OPTIMIZATION OF COCONUT HUSK FERMENTATION BY *Pycnoporus sanguineus* USING TAGUCHI METHOD FOR LACCASE PRODUCTION

IZZATI BT AHMAD FUAD

UNIVERSITI SAINS MALAYSIA

2019

OPTIMIZATION OF COCONUT HUSK FERMENTATION BY *Pycnoporus sanguineus* USING TAGUCHI METHOD FOR LACCASE PRODUCTION

By

IZZATI BT AHMAD FUAD

Thesis submitted in fulfilment of the requirements for the degree of Master of Science

August 2019

ACKNOWLEDGEMENT

First praise is to Allah, the Almighty on whom ultimately we depend for sustenance and guidance. Second, I would like to express my deepest appreciation to all those who provided me the possibility to complete my thesis.

A special gratitude I give to my supervisor, Dr Khairiah Abd Karim whose contribution in stimulating suggestions, ideas and encouragements, helped me to coordinate my project. Without her guidance and persistent help, this thesis would not have been possible.

I would like to express my heart-felt gratitude to my parents and my siblings who loves and always support me for whatever decisions I have made. My deepest gratitude also to my beloved husband who always understanding and spending time to help me finish my thesis.

Furthermore I would like to acknowledge with much appreciation the crucial role of the staff that involved in my project who gave guidance to use all the equipment and the necessary material to complete my lab work. Last but least, a special thanks goes to my friends who help me to assemble the parts and gave suggestions about my project.

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATION

ANOVA	Analysis of variance
ABTS	2,2'-azinobis-3-ethyl-thiazoline-6-sulfonat
BBD	Box behken design
CCD	Central composite design
COD	chemical oxygen demand
DF	Degree of freedom
DOE	Design of experiment
FDA	Food and drug administration
GRAS	Generally regarded as safe
HBT	1-hydroxybenzotriazole
LiP	lignin peroxidase
MnP	manganese peroxidase
NSPI	N-hydro-xyphthalimide
OA	Ortogonal array
OFAT	One factor at a time
PAH	polyaromatic hydrocarbons
RSM	Response surface methodology
RSS	Rubber seed shell
SDA	Sabouraud dextrose agar
S/N	Signal to noise
SmF	Submerged fermentation
SSF	Solid State Fermentation

SS_T	Sum of total
SS_Q	Sum of square
TOA	Taguchi orthogonal array

LIST OF SYMBOLS

β	Product formation constant	
U	Enzyme activity unit	μmol
ε	Dielectric permittivity	F/m
d	Layer thickness	cm
V_S	Volume of enzyme solution	L
V_t	Total volume	L
ΔE	Change in extinction of light	min^{-1}
μ	Specific growth rate	
n_t	Total number of experimental trials	
n_i	Number of trial in i^{th} trial	
$\bar{n}$	Total mean	
yi	Response variable	
ρ	Percentage contribution	
Q	Parameter	

PENGOPTIMUMAN PENAPAIAN SABUT KELAPA OLEH *Pycnoporus sanguineus* MENGGUNAKAN KAEDAH TAGUCHI UNTUK PENGHASILAN ENZIM

ABSTRAK

Pada zaman sekarang, enzim mempunyai permintaan yang tinggi dalam industri dan laccase adalah salah satu daripada enzim yang terdapat secara meluas dalam persekitaran semulajadi. Laccase mempunyai peranan yang penting dalam bioteknologi disebabkan oleh kebolehan mengoksida kedua-dua sebatian yang berkaitan lignin fenolik dan bukan fenolik di samping ia dapat mengatasi masalah pencemaran alam sekitar. Bagi menghasilkan enzim laccase, kulat daripada proses penapaian keadaan pepejal yang menggunakan sisa lignoselulosa telah diperkenalkan. Kajian ini bertujuan mengoptimumkan penghasilan enzim laccase daripada *Pycnoporus sanguineus* melalui *one-factor-at-a-time* (OFAT), kaedah Taguchi dan ANOVA melalui penapaian keadaan pepejal menggunakan sisa lignoselulosa sebagai substrat. Pada awal kajian, beberapa jenis sisa lignoselulosa yang berbeza seperti sabut kelapa, jarum pain, cengkerang biji getah dan habuk kayu disaring bagi memilih substrat yang paling sesuai untuk penghasilan enzim laccase. Bukti menunjukkan sabut kelapa merupakan substrat terbaik dalam penghasilan enzim laccase berbanding substrat yang lain dengan hasil maksima 5134.25 U/L. Selepas proses penyaringan, kajian diteruskan kepada kesan parameter yg dipilih kepada penghasilan enzim laccase menggunakan kaedah OFAT. Parameter yang digunakan adalah hari inkubasi, nisbah substrat dan air (wt/vol), pH, dan suhu. Hasil menunjukkkan bahawa keadaan terbaik untuk penghasilan laccase adalah pada hari ke 18

inkubasi, pH 7, pada suhu 30 ºC dan 1:9 (wt/vol) nisbah substrat dan air dengan maksimum hasil iaitu 8747.4 U/L.

Dalam kajian ini, pengoptimuman seterusnya adalah menggunakan kaedah Taguchi yang diperkenalkan oleh "Minitab 18 Software" dan ciri kualiti yang digunakan ialah " larger is better". L_9 "orthogonal array" telah dibina di dalam pengoptimuman menggunakan kaedah Taguchi kerana ia memerlukan 9 eksperimen untuk 4 parameter dan 3 tahap. 3 tahap yang digunakan dalam kaedah Taguchi ini diperoleh daripada kaedah OFAT yang sebelumnya. Hasil menunjukkan penghasilan laccase yang telah diperoleh iaitu 8747.6 U/L dimana ia menghampiri jumlah yang diperoleh daripada kaedah OFAT. Kaedah Taguchi menunjukkan bahawa suhu adalah parameter yang paling penting dalam penghasilan laccase dengan (36.31%) peratus menyumbang diikuti oleh nisbah substrat dan air (wt/vol) (30.76%), hari inkubasi (27%) dan pH (5..92%). Hasil terakhir daripada kaedah Taguchi menunjukkan bahawa maksima penghasilan laccase diperoleh adalah 8698.1 U/L bersama jumlah peratusan ralat yang kecil (0.5%). Kajian menunjukkan bahawa faktor persekitaran mempengaruhi pertumbuhan kulat untuk penghasilan laccase yang tinggi. Pemilihan kulat dan substrat yang berkebolehan dalam menghasilkan enzim yang tinggi dan kemudian dapat mengoptima keadaan yang sesuai untuk penghasilan enzim merupakan sesuatu yang penting untuk dilakukan. Hal ini kerana, perbezaan kulat, substrat dan keadaan pertumbuhan kulat akan menghasilkan enzim yang berbeza. Oleh itu, tidak hairanlah bahawa enzim ini telah dikaji secara intensif sejak abad ke sembilan belas dan masih menjadi topik kajian yang hangat hari ini.

OPTIMIZATION OF COCONUT HUSK FERMENTATION BY *Pycnoporus sanguineus* USING TAGUCHI METHOD FOR LACCASE PRODUCTION

ABSTRACT

Nowadays, enzymes have high demand in industry and laccase is one of them which present widely in nature. Laccase has an important role in biotechnology due to their ability to oxidize both phenolic and non-phenolic lignin related compounds. It also can be used to overcome environmental pollutions. To produce laccase enzyme, a fungal solid-state fermentation (SSF) process that uses lignocellulosic waste was introduced. This research aims to optimize the production of laccase from *Pycnoporus sanguineus* through one-factor-at-a-time (OFAT), Taguchi method and ANOVA by solid-state fermentation (SSF) of lignocellulosic waste. For the preliminary study, different types of lignocellulosic waste such as coconut husk, pine needle, rubber seed shell and sawdust were screened in order to select the most suitable substrate for laccase production. Coconut husk was proven to be the best substrate for laccase production compared to others, with the highest activity of 5134.25 U/L. After the screening process, studies continued on the effect of selected parameters on production of laccase using OFAT method namely; incubation day, substrate to water ratio (wt/vol), pH and temperature. The results showed that the best conditions for laccase production were at 18^{th} days of incubation, pH 7, temperature of 30℃, and 1:9 (wt/vol) of substrate to water ratio with the highest activity of 8747.4 U/L.

In this study, further optimization by using Taguchi method was proposed by Minitab 18 Software at "larger is better" as a quality character was used. An L_9 orthogonal array was constructed in optimization by using Taguchi method because it needs 9 experiments for 4 parameters operating at 3 levels. The 3 levels used in Taguchi method were obtained from the earlier study of OFAT method. The results showed that the maximum laccase production was obtained as 8747.6 U/L which was almost similar to the OFAT result. Taguchi method has shown that temperature is the most significant factor in optimizing the production of laccase with (36.31%) of percent contribution, followed by substrate to water ratio (wt/vol) (30.76%), incubation day (27%) and pH (5.92%). The final results showed that the maximum laccase production was obtained as 8698.1 U/L with a small value of percentage error (0.5%). The research findings demonstrated that environmental factors influence the fungi growth to produce a high activity of laccase. Selection of strain and substrate that capable of producing high concentrations of an enzyme and then optimize conditions for enzyme production are the important things to do. This is because, different strain, substrate and cultivation condition give a different yield of enzyme production. It is therefore not surprising that this enzyme has been studied intensively since the nineteenth century and yet remains a topic of intense research today.

CHAPTER ONE

INTRODUCTION

1.1 Research Background

1.1.1 Industrial Biotechnology

Development of eco-friendly processes increasing proportionately day by day and still growing as a result of the environmental impact and industrial awareness. Therefore, effort in searching for an enzyme that is capable of substituting conventional chemical methods is actively conducted (Ramirez-Cavazos et al., 2014).

The enzyme is produced by living cells and potentially acts as biocatalyst for a specific biochemical reaction. It has a unique property of biochemical which cause it to be highly demanded in the enzyme's industries due to the continuous growth of sustainable solutions. Microorganisms produce a huge number of such biocatalyst for a wide range of applications such as industry of food, household care, animal feed, biofuels, technical industries, pharmaceuticals and fine chemicals (Brahmachari et al., 2017).

The industrial process has increased their attention to filamentous fungi due to their ability in producing wide types of enzymes in large quantities. Usually, homologous and heterologous fungal protein production by filamentous fungi are effective and recognized as GRAS (generally regarded as safe) (El-Enshasy, 2007; Souza et al, 2014).

Laccase enzyme is one of the widely studied and produced in biotechnological industries. It has the ability to oxidize phenolic and nonphenolic lignin-related compounds. This ability contributes to environmental pollutants removals. Besides, laccase is also applied to detoxify industrial effluents of textile, paper and pulp, and petrochemical industries. These properties also benefit as a medical diagnostic tool, as bioremediation of pesticides and herbicides. Laccase can act as a cleaning agent in a water purification system, as an ingredient in cosmetic and as a catalyst for manufacturing drug (Brahmachari et al., 2017).

1.1.2 Lignocellulosic waste in biotechnology

Lignocellulosic waste is a biological material which is derived from living organisms which include wood, agricultural waste and forestry residues. There are three main compositions of lignocellulose namely; cellulose, hemicellulose and lignin (Wanmolee et al., 2014). Utilization of lignocellulosic materials becomes the most attractive approach instead of conventional fuels recently. Advantages in characteristics of lignocellulose such as renewable, inexpensive, clean and environmental friendly make it interesting alternatives in the production of chemical, materials and fuels which based on biomass feedstock (Wanmolee et al., 2014).

For the past few years and up until now, these potential materials are still treated as waste in many countries. In some developing countries, these materials are being researched due to environmental concern (Dashtban et al., 2010). In Malaysia, lignocellulosic wastes are abundant and readily available. There are a large number of residues produced by the palm oil processing industry, industries of rubberwood products, processing industries of sago starch and others. These residues are either burnt or allowed to decay naturally. Due to environmental concern, researchers have investigated this waste and came out with the production of value-added products such as enzymes (Vikineswary et al., 2006).

The human quest for eco-friendly and green processes in place of chemical processes for the production of industrial products has turned the industrial manufacturing to strongly 'bio-based'. Solid-state fermentation has attained much relevance in this context during the last decade as solid-state fermentation processes offer potential environmental benefits (Thomas et al., 2013). It is also an interesting technology to be applied nowadays as it can be economically feasible for the production of many biotechnological products (Karp et al., 2015). Filamentous fungi are the most important group of microorganisms used in solid-state fermentation compared to bacteria due to their physiological, enzymological and biochemical properties (Mienda et al., 2011).

Until now, there are numerous investigations made by researchers worldwide on the potential of fungal fermentation in lignocellulosic biomass conversion into valuable products such as enzymes. Besides, laccase is one of the enzymes that include a prominent product produced by fungal fermentation. Therefore, utilization of lignocellulosic waste as raw materials in the production of laccase enzymes through fungal fermentation is a good choice as it gives less of negative impact on the environment.

1.2 Problem Statement

Nowadays, researchers around the world give more attention to the search for other affordable and sustainable alternatives for industrial bioprocess due to the rising cost and depletion of natural resources. During production and processing of agricultural products, there are non-product outputs that are beneficial to mankind but due to low economic value compared to cost of collection, transportation and processing for beneficial use, these non-products remain as waste. There are about 998 million tonnes of agricultural waste estimated to be produced yearly (Obi et al., 2016).

In Malaysia, forest and wood processing mass are produced in large scale. There are productions of logging residues in the form of stumps, bark, tops, branches and broken logs during various phases of logging operations (Osman et al., 2014). There are also wastes from a tree that categorized as forestry waste such as needle of a pine tree. A large amount of pine needle form a thick carpet of the forest floor and can cause forest fire even if slightest ember (Sharma, 2014).

It is commonly known that lignocellulosic waste is a great source of energy and is utilized as raw material for the production of high-value products namely; enzymes, bioethanol, organic acids and biodegradable plastics (Ravindran and Jaiswal, 2016). Therefore, application of lignocellulosic waste in the biotechnological industry may be regarded as an innovative avenue for its utilization. If these wastes could be converted into a useful component for the development of a commercially valuable product, it would further boost economic production as well as facilitate waste remediation. Hence, in this study, a proper selection of lignocellulosic wastes prior to the optimization method is necessary.

Generally, laccase was distributed by fungi, plant and some in bacterial species. Among these three sources of laccase which are fungi, plant and bacteria, laccase was reported higher in fungi compared to others (Brijwani et al., 2010; Pannu and Kapoor, 2014).

Among several species of fungi, *P. sanguineus* from white-rot fungi was selected to produce laccase in this study. White rot fungi have potential in producing oxidoreductive enzymes that capable to degrade a variety of textile dyes, and able to detoxify effluents and sludge. Besides, these enzymes also have the ability to oxidize a variety of natural substrates such as phenols and polyphenols. This makes them highly demanded in industrial application such as food, pharmaceutical, bioremediation, textile, paper and chemical industries. Meanwhile, *P.sanguineus* is also known for its high lignocellulolytic potential and laccase produced by *P.sannguineus* also is the main extracellular ligninase or as sole phenoloxidase (Lu et al., 2007; Watanabe, et al., 2012; Pannu and Kapoor, 2014;Marim et al., 2016).

In recent literature reviews, a conventional method was used for the production of the enzyme. This is known as *one-factor-at-a-time* (OFAT) which is time-consuming, laborious process and expensive ordeal (Pundir et al., 2015; Tasar, 2017). Therefore, in order to get high yield, researchers worldwide developed a statistical optimization method as alternatives to conventional methods. It offers more economical and reliable optimization techniques (Tasar, 2017). The frequently applied of statistical design including Response Surface Methodology (RSM), evolutionary algorithm and Taguchi methods (Pundir et al., 2015).

`Furthermore, the use of Taguchi method and analysis of variance (ANOVA) in biotechnology field is still not widely explored. Optimization of laccase production using *P. sanguineus* in fungal fermentation via Taguchi method and ANOVA has not yet been reported. A SSF process that includes screening of several selected lignocellulosic waste and optimization via Taguchi method and ANOVA was presented in this dissertation.

1.3 Research Objectives

The research objectives are as follows;-

1) To screen the potential of lignocellulosic waste as substrates for laccase production through solid-state fermentation (SSF) by using *P. sanguineus.*
2) To study the effects of incubation day, substrate to water ratio (wt/vol), pH of media and temperature on the production of laccase by SSF.
3) To optimize production of laccase by SSF of *P. sanguineus* using Taguchi method and analysis of variance (ANOVA).

1.4 Scope of the study

Different types of lignocellulosic waste namely, coconut husk, pine needle, sawdust and rubber seed shell were screened using SSF method at 16 days of incubation, 1:4 (wt/vol) of solid to water ratio, pH 5 of media and temperature of 30 ºC.

The substrate that showed the highest production of laccase was selected for further analysis. Four parameters such as incubation days, substrate to water ratio (wt/vol), pH and temperature were chosen in order to study the effects on laccase activity by using OFAT method. Three significant levels obtained from each parameter were selected to be used in the Taguchi method. In this study, UV-Vis spectrophotometer was used as analytical tools to determine the laccase activity.

Further optimization study was carried out using the Taguchi method proposed by Minitab 18 Software. Through this method, analyzes done are as follows;-

1. Selection of orthogonal array
2. Analysis of signal to noise (S/N) ratio
3. Main effects plot for S/N ratio
4. Response table for S/N ratio

Analysis of variance (ANOVA) was further carried out in order to analyze the significant levels of parameters selected and their relative contribution for laccase production besides act as supporting result for Taguchi method. Different percentage contribution obtained from each parameter was observed to determine the most significant parameters. This analysis also proposed by Minitab 18 Software.

Interaction plot again proposed by Minitab 18 Software is the final step in this study. It described the interaction between different parameters to obtain the highest activity of laccase produced.

1.5 Organization of thesis

The first chapter introduces the biotechnological industries, lignocellulosic waste in biotechnology and fungal fermentation in enzyme production. Then, problem statements are elaborated followed by the determination on research objectives and thesis organization for this project.

In a literature review (Chapter Two), the background of laccase including characterization, properties, occurrence and utilization of laccase in industries are discussed. Then, reviews on the potential of lignocellulosic waste as a substrate in laccase production with their characterization and properties also presented. Further insight on the capability of white-rot fungi of *P.sanguineus* is also elaborated following by discussion on factors effect laccase activity through SSF method. Reviews on experimental design focusing on Taguchi method are also presented.

Chapter three provides a list of all material and chemicals used in the research. Detailed procedures for screening of substrates and the effect of parameters used in laccase production are presented. This section also illustrates on the Taguchi method and ANOVA in the optimization process of laccase production.

Chapter four discussed all the experimental data obtained. Firstly, the selection of substrates from lignocellulosic waste with the highest production of laccase is presented. It is then followed by a study on the effect of parameters in laccase production using OFAT method. Taguchi method as an optimization process in the determination of the most significant parameters on laccase production is also discussed.

Chapter five concludes concisely all of the major findings in this present research work. Future recommendations for future studies are also presented.