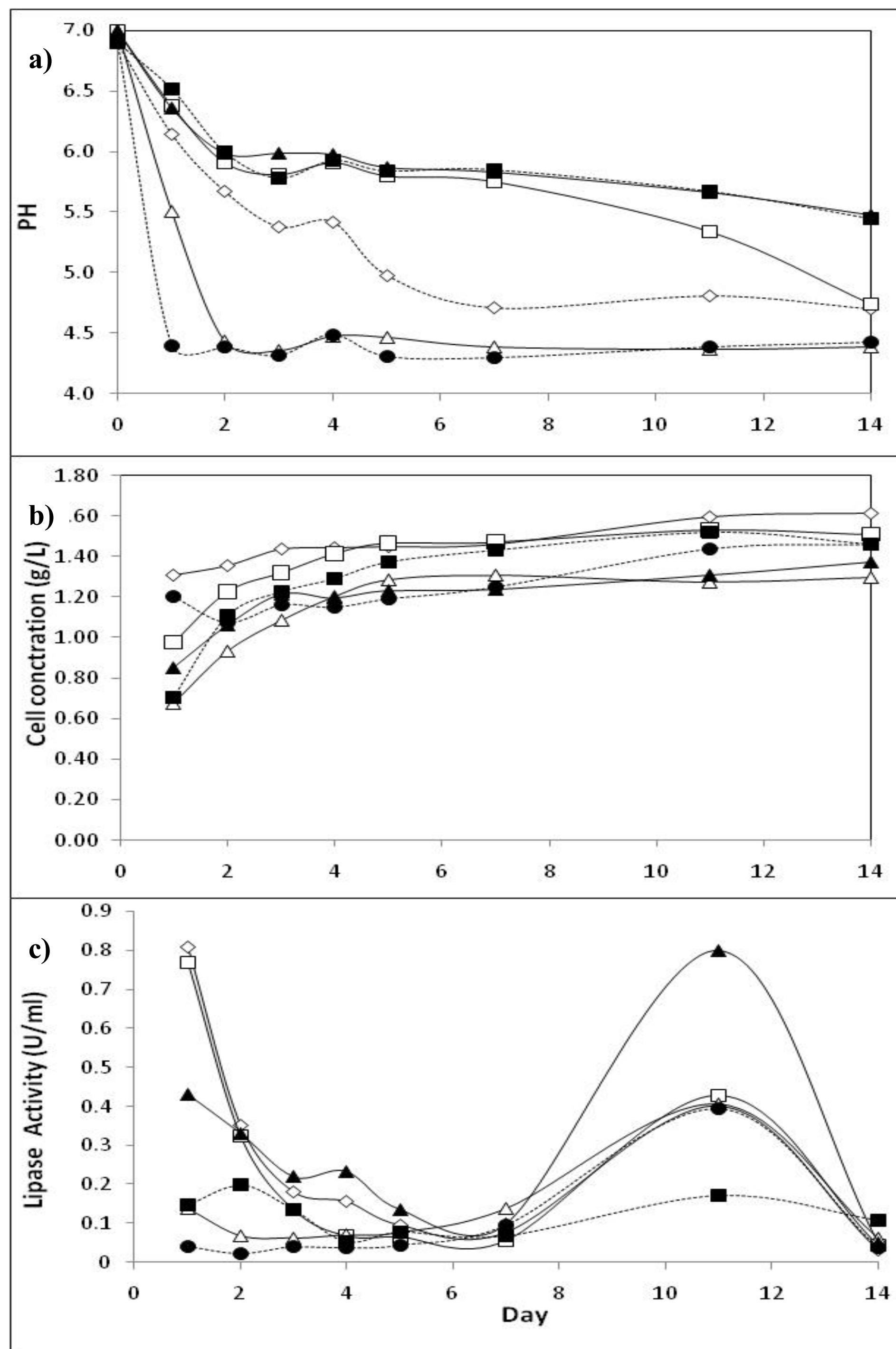

Figure 4.17: Time course of (a) pH, (b) growth, (c) lipase activity for *A. calcoaceticus* cultured in minimal salt broth supplemented with 1 % of crude oil and different metal ion substitution respectively
Symbols: (◇) magnesium, (□) sodium, (△) manganese, (▲) potassium, (■) lithium, (●) ferric

4.4.2.2 Metal Ion Substitution in MSM with 1% Used Cooking Oil

Metal ion substitution test was also carried out with used cooking oil. Magnesium as the metal ion source showed the fastest degradation of cooking oil as compared to other treatments (Figure 4.18). All the tested culture media showed a drastic pH decrease from the original 7.0 to approximately pH 3.6 to 4.2 on day 1 onward (Figure 4.19a). After that, the pH remained between 3.7 to 4.1 for all samples throughout the test period.

The highest growth rate was achieved by ferric chloride and manganese chloride metal ion substitution, 1.5 to 1.8 g/L (Figure 4.19b). Lipase activity could be detected after day 3 in all the tested samples and reached the maximum activity on day 11 (0.4 to 0.8 U/ml) (Figure 4.19c). Magnesium as metal ion source exhibited the maximum lipase activity (0.8 U/ml) as compared to other treatments.

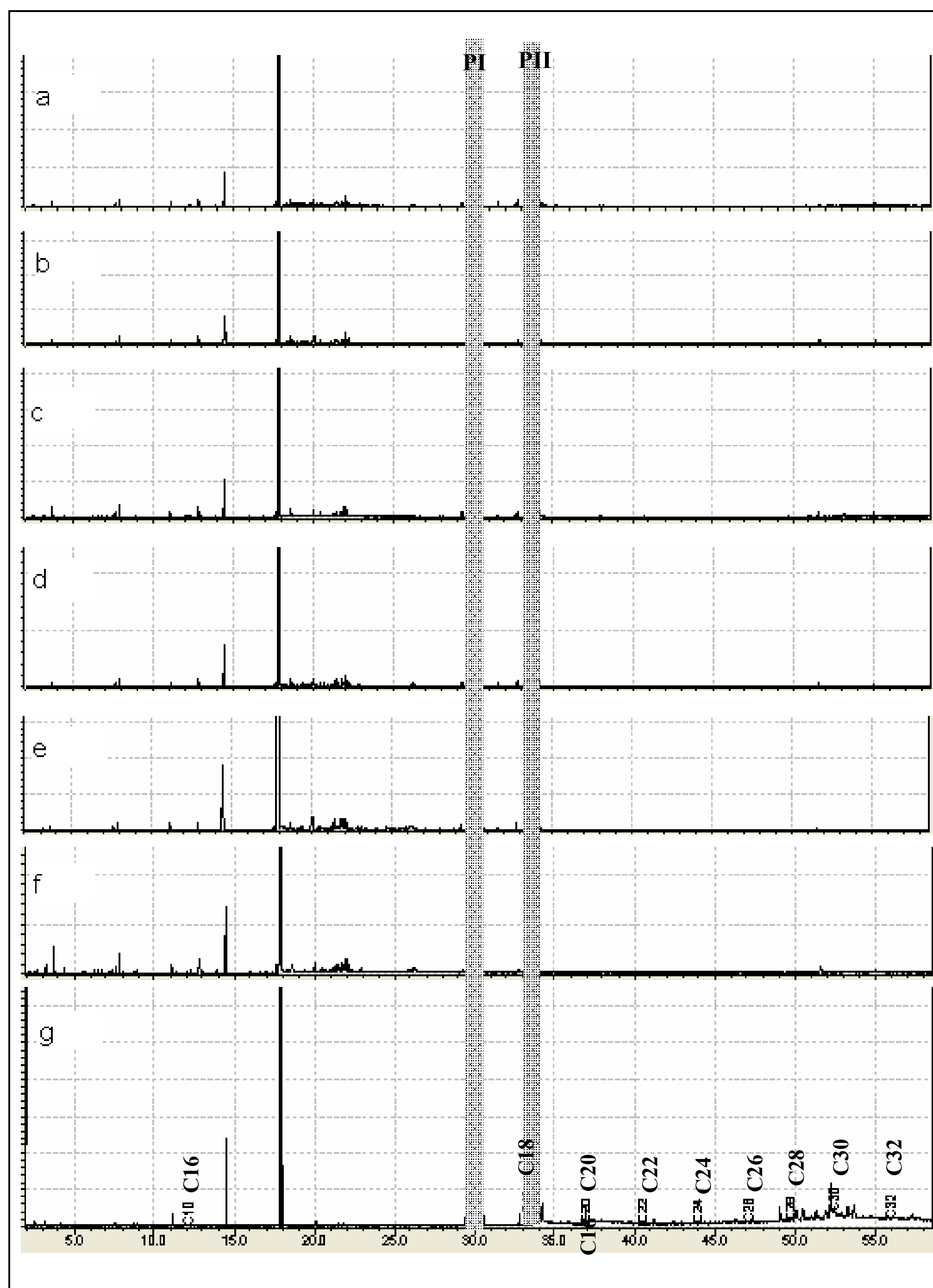

Figure 4.18: Gas chromatography profile for culture medium of *Acinetobacter calcoaceticus* in 1% used cooking oil with different metal ion substitution on day 14: (a) ferric chloride (b) lithium chloride (c) manganese chloride (d) potassium chloride (e) sodium chloride (f) magnesium chloride and (g) control

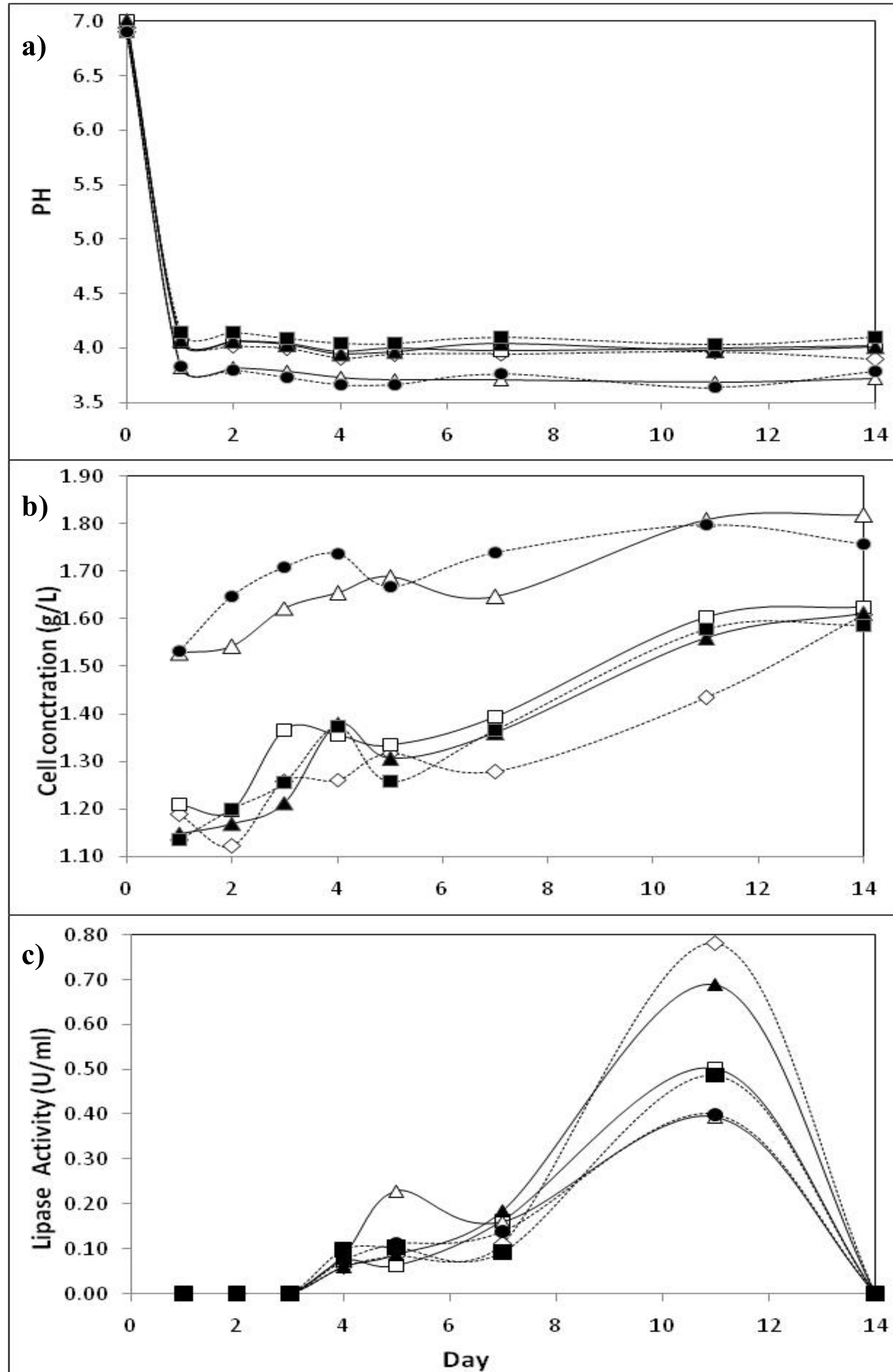

Figure 4.19: Time course of (a) pH; (b) growth; (c) lipase activity for *A. calcoaceticus* cultured in minimal salt broth supplemented with 1 % of used cooking oil and different metal ion substitution respectively Symbols: (◇) magnesium, (□) sodium, (△) manganese, (▲) potassium, (■) lithium, (●) ferric

4.5 Metabolite Analysis

Culture media of *A. baumannii* and *A. Calcoaceticus* in minimal salt broth with 1% crude oil for first three day were centrifuged and precipitated with 80% w/v ammonium sulphate, followed by overnight dialysis against distilled water at 4°C. The dialysed samples were then lyophilised for storage or analysed with SDS PAGE.

4.5.1 SDS-PAGE

Two protein bands of 19 kDa and 29 kDa were observed for *A. baumannii* metabolite (Figure 4.20). Protein band sizes for *A. calcoaceticus* were found to be at 9 kDa, 17 kDa, 18 kDa and 29 kDa (Figure 4.20).

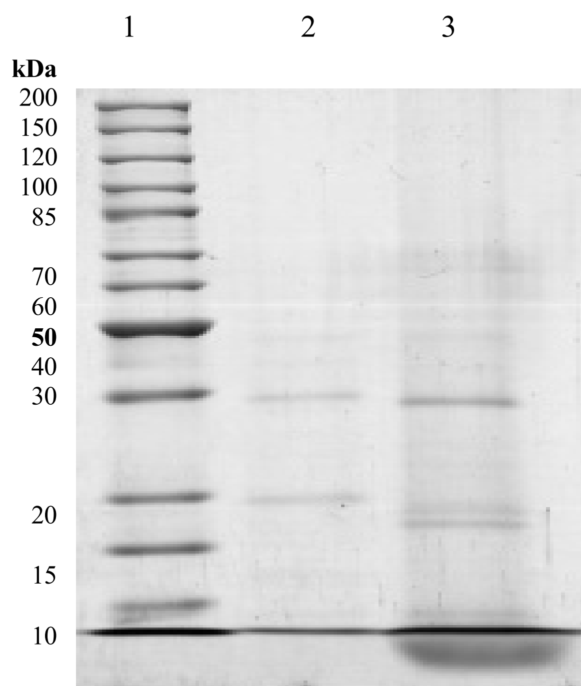

Figure 4.20: SDS PAGE for metabolite of *A. baumannii* and *A. calcoaceticus.* Lane 1- marker, Lane 2- *A. baumannii* sample, Lane 3- *A. calcoaceticus* sample

CHAPTER 5

DISCUSSION

5.1 Isolation and Screening of Hydrocarbon Degrading Bacteria

A total of 12 isolates were selected on the basis of their oil degrading ability. The isolates were adjudged as hydrocarbon-chain-degraders since they were able to grow on minimal salt medium in the absence of any other substrates except spent engine oil. According to Kirk et al. (2004 cited from Ito, Hosokawa, Morikawa & Okuyama, 2008), some bacterial strains cannot grow on plates as in the environment they are exposed to greater stresses in relation to the molecular oxygen or desiccation than in liquid media. Ito et al. (2008) concluded that isolation from lightly polluted soils with vegetation would have a much more abundant and diverse bacterial population than heavily polluted soils because the soils would be relatively rich in nutrients and would have lower toxic effects from the petroleum hydrocarbons.

The observations in the present study also suggested that the isolated bacterial strains were capable of utilising engine oil as the sole source of carbon and energy. The isolates were then grown in minimal salt broth with spent engine oil and floccule formation was observed. According to Whang, Liu, Ma & Cheng

(2008), oil degrading bacteria tend to form floccules accelerating the process of bioremediation. The floccules trap significant amounts of oils, facilitate the utilization of hydrocarbon chain by the bacteria, induce resistance to toxins and provide a way to physically separate the pollutants (Liu & Liu, 2010).

Formation of floccules and growth of bacterial species F1, F2, F4, F10 and F12 suggested that bacteria might have the specific mechanism to utilise spent engine oil and crude oil as sole source of carbon to sustain in the culture. The mechanism for the formation of floccules remained unclear, however floccules formation in other organisms could be triggered by the environmental factors such as physical and chemical stresses and substrate gradient (Whang et al., 2008; Liu & Liu, 2010). Besides, a decrease in the electrostatics repulsion among cells due to a pH drop in their environment was found to trigger floccule formation in *Acinetobacter*. Other than that, an increase in the hydrophobicity of the cell membrane as they utilise petroleum hydrocarbons (e.g. n-hexadecane or diesel oil) could trigger the formation of floccules as well (Liu & Liu, 2010).

Turbidity in the culture flasks was observed (Figure 4.3) and it was postulated that the bacteria were able to grow in the substrate supplemented media. Shoham, Rosenberg & Rosenberg (1983), however, commented that the ability of microorganisms to produce turbidity in culture medium might not necessarily be an indication of its hydrocarbon chain degrading capability. Shoham and colleagues also added that such capability is greatly determined by the ability of

organisms to produce the vital enzymes required to decompose the recalcitrant components of hydrocarbon chains, rather than being nutritionally fastidious. The ability of the bacterial cultures to degrade hydrocarbon chain was further assessed.

The control flask had a clearer medium and distinct layer between the oil and water interfaces and after 10 days of incubation, formation of clump was observed. This is probably due to the weathering of the oil in the incubator shaker which was constantly at 35°C without bacteria to emulsify the oil. Emulsification was observed only when the bacterial cells were present, indicating that it was related to cell hydrophobicity or cell-associated substances (Pizzul et al., 2006).

5.2 Hydrocarbon Utilisation Pattern of Bacterial Species

The ability of the indigenous isolates to hydrolyze hydrocarbon chains was tested. It was found that the degradation pattern of the isolates on the oil used demonstrated the different patterns. The results were clearly shown in the GCMS profile which suggested that the interaction between microorganisms and different oils depend on multiple variables within the interface of a multicomponent system consisting of organic aqueous and inorganic components (Hao, Lu & Zeng, 2004). This is in agreement with Rosenberg et al. (1992) that the essential characteristics that define hydrocarbon chain utilizing microorganisms are membrane-bound group specific oxygenases and mechanisms for optimizing contact between the

microorganisms and the water-insoluble hydrocarbon. On the other hand, Vasconcellos et al. (2009) revealed that bacterial metabolism of hydrocarbon chains occurred through reactions based on oxidation, carbon-carbon bond cleavage and generation of new bonds or by the physical incorporation of these compounds into microbial cell walls.

5.2.1 Degradation Pattern of Spent Engine Oil

In reference to GCMS profile (Figure 4.5), no obvious degradation of spent engine oil was observed. Engine oil was partially solidified in minimal salt broth at 35°C, and solidified engine oil was attached to the wall of flask during incubation. In the control flask, there was phase separation, a clear lower aqueous phase and upper black spent engine oil phase as soon as the culture agitation had stopped. This suggested absence of emulsification during the degradation process.

All three *Acinetobacter* species did not show degradation of spent engine oil, possibly due to the long chain hydrocarbons and c-alkanes in the engine oil. According to Koma et al. (2003), the long chain hydrocarbons and c-alkanes are known to be recalcitrants to microbial degradation, indicating that spills of car engine oil remain for a long time in the environment. Other than that, there are about 5-20% chemical additives in the engine oil selected for specific functions (Jirasripongpun, 2002).

Two *Acinetobacter* species, *A. calcoaceticus* and *A. baumannii* produced some metabolites, which were detected by GCMS analysis at 51.96 min in the case of spent engine oil supplemented flasks. One of the metabolites was found to be Bis [3-(3, 5-di-tert-butyl-4-hydroxyphenyl)propyl] maleate which accounted for 63% similarity with the one available at the MS library. Decanedioic acid produced by *A. calcoaceticus* at 39.81 min was believed to be the by product of substrate breakdown.

On the other hand, 1, 2-benzenedicarboxylic acid bis [(4-hydroxylmethyl cyclohexyl) methyl] ester was detected in the control and sample flasks indicating that the compound was originally present in the engine oil. This is in contrary with Hao et al. (2004) findings. They isolated thermophilic bacteria (strain TH-2) from the reservoir of the Shengli oil field in East China and found the production of metabolites 1,2-benzenedicarboxylic acid-bis ester ($C_{16}H_{22}O_4$), dibutyl phthalate ($C_{16}H_{22}O_4$), and di-n-octyl phthalate ($C_{24}H_{38}O_4$) by the strain while growing in crude oil. They believed that these metabolic products had various effects on crude oil. The ester is a better organic solvent and it possesses low molecular-weight surfactant having lower surface tension. It was by far the most abundant metabolite accounting for about 92% of the total products in their findings.

5.2.2 Degradation Pattern of Crude Oil

Degradation pattern caused by three *Acinetobacter* species was found to be more distinct in the crude oil supplemented medium. The phenomenon of bacterial clumping around the oil droplet as viewed under the phase contrast microscope might due to the substrate uptake mechanism by the bacteria. It has been proposed that the uptake of hydrocarbons as microdroplets is the most common mechanism employed by the unicellular microorganisms (Chen, Cheng & Chen, 1998). Very often the oil degrading microorganisms produce biosurfactants in order to emulsify the free phase hydrocarbons and thereby enhance substrate availability.

The substrate uptake mechanism in the oil degrading bacteria is known to occur by direct contact between the hydrocarbon and the cell surface through hydrophobic interaction. The hydrophobicity of cell membrane is related to the structure and composition of the cell surface. According to Dorobantu, Yeung, Goght & Gray (2004) emulsification might be associated with the surface properties of the cell, as a result of attachment to the oil-water interface by the general hydrophobic interaction rather than specific recognition of the substrate. Therefore the bacterial cells might behave as the fine solid particles at the oil-water interface.

Mishra et al. (2001) concluded that the growth of oil degrading bacteria start with the adherence of bacteria to the oil droplets followed by the

emusification of the substrate in the aqueous medium by the production of an extracellular heteropolysaccharide bioemusifier. On the other hand, Pines & Gutnick (1984) stated that there could be hydrophobic thin fimbriae of approximately 35 Å diameter on the cell surface of *Acinetobacter* species that leads to attachment of oil to the cell surface.

Growth and metabolic activity in the culture medium was also indicated by the decreased pH. The reduction in pH in most of the culture media might be due to the production of metabolites or excretion during the growth of the bacteria cultured. This was in agreement with the finding of Jacques et al. (2007) which stated that the change in pH along with growth of bacteria revealed significant reduction in pH due to acidification. Decrease in pH is probably due to the accumulation of H^+ or other acidic metabolites in the minimal salt medium. This is an indication of the production of acidic metabolites by the bacteria in the cultured medium (Jacques et al., 2007). This was also supported by Sayavedra-Soto et al. (2006). They stated that biodegradation is always accompanied with a significant drop in pH, suggesting an accumulation of acid in the medium.

A. radioresistens did not grow well in the medium as compared to *A. calcoaceticus* and *A. baumannii*. According to Ron & Rosenberg (2002), *A. radioresistens* is unable to use hydrocarbon as the carbon source. Since crude oil is a complex mixture of hydrocarbon chains and other compounds, the wide range of degraded substrates produced by the bacterial species could be a significant

advantage in bioremediation of oil contaminated soils (Zhang et al., 2010). It was concluded that although from the same genus, *Acinetobacter* species varied widely in their ability to degrade crude oil as the sole source of carbon and energy.

5.2.3 Degradation Pattern on Used Cooking Oil

The degraded products of the cooking oil in the culture medium were extracted with hexane for GCMS analysis. Two peaks (PI and PII) were detected in the control sample, whereas, no peaks were detected in the culture medium of *A. calcoaceticus*. It was postulated that *A. calcoaceticus* could degrade both peaks revealed in the control in 17 days.

Cooking oil is glycerol ester of fatty acids. The structure of fatty acid molecule is characterized by the length of the carbon chain and the number of double bonds. The exact position of double bonds will determine the biological reactivity of the fatty acid molecule as well as the lipid containing the fatty acid (Al-Darbi, Saeed & Islam, 2005).

5.3 Culture Medium Modification

The culture medium was optimised with the various nitrogen sources and metal ions to identify the effect of the optimised culture medium on the hydrocarbon degradation by the selected bacterial species. Nutrient deficiency could be a limiting factor in the biodegradation process. According to Braddock, Ruth, Catterall, Walworth & McCarthy (1995), the extent of hydrocarbons biodegradation is mainly conditioned by environmental factors such as temperature and nutrients availability. Nutrient availability is a limiting factor for biodegradation due to high carbon content in hydrocarbons compared to nitrogen or phosphorus chemical species that are essential for microbial growth (Head et al., 2006). Fertilization proved to enhance microbial degradation in different experiments.

5.3.1 Nitrogen Source

The effect of various nitrogen sources on crude oil and cooking oil degradation by *A. calcoaceticus* are shown in section 4.4.1. Organic nitrogen sources are complex, and may play an important role in inducing the oil degradability of *Acinetobacter*. According to Al-Mailem, Sorkhoh, Salamah, Eliyas & Radwan (2010), nitrogen sources have long been recognized as limiting in the bioremediation of oily environments. Nitrogen served as precursor for

vitamins, amino acids and growth factor for cell (Saranya & Shenbagarathai, 2010). Microbes require nitrogen to support the biosynthesis of nitrogenous metabolites, both primary and secondary (Rhodes & Stanbury, 2001). The choice of nitrogen source can have a major effect on the pH drift occurring during microbial growth.

In this experiment, ammonium nitrate was substituted with six organic nitrogen sources. pH fluctuation was observed in the culture medium containing crude oil and substituted organic nitrogen sources (peptone form meat, soypeptone, polypeptone, tryptone, beef extract and yeast extract). According to Rhodes & Stanbury (2001) ammonium salts, such as ammonium sulfate will usually produce acidic conditions as the ammonium ion is utilized and free acid liberated. In contrast, nitrates will cause alkaline drift as they are metabolized. Ammonium nitrate will first cause an acid drift as the ammonium ion is utilized and nitrate assimilation is repressed (Rhodes & Stanbury, 2001). Alkaline drift will only happen when ammonium ion has been exhausted as the nitrate is used as the alternative nitrogen source.

Among six organic nitrogen sources tested, polypeptone was found to be the best that could enhance hydrocarbon degradation (Figure 4.14). Polypeptone is a mixture of peptones, made up of equal parts of pancreatic digest of casein and peptic digest of animal tissue. This might be the reason for polypeptone being a better organic nitrogen source in hydrocarbon degradation as compared to soy

peptone and tryptone, which are made up of one peptone only. Tryptone is a pancreatic digest of casein containing all amino acids found in casein as well as in larger peptide fractions. Another nitrogen source, yeast extract, demonstrated a promising growth of the culture but could not enhance the degradability of the used cooking oil in the medium (Figure 4.15).

According to Tano-Debrah, Fukuyama, Otonari, Taniguchi & Ogura (1999) the degradability of oil was better in medium containing peptone as compared to either ammonium sulphate or potassium nitrate. This finding is in agreement with the results of the present study. Yeast extract on the other hand could act as a growth factor in the present study as the result showed enhancement of cell growth instead of hydrocarbon degradability. Yagafarova et al. (2002) suggested that biodegradation of oil and oil products could be intensified by adding various supplements. Therefore, yeast extract might be added to the culture medium together with polypeptone for the enhancement of the bacterial cell growth and increased biodegradability at the same time.

5.3.2 Metal Ions

Metal ions are trace elements required by living cells in small quantity, often present as contaminants in impure water or media compositions. According to Todar (2009), it is not necessary to add metal ion to culture medium as nutrients.

As metal ions, the trace elements usually act as cofactors for essential enzymatic reactions in the cell. One organism's trace element may be another's required element and *vice-versa*, but the usual cations that qualify as trace elements in the bacterial nutrition are Mn^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+} and Mo^{2+} (Todar, 2009). In the present study, bacteria degradation of crude oil and cooking oil was best observed in culture medium supplemented with Mg^{2+}. Culture flasks with Cd^{2+}, Ni^{2+}, Co^{2+} and Cu^{2+}, on the other hand, did not show any sign of growth. The observation indicated that these elements might intoxicate the bacterial species and retard the growth.

In general, potassium, magnesium and calcium are inorganic cellular cations and cofactors for certain enzymatic reactions. Promising effect was obtained with the addition of Mg^{2+} in the medium, which suggested the improvement of these cations in promoting the production of hydrocarbon degrading metabolites. However, the actual mechanism involved is yet to be studied.

5.4 Lipase Activity

Lipase activity in the culture medium was monitored in this study to reveal whether the hydrocarbon degradation is due to the aid of lipase activity. It is known that lipase activity is a valuable tool to monitor the biodegradation of

petroleum hydrocarbons, such as diesel, in freshly contaminated soil (Margesin, Hammerle & Tscherko, 2007). Lipases (triacylglycerol acylhydrolases) constitute a diverse and ubiquitous family of enzymes that, in biological systems, initiate the catabolism of fats and oils by hydrolyzing the fatty acyl ester bonds of acylglycerols. The enzyme also catalyzes various transformations (Abramic et al., 1999).

Lipase activity was found to be the highest on day 1 and gradually decreased on day 3 in the culture medium containing crude oil and modified with various nitrogen sources. The decrease in lipase activity might be due to the production of proteases in the culture medium. According to Rathi, Goswami, Sahai & Gupta (2002) the decrease in lipase activity might be due to the nutritional limitation, accumulation of unsaturated fatty acids and formation of proteases. On the other hand, the culture medium containing cooking oil and supplemented with various nitrogen sources did not show any lipase activity in the first 4 days. Lipase activity increased on day 5 onwards and peaked at day 10. It was postulated that *A. calcoaceticus* lipase activity might be better in acidic pH as the pH in the culture medium dropped on day 4. However, Chen et al. (1998) revealed that *A. radioresistens* showed poor growth and weak lipase production if the cells were cultivated in the absence of oils or at acidic pH. Reason for the contradictory observation might be due to the source of the bacterial isolates that possess diverse behaviour in the culture environment.

Overall, the similar lipase activity pattern was observed in culture medium containing crude oil supplemented with different nitrogen sources and metal ions. Culture medium containing cooking oil with different nitrogen sources and metal ions demonstrated the same observation as well. Now, it could be postulated that lipase activity was strongly affected by the carbon substrate rather than the component of metal ion or nitrogen source in the culture medium. Further experiments need to be carried out to verify these findings.

5.5 Metabolite Analysis

According to Satpute, Banat, Dhakephalkar, Banpurkar & Chopade (2010), different *Acinetobacter* sp. produce protein polysaccharide complexes that possess surface active properties. This was supported by the work of Yateem et al. (2002). They stated that the ability to secrete biosurfactant production is a common feature among the hydrocarbon-degrading bacteria. Besides biosurfactant, protein based metabolites might be involved in the aid of hydrocarbon chain degradation (Satpute et al., 2010). In this experiment, protein produced by *A. calcoaceticus* was isolated and subjected to SDS-PAGE analysis. The size of protein bands revealed in the gel was determined to be 9, 17, 18 and 29 kDa, whereas in the case of *A.baumannii*, 19 and 29 kDa.

Baldi et al. (1999) commented that cell adhesion to hydrocarbons seemed to proceed mainly via protein in *Acinetobacter* sp strain MJT/F5/199A. The adhension occurs via an acidic protein of 65 kDa, probably a glycoprotein. In *Acinetobacter calcoaceticus* RAG-1, the same occurs via fimbriae and in *Acinetobacter* sp strain A3 via two proteins of 26.5 kDa and 56 kDa (Baldi et al., 1999). The size of the protein bands in the metabolite of the isolated bacterial species in this experiment did not match the available data. It was postulated that the different *Acinetobacter* species might produce different sized metabolite proteins to aid hydrocarbon chain degradation.

CHAPTER 6

CONCLUSION AND FUTURE PERSPECTIVES

6.1 Conclusion

In this investigation, five bacteria were identified to be *Acinetobacter radioresistens*, *Acinetobacter baumannii*, *Acinetobacter calcoaceticus*, *Stenotrophomonas maltophilia* and *Entrerobacter sakazakii*, respectively. Among the three *Acinetobacter* spesies, *A. calcoaceticus* was found to be the best degrader of crude oil and used cooking oil as compared to *A. radioresistents* and *A. baumannii*. It was concluded that although from the same genus, *Acinetobacter* species varied widely in their ability to degrade hydrocarbon chains as the sole source of carbon and energy. Besides, they also showed different ability in clumping oil droplets.

Organic nitrogen source, polypeptone, was found to be a better nitrogen substitution in enhancing crude oil degradation. However, prior to use of organic nutrients as the bioremediation agents further research needs to be conducted to understand their potential environmental impacts.

Out of the six tested metal ions, magnesium was found to enhance oil degradability. Further investigation needs to be carried out to identify and characterise the metabolites produced and possible benefit from such metabolites to human beings and the environment.

6.2 Future Perspectives

Molecular method of detection of hydrocarbon chain degrader is recommended for the improvement of the present research output. Identification of the alkane hydroxylase gene responsible for the hydrocarbon chain degradation can be carried out. PCR-denaturing gradient gel electrophoresis (PCR-DGGE) can be performed to specify the bacteria involved in the hydrocarbon chain degradation.

Besides, consortia of bacterial species could be tried to enhance the rate of hydrocarbon chain degradation as the different bacteria are known to act differentially in the degradation of oil.

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APPENDICES

Appendix A

Minimal Salt Medium (MSM) preparation

Minimal salt medium was prepared by mixing 1 g potassium dihydrophosphate, 1 g dipotassium hydrophosphate, 1 g ammonium nitrate, 0.2 g magnesium sulfate, 0.05 g ferum chloride, 0.02 g calcium chloride and 15 g agar in 1.0 liter of distilled water.

Appendix B

Gram stain procedure

1) Prepared a thin smear of the bacterial population in a drop of water on a clean slide.
2) Heated to fix the smear and then stained with crystal violet solution for 1 min.
3) Washed the slide with distilled water and added iodine for 1 min.
4) Washed the stained slide again with water then blotted dry.
5) Decolourised for 30 sec with 95% ethanol.
6) Washed the slide with tap water and then counter stained with safranin for 10 sec.
7) Washed the slide with tap water, blotted dry and examined under the microscope.
8) Gram positive bacteria appeared as purple colour while Gram negative bacteria stained red colour.

Appendix C

KOH Method (rapid nonstaining method)

1) A drop of 3% aqueous KOH was placed on a glass slide then using a sterile loop, a visible amount of bacterial growth from an agar culture was transferred to the drop of KOH.
2) The cells were mixed thoroughly on the slide by constantly stirring over an area of about 1.5cm in diameter with an inoculation loop.
3) The loop was raised about 1cm from the slide to determine the viscosity.
4) The isolate is Gram negative if the bacterium-KOH suspension becomes markedly viscid or gels within 5 to 60 seconds or an obvious stringiness is present.
5) The isolate is Gram positive if no gelling is observed.

Appendix D

Biolog Identification Method

Biolog has introduced the GN Microplate panel containing 95 tests for the identification of a very wide range of Gram-negative species and the complementary GP MicroPlate for Gram-positive bacteria. The 95 tests are carbon source utilization tests. Utilization reaction is indicated when a purple colour forms in a well. This resulted "breathprint" pattern that can be read either by eye or with an automated microplate (ELISA) reader. Theoretically there are 2^{95} possible "breathprint" patterns that will result from the 95 tests that can either be "positive" or "negative". Microlog 3 computer software was used to perform automated readings.

The GN MicroPlate uses the Gram-negative Data Base, which includes all types of Gram-negative species: enterics, non-fermenters, and fastidious microorganisms whereas Gram-positive Data Base includes cocci, rods, and spore-forming rods. Prior inoculation of the GN and GP MicroPlate, the bacteria were suspended in 0.85% saline. Gram-negative bacteria are inoculated into the GN MicroPlate while Gram positive bacteria are inoculated into GP MicroPlate.

Biolog Test Procedures

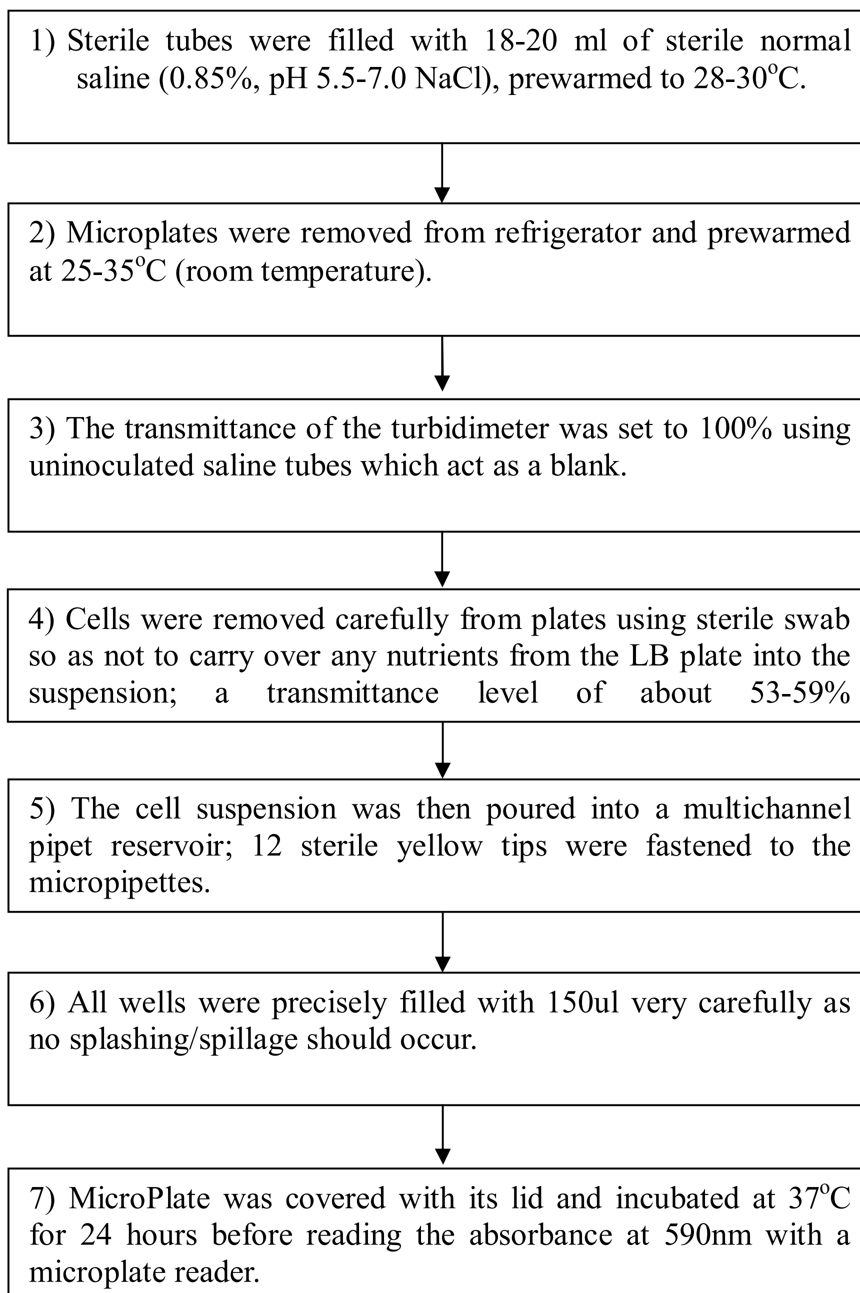
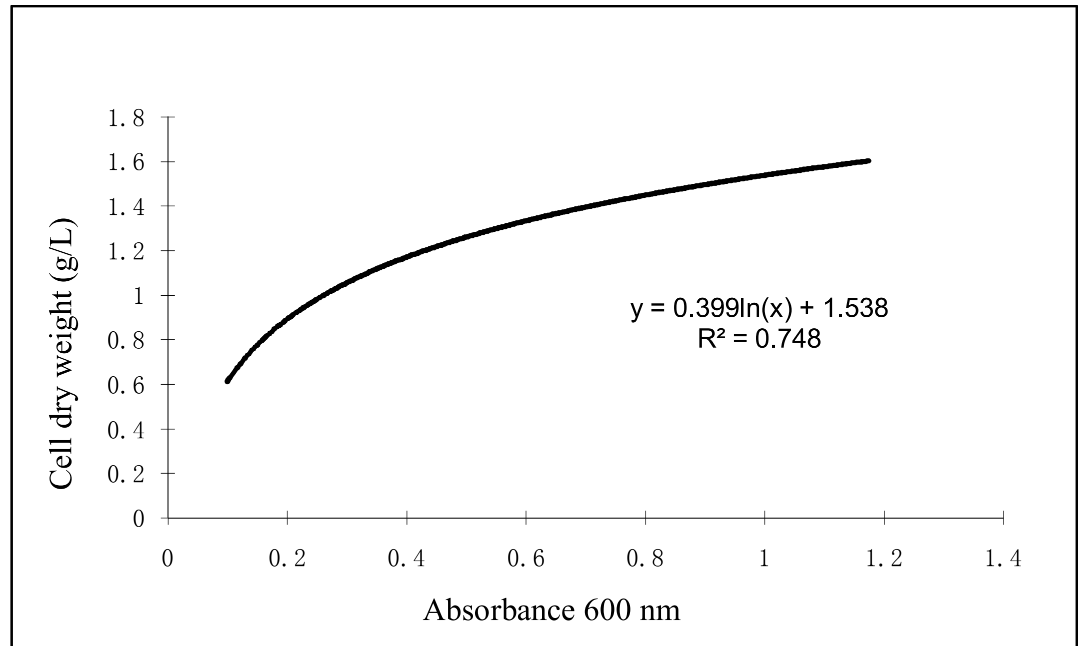


Figure: Flow chart of the inoculum preparation and inoculation of the microplate

Appendix E

Standard Curve For Cell Growth



Appendix F

Total nitrogen content calculation

NH_4NO_3 1g : $\frac{28}{80}$ = 0.35

1g/L X 0.35 X 71.43=25mM

cat 1608.00 soy peptone

TN: **12.54**%

Amount needed in g/L x **0.1254** x71.43 = 25Mm

cat 1700.00 beef extract

TN: 12.48%

cat :1612.00 tryptone

TN: 13.31%

cat: 1702.00 yeast extract

TN: 10.70%

P7750 sigma peptone from meat

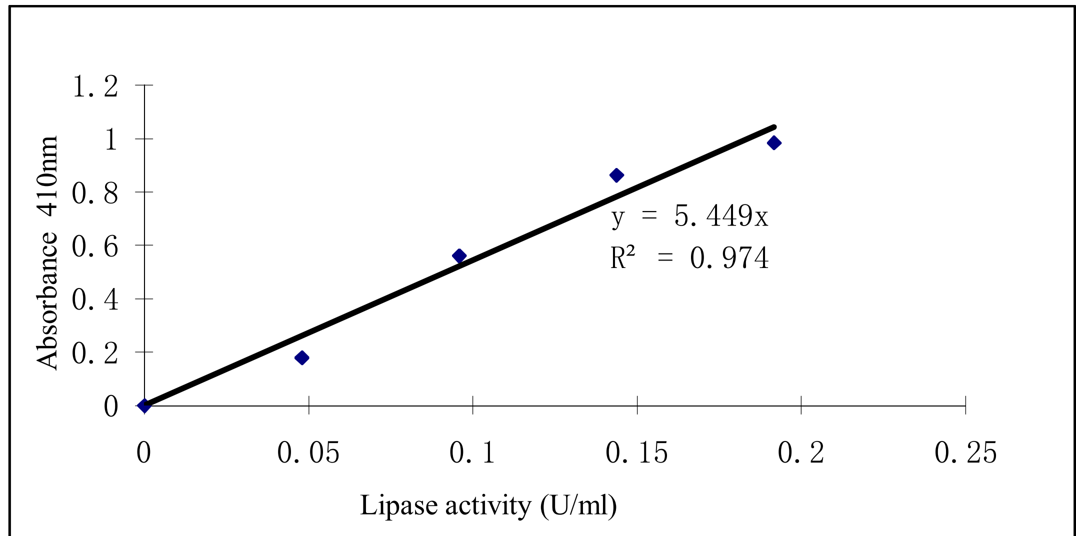
14.2-17.4% =15.8%

polypeptone (BBL Becton Dickison)

13.1% N

Appendix G

Standard Curve for Lipase Activity



Appendix H

Coomassie blue staining procedure

Preparation of coomassie blue staining solution

Methanol 60.0ml

Acetic Acid 15.0ml

Coomasssie blue R-250 0.15g

Add distilled water to make up to 150ml

Preparation of Destaining solution

Methanol	200.0ml
Acetic acid	50.0ml

Add distilled water to make up to 500ml

Staining procedure

Removed polyacrylamide gel from cascade and placed in Coomassie blue staining solution for 15 min with gentle shaking/agitation.

Replaced with destaining solution after 15 min of staining procedure; Repeated the destaining procedure with fresh destaining solution for 3 to 4 times until the clear gel is obtained.

Appendix I

Table: Cell concentration (g/L) for 3 *Acinetobacter* species in 3 different oils

Strain	*A. radioresistens*			*A.calcoaceticus*			*A. baumanii*		
Day	Spent engine oil	Crude Oil	Cooking Oil	Spent engine oil	Crude Oil	Cooking Oil	Spent engine oil	Crude Oil	Cooking Oil
1	0.85±0.02	0.41±0.01	1.01±0.01	0.91±0.03	1.66±0.05	1.03±0.03	0.87±0.01	1.34±0.02	1.15±0.02
2	0.88±0.01	0.47±0.01	1.12±0.02	0.92±0.02	1.72±0.02	1.17±0.02	0.90±0.01	1.43±0.03	1.35±0.07
3	0.88±0.01	0.64±0.02	1.52±0.01	0.94±0.02	1.76±0.01	1.47±0	0.91±0.02	1.50±0.01	1.44±0.02
4	0.90±0.02	0.82±0.04	1.58±0.02	0.94±0.01	1.90±0.01	1.69±0.06	0.92±0.02	1.58±0.05	1.48±0.01
7	0.88±0.01	0.77±0.01	1.61±0.01	0.94±0.02	2.01±0.01	1.72±0.05	0.92±0.03	1.61±0.01	1.62±0.02
12	0.89±0.01	1.06±0	1.62±0.01	0.94±0.01	2.00±0.01	1.72±0.02	0.92±0.01	1.59±0.01	1.63±0.01
17	0.90±0.03	1.06±0.1	1.64±0.02	0.94±0.01	2.01±0.02	1.73±0.04	0.93±0.01	1.59±0.01	1.66±0.01

Table: pH of culture medium for 3 *Acinetobacter* species in 3 different oils together with control and medium

	Spent engine oil					Crude oil					Used cooking oil				
Day	A. r	A. c	A. b	control	Medium	A. r	A. c	A. b	control	medium	A. r	A. c	A. b	control	medium
0	6.9±0.14	6.90±0.08	6.90±0.05	6.90±0.01	6.90±0.02	6.98±0	7.00±0.02	6.90±0.02	6.97±0.03	6.90±0.03	6.85±0.01	6.86±0	6.85±0.01	6.86±0.01	6.83±0
1	6.98±0.26	6.79±0.01	6.78±0.03	6.93±0.05	6.85±0.03	6.94±0.03	6.50±0.01	4.81±0.01	6.91±0.01	6.83±0.03	4.30±0.01	4.64±0.05	4.50±0.08	6.84±0.01	6.83±0.02
2	6.92±0.05	6.65±0.02	6.61±0.06	7.03±0.01	6.90±0.04	6.85±0.04	6.38±0.09	4.48±0.17	6.96±0.05	6.91±0.02	4.30±0.15	6.13±0.03	4.67±0.15	6.85±0.01	6.85±0.01
3	6.89±0.06	6.70±0.20	6.58±0.10	6.96±0.01	6.92±0.02	6.89±0.11	6.39±0.10	4.52±0.08	6.95±0.01	6.95±0.01	4.90±0.30	6.29±0	5.08±0.12	6.86±0.02	6.82±0.01
4	6.82±0.07	6.65±0.13	6.55±0.11	6.95±0.01	6.91±0.15	6.87±0.05	6.35±0.03	4.53±0.01	6.95±0.01	6.92±0.02	4.73±0.05	6.33±0	5.15±0.11	6.83±0	6.80±0.01
5	6.81±0.05	6.62±0.02	6.53±0.08	6.98±0.01	6.91±0.01	6.96±0.03	6.37±0.05	4.52±0.03	6.96±0.05	6.96±0.02	4.98±0.01	6.27±0.18	4.78±0.16	6.84±0.01	6.84±0.01
8	6.79±0.05	6.57±0.04	6.43±0.02	6.97±0.01	6.91±0.03	6.94±0.09	6.35±0.01	4.64±0.01	6.95±0.02	6.92±0.03	4.92±0.01	6.33±0.10	4.77±0.14	6.88±0.03	6.84±0.02
12	6.74±0.19	6.50±0.01	6.37±0.02	6.93±0.01	6.87±0.05	6.46±0.01	6.61±0.02	4.72±0.12	6.92±0.01	6.93±0.01	5.65±0.11	6.37±0.04	4.85±0.50	6.92±0.02	6.90±0
17	6.71±0.11	6.46±0.05	6.34±0.02	6.88±0.03	6.85±0.04	6.70±0	6.29±0.06	4.57±0.31	6.89±0.01	6.85±0.01	5.88±0.15	6.19±0.04	4.90±0.22	6.82±0.02	6.81±0

A. r – *A. radioresistens* A.c – *A. calcoaceticus* A. b – *A. baumanii*

Table: pH, cell concentration and lipase activity for *A. calcoaceticus* in 1% crude oil with different nitrogen source substitution according to day

	Day	0	1	2	3	4	5	7	11	14
pH	Peptone	6.90±0.01	6.61±0.02	6.50±0.02	6.58±0.01	6.58±0.04	6.55±0.01	6.59±0.01	6.59±0.01	6.56±0.02
	soypeptone	7.00±0.01	6.69±0.04	6.68±0.01	6.72±0.02	6.73±0.05	6.67±0.02	6.71±0.03	6.69±0.02	6.70±0.07
	polypeptone	7.00±0.01	6.77±0.25	6.60±0.01	6.66±0.06	6.68±0.02	6.65±0.05	6.70±0.03	6.67±0.02	6.67±0.01
	tryptone	6.90±0.01	6.68±0.01	6.49±0.02	6.59±0.03	6.65±0.11	6.65±0.04	6.66±0.07	6.65±0.05	6.63±0.06
	Beef extract	7.00±0	6.94±0.04	6.67±0.12	6.82±0.09	6.78±0.01	6.89±0.02	6.82±0.08	6.82±00.01	6.83±0.03
	Yeast extract	6.90±0.01	6.89±0.13	6.75±0	6.87±0.02	6.83±0.01	6.89±0.01	6.84±0.03	6.81±0.02	6.82±0.01
Cell Concentration (g/L)	Peptone	-	1.70±0.03	1.72±0.01	1.71±0.02	1.73±0.02	1.72±0.06	1.74±0.01	1.78±0.02	1.82±0.02
	soypeptone	-	1.88±0.03	1.87±0	1.89±0.03	1.91±0.01	1.91±0.01	1.91±0.01	1.92±0.01	1.96±0.02
	polypeptone	-	1.83±0.02	1.83±0.02	1.90±0.02	1.91±0.01	1.90±0.01	1.90±0	1.92±0.01	1.95±0.01
	tryptone	-	1.78±0.01	1.89±0.02	1.93±0.01	1.87±0.02	1.86±0.01	1.87±0.1	1.91±0.04	1.93±0.01
	Beef extract	-	1.68±0.02	1.89±0.01	1.88±0.03	1.88±0.02	1.88±0.01	1.93±0.01	1.97±0	1.99±0.01
	Yeast extract	-	2.01±0.05	2.02±0.03	2.00±0.01	2.02±0.01	2.01±0.02	2.00±0.02	2.02±0.02	2.02±0.02

	Day	0	1	2	3	4	5	7	11	14
Lipase Activity (U/ml)	Peptone	-	0.62±0.01	0.58±0.01	0.34±0.01	0.39±0.01	0.21±0	0.13±0.01	0.66±0.01	0.41±0.01
	soypeptone	-	1.24±0.01	0.95±0.01	0.17±0	0.32±0.02	0.14±0.01	0.15±0.03	0.51±0.01	0.33±0.02
	polypeptone	-	9.16±0.01	5.09±0.01	0.60±0.01	0.30±0.01	0.14±0.01	0.11±0.01	0.56±0.01	0.30±0
	tryptone	-	16.77±0.02	5.96±0	0.09±0.01	0.27±0.01	0.06±0.01	0.10±0.01	0.51±0.02	0.21±0.01
	Beef extract	-	10.99±0.01	4.57±0.02	0.65±0.02	0.96±0.02	0.81±0.01	0.50±0.01	10.05±0.01	2.99±0.01
	Yeast extract	-	2.55±0.01	0.14±0.02	0.16±0.01	0.35±0.01	0.24±0.03	0.18±0.01	0.80±0.01	0.27±0

Table: pH, cell concentration and lipase activity for *A. calcoaceticus* in 1% used cooking oil with different nitrogen source substitution according to day

	Day	0	1	2	3	4	7	10	14
pH	Peptone	7.00±0.10	4.73±0.32	4.10±0	4.05±0.05	4.09±0.03	4.16±0.03	4.17±0.	4.28±0.04
	soypeptone	6.90±0.10	6.06±0.18	5.98±0.6	4.46±0.06	6.64±0.80	7.08±0.40	7.18±0.07	7.19±0.04
	polypeptone	6.90±0.05	5.56±0.35	4.43±0.07	4.66±0.13	4.56±0	4.67±0.01	4.76±0.12	4.78±0.06
	tryptone	7.00±0.08	5.82±0.08	4.39±0.03	4.32±0.05	4.47±0.04	4.62±0.04	4.63±0.04	4.63±0.01
	Beef extract	6.90±0.03	6.32±0.09	6.17±0.03	4.62±0.17	6.37±0.02	6.79±0.04	6.98±0.01	7.04±0
	Yeast extract	6.90±0.04	6.68±0.03	6.16±0.17	5.24±0.01	6.41±0.01	6.47±0.26	6.09±0.48	6.76±0.16
Cell Concentration (g/L)	Peptone	-	2.03±0.03	2.06±0.01	2.06±0.01	2.08±0.03	2.08±0.07	2.14±0.01	2.18±0.01
	soypeptone	-	2.08±0.03	2.10±0.03	2.15±0.04	2.18±0.03	2.21±0.01	2.15±0.01	2.14±0.01
	polypeptone	-	1.98±0.02	2.08±0.01	2.13±0.01	2.14±0.01	2.14±0.01	2.15±0.01	2.14±0.03
	tryptone	-	2.03±0.02	2.06±0.05	2.15±0.02	2.17±0.06	2.18±0.08	2.18±0.04	2.20±0.02
	Beef extract	-	2.09±0.01	2.13±0	2.17±0.03	2.20±0.01	2.24±0.02	2.18±0.02	2.17±0.02
	Yeast extract	-	1.97±0.01	2.14±0.01	2.18±0	2.21±0.01	2.25±0.04	2.28±0.01	2.28±0.01

	Day	0	1	2	3	4	7	10	14
Lipase Activity (U/ml)	Peptone	-	0.55±0.05	0.20±0.01	0.22±0.01	0.22±0.02	0.35±0	0.39±0.03	1.26±0.04
	soypeptone	-	0.64±0.01	0.30±0.02	0.39±0.02	0.34±0.07	8.89±0.02	11.12±0.01	7.31±0.01
	polypeptone	-	0.21±0.01	0.18±0.02	0.18±0.02	0.12±0.01	0.24±0.01	0.30±0.03	0.15±0.03
	tryptone	-	0.28±0	0.28±0.01	0.38±0.01	0.16±0.02	0.13±0.01	0.22±0.03	1.00±0.03
	Beef extract	-	0.25±0.03	0.22±0.04	0.36±0.02	0.31±0.01	1.32±0.01	4.83±0	3.81±0.01
	Yeast extract	-	0.16±0.01	0.18±0.01	0.17±0.02	0.16±0.01	0.34±0.04	1.30±0.03	0.60±0.03

Table: pH, cell concentration and lipase activity for *A. calcoaceticus* in 1% crude oil with different metal ion substitution according to day

	Day	0	1	2	3	4	5	7	11	14
pH	Magnesium	6.90±0.10	6.14±0.18	5.67±0.25	5.38±0.09	5.42±0.18	4.98±0.40	4.71±0.27	4.81±0.30	4.70±0.19
	Sodium	7.00±0.01	6.38±0.01	5.91±0.02	5.81±0.04	5.91±0	5.80±0.17	5.75±0.08	5.34±0.16	4.74±0.05
	Manganese	7.00±0	5.51±0	4.44±0.05	4.36±0.12	4.48±0.25	4.47±0.15	4.39±0.12	4.37±0.09	4.39±0.11
	Potassium	7.00±0.05	6.36±0.02	5.99±0.07	5.99±0.02	5.98±0	5.87±0.07	5.83±0.08	5.66±0.05	5.48±0.03
	Lithium	6.90±0.10	6.52±0.06	6.00±0.05	5.78±0.02	5.93±0.03	5.84±0.10	5.85±0.01	5.67±0.08	5.45±0.03
	Ferric	6.90±0.05	4.40±0.03	4.39±0.05	4.32±0.01	4.49±0.05	4.31±0.04	4.30±0	4.39±0.01	4.43±0.01
Cell Concentration g/L)	Magnesium	-	1.31±0.08	1.35±0.11	1.44±0.14	1.44±0.07	1.45±0	1.46±0.19	1.59±0.17	1.61±0.08
	Sodium	-	0.98±0.03	1.23±0.05	1.32±0.05	1.42±0.09	1.47±0.01	1.47±0.02	1.53±0.05	1.51±0.03
	Manganese	-	0.67±0.05	0.93±0.05	1.09±0.01	1.21±0.09	1.29±0.16	1.31±0.21	1.28±0.17	1.30±0.05
	Potassium	-	0.85±0.17	1.06±0.07	1.21±0	1.20±0.02	1.23±0.02	1.24±0	1.31±0.2	1.37±0.17
	Lithium	-	0.71±0.05	1.11±0.11	1.23±0.12	1.29±0.04	1.38±0.04	1.43±0.10	1.52±0.07	1.46±0.02
	Ferric	-	1.21±0.05	1.08±0.01	1.16±0.01	1.15±0.02	1.19±0.02	1.25±0.02	1.44±0.03	1.46±0.03

Lipase Activity (U/ml)	Day	0	1	2	3	4	5	7	11	14
	Magnesium	-	0.81±0.03	0.35±0.05	0.18±0.02	0.16±0.09	0.10±0.06	0.08±0.01	0.40±0.06	0.03±0.01
	Sodium	-	0.77±0.01	0.32±0.03	0.13±0.01	0.07±0	0.07±0.1	0.06±0.02	0.43±0.01	0.04±0
	Manganese	-	0.14±0.04	0.07±0.03	0.06±0	0.07±0.02	0.08±0.01	0.14±0.02	0.41±0.02	0.06±0.01
	Potassium	-	0.43±0.02	0.33±0.05	0.22±0.01	0.23±0	0.14±0.02	0.10±0.01	0.80±0.01	0.05±0
	Lithium	-	0.15±0.01	0.20±0.01	0.13±0.02	0.05±0	0.08±0.04	0.07±0.04	0.17±0.5	0.11±0.01
	Ferric	-	0.04±0.01	0.02±0.01	0.04±0.01	0.04±0.01	0.04±0.03	0.10±0.02	0.39±0.02	0.04±0.02

Table: pH, cell concentration and lipase activity for *A. calcoaceticus* in 1% used cooking oil with different metal ion substitution according to day

	Day	0	1	2	3	4	5	7	11	14
pH	Magnesium	6.90±0.04	4.09±0.04	4.02±0.02	4.00±0.01	3.91±0	3.95±0.01	3.95±0.02	3.97±0.01	3.90±
	Sodium	7.00±0.05	4.06±0.02	4.07±0.03	4.05±0.02	3.97±0,04	4.00±0,05	3.98±0.06	4.01±0.04	4.03±0.04
	Manganese	6.90±0	3.83±0.07	3.82±0.02	3.79±0.04	3.73±0.06	3.72±0.05	3.71±0.02	3.69±0.04	3.72±0.06
	Potassium	7.00±0.01	4.08±0	4.07±0.02	4.04±0.02	3.95±0.01	3.97±0	4.04±0	3.98±0	4.02±0.04
	Lithium	6.90±0.50	4.15±0.02	4.15±0.02	4.10±0.02	4.05±0	4.05±0.03	4.10±0.03	4.03±0	4.10±0.04
	Ferric	6.90±0.4	3.84±0	3.81±0.05	3.73±0.01	3.66±0.02	3.67±0.01	3.77±0.12	3.64±0	3.79±0.12
Cell Concentration (g/L)	Magnesium	-	1.19±0.15	1.12±0.04	1.26±0.05	1.26±0.07	1.32±0.08	1.28±0.08	1.43±0.01	1.61±0.16
	Sodium	-	1.21±0.03	1.20±0.02	1.37±0.07	1.36±0.01	1.33±0.04	1.39±0.04	1.60±0.05	1.62±0.01
	Manganese	-	1.53±0.04	1.54±0.04	1.62±0.01	1.65±0.13	1.69±0.10	1.65±0.07	1.81±0.02	1.82±0.15
	Potassium	-	1.15±0.04	1.17±0..04	1.21±0.01	1.38±0.13	1.31±0.10	1.36±0.07	1.56±0.02	1.61±0.15
	Lithium	-	1.14±0.07	1.20±0.02	1.26±0.05	1.37±0.09	1.26±0.03	1.37±0.11	1.58±0.03	1.59±0.07
	Ferric	-	1.53±0.05	1.65±0.04	1.71±0.15	1.74±0	1.67±0.24	1.74±0.13	1.80±0.04	1.76±0.31

Day		0	1	2	3	4	5	7	11	14
Lipase Activity (U/ml)	Magnesium	-	0.00	0.00	0.00	0.06±0.02	0.09±0.06	0.11±0.09	0.78±0.14	0.00
	Sodium	-	0.00	0.00	0.00	0.07±0	0.06±0.02	0.16±0.5	0.50±0	0.00
	Manganese	-	0.00	0.00	0.00	0.09±0.01	0.23±0.03	0.16±0	0.40±0.02	0.00
	Potassium	-	0.00	0.00	0.00	0.06±0.02	0.09±0.06	0.19±0.01	0.69±0.06	0.00
	Lithium	-	0.00	0.00	0.00	0.10±0.01	0.10±0.01	0.09±0.03	0.49±0.01	0.00
	Ferric	-	0.00	0.00	0.00	0.08±0.01	0.11±0.01	0.14±0.06	0.40±0..06	0.00

Appendix J

Sequences and BLASTn results for each strains

F1 Strain

```
Query  641  CTCAGCGTCAGTATTAGGCCAGATGGCTGCCTTCGCCATCGGTATTCCTCCAGATCTCTA  700
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Sbjct  750  CTC-GCGTC-GTA-TAGGCCAGATGGCTGCCTTCGCCATCGGTATTCCTCCAGATCTCTA  694

Query  701  CGCATTTCACCGCTACACCTGGAATTCTACCATCCTCTCCCATACTCTAGCCAACCAGTA  760
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Sbjct  693  CGCATTTCACCGCTACACCTGGAATTCTACCATCCTCTCCCATACTCTAGCCAACCAGTA  634

Query  761  TCGAATGCAATTCCCAAGTTAAGCTCGGGGATTTCACATTT-ACTTAAT-GGCCGCCTAC  818
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Sbjct  633  TCGAATGCAATTCCCAAGTTAAGCTCGGGGATTTCACATTTGACTTAATTGGCCGCCTAC  574

Query  819  GCACGCTT-ACGCCCA-TAA-TCCG  840
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Sbjct  573  GCACGCTTTACGCCCAGTAAATCCG  549
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Accession	Description	Max score	Total score	Query coverage	E value	Max ident
FJ608261.1	Acinetobacter radioresistens strain F29 16S ribosomal RNA gene, partial sequence	346	346	100%	2e-92	97%
FJ608259.1	Acinetobacter radioresistens strain F27 16S ribosomal RNA gene, partial sequence	346	346	100%	2e-92	97%
GQ183856.1	Acinetobacter sp. Ch-02 16S ribosomal RNA gene, partial sequence	346	346	100%	2e-92	97%
GQ115953.1	Uncultured bacterium clone nbw644g01c1 16S ribosomal RNA gene, partial sequence	346	346	100%	2e-92	97%
GQ115395.1	Uncultured bacterium clone nbw643f08c1 16S ribosomal RNA gene, partial sequence	346	346	100%	2e-92	97%
GQ115328.1	Uncultured bacterium clone nbw642e03c1 16S ribosomal RNA gene, partial sequence	346	346	100%	2e-92	97%
GQ115302.1	Uncultured bacterium clone nbw642a10c1 16S ribosomal RNA gene, partial sequence	346	346	100%	2e-92	97%
GQ109041.1	Uncultured bacterium clone nbw606g10c1 16S ribosomal RNA gene, partial sequence	346	346	100%	2e-92	97%
GQ108986.1	Uncultured bacterium clone nbw720e01c1 16S ribosomal RNA gene, partial sequence	346	346	100%	2e-92	97%
GQ108843.1	Uncultured bacterium clone nbw718c06c1 16S ribosomal RNA gene, partial sequence	346	346	100%	2e-92	97%

F2 Strain

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Query  651  ATGTTGGTCCAGGTAGCTGCCTTCGCCATGGATGTTCCTCCTGATCTCTACGCATTTCAC  710
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Sbjct  747  ATGTTGGTCC-GGTAGCTGCCTTCGCCATGGATGTTCCTCCTGATCTCTACGCATTTCAC  689

Query  711  TGCTACACCAGGAATTCCGCTACCCTCTACCACATTCTAGTCGCCCAGTATCCACTGCAG  770
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Sbjct  688  TGCTACACCAGGAATTCCGCTACCCTCTACCACATTCTAGTCGCCCAGTATCCACTGCAG  629

Query  771  TTCCCAGGTTGAGCCCAGGGCTTTCACAACGGACT-AAACGACCACCTACGCACGCTT-A  828
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Sbjct  628  TTCCCAGGTTGAGCCCAGGGCTTTCACAACGGACTTAAACGACCACCTACGCACGCTTTA  569

Query  829  CGCCCA-TA-T-CCGAG  842
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Sbjct  568  CGCCCAGTAATTCCGAG  552
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Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EU939967.1	Uncultured bacterium clone D16S_64 16S ribosomal RNA gene, partial sequence	331	331	99%	7e-88	97%
FJ481929.1	Stenotrophomonas maltophilia strain AF-222 16S ribosomal RNA gene, partial sequence	329	329	100%	2e-87	97%
GQ287630.1	Stenotrophomonas maltophilia 16S ribosomal RNA gene, partial sequence	326	326	100%	3e-86	96%
FN428834.1	Uncultured Stenotrophomonas sp. partial 16S rRNA gene, clone K31	326	326	100%	3e-86	96%
AB508873.1	Stenotrophomonas sp. TSH90 gene for 16S ribosomal RNA, partial sequence	326	326	100%	3e-86	96%
AB508865.1	Stenotrophomonas sp. TSH76 gene for 16S ribosomal RNA, partial sequence	326	326	100%	3e-86	96%
GQ024392.1	Uncultured bacterium clone nbw121h08c1 16S ribosomal RNA gene, partial sequence	326	326	100%	3e-86	96%
GQ022436.1	Uncultured bacterium clone nbu312e11c1 16S ribosomal RNA gene, partial sequence	326	326	100%	3e-86	96%
FJ894019.1	Uncultured bacterium clone nbt175b01 16S ribosomal RNA gene, partial sequence	326	326	100%	3e-86	96%
FJ893802.1	Uncultured bacterium clone nbt35f10 16S ribosomal RNA gene, partial sequence	326	326	100%	3e-86	96%

F4 Strain

```
Query  641   CTCAGCGTCAGTATTAGGCCAGATGGCTGCCTTCGCCATCGGTATTCCTCCAGATCTCTA  700
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Sbjct  751   CTC-GCGTC-GTA-TAGGCCAGATGGCTGCCTTCGCCATCGGTATTCCTCCAGATCTCTA  695

Query  701   CGCATTTCACCGCTACACCTGGAATTCTACCATCCTCTCCCATACTCTAGCTCACCAGTA  760
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  694   CGCATTTCACCGCTACACCTGGAATTCTACCATCCTCTCCCATACTCTAGCTCACCAGTA  635

Query  761   TCGAATGCAATTCCCAAGTTAAGCTCGGGGATTTCACATCCGACTTAATAAGCCGCCTAC  820
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Sbjct  634   TCGAATGCAATTCCCAAGTTAAGCTCGGGGATTTCACATCCGACTTAATAAGCCGCCTAC  575

Query  821   GCACGCTT-ACGCCCA-TAA-TCCG  842
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Sbjct  574   GCACGCTTTACGCCCAGTAAATCCG  550
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Accession	Description	Max score	Total score	Query coverage	E value	Max ident
GQ289381.1	Acinetobacter sp. 117 16S ribosomal RNA gene, partial sequence	231	231	100%	7e-58	89%
GQ289380.1	Acinetobacter sp. 109 16S ribosomal RNA gene, partial sequence	231	231	100%	7e-58	89%
GQ289379.1	Acinetobacter sp. 101 16S ribosomal RNA gene, partial sequence	231	231	100%	7e-58	89%
GQ289378.1	Acinetobacter sp. 40 16S ribosomal RNA gene, partial sequence	231	231	100%	7e-58	89%
GQ178059.1	Acinetobacter baumannii strain AZR3410 16S ribosomal RNA gene, partial sequence	231	231	100%	7e-58	89%
GQ178054.1	Acinetobacter sp. 81A1 16S ribosomal RNA gene, partial sequence	231	231	100%	7e-58	89%
GQ178053.1	Acinetobacter sp. 71A1 16S ribosomal RNA gene, partial sequence	231	231	100%	7e-58	89%
GQ178052.1	Acinetobacter sp. 66A1 16S ribosomal RNA gene, partial sequence	231	231	100%	7e-58	89%
GQ178049.1	Acinetobacter sp. 56A1 16S ribosomal RNA gene, partial sequence	231	231	100%	7e-58	89%
GQ178044.1	Acinetobacter sp. 01B0 16S ribosomal RNA gene, partial sequence	231	231	100%	7e-58	89%
GQ200824.1	Acinetobacter baumannii strain KK14 16S ribosomal RNA gene, partial sequence	231	231	100%	7e-58	89%
FJ976578.1	Acinetobacter baumannii strain LCR69 16S ribosomal RNA gene, partial sequence	231	231	100%	7e-58	89%

F10 Strain

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Query  636   TGAGCGTCAGTCTTCGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTAC  695
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Sbjct  754   TGA-CGTC-GTCTTCGT-CAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTAC  698

Query  696   GCATTTCACCGCTACACCTGGAATTCTACCCCCCTCTACGAGACTCAAGCCTGCCAGTTT  755
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Sbjct  697   GCATTTCACCGCTACACCTGGAATTCTACCCCCCTCTACGAGACTCAAGCCTGCCAGTTT  638

Query  756   CAAATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCTGACTTAACAGACCGCCTGCG  815
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Sbjct  637   CAAATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCTGACTTAACAGACCGCCTGCG  578

Query  816   TGCGCTT-ACGCCCAGTA  832
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Sbjct  577   TGCGCTTTACGCCCAGTA  560
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Accession	Description	Max score	Total score	Query coverage	E value	Max ident
FJ906924.1	Cronobacter sakazakii strain Jor184 16S ribosomal RNA gene, partial sequence	359	359	100%	3e-96	99%
FJ906914.1	Cronobacter sakazakii strain Jor160A 16S ribosomal RNA gene, partial sequence	359	359	100%	3e-96	99%
FJ906913.1	Cronobacter sakazakii strain Jor154 16S ribosomal RNA gene, partial sequence	359	359	100%	3e-96	99%
FJ906898.1	Cronobacter sakazakii strain Jor175 16S ribosomal RNA gene, partial sequence	359	359	100%	3e-96	99%
FJ611869.1	Pantoea dispersa strain CIP 102701 16S ribosomal RNA gene, partial sequence	359	359	100%	3e-96	99%
FJ581031.1	Enterobacter sakazakii strain IITRM16 16S ribosomal RNA gene, partial sequence	359	359	100%	3e-96	99%
EU675663.1	Enterobacter sakazakii strain ES31 16S ribosomal RNA gene, partial sequence	359	359	100%	3e-96	99%
EU675661.1	Enterobacter sakazakii strain ES29 16S ribosomal RNA gene, partial sequence	359	359	100%	3e-96	99%
EU675660.1	Enterobacter sakazakii strain ES28 16S ribosomal RNA gene, partial sequence	359	359	100%	3e-96	99%
EU675659.1	Enterobacter sakazakii strain ES27 16S ribosomal RNA gene, partial sequence	359	359	100%	3e-96	99%
EU675658.1	Enterobacter sakazakii strain ES26 16S ribosomal RNA gene, partial sequence	359	359	100%	3e-96	99%
EU675657.1	Enterobacter sakazakii strain ES25 16S ribosomal RNA gene, partial sequence	359	359	100%	3e-96	99%
EU675656.1	Enterobacter sakazakii strain ES24 16S ribosomal RNA gene, partial sequence	359	359	100%	3e-96	99%

F12 Strain

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Query  640   CTCAGCGTCAGTGTTAGGCCAGATGGCTGCCTTCGCCATCGGTATTCCTCCAGATCTCTA  699
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Sbjct  751   CTCA-CGTC-GTG-TAGGCCAGATGGCTGCCTTCGCCATCGGTATTCCTCCAGATCTCTA  695

Query  700   CGCATTTCACCGCTACACCTGGAATTCTACCATCCTCTCCCACACTCTAGCTAACCAGTA  759
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Sbjct  694   CGCATTTCACCGCTACACCTGGAATTCTACCATCCTCTCCCACACTCTAGCTAACCAGTA  635

Query  760   TCGAATGCAATTCCCAAGTTAAGCTCGGGGATTTCACATTT-A-TTAATTAGCCGCCTAC  817
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Sbjct  634   TCGAATGCAATTCCCAAGTTAAGCTCGGGGATTTCACATTTGACTTAATTAGCCGCCTAC  575

Query  818   GCGCGCTT-ACGCCCAG-AA-TCCG  839
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Accession	Description	Max score	Total score	Query coverage	E value	Max ident
FJ688379.1	Acinetobacter sp. KZ-OAlM 16S ribosomal RNA gene, partial sequence	346	346	100%	2e-92	97%
GQ289138.1	Bacterium Lce4 16S ribosomal RNA gene, partial sequence	346	346	100%	2e-92	97%
GQ178061.1	Acinetobacter calcoaceticus strain AZR583 16S ribosomal RNA gene, partial sequence	346	346	100%	2e-92	97%
GQ178060.1	Acinetobacter sp. AZR54 16S ribosomal RNA gene, partial sequence	346	346	100%	2e-92	97%
GQ178056.1	Acinetobacter sp. A06(2009) 16S ribosomal RNA gene, partial sequence	346	346	100%	2e-92	97%
GQ178055.1	Acinetobacter sp. 85A1 16S ribosomal RNA gene, partial sequence	346	346	100%	2e-92	97%
GQ178051.1	Acinetobacter sp. 63A1 16S ribosomal RNA gene, partial sequence	346	346	100%	2e-92	97%
GQ178050.1	Acinetobacter sp. 62A1 16S ribosomal RNA gene, partial sequence	346	346	100%	2e-92	97%
FJ897487.1	Acinetobacter sp. NII-130 16S ribosomal RNA gene, partial sequence	346	346	100%	2e-92	97%
FJ897486.1	Acinetobacter sp. NII-128 16S ribosomal RNA gene, partial sequence	346	346	100%	2e-92	97%
FJ897485.1	Acinetobacter sp. NII-56 16S ribosomal RNA gene, partial sequence	346	346	100%	2e-92	97%
FJ976611.1	Acinetobacter calcoaceticus strain LCR102 16S ribosomal RNA gene, partial sequence	346	346	100%	2e-92	97%

CLUSTAL 2.0.11 multiple sequence alignment

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F1              CTCAGCGTCAGTATTAGGCCAGATGGCTGCCTTCGCCATCGGTATTCCTCCAGATCTCTA 60
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F12             CTCAGCGTCAGTGTTAGGCCAGATGGCTGCCTTCGCCATCGGTATTCCTCCAGATCTCTA 60
F10             -TGAGCGTCAGTCTTCGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCAGATCTCTA 59
F2              ----------ATGTTGGTCCAGGTAGCTGCCTTCGCCATGGATGTTCCTCCTGATCTCTA 50
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F12             CGCATTTCACCGCTACACCTGGAATTCTACCATCCTCTCCCACACTCTAGCTAACCAGTA 120
F10             CGCATTTCACCGCTACACCTGGAATTCTACCCCCCTCTACGAGACTCAAGCCTGCCAGTT 119
F2              CGCATTTCACTGCTACACCAGGAATTCCGCTACCCTCTACCACATTCTAGTCGCCCAGTA 110
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F1              TCGAATGCAATTCCCAAGTTAAGCTCGGGGATTTCACATTT-ACTTAATG-GCCGCCTAC 178
F4              TCGAATGCAATTCCCAAGTTAAGCTCGGGGATTTCACATCCGACTTAATAAGCCGCCTAC 180
F12             TCGAATGCAATTCCCAAGTTAAGCTCGGGGATTTCACATTT--ATTAATTAGCCGCCTAC 178
F10             TCAAATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCTGACTTAACAGACCGCCTGC 179
F2              TCCACTGCAGTTCCCAGGTTGAGCCCAGGGCTTTCACAACGGACTAAACG-ACCACCTAC 169
                ** * **** ****** *** *** * *** *******      * **    ** *** *

F1              GCACGCTTACGCCCATAATCCG- 200
F4              GCACGCTTACGCCCATAATCCG- 202
F12             GCGCGCTTACGCCCAGAATCCG- 200
F10             GTGCGCTTACGCCCAGTA----- 197
F2              GCACGCTT-CGCCCATATCCGAG 191
                *  ***** ******
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Research Publications and Participations

1) Tan, Y. H., Hii, S. L. & Woo, K. K. (2007). Isolation and Characterisation of Locally Isolated Oil Degrading Bacteria. 32nd Annual Conference of the Malaysia Society for Biochemistry and Molecular Biology. Kuala Lumpur, Malaysia. (Poster Presentation)

2) Tan, Y. H., Hii, S. L. & Woo, K. K. (2007). Behavioural Properties of Locally Isolated Oil Degrading Bacteria. 12th Biological Sciences Graduate Congress 2007. Kuala Lumpur, Malaysia. (Poster Presentation)