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BIOLOGY OF TRICHODERMA SP. ISOLATED FROM POLLUTED SEDIMENT AND ITS POTENTIAL IN DEGRADATION OF PHENANTHRENE (PAHs)

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By

SAFIYA YAKUBU

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

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Dedicated to my dearest and irreplaceable mum for molding me into the person that I am today. May Allah (SWT) bless you and grant you peace. May He, the most merciful admit you into Aljannatul Firdaus.

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

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Chair: Associate Prof. Muskhazli Mustafa, PhD
Faculty: Science

Polycyclic Aromatic hydrocarbons (PAHs) as contaminants are one of the most recalcitrant, persistent organic compounds and this has made their fate in the environment of significant interest. PAHs have become a great concern as they are highly ecotoxic with potential of being human carcinogens, making their rapid elimination and detoxification from the environment of necessary importance. This study aimed to isolate and screen indigenous soil and sediment fungi for possible application in the remediation of these contaminants. It also aimed to investigate how two important factors, temperature and pH influence the PAHs degradative capability of the potent fungus using phenanthrene as a model substrate. The study also involves evaluating the suitability of this isolate in qualitative phenanthrene degradation through the detection of metabolites. Soil and sediment samples were collected for fungal isolation. Forty-four isolates were obtained and screened to select the best isolate with the potential to degrade phenanthrene as a substrate. *Trichoderma* sp., a filamentous Sordariomycetes isolated from Bagan Lalang sediment showed the best phenanthrene tolerance with a high growth percentage of 76.9% at 72 hrs and a percentage phenanthrene degradation of 60.4% after 10days of incubation in liquid mineral medium. Evaluation of the effects of environmental factors on phenanthrene degradation showed that both parameters (temperature and pH) had a significant effect on the fungus ability to degrade phenanthrene. Laboratory studies using Mineral Salt Broth (MSB) showed that phenanthrene degradation efficiency was influenced by temperature with the highest degradation of 69.5% occurring at 35°C. This temperature also supported a good biomass accumulation of 62mg dry weight. Less phenanthrene degradation was observed at lower and higher temperatures. Influence of pH on substrate degradation showed thatthe optimum pH for phenanthrene degradation by the fungus was at a neutral pH 7. This pH point facilitated both effective phenanthrene degradation of 76.8% and biomass growth of 48mg dry weight thus, making it evident that higher rates of degradation was somewhat related to a high biomass growth. Qualitative analyses by GC-MS of extracts after incubation showed that *Trichoderma* sp. could degrade phenanthrene; yielding metabolites such as 9,10-dihydro-9,10-dihydroxyphenanthrene, 9,10-dihydrophenanthrene, 9-Phenanthrol, 1,2,3,4-

tetrahydrophenanthrene and phthalic acid. Based on the detected metabolites, the mechanism of phenanthrene degradation by this fungus, suggests the activities of both ligninolytic and non-ligninolytic type of enzymes with major attack on the K-region (C-9 and C-10) of phenanthrene. The metabolite 1,2,3,4-tetrahydrophenanthrene detected in culture extracts suggests that another pathway utilized by the fungus may exist in substrate degradation. Thus, this *Trichoderma* isolate could serve as a potential innoculum that can be utilized in microbial PAHs degradation and environmental factors such as pH and temperature does have an influence on the strains ability to act on phenanthrene.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
Sebagai memenuhi keperluan untuk Ijazah Master Sains

BIOLOGI TRICHODERMA SP. YANG DIISOLAT DARIPADA SEDIMEN TERCEMAR DAN POTENSINYA DALAM DEGRADASI FENANTRENA (PAH)

Oleh

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Polisiklik Aromatik Hidrokarbon (PAH) merupakan salah satu daripada bahan pencemar yang amat rekalsitran, kompaun organik yang tegar dan kesannya terhadap alam sekitar telah meningkatkan perhatian terhadap bahan ini. PAH telah menjadi kebimbangan utama kerana ia sangat ekotoksik dengan potensi sebagai karsinogen kepada manusia; menjadikan penyingkiran dan detoksifikasi PAH daripada alam sekitar adalah sangat penting. Kajian ini dijalankan bertujuan untuk mengisolat dan menyaring kulat yang berasal dari sampel tanah dan sedimen untuk diaplikasi sebagai remidiasi bahan pencemar ini. Kajian ini juga ingin mengkaji pengaruh dua faktor penting, suhu dan pH ke atas kebolehan kulat poten mendegradasi PAH menggunakan fenantrena sebagai model subtrat. Selain daripada itu, Kajian ini juga membabitkan penilaian kesesuaian isolat ini dalam degradasi fenantrena secara kualitatif melalui pengesanan metabolit. Sampel tanah dan sedimen dikumpulkan untuk pemencilan kulat. Empat puluh empat isolat telah diperolehi dan disaring untuk memilih jenis kulat terbaik dengan potensi yang tinggi untuk mendegradasi fenantrena sebagai substrat. *Trichoderma* sp., Sordariomycetes berfilamen yang diisolat daripada sedimen dari Bagan Lalang menunjukkan toleransi terbaik terrhadap fenantrena dengan peratus pertumbuhan yang tinggi iaitu 76.9% pada 72 jam dan peratus degradasi fenantrena sebanyak 60.4% selepas 10 hari dieram dalam media cecair mineral. Penilaian kesan-kesan degradasi fenantrena ke atas alam sekitar menunjukkan kedua-dua parameter (suhu dan pH) memberikan kesan yang signifikan ke atas kebolehan kulat untuk mendegradasi fenantrena. Kajian di makmal menggunakan ‘Mineral Salt Broth’ (MSB) menunjukkan kecekapan degradasi fenantrena dipengaruhi oleh suhu dengan degradasi tertinggi sebanyak 69.5% berlaku pada suhu 35°C. Suhu ini juga menghasilkan pengumpulan biomas yang baik iaitu berat berjumlah 62 mg. Degradasi fenantrena yang lebih rendah dapat diperhatikan pada suhu yang lebih rendah dan tinggi. Kesan pH terhadap degradasi substrat menunjukkan bahawa pH optimum untuk kulat mendegradasi fenantrena adalah pada keadaan neutral, pH 7. pH ini membantu degradasi fenantrena yang efektif iaitu 76.8% dan pertumbuhan biomass dengan 48mg berat kering. Ini membuktikan degradasi yang tinggi adalah berkait dengan pertumbuhan biomas yang tinggi. Analisis kualitatif ekstrak menggunakan GC-MS selepas pengeraman menunjukkan *Trichoderma* sp. boleh mendegradasi fenantrena dan menghasilkan metabolit seperti

9,10-dihidro-9,10-dihidroksifenantrena, 9-10-dihidroksifenantrena, 9-fenantrol, 1,2,3,4-tetrahidrofenantrena dan asid ftalik. Berdasarkan metabolit yang dikesan, mekanisme degradasi fenantrena menunjukkan aktiviti kedua-dua enzim jenis ligninolitik dan bukan ligninolitik menyerang pada rantau-K (C-9 dan C-10) pada fenantrena. Metabolit 1,2,3,4-tetrahidroksifenantrena yang dikesan pada kultur mencadangkan bahawa wujud laluan lain yang digunakan oleh kulat dalam degradasi substrat. Oleh yang demikian, isolat *Trichoderma* ini berpotensi digunakan sebagai inokulum untuk aplikasi mikrobial dalam degradasi PAH dan faktor persekitaran seperti pH dan suhu dapat memberi kesan kepada keupayaan strain untuk bertindak ke atas fenantrena

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I certify that a Thesis Examination Committee has met on 31 October 2014 to conduct the final examination of Yakubu Safiya on her thesis entitled "Biology of *Trichoderma* sp. Isolated from Polluted Sediment and its Potential in Degradation of Phenanthrene (PAHs)" 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 requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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Name of Member of Supervisory Committee: Prof. Mohamad Pauzi Zakaria

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LIST OF ABBREVIATIONS/SYMBOLS

BLAST	Basic Local Alignment Search Tool
N,N-DMF	N,N-Dimethylformamide
FID	Flame Ionizing Detector
GC	Gas Chromatography
GC-MS	Gas Chromatography Mass Spectrometry
HMW	High Molecular Weight
LLE	Liquid-to-liquid extraction
LMW	Low Molecular Weight
MSB	Mineral Salt Broth
Oxy-PAH(s)	Oxygenated Polycyclic Aromatic Hydrocarbon(s)
PAH(s)	Polycyclic Aromatic Hydrocarbon (s)
PDA	Potato Dextrose Agar
POPs	Persistent Organic Pollutants
TES	Trace Element Solution
%	Percentage
µl	Microliter
hrs	Hours
mg	Milligram
min(s)	Minute(s)
ml	Milliliter
mm	Millimeter
rpm	Revolution per minute
ºC	Degree Celsius
$mg.l^{-1}$	Milligram per litre

g	Gram
µg	Microgram

CHAPTER 1

INTRODUCTION

1.1 Background of the study

The past decades has experience a rapid rise in industries, particularly in the oil and gas sectors and as a result, environmental pollution by xenobiotic compounds has been very much prevalent. The exploitation and use of fossil fuels for transportation, energy and raw materials for industries has led to a widespread distribution of Persistent Organic Pollutants (POPs) in the environment. Polycyclic Aromatic Hydrocarbons (PAHs) are highly ecotoxic; accumulating and persisting over long periods with most, being potential human carcinogens (Matsubara *et al*., 2006). They are mutagenic and teratogenic (Verdin *et al*., 2004) and have become of public health concern therefore, making them targets for statutory monitoring and control in environmental samples and industrial effluents (Choi *et al*., 2006; Gammon *et al*., 2002). In Malaysia, for instance there is the Environmental Quality Act, 1974 (EQA), comprising of regulations coined to abate the disposal of effluents containing these compounds. Examples include the Environmental Quality (Industrial Effluents) Regulation of 2009 and the Environmental Quality (Scheduled Waste) Regulations, which are under the implementation and monitoring of the department of environment (DOE) and the maritime department in some cases (DOE, 2010). Based on their abundance and toxicity, the United States Environmental Protection Agency (US EPA) has listed 16 PAHs for monitoring as priority pollutants (Pozdnyakova, 2012) among which include phenanthrene.

Phenanthrene, is a tricyclic aromatic hydrocarbon (Figure 1.1) frequently occurring as a pollutant in soils, estuarine wastes, sediments, terrestrial and aquatic sites affected with PAHs pollution (Mrozik *et al*., 2002).

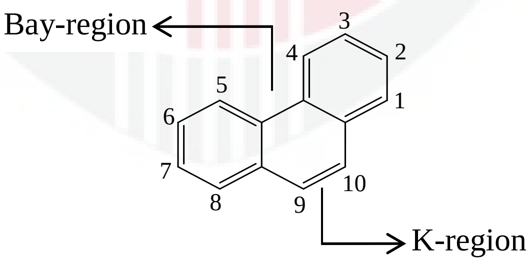

Figure 1.1: Structure of phenanthrene

The tricyclic structure of phenanthrene is found in high molecular weight (HMW) aromatic hydrocarbons and most PAHs containing a phenanthrene moiety are carcinogenic in nature. Phenanthrene is the smallest PAH compound to have a 'bay region' and 'K-region' (Gao *et al.,* 2010; Deveryshetty and Phale, 2009) thus, chosen as a model PAH compound in this research. The bay-region situated between C4 and C5 is a sterically hindered area considered as a prerequisite for the carcinogenic property of PAHs. Bay-region alongside fjord region are structural attributes showing the ability of a PAH compound to bind to DNA and form adducts (Anyakora, 2007).

The K-region on the other hand is a structural region of the compound between C9 and C10 often characterized by a high electron density and bond order, thus associated with chemical and biological reactivity. This makes phenanthrene a suitable model substrate as a carcinogenic PAH representative in microbial biotransformation studies as evident in a number of literatures (Brinda Lakshmi *et al*., 2013; Nasseri *et al*., 2010).

Environmental PAHs sources include both natural (mostly from geochemical processes) and anthropogenic (Cajthalm *et al*., 2002). However, their presence in the environment majorly results from human activities (Hylland, 2006). Anthropogenic petrogenic PAHs are often introduced via processes such as crude and refined petroleum operations involving oil exploration and production (E&P) processes (Liu *et al*., 2009; Yunker *et al*., 2002). Burning and combustion of fossil fuel represents their pyrogenic origins (Zakaria *et al*., 2002). In addition, municipal and urban runoff containing industrial wastes and agrochemicals all add up to their environmental input where they undergo physical, chemical and biological processes (Motelay-Massie *et al*., 2006). In the atmosphere, PAHs tend to adsorb to particulates and eventually deposit in urban terrestrial soils and sediments in rivers, lakes, estuarine and marine waters, thus serving as sinks for such xenobiotics (Hughes *et al*., 1997; Bosma *et al*., 1996). These environments could serve as incubators, harbouring important microbial strains capable of consuming contaminants thus, the idea of isolating effective microbial strains from contaminated epicenter environments could produce microbes that are better adapted for application in PAH remediation science (Korda *et al*., 1997).

PAH, pollution is of great concern to health and the environment owing to the tendency to become sequestered (Suedel *et al*., 1994). They cause negative effects in humans and animals some of which manifest through their immunotoxic, genotoxic and carcinogenic properties (Guo *et al*., 2011). Their elimination from the environment and polluted waste materials becomes of great importance to safeguard the ecosystem. Efforts via the use of modern-day technologies exists for PAHs remediations. These include, high temperature incineration, thermal desorption, immobilization, soil washing, vapour extraction, chemical precipitation, critical fluid extraction and the use of landfills (Semple *et al*., 2007; Rivas, 2006; Vidali, 2001). However, such approaches have many drawbacks in use as they serve mainly for transfer and containment without detoxification of target pollutants from the environment. They are also expensive, technology intensive and not eco-friendly (Haritash and Kaushik, 2009). These limitations have prompted scientists to explore alternative effective and eco-friendly measures and thus, microbial bioremediation as a technique has gained much attention as a potential strategy (Mrozik, 2003). Research efforts on microbial PAH remediation has focused more on the utilization of bacteria (Dash *et al*., 2013; Yu *et al*., 2005; Viñas *et al*., 2005) with a comparatively few attempts to utilize fungi. This owe to the fact that it has seem impossible to achieve a 100% PAHs removal using fungi in contaminated matrices as compared to bacteria (Šašek and Cajthaml, 2005).

Fungi sp. has evolved naturally as decomposers in the environment. They are totipotent heterotrophic eukaryotes and are ubiquitous microbes. Concerning PAH degradation, they possess a superior ability to act on both High Molecular Weight (HML) and Low Molecular Weight (LMW) PAHs whereas bacteria species are more limited to degrading aromatic compounds with fewer rings (Peng *et al*., 2008). This shows a better PAHs susceptibility to fungal degradation as evident in the study by Machín-Ramírez (2010) where fungal cultures were more effective in benzo[a]pyrene degradation even at high concentrations. Such an advantage point out to the fact that fungal enzymes, have low substrate specificities some of which are secreted extracellularly (Casas *et al*., 2009; Torres Duarte *et al*., 2009) thus, offering advantages over bacteria in the wide range of pollutants they are able to degrade (Pointing, 2001). In addition, the structural similarities between PAHs and lignin which comprises of long varried aromatic chain has made it possible for fungi sp. to act on recalcitrant xenobiotics (Mai *et al*., 2004). Despite their capabilities, little attention and research efforts is given to this group of organisms (Sing, 2006, Portin *et al*., 2004; Cerniglia and Sutherland, 2001). Most PAHs mycoremediation studies have focussed on well-utilized fungi species such as *Phanerochaete chrysosporium* and *Pleurotus ostreatus* (Chen and Ding, 2012; Nikiforova *et al*., 2010; Canet, 2001; Bezalel *et al*., 1996a; Bezalel, *et al*., 1996b; Bumpus, 1989). Fungal species with equally or more degradative potential could be found or isolated from the environment for application in PAH remediations.

1.2 Objectives of the study

Soils, sediments and associated waters harbour diverse microbial community. These environments are preferential sites for uptake and preservation of anthropogenic PAHs due to the substantial amount of organic matter. Evaluation of the degradative potentials of inhabiting microorganisms from such polluted sites would provide an opportunity to obtain fungal strains for xenobiotic remediation purposes. Thus, this study aims at isolating and investigating the capacities of autochthonous micro fungal flora from these environments for PAHs bioremediation using phenanthrene as a model PAH and to achieve this goal, three specific objectives have been set.

1. To isolate and screen indigenous fungus from the environment for phenanthrene degradation

2. To determine the effect of pH and temperature on phenanthrene degradation by potent isolate

3. To identify the products of phenanthrene degradation

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