UNIVERSITI PUTRA MALAYSIA

LACCASE PRODUCTION BY LOCALLY-ISOLATED Trichoderma Sp. IS1037 USING RUBBERWOOD DUST FOR BIOBLEACHING OF SODA BAMBOO PULPS

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By

SAJA MAHDEY JABER

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysiin Fulfillment of the Requirements for the Degree of Doctor of Philosophy

July 2017

DEDICATION

This thesis is dedicated to my parents and my husband for their love and supports me. Without them, none of this would have been possible.

Saja Mahdey Jaber
July 2017

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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Chairman : Associate Professor Umi Kalsom Md Shah, PhD
Faculty : Biotechnology and Biomolecular Science

Conventional pulp bleaching of lignocellulosic materials using chlorine and its compounds leads to the generation of toxic compounds including chlorolignins. Therefore a study on biobleaching of lignocellulosic material was carried out as an alternative for pulp bleaching purpose. This study focused on the use of laccase from *Trichoderma* sp. IS1037 for bamboo pulp biobleaching. The laccase enzyme was produced from a local fungi isolate *Trichoderma* sp. IS1037. The possibility of using crude laccase for bleaching bamboo soda pulp by combining it with bleaching chlorine agents was also studied. A total of five native fungi was screened to determine the potential producer of laccase enzyme. The fungi isolate was identified by utilizing the Biolog Kit and molecular assay. Optimization of culture condition for laccase production was carried out using seven selected parameters known as carbon source, nitrogen source, temperature, pH, agitation speed, concentration of $CuSO_4$, and surfactant. The crude laccase produced was used for the pretreatment of bleaching process on unbleached bamboo soda pulp. Optimization of temperature, pH and reaction time on crude laccase pretreatment of bamboo pulp was investigated using response surface method (RSM) followed by chemical bleaching sequences $D_1E_PD_2$. In order to evaluate the decrease in lignin content, adsorbable organic halogens (AOX), hexenuronic acid (HexA) and chemical oxygen demand (COD) were tested. Solid phase extraction was conducted to extract AOX from effluent after bleaching process and HPLC was aplied to determine AOX concentration. HexA was conducted using UV spectrophotometer. The result from screening demonstrated that laccase produced from locally isolated fungi identified as *Trichoderma* sp. IS1037 under optimized condition was 65.0 U/ml which is 11 fold higher than the production of the control culture 5.8 via submerged fermentation strategy. The optimum condition for laccase production was 30 ℃, 4.5 and 150 rpm for temperature, initial pH media and agitation speed, respectively, using rubberwood dust as a carbon source together with organic nitrogen source (peptone, yeast extract and malt extract). The effects of copper sulphate and Tween 80 were found significant in improving the extracellular laccase production. In pretreatment of bamboo pulp using laccase under optimized condition has decreased lignin by 64% and hemicellulose by 50% content was achieved at pH, temperature and reaction time of 4, 50 ℃ and 4 hours, respectively. XRD and FTIR analysis showed an increase in relative crystallinity of cellulose and delignification due to hydroxylation and exfoliation of amorphous regions through laccase pretreatment. In addition, there were significant result found in the reduction of AOX, COD, HexA and lignin by the enzymatic pretreatment. The AOX content of bamboo pulp was reduced by 29%, 34% for biobleaching and modified sample respectively. Moreover, HexA results revealed decrement in content with 1.44% to 1.42% for biobleaching and modified sample respectively. Therefore, the pretreatment of bamboo pulp by laccase has considerable benefit on reducing the organic compound of AOX, COD, HexA and chemical composition in different sequence bleaching. The outcomes presented in this study could contribute to the future study on the production of laccase enzyme from *Trichoderma* sp.IS1037 for various industrial applications including pulp and paper industry.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENGELUARAN LACCASE DARIPADA *Trichoderma* sp. IS1037 PENCILAN TEMPATON MENGGUNAKAN HABUK KAYU GETAH UNTUK BIO-PELUNTUR SODA PULPA BULUH

Oleh

SAJA MAHDEY JABER

Julai 2017

Pengerusi : Profesor Madya Umi Kalsom Md Shah, PhD
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Pelunturan pulpa bahan lignoselulosa konvensional dan sebatiannya membawa kepada penghasilan sebatian toksik termasuk klorolignins. Oleh itu kajian mengenai bio-peluntur dan bahan lignoselulosa telah dijalankan sebagai alternatif untuk tujuan pelunturan pulpa. Kajian ini tertumpu kepada penggunaan enzim laccase daripada *Trichoderma* sp. IS1037 untuk bio-pelunturan pulpa buluh. Enzim laccase dihasilkan daripada isolate kulat *Trichoderma* sp. IS1037. Kemungkinan menggunakan laccase mentah untuk pelunturan pulpa soda buluh dengan menggabungkan ia dengan agen klorin juga dikaji.

Dalam kajian ini, lima kulat tempatan telah disaring untuk menentakar kulat yang berpotensi mengeluarkan enzim laccase. Pencilan kulat telah dikenal pasti dengan menggunakan Kit Biolog dan cerakin molekul. Pengoptimuman keadaan kultur untuk pengeluaran laccase telah dijalankan dengan menggunakan tujuh parameter terpilih yang dikenali sebagai sumber karbon, sumber nitrogen, suhu, pH, kelajuan pergolakan, kepekatan $CuSO_4$, dan surfaktan. Laccase mentah yang dihasilkan telah digunakan untuk rawatan awal proses pelunturan pada pulpa buluh soda yang belum diluntur. Pengoptimuman suhu, pH dan masa tindak balas kepada rawatan awal laccase mentah ke atas pulpa buluh dikaji menggunakan kaedah gerak balas permukaan (RSM) diikuti dengan urutan pelunturan kimia $D_1E_PD_2$. Untuk menilai penurunan kandungan lignin, halogen organik boleh serap (AOX), asid hexenuronik (Hexa) dan permintaan oksigen kimia (COD) telah dujid. Pengekstrakan fasa pepejal telah dijalankan untuk mengeluarkan AOX daripada pelunturan efluen selepas proses pelunturan dan HPLC telah dijalankan untuk menentukan kepekatan AOX. HexA dntentukan dengan menggunakan spektrofotometer UV. Hasil daripada saringan menunjukkan laccase yang dihasilkan dari kulat asingan tempatan dikenal pasti sebagai *Trichoderma* sp. IS1037 pada keadaan optimum adalah 65.0 U / ml iaitu 11 kali ganda lebih tinggi daripada pengeluaran daripada kultur kawalan 5.8 melalui strategi fermentasi tenggelam. Keadaan optimum untuk pengeluaran laccase adalah 30 °C, 4.5 dan 150 rpm untuk suhu, media pH awal dan kelajuan pergolakan, masing-masing, dengan menggunakan habuk kayu getah sebagai sumber karbon bersama-sama dengan sumber organik nitrogen (pepton, ekstrak yis dan ekstrak malt). Kesan kuprum sulfat dan Tween 80 didapati ketara dalam meningkatkan pengeluaran laccase luarsel. Dalam rawatan awal pulpa buluh, menggunakan laccase bawah keadaan optimum telah menurunkan lignin sebanyak 64% dan hemiselulosa dengan kandungan 50% telah dicapai pada pH, suhu dan masa tindak balas 4, 50 °C dan 4 jam, masing-masing. Analisis XRD dan FTIR menunjukkan peningkatan dalam penghabluran relatif selulosa dan delignifikasi kerana penghidroksilan dan pengelupasan kawasan amorfus melalui rawatan awal laccase. Di samping itu, terdapat hasil yang ketara dijumpai di dalam pengurangan AOX, COD, Hexa dan lignin daripada prarawatan bio-pelunturan itu. Kandungan AOX pulpa buluh telah dikurangkan sebanyak 29%, 34%, untuk bio-pelunturan dan sampel yang diubahsuai, masing-masing. Selain itu, keputusan Hexa menunjukken penurunan dalam kandungan dengan 1.42%, dan 1.14%, untuk bio-pelunturan dan sampel yang diubahsuai, masing-masing. Oleh itu, rawatan awal pulpa bambu oleh laccase mempunyai manfaat yang besar dalam mengurangkan kompaun organik AOX, COD, Hexa dan komposisi kimia dalam urutan pelunturan yang berbeza. Hasil dibentangkan dalam kajian ini boleh menyumbang kepada kajian masa depan tentang pengeluaran enzim laccase dari *Trichoderma* sp. IS1037 untuk pelbagai aplikasi industri termasuk industri pulpa dan kertas.

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Saja Mahdey Jaber
July 2017

I certify that a Thesis Examination Committee has met on 18 July 2017 to conduct the final examination of Saja Mahdey Jaber on her thesis entitled "Laccase Production by Locally-Isolated *Trichoderma* Sp. IS1037 Using Rubberwood Dust for Biobleaching of Soda Bamboo Pulps" 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 Doctor of Philosophy.

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LIST OF ABBREVIATIONS

ABTS	2, 2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
AOX	Absorbed organic halogen
COD	Chemical oxygen demand
CTMP	Chemi-thermo-mechanical pulping
D	Sodium chlorite
ECF	elemental chlorine-free
E_P	Alkaline
FTIR	Fourier Transform Infrared Spectroscopy
HexA	Hexeneuronic acid
HPLC	High Performants Liquid Chromatography
KH_2PO_4	Potassium Dihydrogen Phosphate
L	Liter
LiP	Lignin peroxidase
LMS	Laccase mediator system
LWM	Low molecular weight
mL	Milliliter
MnP	Manganese peroxidase
$MgSO_4.7H_2O$	Magnesium Sulfate Heptahydrate
NaOH	Sodium Hydroxide
Nm	Nanometer
mg/mL	milligram/milliliter
Rpm	revolutions per minute
RSM	Response surface method
SEM	Scanning Electron Microscopy
SmF	Submerged fermentation
SSF	Solid state fermentation
U/g	Unite/gram
U/mL	Unite/milliliter
µL	Microliter
XRD	X-ray Diffraction

CHAPTER 1

INTRODUCTION

1.1 General Background

Nowadays, laccase has become increasingly important as an eco-friendly technology in the pulp and paper industry and lead to greater profitability for the industry. With the growing demands for laccase applications in industrial processes, there should be expanding research of microorganisms with high laccase activities and discovering novel laccases with enhanced stability and superior catalytic properties (Desai and Nityanand, 2011; Kiiskinen *et al*. 2004). Although studies about laccase activity in lignin degradation have been reported, laccases cannot catalase the lignin degradation and model compounds without combination with redox mediator compounds, which is increase the range of compounds for oxidation such as non-phenolic substrates. In addition, Laccase–mediator systems enhance the interaction between the laccase and non-phenolic fractions of lignin in wood pulp due to ability to diffuse into cellulose fibres (Barreca *et al*., 2004; Galli and Gentili, 2004; Bajpai *et al*., 2006; Fillat and Roncero, 2010).

The paper and pulp industry involves the process of bleaching pulp which currently uses mostly chemicals such as chlorine dioxide and alkaline extraction (Zhao *et al* ., 2010) which is responsible for generating adsorbed organic halides (AOX) and other chlorine compounds due to chlorine reacts with lignin (Comlekcioglu *et al*., 2014). Traditionally, using a high amount of chlorine in the bleaching process results high brightness paper and large quantity of harmful substances in paper mill effluents into rivers and ponds, which is a potential hazard and contributes to environmental pollution (Morselli *et al.*, 2014; Yao *et al*., 2016). The best way to solve this problem is to adopt an effective bleaching method with low pollution and that is enzymatic bleaching (Pei *et al*., 2016; Gangwar *et al*., 2016). Enzyme technology is gaining global recognition due to the fact which it is high catalytic efficiency, high substrate specificity, shorter reaction time, low energy input, mild reaction conditions and environmentally friendly (Hanana *et al*., 2015; Wong, 2016). Therefore, using laccase treatment in bleaching processes is recommended for the pulp and paper industry to significantly reduce the consumption of chlorine dioxide and generation organic halides, at the same time achieve enhanced paper quality.

Various agricultural wastes have been used as fermentation substrates including wheat bran, rice straw, bagasse, corncobs, banana peels, sunflower receptacle, apricot seed shell, and saw dust in order to produce laccase enzyme (Jesus *et al*., 2015; Hendro *et al*., 2012; Emre and Özfer, 2013). However, there have been some limited studies that used rubber wood dust as a carbon source for laccase production.

Laccases (EC 1.10.3.2) are derived from a family of blue multi-copper oxidases usually associated with polymerisation–depolymerisation reactions that induce phenols and aromatic or aliphatic amines to be oxidised to the corresponding reactive radicals using oxygen as electron acceptor (Riva, 2006). Besides plants, they are also distributed in fungi, bacteria and insects (Janusz, *et al*., 2013; Arca-Ramos *et al*., 2015). In fungi, there has been successful isolation of these enzymes from Ascomycetes, Deuteromycetes, and Basidiomycetes (Brijwani *et al*., 2010), which secrete laccases that contribute to lignin degradation and function essentially in the carbon cycle in the degradation of lignocellulosic material. Many achievements have been reported, but for most of the industrial processes, there is still an obstacle to wider application of laccases due to the high cost of production (Osma *et al*., 2011; Liu *et al*., 2013), which significantly affects production efficiency. Therefore, many researches have been carried out and attempted to produce laccases more efficiently, such as by optimization of fermentation conditions, isolation, and identification of new potential high-level laccase producing strains.

The production of laccase in fungi is low and is unable to meet the demand for practical applications in industry and biotechnology. As a result, there has been much work done to develop a laccase production process that is both cost-effective and efficient. Towards this end, attention has been focused on producing laccase from fungi by utilizing aromatic compounds including xylidine, ferulic acid, and veratric acid which have been found to enhance laccase production during the fermentation process (Yang, *et al.*, 2013; De Souza *et al.*, 2004). Copper has also been known to promote laccase activity in the majority of fungi, in *Trametes versicolor* (Collins and Dobson, 1997) *Pleurotus sajor-caju*, (Soden and Dobson, 2001) *Ceriporiopsis subvermispo*ra (Alvarez, *et al.*, 2009) *Pleurotus ostreatus* (Faraco, *et al.*, 2003) and *Trametes pubescens,* (Galhaup, *et al.*, 2002).

Applying a statistical process such as Response Surface Method (RSM) is very useful in the optimisation of the biobleaching process of unbleached soda pulp, by determining the effects and interactions of pH, temperature, and reaction time on laccase pretreatment, as this is crucial for the reduction of chemical composition content, especially lignin in bamboo. An RSM comprises an empirical modelling system that assesses how a group of experimentally controllable variables is related to an observed response. Limited research has been carried out to examine the benefits of RSM in optimizing laccase pre-treatment of the chemical composition in the bamboo bleaching process.

With increasing scarcity of natural resources, and environmental pollution has raised to demand for natural fiber non wood in paper industry as alternative raw materials for this intent in the future. Bamboo (*Gigantochloa scortechinii*) is a hard non wood plant with superior physical and mechanical properties, it's a rapid growth rate and can give high yields. Therefore, bamboo has long been widely used for constructional purpose and for the pulp and paper in industry (Liu *et al*., 2012; Abdul Khalil *et al.*, 2014).On the other hand, the natural bamboo fibers have great properties such as hygroscopic, which is mean it is able to obtain water from the environment and hold onto it (Erakhrumen and Ogunsanwo 2009). This is important because in the chemical pulping processes, cooking liquor needs to penetrate throughout the chips of a sample such as bamboo (Moradbak *et al*. 2016). However, not much research has been focused on the use of crude laccase on soda bamboo pulp bleaching Therefore, to assess the suitability of utilizing cured laccase, it is suggested that there be a sequential pretreatment before standard elemental chlorine-free (ECF) bleaching that acts on bamboo soda pulp to determine the optical properties prior to and following an accelerated ageing process of the resulting paper sheets.

1.2 Problem Statement

The increasing volume and chemical complexity such as chlorine in the bleaching methods from pulp and paper industry is seriously represented environmental problem today. Chlorine dioxide is the most important bleaching chemical in the elemental chlorine-free (ECF) bleaching of pulp. It has good selectivity for lignin removal, but there will be some adsorbable organic halogen (AOX) formed in the bleaching process (Pei *et al*., 2016; Zhu *et al*., 2016). As a result of reaction between residual lignin from wood fibres and chlorine/alkaline compounds used for bleaching could be discharging these chemical compounds and other toxic as wastewater reason carcinogenic and mutagenic (Gangwar *et al*. 2016) beside that removal of these compounds are costly. To overcome this problem, there is option of cost effective strategy through using enzymatic stage integrated into a bleach sequence (Moldes *et al*., 2010; Gangwar *et al*. 2016). Many researchers have use enzyme in pulping and bleaching process such as xylanase and laccase (Moldes *et al*., 2010; Xu *et al*., 2013; Gangwar *et al*. 2015; Pei *et al*., 2016; Zhu *et al*., 2016) with different type of non-wood.

Laccases are widely distributed multicopper oxidases, and have been isolated from fungal strains belonging to Ascomycetous, Deuteromyctous and Basidiomycetous family. Many of the previous studies have been focused on lignin degrading enzymes from white rot-fungi such as *Phanerochaete chrysosporium*, *Trametes versicolor*, and *Echinodontium taxodii* 2538 (Shi, *et al*., 2014; Kong *et al*., 2015) however, studying the lignolytic enzymes from *Trichoderma* spp. recently there has been a growing interest due to exhibit better lignolytic systems with characterization of their substrates, copper content or amino acid sequence, for use in various biotechnological applications (D'Souza *et al*., 1999; Chakroun *et al*., 2010; Khambhaty *et al*., 2015). Thus, it was of importance to know *Trichoderma* strains with phenol oxidase activity has high efficiency for selective lignin degradation in the biobleaching process, causing modifications in lignin structures and significant decreases organic compounds ratios. In addition, pretreatment with laccase can improve reduction of chlorine dioxide and the other bleaching chemicals usage. Beside that improved paper properties such as brightness, whiteness, burst index, tensile and tear index values (Thakur *et al*. 2012; Andreu and Vidal 2013; Song *et al*., 2016).

1.3 Objectives

This current study was conducted to determine the potential of locally available fungi-produced laccase enzyme with optimum culture conditions of production and to evaluate the effectiveness of laccase as the pulp biobleaching agent. The specific objectives of this study were:

1- To screen and select the potential fungal from local isolate with ability to produced laccase.
2- To determine the optimum culture conditions for laccase production from *Trichoderma* sp. in submerged fermentation and to characterize the laccase produced.
3- To optimize the enzymatic ECF bleaching of soda bamboo using response surface method (RSM) viz pH, temperature, and reaction time.
4- To evaluate the effect of laccase pretreatment on bamboo fibre to reduce the chemical dosage in (ECF) bleaching sequences.

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