STUDYING THE DELIGNIFICATION OF WOOD FIBERS USING LACCASE ENZYMES

NOR ZAWANI BINTI ZAINUDDIN

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ABSTRACT

The process to subtract lignin from the lignocelluloses compound requires a lot of chemical amount, energy contribution and also a huge sum of financial and ecological cost. Furthermore, a large amount of expensive chemical required is not reliable in the long term because of its limited resources. Therefore, the lignin conversion process should be substitute with a better and economical way to convert lignin into marketable product. Fortunately, there is great interest in developing industrial application of enzymes of microorganisms to degrade lignin. Therefore, the study of delignification using laccase enzyme were conducted using different temperature and the pH. In this study, the removal of lignin is from the wood fiber using batch enzymatic hydrolysis. Thus, in the enzymatic hydrolysis, the temperature and the pH value of the buffer solution is being studied by manipulate it into different values (25- 60 ^{o}C), (pH=4-9) respectively. Then, the concentrations of the lignin produced are determined analytically using UV-Vis Spectrophotometer. Consequently, the optimum enzymatic delignification reaction condition is determined. From the study the optimum temperature and pH condition are at 60^{o}C and pH9 respectively.

KAJIAN MENGENAI DELIKNIFIKASI GENTIAN KAYU MENGGUNAKAN ENZIM LACCASE

ABSTRAK

Proses untuk mengasingkan lignin dari sebatian lignocellulos memerlukan sejumlah bahan kimia, sumbangan tenaga dan juga kos kewangan yang tinggi. Tambahan pula, penggunaan bahan kimia yang banyak dikhuatiri akan menyumbang kepada masalah kerana sumbernya yang terhad. Oleh itu, proses memisahkan lignin perlu digantikan dengan cara yang lebih baik dan ekonomik supaya lignin menjadi produk yang boleh dipasarkan. Aplikasi enzim didalam proses deliknifikasi telah mendapat perhatian. Oleh itu, kajian ini telah dijalankan dengan menggunakan enzim laccase pada suhu dan pH yang berbeza. Dalam kajian ini, penyingkiran lignin adalah daripada serat kayu menggunakan hidrolisis enzim. Kajian ini dilakukan dengan memanipulasi suhu dan pH iaitu pada suhu 25 hingga 60 dan pH4 hingga 9. Kemudian, kepekatan lignin yang dihasilkan ditentukan melalui analisis menggunakan UV-Vis Spectrophotometer. Oleh itu, kondisi optimum bagi tindak balas ditentukan. Keputusan kajian mendapati suhu dan pH optimum adalah pada 60^{o}C dan pH9.

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^{o}C	Degree Celsius
(E)	Enzyme concentration
(ES)	Enzyme substrate complex
(E_t)	Total concentration if enzyme in the system
g	Gram
K_M	Michaelis Constant
K_{cat}	Turnover Number
mL	mililiter
(P)	Product concentration
Rpm	Rotation per Minute
(S)	Substrate concentration
V_o	Reaction Velocity
V_{max}	Maximum rate of reaction
%	Percentage

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Wood is composed of three kinds of essential fibers: cellulose, hemicelluloses, and lignin. As wood is released into its three elements, the cellulose still flows into the production of pulp and paper while the hemicelluloses can be extracted and fermented to produces biofuels and energy. Then, Lignin will take its rightful place as a wood fiber with limitless potential as an insulator, an adhesive binder in materials and even a dispersant preventing clumping and settling that degrades the value of numerous mixtures used daily by consumers as well as industry. Nevertheless, during the production of paper from wood, lignin is undesirable as a final product due to its colour, hydrophobicity and poor mechanical properties

(Javor, Buchburger and Tanzcos, 2000). Thus, lignin has to be removed in the process of wood digestion without degrading the cellulosic structure.

However, lignin is the second most abundant terrestrial organic polymer after cellulose which accounting for up to 30% of all vascular plant tissue. Lignin's are essential for structure reliability of the cell wall and stiffness and strength of the stem and root (Ralph, Brunow and Boerjan, 2007). In addition, lignin properties waterproofs the cell wall and at the same time enabling the transport of water and solute through the vascular system and taking a role in protecting plants against pathogen (Boerjan, Ralph and Baucher, 2003). This is because lignin complex forms a hydrophobic matrix which means it repels water and allowing the plant to transport water up through the system. Therefore, it is necessary to get the lignin from the wood fiber so it can be developed to utilize the binding material that glues natural wood together for the binding of wood composites.

To remove the structural polymer lignin from plant tissue there is a process called delignification. The process primarily refers to the chemical process for the removal of pulp from wood and there can also be a mechanical delignification. Traditionally, the removal of lignin from wood has taken place by a delignification method called Kraft process. Then, Oxygen Delignification is introduced as a newer process that removes more lignin and uses fewer chemical. According to Harakava (2005), subtraction of lignin for cellulose manufacture requires bulky amount of chemical and energy contribution, resulting financial and ecological cost. In addition, a large amount of expensive petrochemical required is not reliable in the long term

because of limited resources. So that, the lignin conversion process should be substitute with economical way to convert lignin into marketable product (Milstean, Huttermann, Frund and Ludemann, 1993). Furthermore, efforts are being made worldwide towards the improvement of tree varieties with less modified lignin which would improve wood pulp production efficiency (Harakava, 2005).

Nowadays, there is great interest in developing industrial application of enzymes of microorganisms to degrade and synthesis lignin. Previous research has focused on the use of enzyme phenoloxidases to initiate insolubilization reactions of lignin fragment contained in a model effluent. Several enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP), laccase and also cellobiose dehydrogenases (CDH) may be involve in the delignification (Archibald, Bourbonnais, Jurasek, Paice and Reid, 1997). Therefore, it is important to design and develop an effective and less expensive system for delignification with enzymes and convey the idea to a commercial application. Hence, the aim of this study to analyze the lignin yields from wood fiber using Laccase enzymes which involve the optimization of variables. The variables evaluate are temperature and reaction pH.

1.2 Problem Statement

Subtraction of lignin for cellulose manufacture requires bulky amount of chemical and energy contribution, resulting financial and ecological cost. In addition, a large amount of expensive chemical required is not reliable in the long term because of limited resources so that the lignin conversion process should be substitute with economical way to convert lignin into marketable product. Fortunately, there is great interest in developing industrial application of enzymes of microorganisms to synthesis lignin. Therefore, in order to achieve satisfactory delignification, suitable temperature and pH solution is study for the delignification using Laccase enzyme.

1.3 Objectives of Study

Based on the background of this study, the objectives of this study are listed as following:

1.3.1 To study the removal of lignin from wood fiber.

1.3.2 To study the influence of different reaction pH and Temperature in delignification process.

1.3.3 To study the kinetic on delignification.

1.4 Scope of Study

Based on the objectives of this study, the scopes of study are declared as follows:

1.4.1 To test the result using laccase enzymes that can yield lignin from wood fiber.

1.4.2 To optimize the variables (pH and Temperature) for delignification.

1.4.3 To develop the mathematical model from Michaelis Menten Kinetic equation for the kinetic study on enzymatic delignification.

1.5 Significance of Study

It is necessary to develop the effective and less costly system for delignification. Due to its place as a second most abundant terrestrial organic polymer, the abundance materials, the economical way of lignin conversion should be develop to convert lignin into marketable product. In enzymatic delignification all chemicals used are cheap and environmentally safe. In addition, the using of enzymes in delignification is a possible process. Therefore, it is the economical way of lignin conversion, so lignin can be used as raw materials for the manufacture of value added products.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction to Lignin.

Lignin is the major component of the middle lamella region of wood and most found within the secondary wall of the wood (Figure 2.1 show the illustration of common structure of lignin in plant tissue). Moreover, according to Ralph et. al., (2007), lignin cause the cell wall waterproofs and at the same time enabling the water and solutes transport through the vascular system and plays a role in protecting plants against pathogen. Above all, lignins are fundamental for structural reliability of the cell wall and strength and stiffness of the stem and root.

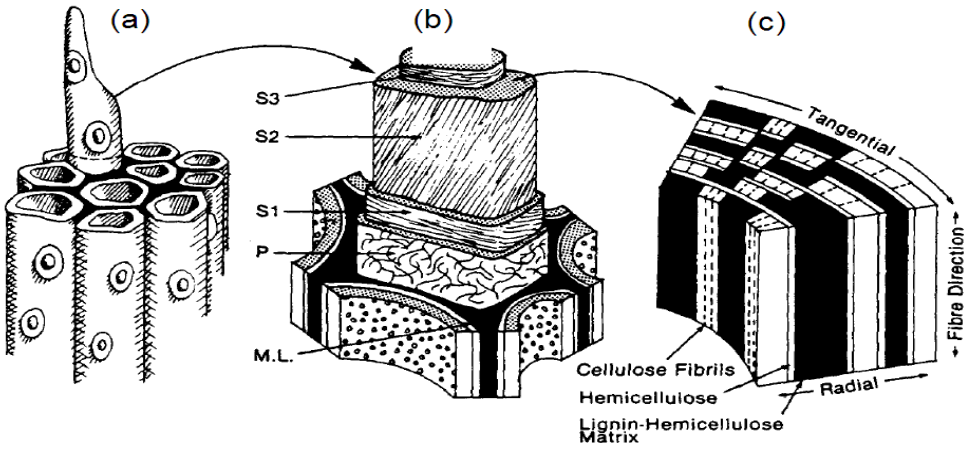

Figure 2.1 Common structures of lignin in plant tissue (a) bundle of contiguous wood cells, (b) wall layer in cut-away view of single cell, and (c) section of secondary wall illustrating the relationship of hemicelluloses and lignin to the cellulose fibril. Cell wall layers are P (primary); and S1, S2 and S3 (secondary). The middle lamella separates the cells. [**Source**: Kirk, 1985].

In chemistry aspect, lignin is an amorphous polymer composed of phenylpropane units with no seemingly systematic order. Moreover, lignin is heterogeneous polymers derived from phenylpropanoid monomers (Frederick et al., 2010). Native lignin is mostly derive principally or made up of three precursor which are coniferyl alcohol, sinapyl alcohol and coumaryl alcohol and the relative amounts of each precursor depends on the origin of the lignin (Ralph et. al, 2007). Figure 2.2 show the three precursor lignin that derived the lignin.

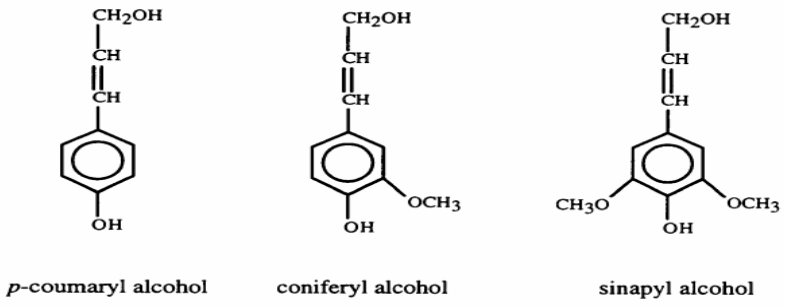

Figure 2.2 The three precursor of lignin [**Source:** Charles, 1998]

Previously, according to Kirk (1985), lignin is polymer of phenylpropane units linked by more than ten different carbon-carbon (C-C) and ether (C-O-C) linkages. Consequently, the precursors are bound to each other by (C-O-C) or (C-C) bonds, with the ether linkages accounting for over two thirds of these bonds (Charles, 1998). Figure 2.3 illustrate the schematic model of the constitution of spruce lignin, showing the many (C-C) and (C-O-C) bonds between lignin precursors.

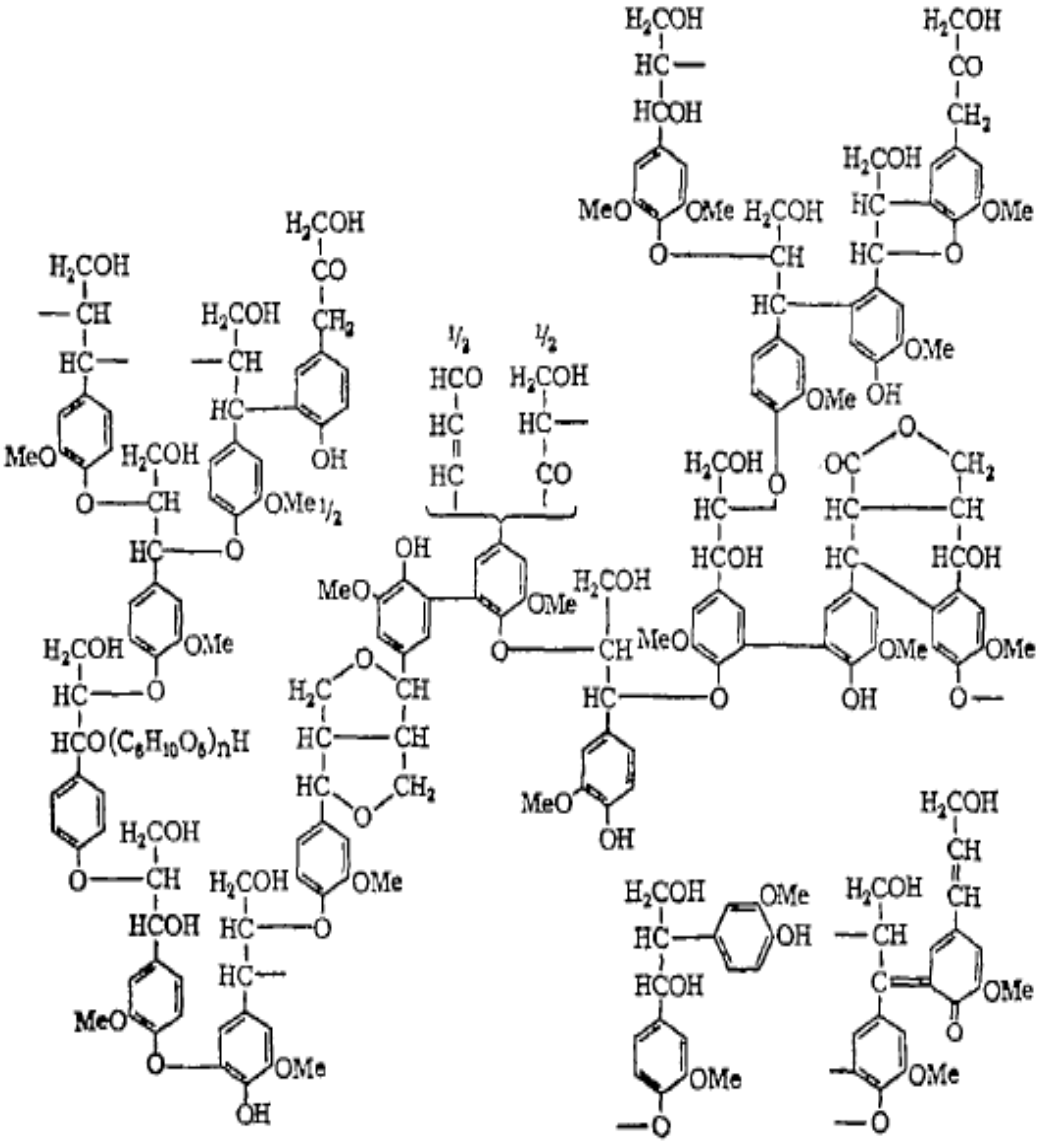

Figure 2.3 Schematic model of the constitution of spruce lignin
[**Source**: Boerjan et. al., 2003]

As shown in schematic model of constitution of spruce lignin (Figure 2.3), the initiate reaction of lignin is difficult to start. However it is still a possibility to separate the lignin from the others biomass component and this is usually accompanied by some degradation.

2.2 Delignification Process.

Delignification primarily refers to the chemical process for the removal of pulp from wood. In the wide understanding, chemical delignification includes all processes consequential in partial or total removal of lignin from wood or unbleached pulps by the action of proper reagents.

The delignification was traditionally taken placed by a Kraft process method where it produces stronger pulp than the previous method and removes 95% of the lignin from wood (Arevalo, 2003). Previously, Archibald et. al. (1996) has studied that the Kraft pulping chemically degrades and removes the middle lamella and primary wall lignin that cements together the fiber walls and during normal process its removed 90-95% of lignin from fiber walls. The process regularly involves digesting wood fiber at high temperature and pressure in the solution of sodium hydroxide and sodium sulfide in water or when combined is known as white liquor. It has become a favorable process because it produces relatively strong fibers and

because pulping chemicals and energy are recycled from the byproducts (Violette, 2003).

Then, Oxygen delignification is a newer process that can remove more lignin and also uses fewer chemicals. The delignification involves treating the pulp with oxygen in a pressurized vessel at high temperature in alkaline solution and followed by washing process with the amount of residual lignin can decreased about 1.5% (Aravelo, 2003). As reviewed by Yang, Lucia Arthur and Jameel (2003), the degradation of lignin in alkaline-oxygen medium arises from integral action of hydroxide and oxygen with phenyl hydroxyl group. Medium consistency oxygen delignification is typically carried out with either softwood or hardwood pulp using sodium hydroxide as an alkali source and pressurized with 400 to 1000 kPa oxygen and previously, the process involves 8 to 12% solids and is heated to 80 ^{0}C to 120 ^{0}C with retention times of 15 to 90 minutes (Violette, 2003).

Presently, there is also great interest in developing industrial application of enzymes in delignification in order to degrade lignin from the wood structure. This is because some fungi are proficient at living on wood since they can produce enzymes such as peroxidases or laccases (Aravelo, 2003). Thus, the existences of those enzymes will catalyze the breakdown of lignin in the presence of oxygen. Hence, there are experiments and research underway to improve the properties of the enzymes for the industrial use of lignin degradation. In addition, the lignin biodegradation using enzymes as industrial biocatalyst in paper pulp manufacture has been largely investigated (Camarero et. al, 2007). Likewise, most attention has

focused on ligninolytic enzymes to replace chlorinated reagents in paper pulp bleaching. From the previous study the ultimate transformation of lignin in nature is performed primarily by white-rot basidiomycetes and particularly by their enzymatic systems (Milstein et.al, 1993). Therefore, it means there are few enzymatic systems that can convert lignin until the transformation is completed.

2.2.1 Enzymatic Hydrolysis

Enzymatic hydrolysis is the catalytic disintegration of a chemical compound by reaction with water, such as the conversion of cellulosic material into fermentable sugars by the addition of the specific enzymes. In this case, the enzymatic hydrolysis is performed to obtain the lignin from the wood fiber which is the lignocelluloses material.

Enzymatic hydrolysis methods have shown distinct advantages over other delignification method where the very mild process conditions give potentially higher yields, high purity, lower utility cost and almost no corrosion problems (Wenjuan , 2010; Argyropoulos et. al, 2002). In addition, the residual lignin isolated by enzymatic hydrolysis has been found to contain a relatively high amount of carbohydrates and protein (Argyropoulos et. al., 2002). According to Wu, (2003) the enzymatic hydrolysis offers lignin at moderate yield with minimal structural

alteration which is up to 32% for various wood species. The two-step methods uses in mild enzymatic treatment obtained the solid residue that contains about 94% of the lignin that formerly present in the pulp (Argyropoulos et. al., 2002).

Furthermore, the enzymatic hydrolysis results are affected by various factors for example the yields obtaining by the hydrolysis are significantly affected by the type of raw material. Otherwise, this method is equally applicable to hardwood and softwoods (Wu et. al., 2003). However, the important factor for the success of the process is the reaction properties such as the pH, mass of enzymes and the temperature. Based on previous study, the enzymatic hydrolysis was carried out at pH 4.5, temperature of 40 ^{0}C and for a period of 48 hours with consistency of 5 % (Argyropoulos et. al, 2002; Wu et. al., 2003). To date, Call, (2005) studies that softwood and hardwood pulp as well as pulp from annual plants can be delignified at pH 4 to 8 but depending on the system within 2 to 4 hours at 50 ^{0}C to 60 ^{0}C and at 10 to 12.5% consistency up to 40% and more by maintaining the strength properties.

2.2.2 Acid Hydrolysis

Acid hydrolysis is usually applied in two-stage acid processes, following the enzymatic pretreatment. The dilute acid process is the oldest technology for converting cellulose biomass to ethanol (Wenjuan, 2010). The purpose performing

acid hydrolysis is to clean the residual lignin isolated from the enzymatic hydrolysis. Nevertheless, the delignification process still generates an effluent that rich in low molecular weight lignin contained in the black liquor that does not precipitate (Anderson, et. al, 1999). If compared to the conventional acidolysis method, the method of two-stage offers more yield by about 25% to 35% and the less acid concentration used during this method will reduces about 0.05m the possibility of structural change induced in lignin (Argyropoulos et. al, 2002). In addition, the residual lignin obtained from the two-stage method is considerably higher purity which is from 93% to 997% and indicative of low carbohydrate and protein contamination.

2.3 Application of Enzymes in Delignification.

Removal of lignin using enzyme has been investigated and currently developed by many researcher. It is obvious that enzymes is able to synthesis lignin by chemically process that are less expensive and often environmental friendly. Previously, the polymerization of phenolic compound by phenoloxidase (type of enzyme) has been reported since the 1950s and usually is related to lignin biosynthesis (Archibald et. al., 1997). Currently, the studies showed the numerous factors that influence the lignin structure such as pH, the presence of polysaccharides, hydrogen peroxide concentration and the cell wall matrix elements in general (Frederik et. al., 2010).

The popular aspirant used in methodological process that involving lignin are the enzymes from white rot fungi which are lignin peroxidase (LiP), managanese peroxidase (MnP) and Laccase. Archibal et. al., (1997) stated that the enzyme family secreted by *Trametes versicolor* (a white-rot basidiomycete fungus) may be involved in delignification which include lignin peroxidase (LiP), managanese peroxidase (MnP), Laccase and cellubiose dehydrogenases (CDH). Nonetheless, besides lignin peroxidase (LiP) and managanese peroxidase (MnP), laccases are the most important lignin degrading and lignin polymerization agent (Euring, Trojanowski, Horstman and Kharazipour, 2011).

The enzyme Laccase are copper-containing, cell wall localized glycoproteins that are encoded by the multigene families in plant (Boerjan et. al., 2003). Moreover, laccases is widely spread and have a quite different physiological purpose. In lignin, only phenolic sub units are attacked by laccase, producing oxygen-centered radicals that can subsequently polymerize or depolymerized (Archibald et. al., 1997). Otherwise, there is a disadvantage of the application of laccases because of it low redox potential make it required a free phenolic groups at the aromatic rings of the lignin for oxidation that normally inhibits their application in the lignin biotechnology (Euring, et, al., 2011).

Lignifications can be imitated by oxidizing monolignols using a peroxidase. Compare to laccase, the peroxidase use hydrogen peroxide, H_2O_2 oxidized the monolignol instead of consume oxygen, O_2 (Boerjan et. al., 2003). There are lignin and manganese peroxidase that act basically the same way to oxidizes phenolic and

also non phenolic lignin sub unit by abstracting one electron and generating cation radicals that are further decomposed non- enzymatically except that manganese peroxidase uses the MnII-MnIII system as redox mediator (Archibald et. al., 1997 and Hutterman, 2001)

CHAPTER 3

METHODOLOGY

3.1 Materials and Methods

The materials used and procedure for the experiment are explain in the next subtopic. At first the experiment were run to determine the optimum temperature and pH for enzymatic delignification. Then, as an addition, the mathematical models for the kinetic study were developed. Thus, the Matlab Program was used for the purposed.