UNIVERSITI PUTRA MALAYSIA

BIODEGRADATION OF DIESEL FUEL BY TWO PSYCHROTOLERANT STRAINS ISOLATED FROM SOUTHERN VICTORIA ISLAND, ANTARCTICA

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

November 2017

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DEDICATION

This thesis is dedicated to my parents.

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

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November 2017

Chairman: Nur Adeela Binti Yasid, PhD
Faculty: Biotechnology and Biomolecular Sciences

Hydrocarbon contamination in Antarctica poses a great threat to the delicate and unique ecosystems of this continent. Bioremediation of hydrocarbon pollutants via utilisation of the indigenous hydrocarbon-degrading bacteria, has been proposed as an environmentally friendly method to clean-up contaminated soils in Antarctica. This study focused on diesel-degrading *Pseudomonas* and *Rhodococcus* species isolated from pristine soils located at the Southern Victoria Island, Antarctica. Isolates were assessed for their ability to grow on diesel as a sole carbon source on solid media at 4°C. Nine isolates showed significant growth in enriched agar after 14 days of incubation. Isolates were then screened to obtain the most promising diesel-degrading strains through colourimetric assay. Two potent isolates that possess rapid utilisation of 0.5% (v/v) diesel were selected and identified as *Pseudomonas* sp. strain ADL15 and *Rhodococcus* sp. strain ADL36. The factors that contribute to the growth of both strains were characterised initially using the conventional 'one-factor-at-a-time' approach. During this stage, the optimal condition for the growth of both ADL15 and ADL36 were at pH 7.0, 20°C, 1.0% (w/v) NaCl, and 1.0 g/L NH_4NO_3. However, strain ADL36 favoured a higher amount of diesel (2.0% (v/v)) for bacterial growth by comparison to ADL15 (1.0% (v/v)). Percentage of dodecane mineralisation was used as the mean to indicate diesel reduction through gas chromatographic analysis. While strain ADL36 showed 83.75% of dodecane mineralisation, the reduction of dodecane by AD15 is merely at 22.39%. Response surface methodology (RSM) based on central composite design (CCD) was used to improve and optimise the effect of significant factors toward the biodegradation of diesel. RSM proved to enhance the reduction of experimented hydrocarbon (dodecane) with a 15% and 16% increment of mineralisation for isolate ADL15 (38.32%) and ADL36 (99.89%), respectively. The results also demonstrated that addition of salt to culture media was the limiting factor in hydrocarbon degradation. Whole genome sequencing showed that ADL15 and ADL36 were closely related to the *Pseudomonas fluorescens* and *Rhodococcus erythropolis* grouping, respectively. Metagenomic analyses revealed the presence of alkane hydroxylases systems which was responsible for alkane degradation in ADL36 but not in ADL15. This founding corresponds to the

gas chromatographic analysis in which ADL36 proved to be a better alkane degrader than ADL15. Detection of the complete pathway of aromatic compound degradation in the latter strain indicates a stronger inclination of the strain to utilise aromatic components in diesel as the carbon source. The presence of putative monooxygenases may also suggest that this strain may utilise specific alkane for their growth. The results from this study showed that strain ADL15 and ADL36 have an excellent potential in bioremediation of aromatics and aliphatics, respectively.

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

BIODEGRADASI MINYAK DIESEL OLEH DUA STRAIN PSIKROTOLERAN YANG DIPENCILKAN DARIPADA PULAU VICTORIA SELATAN, ANTARTIKA

Oleh

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Pencemaran hidrokarbon di Antartika menyebabkan ancaman yang besar kepada ekosistem yang unik dan rapuh di benua ini. Bioremediasi yang menggunakan bakteria yang mampu menguraikan hidrokarbon, telah dicadangkan sebagai kaedah yang mesra alam bagi membersihkan kawasan tercemar di Antartika. Kajian ini memberi tumpuan kepada pengurai minyak diesel daripada spesis *Pseudomonas* dan *Rhodococcus* yang diasingkan daripada tanah yang suci yang terletak di Pulau Victoria Selatan, Antartika. Pencilan dinilai melalui keupayaan mereka untuk menumbuh dengan menggunakan diesel sebagai sumber karbon tunggal pada media pepejal pada 4°C. Sembilan pencilan menunjukkan pertumbuhan yang jelas di dalam agar yang diperkaya selepas 14 hari inkubasi. Pencilan kemudian disaring untuk mendapatkan pencilan pengurai minyak diesel yang paling bagus melalui ujian berwarna. Dua pencilan kekar yang menggunakan 0.5% (v/v) minyak diesel secara pantas telah dipilih dan dikenalpasti sebagai *Pseudomonas* sp. strain ADL15 dan *Rhodococcus* sp. strain ADL36. Faktor-faktor yang menyumbang kepada pertumbuhan kedua-dua strain dicirikan pada mulanya menggunakan pendekatan konvensional 'satu-faktor-pada-satu-masa'. Pada peringkat ini, keadaan optimum pertumbuhan ADL15 dan ADL36 berada pada pH 7.0, 20°C, 1.0% (w/v) NaCl, dan 1.0 g/L NH_4NO_3. Walau bagaimanapun, strain ADL36 menyukai jumlah minyak diesel yang lebih tinggi (2.0% (v/v)) bagi pertumbuhan bakteria berbanding ADL15 (1.0% (v/v)). Peratusan penguraian dodekana digunakan sebagai tanda bagi menunjukkan pengurangan diesel melalui analisis kromatografi gas. Walaupun strain ADL36 menunjukkan 83.75% penguraian dodekana, pengurangan dodekana oleh ADL15 adalah hanya pada 22.39%. Pengkaedahan tindakbalas permukaan (RSM) berdasarkan reka bentuk komposit pusat (CCD) digunakan untuk meningkatkan dan mengoptimumkan kesan faktor-faktor penting kearah penguraian diesel. RSM terbukti dapat meningkatkan pengurangan hidrokarbon (dodekana) yang diuji dengan peningkatan sebanyak 15% dan 16% untuk penurunan dodekana bagi pencilan ADL15 (38.32%) dan ADL36 (99.89%), masing-masing. Keputusan yang diperoleh juga menunjukkan bahawa penambahan garam ke media kultur adalah faktor yang mengurangkan penguraian hidrokarbon. Penjujukan keseluruhan genom menunjukkan bahawa ADL15 dan ADL36 mempunyai kaitan rapat dengan kelompok *Pseudomonas fluorescens* dan *Rhodococcus erythropolis.* Analisis metagenomik mendedahkan

kehadiran sistem alkane hidroksilase yang bertanggungjawab terhadap penguraian alkana di dalam ADL36 tetapi tidak di dalam ADL15. Penemuan ini bersesuaian dengan analisis kromatografi gas dimana ADL36 terbukti menjadi pengurai alkana yang lebih baik daripada ADL15. Pengesanan laluan penguraian hidrokarbon aromatik yang lengkap didalam ADL15 mungkin menunjukkan kecenderungan ADL15 yang lebih kuat untuk menggunakan bahagian aromatik didalam diesel sebagai sumber karbon. Kehadiran monooksigenase putatif mungkin juga menunjukkan bahawa strain ini menggunakan alkana yang khusus untuk pertumbuhan mereka. Keputusan daripada kajian ini menunjukkan bahawa strain ADL15 dan ADL36 mempunyai potensi yang baik didalam bioremediasi hidrokarbon aromatik dan alifatik.

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Syahir Habib, 2017

I certify that a Thesis Examination Committee has met on 30 November 2017 to conduct the final examination of Nur Muhamad Syahir bin Abdul Habib on his thesis entitled "Biodegradation of Diesel Fuel by Two Psychrotolerant Strains Isolated from Southern Victoria Island, Antarctica" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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

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Declaration by Members of Supervisory committee

This is to certify that:

- the research conducted and the writing of the thesis was under our supervision
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LIST OF ABBREVIATIONS

%	Percent
% (v/v)	Percent concentration volume / volume
% (w/v)	Percent concentration weight / volume
ºC	Degree celsius
µl	Microlitre
µm	Micrometre
x g	Relative centrifugal force
bp	Base pair
CCD	Central composite design
CFU	Colony forming unit
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide phosphate
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
et al.,	And friends
g	Gram
GC-FID	Gas chromatography- flame ionisation detector
gDNA	Genomic DNA
g/L	Gram per Litre
HCl	Hydrochloric acid
K	Kelvin
kb	Kilobase
L	Litre
M	Molar
min	Minute
mg/ml	Milligram per millilitre
mM	Milimolar
mm	Millimetre
ng/µl	Nanogram per microlitre
nM	Nanomolar
nm	Nanometer
OD	Optical density
OFAT	One-factor-at-a-time
PAHs	Polycyclic aromatic hydrocarbons
RNA	Ribonucleic acid
rpm	Revolution per minute
rRNA	Ribosomal RNA
RSM	Response surface methodology
sp.	Species (singular)
TAE	Tris-acetate-EDTA

CHAPTER 1

INTRODUCTION

Oil and its refined products represent a significant fraction of the pollution found in the Antarctic region, an area considered as the most pristine in the world (Raymond et al., 2017). The occurrences of pollution are more clustered near former military and industrial spots, scientific research stations, rural communities, and remote airfields, while recent spills and discharges tend to be linked with resource development and mishaps in transportation (Aislabie et al., 2004). Fuel spills are recognised as a potential threat as contamination can cause risks for humans and other living organisms if oil and fuel reach groundwater reservoirs and water bodies (Wang and Bartha, 1990; Jesus et al., 2015). Hydrocarbon contamination in these ecosystems is perceived as damaging as they are more sensitive, being profoundly adapted to extreme conditions (McDonald and Knox, 2014; Yang et al., 2009). Besides, due to the slow natural attenuation rates in cold climates, hydrocarbons can persist for longer periods of time than their temperate counterparts causing a stunted ecosystem recovery (Snape et al., 2008). Although the crude oil extraction from the polar region is declining and a strict legislation such as Antarctic Treaty was introduced, contamination of hydrocarbons in the Antarctic region may still be introduced by the booming numbers of tourists during the austral summer (November to March) period. According to the recent tourism statistics recorded by the International Association of Antarctica Tour Operators (IAATO), there is a 16% increase in the number of tourists landed on Antarctic from the 2015-2016 to 2016-2017 tour (IAATO 2016, 2017). While tourism cannot be solely blamed for their chances of introducing contamination, any small contamination can cause great risks to the environment for a remote and almost pristine land such as Antarctica.

Soil remediation in Antarctica is driven by several critical factors such as cost, strict environmental policy, and remediation constraints (Filler et al., 2006). Attempts to clean-up Antarctic polluted sites using both physical and chemical methods have been done but considered as a minor success. Several methods which highly practical in temperate environments such as thermal incineration, is banned from the Antarctic environment while soil excavation and removal of contaminated soils are often impractical, for the reason of high cost and risks of further damage from excavation (Snape et al., 2008). Bioremediation is widely proposed to remove pollutants from the contaminated Antarctic environment due to the increased interest in using the eco-friendly method as a process of remediating diesel fuel polluted sites (Aislabie et al., 2006; Jesus et al., 2015; Rayner et al., 2007). Bioremediation aids remediation activities being carried out either near or on site, which can be appealing in an isolated contamination spot. However, the effectiveness of this approach depends on strong limitations in temperature, bioavailability, oxygen, toxicity, and soil freeze-thaw cycle (Yang et al., 2009; Delille and Coulon, 2008). Among the factors, temperature plays a significant role in determining the rate and degree of microbial hydrocarbon biodegradation while affecting the volatilisation and viscosity of hydrocarbons (Delille and Coulon, 2008).

Biodegradation of varied components of hydrocarbons at low temperatures in Antarctic soils (Baraniecki et al., 2002; Bej et al., 2000) has been reported and is a result of the degradation capacity of indigenous cold-adapted microorganisms. Cold-adapted microorganisms are able to grow at temperatures around 0°C and have adapted their metabolism to function optimally at low temperatures. These microorganisms play a substantial role in the *in situ* biodegradation of hydrocarbons in cold environments, where ambient summer temperatures often correspond with their growth temperature range. As the Antarctic Treaty prohibits the introduction of non-native organisms, microbes that are indigenous to the Antarctic soil were required for the application of bioremediation (Aislabie et al., 2000). Among microbes, bacterial species play a key role in degrading hydrocarbon pollutants. A large number of hydrocarbon-degrading bacteria from cold soils have been identified, including representatives of gram-negative and gram-positive genera (Aislabie et al., 2000; Ruberto et al., 2005; Shukor et al., 2009). Although large numbers of hydrocarbon-degrading bacteria were isolated from contaminated soils, Margesin et al. (2003) and Stallwood et al. (2005) have observed the occurrence of bacterial species with hydrocarbon-degradative ability in the pristine soil. The addition of indigenous bacteria isolated from Antarctic pristine soil that possess a high competency to degrade diesel fuel may speed up the mineralisation process of petroleum hydrocarbons by several folds when favourable condition is maintained and the regulation of genetic diversity in the bacteria is acknowledged.

Thus, a study was carried out with the following objectives:

1. To isolate and identify bacterial species with hydrocarbon-degrading capacity from the Antarctic pristine soil.
2. To determine the optimum condition for bacterial growth and dodecane mineralisation for isolated strain using statistical analysis and model prediction.
3. To observe the residual hydrocarbon compounds in culture-optimised state qualitatively using gas chromatographic analysis.
4. To analyse the responsible alkane pathways, hydrocarbon-degrading enzymes and their respective genes through bacterial whole genome sequencing.

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