ABSTRACT

YU, ZHIYING. Fundamental Factors Affecting Enzymatic Hydrolysis of Lignocellulosic Biomass. (Under the direction of Dr. Hasan Jameel and Dr. Sunkyu Park).

The objective of this research is to understand the fundamental factors from lignocellulosic biomass and enzymes that affect the bioconversion of biomass to fermentable sugars via enzymatic hydrolysis. This work decoupled various factors, and obtained a significant progress in understanding the recalcitrance of lignocellulose with a focus on two major factors: lignin and biomass-enzyme interactions.

Lignin has a critical adverse impact on enzymatic hydrolysis by non-productive adsorption and physical barrier, especially for softwood lignin. The greater degree of condensation of softwood lignin, which has stronger interaction with enzymes, is considered to be a reason for the more recalcitrant nature of softwood than that of hardwood. However, the impact of lignin on enzymatic hydrolysis can be significantly descended when the lignin content of biomass was reduced to less than 15%, where the critical physical barrier of lignin is removed and sufficient surface area of carbohydrate is accessible to enzymes.

The biomass-enzyme interaction is another important concern determining the efficiency of enzymatic hydrolysis. During enzymatic hydrolysis, the bound enzymes are primarily responsible for enzymatic hydrolysis, while the free enzymes have very limited activity on hydrolyzing carbohydrates due to the loss of synergism effect. Although bound enzymes are generally favorable for enzymatic hydrolysis, the accumulation of those irreversibly bound enzymes which have little activities could block the accessible surface area of cellulose, thus result in a severe decrease of cellulose digestibility.

Fundamental Factors Affecting Enzymatic Hydrolysis of Lignocellulosic Biomass

by
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DEDICATION

I would like to dedicate this dissertation to my parents Shiyuan Yu and Guoying Lu whose patient love and full support enabled me to complete this work.

BIOGRAPHY

Zhiying Yu was born in Sanming, Fujian province on April 10, 1986, China. She grew up in Nanjing, Jiangsu province, China and graduated from Nanjing Forestry University in 2008 with a Bachelor of Engineering degree majored in Biochemical Engineering. She began her Ph.D. study in August 2008 in the Department of Forest Biomaterials at North Carolina State University under the supervision of Dr. Hasan Jameel and Dr. Sunkyu Park.

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CHAPTER 1

Recalcitrance of Lignocellulosic Biomass for Enzymatic Hydrolysis: A Critical Review

Abstract

The bioconversion of renewable resources to biofuels has been proposed to provide a sustainable substitute for fossil fuels. Lignocellulosic biomass, the most abundant renewable material on Earth, has been extensively studied in the past decades to produce transportation fuels via modern biotechnology. However, the recalcitrant nature of lignocellulosic biomass is a major obstacle for achieving a cost-efficient bioconversion through enzymatic hydrolysis. In this paper, the recalcitrant factors that limit the enzymatic hydrolysis of lignocellulosic biomass are reviewed with a particular emphasis on a comprehensive understanding of the physico-chemical characteristics of lignocellulosic biomass. These reactivity considerations include: lignin content, structure and distribution, heterogeneity of hemicellulose, crystalline structure and degree of polymerization of cellulose, particle size and porosity of biomass. Brief introductions of methods for determining the substrate characteristics related to these limiting factors are also summarized. This paper is intended to provide an up-to-date fundamental understanding of biomass recalcitrance that limits the cost-efficient bioconversion of lignocellulosic biomass.

Keywords: Biomass reactivity, Biomass recalcitrance, Lignocellulosic biomass, Lignin inhibition, Hemicellulose heterogeneity, Cellulose crystallinity, Cellulose degree of polymerization, Particle size, Porosity

Introduction

Lignocellulosic biomass, the most abundant renewable resource on Earth, is produced from agriculture (*e.g.* corn stover, sweet sorghum, and bagasse) and forestry resources. The worldwide production of lignocellulosic biomass was estimated to be in the hundreds of billion tons annually, but only 3% is utilized by humans (Lucia, 2008). Since a renewable natural resource is composed of carbon as a backbone element, the development of biofuel and commodity chemicals from lignocellulose is one of the most attractive potential alternatives to our current reliance on fossil fuels (Chandra et al., 2007).

As a natural composite material, lignocellulosic biomass is a chemical complex of cellulose, hemicellulose, lignin, extractives, and inorganic materials. Cellulose provides structural support, while the highly branched hemicelluloses retain water for the biomass. Lignin fills in vacant space and functions as an adhesive between the various cell wall components. Lignin is also important for mechanical support, water transport, and defense against natural invasion (Campbell & Sederoff, 1996; Decker et al., 2008). From the biomass plant to its specific chemical components, the plant cell wall shows a multi-layered structure. A hierarchical structure of woody biomass is shown with the approximate dimensions of each layer in Figure 1.1. The cell wall in wood has a layered structure around lumen, an empty space where the protoplasm is located. Middle lamella (ML), primary wall

(P) and three layered secondary cell wall (S1, S2, and S3) are in the outer part of lumen. The three layers of secondary cell wall (S1, S2, and S3) differ in the orientation of the cellulose fibers (Ioelovich, 2008). The cell walls are composed of fibril. The parallel cellulose is held together by cross-linking and hydrogen bonds of hemicellulose and lignin to form fibrils, which are approximately 10-20 nm in diameter (Donaldson, 2007). A hierarchical structure of woody biomass is shown in Figure 1.1.

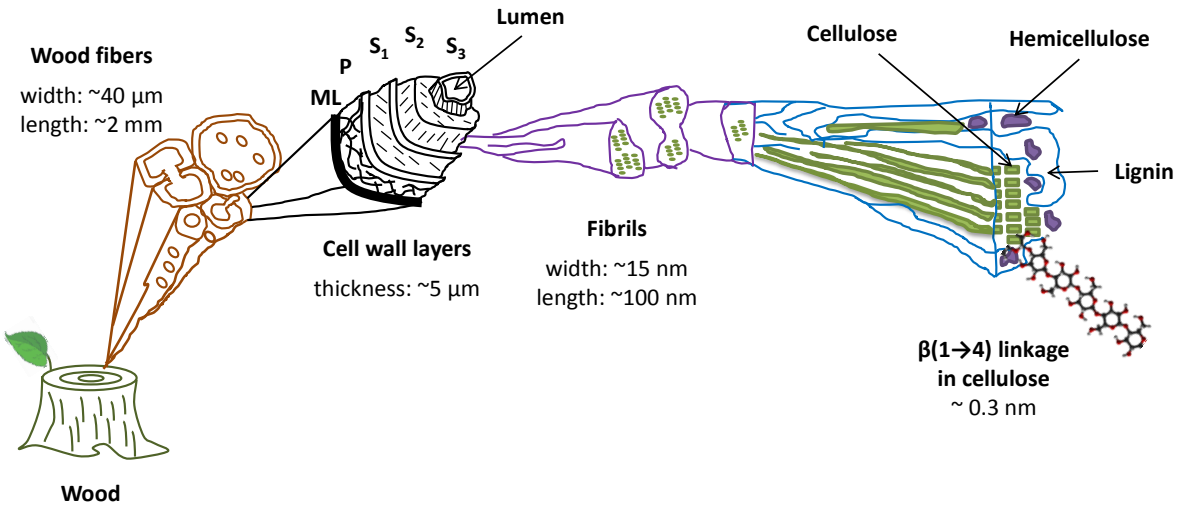

Figure 1.1. Hierarchical structure of woody biomass.

The modern biotechnical process of converting lignocellulosic biomass to ethanol includes pretreatment, enzymatic hydrolysis, and fermentation. A key issue for this process is the bioconversion of carbohydrates in lignocellulosic biomass into fermentable sugars by enzymatic hydrolysis. It is one of the most important processes not only because the yield and concentration of the hydrolysates directly determine the efficiency of fermentation, but

also because the cost of biocatalysts have a great impact on the overall economy of bioethanol production on a commercial scale. In the past few decades, many pretreatment strategies have been developed to make the lignocellulosic substrate more susceptible to enzymatic hydrolysis. In addition, the price and dosages of cellulolytic enzymes have progressively decreased due to the intensive research by enzyme producers (*i.e.* Novozymes and Genencor) (Zhang et al., 2006). Although substantial progress has been achieved to improve the enzymatic hydrolysis of lignocellulosic biomass, the conversion of saccharification is not ideally fast enough and often incomplete, especially at low enzyme loadings.

It has been recognized that for an efficient enzymatic hydrolysis, opening up the structure of lignocellulosic biomass by proper treatment prior to hydrolysis is critical to enhance saccharification. Since enzymes are in the scale of 2.4 nm-7 nm (Cowling & Kirk, 1976), according to the hierarchical organization of lignocellulosic biomass in Figure 1.1, the recalcitrant nature of biomass is emphasized on the configuration of the basic macromolecules of lignocellulose: cellulose, hemicellulose, and lignin. In order for cellulase to efficiently hydrolyze cellulose, they must first be able to access cellulose surfaces. One of the major barriers faced by cellulases is their limited access to cellulose, which is encased within the highly ordered and tightly packed architecture of plant cell wall structure (Arantes & Saddler, 2010; Mansfield et al., 1999). Previous work has shown that the ability of cellulase enzymes, such as *Trichoderma reesei* cellobiohydrolase (CBH), to access the cellulose chains within the microfibrils is significantly limited, probably due to the ability of enzymes to access only the surface layers of the microfibrils (Kurasin & Valjamae, 2010;

Mansfield et al., 1999; Mooney et al., 1999). A microscopic study of the pretreated corn stover showed that the enzyme penetration increased from <1% to 100% of the thickness of secondary cell walls as the severity of dilute-acid pretreatment increased from 20 min at 100°C to 20 min at 150°C (Donohoe et al., 2009). This was direct evidence supporting that the loosening of plant cell wall structure through pretreatment improved the accessibility of cellulolytic enzymes. Rather than opening the cellulose fibrils from the outside of cell walls by physical or chemical pretreatment, it has also been suggested that these inaccessible regions can be disrupted by non-hydrolytic proteins (*e.g.* expansin and expansin-like proteins), which can increase the cellulose surface area and make it more accessible to the cellulases, and consequently enhance enzymatic hydrolysis (Arantes & Saddler, 2010; Baker et al., 2000). The discovery of oxidative enzymes (*e.g.* GH61) has been shown to have boosting and synergetic effects with cellulolytic enzymes. Oxidative enzymes are now present in commercially available cellulases (*e.g.* Cellic CTec2) to improve saccharification yield. One well known oxidoreductase, which can potentiate hydrolysis, is GH 61. It functions to oxidize the glucosidic linkage and produce oxidized gluconic acid. The cleavage of cellulose chain by GH61 is favorable for enhancing hydrolysis rate (Cannella et al., 2012; Quinlana et al., 2011).

In the past decades, an enormous amount of interest and effort has been devoted to the development of various pretreatment strategies. Nowadays, with the great progress in knowledge of biomass recalcitrance, the mechanisms of many pretreatments are known at a fundamental level, and researchers tend to take more rational and logical standpoints to design new processes. Generally, all of these pretreatments or subsequent treatments have the

same goal: to increase the accessibility of substrate and thus, to enhance the digestibility of lignocellulosic biomass. Therefore, it is essential to understand the factors that influence the digestibility of lignocellulosic biomass. It has been recognized that both the properties of substrates (*e.g.* the architecture and component of lignocellulosic biomass such as lignin, crystallinity, the degree of polymerization, particle size, and pore volume) and enzymes (*e.g.* the component, stability, and synergistic effect of enzyme systems) have significant influence on enzymatic hydrolysis (Chandra et al., 2008; Hall et al., 2010; Mansfield et al., 1999; Mooney et al., 1998; Puri, 1984; Studer et al., 2011; Yu et al., 2011).

This paper is attempting to give a comprehensive, up-to-date overview of the physicochemical properties of lignocellulosic biomass that affect its digestibility. These factors include lignin blockage and inhibition, highly order structure of cellulose, heterogeneity of hemicellulose, and spatial accessibility represented by particle size and porosity. Additionally, the approaches for measuring these factors are briefly introduced. This information provides an insightful understanding from the fundamental point of view on the biomass recalcitrance for biofuel and biochemical production, and is of great value for understanding the importance of biomass reactivity and developing a more efficient treatment process to improve enzymatic hydrolysis.

Lignin

Lignin is the most abundant non-carbohydrate component in lignocellulosic biomass. The lignin content varies with the type of biomass and it is usually in the range of 18-25% for hardwood, 25-35% for softwood, and 10-25% for agricultural residues such as corn stover,

switchgrass, wheat straw, and bagasse (Saka, 2001; Zhao et al., 2012; Zhou et al., 2012). The basic structure of lignin is a network aromatic biopolymer composed of phenylpropane (C_9) units of the p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) types, as shown in Figure 1.2. These monomeric units are linked in lignin macromolecule by various ether and C-C bonds. Softwood is composed mainly of guaiacyl units, while the ratio of syringyl and guaiacyl units (S/G ratio) in hardwoods can vary in the range of 1.2-3.2 depending on the species (Santos et al., 2011). Grass lignin contains syringyl, guaiacyl and *p*-hydroxyphenyl units (Sakakibara & Sano, 2001). Since lignin has a network structure, its removal requires an intensive chemical treatment. In addition, lignin is covalently linked to carbohydrates forming lignin-carbohydrate complex (LCC) (Balakshin et al., 2011; Balakshin et al., 2008). The side-groups arabinose, galactose, and 4-O-methylglucuronic acid are most frequently perceived as connecting links to lignin (Fengel & Wegener, 1984).

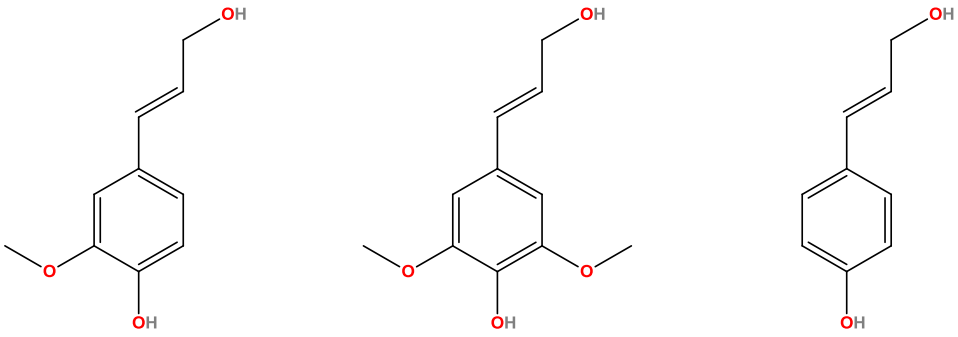

Figure 1.2. Lignin building blocks. (a) coniferyl alcohol (guaiacyl (G) unit), (b) sinapyl alcohol (syringyl (S) unit), and (c) p-coumaryl alcohol (4-hydroxyphenyl (H) unit).

Apparently, biomass with high lignin content has low carbohydrate content, resulting a low maximum sugar recovery based on the total weight of biomass. Even if evaluating the sugar recovery based on the available carbohydrate content, the higher lignin content still possesses a negative effect on enzymatic hydrolysis. Substantial studies have demonstrated the negative impact of lignin content on carbohydrate conversion during enzymatic hydrolysis. A comprehensive modeling study of 147 lignocellulosic substrates showed that compared with crystallinity and acetyl content, lignin content has the greatest impact on enzymatic hydrolysis (Chang & Holtzapple, 2000). It was found that the carbohydrate conversions of enzymatic hydrolysis improved significantly when the lignin content decreased (Mooney et al., 1998; Santos et al., 2012a; Yu et al., 2011). It has also been reported that a decrease in lignin content promotes a higher enzyme adsorption on the substrate, which results in improved efficiency of enzymatic hydrolysis (Santos et al., 2012b). This negative impact of lignin on hydrolysis is thought to be originated from three aspects: 1) Lignin may exist as a surface barrier (physical blockage and lignin carbohydrate complex) that restricts the access of enzymes to the accessible sites of polysaccharides, 2) Lignin may nonproductively adsorb enzymes, and 3) The soluble lignin may deactivate enzymes (Nakagame et al., 2011b; Yang et al., 2011b). A conceptual model of lignin-derived inhibition on enzymatic hydrolysis is shown in Figure 1.3.

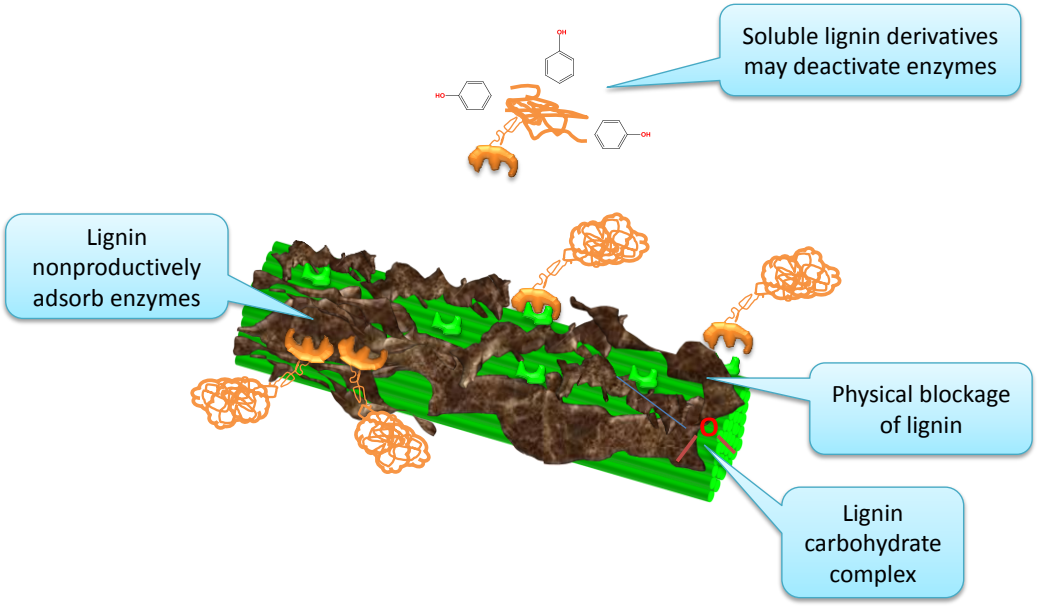

Figure 1.3. A conceptual model of lignin-derived limitation for enzymatic hydrolysis.

Limitation of biomass accessibility by a surface barrier of lignin

Enzymatic hydrolysis requires direct physical contact between an enzyme and substrate. Enzymes must diffuse from the bulk aqueous solution to the substrate surface and diffuse through surface barriers, such as lignin, to absorb on the carbohydrates, and then catalyze the hydrolysis (Chang & Holtzapple, 2000). Yang and coworkers suggested that cellulase could be irreversibly entrapped within the tri-dimensional matrix of lignin, which may be a barrier that significantly reduced the enzyme-cellulose interactions (Yang et al., 2011b). It has been shown that the removal of lignin increased the porosity of pretreated pulps, and consequently improved enzymatic hydrolysis (Koo et al., 2011; Mooney et al., 1998; Yu et al., 2011). The pores created by lignin removal leave cellulose and hemicellulose more accessible and open for enzymes (Taherzadeh & Karimi, 2007).

This lignin barrier not only depends on the actual content of lignin, but is also related to the distribution and macromolecular structure of lignin. A redisposition of unextracted lignin into the accessible pores and onto the cellulose surface was proposed as a greater barrier for the decreased hydrolysis yield after alkaline treatment of steam pretreated pine (Palonen, 2004; Wong et al., 1988). Microscopic techniques, such as SEM and TEM, revealed a range of droplet morphologies that appear on and within cell walls of pretreated biomass. These droplets were shown to contain lignin by FT-IR, NMR, antibody labeling, and cyto-chemical staining analyses. It was proposed that when the temperature of a thermochemical pretreatment reaches above the range for lignin phase transition, the lignin was caused to coalesce into larger molten bodies that migrate within and out of the cell wall, and can redeposit onto the surface of plant cell walls (Donohoe et al., 2008).

It has been widely observed that softwood substrates are more resistant to enzymatic hydrolysis than that of hardwood (Mansfield et al., 1999; Yu et al., 2011). Even though the lignin content of hardwood and softwood obtained at a similar level by pretreatment and delignification; the softwood was more recalcitrant to enzymatic hydrolysis comparing to hardwood (Yu et al., 2011). Since softwood is composed of G units of lignin, while hardwood lignin is a mix of S and G units, it is considered that the difference of S/G ratio might be a reason for the different digestibility of hardwood and softwood (Santos et al., 2012b; Studer et al., 2011). A broad investigation of the correlation between S/G ratio and the enzymatic hydrolysis efficiency was conducted among 47 extreme phenotypes of *Populus trichocarpa* trees. The results showed that a strong negative correlation between sugar release and lignin content was found for pretreated samples with an S/G ratio less than 2. Higher S/G ratios

generally result in higher sugar release and the impact of lignin content is less pronounced (Studer et al., 2011). Santos and coworkers recently found that when the lignin content was the same in several species of hardwood, the higher S/G showed a higher enzyme adsorption and resulted in a more efficient hydrolysis yield (Santos et al., 2012b). A few thoughts have been considered to explain the reason for a higher carbohydrate conversion from the substrate with a higher S/G ratio. Generally, S-rich lignin features predominantly linear chains with less cross-linking than G-rich lignin. This is because the C-5 position is occupied by a methoxyl group in the syringyl unit (Figure 1.4 a). Additionally, the higher occurrence of β-β units (resinols) in S-rich lignin leads to a shorter chain length and thus, a lower molecular weight (Figure 1.4b) (Kishimoto et al., 2010; Stewart et al., 2009). This structure resulted in less 5-5 and β-5 linkages, which is highly stable in lignin (Figure 1.4 c and 1.4 d). Therefore, the more branched guaiacyl units are likely to have a more spreadable distribution that not only act as a surface barrier, but also restrict the swelling of the cellulosic substrate and reduce the accessible surface area available to the enzymes (Ramos et al., 1992).

Besides, lignin-carbohydrate complex (LCC) has been shown to inhibit the availability of the carbohydrates for digestion (Laureano-Perez et al., 2005). It was found that carbohydrate chains or side groups were attached to lignin through the non-reducing moieties and thus, the existence of LCC can limit the action of CBH II, which attacked the cellulose chain from the non-reducing ends (Jeffries, 1990). The steric hindrance of multiple cross-links between the lignin and polysaccharides is believed to be another obstacle for the adsorption of enzymes (Jeffries, 1990). When the carbohydrates have been extensively hydrolyzed, the lack of binding sites for the enzymes could be a reason for the incomplete

digestion. Biely and coworkers showed that the substrate-binding site of endo-1,4-β-xylanase of the yeast *Cryptococcus albidus* had eight to ten subsites for binding pyranose rings and the catalytic groups that possess the active site are located in the center. Therefore, the digestion of LCC with endo-xylanases and endo-cellulases leaves residual polysaccharide oligomers with a DP of four or more attached to the lignin (Biely et al., 1981). Even though carbohydrates are attached to the lignin by O-1 hydroxyl, a single glucose may still linked to lignin fractions after being completely attacked by β-glucosidase, which makes the complete digestion of the carbohydrate to be a great challenge (Jeffries, 1990).

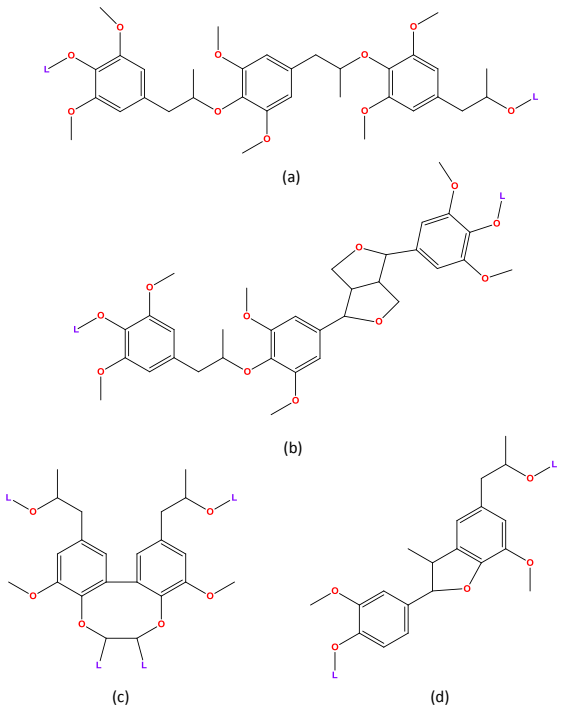

Figure 1.4. Typical structures of lignin in softwood. (a) Linear structure of syringyl lignin, (b) Syringyl lignin with β-β linkage. (c) Highly stable 5-5 linkage in softwood, and (d) Highly stable β-5 linkage in softwood.

Nonproductive adsorption of enzymes on lignin

Numerous evidences indicate that the nonproductive adsorption of enzymes onto lignin, preventing their access to cellulose and hemicellulose, is another reason for the significant decline of hydrolysis efficiency (Berlin et al., 2005; Mansfield et al., 1999; Nakagame et al., 2010). The hydrophobic interaction, or ionic-type interaction, is thought to be the reason for the adsorption of cellulases and hemicellulases onto lignin (Berlin et al., 2006; Eriksson et al., 2002). Both lignin and enzymes are considered overall hydrophobic due to the lack of water-loving functional groups in their structures (etc. -OH). Hydrophobic interaction is an entropy-driven process in which by aggregating together, nonpolar molecules reduce the surface area exposed to water and minimize the disruption of hydrogen bonds in water (Silverstein, 1998). Several indirect evidences indicate this phenomenon. For example, enzymes have been found to be inhibited more by lignins with lower content of carboxyl and aliphatic hydroxyl groups which were supposed to possess higher surface hydrophobicity (Berlin et al., 2006). The observation that the addition of surfactant reduced the nonproductive binding of CBH I to lignocellulose was probably due to the coverage of surfactant on the hydrophobic sites of lignins (Eriksson et al., 2002). Besides, it is speculated that the presence of charged (COOH, OH) or partially charged (CO) functional groups on both lignin and enzyme surfaces can also build ionic-type lignin-enzyme interactions (Berlin et al., 2006). However, it is less likely to be convinced since both enzymes and lignin have overall negative charges under hydrolysis conditions, typically pH ~4.8. It was proposed that the increase of carboxylic content in lignin improved enzymatic hydrolysis because those carboxylic groups contributed to the decrease of hydrophobicity and increase of negative

charge. The negatively charged lignin might further decrease enzyme binding by electrostatic repulsion since cellulase enzymes are also negatively charged at the hydrolysis environment of pH 4.8 (Nakagame et al., 2011a).

Experimental evidence of the impact of lignin nonproductive adsorption is reported in many references. It was found that the lignin isolated from lodgepole pine and steam pretreated poplar significantly decreased the hydrolysis yields when the isolated lignins were mixed with crystalline cellulose (Avicel) (Nakagame et al., 2010). It was also suggested that the extent to which lignin adsorbed enzymes substantially depends on the nature of lignin itself and, lignin adsorption of enzyme is decreased as the severity of pretreatment increases (Nakagame et al., 2010). A study of enzyme binding and deactivation on lignin-rich surface showed a deactivation of lignin-bound enzymes taking place on the surface of softwood lignin at the hydrolysis temperature (Rahikainen et al., 2011).

Deactivation of enzymes by soluble lignin

During pretreatment and enzymatic hydrolysis, some lignin can be dissolved in the liquid. The amount of dissolved lignin depends on the type of pretreatment. The fractions of soluble lignin were found to affect not only microorganisms, but also enzymes such as cellulase, xylanase, and β-glucosidase (Yang et al., 2011a). Soluble lignin containing phenolic-based compounds such as vanillin, syringaldehyde, trans-cinnamic acid, and hydroxybensoic acid (Figure 1.5), were found to inhibit cellulose hydrolysis by endo- and exocellulases, and cellobiose hydrolysis by β-glucosidase. Vanillin showed strongest

inhibition on the mixture of Spezyme CP and Novozyme 188, while hydroxybenzoic acid had the greatest inhibition of these individual commercial enzymes (Ximenes et al., 2011).

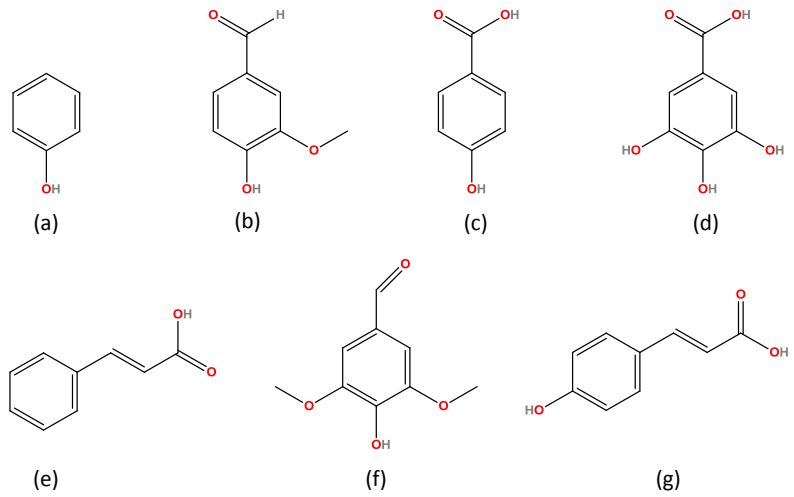

Figure 1.5. Structures of soluble lignin-derived inhibitors for enzymatic hydrolysis. (a) phenol, (b) vanillin, (c) 4-hydroxybenzoic acid, (d) gallic acid, (e) *trans*-cinnamic acid, (f) syringaldehyde, and (g) *p*-coumaric acid.

On the other hand, aldehydes-containing soluble lignins such as vanillin, syringaldehyde, and 4-hydroxybezaldehyde or corresponding carboxylic acids were reported to have minor inhibition on cellulases (Cantarella et al., 2004). Acid-containing soluble lignin, such as p-coumaric acid and gallic acid, were found to exhibit 38% and 47% degree of inhibition based on the original activity of xylanase XynA (Morrison et al., 2011). It was noticed that all these soluble lignins possess an aromatic ring or phenolic structure. Since both enzymes and soluble lignin are hydrophobic in nature, the hydrophobic interaction might be a reason for the inhibition. It is also rendered that the deactivation of enzymes by

phenolic radical attacking is also a concern as the phenolic compounds are strong antioxidants (Kim et al., 2009). During the oxidation of phenolic compounds, the formation of phenolic radicals, which form polymers by polymerization, may deactivate enzymes by hydrophobic adsorption. In another case, it was reported that the covalent bonds between enzyme and phenolic compounds might be the reason for the deactivation of enzymes (Kim et al., 2009).

Characterization of lignin

The most common way to determine the lignin content is by a two-step acid hydrolysis followed by gravimetric measurement and ultraviolet (UV) spectra (Sluiter et al., 2011). Besides, a rapid transmittance near-infrared (NIR) spectroscopic method has been developed to characterize the lignin content of solid wood by Yeh and coworkers (Ting-Feng Yeh et al., 2004). By comparing the NIR spectra of holocellulose and loblolly pine, the ratio of the peak height identified from lignin and assigned to carbohydrates showed a linear relationship with lignin content determined by traditional chemical methods (Hames et al., 2003; Ting-Feng Yeh et al., 2004). In addition, it is reported that lignin content can also be determined by analytical pyrolysis. The ground wood samples were pyrolyzed in a pydrolysis reactor. A custom-built molecular-beam mass spectrameter was used for pyrolysis vapor analysis. The intensities of the major peaks assigned to lignin were summed in order to estimate the lignin content across the range of samples (Sykes et al., 2008).

It is a great challenge to elucidate the chemical structure of lignin, since the isolation of lignin is usually incomplete and the lignin structure may be subject to a significant

modification during the isolation process. The quantitative data for various functional groups and linkage types in lignin are essential to understand lignin structure. UV, infrared (IR), and nuclear magnetic resonance (NMR) spectroscopic techniques, particularly when used in conjunction with chemical modification, have contributed to estimating the frequencies of functional groups and linkage types (Sakakibara & Sano, 2001).

A number of methods can be used to study the distribution of lignin (Saka, 2001). One of the popular and qualitative ways is selective staining, followed by light microscopy analysis. For example, potassium permanganate staining has been used extensively for studying lignin distribution by electron microscopy (Saka et al., 1979). Various electro-microscopic methods have been developed to analyze the lignin distribution quantitatively in the past decades, including ultraviolet (UV) (Fergus et al., 1969; Siqueira et al., 2011), interference microscopy and confocal laser scanning microscopy (Donaldson et al., 2001), transmission electron microscopy (TEM) and scanning electron microscopy (SEM) (Donohoe et al., 2008; Fromm et al., 2003), energy-disperse X-ray analysis (EDXA) (Eriksson et al., 1988; Saka et al., 1982) Fourier transform infrared (FTIR) spectroscopy (Jaaskelainen et al., 2003), and Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS) (Zhou et al., 2011). The quality of the results depends on the absorptivity of the lignin molecule, which is quite sensitive to different structural factors (Fromm et al., 2003).

Lignin issue is the most important and unknown factor governing the digestibility of lignocellulose. Although the lignin content, lignin structure, and lignin distribution are recognized to inhibit enzymatic hydrolysis due to its nonproductive adsorption, surface barrier, and highly recalcitrance of LCC, substantial studies are still needed to provide a clear

elucidation of lignin inhibition. For example, the role and importance of individual lignin factor on enzymatic hydrolysis, and the interactions between each factor need to be further investigated. It should be noted that lignin content or lignin structure itself is not necessarily a key determinant in the negative impact of lignin on enzymatic hydrolysis. The interactions between lignin content and structure or the way that lignin is incorporated with the polyssacharides may also play an important role in limiting enzymatic hydrolysis.

Cellulose

Crystalline structure

Cellulose is a linear polymer of β-1,4-D-glucopyranose units in $4C_1$ conformation with the repeating unit being cellobiose (Figure 1.6). Cellulose found in plants is in the form of microfibril, which dimension is 2-20 nm diameter and 100-40,000 nm long (Nishiyama, 2009).

For a long time, cellulose crystallinity, the amount of crystalline fraction, has been thought to be one of the most important properties of cellulose that influences its enzymatic digestibility (Fan et al., 1980; Mittal et al., 2011). Many studies have shown that completely disordered or amorphous cellulose is hydrolyzed at a much faster rate than partially crystalline cellulose (Dermoun & Belaich, 1988; Fan et al., 1980; Sinitsyn et al., 1991). This observation provides a readily explanation of observed kinetics cellulose hydrolysis. It is believed that enzymes hydrolyze the presumably *easy* amorphous first prior to digesting the *difficult* crystalline portion. Consequently, a highly recalcitrant substrate with an increase in crystallinity is expected as the progress of enzymatic hydrolysis (Reese et al., 1957). Hall and

coworkers found a strong correlation between the initial hydrolysis rate and the crystallinity of cellulose. Factor analysis indicated that initial rates of digestion (up to 24 h) were most strongly correlated with the amount of amorphous fraction (Mittal et al., 2011). However, it was observed that the crystallinity index of Avicel measured by x-ray diffraction did not change with the progress of hydrolysis (Hall et al., 2010; Yu et al., 2012). Furthermore, it was also reported that when some other physico-chemical properties of substrate (*e.g.* surface area, degree of polymerization) are similar, the difference in the degree of crystallinity of the substrate has no effect on enzymatic hydrolysis (Puri, 1984). This discrepancy might be due to the inconsistent observation with different substrates and different measurement techniques (Park et al., 2010). Therefore, experimental correlations of enzymatic hydrolysis with crystallinity index should be interpreted with caution.

On the other hand, it has been recognized that different crystalline structures of cellulose have different effects on enzymatic hydrolysis (Mittal et al., 2011). There are four different crystalline allomorphs of cellulose, *i.e.* cellulose I, III, III, and IV, which are differed in intra- and inter-chain hydrogen bonds within the unit cell and the polarity of adjacent cellulose sheets within the crystallite (Marchessault & Sundararajan, 1983). These differences can be identified by a combination of molecular dynamic simulation and experimental characterization using x-ray diffraction patterns and solid-state ^{13}C NMR spectra. Cellulose I, the most abundant polymer in nature, has two distinct forms: cellulose I_α and cellulose I_β (Atalla & VanderHart, 1984). Cellulose I_α is the predominant form in bacteria and alga, whereas cellulose in higher plants is mostly I_β. The other crystalline structures cellulose II, III, and IV can be obtained from cellulose I by chemical or thermal treatment (Figure 1.7).

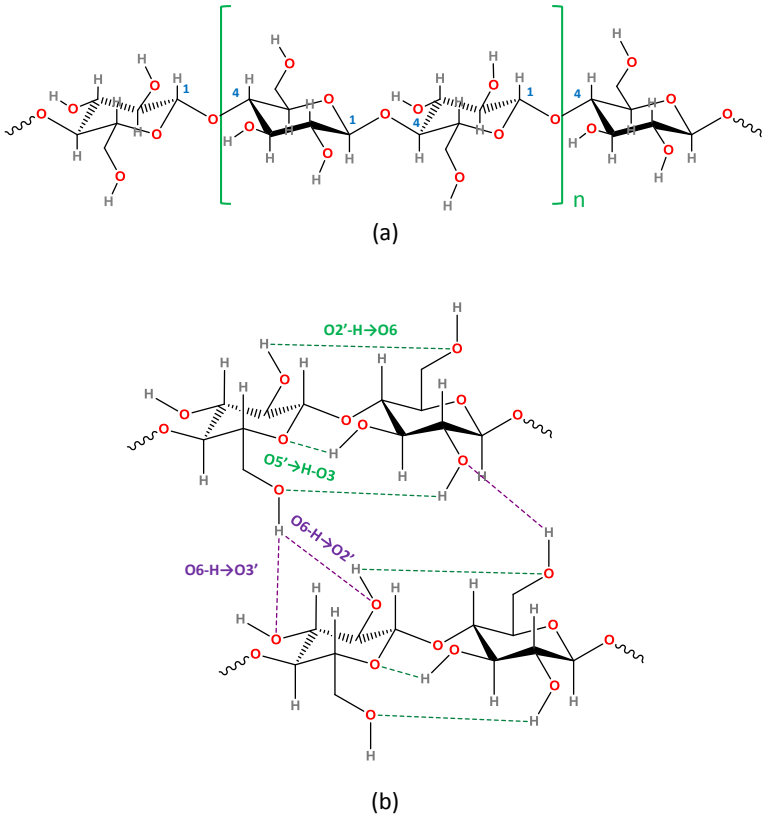

Figure 1.6. Structure of cellulose. (a) Molecular structure of cellulose, and (b) Intra-molecular and inter-molecular hydrogen bond in cellulose I_{β}.

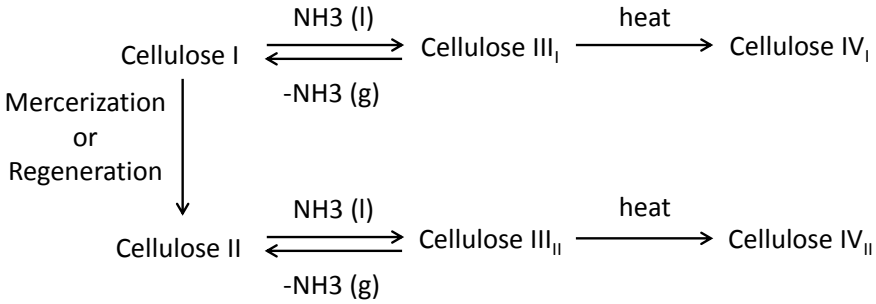

Figure 1.7. Interconversion of the polymorphs of cellulose (O' Sllivan, 1997).

The relative digestibility of cellulose allomorphs has been investigated in the previous studies. Weimer and coworkers compared the digestibility of amorphous cellulose and all four crystalline allomorphs (I, II, III, and IV) of cellulose from cotton fiber (mainly cellulose I_β). It was found that the initial digestion rates for different allomorphs were in the following order: amorphous > III_I > IV_I > III_{II} > I > II. The amorphous cellulose, which possessed 4-fold higher gross specific surface area compared to starting cellulose I, obtained 10-fold hydrolysis rate compared that of cellulose I (Weimer et al., 1991). In addition, the enhanced hydrolysis rate of III_I was due to its reduced crystallinity and the screwed structure which might expose alternating corner chain residues to the exoglucanases to a greater extent than that of cellulose I (Weimer et al., 1991). Igarishi and coworkers converted alga cellulose (mainly cellulose I_α) into cellulose III_I and I_β by supercritical ammonium and hydrothermal treatment, respectively, and found that the enzymatic hydrolysis of different crystalline structures of cellulose was in an order of cellulose III_I >> cellulose I_α > cellulose I_β. It was speculated that the increased hydrolysis rate of cellulose III_I was due to its reduced crystallinity, lower packing density, and greater distances between hydrophobic surfaces of cellulose III_I than that of cellulose I (Igarashi et al., 2007). For the differences of the hydrolysis rates between cellulose I_α and I_β, it was believed that the 0.02 Å shorter in the distance of hydrophobic surface and smaller 4 $Å^3$ in the volume of cellobiose unit of cellulose I_β compared with I_α were sufficient to explain the lower hydrolysis behavior (Igarashi et al., 2007). A recent study, which aimed to elucidate the changes in crystalline structure by alkaline and liquid-ammonia treatment and their effects on enzymatic hydrolysis, showed a hydrolysis order of cellulose II > cellulose III_I > cellulose I. It was found that the

formation of cellulose II produced much less crystalline cellulose which resulted in faster and higher digestion (Mittal et al., 2011). Computational simulation was performed to calculate the intrinsic work required for decrystalize individual chains of different allomorphs of crystalline cellulose. It was found that for edge chains, the order of decrystallization work ranked (highest to lowest) as: I_β, I_α, III_I, II, which indicated the free energy required to deconstruct these celluloses (Gregg T. Beckham et al., 2011). However, it was also noted that the correlation of allomorph type with digestibility was weak among different cellulose sources, but was strongest with cellulose conversion at a later time. Although the cellulose III_I produced at higher temperatures had comparable crystallinity to the initial cellulose I, it achieved higher levels of conversion at longer digestion time (Mittal et al., 2011). Another case regarding the reactivity of different allomorph of cellulose showed that by transforming natural crystalline allomorpho I_β to III_I through ammonia treatment, the amount of cellulose intra-sheet hydrogen bonds was decreased while the amount of inter-sheet hydrogen bonds was increased. As a result, the number of solvent-exposed glucan chain hydrogen bonds with water was increased by 50% accompanied by an enhanced saccharification rate of up to 5-fold (close to amorphous cellulose). The "amorphous-like" nature of the cellulose III_I fibril surface, which was converted from tough crystalline structure into a more accessible one, is thought to be a key to enhance the cellulose reactivity (Chundawat et al., 2011a).

The degree of crystallinity or crystalline structure can be evaluated using several different techniques such as XRD, solid-state ^{13}C NMR, IR (infrared spectroscopy), and Raman spectroscopy. XRD is the most widely used method for crystallinity index (CrI) measurement, which has been used in about 90% of related literatures (Park et al., 2010).

Although these tools have been used for decades, accurate and quantitative characterization is far from perfect and it was found that the CrI varied at a great degree depending on the choice of the measurement technique and the analytical methodology of the data process, especially when non-cellulosic components such as hemicellulose and lignin are present in the substrate. A new analysis method was recently proposed using sum-frequency-generation vibration spectroscopy, which selectively detects crystalline cellulose in lignocellulosic biomass (Barnette et al., 2011; Barnette et al., 2012).

Degree of polymerization

The degree of polymerization (DP) of cellulose refers to the number of monomeric units (glucose) in the cellulose chain. The DP of cellulose varied from hundreds to even 12,000 or more. Many researchers have studied the effect of DP on enzymatic hydrolysis and it has been reported that the reduction in cellulose DP significantly improves its yield of enzymatic hydrolysis. It is considered due to the increased activity of exoglucanase when the number of reducing ends in cellulose is greater at the same mass basis. It was found that by increasing the number of endoglucanase-generated chain ends from 0 to 6 μmol/g in bacterial cellulose, the amount of soluble sugar increased from 0.1 to 0.25 mM (Väljamäe et al., 2001). Furthermore, shorter chains enable cellulose to be more amenable to enzymatic digestion, because they do not form strong hydrogen bonds. The weaker network allows greater possibility for enzyme access (Hallac et al., 2010; Pan et al., 2007; Pan et al., 2008).

Due to the applied pretreatment method, the pretreated substrate will contain a different amount of reducing chain ends. For example, substrates prepared from acidic pretreatment,

like dilute sulfuric acid, have a higher number of reducing ends than alkali pretreatment (AFEX and lime) (Kumar et al., 2009). The study by Puri showed that the enzymatic hydrolysis was improved with the reduction in DP (Puri, 1984). The mixed spruce and pine substrate also showed a higher sugar conversion with the decreased DP. At DP of 200, 40% was achieved as compared to 5% conversion at DP of 400 (Martínez et al., 1997).

There are typically three techniques used for DP measurement: reducing end measurement, viscometry, and the gel-permeation chromatography (GPC) methods (Hallac & Ragauskas, 2011). The determination of cellulose DP by viscometry and GPC require the cellulose to be dissolved in metal complex or to form soluble cellulose derivatives that do not significantly affect its original DP (Trathnigg, 2004). DP can be expressed in number average (DP_N), weight average DP (DP_W), or viscosity average DP (DP_V). In general, the value from viscosity measurement is greater than DPw from GPC methods (Pala et al., 2007).

Hemicellulose

Hemicellulose, the second most abundant polysaccharide in nature, represents about 20-35% of lignocellulosic biomass (Saha, 2003). Hemicellulose is highly branched, substituted, and heterogeneous, composed of a wide variety of subunits, including pentoses (xylose and arabinose), hexoses (glucose, galactose, and mannose), and sugar acids. The major hemicellulose structures in herbaceous plants, hardwood, and softwood are shown in Figure 1.8. Hemicellulose is reported to be linked to lignin through cinnamate acid ester linkages, to cellulose through interchain hydrogen bonding, and to other hemicelluloses via covalent and hydrogen bonds (Decker et al., 2008). Different plant families have different ratios of the

various hemicelluloses. Hemicelluloses of herbaceous plants contain mainly xylan, especially arabinoxylan (Figure 1.8a). The major hemicellulose in hardwood is xylan, more specifically *O*-acetyl-4-*O*methylglucurono-ß-D-xylan (Figure 1.8b). The major hemicelluloses in softwood are galactoglucomannan and arabinoglucuronoxylan (Figure 1.8c) (Decker et al., 2008; Laine, 2005).

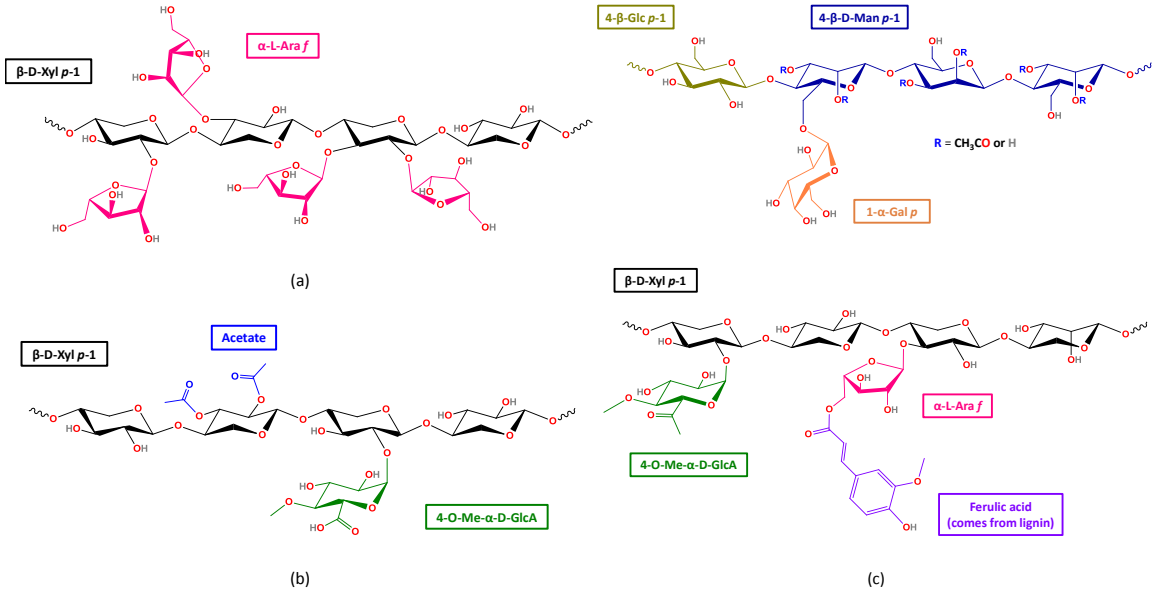

Figure 1.8. Structure of major hemicellulose in herbaceous plant and woody biomass.

Heterogeneity of hemicellulose

Since the structure and chemistry of hemicellulose is complex and heterogeneous, a complete degradation of hemicellulose may require a variety of hemicellulose such xylanase, mannanase, arabinose, and esterases. For example, xylan is the dominant hemicellulose in hardwood and non-woody biomass. Its deconstruction to monomer sugar not only contributes

to the source of sugars, but also enhances the exposure of cell wall to enzyme/chemical hydrolysis. Xylan is known as a heteropolymeric substrate consisting of a repeating β-1,4-linked xylose backbone branched with acetyl, arabinofuranosyl, and 4-O-methyl glucuronyl groups (Figure 1.8b). In addition, xylan may be cross linked to lignin by aromatic esters. In order to efficiently depolymerize xylan to the component monosaccharides, a mixture of different enzymatic functionalities are required, including endo-1,4-β-xylanases that cleaves internal β (1→4) backbone bonds in xylan, β-D-xylosidases that cleaves xylobiose or longer xylooligosaccharides into xylose from the non-reducing end (Xu, 2011), α-L-arabinofuranosidases, α-glucuronidases, acetyl xylan esterases, and ferulic/coumaric acid esterases (Dodd & Cann, 2009). In contrast, mannan is the major backbone hemicellulose in softwood, thus the potential application of mannanase on softwood may help accelerate hydrolysis. Endo-mannanase is able to cleave internal β (1→4) bonds in mannan, while β-mannosidase cleaves mannosyl units from non-reducing ends of mannooligosaccharides (Xu, 2011).

Limitation of cellulase accessibility by physical blockage of hemicellulose

Similar to lignin, hemicellulose is known to coat the cellulose microfibril in the plant cell wall and form a physical barrier to access by hydrolytic enzymes. Therefore, the removal of hemicellulose has been reported to increase the enzymatic hydrolysis of cellulose (Yoshida et al., 2008). Without a supplementary of hemicellulase, the hemicellulose was found to hinder the access of cellulase to the substrate even after delignification (Yoshida et al., 2008). Although cellulose digestibility has been a major consideration for pretreatment design and

optimization, the sugar yields from both hemicellulose and cellulose are essential for an economically-feasible process of ethanol production (Ohgren et al., 2007). Hemicellulase, such as xylanase, shows a great potential in saccharifying various lignocellulosic biomasses to fermentable sugars. Ohgren and coworkers found that almost all the glucose from acid-catalyzed steam pretreated corn stover can be recovered during enzymatic hydrolysis if xylanase of 0.06 g protein/g cellulose was used to supplement 15 FPU cellulase/g cellulose, while xylose conversion reached to 70-74%. As a contract, only 70% glucose yields were obtained without the supplement of xylanase (Ohgren et al., 2007). This is strong evidence indicating that the hydrolysis of hemicellulose by xylanases consequently improved the access of cellulase to cellulose. Similar findings can be found elsewhere (Yang & Wyman, 2004; Yoshida et al., 2008). Since hemicellulose polysaccharides are not tightly constructed like crystalline structure of cellulose, hemicellulose is relatively more accessible to enzymes comparing to cellulose (Saha, 2003).

Since significant portion of sugars is in hemicellulose, it is essential to maintain the hemicellulose in either a solid or soluble form during pretreatment to maximize sugar recovery for fermentation. The cost of hemicellulase is, in general, higher than that of cellulase largely because the substrate used for hemicellulose production is more expensive than cellulase. Developing robust hemicellulases with less cost needs to be emphasized in future research.

Surface Area

Particle size

Particle size of lignocellulosic substrate is another factor for enzymatic hydrolysis. The particle size essentially reflects the available exterior specific surface area for the adsorption of cellulase. Since the adsorption of enzymes onto carbohydrates is the prerequisite step for enzymatic hydrolysis, the surface area would be a critical factor for hydrolysis rate, as higher specific area can provide more available adsorption sites per mass of substrate (Chundawat et al., 2007).

There is evidence to support the correlation between the particle size and hydrolysis rate for lignocellulosic substrates. It was found that for the ammonia fiber explosion (AFEX) pretreated corn stover, after milling and fractionation, particle size reduction (from 0.85-0.5mm to <0.15 mm) of corn stover enhanced glucan and xylan conversions by 15-20% (Chundawat et al., 2007). Similarly, red-oak sawdust with fractionated particle size from 0.033 mm to 0.850 mm showed higher enzymatic reaction rates and the conversions of cellulose to glucose yield with smaller particle size. After 72 hours of enzymatic hydrolysis, about 55% more glucose was produced from the smallest size particles than the largest size ones (Dasari & Berson, 2007).

For the untreated corn stover, the same trend, but less pronounced, was reported with no more than 10% higher conversion when the particle size was reduced from 0.15mm to 0.07 mm (Elshafei et al., 1991). A kinetic of enzymatic hydrolysis of α-cellulose with a particle size ranging from 0.038 to 0.082 mm reported that the smaller particle size yielded over 30% more reducing sugar in the first 2 h, whereas there was little difference in the production of

reducing sugar between the smallest and largest particle size after 20 h of hydrolysis (Gan et al., 2003).

However, a few researches found no relationship between particle size and enzymatic hydrolysis. A research investigating the effect of particle size on enzymatic hydrolysis of newspaper and corrugated cardboard reported that particle size had no correlation with the hydrolysis conversion (Rivers & Emert, 1988). The correlation between the particle size of various cellulose substrates (cotton linter, microcrystalline cellulose, and α-cellulose) and the efficiency of enzymatic hydrolysis was studied and it was found that the average size of cellulose particles (from 17 μm to the length of fiber) did not influence the efficiency of hydrolysis to any noticeable extent (Sinitsyn et al., 1991).

A quick and approximate determination of particle size can be conducted by using a sieve shaker with different meshes (the number of openings per linear inch). A material that can pass through a specific mesh, but is retained by another specific tighter mesh, establishes the range of particle size. The particle size distribution of samples can also be determined by using a laser diffraction particle size analyzer with detecting range of 0.04-2000 μm (Yeh et al., 2010). The samples are suspended and subjected to mild stirring and the average diameters of particles can be calculated with affiliated software.

Porosity

The porosity of the substrate essentially reflects the interior specific surface area of a biomass. Particle size alone might be not an appropriate factor for cellulose adsorption sites since lignocellulosic biomass has a porous structure. The pioneering work was performed by

Stone and coworkers: they estimated the rate limiting pore size of a substrate within the 4-5 nm during enzymatic hydrolysis, while Cowling and Kirk showed that the diameter of most cellulases was in the range of 2.2-7.7 nm (Cowling & Kirk, 1976; Stone et al., 1969). These observations indicated that the 4-7 nm pore size was necessary to accommodate cellulases to catalyze the hydrolysis. Since then, there has been a substantial amount of research addressing the necessity of developing accessible surface area or pores to improve enzymatic hydrolysis.

The pores in the nano-meter scales have been identified by imaging techniques. For example, it was found by scanning electron microscopy (SEM) that nano meter scales pores were clearly visible after the pretreatment of mixed hardwood sawdust in a condition with deionized water and CO_2 at 200°C, 5 min, and 450 psi (Zhu et al., 2011). Tunnel-like networks of nanoporous structures with sizes from 10 to 1000 nm, as visualized by TEM and 3D-electron tomography, were formed within the cell walls of Ammonia fiber expansion (AFEX) pretreated corn stover. It was suggested that the shape, size, and distribution of the pores depended on their location within the cell wall and pretreatment conditions. Furthermore, it was proposed that this highly porous structure greatly enhanced enzyme accessibility into cellulosic microfibrils (Chundawat et al., 2011b).

It has been widely confirmed that the pore volume is important and many literatures supported that the increase of pore volume lead to the improvement of enzymatic hydrolysis (Grethlein, 1985; Koo et al., 2011; Mooney et al., 1998; Yu et al., 2011). It was found that the specific surface areas and total pore volumes of ozone pretreated pulps of newsprint and magazine paper were increased, which resulted in the improved enzymatic hydrolysis. In

contrast, the surface areas and total pore volumes of kraft pulp and bleached kraft pulp were decreased by ozone treatment, and thus the efficiency of enzymatic hydrolysis was decreased (Kojima & Yoon, 2008). It is also recognized that the drying of the wet samples caused the decrease in the digestibility of cellulose due to the irreversible collapse of the pores (Ioelovich & Morag, 2011). The cell walls become less swollen and less flexible compared to the never-dried state; this phenomenon is called hornification (Park et al., 2006b).

The surface area is one of the most important factors affecting enzymatic hydrolysis. However, the current available methods determine the overall surface area, not the accessible surface area of polysaccharides for enzymes. Therefore, whether the measured surface truly represents the accessible surface area to enzymes is a concern. There are many techniques used to determine the accessible surface area or porosity (Chandra et al., 2008; Koo & Park, 2012). In these methods, some measure the surface area or porosity at dry state, such as BET and mercury porosimetry (Chu & Kimura, 1996; Grethlein, 1985). However, the measurement at dry state does not accurately reflect the true porosity because of the honification. This issue has had considerable awareness in recent years and a few methods of measuring porosity at wet-state have been frequently reported, such as thermoporometry using DSC and solid state NMR, staining (*e.g.* Simons' stain), and size exclusion using probe molecules, such as dextran. (Brunauer et al., 1938; . Simons, 1950; Hong et al., 2007; Hopner et al., 1955; Koo et al., 2011; Park et al., 2006a; Simons, 1950; Yu et al., 1995).

In addition, a quantitative method for determining the accessible surface area of cellulose was established based on the maximum adsorption capacity of non-hydrolytic fluorescence fusion protein, which has a similar size to *Trichoderma* cellulase (Hong et al.,

2007; Zhu et al., 2009). It is necessary to determine the difference in surface area between a conventional measurement of surface area and a specific measurement of accessible surface area of enzymes. For lignocellulosic biomass, how to quantify the accessible surface area of polysaccharides without the interference of lignin is another challenge. Using bovine serum albumin (BSA) to block the lignin surface is one approach to screen out the lignin interference with the accessible surface area of carbohydrates since BSA was considered to not bind on microcrystalline cellulose (Yang & Wyman, 2006; Zhu et al., 2009). However, whether BSA has any interaction with the heterogeneous carbohydrate in a pretreated lignocellulosic biomass needs to be verified. In addition, BSA has the similar molecular weight as CBH, it may also be entrapped in the porous structures other than adsorbed on substrate surface.

Coupling effect of various factors

It should be noted that the interactions between the characteristics of biomass, such as lignin, crystalline structure, degree of polymerization, accessible surface area, and particle size, are complex. During the pretreatment, the change in one property is accompanied by changes in other properties. For example, the decrease of lignin content resulted in the increase of accessible pore volume (Yu et al., 2011). The reduction of particle size of microcrystalline cotton cellulose from under 90 μm to submicron scale by media milling also resulted in a significant decrease in crystallinity (Yeh et al., 2010). The removal of hemicellulose in alkaline media leads to the removal of lignin and an increase in porosity. These are all favorable factors for enhancing enzymatic hydrolysis efficiency (Yoshida et al.,

2008). Therefore, it is quite challenging to assess the effect of an individual factor on enzymatic hydrolysis since more than one change usually occurs during pretreatment.

Besides, there is still considerable disagreement in literature regarding the relative importance of each of these factors (Arantes & Saddler, 2010). For lignocellulosic biomass, lignin content and CrI are considered to have the greatest impact on enzymatic hydrolysis (Chang & Holtzapple, 2000). Chang and Holtzapple perceived that the low crystallinity is a more favorable condition for enzymatic hydrolysis regardless of lignin content (Chang & Holtzapple, 2000). However, a study using corn stover showed that the hydrolysis initial rate is most influenced by the cellulose crystallinity, while lignin content mostly governed the extent of hydrolysis at 72 h (Laureano-Perez et al., 2005). It is also rendered that increasing cellulose accessibility was a more important pretreatment consideration than delignification for effectively releasing sugars from lignocellulose at high yield. Extensive delignification without a significant increase in cellulose accessibility did not result in a correspondingly large increase in glucan digestibility (Rollin et al., 2011).

Study of the coupling effect of multi-factors requires future research to determine the relative importance of each individual factor. A pretreatment having moderate limitation on multi-factors, which can achieve an economically feasible hydrolysis yield, will likely be the ultimate goal for establishing a biofuel plant in a commercial scale. Although it is difficult to include all the factors in one model, for researchers to gain a comprehensive understanding of the factors affecting enzymatic hydrolysis, it is worth the effort to continue to develop more robust pretreatment or subsequent treatment strategies to overcome the recalcitrance of lignocellulosic biomass.

Future prospects

The research of biofuel converted from lignocellulosic biomass is a visionary strategic reserve, which possesses an immense value for a worldwide energy security, environmental improvement, and sustainability of human beings. The progress in biofuel research and production needs the intelligent collaboration of experts from all of the related areas, such as pretreatment, enzymatic hydrolysis, and fermentation, as well as the committed support from the government and the enterprises.

From the fundamental prospective of biofuel research, the heterogeneity of insoluble lignocellulosic biomass, which is highly resistant to enzymatic hydrolysis, is a key that limits the production yield of biofuel. There is still much to be studied about the limiting mechanism of enzymatic hydrolysis by lignocellulosic biomass. For example, lignin, one of the most elusive components in lignocellulosic biomass, needs a lot of further examination to unravel its role on enzymatic hydrolysis and how to overcome its negative impact on enzymatic hydrolysis in a less costly way. Besides, rather than study one or two biomass related factors that affect enzymatic hydrolysis, the coupling effects of various factors need to be viewed as the overall picture of the substrate characteristics. The importance of individual factor may vary depending on the type of substrate, the way of pretreatment, and the features of applied enzymes. A systematic study of all these possible factors that affect the saccharification of lignocellulosic biomass will be of great value to improve our understanding of enzymatic hydrolysis. Although progress has been made towards determining the characteristics of the lignocellulosic biomass that affect enzymatic hydrolysis, method development is also important for clarifying some key issues in current

state, such as lignin interference for the measurement of cellulose crystallinity, lignin interference with the measurement of accessible surface area, and the overall lignin structure in the pretreated biomass, that might create confusion or misleading on our current understanding of biomass recalcitrance.

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CHAPTER 2

The Effect of Delignification of Forest Biomass on Enzymatic Hydrolysis

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Abstract

The effect of delignification methods on enzymatic hydrolysis of forest biomass was investigated using softwood and hardwood that were pretreated at an alkaline condition followed by sodium chlorite or ozone delignification. Both delignifications improved enzymatic hydrolysis especially for softwood, while pretreatment alone was found effective for hardwood. High enzymatic conversion was achieved by sodium chlorite delignification when the lignin content was reduced to 15%, which is corresponding to 0.30-0.35 g/g accessible pore volume, and further delignification showed a marginal effect. Sample crystallinity index increased with lignin removal, but it did not show a correlation with the overall carbohydrate conversion of enzymatic hydrolysis.

Keywords: Lignin, dignification, enzymatic hydrolysis, accessible pore volume, crystallinity index

Introduction

The development of second generation biofuels has been receiving a lot of attention due to the limitations of using corn for producing ethanol in the US. Alternative and non-edible

feedstocks, including waste non-food crops and lignocellulosic biomass, have been used to substitute food crop feedstocks, which were used in first generation bioethanol (Luque et al., 2008). However, technologies for developing second generation biofuels encounter greater challenges due to their lower sugar recoveries.

Enzymatic hydrolysis is one of the critical processes for converting lignocellulosic biomass to bioethanol. Considerable research has been done to investigate the characteristics of biomass affecting the efficiency of enzymatic hydrolysis. Several factors are thought to have a significant impact on enzymatic hydrolysis, such as lignin content, lignin distribution and its structure, accessible surface area and pore volume, fiber dimensions, degree of polymerization, crystallinity, and more. Concerning lignin related issue; a few factors have been suggested to be important such as lignin content, the linkage between lignin and carbohydrates, and the hydrophobic interaction between lignin and enzyme (Berlin et al., 2006; Berlin et al., 2005; Chang & Holtzapple, 2000; Lu et al., 2002; Mooney et al., 1998). Some studies also investigated the effect of pore volume on enzymatic hydrolysis. A strong relationship between the biomass substrate pore volume and the enzymatic digestibility of lignocelluloses had been reported (Grethlein, 1985). However, a subsequent loss in accessibility resulted from structure collapse, and further lignin redistribution lead to a corresponding loss in digestibility (Wong et al., 1988). Cellulose crystallinity of biomass is believed to affect enzymatic hydrolysis as well. A good correlation between crystallinity and effect of hydrolysis of relatively pure cellulose has been reported (Yoshida et al., 2008). Recently, Hall and his coworkers found that crystallinity had a linear correlation with the initial rate of enzymatic hydrolysis of pure cellulose (Hall et al., 2010). However, this

relationship is not the same as lignocellulosic biomass, due to its more complicated structure and chemistry.

A substantial research related to improving enzymatic hydrolysis through a specific delignification has been studied. For example, ozone delignification (García-Cubero et al., 2009), sodium chlorite delignification (Hubbell & Ragauskas, 2010), ionic liquid-mediated extraction (Lee et al., 2009), and oxygen delignification (Koo et al., 2011) have been studied to reduce the lignin content in lignocellulosic biomass. Some pretreatments were also found to be effective in decreasing lignin content, such as lime pretreatment (Fuentes et al., 2010; García-Cubero et al., 2009), alkali or ammonia pretreatment (Li & Kim, 2011; Wu et al., 2011), ball milling along with steam explosion (Asada et al., 2011) and organosolv pretreatment (Romaní et al., 2011). However, few works have been reported to study the effect of different delignification methods and the level of lignin removal on enzymatic hydrolysis of forest biomass.

In this study, the effect of sodium chlorite delignification and ozone delignification on enzymatic hydrolysis of wood was investigated. Loblolly pine and mixed southern hardwood chips were pretreated by alkaline green liquor (GL), which is a mixture of sodium carbonate and sodium sulfide, followed by sodium chlorite delignification and ozone delignification, respectively, before enzymatic hydrolysis. Green liquor can be generated from black liquor after multiple evaporators and combustion in a recovery boiler after kraft pulping. It has been demonstrated that the pulps produced by GL pretreatment can be enzymatically hydrolyzed to monosaccharides with a high overall sugar recovery (Jin et al., 2010; Wu et al., 2010). The objective of this study was to determine how different methods and extents of

delignification affect the enzymatic hydrolysis of wood, and to identify the changes of accessible pore volume and sample crystallinity index by delignification methods and their impact on enzymatic hydrolysis.

Materials and Methods

Materials

Fresh loblolly pine and mixed southern hardwood chips (mainly poplar and maple) were obtained from a mill in United States. They were stored at 4°C until used. Uniform sizes of wood chips were prepared by a screening sieve with a 9.5 mm length and 25.4 mm width grid mesh.

Three enzymes, NS50013 (cellulase), NS50044 (hemicellulose) and NS50010 (β-glucosidase), were kindly provided by Novozymes North America, Inc. (Novozymes, Franklinton, NC). Sodium carbonate, sodium sulfide, sodium chlorite, and glacial acetic acid were purchased from Fisher Scientific (Fisher Scientific, Suwanee, GA). Avicel PH-101 was purchased from Sigma-Aldrich (Sigma, St. Louis, MO).

Green liquor pretreatment

Green liquor (GL) solution was prepared by mixing sodium carbonate and sodium sulfide with a sulfidity of 25%. The Total Titratable Alkali (TTA) charge as sodium oxide on oven dried (od) wood chips was 12%. The TTA charge takes into account both the sodium carbonate and the sodium sulfide.

GL pretreatment was carried out in an M/K bath digester (M/K system Inc., Danvers, Massachusetts). The ratio of pretreatment liquor to wood chips (od) was 4 (v/w). The GL pretreatment conditions for softwood and mixed hardwood were an H-factor of 800 at 170°C and H-factor of 400 at 160 °C, respectively. At the end of pretreatment, the solids were collected and washed with tap water overnight to completely remove residual chemicals and dissolved wood components. Then, the pretreated wood chips were refined to pulp at an atmospheric condition, using a Bauer 148-2 disk refiner twice with a disk gap of 0.25 mm and 0.05 mm, followed by fluffy treatment.

Delignification treatment

Sodium chlorite and ozone delignification were conducted on GL pretreated pulps. Sodium chlorite delignification was carried out in an 80 °C water bath for 15 min, except for the completely delignified samples (120min). The sodium chlorite solution was prepared by adding sodium chlorite with acetic acid (w/v=3:1) to 600 ml deionzed water and mixed with 15 g (od) pulp. The amount of sodium chlorite was 3.75, 7.50, 15.0, and 22.5g, respectively. The completely delignified sample was prepared by adding 22.5 g sodium chlorite four times every 30min with a fixed amount of acetic acid of 7.5 g. At the end, the pulps were washed with tap water until washings were colorless, then rinsed again with deionized water for 3 times, and air-dried until the consistency was about 30%.

High-consistency ozone delignification occurred in a gaseous phase reactor, in which a mixture of ozone and oxygen and pulps with about 30% consistency were in contact. The pH of the conditioning solution was adjusted with appropriate buffer systems, such as acetic

acid-sodium acetate, for a pH of 2. Ozone was generated from an ozone generator by passing bulk oxygen through 35kV voltage. GL pulps of 30 g (od) were used for reaction, when the ozone-oxygen stream was stabilized at 80 mg/L ozone with a flow rate of 1L/min. The reaction time was 15 or 30 min with conditioned pH (pH=2) or no conditioned pH (neutral).

Characterization of GL and delignified pulps

Klason lignin (KL) and acid soluble lignin (ASL) were measured by two-stage sulfuric acid hydrolysis. The sample of 0.1 (od) g was swollen in 1.5 ml of 72% H_2SO_4 at room temperature for 2 h, and then the acid was diluted to 3% and heated in an autoclave at 120°C for 1.5 h. After cooling, the solution was filtered with a filtering crucible, and the residue was washed, dried, and weighed as KL. The filtrate was diluted and used for ASL analysis. ASL was determined by absorbance at 205 nm in an HP 8453E UV-VIS spectrometer.

The accessible pore volume, interpreted by the accumulation of freezing bound water, was analyzed by differential scanning calorimetry (DSC) Q100 (TA Instruments, New Castle, DE) in this study. The phenomenon that water in small pores is frozen at lower temperature is called freezing point depression. The depressed freezing temperature has a reciprocal relationship with pore diameters. Therefore, the accessible pore volume can be determined by calculating the accumulation of freezing bound water in different diameters of pores, based on the Gibbs-Thomson equation (Park et al., 2006). In this experiment, the wet pulps before and after delignification were sealed in a hermetic pan and frozen under -70 °C, maintained for 5 min. The temperature was then raised to -49.5 °C. The first segment (-70 to -49.5 °C) was used to determine the sensible heat of the wet fibers, assuming that there was no melting.

The following heating steps to higher temperature (-39.6, -26.4, -19.8, -12.8, -8.8, -5.7, -4.0, -2.8, -2.0, -1.4, -0.9, -0.7, -0.5, -0.3, -0.2 °C) were conducted. In each step, the temperature was increased at 1 °C/min to the target temperature and maintained isothermally until the heat flow returned to the baseline. A typical DSC diagram is shown in Figure 2.1. The integration of each heat-adsorbing segment was used for freezing bound water calculation. The freezing bound water in a pore size of 7 nm was selected, based on the size of enzyme molecule and calculated up to 180 nm (Wong et al., 1988).

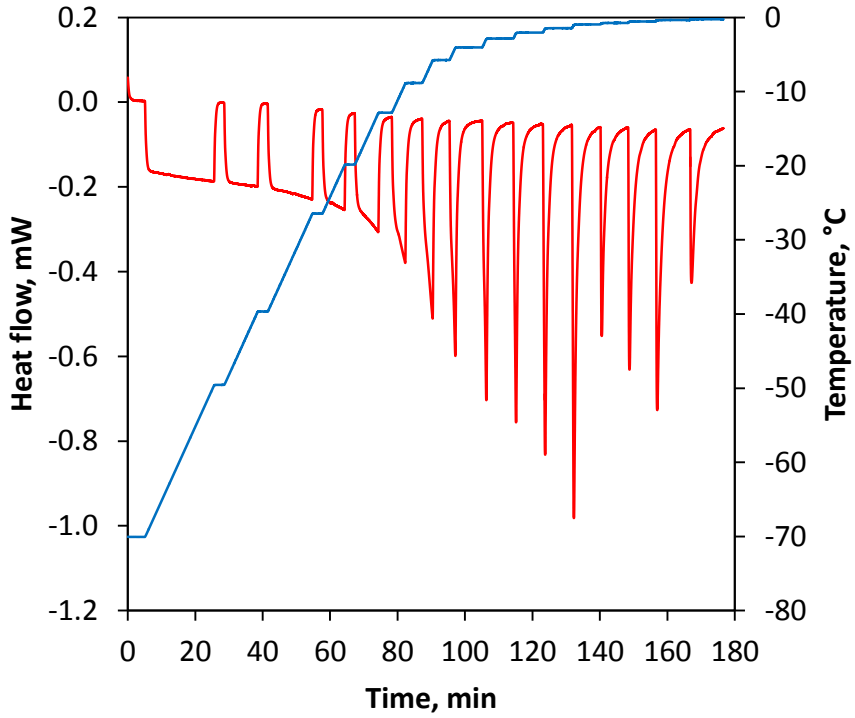

Figure 2.1. DSC diagram of HW-chl-E pulp for freezing bound water analysis.

The microscope images of the biomass samples were taken by a JSM-6400F scanning electron microscope (SEM). The biomass was pre-coated with a thin layer, approximately 10 nm, of 60 Au/40 Pd to enhance the contrast of the images.

Sample crystallinity was determined by X-ray diffraction (XRD). XRD experiments were carried out on freeze dried pulps using a Philips XLF ATPS X-ray diffraction 1000 with OMNI Instruments Inc. customized automount and Cu tube. The radiation was generated at 25 mA and 35 kV. The Bragg angle 2θ was from 10 to 40 with 0.05 step size and 5 seconds retention time in each step. The sample crystallinity index was calculated by Segal's method (Segal et al., 1959).

Enzymatic hydrolysis and sugar analysis

Enzymatic hydrolysis of delignified pulps was performed in 100 mM acetate buffer (pH 4.8) with 5% consistency. The mixture was incubated at 50°C for 48 hours with 180 rpm shaking. The enzymes were a mixture of cellulase, xylanase, and β-glucosidase with an activity ratio of 1 FPU: 1.2 FXU: 1CBU. Different enzymes loadings of 5, 10 and 20 FPU/g of dry mass were conducted, respectively. The sugar contents in acid hydrolysates and enzymatic hydrolysates were analyzed by HPLC (Dionex, Sunnyvale, California) to detect glucose, xylose, galactose, mannose, and arabinose. The column system of HPLC consisted of a Shodex SP0810 column and a de-ashing cartridge pre-column. The temperature was 80°C, the eluent was H_2O, and the flow rate was 0.4 ml/min. The chemical compositions of original wood chips and GL pulps are shown in Table 2.1.

Results and Discussion

Lignin content of delignified pulps

The chemical compositions of the pulps were determined before and after GL and delignification treatments. As shown in Table 2.1, the total lignin content of fresh SW chips was 29.2%. Fresh HW chips had a higher ASL content of 4.0%, but the total lignin content (26.7%) was lower than SW. After GL pretreatment, the total lignin contents were 22.4% (23% lignin removal) in SW and 19.0% (28.8% lignin removal) in HW pulps, indicating some degree of delignification during GL pretreatment. It is noted that all glucan and significant portion of xylan were retained during GL pretreatment, which proves this pretreatment attractive for subsequent enzymatic hydrolysis process.

Table 2.1. Chemical composition of SW (loblolly pine), HW (mixed southern hardwood), SW-GL pulp and HW-GL pulp. All the numbers are based on the initial (od) weight of analyzed sample.

	Yield (%)	Carbohydrate (%)					Lignin (%)			Mass Balance (%)
		Glucan	Xylan	Galactan	Man & Ara	Sum	KL	ASL	Sum	
SW	-	41.2	5.2	2.3	13.5	62.2	28.5	0.7	29.2	91.4
HW	-	47.8	16.3	0	2.4	66.5	22.7	4.0	26.7	93.2
SW-GL pulp	78.5	52.2	6.1	1.3	7.0	66.6	29.8	0.4	30.2	96.8
HW-GL pulp	79.0	54.2	15.1	0	0.8	70.1	22.0	2.6	24.6	94.7

Table 2.2. Lignin and carbohydrate contents of sodium chlorite delignified pulps. The term chl indicated sodium chlorite delignification. SW-GL and HW-GL were pulps prepared by GL pretreatment without delignification. % is based on the (od) weight of pulp.

	Sodium chlorite (g/15 g pulp)	KL (wt %)	ASL (wt %)	Total lignin (wt %)	Total carbohydrates (wt %)
SW-GL	0	29.8	0.4	30.2	69.8
SW-chl-A	3.75	24.2	1.4	25.6	69.3
SW-chl-B	7.5	15.5	2.5	18.0	71.0
SW-chl-C	15	5.7	2.6	8.3	84.0
SW-chl-D	22.5	4.2	2.4	6.6	86.7
SW-chl-E	22.5×4	0	1.4	1.4	92.8
HW-GL	0	22.0	2.6	24.6	76.0
HW-chl-A	3.75	13.8	4.2	18.0	75.1
HW-chl-B	7.5	8.5	4.2	12.7	80.5
HW-chl-C	15	5.5	4.1	9.6	83.8
HW-chl-D	22.5	1.6	3.9	5.5	85.0
HW-chl-E	22.5×4	0	1.4	1.4	96.5

The delignification followed by GL pretreatment changed the lignin content significantly. The decreases in lignin content in pulps after chlorite and ozone delignification are shown in Tables 2.2 and 2.3. In the case of sodium chlorite delignification, the KL lignin contents of SW pulps were reduced from 29.8% to 0%, while the KL lignin contents of HW pulps were decreased from 22.0% to 0%. By contrast, less KL was removed by ozone delignification, in which the lignin contents were decreased from 29.8% to 11.3% in SW and 22.0% to 4.8% in HW pulps. The ASL of HW is generally higher than that of SW after delignification. It was noted that for the pulps treated by ozone delignification with the same

reaction time, there was lower KL in pulp at pH 2 than that at neutral pH. This is due to the fact that the decomposition of ozone in water is favored by hydroxyl ions, and the decomposition is increased at pH values above 4. Thus, the best ozone delignification could be achieved at pH 2~3 (Pan et al., 1984). With the removal of lignin, the carbohydrate content in the delignified pulps generally increased.

Table 2.3. Lignin and carbohydrate contents of ozone delignified pulps. The term oz indicated ozone delignification. SW-GL and HW-GL were prepared by GL pretreatment without delignification. % is based on the (od) weight of pulp.

Samples	Time (min)	pH	KL (wt %)	ASL (wt %)	Total lignin (wt %)	Total carbohydrates (wt %)
SW-GL	0	-	29.8	0.4	30.2	69.8
SW-oz-A	15	7	20.3	1.4	21.7	68.0
SW-oz-B	15	2	17.5	1.6	19.1	71.6
SW-oz-C	30	7	13.1	1.6	14.7	76.5
SW-oz-D	30	2	11.3	1.5	12.8	83.8
HW-GL	0	-	22.0	2.6	24.6	76.0
HW-oz-A	15	7	11.1	2.9	14.0	75.5
HW-oz-B	15	2	9.0	2.7	11.7	77.0
HW-oz-C	30	7	6.2	2.4	8.6	78.6
HW-oz-D	30	2	4.8	2.0	6.8	82.1

Effect of lignin removal on enzymatic hydrolysis

Increased enzymatic hydrolysis of wood biomass was observed with the increased lignin removal. With the enzyme loading of 5, 10, and 20 FPU/g substrate, the 48 h carbohydrate conversion of sodium chlorite delignified SW improved from 13% to 65%, 25% to 84%, and 42% to 88%, respectively, as shown in Figure 2.2 (SW-chl). The improvement in enzymatic hydrolysis of sodium chlorite delignified HW was not remarkable, showing improvements of 43% to 63%, 62% to 88%, and 80% to 89% at 5, 10, and 20 FPU/g substrate, as shown in Figure 2.2 (HW-chl). However, it is noted that green liquor pretreatment alone is an effective pretreatment for HW, especially at high enzyme loadings. The 48 h carbohydrate conversion with 10 FPU/g substrate and 20 FPU/g substrate enzyme loading obtained 62.3% and 81.0%, respectively. Compared to sodium chlorite delignification, ozone delignification was not very effective for SW. The 48 h carbohydrate conversion was 20-30% lower than the sodium chlorite delignified pulps. But ozone delignification for HW showed a comparable effect as the sodium chlorite delignification, which again indicated the effectiveness of GL pretreatment. Generally, the improvement in enzymatic hydrolysis of HW due to the delignification was marginal, especially at a high enzyme loading (20 FPU/g substrate). It is because GL pretreatment produce highly digestible substrates from HW, but not for SW. Therefore, delignification treatment was found much more effective for improving enzymatic hydrolysis of SW than that of HW. However, it was also noted that when the lignin content of sodium chlorite delignified pulps was less than 15%, enzymatic hydrolysis nearly leveled off, even though the lignin was further removed at each enzyme loading level.

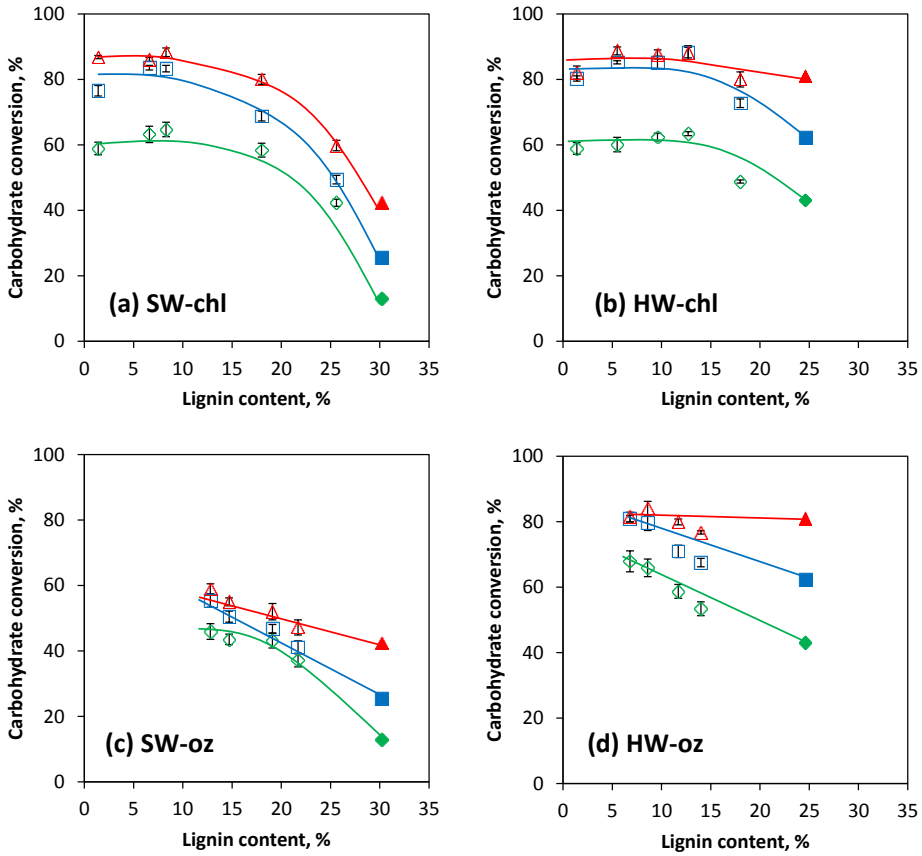

Figure 2.2. Effect of delignification on enzymatic hydrolysis. (◇) 5 FPU/g, (□) 10 FPU/g, (△) 20 FPU/g, (◆) GL pulp w/o delignification 5 FPU/g, (■) GL pulp w/o delignification 10 FPU/g, (▲) GL pulp w/o delignification 20 FPU/g.

When comparing the enzymatic hydrolysis yields to the same lignin basis, sodium chlorite delignification was more effective than ozone delignification. As shown in Figure 2.2 and Tables 2.2 and 2.3, when the lignin content of ozone delignified SW was reduced to 12.8% (SW-oz-D), with 20 FPU/g substrate, the carbohydrate conversion was only 58%. However, when the lignin content of sodium chlorite delignified SW was decreased to 18.0% (SW-chl-B), the carbohydrate conversion was raised to 80%. Moreover, ozone delignification

had very little benefit for the improvement of enzymatic hydrolysis of HW with 20 FPU/g substrate. Although the lignin content of HW was decreased from 25% (HW-GL pulp) to 7% (HW-oz-D), the carbohydrate conversion was about the same. The reason for the difference in enzymatic hydrolysis efficiency between sodium chlorite delignification and ozone delignification was probably due to the different delignification mechanism. It was speculated that because sodium chlorite delignification was carried out in liquid phase, while ozone delignification was conducted in gaseous phase, the lignin removal by ozone delignification was not as uniform as that in sodium chlorite delignification. Ozone delignification is a solid-liquid-gas phase reaction. The gas has to dissolve in the liquid and then diffuse into the reaction sites. However, ozone is so reactive that it might mostly react on the surface of the pulp before penetrating into the inner structure. It is likely that the lignin content on the surface of ozone delignified pulps was less than that inside the pulps. Thus, the difference in lignin distribution between sodium chlorite delignified pulps and ozone delignified pulps may contribute to the different digestibility in enzymatic hydrolysis.

Lignin is one of the major barriers for enzymatic hydrolysis, and the removal of lignin usually improves enzymatic hydrolysis (Chang & Holtzapple, 2000; Mooney et al., 1998). Our results are consistent. The concerns about the lignin barrier for enzymatic hydrolysis are typically from three aspects: lignin blocks the accessibility of enzymes, lignin non-productively adsorbs enzymes, and lignin-carbohydrate complex probably limits enzymatic hydrolysis. Cellulose is embedded in lignin, while lignin acts as a physical barrier to protect cellulose against microbial or chemical degradation. Lignin also has the capability of binding to enzymes, and without delignification, up to 60-70% of the total added enzymes can be

bound to lignin after complete hydrolysis of the cellulose fraction in lignocelluloses (Jørgensen et al., 2007). Therefore, the removal of lignin not only exposes more accessible cellulose, but also reduces the strong surface interaction between lignin and enzyme. Besides, lignin removal has been reported to improve cellulose accessibility effectively by creating pores and by breaking the lignin-carbohydrate complex (Mooney et al., 1998; Yang & Wyman, 2004).

It is well known that SW is more recalcitrant than HW, and the higher lignin content of softwood is always a concern. In this study, it was noted that SW had much lower carbohydrate conversion than that of HW even at the similar lignin contents; for example, under 20 FPU/g substrate enzyme loading, the carbohydrate conversion was 59.8% for SW-chl-A (25.6% lignin) vs. 81.0% for HW GL pulp (24.6% lignin) and 58.9% for SW-oz-D (12.8% lignin) vs. 79.9% for HW-oz-B (11.7% lignin). These observations indicated that the lower digestibility of wood cannot be simply explained by higher lignin content. Lignin structure and distribution should be considered to better understand the recalcitrant nature of biomass. For example, softwood lignin contains only guaiacyl units, while hardwood lignin contains mixed guaiacyl and syringyl units, and it was reported that guaiacyl lignin restricts fiber swelling and enzymatic accessibility more than syringyl lignin (Ramos et al., 1992). It is reported that the carbohydrate conversion was generally higher with a higher syrigyl/guaiacyl (S/G) ratio up to two (Studer et al., 2011). Besides, lignin carbohydrate complex might be another factor for the recalcitrant nature of softwood, and further study is needed (Berlin et al., 2006).

Changes of accessible pore volume by delignifications and its effect on enzymatic hydrolysis

There are many methods for determining surface area and pore volume such as gas permeability, mercury intrusion, and scanning electron microscopy (Chinga & Helle, 2002; Gharpuray et al., 1983). These techniques require dried samples, which suffered from hornification effect, resulting in irreversible changes in pore structure (Maloney & Paulapuro, 1999). There are also a few techniques to test pore volume distribution using never-dried samples. Solute exclusion technique with a series of different size molecular probes can be used to calculate the distribution of accessible pores (Grethlein, 1985). However, pores with very small entrances are not detectable and this test is rather laborious. Based on these considerations, the accessible pore volume of samples was determined in a wet-state by measuring the amount of cumulative freezing bound water using DSC in this study, which is sensitive enough to probe small pores (Park et al., 2006).

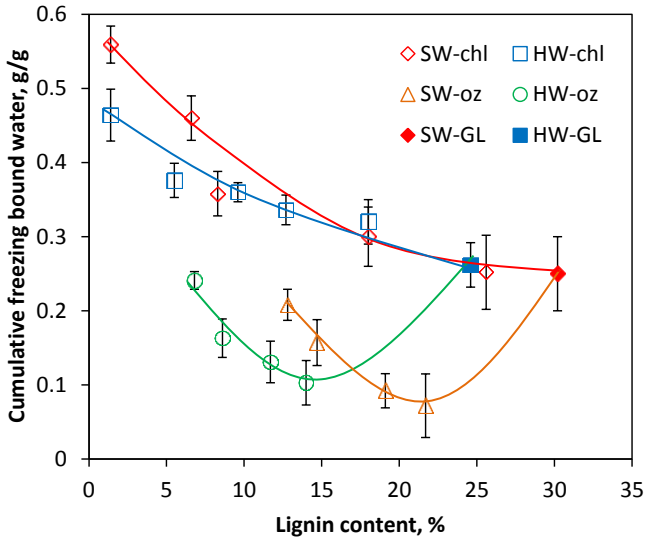

Figure 2.3. Effect of delignification on accessible pore volume of pulps.

Lignin removal in sodium chlorite delignification provided increasing vacancy which increased the accessible pore volume, as shown in Figure 2.3. The cumulative freezing bound water was in the range of 0.25-0.56 g/g in SW and 0.26-0.46 g/g in HW. In this study, it was found that in the case of sodium chlorite delignification, enzymatic hydrolysis was improved with the increasing of accessible pore volume to a certain level. Once the carbohydrate conversion was over 80%, corresponding to 15% lignin content and 0.30-0.35 g/g cumulative freezing bound water, the increase of accessible pore volume did not further improved enzymatic hydrolysis, as shown in Figures 2.3 and 2.4.

These results suggested that there might be some interaction between the effect of pore volume and delignification on enzymatic hydrolysis. It seems that an accessible pore volume between 0.30 to 0.35 g/g, which corresponds to about a 15% lignin content, sets a limit for the improvement of enzymatic hydrolysis. Once lignin content was decreased to less than 15%, the increase of accessible pore volume made no difference for enzymatic hydrolysis. This observation indicated that accessible pore volume at high lignin content is an important factor for enzymatic hydrolysis. Since a substantial fraction of lignin had been removed during delignification, the changes in biomass physical structure were expected. Six samples were examined by SEM including GL pretreated pulps and delignified pulps for both SW and HW (Figure 2.5). The change of the surface structure of the pulp by sodium chlorite delignification was visualized. Some wave drape and fragmented structure was observed on the surface of sodium chlorite delignified pulp, which might be an indication for the vacancy generated through lignin removal. The existence of vacancy and some pore structure on the surface of the pulp was observed.

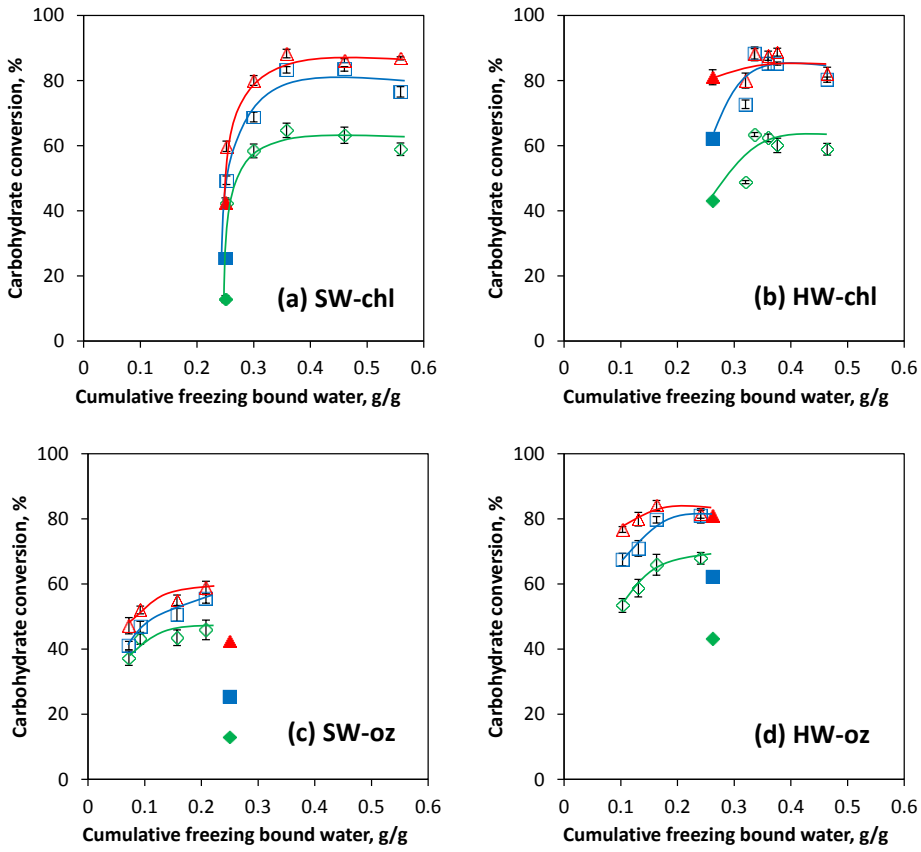

Figure 2.4. Effect of accessible pore volume on enzymatic hydrolysis. (a) Sodium chlorite delignified SW pulp, (b) sodium chlorite delignified HW pulp, (c) ozone delignified SW pulp and (d) ozone delignified HW pulp. (◇) 5 FPU/g substrate enzyme loading, (□) 10 FPU/g substrate enzyme loading and (△) 20 FPU/g substrate enzyme loading.(◆) GL pulp without delignification with 5 FPU/g substrate enzyme loading, (■) GL pulp without delignification with 10 FPU/g substrate enzyme loading and (▲) GL pulp without delignification with 20 FPU/g substrate enzyme loading. Carbohydrate conversion is defined as the amount of hydrolyzed carbohydrate in the enzymatic hydrolysate as a percent of total carbohydrate in the starting substrate for enzymatic hydrolysis.

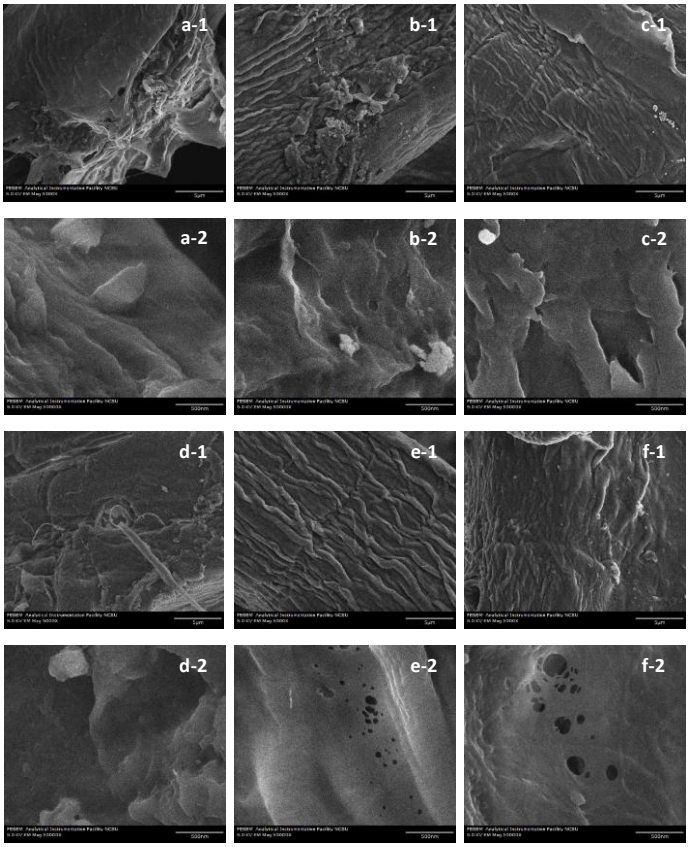

Figure 2.5. SEM images of SW and HW with GL pretreatment alone, GL pretreatment followed by ozone delignification, and GL pretreatment followed by sodium chlorite delignification. The scale bar for the 5,000×magnification is 5 μm, and the scale bar for 50,000× magnification is 500 nm.

The name and magnification factor of each sample is shown below:

a-1. SW-GL (5,000×)	b-1. SW-oz-A (5,000×)	c-1. SW-chl-A (5,000×)
a-2. SW-GL (50,000×)	b-2. SW-oz-A (50,000×)	c-2. SW-chl-A (50,000×)
d-1. HW-GL (5,000×)	e-1. HW-oz-A (5,000×)	f-1. HW-chl-A (5,000×)
d-2. HW-GL (50,000×)	e-2. HW-oz-A (50,000×)	f-2. HW-chl-A (50,000×)

In the case of ozone delignification, it is interesting to note that the accessible pore volume was decreased when the ozone delignification was conducted to the first 20~40% lignin removal. With the further delignification, the accessible pore volume gradually increased, but was still smaller than that of the samples without ozone delignification. The cumulative freezing bound water of SW and HW with ozone delignification was in the range of 0.07 to 0.25 g/g and 0.1 to 0.26 g/g (Figure 2.3). The initial decrease of pore volume and smaller accessible pore volume by ozone delignification might be an indication of structural collapse of fibers. It is likely that ozone rapidly reacted with most of the lignin on the surface of pulps at the initial stage of delignification. As a result, the structure of the surface of the pulps might collapse and the accessible pore volume decreased initially. As more and more ozone penetrated into the pulp with the process of delignification, the increase of the accessible pore volume was not very significant and the total accessible pore volume was still smaller than that in the initial state. The enzymatic hydrolysis of ozone delignified pulps was improved with the increased accessible pore volume, but the undelignified GL pulp was not included in the trend as shown in Figure 2.4. The accessible pore volume of ozone delignified pulp is smaller than sodium chlorite delignified pulp, and none of them were greater than 0.30 g/g. When the lignin content of ozone delignified pulps was decreased to less than 15%, the level-off trend of enzymatic hydrolysis was not observed as that in the case of sodium chlorite delignification. Therefore, whether the conditions of 0.3 to 0.35 g/g accessible pore volume corresponding to 15% lignin content in the substrate are the limiting conditions for the improvement of enzymatic hydrolysis could not be ascertained based on current experimental data. Either some additional data analysis or experiments required to explore

this issue. Some morphological changes of ozone delignified pulps were detected by SEM (Figure 2.5). A clear wave drape morphology was observed for SW-oz-A and HW-oz-A samples. It seems that the surface of the initial ozone delignified pulp has been twisted shrunk severely, which supported the results obtained by DSC that the surface of the pulps might be collapsed in the initial stage of ozone delignification.

Changes of crystallinity index by delignification and its effect on enzymatic hydrolysis

Lignin removal also had an influence on the crystallinity index of woody biomass. It was found that crystallinity index increased with the decreased lignin content, regardless of the origin of samples in Figure 2.6. The increased sample crystallinity index measured by Segal's method was mainly due to the decreased amorphous components because lignin and hemicellulose are amorphous materials (Kim et al., 2003). A method for calculating cellulose crystallinity index based on the quotient of sample crystallinity to cellulose content had been developed by Thygesen and his coworkers (Thygesen et al., 2005). However, this method assumed that the signal contribution between cellulose and non-cellulose constituents has to be linear, which was not justified. A recent progress in analyzing the cellulose crystallinity of lignocelluloses had been achieved by using Sum-Frequency-Generation (SFG) vibration spectroscopy (Barnette et al., 2011). This method could detect crystalline cellulose selectively in lignocellulose without extraction of non-cellulosic materials from biomass or deconvolution of amorphous spectra, and a quantitative calibration between SFG signals and cellulose crystallinity in biomass is not established yet. Based on the experimental results in

this study, it is not clear whether crystallinity index provides a clear indication for the digestibility of lignocellulosic biomass.

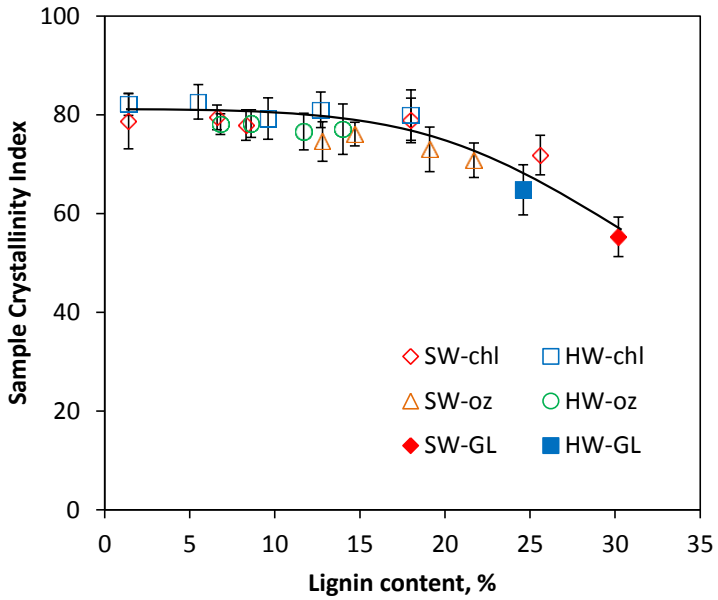

Figure 2.6. Effect of lignin removal on sample crystallinity index.

Conclusions

Enzymatic hydrolysis was enhanced with the decreased lignin content and increased accessible pore volume, especially for softwood and for sodium chlorite delignified pulp. The initial decrease in accessible pore volume for ozone delignified pulp could be due to the collapse of biomass structure. A certain interaction between the effect of accessible pore volume (0.30 to 0.35g/g) and delignification (15% lignin content) on the limitation of enzymatic hydrolysis has been observed in this study. Delignification increased sample

crystallinity index due to the removal of amorphous materials, but no correlation was identified with enzymatic hydrolysis.

Acknowledgements

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CHAPTER 3

The impact of hardwood and softwood lignin on enzymatic hydrolysis

Abstract

The impact of lignin-derived inhibition on enzymatic hydrolysis such as non-productive adsorption and physical barrier were investigated using lignins isolated from raw woods and pretreated wood pulps as milled wood lignins (MWLs). MWLs of 20% were physically mixed with bleached pulps and reconstructed with bleached to assess the impact of non-productive adsorption and physical barrier of lignin. It was found that MWLs obtained from pretreated woods adsorbed 2 to 6 times more cellulase than the MWLs obtained from untreated woods. The higher adsorption of enzymes on lignin agreed with the trend of decreased carbohydrate conversion in enzymatic hydrolysis. One of the characteristics of lignin, the degree of condensation, was found to have a critical impact on cellulase adsorption and enzymatic hydrolysis. The greater degree of condensation of lignin in softwood materials, especially the pretreated softwood substrates, was probably an important reason for the more recalcitrant nature of softwood substrate.

Keywords: lignocellulosic biomass; lignin; hardwood; softwood; milled wood lignin; ^{13}C NMR spectroscopy, enzymatic hydrolysis

Introduction

Enzymatic hydrolysis of lignocellulosic biomass is one of the key steps for sugar production and the subsequent conversion of sugars to chemicals or biofuels. The abundant woody biomasses including both hardwood (HW) and softwood (SW) in North America are good resources for sugar production via enzymatic hydrolysis. Softwood, such as loblolly pine, accounts for 80 % of planted forests in the southeast of America (Nordlie, 2011). However, it has been widely observed that softwood is more recalcitrant in terms of its enzymatic digestibility as compared to hardwood (Taherzadeh & Karimi, 2008; Xue et al., 2012; Yu et al., 2011). The reasons for this difference are usually thought due to the larger fiber dimensions and higher lignin content in SW (Arantes & Saddler, 2011; Smook, 2002). HW is normally 1 mm in length and 20-40 μm in width, while SW is 3 mm in length and 30-50 μm in width (Smook, 2002). Therefore, the larger size of softwood is like to be more resistant to enzymatic hydrolysis that of HW. However, the hydrolysis kinetics of fully bleached HW and SW were found to be almost identical (Yu et al., 2012), although the difference in fiber size still exists in BSW and BHW. Furthermore, many researchers found that even though the lignin content of softwood and hardwood substrates could be controlled to be similar by pretreatment, softwood substrates were still usually more resistant to enzymatic hydrolysis than that of hardwood (Yu et al., 2010; Yu et al., 2011). The degree of differences in hydrolysis between hardwood and softwood at the similar lignin level depends on the lignin content and the methods of pretreatment (Yu et al., 2011). For example, it was found that at the similar lignin content of green liquor and delignification treated hardwood and softwood, when the lignin content was beyond 15%, softwood substrate showed up to

40% lower in carbohydrate conversion than hardwood substrate. Ozone delignified hardwood and softwood substrates had more distinctive digestion than that of sodium chlorite delignified substrates (Yu et al., 2011). Therefore, the difference in lignin chemistry and structural in SW and HW substrates is an important reason for their different digestibility.

Lignin-derived inhibition is a major obstacle restricting the enzymatic hydrolysis of lignocellulosic substrate, especially for softwood (Nakagame et al., 2010). It has been suggested that the inhibition effect of lignin on enzymatic hydrolysis can be classified as three categories: 1) Enzymes are non-productively adsorbed on lignin via hydrophobic interaction, electrostatic interactions, and hydrogen bonding interactions. 2) Lignin in lignocellulosic materials acts as a physical barrier to block the accessible surface of carbohydrates. 3) Lignin carbohydrate complex, which is highly resistant to enzymatic hydrolysis, is a part of the reason for the incomplete digestion of lignocellulosic materials (Berlin et al., 2006; Jeffries, 1990; Nakagame et al., 2011; Pan et al., 2005).

However, the studies on lignin-derived inhibition for enzymatic hydrolysis such as physical barrier of lignin and non-productive binding between lignin and enzymes are very limited (Nakagame et al., 2011). There is a great challenge to delineate the coupling factors of non-productive adsorption and physical barrier of lignin. In addition, the pattern of lignin incorporated with the polysaccharides might be another important factor other than lignin content that limits enzymatic hydrolysis. This research is aimed to understand the impact of lignin of untreated and pretreated woody biomasses on enzymatic hydrolysis by studying the role of physical barrier of lignin and the non-productive adsorption of enzyme onto lignin. Milled wood lignin (MWL), a good representative of native lignin in the substrate, was

isolated from various hardwood (HW) and softwood (SW) materials. A unique technique, the preparation of reconstructed lignocellulose with MWLs and bleached pulps, was established to mimic the physical barrier of lignin on enzymatic hydrolysis. The non-productive adsorption and physically barrier of lignin can be evaluated by comparing the hydrolysis of physically mixed lignin and carbohydrate, and hydrolysis of reconstructed lignocellulose, respectively. This research is of significant importance to elucidate the mechanisms of lignin impact on enzymatic hydrolysis.

Materials and Methods

Materials

Eucalyptus *globulus*, red maple, loblolly pine chips, mixed HW chips (including oak, maple, poplar, and sweet gun), bleached hardwood (BHW) and bleached softwood (BSW) pulps were obtained from a mill in the southeast of US. Commercial enzyme preparations of cellulase (NS50013), xylanase (NS50014) and β-glucosidase (NS50010) were kindly provided by Novozymes (Novozymes, Franklinton, NC).

Pretreatments of woody biomasses

Green liquor (GL) pretreatment and auto-hydrolysis pretreatment, as representatives of alkaline pretreatment and acid pretreatment, were conducted on hardwood and softwood chips. Both pretreatments were carried out in an M/K bath digester (M/K system Inc., Danvers, MA).

GL was a mixture of sodium carbonate and sodium sulfide with a sulfidity of 40%. The total titratable alkali (TTA) charge was 16% and 20% for hardwood and softwood, respectively. The ratio of GL liquor to wood chips was 4:1 (v/w). The pretreatment conditions for softwood were H-factor of 800 at 170°C and for hardwood were H-factor of 400 at 160 °C. After cooking, the solids were washed with running tap water overnight to completely remove residual chemicals and dissolved wood components.

In auto-hydrolysis pretreatment, wood chips of 600 oven-dried (od) g were loaded in each batch. The water to solid ratio was 4:1 (v/w). The pretreatment was conducted at 180 °C for 1h. The time required to reach the target temperature was about 45 min. After cooking, the remaining solid residues were washed under running tap water for at least 2 hours and subsequently soaked in water for 8 hours. The resulting residual chips were then centrifuged to achieve uniform moisture content.

After GL and auto-hydrolysis pretreatment, the pretreated chips were refined to pulps using a Bauer 148-2 disk refiner twice with a disk gap of 0.25 mm and 0.05 mm, followed by fluffy treatment to obtain uniform pulps .

Preparation of milled wood lignins

Milled wood lignins (MWLs) of eucalyptus, maple and loblolly pine, green liquor and auto-hydrolysis pretreated hardwood (HW) and softwood (SW) pulps were isolated according to the procedure Figure 3.1.

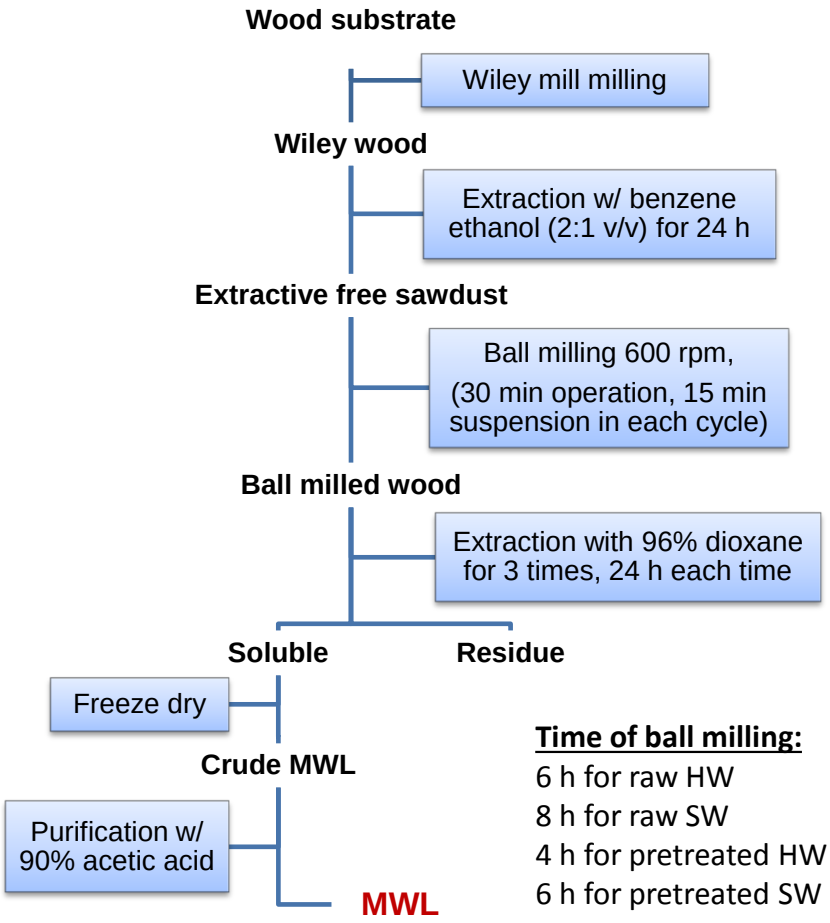


Figure 3.1. Preparation of MWLs from raw woods and pretreated wood pulps.

The chips and pulps were ground to pass a 20-mesh screen and then extracted by 1:2 (v/v) ethanol/benzene for 24 h to remove the extractives. The extractive free sawdust was subjected to ball milling before organosolv extraction. Each 2 od g of sample was milled using 17 zirconium dioxide grinding balls in a 45 ml silicon nitride jar in a planetary micro mill pulverisette 7 classic line (Fritsch, Idar-Oberstein, Germany). The speed was 600 rpm. The grinding time for eucalyptus and maple was 6 h, and for loblolly pine was 10 h. The

pretreated pulps were grinded for a shorter time, which were 4 h for pretreated HW and 6 h for pretreated SW. In every 30 min of grinding, 15 min of suspension time was applied. After ball milling, the ball milled samples were extracted with 96% (v/v) dioxane at 50 °C for 24 h for three times. The solution was collected by centrifugation and concentrated for freeze dry. The crude MWL, isolated by freeze-drying, was dissolved in 90% acetic acid and precipitated into 10 times of water. The precipitated lignin was washed, centrifuged and freeze dried to obtain the purified MWLs.

The total lignin content of all the samples were determined using a modified Klason lignin method derived from the TAPPI standard method T222 pm-88, as previously described (Yu et al., 2011).

NMR analysis

^{13}C spectra were recorded for both non-acetylated and acetylated lignins in order to obtain overall structural information (Figure 3.2). The non-acetylated MWL was the purified MWL. The acetylated MWL was prepared by dissolving 40 mg of MWL in 0.5 ml pyridine and 0.5 ml acetic anhydride and placed in a dark place at room temperature for 2 days to allow a complete acetylation reaction. The acetylated lignin was recovered by rotary evaporation with ethanol for 3 to 5 times to maximally remove the pyridine.

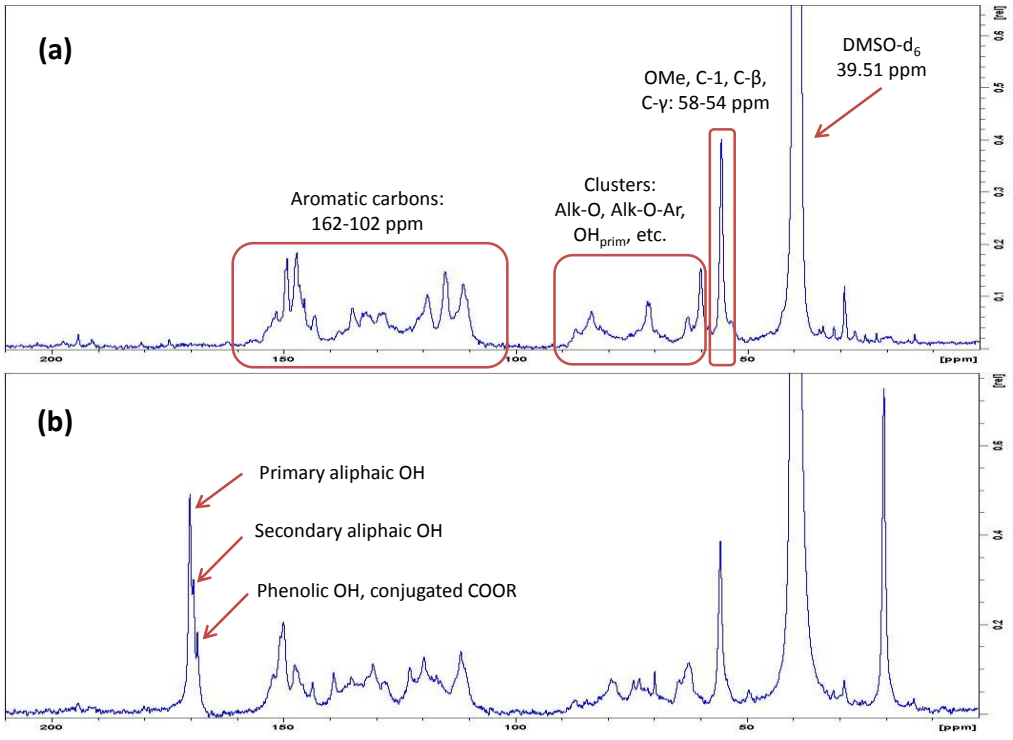


Figure 3.2. ^{13}C NMR spectra of MWL of pine. (a) nonacetylated MWL of pine, (b) acetylated MWL of pine.

MWL sample, either MWL or MWL-Ac, of 40 mg was dissolved in 180 µl DMSO-d6 and 20 µl Chromium (III) acetylacetonate (0.01 M) to obtain a 20% (w/v) lignin concentration. The Chromium (III) acetylacetonate was added as a relaxant to provide complete relaxation of all nuclei, and presence of relaxant was assured to have no impact on the quality of the spectra. The dissolved lignin sample was loaded in Shigemi microtube before being loaded in NMR. The NMR spectra were recorded on a Bruker AVANCE 300 MHz spectrometer at 300 K using DMSO-d6 as the solvent. Chemical shift were referenced to TMS (0.0 ppm). A 90° pulse width, 1.2 s acquisition time, and 1.7 s relaxation delays were used. A total of 25600 scans were collected lasting for about 21 h.

Contact angle analysis

The initial and dynamic water contact angle (WCA) was determined using a Phoenix 300 contact angle analyzer (SEO Co. Ltd, Lathes, South Korea). A multi-layered lignin film was prepared for contact angle analysis. A 0.5 % (w/w) MWL lignin solution was prepared by dissolving MWL in 1,4-dioxane. The lignin was allowed to dissolve for at least 2 days before use. For some of the MWL samples, such as MWL of pine, a small part of the lignin (presumably with very high molecular weight) did not dissolve. Thus all the dissolution was filtered through 0.22 μm nylon syringe filter. A few drops of filtered lignin solution were spread on silicon chips which were pre-coated with polystyrene. The lignin film was spin coated with the following recipe: 400 rpm for 10 s with the acceleration time of 3 s, 500 rpm for 5 s with the acceleration time of 5 s and maximum rotation speed of 1000 rpm for 2 min with the acceleration time of 15 s (Tammelin et al., 2006). The example images of water sphere on lignin films were shown in Figure 3.3.

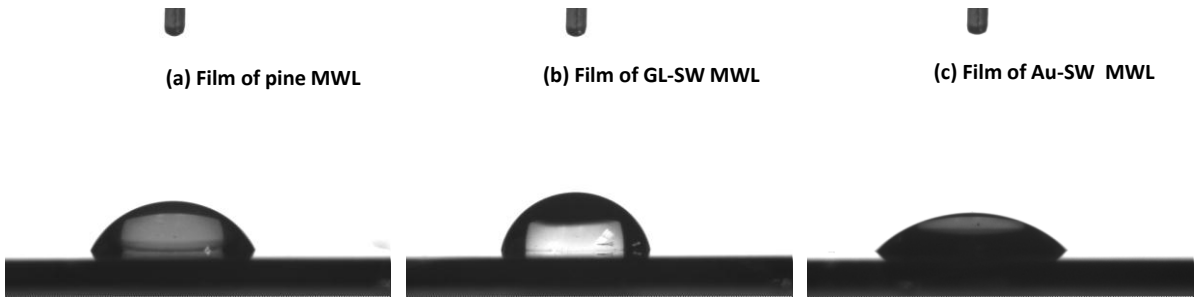


Figure 3.3. Images of water drop on (a) film of pine MWL, (b) film of GL-SW MWL and (c) film of Au-SW MWL.

Measurement of cellulase adsorption on bleached pulps and lignins

The kinetics of cellulase adsorption was conducted by incubating 1 mg/ml of cellulase preparation with bleached pulps or MWLs at 1% consistency for 5, 10, 20, 30 and 60 min at 50 °C with 180 rpm. The protein content of cellulase in the supernatant was measured by ninhydrin assay analysis. Aqueous bovine serum albumin (BSA) was used as reference standard. Protein solutions of 0.1 ml with a maximum protein concentration of 1 mg/ml were mixed with 0.3 ml 10 M NaOH and autoclaved at 121 °C for 20 min for hydrolyzing protein to amino acid completely. After cooling down, 0.5 ml acetic acid and 0.5 ml 2% ninhydrin reagent were added and mixed well, and then boiled for 10 min. Then the samples were diluted with 4.2 ml 99.5% ethanol, and centrifuged to remove by-products and solids before analyzed by UV-Vis at 570 nm wave length (Zhu et al., 2009).

Reconstruction of lignocelulloses

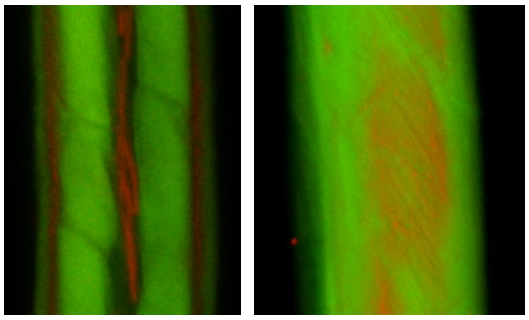
Artificial lignocellulosic pulps were reconstructed using bleached pulps and MWLs. The total lignin content of reconstructed pulps was designed to be about 20%. A certain amount of isolated lignins were firstly dissolved in about 7 ml of 0.1 N NaOH to obtain an alkali-lignin solution. This amount of lignin is calculated as 0.25 od g MWL divided by the lignin content in this sample and again divided by an empirical parameter of reconstruction efficiency. This parameter was determined based on the experiment experience, which was about 75%-80%. Then, 1 od g bleached pulp was mixed with the alkali-lignin solution and make its consistency to be 10%. The mixture was standing overnight to allow the penetration of alkali-lignin solution. After that, 1% sulfuric acid of 6 ml was added into the alkali-lignin pulp to

precipitate the lignin onto the bleached pulps and the pH should be decreased to 2.5. The excessive acid was filtrated after 1 h, and then the pulp was washed with 50 ml DI water to remove the salt. A control of only bleached pulp treated by this reconstruction procedure was conducted as well. The lignin content of the reconstructed lignocelluloses was in the range of 20% to 22%.

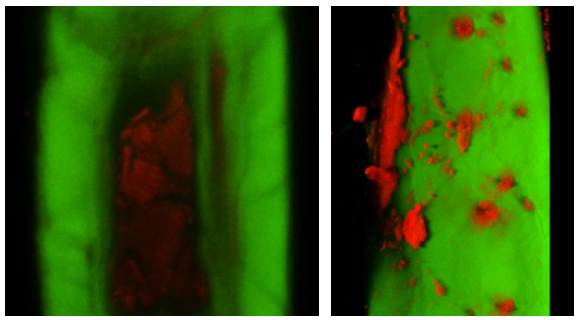
Determination of lignin distribution in the reconstructed lignocellulose

The reconstructed lignincellulose was stained before imaging using confocal laser scanning microscopy (CLSM). Staining of samples with HPLC grad acridine orange (AO) (3,6-Bis(dimethylamino) acridine hydrochloride, Sigma-Aldrich, St. Louis, MO) was performed at room temperature. Approximately 200 mg of sample was placed in a 25 mL vial with 20 mL of 1.25×10^{-5} M AO solution prepared with deionized water. The vial was sealed and placed in a box to avoid light and shaken frequently for 1 hour. After staining the samples were washed three times by carefully pouring the supernatant out of the vial and replacing the volume with deionized water. The samples were then placed on a 3 in. x 1 in. glass slide, covered with a No.1 cover slip, and sealed with wax. The imaging of dyed samples was conducted by using a Carl Zeiss LSM 710 confocal workstation with a C-Apochromat 40x/1.1 water immersion objective lens. An argon laser at 488 nm was used as excitation light. Fluorescence emission between 515-540 nm was collected as the green channel and emissions above 590 nm was collected as the red channel. Image analysis was performed using ZEN lite 2011 image analysis software. The distributions of lignin in some

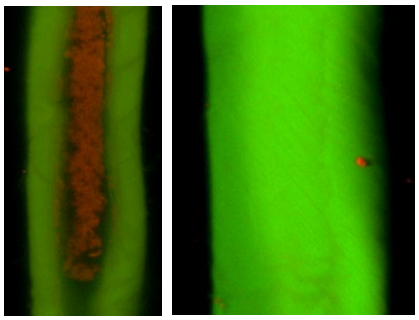
of the reconstructed lignocelluloses were shown in Figure 3.4. The images that appear green in color are rich in carbohydrate and areas that are red in color are rich in lignin.



(a) Reconstructed Pine



(b) Reconstructed Au-SW



(c) Reconstructed GL-SW

Figure 3.4. Confocal images showing the lignin distribution in the reconstructed lignocelluloses.

Enzymatic hydrolysis

The physically mixed substrate, including 20% (w/w) of MWL and 80% (w/w) of bleached pulp was subjected to enzymatic hydrolysis. Likewise the reconstructed lignocellulose with about 20% (w/w) lignin was used for enzymatic hydrolysis. The MWLs obtained from SW was physically mixed or reconstructed with BSW, while the MWLs isolated from HW was processed with BHW. Enzymatic hydrolysis was carried out in 2 ml microcentrifuge tubes with 0.1 od g substrate at 5% consistency in sodium acetate buffer (pH 4.8, 100 mM) and 0.3% sodium azide. The reaction was conducted in an air shaker at 50 °C with shaking speed of 180 rpm. The loading of cellulase was 5 FPU/g carbohydrates. Xylanase and β-glucosidase were used as well to enhance the hydrolysis. The weight ratio of cellulase, xylanase and β-glucosidase was 1:0.3:0.3. Hydrolysis kinetics was obtained by sampling the hydrolysate periodically. The sugar content in the hydrolysate was determined by HPLC (Agilent technology 1200 series, Palo Alto, CA). The column system of HPLC consisted of a Shodex SP0810 column and a de-ashing cartridge filter pre-column. Liquid samples were filtered through 0.22 μm filter before analysis. Milli-Q water was used at a flow rate of 0.5 ml/min. The hydrolysis yield of the substrate was evaluated by carbohydrate conversion, which was defined as the amount of hydrolyzed carbohydrate in the hydrolysates as a percentage of total carbohydrate in the starting substrate for enzymatic hydrolysis. Two parallel samples were used in all analysis, and the data were presented as the mean of the duplicates.

Results and Discussions

Characterization of MWLs

The yield and content of all the MWLs were shown in Table 3.1**.** The total lignin contents of all the MWL were around 90~94%, which was considered as good representatives of pure lignins. MWL has been traditionally used as a representative source of original lignin in the substrates due to the mild preparation conditions that maximally reserve the lignin structures (Ikeda et al., 2002). Though the yield of MWL is usually lower than cellulolytic enzyme lignin (CEL), MWL, unlike CEL in which the residual enzymes may interfere with the further interaction with enzymes, is readily to use for enzyme adsorption test (Nakagame et al., 2010). The yield of purified MWL was a limiting factor, which depends on the extent of ball milling. Increase in the milling time could increase the yield of MWL, but it also resulted in higher amount of lignin degradation (Ikeda et al., 2002).

Table 3.1. Yields and purity of MWL. Yield of MWL is the percentage of the weight of MWL based on the weight of lignin in the extractive free substrates.

Source of MWL	Yield of crude MWL, %	Yield of purified MWL, %	Lignin content of MWL, %		
			KL, %	ASL, %	Total, %
Pine	75.4	37.8	92.0	0.4	92.4
Maple	65.4	32.0	87.3	2.6	89.9
Eucalyptus	65.4	38.6	84.4	4.6	89.0
Auto-HW	48.7	35.1	90.6	2.1	92.7
Auto-SW	32.6	22.6	91.8	0.6	92.4
GL-HW	60.7	41.2	89.8	3.8	93.7
GL-SW	38.7	25.6	93.3	0.8	94.1

Quantitative ^{13}C NMR is recognized as the most used NMR methods for lignin characterization. This technique is rather informative, reliable, and at the same time relatively feasible (Capanema et al., 2004). The advantage of nuclear magnetic resonance (NMR) over other spectroscopic techniques, such as infrared (IR), ultraviolet-visible (UV), and Raman spectroscopy, is that NMR has much higher resolution, enabling a larger amount of information to be obtained (Capanema et al., 2004). An example of nonacetylated and acetylated pine MWL was shown in Figure 3.2. For MWLs obtained from SW, the integral of the 162-102 ppm region was set as the reference, assuming that it includes six aromatic carbons and 0.12 vinylic carbons (Chen, 1998). It follows that the integral value divided by 6.12 is equivalent to one aromatic ring. For MWLs obtained from HW, the reference of integration at 160-102 ppm includes six aromatic carbons because the amount of vinyl carbon atoms in cinnamyl alcohol and cinnamaldehyde in HW MWL is very limited, thus the contribution of vinyl carbons can be neglected (Capanema et al., 2005). A very small resonance of C-1 of carbohydrates at 102-90 ppm was observed in Figure 3.2. Therefore, carbohydrates do not interfere with the analysis of lignin moieties in MWL preparation.

Some of the key lignin structures that could have potential impact on enzymatic hydrolysis were listed in Table 3.2. It was found that after pretreatment, the total OH groups in both HW and SW were decreased. The total OH groups include primary aliphatic OH, secondary aliphatic OH and phenolic OH. The significant decreased of primary and secondary OH groups were the major reason for the decrease of total OH groups. Phenolic OH group, in contract, was increased after pretreatment. Carboxylic groups was rendered to increase hydrogen bonding between lignin and enzyme (Brey, 1978). MWLs obtained from

SW substrates generally possessed higher carboxylic groups than that of HW. However, due to the limited amount of carboxylic groups in MWLs, their impact on lignin-enzyme interaction was not clear. The content of OMe was also decreased after pretreatment, and the S/G ratio of pretreated HW was significantly decreased which might due to the complete loss of both OMe groups of S unit or the conversion of S to G unit by losing one of the OMe group. Degree of condensation for MWLs of SW and HW were calculated in different ways. For MWL of SW, the degree of condensation is denoted as (3.00 - h-units) - [(I125-103)na + M + 2* I]. I125-103 is the region of 125-103 ppm attributed to aromatic methine carbons (CAr-H). The theoretical value for CAr-H in noncondensed guaiacyl units is 3.00, and the difference between it and the integral at I125-103 ppm is usually considered as the degree of condensation. However, some corrections should be made. For example, only two carbons from p-hydroxyphenyl units resonate in this region. The signal of C-6 in vanillin moieties (M) is at 126 ppm. The chemical shifts of C-6 and C-5 in spirodienone (I) moieties are also higher than 125 ppm. In the case of HW, the degree of condensation is defined as (2s + 3g + 2h) - (I125-103). The theoretical amount of CAr-H atoms can be calculated from the h: g: s ratio considering the contribution of two carbons of s and h units and three carbons of g units in this region. The amount of tertiary aromatic carbons (CAr-H) in the lignin preparation was obtained from the integral at 125-103 ppm. Generally, SW MWLs had greater degree of condensation than that of HW MWLs. Since G type of lignin moieties has more non-condensed carbon, it has greater chance to be subjected to condensation. A linear correlation between the total OH groups and the degree of condensation was observed as shown in Figure 3. The major decrease of OH groups were due to the decrease of primary and

secondary OH, thus carbon bonds of CAr-C-α, CAr-C-β or CAr-C-γ were likely to be formed during the pretreatments. The impact of lignin structure on enzyme adsorption and enzymatic hydrolysis is discussed in the following paragraphs.

Table 3.2. Characterization of MWL by ^{13}C NMR.

Structure	Amount (per Ar)						
	Hardwood MWLs				Softwood MWLs		
	MWL of Eucalyptus	MWL of Maple	MWL of Au-HW	MWL of GL-HW	MWL of Pine	MWL of Au-SW	MWL of GL-SW
Total OH	1.70	1.58	1.19	1.18	1.41	1.02	1.11
Aliphatic OH	1.48	1.33	0.69	0.63	1.15	0.68	0.65
Primary OH	0.81	0.72	0.44	0.30	0.81	0.49	0.45
Secondary OH	0.67	0.61	0.25	0.32	0.34	0.19	0.20
Phenolic OH	0.22	0.25	0.51	0.55	0.27	0.34	0.46
OMe	1.62	1.47	1.26	1.34	0.89	0.65	0.77
S/G	2.89	1.34	0.47	0.56	-	-	-
Degree of cond.	0.02	0.23	0.52	0.42	0.48	0.64	0.69

Cellulase adsorption on MWLs and bleached pulps

The non-productive adsorption of cellulase onto the surface of lignin is an important consideration to understand lignin inhibition. The adsorption kinetics of cellulase was conducted on all MWLs, BHW and BSW pulps, respectively (Figure 3.5). All the materials reached their maximum adsorption at about 30 min. BSW and BHW adsorbed almost half the initially loaded cellulase. MWLs of pine and maple had similar adsorption performance which showed about 18% of the total cellulase adsorption, while MWL of eucalyptus

adsorbed about 10% of cellulase. The adsorption of cellulase on lignins from the untreated wood was much lower than that on bleached pulps. However, after pretreatment, the adsorption of all MWLs increased dramatically. The cellulase adsorptions on MWLs of all the pretreated wood pulps were in the range of 40% to 68%. MWL of GL-SW adsorbed the most cellulase, and the adsorption reached to 68%, which was even higher than that of bleached pulps. MWL of GL-HW adsorbed the least amount of cellulase, which was about 40%, compared to the MWLs from the other pretreated wood pulps.

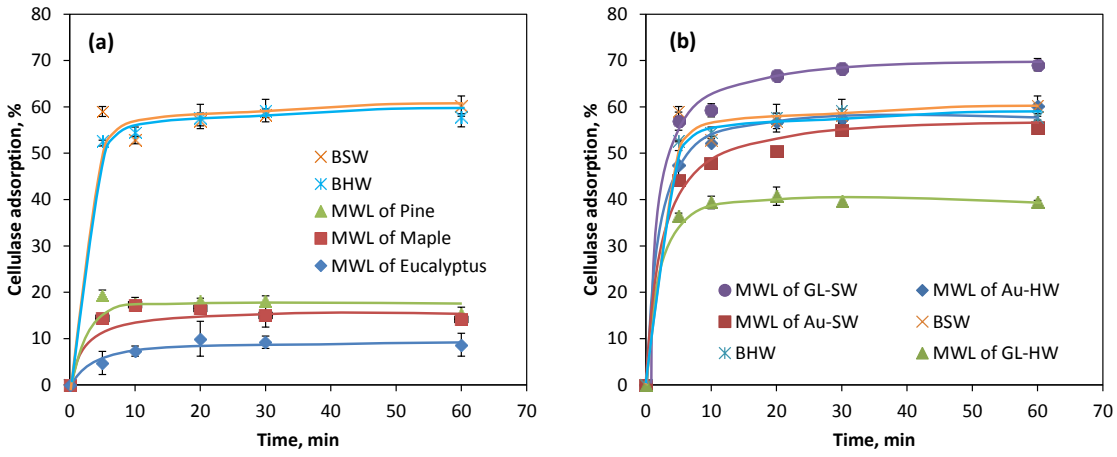


Figure 3.5. Adsorption kinetics of cellulase on MWLs and bleached pulps. (a) MWLs isolated from raw woods and (b) MWLs isolated from pretreated wood pulps.

It is interesting to observe that the characteristics of lignin in terms of non-productive adsorption of cellulase was significant changed after pretreatment. This result might be associated with the changes of the lignin structures by pretreatment. In order to understand the relationship between the extent of non-productive adsorption of cellulase on lignin and

the structures of lignin, the detailed structures of these MWLs were characterized by ^{13}C NMR. As shown in Figure 3.6a, overall, the cellulase adsorption on lignin increased with the decreasing of total OH from 1.7 to 1.0 per aromatic ring. The major contributors of the changes of total OH were aliphatic OH groups, which indicated that the condensation reaction occurred during the pretreatment. Alternatively, the phenolic OH groups increased with the increase of cellulase adsorption (Figure 3.6a). Several studies mentioned that phenolic OH groups could be involved in the adsorption of cellulase to lignin (Pan, 2008; Sewalt et al., 1997). Sewalt and coworkers compared the effect of hydroxypropylated organosolv lignin and steam pretreated lignin on filter paper digestion. They found that the hydroxypropylation of lignin with free phenolic sites removed resulted in increased cellulose hydrolysis (Sewalt et al., 1997). Pan et al. reported that phenolic hydroxyl groups are critical to their inhibitory effects on enzymatic hydrolysis of cellulose. At 10 mM concentration, phenolic compounds showed 1-5% more inhibition than nonphenolic ones. The inhibitory effect of lignin could be significantly removed by using hydroxypropylation to block of free phenolic hydroxyl groups (Pan, 2008). Due to the correlation between OH groups and cellulase adsorption, it is speculated that the changes of OH was associated with the changes of hydrophobicity of lignin. Thus, the analysis of contact angle on lignin materials could be an indication for this issue. As shown in Figure 3.6 and Table 3.3, all the MWLs were considered as hydrophilic because their contact angle were all less than 90°. The higher contact angle indicated a relatively more hydrophobic nature of a material. As shown in Table 3.3, it was found that MWLs obtained from GL pretreatment became more hydrophobic than MWLs obtained from untreated woods, while MWLs isolated from au-hydrolysis pretreated

woods became more hydrophilic. Although the amount of OH groups are an important contributor for the hydrophobicity of a material, other functional groups such as alkane and fats and greasy substances are also contribute to the hydrophobicity of a materials. No correlation between the contact angle and the amount of total OH groups was observed in this study. Besides, the contact angle did not show a correlation with the cellulase adsorption either, which suggested that the lignin-enzyme interaction involved not only hydrophobic interaction, other interactions between lignin and enzyme such hydrogen bonding, electrostatic interaction might played an important role in lignin-enzyme adsorption. A clear trend, cellulase adsorption was increased with the increase of lignin degree of condensation, was observed in this study. A greater degree of condensation of lignin resulted in a more branched lignin structure which probably involve in more interactions with enzyme.

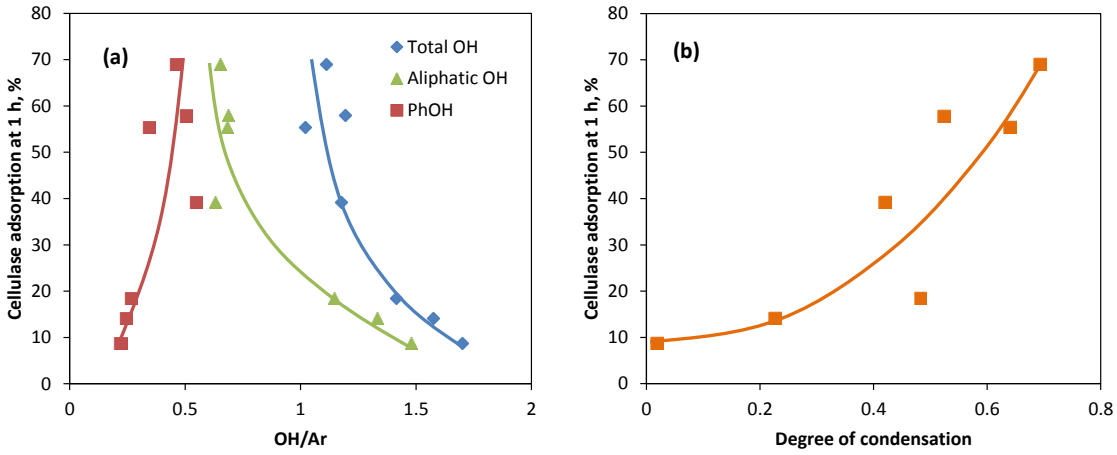


Figure 3.6. The effect of characteristics of MWLs (a) total OH and (b) degree of condensation on cellulase adsorption.

Table 3.3. Water contact angle results of MWLs.

MWL source	Contact angle, °
Pine	66.4
Maple	61.9
Eucalyptus	59.0
Au-HW	49.3
Au-SW	48.7
GL-HW	73.5
GL-SW	72.2

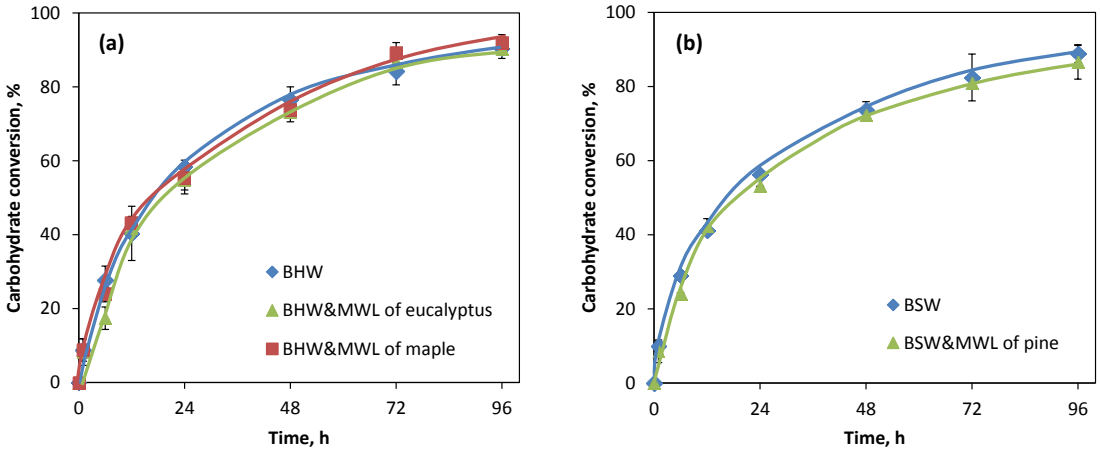


Figure 3.7. Enzymatic hydrolysis of physically mixed bleached pulps and MWLs isolated from raw woods. (a) Hydrolysis of BHW and BHW mixed with MWLs from raw HW. (b) Hydrolysis of BSW and BSW mixed with MWL from raw SW.

The impact of non-productive adsorption of lignin on enzymatic hydrolysis

The impact of non-productive adsorption of lignin on enzymatic hydrolysis was studied by enzymatic hydrolysis of physically mixed substrate with 80% bleached pulps and 20%

MWLs as shown in Figure 3.7 and 3.8. It appears that the presence of 20% MWLs had no impact on enzymatic hydrolysis in the case of physically mixed bleached pulps and MWLs obtained from untreated woods because the hydrolysis kinetics of bleached pulps mixed with MWLs of eucalyptus, maple and pine were mostly the same as that of bleached pulps alone (Figure 3.7). This founding is probably due to two reasons: 1) MWLs isolated from untreated woods adsorbed much less cellulases than bleached pulps and, 2) the amount of MWLs vs. bleached pulps was 20% vs. 80%. Therefore, the impact of non-productive adsorption of MWLs from untreated woods was limited for enzymatic hydrolysis.

In contrast, the presence of 20% MWLs obtained from pretreated biomass showed a significant impact on enzymatic hydrolysis (Figure 3.8). A 4-13% decrease of 96 h carbohydrate conversion was. Particularly, the MWL of GL-SW which had a potential of adsorbing up to 68% of loaded cellulase decreased 96 h carbohydrate conversion from 92.8% to 80.5%. This is the most sever decreased in the case of hydrolysis of physically mixed substrate. Therefore, weather the lignin possess a clear impact on enzymatic hydrolysis is largely due to adsorption properties of lignin towards enzyme. When the lignin alone was capable to adsorb over 40% of the initially loaded cellulase, which accounted to 2/3 of the cellulase that bleached pulps can adsorb, the presence of 20% MWLs showed adverse impact on enzymatic hydrolysis (Figure 3.8). It has been suggested that the extent to which lignin adsorbed enzymes substantially depends on the nature of lignin itself and, lignin adsorption of enzyme is decreased as the severity of pretreatment increases (Nakagame et al., 2010). It was reported that the lignin isolated from lodgepole pine and steam pretreated poplar decreased the hydrolysis yields of Avicel, whereas the other isolated lignins did not appear to

decrease the hydrolysis yields significantly, likely because of the different adsorption properties of the isolated lignins (Nakagame et al., 2010).

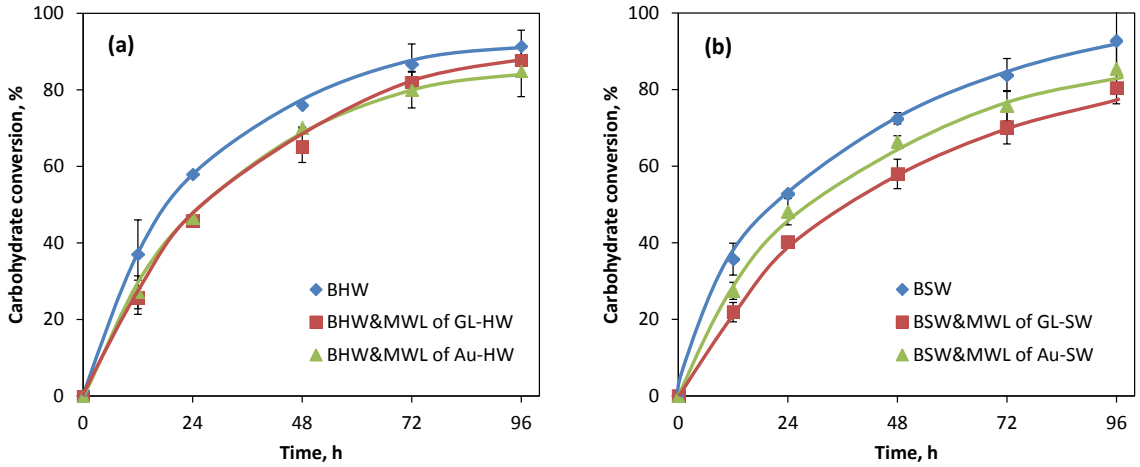


Figure 3.8. Enzymatic hydrolysis of physically mixed bleached pulps and MWLs isolated from pretreated wood pulps. (a) Hydrolysis of BHW and BHW mixed with MWLs from pretreated HW. (b) Hydrolysis of BSW and BSW mixed with MWLs from pretreated SW.

The impact of physical barrier of lignin on enzymatic hydrolysis

The effect of physical barrier of lignin on enzymatic hydrolysis was revealed by the hydrolysis of reconstructed pulps (Figure 3.9). It should be noted that the reconstruction procedure had a minimal change in carbohydrate substrate, where the hydrolysis kinetics of BHW and BSW subjected to reconstruction without the addition of lignin had no difference in hydrolysis kinetics with the original BHW and BSW. The built-up of physical barrier of lignin in reconstructed lignocellulose was confirmed by the imaging of confocal microscopy (Figure 3.4). For all images, the length of the fiber is shown from top to bottom. For each

sample type the image on the left is an image of the two cell walls of a single fiber with the open lumen in the center. The images on the right are images of the outside surface of the reconstructed sample. Portions of the image that appear red and green are rich in lignin and carbohydrates respectively. These images suggest that during reconstruction (pH induced precipitation) the lignin tends to agglomerate in the fiber lumen. The amount of lignin deposition on the outside surface of the fiber seems limited but is non-uniform when it does occur as shown in Figure 3.4. It was speculated that before reconstruction, the lignin was soluble and could easily flow through the bordered pits and into the lumen. As the pH reduced, lignin began to precipitate in the lumen and formed globules that grow to be larger than the bordered pit size. During washing, the precipitated lignin in the lumen is mechanically trapped and therefore appears to be more abundant in this part of the fiber than the bulk of the cell wall of the outside surface. Although this pattern of lignin distribution is different than in real wood, the formation of physical barrier is undoubted.

Compared to the hydrolysis of physically mixed substrate, the reconstructed lignocellulosic substrates were more resistant to be hydrolyzed. For the reconstructed HW with MWLs obtained from untreated woods, the 96 h carbohydrate conversion dropped from 90.4% to 83.2% and 78.4% for the hydrolysis of reconstructed eucalyptus and reconstructed maple, respectively. The reconstructed pine showed a more pronounced decrease in the efficiency of enzymatic hydrolysis. At the end of 96 h hydrolysis, the reconstructed pine pulps obtained 71.3% carbohydrate conversion, while the carbohydrate conversion for BSW was 88.9% (Figure 3.9a). It was found that the order of the decrease of carbohydrate conversion in reconstructed pulps followed the decrease of S/G ratio of MWLs isolated from

untreated woods. With the decreasing of S/G ratio, the reconstructed pulps became more resistant to digestion. This observation indicated that different lignin structure might have different effect of physical blockage to the accessible sites of carbohydrates. The guaiacyl unit of lignin may forme a more cross-linked lignin than that of syringyl unit of lignin, and thus resulted in a greater physical barrier against the access of enzymes (Grabber, 2005).

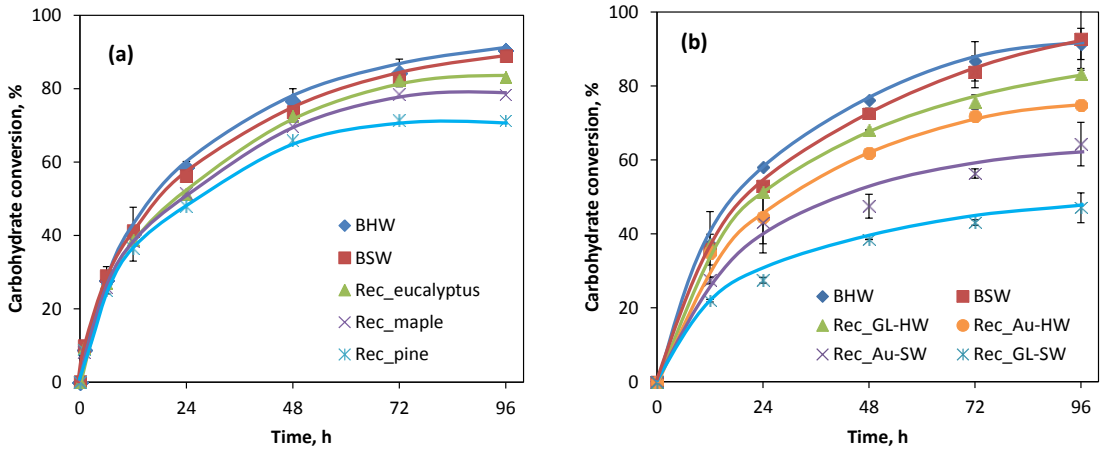


Figure 3.9. Enzymatic hydrolysis of reconstructed lignocellulosic pulps. (a) Hydrolysis of reconstructed substrate using bleached pulps and MWL obtained from untreated woods. The lignin contents in the reconstructed eucalyptus, maple and pine were 21.1%, 21.5% and 21.6%, respectively. (b) Hydrolysis of reconstructed substrate using bleached pulps and MWL obtained from untreated woods. The lignin content in reconstructed Au-HW, Au-SW, GL-HW and GL-SW were 20.6%, 22.3%, 21.5% and 21.9%, respectively.

In the case of reconstructed substrate with MWLs obtained from pretreated woods, in a course of 96 h hydrolysis, the carbohydrate conversion dropped from over 90% to 83.3%

once 20% MWL of GL-HW was reconstructed with bleached HW pulps. For the other reconstructed substrates, the carbohydrate conversion decreased to a greater degree and they are 74.9%, 64.3% and 47.1% for Au-HW, Au-SW and GL-SW, respectively (Figure 3.9b). For the same type of pretreatment, the reconstructed SW substrates always had lower carbohydrate conversion than that of reconstructed HW substrates. This trend was consistent with the observation in the case of hydrolysis of reconstructed lignocellulose using MWLs obtained from untreated wood. The order of decreased hydrolysis of the reconstructed substrates generally agrees with the increase trend of the adsorption capability of the corresponding MWLs (Figure 3.10).

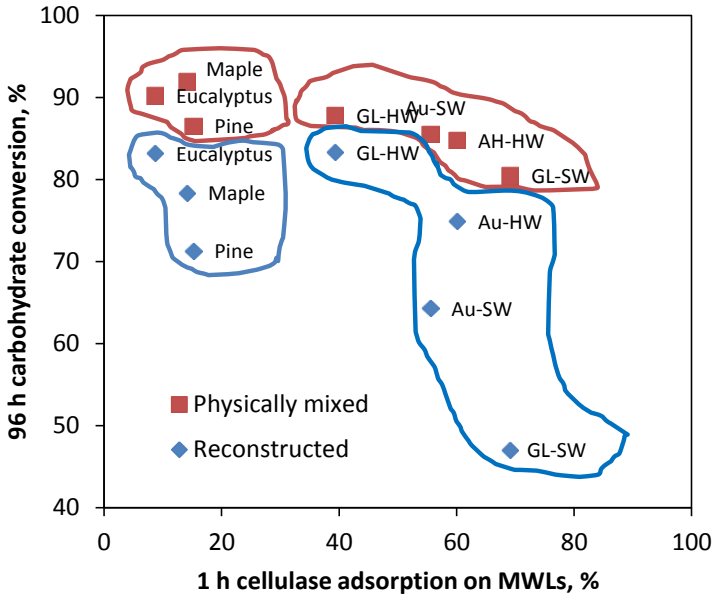


Figure 3.10. The effect of cellulase adsorption on MWLs on enzymatic hydrolysis of physically mixed substrate with bleached pulp and MWLs and reconstructed lignocellulose.

In the physically mixed substrate, lignin and bleached pulp did not bond together. Enzymes could either adsorb on carbohydrate or on lignin by non-productive adsorption. In the reconstructed substrate, lignins were penetrated into the carbohydrate and precipitated on the carbohydrate, thus the lignin formed a physical barrier. This physical barrier could limit the access of enzyme to the carbohydrate surface. If enzymes are not accessible to the carbohydrate, it might adsorb on the lignin surface. Besides, the blockage effect of lignin not only did it limit the accessible area of enzymes, but also could entrap enzyme within the tri-dimensional matrix of lignin and thus enzymes were forced to bound with lignin rather than bound with carbohydrate (Yang et al., 2011). Thus, the impact of non-productive adsorption of enzyme onto lignin might be amplified in the case of reconstructed substrate (Figure 3.11).

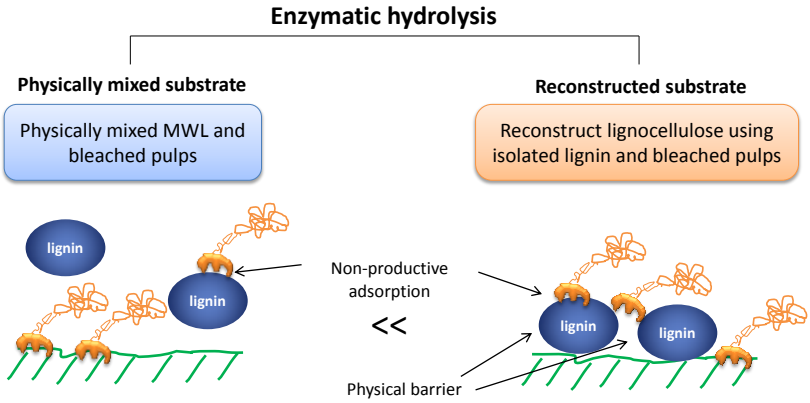


Figure 3.11. Schematic comparison of the lignin-enzyme interaction in the physically mixed substrate and reconstructed substrate.

In real lignocellulosic biomass, the lignins distribute in each cell wall layers and middle lamella (Fromm et al., 2003). The more spreadable distribution of lignin is likely to cover

more surface of carbohydrate, and lead to a greater obstacle to the accessibility of enzyme to carbohydrates. Non-productive adsorption and physical barrier are the two major concerns for the impact of lignin on enzymatic hydrolysis but cannot explain everything. The degree of the impact varied among the lignins obtained from different substrate. One of the lignin characteristics, the degree of condensation, was found to be an important indication for the extent of lignin inhibition on enzymatic hydrolysis. As show in Figure 3.12, the hydrolysis was improved with the decreased of degree condensation. Once the degree of condensation reached to 0.4, the further decreased of the degree of condensation has minimal effect on enzymatic hydrolysis. The larger value of the degree of condensation implied that the C_{Ar}-H bonds in lignin were converted to C-C, C_{Ar}-C_{Ar}, or C_{Ar}-O bonds. This finding was the first time that reported a correlation between the same category of lignin structure in several different lignin samples and the carbohydrate conversion of enzymatic hydrolysis.

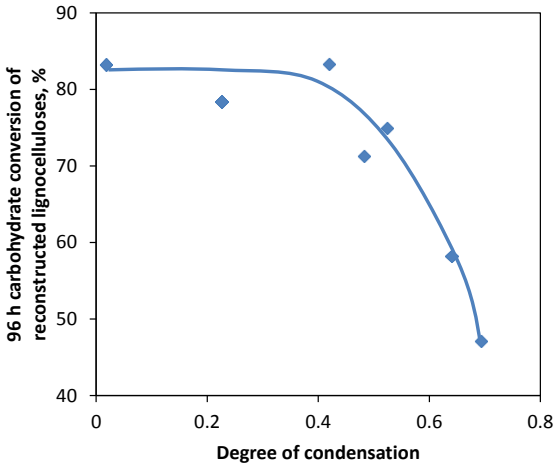


Figure 3.12. The effect of degree of condensation of lignin on 96 h carbohydrate conversion of reconstructed lignocelluloses.

Conclusions

The impact of lignin obtained from untreated woods, acid and alkali pretreated woody biomasses on enzymatic hydrolysis was investigated by non-productive adsorption and physical barrier of lignin. Both non-productive inhibition and physical barrier of lignin had important impact on enzymatic hydrolysis of lignocellulosic biomass.

MWLs obtained from pretreated woody biomass adsorbed 2 to 6 folds more cellulase than MWLs isolated from untreated woods. When the MWLs adsorbed over two thirds of cellulase compared with that of bleached pulps, the presence of 20% MWLs in the physically mixed substrate showed negative impact on enzymatic hydrolysis.

The carbohydrate conversion of reconstructed lignocelluloses with about 20% lignin decreased significantly compared to the hydrolysis of bleached pulps. The reconstructed softwood substrate had lower carbohydrate conversion than the reconstructed hardwood substrate. The degree of condensation of lignin increased significantly after pretreatment, especially with SW lignins. The degree of condensation of lignin was found to have an important impact on cellulase adsorption and enzymatic hydrolysis.

Acknowledgement

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CHAPTER 4

Evaluation of the Factors Affecting Avicel Reactivity Using Multi-stage Enzymatic Hydrolysis

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Abstract

Multi-stage and single-stage enzymatic hydrolysis of cellulose (Avicel PH-101) was conducted to investigate individual factors that affect the rate-reducing kinetics of enzymatic hydrolysis. Understanding factors affecting enzymatic hydrolysis of Avicel will help improve hydrolysis of various biomasses. Product inhibition, enzyme deactivation, and the changes of substrate are potential factors that can affect the hydrolysis efficiency of Avicel. Multi-stage enzymatic hydrolysis resulted in 36.9% and 25.4% higher carbohydrate conversion as compared to a single-stage enzymatic hydrolysis with an enzyme loading of 5 and 20 FPU/g in a 96 h reaction. However, a decline in carbohydrate conversion of 1.6% and 2.6% was observed through each stage with 5 and 20 FPU/g, respectively. This indicated that the substrate became more recalcitrant as hydrolysis progressed. The decreased reactivity was not due to crystallinity because no significant change in crystallinity was detected by X-ray diffraction. Product inhibition was significant at low enzyme loading, while it was marginal at high enzyme loading. Therefore, product inhibition can only partially explain this decreased conversion. Another important factor, enzyme deactivation, contributed to a 20.3% and 25.4% decrease in the total carbohydrate conversion of 96 h hydrolysis. This work

shows that a significant reason for the decreased Avicel digestibility is the effect of enzyme blockage, which refers to the enzymes that irreversibly adsorb on accessible sites of substrate. About 45.3% and 63.2% of the total decreased conversion at the end of the 8th stage with 5 and 20 FPU/g, respectively, was due to the presence of irreversibly adsorbed enzyme. This blockage of active sites by enzymes has been speculated by other researchers, but this paper shows further evidence of this effect.

Keywords: cellulose; enzymatic hydrolysis; product inhibition; substrate reactivity; enzyme blockage

Introduction

The recalcitrant nature of biomass and high cost of enzyme are great barriers for the economic feasibility of producing bioethanol on a commercial scale from lignocellulosic biomass (Wu et al., 2010; Zhang et al., 2006). The kinetics of enzymatic hydrolysis of biomass typically include a rapid initial rate and a substantial decrease in the rate of hydrolysis with time. As a result, incomplete conversion of the carbohydrates to sugars is often observed.

Several hypotheses have been presented to explain this rate-reducing issue including enzyme deactivation, reduction of synergistic effect, changes in substrate reactivity, and product inhibition (Ghadge et al., 2005; Hong et al., 2007; Smith et al., 2010; Xiao et al., 2004; Zhang et al., 1999). Under the conditions of enzymatic hydrolysis, the shear strength and thermal instability of enzymes are the major reasons for enzyme deactivation (Caminal et

al., 1985; Elias & Joshi, 1998; Tejas P. Gunjikar et al., 2001). Although shaking during hydrolysis is supposed to obtain a uniform reaction, it was found that enzymes were deactivated when exposed to an air-liquid interface during shaking or agitation (Basu & Pal, 1956; Elias & Joshi, 1998). However, when the impact of air-liquid interface was eliminated by sealing the reactor after completely filling it, only 5% deactivation occurred after 20 h with 700 rpm agitation, whereas 50% deactivation was observed in the presence of air (Jones & Lee, 1988). For the thermal deactivation of enzymes, enzyme activity decreases with time at the temperature of maximum activity, while enzymes can be stored for months at a lower temperature without losing detectable activity. Thus, this thermal deactivation is considered one of the reasons for the rate-reducing kinetics of enzymatic hydrolysis (Caminal et al., 1985). Another enzyme-related issue is the reduction in the synergistic effect of enzymes due to the irreversible adsorption of cellobiohydrolase I (Ma et al., 2008). Even the loss of only one type of enzyme may result in a substantial drop in the overall reaction rate (Smith et al., 2010). The change in substrate reactivity during enzymatic hydrolysis is also a concern. It is believed that some fraction of cellulose is hydrolyzed preferentially, leaving the rest more and more recalcitrant. It was found that the digestibility of Avicel PH-105 decreased by over 80% as a function of conversion up to 90%, accompanied by a loss of substrate's accessibility to enzyme (Hong et al., 2007). For lignocellulosic biomass, the increased lignin ratio during hydrolysis may result in increased lignin-enzyme binding, which limits the reaction rate (Berlin et al., 2005). Product inhibition, during which the accumulation of sugars results in a decreased reaction rate, is another reason. Both oligosaccharides and monosaccharides can affect the hydrolysis efficiency. It was found that xylooligomers were

strong inhibitors compared to xylan or xylose in terms of initial hydrolysis rate. Compared to the control experiment without sugar addition, even <2 mg/ml xylooligomers resulted in 35% lower final glucose yield from enzymatic hydrolysis of Avicel (Qing et al., 2010). The mechanism of product inhibition is related to the characteristics of both enzyme and substrate (Gusakov & Sinitsyn, 1992).

Although many researchers have studied the factors affecting the kinetics of enzymatic hydrolysis, it is difficult to assess the effect of the individual factor due to its coupling effect with other factors (Park et al., 2010). Our understanding of the mechanisms of enzymatic hydrolysis is still far from complete (Arantes & Saddler, 2010; Eriksson et al., 2002; Hong et al., 2007; Smith et al., 2010; Yang et al., 2006). Based on these considerations, a study of each individual factor that affects the kinetics of enzymatic hydrolysis of pure cellulose may provide insight into a more complicated system with multi-component enzymes and lignocellulosic biomass. In this study, Avicel was used as a cellulose model to investigate the factors affecting the kinetics of enzymatic hydrolysis using the unique experimental design of multi-stage enzymatic hydrolysis. By comparing these processes, *i.e.* single- and multi-stage enzymatic hydrolysis and a multi-stage process with and without sugar addition, the effect of enzyme deactivation and product inhibition can be revealed. Since the same enzyme-to-substrate ratio was used in every stage, the changes in substrate can also be monitored. This fundamental study and kinetics information will be useful to determine dominant rate-reducing factors, which may provide opportunities to improve the current technology by minimizing the limiting factors for enzymatic hydrolysis.

Materials and Methods

Materials

Avicel PH-101 microcrystalline cellulose (97.6% glucan content) was purchased from Sigma-Aldrich (St. Louis, MO). Commercial enzymes, cellulase (NS50013), and β-glucosidase (NS50010) were generously provided by Novozymes North America, Inc. (Franklinton, NC).

Single-stage enzymatic hydrolysis

Single-stage enzymatic hydrolysis was carried out in Erlenmeyer flasks at 50°C and 180 rpm with 100 mM sodium acetate buffer (pH=4.8) and 10 oven-dried (od) g Avicel at 5% consistency. The enzyme was a mixture of cellulase and β-glucosidase with a loading ratio of 1.0:0.3 as recommended by Novozymes. The enzyme loadings were 5 FPU/g substrate (6.3 mg cellulase/g substrate) and 20 FPU/g substrate (25.3 mg cellulase/g substrate). The hydrolysates were taken periodically to separate the solid and liquid by centrifugation. Then the supernatant was used for sugar analysis by HPLC. A three-step mild washing was performed to remove the sugar and enzyme on the surface of the hydrolysis residue, which included flushing with 20 times the initial substrate weight with deionized (DI) water, 10 times with sodium acetate buffer, and another 20 times with DI water. After washing, the solids were freeze-dried and subjected to X-ray diffraction and nitrogen content analysis.

Multi-stage enzymatic hydrolysis

Multi-stage enzymatic hydrolysis includes eight stages. Each stage was conducted for 12 h with an enzyme loading of 5 and 20 FPU/g substrate, respectively. The ratio of fresh enzyme to available substrate was kept the same for each stage, as shown in Table 4.1. The residue was separated by filtration at the end of each stage, and the filtrate was used for sugar analysis. The residue was washed by mild washing as described in single-stage enzymatic hydrolysis. Fresh enzyme and buffer was then added into the washed solids to initiate the next stage. To study product inhibition, glucose was added with fresh enzyme to compensate the sugar loss during the washing step. The amount of added glucose was kept the same as the glucose released from the previous stage.

Extensive washing of substrate

Extensive washing was applied to some of the hydrolyzed residue to completely remove the remaining enzymes that could not be removed by mild washing. Untreated Avicel, some residues from 3rd and 8th stages with the enzyme loading of 5 FPU/g and residues from 6th and 8th stages with 20 FPU/g were processed by extensive washing. Sodium hydroxide of 40 ml with 0.8% (w/v) concentration was mixed with 1 od g sample. After centrifugation, the samples were suspended into 1% sodium dodecyl sulfate (SDS) solution and incubated at 80°C for 15 min. After centrifugation, the Avicel residues were suspended and washed three times by both 75% (v/v) ethanol and distilled water (Hong et al., 2007). The enzyme content of the extensively washed residues was analyzed for nitrogen content.

Compositional analysis

Chemical composition analysis of Avicel and hydrolysis residues were conducted by a two-step sulfuric acid hydrolysis as described in the previous work (Yu et al., 2011). The sugars from acid and enzymatic hydrolysates were measured using high performance liquid chromatography (HPLC, Dionex, Sunnyvale, CA). The concentrations of glucose, xylose, galactose, mannose, and arabinose were determined. The separation was performed by a Shodex SP0810 column with deionized water as the eluent at a flow rate of 0.6 ml/min at 80°C.

Nitrogen content analysis

Nitrogen content of samples was measured by a Perkin Elmer 2400_CHNS/O Elemental Analyzer (Perkin Elmer Corporation, Norwalk, CT). The percentage of protein can be calculated from the nitrogen data using a procedure published by the National Renewable Energy Laboratory to estimate the amount of enzyme (Hames et al., 2008).

Determination of crystallinity index

The cellulose crystalline structure was measured with freeze-dried samples by X-ray diffraction using a Rigaku SmartLab X-ray diffraction (The Woodlands, TX) with a Cu tube (λ=0.15405 nm). The Bragg angle of 2θ was scanned from 10° to 40° with a 0.05° step size and 5 seconds retention time in each step.

Results and Discussion

Enzymatic hydrolysis of Avicel

The kinetics of single-stage enzymatic hydrolysis of Avicel went through a rapid hydrolysis rate in the first 12 h, as shown in Figure 4.1. The carbohydrate conversions in the first 12 h were 25.8% and 38.6% with an enzyme loading of 5 and 20 FPU/g, respectively. After that, the reaction rate decreased significantly. From 12 to 72 h, the carbohydrate conversions were 25.5% and 28.9% for an enzyme loading of 5 and 20 FPU/g, which were less than in the first 12 h, although the reaction time was five times longer. Nearly no increase of carbohydrate conversion was obtained from 72 h to 96 h. The overall 96 h carbohydrate conversions of Avicel were limited to 49.7% and 69.6% with 5 and 20 FPU/g, respectively.

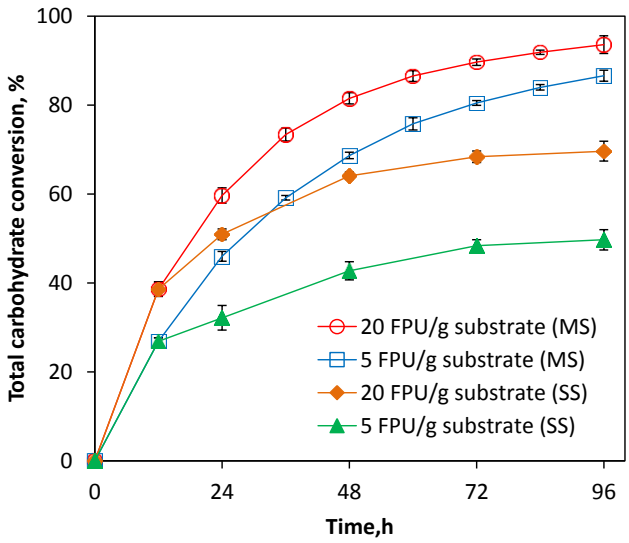

Figure 4.1. Carbohydrate conversion during single-stage (SS) and multi-stage (MS) enzymatic hydrolysis with an enzyme loading of 5 and 20 FPU/g substrate, respectively.

The reasons for the slower and more limited conversion of single-stage enzymatic hydrolysis can be originated from three aspects: enzyme deactivation, product inhibition, and the changes in substrate reactivity. This study was designed to evaluate these factors using multi-stage enzymatic hydrolysis as a process tool. The same ratio of fresh enzyme to substrate was applied in each stage to minimize the impact of enzyme deactivation and allow for an examination of the changes in Avicel reactivity. By conducting multi-stage enzymatic hydrolysis, a significant improvement in carbohydrate conversion was obtained within the same reaction time (96 h), compared to the single-stage (Figure 4.1). At the end of the 8^{th} stage, carbohydrate conversion reached 86.6% and 93.6% with 5 and 20 FPU/g, respectively, which were 36.9% and 25.4% higher than single-stage hydrolysis. One of the advantages of multi-stage enzymatic hydrolysis is that it substantially reduces product inhibition because the enzymes and buffer were replaced in every 12 h (Yang et al., 2010). Therefore, the effect of product inhibition on a multi-stage process can be investigated by adding sugars along with fresh enzyme and buffer at the beginning of each stage, as demonstrated in the following section.

Effect of product inhibition on multi-stage enzymatic hydrolysis

Product inhibition is an important reason for the decreased rate of enzymatic hydrolysis (Converse et al., 1988; Qing et al., 2010; Smith et al., 2010). Sugar products combined with enzyme can inhibit the hydrolysis ability of an enzyme. A frequently used method for studying this phenomenon is to add sugars into substrate suspension at the beginning of the reaction (Lee & Fan, 1983; Qing et al., 2010). Another approach is to use ultrafiltration to

remove sugars while keeping the enzyme in the reactor. However, it was observed that a substantial amount of enzyme leaked from the reactor (Converse et al., 1988). In order to study the impact of product inhibition on enzymatic hydrolysis without the interference of enzyme deactivation, glucose was added into the hydrolysis system along with fresh enzyme and buffer in every stage. Using this approach, product inhibition at low and high enzyme loading was investigated.

Figure 4.2a shows that glucose-supplemented eight-stage enzymatic hydrolysis obtained about 20% lower carbohydrate conversion than regular multi-stage enzymatic hydrolysis at an enzyme loading of 5 FPU/g. However, there was no noticeable difference in the case of 20 FPU/g as shown in Figure 4.2b. The glucose concentrations at the end of the 8^{th} stage were 40.9 g/l and 54.5 g/l with an enzyme loading of 5 and 20 FPU/g. These sugar concentrations can be considered high enough to cause product inhibition, but the higher glucose concentration had no impact on higher enzyme loading (20 FPU/g) in this study. These results implied that product inhibition had a significant impact at low enzyme loading, while this impact was marginal at high enzyme loading. A similar observation is supported by earlier works (Dekker et al., 1987; Hogan et al., 1990; Ramos et al., 1992). This phenomenon might be explained by the mechanism of competitive inhibition. At low enzyme loading, it is considered that a signification portion of enzymes was inhibited by forming an enzyme-inhibitor complex. Compared to the amount of uninhibited enzymes in multi-state enzymatic hydrolysis without glucose-supplemented process, less uninhibited enzymes can bind to substrate to form an enzyme-substrate complex (ES) when glucose was supplemented. At high enzyme loading, however, it was likely that there was still a substantial amount of

enzymes that were not inhibited by sugars to form an ES. When there is an enough amount of ES in the multi-stage process, even with glucose addition in the case of 20 FPU/g, product inhibition can be overcome. Some earlier works have identified that the product inhibition in enzymatic hydrolysis of Avicel is competitive inhibition (Borchert & Buchholz, 1987; Ohmine et al., 1983). Besides, the differences between glucose-supplemented multi-stage enzymatic hydrolysis and single-stage enzymatic hydrolysis were largely due to the effect of enzyme deactivation on the hydrolysis process, which were 20.3% and 25.4% for enzyme loadings of 5 and 20 FPU/g, respectively, as shown in Figure 4.2.

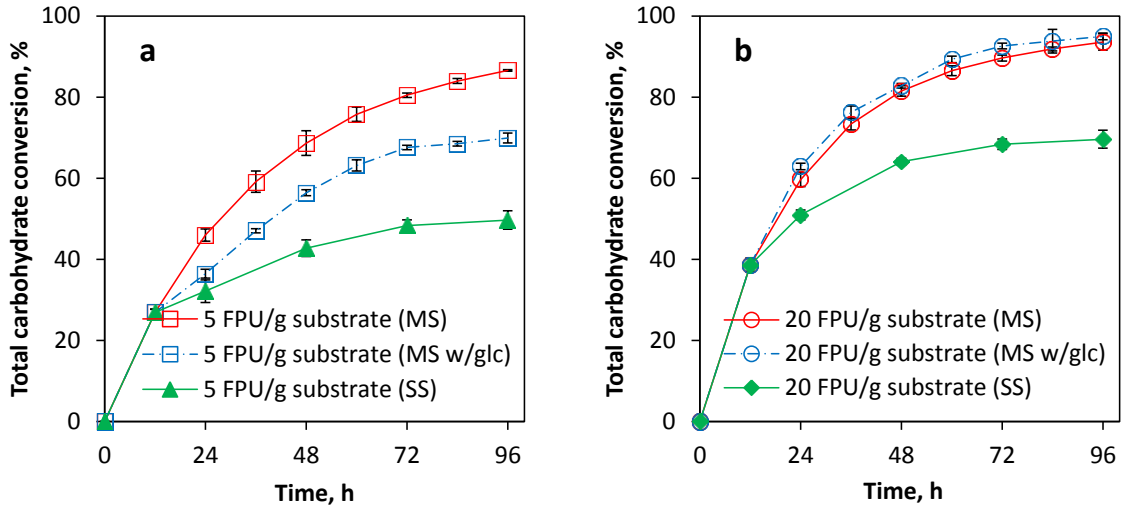

Figure 4.2. Comparison of glucose-supplemented eight-stage enzymatic hydrolysis, eight-stage, and single-stage enzymatic hydrolysis with an enzyme loading of (a) 5 FPU/g, and (b) 20 FPU/g.

It should be noted that determining a definite mechanism for product inhibition is a difficult task. Enzymatic hydrolysis usually takes place in a system contents multiple enzymes (exoglucanase, endoglucanase, and β-glucosidase) and multiple substrates (cellulose is first converted to oligosaccharides, then to monosaccharides), which requires many assumptions in order to make a model. In addition, the insolubility of the substrate is another concern. Michaelis-Menten equations are valid for soluble substrate, but the applicability of this concept to insoluble substrates is not well understood. For insoluble substrates, the substrate architecture (e.g. surface area, pores, and smoothness) and crowding effect (e.g. substrate concentration gradient) can be very different for different substrates and the time of enzymatic hydrolysis. Some studies related to product inhibition of cellulose found that the inhibition pattern could be competitive, noncompetitive, or mixed, depending on the cellulase binding constant, enzyme concentration, maximum adsorption of the enzyme, substrate concentration, and β-glucosidase activity (Gusakov & Sinitsyn, 1992). For a more complicated substrate such as lignocellulosic substrate, noncompetitive inhibition has been reported (O'Dwyer et al., 2007).

Changes of substrate reactivity during multi-stage enzymatic hydrolysis

The change in substrate reactivity during enzymatic hydrolysis is described as the substrate transforming into a less digestible (or more recalcitrant) form as a function of time. These changes in substrate are considered to have a negative impact on the hydrolysis rate. In eight-stage enzymatic hydrolysis, it has been shown that the digestibility of Avicel linearly decreased in each stage (Figure 4.3). With an enzyme loading of 20 FPU/g, the carbohydrate

conversion during the 1st stage was 38.6%, but it dropped to 30.6% during the 4th stage and to 21.2% in the 8th stage. An average decrease of 2.6% in carbohydrate conversion was found every 12 h. A similar trend was observed for the enzyme loading of 5 FPU/g with a 1.6% average decrease every stage. Generally, Avicel digestibility with an enzyme loading of 5 FPU/g decreased more slowly than that with 20 FPU/g. The 12 h carbohydrate conversion declined from 26.9% to 16.3% after the eight stages.

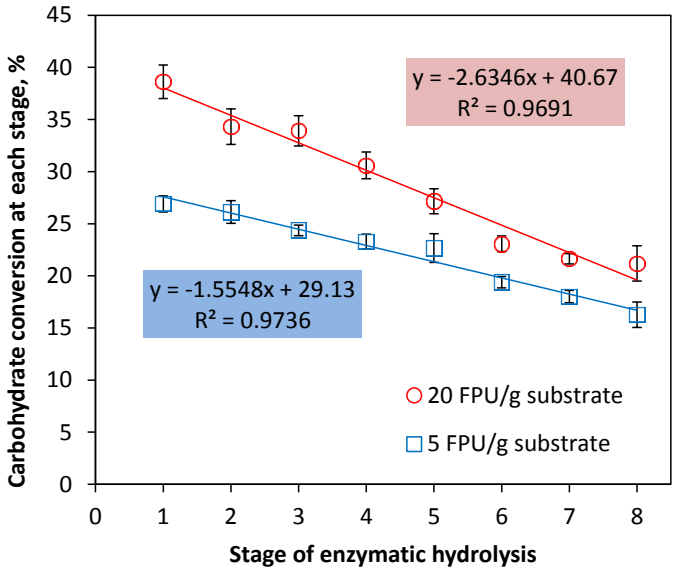

Figure 4.3. Changes in substrate reactivity during multi-stage enzymatic hydrolysis with an enzyme loading of 5 and 20 FPU/g, respectively.

The changes in substrate reactivity have been explained by the changes in crystallinity index, accessible surface area, chemical composition, and enzyme-substrate interaction (Arantes & Saddler, 2010; Jeoh et al., 2007; Lee & Fan, 1983; Sathitsuksanoh et al., 2011;

Smith et al., 2010). Some researchers have investigated the reactivity of Avicel during enzymatic hydrolysis. Two-stage enzymatic hydrolysis was conducted on Avicel PH-101 with completely removal of residual enzyme on the Avicel residue after first stage, and no decline in the reaction rate was observed in the first hour of the second stage (Yang et al., 2006). However, it was found that the reactivity of Avicel PH-105 was significantly reduced due to the decrease of cellulose's accessibility to cellulase, which was measured by the adsorption of nonhydrolytic fusion protein (Hong et al., 2007). The structure of Avicel PH-101 is simple and chemically uniform because it has no extra cell wall components such as hemicellulose and lignin. The degree of polymerization and particle size of Avicel are not expected to increase, which should not negatively affect enzymatic hydrolysis (Hallac & Ragauskas, 2011; Hong et al., 2007; Yeh et al., 2010). For these reasons, the potential increase of crystallinity and the presence of remaining adsorbed enzyme due to the limitation of mild washing are considered to be the major factors that affect the reactivity of Avicel in this study.

Changes in crystallinity during enzymatic hydrolysis

The crystallinity of untreated Avicel and hydrolysis residue from multi-stage enzymatic hydrolysis was measured by XRD. Figure 4.4 shows that the normalized XRD spectrum of untreated Avicel and extensively hydrolyzed Avicel in the 8^{th} stage of hydrolysis with an enzyme loading of 5 and 20 FPU/g mostly overlap, which indicates that the crystalline structure of Avicel had marginal change even after extensive hydrolysis. Small decreases in the crystal face of $1\bar{1}0$ and 110 were observed in the hydrolyzed samples. However, the

significance of this minor change in $1\bar{1}0/110$ face is not clear. It has been reported that no significant change was observed in the degree of crystallinity during enzymatic hydrolysis of Avicel PH-101, up to 90% conversion (Hall et al., 2010). The invariant XRD spectra of untreated and enzymatically treated Avicel also demonstrated that the crystalline structure of Avicel remained nearly unchanged (Figure 4.4). A possible reason for the unchanged crystallinity could be that the structure of Avicel is highly crystalline and chemically uniform. However, one issue about the crystallinity measured by XRD is that it is a bulk measurement and measured in the dry state. For example, the drying process which carried out before crystallinity measurement is known to change the structure of cellulose. This deficiency can be avoided by solid-state ^{13}C nuclear magnetic resonance (NMR) measurement, because the analysis can be conducted in wet state. Although there is some evidence showing that NMR measured crystallinity changes would be similar, the total crystallinity might differ (Park et al., 2009). An *in situ* analysis of crystalline structure under reacting conditions at the localized surface is not available (Barnette et al., 2011) and thus, whether the crystallinity has any significant changes in wet state during enzymatic hydrolysis needs to be further explored (Hall et al., 2010).

Accumulation of irreversibly adsorbed enzyme on Avicel in multi-stage enzymatic hydrolysis

Another factor that may affect the digestibility of Avicel is the accumulation of irreversibly adsorbed enzyme. The irreversibly adsorbed enzyme is referred to the part of enzyme that remains bound to the substrate residue after washing the residue with the same buffer that used during enzymatic hydrolysis (Ma et al., 2008). In this study, the irreversibly

adsorbed enzyme could be visualized in a macro state as the color getting darker after mild washing with the progressed multi-stage hydrolysis. The color of residues with enzyme loading of 20 FPU/g was darker than that with 5 FPU/g (Figure 4.5). It was speculated that these irreversibly adsorbed enzymes may affect the accessibility of Avicel. Table 4.1 shows that the irreversibly adsorbed enzyme on Avicel residue in multi-stage enzymatic hydrolysis increased with the number of stages. With enzyme loading of 5 and 20 FPU/g, the amounts of irreversibly adsorbed enzymes were 1.3 and 3.8 mg/g substrate after the 1st stage. Three stages later, these enzymes increased to 3.1 mg/g and 10.0 mg/g. At the end of the 7th stage, the total irreversibly adsorbed enzymes were 11.3 and 28.8 mg/g.

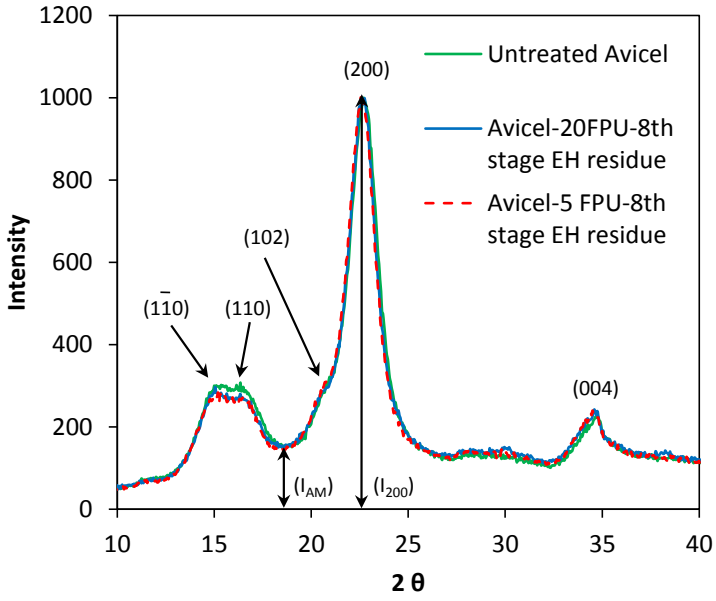

Figure 4.4. X-ray diffraction spectra of untreated Avicel PH-101 and substantially converted Avicel residues from the 8th stage of multi-stage enzymatic hydrolysis with an enzyme loading of 5 and 20 FPU/g, respectively.

Table 4.1. Accumulation of irreversibly adsorbed enzymes on the residues of multi-stage enzymatic hydrolysis.

Samples	Avicel residue, g	Total amount of fresh enzyme added in each stage, mg	Total Fresh enzyme added in each stage, mg/g substrate	Total amount of accumulated irreversibly adsorbed enzyme, mg	Total accumulated irreversibly adsorbed enzyme, mg/g substrate
enzyme loading: 5 FPU/g substrate					
Avicel	100.0	990.6	9.9		
12 h	74.1	734.5	9.9	92.7	1.3
12×2 h	56.2	557.1	9.9	175.7	3.1
12×3 h	43.4	430.2	9.9	135.7	3.1
12×4 h	34.3	340.1	9.9	193.1	5.6
12×5 h	26.9	266.9	9.9	185.2	6.9
12×6 h	22.1	218.7	9.9	179.4	8.1
12×7 h	18.4	182.2	9.9	206.9	11.3
12×8 h	15.5				
enzyme loading: 20 FPU/g substrate					
Avicel	100.0	3962.5	39.6		
12 h	62.3	2470.6	39.6	233.8	3.8
12×2 h	42.3	1677.5	39.6	317.5	7.5
12×3 h	29.5	1169.0	39.6	295.0	10.0
12×4 h	21.2	838.1	39.6	304.0	14.4
12×5 h	15.8	624.4	39.6	295.4	18.8
12×6 h	12.5	497.0	39.6	290.1	23.1
12×7 h	10.0	397.9	39.6	288.7	28.8
12×8 h	8.0				

On the other hand, the accumulation of the irreversibly adsorbed enzyme shows a correlation with the carbohydrate conversion in each stage of multi-stage enzymatic hydrolysis as shown in Figure 4.5. With the accumulation of the irreversibly adsorbed enzyme, the carbohydrate conversion in each stage decreased linearly, and the rate of

decrease for samples with the enzyme loading of 5 FPU/g was greater than that of 20 FPU/g. These results indicated that the irreversibly adsorbed enzyme had a negative impact on enzymatic hydrolysis. A hypothesis of the reason for this negative impact can be described as such: during enzymatic hydrolysis the enzymes adsorb on the accessible surface of the substrate and begin to catalyze the hydrolysis of the substrate. When this part of the substrate has been converted to sugars, the enzymes disengage and seek another accessible site. However, some of the enzymes might lose some activity, so that they are not capable of progressing to the available substrate, resulting in irreversible adsorption. The irreversibly adsorbed enzyme occupy the accessible surface rather than functioning as catalyst. This phenomenon can be called the effect of enzyme blockage. Some indirect demonstration about this enzyme blockage effect has been addressed by modeling in the earlier research. A model addressing the deactivation of adsorbed enzyme-substrate complex which blocked further hydrolysis was proposed (Howell & Mangat, 1978). Similarly, an enzyme transfer model has been suggested, where the negative effect of inactivated enzyme around and within in the substrate residue show a good prediction of product concentration (Converse et al., 1988). One experimental evidence was that the enzymatic hydrolysis rate of cellulose decreased with irreversible adsorption of cellobiohydrolase I (Ma et al., 2008). However, it is not clear that whether the effect of enzyme blockage was the major reason for the decreased hydrolysis rate.

On the other hand, the accumulation of the irreversibly adsorbed enzyme shows a correlation with the carbohydrate conversion in each stage of multi-stage enzymatic hydrolysis as shown in Figure 4.5.

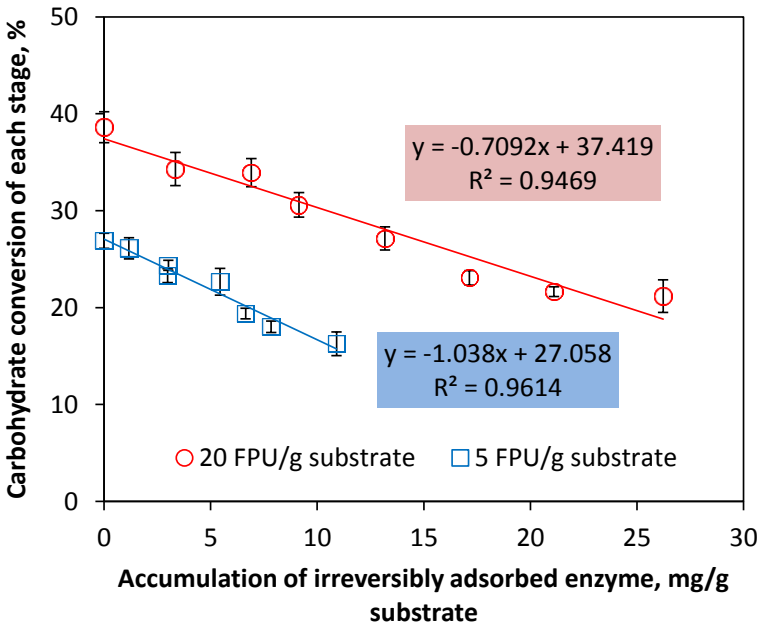

Figure 4.5. Effect of the accumulation of irreversibly adsorbed enzyme on Avicel during multi-stage enzymatic hydrolysis.

With the accumulation of the irreversibly adsorbed enzyme, the carbohydrate conversion in each stage decreased linearly, and the rate of decrease for samples with the enzyme loading of 5 FPU/g was greater than that of 20 FPU/g. These results indicated that the irreversibly adsorbed enzyme had a negative impact on enzymatic hydrolysis. A hypothesis of the reason for this negative impact can be described as such: during enzymatic hydrolysis the enzymes adsorb on the accessible surface of the substrate and begin to catalyze the hydrolysis of the substrate. When this part of the substrate has been converted to sugars, the enzymes disengage and seek another accessible site. However, some of the enzymes might lose some activity, so that they are not capable of progressing to the available

substrate, resulting in irreversible adsorption. The irreversibly adsorbed enzyme occupy the accessible surface rather than functioning as catalyst. This phenomenon can be called the effect of enzyme blockage. Some indirect demonstration about this enzyme blockage effect has been addressed by modeling in the earlier research. A model addressing the deactivation of adsorbed enzyme-substrate complex which blocked further hydrolysis was proposed (Howell & Mangat, 1978). Similarly, an enzyme transfer model has been suggested, where the negative effect of inactivated enzyme around and within in the substrate residue show a good prediction of product concentration (Converse et al., 1988). One experimental evidence was that the enzymatic hydrolysis rate of cellulose decreased with irreversible adsorption of cellobiohydrolase I (Ma et al., 2008). However, it is not clear that whether the effect of enzyme blockage was the major reason for the decreased hydrolysis rate.

In order to prove this hypothesis, extensive washing was applied to remove the irreversibly adsorbed enzyme and, the 12 h digestibility of Avicel was examined. After extensive washing, the enzymes were completely removed from the substrate. It was observed that Avicel residue in the 8^{th} stage of hydrolysis with an enzyme loading of 20 FPU/g showed a brown color after mild washing, whereas the color turned to as white as untreated Avicel after extensive washing (Figure 4.6). The nitrogen content of all the hydrolysis residues was the same as the untreated Avicel, which was less than 0.02% (w/w) nitrogen content. A significant improvement in carbohydrate conversion of these extensively washed Avicel residues was observed compared to the mildly washed residues (Figure 4.7). After extensive washing, the untreated Avicel obtained a carbohydrate conversion of 28.4% and 40.5% in the 1^{st} stage, while the untreated Avicel without extensive washing yielded

26.9% and 38.6% carbohydrate conversion. In the case of the enzyme loading of 20 FPU/g, the 3rd stage residue with extensive washing obtained 39.6% carbohydrate conversion, which was comparable to that of the untreated Avicel, while the carbohydrate conversion of the mildly washed 3rd stage Avicel residue was only 33.9%. This result suggested that the digestibility of Avicel had little change after three stages. However, it was found that the complete removal of the adsorbed enzyme did not bring back the carbohydrate conversion of all the samples to the level of the initial 12 h hydrolysis, especially for 8th stage hydrolysis residues. The 12 h carbohydrate conversion of the extensively washed Avicel from the 8th stage decreased to 32.2%, however, it was still higher compared to the mildly washed residues of the 8th stage of 21.2% carbohydrate conversion. By removing the irreversibly adsorbed enzyme, the carbohydrate conversion was improved by 11% (from 21.2 to 32.2%). A similar trend was found for an enzyme loading of 5 FPU/g. The extensive washing improved enzymatic hydrolysis by 4.8% (from 16.3 to 21.1%). These improvements (11 and 4.8%) corresponded to 63.2% and 45.3%, based on the total decreased conversion at the end of the 8th stage for enzyme loading of 20 and 5 FPU/g, respectively. These results are strong evidence for supporting the effect of enzyme blockage, which has been hypothesized by the research community for a long time. Additionally, a difference in the 12 h carbohydrate conversion between untreated Avicel and extensively washed Avicel 8th stage hydrolysis residue indicated that the reactivity of Avicel decreased when it was subjected to extensive hydrolysis, but this change could not be reflected by the change in crystallinity (Figure 4.4). Other substrate characteristics that affect the reactivity of substrate need to be further investigated.

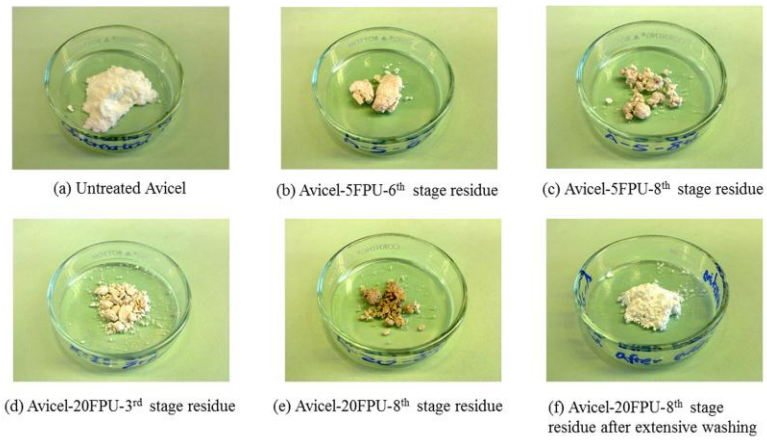

Figure 4.6. Pictures of untreated Avicel and Avicel residues after multi-stage enzymatic hydrolysis with 5 and 20 FPU/g substrate. Samples of (a) ~ (e) were subjected to mild washing. Sample (f) were prepared by extensive washing.

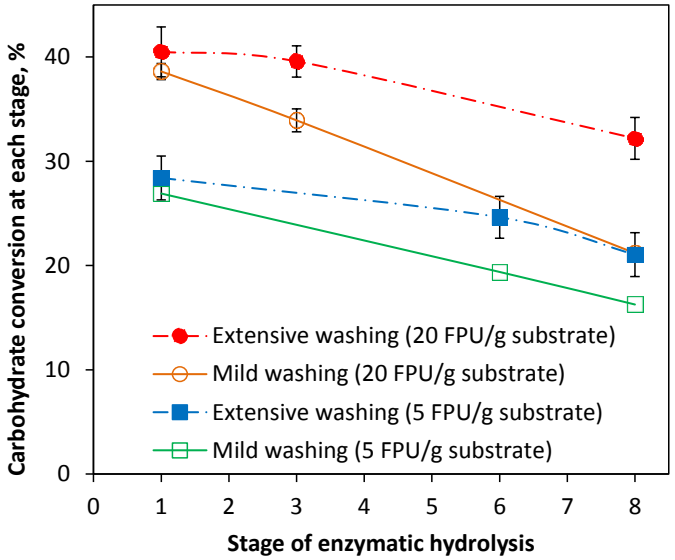

Figure 4.7. Comparison of the 12 h carbohydrate conversion of extensively washed and mildly washed Avicel residues from 1st, 3rd, 8th stage with an enzyme loading of 20 FPU/g and 1st, 6th, 8th stage with an enzyme loading of 5 FPU/g in multi-stage enzymatic hydrolysis.

Conclusions

The individual factors that affect the enzymatic hydrolysis have been evaluated by multi-stage and single-stage enzymatic hydrolysis. These factors include: product inhibition, enzyme deactivation, crystallinity, and blocking of accessible sites. Avicel reactivity evaluated by carbohydrate conversion linearly decreased by 1.6% and 2.6% at each stage for both enzyme loadings, 5 and 20 FPU/g. This indicated that the substrate became more recalcitrant as multi-stage enzymatic hydrolysis progressed. Crystallinity was not a reason for this increased recalcitrance, as no significant difference in crystalline structure was observed during multi-stage enzymatic hydrolysis. Product inhibition was clearly observed at an enzyme loading of 5 FPU/g, while no impact was identified at 20 FPU/g. Comparing to glucose-supplemented multi-stage enzymatic hydrolysis, the 20.3% and 25.4% lower carbohydrate conversion during 96 h single-stage enzymatic hydrolysis with 5 and 20 FPU/g, respectively, was mainly due to enzyme deactivation. It was found that the decreased Avicel digestibility was mostly originated from irreversibly adsorbed enzymes, in which these enzymes are considered to block available sites for enzymatic hydrolysis. This blockage effect was responsible for 63.2% and 45.3% of the total decreased conversion at the end of the 8^{th} stage with 20 and 5 FPU/g, respectively. This finding is important for explaining the decreased digestibility of some substrates.

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CHAPTER 5

Quantification of Bound and Free Enzymes during Enzymatic Hydrolysis and Their Reactivities on Pure Cellulose and Lignocellulose

Abstract

Enzymatic hydrolysis of insoluble biomass is a surface reaction. Part of the initially loaded enzyme adsorb on the surface of the biomass whereas the other part stays in the liquid phase. These two parts of enzyme are known as bound enzyme and free enzyme. In this study, Avicel, bleached hardwood pulp and green liquor pretreated hardwood pulp have been used for studying the characteristics of bound enzyme and free enzyme. In a continuous enzymatic hydrolysis, 35%-65% initial added enzyme became bound enzyme and, 30%-65% became free enzyme. It was found that the enzyme which initially adsorbed on the substrate was primarily responsible for enzymatic hydrolysis. The contributions of free enzymes become insignificant after a certain period of reaction time. Although about half of the initial loaded enzymes were free enzyme, the reactivity of the free enzyme decreased rapidly even after 20 min hydrolysis. SDS-PAGE analysis showed that CBH I was significantly decreased in the free enzyme, which might be the reason for such low hydrolysis ability of free enzyme. In order to improve the hydrolysis ability of free enzyme, a surfactant Tween 80 was used to evaluate its effect on free enzyme. Tween 80 showed limited effect on bound enzyme whereas it had a benefit for improving the hydrolysis of free enzyme on Avicel probably because Tween 80 helped release more CBH I in the free enzyme. This is a great advantage

for the hydrolysis for Avicel (highly crystallized cellulose), because CBH I mainly attach on the chain ends of crystalline region. However, maintaining more CBH I in the free enzyme was not significant favorable for the hydrolysis of pulp. This might be due to the more complicated components and structures of lignocellulosic materials, and their difference requirement for the enzyme cocktails.

Keyword: enzymatic hydrolysis, bound enzyme, free enzyme, SDS-PAGE, synergism effect

Introduction

Lignocellulose is one of the most abundant biomass on earth that can be converted into liquid fuels by modern biotechnology (Chang & Holtzapple, 2000). The development of bioethanol and commodity chemicals from lignocellulose is a promising potential alternative to our current reliance on fossil fuels (Chandra et al., 2007). The biological process of converting lignocellulose to ethanol includes pretreatment, enzymatic hydrolysis, and fermentation. A key issue for this process is the conversion of polysaccharides from lignocellulose into fermentable sugars. Therefore, enzymatic hydrolysis is a critical process not only because the sugar yield and concentration of the hydrolysates directly determine the efficiency of fermentation, but also the cost-sensitive issue of enzymes has a great impact on the feasibility of bioethanol production in a commercial scale.

The efficiency of the enzymatic hydrolysis of lignocellulose is mostly governed by the chemical and physical properties of substrates, and the reactivity and synergism effect of cellulase cocktails. Enzymatic hydrolysis of insoluble biomass is a heterogeneous reaction.

Cellulases firstly adsorb onto solid substrate to cleave cellulose chain and then release soluble cellobiose, which can be further hydrolyzed to glucose by β-glucosidase. For cellulase cocktails that were designed to have a synergistic effect, only some portion of loaded enzymes adsorbs on the substrates as bound enzymes. Cellobiohydrolases (CBH) possess a catalytic core and one or more carbohydrate-binding module connected by a linker. Carbohydrate-binding module is believed to have a high affinity for crystalline cellulose and surface hydrogen bonds are considered to disrupt by a linker. These combined actions can facilitate anchoring the cellulose microfibril into catalytic core (Ding & Xu, 2004). The typical catalytic cycle involves binding at the end of cellulose chain, cleavage of a β-1,4 glycosidic bond to yield cellobiose, and finally translocation along the chain for the next cycle. On the other hand, the other portion of enzyme in the liquid phase, such as β-glucosidases and desorbed CBH, are free enzymes that have flexible mobility and potentially available for recycling. Enzymatic hydrolysis is a dynamic equilibrium process, in which the concentration of bound enzyme and free enzyme is always changing due to the amount of available substrate and its surface characteristics.

In the past two decades, a great progress has been achieved in understanding the mechanism of enzymatic hydrolysis, especially the adsorption of cellulase. Cellulase adsorption is rapid compared to the time required for hydrolysis, with many studies finding that adsorption reaches steady-state within half an hour (Zhang & Lynd, 2004). The adsorption of enzyme proteins onto carbohydrate could involve hydrogen bonds, van der Waals interactions, and ionic polarization or hydrophobic interactions (Ding & Xu, 2004; Lehtio, 2003). The most common description of cellulase adsorption is the Langmuir

isotherm, which measures overall cellulase protein adsorption, assuming a uniform cellulose surface, adsorption enthalpy independent on surface coverage, and negligible interaction among adsorbed molecules. However, the insoluble biomass is far from an ideal substrate model. It has a non-uniform surface due to factors such as its morphology, cellulose crystallinity, and heterogeneous components (*i.e.* hemicellulose and lignin). Besides, this measurement is usually conducted at low temperature (4 °C) since both the adsorption of cellulase and hydrolysis of substrates occurred under the optimum temperature of 50°C. Therefore, the dynamic adsorption of cellulases with respect to the changes of substrate in the real hydrolysis conditions should be much more complicated. Researchers have studied the behavior of adsorbed cellulose in a dynamic process. For example, it was found that adsorbed enzymes are sufficient to digest the cellulose without replenishing of enzyme while the liquid phase containing the sugar and free enzymes was continuously removed. This observation indicated that the adsorbed enzymes rather than free enzymes were mainly responsible for enzymatic hydrolysis (Mandels et al., 1971). Another research compared two scenarios of enzymatic hydrolysis without removing the supernatant and with replacement of supernatant by fresh buffer and the same amount of produced glucose, and they found that a similar trend observed in reducing sugars (Lee & Fan, 1983). However, whether this behavior was consistent with the heterogeneous enzyme cocktails and lignocellulosic materials was not clearly reported. The importance of utilizing the bound enzymes should have been further investigated.

For the process standpoint, hydrolysates from enzymatic hydrolysis are usually subjected to fermentation. However, there are still a significant amount of enzymes are in a

liquid phase as free enzymes. Unabsorbed enzymes such as β-glucosidase are the major components in a liquid phase together with some desorbed enzymes that can be resorbed to fresh substrate. Therefore, it is technically feasible to recover and reuse these free enzymes from hydrolysis suspensions. Some of the previous works showed encouraging results on enzyme recycling. For example, it was found that cellulases can be recovered in high yields by contacting fresh steam-exploded wood with hydrolysis filtrate and by extraction of spent hydrolysis residue with a buffer of pH 7. The recycled enzymes gave hydrolysis rate about equal to those with fresh enzymes (Clesceri et al., 1985). Lu and coworkers found that the free enzymes recycled via ultrafiltration remained relatively active for three rounds of recycle (Lu et al., 2002). However, Hogan and Mes-Hartree found that the recovered enzyme had only 10-20% filter paper activity compared with the original enzymes. When the recovered enzymes were combined with fresh enzyme to hydrolyze steam-exploded aspen, the increase in hydrolysis yield was marginal. It was suggested that the low enzyme activity of the recovery enzyme was due to the combination of thermal inactivation and adsorption of some of the cellulases onto the lignocellulosic residue (Hogan & Mes-Hartree, 1990).

Despite some significant progresses made so far, the activity of bound and free enzymes with the different types of substrates remain unclear. As the hydrolysis time progressed, whether the bound enzymes and free enzymes still have hydrolysis ability and how the hydrolysis changes with respect to the reaction time needs to be understood. From the application perspective, the recovery and reuse of both bound enzyme and free enzyme at the same time has not be investigated previously. Based on these considerations, the objective of this research is to investigate the characteristics of bound and free enzymes during enzymatic

hydrolysis and seek for the potentials of reusing bound and free enzymes for a more efficient and economical application. In this study, the different types of substrates, Avicel, bleached hardwood pulp and alkaline-pretreated hardwood pulp, were used to evaluate the characteristics of bound and free enzymes. Understanding the nature and behavior of bound and free enzymes is of significant importance for not only enhancing the fundamental insights of enzymatic hydrolysis, but also valuable for various industrial applications, including the development of unique process of enzymatic hydrolysis and recycling of enzyme aimed at saving the cost and maximizing the enzyme utilization.

Materials and Methods

Materials

Microcrystalline cellulose (Avicel PH-101) was purchased from Sigma Aldrich (St. Louis, MO). Fully bleached hardwood pulp (BHW) was obtained from a mill in the southeastern United States. The preparation of alkaline-pretreated hardwood pulp with green-liquor (GLHW) using the mixed southern hardwood (oak, maple, poplar, ash, sweet gum) as the resources was conducted in the lab. The green-liquor pretreatment was conducted with 16% total alkali charged green liquor, 40% sulfidity, 400 H-factor, 160 °C, with a liquid to solid ratio of 4. After green liquor pretreatment, the chips were disk-refined to pulps and subjected to oxygen delignification with 3% sodium hydroxide at 110°C for 1 h at an oxygen pressure level of 100 psi. Then, the PFI refining was performed with 4000 revolution counts at 10% consistency. The chemical composition of each substrate was shown in Table 5.1. All the other chemicals were reagent grade and purchased from Sigma Aldrich (St. Louis, MO)

and Fisher Scientific (Pittsburgh, PA), unless noted otherwise. Three enzymes, NS50013 (cellulase), NS50014 (hemicellulose) and NS50010 (β-glucosidase), were kindly provided by Novozymes North America, Inc. (Novozymes, Franklinton, NC).

Table 5.1. Chemical composition of Avicel PH-101, bleached hardwood (BHW) and green liquor pretreated hardwood pulp (GLHW). All the numbers are based on the initial (od) weight of analyzed sample.

	Carbohydrate (%)				Lignin (%)			Mass Balance
	Glc	Xyl	Man & Ara	Sum	KL	ASL	Sum	(%)
Avicel	97.9	-	-	97.9	-	-	-	97.9
BHW	70.4	19.3	0.7	90.3	-	0.5	0.5	90.8
GLHW	61.6	15.0	0.5	77.1	15.1	1.7	16.8	93.9

Preparation of bound and free enzymes by Pre-enzymatic hydrolysis

Avicel, BHW and GLHW were hydrolyzed for 20 min, 3 h, 12 h and 48 h at 5% consistency using 10 od g of substrate mixing with 100 mM sodium acetate buffer (pH 4.8) supplemented with 0.3% sodium azide. The pre-enzymatic hydrolysis (pre-EH) was conducted with an enzyme loading of 5 FPU cellulase and 0.3 weight ratio of β-glucosidase per gram of carbohydrate (6.3 mg proteins of cellulase and 3.6 mg proteins of β-glucosidase per gram of carbohydrate) at 50°C with a shaking rate of 180 rpm. Duplicates of each sample were conducted for weight loss measurement and protein analysis. After pre-EH, the solid and liquid were separated by Bush funnel with Whatman No. 1 filter paper. In the

liquid, the soluble sugar was measured by HPLC and the amount of proteins in a liquid phase was determined by ninhydrin assay analysis. The solids were carefully removed from the filter paper and freeze-dried to measure the weight loss and the amount of bound enzymes.

The hydrolysis ability of bound and free enzymes was investigated by a kinetic hydrolysis, respectively. The hydrolysis ability of bound enzymes was evaluated by replacing the same volume of filtrate with fresh buffer and continuing the hydrolysis for up to 144 h. For determination of the hydrolysis ability of free enzymes, parts of the filtrate was mixed with 5 od g of fresh substrate, and the consistency was adjusted to 5% before continuing the hydrolysis for up to 144 h. In the case of pre-EH supplemented by Tween 80, 12 h pre-EH was conducted on all the substrates with 15 mg/g substrate of Tween 80 added at the beginning of pre-EH, and at 20 min prior to the end of the reaction, respectively. In both of the hydrolysis with bound and free enzymes, the hydrolyzed sample of 1.5 ml was taken at 12 h, 24 h, 48 h, 72 h, 96 h, 120 h and 144 h for sugar analysis by HPLC.

Direct quantification of the bound and free enzymes

The protein concentration of enzymes was measured by ninhydrin assay analysis. Aqueous bovine serum albumin (BSA) was used as reference standard. Protein solutions of 0.1 ml with a maximum protein concentration of 1 mg/ml were mixed with 0.3 ml 13.5 M NaOH and autoclaved at 121 °C for 20 min for hydrolyzing protein to amino acid completely. After cooling down, 0.5 ml acetic acid and 0.5 ml 2% ninhydrin reagent were added, and then boiled for 10 min. The samples after cooling down were diluted with 4.2 ml 99.5% ethanol, and centrifuged to remove by-products and solids before analyzed by UV-Vis

at 570 nm wave length (Zhu et al., 2009). For the solid sample, using 1 mg to 10 mg solids to mix with 0.1 ml buffer before added the 13.5 M NaOH. The exact amount of the solid used for this analysis depends on the extent of enzyme adsorption. The enzymes associated with the substrate, free enzymes in the filtrate and bound enzymes on the filter paper were measured and calculated as the percentage of the total loaded enzyme to evaluate the mass balance of the enzyme during EH. Using filter paper to conduct the separation is fast, which was usually within 30 sec to 2 min. It was found that a small amount of enzymes, about 1.0%-3.5% total loaded enzymes, absorbed on filter paper. Comparing with the amount of the bound and free enzymes, which were 35-65% of the initial loaded enzymes, the amount of enzymes on filter paper was too small to have significant influence in the distribution of bound and free enzymes.

SDS-PAGE

Precast polyacrylamide 4-20% precise protein gels, ImmunoPure lane marker reducing sample buffer, BupH tm) Tris-HEPES-SDS Running Buffer, Imperial Protein Stain, and Fisher BioReagents EZ-Run Protein Marker were all purchased from Fisher Scientific (Fisher Scientific, Suwanee, GA). A drying agent was prepared by mixing glycrol, ethanol, and Milli-Q water at 3%, 20%, and 77% by volume, respectively.

Sample liquid of 200 µl, including the diluted original cellulase solution, the hydrolysis supernatant was mixed with 50 µl of the sample buffer. The reducing buffer served as a lane marker denatures the enzyme proteins and the pink color of this buffer assists to visualize the progress of the electrophoresis run by monitoring the location of the front by pink dye. The

prepared mixtures were incubated for 5 min at 95°C to denature the proteins. After cooling the samples to room temperature, 15 µl samples were placed in gel wells, while 10 µl of denatured protein marker was placed into the first well. The electrophoresis experiment was conducted in a Hoefer SE 260 mini-vertical gel electrophoresis unit (Hoefer Inc, Holliston, MA). The unit tank was filled with running buffer before starting the experiment. One gel was run in each time at 150 V (constant voltage), 240 mA initial current, and approximately 50 min of running time until the front indicator of the samples reached about 0.5 cm above the bottom of the gel. Then the gel was rinsed in Milli-Q water for 20 min by gently shaking in a shaker bath to remove the running buffer. After that, the gel was stained using Imperial blue stain for 2 hours with gentle shaking. The stained gel was again soaked in Milli-Q water with gentle shaking for overnight to reduce the background. For a storage purpose, the gel was dehydrate for 30 min by soaking in the drying agent, followed by was sealing the gel between two wet cellophanes and clamped by drying kit until dried. The dried gel was scanned, and the image was analyzed using Image-Pro Plus software (Hu et al., 2010).

Sugars analysis

The concentration of cellobiose, glucose, xylose, galactose, arabinose and mannose were determined using a high performance liquid chromatography system (HPLC, Agilent technology 1200 series, Palo Alto, CA). The column system of HPLC consisted of a Shodex SP0810 column and a de-ashing cartridge filter pre-column. Liquid samples were filtered through 0.2 µm filter before analysis. Milli-Q water was used at a flow rate of 0.5 ml/min. The hydrolysis yield of the substrate was evaluated by carbohydrate conversion, which was

defined as the amount of hydrolyzed carbohydrate in the enzymatic hydrolysates as a percent of total carbohydrate in the starting substrate for enzymatic hydrolysis. Two parallel samples were used in all analysis, and the data were presented as the mean of the duplicates.

Results and Discussion

Protein mass balance after pre-EH

Three substrates of Avicel, BHW and GLHW were subjected to pre-EH with 5 FPU/g carbohydrates for 20 min, 3 h, 12 h, and 48 h, respectively. After pre-EH, the liquid and solid were separated by filtration, and the protein of enzymes associated with substrate and the free enzymes in the filtrate were determined by ninhydrin assay. The proteins of enzymes associated with substrates are consisted of the proteins of bound enzymes and a small amount of free enzymes within substrate because the substrate residues after filtration was wet that some moisture was existed to keep the consistency of the substrate residues in a certain level. As shown in Table 5.2, in the case of Avicel, the amount of proteins of bound enzymes, free enzymes within substrate residues in the liquid after 20 min were 44.4 mg and 4.7 mg, respectively. With the progress of the pre-EH, the amount of the protein of bound enzymes increased a little to 45.3 mg at 3 h, and then decreased continuously to 34.3 mg at 48 h. The portions of the proteins of enzymes associated with substrates were in the range of 36.2-47.3%, while the proteins of free enzymes in the filtrate were between 49.0-62.5%.

Compared to Avicel, BHW reached the peak of enzyme adsorption which was 62.3 mg proteins of bound enzymes in 20 min, and then the amount of bound enzyme decreased, and 51.9 mg proteins of bound enzymes were detected at 48 h. The portions of the proteins of

enzymes associated with substrates and the free enzymes in the liquid were distributed as 51.6%-66.5% and 30.9%-46.4%, respectively. This indicated that the maximum adsorption of enzymes on BHW was very fast which was within 20 min, and BHW had stronger affinity with the enzymes than that of Avicel.

Table 5.2. The amount of bound and free enzymes characterized by protein content on Avicel, BHW and GLHW residues after Pre-EH 20 min, 3 h, 12 h and 48 h.

Sample	Pre-EH, h	Enzymes associated with substrate, mg		Substrate residue, g	Enzyme associated with substrate, mg/g	Enzyme associate with substrate, %	Free enzyme, %	Enzyme on filter paper, %	Mass balance, %
		Bound enzyme, mg	Associated not bound enzyme, mg						
Avicel	0.3	44.4	4.7	9.6	5.1	46.2	52.5	2.2	100.9
	3	45.3	4.9	9.0	5.6	47.3	49.0	3.5	99.8
	12	38.7	5.2	8.1	5.4	41.4	55.0	1.4	97.8
	48	34.3	4.2	7.0	5.5	36.2	62.5	2.1	100.8
BHW	0.3	62.3	8.5	9.8	7.2	66.6	30.9	1.9	99.4
	3	55.6	5.5	8.4	7.3	57.5	36.5	1.4	95.4
	12	53.1	5.2	4.9	11.9	54.9	46.4	3.0	104.3
	48	51.9	2.9	3.0	18.3	51.6	43.3	1.2	96.1
GLHW	0.3	34.5	6.8	9.3	4.4	50.4	39.6	1.1	91.1
	3	47.3	2.5	7.8	6.4	60.7	30.5	2.6	93.8
	12	44.3	4.0	6.4	7.5	58.8	37.0	3.6	99.4
	48	42.7	2.6	5.6	8.1	55.2	40.4	1.2	96.8

The case of GLHW, the adsorption of enzymes was slower than both Avicel and BHW, only 34.5 mg of the protein of bound enzymes adsorbed on substrate after 20 min hydrolysis. The maximum adsorption occurred at 3 h which as 47.3 mg protein of bound enzymes, and then the amount of the proteins of bound enzymes decreased until 42.7 mg at 48 h. The portions of the proteins of enzymes associated with substrates and free enzymes in the filtrate were varied from 50.4-60.7% and 30.5-40.4%, respectively. When considering the ratio of

the proteins of enzymes associated with substrate residues (mg/g) as shown in Table 5.2, it was interesting to observe that this ratio had no significant variations from 20 min to 48 h pre-EH, which was between 5.1 to 5.6 mg/g. However, for the other two substrates, the ratio of bound enzyme on substrate residues increased a lot from 20 min to 48 h, which were from 7.2 mg/g to 18.3 mg/g and 4.4 mg/g to 8.1 mg/g for BHW and GLHW, respectively. This observation indicated that the pulp structure of BHW and GLHW could retain much more enzymes that the simple crystalline structures of Avicel particles. It was speculated that the adsorption of enzyme on Avicel mostly covered on the surface of the particle, while the enzyme not only adsorb on the surface but maybe also trapped in the structure of pulps and therefore resulted in much higher ratio of the proteins of bound enzymes on substrate.

Enzymatic hydrolysis of biomass by enzymes associated with substrates

After pre-EH, the same amount of fresh buffer without enzymes were added in to the wet substrate residues of 20 min, 3 h, 12 h, 48 h pre-EH. The volume of the added fresh buffer was the same as the volume of the filtrate. Therefore, the carbohydrate conversion of the subsequent enzymatic hydrolysis entirely relied on the bound enzymes along with the small amount of free enzymes within the substrate residues. The hydrolysis performance of the enzymes associated with substrates is shown in Figure 5.1. The total carbohydrate conversion was a summary of the carbohydrate conversion from pre-EH and the carbohydrate conversion from the subsequent hydrolysis using enzymes associated with substrates only.

In the case of Avicel as shown in Figure 5.1a, it was found that compared with enzymatic hydrolysis without pre-EH, the kinetics of hydrolysis with pre-EH 20 min was significant lower and ended with about 20% lower carbohydrate conversion after 144 h, while the other cases were very close with no pre-EH. For BHW, the curve of the kinetics of hydrolysis with pre-EH 12 h was almost overlapped with the case of no pre-EH. The hydrolysis with pre-EH 48 h was about 5% higher in terms of the kinetics of carbohydrate conversion than no pre-EH after 48 h reaction time. In contrast, the hydrolysis behavior of pre-EH 3 h and pre-EH 20 min were quite similar, and both of them were about 20% lower in carbohydrate conversion than no pre-EH. GLHW shows close hydrolysis of no pre-EH, pre-EH 12 h and pre-EH 48 h, while pre-EH 3 h was 5% lower than no pre-EH and pre-EH 20 min was even lower which resulted in over 10% lower of carbohydrate than no pre-EH. Comparing the carbohydrate conversions of all the three substrates, it was noted that ever since 12 h pre-EH was conducted, the total carbohydrate conversion became very close as the hydrolysis without pre-EH regardless of the types of substrates.

However, if the pre-EH was only conducted for 20 min, the total carbohydrate conversion was about 20% lower than that without pre-EH for all of the three substrates. The total conversion of 3 h pre-EH was dependent on the type of substrates. For Avicel, the total carbohydrate conversion with 3 h pre-EH was very close to that with 12 h pre-EH, while it was almost the same as that with 20 min pre-EH for BHW. For GLHW, it was in the middle between 20 min and 12 h pre-EH. All of these observations indicated that after a certain time of enzymatic hydrolysis, the free enzymes in the liquid phase became insignificant. The almost same total carbohydrate conversion yield can be achieved by replacing the free

enzyme and buffer with the only the fresh buffer. Almost the same amount of sugars could be obtained through this technology, although more volume of buffer was needed. It appears to imply that the enzyme initially adsorbed onto the cellulose was primarily responsible for biomass digestions. The total hydrolysis yield could be as good as the hydrolysis without removing the supernatant by replacing the filtrate with buffer at an optimal time.

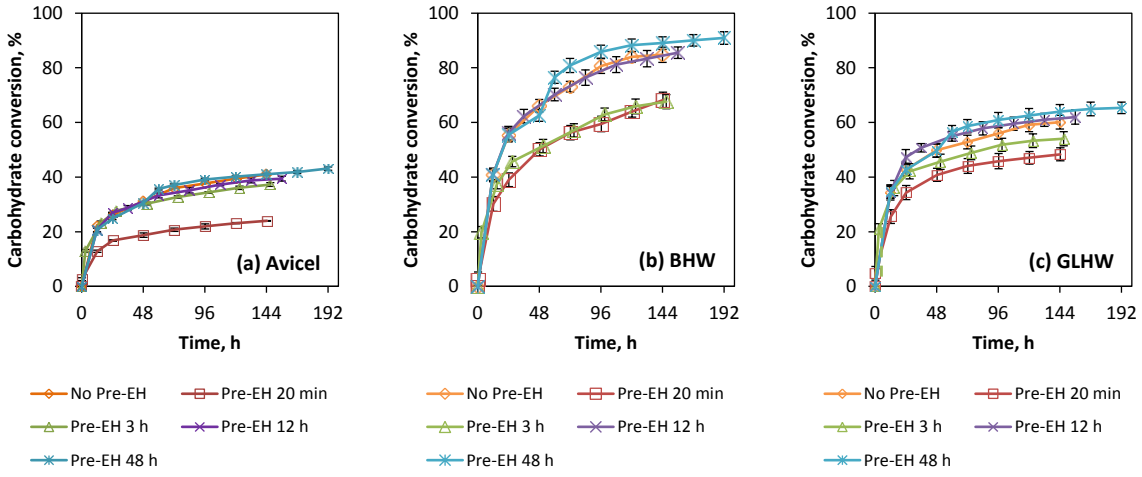


Figure 5.1. Total carbohydrate conversion by pre-EH and subsequent enzymatic hydrolysis by bound enzyme of (a) Avicel, (b) BHW and (c) GLHW. The total carbohydrate conversion is the summary of the carbohydrate conversion of pre-EH for 0 h, 20 min, 3 h, 12 h, 48 h and the carbohydrate conversion for up to 144 h by bound enzyme after separating the bound and free enzyme.

Similar experimental observations have been in the past research (Lee & Fan, 1983; Mandels et al., 1971). For a long time, it is believed that the dynamic equilibrium of bound enzymes and free enzymes in a hydrolysis system is important for an effective digestion.

However, the recognition of the contribution of the enzymes associated with substrates which mostly contains the bound enzymes challenged the function of equilibrium of enzymes during hydrolysis reaction. Although it was noted that the separated solid residues after pre-EH were wet with a consistency of 30%-40%, this small amount of liquid containing non-adsorbed enzyme (i.e. β-glucosidase) with and around the residues. However, this amount of free enzymes within the substrates was quite small as shown in Table 5.2, which was not sufficient to challenge the importance of bound enzymes. The associations of bound enzymes and the free enzymes within the substrates are enough for processing the ongoing hydrolysis as well as having both bound and free enzymes in the system. Therefore, the effective digestion of biomass by the enzymes associated with substrate offers a fundamental support for a promising application to realize a continuous digestion by conducting multi-stage EH. The details of the related application were under process.

Enzymatic hydrolysis by free enzyme

Besides the enzymes associated with the substrates, the fact that about 30.5%-62.5% of the initially loaded enzymes were in the free enzymes also provided a potential for the enzymes recycling (Table 5.2). It is expected that this amount of free enzyme still can convert a significant amount of substrate to sugars. In order to evaluate the hydrolysis of the free enzymes from filtrate, fresh substrates of Avicel, BHW and GLHW were mixed with the correspondent filtrate from the pre-EH of 20 min, 3 h, 12 h and 48 h with 5% consistency and a total of 144 h hydrolysis time. The hydrolysis efficiency by of free enzyme was shown in Figure 5.2. Unfortunately, the carbohydrate conversions by the free enzymes were much

lower than the expected conversion. For example, the initial loaded enzyme without pre-EH ended at 41.0% carbohydrate conversion of Avicel after 144 h reaction, but the free enzyme after 20 min pre-EH containing 52.5% initial loaded enzymes only obtained 9.6% carbohydrate conversion at 144 h. The free enzyme of 48 h pre-EH of Avicel even had 66.5% of initial loaded enzymes, but the 144 h carbohydrate conversion was barely 3.1%. Although the recalcitrance of different substrates could be very different, similar trends were observed in the case of BHW and GLHW. The effectiveness of free enzyme was in a decreasing order by BHW, GLHW and Avicel.

In addition, with the increasing time of pre-EH, the hydrolysis ability of free enzyme was getting worse regardless of the amount of free enzyme. This implies that the recycling of the free enzyme would not be very helpful for the new batch of enzymatic hydrolysis by using the current enzyme cocktails and substrates. Due to the fact that the sugars in the hydrolysates were not separated away from the free enzymes, the first concern of the low hydrolysis ability of the free enzyme was originated from the sugar inhibition. Nevertheless, the sugar concentration was quite low in the hydrolysates of 20 min pre-EH, which was only 1.5 g/l, 1.4 g/l and 2.1 g/l of Avicel, BHW and GLHW, respectively. Such a low concentration of sugar was not like to cause a significant sugar inhibition, but even the free enzymes from 20 min pre-EH had very low hydrolysis ability. These results indicated that the characteristics of the free enzymes rather than the quantities of the free enzymes or product inhibition probably play a more important role in determining the hydrolysis efficiency of the free enzymes. In addition, these characteristics of free enzyme were somehow related to the pre-EH time of the free enzymes.

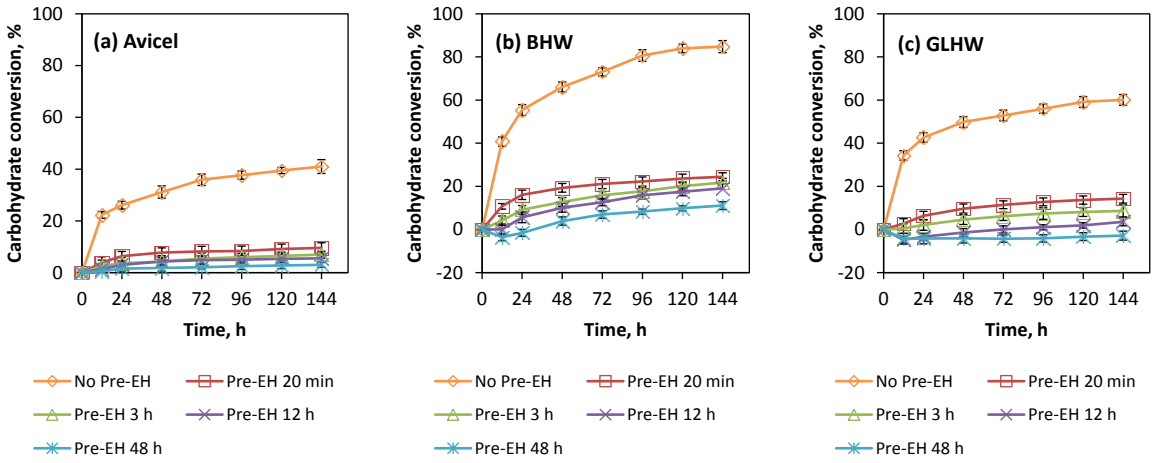


Figure 5.2. Total carbohydrate conversion by free enzyme after Pre-EH for (a) Avicel, (b) BHW, and (c) GLHW. The free enzyme is separated from bound enzyme after Pre-EH for 0 h, 20 min, 3 h, 12 h, and 48 h.

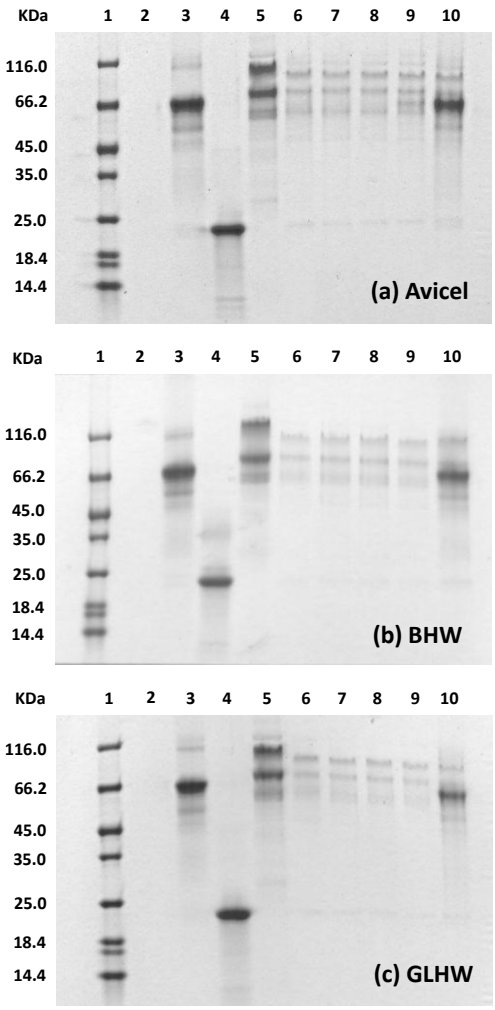
Characterization of free enzymes by SDS-PAGE

In order to investigate the characteristics of the free enzyme that limit their hydrolysis ability, the components of the initial added enzymes and free enzymes after pre-EH were characterized by SDS-PAGE experiment as shown in Figure 5.3. In all the three images of Figure 5.3, lane 3 to 5 shows the components of cellulase, xylanase and β-glucosidase, respectively. The major components in cellulase, xylanase and β-glucosidase are within a molecular weight of about 65 KDa, 20 KDa and 70-110 KDa. The compositions of the initially loaded enzymes were shown in Lane 10 in each of the images. The images composed exclusively of shades of gray varying from black at the strongest intensity to white at the weakest intensity. The scale ranges from 0 to 255, while 0 is black and 255 are white.

The higher intensity, which correlated with lower grayscale value, indicates higher concentrate of the proteins.

Comparing with intensities of the components in the initially loaded enzymes and the free enzymes, the concentration of one of the major cellulase components, which molecular weight was about 65 KDa, was significantly decreased in the free enzymes asfter pre-EH as shown in the lane 6-9 in each image of Figure 5.3. Another cellulase component which appears at about 50 KDa was also decreased. According to literatures with more specific analysis, the major components of cellulase at 65 KDa were CBH (Chundawat et al., 2011). CBH has a cellulose binding module (CBM) which is non-catalytic module but it has the function to locate and bind to the active surface of cellulose and introduce enzyme proteins to hydrolyze the substrates. Therefore, the decreased concentration of CBH was very likely due to the adsorption of CBH on the substrate. In the cases of pulps, there were no significant differences among the CBH concentrations in the free enzyme of BHW with difference pre-EH time which grayscales were from 222 to 227. However, for GLHW, the CBH concentrations seem decreased with the increasing of pre-EH time which grayscales were increased from 203 to 222. These results indicated that there were less extents of desorption of CBH occurred in the pre-EH of pulps compared with that of Avicel, even though the amount of substrate residue decreased. It again indicated that the pulp structures of BHW and GLHW might trap CBH or there might be more irreversible adsorption of CBH within the pulp. Considering the behavior of bound enzymes and free enzymes, in order to guarantee the effectiveness of bound enzymes and to reserve high hydrolysis ability of free enzyme, the separation time of bound and free enzyme should be no later than 12 h of pre-EH. More

strategies are needed to be developed for improving the hydrolysis ability of free enzymes based on enhancing the synergism effect of different enzyme components.



Comparison of cellulase major component (65 KDa)

Lane No.	Grayscale
6	207 ± 6
7	209 ± 3
8	208 ± 2
9	178 ± 14
10	81 ± 7

Comparison of cellulase major component (65 KDa)

Lane No.	Grayscale
6	227 ± 1
7	222 ± 2
8	222 ± 2
9	223 ± 1
10	109 ± 4

Comparison of cellulase major component (65 KDa)

Lane No.	Grayscale
6	203 ± 2
7	214 ± 1
8	219 ± 2
9	222 ± 2
10	117 ± 9

Figure 5.3. SDS-PAGE analysis of free enzyme of (a) Avicel, (b) BHW and (c) GLHW. Lane 1: protein marker; 2: sodium acetate buffer; 3: cellulase: 4: xylanase; 5: β-glucosidase; 6: Pre-EH 20 min; 7: Pre-EH 3 h; 8: Pre-EH 12 h; 9: Pre-EH 48 h; 10: Initial loaded enzyme (No Pre-EH).

Table 5.3. The amount of bound and free enzymes characterized by protein content on Avicel, BHW and GLHW residues after Pre-EH 12 h without adding tween 80, Pre-EH 12 h with tween 80 added 20 min before separation and Pre-EH 12 h with tween 80 added initially.

Sample	Pre-EH, h	Proteins of enzymes associated with Avicel residue, mg/g		Enzyme associated with substrate residue, mg/g	Enzyme associated with substrate, %	Free enzyme, %	Enzyme on filter paper, %	Mass balance, %
		Bound enzyme, mg	Enzyme associated with substrates, mg					
Avicel	12 h w/o T80	38.7	5.2	5.6	41.4	55.0	1.4	97.8
	12 h w/ T80 for 20 min	37.3	3.6	5.2	38.5	54.0	3.0	95.5
	12 h w/ T80	32.0	4.1	4.5	34.0	61.6	4.3	99.9
BHW	12 h w/o T80	53.1	5.2	9.6	54.9	46.4	3.0	104.3
	12 h w/ T80 for 20 min	42.8	4.5	7.9	44.6	54.0	2.8	101.4
	12 h w/ T80	33.1	5.4	6.5	36.2	64.2	2.8	103.2
GLHW	12 h w/o T80	43.1	5.2	7.0	58.8	37.0	3.6	99.4
	12 h w/ T80 for 20 min	36.1	3.2	5.8	47.9	44.0	3.7	95.6
	12 h w/ T80	32.5	3.6	5.4	44.0	50.1	3.8	97.9

The effect of Tween 80 on hydrolysis ability of bound enzymes and free enzymes

Previous research has shown that adding non-ionic surfactants such as Tween 80 and Tween 20 can effectively improve the efficiency of enzymatic hydrolysis of various

substrates including pure cellulose and lignocellulosic materials (Tu & Saddler, 2009; Yang et al., 2011). The mechanisms of the improvement of enzymatic hydrolysis by the addition of surfactant have been attributed from three categories: (1) the surfactants can reduce the unproductive binding of enzymes to lignin due to hydrophobic interaction of surfactant with lignin on the lignocellulose surface (Tu & Saddler, 2009), (2) the surfactants increase the stability of enzymes. It has been reported that the enzymes are deactivated or denatured at the air-liquid interface, and this effect could be greatly enhanced by the shear forces during rotation (Elias & Joshi, 1998). Surface active agents such as surfactants are effective in reducing the extent of enzyme deactivation by decreasing the concentration of enzymes at the air-liquid interface (Kim et al., 1982). (3) The surfactants change the ultra-structure of the substrate, making the substrate more available to enzyme attack (Tu et al., 2009). In the past work, most of the research focused on adding surfactant in batch enzymatic hydrolysis and testing its effect on the improvement of enzymatic hydrolysis. It has also been reported that non-ionic surfactants potentially promote enzyme recycle during enzymatic hydrolysis of lignocellulose (Tu & Saddler, 2009). Therefore, Tween 80 was used in this study to test its function for enhancing the hydrolysis of bound and free enzymes.

However, for the carbohydrate conversion of free enzyme from BHW and GLHW, Tween 80 had marginal advantages. From Figure 5.5b BHW and Figure 5.5c GLHW, the addition of Tween 80 had very limited effect on the hydrolysis by free enzymes, which was completely different from that of Avicel. BHW is relatively an easier digestive substrate because its cellulose has more amorphous region than Avicel, and there is no lignin interference. The initial loaded enzyme obtained 84.8% carbohydrate conversion at 144 h,

while the free enzyme of pre-EH 12 h only made 20.5% carbohydrate conversion at 144 h. With the addition of Tween 80, the hydrolysis by free enzyme did not have significant improvement. The 144 h carbohydrate conversion by free enzyme of Pre-EH 12 h w/T_20 min and free enzyme of Pre-EH 12 h w/T only reached 24.2 % and 26.8%, respectively. GLHW, which was also a substrate of hardwood, had 16.8% lignin content. The effect of Tween 80 on free enzyme in this case was even more limited. The 144 h carbohydrate conversion was 60.1% for initial loaded enzyme, while it was only 7.7% by free enzyme from pre-EH 12 h. The 144 h carbohydrate conversion by free enzyme of pre-EH 12 h w/T_20 min and pre-EH 12 h w/T were 11.0% and 11.6%, respectively. Although the trends of the effect of Tween 80 on hydrolysis ability of free enzyme of BHW and GLHW were the same as that in Avicel, the extents of improvement for BHW and GLHW were much less.

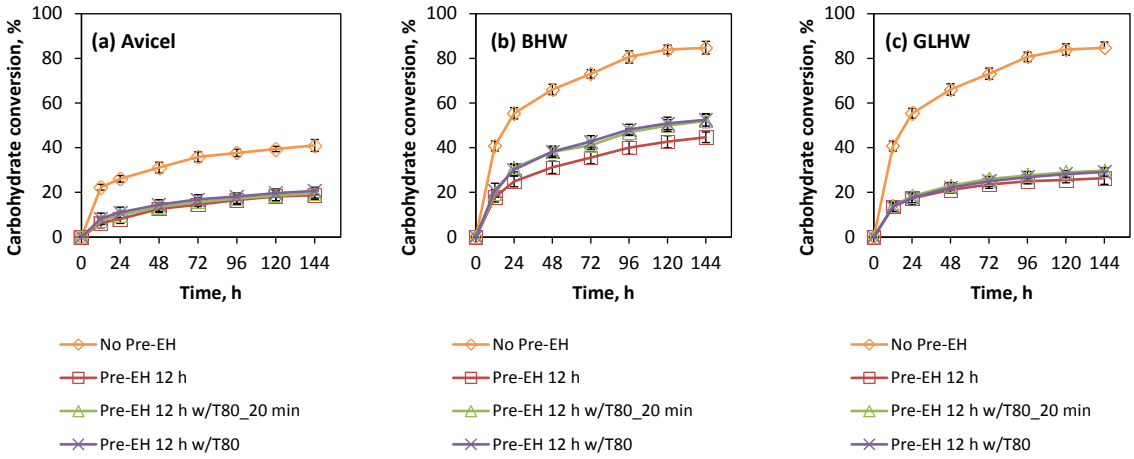


Figure 5.4. Comparison of carbohydrate conversion by Initial added enzyme and bound enzyme of Pre-EH 12 h, Pre-EH 12 h with Tween 80 added 20 min before separation and Pre-EH with Tween 80 from (a) Avicel, (b) BHW and (c) GLHW.

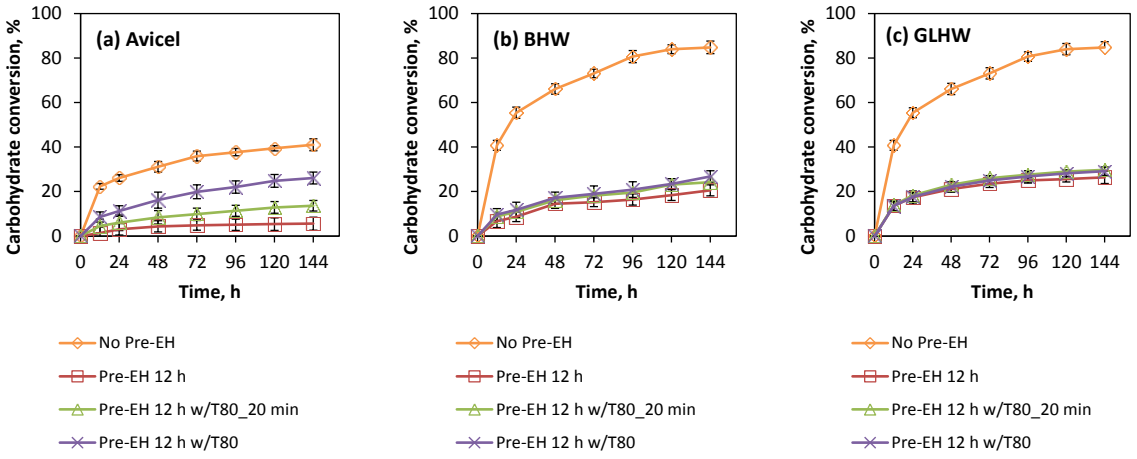
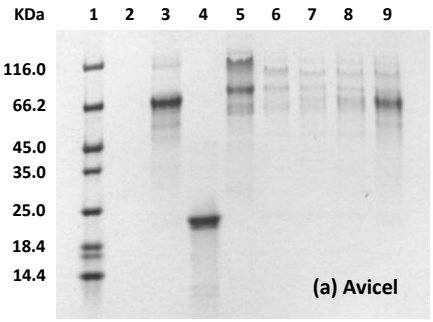


Figure 5.5. Comparison of carbohydrate conversion by free enzyme of No Pre-EH, Pre-EH 12 h, Pre-EH 12 h with Tween 80 added 20 min before separation, and Pre-EH 12 h with Tween 80 from (a) Avicel, (b) BHW and (c) GLHW.

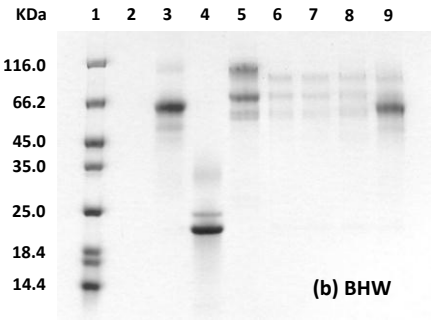
Characterization of free enzymes from filtrate of 12 h pre-EH with addition of Tween 80 by SDS-PAGE

It is clear that the addition of Tween 80 had more or less influence on the hydrolysis by free enzyme. In order to explain this phenomenon, the components of the free enzymes have analyzed by SDS-PAGE experiment as shown in Figure 5.6. It was found that the concentration of CBH at 65 KDa had a significant changes compared with the initial loaded enzyme of all of the three substrates. With the addition of Tween 80 at the beginning of pre-EH, more CBH were kept in the free enzyme which was in the order of pre-EH 12 h w/T80, pre-EH 12 h w/T80_20 min and pre-EH 12 h.



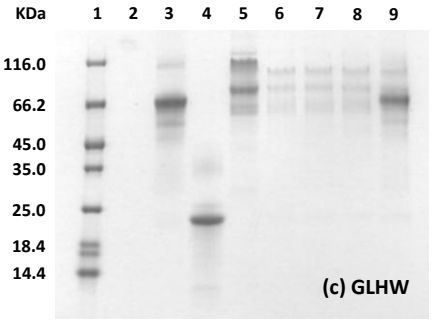
Comparison of cellulase major component (65 KDa)

Lane No.	Grayscale
6	215 ± 2
7	210 ± 2
8	196 ± 4
9	123 ± 10



Comparison of cellulase major component (65 KDa)

Lane No.	Grayscale
6	234 ± 2
7	233 ± 2
8	222 ± 2
9	113 ± 6



Comparison of cellulase major component (65 KDa)

Lane No.	Grayscale
6	231 ± 2
7	223 ± 1
8	214 ± 1
9	133 ± 2

Figure 5.6. SDS-PAGE analysis of free enzyme of pre-EH with and without Tween 80 from (a) Avicel, (b) BHW and (c) GLHW. Lane 1: protein marker; 2: sodium acetate buffer; 3: cellulase; 4: xylanase; 5: β-glucosidase; 6: Pre-EH 12 h; 7: pre-EH 12 h with Tween 80 added 20 min before separation; 8: pre-EH 12 h with Tween 80; 9: Initial loaded enzyme.

It was known that CBH, including CBH I and CBH II attach from the reducing end and non-reducing end of cellulose chain (Ding & Xu, 2004). CBH I prefers to attach from the

chain ends of crystalline cellulose (Chundawat et al., 2011; Lehtio, 2003). Given Avicel is highly crystalline cellulose which crystallinity index was up to 85 (Yu et al., 2011), and its crystallinity index was constant with respect to the progressing of hydrolysis time (Yu et al., 2012) The increasing of CBH was significant favorable for enhancing the hydrolysis ability of free enzyme on Avicel substrate. However, this advantage was not remarkable in the case of BHW and GLHW probably because the crystallinity was not a major factor that governs their recalcitrant nature. Therefore, other reasons which may be related to the different architecture and different chemical components of BHW and GLHW are possible for the limited improvement of hydrolysis ability of their free enzymes.

Conclusions

Enzyme initially adsorbed on the substrate was primarily responsible for enzymatic hydrolysis. The contribution of free enzymes became insignificant after a certain period of reaction time. Although about half of the initial loaded enzymes were free enzymes, the reactivity of the free enzymes decreased rapidly even after 20 min hydrolysis. A cellulase component CBH I was significantly decreased in the free enzymes, which might be the reason for a low hydrolysis ability of free enzymes.

The addition of non-ionic surfacnatnt, such as Tween 80, is known to improve enzymatic hydrolysis. Thus the effect of Tween 80 on the improving the activity of bound and free enzyme was investigated in this study. It was found that the hydrolysis ability of free enzymes on Avicel was significantly improved by adding surfactant Tween 80, but Tween 80 had limited effect on the bound enzymes. For BHW and GLHW, adding Tween 80 had

marginal improvement for the hydrolysis of both free and bound enzymes. It was evidenced by SDS-PAGE analysis that Tween 80 assisted in releasing more CBH I into the free enzymes, which attack the chain ends of crystalline region. This is a great advantage for the hydrolysis for Avicel (highly crystallized cellulose). However, maintaining more CBH I in the free enzymes was not significantly favorable for the hydrolysis of pulp. This might be due to the more complicated components and structures of lignocellulosic materials, and their difference requirement for the enzyme cocktail.

Acknowledgements

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CHAPTER 6

The investigation of enzyme deactivation by the factors of the hydrolysis conditions

Introduction

The deactivation of cellulase under the hydrolysis condition is an important concern during enzyme production, enzymatic hydrolysis and simultaneous saccharification and fermentation (Charm & Wong, 1970; Elias & Joshi, 1998; Ghadge et al., 2005).

The deactivation of enzymes may occur due to a number of reversible and irreversible processes. The reversible process includes the dissociation and denaturation due to changes in the tertiary structure of enzyme proteins with changes in physical conditions such as pH and salt concentration. The irreversible reactions involve decomposition, aggregation and coagulation which occur due to the changes in the secondary structure or chemical modification of the amino acid residues of the native protein molecule (Elias & Joshi, 1998).

For enzyme deactivation during enzymatic hydrolysis, besides the impact of the biomass substrates, the factors of the hydrolysis conditions such as the liquid component, temperature, and shaking due to hydrodynamic shearing stress may also affect the stability and activity of enzymes and consequently have an important impact on the hydrolysis efficiency. In order to prevent the growing of microorganism that consumes the produced sugars in the hydrolysate, antibiotics, such as sodium azide is usually adeed in the buffer. Whether the presence of antibiotics has negative impact on enzymes is not for sure. The deactivation of enzymes at hydrolysis temperature is another concern. Although cellulase has the maximum activity at

hydrolysis temperature (50 °C), the stability of cellulase is not guaranteed. Thus, cellulase needs to be stored at refrigerator (under 4 °C) for keeping its performance stable (Yu et al., 2012). Shaking or mixing, which aimed to obtain a uniform hydrolysis, is usually applied during enzymatic hydrolysis. However, the generation of hydrodynamic shear stress may break the three dimensional structure of enzymes and make them deactivated. It was reported that the cellulase was deactivated due to shaking in flasks during enzymatic hydrolysis of cellulose carried out in shaking flasks (Basu & Pal, 1956). However, a comparison of the importance of these factors that affect enzyme deactivation was not found in the literatures. The objective of this work is to study the enzyme deactivation by the factors of the hydrolysis conditions: The type of liquid media, incubation temperature, and shaking.

Materials and Methods

Materials

Microcrystalline cellulose (Avicel PH-101) was purchased from Sigma Aldrich (St. Louis, MO). Cellulase (NS50013, 72.1 FPU/g, 103 mg protein/g) and β-glucosidase (NS50010, 196.5 mg protein/g) were kindly provided by Novozymes North America, Inc. (Novozymes, Franklinton, NC).

Enzymes incubation without substrate

Cellulase and β-glucosidase with a weight ratio of 1:0.3 was used to prepare an enzyme cocktail. The loading of enzyme is 5 FPU/g Avicel with 5% consistency. However, no Aivcel was loaded in the mixing conditions before hydrolysis. Three different types of liquid media

were used. They are dionized water, sodium acetate buffer (pH 4.8, 100mM), and sodium acetate buffer (pH 4.8, 100mM) with 0.3% (w/w) sodium azide. The enzymes and liquid media were well mixed and subjected to shaking incubation in an air bath for different time. The incubation temperature was 4 °C and 50 °C, respectively. At 50 °C, the incubation was conducted without mixing and with mixing at 180 rpm, respectively. After incubation, the aggregation of enzymes was observed in some solutions as shown in Figure 6.1. The solution was centrifuged and the protein content in supernatant, which is called free enzymes, was analyzed by ninhydrin assay.

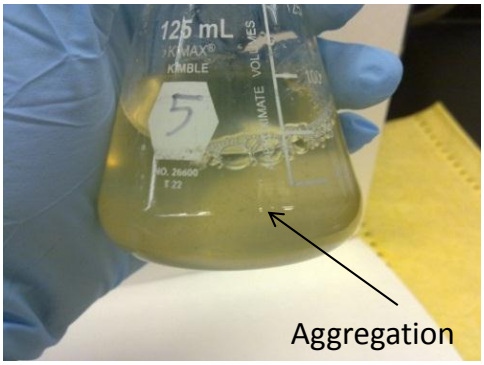

Figure 6.1. The observation of protein aggregation which made the enzyme and buffer solution in a cloudy form.

Avicel hydrolysis by pre-incubated enzymes

The enzyme preparation of cellulase and β-glucosidase with a weight ratio of 1:0.3 was pre-incubated at 50 °C with mixing speed of 180 rpm for 0, 1, 4, 12 and 48 h, respectively. The loading of enzyme was 5 FPU/g Avicel with 5% consistency, although no Aivcel was

loaded in the pre-incubation stage. Before loading of Avicel, a small portion of the pre-incubated solution was used for protein concentration analysis and SDS-PAGE analysis by take the supernatant of the solution after centrifugation.

After the pre-incubation of enzymes, the Avicel substrate is loaded into the incubated enzyme and buffer with 5% consistency, and the hydrolysis was started and lasted for up to 144 h. The hydrolysates were sampled periodically for sugar analysis by HPLC. The process of this experiment is shown in Figure 6.2.

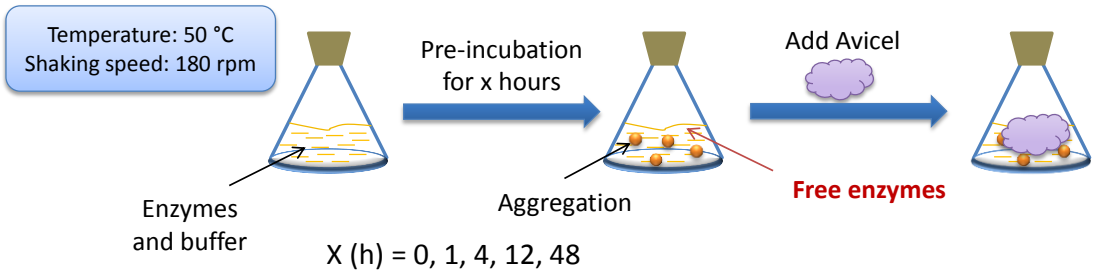

Figure 6.2. Schematic diagram of Avicel hydrolysis with pre-incubated enzymes.

Ninhydrin analysis of enzyme proteins

The protein concentration of enzymes in the liquid was measured by ninhydrin assay analysis. Aqueous bovine serum albumin (BSA) was used as reference standard. Protein solutions of 0.1 ml with a maximum protein concentration of 1 mg/ml were mixed with 0.3 ml 13.5 M NaOH and autoclaved at 121 °C for 20 min for hydrolyzing protein to amino acid completely. After cooling down, 0.5 ml acetic acid and 0.5 ml 2% ninhydrin reagent were added and mixed well, and then boiled for 10 min. The samples after cooling down were

diluted with 4.2 ml 99.5% ethanol, and centrifuged to remove by-products and solids before analyzed by UV-Vis at 570 nm wave length (Zhu et al., 2009).

SDS-PAGE

Precast polyacrylamide 4-20% precise protein gels, ImmunoPure lane marker reducing sample buffer, BupH tm) Tris-HEPES-SDS Running Buffer, Imperial Protein Stain, and Fisher BioReagents EZ-Run Protein Marker were all purchased from Fisher Scientific (Fisher Scientific, Suwanee, GA). A drying agent was prepared by mixing glycrol, ethanol, and Milli-Q water at 3%, 20%, and 77% by volume, respectively.

Sample liquid of 200 µl, including the diluted original cellulase solution, the hydrolysis supernatant was mixed with 50 µl of the sample buffer. The reducing buffer served as a lane marker denatures the enzyme proteins and the pink color of this buffer assists to visualize the progress of the electrophoresis run by monitoring the location of the front by pink dye. The prepared mixtures were incubated for 5 min at 95 °C to denature the proteins. After cooling the samples to room temperature, 15 µl samples were placed in gel wells, while 10 µl of denatured protein marker was placed into the first well. The electrophoresis experiment was conducted in a Hoefer SE 260 mini-vertical gel electrophoresis unit (Hoefer Inc, Holliston, MA). The unit tank was filled with running buffer before starting the experiment. One gel was run in each time at 150 V, 240 mA initial current, and approximately 50 min of running time until the front indicator of the samples reached about 0.5 cm above the bottom of the gel. Then the gel was rinsed in Milli-Q water for 20 min by gently shaking in a shaker bath to remove the running buffer. After that, the gel was stained using Imperial blue stain for 2

hours with gentle shaking. The stained gel was again soaked in Milli-Q water with gentle shaking for overnight to reduce the background. For a storage purpose, the gel was dehydrate for 30 min by soaking in the drying agent, followed by was sealing the gel between two wet cellophanes and clamped by a SDS-PAGE drying kit for overnight until dried. The dried gel was scanned, and the image was analyzed using Image-Pro Plus software (Hu et al., 2010).

Analysis of sugars

The concentration of cellobiose, glucose, xylose, galactose, arabinose and mannose were determined using a high performance liquid chromatography system (HPLC, Agilent technology 1200 series, Palo Alto, CA). The column system of HPLC consisted of a Shodex SP0810 column and a de-ashing cartridge filter pre-column. Liquid samples were filtered through 0.2 μm filter before analysis. Milli-Q water was used at a flow rate of 0.5 ml/min. The hydrolysis yield of the substrate was evaluated by carbohydrate conversion, which was defined as the amount of hydrolyzed carbohydrate in the enzymatic hydrolysates as a percent of total carbohydrate in the starting substrate for enzymatic hydrolysis. Two parallel samples were used in all analysis, and the data were presented as the mean of the duplicates.

Results and Discussion

The impact of liquid media, temperature and shaking on enzymes stability

The effect of buffer component, temperature and shaking on enzyme stability was investigated, and the results are shown in Figure 6.3. In the three types of liquid media, the influence of different media type had marginal impact on enzymes stability. The presence of

sodium acetate and sodium azide had no negative impact on the protein content of free enzyme. On the contrary, this media is slightly more favorable for maintaining the free enzymes under shaking conditions at 50 °C. About 2% higher of free protein content was observed in the case of enzymes incubation with sodium acetate buffer and sodium azide than the other two cases. Compared to the impact of liquid media on enzymes stability, the incubation temperature and mixing had more significant influence. In a course of 24 h incubation, the protein content of free enzyme at 4 °C had no change, whereas the incubation at 50 °C resulted in a minor loss of enzyme proteins. In contrast, when enzymes were incubated at 50 °C, the content of free enzymes dropped quickly as a function of time, especially in the first 6 h. This observation indicated that the mixing during the hydrolysis condition is a major concern for enzyme instability.

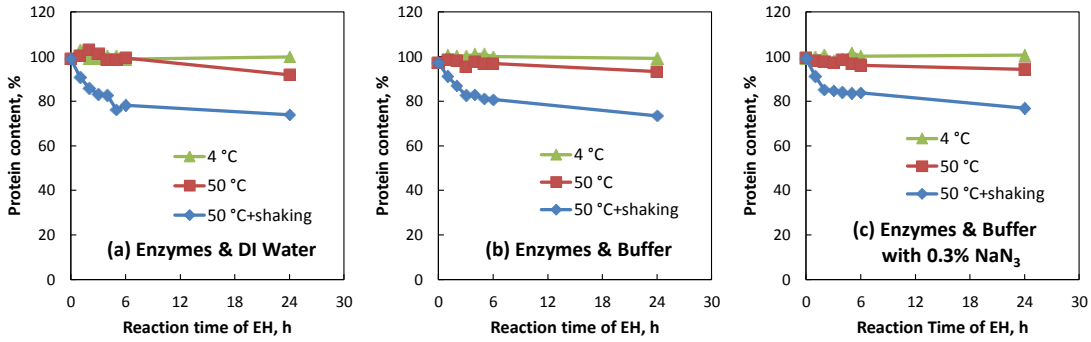

Figure 6.3. The changes of proteins of free enzyme at 4 °C, 50 °C, and 50 °C with 180 rpm shaking in the media of (a) deionized water, (b) buffer (pH 4.8, 100 mM), and (c) sodium acetate buffer (pH 4.8, 100mM) with 0.3% sodium azide. The starting enzyme loading was 20 FPU/g Avicel at a consistency of 5% without loading of Avicel.

The impact of pre-incubation of enzymes on Avicel hydrolysis

Although some precipitations were observed during the pre-incubation with mixing, it is not clear if they have been deactivated. Thus, the Avicel substrate with 5% consistency was loaded into enzyme and buffer solutions which were previously incubated with 180 rpm shaking at 50 °C for 0, 1, 4, 12, 48 h. The hydrolysis kinetics was conducted. A sever decrease of carbohydrate conversion was observed after 4 h shaking incubation prior to hydrolysis as shown in Figure 6.4a. The hydrolysis ability of enzymes which were incubated with shaking for 0 to 4 h before hydrolysis was not significant changed, for the 144 h carbohydrate conversions stayed around 40%. However, a longer shaking incubation prior to hydrolysis resulted in a significant deactivation of the enzymes. The 144 h carbohydrate conversion decreased to 36.8% and 29.4% when the applied enzymes were previous incubated with shaking at 50 °C for 12 h and 48 h, respectively (Figure 6.4). On the other hand, Figure 6.4b indicated that the enzyme deactivation possessed a major impact on the initial hydrolysis rate (average hydrolysis in the first 24 h) whereas no significant change in hydrolysis rate after 24 h was observed. This observation indicated that the deactivation of enzymes had the most important impact on the initial hydrolysis rate. The later hydrolysis rate is mainly governed by any other factors such as substrate characteristics.

In addition, as shown in Figure 6.5, a linear correlation was observed between the proportion of free enzymes and the initial hydrolysis rate implying that the free enzymes which stayed in the liquid were primarily responsible for the hydrolysis of Avicel, while the precipitated enzyme proteins were subjected to a sever deactivation and could not contribute

an adequate function for hydrolysis. With the progressing of the incubation time, the enzymes which form aggregation were deactivated in a greater degree.

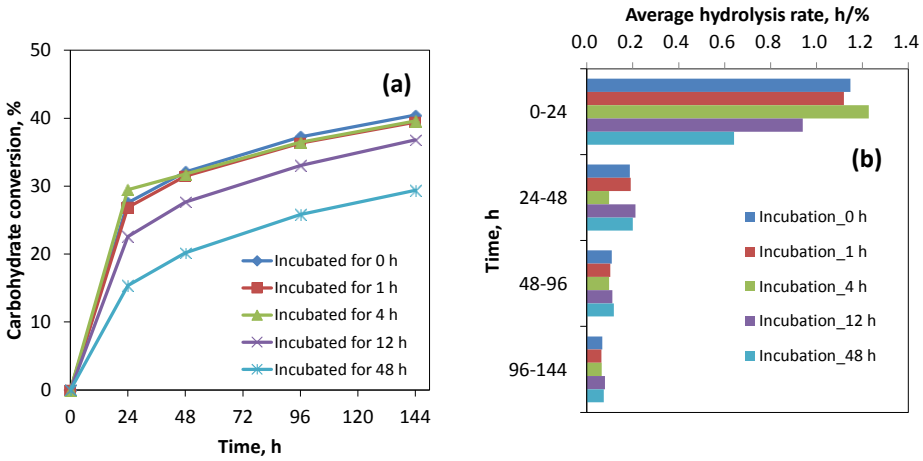

Figure 6.4. The effect of deactivated enzymes on hydrolysis (a) hydrolysis kinetics, and (b) average hydrolysis rate.

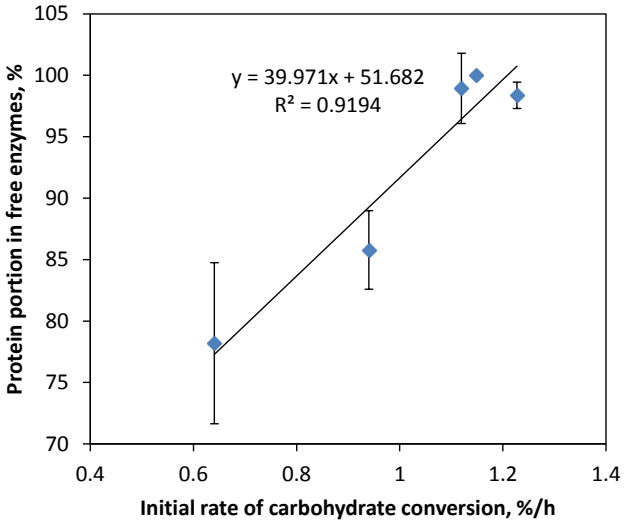

Figure 6.5. Effect of thermal and shearing on the hydrolysis ability of enzymes.

Furthermore, in order to know if the shaking is detrimental to a certain type of enzymes (cellulase or β-glucosidase) or all the enzyme components, the changes of enzyme composition in the free enzymes after shaking incubation were determined by SDS-PAGE, and the analysis was shown in Figure 6.6. It can be visually observed that the intensity of the protein bands of enzyme was decreased with the increasing of shaking time (Figure 6.6a). For a more precise determination of the changes of protein content, the intensity of the protein bands on SDS-PAGE gels were converted to spectra using Image Pro Plus software as shown in Figure 6.6b. For the protein concentration of cellulase, it was transformed to the information of grey level. The colors from black to white were divided to 255 parts. The value of 0 is means black and the value of 255 corresponds of white. The increasing value of gray level indicated the color of protein band is getting lighter and the protein concentration is decreased. Thus, for cellulase, it is clear that the cellulase in free enzymes decreased with the progress of pre-incubation time. The intensity of protein bands was converted to spectrum for a more close investigation of the changes of each protein components in the free enzymes. In In Figure 6.6b, each peak represents one component of enzyme protein. Compare with the spectrum of enzyme solution at 0 h, it was found that the concentration of each component of both cellulase and β-glucosidase proteins were reduced with the increasing of pre-incubation time. The loss of enzyme proteins was in the protein aggregations. Therefore, it is assured that shaking had a wide impediment on the stability of all the enzyme proteins.

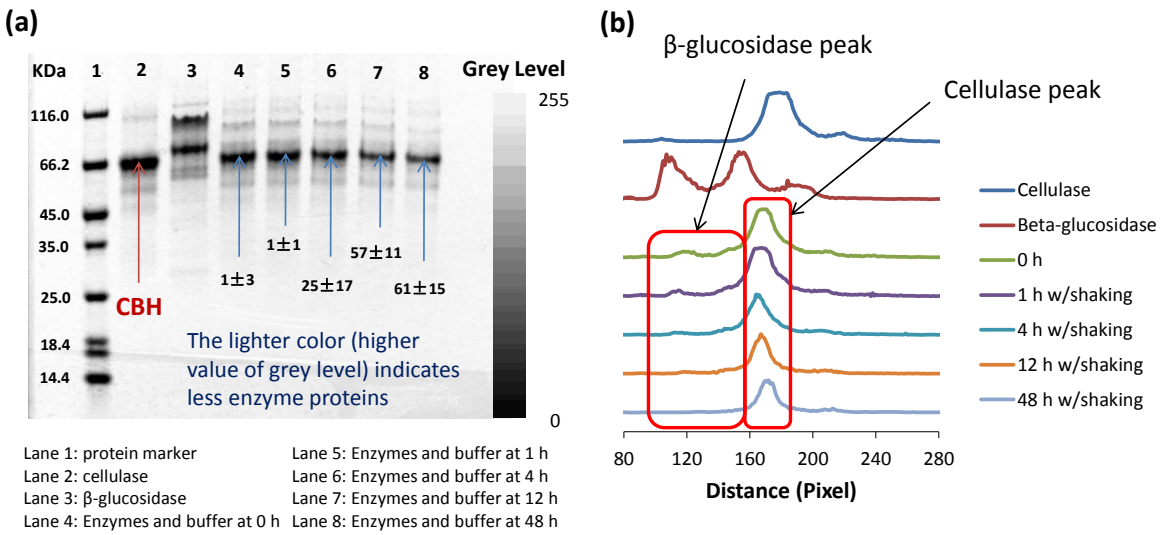

Figure 6.6. Analysis of the components of free enzyme after shaking and incubation by SDS-PAGE. Lane 1: protein marker; Lane 2: cellulase; Lane 3: β-glucosidase; Lane 4: 0 h shaking incubation; Lane 5: 1 h shaking incubation; Lane 6: 4 h shaking incubation; Lane 7: 12 h shaking incubation; Lane 8: 48 h shaking incubation.

Conclusions

Although shaking during reaction is aimed to obtain uniform hydrolysis, it was found that cellulases were deactivated during shaking when it was mixed with buffer solution, and the degree of deactivation increased with the progressing of incubation time. This will reduce the efficiency of enyzme performance during enzymatic hydrolysis especially for a long time. The deactivation of enzymes occurred in both cellulase and β-glucosidase in this study. The deactivation of enzymes resulted in a significant decrease of the initial hydrolysis rate, but the hydrolysis rate after 24 h had little change compared with the enzymes without shaking incubation.

Future work

It has been reported that enzymes were deactivated when exposed to an air-liquid interface and this effect was greatly enhanced under an air-liquid interface occurs along with shaking or agitation (Elias & Joshi, 1998; Kim et al., 1982). It was also reported that adding additives, such as Zonyl, Triton X-100 and BSA could minimize the concentration of enzyme present at the air-liquid interface, and improve enzymatic hydrolysis (Kim et al., 1982). Therefore, the elimination of air-liquid interface or the application of surfactant which adsorbs at interfaces between air and water may reduce the deactivation of enzymes. To investigate the effect of gas-liquid interface on enzymes deactivation, several different environment of interface was proposed for the future work. They are air-liquid interface, N_2-liquid interface, vacuum-liquid interface, applying surfactant in hydrolysate, and no air-liquid interface (Figure 6.7).

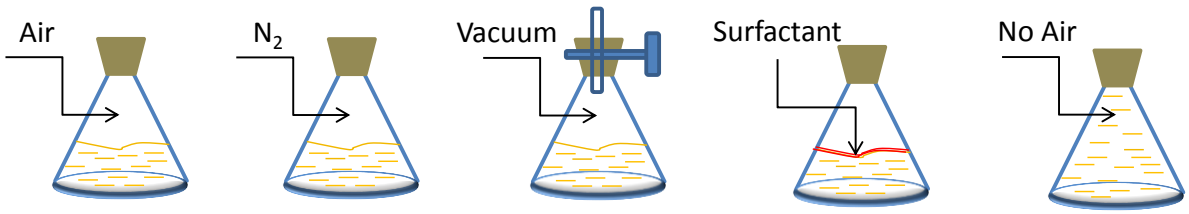

Figure 6.7. Investigation of the effect of gas-liquid interface on enzymes deactivation. 1: Air-liquid interface, 2: N_2-liquid interface, 3: Vacuum-liquid interface, 4: Applying surfactant in hydrolysate, 5: No air-liquid interface.

Besides, in the case of hydrolysis with substrate, not all the enzymes stay in the liquid phase. Some enzymes will adsorb on the surface of biomass, then how is the situation of enzyme deactivation due to shaking with the presence of biomass needs to be understood.

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CHAPTER 7

Summary

The fundamental factors of lignocellulose that affect enzymatic hydrolysis of lignocellulosic biomass include the amount and characteristics of lignin, hemicellulose, cellulose, and accessible surface area of the substrate. During the pretreatment, the change in one factor is usually accompanied by changes in other factors. All these factors are coupled with each other making the assessment of the effect of individual factor on enzymatic hydrolysis a great challenge. The major innovation and contribution of this work is that it endeavored on decoupling the coupled factors, and obtained a progress in understanding on the recalcitrance of lignocellulose focused on addressing two factors: lignin and biomass-enzyme interactions.

Milled wood lignin (MWL), as a representative of lignin, has a critical impact on enzymatic hydrolysis by both non-productive adsorption and physical barrier. After alkali and acid pretreatment, the MWL could absorb 2 to 6 times more cellulases than the MWL obtained from untreated woods. Once the MWL adsorb more than two thirds of cellulases that bleached pulp can adsorb, the impact of lignin in terms of non-productive adsorption and physical barrier was significantly increased. One of the reasons for the more recalcitrant nature of softwood than that of hardwood was due to the greater degree of condensation of softwood lignin. The impact of more condensed lignin was found to associate with stronger adsorption of cellulases on lignin, and thus lower carbohydrate conversion of both physically

mixed lignocellulose and reconstructed lignocellulose during enzymatic hydrolysis. The greater degree of condensation might result in a more branched structure of lignin that could either trap the cellulases or block the accessible surface area of carbohydrate. However, the impact of lignin on enzymatic hydrolysis can be significantly limited when the lignin content of biomass was reduced to less than 15%, where the critical physical barrier of lignin was removed and enough surface of carbohydrate was accessible to enzymes.

The biomass-enzyme interaction is another important concern determining the efficiency of enzymatic hydrolysis. During enzymatic hydrolysis, bound and free enzymes were evaluated for their kinetic activity on Avicel, bleached hardwood and green liquor pretreated hardwood. Although 34%-62% of the initially loaded enzymes were bound enzymes while the others were free enzymes, the bound enzymes were primarily responsible for enzymatic hydrolysis. However, the free enzymes had very limited activity due to the loss of synergism effect since one of the cellulases component cellobiohydrolase I (CBH I) were mostly involved in bonding with the substrate. Thus, the maximal utilization of bound enzymes as well as recovery and modifying the free enzyme cocktails are considered to be an efficient and cost-saving process for enzymatic hydrolysis.

Although bound enzymes are generally favorable for enzymatic hydrolysis, the accumulation of those irreversibly bound enzymes which have little activities could block the accessible surface area of cellulose, thus result in up to 63% decrease of cellulose digestibility in a course of eight stage enzymatic hydrolysis with 96 h as a total reaction time. In a condition of enzymatic hydrolysis without substrate, both cellulase and β-glucosidase could be deactivated and partially precipitated due to shaking during enzymatic hydrolysis.

Weather the presence of substrate during enzymatic hydrolysis functions as a buffer to prevent the deactivation of enzymes need to be further understood.

CHAPTER 8

Suggested Future Research

The fundamental understanding of biomass recalcitrance as well as the interactions between lignocellulosic biomass and enzyme for sugar production is the key for designing the most efficient and applicable process for a commercial scale biorefinery platform. Based on the work of this dissertation, several interesting subjects are suggested to be carried on in the future research.

The effect of individual enzyme components (EG, CBH I, CBH II, BG) and their synergism effect on enzymatic hydrolysis

Most of the enzymes used in the current research of lignocellulose saccharification are commercial enzyme cocktails. However, these enzyme cocktails may not be the most desirable mixture for a specific type of biomass. Studying and optimizing the synergism effect of these enzyme components could result in a great improvement of enzymatic hydrolysis of lignocellulosic biomass (Beldman et al., 1988; Medve et al., 1998). The synergism effect of enzyme cocktails could be further improved by understanding the effect of each individual enzyme on altering the characteristics of biomass, followed by a systematically trials to determine the optimal dosage of each enzyme in the cocktail. A semi-quantitative analysis of the composition and the molecular weight of several enzyme cocktails are shown in Figure 8.1.

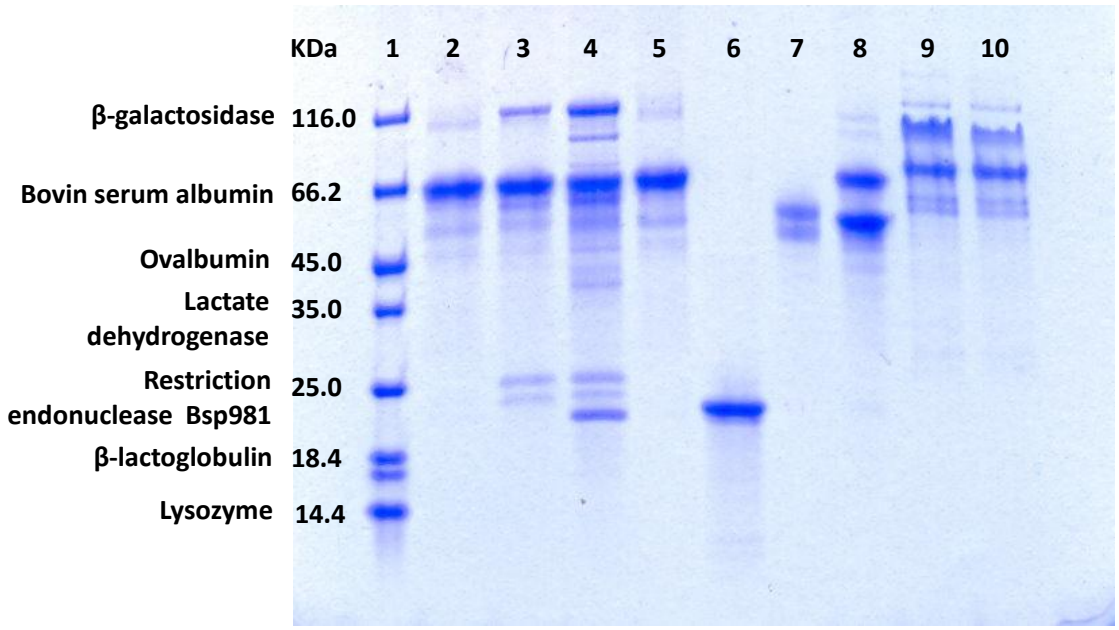


Figure 8.1. SDS-PAGE analysis of various enzyme cocktails. The original enzymes of 2, 3, 4, 5, 7, 8, 9, and 10 have been diluted for 200 times. The xylanase NS50014 has been diluted for 10 times. Lane 1: Protein marker. Lane 2: cellulase (NS50013). Lane 3: Ctec. Lane 4: Ctec 2. Lane 5: cellulase Sigma. Lane 6: xylanase (NS50014). Lane 7: Htec. Lane 8: Htec 2. Lane 9: β-glucosidase (NS50010). Lane 10: Novozymes 188 (β-glucosidase).

Using a lignin-free substrate as a biomass model (e.g. bleached hardwood or bleached softwood) to understand the effect of the individual enzyme on changing the cellulose structure, such as crystallinity or DP, and the extent of hydrolysis would be a good start. A preliminary work has been initiated by using bleached softwood as the substrate and pure enzymes including EG II and CBH I obtained from Megazyme (Megazyme International Ireland, Wicklow, Ireland). The bleached softwood pulps were subjected to 48 h enzymatic hydrolysis at 50 °C, 180 rpm by 20 mg/g EG II supplemented with 40 mg/g BG and 20 mg/g

CBH II supplemented with 40 mg/g BG, respectively. The addition of BG is to avoid the sever end-product inhibition by the oligosaccharides (Qing et al., 2010). Alternatively, a trial using 20 mg/g EG II, 20 mg/g CBH I and 80 mg/g BG was conducted to investigate the synergism effect of EG II and CBH I. The extent of hydrolysis was reported as weight loss based on the starting weight of substrate for enzymatic hydrolysis. As shown in Figure 8.2, the individual EG II and CBH I supplemented with BG resulted in 39.2% and 22.9% weight loss, respectively.

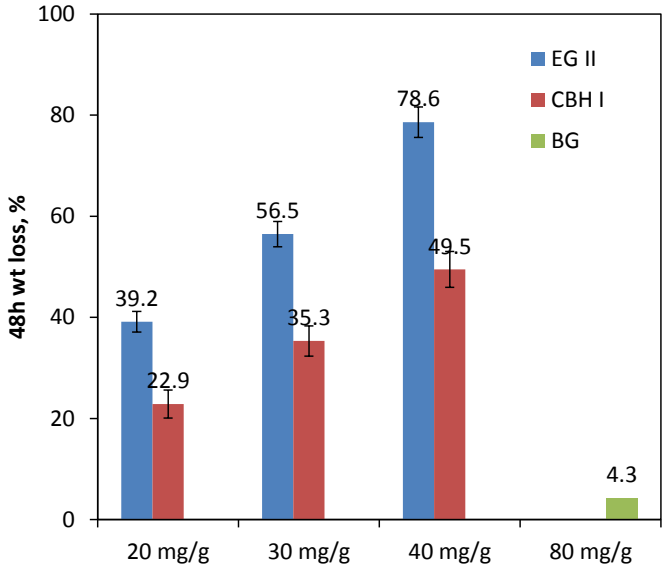


Figure 8.2. Enzymatic hydrolysis of bleached softwood by pure enzymes. Substrate: bleached pine pulps. Enzymatic hydrolysis conditions: 1% consistency, 50 °C, 180 rpm, 48 h. Pure enzymes were obtained from Megazyme. Enzymatic hydrolysis with EG II or CBH I was supplemented with two folds amount of BG to overcome the strong end-product inhibition caused by oligosaccharides (e.g. 20 mg/g EG II+ 40 mg/g BG-NS50010).

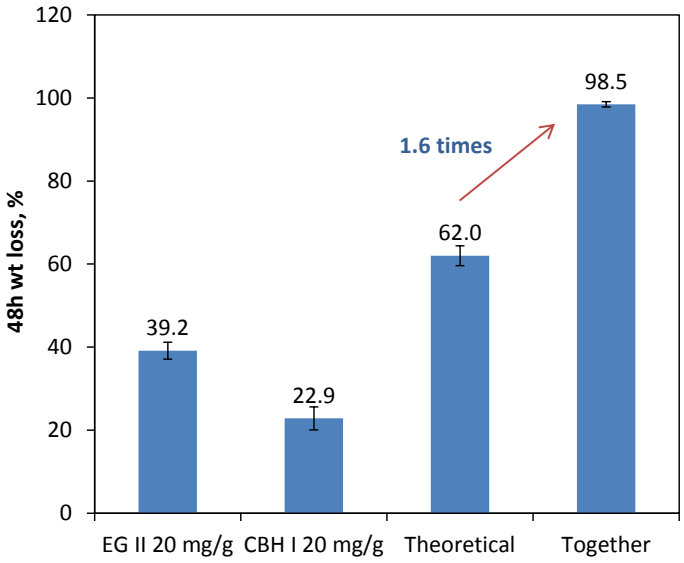


Figure 8.3. Synergism effect of EG II and CBH I. Substrate: bleached pine pulps. Enzymatic hydrolysis conditions: 1% consistency, 50 °C, 180 rpm, 48 h. Pure enzymes were obtained from Megazyme. Enzymatic hydrolysis with EG II or CBH I was supplemented with two folds amount of BG to overcome the strong end-product inhibition caused by oligosaccharides (e.g. 20 mg/g EG II+ 40 mg/g BG-NS50010).

In theory, the combined weight loss should be 62.0%. However, the synergism effect of EG II and CBH I enabled an almost complete digestion of bleached softwood (Figure 8.3), showing 1.6 times higher in weight loss than that of the theoretical weight loss. Therefore, utilizing the powerful synergism effect of enzymes to improve enzymatic hydrolysis could be a very promising strategy and should be further pursued. In the past research, the synergism effect of cellulases and xylanase had been reported (Hu et al., 2011; Kumar & Wyman, 2009). This synergism effect could be further evaluated by comparing on the optimal ratio of

cellulase and xylanase on the substrate with different chemical composition (e.g. hardwood vs. softwood). In addition, since the major hemicellulose component of softwood substrate is mannan, the synergism effect of cellulase and mannase or even together with xylanase would be a promising research for elucidating the role of synergism effect of multiple enzymes for softwood hydrolysis. It also holds a potential to provide a breakthrough in enzymatic digestion of softwood substrate.

The effect of lignin structure on enzymatic hydrolysis

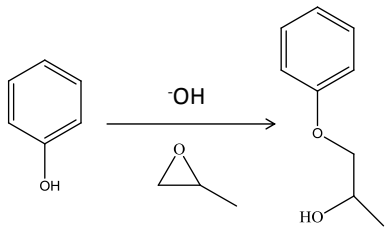
The study of lignin inhibition on enzymatic hydrolysis needs to be continued. The exact mechanisms of how cellulases interact with lignin as well as the inhibition of enzymes by lignin have not been fully understood. For example, it was reported that some functional groups of lignin, such as methoxyl groups, phenolic hydroxyl groups, aliphatic hydroxyl groups, carboxylic groups were critical for enzyme inhibition. Nevertheless, there are some discrepancies regarding the relative importance of these functional groups of lignin on enzymatic hydrolysis. For example, Pan and reported that the phenolic hydroxyl groups of lignin had negative impact on enzymatic hydrolysis (Berlin et al., 2006; Pan, 2008), whereas another study did not find a correlation between the amount of phenolic hydroxyl groups in lignin and the non-productive adsorption of enzymes (Kawamoto et al., 1992). Besides, the inhibitory mechanism has not been clearly evaluated as compared with physical barriers or non-specific adsorptions. Therefore, a systematic evaluation of the effect of some functional groups of lignin on enzymatic hydrolysis is suggested to carry out by modifying the functional groups in the isolated lignins, and test the lignin-enzyme interaction using the

modified lignins. The modification methods include: hydroxypropylation of phenolic hydroxyl groups (Pan, 2008), acetylation of hydroxyl groups (Adler et al., 1987), periodate oxidation of phenolic hydroxyl groups to carbonyl groups (Lai et al., 1990), $NaBH_4$ reduction of the carbonyl groups to hydroxyl groups (Adler et al., 1987) (Figure 8.4). Moreover, from the overall structure of lignin, the degree of condensation of lignin was found to be an important indication for its inhibition on enzyme adsorption and enzymatic hydrolysis. A valuable future work could be conducted by varying the severity of a specific pretreatment to obtain a set of lignin samples with different degree of condensation. Evaluating the effect of degree of condensation of lignin prepared by a specific pretreatment method on enzymatic hydrolysis will provide a further validation of the role of degree of condensation on enzymatic hydrolysis.

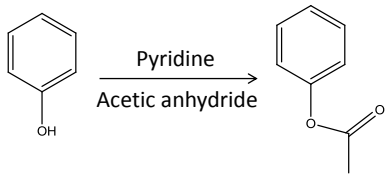
The effect of lignin distribution on enzymatic hydrolysis

The physical barrier of lignin is another limitation for enzymatic hydrolysis. At similar lignin content, whether the lignin distribution affect enzymatic hydrolysis is an importance concern. To track the changes of lignin distribution during a kinetic pretreatment by confocal image analysis would be a good start-up. Furthermore, if several samples with similar lignin content can be prepared under different pretreatment methods, it would be a very interesting study to compare the differences in lignin distribution among these samples. If certain trends or even an evaluation method which correlate the pattern of lignin distribution and its impact on enzymatic hydrolysis could be established, it would be a significant insightful

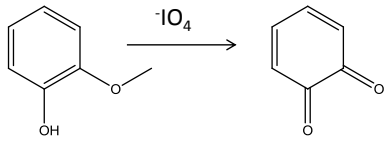
contribution for a better understanding of the inhibition mechanism of the lignin on enzymatic hydrolysis.



(a) Hydroxypropylation of phenolic hydroxyl groups



(b) Acetylation of phenolic hydroxyl groups



(c) Periodate oxidation of phenolic hydroxyl and methoxyl groups

Figure 8.4. Suggested reactions for modifying the functional groups of lignin.

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