



UNIVERSITI PUTRA MALAYSIA

**BIODEGRADATION OF DIESEL BY A LOCALLY ISOLATED
*ACINETOBACTER SP.***

FARRAH AINI BT. DAHALAN

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**BIODEGRADATION OF DIESEL BY A LOCALLY ISOLATED
ACINETOBACTER SP.**

By

FARRAH AINI BT. DAHALAN

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in
Fulfilment of the Requirements for the Degree of Master of Science**

March 2007



Dedicated to my parents...



Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Master of Science

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Chairman: Mohd Yunus b. Abd Shukor, PhD

Faculty : Biotechnology and Biomolecular Sciences

Local bacteria isolated from oil-contaminated soils from various locations in Malaysia were screened for their ability to degrade commercial diesel fuel. Enrichment culture from soil samples yielded several isolates capable of degrading diesel. Of these, Isolate 1 was selected for further studies based on its best growth performance compared to the other isolates. Biodegradation studies were conducted using head-space solid-phase microextraction (HS-SPME: 110°C, 10 min, 7 µm PDMS fiber and 25% NaCl) coupled to gas chromatography equipped with flame ionization detector (GC-FID). The isolate was identified as *Acinetobacter* sp. (1470 bp) (98% sequence homology) using 16s rRNA molecular phylogenetic analysis. Isolate 1 exhibited optimum growth at 37°C in media containing 4% (v/v) diesel, and is able to degrade 51.7% of diesel in 6 days. Isolate 1 was grown on various nitrogen sources such as NH₄Cl, NH₄SO₄, NaNO₃, and KNO₃. The best nitrogen source is potassium nitrate (KNO₃) at 0.9% (v/v). Its optimized optimum pH for growth is pH 7.5. Five different diesel-degrading enzymes were



detected: alkane-oxidizing enzyme ($0.2.8 \mu\text{mol min}^{-1} \text{ml}^{-1}$), alcohol dehydrogenase ($9.0956 \mu\text{mol min}^{-1} \text{ml}^{-1}$), aldehyde dehydrogenase ($4.234 \mu\text{mol min}^{-1} \text{ml}^{-1}$), pyridine nucleotide-independent dehydrogenase ($4.229 \mu\text{mol min}^{-1} \text{ml}^{-1}$) and aldehyde reductase ($8.126 \mu\text{mol min}^{-1} \text{ml}^{-1}$).



Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

**BIODEGRADASI DIESEL DENGAN MENGGUNAKAN SEJENIS ISOLAT
*ACINETOBACTER SP. TEMPATAN***

Oleh

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Bakteria tempatan yang berupaya mengurai minyak diesel telah dipencilkan daripada sample tanah yang diambil dari beberapa lokasi di Malaysia. Isolat pengurai diesel telah diperolehi daripada teknik penggalakkan kultur pertumbuhan. Isolat 1 telah dipilih untuk kajian lanjutan berdasarkan pertumbuhannya yang menggalakkan. Isolat ini kemudiannya telah diuji tahap keberkesanan dan keupayaannya dalam penguraian diesel dengan menggunakan ‘head-space solid-phase microextraction’ (HS-SPME: 110°C, 10 min, 7 µm PDMS fiber and 25% NaCl) digandingkan dengan ‘gas chromatography-flame ionization detector’ (GC-FID). Melalui penganalisan filogenetik molekul 16S rRNA, isolat ini telah dikenalpasti sebagai *Acinetobacter sp.* (1470 bp) (98% jujukan homologi). Isolat ini telah mempamerkan kadar pertumbuhan yang optimum pada suhu 37°C di dalam media yang mengandungi kepekatan diesel sebanyak 4% (v/v) dan ia juga berupaya untuk mengurai 51.7% diesel dalam tempoh 6 hari. Ia telah dibiakkan di dalam pelbagai sumber nitrogen seperti NH₄Cl, NH₄SO₄, NaNO₃, and KNO₃. Ia didapati memerlukan sumber nitrogen iaitu kalium nitrat (KNO₃) dengan kepekatan 0.9% (v/v)



dan pH optimum pada 7.5. Lima jenis enzim pengurai diesel telah dapat dikesan kehadirannya di dalam Isolat 1 iaitu; enzim pengurai alkana ($0.2.8 \mu\text{mol min}^{-1} \text{ml}^{-1}$), dehidrogenas alcohol ($9.0956 \mu\text{mol min}^{-1} \text{ml}^{-1}$), dehidrogenas aldehida ($4.234 \mu\text{mol min}^{-1} \text{ml}^{-1}$), enzim penurun aldehida ($4.229 \mu\text{mol min}^{-1} \text{ml}^{-1}$) dan dehidrogenas piridina nukleotida-kendiri ($8.126 \mu\text{mol min}^{-1} \text{ml}^{-1}$).



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I certify that an Examination Committee met on **date of viva** to conduct the final examination of Farrah Aini bt. Dahalan on her Master of Science thesis entitled “Biodegradation of Diesel by Using Tropical Microbes” in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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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 duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

FARRAH AINI BT. DAHALAN

Date: 25th APRIL 2007



LIST OF TABLES

Table		Page
1	Reported off-shore oil pollution cases since 1976-2005	9
2	Status of marine water quality of oil & grease in Malaysia for year 2002	10
3	Classification of hydrocarbon	13
4	Advantages and disadvantages of bioremediation	20
5	Material Safety Data Sheet For Diesel	21
6	Diesel components	21
7	Various genus of hydrocarbon degraders	33
8	Sampling sites of diesel-degrading bacteria	44
9	The composition of major elements in the Basal Salt Medium (BSM)	45
10	The components of major elements in Trace Elements (TE)	45
11	Isolated diesel-degrading bacteria and their sampling sites	70
12	Biodegradation studies of Isolate 1 incubated on orbital shaker (150 rpm) at room temperature for 6 days.	92



LIST OF FIGURES

Figure		Page
1	Marine water quality for oil & grease (O&G), 2000-2002	11
2	Aliphatics and aromatic hydrocarbon compound	15
3	Steps in oxidation of an aliphatic hydrocarbon	35
4	Pathways for the degradation of alkanes	36
5	Solid-Phase Microextraction (SPME) device	51
6	Growth curve of eight isolates on 10 ml DBM liquid medium	72
7	The effect of diesel concentration on the growth of Isolate 1	74
8	The effect of temperature for the growth of Isolate 1	77
9	The effect of pH on the growth of Isolate 1	78
10	The effect of different nitrogen source on the growth of Isolate 1	81
11	The effect of potassium nitrate concentration on the growth of Isolate 1	82
12	The effect of detergents on growth of Isolate 1	84
13	The effect of HS-SPME absorption on temperature	86
14	The effect of HS-SPME absorption on fiber's exposure time	88
15	The effect of different fiber used for diesel absorption	89
16	The effect of NaCl concentration on HS-SPME absorption	90
17	The degradation of diesel by Isolate 1	93
18	Detection of enzyme involved in alkane degradation of Isolate 1	99
19	Photomicrograph of Isolate 1	100
20	Extraction of genomic DNA from bacterial Isolate 1	102



21	16s rRNA gene of bacterial Isolate 1 amplified via PCR	103
22	The region of homology of Isolate 1	104
23	Neighbour-joining method cladogram	106
24	The accession number of 16s rRNA sequence of Isolate 1	109



TABLE OF CONTENTS

	Page
DEDICATION	ii
ABSTRACT	iii
ABSTRAK	iv
ACKNOWLEDGEMENTS	vi
APPROVAL	viii
DECLARATION	x
LIST OF TABLES	xi
LIST OF FIGURES	xii
CHAPTER	
1 INTRODUCTION	1
2 LITERATURE REVIEW	
2.1 Petroleum	5
2.2 Exploration of Petroleum	6
2.3 Petroleum Pollutions	7
2.4 Oil Pollution In Malaysia	8
2.5 Effects of Oil Pollution	11
2.5.1 Animals	11
2.5.2 Human	12
2.6 Hydrocarbon	13
2.7 Analysis and Quantification of Hydrocarbon	16
2.8 Bioremediation	17
2.8.1 Advantages and Disadvantages of Bioremediation	19
2.9 Diesel	20
2.10 Biodegradation of Diesel	22
2.10.1 Significance of Studying Biodegradation of Diesel	25
2.11 Physicochemical Factors of Biodegradation of Diesel	26
2.11.1 General Overview	26
2.11.2 Oxygen	27
2.11.3 Carbon	27
2.11.4 Macronutrients and Micronutrients	29
2.11.5 pH	31
2.11.6 Temperature	31
2.12 Diesel-Degrading Bacteria	32
2.13 Metabolism Diesel Degrading Bacteria	34
2.14 Distribution of Diesel Degrading Enzymes	39
2.15 Characteristics and Distribution of <i>Acinetobacter</i>	42



3	MATERIALS AND METHODS	43
3.1	Sampling of Diesel-Degrading Bacteria	43
3.2	Preparation of Culture Growth Medium	44
3.3	Screening and Isolation of Diesel-Degrading Bacteria	45
3.4	Determination of Diesel-Degrading Bacterial Growth Profile	46
3.5	Growth Optimization of Isolate 1	47
	3.5.1 Diesel as Sole Carbon Source	47
	3.5.2 Various Temperatures	47
	3.5.3 Different pH	47
	3.5.4 Nitrogen Sources	48
	3.5.5 KNO ₃	48
	3.5.6 Surfactants	49
3.6	Diesel Analysis Using gas Chromotography	49
3.7	Solid-Phase Microextraction (SPME)	50
	3.7.1 Injection Technique	52
	3.7.2 Fibers Maintenance	53
	3.7.3 Extraction of Diesel By SPME	53
	3.7.4 Vials Characteristics	54
3.8	Optimization of SPME Absorption Conditions	54
	3.8.1 Temperature	54
	3.8.2 Absorption Time	55
	3.8.3 Fibers	55
	3.8.4 Effect of Salts	56
3.9	Surrogate Working Standard	56
3.10	Degradation Studies	57
3.11	Diesel-Degrading Enzymes Detection	57
3.12	Preparation of Buffers	58
3.13	Preparation of Substrate Solution For The Enzyme Assay	58
3.14	Obtaining Cell-free Extracts	58
3.15	Determination of Protein Concentration	59
3.16	Diesel-Degrading Enzymes Detection	60
	3.16.1 n-Alkane-Oxidizing Enzyme	60
	3.16.2 Alcohol Dehydrogenase	60
	3.16.3 Aldehyde Dehydrogenase	61
	3.16.4 Aldehyde Reductase	61
	3.16.5 Pyridine-Nucleotide Independent Dehydrogenase	61
3.17	Identification of Isolate 1	61
	3.17.1 Morphological Studies	61
	3.17.2 Gram Staining	62
	3.17.3 16S rRNA Gene Sequencing and Phylogenetic Analysis	63



4	RESULTS AND DISCUSSION	
4.1	Screening and Isolation of Diesel-Degrading Bacteria	69
4.2	Determination of Diesel-Degrading Bacteria Growth Profile	71
4.3	Growth Optimization of Isolate 1	72
	4.3.1 General Overview	72
	4.3.2 Growth Optimization of Isolate 1 Using Diesel As Sole Carbon Source	73
	4.3.3 Growth Optimization of Isolate 1 at Various Temperatures	75
	4.3.4 Growth Optimization of Isolate 1 on Different pHs	77
	4.3.5 Growth Optimization of Isolate 1 on various Nitrogen Sources	79
	4.3.6 Growth Optimization of Isolate 1 on KNO ₃	81
	4.3.7 Growth Optimization of Isolate 1 on Surfactants	82
4.4	Diesel Analysis Using Gas Chromatography	85
	4.4.1 Optimization of SPME Absorption Conditions	85
	4.4.1.1 Temperature	85
	4.4.1.2 Absorption Time	86
	4.4.1.3 Fibers	88
	4.4.1.4 Effect of Salts	89
	4.4.2 Surrogate Working Standards	90
	4.4.3 Degradation Studies	91
4.5	Diesel-degrading Enzymes Detection	95
4.6	Identification of Isolate 1	100
4.6.1	16S rRNA Gene Sequencing and Phylogenetic Analysis	101
	4.6.1.1 Genomic Extraction	101
	4.6.1.2 Polymerase Chain Reaction (PCR)	102
	4.6.1.3 Sequence Analysis	103
	4.6.1.4 Phylogenetic Analysis	105
5	CONCLUSIONS	110
	REFERENCES	112
	APPENDICES	125
	BIODATA OF THE AUTHOR	143



CHAPTER 1

INTRODUCTION

The exploration of petroleum or hydrocarbons started thousands of years ago. Since then, the frontiers of oil exploration worldwide have been expanding, producing medium and light crude oil with a wide range of petroleum products. However, the abundance of hydrocarbons in areas such as in marine environments and soils cause massive pollution, many of which must be eliminated due to their high toxicity effects.

Many countries have battled oil pollution by applying chemicals in order to breakdown the hydrocarbons into smaller components, which relatively will decrease the pollution rate. But, these physical and chemical methods commonly used are not totally effective in eliminating them and may even create additional polluting compounds.

In the past, there was a limited use of innovative remedial technologies. Recently, several approaches are used throughout the world to reclaim contaminated soils, among which bioremediation or biodegradation is one of the most important methods. Bioremediation, which utilizes microorganisms to degrade various chemical substances in order to facilitate their removal from the environment, is not a new science. This has become an important technology and a promising tool for dealing with environmental contamination caused by industrial pollutants. Bioremediation is a rapidly developing field of environmental restoration by utilizing



natural activity to reduce the concentration and toxicity of various hazardous substances, such as petroleum products, aliphatic and aromatic hydrocarbon (including polyaromatic hydrocarbons and polychlorinated biphenyls), industrial solvents (phenols, benzene, acetone etc.), battery liquids, pesticides, and heavy metals into less toxic or non-toxic substances to environmentally safe levels (Korda *et al.*, 1997).

Bioremediation is a natural process carried out by numerous soil and aquatic microorganisms, mostly bacteria and fungi. These microorganisms feed on the contamination, deriving nutrition for growth and reproduction. But it is only during the last decades that humans have started to learn and understand more about microorganisms and their important roles in natural degradation processes. Due to the environment, several microorganisms have adapted specific enzyme systems and are able to degrade most of hydrocarbons compounds. A compound may be degraded by different pathway by one bacterium depending on the permitted surrounding conditions (Hostettler and Kvenvolden, 2002).

Usually bacteria are preferably used in order to digest hydrocarbon compared to other microorganisms as they reproduce more rapidly by binary fission which will certainly increase the biodegradation rate. Other microorganisms such as fungus are not widely used because they grow in the form of branched hyphae which require more time for reproduction and will trigger slow biodegradation. Certain bacterial strains have demonstrated the ability to break down or transform the chemical substances present in petroleum products to support their life cycle. There are several genus that have been reported as hydrocarbon utilizers, such as *Pseudomonas*,

Bacillus, *Proteus*, *Aeruginosa*, *Klebsiella* (Odokuma and Dickson *et al.*, 2003), *Aeromonas*, *Micrococcus*, *Serratia*, *Acinetobacter*, and *Flavobacterium* (Vinas *et al.*, 2002).

Oil-spill bioremediation methods aim at providing favourable conditions of oxygen, pH, temperature and nutrients to maximize biological hydrocarbon breakdown. Such methods are being proposed as a treatment option at many hydrocarbon-contaminated sites such as seashores, airports, soil and power plants (Korda *et al.*, 1997).

The effectiveness of biodegradation is often a function of the microbial population or consortium and how it can be enriched and maintained in the environment. Microorganisms with the ability to degrade hydrocarbon are ubiquitously distributed in the soil and marine environment. The most important aspect is that this technology will permit discrimination of critical biological variables that must be quantified to develop more efficient or controllable biodegradation processes (Sayler and Fox, 1991).

Researches (Baldi *et al.*, 1999; Bento *et al.*, 2003; Bundy *et al.*, 2002; Margesin and Schinner, 2003) have been done internationally on hydrocarbon degradation but not much particularly on diesel degradation. It is worth to consider diesel as a study model since it comprises various species of hydrocarbons. To date, a specific study on diesel has not been done locally in Malaysia. Therefore, this is a good opportunity to study diesel biodegradation using locally isolated strains.

There are five major accomplished objectives in this study. The objectives are:

- 1) To screen, isolate and identify locally available diesel-degrading bacteria.
- 2) To determine the condition of optimum growth of an isolated diesel-degrading microbe.
- 3) To study the degradation of diesel and detect the presence of diesel-degrading enzymes in the isolated strain.

CHAPTER 2

LITERATURE REVIEW

2.1 Petroleum

Petroleum or fossil fuel is formed from incomplete biological decomposition of naturally-occurring organic debris of ancient organisms and microorganisms, mainly plankton and simple plants, which were buried deep within the earth over geologic time (Gauthier *et al.*, 2003).

These organic sediments were slowly decayed under anaerobic conditions where they underwent some physical, chemical, and biological processes due to high temperatures and pressure to produce oil. As rocks were compacted, oil and water were forced out and slowly migrated to porous reservoir rocks, mostly sandstone or limestone. Finally, secondary migration occurred within the reservoir as the oil merged to form a pool, generally capped by impervious strata and often associated with natural gas (Harkavy, 1996). The formation of petroleum requires long periods in the order of millions of years and is still occurring. But, petroleum from different reservoirs varies widely in compositional and physical properties (Hamme *et al.*, 2003).

Since the original decayed organisms contained different elements other than carbon and hydrogen, virtually all crude oils and petroleum products are very complex mixtures. For example, jet fuels can contain over 300 different hydrocarbons

(Cookson, 1995). These hydrocarbons include many indeterminate structures. Various deposits containing oxygen, sulphur, nitrogen, hydrogen and small trace amount of metals, such as lead, manganese, copper and various other additives could also be found (Cole, 1994). After the removal of salt and water, petroleum is refined by fractional distillation, producing a wide range of hydrocarbon-based substances such as natural gas, gasoline, kerosene, diesel oil, fuel oil, lubricating oil, and asphalt (Harkavy, 1996). Petroleum and petroleum derivatives are reported to be the most widely used chemicals in history (Potter, 1993).

2.2 Exploration of Petroleum

The world's first discovery of petroleum is reported to have started ages ago as early as 20,000 B.C. by earliest Mesopotamian civilizations to light torches made from pieces of wood. The Chinese apparently started at around 5,000 B.C when they found oil and used them in medication, waterproofing and warfare. Then the usage of petroleum became widespread by 3,000 B.C in the Middle East where oil seeps were found abundantly on the earth's surface (Harkavy, 1996).

The first oil well in the world was drilled in Titusville, western Pennsylvania in 1859 by Colonel Edwin Drake, a railroad conductor. Since then, the industry now supplies about half of the world's energy, as well as the raw materials for petrochemicals. The chief world oil-producing regions are the Persian Gulf, the U.S., the USSR, northern and western Africa, Mexico, Indonesia and Venezuela (Harkavy, 1996).



Malaysia started the petroleum industry in 1972 when they discovered oil fields offshore in Sabah, Sarawak and Terengganu. The first oil well was drilled by Shell in Miri, Sarawak. The industry has contributed much since then to the Malaysian economy particularly in those three states. This led to the formation of national petroleum company of Malaysia, Petroliam Nasional Berhad (PETRONAS).

2.3 Petroleum Pollutions

Oil and oil products are the most common large-scale environmental pollutants. The widespread use and storage of petroleum fuels has made petroleum hydrocarbons the most dangerous and prevalent contaminant in the biosphere, as they are threatening the health and sustainability of the environment. Gasoline, diesel, fuel oil and also hazardous petroleum compounds such as benzene, toluene, xylene, naphthalene, and some polynuclear aromatics are the most common oil pollutants.

There are two major sources of oil pollution, which have contributed to aquatic environments. One of the sources is the spillage and dumping of waste oil and also from the leakage of oil tank from vehicles onto road surfaces (Zakaria *et al.*, 2002). The other source of oil pollution results from a variety of human activities. These include, manufacturing, machinery and engineering products, bilge cleaning, uncontrolled disposal of oil brines, accidental spillage of industrial oil, domestic and industrial wastes, recreational boating, spills from shipping accidents (Nadim *et al.*, 2001), runoff from land, automobile industrial activities, atmospheric depositions and most frequently caused by mishandling and disposal of organic chemical products (Petrikevich *et al.*, 2003). Furthermore, organic chemicals are also widely