UNIVERSITI PUTRA MALAYSIA

CHARACTERISATION OF TEXTILE DYE-DEGRADING PROPERTIES OF LIGNINOLYTIC FUNGUS ISOLATE 5-UPM

ARIFF BIN KHALID.

FBSB 2005 21

CHARACTERISATION OF TEXTILE DYE-DEGRADING PROPERTIES OF LIGNINOLYTIC FUNGUS ISOLATE 5-UPM

By

ARIFF BIN KHALID

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

May 2005

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

CHARACTERISATION OF TEXTILE DYE-DEGRADING PROPERTIES OF LIGNINOLYTIC FUNGUS ISOLATE 5-UPM

By

ARIFF BIN KHALID

May 2005

Chairman: Mohd. Yunus Abdul Shukor, PhD

Faculty : Biotechnology and Biomolecular Sciences

Local white-rot fungi isolated from soil and wood samples were screened for their ability to degrade textile azo dyes. Sixty three white-rot fungi cultures isolated from various Peninsular Malaysia locations in Selangor, Kelantan, Perak and Terengganu were screened for the ability to degrade four textile azo dyes; Ponceau 2R (C.I. 16450), Orange G (C.I. 16230), Direct Blue 71 (C.I. 34140) and Biebrich Scarlet (C.I. 26905). Forty isolates gave positive results with varying degrees of degradation. Based on these results, an unidentified white-rot fungus (Isolate 5-UPM) isolated from Universiti Putra Malaysia (UPM) Selangor campus was selected for further studies due to its ability to completely degrade all four azo dyes in the minimum amount of time. Nutritional studies on defined solid medium showed that Isolate 5-UPM was only able to degrade the four azo dyes under nitrogen-limiting conditions and an additional carbon source such as glucose was needed to provide sufficient

energy for the degradation to occur. When grown in two-stage liquid cultures, Isolate 5-UPM was able to degrade 93 to 99 % of 0.2 g/L azo dyes in two to eight days with each dye being degraded at different rates. Direct Blue 71 was degraded the fastest followed by Orange G, Ponceau 2R and Biebrich Scarlet. Generally, azo dye degradation rates were shown to be higher in agitated cultures compared to static cultures, with rates almost twice those in static cultures. Isolate 5-UPM degraded the four azo dyes optimally when incubated at 35 to 45 °C in static cultures. The initial degradation medium (pH 4.5 to 5.9) did not have any significant effects on the degradation rates except for Ponceau 2R cultures where the degradation rate was highest at pH 4.5. However, the final pH of all cultures dropped to approximately pH 4.0. Assays for lignin-modifying enzymes (LMEs) involved in azo dye degradation showed only the presence of laccase (E.C. 1.10.3.2) while lignin peroxidase (E.C. 1.11.1.14) and manganese peroxidase (E.C. 1.11.1.13) were not detected. Laccase activity profile in static liquid degradation cultures showed correlation to the azo dye degradation profile and was highest in cultures incubated at room temperatures except for Biebrich Scarlet cultures, which was highest at 30 °C. The initial pH of the degradation medium (pH 4.5 to 5.9) did not have any significant effect on laccase activity except in Direct Blue 71 culture where it is highest at pH 5.9. Laccase produced by Isolate 5-UPM during azo dye degradation was partially purified and when 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS) was used as the substrate, it was shown to have a K_m value of 0.1 mM, optimum activity at 50 to 70 °C and pH 3.5 to 4.0 while being most stable at room temperature and pH 6.0 to 7.0. Laccase was proven to directly degrade the four azo dyes with the K_m values of 1.5 x 10^{-3} mM, 9.8 x 10^{-4} mM, 1.8 x 10^{-4} mM and 1.8 x 10^{-4} mM for Ponceau 2R, Orange

G, Direct Blue 71 and Biebrich Scarlet, respectively although the latter azo dye inhibited laccase activity at concentrations higher than 0.8 mg/L.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Master Sains

PENCIRIAN SIFAT PENDEGRADASI PEWARNA AZO OLEH KULAT LIGNINOLITIK ISOLATE 5-UPM

Oleh

ARIFF BIN KHALID

Mei 2005

Pengerusi: Mohd. Yunus Abdul Shukor, PhD

Fakulti : Bioteknologi dan Sains Biomolekul

Kulat reput-putih tempatan yang di pencilkan dari sampel tanah dan kayu telah disaring untuk keupayaan mengurai pewarna tekstil azo. Enam puluh tiga kultur kulat reput-putih telah dipencilkan dari beberapa lokasi di Selangor, Kelantan, Perak dan Terengganu dan disaring untuk keupayaan mengurai empat pewarna tekstil azo; Ponceau 2R (C.I. 16450), Orange G (C.I. 16230), Direct Blue 71 (C.I. 34140) dan Biebrich Scarlet (C.I. 26905). Empat puluh kultur pencilan telah memberikan keputusan positif yang berbeza-beza tahap penguraiannya. Berdasarkan keputusan ini, satu kultur kulat reput-putih yang tidak dikenalpasti (Isolat 5-UPM) yang telah di pencilkan dari sampel di kampus Universiti Putra Malaysia (UPM) Selangor telah dipilih untuk kajian seterusnya kerana keupayaanya mengurai ke empat-empat pewarna azo yang digunakan dalam masa yang tersingkat. Kajian nutrisi menggunakan media kultur pejal terperinci menunjukkan Isolat 5-UPM hanya mampu mengurai ke empat-empat pewarna azo tersebut ketika berada di dalam keadaan kekurangan nitrogen dan sumber karbon tambahan seperti glukosa diperlukan untuk

membekalkan tenaga yang cukup bagi proses penguraian untuk berlaku. Apabila ditumbuhkan di dalam kultur cecair dua peringkat, Isolat 5-UPM mampu mengurai dari 93 hingga 99 % 0.2 g/L pewarna azo dalam dua hingga lapan hari dengan kadar penguraian yang berbeza. Direct Blue 71 telah diurai terpantas, diikuti oleh Orange G, Ponceau 2R dan Biebrich Scarlet. Secara amnya, kadar penguraian pewarna azo adalah lebih tinggi di dalam kultur goncang berbanding di dalam kultur pegun, dengan kadarnya hampir dua kali ganda di dalam kultur pegun. Isolat 5-UPM mengurai pewarna-pewarna azo tersebut secara optimum apabila dieramkan pada suhu 35 to 45 °C di dalam kultur pegun manakala pH awal media penguraian (pH 4.5 hingga 5.9) tidak mempunyai kesan yang bermakna ke atas kadar penguraian kecuali di dalam kultur Ponceau 2R di mana kadar penguraian yang tertinggi berlaku pada pH 4.5. Walaubagaimanapun, pH akhir kesemua kultur telah menurun ke sekitar pH 4.0. Pencerakinan untuk enzim-enzim pengubah lignin yang terlibat dengan penguraian pewarna azo hanya menunjukkan kehadiran laccase (E.C. 1.10.3.2) manakala lignin peroksidase (E.C. 1.11.1.14) dan mangan peroksidase (E.C. 1.11.1.13) tidak dikesan. Profil aktiviti laccase di dalam kultur cecair pegun menunjukkan korelasi dengan profil penguraian pewarna azo dan adalah tertinggi di dalam kultur yang dieram pada suhu bilik kecuali kultur Biebrich Scarlet (30 °C). pH awal media penguraian (pH 4.5 hingga 5.9) tidak mempunyai kesan yang bermakna ke atas aktiviti laccase kecuali di dalam kultur Direct Blue 71 di mana ia adalah tertinggi pada pH 5.9. Laccase yang telah dihasilkan oleh Isolat 5-UPM semasa penguraian pewarna azo telah di separa-tulenkan dan apabila 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS) digunakan sebagai substrat, didapati mempunyai nilai K_m 0.1 mM, aktiviti optimum pada 50 hingga 70 °C dan pada pH 3.5 hingga 4.0 manakala ia adalah paling stabil

pada suhu bilik atau kebawah dan pada pH 6.0 dan 7.0. Laccase ini telah dibuktikan mampu mengurai pewarna azo secara langsung dengan nilai-nilai K_m 1.5×10^{-3} mM, 9.8×10^{-4} mM, 1.8×10^{-4} mM dan 1.8×10^{-4} mM untuk Ponceau 2R, Orange G, Direct Blue 71 dan Biebrich Scarlet mengikut turutan. Walaubagaimanapun, Biebrich Scarlet didapati menyekat aktiviti laccase pada kepekatan yang lebih tinggi dari 0.8 g/L.

ACKNOWLEDGEMENTS

My sincerest gratitude to Dr. Mohd. Yunus Abdul Shukor, Prof. Dr. Mohd. Arif Syed and Dr. Shuhaimi Mustafa for their supervision and kind advice during the course of this research. I would also like to acknowledge the assistance provided by many laboratory assistants from the Faculty of Biotechnology and Biomolecular Sciences, my lab mates, Sim, Kak Su, Kak Ilah, Fazu and Surini and countless undergraduates. I'm also thankful to my wife for putting up with myself being a weekend husband. Finally, I would like to express my gratitude to the Ministry of Science, Technology and Innovation for sponsoring my Masters degree.

"Research is the act of going up alleys to see if they are blind".

Plutarch

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

2,6-DMP	2,6-dimethoxyphenol
ABTS	2,2'- azinobis (3-ethylbenzothiazoline-6-sulfonate)
BS	Biebrich Scarlet
C.I.	Colour Index
CAS	Chemical Abstracts Service
COD	Chemical Oxygen Demand
DB71	Direct Blue 71
DNA	Deoxyribonucleic acid
E.C.	Enzyme Commission
EDTA	Ethylenediaminetetraacetic acid
GPS	Global Positioning System
HPLC	High Performance Liquid Chromatography
IUPAC	International Union of Pure and Applied Chemistry
$K_{m\ (app)}$	Apparent Michaelis-Menten Constant
LME	Lignin Modifying Enzyme
OG	Orange G
PDA	Potato Dextrose Agar
P2R	Ponceau 2R
SAAA	Sodium acetate-acetic acid buffer
TE	Trace Elements

$V_{max\ (app)}$	Apparent maximum enzyme velocity

CHAPTER 1

INTRODUCTION

The modern world will be a drab and colourless place without the use of synthetic dyes. Indeed, synthetic dyes have largely replaced natural dyes, especially in the textile industry due to their generally superior qualities such as range of colours, colour intensity, ease of manufacture, fastness, and resistance to fading by physical, chemical and microbial agents (Wesenberg *et al.*, 2003).

Despite the advantages of synthetic dyes over natural dyes, synthetic dyes present their own new set of problems. The most obvious problem is aesthetic pollution of waterways caused by the presence of dyes leached from textile factories since they are visible even at low concentrations (Banat *et al.*, 1996). In addition, the presence of dyes could also potentially reduce the amount of sunlight reaching the bottom of rivers and lakes and thus affects the ability of aquatic plants to carry out photosynthesis (Banat *et al.*, 1996; Torres *et al.*, 2003; Wesenberg *et al.*, 2003). This will have the net effect of reducing the availability of oxygen in the water to other aquatic animals (Yesilada *et al.*, 2003). Another more insidious problem is production of potentially carcinogenic aromatic amine compounds from the partial cleavage of synthetic dyes, especially from the azoic dye group, so called because of the presence of (-N=N-) azo bond by anaerobic bacteria found in wastewater treatment plants.

Current azo dye removal methods usually involve physical and/or chemical treatments. Conventional wastewater treatment such as activated sludge and trickling filters generally fail to decolourise these dye effluents (Kasinath *et* al., 2003; Wesenberg *et al.*, 2003) and as stated above, they might actually worsen the problem. These methods have many disadvantages. Chemical treatments produce large amounts of chemical sludge with the attendant disposal problems while ozone is very expensive to produce (Supaka *et al.*, 2004). Physical treatments are also very expensive due to the high operating expenses to produce and regenerate activated carbon (Shen *et al.*, 1992). For these reasons, biological treatments such as utilizing the biodegradative ability of bacteria and ligninolytic fungi are being investigated as a viable and cost effective alternative.

Research into bioremediation, or the use of microorganisms or their enzymes to biotransform the contaminated environments to their original state (Thassitou and Arvanitoyannis, 2001) are currently still in the early stages. In our case, many investigators have isolated fungi from the environment for the biodegradation of textile dyes for the past 20 years or so. Fungi such as *Phanerochaete chrysoporium* and *Tinctporia sp.*, both belonging to the ligninolytic white-rot group, are among the first to have been shown to have the ability to degrade azo dyes (Awaluddin *et al.*, 2001). Until recently however, most published research, including those that have been done in Malaysia have focused on these temperate species (Awaluddin *et al.*, 2001; Levin *et al.*, 2004) while ignoring the rich biodiversity available in our tropical country.

CHAPTER 2

LITERATURE REVIEW

2.1 Production of Synthetic and Azo Dyes by Global and Malaysian Industries

A dye is a chemical that is used to impart colour onto a material and is soluble in some stages during the application process (World Bank Group, 1999). There are more than ten thousand different types of synthetic dyes that are being used by the textile industry throughout the world (Levin *et al.*, 2004) with production exceeding 700 000 metric tones annually (Toh *et al.*, 2003).

The main synthetic dye producers of the world are China, India, Russia, Eastern Europe, South Korea and Taiwan with China producing the largest amount of dyes at 200 000 metric tones annually (Wesenberg *et al.*, 2003). In 1999, the total global dye production is valued at USD 6.6 billion with Asia as the largest dyestuff market at 42 % of the total market (Wesenberg *et al.*, 2003). Out of this percentage, 60 to 70 % of the total dyes produced globally are from the azo dyes group (Pinhiero *et al.*, 2004), thus making this class of dye environmentally significant. Azo dyes are used by the textile, printing, leather, papermaking, drug and food industries due to its ease of manufacture, relatively non-toxic in their original chemical form, stability to degradation by light, temperature, detergents, oxidizers and microbial attack (Torres *et al.*, 2003; Wesenberg *et al.*, 2003).