BIOREMEDIATION OF ANTHRACENE AND REMAZOL BRILLIANT BLUE R BY FUNGI

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For the loved one and His Prophet,

My beloved family; husband and son

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ABSTRACT

Fungi are reported as a potent tool to biodegrade many type of organic pollutants for instance polyaromatic hydrocarbons (PAHs) and synthetic dyes that caused severe pollution to the environment. This research emphasized on biodegradation and biosorption process of PAHs and synthetic dyes; anthracene and Remazol Brilliant Blue R (RBBR) collected from decayed wood and soil. *Trichoderma citrinoviride* W04, *Trichoderma koningiopsis* W14 and *Pestalotiopsis* W15 were selected among twenty fungi species based on faster growth rate after 5 days of incubation. The identification of fungal species was done by morphology characterization and 18S rRNA sequence analysis. According to phylogenetic tree, W04, W14 and W15 were classified as *Trichoderma citrinoviride, Trichoderma koningiopsis,* and *Pestalotiopsis* sp. These three fungi species were used further to study the environmental effects; agitation, carbon and nitrogen sources and pH were investigated to choose optimum parameters. Optimization parameters are compulsory to obtain better results. The biodegradation and biosorption process were investigated using UV-Vis spectrophotometer, gas chromatography (GC), Fourier Transform Infrared Spectroscopy (FTIR) and Field Emission Scanning Electron Microscopy (FESEM). The optimum parameters for RBBR by *Pestalotiopsis* sp. W15 in this study were glucose (86.7%), yeast (91.9%), agitation condition (71.1%) and pH 5 (88.4%). Meanwhile, *Trichoderma koningiospsis* W14 had the highest degradation of anthracene and optimum in galactose (55.34%), ammonium chloride (55.2%), agitation (46.8%) and pH 5 (26.2%) culture condition. Isotherm and kinetic studies for RBBR and anthracene showed the adsorption process best fit the Langmuir with R^2 of 0.93 and 0.90 with maximum biosorption capacity; 0.17 and 0.78 and followed pseudo second order models with rate constant of 0.19 x 0.1/min and 0.47 x 0.1/min. This result demonstrated that fungi are promising biosorbent material to treat RBBR and anthracene.

ABSTRAK

Kulat dilaporkan sebagai kaedah kuat untuk biodegradasi pelbagai jenis bahan pencemar misalnya hidrokarbon poliaromatik (PAHs) dan pewarna sintetik yang menyebabkan pencemaran teruk terhadap alam sekitar. Kajian ini memberi penekanan kepada proses biodegradasi dan penjerapan PAHs dan pewarna sintetik; anthracene dan Remazol Brilliant Blue R (RBBR) yang dipungut daripada kayu reput dan tanah. *Trichoderma citrinoviride* W04, *Trichoderma koningiopsis* W14 dan *Pestalotiopsis* sp W15 dipilih daripada dua puluh spesies kulat berdasarkan kadar pertumbuhan yang lebih cepat selepas 5 hari pengeraman. Pengenalpastian spesies kulat telah dilakukan dengan pencirian morfologi dan analisis urutan 18S rRNA. Menurut pokok filogenetik, W04, W14 dan W15 dikelaskan sebagai *Trichoderma citrinoviride*, *Trichoderma koningiopsis* dan *Pestalotiopsis* sp. Tiga spesies kulat ini digunakan untuk kajian lanjutan dan mengkaji kesan alam sekitar; agitasi, sumber karbon dan nitrogen serta pH untuk memilih parameter yang optimum. Pengoptimuman parameter wajib untuk mendapatkan keputusan yang lebih baik. Proses biodegradasi dan biopenjerapan dianalisis menggunakan spectrometer UV-Vis, kromatografi gas (GC), spektroskopi inframerah transformasi fourier (FTIR) dan mikroskop imbangan electron pancaran medan (FESEM). Parameter yang optimum untuk RBBR oleh *Pestalotiopsis* sp. W15 dalam kajian ini ialah glukosa (86.7%), yis (91.9%), agitasi (71.1%) dan pH 5 (88.4%). Sementara itu, *Trichoderma koningiospsis* W14 menunjukkan degradasi anthracene tertinggi dan optimum dalam keadaan pengkulturan dengan galaktosa (55.3%), ammonium klorida (55.2%), agitasi (46.8%) dan pH 5 (26.2%). Kajian isoterma dan kinetik untuk RBBR dan anthracene menunjukkan proses penjerapan terbaik sesuai dengan Langmuir R^2 pada 0.93 dan 0.90 dengan kapasiti penjerapan maksimum; 0.17 dan 0.78 dan pseudo model peringkat kedua dengan kadar tetap 0.19 x 0.1/min dan 0.47 x 0.1/min. Keputusan ini menunjukkan kulat adalah bahan biopenjerap yang meyakinkan untuk merawat RBBR dan anthracene.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

DDTs	-	Dichlorodiphenyltrichloroethanes
DNA	-	Deoxyribonucleic acid
dNTP	-	Dideoxynucleotide triphosphate
EPA	-	Environmental Protection Agency
FESEM	-	Field Emission Scanning Electron Microscopy
FID GC	-	Flame Ionization Detector Gas Chromatography
FRs	-	Flame Retardant
FTIR	-	Fourier Transform Infrared Spectroscopy
GC	-	Gas Chromatography
GI	-	Germination Index
ID	-	Compound Identification
IR	-	Infrared
MB	-	Methylene Blue
MEA	-	Malt Extract Agar
MEGA	-	Molecular Evolutionary Genetic Analysis
NCBI	-	National Centre for Biotechnology Information
OCPs	-	Organochlorine Pesticides

PAHs	-	Polyaromatic Hydrocarbons
PCBs	-	Poly-chlorinated Biphenyls
PCDD/Fs	-	Polycyclic aromatic hydrocarbons
PCR	-	Polymerase Chain Reaction
PDA	-	Potato Dextrose Agar
PFCs	-	Poly- and Perfluorinated Compounds
POPs	-	Persistent Organic Pollutants
RB	-	Remazol Blue
RBB	-	Reactive Black B
RBBR	-	Remazol Brilliant Blue R
RRB	-	Reactive Red B
rRNA	-	Ribosomal ribonucleic acid
UV	-	Ultraviolet
UV-VIS	-	Ultraviolet-visible Spectroscopy

LIST OF SYMBOLS

%	-	Percent
~	-	Approximately
λ_{max}	-	Maximum Wavelength
μL	-	Microliter
μm	-	Micrometer
ι	-	Path Length
ε	-	Molar Extinction
°C	-	Degree Celcius
A	-	Absorbance
A	-	Temkin Equilibrium
B	-	Temkin Constant
bp	-	Based Pair
C_0	-	Initial Concentration
C_e	-	Equilibrium Concentration
C_t	-	Given Time Concentration
C	-	Carbon
C	-	Dye Concentration

C	-	Intercept
cm	-	Centimeter
d	-	Day
g	-	Gram
g/L	-	Gram per liter
g/mol	-	Gram per mol
h	-	Hour
H	-	Hydrogen
K_1	-	Pseudo First Order Rate Constant
K_2	-	Pseudo Second Order Rate Constant
K_{diff}	-	Intraparticle Diffusion Rate Constant
K_L	-	Langmuir Constant
K_F	-	Freundlich Constant
kPa	-	Kilopascal
M_W	-	Molecular Weight
m	-	Medium
m	-	Meter
mg	-	Milligram
mg/g	-	Miligram per gram
mg/L	-	Milligram per liter
min	-	Minute
mL	-	Milliliter

mL/min	-	Milliliter per minute
mm	-	Millimeter
n	-	Empirical parameter
N	-	Nitrogen
ng/g	-	Nanogram per gram
nm	-	Nanometers
O	-	Oxygen
ppm	-	Part per million
q_e	-	Adsorption capacity at equilibrium
q_m	-	Maximum adsorption capacity
q_t	-	Adsorption capacity at time
rpm	-	Revolution per min
R^2	-	Regression
s	-	Second
s	-	Strong
S	-	Sulphur
sp.	-	Species
t	-	Time
$t^{1/2}$	-	Half time
V	-	Volume
w	-	Weak
W	-	Fungal biomass

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Background of Study

The polycyclic aromatic hydrocarbons (PAHs) and synthetic dyes had developed international concern among environmentalist and public due to the toxicity, colour and bioaccumulation of the chemical substance in the environment (Wania and Mackay, 1999; Wu *et al.*, 2014). PAHs were categorized as organic pollutants that occur when incomplete combustion of coal, oil and gas happened. In general, PAHs are ubiquitously present and their derivatives are toxic, mutagenic and carcinogenic to human and wildlife (Haritash and Kaushik, 2009; Lee *et al.*, 2003).

From the previous literature (El-Shahawi *et al.*, 2010), the stable; carbon-carbon bonds that made of carbon and hydrogen (Ye *et al.*, 2011) caused PAHs has high resistance from degradation. PAHs exist in linear, cluster or angular that has two or more fused benzene rings which exist in more than hundred combinations (Hadibarata and Teh, 2014; Poonthrigpun *et al.*, 2006; Zakaria *et al.*, 2002). Previous study reported that PAHs can be degraded by microorganism because of the ability of biotransformation and secretion of enzyme properties to break down very strong PAHs structure compound (Hadibarata and Kristanti, 2013).

Anthracene is mostly discovered in high concentrations in PAH-contaminated sediments, surfaces soils and waste sites. Anthracene is toxic, mutagenic, carcinogenic PAHs because it exist in benz[a]anthracene and benzo[a]pyrene that endangered environment ecosystem (Cui *et al.*, 2014; Mohammadi and Nasernejad, 2009; Krivobok *et al.*, 1998). Anthracene has three fused benzene rings which widely used in dye industry such as an alizarin. It has colourless to pale yellow crystal-like solid. Anthracene is used in dye manufacturing, plastic and pesticides. Thus, the improper treatment of anthracene may lead to ecosystem damage. The ineffectiveness of conventional physical-chemical treatment methods force the rising of biological treatment to degrade anthracene that resist in the environment (Teh *et al.*, 2014).

Meanwhile, synthetic dyes are also pollutants that widely used in cosmetics, pharmaceutical, paper, textile and food industries. Heterocyclic, triphenylmethane, anthraquinone and phthalocyanine dye can be categorized as synthetic dyes. Annually, starting from 1856 over 10^5 of different dyes were produced about 7 x 10^5 metric tons (Ali, 2010; Taha *et al.*, 2014; Toh *et al.*, 2003) and about 10.0-15.0% of dyes were lost throughout dyeing process (Levin *et al.*, 2010; Selvam *et al.*, 2003).

In the textile industry, the effluent release into water bodies are in a vast amount. They are highly colored and if the effluents do not go into major treatment, it will affect the aquatic ecosystem that caused water pollution. The high toxicity of dye released can caused abnormal growth for phytoplankton and decrease in photosynthesis due to the lack of light penetration in water (Chacko and Subramaniam, 2011). Therefore the aquatic flora barely can produce food for aquatic organisms (Ali, 2010). The degradation of synthetic dyes is crucial to avoid ruinous effects to the environment (Adnan *et al.*, 2014). Other than that, the preservation of water quality turn to be public concern in order to maintain economic growth and at the same time enable the water to be safe for drinking (Bao *et al.*, 2012). This is the important of effluent treatment.

After azo dyes, anthraquinone is the most significant dyes that widely used in textile industry (Osma *et al.*, 2010). In the production of polymeric dyes, Remazol Brilliant Blue R (RBBR) is one of anthraquinone dyes as starting material. Structure of RBBR consists of anthracene derivative that can be categorized in toxic and recalcitrant organopollutants class. (Mechichi *et al.*, 2006; Osma *et al.*, 2010; Soares *et al.*, 2001).

Decolourization of these dyes by physical or chemical methods including adsorption and precipitation methods, chemical degradation or photo-degradation gave various drawbacks for example these methods are costly, time-consuming and mostly not very effective. These methods inclined to accumulate and concentrate the toxic dyes instead of degrade them (Adnan *et al.*, 2014). Another previous study stated that the environmental friendly method; biological methods are suitable used as it can accomplish mineralization of persistent organic pollutants at very low cost and reduced water consumption when compare to physicochemical method (Ali, 2010; Imran *et al*., 2014; Saratale *et al.*, 2011).

Many microorganisms capable of metabolizing PAHs were discovered including bacteria, fungi and algae but when the rings are more than five, the biodegradation process by microorganism will be more challenging (Matsubara *et al.*, 2006). Other than biodegradation, biosorption is significant method in dye decolorization using living fungi (Srinivasan and Viraraghavan, 2010). Biosorption is an alternative eco-friendly technology in removing synthetic dye by using fungi. This method combines active and passive transport mechanism which the adsorbed components diffused to the surface of microbial cell (Aksu and Tezer, 2000). The advantages of this method are simple in design, simplicity of operation, not sensitive towards toxic substance and capable to completely remove pollutants even though in dilute solutions (Dotto *et al*., 2012).

On the other hand, fungi has an ability for biosorption of dye with fungal biomass and biodegradation or biotransformation of dye into less toxic compound using extracellular enzymes (Adnan *et al.*, 2014). Fungi are essential in forest ecology because it has the ability to decompose and degrade wood. Fungal mycelia are promising microorganisms used as bioremediation to breakdown organic pollutants and degrade into less harmful products or usable product. This method is inexpensive somehow and the products are less toxic (Ali, 2010). Therefore, several studies have concentrated to extensively study on microorganisms that had capabilities decolorize and biodegrade these dyes effectively (Wesenberg *et al.*, 2003).

The study aims at screening fungi that having ability in degradation of PAH and synthetic dyes. Treatment of pollutant using fungi is the development of biotechnological process and environmentally friendly which can be green solution to improve environment and water pollution for next generation (Ali, 2010). The study of environmental effects is performed in this study to obtain the optimum condition for degradation of fungi. Thus, it can give the best result for biodegradation rate. The liquid culture of filamentous fungi will be analyzed using UV-Vis Spectrophotometer (UV-Vis), Gas Chromatography (GC), Fourier Transform Infrared Spectroscopy (FTIR) and Field Emission Scanning Electron Microscopy (FESEM). Finally, the better understanding of the characterization of the degradation of PAHs and synthetic dyes using fungi in environments is obtained.

1.2 Problem of Statements

PAHs and synthetic dyes are the major problems to the world nowadays because it can cause the deterioration of environment by its colour and chemical substances released as effluents to the ecosystems. The resistance of PAHs to degradation is due to the low aqueous solubility of structure compound made them stay in the environment (Acevedo *et al.*, 2011; Sartoros *et al.*, 2005). This multilateral environmental agreements depict that the release of PAHs to the environment must be controlled to avoid more pollution occur to the surrounding (Hung et al., 2013).

The physical and chemical methods face severe limitations such as high cost, low efficiency and produce secondary pollution (Ali, 2010). Meanwhile, the use of fungi which is low cost and ecofriendly technology is much preferred in this treatment. Thus, the development of biotechnological process is more favorable which cheaper and environmental friendly method. This research will be the first that explores the application of fungi for biosorption and biodegradation of PAHs and synthetic dyes in the environment especially in soil.

1.3 Objective

This study is to determine the ability of fungi for bioremediation of anthracene and Remazol Brilliant Blue R (RBBR). The objectives of this study are:

1) To screen and identify fungi collected from nature that having ability in degrading anthracene and RBBR.

2) To characterize the physical and chemical properties of fungi using FTIR and FESEM.

3) To investigate the optimum conditions for the removal of anthracene and RBBR by fungi.

4) To determine the adsorption behavior of anthracene and RBBR into the fungi by isotherm and kinetic model.

1.4 Scope of Study

The study is to determine the growth of fungi from a tropical rainforest. The screening method used to test fungi, based on speed to choose the most preferable fungi for degradation of anthracene and PAHs. Then, fungi will be incubated for 30 days in liquid medium to degrade pollutants; RBBR and anthracene. The identification of selected fungi was performed using 18S rRNA sequence and microscopic observation.

The optimization of fungi is based on the carbon and nitrogen sources, pH value, concentration of pollutants and stationary and mobile condition of fungi. The fungi growth conditions are important for better performance in the bioremediation process of pollutants in this study. The liquid culture medium will be centrifuged, filtered and analyzed by using UV-Vis spectrophotometer and Gas Chromatography

(GC). Before any further, samples that will be analyzed using GC must undergo extraction and purification process using separation and chromatography technique. The characteristics of fungi biosorption will be investigated by using FTIR and FESEM. Last but not least, the adsorption isotherm and kinetic modelling were studied by using concentration and contact time of experiment.

1.5 Significance of Study

The significance of this current study is to biosorp and degrades PAHs and synthetic dyes in the ecosystem or environment by using natural, harmless and environmental method using fungi. This is as an alternative way to replace other high cost method to degrade recalcitrant dye, RBBR and anthracene by using fungi collected from a tropical rainforest. This study also explores new species of fungi that exhibit great performance for biosorption and biodegradation.

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