PRODUCTION OF EFFICIENT LIGNIN DEGRADING ENZYMES BY NEWLY ISOLATED *CERRENA* SPECIES

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DEDICATION

Dedicated to my beloved parents, husband, kids and siblings

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ABSTRACT

White rot fungi have attracted global attention because of their potential application in biotechnology industry. Of all ligninolytic exoenzymes produced by white rot fungi, laccases are one of the most investigated enzymes related to a variety of green oxidation processes. The study of ligninolytic enzymes has been hampered by the difficulty in producing these enzymes in sufficient quantities due to a large number of factors contribute to the development of morphological forms that will influence the enzymes production optimization. In this study, the identification of white rot fungi producing ligninolytic enzymes from local soils, optimization study, purification and characterization work aiming to laccase were carried out. Eight isolates white rot fungi were identified as potentially useful over 119 isolates screened. *Cerrena* sp. WICC F39 was chosen based on the high laccase activity of enzyme production. Optimization was carried out using one-factor-at-time (OFAT) and statistical approach using response surface methodology (RSM). Laccase enzymes produced in selected unoptimized screened medium composed of (g L^{-1}): mannitol, 10.0; ammonium tartrate, 2.0; potassium dihydrogen phosphate, KH_2PO_4, 0.8; dipotassium hydrogen phosphate, K_2HPO_4, 0.6; magnesium sulphate heptahydrate, $MgSO_4 \cdot 7H_2O$, 0.5; copper sulphate pentahydrate, $CuSO_4 \cdot 5H_2O$, 0.1 mM; Manganese sulphate pentahydrate, $MnSO_4 \cdot 5H_2O$, 0.1 mM and yeast extract, 3.0 (pH 6.0), which produced highest laccase at 199.67 U ml^{-1}. The medium optimized using OFAT composed of (g L^{-1}): rice straw, 200.0; starch, 5.0; peptone, 1.5; ammonium tartrate, 2.0; potassium dihydrogen phosphate, KH_2PO_4, 0.8; K_2HPO_4, 0.6; $MgSO_4$, 0.5; $CuSO_4$ (1 mM); $MnSO_4$ (1 mM) produced laccase of 552.31 U ml^{-1}. Next, optimization using RSM contributed to maximum laccase production of 496.89 U ml^{-1} with optimum medium concentration (in g L^{-1}): rice straw, 179.3; starch, 11.8; peptone meat, 3.5; ammonium tartrate, 0.1 and KH_2PO_4, 0.2. Further cultivation of *Cerrena* sp. WICC F39 was carried out using batch mode in a 5-L bioreactor. Using OFAT optimized medium, copper added resulted maximum laccase production 478 U ml^{-1} after 72 hours cultivation while non-copper added medium produced 189 U ml^{-1} after 48 hours cultivation. Laccase from *Cerrena* sp. WICC F39 was purified by anion-exchange chromatography and gel filtration chromatography resulted with the fold of purification about 5834.68 times and 158.6% recovery and molecular weight of 62 kDa. The calculated K_m and V_{max} value of the enzyme using ABTS as substrate were 0.107 mM and 77101.00 S^{-1} mM^{-1}, respectively. The optimum pH, optimum temperature, pH stability and thermal stability of laccases were 2.5, 60°C, 4–6, 20-80°C, respectively. Sodium azide was an inhibitor for laccases from *Cerrena* sp. WICC F39. In accordance to the results showed in this study, such high level secretion of laccase and other ligninolytic enzymes make *Cerrena* sp. WICC F39 as a potential candidate for enhanced bioremediation.

ABSTRAK

Kulat reput putih telah menarik perhatian global kerana potensinya dalam aplikasi industri bioteknologi. Daripada semua eksoenzim ligninolitik yang dihasilkan oleh kulat reput putih, lakase yang merupakan enzim yang kerap dikaji di dalam proses pengoksidaan hijau. Kajian terhadap enzim ligninolitik ini adalah terhad berikutan kesukaran menghasilkan enzim-enzim ini didalam kuantiti yang mencukupi disebabkan oleh pelbagai faktor yang mempengaruhi pembentukan morfologi kulat yang akan mempengaruhi pengoptimuman enzim. Dalam kajian ini, penentuan kulat reput putih yang menghasilkan enzim ligninolitik dari tanah tempatan, proses pengoptimuman, penulenan dan pencirian terhadap enzim lakase telah dijalankan. Lapan pencilan kulat reput putih telah dikenal pasti sebagai berpotensi digunakan daripada 119 pencilan yang dipilih. *Cerrena* sp. WICC F39 telah dipilih berdasarkan kepada aktiviti pengeluaran lakase enzim yang paling tinggi. Pengoptimuman telah dijalankan menggunakan kaedah satu-faktor-dalam-satu-masa (OFAT) dan kaedah statistik sambutan permukaan (RSM). Enzim lakase yang dihasilkan dalam penyaringan media terpilih sebelum pengoptimuman mengandungi (g L^{-1}): manitol, 10.0; ammonium tartrat, 2.0; kalium dihidrogen fosfat, KH_2PO_4, 0.8; kalium hidrogen fosfat, K_2HPO_4, 0.6; magnesium sulfat heptahidrat, $MgSO_4 \cdot 7H_2O$, 0.5; kuprum sulfat pentadhidrat, $CuSO_4 \cdot 5H_2O$, 0.1 mM; mangan sulfat pentahidrat, $MnSO_4 \cdot 5H_2O$, 0.1 mM dan yis ekstrak, 3.0 (pH 6.0) ialah 199.67 U ml^{-1}. Medium yang telah dioptimumkan menggunakan OFAT mengandungi (g L^{-1}): jerami padi, 200.0; kanji, 5.0; pepton, 1.5; ammonium tartrat, 2.0; kalium dihidrogen fosfat, KH_2PO_4, 0.8; kalium hidrogen fosfat, K_2HPO_4, 0.6; magnesium sulfat heptahidrat, $MgSO_4 \cdot 7H_2O$, 0.5; kuprum sulfat pentadhidrat, $CuSO_4 \cdot 5H_2O$ (1 mM); mangan sulfat pentahidrat, $MnSO_4 \cdot 5H_2O$ (1 mM) menghasilkan lakase pada 552.31 U ml^{-1}. Seterusnya, pengoptimuman menggunakan RSM menyumbang kepada pengeluaran maksimum enzim lakase pada 496.89 U ml^{-1} dengan kepekatan medium optimum (g L^{-1}): jerami padi, 179.3; kanji, 11.8; pepton meat, 3.5; ammonium tartrat, 0.1 dan kalium dihidrogen fosfat, KH_2PO_4, 0.2. Pengkulturan *Cerrena* sp. WICC F39 selanjutnya telah dijalankan menggunakan mod kelompok dalam tangki teraduk 5-L. Menggunakan medium optimum OFAT, penambahan kuprum menghasilkan maksimum lakase 478 U ml^{-1} selepas 72 jam pengkulturan manakala medium yang tiada penambahan kuprum menghasilkan lakase 189 U ml^{-1} selepas 48 jam pengkulturan. Lakase daripada *Cerrena sp.* WICC F39 telah ditulenkan menggunakan kromatografi penukar ion dan kromatografi penapisan gel menghasilkan jumlah penulenan kira-kira 5834.68 kali dan 158.6% pemulihan dan jisim molekul lakase pada 62 kDa. Pengiraan nilai K_m dan V_{max} enzim menggunakan ABTS sebagai substrat ialah masing-masing 0.107 mM dan 77101.00 S^{-1} mM^{-1}. PH dan suhu optimum, kestabilan pH dan kestabilan termal lakase yang diperolehi adalah masing-masing pada 2.5, 60 ° C, 4-6, 20-80 ° C. Natrium azide merupakan perencat untuk lakase *Cerrena* sp. WICC F39. Selaras dengan keputusan yang ditunjukkan didalam kajian ini, rembesan tahap tinggi lakase dan enzim ligninolitik lain menjadikan *Cerrena sp.* WICC F39 berpotensi sebagai agen bioremediasi yang boleh dipertingkatkan.

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LIST OF ABBREVIATIONS

ABTS	-	2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid
ANOVA	-	Analysis of Variance
BBD	-	Box Behnken Design
$CaCO_3$	-	Calcium Carbonate
CO_2	-	Carbon Dioxide
$CaCl{\cdot}2H_2O$	-	Calcium Chloride dehydrate
$CuSO_4{\cdot}2H_2O$	-	Calcium Chloride dihydrate
CFU	-	Colony Forming Unit
CDW	-	Cell Dry Weight
DO	-	Dissolved Oxygen
DMP	-	2,6-dimethyhoxyphenol
EH	-	Enzyme Hydrolysis
FDA	-	Food and Drug Administration
$FeSO_4{\cdot}7H_2O$	-	Ferrous Sulphate Heptahydrate
GRAS	-	Generally Recognize as Safe
H_2O_2	-	Hydrogen Peroxide
IBD	-	Institute of Bioproduct Development
ITS	-	Internal Transcribed Spacer
Lac	-	Laccase
LiP	-	Lignin Peroxidase
LMS	-	Laccase Mediated System
LSW	-	Liftshitz-Slezov-Wagner
MCT	-	Medium Chain Triglycerides
MnP	-	Manganese Peroxidase
KH_2PO_4	-	Potassium Dihydrogen Phosphate
K_2HPO_4	-	Dipotassium Hydrogen Phosphate
$MgSO_4{\cdot}7H_2O$	-	Manganese Sulphate Heptahydrate
$MnCl_2$	-	Manganese Chloride
$MnSO_4{\cdot}7H_2O$	-	Manganese Sulphate
Mwt	-	Molecular Weight

NaCl	-	Sodium Chloride
Na_2HPO_4	-	Sodium Phosphate dibasic
$NaNO_3$	-	Sodium Nitrate
NaOH	-	Sodium Hydroxide
NCBI	-	National Center for Biotechnology and Information
NaH_4Cl	-	Ammonium Chloride
$(NH_4)_2SO_4$	-	Ammonium Sulphate
$(NH_4)_2HPO_4$	-	Ammonium Hydrogen Phosphate
$NANH_4 \cdot HPO_4 \cdot 4H_2O$	-	Ammonium Sodium Hydrogen Phosphate Tetrahydrate
18S	-	18S Ribosomal RNA
OD	-	Optical Density
OD600	-	Optical Density at 600 nm
OFAT	-	One Factor At Time
QR	-	Quinone Reductase
rRNA	-	Ribosomal RNA
RSM	-	Response Surface Methodology
SSF	-	Solid Sate Fermentation
sp.	-	Species
TEM	-	Transmission Electron Microscopy
TEMED	-	Tetramethylethylenediamine
UK	-	United Kingdom
US	-	Unites States
UTM	-	Universiti Teknologi Malaysia
UV-Vis	-	Ultraviolet-Visible
VA	-	Veratryl Alcohol
VP	-	Versatile Peroxidase
VVM	-	Volume of Air per Litre of Medium per minute
WICC	-	Wellness Centre of Collection
$ZnSO_4 \cdot 4H_2O$	-	Zinc Sulphate

LIST OF SYMBOLS

%	-	Percentage
µg/ml	-	Micro Gram Over Mililiter
D	-	Bul-Phase Solubility
g	-	Diffusion
hr	-	Gram
Hz	-	Hour
M	-	Hertz
min	-	Molar
min	-	Minte
ml	-	Milililiter
nm	-	Nanometer
°C	-	Degress Celsius
$p \leq 0.05$	-	Significant Vales Less Than 95%
R	-	Gas Constant
r	-	Particle Radius
T	-	Absolute Temperature
Vm	-	Molar Volume of the Internal Phase
w/v	-	Weight Over Volume
ρ	-	Density of Internal Phase
ω	-	Frequency of Rupture per Unit Surface of Particle Film
U	-	Enzymes Activity (µmol min^{-1} ml^{-1})

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CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Fungi have been reported as a good producer for lignin-degrading enzymes (Harith *et al.*, 2014). The well-known fungi which degrade lignin to their certain extent are white, brown, soft-rot fungi, and Deuteromycetes (Bugg and Rahmanpour, 2015). Amongst these, white rot fungi are extensively reviewed as the most efficient bio-degrader in nature (Manavalan *et al.*, 2015). The unique characteristic that differentiates from most other microorganisms is their capability to mineralize all components of lignin to carbon dioxide and water (Sigoillot *et al.*, 2012). White rot fungi degrade lignin and cellulose that commonly cause the rotted wood to become moist, soft, spongy or stringy with white or yellow appearance during the deterioration process (Godliving and Mtui, 2012). A perusal of literature revealed various studies dealing with lignin biodegradation of white rot fungi (Datta *et al.*, 2017; Madadi and Abbas, 2017).

In biotechnology, white rot fungi turn into an important interest when the lignin degradation was being systematically explored and its mechanisms are revealed (Novotný *et al.*, 2009). White rot fungi constitute the biodegradation by penetrating the wood with its versatile machinery of enzymes. These multi enzymatic processes led to easily metabolize carbohydrates complexes and attack directly the lignin barrier with separate or cooperative enzymes function (Acharya *et al.*, 2010). The powerful extracellular heme peroxidases enzymes which included in lignin biodegradation are lignin peroxidase, LiP (EC 1.11.1.14) (Tien and Kirk, 1988), manganese peroxidase, MnP (EC 1.11.1.13) (Paszczyński *et al.*, 1988) and one glycosylated blue multi-copper phenol oxidase known as laccases, Lac (EC 1.10.3.2) (Mayer and Staples, 2002).

The degradation of lignin is dependent on carbohydrate-active enzymes, whose functions do not overlap. Therefore, usually more than one lignin-modifying enzymes (LMEs) are secreted by white-rot fungi in addition to other compounds for effective lignin degradation (Coconi-Linares *et al.*, 2014). Due to their metabolic diversity, high production capacity, secretion efficiency and adaptable to post-translational modifications, white rot fungi have been widely used in the fermentation industry (Krull *et al.*, 2013). Furthermore, heightened interest in industrial relevance for using ligninolytic enzymes as biocatalyst in chemical substituting processes such as in the textile, pulp and paper as well as in pharmaceutical field promoted safe and green chemical removal of lignin (Mate and Alcalde, 2016). The novelty of the present study is the identification of new isolated white rot fungus of *Cerrena sp.* WICC F39 and its efficient, low-cost schemes and optimized fermentation process; focusing on availability, high enzyme activity productivity and purity of laccase obtained.

1.2 Problem Statements

In past years, white rot fungi are well known as a source of powerful enzymes (Quintanilla *et al.*, 2015). This filamentous structures (hyphae) can explore large volumes of substrates and given wide contact area for nutrient uptake (Liu *et al.*, 2008). However, screening for ligninolytic enzymes in local soil is limited and hence, little is known about these enzymes from Malaysian soil (Cheng *et al.*, 2016). Even there have been many efforts empirically precise about the application of these fungi (Ibrahim, 2008; Mangamuri *et al.*, 2012; Mohamed *et al.*, 2013), however, there is still largely unexplored and many novel ligninolytic enzymes may await discovery. Since compile literature on the instability of *Phanerochaete chrysosporium* during production and poor understanding of its degradation mechanism have been reported, the response of other potential organism need to be discovered (Chen *et al.*, 2011; Singh and Chen, 2008). However, the application of enzyme extracts into commercially successful instruments has been hampered mainly by their poor stability and high operation cost. Furthermore, even it is well known

that lignin biodegradation is carried out mainly by white rot fungi, but in fact actual knowledge in the production of these enzymes in large scales still reveals large gaps.

Development of a novel economic design of the full-scale submerged fermentation process is required for the economic feasibility of the process (Babič *et al.*, 2012). However, the process performed by the filamentous fungi embraced difficulties mainly in unravelling the link between morphology and its physiology during cultivation. In submerged cultures, the growth morphological of filamentous fungi could vary from compact pellet to suspension of dispersed mycelia (filaments). Filaments leads to high viscosity of the cultivation growth and insufficient mixing thus resulted in low nutrient supply (Krull *et al.*, 2010). While distinct pellets showed Newtonian flow behaviour raised to limited nutrient availability within the inner part of the bio-pellets. Even many studies opposing important concerns of good control of mycelial morphology during the fermentation process and yet, limited to certain types of strains only (Papagianni, 2014; Xiong *et al.*, 2012).

As the complex relationship between morphology and productivity always become a bottleneck in the process using filamentous fungi, the monitoring and morphological control during the cultivation process required identification of the following parameters; the dependent strain characterization, medium composition and cultural condition. These results may represent significant progress toward the stable production of ligninolytic enzymes and the development of an effective fungal strain with promising biotechnological applications.

Previous studies describe that enzymes are manufactured in practice for commercial use. Some applications such as dye decolourization, phenol degradation, and bio-bleaching require only crude preparations of the enzyme. However, in certain cases, crude enzymes have to be purified for further use. Such application including in the production of sweetening agents, modification of antibiotics, ingredients in cleaning products, forensic and other applications (Robinson, 2015). Therefore, protein purification is vital to acquire knowledge about its functional properties, structure and interactions in order to foretell the potential applications.

1.3 Objectives

In order to tackle the problems discuss above, the objectives of this study could be summarized as follows:-

1.3.1 To isolate and identify a novel new-biofactories belong to white rot fungi from local soil with a high potency of lignin-degrading enzymes.

1.3.2 To optimize the culture medium for ligninolytic enzyme production using One Factor at Time Approach (OFAT) and statistical method and their correlation with cell morphology.

1.3.3 To optimize the productivity of ligninolytic enzymes through batch fermentation using 5-L stirred tank bioreactor.

1.3.5 To isolate, purify and characterize the produced enzyme from selected white rot fungus isolates.

1.4 Scope of Research

In order to achieve the objectives of the research, the work is focused as below:

1.4.1 Isolation, screening and selecting of the most potent lignin degrading white rot fungi from different niches of soil samples.

1.4.2 Molecular identification of potent ligninolytic enzyme producer of white rot fungal isolates.

1.4.3 Media optimization for enhancement of the ligninolytic enzyme productivity using One Factor at Time and statistical approaches.

1.4.4 Growth kinetics of the selected strain in shake flasks cultivation in 5-L stirred tank bioreactor.

1.4.5 Characterization of cell morphology using image analysis and biological staining methods and their correlative effect on growth and productivity resulted from different stages of cultivation.

1.4.6 Purification laccase from the white-rot fungus *Cerrena sp.* WICC F39 using anion-exchange chromatography and gel filtration technique.

1.4.7 Determination of the laccase enzyme properties included molecular mass, the effect of temperature, pH, reaction temperature, storage stability, metal cation, chelating agents, inhibitors, solvent, decolourization, substrate specificity as well as their kinetic characteristics towards laccase activity.

1.5 Organization of the Thesis

This thesis is divided into 5 chapters. Each chapter describes the sequence of the research and represent a valuable as well as tangible information about the research study.

Chapter 1 briefs the general background, statement of the problems, objectives of the study, the significance and the organization of the study.

Chapter 2 deals with the review of the related literature and studies. The chapter explains about white rot fungi and their principles enzymes in detail including the source of fungi, isolation and identification, enzymes properties, enzyme applications and scale up studies for enzyme production correlated with macro- and micromorphology of growth.

Chapter 3 concerned with the methodology adapted to the study to be carried out.

Chapter 4 presents the results relating to each research question.

Chapter 5 summarized the research work performed. It develops a clear view of the contributions of the study and critique of the findings. Finally, the future works arising from these studies are discussed. The remaining part of the thesis proceed references and appendixes.

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