BIODEGRADATION OF HIGH MOLECULAR WEIGHT POLYCYCLIC AROMATIC HYDROCARBON BY ISOLATED FUNGI

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To my beloved mother,

To my family,

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are recalcitrant pollutants with two or more fused benzene rings. Almost 90% of PAHs emission to the environment is anthropogenic, causing carcinogenic and mutagenic effects in humans. These PAHs are subject to removal by biological treatment. However, because of the physio-chemical characteristics of PAHs and especially high molecular weight (HMW-PAHs), the biodegradation by microorganisms is difficult. Fungi were collected from contaminated soil and rain forest in Malaysia, isolated based on their ability to decolorize RB5 and RBBR due to the similarity in chemical structure and ease in its measurement on agar medium. The best-performing fungi were identified based on the DNA sequence and phylogenetic tree. Three fungi were identified in the lab as *Candida* sp. S1, *Meyerzoma* sp. S7 and *Rhizoctonia zeae* SOL3. The biodegradation of PAHs by these fungi have been studied in 7, 15, 21, 30 days of incubation in liquid medium. Among the screened and collected fungi, *R.zeae* SOL3 showed the highest degradation of pyrene in 15 days (42%). Parameters such as temperature, glucose concentration, NaCl, pyrene concentration, agitation and pH were investigated to show their effect on the biodegradation by *Candida* sp. S1 and *R.zeae* SOL3. The results showed that these fungi are mesophilic and halophilic. The degradation of pyrene by *Candida* sp. S1 and *R.zeae* SOL3 have been optimized based on the response surface method (RSM), the predicted values from the model were very close to the actual data from the experiments. This indicated the suitability of the model in prediction of the experiment. The metabolites of pyrene biodegradation by *R.zeae* SOL3 were identified by GC-MS as 4-hydroxy benzoic acid, benzoic acid and butanedioic acid. These fungi showed a good ability to remove HMW-PAHs from the liquid medium in extreme saline and acidic conditions, producing metabolites less dangerous than the parent compound, which can be used in the removal of PAHs in industrial wastewater.

ABSTRAK

Hidrokarbon aromatik polisiklik (PAH) adalah pencemaran yang sukar ditangani yang berstruktur dua atau lebih daripada dua gelang benzena. Hampir 90% daripada pelepasan PAH kepada alam sekitar adalah antropogenik, menyebabkan kesan karsinogenik dan mutagenik kepada manusia. PAH ini tertakluk kepada penyingkiran dengan olahan biologi. Walau bagaimanapun, ciri-ciri fizik-kimia PAH dan terutamanya berat molekul yang tinggi (HMW-PAH), menyebabkan biodegradasi oleh mikroorganisma adalah sukar. Kulat dikumpulkan dari tanah tercemar dan hutan di Malaysia, di isolasi berasaskan kepada keupayaan mereka dalam pengingkiran warna RB5 dan RBBR kerana persamaan dalam struktur kimia dan kebolehupayaan dalam pengukuran di medium agar. Jenis kulat yang dikenalpasti berdasarkan jujukan DNA dan pokok filogenetik. Tiga kulat telah dikenalpasti di makmal, dikenali sebagai *Candida* sp. S1, *Meyerzoma* sp. S7 dan *Rhizoctonia zeae* SOL3. Biodegradasi PAH oleh kulat telah dikaji menerusi 7, 15, 21, 30 hari pengeraman di dalam medium cecair. Antara kulat yang dikumpul and diperiksa, *R.zeae* SOL3 menunjukkan degradasi tertinggi untuk pirena dalam masa 15 hari (42%). Parameter seperti suhu, kepekatan glukosa, NaCl, penumpuan pirena, agitasi dan pH telah disiasat untuk menunjukkan kesannya terhadap biodegradasi oleh *Candida* sp. S1 dan *R.zeae* SOL3. Hasil eksperimen menunjukkan bahawa kulat ini adalah mesofilik dan halofilik. Degradasi pirena oleh *Candida* sp. S1 dan *R.zeae* SOL3 telah dioptimumkan berdasarkan kaedah gerak balas permukaan (RSM), dengan nilai yang diramalkan daripada model adalah hampir sama dengan data eksperimen. Ini menunjukkan kesesuaian model dalam ramalan percubaan. Metabolit-metabolit yang terhasil daripada pirena dibiodegradasi oleh *R.zeae* SOL3 telah dikenal pasti oleh GC-MS sebagai asid benzoik 4- hidroksi, asid benzoik dan asid butanedioik. Kulat ini menunjukkan keupayaan yang tinggi dalam degradasi HMW-PAH dalam keadaan medium cecair yang terlampau masin dan keadaan berasid, menghasilkan metabolit kurang berbahaya daripada sebatian induk, yang boleh digunakan dalam penyingkiran PAH dalam air sisa industri.

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LIST OF SYMBOLS

AMU	-	Atomic mass unit
BLAST	-	Basic Local Alignment Search Tool
CCD	-	Central composite design
CMC	-	Critical micelle concentration
DCM	-	Dichloromethane
DOE	-	Design of experiment
DMF	-	N,N-Dimethylmethanamide
EA	-	Ethyl acetate
eV	-	Electron volt
FID	-	Flame ionization detector
GC	-	Gas Chromatography
LM	-	Liquid medium
M^+	-	Molecular ion
MEA	-	Malt extract agar
MS	-	Mass Spectrophotometer
m/z	-	Mass to charge ratio
NCBI	-	National Center for Biotechnology Information
PAH	-	Polycyclic Aromatic Hydrocarbon
PCR	-	Polymerase Chain Reaction
RB5	-	Reactive Black 5
RBBR	-	Remazol Brilliant blue R

R_f	-	Retention factor
RSM	-	Response Surface Methodology
rRNA	-	Ribosomal ribonucleic acid
SEM	-	Scanning Electron Microscopy
TCA	-	Tricarboxylic acid
TLC	-	Thin Layer chromatography
TMS	-	Trimethyl-silylation
UV	-	Ultraviolet

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CHAPTER 1

INTRODUCTION

1.1 General

Contamination of water is found frequently, this can be caused from industry discharge or accidentally such as pipeline leaks, ship wreckages, tank ruptures and transport accidents. Polycyclic aromatic hydrocarbons (PAHs) belong to the group of persistent organic pollutants (POPs). In Malaysia, the two major routes PAHs enter into the aquatic environment have been identified as: (i) leakage of crankcase oils from vehicles onto road surfaces, with the subsequent washout by street runoff, (ii) spillage and dumping of waste crankcase oil (Zakaria *et al*., 2002). PAHs are characterized by high toxicity, high environmental stability, and high hydrophobicity (Harvey, 1997). PAHs can persist in the ecosystem for long periods (Painter, 1996), resulting in their accumulation in the food chain with final destination, the human tissue and body fluids as shown in Figure 1.1. The distributions of PAHs in the environment and potential human health risks have become the focus of much attention. Their presence combined with other potentially toxic compounds can result in negative effects. The U.S. Environmental Protection Agency (EPA) has identified 16 of PAHs as Priority Pollutant List.

From this demand to remediate PAHs, treatment methods appear to be essential. These methods include volatilization, oxidation, adsorption and biodegradation. However, these methods are expensive and PAHs either confines or transfers to another phase without its destruction, or producing new dangerous metabolites (Mueller *et al.,* 1996). Therefore, bioremediation is emerging as a green technology intended to achieve the remediation of water and soil via biodegradation by microorganisms. By far, studies on biodegradation of PAHs have mainly focused on bacteria rather than fungi (Raghukumar *et al*., 2006; Stringfellow and Alvarez-Cohen, 1999).

Although remediation of PAHs by fungi has been investigated, the emphasis on fungi that could biodegrade high molecular weight PAHs (HMW-PAHs) ($\geq$ 4 fused benzene rings) without producing dangerous metabolites has been limited. The biodegradation of HMW-PAHs is yet to be demonstrated consistently, due to the weak potential for biodegradation by microorganisms (Harayama, 1997). The aqueous solubility of PAHs decrease almost logarithmically with increasing molecular weight, however microorganisms can degrade PAHs only if they are dissolved in water (Johnsen *et al.*, 2005). This failure to demonstrate consistent degradation has made identifying the degradation of HMW-PAHs (e.g. chrysene, pyrene and benzo[*a*]anthracene) an immediate research priority to the development of appropriate bioremediation strategies.

Among microorganisms, fungi have proven to have promised ability to biodegrade HMW-PAHs. They can oxidize PAHs to give CO_2 and largely uncharacterized polar metabolites. Although the xenobiotic oxidation of fungi are not rapid, but they are very unspecific (Hammel, 1995). Many studies that have used fungi to degrade HMW-PAHs have indicated an increase in the medium toxicity, this is because the metabolite products by some fungi are more toxic than the parent compounds.

As the environmental goal is to optimize the removal rate, an attempt to speed up and enhance the biodegradation rate of HMW-PAHs is made. Parameters such as temperature, pH, salinity, agitation, glucose concentration were varied to investigate their effect on the biodegradation rate. These data were analyzed mathematically using Design Expert® software to optimize the process.

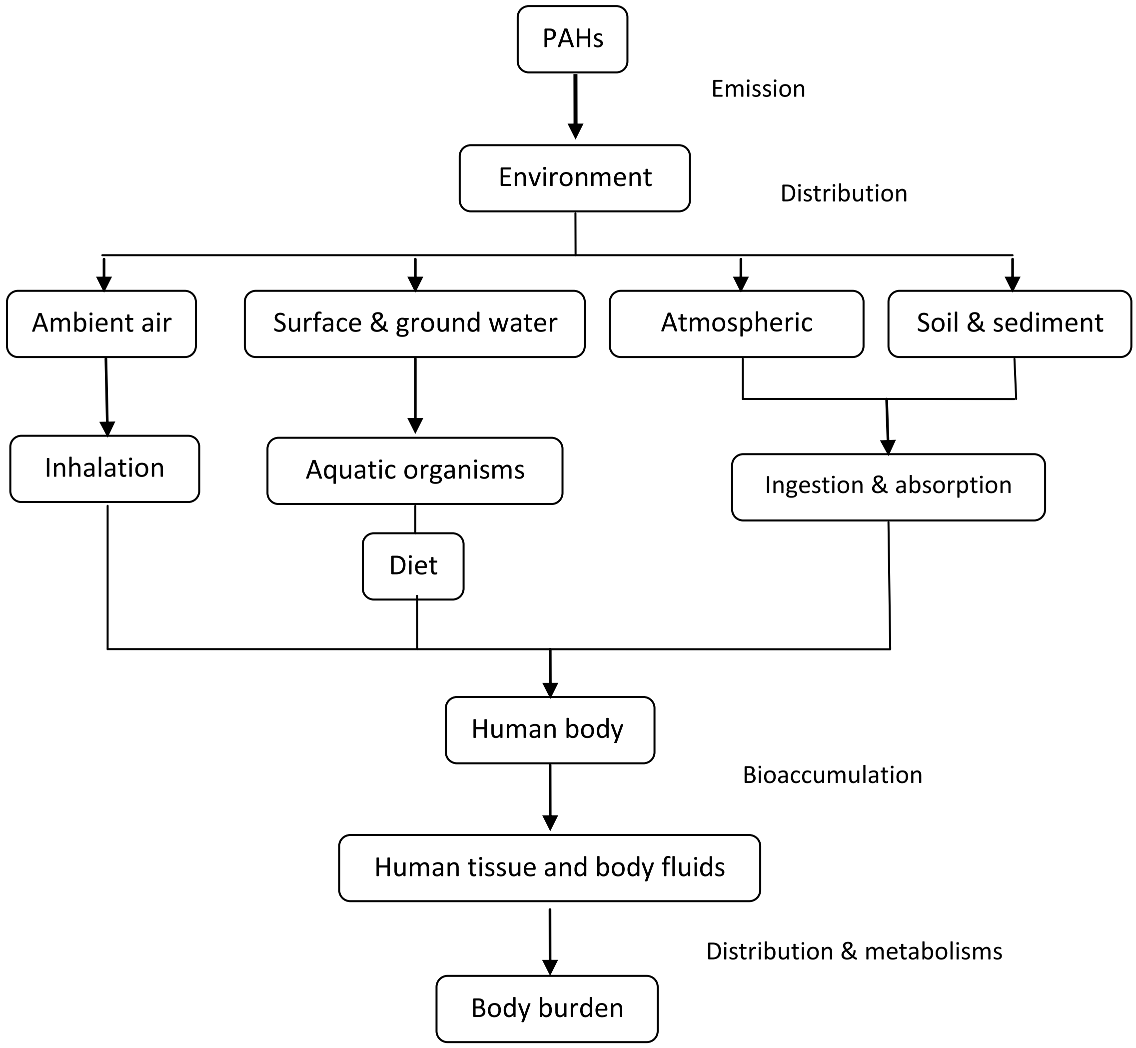

Figure 1.1 The environmental fate and distribution of PAHs (Król *et al*., 2013)

1.2 Problem Statement

Due to human activities, the environment is facing continuous risk coming from pollutants. These pollutants (including PAHs) have the potential to cause adverse environmental effects. They can persist over long periods and difficult to remove by traditional methods because of their physical-chemical properties.

The bioremediation method by microorganisms and especially fungi, have shown promise to biodegrade HMW-PAHs. Isolating new species that have the ability to biodegrade HMW-PAHs and optimizing the degradation rate have gotten a large interest recently. However, the degradation pathways of HMW-PAHs by fungi are still not clear. Furthermore, the metabolite products may be more dangerous than the parent compounds. By optimizing the degradation rate and understanding the degradation pathway, can introduce a new method of PAHs treatment of contaminated water.

1.3 Research objectives

The objectives of this research are as follows:

1. To isolate and identify fungi from nature capable of degrading HMW-PAHs.
2. To investigate the environmental conditions effect on the biodegradation of pyrene by fungi.
3. To propose a RSM model showing the relationship between biodegradation of pyrene and its parameters.
4. To maximize the biodegradation rate of pyrene by fungi
5. To examine the metabolic pathway of pyrene by fungus that has already been isolated and identified.

1.4 Significance of the Study

This study is carried out in order to find an alternative method in treatment of persistent pollutants that have adverse effects on humans and the environment. The traditional methods, including the physical-chemical method, have negative effects compared to biological treatment. Although bioremediation by fungi have been studied before, the finding of new species of fungi that have good ability to biodegrade HMW-PAHs is a big challenge.

This work will be relevant to the industrial sector that produces wastewater that contain PAHs. Furthermore, this method can be used to treat water in accidental petroleum spillage under extreme conditions. As a whole, this study presents a potential environmental benefit, which can contribute to both the economical and environmental aspects.

1.5 Thesis organization

This thesis is divided into five chapters. Chapter One gives an overview of this study. It gives introduction to the background of the problem and a set of objectives of this study. In Chapter Two, the main outlines of the literature review are presented as follows:

- PAHs properties
- Effect of PAHs on human health and the environment
- PAHs source
- PAHs removal
- Biodegradation of PAH by fungi
- Parameters that effect on the biodegradation rate
- PAH metabolites
- Optimization by Design of Experiment software.

Chapter Three shows the methodology that has been used to get the data, whereas Chapter Four contains the results of this research. The main outlines of this chapter are:

- Screening and isolation of fungi based on its ability to decolorize dyes.
- Identifying the best three fungi according to DNA sequence.
- The degradation of pyrene by fungi.
- The degradation of pyrene in co-culture of fungi.

- The degradation of individual and mixed PAHs by fungi.
- Some parameters that effect on the degradation rate of pyrene by fungi
- Pyrene metabolites
- Optimization of pyrene degradation using DOE/RSM software.

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