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

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By

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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.

CHAPTER TWO

LITERATURE REVIEW

2.1 Historical background of laccase

Laccase has firstly used in industry in the 1990s. Since then, a careful investigation of a reaction catalyzed in vivo by fungal laccase has contributed to the increasing number of biotechnological applications (Pezzela et al., 2015). Deep investigations of laccase enzyme have started in 1883 by Yoshida after he extracted laccase from exudates of the Japanese lacquer tree, *Rhus Vernicifera* (Kunamneni et al., 2007). This enzyme contains about 15-30% carbohydrate with the molecular mass of 60-90 kDa and was reported as the oldest enzyme and had been the most studied in the enzymatic system (Shradda et al., 2011).

2.1.1 Characterization and properties of laccase

Laccase is known as polyphenol oxidases which part of broad group enzymes. It contains copper atoms in the catalytic center and makes it also known as multicopper oxidases. There are three types of copper atoms in laccase in which one of them has responsibility for their blue color characteristics. The enzyme is called as yellow or white laccase when it is lacked in a blue copper atom. Generally, the process of laccase catalysis occurs with the reduction of oxygen to water associated with the oxidation of substrate (Brijwani et al., 2010; Pannu and Kapoor, 2014). Oxidation of substrate cause loss of a single electron and result on free radical form. These form of free radical may go under further oxidation or non-enzymatic reaction namely; disproportionation, hydration and polymerization (Shradda et al., 2011).

Laccase has interesting advantages due to their potential on catalyzing oxidation variety of organic compound such as aromatic compound especially and also inorganic compounds (Morozova et al., 2007). According to Couto and Herrera, (2006), laccase is categorized as oxygen oxidoreductase which has an advantage in catalyzing the oxidation of ortho- and paradiphenols, lignins, plyamines, aminophenols, polyphenols, aryl diamines and some inorganic ions coupled to the reduction of molecular dioxygen to water. Aside from phenolic substrate, non-phenolic compound also can be catalyzed by laccase with the inclusion of mediators such as 2,2'-azinobis-3-ethyl-thiazoline-6-sulfonat (ABTS), N-hydro-xyphthalimide (NSPI) and 1-hydro-xybenzotriazole (HOBT) (Brijwani et al., 2010).

Besides oxidize phenolic and methoxyphenolic acids, laccase also has potential to decarboxylate them and able to attack their methoxy group (demethylation). It is also capable to oxidize many recalcitrant substances namely; chlorophenols, lignin-related structures, polyaromatic hydrocarbons (PAH) and organophosphorus compound (Kuhad et al., 2013).

Laccase uses molecular oxygen as the oxidant and only water as by-product make it interesting as catalytic characteristic as well as become an excellent catalyst (Pannu and Kapoor, 2014).

2.1.2 Occurrence of Laccase

Late in 19th century, laccase was first found in the sap of lacquer tree namely *R. vernicifera* which is plant origin. Then, studies on laccase from plant origin were continuously done and lead to invention laccase in other plant species known as *Rhus succedanea, Pinus taeda, Acer pseudoplatanus, Populus euramericana, Nicotiana tonacco, Liriodendron tulipifera, Zea mays* and *Lolium perenne* (Morozova et al., 2007).

Laccase is produced by different sources such as from insect, bacteria, plant and fungi. Among them, laccases produced by fungi are the one that is mostly used in the industrial (Brijwani et al., 2010; Obinno, 2015). Investigation on laccase produced by a wide number of fungi has great variability in terms of induction mechanisms, expression of different isoenzymes, degree of polymorphism and physic-chemical and catalytic properties (Obinno, 2015).

Sources of laccase production either from fungal, plant or bacteria determine its biological function. Plant laccase participates in lignin polymerization and in wound responses. Bacterial laccase involves in morphogenesis, pigmentation, oxidation of toxin, protection against UV light and against the oxidizing agent. Meanwhile, fungal laccase has potential in stress defense, fruit body formation, pathogenicity, lignin degradation, fungal plant-pathogen and morphogenesis (Kuhad et al., 2013; Mate and Alcalde, 2016).

Besides, fungal laccase also has higher redox potential which is about +800mV compared to laccase produced from plants and bacteria (Morozova et al., 2007). Plant and bacterial laccase were reported have +460mV of redox potential. In the biotechnological application that involves in lignin degradation, high redox potential is an important factor because it allows them to oxidize a wide range of substrates compared to low and medium redox potential (Mate and alcalde, 2016).

Production of laccase by fungi is divided into two groups of lignocellulolytic enzyme, which classified based on their compound of degradation. The first group degrades cellulose, xylan, β-glucosidase, endo-1,4-β-glucanase, cellobiohydrolase and xylanases. The second group includes the entire lignin oxidative complex, which composed of laccase, lignin peroxidase (LiP) and manganese peroxidase (MnP) (Gutirrez-Soto et al., 2015).

2.1.3 Industrial use of laccase

In the biotechnological area, deep investigations of laccase cause the widespread application of laccase in industries. Contribution of laccase in industries has a broad usage such as detoxification of pulp bleaching, effluent decolourisation, organic synthesis, act as dye transfer blocking functions in washing powder, synthesis of complex medical compound, as removal of phenolics from wines and xenobiotics substances, grafting reaction, as forest product industry and bioremediation (Kunamneni et al., 2007; Eugenio et al., 2010; Mate and Alcalde, 2016).

Laccase as biocatalyst is useful in pharmaceutical industry due to their potential to catalyze a wide range of synthetic reactions. Laccase can be used to synthesize complex medical product such as antibiotics, anti-cancer drug, anti-inflammatory, anesthetics, and immunosuppressors (Pannu and Kapoor, 2014; Mate and Alcalde, 2016

In the paint industry, alkyd resin is used as binding agents in coating. These resin need heavy metal-catalyzed in the chemical drying process. Investigation on laccase reported that laccase is environmentally friendly and less toxic compared to the heavy metal-catalyzed as well as cross-link the alkyd resin (Mate and Alcalde, 2016).

In the furniture industry, formaldehyde is one of the ingredients used in medium-density fiberboards. Study of laccase showed that laccase can act as glue lignin-based materials in furniture industry instead of using formaldehyde (Mate and Alcalde, 2016).

The ability of laccase to catalyze electron transfer directly makes it useful in the development of biosensor. As in biosensor, laccase has potential to reduce oxygen to water and then the consumption of oxygen during analyte oxidation is recorded by biosensors. This

ability contributes to developing biomedicine as laccase-based biosensors able to detect insulin, codeine and morphine (Mate and Alcalde, 2016). **Table 2.1** summarizes the application of laccase produced by fungi in industries.

Investigation of the scientific literature published on laccase since the last 10 years until now reveals a continuous growth in the research of laccase application in several industrial followed by the publication of a number of patents. This show that laccase has a "never-ending story' (Pezzella et al., 2015). Different variability gave differents application of the enzyme. Therefore, studies in fungal laccase with different properties and potential application are still an on-going process. Hence, it is expected to oxidize a new substrate for a new application or use for wide applications in a different way (Brijwani et al., 2010).

2.2 Lignocellulosic waste as potential substrates for laccase production

Although laccase becomes highly demand in industries, high costs of laccase production are the main hindrance for market application. Over the last years, researchers from all over the world have made several attempts to solve these problems. It is also estimated to compete with other chemical processes. Therefore, in order to achieve low cost in this biocatalyst production, efforts have been made by using lignocellulosic waste as substrate and their modification by protein engineering to achieve more active enzymes. Method of production is the important factor in reducing cost besides offering high benefit for the practitioner (Demir et al., 2011).

Table 2.1: Application of laccase produced by fungi in industries

Fungal	Application of laccase	Industry	References
Trametes hirsuta	Reduce dough extensibility in both gluten and flour dough	Food industries	Pannu and Kapoor, 2014
M.thermophila	Able to oxidize phenols and the released phenoxyl radicals will undergo non-enzymatic homopolymerization. It is in order to avoid 2,4,6-trichloroanisole which responsible to the cork taste. This oxidation of polymerization able to modify cork surface as well as increasing the hydrophobicity and reduced the substances extraction into the wine	Wine industries	Conrad et al., 2000
M.thermophila	Able to scavenges oxygen which otherwise could react with amino acids, fatty acids, alcohols and proteins in order to form off-flavour precursor.	Food idustries	Osma et al., 2010
T.versicolor	Used in clarification of fruit juice which lightening the colour of original juice to 61% and remove 29% of its turbidity.	Beverages industries	Bezzera et al., 2015
Pleurotus ostreatus	As fruit juice clarification which reduced 45% of phenol	Beverages industries	Mate and Alcalde, 2016
P.cinnabarinus	Used in treatment of unbleached flax fibres	Pulp and paper industries	Fillat et al, 2012
Ganoderma lucidum	Potential to remove 84% of phenolic content in corn stover hydrolysate which lead to improved ethanol yield by 10 %	Biofuels	Fang et al., 2015
Trametes sp.	Used to degrade 17ß-estradiol 3-(ß-D-glucuronide) and eliminate this compound and its intermediate 17 ß-estradiol efficiently	Enzymatic bioremediation	Mate and Alcalde, 2016

Fungal	Application of laccase	Industry	References
Trametes villosa *Coriolopsis rigida* *Pycnoporus cocconeus*	Able to decolourize the flexographic inks for paper recycling	Pulp and paper industries	Fillat et al, 2012
Thermobifida fusca *T.versicolour* *Flammulina velutipes*	Able to oxidize dye intermediates that used widely in hair colouring	Cosmetics	Mate and Alcalde, 2016; Shradda et al., 2011
T.versicolour	Treat black liquors discharge for detoxifying in the form of pellets Reduce colour, chemical oxygen demand (COD) and aromatic compounds.	Textile industry	Shradda et al., 2011

Selection of a suitable substrate is an important aspect for solid-state fermentation (SSF). In SSF, substrate acts as carbon and energy sources besides provide solid support in fermentation process. There are several factors that influence the selection of substrate in SSF such as availability, cost and heterogeneity of the substrate. SSF system can be categorized into two types based on the nature of the substrate. The first one is the most common substrate that is a natural substrate. It provides carbon and offers energy source in SSF. The second category is called as inert material which supplied mineral medium that only provides a solid support for SSF. Therefore, based on these statements and also according to several works of literature, lignocellulosic waste which is a natural substrate is the best choice as solid support for microorganism growth (Wittman and Liaou, 2017). **Table 2.2** summarized several lignocellulosic wastes used by fungi in enzyme production.

Commonly, fungi culture media is classified into two types which are natural and synthetic. Natural media are composed of the natural substrate, for example, herbaceous, seeds, woody stems, cornmeal, oatmeal, and others. This medium is easy to prepare but has unknown composition. Synthetic media are types of media that contain a defined amount of carbohydrate, nitrogen and vitamin sources. For example, Sabouraud dextrose agar (SDA), Czapex-Dox medium, glucose-asparaginase and others. Production of enzymes in large scale is considered by using a cost-effective medium such as natural medium (Basu et al., 2015).

Since lignocellulosic waste is from major products of forestry, agriculture, urban refuge and food waste, they become the most abundant of renewable organic compounds. This gives advantages to the biotechnological process of enzyme and other compound production as they have potential to use as a substrate in fermentation media (Kuhad et al., 2013; Gutierrez-Soto et al., 2015). Industries nowadays have increased development in searching for methods to lower the cost of production. In SSF techniques, selection of cheap substrate from organic waste such as residues from agricultural, forestry and food industries

Table 2.2: Lignocellulosic wastes used by fungi in enzyme production

Lignocellulosic waste	Fungal	Enzyme	Refereces
Grapevine sawdust	*P.ostreatus*	laccase	Stajic et al., 2006
Tangerine peels Banana peels	*P.coccineus*	cellulose xylanase	Gutierrez-Soto, 2015
Starch/wheat bran	*P.sanguineus*	amylase	Gutierrez-Soto, 2015
Rice straw	*Marasmius sp.*	laccase	Risdianto et al., 2010
Mandarine peels	*P.eryngii*	laccase	Stajic et al., 2006
Kenaf chips	*Oxyporus latemarginus*	lignin peroxidase manganese peroxidase	Halis et al., 2012
Wheat straw Oil palm frond parenchyma Oil palm biomass chips Rice husk Kenaf chips Sugarcane molasses	*P. sanguineus*	laccase	Eugenio et al., 2010;Suffian et al., 2010;Teck Nam, 2013; Halis et al., 2012; Singh et al., 2012; Marim et al., 2016

to produce ligninolytic enzyme are the crucial part to cut the cost of production. These wastes act as an inducer for the ligninolytic enzyme due to their lignin, cellulose and hemicellulose composition and also offer a solution to the environmental problem (Demir et al., 2011).

2.2.1 Characterization and properties of lignocellulosic waste

Biomass including agricultural waste, forest, crop residues, municipal solid waste, clipping, wood chips and others is the largest renewable energy sources. Biomass is derived from plant material. It comprises cellulose, carbohydrate polymers, hemicellulose and aromatic polymer that is known as lignin. Cellulose and hemicellulose categorized as carbohydrate while lignin fraction categorized as a non-sugar molecule (Agrawal et al., 2014).

In developing new technologies, biomass is a natural resource that plays a crucial role in reducing economic and environmental impacts. The main components of lignocellulosic biomass are cellulose, hemicellulose and lignin which represent 90% of the biomass dry mass and 10% represent ashes and extractives (Cabral et al., 2016). Lignocellulosic biomass also has potential as renewable energy which has been evaluated worldwide over many decades (Mezula et al., 2015).

In plant or in lignocellulosic waste, laccase involves in lignin synthesis. Laccase catalyzes the free radical polymerization of lignin structural units namely p-coumaryl, sinapyl and coniferyl alcohols (Obinno, 2015). After cellulose, lignin is the most abundant source of carbon in the soil (Datta et al., 2017).

Lignin provides physical strength to the wall of a plant cell. Actually, lignin present in lignocellulosic waste can cause difficulty of enzyme degradation and also in the utilization of fermentable sugars by microbes including fungal and bacteria (Ravindran and Jaiswal,

2016). Rencoret et al. (2016) who studied on pre-treatment of lignin claimed that *P. cinnabarinus* laccase and 1-hydroxybenzotriazole (HBT) as mediator followed by alkaline peroxide extraction resulting up to 48% lignin removal from ground wheat straw (Datta et al., 2017).

The complex structure of lignin including heterogeneity of the monomers and linkages cannot be hydrolyzable, so, biocatalyst that able to degrade or modify lignin must be oxidative and non-specific (Kuhad et al., 2013; Rencoret et al., 2016). Degradation of lignin by white-rot fungi is known as an oxidative process which involving oxidative enzymes namely; lignin peroxidase, manganese peroxidase and laccase (Kuhad et al., 2013).

Studies on *P. pulmonarius* also showed that utilization of numerous phenolic and aromatic compounds that structurally related to lignin as culture media has high potential in the production of laccase which also is the main element of ligninolytic enzymes (Imran et al., 2012).

In this study, four different types of lignocellulosic wastes namely coconut husk, sawdust, pine needle and rubber seed shell were chosen according to several reasons.

2.2.1.1 Coconut husk

Coconut husk is classified as lignocellulosic biomass and produced annually by most countries which make it as one of the abundant agricultural residues. Coconut husk is also known as fibrous biomass due to their composition of pithy tissue and short length fiber. Originally, coconut husk comes from the coconut palm tree or known as *Cocos nucifera L.* (Palmae). It is present mostly in coastal areas of tropical countries. Coconut husk actually is the outer shell of coconut fruit (mesocarp) (Ngadi et al., 2014).

Approximately, there are about 55 billion of the world coconut productions per year (Ngadi et al., 2014). By the year 2008, production of coconut had reached 54, 716, 444 tonnes (Ding et al., 2012). However, from this amount, only 15% of the husk fiber being used and the rest are being unconsumed and result in environmental pollution (Ngadi et al., 2014).

Coconut husk has fiber tougher and stiffer of physical characteristics due to their high content of lignin which is 46 % on average (Ngadi et al., 2014). According to Cabral et al. (2016), coconut husk has a composition of 40.10% lignin, 24.70% cellulose and 12.26% hemicellulose. Ding et al. (2012) reported on composition of coconut husk with 21.26 ± 1.51% cellulose, 17.33 ± 0.74% hemicellulose and 46.36 ± 0.57 % lignin.

The high lignin content of coconut husk gives the advantage to be a potential candidate as a support matrix in the growth of fungal and also capable to be a natural inducer for production of fungal ligninolytic enzymes like laccase. It also offers economically and environmental friendly alternative of laccase enhancer (Karim and Annuar, 2009).

2.2.1.2 Sawdust

Sawdust which is also known as wood dust is a by-product of grinding, sanding, drilling, cutting or otherwise pulverizing the wood by using a saw or other tool. Production of sawdust is in small fragments of wood or in small discontinuous chips during operation of log or timber sawing into different sizes. It results in producing chips flow from the cutting edges of saw blades (Kumar et al., 2014).

Sawdust has potential application in several industries. It is used as fuel, scatter, serve as mulch, in artistic displays, as an ingredient in concrete making and often used in icehouses in order to keep the ice frozen during summer (Kumar et al., 2014). In biotechnological

industries, chemical characteristics of sawdust make it useful in as carbon source for the fermentation process (Zhang et al., 2017). It also used as a biomixture and microbial supporting matrix in biological treatment of new system such as in order to treat the wastewater from potato chips of factory's producing. Sawdust also has a rough surface that make it suitable for microbial growth or for cells attack as well as in the fermentation process (Azab, 2008).

Sawdust is also choses as a carbon source in yield of oyster mushroom (*Pleurotus ostreatus*) due to their large amount of production by wood industries and rich with cellulose, hemicellulose and lignin composition (Soliman et al., 2011). According to Shulga et al., (2007) and Abdul Rahman and Abdul Munaim, (2018), chemical composition of cellulose, hemicellulose and lignin in sawdust are cellulose (47.72±0.05 - 58.2%), hemicellulose (24.88±0.57%), lignin (27.75±2.02 – 28.4%), moisture (4.8 – 5%) and ash (0.18-0.33).

2.2.1.3 Pine needle

Pine needle is an adult leaves that green bundled in fascicles (Lal et al., 2013). Pine trees are an important source for producing timber. There are about 600 million are chopped down in the European Union (EU) for each year. However, this production cause waste for billions of pine needles (Schwab, 2016). Besides, fallen of pine needles for enormous amounts also presence as waste biomass (Ghosh and Ghosh, 2011). On the forest floor, pine needles forming a thick carpet that could easily cause forest fire (Sharma, 2014). These forest fires that occur periodically are one of the dominants events of ecological. It threatens the habitat of animal and plant in the coniferous forest (Ghosh and Ghosh, 2011).

Therefore, several researchers worldwide had investigated on pine needle to be a value-added product. In biotechnological application, pine needle has potential in producing essential oil with antioxidant and antimicrobial properties for food industry (Zheng et al., 2012). It also used as bed material in the production of value-added chemical like lactic acid through the SSF method (Ghosh and Ghosh, 2011).

Lal et al. (2013) reported on composition of pine needle with a range of 41-50% of cellulose, 31-35% of lignin, 68.5% hollocellulose, 4.56% of extractives and 3.2% of ash content. On the other hand, Räisänen and Athanassiadis, (2013) claimed that pine needles have a composition of 29.1 % cellulose, 24.9 % hemicellulose, 6.9% lignin and 39.6% extractives. Dried fallen pine needles content 67.3% holocellulose, 33.4% lignin, 15% extractives and 2.71% ash (Ghosh and Ghosh, 2011). Pine needle also reported contained the highest concentration of C, N, P, K, Mg, S, Mn, Na, and Fe compared to others parts of pine tree (Skonieczna et al., 2014).

2.2.1.4 Rubber seed shell

Malaysia is one of the largest producers of natural rubber in the world. Approximately, there are 1.3 million ha of rubber plantation area and varying with more than 20 of rubber trees clones. Rubber tree and its seed have value-added as it is used as a source of natural rubber and presence to be rich in oil respectively (Hassan et al., 2014). Besides, production of biodiesel from refined rubber seed oil has generated solid waste in large amount especially rubber seed shell (Borhan and Kamil, 2012). In biotechnological industries, there are some reported on usages of rubber seed shell such as act as a raw material in production of activated carbon (Lam and Zakaria, 2008; Hassan et al., 2014).

Rubber seed shell compositions are reported content 48.8% carbon (C), 5.9% hydrogen (H), 1.5% nitrogen (N), 0.1 %sulphur (S) and 43.7 %oxygen (O). It also has 7.8 % of extractives. Extractives actually derived from lignocellulosic materials that protect the plant from termites and other microorganisms. Rubber seed shell contained 66.4 % hemicellulose and 25.8 % cellulose (Hassan et al., 2014). Composition of rubber seed shell that obtained from Southwest China is consist of 71.98% cellulose, 24.25% hemicellulose, 2.92% lignin and 0.85% ash (Xu et al., 2016). Ekebafe et al. (2012) had reported on rubber seed shell composition with 60-80% cellulose, 5-20% lignin and more than 20% of moisture. Besides, they also reported on metal content of rubber seed shell which is 0.673% magnesium (Mg), 0.014% sodium (Na), 0.01% potassium (K) and 0.32% calcium (Ca).

In microbial culture media including fungal and bacteria, there are 10 macroelements that required by microorganisms to growth namely (C, O, H, N, S, P, K, Ca, Mg and Fe). C, O , H, N, S and P are important in the synthesis of lipids, carbohydrate, nucleic acid and proteins while the rest macroelements have their role in the cell as cations. Besides, microorganisms also need microelements such as Mn, Co, Ni, Mo, Cu, and Zn as there are part of enzymes and cofactor. The organic compound also needed by microorganism as a growth factor (Basu et al., 2015). **Table 2.3** summarizes the composition of cellulose, hemicellulose and lignin in coconut husk, sawdust, pine needle and rubber seed shell.

2.3 White rot fungi

White rot fungi basidiomycetes are characterized by its potential in degrading lignocellulose through the biosynthesis of a complex set of extracellular oxidative and hydrolases enzymes. Among other fungi namely; brown rot fungi and soft rot fungi, white-rot

Table 2.3: Composition of cellulose, hemicellulose and lignin in coconut husk, sawdust, pine needle and rubber seed shell.

Lignocellulosic waste	Chemical composition (%)			References
	Cellulose	Hemicellulose	Lignin	
Coconut Husk	18.19-21.26%	17.33-11.34%	46.53-53.08%	Ding et al., 2012
	-	-	46%	Ngadi et al., 2014
	24.70%	12.26%	40.10%	Cabral et al., 2016
Sawdust	51.7-58.2%	-	20.2-28.4%	Shulga et al., 2007
	47.72%	24.88%	27.75%	Abdul Rahman and Abdul Munaim, 2018
	40.99-48.33%	-	27.28-29.99%	Pratiwi et al., 2018
Pine needle	30.84%	-	43.24%	Lal et al., 2013
	29.1%	24.9%	-	Räisänen and Athanassiadis, 2013
	-	-	33.4%	Ghosh and Ghosh, 2011
	-	-	33.37%	Singha and Thakur, 2009
	41%	-	31.0%	Sahin and Yalcin, 2017
Rubber seed shell	60-80%	-	5-20%	Ekebafe et al., 2012
	33.54%	-	33.54%	Pratiwi et al., 2018