UNIVERSITI PUTRA MALAYSIA

BIODEGRADATION OF PETROLEUM HYDROCARBON BY INDIGENOUS BACTERIA ISOLATED FROM TARBALL AT TERENGGANU BEACH, MALAYSIA

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By

NKEM BRUNO MARTINS

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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DEDICATION

Dedicated to Almighty God and my family

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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June 2020

Chairman : Normala bt. Halimoon, PhD
Faculty : Forestry and Environment

Oil spills occur during exploration, production, vessel operation or accidents, discharge of industrial and municipal wastes. Spilled oil form Tarballs that are deposited on beaches. Oil spill triggers growth of oil-degrading bacteria which can be used for bioremediation by delivering sufficient nutrients and optimizing environmental conditions favorable for their growth. Petroleum pollution negatively impacts on human health, ecosystem and economy by causing birth defects, disrupting food chain, killing fishes and discouraging tourism. This study will aim to isolating and identifying indigenous hydrocarbon-degrading bacteria from tarball in Terengganu Beach, Malaysia. Hydrocarbon-degrading ability of isolated strains were compared to select the most efficient oil-degrading isolate. Diesel-oil biodegradation were optimized using single isolate and consortium of all isolated bacteria by Taguchi method. Biodegradation of diesel-oil was investigated using single most efficient isolate under optimized conditions on laboratory scale. Bioreactor was used to investigate diesel-oil biodegradation using consortium of isolated bacteria under optimized. Isolates were identified by biochemical characteristics and 16S rRNA gene sequence as *Pseudomonas stutzeri* DSM 5190, *Cellulosimicrobium cellulans* ATCC 12830, *Acinetobacter baumannii* CIP 70.34 and *Pseudomonas balearica* SP1402. All isolates are hydrocarbon-degraders but *C. cellulans* exhibited maximum diesel-oil removal of 64.4% in 10 days. Taguchi optimization by *C. cellulans* generated maximum diesel-oil removal of 88.4% and optimal parameters were 2% (v/v) initial diesel concentration, 30.0 gL^{-1} NaCl concentration, 1.0 gL^{-1} NH_4NO_3 concentration, 7.0 pH, 40ºC temperature, 100 rpm agitation speed and 14 days incubation time. However, combined effect of these parameters on diesel-oil degradation was not statistically significant with $P > 0.05$. Biodegradation of diesel-oil by *C. cellulans* recorded 94.6% diesel-oil removal under these optimized conditions after 30 days. The second Taguchi optimization by consortium generated maximum diesel-oil removal of 93.4%. Optimal parameters were 12% (v/v) initial diesel concentration, 40.0 gL^{-1} NaCl concentration, 1.0 gL^{-1} NH_4NO_3 concentration, 7.0 pH, 42^oC temperature, 150 rpm agitation speed and 2.5 mL inoculum size. These parameters combined significantly to improve diesel-oil removal by 90.89% (R^2) using consortium. Bioreactor treatment of diesel-oil using consortium of isolated bacteria removed 98.82% under optimized conditions after 60 days. Bioreactor parameters such as time (days), temperature, dissolved oxygen, pH and bacteria growth combined to significantly improve diesel-oil removal by 69.37% (R^2). Treatment time (days) and bacteria growth were the most significant individual contributors to this improvement. Indigenous hydrocarbon-degraders isolated from tarball exhibited diesel-oil degradation potentials implying they can degrade several hydrocarbon pollutants. Taguchi design produced optimum parameter settings required to achieve the best biodegradation response within the shortest time. Bioreactor treatment using consortium was very effective because different parameters can be manipulated to optimal settings established using Taguchi approach, to achieve maximum bioremediation response. Results from this study can be used to develop unique bioremediation strategy for efficient decontamination of oil polluted sites in minimal time either *ex situ* using bioreactor, or *in situ* by direct application of consortium to contaminated sites.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

BIOPENGURAIAN HIDROKARBON PETROLEUM OLEH BAKTERIA TEMPATAN YANG DIPENCILKAN DARIPADA BEBOLA TAR DI PANTAI TERENGGANU, MALAYSIA

Oleh

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Tumpahan minyak biasanya berlaku ketika eksplorasi, proses pengeluaran, operasi kapal atau kemalangan serta hasil buangan sisa industri dan perbandaran. Minyak yang tertumpah akibat salah satu daripada perkara di atas akan membentuk bebola tar yang akan dihanyutkan ke pantai. Tumpahan minyak ini akan mencetuskan pertumbuhan bakteria yang menguraika minyak yang dapat digunakan untuk bioremediasi dengan memberikan nutrien yang mencukupi dan mengoptimumkan keadaan persekitaran yang baik untuk pertumbuhannya. Apabila berlaku pencemaran minyak, kesihatan manusia, ekosistem dan ekonomi akan terjejas kerana ia akan menyebabkan kecacatan semasa lahir, mengganggu rantai makanan, membunuh ikan dan mengurangkan aktiviti pelancongan. Oleh itu, kajian ini dilakukan untuk mengurangkan kesan negatif tumpahan minyak dengan mengasingkan dan mengenal pasti bakteria yang boleh menguraika hidrokarbon asli daripada bebola tar di Pantai Terengganu, Malaysia. Keupayaan menguraika hidrokarbon daripada *strain* yang diasingkan dibandingkan dengan memilih isolat uraikan minyak yang paling berkesan. Biopenguraian minyak diesel dioptimumkan menggunakan isolat tunggal dan konsortium semua bakteria yang dipencilkan dengan kaedah Taguchi. Biopenguraian minyak diesel dikaji menggunakan satu isolat paling berkesan dalam keadaan yang dioptimumkan pada skala makmal dan bioreaktor digunakan untuk mengkaji Biopenguraian minyak-diesel menggunakan konsortium bakteria terpencil di bawah keadaan yang dioptimumkan. Isolat telah dikenal pasti oleh ciri biokimia dan urutan gen 16S rRNA sebagai *Pseudomonas stutzeri* DSM 5190, *Cellulosimicrobium selulans* ATCC 12830, *Acinetobacter baumannii* CIP 70.34 dan *Pseudomonas balearica* SP1402. Semua isolat adalah pengurai hidrokarbon tetapi *C. selulans* menunjukkan penyingkiran minyak diesel maksimum sebanyak 64.4% dalam tempoh 10 hari. Pengoptimuman Taguchi oleh *C. selulans*

menghasilkan penyingkiran minyak diesel maksimum 88.4% dan parameter optimum adalah kepekatan diesel awal 2% (v / v), kepekatan NaCl 30.0 gL^{-1}, kepekatan 1.0 gL^{-1} NH_4NO_3, pH 7.0, suhu 40°C, kelajuan kocakan 100 rpm dan masa inkubasi 14 hari. Walau bagaimanapun, kesan gabungan parameter ini terhadap pengradasian minyak-diesel tidak signifikan secara statistik dengan P> 0,05. Biopenguraian minyak diesel oleh *C. selulan* mencatatkan penyingkiran minyak diesel sebanyak 94.6% setelah 30 hari dalam keadaan yang dioptimumkan ini. Pengoptimuman Taguchi kedua oleh konsortium menghasilkan penyingkiran minyak diesel maksimum sebanyak 93.4%. Parameter optimum ialah kepekatan diesel awal 12% (v / v), kepekatan NaCl 40.0 gL^{-1}, kepekatan NH_4NO_3 1.0 gL^{-1}, pH 7.0, suhu 42°C, kelajuan kocakan 150 rpm dan ukuran inokulum 2.5 mL. Parameter ini digabungkan secara signifikan untuk meningkatkan penyingkiran minyak diesel sebanyak 90.89% (R^2) menggunakan konsortium. Rawatan bioreaktor diesel-minyak menggunakan konsortium bakteria terpencil dikeluarkan 98.82% dalam keadaan yang dioptimumkan setelah 60 hari. Parameter bioreaktor seperti masa (hari), suhu, oksigen terlarut, pH dan pertumbuhan bakteria digabungkan untuk meningkatkan penyingkiran minyak diesel sebanyak 69.37% (R^2). Masa rawatan (hari) dan pertumbuhan bakteria adalah penyumbang individu yang paling penting untuk peningkatan ini. Pengradasi hidrokarbon asli yang diasingkan daripada bebola tar menunjukkan potensi degradasi minyak-diesel iaitu ia boleh menurunkan beberapa pencemaran hidrokarbon. Reka bentuk Taguchi menghasilkan tetapan parameter optimum yang diperlukan untuk mencapai tindak balas Biopenguraian terbaik dalam masa terpendek. Rawatan bioreaktor menggunakan konsortium sangat berkesan kerana parameter yang berbeza dapat dimanipulasi ke pengaturan optimum yang dibuat menggunakan pendekatan Taguchi, untuk mencapai tindakbalas bioremediasi maksimum. Hasil daripada kajian ini dapat digunakan untuk membina strategi bioremediasi yang unik untuk penyahcemaran bahan-bahan yang tercemar secara efektif dalam waktu minima sama ada *ex situ* menggunakan bioreaktor, atau *in situ* dengan aplikasi konsortium langsung ke lokasi yang tercemar.

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

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This is to confirm that:

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LIST OF ABBREVIATIONS

µL	Microliter
µm	Micrometer
BLAST	Basic Local Alignment Search Tool
BTEX	Benzene, Toluene, Ethylbenzene and Xylene
°C	Degree Celsius
$CFUmL^{-1}$	Colony Forming Unit per Milliliter
Conc.	Concentration
D_C	Total concentration of extracted diesel-oil hydrocarbon in control.
D_S	Total concentration of extracted diesel-oil hydrocarbon in experiment.
DCM	Dichloromethane
DHA	Dehydrogenase Activity
DNA	Deoxyribose Nucleic Acid
dNTP	Deoxynucleoside triphosphates
DOE	Department of Environment
DOE	Design of Experiment
EDTA	Ethylenediaminetetraacetic Acid
EEZ	Exclusive Economic Zone
EPA	Environmental Protection Agency
EOR	Enhanced Oil Recovery
EtBr	Ethidium bromide
FID	Flame Ionization Detector
GC	Gas Chromatography
GC-FID	Gas Chromatography Flame Ionization Detector

GC-MS	Gas Chromatography Mass Spectrometry
gL^{-1}	Gram per liter
h	hours
H_2S	Hydrogen sulfide gas
HDB	Hydrocarbon Degrading Bacteria
HMC PAH's	High Molecular Weight PAH's
IIS	Internal Injection Standard
IOC's	International Oil Companies
LB	Luria Bertani
LMW	Lower Molecular Weight
MARPOL	Marine Pollution Convention
mins	Minutes
MIU	Motility Indole Urease Base Medium
MSM	Minimal Salt Medium
NB	Nominal is best
NCBI	National Center for Biotechnology Information
O and G	Oil and Grease
OD_{600nm}	Optical densities at 600 nm
PAH's	Polycyclic Aromatic Hydrocarbons
PCR	Polymerase Chain Reaction
ppm	Parts per million
PRB	Phenol Red Broth
rRNA	Ribosomal RNA
rpm	Revolutions per minute
SIS	Surrogate Internal Standard
sp.	Species

SMS	Smart Model Selection
SN	Signal Noise
TAE	Tris base, acetic acid and EDTA
TPH	Total Petroleum Hydrocarbon
TSI	Triple Sugar Iron Slant
UCM	Unresolved Complex Mixture
UNCLOS	United Nations Convention on Law of the Sea
WSF	Water Soluble Fractions
v/v	Volume to volume
X_D and R_D	Biodegradation rate
X_C	Hydrocarbon concentration in diesel-oil control culture
X_S	Hydrocarbon concentration of diesel-oil in experimental runs

CHAPTER 1

INTRODUCTION

1.1 Background

The quality of life on earth is inevitably linked to long-term environmental quality. Incessant toxic and bioaccumulative chemicals are persistently released into the environment with pernicious impacts on the environment and human (Beyer *et al*., 2016; Varjani, *et al*., 2017). Petroleum hydrocarbon infiltrates the environment through various routes during exploration, vessel operation, activities of production industries, seeps from pipelines or natural sources, tanker accidents, anthropogenic activities such as effluent wastewater, industrial and municipal wastes (Varjani *et al*., 2017). Petroleum refined products such as asphalt, gasoline, diesel-oil, natural gas, fuel, kerosene and lubricating oil have been reported to be the most widely used chemicals in history (Chaudhuri, 2016). Interest in the use of petroleum and its products in Malaysia elevated rapidly within the past decade owing to rise in population, industrialization and urbanization. Individual car ownership has quadrupled over the years (Zakaria *et al*., 2018).

Malaysian coastal water experienced multiple cases of shipping accidents with tons of crude oil discharged into the surrounding marine waters. Shipping lane of the Straits of Malacca is a regularly used route for vessels with an average of 150 ships passing through each day, mostly comprising of tankers, cargo ships and other vessels (Ishak and Mohalid, 2018; Keshavarzifard and Zakaria, 2015; Omar, 2015). Oil spilled from these sources oil breaks up into slicks and form Tarballs which are transported several distances from the original source (Bacosa *et al*., 2016). Tarballs are formed from oil slicks which accumulate debris, aggregate to form dark-colored, mousse-like balls. Tarballs contain several recalcitrant hydrocarbons that persist in the environment and eventually deposited on coastal areas (Payne and Phillips, 2018).

Tarballs are part of petroleum-based pollutants ranked amongst the most common causes of environmental degradation in the modern world (Payne and Phillips, 2018). In enormous concentrations, their hydrocarbon components are extremely harmful to numerous life forms, including humans (Chaqda *et al*., 2019). Impacts on humans are associated with pollution of groundwater and contamination of drinking water. This introduces carcinogens to the human body when consumed. It also causes birth defects and degenerative diseases such as neuropathy (Mobilik and Hassan, 2016). Economic impacts of oil pollution include impairment of infrastructures, tourism, and aquaculture within a locality with persistent long-term effects (Rengarajan *et al*., 2015).

Ecological impacts of oil pollution are associated with impairment of marine lifeforms which includes birds, marine organisms, fishes and plants (Varjani, 2017). Oil slicks cause breakdown of feathers and furs of seals, otters or other birds causing hypothermia and even death (Adzigbli and Yuewen, 2018). Tarballs look like food for some marine life or fishes. Ingestion of as little as 4000 parts per million (ppm) is lethal to these organisms (Payne and Phillips, 2018). Primary producers within the marine ecosystem are also killed by oil, thereby threatening the existence of some marine organisms (Varjani, *et al*., 2017). Eggs of some fishes, birds or reptiles become thinner after prolonged exposure to oil. Sea grass and algae become polluted causing long-term effects on the ecosystem (Ishak *et al*., 2018; Adzigbli and Yuewen, 2018).

Introduction of petroleum hydrocarbon in the environment triggers growth of hydrocarbon-degrading microorganisms (Al-Hawash *et al*., 2018; Varjani, 2017). The practice of utilizing indigenous or deliberately introduced microbes for removal of pollutants is termed bioremediation (Xue *et al*., 2016). Bioremediation techniques are green, cost-effective alternative remedy for efficient decontamination of hydrocarbon polluted environments (Al-Hawash *et al*., 2018; Varjani, 2017). Some commonly known hydrocarbon-degraders include *Acinetobacter species*, *Pseudomonas* sp., *Achromobacter* sp., *Norcadia* sp. and *Vibrio* sp., *Marinobacter* sp., *Actinomycetes* sp., *Alcanivorax* sp., *Arthrobacter* sp., *Bacillus* sp., and *Geobacillus* sp. (Catania *et al*., 2018; Crisafi *et al.,* 2016; Varjani, 2017).

Current bioremediation strategies used in Malaysia based on site application are grouped into *In situ* or *Ex situ* methods. *In situ* techniques includes pump-and-treat method, soil vapor extraction, air stripping and thermal desorption (Guarino *et al*., 2017). Pump-and-treat systems are used to clean-up hydrocarbon polluted groundwater using drilled pump wells through which polluted water are brought to the surface for treatment. This system is inefficient because many contaminants are trapped in the subsurface, requiring complete flushing of enormous water volumes over a long period of time (Azubuike *et al*., 2016; Borden, 2017). Soil vapor extraction and air stripping have also been used to clean-up contaminated soil and water, respectively. In both cases, treatment of hydrocarbon polluted soil or water is carried out by drawing air through pumps into the soil or water to mix with hydrocarbon pollutants which are then drawn out as contaminated vapors (Azubuike *et al*., 2016; Lim *et al*., 2016). This method transfers pollutants to either land, water or air rather than destroying them completely. Some polluted air might escape without treatment into the atmosphere thereby endangering public health. It also requires large portions of land, implying the technique is expensive to operate (Islam, 2015; Azubuike *et al*., 2016). Thermal desorption is another method currently used to treat oil-contaminated soil or sediments by vaporizing the oil pollutant with heat. This method also endangers public health, requires manual labor, intensive monitoring and high cost of maintenance (Liu *et al*., 2019a; Brown *et al*., 2017; Crisafi *et al*., 2016). In general, *In* situ bioremediation methods are limited by their requirement of enormous land mass that is expensive to acquire. They can also be time-consuming and site disrupting. Vapor or gas emissions could also impact workers with long-term public health concerns (Liu *et al*., 2019a; Adams *et al*., 2015).

Recent trends aimed at improving effectiveness of *In situ* methods through natural attenuation, biostimulation and bioaugmentation also have some limitations. Natural attenuation involves elimination of hydrocarbon pollutants with naturally occurring microbes. However, it is limited by presence or absence of nutrients as well as favorable environmental conditions (Borden, 2017; Chikere *et al*., 2017). Biostimulation reinforces contaminated sites with nutrients needed to support growth and metabolic activities of indigenous hydrocarbon degraders. The main limitation is dependency on local geologic profile of the subsurface which determines the effectiveness of nutrient delivery to subsurface microbes (Abed *et al*., 2015; Dias *et al*., 2015). Bioaugmentation requires the introduction of specific microbes with proven ability to degrade pollutants into a contaminated site. This can be limited by the fact that introduced microbes might not be adapted to the new ecosystem (Abtahi *et al*., 2020). These *In situ* strategies might not be effective because pollutants could sorb to surfaces making them difficult to be acted upon by either naturally-occurring or introduced microbes. This means they are not effective for highly impacted sites (Azubuike *et al*., 2016; Adams *et al*., 2015).

Ex situ bioremediation methods currently used are land farming, biopiling and composting (Balseiro-Romero *et al*., 2019). Land farming involves removal of oil pollutants by removing pollutants to a new site where they are spread on soil to be degraded by indigenous hydrocarbon-degrading bacteria (Kuppusamy *et al*., 2020). Biopiling is like land farming but the new sites are equipped with punctured sub-drain that gathers leachate, suck air into the biopile by vacuum application and invigorate microbial development (Smith *et al*., 2015). Composting involves adding soil amendments in form of nutrients to arouse microbial growth (Pereira *et al*., 2018). These *ex situ* methods are limited by their requirement of enormous labor, and large land mass which makes them expensive. They are also difficult to control leading to incomplete degradation of heavy pollutants since certain conditions can’t be manipulated. Potential exposure of on-site workers to emissions pose public health concerns (Abatenh *et al*., 2017; Roy *et al*., 2018).

This study proposes promising bioremediation strategy where bioreactor systems are operated under optimized environmental parameters developed by Taguchi model to degrade hydrocarbon-pollutants using indigenous bacteria consortium isolated from Tarball (Arora *et al*., 2018). This strategy is more efficient and environmentally friendly since it employs naturally occurring bacteria in Tarball. This strategy is relatively cheap, less labor-intensive and does not require additional chemicals or large land mass. However, it guarantees public health safety with complete destruction of pollutants achieved within the shortest possible time (Safdari *et al*., 2018). This bioremediation strategy is relatively new due to combination of indigenous bacteria in tarball, Taguchi optimization and bioreactor treatment for degradation of hydrocarbon pollutants.

1.2 Justification

Past studies have indicated that indigenous bacteria isolated from oil-impacted sites composed of heavy and light hydrocarbon fractions had capacity to degrade hydrocarbons (Cappello *et al*., 2015). Indigenous bacteria from these sites can utilize specific hydrocarbons found on those sites for growth while degrading them (Brown *et al*., 2017; Prince *et al*., 2017). The present study highlights potential use of uniquely adapted hydrocarbon-degrading bacteria from Tarball for development of highly efficient bioreactor-based bioremediation strategy. In this study, indigenous oil-degrading bacteria isolated from Tarball could potentially have greater hydrocarbon-degrading capacity than those isolated from other polluted sites since they thrived on heavily weathered hydrocarbon fractions (Priyanka *et al*., 2019). These bacteria are uniquely adapted to harsh environmental conditions which resulted in loss of volatile hydrocarbon fractions through evaporation, dissolution, photooxidation, emulsification, sedimentation, and wind dispersal (Payne and Phillips, 2018; Rekadwad and Khobragade, 2015).

This study also proposes the use of Taguchi approach for optimization hydrocarbon biodegradation by manipulating different growth and environmental parameters. Currently used optimization methods studies are tedious and time-consuming because parameters are manipulated one by one (Freddi and Salmon, 2019). This means several experiments must be undertaken which increases the margin for error. Taguchi method uses accurately designed model to manipulate several parameters at the same time using fewer experiments. This reduces margin for error and increases possibility of real-time application in the field (Notowidjaja *et al*., 2019). Taguchi method can be used to predict maximum or minimum hydrocarbon removal by set of parameter settings that can be related to large-scale application (Dhawane *et al*., 2017). Taguchi method can also be used to quantitatively estimate the size and significance of contributions for each investigated parameter with minimal number of experiments (Messaoudene, 2010). Knowledge acquired from this optimization method can be used to eliminate or select the most important parameters that significantly improve bioremediation process.

This study, however, focus on bioreactor treatment of pollutants which involves breakdown of pollutants in a tank using indigenous bacteria isolated from Tarball. This means parameters such as bacteria growth, environmental conditions and nutrient delivery can all be manipulated to achieve efficient pollutant removal (Safdari *et al*., 2018). This strategy is more efficient than currently implemented methods described in the previous section because it assures long term public health protection by guaranteeing complete pollutant removal while minimizing exposure of workers to pollutants (Bhattacharya *et al*., 2015). It also reduces duration for treatment by selectively manipulating parameters that significantly improved bioremediation. Labor requirement is minimal and it does not require large land masses (Kuyukina *et al*., 2020; Arora *et al*., 2018; Bhattacharya *et al*., 2015). This strategy is an improvement on currently used methods because it combines the metabolic ability of indigenous bacteria in tarball with delivery of optimal nutrient and environmental conditions in bioreactor system where these conditions can be manipulated to achieve complete destruction of pollutants rather than transforming them to other forms. Environmental conditions such as pH, temperature, dissolved oxygen, salinity and specific nutrient formulations can be regulated to achieve optimal biodegradation using this strategy.

1.3 Research Objectives

This study is aimed at isolating and identifying indigenous hydrocarbon-degraders from oil-polluted tarball, establish optimal environmental and growth conditions favorable for their metabolic activities and evaluate their biodegradation capacity in a bioreactor. The specific objectives are:

1. To isolate and identify indigenous hydrocarbon-degrading bacteria from oil-impacted Tarball.
2. To investigate potential hydrocarbon-degrading ability of bacteria isolated from Tarball.
3. To optimize factors affecting diesel-oil biodegradation using single and consortium of isolated bacteria by Taguchi method.
4. To evaluate diesel-oil biodegradation by consortium of isolated bacteria under optimized conditions in Bioreactor.

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BIODATA OF STUDENT

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LIST OF PUBLICATIONS

Nkem, B. M., Halimoon, N., Yusoff, F. M., and Johari, W. L. W. (2019). Isolation and optimization of diesel-oil biodegradation using cellulosimicrobium cellulans from tarball. *Pertanika Journal of Science and Technology*, *27*(3), 1031–1040.

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