UNIVERSITI PUTRA MALAYSIA

BIODEGRADATION OF PHENANTHRENE AND PYRENE USING BACTERIA ISOLATED FROM USED VEHICLE LUBRICANTCONTAMINATED SOIL

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By

UMAR ZUBAIRU DARMA

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

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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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Chairman : Associate Professor Muskhazli Mustafa, PhD
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Petroleum industry contributes significantly towards global industrial civilization. Used vehicle lubricant oil being a petroleum product portrays dangerous impact on the environment as it generates hazardous polycyclic aromatic hydrocarbons (PAHs) through incomplete combustion. The rampant discharge of this oil in Malaysia causes serious environmental concern as PAHs are dangerous organic pollutants that cause mutation and cancer to living cells. The most commonly hazardous PAHs from used lubricating oil were phenanthrene and pyrene belonging to the low and high molecular weights PAHs. Biological removal of phenanthrene and pyrene provides an environmentally sound and state-of-the-art technique that employs natural biological processes for the complete elimination of such pollutants from the environment. However, slow degradation duration is a major PAHs biodegradation limitation. Therefore, the research objectives involved isolation of effective phenanthrene and pyrene degrading bacteria, optimizing the biodegradation conditions, analysing the biodegradation intermediates and evaluating the effects of heavy metals on the biodegradation effectiveness.

Phenanthrene and pyrene degrading bacteria were initially isolated from used lubricating oil contaminated soil using enrichment and plating techniques where both PAHs served as the sole bacteria carbon and energy sources. The effective phenanthrene and pyrene degrading bacteria were screened based on spray plate technique and colorimetric assay followed by 16S rRNA gene identification using universal primers. The biodegradation conditions of such identified bacteria were then optimized using single factor optimization before subjected to response surface methodology based on full factorial central composite design ($p \leq 0.05$). This was preceded with the analyses of phenanthrene and pyrene degrading metabolites using gas chromatography mass spectrophotometer (GC-MS). The identified bacteria were finally used for the formulation of bacteria consortium after the compatibility testing

based on cross spread technique and permutation assessments through colorimetric assay. The consortium was then used to degrade 500 mg/L phenanthrene and 250 mg/L pyrene in a complex culture that contained varying concentrations (2 mg/L to 12 mg/L) of Nickel, Lead, Vanadium and Cadmium separately.

Total of 93 different strains of bacteria were isolated from the enrichment technique. Among these bacteria, only 53 strains initiated the degradation of 5 g/L phenanthrene in 72 hours based on spray plate assessments ($n = 3$). Further screening by colorimetric assay indicated only two strains named MM045 and MM087 were able to degrade 75.2% and 80.2% of 500 mg/L phenanthrene in addition to 54.3% and 59.7% of 250 mg/L pyrene within 24 hours respectively ($n = 3$). These strains were then identified as *Cronobacter sakazakii* MM045 and *Enterobacter* sp. MM087 with accession numbers KT933253 and KT933254 respectively. The phenanthrene and pyrene degradation capabilities of *C. sakazakii* MM045 and *Enterobacter* sp. MM087 was statistically optimized through response surface methodology ($p \leq 0.05$). This optimization showed the combined efforts of the independent variables from each of *C. sakazakii* MM045 and *Enterobacter* sp. MM087 culture resulted in 100% degradations of both phenanthrene (500 mg/L) and pyrene (250 mg/L) in 24 hours. These were validated experimentally using the numerical optimization analyses ($n = 3$). The phenanthrene and pyrene biodegradation intermediates were then identified using the GC-MS analyses ($n = 3$). The pyrene identified metabolites from the *C. sakazakii* MM045 and *Enterobacter* sp. MM087 degradation cultures include pyrene *cis*-4,5-dihydrodiol, 3,4-dihydroxyphenanthrene, phthalic acid, pyruvic acid, acetic acid, lactic acid and formic acid. Additionally, phenanthrene identified metabolites from both cultures were 3,4-dihydroxyphenathrene, phthalic acid, pyruvic acid, acetic acid and oxalic acid. These metabolites established the degradation pathways undergone by both bacteria. The bacteria were also found to tolerate more than 6 mg/L of Nickel, Cadmium, Lead and Vanadium which significantly exceeded the hazardous metals concentrations in the natural habitats were both bacteria survived prior to the isolation.

Considering the effective phenanthrene and pyrene degradation responses of *C. sakazakii* MM045 and that of *Enterobacter* sp. MM087 in 24 hours, both isolates can be used for commercial applications of degrading PAHs from contaminated environments. This commercial application could be possible as all the optimized independent variables are achievable within natural environments such as soil and water bodies. Furthermore, environmental availability of the *C. sakazakii* and *Enterobacter* sp. portrays another desirable character that made their choice as the best degradation alternative. Additionally, both bacteria can maintain their degradation effectiveness even in a complex environment that contained concurrent contamination of PAHs and heavy metals. Therefore, *C. sakazakii* MM045 and *Enterobacter* sp. MM087 can effectively and rapidly degrade phenanthrene and pyrene from used vehicle lubricant contaminated environment.

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

BIODEGRADASI PHINANTRIN DAN PIRIN MENGGUNAKAN BAKTERIA TERASING DARIPADA MINYAK LUBRIKAN KENDERAAN TERKONTAMINASI

Oleh

UMAR ZUBAIRU DARMA

Julai 2017

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Industri petroleum menyumbang secara signifikan ke arah tamadun perindustrian global. Minyak pelincir kenderaan yang digunakan sebagai produk petroleum memberikan gambaran kesan berbahaya terhadap alam sekitar kerana ia menghasilkan hidrokarbon aromatik polisiklik (PAHs) yang berbahaya melalui pembakaran tak lengkap. Pelepasan minyak ini secara meluas di Malaysia menyebabkan kebimbangan yang serius terhadap alam sekitar kerana PAHs adalah bahan pencemar organik berbahaya yang mampu menyebabkan mutasi dan kanser terhadap sel hidup. Jenis PAH yang paling kerap diperolehi dari minyak pelincir yang digunakan adalah phenantren dan pirin dari kumpulan PAHs dengan berat molekul yang tinggi dan rendah. Penyingkiran phenantren dan pirin secara biologi adalah teknik yang mesra alam dan terkini menggunakan proses biologi semula jadi untuk menghapuskan bahan pencemar sedemikian secara menyeluruh dari alam sekitar. Walau bagaimanapun, tempoh biodegradasi yang perlahan adalah pengehadan biodegradasi PAH yang utama. Oleh itu, objektif penyelidikan ini melibatkan pemisahan bakteria efektif yang merendahkan phenantren dan pirin, mengoptimumkan keadaan biodegradasi, menganalisis perantaraan biodegradasi dan menilai kesan logam berat terhadap keberkesanan biodegradasi.

Bakteria phenantren dan pirin yang terdegradasi pada mulanya diasingkan dari tanah yang tercemar oleh minyak pelincir yang digunakan menggunakan teknik pengayaan dan penyaduran di mana kedua-dua PAH berfungsi sebagai karbon tunggal dan sumber tenaga bakteria. Bakteria phenantren dan pirin yang merosakkan bakteria ditapis menggunakan teknik plat semburan dan cerakin kolorimeter diikuti pengenalan gen rRNA 16S menggunakan primer universal. Keadaan biodegradasi bakteria yang dikenal pasti kemudian dioptimumkan menggunakan pengoptimuman faktor tunggal sebelum diletakkan pada metodologi respon permukaan berdasarkan reka bentuk komposit pusat faktorial penuh ($p \leq 0.05$). Ini didahului dengan analisis metabolit yang

merendahkan phenantren dan pirin menggunakan spektrofotometer jisim kromatografi gas (GC-MS). Bakteria yang dikenal pasti akhirnya digunakan untuk perumusan konsortium bakteria selepas ujian keserasian berdasarkan teknik rebakan silang dan penilaian permutasi melalui ujian warna. Konsortium kemudiannya digunakan untuk menurunkan phenantrena 500 mg/L dan 250 mg/L pirin dalam kultur kompleks yang mengandungi Nikel, Plumbum, Vanadium dan Kadmium dengan kepekatan yang berbeza-beza (2 mg/L hingga 12 mg/L) secara berasingan.

Sejumlah 93 jenis bakteria yang berbeza telah diasingkan menggunakan teknik pengayaan. Antara bakteria ini, hanya 53 strain sahaja memulakan degradasi 5 g/L phenantrena dalam 72 jam berdasarkan penilaian plat semburan (n = 3). Penyaringan selanjutnya oleh cerakin kolorimeter menunjukkan hanya dua strain iaitu MM045 dan MM087 yang mampu menurunkan 75.2% dan 80.2% daripada 500 mg/L phenantrena serta 54.3% dan 59.7% daripada 250 mg/L pirin dalam tempoh 24 jam (n = 3). Strain ini kemudian dikenal pasti sebagai *Cronobacter sakazakii* MM045 dan *Enterobacter sp*. MM087 dengan nombor kemasukan KT933253 dan KT933254 masing-masing. Keupayaan degradasi phenantren dan piren oleh *C. sakazakii* MM045 dan *Enterobacter sp*. MM087 dioptimumkan secara statistik melalui metodologi permukaan tindak balas ($p \leq 0.05$). Pengoptimuman ini menunjukkan usaha gabungan pembolehubah bebas dari kultur *C. sakazakii* MM045 dan *Enterobacter sp.* MM087 dalam mendegradasi 100% phenantrena (500 mg/L) dan pirin (250 mg/L) dalam masa 24 jam. Ini telah disahkan secara eksperimen menggunakan analisis pengoptimuman berangka (n = 3). Perantara biodegradasi phenantren dan pirin kemudiannya dikenal pasti menggunakan analisis GC-MS (n = 3). Metabolit pirin yang dikenal pasti dari kultur degradasi *C. sakazakii* MM045 dan *Enterobacter sp*. MM087 termasuk pirin cis-4,5-dihydrodiol, 3,4-dihydroxyphenantren, asid ftalik, asid piruvik, asid asetik, asid laktik dan asid formik. Selain itu, metabolit phenantren yang dikenal pasti dari kedua-dua kultur adalah 3,4-dihydroxyphenantren, asid ftalik, asid piruvik, asid asetik dan asid osalik. Metabolit ini menubuhkan laluan degradasi yang dialami oleh kedua-dua bakteria. Bakteria juga didapati menoleransi lebih daripada 6 mg/L Nikel, Kadmium, Plumbum dan Vanadium yang melebihi kepekatan logam berbahaya di habitat semulajadi. Kedua-dua bakteria terselamat sebelum pengasingan.

Disebabkan keberkesanan tindak balas degradasi phenantren dan pirin oleh *C. sakazakii* MM045 dan *Enterobacter sp*. MM087 dalam masa 24 jam, kedua-dua pencilan boleh digunakan untuk aplikasi komersil dalam merendahkan PAH daripada persekitaran yang tercemar. Aplikasi komersil ini boleh dilakukan memandangkan semua pemboleh ubah bebas yang optimum dapat dicapai dalam persekitaran semula jadi seperti tanah dan hidupan air. Tambahan pula, ketersediaan alam sekitar *C. sakazakii* dan *Enterobacter sp.* menggambarkan ciri terpilih yang menjadikan mereka sebagai pilihan degradasi alternatif yang terbaik. Selain itu, kedua-dua bakteria boleh mengekalkan keberkesanannya walaupun dalam persekitaran yang kompleks yang mengandungi pencemaran serentak PAH dan logam berat. Oleh itu, *C. sakazakii* MM045 dan *Enterobacter sp.* MM087 dengan berkesan dan cepat boleh menghancurkan phenantren dan pirin daripada persekitaran tercemar oleh pelincir kenderaan yang digunakan.

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This thesis was submitted to the Senate of 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:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
As	Arsenic
ATSDR	Agency for Toxic Substances and Disease Registry
BHA	Bushnell Haas Agar
bp	Base pairs
CC	Column Chromatography
CCD	Central Composite Design
Cd	Cadmium
CFU	Colony forming unit
cm	Centimetre
Co	Cobalt
Cr	Chromium
CRMs	Certified reference materials
Cu	Copper
CV	Coefficient of variation
^{0}C	Degree Celsius
DF	Degrees of freedom
DCPIP	Dichlorophenolindophenol
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotides
EU	European Union
F-primer	Forward primer
FRLI	Federal Register of Legislative Instruments
g	Gram
GC-MS	Gas Chromatography- Mass Spectrometry

GDP	Gross domestic products
h	Hour
HMW	High Molecular Weight
HPLC	High performance liquid chromatography
HSD	Honest significant difference
ICP-OES	Inductively coupled plasma optical emission spectrophotometer
ISO	International standard organisation
IV	Inoculums volume
kDa	Kilo Dalton (unified atomic mass unit)
kg	Kilogram
L	Litre
LB	Luria-Bertani
LMW	Low Molecular Weight
LOQ	Limit of quantification
mΩ	Milliohm
mm^3	Millimetre cube
$MgCl_2$	Magnesium Chloride
MEGA	Molecular Evolutionary Genetics Analysis
MEQR	Malaysian environmental quality report
mg	Milligram
min	Minute
mL	Millilitre
Mn	Manganese
MS	Mean square
MSM	Mineral Salts Medium
MT	Metric tonne

m/z	Mass per charge
NaCl	Sodium chloride
NADP	Nicotinamide adenine dinucleotide phosphate
NCBI	National Centre for Biotechnology Information
Ni	Nickel
nm	Nanometer
NIST	National institute of standards and technology
OD_{600}	Optical Density at 600nm wavelength
PAHs	Polycyclic Aromatic Hydrocarbons
Pb	Lead
PBS	Phosphate buffer salinre
PCR	Polymerase Chain Reaction
pH	Hydrogen ion concentration
PHN	Phenanthrene
PYR	Pyrene
R^2	Coefficient of determination
rpm	Revolution per minute
R-primer	Reverse primer
rRNA	Ribosomal ribonucleic acid
RSM	Response Surface Methodology
sec	Second
SFO	Single factor optimization
SRM	Standard reference material
SS	Sum of the square
TAE	Tri-acetic acid-Ethylenediaminetetraacetic acid
TRI	Texas Research Institute

U	Unit
US EPA	United States Environmental Protection Agency
US FDA	US Food and drugs administration
V	Vanadium
v/v	Volume per volume
WHO	World Health Organization.
wt	Weight
w/v	Weight per volume
Zn	Zinc
α	Alpha
β	Beta
3D	Three dimension
%	Percent
µL	Microlitre

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Polycyclic aromatic hydrocarbons (PAHs) comprised large group of hazardous chemicals that resulted from incomplete combustion of organic products (Zhao *et al*., 2008). Naturally, PAHs are sourced from forest fires, oil seepage and volcanoes while anthropogenic sources involved incineration, internal engine combustion, tobacco cigarette, carbon black, coal tar and asphalt (Feng *et al*., 2012). These sources directly deposit toxic PAHs into the environment which persist for long duration due to chemical stability and low water solubility (Maliszewska, 1999). Such persistence has tremendously contributed in the bioaccumulation, adsorption, desorption and re-suspension of PAHs within different ecosystems (Arulazhagan and Vasudevan, 2011). However, very little proportion of such toxic PAHs undergo natural transformations while the larger part posed serious environmental concern as human health is the most critically affected (Lundstedt *et al.*, 2007; Bispo *et al*., 1999). Hence, the current environmental challenges on PAHs need compliance with international best practices that eliminate lasting hazardous effects.

Environmental pollution involving used lubricating oil were reported in different parts of Malaysia where Selangor State recorded the highest reported cases (Mohd Radzi *et al*., 2016; Keshavarzifard and Zakaria, 2015; Sany *et al*., 2014). This created public health concerns due to the frequency of its occurrence and the severity it affects plants, animals, humans and the environments (Spinelli and Freitas, 2005; Akintunde *et al.*, 2015). Such oil damages animals' central nervous system and plants nutritional pathway (Todd *et al.*, 1999). Human being is also affected by used lubricating oil through the skin contact which absorbs PAHs into the bloodstream and eventually converts them into electrophilic derivatives that become cancer (Todd *et al.*, 1999). Additionally, humans are also affected on their central nervous systems which create disorderliness on the nerve, a situation termed as peripheral neuropathy (Todd *et al.*, 1999). Such situation causes feet and legs numbness which eventually becomes paralysis (Todd *et al.*, 1999). However, inadequate information on the toxicity of used lubricating oil and its post-disposal behaviour has further complicated issues as even sites not intended for its disposal are grossly affected (Oa and Lee, 2009; Park *et al.*, 2009; 2010).

Among the most hazardous PAHs commonly found in used lubricating oil are phenanthrene and pyrene which contained three and four aromatic structures arranged in angular and cluster forms respectively (Hossain and Salehuddin, 2012). These were classified as low and high molecular weights PAHs considering their number of aromatic rings (Wick *et al*., 2011). Phenanthrene was reported to be a dangerous animal's skin photosensitizer which is characterized with mild allergen while pyrene has much toxicity effect on animal's body as it causes neurological disorder, lesions

on the kidney and reduces body weight (CCME, 2010; Patri *et al*., 2010; Moody *et al*., 2001).

The phenanthrene and pyrene conventional degradations were faced with high technological implication which generates more toxic intermediates (Park *et al.,* 2009; 2010). The biological removal is the scientifically proven technique due to its complete removal response and low cost implication when compared to conventional alternatives (Abdulsalam and Omale, 2009; Karamalidis *et al.,* 2010). In the biological removal, efficient microorganisms are utilized due to high abundance, species diversity, catabolic versatility and adaptation capability to adverse conditions (Moraes *et al.*, 2009). Bacteria were reported as the best microorganisms that that occupy suitable niche in used lubricating oil contaminated site due to broad enzymes possession that enable them utilize the PAHs as sole carbon sources (Madigan *et al*., 1998). Previous studies indicates the PAHs removing capabilities of bacteria involving *Mycobacterium, Arthrobacter, Burkholderia, Sphingomonas,* and *Pseudomonas* (Baboshin *et al*., 2008; Seo *et al.*, 2006; Kim *et al*., 2003).

1.2 Problem statement

Despite the global acceptability of PAHs biodegradation, many degrading bacteria are faced with challenges that limit their rapid degradation responses (Seo *et al*., 2009). Such limitations were caused by PAHs resistance to microbial attack, extended lag growth phase of the degrading isolates and unfavorable biodegradation conditions (Fankhauser, 2004). Among the most dangerous PAHs classified by USEPA were phenanthrene and pyrene which contained threne and four benzene rings respectively (ASTDR, 2005). These phenanthrene and pyrene causes skin photosensitization, kidney and brain damages to human while destroying the soil and water ecosystems (Mesdhaghinia *et al.,* 2005; Patri *et al.,* 2010).

All the phenanthrene and pyrene degrading bacteria are very sensitive to physico-chemical conditions whose little alteration will negatively affect their metabolic activities (Leahy and Colwell, 1990). Such alteration will minimize degradation efficiency of the bacteria which subsequently will cause low enzymes production that may extend the isolates lag growth phase (Fankhauser, 2004; Richnow *et al*., 1994). In this situation, PAHs intracellular localization is reduced which further complicates the entire biodegradation process as many bacteria survived at sole expense of PAHs (Chauhan *et al*., 2008). However, optimizing the physico-chemical biodegradation conditions using statistically based approach is the best alternative to enhance degradation outcomes (Richnow *et al*., 1994; Szabó *et al*., 2007).

Another limitation that considerably affects phenanthrene and pyrene biodegradation is heavy metal co-contaminants whose complex interactions with the PAHs increase hazardous environmental risks (Markowicz, *et al*., 2016; Sarma *et al*., 2016). This negatively suppresses the bacterial metabolic activities due to combined effects from both PAHs and the heavy metals (Lu *et al*., 2013; Thavamani *et al*., 2012). Currently,

there are limited studies on the bacterial degradation response for PAHs in a mixed presence with heavy metals (Markowicz *et al*., 2016; Sarma *et al.*, 2016).

Based on the prevailing slow phenanthrene and pyrene degradation limitation experienced by the bacteria, research questions that instigated the conduct of this study include; does used vehicle lubricant bioavailability perform effective phenanthrene and pyrene degradations at much faster rate? How do we determine the desirability of such microorganisms in a complex situation that involved PAHs and hazardous heavy metals?

1.3 Significant of the study

Effective biodegradation of phenanthrene and pyrene provides avenue of tackling used lubricating oil and its environmental challenges in Malaysia. This creates opportunity of using the locally isolated bacteria from the commonly natural inhabitants of used lubricating oil contaminated sites as fast effective biodegraders. The previously isolated bacteria were shown to degrade less than 120 mg/L phenanthrene and pyrene in more than 20 days of incubation (Abdul-Talib *et al.*, 2015; Sarma *et al.*, 2004). Therefore, isolating effective phenanthrene and pyrene degrading bacteria that can remove more than higher concentrations in 24 hours is much needed in Malaysia. This will improve the environmental condition which receives considerable amounts of used lubricating oil on daily basis. Additionally, opportunity will be provided to confirm the attainment of suitable niche by such bacteria in a complex system that is co-contaminated with heavy metals pollutants.

1.4 Objectives of the study

The main aim of the present study is to identify efficient bacteria that rapidly degrade phenanthrene and pyrene belonging to low and high molecular weight PAHs. The bacteria were enhanced by statistical based approach and tested for the biodegradation efficiency in complex mixture with heavy metals. The specific objectives designed to achieve this aim include:

1. To isolate and identify phenanthrene and pyrene degrading bacteria from used vehicle lubricant contaminated soil.
2. To optimize phenanthrene and pyrene biodegradation conditions.
3. To analyse phenanthrene and pyrene biodegradation intermediates.
4. To evaluate the effects of heavy metals co-contaminants on the phenanthrene and pyrene biodegradation.

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