UNIVERSITI PUTRA MALAYSIA

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

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FBSB 2007 5

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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 NH_4Cl, NH_4SO_4, $NaNO_3$, and KNO_3. The best nitrogen source is potassium nitrate (KNO_3) 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 μmol min^{-1} ml^{-1}), alcohol dehydrogenase (9.0956 μmol min^{-1} ml^{-1}), aldehyde dehydrogenase (4.234 μmol min^{-1} ml^{-1}), pyridine nucleotide-independent dehydrogenase (4.229 μmol min^{-1} ml^{-1}) and aldehyde reductase (8.126 μmol min^{-1} 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

FARRAH AINI BT. DAHALAN

Mac 2007

Pengerusi: Mohd Yunus b. Abd Shukor, PhD

Fakulti : Bioteknologi dan Sains Biomolekul

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 penganalisaan 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 sunber nitrogen seperti NH_4Cl, NH_4SO_4, $NaNO_3$, and KNO_3. Ia didapati memerlukan sumber nitrogen iaitu kalium nitrat (KNO_3) 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 μmol min^{-1} ml^{-1}), dehidrogenas alcohol (9.0956 μmol min^{-1} ml^{-1}), dehidrogenas aldehida (4.234 μmol min^{-1} ml^{-1}), enzim penurun aldehida (4.229 μmol min^{-1} ml^{-1}) dan dehidrogenas piridina nukleotida-kendiri (8.126 μmol min^{-1} ml^{-1}).

ACKNOWLEDGEMENTS

In the name of Allah, the Beneficient, the Merciful…

First and foremost, I would like to extend my greatest and deepest gratitude to my supervisor, Dr. Mohd Yunus b. Abd Shukor for his invaluable guidance throughout the preparation of this project until successful completion. My warmest gratitude also goes to Assoc Prof. Dr Radzali b. Muse for his thorough and diligent supervision, guidance, advice, constructive suggestions and review of my work during the period of this study.

I would also like to express my sincere appreciation to the Academy Sciences of Malaysia, Universiti Putra Malaysia and also the Antarctica Research Programme (Grant no: 63597) for financing my entire research.

I would also like to take this opportunity to thank all members, postgraduates as well as the undergraduates of Enzymology and Bioremediation Lab (115 and 204) and especially my fellow seniors: Neni Gusmanizar, Lailatul Juma'yah bt. Sallehuddin, Shahizal Rizwan b. Ahmat Raslan, Nina Suhaity bt Azmi, Norazlan b. Baharom, my course associates: Nor Ayshah Alia bt Ali Hassan, Mohd Nubli b. Suhaimi, Siti Aqlima bt. Ahmad, Wan Surini bt. Wan Hussin, Fazuriana bt Ahmad, Sim Han Koh, En. Ariff b. Khalid, and Natarajan, for their kind assistance and for sharing their experiences and knowledge, directly or indirectly with me.

Also, I would like to render my highest recognition for the contribution of my beloved parents: Dahalan b. Hj. Embun and Norlina Hew bt. Abdullah for their untiring sacrifices, physically, mentally, and spiritually towards the achievement of my academic goal. To both of you my love and admiration, thank you for believing in me and for making me the person I am today.

Lastly, heartfelt appreciation to my beloved husband, Mohamad Syaiful b. Sabtu for his love and support, encouragement who share much of my joy and sorrow.

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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This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee are as follows:

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Date : 14th JUNE 2007

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

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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