A SYSTEMATIC UNDERSTANDING OF BIOMASS RECALCITRANCE
FROM FIBER, FIBRIL AND MOLECULAR LEVELS

By

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ABSTRACT

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Deep understanding of “biomass recalcitrance” is a key to the development of cost effective pretreatment and discovery of highly efficient polysaccharide degrading enzymes. This thesis presents a systematic investigation of biomass recalcitrance across plant cell wall structures from fiber, fibril and molecular levels (Figure below). To achieve this goal, a set of biomass reference substrates with controlled levels of physico-chemical properties were prepared, and a number of analytical techniques to investigate specific substrate characteristics were developed.

The effects of fiber size, fiber swelling, and resulting surface area changes on biomass substrate digestibility were investigated by using reference substrates (Chapter Two). Results showed that fiber size changes have negligible influence while swelling changes have significant influence on enzymatic hydrolysis efficiency. At the fibril level, the interactions between different cell wall components, cellulose, hemicellulose and lignin and their effects on biomass recalcitrance were revealed. X-ray photoelectron spectroscopy was established to quantify the amount of lignin on biomass substrate surface. Apart from its hindrance effect, xylan was found to enhance fibril swelling and thus generate more accessible surface area to facilitate enzyme and
substrate interactions. Surface lignin has a direct impact on enzyme adsorption kinetics and hydrolysis rate (Chapter Three). Cellulose crystallinity is the one of the major factor contributing to biomass recalcitrance from molecular level. In this thesis, refined X-ray diffraction was developed to monitor changes in the crystallite structure of NCC (Chapter Four). Results provide strong evidence to demonstrate that the decrystallization of cellulose does not involve a "swelling component" to disrupt hydrogen bond in cellulose crystallite which has been a main hypothesis to explain cellulose hydrolysis mechanism. Our research supports that cellulose decrystallization proceed through delamination mechanism which can be enhanced by putative oxidative enzymes (Chapter Five).

Finally, reference substrates have been applied to evaluate the performance of commercial cellulase as well as cellulase mutants toward biomass hydrolysis (Chapter Six). It is apparent that biomass references substrates and the methodology established by this thesis can provide an effective and practical approach to identify highly efficient plant cell-wall degradation enzyme to overcome biomass recalcitrance.
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Dedication

For Brett
CHAPTER ONE: INTRODUCTION

1.1 Energy crisis, lignocellulosic biomass and a green economy

Billions of people on this planet enjoy a splendid standard of living because of the presence of oil. Americans consume 18.6 million barrels of oil daily while the rest of the world burns 70.6 million barrels (2012) (US Energy Information Administration). Every hour, four million barrels of crude oil was burned for various means supporting the life style of human beings. Consumption is rising so fast that oil companies must discover a new billion-barrel oil field every two weeks – or the equivalent of a new North Sea every eighteen months (Kilian 2009; Alquist and Kilian 2010; Kilian and Murphy 2013). What's now clear, however, is that this pace of consumption is outpacing production. Oil companies are struggling to find new oil fast enough to replace the oil they're currently pumping. Prolific oil regions have hit production peaks and are now in decline. What's needed is a viable, cost-effective strategy for moving our economy beyond oil. Bridging the gap between the current oil economy and an as-yet-undefined clean-energy economy will not be easy. Alternative systems, such as wind power, hydro power, solar power, and hybrid energy, may be vastly more sustainable someday. But “sustainability” is an economic concept as much as it is an environmental one. People will always prefer cheap energy to expensive energy. And the process of making alternative energy systems affordable will be long and uncertain, in part because the oil-based systems they must compete against will themselves become even more efficient and alluring.

United States is blessed with abundant and diverse agricultural and forest resources, unused cropland and favorable climates (DOE report 2001). We have used biomass energy, or "bioenergy"—the energy from plants and plant-derived materials—since people began burning
wood to cook food and keep warm. However, combusting biomass feedstock to create electricity does not contribute to global warming and helps avoid the release of other harmful emissions. Typical biomass fuels include a wide variety of material. Wood is still the largest biomass energy resource today, but other sources of biomass can also be used. These include food crops, grassy and woody plants, residues from agriculture or forestry, oil-rich algae, and the organic component of municipal and industrial wastes. Even the fumes from landfills (which are methane, the main component in natural gas) can be used as a biomass energy source. In the 21st century, use of biomass—plants and plant-based materials, produced by photosynthesis within biological rather than geologic time—will offset this petrochemical dependence. Biomass can’t fully replace the huge volumes of petroleum and other fossil fuels that we now use, but it can provide fuels and chemicals comparable to those derived from petroleum. There are also many next generation biomass feedstocks in different stages of commercialization. These new fuels have the potential to dramatically scale plant sizes and the feedstock supply chain, creating significant opportunities for additional generation fleet expansion. Together with a remarkable talent to develop new technologies, we have a tremendous opportunity to use domestic, sustainable resources from plants and plant-derived resources to augment our domestic energy supply.

One out of eight gallons of gasoline sold in the United States already includes ethanol as an additive (Anderson 2012). Ethanol is made by fermenting sugar, most of which is derived from starch in corn kernels. In contrast, instead of starting with sugar, advanced bioethanol technology depends on technologies breaking down lignocellulosic biomass to sugars for fermentation. In addition to ethanol, the sugars, or intermediate breakdown products, can be fermented, polymerized, or otherwise processed into any number of products. Lignin, one of the
major components of biomass, can fuel the process or be used to produce a slate of different chemicals, expanding the number of products for the sugar-lignin platform biorefinery.

The cell wall material from the plants, known as lignocellulosic biomass, is composed of cellulose, hemicelluloses and lignin. Cellulose and hemicelluloses comprise 65-75% of the overall biomass dry weight and can be biologically converted to sugars and their fermentation products for biofuel production. Biomass presents an abundant and renewable resource for sustainable production of low cost, none-food based sugars for biofuel applications. However, strategies for feedstock supply, handling and processing and the technologies requires for the large scale production of lignocellulosic biomass into fuels and chemicals have not yet been fully developed. As a result, large commercial biofuels from lignocellulosic feedstock has not been implemented. A significant amount of research and development is still required to address the techno-economic feasibility of the every major process steps on the biomass conversion.

My thesis attempted to address a systematic understanding of biomass recalcitrance during deconstruction of lignocellulosic biomass followed by enzymatic hydrolysis to produce

![Figure 1.1. Illustration of different fiber structural levels of plant cell wall.](image)
fermentable sugars. The ultimate goal is to provide clear guidance toward overcoming technical and economic challenges during biomass conversion process and design highly efficient and cost effective cell-wall degrading enzymes. From an anatomical viewpoint, a lignocellulosic biomass fiber can be represented at several structural levels, fiber, fibril and molecular levels (Figure 1.1). The fiber level presents the highest magnitude of an intact fiber cell. The morphological properties of fiber cells such as length, width, cell wall thickness and lumen diameters etc. are revealed at this level. A typical fiber consists of primary (P) and secondary (S) cell wall layers. The secondary cell wall layer can be further separated into S1, S2 and S3 layers. Each of these layers is composed of numerous fibrils which encompass macro-fibril, micro-fibril and elementary fibril. At the fibril level, the interactions between different cell wall chemical components, cellulose, hemicellulose and lignin (macro fibril level) as well as the arrangement of amorphous and crystalline cellulose can be revealed (microfiber/elementary fibril level). The molecular level describes molecular interaction and chemical bonding between monomeric constituents (e.g. glucose, xylose, phenolics) of cell wall components. When the substrate parameters contributing to biomass resistance to break down are examined across these structural levels, complexity arises.

1.2 Biomass recalcitrance

There is little doubt lignocellulosic biomass is the most promising and sustainable source for supplying non-food sugar as building blocks for fuel and chemicals conversions. However, an economically feasible biomass-to-biochemical and biofuel conversion process has yet to be developed and demonstrated. Deconstruction of lignocellulosic biomass followed by enzymatic hydrolysis to produce fermentable sugars is a primary, near-term option for commercial liquid
fuels production from renewable sources. However, technical and economic challenges come from the strong structural resistance of lignocellulosic biomass to physical, chemical and microbial deconstruction. The lack of highly efficient and cost effective cell-wall degrading enzymes is a major obstacle preventing the commercialization of a biologically based lignocellulosic biomass conversion process.

Plant biomass has evolved complex structural and chemical mechanisms for resisting assault on its structural sugars from the microbial and animal kingdoms. Here, we consider the natural resistance of plant cell walls to microbial and enzymatic deconstruction, collectively known as “biomass recalcitrance”. It is this property of plants that is largely responsible for the high cost of lignocelluloses conversion (Himmel, Ding et al. 2007). Biomass recalcitrance is a complex phenomenon that is governed by physicochemical properties spanning several orders of magnitude from the macroscopic to the molecular scale and by the hierarchical structure of the plant cell wall. Biomass recalcitrance caused by substrate characteristics, such as: fiber size, specific surface area, xylan and lignin content, as well as cellulose crystallinity are among the most frequently cited parameters influencing enzymatic hydrolysis and sugar production. However, from literature, conclusions regarding to their effect on enzyme hydrolysability is commonly debated from contradictory observations. While during most biomass pretreatment methods, a random disruption of fiber cell wall typically lead to a heterogeneous nature of pretreated substrates, specific character tics of biomass becomes difficult to be investigated individually.
1.3 Factors contributing to biomass recalcitrance

1.3.1 Heterogeneous nature of pretreated substrates

The heterogeneous nature of pretreated substrates prevents a systematic approach to deeper understanding of biomass recalcitrance. Chang and Holtzapple have used peracetic acid, potassium hydroxide and ball milling methods to treat poplar, switch-grass and bagasse in order to produce model substrates with variable amount of lignin, acetyl group and cellulose crystallinity. The study generated 147 biomass substrates and significant amount of information on their hydrolysability (Chang and Holtzapple 2000). The objective of the study was to produce these substrates with independent variables such as lignin content, acetyl content, and crystallinity. However, it should be noted that both peracetic acid and ball mill treatments can bring profound changes to the chemical composition and morphological structure of the cell wall. Previous work has shown that ball milling of lignocellulose can significantly affect cellulase digestibility due to a range of morphological changes in cell wall structure other than crystallinity alone (Fukazawa, Revol et al. 1982). Although peracetic acid has a high selectivity for lignin, extended delignification will inevitably lead to significant changes in carbohydrate content, cellulose viscosity, and functional groups on hemicelluloses (Sundquist and Poppius-Levlin 1997; Zhao, Wang et al. 2008). It is conceivable that peracetic acid, potassium hydroxide and ball milling treatments will all result in increases in available surface area of the substrates which is a significant factor related to enzymatic hydrolysability. These are probably some of the main reasons that explain the limitations associated with using these model substrates to predict substrate digestibility. Also, the ground biomass samples used in the above study do not necessarily reflect the nature of “biomass recalcitrance” at the fiber level. Our poor understanding of intricate substrate and enzyme interactions is one of the main factors which
impede the research efforts to reduce enzyme cost by improving enzyme performance (Himmel 2008). A systematic investigation of biomass recalcitrance requires the use of reference substrates with integral fiber structure and homogeneous and well characterized chemical and physical properties at all atomic levels. New methodology is needed to create these “reference substrates”.

1.3.2 Lignin

Lignin, one of the major components of lignocellulosic biomass presents both chemical and physical barriers to the enzymatic hydrolysis of pretreated substrates (Chang and Holtzapple 2000). The two primary mechanisms include lignin role in limiting access of the enzymes to the cellulose by its physical/mechanical role and location within the substrate and through the non-productive binding of cellulases to lignin (Eriksson, Borjesson et al. 2002; Palonen, Tjerneld et al. 2004; Berlin, Balakshin et al. 2006). Although hydrophobic, electrostatic and hydrogen bonding interactions have been implicated in these cellulase-lignin interactions, due to the heterogeneous distribution of lignin in most pretreated substrates, a quantitative description of the impact of lignin on cellulase inhibition remains to be more fully elucidated (Seiji, Richard et al. 2001). In addition to affecting the amount of lignin in the substrate, the choice of pretreatment will also affect the physical and chemical structure of lignin, altering both the accessibility to the cellulose and the interaction of cellulase with the pretreated substrate. It is likely that acidic pretreatments such as steam and dilute acid treatment would result in a net increase in condensed lignin, although simultaneous lignin depolymerization and condensation reaction are known to occur. It has also been suggested that “external lignin” that is often observed as droplets or re-precipitates on the surface of pretreated substrates act as physical barrier to cellulases. In the case of alkaline pretreatments, lignin is solubilized by the deprotonation of the phenolic lignin
subunits combined with alkaline induced breakage of α and β-aryl ether bonds. Solvent pretreatments such as ethanol organosolv result in the cleavage of α and β-aryl ether bonds which solubilized the lignin and increases the free phenolic groups present in the solid substrates (Berlin, Balakshin et al. 2006).

1.3.3 Hemicelluloses

As a non-cellulose component, hemicellulose has been generally considered as a physical hindrance to cellulase enzymes. A number of studies have shown that the addition of xylanase to cellulase preparation can enhance cellulose hydrolysis rate. It has been postulated that native plant cellulose fibrils are embedded in hemicellulose and lignin matrix, and after pretreatment, hemicellulose remains coated on the cellulose fibril and/or bound with cellulose and thus limit the access of cellulase enzyme to cellulose. However, on the other hand, it is well recognized that hemicellulose in chemical pulps plays an important role in improving cellulose fiber swelling (Hubbe, Venditti et al. 2007), increasing the fiber saturation point, preventing cellulose hornification (Kohnke, Lund et al. 2010), and enhancing specific fiber surface area, as well as wet fiber flexibility (Oksanen, Buchert et al. 1997; Kohnke, Lund et al. 2010) while all of these effects improve cellulose hydrolysability. A pretreated biomass substrate typically contains a large amount of unseparated and disrupted fibers, therefore, the positive effect of xylan on cellulose swelling might not be revealed.

In majority of previous studies, pretreated biomass substrates are typically used to investigate the xylan effects on enzyme hydrolysis. Pretreatment typically causes a disruption of fiber cell wall structure that leads to a heterogeneous distribution of chemical components throughout different structural levels. It is likely that a significant portion of the cellulose in these substrates is entangled with the hemicellulose and lignin matrix. Although the beneficial effect of
xylanase addition has been frequently observed, a direct relationship between xylan removal and cellulose hydrolysability does not always exist (Garcia-Aparicio, Ballesteros et al. 2007; Kumar and Wyman 2009). A previous investigation of xylanase leverage effect on enzymatic hydrolysis of corn stover substrates prepared by several pretreatment methods concluded that the removal of xylan does not always enhance cellulose conversion (Kumar and Wyman 2009). Besides the interaction with cellulose, the removal of xylan in many pretreated biomass substrates is often associated with changes in other substrate parameters such as lignin content, porosity, etc. It remains a great challenge to evaluate the effect of xylan as an isolated factor using pretreated biomass substrates with heterogeneous properties.

1.3.4 Cellulose

The plant cellulosics are synthesized by cellulase synthases (CESA), which are organized in rosette complexes containing 36 individual proteins (Williamson, Burn et al. 2002; Mutwil, Debolt et al. 2008). Plant cellulose never exists as a single glucan chain but rather forms fibrils. An elemental cellulose fibril contains 36 glucan chains in a hexagon shape with a width about 5 nm. The hydroxyl groups on glucan chains can form hydrogen bonds both between and within the glucan chains. A well-organized H-bonds network in combination with van de Waals forces between the chains give rise to compact and highly ordered crystalline regions. The crystalline cellulose regions are alternating with less ordered or disordered amorphous regions to form macromolecular cellulose. Cellulose also has several polymorphs. The polymorphism is most typical for crystals of organic compounds whose molecules contain groups capable of hydrogen bonding. Due to the different mutual arrangements of the pyranose rings and possible conformational changes of the hydroxymethyl groups, cellulose chains can exhibit different crystal packing. Several cellulose polymorphs, such as cellulose I, II, III and IV and their
varieties Iα, Iβ, IIIi and IVii, are known (Okano and Sarko 1984; Kovalenko 2010; Perez and Samain 2010). Polymorph Iα prevails in the major part of cellulose isolated from bacteria and in cellulose from fresh water algae. Polymorph Iβ is the major form in cotton, wood, and ramie cellulose. Hydrothermal treatment can lead to the irreversible transformation of cellulose Iα to Iβ. Cellulose I can also be mercerized or regenerated to produce cellulose II. The treatment of cellulose I and II with liquid ammonia at -80 °C leads to their transformation into cellulose III (Kovalenko 2010). Cellulose Iβ, cellulose II and cellulose IIIi are the most commonly obtained cellulose polymorphs after variety of lignocellulosic biomass pretreatment process, with cellulose Iβ as dominant, representing the native crystalline cellulose in lignocellulosic biomass unaltered.

It is well recognized that the hydrolysis rate of crystalline cellulose is much slower than the rate with amorphous cellulose (Teeri 1997). However, a positive correlation between low crystallinity and hydrolysability is not always observed (Puri 1984; Gregg and Saddler 1996; Mansfield, Dionne et al. 2003). It is acknowledged that altering the crystallinity of pretreated substrates also results in changing accessible surface area to the enzyme which makes it difficult.

![Figure 1.2](image)

**Figure 1.2.** NCC appearance: (A) Nanocrystalline cellulose powder; (B) NCC in solution; (C) AFM image of dispersed NCC; (D) SEM images of freeze-dried NCC (all NCC showed here were from poplar).
separate the effects of accessible surface from those of crystallinity on enzymatic hydrolysis efficiency (Mansfield and Meder 2003). Investigating the mechanism of enzyme degradation of crystalline cellulose have been pursued using pure cellulose substrates which processed mainly cellulose Iβ, such as microcrystalline cellulose (e.g. Avicel, Sigmacell), bacterial or algae cellulose (cellulose Iα). These substrates are at or above micro-scale in size and contain an aggregate of elementary cellulose crystallites which are entwined with amorphous and crystalline regions. Therefore it has been a great challenge to use these substrates to achieve a clear understanding of the mechanism of enzyme degradation of crystalline cellulose (Zhang and Lynd 2004; Zhang, Himmel et al. 2006). New cellulose substrate is needed to gain an advanced molecular understanding of cellulose decrystallization.

A procedure has been recently developed in our lab to extract nanocrystalline cellulose from poplar and wheat straw cellulose. These nanocrystalline celluloses (NCC) represent the unit dimension of the elemental crystalline cellulose with a width between 5-20 nm and an average length of 200 nm. Unlike all other pure crystalline cellulose analogues (e.g. microcrystalline cellulose, algae or bacterial crystalline cellulose) that are insoluble in water, NCC forms a colloid dispersant in aqueous solution, thereby providing an ideal system to determine the kinetic parameters to help understand reaction mechanisms (Figure 1.2). In the following thesis, advanced instrumentation was applied to carry out a systematic approach to elucidate the mechanism of enzyme decrystallize cellulose at the molecular level by using nanocrystalline cellulose as a model substrate. The goal is to investigate the effect of enzymatic hydrolysis of NCC with the purpose to find new insights into the limiting “decrystallization” factors, especially related to the substrate structure, responsible for the gradual slowing down of the process.
1.3.5 Available surface area

Among all potential factors contributing to biomass recalcitrance, the accessible surface area or available surface area to enzymes has been regarded as one of the most important hydrolysis limiting factors. It is apparent the degree of crystallinity and the amount of lignin and hemicellulose at the fibril level and the cell wall thickness and lumen diameter at the fiber level all have a significant impact on the accessible surface area to enzymatic treatments. It becomes impossible to attribute “accessible surface area” to any single factor unless we can create substrates that vary by only one or a few physical and chemical properties. Pretreatment typically causes a random disruption of fiber cell wall structure which makes it difficult to maintain the integrity of fiber morphology and to obtain an even distribution of chemical composition throughout different structural levels. Developing a correlation between each of the specific substrate features and enzymatic hydrolysis efficiency has proven to be problematic when pretreated substrates are used.

1.4 Enzymatic hydrolysis of lignocellulosic substrates

Deconstruction of lignocellulosic biomass followed by enzymatic hydrolysis to produce fermentable sugars is a primary, near-term option for commercial liquid fuels production from renewable sources. However, technical and economic challenges come from the strong structural recalcitrance of cellulose to physical, chemical and microbial deconstruction (Himmel, Ding et al. 2007). The exceptional mechanical and structural properties are derived from a molecular-level network of hydrogen bonds and hydrophobic interactions present in crystalline cellulose, making deconstruction of this polymer difficult. Numerous research efforts (Himmel, Ruth et al. 1999; Percival Zhang, Himmel et al. 2006; Jorgensen, Kristensen et al. 2007; Rubin 2008; Gupta
and Lee 2009) have been directed toward developing the known cellulose-degrading enzymatic pathways found in fungi. Understanding the molecular-level mechanisms that enzymes employ to deconstruct plant cell walls is a fundamental scientific challenge with significant ramifications for renewable fuel production from biomass (Mansfield, Mooney et al. 1999; Gupta and Lee 2009).

The C_{1}-C_{x} concept proposed by Mandel and Reese (Reese, Siu et al. 1950; Mandels and Reese 1999) established the first mechanistic model for enzymatic hydrolysis of cellulose. They postulated that C_{1} as an unknown component of the cellulase system (so-called “swelling factor”) opens up the cellulose supermolecular structure to allow subsequent cellulose depolymerization by the C_{x}, a group of hydrolytic enzymes. So far many hydrolytic enzymes that could count for the suggested C_{x} actions have been identified and characterized (Desrochers, Jurasek et al. 1981; Henrissat, Driguez et al. 1985; Wood, Bhat et al. 1988; Ting, Makarov et al. 2009; Zhang 2011). The classical C_{x} scheme for cellulose degradation involves the synergistic actions of at least three classes of enzymes: endo-1,4-β-glucanase (EG) that randomly cleave internal bonds in the chain, Exo-1,4-β-glucanases (Exo) or cellobiohydrolase (CBH) that attack the reducing or non-reducing end of cellulose polymer, and β-glucosidase (BG) that convert cellobiose to glucose. Despite these traditionally accepted hydrolytic enzymes involved in the solubilisation and depolymerization of carbohydrates within the lignocellulosic biomass matrix, it remains difficult to understand how the glycoside hydrolases could act on a polysaccharide chain in its crystalline environment as the role of C_{1} factor. Researchers have speculated about the existence of a substrate-disrupting factor that could make the crystalline substrate more accessible to hydrolytic enzymes. In 1985, Coughlan (Coughlan 1985) first came up with the term “amorphogenesis” to illustrate a possible mechanism by which the dispersion, swelling or
delamination of cellulosic substrate occurred, resulting in a reduction on the degree of fibrillar aggregation and/or crystallinity. Cosgrove et al. (Mcqueenmason and Cosgrove 1994) identified a novel protein with ability to induce extension by disrupting the hydrogen bonding between plant cell wall polymers. Carbohydrate binding domain (CBD) of the carbohydrate-hydrolyzing enzymes, along with a number of other proteins (expansin-like proteins, swollenin), has also been suggested (Cosgrove 2000; Yuan, Wu et al. 2001; Saloheimo, Palohelmo et al. 2002; Kim, Lee et al. 2009; Arantes and Saddler 2010; Chen, Ishida et al. 2010; Lee, Lee et al. 2010) to be able to non-hydrolytically loosen or disrupt the packaging of the cellulose fibril network by weakening or splitting hydrogen bonding between cellulose fibers (Arantes and Saddler 2010). On the other hand, Harris et al. (Harris, Welner et al. 2010) first demonstrated that a novel enzyme class glycoside hydrolases family 61(GH61) greatly increases the performance of cellulases from *Trichoderma reesei* in lignocelluloses hydrolysis. Gustav et al. (Vaaje-Kolstad, Westereng et al. 2010) proposed that GH61 proteins may facilitate the hydrolysis by introducing chain breaks in the most inaccessible and rigid parts of crystalline polysaccharides (Harris, Welner et al. 2010). These proteins have flat substrate-binding surfaces and are capable of cleaving polysaccharide chains in the crystalline contexts using an oxidative mechanism (Horn, Vaaje-Kolstad et al. 2012). However, the non-hydrolytic activity demonstrated by swollenin/expansin-like proteins or GH61 families is difficult to identify because products have low solubility and potentially a high tendency to remain attached to the crystalline material.

### 1.5 Thesis objectives

A deeper understanding of “biomass recalcitrance” is a key to the development of cost effective pretreatment methodologies and discovery of highly efficient cell wall polysaccharide degrading enzyme systems. The objectives of this dissertation are to demonstrate a systems-level
understanding of the recalcitrant nature of lignocellulosic biomass from fiber level, fibril level to molecular level, respectively.

In order to achieve the objectives, the research will start by establishing a system of reference substrates from poplar with controlled levels of physico-chemical properties including bulk/surface lignin and hemicellulose content, cellulose DP and crystallinity, inorganic content, available surface area, fiber length and coarseness, etc (Figure 1.3). Novel pulping methods will be developed and applied to achieve such goal. Modified chemical and mechanical pulping methods, such as kraft pulping, sulfite pulping, organosolv pulping, and PFI swelling, will ensure the establishment of systematic reference substrates with controlled level of individual characteristics. Substrates characteristics will be further investigated and confirmed to ascertain the individual unique factors influencing biomass recalcitrance. During the characterization process, advanced instrumentation will be developed and applied for characterization biological material. Fiber Quality analysis will be utilized to characterize fiber level characteristic control. X-ray photoelectron spectroscopy (XPS) is generally used for characterization of inorganic materials. In this study, it will be further developed to quantify surface lignin coverage for biomass, as well as surface chemical characterization of polysaccharides. X-ray diffraction (XRD) techniques for characterizing molecular structural changes will also be established in this dissertation to insure investigation of cellulose decrystallization mechanism. During the process of the study, it was found that cellulose crystalline structure is one of the most intriguing aspects of “biomass recalcitrance” at the fibril/molecular scale. The research will further attempt to acquire a deeper understanding of the mechanism of enzymatic decrystallization of cellulose at molecular level by utilizing newly developed nanocrystalline cellulose. After characterizing the substrates, the subsequent research will test their hydrolysability by different commercial
cellulase enzyme preparations. This step will allow us to quantitatively determine the effects of each individual substrate characteristic on enzymatic hydrolysis efficiency.

![Figure 1.3. “Reference substrates” preparation diagram (bold text indicates treatment methods, variable substrate factors are shown in the brackets).](image)

1.6 **Organization of the dissertation**

This dissertation represents manuscripts that I have written during the course of my Ph.D. The first chapter, which consisted of this introduction, covers the essential information such as backgrounds, hypothesis and objectives.

**Chapter Two** is a published manuscript studying the specific effects of fiber size and fiber swelling on biomass substrate surface area and enzymatic digestibility. My goal of this study is to provide updated understanding of biomass recalcitrance from fiber level. For the purpose, the specific effects of biomass particle size and swelling on substrate surface areas changes were studied by applying a set of reference substrates with controlled chemical composition and morphological properties. Our results 1) demonstrated a new approach to prepare representative substrate for detail investigation of biomass recalcitrance; 2) confirmed that the change of substrate external surface area resulted from particle size reduction had
insignificant effect on enzymatic digestibility; 3) gained evidence to support that fiber swelling can improve surface internal surface area as well as substrate reactivity. I mainly designed and performed the experiments and analyzed the data with the help of undergraduate student Courtnee Grego for performing part of hydrolysis experiment. I wrote the manuscript with the guidance of Dr. Zhang.

Chapter Two also includes extensive study from a ready-to-submit manuscript studying the effects of cutting orientation in woody biomass size reduction on enzymatic hydrolysis sugar yield. The goal of this manuscript was to answer fundamental questions in woody biomass size reduction, since in pulping, wood chipping orientation has impact on conversion rate or similar but no reported studies on this issue in the field of enzymatic hydrolysis for biofuel purpose. It is agreed with Chapter two that enzyme accessible area is more important than particle size. It is not necessary to invest a huge amount of energy to produce very fine particles, if particles produced by size reduction already have very accessible surfaces to enzymes. The manuscript is written by Meng Zhang. I conducted the experiments and data analysis on hydrolysis yield and available surface area and contributed to the manuscript discussion on this section.

Chapter Three is a published manuscript investigating specific effects of xylan and surface lignin on enzymatic digestibility. My goal of this study is to gain advanced understanding of biomass recalcitrance from fibril level. For the purpose, an innovative approach was taken to create realistic biomass substrates with controlled morphological and chemical properties that gains new insight into the efforts of xylan and surface lignin on enzymatic hydrolysis. Our results 1) Established XPS procedure to quantify surface lignin content on realistic lignocellulosic biomass substrates; 2) Gained new insight into the effect of biomass xylan on cellulose digestibility: beside its physical barriers to hinder enzyme access to cellulose,
xylan has positive effect on enhancing fiber swelling; 3) provided new evidence to demonstrate the non-productive binding between hydrophobic lignin and cellulase enzymes. Dr. Mark Engelhard provides extensive guidance about XPS. I mainly designed and performed the experiments and analyzed the data. The manuscript is written by me with the guidance of Dr. Xiao Zhang.

**Chapter Four** is a manuscript ready to submitted developing accurate determination of crystallinity index and crystallite size of cellulose I material by refined X-ray diffraction. The goal of this study is to develop a more accurate crystallinity index calculation and crystallite size determination by XRD. This research will help characterize Nanocrystalline cellulose structure changes during depolymerization, which eventually provides technical support for the understanding of biomass recalcitrance from molecular level in the following chapter. A more accurate determination of the true crystallinity index (CrI) and crystallite structures for cellulose Iβ by refined X-ray diffraction method was developed in this study. The method for the first time identified and applied two amorphous broadenings and extended the XRD spectrum up to 140° to resolve the calculation of CrI based on Rietveld modeling. Nanocrystalline cellulose (NCC) was utilized as a model compound to determine the crystallite structures of cellulose Iβ. The refined CrI represents the true portion of crystalline cellulose separating apart of paracrystalline and amorphous cellulose. Crystallite parameters calculated from the refined method provides a more reliable reference for the future studying of cellulose structural changes during chemical and biological treatment. Dr. Mark Bowden provides extensive guidance about XRD. I mainly designed and performed the experiments and analyzed the data. The manuscript is written by me with the guidance of Dr. Mark Bowden and Dr. Xiao Zhang.
Chapter Five is a ready-to-submit manuscript about mechanisms of cellulose decrystallization at the molecular level. The goal of this work is to complete the understanding of biomass recalcitrance from molecular level. Such goal is achieved by studying the mechanisms of cellulose decrystallization by their molecular interaction with cellulase enzymes. The results demonstrated that cellulase decrystallization cellulose proceeded through a delamination mechanism instead of by disruption of hydrogen bond network of the crystallite to cause swelling. Further evidence showed from this study showed a likely redox mechanism of decrystallization which could facilitate the decrystallization process by weakening the hydrogen bonds. Confirmation of such decrystallization action introduced by oxidative enzymes renders a new perspective regarding to further biomass pretreatment development as well as effective enzyme system design, which will lead toward a more efficient and environmental-friendly energy production. This is a collective research work. I am one of the main contributors to the experimental design and have conducted most experiments and data analysis. Dr. Mark Bowden, Dr, Chulkee Kang, Dr. Jack Saddler and Keith Gourlay provide technical guidance. The manuscript is written by me with the guidance of Dr. Xiao Zhang.

Chapter Six is a submitted manuscript investigating commercial cellulase performances toward specific biomass recalcitrance factors using reference substrates. My goal of the study is to evaluate several fungal cellulase commercial preparations from Trichoderma species for their hydrolytic activities by reference substrates prepared from chapter 2 and 3. For the purpose of the study, influence of low molecular weight (LMW) phenolic compound, kraft lignin, lignosulfonates, xylan and high crystalline cellulose for commercial cellulase preparations were evaluated by reference substrates prepared from modified organosolv, kraft and sulfite pulping pretreatment, as well as newly developed Nanocrystalline cellulose. Results showed that
cellulose depolymerization by enzymes are one of the detrimental influences of low cellulase hydrolytic efficiency. Lignin, as a major hindrance of cellulase accessibility to cellulose, exhibited different levels of inhibition depending on its type and distribution. Different commercial enzymes showed various levels of capacity overcoming such hindrance. Nanocryalline cellulose was applied in this study to evaluate enzyme efficiency of decrystallization. Novel group of glycoside hydrolases is suspected to significantly improve such efficiency. These results indicate that the individual structure features of chemical pulped poplar can be applied to evaluate commercial cellulase hydrolysis efficiencies toward real pretreated substrates. Such system of reference substrates would help to screen “weak-lignin binding” cellulases as well as cellulases with high decrystallization efficiency. I mainly designed and performed the experiments and analyzed the data. The manuscript is written by me and edited by Dr. Xiao Zhang.

**Chapter Six** also includes part of the results from a published manuscript about impact of *alg3* gene deletion on growth, development, pigment production, protein secretion, and functions of recombinant *Trichoderma reesei* cellobiohydrolases in *Aspergillus niger*. My goal of this study is to evaluate the enzymatic activity of *alg3* gene deleted recombinant *Trichoderma reesei* cellobiohydrolase (rCel7A) expressed in *Aspergillus niger*. For the purpose of study, enzyme assays of Cel7A and rCel7A on nanocryalline cellulose and bleached kraft pulp were evaluated. Results demonstrated that the rCel7As have improved activities on hydrolyzing the nanocryalline cellulose. These results further confirms that the reference substrates provides a powerful tool screening and evaluating genetic modified enzymes with enhanced activity. The manuscript is written by Dr. Ziyu Dai. I conducted the experiments and data analysis of enzyme hydrolysis by NCC and bleached kraft pulp.
Finally, I summarized my work and the main conclusions as well as recommendations for future studies in *Chapter Seven*.
1.7 List of papers

This thesis is based on the following original research papers which are referred to in the text as Chapter Two to Chapter Six. The publications are reproduced with kind permission from the publishers. Additional unpublished material is also presented.


4. Ju, X., Bowden, M., Zhang, X., Development of accurate determination of crystallinity index and crystalline size of cellulose I material by refined X-ray diffraction. To submit to ACS Macro letters.


CHAPTER TWO: SPECIFIC EFFECTS OF FIBER SIZE AND FIBER SWELLING ON BIOMASS SUBSTRATE SURFACE AREA AND ENZYME DIGESTIBILITY

2.1 Abstract

To clarify the specific effect of biomass substrate surface area on its enzymatic digestibility, factors of fiber size reduction and swelling changes were investigated by using poplar substrates with controlled morphological and chemical properties after modified chemical pulping. Results showed that fiber size changes had insignificant influence on enzymatic hydrolysis, although the external surface area increased up to 41% with the reduction of fiber size. Swelling changes caused by increased biomass fiber porosities after PFI refining showed a significant influence on the efficiency of enzymatic hydrolysis. It is also found that chemical properties such as xylan and lignin content can influence the swelling effect. Xylan is confirmed to facilitate substrate hydrolysability by swelling, while lignin restricts swelling effect and thus minimizes the enzyme accessibility to substrates.

Keywords: Biomass recalcitrance; enzymatic hydrolysis; fiber size; swelling; surface area

2.2 Introduction

Discovering and identifying highly efficient and cost effective plant cell wall degrading enzymes is one of the most important research topics in developing an economically viable lignocellulosic biomass to fuel and chemical conversion process. Plant cell walls are constructed by nature to resist chemical, physical and microbial degradation. Understanding the relationship between lignocellulosic biomass substrate characteristics and their impact on enzymatic
digestibility has attracted a significant amount of research activities. Among various factors, the amount of substrate surface area (available or accessible surface area) has been cited as a key substrate characteristic closely related to enzymatic hydrolysability of the substrate (Gregg and Saddler 1996; Wyman 1999; Zhang and Lynd 2004). Lignocellulosic biomass is assembled by fibrous elements constructed in a matrix mainly consists of cellulose, hemicellulose and lignin. The amount of total substrate surface area is intricately linked to morphological characteristics across biomass fiber structural levels. Surface area of pulp fibers can be divided into external surface area affected mainly by fiber length and width, or internal surface area, which is governed by the size of the lumen and the volume of pores and other void within fiber cell wall. The varying fiber lengths and widths produced during pulping can be viewed in a similar manner as the array of particle sizes produced during the pretreatment of lignocellulosic substrates for bioconversion (Chandra, Bura et al. 2007). Changing the fiber length, width, or lumen diameter of fiber cells in biomass will collectively affect substrate particle size, resulting in changes of the substrate external surface area. Disruption of physical and chemical interactions among cellulose, hemicellulose and lignin can influence layer and porous structures within fiber cell walls, leading to changes in substrate internal surface area. A precise measurement of external, internal, or even total surface area of biomass substrate associated with cellulase accessibility is proven to be difficult to attain. The generally used external surface measurement for biomass substrate by Brunauer-Emmett-Teller (BET) method is proven to be difficult to represent the cellulase accessibility to substrate (Zhang and Lynd 2004). Measurement of the pore volume or porosity has been frequently used as an alternative to represent the amount of accessible substrate surface area to cellulase enzymes (Ogiwara and Arai 1968; Stone J, Scallan A et al. 1969; Grethlein 1985; Esteghlalian, Bilodeau et al. 2001; Chandra, Ewanick et al. 2008).
A number of studies have demonstrated that reduction in substrate size can help improve the enzymatic digestibility of biomass. For example, both Dasari et al. and Yeh et al., have reported that reducing the size of sawdust or microcrystalline cellulose by ball milling improved sugar and reduced the total hydrolysis time for enzymatic hydrolysis (Dasari and Berson 2007; Yeh, Huang et al. 2010). However, ball milling has been shown to reduce cellulose DP and crystallinity in addition to particle size (Yeh, Huang et al. 2010). In contrast to these reports of the beneficial effect of particle size on hydrolysis efficiency, Ballesteros has shown that particle size reduction does not necessarily lead to improvement in enzymatic digestibility since larger steam-exploded chips (8–12 mm) exhibited higher enzymatic digestibility (Ballesteros, Oliva et al. 2000). It is inevitable that, along with the biomass substrate size changes, other substrate characteristics were also modified which likely affected substrate hydrolysability. Mansfield has shown that with the reduction in particle size of the pretreated biomass substrate, there is also an increase in the amount of surface lignin coverage which would hinder enzymatic hydrolysis (Mansfield, Mooney et al. 1999). Besides these two contradictory observations, Sinitsyn et al. have also reached to a conclusion that “particle average size” does not noticeably influence the efficiency of enzymatic hydrolysis since the mixture of geometrically large and small particles may distort the size measurement (Sinitsyn, Gusakov et al. 1991). In all the previous studies, biomass substrates pretreated by physical or chemical methods were used. Selectively removing hemicellulose and/or lignin as well as drying the biomass during pretreatment has been shown to alter the substrate porosity. It is conceivable that the particle size reduction would primarily change the external surface area of the biomass substrate, while fiber internal structural changes will have direct impact on the porosity. In general, an increase in substrate porosity would likely lead to improved enzymatic digestibility. However, the specific effect of substrate porosity
changes without the interference of other substrate factors is difficult to determine. Due to the complex structural properties and heterogeneous surface topology of biomass substrate, especially after chemical pretreatments, it is a challenge to draw a quantitative relationship between substrate surface area (or available/accessible surface area) and its enzymatic digestibility.

Based on reviewing of existing information, it becomes apparent that a further understanding and clarification is needed for the effects that changes in substrate surface area, either through changes in fiber size or porosity, have on the biomass substrate hydrolysability. This information will provide critical guidance to the development of biomass construction methods to produce “ideal” substrates for efficient enzymatic hydrolysis. In a previous study, a methodology to prepare biomass “reference substrates” with controlled parameters was developed to allow the investigation of individual substrate factors and their effect on enzymatic hydrolysis (Ju, Engelhard et al. 2013). The main objective of this study was to attain a clear understanding of the effect of fiber size and porosity on the enzyme accessibility and hydrolysis efficiency. In this study, representative reference substrates with different fiber size and porosities were prepared by fiber fractionation and PFI refining. All the other substrates characteristics were maintained at a similar level. These substrates allowed us to investigate the individual effects of fiber size and swelling on substrate surface area and their enzymatic digestibility.
2.3 Materials and methods

2.3.1 Substrate preparation

Reference substrates prepared by modified kraft pulping of poplar, KP0 and KP13, as well as modified sulfite pulping of poplar SP0 and SP13 (Ju, Engelhard et al. 2013), were used to produce substrates with different fiber size and swelling fibers. KP0 is a lignin-free substrate with 82.20% glucan and 16.78% xylan, while KP13 consists of 79.91% glucan, 16.05% xylan and 2% lignin (Kappa number is 13). SP0 is a lignin free substrate with 87.41% glucan and 9.65% xylan, while the SP13 consists of 85.65% glucan, 9.52% xylan and 2% lignin (Kappa number is 13).

Fractionation of KP0 and SP0 to different fiber lengths (long, medium and short) was carried out in a Bauer-McNett fiber classifier fitted with 14-, 28-, 48-, 100-, and 200-mesh screens following Technical Association of Pulp and Paper Industry (TAPPI) standard method T222. Laboratory beating of substrates KP0, SP0, KP13 and SP13 was conducted on a PFI mill following TAPPI standard method T248. Two different revolution counts were applied for the PFI refining in this study (5,000 and 10,000 revolutions).

2.3.2 Chemical analysis

Moisture content of all substrates was determined by the mass loss after drying to constant weight at 105 °C in a convection oven. Kappa number was determined using the procedure described in TAPPI standard method T236 om-06. Monosaccharides were determined with a Perkin Elmer HPLC system with HPX-87P column following NREL/TP-510-42623 protocol.
2.3.3 Substrate characterization

The fiber length distribution and coarseness of all substrates were measured using a Fiber Quality Analyzer (FQA) as previously described (Robertson, Olson et al. 1999). All FQA measurements were the average of 5,000 fibers. Viscosity (measurement of degree of polymerization of cellulose) of all delignified substrates was measured according to TAPPI standard method T230 om-04 and American Society for Testing and Materials (ASTM) D4243-99. The viscosity of substrate solutions containing 0.05% (w/v) cellulose solution in 0.5 M cupriethylenediamine was measured on a viscosity rheometer (DV-III ULTRA, Brookfield engineering laboratories, Inc, MA). The intrinsic viscosity based average cellulose degree of polymerization (DPv) was calculated as described in ASTM standard method D4243-99. All viscosity measurements were performed using triplicate samples with triplicate measurements of each sample. The Simons’ stain (SS) of all samples was performed as previously described (Chandra, Ewanick et al. 2008). Orange dye was purified by 10K Amicon membrane under pressure of 35 psi (Chandra, Ewanick et al. 2008). The purpose of ultrafiltration is to remove the small particles in orange dye to ensure its absorption to represent enzyme. The fraction collected above 10 K was analyzed for particle size distribution. It was shown the particle size (hydrodynamic radius) of orange dye ranges from 70 nm to 1,100 nm with three peaks at 70-90 nm, 170-270 nm and 670-1,100 nm, respectively. The size of the orange dye is larger than the hydrodynamic radius of cellulase enzyme (>5 nm) (Yu, Minor et al. 1995).

The substrate crystallinity was measured by the general purpose X-ray diffractometer with Philips X'Pert MPD system and a vertical Θ-Θ goniometer (190 mm radius). The X-ray source was a ceramic X-ray tube with Cu anode. Operating power was 40 kV, 50 mA (2.0 kW). X-ray diffraction pattern of samples obtained after freeze-drying were recorded at room
temperature from 10° to 75°. The scan was carried out with a step size of 0.05°. Crystallinity index (CrI) was calculated from the height ratio between the intensity of the crystalline peak (I_{002} - I_{AM}) and total intensity (I_{002}) after subtraction of the background signal measured without substrate, using the Segal method (Segal, Creely et al. 1962).

2.3.4 Enzymes and enzymatic hydrolysis

Celluclast® 1.5 L (cellulase activity), and Novozyme 188® (β-glucosidase, BG) were obtained from Novozymes North America (Franklinton, NC). The activity of Celluclast is 80 FPU/mL, and the protein concentration of 97.53 mg/mL. Novozyme 188® has a protein concentration of 117 mg/mL and an activity of 487 CBU/mL. Cellulase was supplemented with β-glucosidase (1 FPU: 2 CBU) to avoid product inhibition caused by cellobiose accumulation.

Hydrolysis experiments were performed both in 250-mL flasks and in 2-mL Eppendorf tubes with reaction volume of 100 mL and 1.5 mL. The reaction solution contained 50 mM sodium acetate buffer (pH 4.8). Enzymatic hydrolysis was carried out at 50 °C for 72 hours. The results showed no difference between small and large volume hydrolysis, so averaged data was taken to calculate conversion yield. The enzymatic digestibility of reference substrates were determined and compared by enzymatic hydrolysis using 2% (w/v) substrate consistency. Such consistency was chosen to minimize the effect from glucose inhibition as suggested by previous research (Xiao, Zhang et al. 2004). The cellulose conversion yield is defined as the glucose amount in the hydrolysate divided by the cellulose content (as glucose) in the substrate.

Duplicate samples were taken at each time point. Aliquots were taken periodically from the hydrolysate to determine the glucose concentration. Glucose in the hydrolysate was measured using the glucose oxidase/peroxidase (GOPOD) assay kit from Megazyme (Wicklow, Ireland) that employs high purity glucose oxidase and peroxidase and can be used for the specific
measurement of D-glucose in extracts of plant materials or foods. Sugar analysis from HPLC as suggested by NREL (NREL/TP-510-42623) and microplate assay were compared by using a calibration curve generated from pure glucose. The 96-well microplate assay and HPLC analysis yielded identical conversion results. All samples were first centrifuged followed by filtration through a 0.45μm membrane prior to the analysis. All results reported for glucose analysis from enzymatic hydrolysis were mean values based on triplicate repeated experiments under the same conditions.

2.3.5 Cellulase adsorption isotherm

Cellulase adsorption on reference substrates was performed at 25 °C in 50 mM sodium acetate buffer (pH 4.8) as described by Tu (Tu, Chandra et al. 2007). Celluclast was used for the adsorption isotherm experiments instead of the mixture of cellulase and β-glucosidase. Enzyme solutions with concentrations ranging from 0.01 mg/mL to 3 mg/mL were added to the buffer with substrates at 2% consistency. Calculations followed the procedure described previously (Ju, Engelhard et al. 2013).

2.4 Results and discussion

2.4.1 Preparation and characterization of substrates with different fiber size

As mentioned in the previous work (Ju, Engelhard et al. 2013), KP0 and SP0 are delignified substrates with differing xylan content. The morphological properties of KP0 and SP0 were similar, with an average fiber length of 0.8 mm and an average fiber width of 22 μm. Three fractions from each substrates were collected to represent three levels of fiber lengths (L: long, M: medium; S: short). Such fiber fractionation is achieved by chambers fitted progressively with smaller screen mesh, which can act as mechanical barriers to separate fibers based on their
length. KP0-L and SP0-L were the combined fractions retained on 14 and 28 meshes from respective KP0 and SP0. KP0-M and SP0-M were collected between 28 and 48 meshes while KP0-S and SP0-S were collected between 48 and 100 meshes. The fiber lengths and widths were measured (Table 2.1). The degree of polymerization (DP\textsubscript{v}) and crystallinity index (CrI) of these samples were measured by viscosity and XRD methods respectively. The DP\textsubscript{v} estimated by viscosity are shown in Table 2.1. Statistical analysis showed that the difference between KP0, KP0-L and KP0-M were negligible while the KP0-S has an insignificantly lower DP\textsubscript{v} (ANOVA p-value = 0.07). As shown in Table 2.1, the DP\textsubscript{v} difference among the SP0 samples was negligible (ANOVA p-value = 0.86). No significant difference in CrI was detected (average CrI at 0.82 ± 0.03, ANOVA p-value = 0.69).

Table 2.1. Fiber characterization of reference substrates before and after fiber size fractionation.

Numbers in parentheses indicate the standard deviation (n≥3).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Length (mm)</th>
<th>Width (um)</th>
<th>DP\textsubscript{v}</th>
<th>Γ\textsubscript{max} (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP0</td>
<td>0.824 (0.010)</td>
<td>22.2 (0.2)</td>
<td>501 (72)</td>
<td>69 (1)</td>
</tr>
<tr>
<td>SP0-L</td>
<td>1.235 (0.014)</td>
<td>24.4 (0.2)</td>
<td>477 (72)</td>
<td>58 (1)</td>
</tr>
<tr>
<td>SP0-M</td>
<td>1.053 (0.007)</td>
<td>23.0 (0.2)</td>
<td>477 (48)</td>
<td>57 (3)</td>
</tr>
<tr>
<td>SP0-S</td>
<td>0.770 (0.007)</td>
<td>21.4 (0.2)</td>
<td>453 (96)</td>
<td>126 (1)</td>
</tr>
<tr>
<td>KP0</td>
<td>0.833 (0.008)</td>
<td>22.0 (0.2)</td>
<td>619 (91)</td>
<td>102 (6)</td>
</tr>
<tr>
<td>KP0-L</td>
<td>1.281 (0.016)</td>
<td>25.5 (0.3)</td>
<td>573 (96)</td>
<td>106 (2)</td>
</tr>
<tr>
<td>KP0-M</td>
<td>1.076 (0.006)</td>
<td>22.9 (0.1)</td>
<td>643 (48)</td>
<td>105 (1)</td>
</tr>
<tr>
<td>KP0-S</td>
<td>0.834 (0.007)</td>
<td>21.8 (0.2)</td>
<td>453 (72)</td>
<td>118 (4)</td>
</tr>
</tbody>
</table>
2.4.2 Specific effect of fiber size changes on substrate external surface area and enzymatic digestibility

When the hydrolysis profiles of these substrates were compared (Figure 2.1A), it was apparent that the difference in fiber size resulted in little change in hydrolysis yields during the 72-hour enzymatic hydrolysis. At enzyme loading of 2 FPU per gram of cellulose in substrate,

![Graph A](image1)

![Graph B](image2)

**Figure 2.1.** (A) Enzymatic hydrolysis of fiber fractionated reference substrates SP0 and KP0; (B) The dye adsorption measurement of fractionated reference substrates using the Simons’ Stain (SS) technique. Hydrolysis conditions: 2% solids content, 2 FPU cellulase enzyme loading per gram of cellulose in substrates (Celluclast: BG =1 FPU; 2 CBU), 50mM sodium acetate buffer pH 4.8, 50°C. Error bars indicate the standard deviation (n=3).
only a 2.8% increase in cellulose hydrolysis yield was observed for the shortest fraction SP0-S (44%) compared to that of the longest fraction, SP0-L (41%). The hydrolysis yield difference between longest (KP0-L) and shortest (KP0-S) fractions of kraft pulp was also negligible, 62% vs 64%. ANOVA p-value for SP0 set and KP0 set hydrolysis yield was 0.08 and 0.69 respectively, indicating no significant digestibility difference among substrates with various fiber lengths. Enzymatic hydrolysis was also performed at higher dosage ranging from 5 FPU.g\(^{-1}\) to 20 FPU.g\(^{-1}\). However, higher enzyme loadings resulted in faster hydrolysis rate and further minimized the hydrolysability difference among substrates with various fiber lengths (data not shown). It is noticed that KP0 samples all showed a better hydrolysability than SP0 samples with similar fiber length, substantiating the previous finding of the effect of xylan since KP0 has a higher xylan content than SP0 (Ju, Engelhard et al. 2013). However, despite an observation of 54-60% length difference between long and short fractions of fibers (e.g. KP0-L vs. KP0-S, SP0-L vs. SP0-S), the difference in hydrolysability was insignificant.

It is recognized that various biomass fiber size reduction methods may bring in subsequent changes regarding to fiber chemical and physical properties (Chandra, Bura et al. 2007; Vidal, Dien et al. 2011; Leu and Zhu 2012). Ball milling, one of commonly used techniques for fiber size reduction, can cause cellulose crystallinity decrease due to the fraction damage of the crystalline structure (Sinitsyn, Gusakov et al. 1991). Separating fibers of different sizes through the screening of refined substrates from either raw or pretreated biomass can also result in heterogeneous distribution of chemical compositions (e.g. surface lignin content) in different size fractionations (Mooney, Mansfield et al. 1999; Del Rio, Chandra et al. 2012).

In this study, both lignin-free KP0 and SP0 sets of samples have well controlled chemical and physical properties, with fiber size as the only significant variable between them. It is
apparent that this isolated factor did not have a significant impact on enzymatic digestibility. Simons’ stain method was applied to measure the surface area of the KP0 and SP0 samples. The technique is based on the competitive adsorption of two dyes (blue and orange) where the orange dye has a higher molecular weight and affinity to cellulose (Yu, Minor et al. 1995; Esteghlalian, Bilodeau et al. 2001; Chandra, Ewanick et al. 2008). Macromolecules such as protein in solution are non-spherical, dynamic (tumbling), and solvated. As such, the radius calculated from the diffusional properties of the particle is indicative of the apparent size of the dynamic hydrated/solvated particle. Hence, it is called “hydrodynamic radius”. Cellulase enzymes were calculated to have hydrodynamic radii bigger than 5 nm (Grethlein 1985). The hydrodynamic radius of the orange dye is comparable to those of cellulase enzymes, while the blue dye consists of much smaller molecules. Thus, the total dye adsorption can be used to represent the total surface area of fibers, while the orange dye adsorption can indicate amount of surface area that can be accessed by enzymes. As shown in Figure 2.1B, the total dye adsorbed on SP0-S and KP0-S were approximately 22% and 41% higher than the respective SP0-L and KP0-L, suggesting a significant increase in the surface-to-weight ratio from fiber size reduction. However there was not a clear trend in the amount of orange dye adsorption on different substrates.

The change in fiber size predominantly brings changes in external surface area at the fiber level, while the ultrastructure such as cellulose-cellulose, hemicellulose-cellulose interactions within fiber cell remains unaltered. During the hydrolysis of lignin-free substrates such KP0 and SP0, it is likely that the amount of reactive substrate surface is in excess to the amount of enzyme presented. Therefore a 22-41% increase in total surface area may not be sufficient to cause a significant impact on enzyme hydrolysis rate. These results are in agreement
with the previous findings using pure cellulose substrates (Peters, Walker et al. 1991; Sinitsyn, Gusakov et al. 1991). A recent work by Del Rio et al. has also shown that fiber size had little influence on enzyme hydrolysis of organosolv-pretreated softwood, which is a substrate susceptible to enzymatic hydrolysis (Del Rio, Chandra et al. 2012). However, during the hydrolysis of more recalcitrant biomass substrates, such as in sawdust with considerable amount of non-cellulose components on substrate surface (e.g. lignin), an increase in surface area by size reduction would expose more cellulose for enzyme and hence increase substrate hydrolysability (Chang and Holtzapple 2000).

As mentioned earlier, it is confirmed that changes in fiber size primarily affect substrate external surface area. It is well understood that the enzymatic hydrolysis of cellulose proceeds in two phases, an initial fast hydrolysis phase and a second slow-down phase. The difference of external surface area would likely play a role during the initial hydrolysis phase. Del Rio et al. has shown that after 12 hours of enzymatic hydrolysis, the fiber size from different fiber length fractions becomes identical (Del Rio, Chandra et al. 2012). Therefore, it becomes clear that the influence of fiber size on substrate digestibility is essentially associated with the recalcitrance from the biomass substrate itself. Fiber size will have little effect on a substrate that is already susceptible to enzymatic hydrolysis, while size reduction may be useful for improving the digestibility of substrates that have limited reactive cellulose surface to enzymes. It is also conceivable that biomass size reduction technique aiming at fiber separation (e.g. disk refining) instead of fiber cutting will produce substrates more suitable for enzyme hydrolysis.

2.4.3 Preparation substrates with different levels of fiber swelling

While fiber size reduction primarily affected the external surface area, a change in the macro or micro porous structures within fiber wall at the fibril level will likely alter the
interactions between hemicellulose and cellulose and/or between cellulose fibrils. Increases in substrate porosity have often been observed after the chemical pretreatment of biomass substrate along with improvements in their enzymatic digestibility of the substrate (Sinitsyn, Gusakov et al. 1991; Esteghlalian, Bilodeau et al. 2001). To better determine the porosity effect on substrate hydrolysability, we treated lignin-free substrates KP0 and SP0 as well as low-lignin content substrates KP13 and SP13 by PFI refining at two different intensities, 5,000 and 10,000 revolutions, to create a new set of substrates with different degrees of swelling, namely SP0-5,000, SP0-10,000, KP0-5,000, KP0-10,000, SP13-5,000, SP13-10,000, KP13-5,000, and KP13-10,000. An attempt to specifically change substrate porosity without altering other substrate characteristics has not been successfully pursued previously. A recent work by Del Rio et al. using PFI milling to swell organosolv pretreated lodgepole pine fiber (Del Rio, Chandra et al. 2011) leads to fiber length reduction and decrease in crystallinity using refining intensities between 2,500-20,000 revolutions. Despite these changes, the substrate digestibility does not change significantly. High lignin content is suspected to play a more significant role in hindering substrate digestibility. We have chosen lignin-free (KP0 and SP0) as well as low-lignin content substrates (KP13 and SP13) in this study to minimize the “lignin effect”. Careful operation of PFI refining in this study helped to avoid significant fiber cutting and minimize fiber length changes, as shown previously (Kerekes 2005). In addition, we have shown in the previous section that fiber size reduction did not affect enzymatic digestibility of KP0 and SP0 samples. The crystallinity indices of PFI treated samples were compared with untreated samples and have shown little changes (Table 2.2) when differing in PFI refining intensities between 5,000 and 10,000 revolutions. There was a decrease in DP, after PFI treatment using 10,000 revolutions for both KP0 and SP0.
Table 2.2. Fiber characterization and Langmuir constants from Celluclast on reference substrates at 25 °C. Numbers in parentheses indicate the standard deviation (n≥3). Protein loading from 0.01 mg/mL to 3.0 mg/mL to ensure a full adsorption.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>CrI (%)</th>
<th>DPv</th>
<th>( \Gamma_{\text{max}} ) (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP0</td>
<td>0.80 (0.01)</td>
<td>501 (72)</td>
<td>69</td>
</tr>
<tr>
<td>SP0-5,000</td>
<td>0.76 (0.06)</td>
<td>429 (48)</td>
<td>83</td>
</tr>
<tr>
<td>SP0-10,000</td>
<td>0.77 (0.06)</td>
<td>333 (72)</td>
<td>116</td>
</tr>
<tr>
<td>KP0</td>
<td>0.77 (0.01)</td>
<td>619 (91)</td>
<td>102</td>
</tr>
<tr>
<td>KP0-5,000</td>
<td>0.76 (0.03)</td>
<td>549 (45)</td>
<td>129</td>
</tr>
<tr>
<td>KP0-10,000</td>
<td>0.75 (0.07)</td>
<td>453 (96)</td>
<td>142</td>
</tr>
</tbody>
</table>

2.4.4 Specific effect of fiber swelling on substrate surface area and enzymatic digestibility

Enzymatic hydrolysis of SP0, SP0-5,000, SP0-10,000, KP0, KP0-5,000, KP0-10,000, SP13, SP13-5,000, SP13-10,000, KP13, KP13-5,000, and KP13-10,000 were carried out for 72 hours at different enzyme dosages ranging from 2 FPU to 20 FPU per gram of cellulose. It was apparent that the increase in PFI refining intensities improved the hydrolysability of most of the samples. As shown in Figure 2.2A, the 72-hour enzymatic hydrolysis yield of refined substrates from sulfite pulping after 10,000 PFI revolutions was 13%-14% higher than untreated substrates (2 FPU enzyme loading per gram of cellulose). The 72-hour enzymatic hydrolysis yield of refined substrates from kraft pulping after 10,000 PFI revolutions was 24%-26% higher than substrates without refining from the same pulping process regardless of lignin content. The PFI
refining had a more significant impact on kraft pulped substrates due to their higher xylan content, which made them more susceptible to fiber swelling. Simons’ stain method was applied.

Figure 2.2. (A) Enzymatic hydrolysis of PFI refined reference substrates; (B) Orange dye adsorbed on PFI refined substrates measured by Simons’ stain (SS). Hydrolysis conditions: 2% solids content, 2 FPU cellulase enzyme loading per gram of cellulose in substrates (Celluclast: BG =1 FPU; 2 CBU); 50mM sodium acetate buffer pH 4.8, 50°C. Error bars indicate the standard deviation (n=3).
to analyze substrate porosity changes resulted from PFI refining. As shown in Figure 2.2B, a consistent increase in orange dye adsorption was observed along with the increase in refining intensities among all samples. 10,000 PFI revolutions led to a 2-3 times increase in orange dye adsorption compared to untreated samples. PFI milling is a technique to treat plant fibers in water by mechanical compression force to create fibrillation on the surface and within cellulose fibrils (Stephansen 1948; Laine, Wang et al. 2004; Kerekes 2005). PFI refining can open up pore structure in the fiber walls leading to fiber swelling and internal pore volume increases. The observed improvement in orange dye adsorption confirmed that the increase in porosity resulting from PFI refining is likely associated with increased internal surface area available to accommodate more cellulase enzymes.

A closer investigation of hydrolysis profiles in Figure 2.2A revealed that this “porosity change effect” caused by PFI refining apparently affected both initial hydrolysis rate and final hydrolysis yield. This appeared to suggest that after PFI refining, the reactivity of the substrate changed in addition to the increase in surface area. Besides intrinsic reactivity related to the chemical nature of cellulose, a number of other physical parameters may also affect cellulose reactivity. Native cellulose is a compacted water insoluble macromolecule that is constructed by nature to resist chemical and microbial penetration. Creating more accessible chain ends and increasing fraction of glucose-glucose bonds accessible to cellulase enzymes within the cellulose crystalline structure will help improve cellulose reactivity (Zhang and Lynd 2004). The PFI refining primarily caused swelling of cellulose macromolecules and internal separations between macro and microfibrils, in another words, fibrillation. This explained why the orange dye adsorption increased more significantly after PFI refining than fiber size reduction. Reducing fiber size only brought limited internal surface area enhancement, while fibrillation swelling led
to more profound changes to the pore structures and thus the exposure of more layers of reactive substrate surface within the fiber. Therefore, an increase in the initial hydrolysis was observed. More importantly, improved hydrolysis efficiency was observed during the entire hydrolysis course which led to a higher conversion yield.

While orange dye adsorption measured by Simons’ stain has been demonstrated as an effective technical method to quantify the amount of substrate surface area that would be accessible to cellulase enzymes based on their physical dimension, the actual enzyme-substrate interactions during enzymatic hydrolysis could be different due to surface and chemical properties of specific cellulase enzymes (Mansfield, Mooney et al. 1999). Enzyme adsorption isotherm was further evaluated to determine maximum enzyme adsorption capacity $\Gamma_{\text{max}}$ for the substrates before and after PFI refining. As shown in Table 2.2, a general increase in $\Gamma_{\text{max}}$ was observed with the increase in refining intensities. Compared to KP0 and SP0, the $\Gamma_{\text{max}}$ values of KP0-10,000 and SP0-10,000 after 10,000 revolutions PFI refining increased by 38% and 68% respectively. While such improvement was significant, these percentages were much smaller than those observed in the available surface area according to the amount of orange dye adsorption during Simons’ stain, which was between 2-3 times greater. This finding suggested that a significant increased portion of the substrate surface area resulted from PFI refining was not readily accessible to the cellulase enzymes at the beginning of the hydrolysis. These embedded “reactive surface areas” were likely to be sequentially exposed during progressive enzymatic hydrolysis, which was demonstrated by a constantly improving hydrolysis rate detected during Simons’ stain, which was between 2-3 times greater. This finding suggested that a significant increased portion of the substrate surface area resulted from PFI refining was not readily accessible to the cellulase enzymes at the beginning of the hydrolysis. These embedded
“reactive surface areas” were likely to be sequentially exposed during progressive enzymatic hydrolysis, which was demonstrated by a constantly improving hydrolysis rate detected during the entire hydrolysis course.

The results obtained from this study confirmed that mechanical treatment such as PFI refining can increase biomass substrate surface area and result in improved enzymatic digestibility as observed by a number of previous studies (Mooney, Mansfield et al. 1999; Koo, Treasure et al. 2011). It is interesting to see that the extent of cellulose-to-glucose conversion yield improvements resulting from the refining differs among substrates. After 10,000 revolutions refining, the conversion yield of SP0 improved from ~ 38% to ~ 50% (approximately 31% increase) while that of KP0 improved from 58.5% to 82% (approximately 40% increase). This suggests that the efficacy of the refining on substrate digestibility also depends on the nature of the substrates. As reported previously, the presence of xylan in cellulosic substrates can facilitate fiber swelling and enhance enzymatic hydrolysis (Ju, Engelhard et al. 2013). Comparing the results between SP0 and KP0 before and after PFI refining shown in Figure 2.3 further substantiated this conclusion. The presence of xylan in the substrate is critical to cellulose fibril swelling (Figure 2.3). The organosolv-pretreated substrates used in the Del Rio et al. study were treated by ethanol solution with sulfuric acid as a catalyst (Del Rio, Chandra et al. 2011; Del Rio, Chandra et al. 2012). The majority of xylan was degraded with little remaining in the substrate. This is probably one of the reasons why little improvement of substrate digestibility was observed after PFI refining. On the other hand, the presence of a significant amount of lignin in the organosolv-pretreated substrates may also diminish the potential “swelling effect”. As shown by Del Rio et al., either partial removal of lignin or changing lignin hydrophobicity by sulfonation led to a significant improvement in substrate digestibility. Therefore the lack of xylan
and the presence of hydrophobic lignin led to a minimized effect of PFI refining on substrate digestibility improvement (Figure 2.3).

As shown in our previous study (Ju, Engelhard et al. 2013), the presence of the surface lignin in substrate SP13 and KP13 (containing approximately 16.5% and 29.4% of surface lignin coverage respectively) clearly limited the substrate swelling effects after 5,000 and 10,000 PFI refining. The orange dye adoptions of the SP13 increased by 33.8% and 89.57% for 5,000 and
10,000 resolutions refining compared to 112.8% and 193.7% increase from SP0 after the same treatment conditions. A similar trend was observed between KP0 and KP13. The hydrolysis rate increases on SP13 were smaller than those obtained from SP0. Increasing PFI refining intensity from 5,000 revolutions to 10,000 revolutions brought little change to KP13 hydrolysability.

Therefore it becomes clear that substrate swelling can enhance substrate hydrolysability. This effect is much more significant on a lignin-free substrate with considerable amount of xylan than on substrates with high lignin content (Figure 2.3). The amount of orange dye adsorption has been shown to have a strong correlation to enzymatic digestibility (Koo, Treasure et al. 2011). A similar correlation is observed in this study within each set of substrate SP0 and KP0. However comparing between the SP0 and KP0, it became difficult to draw a quantitative relationship among different types of substrates. The orange dye adsorption value obtained from Simons’ stain assay provides good quantification of substrate surface area accessible to enzyme according to their physical dimension (Chandra, Ewanick et al. 2009).

It is conceivable that different substrates with different chemical properties would exhibit different reactivity to cellulase enzymes. Although the surface area determined by orange dye adsorption of SP0-10,000 is comparable to that of KP0-5,000, the hydrolysability difference between these two samples was apparent. It also needs to be pointed out that the PFI refining did cause some reduction in cellulose DPv measured by viscosity. In KP0 samples, the level of DPv reduction was similar to that observed among different fiber sizes. Therefore, it is likely this potential DPv difference did not contribute significantly to hydrolysability improvement. It was also apparent that the initial 5,000 revolutions brought more significant impact on both SP0 and KP0 samples regarding both surface area and enzymatic digestibility increases compared to the
additional 5,000 revolutions. This is in agreement with the observations from Koo et al. (Koo, Treasure et al. 2011).

The effects of cutting orientation in woody biomass size reduction on enzymatic hydrolysis sugar yield were also investigated (Figure A2.1). Since in pulping industry, wood chipping orientation has impact on conversion rate or similar but no reported studies on this issue in the field of enzymatic hydrolysis for biofuel purpose. Three cutting orientations were studied. The longitudinal direction is parallel to the long axis of the stem, the radial direction is perpendicular to both wood annual rings and the long axis of the stem, and the tangential direction is tangent to wood annual rings (Figure A2.2). Results of different cutting orientation chips with different same size showed significant difference regarding to the sugar yield (Figure A2.3). It is agreed with previous study that enzyme accessible area is more important than particle size (Figure A2.4). Thus it is concluded that it is not necessary to invest a huge amount of energy to produce very fine particles, if particles produced by size reduction already have very accessible surfaces to enzymes.

2.5 Conclusions

This study confirms that fiber size reduction leads to a change in substrate external surface area. However the impact of this change on the hydrolysability improvement is limited, especially for substrates with good digestibility. PFI refining can effectively swell fiber cell wall structures, resulting in the increases of both external and internal surface areas as well as substrate reactivity. The efficacy of substrate swelling on enzymatic digestibility is also influenced by substrate chemical properties. The presence of xylan facilitates swelling and thus
improves enzymatic digestibility, while the presence of lignin minimizes the beneficial effect of fiber swelling on its digestibility.
CHAPTER THREE: AN ADVANCED UNDERSTANDING OF THE SPECIFIC EFFECTS OF XYLAN AND SURFACE LIGNIN CONTENTS ON ENZYMATIC HYDROLYSIS

3.1 Abstract

In this study, chemical pulping techniques were applied to create a set of biomass substrates with intact lignocellulosic fibers and controlled morphological and chemical properties to allow the investigation of the individual effects of xylan and surface lignin content on enzymatic hydrolysis. A high resolution X-ray photoelectron spectroscopy technique was established for quantifying surface lignin content on lignocellulosic biomass substrates. The results from this study show that, apart from its hindrance effect, xylan can facilitate cellulose fibril swelling and thus create more accessible surface area, which improves enzyme and substrate interactions. Surface lignin has a direct impact on enzyme adsorption kinetics and hydrolysis rate. Advanced understanding of xylan and surface lignin effects provides critical information for developing more effective biomass conversion process.

Keywords: Biomass recalcitrance; X-ray photoelectron spectroscopy; enzymatic hydrolysis; xylan; surface lignin

3.2 Introduction

There is little doubt that sugar will become a primary currency in the future bioeconomy, and lignocellulosic biomass has been regarded as the most promising and sustainable source for supplying non-food sugar (Himmel, Ding et al. 2007). However, the lack of highly efficient and cost effective cell-wall degrading enzymes is a major obstacle that prevents commercialization of
a biologically-based lignocellulosic biomass conversion process. The effectiveness of enzymatic hydrolysis is inextricably linked to the structural and chemical characteristics of the biomass material. Cellulose crystallinity, degree of polymerization (DP), lignin and hemicellulose content, and their distributions, particle size, and accessible surface area are among the most frequently cited substrate parameters associated with this effectiveness (Gregg and Saddler 1996; Wyman 1999; Zhang and Lynd 2004). From an anatomical viewpoint, lignocellulosic biomass fiber can be represented at the fiber, fibril, and molecular level. The morphological properties of fiber cells such as length, width, cell wall thickness, and lumen diameters, etc. are revealed at the fiber level. A typical fiber consists of primary (P) and secondary (S) cell wall layers; each of these layers is composed of numerous fibrils which encompass macro, micro and elementary fibril (Ding and Himmel 2006; Chinga-Carrasco 2011). At the fibril level, the interactions between cellulose, hemicellulose and lignin can be revealed (Gibson 2012), as well as the arrangement of amorphous and crystalline cellulose (microfibril/elementary fibril level) (Frey-Wyssling 1954; Heyn 1969). When the substrate parameters are examined across these structural levels, complexity arises. While Chapter two delineate biomass recalcitrance factors such as fiber size and available surface effects on enzyme hydrolysability, this chapter focuses on clarifying the role of biomass recalcitrance influences from fibril level.

A considerable amount of research effort has been directed toward delineating the relationship between specific biomass substrate properties and the efficacy of cellulase enzyme components (Wyman 1999; Zhang and Lynd 2004; Himmel, Ding et al. 2007; Himmel 2008). These studies have significantly advanced the knowledge of major factors that limit enzymatic hydrolysis. However, it is recognized that pretreated biomass substrates have intricate morphological structure and heterogeneous chemical composition. These characteristics have
significantly impeded a complete understanding of the specific effect of individual biomass components on enzyme hydrolysability. For example, while the removal of xylan during pretreatment enhances cellulose enzymatic digestion by reducing xylan coating and linkages to cellulose at the fibril level (Hendriks and Zeeman 2009; Lee, Rodrigues et al. 2010), the mechanism by which xylan impacts cellulose digestion is still unclear. Furthermore, xylan is inextricably embedded in the cell fiber matrix with lignin; thus, the individual xylan impact can hardly be separated and studied from other substrate parameters. On the other hand, substrate lignin content has also been considered as one of the most influential substrate factors that limit enzymatic hydrolysis (Chang and Holtzapple 2000; Palonen, Tjerneld et al. 2004; Berlin, Balakshin et al. 2006). Lignin can physically impede access of an enzyme to cellulose and/or reversibly or irreversibly adsorb cellulase enzymes, which impairs their activity (Eriksson, Borjesson et al. 2002; Palonen, Tjerneld et al. 2004; Berlin, Balakshin et al. 2006). In previous studies, bulk lignin content is often the only parameter used to interpret the “lignin effect” on enzyme hydrolysability. However, there has been great difficulty in drawing a clear correlation between bulk lignin content and enzyme hydrolysability. Previous work has shown that different lignin contents in either grass or hardwood substrate did not significantly influence enzyme digestibility (Sathitsuksanoh, Zhu et al. 2012).

During the pretreatment, lignin can migrate to the fiber surface and lead to a redistribution throughout the biomass substrate. Enzymatic hydrolysis of lignocellulosic biomass starts with the adsorption of enzymes onto the fiber surface. The topographic characteristics of the substrates have a dominant effect on the hydrolysis rate. An understanding of the specific effect of surface lignin on enzymatic hydrolysis is lacking.
In this study, modified chemical pulping technique was applied to create a set of biomass substrates with controlled fiber properties (length, width, cell wall thickness, lumen diameter) and selectively altered substrate parameters (xylan, surface and bulk lignin contents) at the fibril level. Chemical pulping specifically targets the lignin-enriched compounded middle-lamella layer and thus separates adjacent fiber cells. Maintaining fiber integrity is one of the most important objectives of chemical pulping. Pulped substrates can serve as a better model to mimic the structural complexity of the plant cell wall with controlled chemical composition and surface properties. The objective of this study is to gain an advanced understanding of the specific effects of xylan and surface lignin on enzyme hydrolysability with these substrates.

### 3.3 Materials and methods

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Pulping chemicals (% on wood)</th>
<th>Temperature (°C)</th>
<th>Total time/time at temperature (min/min)</th>
<th>Liquid: wood ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>KP13</td>
<td>16% Na₂O (active alkaline) and 25% Na₂S (sulfidity)</td>
<td>170</td>
<td>160/70</td>
<td>4:1</td>
</tr>
<tr>
<td>KP40</td>
<td>16% Na₂O (active alkaline) and 25% Na₂S (sulfidity)</td>
<td>170</td>
<td>107/37</td>
<td>4:1</td>
</tr>
<tr>
<td>SP13</td>
<td>5.46 % Total SO₂ (Mg₂SO₄), pH: 2.88</td>
<td>165</td>
<td>218/92</td>
<td>6:1</td>
</tr>
<tr>
<td>SP40</td>
<td>5.46 % Total SO₂ (Mg₂SO₄), pH: 3.8</td>
<td>165</td>
<td>160/40</td>
<td>6:1</td>
</tr>
</tbody>
</table>

#### 3.3.1 Substrate preparation and characterization

Poplar was used to produce substrates in this study. As shown in Table 3.1, Two kraft pulping conditions were chosen to produce substrates with kappa numbers at 13 (KP13) and 40 (KP40), respectively. Kappa number is a measurement of the bulk lignin content of substrates that have relatively low lignin content. An empirical conversion factor of kappa number multiplied by 0.147 can be used to estimate Klason lignin. Pulping to a higher kappa number was
also attempted by lowering the pulping severity (H factor); however, this condition was insufficient for attaining a fiber separation point, which resulted in a pulp sample with a significant amount of unseparated fiber (rejects). Pulping poplar at a higher H factor led to severe damage to the cellulose fiber. To obtain a lignin free substrate KP0, KP13 was treated by acid chlorite solution at room temperature for 24 h following a previously described procedure (Browning 1967). Two sulfite pulping conditions were used to produce substrates SP13 and SP40 to attain similar lignin contents as KP13 and KP40, respectively. After pulping, the substrates were screened through a laboratory flat screen plate (0.25 mm wide slots) to remove shives and uncooked material. The chemical compositions of all substrates were determined by standard Tappi procedures (T236, T204, T222, T211, and T249). Fiber length and coarseness were measured by fiber quality analysis (FQA). The available surface areas of the substrates were evaluated by Simons’ staining (SS) procedure (Chandra, Ewanick et al. 2008). The crystallinity of substrates was measured by the general purpose X-ray diffractometer with Philips X’Pert MPD system and a vertical Θ-Θ goniometer (190 mm radius). The X-ray source was ceramic X-ray tube with Cu anode. Operating power was 40 kV, 50 mA (2.0 kW). X-ray diffraction pattern of samples obtained after freeze-drying were recorded at room temperature from 10° to 75°. The scan was carried out with a step size of 0.05°. Crystallinity index (CrI) was calculated from the height ratio between the intensity of the crystalline peak (I_{002} - I_{AM}) and total intensity (I_{002}) after subtraction of the background signal measured without substrate, using the Segal method.

3.3.2 X-ray photoelectron spectroscopy (XPS) measure of surface lignin coverage

The amount of surface lignin on the substrates was determined using XPS. As XPS can be used to determine surface lignin content on cellulose substrates by either O/C ratio or high
resolution C1s spectra, both methods were employed in this study to determine the differences in surface lignin content in all substrates. A procedure described by Johansson (Johansson, Campbell et al. 1999) was used to measure surface lignin contents of KP0, KP13, KP40, SP0, SP13, and SP40. The O/C ratio was used to estimate surface lignin content via a linear relationship between the O/C ratio of 0.74 for pure cellulose (Whatman filter paper) and an O/C ratio of 0.33 for lignin. Prior to XPS analysis, the samples were extracted with acetone to remove lipophilic extractives and then air dried. No plastic containers were used to avoid possible hydrocarbon contamination. Samples were pumped inside the chamber overnight to eliminate any residual moisture content. The XPS analysis was carried out with Kratos Axis Ultra spectrometer, using a monochromatic Al K (alpha) source (10mA, 15kV). XPS has capability to probe the surface of the sample to a depth of 5-7 nm, and has detection limits that range from 0.1 to 0.5 atomic percent depending on the element. The Kratos charge neutralizer system was used on all specimens. Survey scan analysis was carried out with an analysis area of 300 µm × 700 µm and pass energy of 160 eV. High resolution scan was performed with the same analysis area of 300 µm × 700 µm and pass energy of 20 eV. Measurements were taken at three different spots on each sample to attain an average over the heterogeneity of the samples.

3.3.3 Enzymes and enzymatic hydrolysis

CelliC® Ctec2, Celluclast® 1.5 L (cellulase activity), and Novozyme 188 (β-glucosidase, BG) were obtained from Novozymes North America (Franklinton, NC). The activity of Ctec2 is 120 filter paper units (FPU)/ml; the xylanase activity, 11.5 international units (IU)/ml; and the protein concentration, 257 mg/ml. The activity of Celluclast is 80 FPU/ml, and the protein concentration, 97.53 mg/ml. Novozyme 188 has a protein concentration of 117 mg/ml and an
activity of 487 CBU/ml. Different levels of enzyme dosage (unit per gram of cellulose in the substrate) were used in the hydrolysis as specified in the text.

Hydrolysis experiments were performed both in 250-ml flasks and in 2-ml Eppendorf tubes. The reaction solution contained 50mM sodium acetate buffer (pH 4.8). Enzymatic hydrolysis was carried out at 50 °C for up to 120 hours. Compared results showed no difference between small and large volume hydrolysis, so averaged data was taken to calculate conversion yield. The hydrolysability of the substrates were determined and compared by enzymatic hydrolysis using 2% (w/v) substrate consistency. Such consistency was chosen to minimize the effect from glucose inhibition as suggested by previous research (Xiao, Zhang et al. 2004). The cellulose-to-glucose conversion yield is defined as the glucose amount in the liquid phase product divided by the cellulose content (as glucose) in the substrate.

Aliquots of 0.1 ml were taken periodically from the hydrolysate to determine the glucose and reducing sugar concentration. Glucose in the hydrolysate was measured by glucose oxidase/peroxidase (GOPOD) assay kit from Megazyme (Wicklow, Ireland) that employs high purity glucose oxidase and peroxidase and can be used for the specific measurement of D-glucose in extracts of plant materials or foods. Glucose and xylose content in the hydrolysate were also measured by HPLC method according to NREL/TP-510-42623 protocol. Reducing sugar content was applied to determine the initial hydrolysis rate (in the first hour) as the hydrolysate likely contains a mixture of glucose and its oligomers. The reducing sugar content was measured using a modified 96-well microplate DNS assay. All samples were filtered through a 0.45μm first centrifuged to prior to the analysis.
3.3.4 Cellulase adsorption isotherm

Cellulase adsorption on substrates was performed at 25 °C in 50 mM sodium acetate buffer (pH 4.8) as described by Tu (Tu, Chandra et al. 2007). Celluclast was used for the adsorption isotherm experiments instead of Ctec2, since the latter contains high amounts of β-glucosidase. Enzyme solution with concentrations ranging from 0.01 mg/ml to 3 mg/ml was added to the buffer with substrates at 2% consistency. The mixture was incubated at 25 °C for 1 h to reach equilibrium, after which protein content in the supernatant was determined by Bradford assay using bovine serum albumin (BSA) as standard. The adsorbed protein was calculated by taking the difference between the initial cellulase content and free cellulase content in the supernatant. Classical Langmuir adsorption isotherm was applied to cellulase adsorption on substrates. The surface concentration of adsorbed enzymes (Γ) was given by equation (3.1)

$$\Gamma = \frac{Γ_{max}KC}{1+KC}$$  \hspace{1cm} (3.1)

where Γ_{max} is the surface concentration of protein at full coverage, K is the Langmuir constant, and C is the free protein concentration in the bulk solution.

3.3.5 Data analysis and number of sample replicates

All results reported for glucose and reducing sugar analysis from enzymatic hydrolysis determined by 96-well microplate are the mean values of four replicates based on two batches of repeated experiments under the same conditions. Duplicate samples were taken for each time point. The mean and standard deviation were calculated using Origin® Pro 8 (OriginLab Corp., Northampron, MA). Sugar analysis from HPLC as suggested by NREL (NREL/TP-510-42623) and microplate assay were compared by using a calibration curve generated from pure glucose and cellobiose standards. The 96-well microplate assay and HPLC analysis yielded identical conversion results based on at least four replicates from repeated hydrolysis batches.
3.4 Results and discussion

3.4.1 Characterization of substrates prepared from chemical pulping

Kraft pulping and sulfite pulping are the two main methods used for pulping woody biomass. Kraft pulping employs sodium sulfate and sodium hydroxide and the lignin is significantly degraded and fragmented during this process (Smook 2002). In sulfite pulping, HSO₃⁻ (in salt forms with Ca²⁺, Na⁺, Mg²⁺, or NH₄⁺) is the main delignifying agent. During sulfite pulping, lignin is converted to lignosulfonates that are highly soluble in water and thus can be removed from the pulp fiber. Due to its acidic pulping condition, sulfite pulp also has a lower hemicellulose content compared to kraft pulp at the same lignin content, but typically with comparable cellulose DP and crystallinity. These pulp samples afford a unique set of substrates that can be used to quantify the specific effects of biomass xylan and surface lignin on enzyme hydrolysability. The chemical compositions of this unique set of substrates (reference substrates), KP0, KP13, KP40, SP0, SP13 and SP40, are shown in Table 3.2. Both KP and SP series of samples had three different levels of bulk lignin content (0%, 1.9% and 5.9%). A distinctive difference in xylan content between KP and SP samples was observed. Xylan content

<table>
<thead>
<tr>
<th>Reference substrates</th>
<th>Chemical composition</th>
<th>Surface characterization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kappa number</td>
<td>Klason lignin (%)</td>
</tr>
<tr>
<td>KP0 (lignin free KP13)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>KP13</td>
<td>13</td>
<td>1.9</td>
</tr>
<tr>
<td>KP40</td>
<td>40</td>
<td>5.9</td>
</tr>
<tr>
<td>SP0 (lignin free SP13)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SP13</td>
<td>13</td>
<td>1.9</td>
</tr>
<tr>
<td>SP40</td>
<td>40</td>
<td>5.9</td>
</tr>
</tbody>
</table>

*ND: non-detectable. All analyses were done in triplicates with a standard error within ±5%.
of KP0 was ~70% higher than that of SP0, while both KP0 and SP0 were lignin free. KP13 and KP40 also contained significantly higher xylan compared to SP13 and SP40, respectively, with similar lignin content. Poplar substrates with higher lignin content (kappa number > 40) were also prepared, but were not used, since the morphologic characteristics of these samples were significantly different due to the presence of a large amount of unseparated fibers. Figure 3.1 shows the color differences among these samples, with KP40 > KP13 > SP40/SP13 > KP0/SP0 (dark to light). Although the lignin content does not necessarily correlate to the amount or intensity of chromospheres, the different color indicates different surface lignin content. All of the substrates used in this study had similar morphologic features, similar average fiber length of 0.67 mm, and fiber coarseness (weight at a given length) of 0.088 mg/m (Figure 3.2). One-way analysis of variance (ANOVA) gave a value of p factor less than 0.05 for both fiber lengths and coarseness for all substrates (6.97×10⁻⁴ and 7.81×10⁻⁶ respectively), indicating a similar fiber morphology. All the substrates have similar fiber size distribution as well. Crystallinity Index (Crl) of KP0 and SP0 were comparable, at 77.40% ± 1.47% and 79.97% ± 1.43% respectively.
Figure 3.2. Fiber quality analysis of reference substrates: (A) fiber average length (mm) and (B) fiber coarseness (mg/m fiber).

3.4.2 XPS analysis of surface lignin coverage of reference substrates

It has long been recognized that XPS can be used to determine surface lignin content on cellulose substrates by either O/C ratio or aliphatic carbon percentage to the total carbon amount. Johansson, et al. evaluated 250 chemical and mechanical pulp samples with XPS using these two different analysis and both gave consistent indications of surface lignin content (Johansson, Campbell et al. 1999). The representative spectra of sample KP13 collected from XPS is shown in Figure 3.3A. Carbon and oxygen were the predominant elements detected on all sample surfaces. Trace amount of sulfur was also detected as a result of the sulfur containing chemicals used in the treatment process. Deconvolution of C1s yielded four peaks: C-C, C-O, C=O, and COOH (Figure 3.3B). The aliphatic carbon C-C region was centered at 285.0 eV. Ideally, pure cellulose is devoid of C-C because of its polysaccharide structure, while in milled wood lignin, 49% of carbon atoms are reported to be of C-C type. As shown in Figure 3.3B, both KP0 and SP0 exhibit negligible C-C peaks while KP13, KP40, SP13 and SP40 contain different amounts of C-C. In the oxygen spectra all substrates exhibit similar sizes and binding energies.
3.3C), indicating that the oxygen bond type remained relatively the same on the surface. Theoretically, the surface lignin coverage of the substrates can be calculated by either the O/C ratio or the C-C peak area after deconvolution. However, it was found that the results obtained

![Graph showing XPS intensity vs. Binding Energy](image)

**Figure 3.3.** (A) Representative XPS survey spectra of substrate KP13; (B) high energy resolution C 1s with component-peaks fitted carbon regions and (C) high energy resolution O 1s spectra with component-peaks fitted oxygen regions for all pulp substrates.

from carbon peak deconvolution were significantly influenced by the chemical nature of the reference lignin selected (i.e., lignosulfonates or milled lignin as the reference). Quantification using O/C ratio provided a more consistent surface lignin coverage measurement of biomass substrates. When comparing substrates from different pretreated methods, O/C ratio approach
gives more reliable results which are less influenced by the heterogeneous characteristics of the lignin type and fiber surface.

A linear correlation between surface lignin content and O/C can be established by using theoretically pure cellulose and pure lignin. A Whatman filter paper was used to represent pure cellulose which had O/C ratio of 0.74 and an O/C of 0.33 was used for lignin as suggested by Johansson, et al. The calculated surface lignin contents of the reference samples are listed in Table 3.2. Although the bulk lignin content was relatively small, a significant difference in surface lignin content was observed among the substrates. The KP samples had higher surface lignin coverage than SP samples. For bulk lignin content around 1.9%, the surface coverage for KP13 was 29%, while the coverage for SP13 was only 16%. At 5.9% bulk lignin content, the surface lignin went up to 45% in KP40 and 39% in SP40 respectively. The O/C ratio of KP0 and SP0 is between 0.71 and 0.72 with a standard deviation of ± 2. Therefore, their surface lignin converge is negligible compared to the rest of the samples. Kraft pulping aims at extensive depolymerization of lignin and dissolving lignin fragments in concentrated alkali pulping liquor, however, the lower temperature and lower alkalinity at the end of pulping stage lead to a redeposition of dissolved lignin onto pulp fiber surfaces. Kraft pulp typically has higher surface lignin content than other pulping method. SP13 and SP40 were generated to have similar bulk lignin contents to KP13 and KP40 respectively. In contrast to kraft lignin, lignosulfonates are much more soluble in water. A redeposition of lignin on fiber surface is expected to be less significant. Therefore surface lignin contents on SP13 and SP40 are considerably lower than the respective KP13 and KP40. It is likely that SP surface lignin is much more hydrophilic than KP surface lignin.
3.4.3 Enzymatic hydrolysis of references substrates

The physical, chemical and surface characteristics of KP0, KP13, KP40, SP0, SP13 and SP40 provided a unique set of references substrates to detail investigate the specific effect of xylan and surface lignin content on enzymatic hydrolysis of cellulose. Enzymatic hydrolysis of these substrates at a series of enzyme (Ctec2) dosages (0.5-32 FPU per gram of cellulose in the substrate) was carried out and the cellulose-to-glucose conversion yield was determined.

3.4.3.1 Xylan influence on enzymatic hydrolysis of reference substrates

Comparing the enzyme hydrolysability of KP and SP samples helped reveal the individual effect of xylan on enzymatic hydrolysis. The initial enzyme hydrolysis rates of KP0 and SP0 were determined by measuring the reducing sugars released during the first hour of hydrolysis. As shown in Figure 3.4A, KP0 exhibited a consistently higher (0.5 to 2 fold) initial conversion rate than that of SP0 at all enzyme dosages. As an example, the initial hydrolysis of KP0 with 7.13%
more xylan than SP0 is 40% higher than that of SP0 at an enzyme dosage of 8 FPU/g. However, when the hydrolysis profiles of KP0 and SP0 were compared after 72 hours, a different trend was observed (Figure 3.4B). At lower enzyme dosages (i.e. 0.5 and 1 FPU/g), there was still a significant difference in the cellulose conversion rates between KP0 and SP0 after 48 hours. However, at higher enzyme dosages (i.e. 3, 8, 16 and 32 FPU/g), the rate difference between KP0 and SP0 becomes smaller much smaller. Also shown in Figure 3.5 (top lines in left graphs vs top lines in right graphs), at lower enzyme dosages (i.e. 0.5 and 1 FPU/g), the cellulose-to-glucose conversion yields of KP0 are higher than those of SP0. Whereas at higher enzyme dosages (i.e. 16 and 32 FPU/g), the conversion yields of KP0 and SP0 approached to a similar level along with the increase in hydrolysis time.

As a non-cellulose component, xylan has been generally considered as a physical hindrance to cellulase enzymes. A number of studies have shown that the addition of xylanase to cellulase preparation can enhance cellulose hydrolysis rate. It has been postulated that native plant cellulose fibrils are embedded in hemicellulose and lignin matrix, and after pretreatment, hemicellulose remains coated on the cellulose fibril and/or bound with cellulose and thus limit the access of cellulase enzyme to cellulose. The higher xylan content in KP0 compared with SP0 led to a higher cellulose-to-glucose conversion rate is a striking finding compared with these previous studies. This suggested that, besides its physical hindrance effect, xylan may contribute to a positive effect on enhancing cellulose hydrolysis.

In majority of previous studies, pretreated biomass substrates are typically used to investigate the xylan effects on enzyme hydrolysis. Pretreatment typically causes a disruption of fiber cell wall structure that leads to a heterogeneous distribution of chemical components throughout different structural levels. It is likely that a significant portion of the cellulose in these
Figure 3.5. Hydrolysis profiles of reference substrates under different enzyme (Ctec2) loadings.
substrates is entangled with the hemicellulose and lignin matrix. Although the beneficial effect of xylanase addition has been frequently observed, a direct relationship between xylan removal and cellulose hydrolysability does not always exist (Garcia-Aparicio, Ballesteros et al. 2007; Kumar and Wyman 2009). A previous investigation of xylanase leverage effect on enzymatic hydrolysis of corn stover substrates prepared by several pretreatment methods concluded that the removal of xylan does not always enhance cellulose conversion (Kumar and Wyman 2009). Besides the interaction with cellulose, the removal of xylan in many pretreated biomass substrates is often associated with changes in other substrate parameters such as lignin content, porosity, etc. It remains a great challenge to evaluate the effect of xylan as an isolated factor using pretreated biomass substrates with heterogeneous properties. KP0 and SP0 substrates used in this study had similar fiber length, fiber coarseness and non-detectable amount of lignin. The difference in CrI value of substrates KP0 and SP0 is also negligible. The most distinctive compositional difference between these two substrates was the xylan content; 16.78% in KP0 vs. 9.65% in SP0. The use of KP0 and SP0 provided a unique opportunity to gain new insight into the effect of xylan on biomass recalcitrance. It is well recognized that hemicellulose in chemical pulps plays an important role in improving cellulose fiber swelling (Hubbe, Venditti et al. 2007), increasing the fiber saturation point, preventing cellulose hornification (Kohnke, Lund et al. 2010), and enhancing specific fiber surface area, as well as wet fiber flexibility (Oksanen, Buchert et al. 1997; Kohnke, Lund et al. 2010) while all of these effects improve cellulose hydrolysability. A pretreated biomass substrate typically contains a large amount of unseparated and disrupted fibers, therefore, the positive effect of xylan on cellulose swelling is not revealed. In chemical pulp, fibers are well separated and xylan promotes macrofibrils swelling by forming a hydrophilic xylan layer on the fibril surface. Since more hydrophilic layer is formed on the
surface of fibrils, strong interactions between crystalline regions of adjacent fibrils are prevented from forming, which also provides better accessibility for the cellulase enzymes to make contact with cellulose. To confirm the effect of xylan on fiber swelling, the amount of surface area in KP0 and SP0 was determined by Simon’s staining by the amount of orange dye adsorbed on the substrates. It is apparent that KP0 (58.1 ± 2.9 mg/g) has 34% more accessible surface area than SP0 (43.1 ± 2.2 mg/g). Also shown by enzyme isotherm results (Figure 3.6), KP0 has a higher affinity to cellulase than SP0 (Langmuir constant at 0.398 mL/mg and 0.600 mL/mg respectively).

To further confirm the swelling effect on hydrolysability improve of the substrate, SP0 was treated by PFI milling, a common pulp treatment technique for increasing fibrillation on cellulose fibril surface similar to the presence of xylan fragments. PFI milling was carried out by a laboratory PFI beater following Tappi Standard procedure T248. SP0 after 10,000 revolutions of PFI milling (SP0-10,000) was subjected to Ctec2 hydrolysis at 1 and 8 FPU enzyme loadings and compared with KP0 and SP0. As shown in Table 3.3, the initial hydrolysis rates of SP0-10,000 increased substantially after PFI milling at both enzyme dosages and were slightly higher than those obtained from KP0. After 72 hours of hydrolysis, SP0-10,000 maintained a higher conversion rate than that of SP0 at lower enzyme loading (1 FPU). However, the conversion rates of all three samples (KP0, SP0, SP0-10,000) approached to a similar level after 72 hours at the higher enzyme loading. This is the same trend as shown between KP0 and SP0 confirming that the beneficial effect of xylan on enzyme hydrolysis is likely owing to its ability to facilitate cellulose fibril swelling. It is apparent that this effect on the hydrolysis rate is more pronounced at the beginning of the hydrolysis phase. As hydrolysis proceeds, the substrate characteristics change and the amount of readily accessible and hydrolysable substrates are reduced. As a result,
Figure 3.6. Correlations between (A) Langmuir constant and initial rate; and (B) maximum adsorbed enzyme on the surface of reference substrates and initial rate; under different enzyme (Celluclast) dosages.
Table 3.3. Hydrolysis rate comparison of KP0, SP0, SP0-10,000 after PFI milling treatment under different enzyme (Ctec2) loadings.

<table>
<thead>
<tr>
<th></th>
<th>KP0</th>
<th>SP0</th>
<th>SP0-10,000</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Initial 1hr hydrolysis rate</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(g.g⁻¹.h⁻¹)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 FPU/g</td>
<td>4.10 ± 0.02</td>
<td>3.48 ± 0.27</td>
<td>4.40 ± 0.06</td>
</tr>
<tr>
<td>8 FPU/g</td>
<td>5.37 ± 0.13</td>
<td>4.31 ± 0.53</td>
<td>6.44 ± 0.12</td>
</tr>
<tr>
<td><strong>72 hr hydrolysis conversion rate</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(g.g⁻¹.h⁻¹)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 FPU/g</td>
<td>0.13 ± 0.01</td>
<td>0.06 ± 0.01</td>
<td>0.10 ± 0.01</td>
</tr>
<tr>
<td>8 FPU/g</td>
<td>0.31 ± 0.01</td>
<td>0.29 ± 0.01</td>
<td>0.27 ± 0.01</td>
</tr>
</tbody>
</table>

*Hydrolysis rate is expressed in g.g⁻¹.h⁻¹: grams of glucose produced from 1 gram of cellulose per hour.*

the hydrolysis rate of KP0 and SP0 became similar because of similar cellulose fibril properties. The xylan leverage effect was also less significant at higher enzyme dosages as the excessive cellulase loading potentially overcomes swelling and surface area limitations.

Ctec2 preparation contains a significant level of xylanase activity, which would help remove xylan on cellulose and alleviate the xylan hindrance effect. The amount of xylose released during substrate hydrolysis was also determined. It was found that xylose was released at a similar rate as glucose from the substrates during enzymatic hydrolysis by Ctec2. No selective removal of xylan from any of the substrates was observed, indicating a homogenous distribution of xylan in macrofibrils of the substrates. Supplementation of xylanase during cellulase hydrolysis of substrates was not tested in this study as the Ctec2 preparation already contains a significant amount of xylanase activity. Instead, a mixture of cellulase (Celluclast) and β-glucosidase (β-G) with FPU/CBU activities equivalent to Ctec2 was prepared and used to hydrolyze KP0 and SP0. The initial cellulose hydrolysis rates obtained from Celluclast/β-G
mixture at a range between 5-20 FPU/g were 13-26% lower than those obtained from Ctec2 at the same enzyme dosages. This result agrees with previous studies that xylanase supplementation has a beneficial effect. However, in substrates such as KP0 and SP0, with homogenous distribution of xylan in macrofibrils, the swelling effect of xylan prevails.

A similar xylan effect was also observed when comparing the enzymatic hydrolysability between KP13 and SP13, as well as KP40 and SP40 (Figure 3.5) in the presence of lignin. Using this unique set of biomass substrates, it was demonstrated for the first time that the presence of xylan can afford cellulosic fibrils with improved swelling and better accessibility toward cellulase hydrolysis.

3.4.3.2 The effect of surface lignin content on enzymatic hydrolysis of reference substrates

It is perhaps indisputable with current knowledge that lignin impedes cellulase hydrolysis of cellulose (Gregg and Saddler 1996; Mooney, Mansfield et al. 1998; Berlin, Balakshin et al. 2006; Obama, Ricochon et al. 2012). Lignin has been shown to both physically hinder the accessibility of enzymes and bind with cellulase leading to its deactivation (Kumar, Arantes et al. 2012). Despite intensive research efforts, it remains a challenge to draw a clear correlation between hydrolysis rate and bulk lignin content in biomass substrates. Enzymatic hydrolysis of lignocellulosic biomass starts with adsorption of enzymes onto the fiber surface. The topographic characteristic of the substrates has a dominant effect on the initial hydrolysis rate. A number of previous studies have shown that lignin redistributes and migrates to substrate surface during biomass pretreatment (Donohoe, Decker et al. 2008). Thus, substrate surface lignin is likely more closely related to enzyme hydrolysis efficiency than the bulk lignin content. So far, there is a lack of a quantitative approach to determine biomass surface lignin and its effect on enzymatic hydrolysis of lignocellulosic substrates.
Comparing enzymatic hydrolysis among KP and SP sets of samples helped to gain insight into the specific effect of biomass surface lignin on enzymatic hydrolysis. The hydrolysis profiles of KP0, KP13, KP40, SP0, SP13 and SP40 were shown in Figure 3.6.

Despite a similar bulk lignin content gradient in both KP and SP sets of substrates, it is evident that the cellulose-to-glucose conversion rate differences among KP samples were more significant than those observed among SP samples (Figure 3.6 and Table 3.4). There is about 9% yield difference between KP0 and KP40 while the difference between SP0 and SP40 is between

**Table 3.4.** Hydrolysis conversion yield at 48 hours of reference substrates under low and high enzyme loadings.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Klason Lignin* (Calculated from Kappa)</th>
<th>48 hours conversion yield</th>
<th>48 hours Conversion yield difference based on lignin-free pulp</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Low enzyme: 0.5 FPU/g</td>
<td>High enzyme: 16 FPU/g</td>
</tr>
<tr>
<td>KP0</td>
<td>0%</td>
<td>22±0.99%</td>
<td>78±0.17%</td>
</tr>
<tr>
<td>KP13</td>
<td>1.9%</td>
<td>16±0.92%</td>
<td>74±1.43%</td>
</tr>
<tr>
<td>KP40</td>
<td>5.9%</td>
<td>13±0.96%</td>
<td>69±1.99%</td>
</tr>
<tr>
<td>SP0</td>
<td>0%</td>
<td>12±0.67%</td>
<td>80±3.80%</td>
</tr>
<tr>
<td>SP13</td>
<td>1.9%</td>
<td>10±0.46%</td>
<td>79±2.92%</td>
</tr>
<tr>
<td>SP40</td>
<td>5.9%</td>
<td>8±0.94%</td>
<td>75±2.89%</td>
</tr>
</tbody>
</table>
4-5% after 48 hours of enzyme hydrolysis. This result reaffirmed the difficulty to use bulk lignin content to predict substrate digestibility. However, it became apparent that substrate surface lignin content may have a more direct impact on hydrolysis rate.

To better understand the specific effect of surface lignin on cellulase adsorption and cellulose hydrolysis, Langmuir adsorption isotherms of cellulase on KP and SP substrates under a wide range of enzyme concentrations were measured. The data was fitted using nonlinear least squares regression to obtain a high goodness-of-fit either by smallest vertical error (v-NLLS) or smallest normal error (n-VLLS). The Langmuir constant (K) determines the affinity between the enzyme and substrate. Cellulase binds preferentially on cellulose than on lignin. More surface lignin coverage led to a lower binding affinity and a lower K value. A good linear correlation between surface lignin coverage with Langmuir constant K was observed for kraft pulp substrates with a R² of 0.97 (p value = 0.07 by one-way ANOVA). It is statistically significant that higher surface lignin coverage can lead to lower binding affinity. Comparison of initial rate data among KP samples revealed a higher binding affinity (which means lower surface lignin coverage), and a higher reducing sugar rate (Figure 3.6A). The linear correlations were tested under different enzyme loadings, which yielded a R² range between 0.87-0.99 and p values below 0.05. These results demonstrated a strong correlation between the substrate surface lignin content and initial hydrolysis rate. The maximum adsorbed enzyme Γₘₐₓ is another parameter obtained from adsorption kinetics analysis. Different from the K value, Γₘₐₓ measures the total amount of enzymes can be absorbed on the substrate surface when enzyme is saturated. Higher Γₘₐₓ means more enzymes were adsorbed on the surface of substrates when the enzyme was saturated. As shown in Figure 3.6B, with the exception of KP40, the increase in maximum adsorbed enzyme on the substrate led to the increase in the initial hydrolysis rate under enzyme
dosage ranging from 1 FPU/g to 32 FPU/g (R² > 0.90 and p values below 0.001). This correlation is in agreement with previous research investigations on the relationship between the initial hydrolysis rate and the extent of soluble protein adsorption (Lee and Fan 1982). It was observed that, for all enzyme loadings, KP samples exhibited a higher Γ_max than all SP samples. While the higher surface area of KP substrates measured by Simon’s staining is likely to contribute to their high Γ_max compared to SP samples, the differences in the chemical nature between KP and SP lignin also played a major factor.

As mentioned earlier, kraft lignin is more hydrophobic than lignosulfonate. Hydrophobic interactions were postulated to play an important role in the adsorption of cellulases on lignocelluloses and subsequent cellulose hydrolysis efficiency (Eriksson, Borjesson et al. 2002; Palonen, Tjerneld et al. 2004; Berlin, Balakshin et al. 2006). Major cellulase enzymes all have hydrophobic amino-acid exposed on the surface (Reinikainen, Teleman et al. 1995), which can interact with hydrophobic lignin and result in non-productive binding of cellulase enzymes. An increase in lignin hydrophobicity has shown to lead to a stronger binding toward enzymes (Nakagame, Chandra et al. 2010). Applying surfactants to block hydrophobic sites on lignocellulosic substrate has been shown to reduce non-productive binding of T.reesei CBHI (Eriksson, Borjesson et al. 2002). The enzyme adsorption isotherm showed a constant higher maximum enzyme adsorption for KP13 and KP40 than for SP13 and SP40 which provided direct evidence to support these previous observations (Eriksson, Borjesson et al. 2002; Palonen, Tjerneld et al. 2004; Berlin, Balakshin et al. 2006). It was intriguing to find that, despite a lower affinity (K) to cellulase, KP40 has a higher Γ_max value than KP13 (Figure 4B). The enzyme adsorption experiment was carried out at a condition (25°C for 1 hour) to reach adsorption equilibrium between enzyme and cellulose. The increase in the Γ_max is likely due to irreversible
binding of cellulase enzymes with the surface lignin on the substrate during the incubation. KP40 has over 45% surface coverage of hydrophobic lignin, thus the irreversible binding of enzyme accumulated on substrate surface becomes significant. This irreversible binding of cellulase is likely the main reason attributing to the significant differences in the hydrolysis rates observed among KP substrates. Lignosulfonates appear to have less non-productive binding effects than the kraft lignin. Therefore the hydrolysis rate difference among SP samples with different lignin content is smaller. These results suggest that surface lignin content is a key characteristic of biomass recalcitrance. Controlling lignin distribution and hydrophobicity during biomass deconstruction will help improve the enzyme hydrolysability of biomass substrates.

3.5 Conclusions

A modified chemical pulping process was successfully applied to poplar to create a unique set of substrates to investigate the specific effects of xylan and surface lignin content on enzymatic hydrolysis. Apart from a physical barrier to cellulose hydrolysis, xylan was shown to facilitate cellulose fibril swelling and enhance subsequence enzymatic hydrolysis rate. XPS was demonstrated as an effective tool to quantify surface lignin content on lignocellulosic biomass substrates. Surface lignin has a direct impact on hydrolysis rate. Higher surface lignin content, especially from hydrophobic lignin, led to lower cellulase affinity to the substrate and lower initial hydrolysis rate.
4.1 Abstract

We show in this work a more accurate and applicable determination of the crystallinity index (CrI) and crystallite structures for cellulose Iβ by refined X-ray diffraction method. Rietveld refinement was applied with consideration of March-Dollase preferred orientation at the (001) plane. The method differentiated background scattering from the amorphous phase, the latter of which was represented by three broad peaks identified from appropriate experimental measurements. A suitable integration range of 2θ from 10° to 75° was determined by investigating the relations between choice of diffraction angle and calculated CrI. Crystallite structures of newly developed model cellulose Iβ compound nanocrystalline cellulose (NCC) was examined using developed XRD refinement. CrI and crystallite parameters calculated from the refined method provide a more reliable and universal reference for the future studying of cellulose structural changes during chemical and biological treatment.

Keywords: Cellulose; X-ray diffraction; Nanocrystalline cellulose; amorphous cellulose, crystallite structure.

4.2 Introduction

Chapter Two and Three mainly focused on understanding biomass recalcitrance from fiber and fibril level, investigating individual factors effect on enzymatic hydrolysis. When biomass recalcitrance was investigated from molecular level, cellulose crystallinity plays a major role
hindering a comprehensive understanding. Cellulose crystallinity has been identified as an important factor influencing the physical, chemical and mechanical properties of cellulose-based materials (Ryu, Lee et al. 1981; Soltes 1983; Tanahashi, Goto et al. 1989; Weimer, Hackney et al. 1995; Andersson, Wikberg et al. 2004). In many situations it is important to quantify the amount of crystalline cellulose, termed as crystallinity index (CrI), by analytically separating the crystalline fraction of cellulose from other matter, including amorphous cellulose. CrI of cellulose has been used more than five decades to interpret changes in cellulose structure after physicochemical and biological treatment (Chanzy, Marechal et al. 1999; Andersson, Serimaa et al. 2003; Cao and Tan 2005; Ai-Zuhair 2008; Hall, Bansal et al. 2010). Among numerous technologies for the determination of CrI such as 13C nuclear magnetic resonance (NMR), Fourier transform (FT)-IR and X-ray diffraction (XRD), XRD method stands out as a preferential technique since the definition of absolute crystal relies on the presence of identifiable peaks in the diffraction pattern (Teeaar, Serimaa et al. 1987; Evans, Newman et al. 1995; Liitia, Maunu et al. 2000; Deraman, Zakaria et al. 2001; Liitia, Maunu et al. 2003; Agarwal, Reiner et al. 2010; Bansal, Hall et al. 2010; Park, Baker et al. 2010; Kljun, Benians et al. 2011).

Based on literature reports (Thygesen, Oddershede et al. 2005; Bansal, Hall et al. 2010; Park, Baker et al. 2010), there are at least three methods commonly used to calculate the CrI of cellulose from the XRD pattern, namely, the peak height method (Segal, Creely et al. 1962; Cao and Tan 2005; French and Cintron 2013), peak deconvolution method (Rietveld 1969; Tanahashi, Goto et al. 1989; Driemeier and Calligaris 2011) and amorphous subtraction method (Ruland 1961; Rowe, Mckillop et al. 1994). The peak height method developed by Segal et al(Segal, Creely et al. 1962) is based on the ratio of the height of the (200) peak (I_{200}) and the
height of the minimum ($I_{AM}$) between the (200) and (110) peaks. The peak height method stands as the most simple and popular CrI calculation and offers a comparison between similar samples. However, since the true quantity of a compound is proportional to the area and not the height of its diffraction pattern, the peak height method is unlikely to represent the true crystalline fraction.

The peak deconvolution method models the crystalline portion by a series of peaks corresponding to the most intense peaks expected for cellulose (e.g. (110), (1\(\bar{1}\)0), (102), (200), and (004) for cellulose I\(\beta\)), or by a complete diffraction profile calculated from a known crystal structure. The amorphous component is typically modeled by a further, broader peak and CrI is calculated from the ratio of the area of the crystalline fraction to the total of the two components. The accuracy of this method relies on how well the groups of peaks used correspond to the actual diffraction contributed by each fraction. In the case of the profile calculated from crystal structures it is likely to be a reasonable representation, but the amorphous peak is often arbitrarily refined and may not match that given by amorphous cellulose.

The amorphous subtraction method outlined by Ruland et al. (Ruland 1961) fits an intensity profile representing the amorphous fraction scaled so that it just remains below all the observed intensity from the experimental sample pattern. The CrI is determined from the ratio of the area above the amorphous profile to the total area. The amorphous profile is obtained either from a polynomial function or a pattern measured from experimentally prepared material believed to be entirely amorphous (e.g., ball-milled cellulose, regenerated cellulose, xylan or lignin powder) (Thygesen, Oddershede et al. 2005; Bansal, Hall et al. 2010).

Each of the methods described above has their strengths and weaknesses. Most are useful for comparative measures of crystallinity between similar samples, but may not give accurate absolute measures because of deficiencies in modeling at least one of the cellulose components.
CrI is not straightforward to measure because the small crystallite size of cellulose (typically <10 nm for plan-derived material) generates broad Bragg peaks that are difficult to separate from the scattering from amorphous material and the background.

The scope of this paper is twofold. Firstly, we want to establish a more accurate procedure for measuring cellulose crystallinity from XRD patterns. For this purpose, four aspects were considered: 1) The model used to represent the crystalline diffraction pattern; 2) how best to model the amorphous fraction; 3) additional background intensity from e.g. diffuse and air scattering; 4) a suitable 2θ range which will give reliable crystallinity estimates without extensive data collection times. Our goal was to establish a protocol which gives as true representation of the crystalline fraction as possible, yet be relatively simple to calculate and able to be implemented in most XRD laboratories. Secondly, we want to analyze the model cellulose crystallinity indices and crystallite structures by using this developed procedure. Three most representative cellulose compounds were used in the study to ascertain this purpose: newly developed nanocrystalline cellulose (NCC) with pure cellulose crystalline structure at nanoscale; classical microcrystalline cellulose Avicel, and bleached kraft pulp (BKP) as a representation of pretreated lignocellulosic biomass and starting material for NCC.

4.3 Materials and methods

4.3.1 Substrate preparation

Bleached kraft pulp (BKP) was prepared following previously described procedure (Ju, Engelhard et al. 2013). Nanocrystalline cellulose was prepared by acid extraction (Hamad and Hu 2010). In brief, 64.5% sulfuric acid was used to extract nanocrystalline cellulose from BKP, followed by dilution to stop reaction. The mixture was filtered through 3K Dalton RC membrane.
using AMICON ultrafiltration 8400 system. Nanocrystalline cellulose collected above the membrane after repeated washing and filtering was freeze-dried for further analysis. Microcrystalline cellulose (Avicel PH101) was purchased from Sigma-Aldrich (St Louis, USA). Phosphoric acid swollen amorphous cellulose (PASC) was prepared using phosphoric acid treating NCC as well as Avicel following procedure as previously described (Zhang, Cui et al. 2006).

4.3.2 Nanocrystalline cellulose characterization

NCC was analyzed by FTIR (Fourier Transform Infrared Spectroscopy) and X-ray photoelectron spectroscopy (XPS) for confirmation of successful preparation. For FTIR, the spectral resolution was 4 cm\(^{-1}\) and the number of average scans was 128. The measured wave number was from 4000 to 600 cm\(^{-1}\). DRIFTS-IR focuses on the surface of the sample, and the specific area where the measurement takes place was small. The samples were freeze-dried and ground to homogeneous prior to FTIR analysis. At least three replicate samples were probed. The XPS analysis was carried out with Kratos Axis Ultra spectrometer, using a monochromatic Al K (alpha) source (10 mA, 15 kV). XPS has capability to probe the surface of the sample to a depth of 5–7 nm, and has detection limits that range from 0.1 to 0.5 atomic percent depending on the element. The Kratos charge neutralizer system was used on all specimens. Survey scan analysis was carried out with an analysis area of 300 \(\times\) 700 \(\mu\)m and passenergy of 160 eV. Obtained NCC particle sizes were determined by a BI-90 Plus Dynamic Light Scattering Particle Size Analyzer at 25 °C in Milli-Q water at pH 4.9 (adjusted with HCl). The diluted NCC/water or ONCC/water mixture was centrifuged for 0.5 h at 500 g prior to particle size analysis. Three diluted mixtures were prepared for replicate data. NCC was characterized by Atomic force microscopy (AFM). Samples were air-dried and subjected to tapping mode AFM for surface
imaging. A silicon MPP-11100 AFM probe with a resonance frequency around 300–400 kHz was used. The scan angle was maintained at 0°, and 1 × 1 μm² images were captured in the trace direction with a scan rate of 1.0 Hz with 256 lines per image. Scanning was done in multiple spots on the sample surfaces to capture indented and nonindented spots. X-ray diffraction (XRD) pattern of substrates were obtained by the general purpose X-ray diffractometer with Philips X’Pert MPD system and a vertical H–H goniometer (190 mm radius). The X-ray source was ceramic X-ray tube with Cu anode. Operating power was 40 kV, 50 mA (2.0 kW). X-ray diffraction pattern of samples obtained after freeze-drying were recorded at room temperature from 10° to 140°. The scan was carried out with a step size of 0.05°.

4.3.3 XRD data refinement

XRD data refinement was carried out by TOPAS software. Detailed refinement parameter setup was described in the 5.4 Results and Discussion. Crystallinity index (CrI) based on the “Segal method” was calculated from the height ratio between the intensity of the crystalline peak (I_{200}–I_{AM}) and total intensity (I_{200}) after subtraction of the background signal measured without substrate. Peak separations of the refined XRD profiles were carried out using the nonlinear least squares fitting program, where Voigt function and a three-parameter 2nd order polynomial function for background profile for each crystalline peak were used, as described in previous papers (Wada, Okano et al. 1997; Wada, Ike et al. 2010). The d-spacing were calculated using the Bragg’s equation (4.1) and the crystallite sizes were calculated using the Scherrer equation (4.2):

\[ n\lambda = 2d \sin \theta \]  \hspace{1cm} (4.1)

\[ L = 0.9\lambda/(H \cos \theta) \]  \hspace{1cm} (4.2)
where $n$ is an integer; $\lambda$ is the wavelength of incident wave length; $d$ is the spacing between the planes in the atomic lattice, $\theta$ is the angle between the incident ray and the scattering planes; $L$ is the crystallite size perpendicular to the plane and $H$ is the full width at half-maximum (FWHM) in radians.

4.4 Results and discussion

The Rietveld method was chosen as the calculation method in this study, which uses a well-defined crystal structure model to quantify the crystalline portion. A number of Rietveld refinement programs are readily available so this is achievable for most laboratories (Wiles and Young 1981; Young, Sakthivel et al. 1995). The method requires knowledge of the crystal structure (unit cell, space group and atomic coordinates) as starting parameters. The crystallographic information is well studied and cellulose Iβ structure published by Nishiyama et al (Nishiyama, Langan et al. 2002) has been cited frequently as the physical crystal model in cellulose Rietveld refinement (Thygesen, Oddershede et al. 2005; Driemeier and Calligaris 2011). The diffraction patterns calculated from this structure were in good agreement with the measured patterns in our study. Using published well-defined crystal structures means the same procedure can be used universally for other cellulose polymorphs, such as cellulose Iα (Nishiyama, Sugiyama et al. 2003) and cellulose II (Langan, Nishiyama et al. 2001). A recent publication by Driemeier and Calligaris (Driemeier and Calligaris 2011) examined the application of Rietveld refinement to cellulose crystallinity, and we have taken account of some of their findings here. The main difference is that these authors used a different diffractometer geometry and enclosed their samples in glass capillaries. This necessitated subtraction of a reference measurement from an empty capillary; following this they refined intensities from the
crystal structure and a high-order polynomial representing the amorphous fraction. In our work, we chose to model each of the two fractions explicitly, avoiding the need for an empty sample measurement.

Since cellulose frequently forms in long fibrils, it is important to account for the preferred orientation that is almost invariably present in diffraction samples. There are two main computational options: 1) a spherical harmonics approach. We had most success in our study with a 4th order correction which requires eight refinable parameters; 2) the March-Dollase approach (Dollase 1986), which requires identification of either one or two preferred orientation directions (Zolotoyabko 2009). After some experimentation, it was decided that the March-Dollase approach was preferable. Although the spherical harmonics approach gave better fits in some cases, the larger number of parameters meant it could become unstable occasionally. This resulted in negative intensities for some peaks, which is clearly not realistic and was offset by large positive backgrounds or amorphous peaks. We found more reliable results and only slightly poorer fits using a single (001) March-Dollase correction.

It is likely that more accurate crystallinity estimates will result if the intensity used to model the amorphous fraction of samples has a close resemblance to real amorphous cellulose. For this reason we chose to base our model on amorphous experimental samples rather than arbitrary peaks or background functions. We represented these experimental patterns with a series of pseudo-Voigt peaks since this method is able to be implemented in all common Rietveld programs. Although some studies chose xylan or lignin powder to represent the amorphous fraction because of their presence in lignocelluloses (Andersson, Serimaa et al. 2003; Thygesen, Oddershede et al. 2005), these substrates did not reflect the true pattern of amorphous cellulose. In this study, regenerated amorphous cellulose (RAC) was prepared from two forms of
pure cellulose, microcrystalline cellulose Avicel and nanocrystalline cellulose, to determine the cellulose amorphous phase (Zhang, Cui et al. 2006). The regeneration process uses concentrated phosphoric acid to dissolve homogeneous cellulose rather than forming the swollen heterogeneous cellulose. During precipitation, cellulose with disrupted hydrogen bonds is reformed without recrystallization.

Three broad peaks could be seen in the diffraction patterns of both RACs experimentally. The positions which were fitted with pseudo-Voigt profiles centered at approximately 20.53°, 38.87° and 80.90° 2θ (Figure 4.1). Previous research has generally used a single amorphous peak centered at 20° for the amorphous subtraction method (Bansal, Hall et al. 2010; Park, Baker et al. 2010; Driemeier and Calligaris 2011). The other peaks may not have been observed because many workers have collected data over a restricted range of diffraction angles, almost always less than 75° 2θ and sometimes less than 45° 2θ. Even the 40° peak is relatively weak and broad, so that it may not be distinguished from the background below 70°. Bansal et al showed that there was a less distinguishable bump around 35° 2θ, however, without subtracting other background scattering, such a peak is less obvious. The work presented here is the first to utilize more than one amorphous peak for crystalline cellulose analysis. This provides a more accurate representation of amorphous cellulose separate from the background.
Figure 4.1. Experimentally generated amorphous cellulose materials from NCC and Avicel shows three broadening peaks centered at 20.3°, 38.87° and 80.90°, respectively.
It is important to distinguish between the intensity from amorphous cellulose and that from background scattering processes. These include diffuse scattering, scattering from crystal defects including the mosaic nature of most real crystals, and instrumental scattering including that from interaction of the X-ray beam with air. Incoherent scattering is not significant in this study, since a monochromator was used to filter out radiation scattered at energies different from the incident radiation. The need to separate the background from amorphous material is illustrated by Figure 4.2. This shows a Rietveld fit of the cellulose Iβ structure with (001) preferred orientation to experimental data collected to 140°. The calculated peak intensities for cellulose (shown in green) become smaller with increasing diffraction angle, such that hardly intensity is expected for diffraction higher than 80°. This is typical behavior, especially for crystals of poorly crystalline organic compounds. However, the measured intensity (shown in blue) remains relatively constant at about 5% of the maximum intensity at these diffraction angles. If this intensity is apportioned to amorphous cellulose, as in some procedures (Driemeier and Calligaris 2011), then the estimated amount of amorphous material (which is represented by the difference between the blue and green lines) will become greater with increasing diffraction angle.

We therefore fitted a separate background to the data in this study. While Thygesen et al (Thygesen, Oddershede et al. 2005) applied a 10 parameter Chebychev polynomial background, we found that using a higher order polynomial risks confusing this background with amorphous cellulose. In order to choose a suitable background, a diffraction pattern of glucose monohydrate was collected, since this sample was believed to be 100% crystalline and has a chemical composition similar to cellulose. A 2 parameter Chebychev polynomial was found to model the background satisfactorily in both the glucose and cellulose samples.
Figure 4.2. XRD spectra up to 140° of Rietveld fit of NCC with single amorphous peak and scattering background.
French and Cintrón (French and Cintron 2013) demonstrated that the Segal CrI calculation depends largely on the simulated crystal sizes for a given cellulose polymorph. They proposed that a reliable determination of the crystal structure would contribute a better understanding of structural aspects of cellulose than CrI. Different originated cellulosic materials have widely varying crystallite sizes. Nanocrystalline cellulose (NCC) and cellulose nanofibers (CNFs) are newly developed macromolecules which generated remarkable excitement in the community of cellulose science and technology (Hamad 2006; Moon, Martini et al. 2011; Way, Hsu et al. 2012; Brown, Hu et al. 2013; Han, Zhou et al. 2013). NCC exhibits unique physicochemical properties and has been used as reinforcing components in nanocomposites (Samir, Alloin et al. 2005; Habibi, Lucia et al. 2010). Derived from renewable and sustainable biomass, NCC comprises particles with a width between 5-20 nm and an average length of 100-200 nm (Hamad 2006; Habibi, Lucia et al. 2010) (Figure A4.1). The self-assembled parallelepiped rod-like structure of the NCC crystallites approaches the closest attribute to perfect cellulose chains (Moon, Martini et al. 2011). In a previous study we found that NCC exhibits unique hydrolysis kinetics related to its crystalline structural feature other than generally used cellulose model compound, such as microcrystalline cellulose (e.g. Avicel). NCC shows a crystallite size comparable to the crystal size along the chain layer directions. Numerical calculations from previous research also indicate that the morphological dependence is less pronounced with increasing degree of molecular orientation and crystallinity (Matsuo, Sawatari et al. 1990). Thus, it is concluded that fibers with a high degree of molecular orientation and a higher crystallinity should be used as test specimens for measuring crystal lattice by X-ray diffraction.
A fit to NCC over the full diffraction range (from 10° to 140°) by the refined XRD calculation described in this research is shown below (Figure 4.3). This is essentially the same as Figure 4.1 but with background contributions from both amorphous material (black peaks) and other scatterings (smooth grey line). In order to justify the relations between choice of 2θ diffraction ranges and calculated CrI, the fit was repeated with data truncated at different diffraction angles and the resulting crystallinity shown in Figure 4.3 (insertion). The estimated fraction of crystalline cellulose decreased rapidly from 84% to 77% above 40° diffraction range.

As a practical consideration, we sought a suitable diffraction range for determination of cellulose crystallinity. This range should cover the significant areas where amorphous and crystalline material diffract, but be sufficiently small to allow reasonable data collection times. A fit to NCC over a wide range (from 10° to 140° 2θ) which uses the crystalline, amorphous, and background components discussed above is shown in Figure 4.3. The degree of crystallinity was determined from the area of the crystalline portion divided by the total of crystalline and amorphous fractions. In order to determine a practical range, the fit was repeated with data truncated at different diffraction angles and the resulting crystallinity inset in Figure 4.3. We used 3 amorphous peaks with their positions, widths, and relative intensities constrained according to the measurement of amorphous cellulose described above. This most closely modeled the experimentally observed amorphous pattern, and gave more consistent results than a similar approach where the peak intensities were refined independently.

The estimated fraction of crystalline cellulose remained consistent at approximately 77% when the diffraction range extended from 10 to between 60 and 100 °2θ. The CrI rose rapidly when the largest diffraction angle was less than 60 °2θ, reaching 84% at 40 °2θ. This is because crystalline cellulose has its most intense peaks below 30 °2θ, whereas the more intense broad
Figure 4.3. XRD refined spectra of Rietveld fit of NCC up to 140 ° with two experimentally defined amorphous peaks (Insertion: variation of determined CrI by refined method vs. the maximum 2θ angle used for calculation).
features of amorphous cellulose extend beyond 60°2θ (compare Figures 1 and 2). Unexpectedly, CrI dropped when the diffraction range extended past 100°2θ. There was a broad region of fairly weak intensity near 100°2θ in the NCC sample which was not observed in the amorphous sample (see Figure 4.1) and did not appear in the pattern calculated from the crystal structure. Most of the studies of cellulose CrI examined XRD pattern only to a maximum 2θ of 50°, partially because the last major crystalline peaks are centered at 35° (Bansal, Hall et al. 2010; Hall, Bansal et al. 2010; Driemeier and Calligaris 2011; French and Cintron 2013). However, our findings indicate that considerable intensity from amorphous cellulose can extend all the way to 70°, making a significant contribution when calculating CrI. It is apparent that different choices of 2θ range can result in different refinements and consequently different CrI. The choice of a maximum 2θ of only 40° as used in most studies cannot be justified since the CrI calculation is biased toward higher values. Based on the results from this study, we highly recommend a choice of diffraction angle range up to 75° should be used for calculating CrI.

In combination with the refinement up to 75° and the background/amorphous deconvolution, a better understanding of the crystalline structure of cellulose can be obtained from the XRD spectrum. The method was applied to three cellulose model compounds, bleached kraft pulp (BKP) prepared from poplar, microcrystalline cellulose Avicel and nanocrystalline cellulose. Figure 4.4 showed the refined XRD patterns for these cellulose Iβ model compounds with five distinctive peaks, (110), (110), (102), (200) and (004) (Figure A4.3) illustrates cellulose Iβ unit cell structure corresponding to the distinctive peaks).
Figure 4.4. XRD spectra of model cellulose compounds: NCC, Avicel and BKP.

In order to compare the CrI calculation based on refined XRD with published data, the commonly employed peak-height method was also evaluated. Table 4.1 summarizes the results. It is noteworthy that Segal CrI differences between NCC and Avicel is 8%. While when calculated by refined XRD method, the CrI difference increases to 17% between NCC and Avicel. Such differences are due to the sharp (200) peak of NCC which is resulted from the highly ordered crystallite structure of NCC. In Avicel, the peak width was decreased without significantly increasing the I_{200} intensity. It has long been recognized that peak height CrI overestimated the true crystallinity of cellulose Iβ\(^{13}\). In this study, higher CrI was also observed for three tested materials calculated by the peak height method than by refined XRD method.
**Table 4.1.** Crystallinity index (CrI) calculated for model compounds NCC, Avicel and BKP.

<table>
<thead>
<tr>
<th>Crystallinity index (CrI)</th>
<th>NCC</th>
<th>Avicel</th>
<th>BKP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Segal method</td>
<td>96%±1%</td>
<td>88%±1%</td>
<td>71%±2%</td>
</tr>
<tr>
<td>Refined area method</td>
<td>77%±3%</td>
<td>60%±4%</td>
<td>50%±3%</td>
</tr>
</tbody>
</table>

Although absolute value of CrI is hard to determine, the crystallite sizes are believed to correlate to CrI from simulated computation. Table 2 showed the calculation of crystallite size dimensions along the cellulose microfibril direction as well as the direction indicating glucan chain thickness.

**Table 4.2.** Crystallite structures of model cellulose compounds calculated from refined method index (CrI) calculated for model compounds NCC, Avicel and BKP.

<table>
<thead>
<tr>
<th>Planes</th>
<th>Crystallite structure</th>
<th>NCC</th>
<th>Avicel</th>
<th>BKP</th>
</tr>
</thead>
<tbody>
<tr>
<td>d-spacings</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(110)</td>
<td>0.593</td>
<td>0.607</td>
<td>0.588</td>
<td></td>
</tr>
<tr>
<td>(200)</td>
<td>0.531</td>
<td>0.544</td>
<td>0.539</td>
<td></td>
</tr>
<tr>
<td>(004)</td>
<td>0.389</td>
<td>0.399</td>
<td>0.396</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.259</td>
<td>0.263</td>
<td>0.259</td>
<td></td>
</tr>
<tr>
<td>Average crystallite size (nm)</td>
<td>4.5±0.1</td>
<td>5.6±0.1</td>
<td>4.0±0.1</td>
<td></td>
</tr>
</tbody>
</table>
Pure cellulose was reported to have a $d$-spacing around 0.394 nm along (200) plane (Kim, Eom et al. 2010). The $d$-spacing along (200) direction of NCC is the smallest (0.389 nm) compared to those of Avicel (0.399 nm) and BKP (0.396 nm). If the detailed crystallite structure is examined (Figure A4.3), it is reasonable to assume that the larger $d$-spacings of Avicel and BKP indicate a somehow expanded distance in between layers. It is noteworthy that even though BKP is the starting material of NCC (Figure A4.2), the crystallite thickness distances were significantly changed after extracting NCC from BKP. The crystallites of cellulose contain a highly ordered crystalline core and lower-ordered surface paracrystalline layers surrounding the core (Newman, Ha et al. 1994; Ioelovich, Leykin et al. 2010). Amorphous cellulose domains are in-between the crystallites along the microfibril chain direction (Lemke, Dong et al. 2012) (as indicated in the abstract scheme). Since XRD measured the average crystalline structure of the bulk material, it is hard to differentiate the paracrystalline layers with the amorphous embedded in the crystalline structure. Both materials contribute to the amorphous portion of CrI calculation. Distance difference between BKP and NCC can reach to ~70 pm, which is close to the distance of H-H and H-O covalent bonds (~74 and 96 pm, respectively). Such $d$-spacing difference is suspected to be caused by one imperfect paracrystalline layer on the surface, although detailed investigation is still needed to validate such hypothesis.

Structural features of crystallite sizes have been applied for studying the age of the wood as the crystallite sizes change. However, such study used the substrates with blend of lignin and hemicellulose which impedes the refinement of crystalline cellulose (Andersson, Wikberg et al. 2004; Cao and Tan 2005). Judging from the scheme of structural arrangement of pure cellulose, CrI calculated by XRD indicates the pure and perfect crystallite portions which result into sharp peaks, although amorphous portion and paracrystalline phase cannot be differentiated. On certain
extent such structure also changed the average crystallite sizes reflected by XRD, and such changes can be quantified once the XRD pattern is refined by subtracting reasonable background broadening. The characterization of crystallite sizes along with the CrI calculated after refinement are in agreement and better reflect the true crystalline structure of cellulose materials.

4.5 Conclusions

In conclusion, we have shown that a refined Rietveld XRD analysis can better determine the true crystallinity index of pure crystalline cellulose by subtracting experimentally determined amorphous background, including preferred orientation as well as extending the measuring 20 angle to 75°. Although such refinement cannot distinguish between the paracrystalline structure and amorphous regions, a true CrI is accurately determined and such procedure is universally applicable even for different cellulose polymorphs in most XRD laboratories as long as the crystallography information is available. When using the developed refinement to extract information regarding to crystallite structures, nanocrystalline cellulose provide a better model compound to differentiate the paracrystalline layers on the surface. Such information provides a better judgment regarding determination of the true structure of crystalline cellulose. Such analysis provides future possibilities of studying the true crystallinity indices as well as crystalline structural changes during various biological and chemical treatment.
CHAPTER FIVE: MECHANISMS OF CELLULOSE DECRYSTALLIZATION

AT THE MOLECULAR LEVEL

5.1 Abstract

Cellulose decrystallization is becoming increasingly recognized as one of the key steps in the enzymatic deconstruction of cellulosic biomass when used as a feedstock for fuels and chemicals production. In this work, techniques for quantifying molecular level changes in cellulose structures was developed and a new refined X-ray diffraction (XRD) method was used to track specific changes of the crystallite structure of NCC during depolymerization. Results from XRD characterization showed that the $d$-spacing along (200) plane, which represents the distances between hydrophobic sheets, stayed constant. No swelling effect during decrystallization process was observed in all enzyme systems tested here. On the other hand, our study found that the depolymerization process of NCC can be mediated by the presence of oxidoreductive enzymes. Such reaction greatly facilitates hydrolysis process by creating more enzyme accessible sites on the crystalline surface. The results from this study provided strong evidence that the enzyme decrystallization mechanism does not require a pre-swelling to disrupt hydrogen bond network in the crystallite. A surface delamination is a more prevail enzyme decrystallization mechanism in the enzyme tested.

Keywords: Nanocrystalline cellulose; decrystallization, swelling, delamination; enzymatic hydrolysis.

5.2 Introduction

Deconstruction of lignocellulosic biomass followed by enzymatic hydrolysis to produce fermentable sugars is a primary, near-term option for commercial liquid fuels production from
renewable sources. However, technical and economic challenges come from the strong structural recalcitrance of cellulose to physical, chemical and microbial deconstruction (Himmel, Ding et al. 2007). Chapter Two and Three investigated biomass recalcitrance from fiber and fibril levels, respectively. At the molecular level, the presence of a large percentage of crystalline cellulose is a major recalcitrant factor hindering a fast and efficient hydrolysis of plant cellulose by enzymes. Such exceptional mechanical and structural resistances are derived from a molecular-level network of hydrogen bonds and hydrophobic interactions making deconstruction of this polymer difficult (Kovalenko 2010). Understanding the molecular-level mechanisms that enzymes employ to deconstruct plant cell walls is a fundamental scientific challenge with significant ramifications for renewable fuel production from biomass. Numerous researches have been directed toward developing the known cellulose-degrading enzymatic pathways found in fungi (Wyman 1999; Chang and Holtzapple 2000; Zhang and Lynd 2004). Despite a considerable amount of efforts, a detailed molecular level of understanding the mechanism of enzyme degradation remains elusive.

Crystalline cellulose is a highly compact and well organized structure which is impermeable to enzymes. Disruption of this orderly structure of the crystallite to free glucan chains is perquisite to the subsequent cellulose depolymerization. It has long been proposed that a two-step process is involved in complete hydrolysis of crystalline cellulose, as illustrated by the prevailing C₁-C₅ model by Mandel and Reese (Mandels and Reese 1964). C₁ is postulated as an unknown component of the cellulase system (sometime called “swelling factor”) which primarily response for opening up the cellulose crystallites and free glucan chains to be depolymerized by the hydrolytic enzymes (C₅).
Over 60 years research following C₁-Cₓ theory, Cₓ hydrolytic enzymes have been identified and characterized. However, the existence and nature of C₁ enzyme is still in debate (Din, Damude et al. 1994; Arantes and Saddler 2010). It has long been speculated that an enzyme component with non-hydrolytic activity exists in cellulase mixture which has a specific function to disrupt cellulose crystal structure. Several proteins/enzymes, including expansin and swollenin, have shown their potential activities toward disruption of the hydrogen bonds in plant cellulose. In 1985, Coughlan (Coughlan 1985; Coughlan, Honnami et al. 1985) first used “amorphogenesis” as a term to define “the disruption of the crystals takes place by erosion and fibrillation-splitting of lateral hydrogen bonds yielding narrower crystalline elements that retained their original length”. In another word, the dispersion, swelling or delamination of cellulosic substrate occurred, resulting in a reduction on the degree of fibrillar aggregation and/or crystallinity. Cosgrove et al. (Mcqueenmason and Cosgrove 1994) identified a class of proteins with ability to induce extension by distributing the hydrogen bonding between plant cell wall polymers. Carbohydrate binding domain (CBH) of the carbohydrate-hydrolyzing enzymes, along with a number of other proteins (expansin-like proteins, swollenin), has also been suggested to be able to non-hydrolytically loosen or disrupt the packaging of the cellulose fibril network by weakening or splitting hydrogen bonding between cellulose fibers (Arantes and Saddler 2010). Although indirect evidence showed that addition of swollenin like enzymes helped boost enzymatic hydrolysis of pretreated substrates, there is no direct evidence that the enhanced hydrolysability relates to swelling crystalline cellulose by disruption of hydrogen bonds. Recently, another group of enzymes has been proposed to enhance hydrolysability of biomass. Harris et al. (Harris, Welner et al. 2010) first demonstrated that a novel enzyme class glycoside hydrolases family 61 (GH61) greatly increases the performance of cellulases from Trichoderma
*reesei* in lignocelluloses hydrolysis. Gustav *et al.* (Vaaje-Kolstad, Westereng *et al.* 2010) proposed that GH61 proteins may facilitate the hydrolysis by introducing chain breaks in the most inaccessible and rigid parts of crystalline polysaccharides. These proteins have flat substrate-binding surfaces and are capable of cleaving polysaccharide chains in the crystalline contexts using an oxidative mechanism (Horn, Vaaje-Kolstad *et al.* 2012). However, most of previous research focuses mainly on studying protein functions and enzyme activity, so far none of these abovementioned mechanisms have been certified from the perspective of enzyme-crystalline cellulose interaction. It has been reported that thermal treatment (increasing temperature up to 200 °C) of the unit cell parameters of cellulose Iβ exhibited a good swelling expansion along hydrophobic stacking sheet direction (Hori and Wada 2006; Kovalenko 2010), so if the swelling mechanism is dominate, it is expected that unit cell parameter changes will become apparent.

The difficulties in elucidating the cellulose deocrystallization mechanisms were due to two main factors: a lack of substrate representing a unit cellulose crystallite and a lack of analytical technique to investigate cellulose crystallite changes during enzyme treatment. Investigating the mechanism of enzyme degradation of crystalline cellulose has been pursued using pure cellulose substrates, such as microcrystalline cellulose (e.g. Avicel, Sigmacell), bacterial or algae cellulose. While bacterial or algae cellulose contains cellulose Iα crystals which differs from the dominant cellulose Iβ allomorphs in lignocellulosic biomass, other substrates are at or above micro-scale in size and contain an aggregate of elementary cellulose crystallites which are entwined with amorphous and crystalline regions. Therefore it has been a great challenge to use these substrates to achieve a clear understanding of the molecular-level mechanism of enzyme degradation of crystalline cellulose (Zhang and Lynd 2004; Zhang, Himmel *et al.* 2006).
In this study, we have applied nanocrystalline cellulose (NCC) as a model substrate extracted from poplar cellulose, which represent the unit dimension of the elemental crystalline cellulose with a width between 5-20 nm and an average length of 200 nm. We report molecular-level structure changes of nanocrystalline cellulose during enzymatic decrystallization. A refined X-ray diffraction (XRD) technique enables the accurate determination of crystalline structure of NCC. In this study, we assess experimentally whether decrystallization of NCC happens through swelling between hydrophobic stacking layers or by introducing oxidized chain end. We further identify ascorbate as cofactors for proposed GH61 redox reaction and followed crystallite changes during enzyme decrystallization. The results elucidated for the first time that cellulase decrystallization cellulose proceeded through a delamination mechanism instead of disrupting hydrogen bond network of the crystallite to cause swelling. The presence of oxidoreductase in commercial cellulase mixture promotes the delamination of crystalline cellulose via direct enzyme oxidation mechanism.
5.3 Material and Methods

5.3.1 Preparation of model compound substrates

All chemicals used in this study were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise mentioned. Bleached kraft pulp (BKP) was prepared following previously described procedure (Ju, Engelhard et al. 2013). Nanocrystalline cellulose was prepared by acid extraction (Hamad and Hu 2010). In brief, 64.5% sulfuric acid was used to extract nanocrystalline cellulose from BKP, followed by dilution to stop reaction. The mixture was filtered through 3K Dalton RC membrane using AMICON ultrafiltration 8400 system. Nanocrystalline cellulose collected above the membrane after repeated washing and filtering was freeze-dried for further analysis. Microcrystalline cellulose (Avicel PH101) was purchased from Sigma-Aldrich (St Louis, USA). Phosphoric acid swollen amorphous cellulose (PASC) was prepared using phosphoric acid treating NCC as well as Avicel following procedure as previously described (Zhang, Cui et al. 2006).

5.3.2 Enzymatic decomposition of nanocrystalline cellulose

Hydrolysis experiments were performed in 2-mL Eppendorf tubes with reaction volume of 1.5 mL as well as in 250-mL Erlenmeyer flasks with reaction volume of 100 mL. The reaction solution contained 50 mM sodium acetate buffer (pH 4.8). The enzymatic digestibility of NCC was determined and compared by enzymatic hydrolysis using 0.2% (w/v) substrate consistency, unless otherwise mentioned. Enzymatic hydrolysis was carried out at 50 °C for 96 hours. Novozyme Cellic Ctec2, DSM Cytolase CL, Dupont Accellerase 1500 and Novozyme Celluclast were used as commercial cellulase hydrolysis experiment with supplementation concentration at 20 FPU/g of substrate. Cytolase CL was supplemented with Novozyme 188 (β-glucosidase) at the ratio of 1FPU: 2 CBU to avoid product inhibition caused by celllobiose accumulation. The
enzymatic activity and protein concentrations of enzymes used are mentioned in Chapter 4. Single component enzymes such as Cellobiohydrolase I (CBHI) and endoglucanase II (EGII) were purchased from Megazyme (Wicklow, Ireland). Prior to hydrolysis, CBHI and EGII were dialyzed against sodium acetate (100mM, pH 4.8) buffer by ultrafiltration in an Amicon stir cell using 1KDa Millipore membrane at 4°C overnight. Both enzymes were loaded at a protein concentration of 4mg/g substrates separately. When enzyme mixture of CBHI and EGII was used, CBHI and EGII were blended as a ratio of equal protein concentration with a final concentration of 4mg/g. For experiments with ascorbic acid supplementation, a final concentration of 1mM ascorbic acid was added to the reaction before the hydrolysis started.

The remaining NCC after various enzyme treatments was first centrifuged at 10,000 rpm and then followed by water rinse. Solid fractions were freeze-dried for further analysis. The cellulose conversion yield is defined as the glucose amount in the hydrolysate divided by the cellulose content (as glucose) in the substrate. Duplicate samples were taken at each time point. Aliquots were taken periodically from the hydrolysate to determine the glucose concentration. Glucose in the hydrolysate was measured using the glucose oxidase/peroxidase (GOPOD) assay kit from Megazyme (Wicklow, Ireland) as well as was measured by HPLC as suggested (NREL/TP-510-42623). Glucnoic acid was measured by HPLC as described by previous procedure (Horn, Vaaje-Kolstad et al. 2012). The reducing sugar content was measured using a modified 96-well microplate DNS assay. All samples were first centrifuged followed by filtration through a 0.45μm membrane prior to the analysis. All results reported for glucose analysis from enzymatic hydrolysis were mean values based on triplicate repeated experiments under the same conditions.
5.3.3 Nanocrystalline cellulose characterization

X-ray diffraction (XRD) pattern of substrates were obtained by the general purpose X-ray diffractometer with Philips X’Pert MPD system and a vertical H–H goniometer (190 mm radius). The X-ray source was ceramic X-ray tube with Cu anode. Operating power was 40 kV, 50 mA (2.0 kW). X-ray diffraction pattern of samples obtained after freeze-drying were recorded at room temperature from 10° to 140°. The scan was carried out with a step size of 0.05°. NCC crystalline structure characterization by X-ray diffraction (XRD) was followed by the refined procedure developed in Chapter four. NCC was analyzed by FTIR (Fourier Transform Infrared Spectroscopy) and X-ray photoelectron spectroscopy (XPS) for confirmation of successful preparation. For FTIR, the spectral resolution was 4 cm⁻¹ and the number of average scans was 128. The measured wave number was from 4000 to 600 cm⁻¹. DRIFTS-IR focuses on the surface of the sample, and the specific area where the measurement takes place was small. The samples were freeze-dried and ground to homogeneous prior to FTIR analysis. At least three replicate samples were probed. The XPS analysis was carried out with Kratos Axis Ultra spectrometer, using a monochromatic Al K (alpha) source (10 mA, 15 kV). XPS has capability to probe the surface of the sample to a depth of 5–7 nm, and has detection limits that range from 0.1 to 0.5 atomic percent depending on the element. The Kratos charge neutralizer system was used on all specimens. Survey scan analysis was carried out with an analysis area of 300 × 700 μm and passenergy of 160 eV.

5.3.4 Cell chamber membrane cut-off NCC hydrolysis

A customized cell chambers set was applied for the membrane cut-off experiments. NCC and Ctec2 loaded as 20FPU/g of substrate in 50mM sodium acetate buffer were injected to the upper side of the half chamber, with layers of 8K Dalton cut-off membranes separating the
chamber from the lower part. The second lower part of the chamber was injected with 0.2% NCC. The reaction system was 1.5 ml, respectively. For some experiments with α-tocopherol supplementation, 100 μL of pure α-tocopherol was injected to the lower part of the cell chambers. For experiments with superoxide dismutase, a final concentration of 200 unit/ml was injected to the lower part of the cell chambers. After 96hr of hydrolysis, mixture in both cell chambers were collected and centrifuged. Solid fractions was washed by Milli-pure water and followed by freeze drying. Samples were collected after freeze-dry for further analysis.

5.4 Results and Discussion

5.4.1 NCC as ideal model substrate for the investigation of cellulose decrystallization

During extraction by sulfuric acid from wood pulp, cellulose fibrils were cleaved at structural defects resulting in short nanocrystals (Revol, Bradford et al. 1992). Strong acid hydrolysis helped to remove the amorphous region and gave explosion of nanowhisker of cellulose. As the closest attainable attribute to perfect cellulose chains with microfibril size diameter less than 10 nm, NCC reached to a lower degree of polymerization less than 100 and with its crystallinity around 90% (peak height method), approaching the theoretical limit of the cellulose chains (Moon, Martini et al. 2011). NCC is a self-assembled parallelepiped rod-like structure of the crystallites, which possess cross-sections in the nanometer range and lengths orders of magnitude larger (Hamad and Hu 2010).

It has been reported that by Hall et al. (Hall, Bansal et al. 2010) that during enzymatic hydrolysis of Avicel, the crystallinity index (CRI) and structural changes of cellulose were negligible, except a preferential attacking of (102) plane. XRD data were collected from 10° to 140° for experimental regenerated amorphous cellulose (RAC), and two major peaks centered at
20.3° and 39.5° were identified as amorphous contribution from both NCC and Avicel. The decreasing intensity of (102) plane for Avicel could be resulted from contribution of major amorphous peak located at 20.3°, indicating an amorphous part of cellulose removal during Avicel hydrolysis, while such changes were not observed for NCC. A substantial Crl change of NCC was observed during 96hr hydrolysis profile, however, such change is not statistically significant (Figure 5.1). This uncertainty corresponds to most of previous researches indicating Crl parameter are less reliable interpreting the structure changes of cellulose during enzymatic hydrolysis (Arantes and Saddler; Zhang and Lynd 2004; Chen, Stipanovic et al. 2007; Bansal, Hall et al. 2010; Hall, Bansal et al. 2010; Park, Baker et al. 2010). On the other hand, the structural changes during hydrolysis cannot be observed from microcrystalline cellulose like Avicel, since the imperfection of microcrystalline structure as well as aggregation of cellulose microfibrils. Thus in order to investigate a detailed mechanism, in the following study we employed NCC as model cellulose for further assessment.
Figure 5.1. Enzymatic hydrolysis glucose conversion rate of NCC and its crystallinity index change at enzyme Ctec2 loading at 20 mg protein/g of substrate.

5.4.2 Cellulose crystalline structural changes before and after cellulase hydrolysis

An X-ray diffraction profile of NCC with very well oriented nanofibrils was observed with four typical crystalline peaks from Cellulose Iβ (Nishiyama, Langan et al. 2002) after refinement. The four peaks observed in this profile were indexed as (110), (110), (200) and (004) respectively. These peak positions of XRD profile have been widely used as a guide to calculate the $d$-spacing parameter through the Bragg equation which relates peak position inversely to the distance between the adjacent lattice planes along directions perpendicular to these planes. While the $d$-spacing of (200) planes reflected the distance between cellulose hydrophobic stacking sheets linked by hydrogen bonds, the $d$-spacing of (110) and (110) planes represent the relative distances between intrasheet chain hydrogen bonding; (004) reflection represented the lattice planes along the glycosidic bond extension direction.
Studies of the expansion or contraction of crystallites of NCC with commercial enzymes (Novozyme Cellic Ctec2) treatment showed that, the distances between the sheets of the molecules stayed statistically constant during each time point where samples were collected along 96-hour time course, reflected by the \(d\)-spacing from (200) planes (Figure 5.2). Final 96 hour hydrolysis resulted in 40% of glucose conversion; with the \(d\)-spacing (0.39 nm) remaining unchanged as cellulose I\(\beta\) structure proposed by Nishiyama et al. (Nishiyama, Langan et al. 2002), as well as NCC original material. Other commercial enzymes were all evaluated respectively, such as Celluclast, Accellerase and Cytolase, as well as single cellulase component from *Trichoderma reesei* such as cellulohydrolase I (CBHI) and endoglucanase II (EGII), with no observation of (200) \(d\)-spacing changes compared to starting material (Table 5.1). It has been shown that that thermal treatment (increasing temperature up to 200 °C) of the unit cell

![Figure 5.2. X-ray diffraction spectrum of NCC treated by Ctec2 at different hours; (insertion: \(d\)-spacing of (200) plane for different time treated NCC.](#)
parameters of cellulose Iβ exhibited a good swelling expansion in between hydrophobic stacking sheet (Kovalenko 2010), while our study that no changes was observed at this direction during enzymatic decomposition process.

Table 5.1. NCC treated by different enzymes and their d-spacings measured by XRD.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>(110)</th>
<th>(110)</th>
<th>(200)</th>
<th>(004)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulose Iβ (Nishiyama)</td>
<td>0.58</td>
<td>0.53</td>
<td>0.39</td>
<td>0.26</td>
</tr>
<tr>
<td>NCC original</td>
<td>0.59</td>
<td>0.53</td>
<td>0.39</td>
<td>0.26</td>
</tr>
<tr>
<td>NCC + Ctec2-96hr</td>
<td>0.58 (↓)</td>
<td>0.52 (↓)</td>
<td>0.39 (-)</td>
<td>0.26 (-)</td>
</tr>
<tr>
<td>NCC + Ctec2+AA-96hr</td>
<td>0.59 (-)</td>
<td>0.52 (↓)</td>
<td>0.39 (-)</td>
<td>0.26 (-)</td>
</tr>
<tr>
<td>NCC + CBHI-96hr</td>
<td>0.56 (↓)</td>
<td>0.50 (↓)</td>
<td>0.39 (-)</td>
<td>0.26 (-)</td>
</tr>
<tr>
<td>NCC + EGII-96hr</td>
<td>0.63 (†)</td>
<td>0.56 (†)</td>
<td>0.39 (-)</td>
<td>0.26 (-)</td>
</tr>
<tr>
<td>NCC + Celluclast &amp;BG-96hr</td>
<td>0.60 (†)</td>
<td>0.54 (†)</td>
<td>0.39 (-)</td>
<td>0.26 (-)</td>
</tr>
<tr>
<td>NCC + ACC-96hr</td>
<td>0.60 (†)</td>
<td>0.54 (†)</td>
<td>0.39 (-)</td>
<td>0.26 (-)</td>
</tr>
<tr>
<td>NCC + DSM-96hr</td>
<td>0.60 (†)</td>
<td>0.54 (†)</td>
<td>0.39 (-)</td>
<td>0.26 (-)</td>
</tr>
</tbody>
</table>

* ND: non-detectable peak below 0.01; arrow indicates comparison with NCC original.

An average of 99.27% fitting accuracy was achieved for such analysis. Experiment was repeated three times with similar results.

The negligible changes observed along (004) reflections were ensured by the stable unaltered glycosidic bonds distances, while the alcohol confirmation might be one of the reasons contributing to such variation. Another structural changes observed during enzymatic decomposition of NCC is that the variation of intrachain distances resulted from peak shifting of (110) and (110) reflections. The d-spacing along (110) and (110) for NCC were in agreement with
the reported model of cellulose I\(\beta\). In both structures of CBHI and Ctec2 treated NCC, the molecular chains along \((1\overline{1}0)\) and \((110)\) were stretched insignificantly. For EGII treated NCC, however, the hydrophilic surfaces are 5.1-5.7% more separated along \((1\overline{1}0)\) and \((110)\) directions by EGII interchain enzyme cleavages. Another peak indexed as \((102)\) plane representing disordered cellulose emerged only during CBHI and EGII treated NCC (Figure 5.3), indicating disrupting mechanism of single cellulase component treatment for cellulose.

**Figure 5.3.** X-ray diffraction spectrum of original and different enzyme treated NCC (Insertion: enlarge spectrum of CBHI and EGII treated NCC at the region of 18°-27°).
In summary, the $d$-spacing changes at (110), (110) and (004) were observed during enzymatic hydrolysis of NCC, while $d$-spacing at (200) stayed constant. It is inarguable the constant $d$-spacing parameter between (200) planes showed evidence for the first time that the enzyme decrystallization mechanism may happen in another fashion rather than swelling between hydrophobic sheets by weakening hydrogen bonding. No swelling effect during decrystallization process was observed in all enzyme systems tested here. Although variations for other molecular structure changes could stay inside the error margins, we caution against neglecting such structural changes observed from nanocrystals, since for the first time changes were observed using NCC representing the true crystal structure of cellulose Iβ from lignocellulosic biomass. Detailed mechanisms of how such changes happened require higher-resolution crystallographic means, which can give a better clarification of the detailed observation of cellulose unit cell structure.

5.4.3 Crystallite size changes of cellulose Iβ during NCC enzymatic decomposition

The apparent crystallite size (ACS) determined from (200) reflections of different substrates has been reported to decrease, remain unchanged, or even increased during the hydrolytic course by different researchers (Gama and Mota 1997; Wang, Zhang et al. 2006). A logical explanation for such changes observed would be that the smaller and readily hydrolysable fiber particles in microcrystalline cellulose would be hydrolyzed faster, leaving proportionally more crystalline cellulose fibers behind. Since the average diameter of cellulytic enzymes is as high as 5.9 nm while the average diameter of NCC is around 5-10 nm, the accessibility of enzymes to NCC is impeded by the roughness of the surface or on the pore structure of particles than microcrystalline cellulose (Avicel). Crystallite size changes happened on independent
crystal particle of NCC along different reflections could be analyzed directly from microfibril level, eliminating intact cellulose fiber relevant effects.

The average crystallite size determined from refined XRD pattern and calculated crystal sizes in four directions are shown in Table 5.2. NCC hydrolyzed by Ctec2 showed a significant decrease in crystallite size compared to original NCC. When looking from the number of layers

Table 5.2. NCC treated by different enzymes final degree of hydrolysis and their crystalline size corresponding to reflections measured by XRD.

<table>
<thead>
<tr>
<th></th>
<th>Crystallite size (nm) average</th>
<th>Crystallite size (nm) corresponding to reflections*</th>
<th>Degree of hydrolysis (RD) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(nm) average</td>
<td>(110)</td>
<td>(110)</td>
</tr>
<tr>
<td>NCC original</td>
<td>4.5±0.1</td>
<td>4.32</td>
<td>4.41</td>
</tr>
<tr>
<td>NCC + Ctec2-96hr</td>
<td>3.7±0.1</td>
<td>3.21</td>
<td>4.46</td>
</tr>
<tr>
<td>NCC + Ctec2+AA</td>
<td>3.5±0.1</td>
<td>3.24</td>
<td>3.89</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCC + CBHI-96hr</td>
<td>4.6±0.2</td>
<td>3.87</td>
<td>5.59</td>
</tr>
<tr>
<td>NCC + EGII-96hr</td>
<td>4.3±0.2</td>
<td>5.28</td>
<td>8.24</td>
</tr>
</tbody>
</table>

*ND: non-detectable peak below 0.01 nm.

* Calculation derived from XRD raw patterns without refinement and is a subjective measurement for crystallite sizes compared to the average crystallite sizes.
(crystallite size/d-spacing) remained from different reflections, difference arises between CBHI and EGII (Figure 5.4). The crystallite size increase resulted by CBHI treatment was mainly

**Figure 5.4.** Numbers of layer changes of enzyme treated NCC at different reflections.
contributed from crystallite increase of (110) and (004) reflections. Since CBHI hydrolyze single cellulose chain in a possesive manner from non-reducing end, it is reasonable to expect the results of a more ordering manner along (004) direction. More significantly, changes along (110) reflections could be evidence of the more favorable hydrolyzing location at edge and corner chains rather than middle chains, as suggested by molecular dynamic simulation of CBHI on cellulose crystals (Beckham, Matthews et al. 2011; Payne, Matthews et al. 2011). Chains near the side of a cellulose crystal model along (110) directions has lower free energies and are believed to be more easily accessed for CBHI, thus threading into the catalytic tunnel of enzyme for further hydrolysis. While the increasing numbers of crystallite layers from all directions by EGII treatment showed a less preferential attacking site for NCC.

Comparing to the increasing crystallite size of CBHI and EGII treated NCC, NCC from Ctec2 hydrolysis showed an opposite direction of crystallite size change. Contradictory to the hypothesis that smaller particles being hydrolyzed first leading to bigger crystal left, ~11% of ACS reduction was observed after Ctec2 hydrolysis of NCC. Specifically, reducing number of layers were observed along (200) reflection for Ctec2 treated NCC. Such reduction is hard to be explained by the traditionally proposed cellulase hydrolysis theory, where more enzyme-accessible cellulose could be hydrolyzed first. If “chain-disrupting” factors existed during cellulose decrystallization, the flat surface of hydrophobic layers of cellulose could be disrupted, resulting to more and more less-ordered chains along (200) reflections (Himmel, Ding et al. 2007; Vaaje-Kolstad, Westereng et al. 2010; Quinlan, Sweeney et al. 2011). Since the hypothesis of GH61 enzyme family being identified from Ctec2 enzyme preparation, our experiment for the first time supported that GH61 in Ctec2 enzyme preparation could act as a “chain disrupting” factor, resulting to a decrystallization of highly ordered crystalline cellulose hydrolysis.
5.4.4 Ascorbic acid potentiate Ctec2 activity during decomposition of NCC

In our previous research, Ctec2 enzyme tends to work more efficiently on NCC substrate than other commercial enzymes, resulting to at least 10% more sugar yield (detailed description see Chapter 4). It has been postulated that the novel enzyme component like GH61 existed in Ctec2 (Cannella, Hsieh et al. 2012; Horn, Vaaje-Kolstad et al. 2012) can promote efficiency of the classical hydrolytic enzymes (cellulases) by acting on the surfaces of the insoluble substrate, without the need of “extracting” polysaccharide chains from their crystalline matrix. Direct oxidation mechanism of such enzyme depends on the presence of divalent metal ions and an electron donor to introduce breaking chain ends (Vaaje-Kolstad, Westereng et al. 2010; Quinlan, Sweeney et al. 2011), such as ascorbic acid and gallate. Analysis of reaction products of GH61 shows a variety of native as well as oxidized celledextrins as a result of the oxidized glycosidic bond cleavage. Gluconic acid is one of the detected aldonic acid produced by oxidative enzyme which generates a chain end comprising C1-oxidized sugar at a lower PH (~5).

We demonstrated that with the presence of ascorbic acid, the hydrolysis of Ctec2 on crystalline cellulose was potentiated to ~18% of glucose production (Figure 5.5), which agrees with most of the studies by the effect of ascorbic acid as an external electron donor (Vaaje-Kolstad, Westereng et al. 2010; Quinlan, Sweeney et al. 2011). When ascorbic acid was added after 48 hours of hydrolysis, the sugar release can be boosted up to the almost same level as added in the beginning of hydrolysis during a 96 hr hydrolysis course. At the same time, 13% of glucose resulted into gluconic acid from NCC besides glucose release, when ascorbic acid is added for enzymatic hydrolysis. The gluconic acid peak detected by HPLC is separated with glucose using a method described in the experimental part, and the glucose amount was further tested by glucose-specific enzyme kit, yielding the same results of glucose concentration. Such
Figure 5.5. Effect of ascorbic acid on NCC hydrolysis by Ctec2 at 20 FPU/g of substrates: (A) hydrolysis profile as glucose yield from NCC with or without addition of ascorbic acid; (B) final product yield of glucose and gluconic acid without addition of ascorbic acid.

Product analysis ensured that the gluconic acid detected by HPLC is not included in the glucose peak, which is a concern during traditional standard HPLC measurement (Cannella, Hsieh et al. 2012). While analysis of the product solubilized in the liquid fraction confirmed the promotion of nanocrystalline cellulose hydrolysis by oxidative mechanism of GH61 and the cofactor effects, further structural analysis were carried out for the solid fractions from hydrolysis.

In agreement with the changes observed previously from XRD data, no $d$-spacing swelling along (200) reflection of the crystalline structure was detected during the whole time course of ascorbic acid added Ctec2 enzymatic hydrolysis. Furthermore, the same reduction of crystallite size perpendicular to (200) plane was observed when ascorbic acid was presented as purely hydrolyzed by Ctec2. An average of one more layer along (200) plane was removed when ascorbic acid presented together with Ctec2 from the beginning of hydrolysis (Figure 5.4). Such observation further supported the efficiency of novel enzyme component like GH61 being able
to act on flatten hydrophobic surface and bring into more chain disruptions, thus the whole cellulose hydrolysis is potentiated by more non-reducing chain end generated, leading to an improved decrystallization process.

5.4.5 Surface characterization of original and different enzyme treated NCC

X-ray photoelectron spectroscopy (XPS) is a quantitative spectroscopic technique that measures the elemental composition, chemical state and electronic state of the elements that exist within a material. While XPS has been a ubiquitous method for characterization and investigation in the field of classical materials for a long time, its application to systems of biological nature or origin is still rather limited. This is mainly due to the requirement of bringing the samples under high vacuum. On the other hand, it is in contradiction with the fact that living systems and environments are by essence made of interfaces and that solid materials of biological origin (e.g. cellulose, starch) are mesomorphic. Nevertheless, XPS provides a good balance between qualitative information, quantification and surface selectivity, to investigate the spatial distribution of components and to understand biological material properties and processes (Johansson, Campbell et al. 1999; Li, Liu et al. 2011; Rouxhet and Genet 2011). In this study we applied XPS to characterize the possible biological modifications on the surface functional groups before and after NCC decrystallization.

Figure 5.6 depicts the deconvoluted XPS carbon and oxygen high resolution spectra from original NCC and several biologically treated NCC. According to the survey scans, all sample surfaces consisted mainly of carbon and oxygen while hydrogen is not detectable by XPS. The deconvolution used Voigt peak shapes and integrated background subtraction. For the fits, the
Figure 5.6. XPS surface characterization of original and different enzyme treated NCC.
peak positions were fixed according to tabulated chemical shifts and guidelines established for cellulose (Johansson, Campbell et al. 1999; Li, Liu et al. 2011; Rouxhet and Genet 2011). The difference in the $\text{C}\text{l}s$ peak intensity between the spectra in Figure 5.6A and Figure 5.6B is the increased peaks of oxidized carbon in the form of carbonyl groups, with possibilities such as aldehyde ($\text{RCHO}$), ketone ($\text{RCOR'}$), ester ($\text{RCOOR'}$) or carboxylic acid ($\text{RCOOH}$). Experiments were repeated to verify such observations. Since XPS scan analysis was carried out with an analysis area of $300 \times 700 \mu\text{m}$, the NCC surface exhibited heterogeneous characteristics, with only few sites observed as oxidized spots. Original NCC is a polymer whose macromolecule consists of $\beta-$d-glucopyranose rings. The repeating unit of the polymer chain of cellulose is composed of two $\beta-$d-glucopyranose rings rotated with respect to each other. The spotted regions of oxidation surface are highly suspected as a result of the direct cleavage by redox mechanism as schemed in Figure 5.7.

![Proposed Redox reaction mechanism by Ctec2](image)

**Figure 5.7.** Proposed scheme of oxidoreductive reaction mechanism by Ctec2.
5.4.6 NCC surface structure changes caused by oxidization mechanism

According to the proposed mechanism, certain intermediate are in responsible for the oxidation reaction to begin, such as free radicals generated during reaction or redox chemical species. Free radicals generally have a short life and can act as initiations for continuous reactions. In order to verify the effect of intermediate, membrane cut-off experiments were designed by using a set of customized cell chamber as shown in Figure 5.8. In summary, NCC with Ctec2 reaction was injected from one side of the cell chamber, with original NCC injected to the other side. 8,000 Dalton cut-off dialysis membranes were placed in between, allowing only small molecules to pass through reaching equilibrium. XRD and Fourier transform (FT)-IR were applied to analyze the structural changes of the NCC extracted from the original NCC after 8 hours incubation in the membrane cut-off apparatus. Results showed that after such treatment, the original NCC structures were significantly changed. Crystallinity index (Crl) calculated from refined XRD methods as described in Chapter 4 indicated a considerable amount of amorphous
cellulose generated after membrane experiment. Approximately 16% of less-ordered cellulose was generated. This was also confirmed by the FT-IR calculated indirect CrI showing at least 3% of decrease for crystalline region on the surface. NCC after cut-off membrane treatment was extracted and applied for hydrolysis again by cellulase components of CBIII and EGII mixture. Results showed that the treated NCC was much easier to be hydrolyzed by this single component of cellulase, with 10% of glucose release increase and 16% of reducing sugar release increase (Table 5.3). Such increase was apparently caused by the cleavage of oxidized surface which provide more accessibility for enzymes to work on.

**Table 5.3.** Glucose and reducing sugar released after 96 hour of CBH+EG enzymatic hydrolysis.

<table>
<thead>
<tr>
<th>Enzyme added:</th>
<th>Glucose</th>
<th>Reducing sugar</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBH+EG(protein=1:1)</td>
<td>(%</td>
<td>(%)</td>
</tr>
<tr>
<td>NCC substrates from cell chamber</td>
<td>11.71 ± 1.62</td>
<td>19.62 ± 0.24</td>
</tr>
<tr>
<td>NCC control</td>
<td>2.10 ± 0.54</td>
<td>3.61 ± 0.63</td>
</tr>
</tbody>
</table>

To further verify the possible involvement of small molecule free-racial in the reaction which could be transferred from the side of reaction to the NCC original solution, free-radical inhibitors, α-tocopherol and superoxide dismutase, were added to the NCC original solution. Tocopherol, known as Vitamin E, has been claimed to be the most important lipid-soluble antioxidant, and that it protects membranes from oxidation by reacting with lipid radicals produced in the lipid peroxidation chain reaction. This removes the free radical intermediates and prevents the propagation reaction from continuing. Superoxide dismutases are enzymes that catalyze the dismutation of superoxide (O$_2^-$) into oxygen and hydrogen peroxide. Thus, they are
Table 5.4. Crystallinity index for original and different treated NCC, calculated by XRD as well as FTIR.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Crystallinity index (CrI)</th>
<th>Crystallite size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peak height</td>
<td>Refined XRD</td>
</tr>
<tr>
<td>NCC</td>
<td>80.10%</td>
<td>72.26%</td>
</tr>
<tr>
<td>NCC-Ctec2</td>
<td>72.27%</td>
<td>97.59%</td>
</tr>
<tr>
<td>NCC membrane</td>
<td>79.01%</td>
<td>55.83%</td>
</tr>
<tr>
<td>NCC2-Membrane -tocopherol</td>
<td>80.92%</td>
<td>70.12%</td>
</tr>
<tr>
<td>NCC2-Membrane -Superoxide dismutase</td>
<td>82.04%</td>
<td>76.10%</td>
</tr>
</tbody>
</table>

*Note: FTIR crystallinity was calculated based on area 1370 cm⁻¹ assigned as CH bending (1400 cm⁻¹ -1350 cm⁻¹) to the ratio of area 2900 cm⁻¹ assigned as CH stretching (3010 cm⁻¹ -2780 cm⁻¹).

an important antioxidant defense in nearly all cells exposed to oxygen. Results indicated by adding both antioxidants, the oxidization of NCC generating amorphous cellulose were minimized (Table 5.4). The CrI remains similar as original NCC. Analysis of the NCC structure changes by FT-IR were shown in Figure 6.9. Regions between 1800 and 1500 cm⁻¹ were further investigated. Original NCC exhibited a significant peak located at 1645 cm⁻¹, representing the C-O bonds in the glucose ring. However, as shown in Figure 5.9, such regions gradually disappear and merged into a new peak located at 1660 cm⁻¹. Such peak formation represents the oxidized carbon groups in the forms of the oxidized carbon such as carbonyl groups. Such peaks were observed from NCC treated after Ctec2, Ctec2 treated NCC in addition of ascorbic acid, as well as membrane cut-off treated NCC. It was also shown that the oxidative functional groups on the surface of NCC disappeared after adding antioxidants (Figure 5.9). No peaks centered at 1660 cm⁻¹ was observed once antioxidants such as α-tocopherol and superoxide dismutase were added in the original solution. Although these data provides possible explanations that certain chemical
Figure 5.9. FTIR 1800-1500 cm\(^{-1}\) comparison of original and different treated NCC.
species may pass through membrane that leading to a direct interaction with NCC. Exact mechanism of NCC interaction with such chemical species is under investigation. A number of recent studies have demonstrated that Ctec2 contains polysaccharides monooxygenases (PMOs) (Harris, Welner et al. 2010; Beeson, Phillips et al. 2012; Holm, Vaaje-Kolstad et al. 2012; Zifcakova and Baldrian 2012) which can boost the hydrolytic enzyme activities during lignocellulose hydrolysis. The results from NCC depolymerization provided the first evidence that this group of PMO can potentially enhance the efficiency of enzyme decrystallize cellulose.

5.5 Conclusions

Previous attempts to try and identify the cellulose decrystallization mechanism by either “swelling/amorphogenesis step” or “cellulose surface delamination” have tended to make use of a suite of complementary qualitative and semi-quantitative techniques. While these techniques have provided some useful information regarding the functionality of these proteins, they have typically provided little insight into the mechanism of cellulose-protein interaction from substrate perspective. By applying newly developed nanocrystalline cellulose coupled with complex surface characterization techniques, the decrystallization changes of the substrate during enzymatic hydrolysis can be tracked quantitatively. Structure changes of NCC indicate the previously suggested swelling mechanism is unlikely to play a role in the non-hydrolytic mechanisms of “cellulose structure open-up”. A mechanistic understanding of decrystallization process is further identified to proceed through delamination mechanism. The surface of crystalline cellulose was heterogeneously oxidized, creating more reducing ends for hydrolytic enzymes to access. The enhanced decrystallization process of NCC is attributed to the favorable individual enzyme function likely to be glycoside hydrolases 61 (GH61) families, which recently
has been classified as polysaccharide monooxygenases (PMOs). Future research should be oriented to better characterize individual PMOs interaction with NCC by identifying the generation free radicals during depolymerization. Such effort will open up avenues to engineer more efficient cellulases in concert with both native and nonnative (i.e., chemically pretreated) celluloses.
CHAPTER SIX: INVESTIGATING COMMERCIAL CELLULASE
PERFORMANCES TOWARD SPECIFIC BIOMASS RECALCITRANCE
FACTORS USING REFERENCE SUBSTRATES

6.1 Abstract

Three commercial cellulase preparations, Novozymes Celic® Ctec2, Dupont Accellerase® 1500, and DSM Cytolase® CL, were evaluated for their hydrolytic activity using a set of reference biomass substrates with controlled substrate characteristics. It was found that lignin remains a significant recalcitrance factor to all the preparations, although different enzyme preparations respond to the inhibitory effect of lignin differently. Also, different types of biomass lignin can inhibit cellulase enzymes in different manners. Enhancing enzyme activity toward biomass fiber swelling is an area significantly contributing to potential improvement in cellulase performance. While the degree of polymerization of cellulose in the reference substrates did not present a major recalcitrance factor to Novozymes Celic® Ctec2, cellulose crystallite has been shown to have a significant lower reactivity toward all enzyme mixtures. The presence of polysaccharide monooxygenases (PMOs) in Novozymes Ctec2 appears to enhance enzyme activity toward decrystallization of cellulose. This study demonstrated that reference substrates with controlled chemical and physical characteristics of structural features can be applied as an effective and practical strategy to identify cellulosic enzyme activities toward specific biomass recalcitrance factor(s) and provide specific targets for enzyme improvement.

Keywords: Cellulase; hydrolytic efficiency; reference substrates; lignin; nanocrystalline cellulose; PMOs.
6.2 Introduction

Lignocellulosic biomass provides a promising and renewable source of non-food sugar for sustainable biofuel production. Microbial enzymes play a vital role in releasing sugars from biomass substrate for fuel conversion. A considerable amount of effort has been directed to identifying and developing highly efficient and cost effective plant cell-wall degrading enzymes, and has resulted in the significant improvements in enzyme activities and reductions in enzyme production cost (Merino and Cherry 2007). Despite these achievements, the cost of enzyme remains a major economic bottleneck in the production of lignocelluloses biofuel and “a significant effort is still required to lower the contribution of enzyme to biofuel production” (Klein-Marcuschamer, Oleskowicz-Popiel et al. 2012).

Due to their high extracellular enzyme productivity needed for efficient cellulose hydrolysis, filamentous fungi (e.g. Trichoderma, Aspergillus species), is the preferred source for industrial cellulase preparation (Coughlan 1985; Persson, Tjerneld et al. 1991; Pandey, Selvakumar et al. 1999). They produce a spectrum of enzymes including endoglucanases (1,4-β-D-glucan glucanohydrolases), exoglucanases (1,4-β-D-glucan cellbiohydrolases) and accessory enzymes which are essential for lignocellulose hydrolysis (Berlin, Gilkes et al. 2005; Berlin, Maximenko et al. 2007). Most of the previous attempts to improve the fungal cellulase activity have been focused on improving their hydrolytic activity on cellulose using pure cellulose/dextrin or their analogs in soluble or insoluble forms (Rosgaard, Pedersen et al. 2006; Zhang, Himmel et al. 2006; Martinez, Berka et al. 2008). It has been increasingly recognized that the hydrolytic efficiency of fungal cellulases determined by these model cellulosic substrates does not provide a reliable prediction of their performance on pretreated lignocellulosic
substrates (Berlin, Gilkes et al. 2005; Kabel, van der Maarel et al. 2006). The efficacy of enzyme depolymerization of plant cell wall polysaccharide is affected by the characteristics of biomass substrate and the catalytic efficacy of enzymes themselves. The complexity of plant cell structure and the heterogeneous nature of lignocellulosic substrate make it a formidable challenge to design practical enzyme screening and selection strategies to overcome biomass recalcitrance.

In pretreated biomass substrates, biomass recalcitrance is compounded by a number of factors often interwoven with each other (Zhang, Himmel et al. 2006; Himmel, Ding et al. 2007). Applying heterogeneous pretreated biomass substrates for enzyme evaluation has encountered a great difficulty to achieve an aggregated understanding of the interactions of enzymes and substrates (Chang and Holtzapple 2000; Ju, Engelhard et al. 2013). Designing biomass substrates that can detect the deficiencies of the enzymes toward individual biomass recalcitrance factors (e.g. lignin content, crystallinity etc.) will offer specific guidance to selecting and screening highly efficient enzymes for lignocelluloses conversion. In the previous chapters, we have demonstrated the use of pulping technologies to create biomass "reference substrates" with controlled physicochemical properties (e.g. length, width, cell wall thickness, lumen diameter, cellulose degree of polymerization, etc.) and at the same time selectively alter one or a few substrate parameters (e.g. lignin, xylan, swelling, etc.). These substrates enabled us to gain advanced understanding of specific substrate recalcitrance factors on cellulase hydrolysis efficiency (Ju, Engelhard et al. 2013; Ju, Grego et al. 2013). These substrates would also likely allow us to determine the deficiencies of cellulase enzymes toward specific biomass substrate recalcitrance properties and help identify targeted areas for further enzyme improvement/engineering. Chapter Two, Three and Five also delineate the relations between biomass recalcitrance and enzymatic hydrolysis, from three different levels, fiber, fibril and
molecular levels, respectively. In this paper, we demonstrate the application of these biomass substrates to evaluate the performance of three commercial cellulase mixtures, Novozyme Cellic® Ctec2, Dupont Accellerase® 1500 as well as DSM Cytolase CL, and identify specific areas for improvement of these commercial enzyme preparations.

6.3 Methods

6.3.1 Pretreated substrate preparation and characterization

Reference substrates OPP, KP40, and SP40 were prepared from poplar by modified chemical pulping as described previously (Zhang, Qin et al. 2009; Ju, Engelhard et al. 2013). In summary, OPP was prepared through organosolv pretreatment, KP40 derived from modified kraft pulping pretreatment with lignin kappa number of 40, and SP40 was prepared by modified sulfite pulping pretreatment with lignin kappa number of 40. Lignin-free substrates, OPP0, KP0 and SP0, were prepared by treating respective OPP, KP40 and SP40 with acid chlorite solution at room temperature for 24h following a procedure described previously (Browning 1967). Microcrystalline cellulose (Avicel PH101) was purchased from Sigma–Aldrich (St Louis, USA). Nanocrystalline cellulose (NCC) was prepared from bleached poplar pulp following a previously described procedure (Hamad and Hu 2010).

6.3.2 Substrate compositional analysis and characterization

The chemical compositions of the substrates were analyzed by standard TAPPI test methods (T236 cm-06, T204 cm-07, T222 om-11, T211om-12 and T249 cm-09) (TAPPI). The fiber length distribution and coarseness were measured using a fiber quality analyzer (FQA, OpTest Equipment Inc., Hawkesbury, Canada) with average of 5,000 fibers analyzed as previously described (Ju, Grego et al. 2013). The viscosity of substrate solutions containing
0.05% (w/v) cellulose solution in 0.5 M cupriethylenediamine was measured on a viscosity rheometer (DV-III ULTRA, Brookfield engineering laboratories, Inc, MA). The intrinsic viscosity based average cellulose degree of polymerization (DPv) was calculated as described in ASTM standard method D4243-99. All viscosity measurements were performed using triplicate samples with triplicate measurements of each sample. The substrate crystallinity was measured using a Philips X’Pert MPD X-ray powder diffractometer (PANalytical Inc., Westborough, MA). The X-ray source was a ceramic X-ray tube with Cu anode, operated at 40 kV, 50 mA (2.0 kW). X-ray diffraction patterns of samples obtained after freeze-drying were recorded at room temperature from 10° to 75° 20 with a step size of 0.05°. Crystallinity index (CrI) was calculated from the height ratio between the intensity of the crystalline peak (I002 - IAM) and total intensity (I002) after subtraction of the background signal measured without substrate, using the Segal method (Segal, Creely et al. 1962). Surface lignin coverage and functional group analysis were characterized by X-ray photoelectron spectroscopy (XPS, Physical Equipment Inc., Chanhassen, MN). The experiments were carried out following a procedure described previously (Ju, Engelhard et al. 2013). XPS was also used to characterize surface changes of NCC before and after enzymatic hydrolysis. The deconvolution used Voigt peak shapes and integrated background subtraction. For the fits, the peak positions were fixed according to tabulated chemical shifts and guidelines established for cellulose (Johansson, Campbell et al. 1999; Li, Liu et al. 2011; Rouxhet and Genet 2011). Measurement was taken at three different spots on each sample to attain an average over the heterogeneity of the samples. NCC particle sizes were determined by a BI-90 Plus dynamic light scattering particle size analyzer (Brookhaven instrument cooperation, Holtsville, NY) at 25 °C in Milli-Q water at pH 4.9 (adjusted with HCl). Three diluted mixtures were prepared for replicate data. The Atomic Force Microscopy (AFM)
measurements were conducted using Digital Instruments Multimode Nanoscope III (Veeco Instruments Inc., Plainview, NY) as described previously (Brown, Hu et al. 2013). Samples were air-dried and subjected to tapping mode AFM for surface imaging. A silicon MPP-11100 AFM probe with a resonance frequency around 300–400 kHz was used. The scan angle was maintained at 0°, and 1 × 1 μm² images were captured in the trace direction with a scan rate of 1.0 Hz with 256 lines per image. Scanning was done in multiple spots on the sample surfaces to capture indented and non-indented spots. The surface area of Avicel and NCC was measured by the Brunauer-Emmett-Teller (BET) method using nitrogen adsorption following a previously described procedure (Lee, Kim et al. 1983).

6.3.3 Enzyme and enzyme assays

Celic® Ctec2 (Ctec2), and Novozyme 188® (β-glucosidase, BG) were obtained from Novozymes North America (Franklinton, NC). Accellerase® 1500 (ACC1500) was received from Dupont Danisco (Rochester, NY). Cytolase® CL (Cytolase) derived from Trichoderma longibrachiatum and Aspergillus niger was obtained from DSM (Seclin, France).

Filter paper activity was determined at 50°C by filter paper unit measurement (Ghose 1987) and expressed as FPU/mL. Beta-glucosidase was assayed at 40°C by monitoring the release of p-nitrophenol from p-nitrophenyl-β-D-glucoside and expressed as CBU/mL. The protein concentration in enzyme preparation was determined using the Pierce™ BCA protein assay microplate procedure (Thermo scientific, Rockford, IL) with reference to bovine serum albumin. Enzyme activity and protein concentration is shown in Table 6.1.

Table 6.1. Enzyme protein concentration and activities in commercial cellulase preparations.
<table>
<thead>
<tr>
<th>Enzyme commercial name</th>
<th>Abbrev.</th>
<th>Supplier</th>
<th>Protein concentration (mg/mL)</th>
<th>Cellulase activities</th>
<th>Specific activity (FPU/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellic® Ctec2</td>
<td>Ctec2</td>
<td>Novozymes</td>
<td>257</td>
<td>FPA 119</td>
<td>0.46</td>
</tr>
<tr>
<td>Accellerase® 1500</td>
<td>ACC1500</td>
<td>Dupont (Genencor)</td>
<td>114</td>
<td>57</td>
<td>0.50</td>
</tr>
<tr>
<td>Cytolase CL</td>
<td>Cytolase</td>
<td>DSM</td>
<td>143</td>
<td>117</td>
<td>0.82</td>
</tr>
<tr>
<td>Novozyme 188®</td>
<td>BG</td>
<td>Novozymes</td>
<td>117</td>
<td>487 (CBU/mL)</td>
<td>N/A</td>
</tr>
</tbody>
</table>

### 6.3.4 Batch enzymatic hydrolysis of pretreated substrates

Hydrolysis experiments were performed in 2-mL Eppendorf tubes with reaction volume of 1.5 mL. The reaction solution contained 50 mM sodium acetate buffer (pH 4.8). Enzymatic hydrolysis was carried out at 50 °C for 72 hours. Cytolase CL was supplemented with Novozyme 188 (β-glucosidase) at the ratio of 1 filter paper unit (FPU): 2 cellobiase units (CBU) to avoid product inhibition caused by cellobiose accumulation. Two enzyme loadings based on protein equivalence at 4 and 20 mg protein per gram of substrate (dry weight basis) were chosen for enzymatic hydrolysis, unless otherwise mentioned. The enzymatic digestibility of reference substrates were determined and compared by enzymatic hydrolysis using 2% (w/v) substrate consistency, unless otherwise mentioned. Such consistency was chosen to minimize the effect from glucose inhibition as suggested by previous research (Xiao, Zhang et al. 2004). The cellulose conversion yield is defined as the glucose amount in the hydrolysate divided by the cellulose content (as glucose) in the substrate. In order to compare the performance of different cellulase preparations on various reference substrates, two indices, the enzymatic hydrolysis initial rate and final conversion yield were applied. In all experiments described below, the initial
rate was calculated based on the first hour hydrolysis rate of glucose release and the final conversion yield was determined based on the amount of glucose released after 72 hours incubation.

Duplicate samples were taken at each time point. Aliquots were taken periodically from the hydrolysate to determine the glucose concentration. Glucose in the hydrolysate was measured using the glucose oxidase/peroxidase (GOPOD) assay kit from Megazyme (Wicklow, Ireland). Xylose in the hydrolysate was measured by High-performance liquid chromatography (HPLC) as suggested by NREL (NREL/TP-510-42623). All samples were first centrifuged followed by filtration through a 0.45μm membrane prior to the analysis. All results reported for sugar analysis from enzymatic hydrolysis were mean values based on triplicate repeated experiments under the same conditions.

6.3.5 Enzyme adsorption experiments

Cellulase adsorption on reference substrates was performed at 25 °C in 50 mM sodium acetate buffer (pH 4.8) as described previously (Tu, Chandra et al. 2007). Enzyme solutions with concentrations of 0.08 mg/mL and 0.4 mg/mL (equivalent to 4 and 20 mg protein/g substrate enzyme loading for hydrolysis) were added to the buffer with substrates at 2% consistency, respectively. Calculations followed the procedure described previously (Ju, Engelhard et al. 2013).
6.4 Results

6.4.1 Chemical composition and characterization of pretreated reference substrates

![Image](image.png)

**Figure 6.1.** Reference substrates used in this study. Substrates from left to right at the first line: OPP, OPP0, KP40 and KP0, substrates from left to right at the second line: SP40, SP0, Avicel and NCC.

Eight reference substrates, KP40, SP40, OPP, KP0, SP0, OPP0, Avicel and NCC, were prepared and used in this study (Figure 6.1). Among these substrates, KP40, SP40 and OPP contain an appreciable amount of lignin. Previous work has shown that, KP40 and SP40 have exhibited similar morphological properties regarding to their fiber sizes, cellulose CrI and DP. However, the xylan composition and surface lignin properties between SP40 and KP40 are significantly different (Ju, Engelhard et al. 2013). OPP was prepared from organosolv pretreatment of poplar with a lower cellulose DP and crystallinity (Pan, Gilkes et al. 2006; Zhang, Qin et al. 2009). OPP contains approximately 80.5% of cellulose and 2.4% of lignin (Table 6.2). Research showed that the residual lignin remaining in the OPP is more hydrophilic.
and contains more hydroxyl and carboxyl groups than the organosolv dissolved lignin (Berlin, Balakshin et al. 2006). However, a very high amount of acetone extractives (~15%) was detected on OPP, in agreement with a previous report (Zhang, Qin et al. 2009). GC/MS analysis showed that most of these extractives are the low molecule weight phenolic compounds (LMWPC) generated from lignin depolymerization. The high surface lignin content of OPP (~44%) is likely due to deposition of these LMWPC on the fiber surface.

Three lignin-free substrates, KP0, SP0 and OPP0, were prepared by sodium chlorite delignification of the respective substrates, KP40, SP40 and OPP. As demonstrated previously (Ju, Engelhard et al. 2013; Ju, Grego et al. 2013), KP0 and SP0 have comparable fiber morphological properties but with different xylan content. Compared to KP0 and SP0, OPP0 has little residual xylan content and significantly higher cellulose content. However, the DP of OPP0 cellulose is much lower than those of KP0 and SP0 (Table 6.2). Organosolv pretreated substrates also have a lower cellulose crystallinity (Zhang, Qin et al. 2009). Since cellulose crystallinity is

Table 6.2. Chemical compositions and characterizations of reference substrates.

<table>
<thead>
<tr>
<th>Reference substrates</th>
<th>Chemical composition (%)</th>
<th>Characterization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lignin</td>
<td>Cellulose (as glucan)</td>
</tr>
<tr>
<td>KP40&lt;sup&gt;+&lt;/sup&gt;</td>
<td>5.90</td>
<td>77.23</td>
</tr>
<tr>
<td>SP40&lt;sup&gt;+&lt;/sup&gt;</td>
<td>5.90</td>
<td>82.87</td>
</tr>
<tr>
<td>OPP</td>
<td>2.36</td>
<td>80.53</td>
</tr>
<tr>
<td>KP0&lt;sup&gt;+&lt;/sup&gt;</td>
<td>N/D</td>
<td>82.20</td>
</tr>
<tr>
<td>SP0&lt;sup&gt;+&lt;/sup&gt;</td>
<td>N/D</td>
<td>87.41</td>
</tr>
<tr>
<td>OPP0</td>
<td>N/D</td>
<td>91.97</td>
</tr>
<tr>
<td>Avicel</td>
<td>N/D</td>
<td>100.00</td>
</tr>
<tr>
<td>NCC</td>
<td>N/D</td>
<td>100.00</td>
</tr>
</tbody>
</table>
recognized as a key substrate factor affecting enzyme hydrolysis efficiency, two pure cellulose substrates with higher crystallinity were also used. Microcrystalline cellulose (Avicel) has been commonly used as pure cellulose model substrate for evaluating enzymatic hydrolysis efficiency (Bansal, Hall et al. 2010; Hall, Bansal et al. 2010). Avicel is prepared by acid hydrolysis of pure cellulose until a leveling off DP is reached (Zhang and Lynd 2004). It has been shown Avicel particles contain multiple layers of cellulose crystallites interwoven with paracrystalline and/or amorphous regions (Krassig 1993). A nanocrystalline cellulose substrate was prepared from bleached poplar kraft pulp following the procedure described previously (Hamad and Hu 2010; Moon, Martini et al. 2011). Although the crystallinility index calculated by Segal method showed that Avicel and NCC have comparable CrI, (84% vs. 87%), the particle size of these two substrates was significantly different: 1 μm (Avicel) vs. 60 nm (NCC). Compared to Avicel, nanocrystalline cellulose more closely represents the dimension of an elemental cellulose crystallite which typically has a cross dimension of 5 nm.

6.4.2 Evaluation of commercial cellulase efficiency for releasing sugars by lignin-free reference substrates

The hydrolytic efficiencies of three commercial cellulase mixtures on lignin-free substrates KP0, SP0 and OPP0, were first evaluated. As shown in Figure 6.2, at both enzyme dosages of 4 mg/g and 20 mg/g, OPP0 exhibited the highest initial and final cellulose-to-glucose conversion yield among the three substrates by all three enzyme mixtures. After 72 hours, of the glucose yields from OPP0 hydrolysis by Ctec2, ACC1500 and Cytolase at the low enzyme dosage were 16, 14, 15 g/L, respectively; while at the higher enzyme dosage (20 mg/g), glucose concentrations reached to 22, 22, 21 g/L, respectively. Thus, the difference among the hydrolytic
efficiency of three enzyme mixtures on OPP0 is insignificant. The low DP, fiber length and crystallinity of OPP0 likely make it an ideal substrate for cellulase hydrolysis.

KP0 is the second hydrolysable substrate among the three to the enzyme mixtures. KP0 has a noticeably higher cellulose DP and CrI compared to OPP0, which are likely the factors contributing to the difference in the hydrolysability. It is interesting to observe a similar conversion rate of KP0 and OPP0 at higher Ctec2 dosage, while a distinctive gap was observed consistently between KP0 and OPP0 hydrolysis curves from both ACC1500 and Cytolase treatment at both enzyme dosages. This result suggests that Ctec2 is more effective in depolymerizing cellulose than ACC1500 and Cytolase.

Among the three substrates, SP0 exhibited the lowest hydrolysability by all enzyme treatments. Previous work has showed that higher xylan content in KP0 than in SP0 can facilitate cellulose fibril "swelling" which resulted in an improved hydrolysability by Ctec2 treatment (Ju, Grego et al. 2013). This trend was also observed during both ACC1500 and Cytolase hydrolysis. It should be mentioned, based on the results from OPP0, this “xylan effect” could be diminished when cellulose characteristics (such as DP and CrI) were significantly altered.

6.4.3 Effect of lignin type and low molecule weight phenolic compounds on commercial cellulase efficiency

Comparing the hydrolysis of lignin-free substrates with correspondent lignin-containing samples (i.e. KP0 vs KP40, SP0 vs SP40, OPP0 vs OPP) can provide a direct measurement of the effect of different types of lignin on the hydrolysability of each commercial enzyme mixture. KP40 and KP0 were applied to determine the effect of kraft lignin on enzyme hydrolysability. KP40 and KP0 have similar fiber length, width, coarseness, CrI and DP (Ju, Engelhard et al. 2013). The major difference between KP40 and KP0 is the lignin content: KP40 contains 5.9%
Figure 6.2. Hydrolysis profiles of KP0, SP0 and OPP0 by three commercial cellulase enzymes at different loadings: (A) Ctec2 at 4 mg/g protein; (B) ACC1500 at 4 mg/g protein; (C) Cytolase+ BG at 4 mg/g protein; (D) Ctec2 at 20 mg/g protein; (E) ACC1500 at 20 mg/g protein; and (F) Cytolase+ BG at 20 mg/g protein; Hydrolysis conditions: 2% solid content, 50 mM sodium acetate buffer pH 4.8, 50°C. Error bars indicate the standard deviation (n=3).
of bulk lignin with 45.5% of surface lignin coverage, while KP0 is a lignin-free substrate (Table 6.2). Cellulose-to-glucose hydrolysis profiles of KP40 and KP0 by three enzymes mixtures are shown in Figure 6.3. At the low enzyme loading (Figure 6.3A), the presence of lignin in KP40 led to a final cellulose-to-glucose conversion yield decrease by 3, 6 and 3 g/L compared to KP0 for Ctec2, ACC1500 and Cytolase, respectively. Kraft lignin is apparently a significant recalcitrance factor to all three enzyme mixtures. However, at the higher enzyme loading (Figure 6.3B), this adverse effect became alleviated after 72 hours hydrolysis. There was less than 1 g/L of glucose conversion difference between KP40 and KP0 hydrolyzed by Ctec2, while 2 g/L and 1 g/L for ACC1500 and Cytolase. The results suggested that, increasing enzyme loadings can minimize the hydrolysability difference between KP40 and KP0 during Ctec2, ACC1500 and Cytolase hydrolysis. Since enzyme adsorption on the surface of substrate is the first step for hydrolysis, it is likely the high surface lignin coverage on KP40 substrate (45.53%) can cause significant binding to the enzymes which reduce and deactivate their hydrolysability. Therefore, increasing enzyme dosages can reduce this adverse effect. The amount of protein adsorption on the substrates from the three cellulase preparations was determined. At the 4 mg/g protein loading, significantly higher ACC1500 enzyme adsorption on KP40 was detected, 50% more than those of Ctec2 and Cytolase. At the 20 mg/g loading, ACC1500 still showed a high adsorption, 25% more (Figure A6.1). The high substrate binding of ACC 1500 on KP40 did not lead to increased cellulose conversion. When enzyme adsorption was determined using KP0, all three enzymes showed comparable substrate affinity with less than10% protein adsorption difference among them was observed. These findings suggested that ACC1500 may have a higher binding affinity toward kraft lignin.
Figure 6.3. Hydrolysis profiles of KP0 and KP40 by three commercial cellulase enzymes at (a) 4 mg/ protein/mg substrate and (b) 20 mg protein/g substrate loadings. Hydrolysis conditions: 2% solid content, 50 mM sodium acetate buffer pH 4.8, 50°C. Error bars indicate the standard deviation (n=3).
The effect of lignosulfonates on the hydrolysability of three enzymes was also determined by comparing hydrolysis of SP0 and SP40. As seen in Figure 6.4A, at the lower enzyme loading, the gaps between SP40 and SP0 hydrolysis curves obtained from all three enzymes were smaller than those observed between KP40 and KP0. Unlike the trend observed in KP substrates, increasing enzyme dosages did not lead to closing these gaps (except for Ctec2). Instead, the gaps between SP40 and SP0 curves from ACC1500 and Cytolase hydrolysis appear to be widened (Figure 6.4B).

While the OPP had lower lignin content, a significant amount of low molecule phenolic compounds was present. This, in fact, provided a “different type of lignin” covering on substrate surface. The initial and final OPP and OPP0 hydrolysis rates by the three enzyme mixtures were shown in Figure 6.5. It is striking to find that the presence of a considerable amount of LMWPC contaminants in OPP did not have a significant impact on enzyme hydrolysability. Although at the lower enzyme loading (4 mg/g), Ctec2 exhibited a higher hydrolysis rate than ACC1500 and Cytolase, all three enzyme mixtures showed similar hydrolysability on both OPP and OPP0 at higher enzyme loading (20 mg/g) with a complete hydrolysis after 72 hours hydrolysis (Figure 6.5B).
Figure 6.4. Hydrolysis profiles of SP0 and SP40 by three commercial cellulase enzymes at (a) 4 mg/protein/mg substrate and (b) 20 mg protein/g substrate loadings. Hydrolysis conditions: 2% solid content, 50 mM sodium acetate buffer pH 4.8, 50°C. Error bars indicate the standard deviation (n=3).
Figure 6.5. (A) Initial rate and (B) final cellulose-to-glucose conversion yield for hydrolysis of OPP and OPP0 by three commercial cellulase preparations at low (4 mg protein/g substrate) and high (20 mg protein/g substrate) protein loadings. Hydrolysis conditions: 2% solid content, 50 mM sodium acetate buffer pH 4.8, 50°C. Errors bar indicate the standard deviation (n=3).

6.4.4 Evaluation of commercial cellulase efficiency by pure cellulose substrates

Investigating the cellulase efficiency on crystalline cellulose has been previously attempted using pure cellulose substrates, such as microcrystalline cellulose (e.g. Avicel, Sigmacell), bacterial or algae cellulose (Mansfield, Mooney et al. 1999; Chen, Stipanovic et al. 2007; Gupta and Lee 2009). None of these substrates can truly represent the characteristics of plant cellulose crystallites. Bacterial or algae cellulose contains predominantly cellulose Iα crystals which differs from the dominant cellulose Iβ in lignocellulosic biomass, while microcrystalline cellulose substrates such as Avicel have particles size several magnitudes higher
than that of elementary cellulose crystallites which is believed to be 5 nm (Moon, Martini et al. 2011). It has been a great challenge to use these substrates to elucidate and quantify the precise effect of cellulose crystallinity on enzyme hydrolysis (Zhang and Lynd 2004; Zhang, Himmel et al. 2006).

The NCC from bleached poplar pulp clearly showed rod-like dispersed cellulose nanofibrils with a length of around 200 nm determined by AFM imaging. The particle size distribution of NCC showed an average particle size around 60 nm. Highly crystallized nanostructure of NCC provides an ideal model substrate for evaluating cellulase activity toward cellulose decrystallization. Since NCC can form gel-like solutions above a concentration of 2%, Reactions of both Avicel and NCC were carried out at a consistency of 0.2%. As shown in Figure 6.6, a higher conversion rate was observed on Avicel than on NCC by all three enzyme mixtures, demonstrating NCC is more recalcitrant than Avicel. It deserves to mention that the total surface area of NCC is more than 10 times larger than that of Avicel based on BET analysis. This is not surprising given the smaller particle size of NCC compared to microcrystalline cellulose. Consistent with other substrates, Ctec2 hydrolyzed both Avicel and NCC more efficiently than ACC1500 and Cytolase. Increasing the enzyme dosage from 4 to 20 mg/g led to a significant increase in glucose release (from 1.6 to 2.5 folds after 72 hours) from Avicel by all enzymes. The same enzyme dosage increase had less impact on glucose release from NCC. Approximately 28% more glucose was released from NCC at 72 hour after increasing Ctec2 loading from 4 to 20 mg/g. Increasing both ACC1500 and Cytolase loadings has a much smaller effect on hydrolysis yield on NCC. It was observed that the Avicel hydrolyzed by different enzyme mixtures followed the typical hyperbolic hydrolysis kinetics which consists of a rapid rise phase followed by a slow-down retardation phase due to the
**Figure 6.6.** Hydrolysis profiles of Avicel and NCC by three commercial cellulase enzymes hydrolysis at both low and high protein loading: (A) Avicel at protein loading of 4 mg protein/g substrate; (B) Avicel at protein loading of 20 mg protein/g substrate; (C) NCC at protein loading of 4 mg protein/g substrate; (D) NCC at protein loading of 20 mg protein/g substrate. Hydrolysis conditions: 0.2% solid content, 50 mM sodium acetate buffer pH 4.8, 50°C. Errors bar indicate the standard deviation (n=3).
cellulose recalcitrance (Xiao, Zhang et al. 2004). However, such biphasic kinetics was not observed for NCC hydrolysis, which instead exhibited steady and almost linear conversion kinetics after a short initial relatively fast hydrolysis phase.

Samples of NCC after enzymatic hydrolysis by three commercial cellulase mixtures were collected and analyzed for surface changes by XPS. Figure 6.7 depicts the deconvoluted XPS carbon and oxygen high resolution spectra from original NCC and Ctec2 treated NCC. According to the survey scans, all sample surfaces consisted mainly of carbon and oxygen while hydrogen is not detectable by XPS. The difference in the CIs peak intensity between the spectra in Figure 6.7A and Figure 6.7B is attributed to an increase of oxidized carbon in the form of carbonyl groups, with possibilities such as aldehyde (RCHO), ketone (RCOR'), ester (RCOOR') or carboxylic acid (RCOOH). High resolution oxygen spectra confirmed the carbon oxidative

**Figure 6.7.** XPS surface characterization of original NCC and NCC treated by Ctec2 for 72 hr: (A) high-resolution carbon region for NCC; (B) high-resolution carbon region for NCC treated by Ctec2 after 72 hr; (C) high-resolution oxygen region for NCC; (D) high-resolution oxygen region for NCC treated by Ctec2 after 72 hr.

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observation (Figure 6.7C and D). Detailed peak fitting parameters regarding to the peak binding energy and peak assignments were listed in Table A6.1. The oxidation was only observed for Ctec2-treated NCC compared with ACC1500 and Cytolase treated NCC. The Ctec2 treated NCC surface exhibited heterogeneous characteristics, with selected sites observed as oxidized spots. It is suspected that such unique phenomena is caused by the novel group of enzyme component in Ctec2, namely, polysaccharide monooxygenases (PMOs), which is believed to decrystallize cellulose via an oxidoreductive mechanism (Beeson, Phillips et al. 2012; Horn, Vaaje-Kolstad et al. 2012). This is the first time that oxidation characteristics was observed from enzyme treated crystalline cellulose substrates.

6.5 Discussion

This is the first study to assess the efficacies of commercial cellulase preparations using biomass reference substrates with controlled substrate characteristics to identify the specific factor of enzyme deficiency. Overall, Ctec2 demonstrated a higher hydrolysability on all the substrates tested based on the same protein loading compared to ACC1500 and Cytolase. The similar hydrolysability obtained from Ctec2 hydrolysis of KP0 and OPP0 substrates suggests that the cellulose DP and CrI within the testing range did not present a major recalcitrance factor to Ctec2 enzyme. However, these substrates factors showed measurable effect on the efficacy of the ACC1500 and Cytolase. Comparing the enzyme hydrolysability on SP0 and KP0 determines the
effect of substrate swelling. All enzyme mixtures showed a preferential hydrolysis on KP0 to SP0, confirming substrate swelling is one of the key factors to enzyme efficiency. Enhancing enzyme activity toward biomass fiber swelling can lead potentially further improvement in cellulase performance. Devising pretreatment strategies to maximize biomass fiber swelling will also be important to improve substrate digestibility. Several putative enzymes or proteins, such as expansin, expansin-like proteins and swollenin, have been proposed to have specific substrate "swelling" activities (Mcqueenmason and Cosgrove 1994; Arantes and Saddler 2010). Expression or incorporation of these "swelling" components in the commercial enzyme mixtures may improve enzyme efficiency by increasing the access of cellulases to cellulose chains and promoting dispersion of cellulose aggregations (Saloheimo, Paloheimo et al. 2002; Kerff, Amoroso et al. 2008).

Our results continuously substantiate that lignin is a major recalcitrance factor to enzyme hydrolysis of lignocellulosic substrates. Lignin can act as a physical barrier to limit the access of the enzymes to cellulose as well as bind cellulase enzyme to reduce its activity, depending on its type and distribution. Cellulase enzymes have been postulated to adsorb on lignin via hydrophobic, ionic bond and hydrogen bonding interactions (Seiji, Richard et al. 2001; Berlin, Balakshin et al. 2006). It has also been recognized that cellulase enzymes produced from different microbial origins respond differently to the lignin inhibition effects due to the difference of protein hydrophobicity (Sewalt, Glasser et al. 1997; Berlin, Gilkes et al. 2005; Berlin, Gilkes et al. 2005; Berlin, Maximenko et al. 2007; Ximenes, Kim et al. 2011). Our results clearly demonstrated that different types of lignin had different levels of hindrance effects on enzyme mixtures.
As shown by the hydrolysability of KP substrates, surface lignin has a direct effect on reducing enzyme activity by non-productive binding. Ctec2 showed the strongest capacity among the three preparations to overcome this impediment, whereas ACC1500 appeared to have a higher affinity toward kraft lignin and thus more susceptible to lignin inhibition effects. The KP substrates provide a suitable set of reference substrates for screening weak-lignin binding enzymes. The effect SP lignin on enzyme hydrolysability appeared to be more complicated. Previous results showed that the amount of enzyme adoption on SP substrates does not correlate to lignin content (Ju, Engelhard et al. 2013). The smaller gaps between SP0 and SP40 hydrolysis curves at low protein loading also suggested that non-productive binding effect of the SP lignin is less significant. It was interesting to observe that the hydrolysability difference between SP0 and SP40 by ACC1500 and Cytolase seemed to become distinctive at the high enzyme loading. The exact reason for this observation is not clear. Unlike kraft lignin which may distributed predominant on fiber surface, a significant amount of SP lignin may remain with fiber structure presenting as physical hindrance to enzyme to access cellulose. Increasing cellulase enzyme did not help overcome this "physical barrier" whereas in lignin-free substrates, increasing enzyme loading lead to more significant cellulose conversion.

Despite a significantly higher LMWPC content in OPP compared to OPP0, the difference between the hydrolysability of these two substrates by all enzymes is insignificant. This result is intriguing as several previous studies have clearly demonstrated that phenolic compounds have a significant adverse effect on enzyme hydrolysability (Pan 2008; Ximenes, Kim et al. 2010; Tejirian and Xu 2011; Ximenes, Kim et al. 2011). It should be noted that the approaches employed in all these studies were based the addition of phenolic compounds to the enzyme mixtures. In the present study, the LMWPC was naturally presented in the substrate. It has been
pointed out in previous research that to affect high protein binding, phenolic compounds need to be small enough to penetrate inter-fibrillar regions of protein molecules, but large enough to crosslink peptide chains at more than one point (Ozdal, Capanoglu et al. 2013). The irreversible protein-phenolic binding is mainly caused by the oxidation of the diphenol moiety of a polyphenol which reacts with the side chains of protein polypeptides to form covalent bonds. It was also recognized that the conformational mobility and flexibility of phenolic compound molecules contributed greatly to the phenol-protein interaction (Le Bourvellec and Renard 2012). It is likely that LMWPC present in the substrates has different structural properties compared to free phenolic compounds. It is probable that LMWPC were linked with carbohydrates in the OPP substrate which could restrict their mobility and flexibility to bind with protein. Therefore, these LMWPC may no longer have a high affinity to protein. In this study, nanocrystalline cellulose was used as a new model substrate to determine cellulase hydrolysability toward crystalline cellulose. NCC has an average degree of polymerization of about 100 and a crystallinity of 87%, approaching the theoretical limit of the cellulose chains (Moon, Martini et al. 2011). When comparing the hydrolytic activities of commercial enzymes on Avicel and NCC, it is surprising to find that there is little enzyme “dosage effect” on ACC1500 and Cytolase hydrolysis of NCC: despite a 5-fold increase in enzyme loading (from 4 mg/g to 20 mg/g), the final sugar yield remained almost the same. This finding implies that NCC may possess a very small amount of "reactive surface" to cellulase enzymes. Therefore, at the low enzyme loading, the amount of enzyme is already in excess compared to the amount of reactive sites in NCC. The relatively fast initial glucose release of NCC is probably due to the impurity cellulose fragments attached on the cellulose surface resulting from the preparation process. NCC has a much large surface area (~10 times) than that of Avicel as measured by BET. Specific surface area or accessible surface
area has been frequently cited as a global substrate characteristic to correlate and predict substrate hydrolysability (Gregg and Saddler 1996; Wyman 1999; Zhang and Lynd 2004; Ju, Grego et al. 2013). Although useful in many realistic substrates such as pretreated biomass, it is clear from this study that this parameter is not universally applicable to all substrates. Results showed that Ctec2 hydrolysis of both NCC and Avicel fit into pseudo-first order reaction kinetics which is in agreement with previous studies (Drissen, Maas et al. 2007; Kumar and Murthy 2013). When hydrolysis is regarded as one-step reaction, the enzyme catalytic constants calculated for Avicel and NCC hydrolyzed by Ctec2 was 0.2 and 0.05, respectively. Although enzyme catalytic constant is related to the synergy of complex cellulase system with consideration of enzyme adsorption and dissociation (Levine, Fox et al. 2011; Fox, Levine et al. 2012), when specific enzyme is evaluated, such reaction constants can be used as representation of the substrate reactivity. Avicel exhibited a 4-times higher reactivity than NCC. This confirms that for crystalline cellulose such as Avicel and NCC, cellulose reactivity becomes the major factor for hydrolysis. It is likely NCC is a more suitable substrate to specific evaluate enzyme activity to cellulose decrystallization.

Our recent study demonstrated using NCC and BKP to evaluate the effects of *alg3* gene deletion (*alg3Δ*) on recombinant *Trichoderma reesei* cellobiohydrolase (rCel7A) expressed in *Aspergillus niger* (Dai, Aryal et al. 2013). The extent of hydrolysis of BKP and NCC by *T. reesei* Cel7A and rCel7A was evaluated (Figure A6.2). BKP digestibility was similar between *T. reesei* Cel7A and rCel7As. In contrast, rCel7A variants from all three strains exhibited relatively higher NCC activity over *T. reesei* Cel7A. The improvement of rCel7A on highly crystallized NCC hydrolysis will be the subject of further studies. Enzyme assays of Cel7A and rCel7A on
nanocrystalline cellulose and bleached kraft pulp demonstrated that the rCeI7As have improved activities on hydrolyzing the nanocrystalline cellulose.

It is very interesting to observe that the slope of second phase for Ctec2 is much steeper than ACC1500 and Cytolase, indicating a faster hydrolysis rate for Ctec2. More interestingly, increasing Ctec2 dosage appears to lead to enhancing glucose release from NCC. Elucidating the precise mechanism is beyond the scope of this study. A number of recent studies have demonstrated that Ctec2 contains polysaccharides monooxygenases which can boost the hydrolytic enzyme activities during lignocellulose hydrolysis (Harris, Welner et al. 2010; Beeson, Phillips et al. 2012; Horn, Vaaje-Kolstad et al. 2012; Zifcakova and Baldrian 2012). The higher hydrolysability of Ctec2 toward NCC hydrolysis suggested Ctec2 may possess unique activity toward cellulose decrystallization. The surface chemistry changes after enzyme treatment of NCC was investigated by XPS analysis. An apparent increase in carbonyl group on the surface Ctec2 treated NCC was detected. This result seems to suggest oxidation reaction may be involved in cellulose decrystallization. A detail understanding to this aspect is under current investigation.

6.6 Conclusions

In summary, a set of reference substrates with individual structural features that hinders enzyme hydrolysability is available to evaluate commercial cellulase deficiency toward pretreated lignocellulosic biomass. Cellulase hydrolytic activities toward LMWPC, lignin non-productive binding, lignin structural hindrance and crystalline cellulose reactivity have been evaluated individually. It appears that the lignin hindrance and cellulose reactivity are the major recalcitrance toward efficient hydrolysis. Further studies are necessary to develop weak-lignin
binding enzymes through genetic engineering and high throughput screening. Understanding the decrystallization process of crystalline cellulose is a critical step developing more efficient bioconversion process. Therefore we propose that employment of reference substrates as the sole carbon source during mutant screening could be a powerful tool selecting higher and specific cellulase hydrolytic activity toward realistic pretreated lignocellulosic substrates.
CHAPTER SEVEN: CONCLUSIONS AND FUTURE WORK

7.1 Conclusions

The overall goal of the work described in this thesis was to investigate and understand biomass recalcitrance from fiber, fibril and molecular levels. In this work, pulping methodology was applied to create a set of biomass substrates, “reference substrates” which can systematically represent the major key biomass characteristics at the fiber, fiber topographic, fibril and molecular levels. The working hypothesis was that (through a combination of modified chemical and mechanical pulping process to create reference substrates), applying these “reference substrates” to examine individual substrate characteristics and enzyme interactions will greatly enhance an aggregated understanding of “biomass recalcitrance”. At the same time we assessed alternative strategies to characterize individual substrate characteristics from systematic levels, such as assessing the substrate-related properties that influenced the pretreated substrate’s susceptibility to enzymatic hydrolysis. We anticipated that, by developing innovative approaches to a comprehensive understanding of biomass recalcitrance, it will allow the implementation of sustainable bioenergy and bioproduct processes at an industrial scale.

The preliminary work (Chapter Two) clarifies the effect of fiber size and related surface area on enzymatic hydrolysis at the fiber level. The reference substrates with controlled morphological and chemical properties allow the investigation of the individual effects of fiber size and surface areas on enzymatic hydrolysis. Fiber size reduction leads to a change in substrate external surface area. However the impact of this change on the hydrolysability improvement is limited, especially for substrates with good digestibility. It is demonstrated that the change of substrate external surface area resulted from particle size reduction had
insignificant effect on enzymatic digestibility. PFI refining can effectively swell fiber cell wall structures, resulting in the increases of both external and internal surface areas as well as substrate reactivity. The efficacy of substrate swelling on enzymatic digestibility is also influenced by substrate chemical properties. Fiber swelling can improve surface internal surface area as well as substrate reactivity. The information from this study helps clarify the diverged observations to the effects of substrate particle size and porosity changes on enzyme digestibility.

Subsequent work (Chapter Three) investigated the possible effect of xylan and surface lignin content on enzymatic hydrolysis at biomass fibril level. A high resolution X-ray photoelectron spectroscopy technique was established for quantifying surface lignin content on lignocellullosic biomass substrates. The results from this study show that, apart from its hindrance effect, xylan can facilitate cellulose fibril swelling and thus create more accessible surface area, which improves enzyme and substrate interactions. These results provide new insight to the effect of biomass xylan on cellulose digestibility: beside its physical barriers to hinder enzyme access to cellulose, xylan has positive effect on enhancing fiber swelling. Surface lignin has a direct impact on enzyme adsorption kinetics and hydrolysis rate. The set of reference substrates here provided direct experimental evidence to demonstrate the effects of surface lignin on enzyme adsorption kinetics and hydrolysis rate. Advanced understanding of xylan and surface lignin effects provides critical information for developing more effective biomass conversion process.

Cellulose crystallinity (CrI) is one of the most important factors contributing to biomass recalcitrance. However, a reliable measurement of CrI especially during enzyme treatment is lacking. Chapter Four described the development of X-ray diffraction analysis for accurate determination of crystallinity and cellulose crystalline structures. This technique is cited to
further understand biomass recalcitrance at a molecular level. The results showed a more accurate and applicable determination of the crystallinity index (CrI) and crystallite structures for cellulose Iβ by refined X-ray diffraction method. Rietveld refinement was applied with consideration of March-Dollase preferred orientation at (001) plane. It has been shown that a refined Rietveld XRD analysis can better determine the crystallinity index of pure crystalline cellulose by subtracting experimentally determined amorphous background, including preferred orientation as well as extending the measuring 2θ angle to 75°. Although such refinement cannot distinguish between the paracrystalline structure and amorphous regions, the true CrI can accurately be measured and the procedure will be universally applicable for different cellulose polymorphs in most XRD laboratories as long as the crystallography information is available. When using the developed refinement to extract information regarding to crystallite structures, nanocrystalline cellulose provide a better model compound to differentiate the paracrystalline layers on the surface. Such information provides a better judgment regarding determination of the structure of crystalline cellulose. Such analysis provides future possibilities of studying the true crystallinity indices as well as crystalline structural changes during various biological and chemical treatment.

With the refined XRD method and nanocrystalline cellulose model compound, a better understanding for mechanism of cellulose decrystallization at molecular level was described in Chapter Five. It is demonstrated that NCC served as an ideal model for crystalline structure study by a refined X-ray diffraction analysis during enzyme treatment. More emphasis has been focused on the solid cellulose fraction left after hydrolysis rather than solubilized products. Structural analysis from nanometer scale favors more toward the hypothesis of “chain-disrupting” factor like GH61, which can possible work on the flat and compact hydrophobic
surface and introduce breaking chain ends by oxidative mechanism. No evidence was observed showing any swelling factors during different enzyme treatments of the process. This suggests that expansin/swollenin-like proteins which facilitate the decrystallization process by weakening the hydrogen bonds is a major contribution to the enzyme degradation of crystalline structure. Confirmation of such decrystallization action introduced by oxidative enzymes renders a new perspective regarding to further biomass pretreatment development as well as effective enzyme system design, which will lead toward a more efficient and environmental-friendly energy production.

Based on the systematic understanding of biomass recalcitrance affecting enzymatic hydrolysis, a set of reference substrates with individual structural features that hinders enzyme hydrolysability is available to evaluate commercial cellulase deficiency toward pretreated lignocellulosic biomass in Chapter Six. Cellulase hydrolytic activities toward low molecular phenolic compounds, lignin non-productive binding, lignin structural hindrance and crystalline cellulose reactivity have been evaluated individually. It appears that the lignin hindrance and cellulose reactivity are the major recalcitrance toward efficient hydrolysis. Further studies are necessary to develop weak-lignin binding enzymes through genetic engineering and high throughput screening. Understanding the decrystallization process of crystalline cellulose is a critical step developing more efficient lignocellulose bioconversion process. Applying reference substrates for enzyme screening presents powerful tool to discover and identify highly efficient and cost effective cellulases for plant cellulose degradation.

The work described in this thesis provides advanced understanding of biomass recalcitrance and the interactions between cellulases and individual biomass recalcitrance factors. Pretreatment targeting specific individual characteristics, such as lignin distribution,
fiber swelling and cellulose decrystallization, are among the preferable routes for further improvement. Utilizing reference substrates for future enzyme screening is a powerful tool to reduce enzyme production cost by enhancing enzyme efficiency. It is confirmed that crystalline cellulose swelling is not the major mechanism during decomposition. Decrystallization of cellulose requires specific enzyme components that deconstruct recalcitrant crystalline cellulose via oxidoreductive mechanism. Identifying possible accessory enzymes with redox activity is the prominent directions lowering enzyme cost for biofuel production.

7.2 Recommendations for future studies

As mentioned in section 7.1, the work described in the thesis addressed some of the key aspects governing biomass recalcitrance effect on enzymatic hydrolysis. However, several questions remain unanswered from both a fundamental and an applied perspective. The following suggested work might help guide future research.

7.2.1 Identifying lignin-protein interaction by using model lignin compound

The research outcomes will advance the knowledge needed to understand the effects of specific substrate characteristics on the effectiveness of enzymatic hydrolysis of lignocellulose. However, the use of these fibrous “reference substrates” may not explicitly illustrate biomass recalcitrant effects generated at elementary fibril and molecular structural of cell wall components, such as lignin-carbohydrate linkages. The formation of lignin carbohydrate complex (LCC) has been an intriguing topic in pulping chemistry. It has been demonstrated that formation of hexenuronic acid (HexA) on xylan facilitates the bonding between hemicelluloses and lignin to generate recalcitrant LCC structure in pulp fibers (Jiang, Bouchard et al. 2006). Therefore, it is conceivable that the synthesis of a hexenuronic acid, xylan and lignin substrate
(HaXL) will provide a model compound which resembles the realistic LCC complex formed during alkaline chemical pretreatment of biomass. A scheme to produce hexenuronic acid and xylan complex representing LCC structure has been described previously (Jiang, Van Lierop et al. 2000). This model compound can be further used to investigate the efficacy of two cellulase enzymes for breaking LCC linkages.

7.2.2 Distinguish the oxidation mechanisms of cellulose decrystallization by individual enzyme component

Based on preliminary results, further focus is emphasized on the oxidation mechanism by delineating the oxygen and electron donor effect on oxidative enzyme GH61 involved in the decrystallization process by Re-doxy reaction. NCC structural changes by interacting with single component of hydrolytic cellulase, and the prevailing “disrupting factor” expansin enzyme and GH61 oxidative enzyme will be investigated and monitored during the time course of decrystallization. The Small Angle X-ray scattering (SAXS) measurement will be carried out on the same samples as Wide Angel X-ray diffraction Scattering (WAXS) to examine the fraction of ramified cellulose after different single enzyme treatment. Refining NMR analysis will also help to identify the transition of crystalline cellulose to paracrystalline cellulose during decrystallization process. In this step, attempt will be made to acquire a final clarification whether crystalline cellulose proceeds with oxidization mechanism by disruption or amorphogenesis by swelling prior to hydrolysis.

7.2.3 Visualization of enzyme and nanocrystalline cellulose polymorphs interaction

Enzymatic hydrolysis of lignocellulosic biomass starts with the adsorption of protein component on the surfaces of crystalline cellulose. The research at this stage will focus on elucidating the enzyme-substrate binding mechanism and how decrystallization proceed on
single crystal cellulose once the enzyme is bounded on the surface. Different crystalline cellulose polymorphs (cellulose Iβ, cellulose II, and cellulose III) will be produced from nanocrystalline cellulose representing different pretreatment effect on crystalline cellulose. The nanoscale visualization of surface morphology and crystalline structure changes with all these nanocrystalline polymorphs during different time point of decrystallizing NCC will be characterized by Helium Ion Microscopy. X-ray Photoelectron Spectroscopy will be applied for quantification of protein adsorption on the surface of NCC. Protein adsorption kinetics and decrystallization reaction kinetics will also be incorporated to establish a molecular explanation of crystalline cellulose decomposition.

7.2.4 Kinetics and reactivity study of single enzyme interaction with model cellulose substrates

NCC has been proved to be an ideal material for kinetic study since it can form homogeneous solution. It has been demonstrated that NCC exhibited specific recalcitrant reactivity during enzymatic hydrolysis, which is highly suspected to be caused by its ordered crystalline structure. The reactivity of cellulose is suspected to play a major role for the slowdown of hydrolysis kinetics. In our previous research, commercial cellulose enzymes were applied to study the reactivity of crystalline cellulose degradations. However, difficulties arose when using such mixture enzyme system, since the synergistic kinetics cannot be easily quantified. It is hypothesized that by using single-enzyme component such as CBHI, EGII, as well as GH61, etc., the reactivity of degradation process will be further quantified. Further studies needs to be carried out comparing NCC with model cellulose substrates such as Avicel and BKP hydrolyzing by single enzyme component, to compare their reactivity effect on
cellulose hydrolysis kinetics. Further understandings of the reactivity and kinetics regarding to
decrystallization process will be obtained from this set of study.
SUPPLEMENTARY ENTRIES

A.2 Chapter four supplemental entries

Characterization of NCC particle sizes by AFM and particle size analyzer.

Figure A4.1. Obtained NCC size characterization: (A) AFM surface imaging shows that NCC dispersion particles showed a general particle size of 100-200 nm in length and 5-20 nm in width; (B) particle size distribution of NCC showed an average of particle size at 60 nm.
Determination of the successful preparation of NCC.

**Figure A4.2.** Indication of successful preparation of nanocrystalline cellulose from bleached kraft pulp: (A) FTIR spectra (B) XPS spectra of NCC.
Figure A4.3. Illustration to show $d$-spacing and glucan chain organization in a unit cellulose crystallite $\text{I}_\beta$. 
A.3 Chapter six supplemental entries

Figure A6.1. Commercial cellulase proteins (Ctec2, ACC1500 and Cytolase + BG) adsorbed on the surface of substrates at enzyme loadings of 4 and 20 mg protein/g substrate: (A) KP40 and (B) KP0.
Table A6. 1. X-ray photoelectron spectroscopy high energy resolution C\textit{Is} and O\textit{Is} spectra peak fittings for Original NCC and Ctec2 treated NCC. Peak assignments were based on references mentioned in “Methods”.

<table>
<thead>
<tr>
<th>Assignment</th>
<th>NCC original</th>
<th>NCC Ctec2 treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Binding energy (eV)</td>
<td>Full width at half maximum (FWHM)</td>
</tr>
<tr>
<td>Carbon</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-C(H)</td>
<td>284.6</td>
<td>1.3</td>
</tr>
<tr>
<td>C-O</td>
<td>286.3</td>
<td>1.2</td>
</tr>
<tr>
<td>O-C(1)-O</td>
<td>287.6</td>
<td>1.6</td>
</tr>
<tr>
<td>C=O-O-C</td>
<td>289.3</td>
<td>1.8</td>
</tr>
<tr>
<td>C=O-NH</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Oxygen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(C=O*)-O-C(H)</td>
<td>531.9</td>
<td>1.4</td>
</tr>
<tr>
<td>C-O*H</td>
<td>532.6</td>
<td>1.4</td>
</tr>
<tr>
<td>C=O)-O*-C(H)</td>
<td>533.4</td>
<td>1.4</td>
</tr>
<tr>
<td>O*-N</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>O*-N</td>
<td>N/A</td>
<td>N/A</td>
</tr>
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</table>
Figure A6.2. Extent of hydrolysis on bleached kraft pulp (BKP) and nanocrystalline cellulose (NCC) by *T. reesei* Cel7A and recombinant Cel7A (rCel7A) isolated from wild-type (11414-rCel7A), parent (11414kusA-rCel7A), and *alg3Δ* (*alg3Δ*-rCel7A) strains of *A. niger*. Panel (A) is the average glucose (g/L) released from BKP or NCC. BG is Novozyme 188 (β-glucosidase). The hydrolysis was done in triplicates. Panel (B) is the cellulose crystalline structure analyzed by x-ray diffraction.
REFERENCES


Arantes, V. and J. N. Saddler "Cellulose accessibility limits the effectiveness of minimum cellulase loading on the efficient hydrolysis of pretreated lignocellulosic substrates." Biotechnol Biofuels 4: 3.


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