UNIVERSITI PUTRA MALAYSIA

ISOLATION AND CHARACTERIZATION OF DIESEL – DEGRADING BACTERIA FROM THE ANTARCTICA

NOR AYSHAH ALIA BINTI ALI HASSAN

FBSB 2006 32

ISOLATION AND CHARACTERIZATION OF DIESEL – DEGRADING BACTERIA FROM THE ANTARCTICA

By

NOR AYSHAH ALIA BINTI ALI HASSAN

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

November 2006

DEDICATED TO MY FAMILY

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

ISOLATION AND CHARACTERIZATION OF DIESEL – DEGRADING BACTERIA FROM THE ANTARCTICA

By

NOR AYSHAH ALIA BINTI ALI HASSAN

November 2006

Chairman : Mohd. Yunus Bin Abd. Shukor, PhD

Faculty : Biotechnology and Biomolecular Sciences

Several isolates of bacteria with diesel-degrading capability have been isolated and characterized from Antarctica. Three strains; isolates J2(p), J7(p) and G(k) were isolated and selected for further investigations. Microbiological analysis such as gram staining and molecular phylogenetics were used to identify the bacteria. Bacterial growth optimization was studied based on carbon source, nitrogen source, pH and temperature. Biodegradation of diesel oil was monitored by quantitative gas chromatography (GC) analysis. The log phase for isolate J2(p) and G(k) were shown in between day four and day six while the log phase for isolate J7(p) was found in between day six until day eighth. Isolate J2(p), J7(p) and G(k) were identified as *Pseudomonas stutzeri*, *Pseudomonas fluorescens* and *Rhodococcus* sp., respectively using substrate utilization patterns (Biolog). Isolate J2(p) and G(k) showed optimum growth at 3% diesel concentration whilst isolate J7(p) was optimum at 2.5% diesel. Isolate J2(p) exhibits an optimum concentration of ammonium sulphate at 2% whilst isolate J7(p) and G(k) exhibit an optimum concentration of ammonium sulphate at 1%. The optimum pH for growth of all isolates J2(p), J7(p) and G(k) were pH 7.13, 7.15 and 7.23, respectively. All the isolates showed that 10°C was the optimum temperature

for bacterial growth. The biodegradation of diesel oil by isolate J2(p) increased in efficiency from the second to the sixth day of incubation, increasing from 1.4 to 18.8% and remain constant until the eighth day. The biodegradation efficiency decreased from 18.8 to 6.3% after the eighth day. The Biodegradation efficiency (BE) of isolate J2(p) was negligible until day 2 where a linear increase in BE to 19% on the sixth day occurs. For isolate J7(p), the biodegradation efficiency was between the second and eighth day of incubation, which increasing from 0.8 to 18.7%. Then it decreased from 18.7 to 9.4% between the eighth day and tenth day of incubation. The biodegradation efficiency of isolate G(k) increase from 0.9 to 17.4% from the second to the sixth day of incubation before decreasing to day ten from 17.4 to 9.9%. Isolate J2(p) was chosen for the screening of enzyme assays. Activity was detected in isolate J2(p). The enzymes activity was distributed in cell-free extracts, soluble fraction and particulate fraction with respective activities for each fraction for *n* - alkane oxidizing enzyme at 0.04, 0.19 and 0.23 µmol/min^{-1}, DCPIP-dependent dehydrogenase at 0.002, 0.006 and 0.02 µmol/min^{-1} and aldehyde reductase activity at 0.02, 0.09 and 0.24 µmol/min^{-1}.

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

PEMENCILAN DAN PENCIRIAN BAKTERIA DEGRADASI DIESEL DARI ANTARTIKA

Oleh

NOR AYSHAH ALIA BINTI ALI HASSAN

November 2006

Pengerusi : **Mohd. Yunus Bin Abd. Shukor, PhD**

Fakulti : **Bioteknologi dan Sains Biomolekul**

Beberapa strain bakteria dari Antartika yang berkeupayaan mendegradasi diesel telah dipencilkan dan di karakterasi. Tiga strain bakteria iaitu strain J2(p), J7(p) dan G(k) telah dipencilkan dan dipilih bagi kajian selanjutnya. Analisis mikrobiologi seperti pewarnaan gram, profil pertumbuhan karbon dan molekular filogenetik telah digunakan bagi mengenalpasti bakteria. Pertumbuhan bakteria yang optimum telah dikaji berdasarkan sumber karbon, sumber nitrogen, pH dan suhu. Biodegradasi diesel telah dikaji menggunakan kromatografi gas. Fasa log bagi strain J2(p) dan G(k) adalah di antara hari keempat dan hari keenam manakala fasa log bagi strain J7(p) adalah di antara hari keenam dan hari kelapan. Dengan menggunakan corak penggunaan substrat (Biolog), strain J2(p), J7(p) dan G(k) telah dikenalpasti sebagai *Pseudomonas stutzeri*, *Pseudomonas fluorescens* dan spesis *Rhodococcus*. Pertumbuhan optimum bagi strain J2(p) dan G(k) berlaku pada 3% kepekatan diesel manakala pertumbuhan strain J7(p) pula optimum pada 2.5% kepekatan diesel. Pertumbuhan strain J2(p) telah dioptimumkan oleh 2% ammonium sulfat manakala 1% ammonium sulfat telah mengoptimumkan pertumbuhan strain J7(p) dan G(k). pH optimum bagi pertumbuhan strain J2(p), J7(p) dan G(k) ialah pH 7.13, 7.15 dan 7.23 . Suhu 10°C adalah optimum untuk pertumbuhan semua strain bakteria. Biodegradasi diesel bagi strain J2(p)

meningkat secara efisien dari hari kedua hingga hari keenam inkubasi dengan peningkatan dari 1.4 hingga 18.8% dan kemudian menjadi sekata sehingga hari kelapan. Biodegradasi secara efisien mengalami penurunan dari 18.8 kepada 6.3% pada hari seterusnya. Bagi strain J2(p), biodegradasi secara efisien menunjukkan peningkatan linear sehingga 19% pada hari keenam. Biodegradasi secara efisien bagi strain J7(p) adalah di antara hari kedua dan hari kelapan inkubasi, meningkat dari 0.8 hingga 18.7%. Kemudian ia mengalami penurunan dari 18.7 hingga 9.4% di antara hari kelapan dan hari kesepuluh inkubasi. Bagi strain G(k), biodegradasi secara efisien meningkat dari 0.9 hingga 17.4% di antara hari kedua hingga hari keenam inkubasi sebelum mengalami penurunan hingga hari kesepuluh iaitu sebanyak 17.4 hingga 9.9%. Strain J2(p) telah dipilih bagi pengassaian enzim. Aktiviti enzim dicerap di dalam ekstrak bebas – sel, fraksi yang larut dan fraksi partikulat iaitu aktiviti bagi setiap fraksi; bagi enzim pengoksidaan *n* – alkane ialah pada 0.04, 0.19 dan 0.23 µmol/ min^{-1} masing-masing, bagi enzim DCPIP – dehidrogenase ialah pada 0.002, 0.006 dan 0.02 µmol/ min^{-1} dan bagi enzim reduktase ialah pada 0.02, 0.09 dan 0.24 µmol/ min^{-1}.

ACKNOWLEDGEMENTS

First and foremost, I am very thankful to Allah for giving me the spirit, health and good wellbeing for me to do this thesis.

I owed my greatest thanks, appreciation and admiration to my family. They have given me their endless support, motivation and love not only during the completion of this thesis, but throughout my lifetime. I'm sure they will continue showering me with their love as I pursuing all my dreams in life. They inspired me to be a better person.

I would like to convey my deepest gratitude and appreciation to my main supervisor, Dr. Mohd. Yunus Abd. Shukor and also not forgotten to my fellow supervisors, Associate Professor Dr. Radzali Muse and Professor Mohd. Arif Syed. Their advices, priceless guidance and patience that they have given me throughout the entire duration has make me become more passionate and dedicated to accomplish this thesis. Their valuable experiences and ideas have helped me a lot for this thesis. Without their help I couldn't possibly finish this thesis. Again, thank you so much.

I also want to thanks my fellow lab 204 members' kak neni, che wan, nubli, lan, farrah, aqlima and fadhil. They helped a lot by sharing their knowledge, ideas and experiences. They also helped me to overcome the obstacles and difficulties that I been through with this thesis.

I certify that an Examination Committee has met on date of viva to conduct the final examination of Nor Ayshah Alia Binti Ali Hassan on her Master of Science thesis entitled "Isolation and Characterization of Diesel – Degrading Bacteria from Antarctica" 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:

Chairman, PhD
Professor
Faculty of Graduate Studies
Universiti Putra Malaysia
(Chairman)

Examiner 1, PhD
Professor
Faculty of Graduate Studies
Universiti Putra Malaysia
(Internal Examiner)

Examiner 2, PhD
Professor
Faculty of Graduate Studies
Universiti Putra Malaysia
(Internal Examiner)

External Examiner, PhD
Professor
Faculty of Graduate Studies
Universiti Putra Malaysia
(External Examiner)

HASANAH MOHD. GHAZALI, PhD
Professor / Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: June 2006

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:

Mohd Yunus Abd. Shukor, PhD
Lecturer
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Radzali Muse, PhD
Associate professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

AINI IDERIS, PhD
Professor / Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 8 March 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.

NOR AYSHAH ALIA BINTI ALI HASSAN

Date: 19 February 2007

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

%	Percentage
°C	Degree centigrade
°F	Fahrenheit degree
µl	Micro liter
µmol	Micromole
BE	Biodegradation efficiency
bp	Base pair
Btu / LB	British thermal unit
C	Carbon
Ca.	Calcium
$CaCl_2$	Calcium chloride
cfu/ml	Colony forming unit per milliliter
cm	Centimeter
CO_2	Carbon dioxide
CoA	Coenzyme A
$CoCl_2 . 6H_2O$	Cobalt chloride hexahydrate
$CuCl_2 . 2H_2O$	Copper (II) chloride dihydrate
$CuSO_4$	Copper sulfate
DCPIP	2-6-Dichlorophenolindophenol
DNA	Deoxyribonucleic acid
DNADIST	Program to compute distance matrix from nucleotide sequences
EDTA	Ethylenediaminetetraacetic acid

$Fe_2SO_4 . 2H_2O$	Ferrous sulfate dehydrate
FID	Flame ionization detector
g / cm^3	Grams per cubic centimeter
g	Gram
GN / GP	Gram negative / Gram positive
H_2O	Water molecule
$H_2PO_4^{-1}$	Dihydrogen phosphate
HAB	Heterotrophic aerobic bacteria
K_2HPO_4	di-potassium phosphate
kg	Kilogram
KH_2PO_4	Potassium orthophosphates
KNO_3	Potassium nitrate
l	Liter
M	Molar
mg	Milligram
$MgCl_2$	Magnesium (II) chloride
$MgSO_4$	Magnesium sulfate
min	Minute(s)
ml	Milliliter
mM	Millimolar
mmol	millimole
$MnCl_2 . 4H_20$	Manganese chloride tetrahydrate
mv	Millivoltan
MW	Molecular weight
N	Nitrogen

Na_2HPO_4	disodium phosphate
NaCl	Sodium chloride
NAD^+	The oxidized form of NAD
NADH	Nicotinamide Adenine Dinucleotide plus Hydrogen
$NADP^+$	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Reduced form of Nicotinamide Adenine Dinucleotide
NH_4SO_4	Ammonium sulfate
nm	Nanometer
O_2	Oxygen molecule
P	Phosphorus
PAH	Polycyclic aromatic hydrocarbons
PCB	Polychlorinated biphenyls
PCR	Polymerase chain reaction
pg	Picogram
pH	Potential of hydrogen ($-\log_{10} [H^+]$)
ppm	Parts per million
rDNA	16S ribosomal DNA
RNA	Ribonucleic acid
rpm	Revolutions per minute
Rrna	Ribosomal RNA
sp.	Species
TCA	Tricarboxylic acid cycle
TPH	Total petroleum hydrocarbon
UCM	Unresolved complex mixture
UPGMA	Unweighted Pair Group Method with Arithmatic Mean

uv	Ultraviolet
W	Watt
x g	Times the force of gravity
$ZnSO_4 . 7H_2O$	Zinc sulphate heptahydrate

CHAPTER I

INTRODUCTION

Antarctica is one of the largest and most pristine wilderness areas left on earth. The main human activities in this area are scientific research, tourism and fishing, and all these activities require fuels for transport and energy. An important fraction of the petroleum hydrocarbon produced worldwide are extracted and processed in cold areas (Ruberto *et al*., 2003). In the Antarctic continent, although petroleum exploitation is not permitted, the important scientific and logistic activities represent high risk of pollution in an environment where temperature and other climate factors, strongly limit bacterial growth and activity.

Antarctic waters are now crossed regularly in summer by tourist, supply and fishing vessels. According to Ferguson *et al*. (2003), oil contamination of soil was also a consequence of the Dry Valley Drilling Project. Three shipping incidents brought the risk of hydrocarbon pollution of polar water into focus. The wreck of the oil tanker *Exxon Valdev* off the Coast of Alaska on 24 March 1989, resulted in the release of large quantities of crude oil. In the Antarctic, the supply ships *Nella Dan* and *Bahia Paraiso* ran aground and subsequently sank off Macquarie Island and near Antarctic Peninsula (Smith and Simpson, 1995; Karl, 1992; Kennicutt *et al*., 1991).

Cripps and Priddle (1991) reported that accidental fuel spills on land occur mainly near scientific stations where storage and refueling of aircraft and vehicles can result in spills. Petroleum hydrocarbons have been detected in soil from McMurdo Station, Scott Base, the former Vanda Station and the old Marble Point camp site within the McMurdo Dry Valley Region, and in soil from Henry Arctowski Polish Station and Palmer Station on the Antarctic Peninsula (Aislabie *et al*., 1998; Tumeo and Wolk, 1994; Kennicutt *et al*., 1992; Krzyszowska, 1990). Generally the areas contaminated by terrestrial fuel spills are localized; however runoff, from soil has contaminated sub-tidal sediments. These were comparatively minor incidents but significant amounts of diesel fuel and lubricating oil were released into the sea and washed ashore.

With the increasing attention towards the preservation of the environment, the applied technologies gained increasing interest. Biodegradation of hydrocarbon-contaminated soils, which exploits the ability of microorganism to degrade and / or detoxify organic contamination, has been established as an efficient, economic, versatile and environmentally sound treatment. Extensive works have been done on isolating hydrocarbon-degrading microbes from polar environments, especially the northern pole for bioremediation purposes. Despite this, the distribution of works on both poles is skewed towards the North Pole with fewer works carried out on the South Pole or the Antarctic. In a previous Antarctic expedition, soils contaminated with diesel have been brought back to Malaysia and the existend of potential diesel-degrading isolates is expected.

Thus, the main objectives of this study are:

1. To isolate diesel-degrading bacteria from soil samples in Antarctica
2. To identify the diesel-degrading bacteria
3. To optimize the growth of the diesel-degrading bacteria
4. To detect diesel-degrading enzymes activities from the selected isolate

CHAPTER II

LITERATURE REVIEW

2.0 Bioremediation

Bioremediation has been defined by Madsen (1991) as "a managed or spontaneous process in which biological, especially microbial, catalysis acts on pollutant compounds, thereby remedying or eliminating environmental contamination." Harmful hydrocarbon contaminants may be assimilated by microorganisms and converted into biomass or transformed by cells or cell-free enzymes (Babel, 1994). These are normal processes in the environment. Amdur and Clark-Clough (1994) reported that under certain conditions, petroleum hydrocarbons, gasoline, diesel fuel, jet fuel, and motor oil can be bioremediated rapidly and at low cost. Bacteria capable of biodegrading petroleum hydrocarbons may commonly be found in subsurface soils. However, natural breakdown of the compounds will occur slowly without intervention to prevent accumulation of the pollutants to unacceptable levels (Lyman *et al*., 1990).

Bioremediation refers to the enhancement of this native capability of the microorganism. According to Catallo and Portier, (1992), the indigenous microbes can be stimulated, or specially developed microorganisms can be added to the site to degrade, transform, or attenuate organic and organometallic compounds to low levels and nontoxic products. Petroleum contaminants can be converted by this method to inert or less harmful materials (Ram *et al*., 1993). Oxygen and nutrients are added to the system to support biological growth and improve the degradation.

Fouhy and Shanley (1992) reported that unlike other techniques that temporarily displace the problem or transfer the contaminants to another medium, bioremediation attempts to render the contaminants into harmless substances. Bioremediation has been found to restore fuel spill-contaminated soil to the point where it could support plant growth in four to six weeks, with complete recovery of the soil in twenty weeks (Wang and Bartha, 1990). Polycyclic aromatic hydrocarbon (PAH) components of diesel oil were completely eliminated in twelve weeks (Wang *et al*., 1990).

According to Piotrowski (1991), bioremediation is receiving considerable attention as a remediation option for sites contaminated with hazardous organic compounds. There are two forms of bioremediation: the microbiological approach and the microbial ecology approach (Piotrowski, 1991). The microbiological approach involves supplying microorganisms that have been conditioned to degrade target compounds, along with appropriate nutrients, to the subsurface. These organisms could be prepackaged "superbugs", which are strains developed in the laboratory and shipped to contaminated area, or they could be site-specific superbugs, which have been isolated from the affected area itself and reintroduced at higher concentrations. The microbial ecology approach, on the other hand, involves altering the environment of the indigenous organisms to optimize their biodegradation of the contaminants.

Bioremediation can also be expressed as being engineered or intrinsic. Any modification of the bioremediation process is considered engineered bioremediation, and the lack of intervention is intrinsic bioremediation, or natural attenuation (Hart, 1996). Morin (1997) reported that intrinsic remediation results from several natural processes, such as biodegradation, abiotic transformation, mechanical dispersion, sorption, and dilution that reduce contaminant concentrations in the environment. For natural attenuation to be viable approach, the site must have a high natural supply of nutrients and oxygen, and the source of contamination must be small (Hart, 1996). Significant evaluation up front and follow-up monitoring are necessary to ensure removal of contaminants of concern at reasonable rates.

2.0.1 Effect of low temperatures on bioremediation

Generally, degradation rates are known to decrease with decreasing temperature. This is believed to be a result, primarily of decreased rates of enzymatic activity the Q10 effect. According to Morita (1975), cold-adapted, psychrophilic and psychrotrophic microorganisms are characterized between $\leq$ 0°C and 20°C, and between 0°C and 35°C, respectively. Since a big part of the biosphere is characterized by cold temperatures, such organisms occur frequently in nature. The biodegradation of many components of petroleum hydrocarbons has been reported in a variety of terrestrial and cold marine ecosystems.

Comparatively, little is known about biodegradation of aromatic and polynuclear aromatic compounds under cold conditions. Whyte *et al*. (1996) reported that