UNIVERSITI PUTRA MALAYSIA

OPTIMIZATION OF DECOLOURISATION OF TEXTILE DYES BY A LOCALLY ISOLATED LIGNINOLYTIC FUNGUS

SIM HAN KOH

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OPTIMIZATION OF DECOLOURISATION OF TEXTILE DYES BY A LOCALLY ISOLATED LIGNINOLYTIC FUNGUS

By

SIM HAN KOH

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

January 2007

Dedicated to my father, Sim Wan Chai

and mother, Lian Kah Lang,

to my elder brother, Sim Han Teck

and my two younger sisters, late Sim Hwee Ting

and Sim Hwee Min,

and to the teachers and lecturers who

have taught me everything…

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

OPTIMIZATION OF DECOLOURISATION OF TEXTILE DYES BY A LOCALLY ISOLATED LIGNINOLYTIC FUNGUS

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Chairman: Professor Mohd Arif Syed, PhD

Faculty : Biotechnology and Biomolecular Sciences

Water pollution by textile azo dyes is a serious problem worldwide. Local white-rot fungi isolated from soil and wood samples were screened for the ability to degrade textile azo dyes. Seventy one white-rot fungi isolated from various locations in Peninsular Malaysia such as Selangor, Kelantan, Perak and Terengganu were screened for their ability to degrade four textile azo dyes namely Orange G (C.I. 16230), Ponceau 2R (C.I. 16450), Biebrich Scarlet (C.I. 26905) and Direct Blue 71 (C.I. 34140). Forty five isolates gave positive results with varying degrees of degradation. Based on these results, an unidentified white-rot fungus (Isolate S17-UPM) isolated from Universiti Putra Malaysia (UPM) campus in Selangor was selected for further studies due to its ability to completely degrade all four azo dyes in the shortest time. Nutritional studies on defined solid media showed that Isolate

S17-UPM was only able to degrade the four azo dyes under nitrogen-limiting conditions and an additional carbon source in the form of glucose was needed to provide sufficient energy for the degradation to occur. When grown in two-stage liquid culture, Isolate S17-UPM was able to degrade 84 to 99% of 0.2 g/L azo dyes in one to ten days with each dye being degraded at different rates. Orange G was degraded the fastest followed by Ponceau 2R, Direct Blue 71 and Biebrich Scarlet. Generally, azo dye degradation rates were shown to be higher in shake cultures compared to static cultures, with rates almost twice those in static cultures. Isolate S17-UPM degraded the four azo dyes optimally when incubated at temperature between room temperature to 30°C in static cultures. The initial pH of the degradation medium (pH 4.0 to 5.9) had significant effects on the degradation rates, where the highest degradation rate was found to be at pH 4.5. The final pH of all cultures dropped to approximately 4.0. Optimum degradation of the four azo dyes was observed when glucose, sucrose, maltose, lactose and fructose were used separately as additional carbon source. The degradations rates were higher at lower concentrations (0.05 g/L) as compared to higher concentrations (1 g/L) except for Biebrich Scarlet. Assays for lignin-modifying enzymes (LMEs) involved in azo dye degradation showed the presence of laccase (E.C. 1.10.3.2) only 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 Orange G cultures, which was highest at 30 °C. The initial pH of the degradation medium (pH 4.0 to 5.9) did not have any significant

effect on laccase activity except in Ponceau 2R and Biebrich Scarlet cultures where it is highest at pH 5.9. Additional carbon sources such as glucose (6C), sucrose (12C), maltose (12C), lactose (12C) and fructose (6C) which were used separately in cultures incubated with Orange G, Ponceau 2R and Direct Blue 71 gave much higher laccase activity compared to other carbon sources used. Dye concentrations ranging from 0.05 to 1.00 g/L have significant effects on the laccase activity especially Ponceau 2R. Staining activities of laccase in non-denaturing sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE) showed highlighted green bands around 66 kDa. Laccase produced by Isolate S17-UPM during azo dye degradation was partially purified using Macro-Prep High-Q^{TM} strong-anion exchanger and Superose^{TM} gel filtration column, when 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS) was used as the substrate, it was shown to have a $K_{m\ (app)}$ value of 1.6 mM, $V_{max\ (app)}$ value of 16.5 µmol/min.ml, optimum activity at 55 to 75°C and pH 2.0 to 3.0 while being most stable at room temperature and pH 6.0 to 7.0. Conclusively, an azo dye-degrading fungus was isolated and the decolourisation process was optimized, while the enzyme involve was partially purified and characterized.

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

PENGOPTIMUMAN PENYAHWARNAAN PEWARNA TEKSTIL OLEH SEJENIS KULAT LIGNINOLITIK TEMPATAN

Oleh

SIM HAN KOH

Januari 2007

Pengerusi: Profesor Mohd Arif Syed, PhD

Fakulti : Bioteknologi dan Sains Biomolekul

Pencemaran air oleh pewarna tekstil azo merupakan satu masalah seluruh dunia. Kulat reput-putih tempatan yang dipencilkan dari sampel tanah dan kayu telah disaring untuk keupayaan mengurai pewarna tekstil azo. Tujuh puluh satu kultur kulat reput-putih telah dipencilkan dari beberapa lokasi di Selangor, Kelantan, Perak dan Terengganu dan disaring untuk keupayaan mengurai empat pewarna tekstil azo; Orange G (C.I. 16230), Ponceau 2R (C.I. 16450), Biebrich Scarlet (C.I. 26905) dan Direct Blue 71 (C.I. 34140). Empat puluh lima kultur pencilan telah memberikan keputusan positif yang berbeza-beza tahap penguraiannya. Berdasarkan keputusan ini, satu kultur kulat reput-putih yang tidak dikenalpasti (Isolat S17-UPM) yang telah dipencilkan dari sampel di kampus Universiti Putra Malaysia (UPM) Selangor telah dipilih untuk kajian seterusnya kerana keupayaanya mengurai keempat-empat pewarna azo yang digunakan dalam masa yang tersingkat. Kajian nutrisi menggunakan media kultur pejal terperinci menunjukkan Isolat S17-UPM hanya

mampu mengurai keempat-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 S17-UPM mampu mengurai 84 hingga 99% 0.2 g/L pewarna azo dalam satu hingga sepuluh hari dengan kadar penguraian yang berbeza-beza. Orange G telah diurai terpantas, diikuti oleh Ponceau 2R, Direct Blue 71 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 S17-UPM mengurai pewarna-pewarna azo tersebut secara optimum apabila dieramkan pada suhu bilik hingga 30°C di dalam kultur pegun manakala pH awal media penguraian (pH 4.5 hingga 5.9) mempunyai kesan yang bermakna ke atas kadar penguraian di dalam semua kultur di mana kadar penguraian yang tertinggi berlaku pada pH 4.5. Walaubagaimanapun, pH akhir kesemua kultur telah menurun ke sekitar pH 4.0. Degradasi optimum dapat diperhatikan apabila glukosa, sukrosa, maltosa, laktosa dan fruktosa digunakan secara berasingan sebagai sumber karbon tambahan. Degradasi untuk pewarna yang diuji adalah lebih tiggi pada kepekatan rendah (0.05 g/L) berbanding dengan kepekatan tinggi (1.00 g/L), kecuali Biebrich Scarlet. Pencerakinan untuk enzim-enzim pengubah lignin yang terlibat dengan penguraian pewarna azo hanya menunjukkan kehadiran lakase (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 dapat 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 Orange G (30 °C). pH awal media penguraian (pH 4.5 hingga 5.9) tidak mempunyai kesan yang bermakna ke atas aktiviti lakase kecuali di dalam kultur Ponceau 2R dan Biebrich Scarlet di mana ia adalah tertinggi pada pH 5.9. Sumber karbon tambahan seperti glukosa, sukrosa, maltosa, laktosa dan fruktosa yang digunakan secara berasingan dalam kultur Orange G, kultur Ponceau 2R dan kultur Direct Blue 71 menghasilkan aktiviti lakase yang lebih tinggi berbanding dengan kultur yang menggunakan sumber karbon yang lain. Kepekatan pewarna yang digunakan (0.05 hingga 1.00 g/L) mempunyai kesan yang bermakna kepada aktiviti lakase terutamanya dalam kultur Ponceau 2R. Pewarnaan aktiviti laccase menerusi gel elektroforisis sodium dodesil sulfat-poliakrilamida tanpa urai menunjukkan garisan berwarna hijau di sekitar 66 kDa. Lakase yang telah dihasilkan oleh Isolat S17-UPM semasa penguraian pewarna azo telah ditulenkan separa menggunakan kolum penukar anion kuat Macro-Prep High-Q^{TM} dan kolum penurasan gel Superose^{TM}. Apabila 2,2'-azinobis (3-etilbenzothiazolin-6-sulfonat) (ABTS) digunakan sebagai substrat, ia didapati mempunyai nilai K_m 1.6 mM, nilai V_{max} 16.5 μmol/min.ml, aktiviti optimum pada 55 hingga 75°C dan pada pH 2.0 hingga 3.0 manakala ia adalah paling stabil pada suhu bilik atau ke bawah dan pada pH 6.0 dan 7.0. Kesimpulannya, sejenis kulat pengurai pewarna azo telah dipencilkan dan proses penyahwarnaan telah dioptimumkan, manakala enzim yang terlibat telah ditulenkan separa dan dicirikan.

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I certify that an Examination Committee met on 9th January 2007 to conduct the final examination of Sim Han Koh on his Master of Science thesis entitled "Optimization of Decolourisation of Textile Dyes by a Locally Isolated Ligninolytic Fungus" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

Raha Abdul Rahim, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Faridah Abdullah, PhD
Associate Professor
Faculty of Sciences
Universiti Putra Malaysia
(Internal Examiner)

Tong Chow Chin, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Internal Examiner)

Vikineswary Sabaratnam, PhD
Professor
Institute of Biological Sciences
Universiti of Malaya
(External Examiner)

_____

HASANAH MOHD GHAZALI, PhD
Professor/Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date :

This thesis 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 are as follows:

Mohd. Arif Syed, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Mohd. Yunus Abdul Shukor, PhD
Lecturer
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

Shuhaimi Mustafa, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

_____
AINI IDERIS, PhD
Professor/Dean
School of Graduate Studies
Universiti Putra Malaysia

Date : 10 MAY 2007

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

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SIM HAN KOH

Date: 23 APRIL 2007

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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	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}	Maximum enzyme velocity

CHAPTER 1

INTRODUCTION

The use of synthetic dyes makes this modern world an interesting and colourful place for mankind. Synthetic dyes have mostly replaced natural dyes, especially in the textile industry as a result of 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).

Regardless of the advantages of synthetic dyes over natural dyes, synthetic dyes present their own new set of problems. The most noticeable is the aesthetic pollution of waterways caused by the presence of dyes leached from textile factories since they are visible even in minute amounts (Banat *et al.*, 1996). Not only that, 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 water 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 dangerous problem is the production of potentially carcinogenic aromatic amine compounds from the partial cleavage of synthetic dyes by anaerobic bacteria found in wastewater treatment plants (Pinheiro *et al*., 2004), especially from the azoic dye group.

Existing 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 shortcomings. Chemical treatments produce large amounts of chemical sludge with the attendant disposal problems while production of ozone is very costly (Supaka *et al*., 2003). 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. 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 fungi, are among the first to have been shown to have the ability to degrade azo dyes (Awaluddin *et al*., 2001). However, until recently, most published research, including those that have been carried out 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.

Despite good degradation, microbes from foreign locations may cause ecological diseases. Thus, local isolates as the best candidates for bioremediation.

Azo dyes, which were designed to be very resistant to physical and biological degradation, are widely used colorants in various industries especially in textile industry. Its ubiquity arises due to its ease of manufacture, low production costs and excellent colours. However, it is now realized that contamination of waterways by azo dyes could lead to some potentially serious ecological and health problems. Current conventional water treatment methods are unable to remove them efficiently or are too expensive to apply in large-scale plants. To biodegrade the azo dyes efficiently, economically and at the same time being environmentally friendly, bioremediation offers an attractive solution.

Biodegradation of azo dyes by white-rot fungi presents a great potential for large-scale applications after many bioremediation processes being investigated for this purpose. Its biodegradation system, comprising of lignin modifying enzymes are not only efficient but also have a wide substrate range. At this time, most research are focused on a narrow range of well-known white-rot fungi while the rich biodiversity of fungi found in tropical forests such as in Malaysia is ignored most of the time. There are reasons to believe that these undiscovered species might have greater azo dye degrading abilities compared to the ones that are being studied now. Hence, the processes to isolate and screen new white-rot fungi for the biodegradation of azo dyes have to be done intensively.