BIODEGRADATION AND METABOLIC PATHWAY OF CRESOL RED DYE BY SELECTED FUNGI

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To my beloved mother, father, brother and sisters

Thanks for all your support, love and care…

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ABSTRACT

Massive amount of dye concentrations has been released into the environment as wastewater. Cresol red dyes remains as one of the most difficult dyes to be degraded. In this study, 20 different types of white rot fungi were isolated from a decayed wood in UTM forest andlabelled as M 01-M 20. These fungi were screened fortheir ability to degrade cresol red dye after 30 days ofincubation. New metabolic pathways of degradation of cresol red dyes by fungi M06 and M15 were explored in this research. Both M06 and M15 were chosen based on their ability to decolorize 10 ppm cresol red dye on agar and liquid medium. These fungiwere further tested for their ability to degrade cresol red dye inliquid medium under several conditions which is carbon and nitrogen sources, pH, salinity and effect of different concentration of surfactant (Tween 80). From decolorization, M06 showed efficient decolorization with addition of glucose (60.01%), ammonium sulphate (67.22%), Tween 80 0.1 mL (53.82%), 50 g/L of NaCl concentration (59.64%) and pH 3 (67.50%). Meanwhile, M15 showed efficient degradation of cresol red dye with addition of glucose (72.85%), ammonium nitrate (82.40%), Tween 80 0.1 mL (74.35%), 150 g/L of NaCl concentration (78.64%) and pH 3 (85.46%). These fungi were then identified using 18s rRNA sequence analysis method. From the phylogenetic tree, fungi M06 belongs to *Trichdermaharzianum* and fungi M15 belongs to *Absidiaspinosa*. Identification of metabolites was performed by combining several series of experiment through the use of UV-vis Spectrophotometer, Thin Layer Chromatography, and Gas Chromatography Mass Spectrophotometer. The analytical results showed that 2-hydrozybenzoic acid, and 2, 4-dyhidroxybenzoic acid were formed during the degradation of cresol red dye by *Trichodermaharzianum*M06. In the case of *Absidiaspinosa*M15, benzoic acid and benzeneacetic acid were identified as the metabolic products produced during the degradation of cresol red dye. From the study, it is found that M 06 and M 15 have the capability to degrade cresol red dye.For further investigation, several experiments can be carried out to enhance the use of the isolated fungi in textile wastewater treatment.

ABSTRAK

Terdapat sejumlah besar pewarna yang telah disingkirkan ke alam sekitar sebagai air sisaan. Salah satunya adalah pewarna 'cresol red' dan ia adalah salah satu pewarna yang sukar untuk disingkirkan. Dalam kajian ini, 20 kulat yang berlainan jenis telah dipilih dari hutan yang terdapat di sekitar kampus UTM dilabel sebagai M01 - M20. Kulat-kulat ini telah disaring berdasarkan keupayaannya untuk menyingkirkan pewarna 'cresol red'selepas 30 hari pengeraman. Laluan metabolik degradasi untuk pewarna 'cresol red' oleh kulat M06 dan M15 telah dikaji dalam penyelidikan ini. Selepas itu, keupayaan kedua-duakulat M06 dan M15 ini untuk meyingkirkan pewarna 'cresol red' diuji dalam keadaan pepejal dan cecair dengan kepekatan pewarna 'cresol red' sebanyak 10 ppm. Kulat ini seterusnya telah diuji keupayaannya untuk menyingkirkan pewarna 'cresol red'dalam keadaan cecair dibawah kepelbagaian keadaan seperti sumber karbon dan nitrogen, pH, kemasinan dan kesan kepekatan surfaktan (Tween 80) yang berbeza. M06 menunjukkan penyingkiran warna efisien dengan penambahan glukosa (60.01%), ammonium sulfat (67.22%), Tween 80 0.1 mL (53.82%), 50 g / L kepekatan NaCl (59.64%) dan pH 3 (67.50%). Sementara itu, M15 menunjukkan degradasi efisien pewarna 'cresol red' dengan penambahan glukosa (72,85 %), ammonium nitrat (82,40 %), Tween 80 0.1 mL (74,35 %), 150 g / L kepekatan NaCl (78,64 %) dan pH 3 (85.46 %). Kulat-kulat ini telah dikenalpasati menggunakan kaedah 18s rRNA. Berdasarkan pokok filogenetik, kulat M06 adalah dari jenis *Trichderma harzianum* dan kulat M15 adalah dari jenis *Absidia spinosa.* Seterusnya pengenalpastian produk metabolit telah dijalankan menggunakan UV-vis Spectrophotometer, Thin Layer Chromatography, dan Gas Chromatography Mass Spectrophotometer. Dari hasil analisis, dua produk metabolit iaitu 2, asid dihidrozybenzoik acid, dan 2-4 asid dihidroxybenzoik telah terhasil semasa penyingkiran pewarna 'cresol red' oleh *Trichodema harzianum* M06. Untuk kulat M15, produk metabolit dijumpai adalah asid benzoic dan asid benzeneasetik semasa degradasi pewarna cresol red. Berdasarkan kejadian ini, didapati bahawa M 06 dan M 15 mempunyai keupayaan untuk menyingkirkan pewarna 'cresol red'. Untuk siasatan lanjut, beberapa eksperimen boleh dijalankan untuk meningkatkan keupayaan penggunaan kulat terpencil dalam rawatan air sisa tekstil.

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LIST OF ABBREVIATIONS

%	-	Percent
λ max	-	Lambda Maximum
-C=O	-	Carbonyl
$(NH4)_2SO_4$	-	Ammonium Sulfate
°C	-	Celsius
°C/min	-	Celsius per Minute
$C_6H_{12}O_6$	-	Sugar
$C_7H_6O_2$	-	Benzoic Acid
$C_6H_4(OH)_2$	-	Catechol
$C_7H_6O_3$	-	Salicylic Acid
C	-	Carbon
CH_3	-	Methyl
C.I.	-	Color Index
Cl	-	Chloride
Cl_2	-	Chlorine
cm	-	Centimeter
COOH	-	Carboxyl
Cu^+	-	Copper Ion
Cu^{2+}	-	Copper (II) Ion
DNA	-	Deoxyribonucleic Acid
E'_0	-	Redox Potential
e^-	-	Electron
Fe^{3+}	-	Iron (III) Ion
g/L	-	Gram per Liter
g/mol	-	Gram per Mole

GC	-	Gas Chromatography
GC-MS	-	Gas Chromatography-Mass Spectrometry
H_2O_2	-	Hydrogen Peroxide
H_2O	-	Water
H^+	-	Hydrogen Ion
HCL	-	*Hydrochloric Acid*
kPa	-	*Kilopascal*
Lac	-	*Laccase*
LMEs	-	Lignin-Modifying Enzymes
LiP	-	Lignin Peroxides
M^+	-	Molecular Ion (peak)
M	-	Molar
MAE	-	Malt Extract Agar
Min	-	Minutes
mL	-	Milliliter
mg/L	-	Milligram Per Liter
mM	-	*Millimolar*
mm	-	*Milliliter*
MnP	-	Manganese Peroxides
Mn^{2+}	-	*Manganese (II) Ion*
Mn^{3+}	-	*Manganese (III) Ion*
MnO_4	-	*Permanganate*
m/z	-	*Mass Spectrum (*Mass-to-Charge Ratio)
N	-	*Nitrogen*
NaOH	-	Sodium Hydroxide
NH_2	-	Amines
NH_4NO_3	-	Ammonium Nitrate
NO	-	Nitro
NO_2	-	Amino
Nm	-	Nanometer
O_2	-	Oxygen
O_3	-	Ozone

OH	-	Hydroxyl
OH^-	-	Hydroxyl Radical
PAH	-	Polycyclic Aromatic Hydrocarbons
PCR	-	Polymerase Chain Reaction
pH	-	Power Hydrogen
ppm	-	Part per Million
R_f	-	Retention Factor
rpm	-	Rotor per Minute
rRNA	-	Ribosomal Ribonucleic Acid
S	-	Sulfur
Si	-	Silica
SO_3	-	Sulfur Trioxide
TLC	-	Thin Layer Chromatography
TMS	-	Trimethylsilylation
t_R	-	Retention Time
UV	-	Ultraviolet
UV-Vis	-	Ultraviolet-Visible
v/v	-	Volume per Volume
w/v	-	Weight per Volume
µL	-	Microliter
µg	-	Microgram

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Synthetic dyes are widely used in many areas. It has been used in various branches of textile industry (Gupta *et al*., 1992; Sokolowska-Gajda *et al*., 1996), of the leather tanning industry (Tu¨nay *et al*., 1999; Kabadasil *et al*., 1999) in paper production (Ivanov *et al*., 1996), in food technology (Bhat and Mathur, 1998; Slampova *et al*., 2001), in agricultural research (Cook and Linden, 1997; Kross *et al*.,1996), in light-harvesting arrays (Wagner and Lindsey, 1996), in photoelectrochemical cells (Wrobel *et al*., 2001), and in hair colorings (Scarpi et al., 1998). Moreover, synthetic dyes have been used for the control of the efficacy of sewage (Morgan-Sagastume *et al*., 1997) and wastewater treatment (Hsu and Chiang, 1997; Orhon *et al*., 1999), for the determination of specific surface area of activated sludge (Sorensen and Wakeman, 1996) for ground water tracing (Field *et al*., 1995). The metabolic pathway during the degradation of cresol red dye is also been investigated through different series of steps.

Due to rapid growth of industrialization and urbanization, many chemicals including dyes are produced and used in day-to-day life. Dyes are synthetic and aromatic molecular structural compounds. According to their dissociation in an aqueous solution, dyes can be classified as acid, direct reactive dyes (anionic), basic dyes (cationic) and disperse dyes (nonionic) (Sathiya *et al*., 2007). Dye solutions held together by physical adsorption through metals and salts using covalent bonds. Syhthetic dyes have been used increasingly in textile and dyeing industries because

of their ease and cost effectiveness in synthesis, firmness and variety in color, compared to that of natural dyes

The exact amount of dyes produced in the world is not known. It is estimated to be over 10,000 tons per year. Exact data on the quantity of dyes discharged in the environment are also not available. It is assumed that a loss of 1–2% in production and 1–10% loss in use are a fair estimate. For reactive dyes, the loss can be about 4% (Forgacsa *et al*., 2004). Due to large-scale production and extensive application, synthetic dyes can cause considerable environmental pollution and are serious health-risk factors. Although, the growing impact of environmental protection on industrial development promotes the development of ecofriendly technologies (Desphande, 2001), reduced consumption of freshwater and lower output of wastewater (Knittel and Schollmeyer, 1996; Petek and Glavic, 1996), the release of important amounts of synthetic dyes to the environment causes public concern, legislation problems and are a serious challenge to environmental scientists.

Triphenylmethane (TPM) dyes are used extensively in textile industries for dyeing nylon, polyacrylonitrile modified nylon, wool, silk, and cotton. Dyes are recalcitrant molecules which are difficult to degrade biologically. From the treatment point of view, the degradation of dyes has received considerable attention. Conventional wastewater treatment processes are suitable for stabilization of nonxenobiotic compounds whereas these processes do not work well with the xenobiotic compounds. Environmental regulations in most of the countries now have made it mandatory to decolorize the dye wastewater prior to discharge. Increasing concerns about color in the effluents are leading to the worldwide efforts to develop more effective color removal processes (Azmi *et al.,* 1998).

Cresol red dyes belong to the group of triphenylmethane dye. These dyes have been released to the environment as synthetic dyes (Gill *et al*., 2012). TPM dyes has been extensively used in cloth industry as dyeing silk, polyacrylonitrile customize nylon, coat, nylon, and cotton. They also are being used in the biological area as biological stains and marker. Leather and paper industries are also the major consumers of the TPM dyes. These groups of dyes are also used for coloring plastics,

varnish, gasoline, fats, oil, and waxes. Different types of triphenylmethane dyes are also used in cosmetic and food industries (Azmi *et al.,* 1998).

The majority of dyes are extremely poisonous and mutagenic. Artificial sources and complex aromatic configuration enable them to be more resistant to biodegradation activities (Godlewska *et al.*, 2009). Some of TPM dyes have been found in the soil due to improper waste disposal (Gill *et al.*, 2012). Therefore, innovative treatment technologies are needed to be investigated. Decolorization of dye wastewater by fungal metabolic activities is the subject of many studies. Fungi from the Basidiomycetes group, known as white rot fungi are a hetereogenous group of microorganisms but have a common capability to degrade lignin, as well as, other wood components (Kirk and Farrell, 1987). The white rot fungi are by far the most efficient ligninolytic microorganisms. They are able to degrade various recalcitrant pollutants including dyes (Sathiya *et al.*, 2007**).**

Extensive studies on biodegradation of synthetic dyes by ligninolytic fungi has been done with *Phanerochaete chyrsosporium* (Paszczynski and Crawford 1995). White rot fungus are capable to discard dyes from industrial effluents. This fungus has been investigated for their ability to degrade resistant organic pollutants such as polyaromatic hydrocarbons, chlorophenols and polychlorinated biphenyl. The decolorization of phenol red, methylene blue, coomassive blue, and dextran blue has been applied to determine ligninolytic activity (Sathiya *et al.*, 2007).

The purpose of this study is to identify the isolated fungi using 18s rRNA method to know the identity of the targeted fungi by using bioinformatics tools. The isolated fungi are screened to determine their ability to degrade and decolorize cresol red dyes. The metabolic pathway during the degradation of cresol red dye is also being investigated through different series of steps.

1.2 Problem Statement

In Malaysia, textile industry sectors has been the seventh largest contributor to total earnings from manufactured exports, due to its high demanding market either from inside and outside Malaysia (Pang and Abdullah, 2013). Resulting from this, there are massive amount of dye concentrations that has been discharged to the environment as wastewaters. About 743.99 metric tons per year of waste has been released by the textile industry according to a report from Department of Environment of Malaysia (Environmental Quality Report, 2007). Approximately, 40-65 L of textile sewage is produced per kg of fabric formed by the textile industry. For the time being, there is no effective procedure to treat these waste water materials (Azmi *et al.,* 1998). In much worse scenario, the wastewater materials were discharged into the environment at high concentrations by the industries. Dyes may affect photosynthetic activity in water because they prevent light access through the water surface thus causing shortage of oxygen and subsequently decrease the survival rate of aquatic organism in the environment. Most of the dyes produced by textiles industries are greatly toxic and mutagenic.

The release of dyes into wastewaters by various industries poses serious environmental problems due to various dyes' persistent and recalcitrant nature. In Malaysia, a lot of industries including the textile industries also increased the water pollution level. From this industry, the water pollution has become worst especially in colour point of view. The effluent from the textile industry has a high level of colour concentration. Because of this, the best water treatment process is needed to encounter this pollution problem so that the best result will be achieved.

Presently, most of the processes used for the treatment of dye wastewater are chemical processes such as adsorption, coagulation, ion exchange and reduction. These processes are costly, produce large amount of sludge, and less efficient. Since then, biological processes including microorganisms application are getting extensive attention because they are cost effective, environmental friendly, and produce small quantities of sludge (Azmi *et al.,* 1998). In this study, fungi isolated from the enviroment were investigated for their potential in treating cresol red dye.

1.3 Research Objectives

Several objectives are constructed as listed below. These objectives serve as guidelines in completing this study.

1. To screen, isolate, and identify fungi from nature that have the ability to degrade Cresol Red dyes.
2. To investigate the effect of different parameters to the degradation of Cresol Red dyes.
3. To investigate the metabolic pathway for cresol red dyes

1.4 Scope of the Study

In this study, the degradation of cresol red dye by the selected fungi was investigated by measuring its decolorization percentage during the incubation period. After that, the capabilities of the isolated fungi to degrade cresol red dyes were further tested under different conditions with alteration of carbon sources, nitrogen sources, effect of surfactant (Tween 80), salinity and different initial pH. After that, the fungi were identified using 18s rRNA methods. Subsequently, the metabolic pathway employed by the fungi in degrading cresol red dye was also investigated using series of extraction methods. These methods were tested using UV-Spectrophotometre, TLC, Column Chromatography and GC-MS.

1.5 Significance of the Study

The significance of this study is to provide an alternative method in term of decolorization and degradation of cresol red dyes wastewater using fungi which was isolated from the forest. In addition, biological process is more effective and more environmental friendly in the degradation of the synthetic dyes and can become an effective method in solving the problem of textile wastewater treatment.

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