ABSTRACT


The lignocellulosic biorefinery concept provides an attractive alternative to energy, fuels and chemical production from petroleum-derived and other non-renewable resources. However, the realization of this technology is limited by the economic climate and the technical challenges of maximizing the biorefinery production yield.

This dissertation is an investigation of utilizing targeted Engineered Biomass Deconstruction (EBD), or mechanical refining, to overcome the inherent recalcitrance of the lignocellulosic biomass. This recalcitrant nature is often considered the limiting factor for the commercialization of cellulosic biorefineries – including second generation cellulosic ethanol production facilities – which increases the direct costs for the process inputs of the deconstruction steps. This includes requirements of high temperature and chemical charges during pretreatment and high enzyme dosages during enzymatic hydrolysis unit operations.

First, the effects of mechanical refining on the digestibility lignocellulosic biomass is explored at the laboratory scale. Comparisons of two common laboratory scale refiners, PFI mill and valley beater, confirm improvements in enzymatic hydrolysis with increased mechanical refining severity for all biomass pretreatments; including, kraft (NaOH, Na₂S), green liquor (Na₂CO₃, Na₂S), and sodium carbonate (Na₂CO₃) pretreatments. A maximum in refining improvement is observed, highlighting the ability of EBD to generate the most value for the lignocellulosic biorefinery at moderate pretreatment severities and hydrolysis conditions.
Second, Engineered Biomass Deconstruction is compared at lab, pilot and industrial scales. Using the same industrially sourced sodium carbonate pretreated biomass, similar enzymatic hydrolysis kinetics and their respective improvements with mechanical refining were observed for all mechanical refining scales, with the most similar kinetics being between commercial scale and pilot scale refining. Successful simulation of industrial scale refining allows the use of pilot scale refining for optimization of Engineered Biomass Deconstruction at the pilot scale.

Third, utilizing the same commercial sodium carbonate biomass, the pilot scale mechanical refining conditions were optimized. Close to theoretical maximums in enzymatic hydrolysis conversion were achieved using pilot scale EBD compared to the total carbohydrate conversion of 39% for unrefined hardwood sodium carbonate biomass. Mechanical refining conditions of temperature, plate gap width, and consistency were controlled to optimize the Engineered Biomass Deconstruction process. Optimum conditions for the pilot refiner were found to be to 0.13 mm plate gap width, and 20% biomass consistency, at ambient temperature, which produced a total carbohydrate conversion of 90%.

Following the optimization of EBD conditions, efforts were made to fundamentally understand the reason for the improvement in biomass digestibility with mechanical refining. The motivation of this understanding would facilitate the development and application of engineered biomass deconstruction technologies within the lignocellulosic biorefinery concept. Non-hydrolytic fluorescent recombinant protein probes with carbohydrate binding modules of similar size to commercially available cellulases were used a model for the enzyme adsorption process for the initial stages of enzymatic hydrolysis. Model substrates...
were used to confirm the selective binding of the fluorescent protein probes to cellulose. Confocal laser scanning microscopy allowed for visualization and quantitative imaging of the fluorescent markers within the lignocellulosic biomass matrix. Relationships between the maximum fluorescent intensities and the different lignocellulosic biomass were observed. The distribution of adsorbed enzymes in the cell wall were altered by the mechanical refining actions of external fibrillation, internal delamination, and cutting. This indicates that improved biomass accessibility to enzymes throughout the lignocellulosic biomass matrix is related to enhanced enzymatic hydrolysis.

This work highlights the effectiveness of Engineered Biomass Deconstruction and its benefits when applied within the lignocellulosic biorefinery concept. Future research should be targeted for further optimization of mechanical refiner operating conditions including specific development of new refiner plate designs for application in a lignocellulosic biorefinery.
Engineered Biomass Deconstruction: A Multidisciplinary Investigation Towards Understanding Mechanical Refining and its Applications in Lignocellulosic Biorefineries

by
Brandon Wesley Jones

A dissertation submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Forest Biomaterials

Raleigh, North Carolina

2017

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DEDICATION

This dissertation is dedicated to my beautiful wife, Katherine, for her inexhaustible patience and her unconditional love. I am forever grateful and indebted to her for the sacrifices she has made for me.

Thank you for always understanding, always encouraging, and always believing in me.

I love you. I will always love you.

Second, I dedicate this dissertation to time. Enjoy every moment.

“It is never too late or too soon. It is when it is supposed to be.”

— Mitch Albom, The Time Keeper
BIOGRAPHY

Brandon Wesley Jones was born on April 27, 1988 in Greensboro, North Carolina, USA. He is the first-born son of Brian Wesley and Lisa Gerringer Jones. After living briefly in Mebane, North Carolina, the family moved to Burlington, North Carolina where Brandon and his younger brother, William “Mitchell”, would be raised. He was actively involved in Boy Scouts of America, Troop 17, earning his Eagle Scout Award, the highest level in scouting. Brandon graduated from Walter M Williams High School in 2006, lettering in both Soccer and Tennis.

In the Fall of 2006, he enrolled at North Carolina State University at Raleigh, where he studied Chemical and Biomolecular Engineering and Paper Science and Engineering. He was a proud recipient of the Pulp & Paper Foundation Scholarship. His first introduction to bioenergy and the biorefinery concept was a special topics course in Chemical Engineering about the “Engineering Challenges on the Energy Frontier” taught by Professor Wesley Henderson. Brandon spent the Summer of 2009 as an Undergraduate Research Assistant in the Henderson ILEET (Ionic Liquids & Electrolytes for Energy Storage) Laboratory, at NCSU with Xinglian Geng researching a combined ionic liquid dissolution with alkali extraction biomass pretreatment technology.

The following year, Summer 2010, he interned in the Biomass Applications and Discovery group at Novozymes North America in Franklinton, North Carolina. His passion for research in the biomass, biofuels, and biorefinery fields had by then been solidified. He
graduated in December 2010 with dual Bachelor of Science degrees in CHE and PSE, with a concentration in Green Chemistry.

Brandon began his graduate level studies the following semester, Spring 2011, at North Carolina State University in the College of Natural Resources, Department of Forest Biomaterials under co-advisors Dr. Richard Venditti and Dr. Sunkyu Park. He was granted a National Needs Fellowship, awarded by the United States Department of Agriculture, National Institute of Food and Agriculture. The Grant (2010-38420-21828), titled a “Multidisciplinary Doctoral Education Program in Lignocellulosic Biofuels Science and Engineering” was the first and primary sponsor for his Doctor of Philosophy studies. While focusing his research on Engineered Biomass Deconstruction (mechanical refining) within the biorefinery concept, he also pursued a Graduate Minor in Biomanufacturing at the Golden LEAF Biomanufacturing Training and Education Center (BTEC) to gain fundamental background in all biorefinery process operations.

Brandon met his wife, Katherine Thomas Jones, in Raleigh during his first year in graduate school, and were married October 10, 2015. He also began his career in the private sector working full time as a Quality Control Chemist at Novozymes, and moved into his first house in Raleigh with his wife – all within two weeks of his wedding. After completing his degree, Brandon is looking forward to continue working in the bioeconomy and related fields and excited to see the new opportunities and experiences he can share with his family and friends.
ACKNOWLEDGMENTS

Philippians 4:13 “I can do all this through Him who gives me strength.”

There are so many other people I need to thank for all the guidance and love and support shown to me throughout my graduate career. It has been a long time coming, but I couldn’t have made it without the help of this loving team around me. I have always felt that I am not my own accomplishments, but the product of my surroundings. I feel so blessed to be surrounded by such wonderful people that care about me and push me every day to become the best man, son, brother, husband, and friend I can be. Thank you all!

I’ll do my best, but it is impossible to thank all the influential people that have helped me along my journey. In no particular order, I would like to thank these following people for their support during my graduate career at North Carolina State University:

My Advisors and my Committee – Dr. Richard Venditti, Dr. Sunkyu Park, Dr. Hasan Jameel, and Dr. John Sheppard – You have always believed in me and afforded me every opportunity to succeed within this graduate program. This experience is one that I will cherish forever and I am honored to have spent it with you all. Thank you for your mentorship, for teaching me the art of the scientific method, for challenging me, for pushing me to failure then helping me to learn and grow. You have built me into the scholar, scientist, and citizen that I am today. Thank you.

I could have never been able to succeed in this work without the guidance from some great Biomanufacturing professors and staff, Dr. Gisele Gurgel, Dr. Jennifer Pancorbo, Michael Ray, Chris Smith and Dr. Driss Elhanafi. Thank you for always taking the time to
help me plan and execute my graduate minor and my protein production and purification. I always enjoyed my time spent with you in BTEC.

I had the pleasure to work with Dr. Eva Johannes and benefit from the use the equipment at the Cellular and Molecular Imaging Facility (CMIF). This state of the art microscopy lab provided invaluable quantitative data and visualizations for my work, but more importantly I am thankful for the friendly discussions and time shared with Eva.

I would also like to thank the Forest Biomaterials Professors and Staff that built a wonderful academic atmosphere for learning and comradery. To the entire Biomass Research Lab, and my Graduate Student Colleagues including Dr. Bonwook Koo, Dr. Qiang Han, Dr. Hui Chen, Dr. Jiajia Meng, Dr. Trevor Treasure, Dr. Emmy Yu, Dr. Jun Park, Dr. Grant Culbertson, thank you for the shared experience and the comradery. Every day in graduate school was better because of you. My Office Mates, Seunghyun Yoo, Lu Liu, and Ved Naithani – thank you for being more than just co-habitants, but great comrades.

A very special thanks to Andrew Moore – we met in 2007 and we’ve done everything together ever since then: PSE, CHE, FB grad school, ultimate, and more. Thanks for being my colleague, my teammate and my friend for the past 10 years. I don’t know what I would have done without you.

The present and future Graduate Students who carry on the tradition of excellence in Forest Biomaterials; best of luck to you all in the future. Thank you to North Carolina State University, The Graduate School and The Raleigh Community for just being a great place to be.
I would be remiss to not acknowledge the many funding sources that I was extremely proud to serve. USDA, NIFA, NNF Grant 2010-38420-21828, thank you for challenging me to diversify my graduate experience and become a multidisciplinary scholar. Thank you to the consortiums WERC (Wood-to-Ethanol Research Consortium, 15 members) and BtB2 (Biomass to Biochemicals and Biomaterials, 8 members), and especially to the industry members. It is inspiring to see the passion you bring to providing valuable solutions to the bio-based economy and I hope to be an integral part of these solutions in the future.

My Friends, who I can’t even begin to name all of them, thank you for always supporting me and encouraging me along the way. I’m sorry for all the trips and outings and hangouts that were put on hold. I’ll spend a lifetime making it up to you, and I’m looking forward to it.

Mom and Dad – you have always supported me in everything I do, thank you for giving me every opportunity I could ask for in life. I may have never pursued a graduate career if it wasn’t for the encouragement from my little brother, Mitchell. Our overlapping years at NC State we’re some of the best times of my life. Thank you.

My Grandparents, Hulon and Frances Gerringer and Helen and Goble Jones, for being the example of what lifetime of hard work and dedication to your family and your passions looks like. I am proud to be your grandson and I hope that I make you proud too.

Last and most certainly not least, I am forever thankful for my wife Katherine. You have loved me and supported me from the very beginning of my journey through graduate school. You are my everything. Thank you for being there for me.
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1 CHAPTER ONE: INTRODUCTION

1.1 Biorefinery Concept Overview

In 2008, the National Academy of Engineering introduced 14 Grand Challenges for Engineering in the 21st Century that face the future sustainability, health, security and joy of the world (National Academy of Engineering 2017). Overcoming these barriers provide the driving force for advancing civilization, and each challenge is met with its own social, environmental and economic implications. Many of these grand engineering challenges include the responsible use of the world’s resources to meet the energy demands of the ever-growing global population.

The biorefinery concept has been identified as a process that has the potential to meet some of these energy challenges by utilizing renewable biomass resources to produce bio-based fuels, chemicals, materials and power which would otherwise be produced from non-renewable petroleum refineries and other resources (Cherubini 2010).

1.1.1 Benefits

There are many benefits associated with the second-generation biorefinery process. Compared to a first-generation biorefinery which uses that starch from corn to produce a fuel ethanol, a lignocellulosic biorefinery can utilize non-food feedstocks or wastes from other processes, i.e. corn stover, forest residues, etc. to produce a variety of valuable fuels, chemicals and other products. Life cycle assessments of first- and second-generation biorefineries suggest that net greenhouse gas emissions (GHG, gCO₂-eq/MJ) can be significantly reduced compared to the baseline of gasoline, with the biggest improvements
expected from cellulosic feedstocks that require minimal fossil energy during growth and harvesting (Wang et al. 2012)

Biorefineries allow for the diversification of fuel and chemical production, by bringing the feedstock and end user closer together creating favorable energy security and flexibility. Ethanol and other lignocellulosic biorefinery fuel products can be integrated into the current transportation fuel sector supply chain as refineries, distribution pipelines, blending stations, fuel transit, and end-use vehicles are all already equipped to handle liquid fuels like bioethanol. Also, the byproducts of a biorefinery can be upgraded to platform bio-based chemicals and polymers that can serve as renewable alternatives to petroleum-based products, and provides additional benefits for the lignocellulosic biorefinery. The value-added byproducts include nano-cellulose and cellulose derivative based applications (CMC), lignin-based binders and adhesives, and carbohydrate-based chemical solvents, lubricants, and polymers (Jong et al. 2012).

1.1.2 Challenges

Current challenges facing the lignocellulosic biorefinery include various political climates and regulatory constraints and industry uncertainties not conducive to risk mitigation or facilitation new capital investment. The demand for biofuels in the US has been mandated by the EPA declaring the Renewable Fuels Standard (RFS2) production volumes to be met each year from 2009 to 2022 under the Energy Independence and Security Act (EISA) of 2007 (US Environmental Protection Agency 2017). Under the RFS2 mandates, the EPA has capped the production of conventional (starch-based) ethanol at 15 billion gallons,
and target 36 billion gallons of renewable fuel, with 16 billion from cellulosic biomass sources, Figure 1-1.

Figure 1-1. RFS2 mandated biofuel production volumes from 2005-2020.

Compounding the risks and uncertainties of the future fuel sector, there are also technical risks associated with the biorefinery. Unproven commercial applications of bio-based alternatives to chemicals and fuels, potential high delivered biomass feedstock costs, and few demonstrations of process yield at scale all offer some economic uncertainty related to biorefinery production costs and realized biorefinery product value.
Biomass recalcitrance, or the natural resistance of biomass to be broken down through biological, chemical and thermomechanical processes, remains one of the greatest challenges for widespread biorefinery commercialization. However, overcoming biomass recalcitrance, and increasing the biorefinery process yields provides the greatest opportunity to reduce the market barriers to entry and facilitate industrial lignocellulosic biorefinery applications.

1.1.3 Biomass Feedstocks

Biomass feedstocks include any wood, agricultural residue, or municipal waste resource derived from organic matter to produce energy through combustion or other chemical processes (US Department of Energy 2017a). Lignocellulosic biomass is a sub-category of biomass feedstocks that predominantly contain cellulose, hemicellulose and lignin. This plant material is composed of approximately 40-50% cellulose, 20-30% hemicellulose, and 18-28% lignin (US Department of Energy 2017b). Cellulose is the main structural component for the rigidity of plant cell walls and is a β-1-4 glycosidic bound linear polymer of D-glucose. Hemicellulose is a branched heteropolymer of glucose, galactose, mannose, arabinose, xylose, and other minor carbohydrates, most commonly xylans and glucomannans in hardwood and galactoglucomannans in softwoods, for cross-linking between polysaccharides and other components of the cell wall. Lignin is a type of complex network polymers of phenylpropanoids p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units heterogeneously bound together to provide reinforcement within plant cell walls. Figure 1-2 shows a diagram of the lignocellulosic biomass construction (Isikgor et al. 2015).
The Billion-Ton Study performed by the United States Department of Energy, evaluated the technical feasibility of the agricultural and forestry industries to supply the bioproducts industry with enough biomass resources to displace 30% of the 2005 US petroleum consumption (Perlack et al. 2005). This study was updated in 2011 with the economic availability for 20 year projections of 23 candidate biomass feedstocks, and again in 2016 with interactive assessments of national biomass availability to help drive bioenergy
policies, research and commercial deployment an also assess the environmental sustainability impacts of biomass supply scenarios (Perlack et al. 2011; US Department of Energy 2016a).

1.2 Motivation

The largest barrier to market entry for the lignocellulosic biorefinery is the challenge to generate low cost carbohydrates which can be upgraded or processed into direct replacement platform chemicals or suitable alternative bioproducts. Mechanical refining, a technology that can be used for focused engineered biomass deconstruction (EBD), has shown the potential to increase the overall biomass to carbohydrate conversion efficiency and overcome the natural lignocellulosic biomass recalcitrance, or resistance to biological deconstruction. Optimizing biomass digestibility to generate value-added products from lignocellulosic biomass is critical to meeting the biofuel mandates in the US bioeconomy and meeting the populace’s needs in an environmentally and economically sustainable way with bioproducts from the lignocellulosic biorefinery.
1.3 Mechanical Refining

1.3.1 Types of Mechanical Refiners

It is important to define which type of mechanical action that is being applied to the biomass. When applied in the dry state before pulping (pretreatment), it is called dry milling and is commonly used for mechanical size reduction. If the mechanical action is applied in the wet state after pulping (pretreatment), it is called wet milling and used for fiber development. Mechanical pulping combines mechanical size reduction and fiber development into one step. These three main categories of mechanical action – dry milling, wet milling, and mechanical pulping – are described below.

1.3.1.1 Dry milling

After harvest and delivery to a pulp and paper production facility the biomass must be prepared for use within a pulp and paper mill or a biorefinery. Typical the dry milling end of the wood handling process includes a debarking drum that rotates and rolls logs against the walls of the drum and against each other to strip the bark away from the rest of the round wood. Then the debarked logs are either chipped or milled in smaller sizes and the screened to remove undersize pieces and reprocess oversize pieces. This allows for easier process flow and biomass handling. There are many different types of chippers – drum chipper, disk chipper – and biomass mills – hammer mill, ball mill, knife mill, Wiley mill. Each of these dry milling units accomplish mechanical size reduction in a slightly different way. It should be noted that no dry milling optimization was considered in this work; however, dry milling
is an important consideration for the operation of any pulp and paper mill or lignocellulosic biorefinery.

1.3.1.2 Wet milling

The second group of mechanical refining encompasses all refining action that occurs in the wet state. The current major manufacturers for high and low consistency refiners as well as their refiner plates are Andritz and Valmet. The most common type of industrial mechanical refining is disk refining. A single disk refiner involves the biomass passing through the center of a single stationary plate (stator) while a single rotating plate (rotor) spins to impart the mechanical refining actions on the biomass while it moves through the refining zine by centrifugal force. A similar double disk design has two stators on the outside of a double-sided floating rotor, Figure 1-3 (Aikawa Fiber Technologies 2001).
Figure 1-3. Center section orthographic side view of a generic double disk mechanical refiner. Biomass inlet and outlet occurs at large black arrows at the left and right, respectively.

The biomass is fed into the refining zone and travels into the refining zone on either side of the stator then exits the refiner. Both single and double disk refining can be pressurized or operated at atmospheric pressure. In papermaking, high consistency refining can sometimes be referred to as “blow-line” or “hot stock” refining, as this is the location in the pulp mill where a high consistency disk refiner is used to disintegrate fiber bundles. Low consistency refining similarly is referred to as “paper machine” refining, as this is where the fiber properties for papermaking are developed.
In addition to the most common disk-type refiners, there are other refiner types used in various lab and other niche applications. These include conical refiners, valley beaters (or Hollander beater), PFI mills, and Szego mills among others. All mechanical refining research and discussions herein refer to wet milling mechanical refining after the biomass has been pretreated.

1.3.1.3 Mechanical pulping

In the pulp and paper industry, mechanical pulping combines whole log and chip handling can be with pulping or pretreatment into a single unit operation to prepare biomass for papermaking. Stoneground Wood (SGW) and Pressurized Stoneground Wood (PGW) separate whole logs into individual fibers and fiber bundles by applying the logs against a rotation coarse stone. Refiner Mechanical Pulp (RMP), Thermomechanical Pulp (TMP), Advanced Thermomechanical Pulp, and Chemi-Thermomechanical Pulp (CTMP) all utilize biomass chips and a type of disk refiner to administer the pulping chemicals and temperature if necessary, break up fiber bundles. Some TMP refiner plates have tapered zones such that increased refining action can be achieved as the biomass moves out radially from the center of the disk refiner. This mechanical pulping type of mechanical treatment, along with other mechanical processes like steam explosion, or ammonia fiber explosion (AFEX), etc. could be very useful to the biorefinery industry, but is not included within the scope of this dissertation.
1.3.2 *Mechanisms of the Mechanical Refining Action*

During mechanical refining, there is a combination of adsorption and desorption forces, compression and decompression forces, tangential surface shear forces, and normal tension forces applied by the mechanical refiner to the biomass fiber. These actions cause several different biomass fiber changes. To adequately describe the mechanical refining actions, it is important to understand the different parts of a lignocellulosic wood fiber. A macroscopic diagram of the major components are shown in Figure 1-4 (Hubbe 2017).

![Figure 1-4](https://example.com/image.png)

*Figure 1-4. Schematic of the lignocellulosic biomass fiber cell wall sublayers. Diagram shows the Middle Lamella, P=primary cell wall, S=secondary cell wall with layers 1, 2, and 3, and the Lumen.*

Mechanical refining facilitates suspension homogeneity by separating bundles of biomass fibers, or shives, into individual fibers at the middle lamella. Once individual fibers are released into suspension, forces around the circumference of the cell wall cause peeling
of the primary and secondary (S1) cell wall layers of the biomass fiber. This external fibrillation can cause these cell wall layers to become fibrous structures that can extend away from the center axis of the biomass fiber. The larger middle secondary cell wall layer (S2) is where internal compression forces cause internal delamination of the orderly packed biomass structure. Internal delamination of this layer allows the fiber to swell. Additionally, fiber shortening can occur from axial tensile force fiber failure or can be caused by radial fiber cutting. Lastly, mechanical forces exerted on the fiber during mechanical refining causes the lumen to collapse and the fiber to become flat and more flexible and ribbon-like.

The three major mechanical refining mechanisms discussed within this dissertation are external fibrillation, internal delamination, and fiber cutting. All mechanical refiners cause some varying degree of these actions on the biomass fiber structure. One single instance of the step-wise mechanical refining action imparted by a refiner bar to a biomass fiber as the rotor bar passes a stator bar is shown in Figure 1-5 (Lumiainen 1998).
1.3.3 Variables that impact the Mechanical Refining Operation

When optimizing mechanical refining there are 3 major areas that can impact the mechanical refining performance and should be considered; 1) refiner filling, 2) process conditions, and 3) biomass characteristics (Lumiainen 1998).

Refiner filling encompasses of the variables that are directly related to the internal equipment including the refiner filling material or the refiner plate and its specific design of bar height, width, angle, groove dam height for flow restriction. This also includes the material of the refining equipment. These refiner fillings are usually more difficult to change,
but can have a significant impact on the refining performance when optimized for the specific refining application.

Some, but not all the following process conditions can be controlled in every refiner type to vary the mechanical refining performance: motor load (power, kW), rotational speed (rpm), temperature (°C) by indirect steam, dilution rate by direct steam or water application (lpm), pressure (psi), and plate gap width (mm, distance between the refining surfaces). Other process conditions include the flow rate (ton/hour) or production rate of refined biomass and the biomass recirculation rate (%). More details about specific refiner operation conditions are described in the methods of each chapter.

Additionally, the biomass characteristics can be changed to impact the refining performance. The biomass consistency or ratio of oven-dry biomass to the total biomass and water suspension can be adjusted to change the relative fiber-to-bar and fiber-to-fiber impacts. The consistency, along with the biomass viscosity which is related to the degree of polymerization of cellulose in the fiber impact the pumpability and the residence time of the biomass within the refining zone. Changing the biomass chemistry during pulping or pretreatment, and using additives like enzymes will also impact the efficiency and operation of the mechanical refiner. A summary of all the factors affecting the refining result are summarized nicely in Figure 1-6 (Lumiainen 1998).
1.3.4 Energy Calculations in Mechanical Refining

In general, mechanical refining action is caused by refiner bar impacts with the biomass fiber. Since the specific energy used during refining cannot be directly measured, multiple models have been developed to describe the mechanical refining action of biomass suspensions. The two major factors include specific refining energy (SRE) and refining intensity. Most theories characterize the refining intensity by relating the specific refining energy to the number of impacts and the energy of each impact.

The most common parameter for measuring and comparing overall mechanical refining energy is the specific (net) refining energy consumption as measured by a totalizer.
energy meter during mechanical refining. The ISO naming standard for the specific (net) refining energy consumption is SRE, but is equivalent to other abbreviations NSE, and SEC (International Organization for Standardization 2013). This is a machine parameter measurement, and is the difference between the total load power ($P_{tot}$, kW) required during mechanical refining, and the no-load power ($P_0$, kW) which is required to run the equipment without any biomass. Ideally, the no-load power considers the friction and pumping energy requirements of the biomass suspension without applying any mechanical refining action. In practice, the no-load power can be measured for low consistency refining by measuring the power requirement of pumping water through the refiner at the same flow rate with an open gap. For high consistency refining, the no-load power the energy is often simply the energy required to operate the refiner and defined machine speeds. When comparing different refiner SRE it is important to understand the no-load requirements and differences between each type of refiner and refining condition. The net refining power ($P_{net}$, kW) is a representation of the energy transferred to the biomass to cause the physical changes during refining, and is divided the oven-dry biomass flow rate ($F$, oven-dry tons/hour) to normalize to the net specific refining energy input (kilowatt-hours/oven-dry ton).

Equation 1-1. Specific Refining Energy

$$SRE = P_{tot} - P_0 / F$$

Another important refining measurement is the intensity. The most widely adopted theory for describing the mechanical refining intensity is the specific edge load (SEL) theory.
The SEL assumes that all refining energy is transferred from the refiner filling bar edges to the biomass. The SEL theory is most useful when comparing the exact same biomass and mechanical refiner when optimizing conditions for refined biomass characteristics. The SEL is the energy per bar length per bar crossing. To calculate the SEL, the refiner filling properties or the refiner plate dimensions must be known. The cutting edge length (CEL, sometimes called the bar edge length, BEL) is a property of the refiner plate and is equal to the total length of bar edges for both the rotor and stator plate per one revolution (km/rev). The simplest calculation for cutting edge length is the number of stator bars multiplied by the number of rotor bars, multiplied by the bar length. This becomes more complex at the refiner plate bars have different lengths and are set at varying angles to the radius of rotation in multiple different refining zones, Figure 1-7 (Olson 2013).

Equation 1-2. Refiner Plate Cutting Edge Length.

$$CEL = Z_{st} \times Z_{r} \times l$$

$Z_{st}$= number of stator bars in a specific zone

$Z_{r}$= number of rotor bars in a specific zone

$l$= bar length in a specific zone
These refining energy measurements provide good standardizations for comparing and optimizing different types, scales, and conditions of mechanical refining applications. Additional calculations to more accurately quantify the mechanical refining action have been described by Kerekes, et al. (C-factor, etc.) which consider not only the specific energy, but also the refiner geometry, rotational speed, plate gap width, biomass consistency, and other fiber properties including length and coarseness. These measurement models are limited in their ability to characterize refining actions at diverse operation conditions, as they have mostly been applied to low consistency wood pulp mechanical refining.

1.3.5 Mechanical Refining in the Biorefinery Concept

It is not a new concept to utilize mechanical refining energy to prepare the lignocellulosic biomass substrate for enzymatic hydrolysis within the biorefinery concept. Mechanical refining has been shown for many different biomass types and pretreatments to
be an effective method to improve total process carbohydrate yields. Extensive studies for optimizing the dilute acid pretreatment of corn stover combined with mechanical refining and deacetylation significantly improve the enzymatic hydrolysis yield (Chen et al. 2014). Mechanical refining has been effective for increased digestibility or reduced enzyme consumption of alkaline pretreated hardwood and softwood (Koo et al. 2011). Hydrolysis of other biomass, like autohydrolysis wheat straw, can be improved with mechanical refining (Ertas et al. 2014).

However, major gaps in understanding the applications for mechanical refining still exist. Although refining is a well-known unit operation within the pulp and industry, this mechanical refining technology has not been specifically redesigned for use within a biorefinery which has many different objectives than a traditional forest products mill. Most research to date has been performed at lab scale and it is critical for risk mitigation that the effectiveness of the mechanical refining technology is demonstrated at commercial scales, ideally within an operating lignocellulosic biorefinery. It is still unclear which refining mechanisms have the most desirable mechanical refining for improved biomass digestibility. Gaining an understanding in this area would lead to more effective optimization of the mechanical refining process conditions and plate designs for maximum engineered biomass deconstruction in a lignocellulosic biorefinery.

There is a strategic advantage for the pulp and paper industry to leverage expertise in supply chain management to facilitate growth of the lignocellulosic biorefinery. Pulp and paper mills are already physically located near available biomass, and geographically
favorable locations for alternative biorefinery product consumers outside the corn belt. There are also many transferable unit operation technologies that are useful in the lignocellulosic biorefinery concept including biomass handling, pulping (pretreatment), mechanical refining, chemical recovery or power generation, and waste water treatment, among others. Co-location of an integrated biorefinery is also possible by adding pretreatment to a conventional (starch-based) ethanol facility or by utilizing pretreatment and boiler/recovery at a pulp and paper mill while adding enzyme hydrolysis and distillation.
Figure 1-8. Comparison of the current state for paper mills, ethanol plants, and their respective feedstock locations. Geographic Information Systems (GIS) data collected from various sources for a) corn production (bu/acre) (US Department of Energy 2016b), b) ethanol plant locations, colored by feedstock and sized by capacity (Mgy) (Renewable Fuels Association 2017), c) hardwood and softwood harvest (ton/acre) (US Department of Energy 2016b), and d) pulp and paper mill locations (RISI 2014).
1.4 References


2 CHAPTER TWO: RESEARCH OBJECTIVES

The motivation of this research is to use a multi-disciplinary approach to gain insight and understanding about engineered biomass deconstruction so that the knowledge can be applied to the facilitation and advancement of mechanical refining technology within a lignocellulosic biorefinery concept.

The first objective is to develop knowledge around the differences in mechanical refining technologies and how different equipment and refiner designs impact enzymatic hydrolysis efficiencies differently.

Second, is to demonstrate the effectiveness of mechanical refining at multiple scales and then optimize the mechanical refining conditions at the pilot scale for targeted engineered biomass deconstruction.

The final objective is to combine the knowledge gained with the many mechanical refining applications with bioprocessing tools to illuminate new understanding about mechanical refining and its use in a lignocellulosic biorefinery.
3  CHAPTER THREE: ENHANCEMENT IN ENZYMATIC HYDROLYSIS BY LAB SCALE MECHANICAL REFINING FOR PRETREATED HARDWOOD LIGNOCELLULOSICS

3.1  Abstract

This study investigated the effectiveness of mechanical refining to overcome the biomass recalcitrance barrier. Laboratory scale refining was conducted via PFI mill and valley beater refiners using green liquor and Kraft hardwood pulps. A strong positive correlation was determined between sugar recovery and water retention value. Refining produced significant improvements in enzymatic hydrolysis yield relative to unrefined substrates (e.g. sugar recovery increase from 67% to 90%, for 15% lignin Kraft pulp). A maximum absolute enzymatic hydrolysis improvement with refining was observed at enzymatic hydrolysis conditions that produced intermediate conversion levels. For a 91% target sugar conversion, PFI refining at 4,000 revolutions allowed for a 32% reduction in enzyme charge for 15% lignin content hardwood Kraft pulp and 96 hour hydrolysis time, compared to the unrefined material.
3.2 Introduction

The natural recalcitrance of plant biomass feedstock to resist the breakdown of its fibers to structural carbohydrates by enzymatic hydrolysis is a major issue in the commercialization of cellulosic biofuels (Zhao et al. 2012). These natural recalcitrant properties include the waxy outer skin of the plant body, dense packing of the fiber bundles, thick fiber wall, degree of lignification, and structural complexity of the cell wall (Himmel et al. 2007). These issues, combined with the difficulty for soluble cellulase enzyme proteins to act on insoluble cellulose, make the processing of lignocellulosic biomass very difficult and reduces the subsequent enzymatic hydrolysis efficiency. It has been recognized that a pretreatment operation is critical for increasing the lignocellulosic biomass enzymatic digestibility by opening up the biomass structure to increase the cellulose accessibility to enzymes (Zheng et al. 2009; Zhao et al. 2012). Unfortunately, severe pretreatment conditions of high temperature and chemical charge are needed to achieve high sugar conversions, which generate lower pretreatment yields and higher fermentation inhibiting compound concentrations. It has been determined that the overall saccharification efficiency is governed by three basic processes: 1) substrate accessibility of cellulose—component removal and size reduction, 2) substrate and cellulase reactivity—limited by inhibitors, and 3) reaction conditions—optimizing and controlling pH, temp, etc. (Leu et al. 2012).

The combination of chemical pretreatment followed by a mechanical refining post-treatment operation provides an opportunity to generate improved fiber characteristics for enzyme digestibility while attempting to reduce the pretreatment severity and inhibitory
compounds generated. It is widely known that the high temperature and pressures experienced during pretreatment can generate carbohydrate- and lignin-derived degradation products, “inhibitors” (Klinke et al. 2004). More severe pretreatment conditions generate higher concentrations of these compounds; such as acetic acid, furfural, and phenolic compounds; that can reduce the performance of enzymatic hydrolysis and fermentation. Mechanical refining has been used in the pulp and paper industry to separate chemically pulped fibers after pulping and also to improve papermaking properties (Smook 1982). Papermaking properties are improved with mechanical refining by externally fibrillating the exterior wall of the fiber and by delaminating the interior cell wall of the fiber to increase swelling and flexibility. In some cases, refining offers some cutting of longer fibers to provide a more uniform sheet formation (Annergren et al. 2009).

It is expected that the shearing action in mechanical refining will allow for more internal delamination and surface fibrillation, which would disrupt the crystalline structure of the cellulose micro-fibrils, increase the accessible specific area and allow for a more efficient biomass digestibility. In addition to an expected increase in enzymatic hydrolysis yield, refining may also have the potential to allow for a less severe pretreatment. This reduction in severity can reduce the operation costs associated with pretreatment, as well as reduce the amount of by-products formed that would inhibit fermentation efficiency.

The three dominant refining mechanisms of i) external fibrillation – creating fibrils on fiber surface; ii) internal delamination – fiber swelling and loosening of internal structures; and iii) fiber cutting – fiber shortening due to shearing action, can be observed to different
extents depending on the refining apparatus and conditions (Kerekes 2005). Understanding which of these mechanisms drives the optimum scenario for enzyme hydrolysis improvement, in addition to key target fiber properties changed by refining, will allow for the most effective use of refining as a post-treatment in biofuels production.

Mechanical refining has been shown to have a positive effect on the enzymatic digestibility of dilute acid pretreated corn stover (PCS) (X. Chen et al. 2012; Tao et al. 2012), green liquor (GL) pretreated hardwood (Koo et al. 2011) and loblolly pine (Wu et al. 2012), sulfite pretreated softwood (Zhu et al. 2010), and recovered office printing paper (H. Chen et al. 2012). It has also been shown that micro- and nano-scale mechanical refining of hardwood (Endo 2010) and softwood (Hoeger et al. 2013) fibers that have not been thermo-chemically pretreated are especially effective in lignocellulose cell wall breakdown in order to improve the substrate enzymatic digestibility.

The PCS study exhibited increased glucan and xylan conversions after a laboratory scale refining process (PFI mill with 4,000 revolutions), increasing the glucose yield to over 90% compared to the unrefined control at 70% (X. Chen et al. 2012). PFI refining (8,000 revolutions) after green liquor pretreatment of hardwood allowed about a 50% decrease in enzyme charge at the same hydrolysis conversion of a non-refined sample, highlighting the ability of refining to reduce the required enzyme charge (Koo et al. 2011). In the same study, the refining showed an increase in sugar conversion of GL hardwood from 51% to 72% at the same enzyme dosage. Softwood has been found to be very recalcitrant to enzymatic hydrolysis, but PFI refining at 9,000 revolutions increased the sugar conversion from 41% to
around 55% - 60% and was further increased to 78% when mechanical refining was used in combination with oxygen delignification (Wu et al. 2012). The sulfite pretreatment on softwood combined with disk milling proved to have cellulose saccharification efficiencies of over 90% in 48 hours at a cellulase loading of 15 FPU/g-substrate, an increase from 77% without refining (Zhu et al. 2010). Recovered office printing paper showed an average percent increase of sugar conversion with 2-8 FPU/g-substrate of approximately 10% with PFI refining at 5,000 revolutions (H. Chen et al. 2012). In that study, the combination of ash removal from the recovered paper and refining resulted in enzyme conversions of 97%. All of these reports indicate the effectiveness of mechanical refining for improving the biomass enzymatic hydrolysis sugar conversion. However, each of these studies has different refining processes and none of them compare two or more refining methods within a single study.

The objective of this study is to determine if the post-treatment of mechanical refining improves the enzymatic hydrolysis sugar conversion efficiency in green liquor and Kraft pretreated hardwood pulps. This was accomplished by mechanically refining pulps using two types of refining laboratory equipment at different refining severities and subsequently hydrolyzing the pulps with enzymes. The results showed improvements in enzymatic hydrolysis due to refining that were a maximum at moderate enzymatic hydrolysis conditions of time and enzyme dosage. It was also observed that the different refining actions achieved by using different refining apparatuses, made a difference for the enzymatic hydrolysis improvement.
3.3 **Experimental**

3.3.1 **Hardwood biomass characterization**

All pulps were made using a mixture of various species of southern hardwood chips. Compositional analyses of the hardwood chips and the various pulps were measured using a modified version of the NREL standard procedure (Sluiter et al. 2008). A quantity of 0.1g OD powder of wood (40 mesh) was hydrolyzed with 72% H$_2$SO$_4$ for 2 hours. The hydrolyzed biomass was diluted to 3% H$_2$SO$_4$ and autoclaved at 120°C for 1.5 hrs. The hydrolysate was filtered and oven-dried to determine the insoluble solids, and the filtrate was collected for the determination of acid soluble lignin (UV-Vis (Lambda XLS, Perkin Elmer, Waltham, MA, USA) at 205 nm and 110 Abs coefficient) and carbohydrate content analysis using HPLC (filtered with 0.2 μm syringe filter).

3.3.2 **Biomass pretreatment procedure**

The green liquor pretreatment of hardwood chips (GL) was carried out in a 7-liter M&K Batch Digester (MK Systems, Inc., Peabody, MA) with 800 OD grams of chips (Jin et al. 2010). The chips were cooked with green liquor (Na$_2$CO$_3$ and Na$_2$S) at a fixed alkali charge of 16% total titratable alkali (TTA) as Na$_2$O to the target H Factor of 800 at a maximum temperature of 160°C. The liquor to wood ratio was 4:1 and the sulfidity (based on TTA) was 25%. This pulp was used for the increasing PFI and valley beater refining severity experiments.

Kraft pulping is commonly used in the pulp and paper industry and is known for its efficient delignification of wood chips to make high quality pulps for papermaking.
Changing the cooking strength by varying the H-factor (400, 600, 700, 1000), and white liquor (NaOH and Na₂S) chemical charge (10, 12, 13, 15 %TTA) at the same temperature (160°C), liquor to wood ratio (4:1), and sulfidity (25%) generated a spectrum of similar Kraft pulps with different lignin contents. These pulps will be used to understand how lignin content changes the effect of mechanical refining on enzymatic hydrolysis.

After pulping, the chips were washed thoroughly with tap water, centrifuged (Fletcher, Sharples Corp, Philadelphia, PA, USA), and the yield of insoluble solids was determined by total weight and the moisture content. The chips were then disintegrated using a disk refiner (148-2, Bauer, Springfield, OH, USA) at 0.13 mm (0.005 inch) gap and then screened with a laboratory flat screen (Custom, NCSU, Raleigh, NC, USA) using a 0.20 mm (0.008 inch) screen plate. The rejects were refined with a disk gap of 0.03 mm (0.001 inch) and then added back to the accepts from the first screen. The pulp was then centrifuged and fluffed for further processing. Detailed pulping conditions and the resulting biomass compositions of the hardwood GL pulp and the hardwood Kraft pulps are shown in Table 3-1.
Table 3-1. Hardwood (HW) chip and pulp composition analyses. Glc is the glucan content; Xyl is the xylan content; arabinose, mannose, and all other minor sugars were below the detectable limit. K. lignin is the acid insoluble residue measured by the standard Klason lignin analysis, and ASL is the resulting acid soluble lignin. The Kappa numbers of the hardwood Kraft pulps were determined using the TAPPI standard test method, and are indicated after “K#” in the sample description. All values expressed as percent of total oven dry biomass. Calculated error is the standard deviation of replicates indicated by “±” and shown below the mean value for each component.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Carbohydrate</th>
<th>Lignin</th>
<th>Ash</th>
<th>Balance</th>
<th>Pulping Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glc</td>
<td>Xyl</td>
<td>Sum</td>
<td>K. lignin</td>
<td>ASL</td>
</tr>
<tr>
<td>HW chip</td>
<td>44.4</td>
<td>18</td>
<td>62.4</td>
<td>23.6</td>
<td>3.3</td>
</tr>
<tr>
<td>Hardwood GL</td>
<td>55.3</td>
<td>13.7</td>
<td>69.0</td>
<td>17.8</td>
<td>2.5</td>
</tr>
<tr>
<td>±1.0</td>
<td>±0.7</td>
<td>±0.3</td>
<td>±1</td>
<td>±1.6</td>
<td>±0.1</td>
</tr>
<tr>
<td>HW Kraft-K#112</td>
<td>58.7</td>
<td>14.0</td>
<td>72.7</td>
<td>18.3</td>
<td>2.5</td>
</tr>
<tr>
<td>±1.0</td>
<td>±0.3</td>
<td>±0.6</td>
<td>±0.9</td>
<td>±0.4</td>
<td>±0.04</td>
</tr>
<tr>
<td>HW Kraft- K#70.7</td>
<td>63.5</td>
<td>15.2</td>
<td>78.7</td>
<td>13.1</td>
<td>2.0</td>
</tr>
<tr>
<td>±1.0</td>
<td>±0.4</td>
<td>±1.4</td>
<td>±0.3</td>
<td>±0.03</td>
<td>±0.33</td>
</tr>
<tr>
<td>HW Kraft- K#46.9</td>
<td>67.9</td>
<td>16.3</td>
<td>84.2</td>
<td>9.3</td>
<td>1.7</td>
</tr>
<tr>
<td>±1.0</td>
<td>±0.5</td>
<td>±0.1</td>
<td>±0.6</td>
<td>±0.2</td>
<td>±0.04</td>
</tr>
<tr>
<td>HW Kraft- K#23.3</td>
<td>72.2</td>
<td>17.6</td>
<td>89.8</td>
<td>3.6</td>
<td>1.5</td>
</tr>
<tr>
<td>±1.0</td>
<td>±0.3</td>
<td>±1.3</td>
<td>±1.2</td>
<td>±0.04</td>
<td>±1.24</td>
</tr>
</tbody>
</table>

Extractives were determined only for the unrefined hardwood GL biomass to be 3.4±1.2% using an 8 hour benzene: ethanol (2:1) solvent soxhlet extraction (Sluiter et al. 2005). The Kappa numbers of the hardwood Kraft pulps were determined using the TAPPI standard test method, and are indicated after “K#” in the sample description (TAPPI 1993).

3.3.3 PFI refining

PFI refining (or milling) is a batch refining procedure where pulp is held against a bedplate using centrifugal force and the spinning refiner rotor is forced against the bedplate imparting a mechanical shearing action on the fibers (TAPPI 2008). A quantity of 30 g OD
pulp was refined in a PFI mill (Norwegian Pulp and Paper Institute (PFI), Oslo, Norway) at 10% consistency at selected refining intensities between 2,000 and 10,000 revolutions, in increments of 2,000 revolutions. After refining, pulp was collected and stored in the refrigerator without washing. This procedure was the same for both hardwood GL and Kraft pulps. The PFI refining energy was estimated as 0.18 kWh/ton-rev (Kerekes 2005).

3.3.4 Valley beating refining

Valley beating is a batch refining process in which low consistency pulp is looped around a well and forced between a rotor bar and loaded bedplate to generate mechanical shearing action (TAPPI 2001). Hardwood GL pulp was loaded into the valley beater (Valley Iron Works, Appleton, WI, USA) and refined at 1% consistency with a 10 kg load for times selected between 15 and 90 minutes, at 15 minute intervals. Samples were collected and filtered using Whatman No. 1 (Grade 1, Whatman, Maidstone, UK) filter paper as to avoid the loss of fines, then transferred into a sealable container and stored in a refrigerator for later analysis. The valley beater refining energy in kWh/kg was estimated using drainage versus energy data on bleached eucalyptus (Atic et al. 2005). This data was combined with experimental results of drainage and WRV determined herein to produce an empirical relationship for the beater refining energy in kWh/kg as equal to 1.132 × ln(WRV [g/g]) – 0.9.

3.3.5 Enzymatic Hydrolysis

The conversion of cellulose and hemicelluloses into monomer sugars was performed using 1, 3, and 5 FPU/ OD g of substrate Novozymes Cellic® CTec2 cellulase enzyme and
1/9 v/v Cellic® HTec2 hemicellulase enzyme (Novozymes NA, Franklinton, NC, USA). The activity of the CTec2 cellulase was determined to be 139 FPU/mL according to a standard method (Ghose 1987). The activity of the HTec2 hemicellulase was not known, and was charged based on supplier recommendation. One FPU (Filter Paper Unit) is defined as the amount of enzyme that releases 1 μmol of glucose equivalents from Whatman No. 1 filter paper per minute. Experiments were conducted on a 1 OD g basis in a 50 mL conical tube and a 20 mL working volume (5% w/v consistency). A 1 mM sodium acetate buffer solution (pH 4.8) with 0.1% sodium azide antibiotic was used for pH and microbial control. The rotating hybridization incubator (FinePCR COMBI-D24, Seoul, Korea) was controlled to a temperature of 50°C and 12 rpm. Samples were collected using 0.5 mL aliquots from the well–mixed hydrolysis at four different residence times (12, 24, 48, and 96 hours after inoculation). The solids were separated via micro centrifugation for 10 minutes at 13,000 rpm, and then the supernatant was diluted with deionized water to 5x and filtered at 0.2 μm into a HPLC vial for sugar recovery analysis.

### 3.3.6 Sugar Analysis

Sugar analysis was carried out following a modified NREL Laboratory Analytical Procedure (Sluiter et al. 2008), via ligand-exchange chromatography (SP0810, Shodex, Kawasaki, Japan) on the high-performance liquid chromatography (HPLC) system (Agilent 1200, Agilent, Santa Clara, CA, USA) with an Agilent guard column, degasser, pump, and refractive index detector (RID). HPLC grade milliQ water was used as the mobile phase. Aliquots of Klason lignin filtrate and enzyme hydrolysate were used to determine the
carbohydrate content for hardwood pulps and enzymatic hydrolysates, respectively. Samples were analyzed with a 20μL injection volume and 0.5 mL/min flow rate at a column temperature of 80°C. The sugars detected were glucose and xylose, and solutions with mixtures of known sugar concentrations were measured to generate a calibration curve for each sugar. The minor sugar concentrations of galactose, arabinose, and mannose were below the detectable limit and therefore not reported. The digestibility is reported as sugar conversion:

\[
\text{sugar conversion(\%)} = \frac{\text{total sugar released(g)}}{\text{carbohydrate content after pretreatment(g)}} \times 100\%
\]

Equation 3-1. Determination of biomass digestibility.

3.3.7 Fiber properties

Water retention value (WRV) is defined as the water absorbed by the substrate relative to the oven-dried mass of the substrate (TAPPI 2011). For measurement of the WRV a quantity of approximately 0.30 OD g of sample was placed into centrifuge filtering tube (Amicon Centriplus, Millipore Corp., Bedford, MA, USA) with a glass frit (25mm, med porosity borosilicate fritted disc, Chemglass, Vineland, NJ, USA) and centrifuged for 30 min at 0.9 relative centrifugal force (\(\text{rcf}=\text{rw}^2/\text{g}\)), or the acceleration (radius times the square of the angular velocity) relative to gravity. The recorded weights of the wet centrifuged sample \((m_1)\) and the oven-dried at 105°C sample \((m_2)\) were used to calculate the water retention value (WRV). The basis weight of the final pads was approximately 700 g/m².

\[
\text{WRV (g/g)} = \frac{(m_1 - m_2)}{m_2}
\]

The HiRes Fiber Quality Analyzer (FQA) from OpTest Equipment, Inc. (Hawkesbury, ON, Canada) was used to measure fiber length and percent fines (1999). Well-mixed dilute fiber suspensions (~1mg/L) of the pulp samples were prepared. The fiber length is the true contoured length of the fiber and is reported as the length weighted value average. Fines are defined as any particle less than 0.2 mm in size and are reported as a percentage of the total number of particles counted.

3.4 Results and Discussion

3.4.1 Effect of refining on fibers

Initial experiments were intended to explore the effects of different laboratory scale refining equipment on fiber properties. As the refining severity of the PFI mill and valley beater were increased – more PFI mill revolutions and longer valley beater retention times, respectively – significant trends in fiber properties were observed, Figure 3-1. There was no significant change in the percent fines or the mean length of the pulp refined by the PFI mill; however, the valley beater refined pulps drastically increased the amount of fines in the pulp and decreased the fiber length with increased valley beater processing time.
Figure 3-1. Fiber Quality Analyzer PFI and Valley Beater biomass properties versus Refining Severity Index. FQA results for percent fines (hollow) and mean length (filled) are plotted versus the arbitrary Refining Severity Index (RSI; x2000 PFI revolutions; x15 minutes valley beating residence time) for unrefined (circle), PFI 2-10K refined (square), and VB 15-90min (triangle) hardwood GL biomass.

There was also a linear increase in water retention value as the refining severity increased for both refiner types as shown in Figure 3-2a, which indicates that at least some mechanism of refining is occurring at a linear rate in each of these apparatuses for the time studied. These fiber property results indicate that the PFI mill mechanism is dominated by internal fibrillation or swelling, whereas the valley beater exhibits much more fiber cutting, comparatively. These mechanical refining actions have been confirmed previously for the PFI mill (Garcia et al. 2002) and the valley beater (Park et al. 2006a).
Figure 3-2. Water Retention Value of biomass versus Refining Severity Index and Lignin Content. A) (top) Correlation of WRV increase with increased refining severity for unrefined (circle), PFI 2-10K refined (square), VB 15-90min (triangle) hardwood GL biomass with respect to the arbitrary index (RSI; x2000 PFI revolutions; x15 minutes valley beating residence time). B) (bottom) Correlation of WRV decrease with increased lignin content hardwood Kraft biomass for unrefined (circle) and PFI 4K refined (square) pulps.

Graph a:
- $y = 0.462x + 2.656$
- $R^2 = 0.980$
- $y = 0.257x + 2.634$
- $R^2 = 0.938$

Graph b:
- $y = -0.416x + 4.053$
- $R^2 = 0.054$
- $y = -1.174x + 3.377$
- $R^2 = 0.796$
3.4.2 Impact of refining on the enzymatic hydrolysis sugar conversion

Total sugar conversions are plotted versus WRV in Figure 3-3 for both PFI and valley beating mechanical refining. As expected, higher enzymatic hydrolysis times increase the sugar conversion. Increases in sugar conversion with increased refining and WRV are observed with low amounts of refining. Xylose conversions were much higher than glucose conversions for these conditions, likely due to a higher relative charge of the hemicellulase enzyme, but similar trends in hydrolysis were observed versus refining and WRV for both the glucose and xylose. This is in accordance with other studies showing increases in hydrolysis with refining (Endo 2010; Zhu et al. 2010; Koo et al. 2011; H. Chen et al. 2012; X. Chen et al. 2012; Wu et al. 2012; Hoeger et al. 2013). The pretreatment refining energy efficiency defined as the kg of glucose per unit of refining energy in kWh (Zhu et al. 2010) provides a metric for the efficiency of mechanical action on improved biomass digestibility with higher refining energy efficiency being economically desirable. The refining energy efficiency for this study was determined to be in the range of 0.1-1.0 for the PFI refining and 0.3-1.3 for the valley beating. The upper values of pretreatment refining energy efficiency are in the mid to upper range as those reported by (Barakat et al. 2013) from a review of several studies with various biomass and refining types. The results herein are in close agreement with data utilizing a hot water pretreatment and wed disc milling on eucalyptus which had a refining energy efficiency of 1.0 (Barakat et al. 2013).
Figure 3-3. The effect of mechanical refining as measured by WRV on the enzymatic hydrolysis at 5 FPU/g. The legend indicates refining apparatus and hydrolysis residence time in hours. The trend lines shown are best fit third order polynomials for each hydrolysis time as indicated on the lines. Error bars for sugar conversion are expressed as 95% confidence intervals of triplicate experiments. Valley beating times were 15-90 minutes and PFI refining revolutions were 2-10k.

The total sugar conversions are slightly lower for PFI than valley beating in the ranges where the data overlaps, Figure 3-3. This indicates that the different refining processes cause an increase in the hardwood GL biomass digestibility that is not completely described by WRV, and that these different refining processes and their mechanisms have different levels of impact on the increase in sugar conversion. Other researchers have reported that mechanical actions effect particle size, crystallinity of cellulose, accessible specific surface area, and pore size, all impacting the digestibility (Barakat et al. 2013). The complex relationship between all of these different variables and WRV make it
understandable that WRV alone would not uniquely define digestibility. These results indicate that thorough study of refining mechanisms and the resulting biomass digestibility are warranted. The refining mechanisms – (cutting, internal delamination and external fibrillation – can be altered with industrial or pilot plant disk refiners by changing both equipment design parameters (plate design features like the cutting edge length which involves the number, spacing and geometry of bars) as well as the operating parameters (solids consistency, rotational speed of disks, temperature, disk gap width, specific energy input). Concepts reviewed pertaining to pore characteristics, surface area determination and cellulose accessibility might then explain the differences in refining mechanisms and how they relate to biomass digestibility (Zhao et al. 2012). This is ongoing research in this study.

It is of interest to note that a maximum sugar conversion is achieved for both mechanical refining processes for several of the data curves, especially prominent at high total sugar conversions. This suggests that over refining may have a negative impact on enzymatic hydrolysis efficiency. The negative impact of over refining on enzymatic hydrolysis is typically not identified in the literature but it has been discussed that refining can cause a collapse of the fiber structure (Hui et al. 2009). Fiber drying, which has also been shown to collapse portions of the fiber pore matrix (Park et al. 2006b), can negatively impact the subsequent enzymatic hydrolysis (Fan et al. 1981; H. Chen et al. 2012). Measures of fiber cutting, i.e. fiber length and percent fines content, did not correlate with sugar conversion and as such are considered less important than WRV for improving biomass digestibility.
3.4.3 Impact of lignin content on refining benefit

In order to gain a fuller understanding of the impacts of refining on the enzymatic hydrolysis kinetics, digestibility experiments were conducted at enzyme dosages of 1, 3, and 5 FPU/g-biomass with a range of lignin contents from Kraft pretreated substrates from 5% - 20% lignin. The preceding results were with green liquor pulps of approximately 20% lignin. These Kraft pretreated hardwood pulps were refined at PFI 4K and the WRVs significantly increased, Figure 3-2b. The WRV also increased with decreasing lignin content (or more severe pretreatment). Figure 3-4 shows that the more intense the pretreatment and the lower the lignin content, the higher the biomass digestibility. Increased delignification for both softwood and hardwoods has been shown previously to improve the enzymatic hydrolysis conversion (Yu et al. 2011), which is in agreement with the results reported here.
Refining increased the total sugar conversion for 5 FPU/g in all cases for 11%, 15%, and 20% lignin content, Figure 3-4. However, at 5% lignin and long hydrolysis times, the total sugar conversion decreased with refining relative to the corresponding unrefined samples, an unexpected result. Similar trends were observed for the lower enzyme charges, as shown in Table 3-2, but as expected, the total conversions reached by 1 and 3 FPU/g were lower than the 5 FPU/g condition. Again, unexpectedly, for 3 FPU/g at 96 hours enzymatic hydrolysis time a decrease in sugar conversion with refining was observed for the 5% lignin.
content sample. In general, it has been reported that mechanical refining improves the hydrolysis, but the results in this report indicate that overly severe pretreatment combined with refining can create a negative impact on the biomass digestibility. Further investigation is needed to understand if these negative effects of severe pretreatment and over refining are significant.

Table 3-2. Percent sugar conversion of unrefined and refined Kraft pulps with different lignin content and different enzyme charges (FPU/OD g biomass). Cells in grey indicate conditions with lower sugar conversions with refined pulps relative to unrefined pulps.

<table>
<thead>
<tr>
<th>Lignin</th>
<th>Time (hr)</th>
<th>12</th>
<th>24</th>
<th>48</th>
<th>96</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FPU/g</td>
<td>unref</td>
<td>PFI 4k</td>
<td>unref</td>
<td>PFI 4k</td>
</tr>
<tr>
<td>5%</td>
<td>1</td>
<td>19.8</td>
<td>24.4</td>
<td>27.1</td>
<td>36.6</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>42.3</td>
<td>49.3</td>
<td>59.4</td>
<td>67.2</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>48.4</td>
<td>58.4</td>
<td>68.0</td>
<td>77.9</td>
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<tr>
<td>11%</td>
<td>1</td>
<td>19.6</td>
<td>24.6</td>
<td>26.6</td>
<td>36.3</td>
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<td></td>
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<td>38.2</td>
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<td></td>
<td>5</td>
<td>44.6</td>
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<td>15%</td>
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<tr>
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</tr>
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<td>41.7</td>
<td>51.4</td>
<td>53.8</td>
<td>64.1</td>
</tr>
</tbody>
</table>

The trends observed for all enzyme charges in Table 3-2 showed that the improvement is greatest at shorter hydrolysis times. Significant improvements in biomass digestibility are observed at longer hydrolysis times with the exception of the 5% lignin content sample at hydrolysis times of 48 and 96 hours. The results in Table 3-2 also indicate
that refining significantly improves the initial hydrolysis rate and has the best improvement potential at medium lignin contents.

3.4.4 Enzymatic hydrolysis conversion improvement vs. unrefined sugar conversion

The “absolute enzymatic hydrolysis improvement” (defined as the PFI 4k refined sugar conversion minus the unrefined sugar conversion) of all conditions can be plotted versus the sugar conversion at the same conditions without refining, Figure 3-5. There is a maximum absolute sugar conversion improvement versus percent unrefined sugar conversion. At low levels of unrefined sugar conversion, the refining improvement is limited by the kinetics affected by the biomass recalcitrance, which hinders efficient enzymatic hydrolysis. At high levels of unrefined sugar conversion, the refining improvement is limited by the maximum total conversion.
Figure 3-5. Absolute enzymatic hydrolysis improvement on refined Kraft pulps versus the unrefined pulps sugar conversion. The dotted line shown is the best fit quadratic, but is used to qualitatively visualize the general trend of a maximum in absolute conversion improvement with refining. Hollow points indicate conditions with lower sugar conversions of refined pulps relative to unrefined pulps. Legend shows the data points at different hydrolysis time and enzyme charge.

Again, notice that refining at the extreme enzymatic hydrolysis conditions of long time and high enzyme dosage for the low lignin content sample have negative impacts on enzyme hydrolysis relative to unrefined material. This is counter-intuitive since the refined materials have significantly greater WRV suggesting increased enzyme accessibility. It is hypothesized that this observed maximum and width of the curve would shift relative to different biomass substrates, and different refining intensities. To our knowledge this maximum in sugar conversion improvements on refining and decreases in sugar conversion on refining have not been reported previously. The glucose conversion follows the same
trend of maximum improvement with refining as the total sugar conversion, whereas the xylose conversion improvement with refining did not correlate and had a lower relative improvement.

It may be that the low lignin content pulps that were exposed to the highest intensity chemical pretreatment were rendered into a physical form that impacted the mixing and transport of enzyme and substrate enough to negatively impact the sugar conversion. More research is required to investigate this.

### 3.4.5 Possible benefits realized from refining

The improvement in enzymatic hydrolysis by mechanical refining can be realized in two ways; either by savings in hydrolysis time or by reductions in enzyme dosage. Time savings can either be used to increase process throughput or reduce the enzymatic hydrolysis reaction volume, thus reducing the capital costs. Time savings is the amount of time (hours) that could be reduced for the same condition of Kraft pulp pretreatment severity (lignin content) and enzyme charge (FPU/g) by using mechanical refining.

Similarly, enzyme reduction is the amount of enzyme saved (% FPU) by refining for the same condition of Kraft pulp pretreatment severity (lignin content) and hydrolysis time. Enzyme savings were calculated by fitting a curve to the unrefined and refined hydrolysis results with the axes of sugar conversion (%) versus enzyme dosage (FPU/g) for each condition of lignin content and hydrolysis time. Then the potential enzyme reduction was calculated for multiple target total sugar conversions selected within the range of the minimum refined conversion and the maximum unrefined conversion. These percent enzyme
savings were plotted versus their target sugar conversions for each lignin content and enzymatic hydrolysis retention time, Figure 3-6.

![Figure 3-6](image)

Figure 3-6. Potential enzyme savings with refining versus target sugar conversion. Legend [%-h] shows the data points at different percent lignin content and hours hydrolysis time.

A maximum in percent enzyme reduction occurs around a 50% target total sugar conversion, Figure 3-6. There is a lower enzyme reduction potential (lower maximum) for low lignin content pulp as compared to higher lignin content pulp. This analysis elucidates the ability of mechanical refining to reduce the required processing time and/or enzyme dosage. These savings are maximized at higher lignin contents (less severe pretreatment). It is observed that enzyme reductions of 20-50% can be achieved with modest refining. This is
very significant, since a recent study shows that the enzyme cost is approximately 20% of the overall costs (including capital) in the production of ethanol from biomass, second only to the feedstock cost (Gonzalez et al. 2011).

It should be noted that enzymatic hydrolysis conditions in practice should almost always target a total sugar conversion of 100%. The reason for high enzymatic hydrolysis sugar conversions is to ensure that the process would convert close to the theoretical maximum of available cellulose and hemicellulose to monomer sugars, which can be utilized downstream in a biorefinery to produce ethanol via fermentation of these sugars. Targeting lower sugar conversions, although more time or enzyme savings might be possible, is usually not preferred since the goal for most biorefineries is to utilize as much of the starting biomass as possible at the lowest processing cost.

Another possible benefit from the combined application of pretreatment (delignification) and the mechanical refining on woody biomass is that the more digestible woody material produced could be seamlessly blended with easier to digest agricultural materials into a biomass to biofuel process. By reducing the woody biomass recalcitrance, the treated woody biomass and the agricultural biomass could be processed within the same equipment under the same conditions. This would make the system simple in operation, have lower capital (than separate treatment lines) and provide a plant with an ability to be robust with respect to different biomass feedstock availability (for instance seasonal availability of biomass types) and to be flexible with respect to accepting different biomass feedstocks.
depending on their relative costs and availability at any period in time as influenced by supply and demand.

The combination of woody and agricultural biomass as a feedstock to a biorefinery (70/30 ratio of woody to agricultural biomass) is expected to lead to the lowest total biomass feedstock delivery cost (Sultana et al. 2011). This can be very significant to the plant economics since the delivered feedstock cost can be 35-50% of the total production costs of biofuel. In addition, the authors point out that for very large biorefineries, local constraints prevent one single biomass source to generally meet the biorefinery feedstock demand.

3.5 Conclusions

Mechanical refining is a promising post-treatment to improve the bioconversion of lignocellulosics. It was observed that water retention value positively correlates with the hydrolysis improvements by refining. Different refining mechanisms affect the hydrolysis process differently, indicating that future research on refining to optimize refining plate design is useful. Maximum improvements of sugar conversion between refined and unrefined materials occur at moderate sugar conversions where hydrolysis is neither limited by recalcitrance nor total conversion. The improvement in sugar conversion with refining can allow decreased enzyme charge, enzymatic processing time, or process pretreatment severity.
3.6 Acknowledgements

Special thanks to United States Department of Agriculture as a part of the National Needs Foundation for providing the funding for this research (Grant number 2010-38420-21828).
3.7 References


TAPPI. (1993)."TAPPI Test Methods." *Kappa number of pulp.* Atlanta, GA, USA. TAPPI: T 236 cm-85.


CHAPTER FOUR: COMPARISON OF LAB, PILOT, AND INDUSTRIAL SCALE LOW CONSISTENCY MECHANICAL REFINING FOR IMPROVEMENTS IN ENZYMATIC DIGESTIBILITY OF PRETREATED HARDWOOD

4.1 Abstract

Mechanical refining has been shown to improve biomass enzymatic digestibility. In this study, industrial high-yield sodium carbonate hardwood pulp and was subjected to lab, pilot and industrial refining to determine if the mechanical refining improves the enzymatic hydrolysis sugar conversion efficiency differently at different refining scales. Lab, pilot and industrial refining increased the biomass digestibility for lignocellulosic biomass relative to the unrefined material. The sugar conversion was increased from 36% to 65% at 5 FPU/g of biomass with refining at 67.0 kWh/t. There is a maximum in the sugar conversion with respect to the amount of refining energy. Water retention value is a good predictor of improvements in sugar conversion for a given fiber source and composition. Improvements in biomass digestibility with refining due to lab, pilot plant and industrial refining were similar with respect to water retention value.
4.2 Introduction

The natural recalcitrance of plant biomass feedstock to resist the breakdown of its fibers to structural carbohydrates by enzymatic hydrolysis is a major issue in the commercialization of cellulosic biofuels (Zhao et al. 2012). These natural recalcitrant properties include the waxy outer skin of the plant body, dense packing of the fiber bundles, thick fiber wall, degree of lignification, and structural complexity of the cell wall (Himmel et al. 2007). All of these issues, combined with the difficulty for soluble cellulase enzyme proteins to act on insoluble cellulose, make the processing of lignocellulosic biomass complex and reduce the subsequent enzymatic hydrolysis efficiency.

It has been recognized that a pretreatment operation is critical for increasing the lignocellulosic biomass enzymatic digestibility by opening up the biomass structure to increase the cellulose accessibility to enzymes (Zheng et al. 2009; Zhao et al. 2012). Unfortunately, severe pretreatment conditions of high temperature and chemical charge are needed to achieve high sugar conversions, which generate lower pretreatment yields and higher fermentation inhibiting compound concentrations. It has been determined that the overall saccharification efficiency is governed by three basic processes: 1) substrate accessibility of cellulose—component removal and size reduction, 2) substrate and cellulase reactivity—limited by inhibitors, and 3) reaction conditions—optimizing and controlling pH, temp, etc. (Leu et al. 2012).

Mechanical refining has been shown to combat the natural biomass recalcitrance and have a positive effect on the enzymatic digestibility of dilute acid pretreated corn stover
(PCS) (X. Chen et al. 2012; Tao et al. 2012), green liquor (GL) pretreated hardwood (Koo et al. 2011), Kraft hardwood (Jones et al., 2013), and loblolly pine (Wu et al. 2012), sulfite pretreated softwood (Zhu et al. 2010), and recovered office printing paper (H. Chen et al. 2012). It has also been shown that micro- and nano-scale mechanical refining of hardwood (Endo 2010) and softwood (Hoeger et al. 2013) fibers that have not been thermo-chemically pretreated are especially effective in lignocellulose cell wall breakdown in order to improve the substrate enzymatic digestibility.

The PCS study exhibited increased glucan and xylan conversions after a laboratory scale refining process (PFI mill with 4,000 revolutions), increasing the glucose yield to over 90% compared to the unrefined control at 70% (X. Chen et al. 2012). PFI refining (8,000 revolutions) after green liquor pretreatment of hardwood allowed about a 50% decrease in enzyme charge at the same hydrolysis conversion of a non-refined sample, highlighting the ability of refining to reduce the required enzyme charge (Koo et al. 2011). In the same study, the refining showed an increase in sugar conversion of GL hardwood from 51% to 72% at the same enzyme dosage.

Softwood has been found to be very recalcitrant to enzymatic hydrolysis, but PFI refining at 9,000 revolutions increased the sugar conversion from 41% to around 55% - 60% and was further increased to 78% when mechanical refining was used in combination with oxygen delignification (Wu et al. 2012). The sulfite pretreatment on softwood combined with disk milling proved to have cellulose saccharification efficiencies of over 90% in 48 hours at a cellulase loading of 15 FPU/g-substrate, an increase from 77% without refining (Zhu et al. 2010).
2010). Recovered office printing paper showed an average percent increase of sugar conversion with 2-8 FPU/g-substrate of approximately 10% with PFI refining at 5,000 revolutions (H. Chen et al. 2012). In that study, the combination of ash removal from the recovered paper and refining resulted in enzyme conversions of 97%.

Multiple lab scale refining apparatuses have been shown to improve the biomass enzymatic hydrolysis sugar conversion of Kraft and Green liquor pretreated hardwoods (Jones et al. 2013). It was determined that the mechanical refiners with different mechanisms affected the sugar conversion differently. The increase in biomass digestibility was related to the water retention value of the biomass, which is a quick indirect measurement or indication of the biomass surface area or ability to swell and hold water. Additionally, a maximum in the absolute enzymatic hydrolysis improvement due to refining was observed at enzymatic hydrolysis conditions that produced intermediate hydrolysis conversions.

All of these reports indicate the effectiveness of mechanical refining for improving the biomass enzymatic hydrolysis sugar conversion. However, the effectiveness of mechanical refining processes has not been sufficiently validated at the industrial scale and none of these studies compare lab scale refining methods to pilot and large-scale industrial refining within a single study. It is critical to understand the differences and similarities between these refining scales in order to effectively scale up the utilization of refining for biomass to biofuels conversion.

In this study industrially produced sodium carbonate hardwood unrefined pulps were subjected to lab, pilot and industrial scale refining. The objective of this study was to
determine if industrial mechanical refining improves the enzymatic hydrolysis sugar conversion efficiency at reasonable energy inputs. It was also of interest to determine if refining has the same effect at different refining scales. The results of this study show that increases in WRV from the unrefined condition improved the biomass digestibility in a similar manner for lab, pilot and industrial scale refining.

4.3 Experimental

4.3.1 Hardwood biomass characterization

The sodium carbonate pulp was made using a mixture of various species of southern hardwood chips. Compositional analyses of the hardwood chips and the pulp were measured using a modified version of the NREL standard procedure (Sluiter et al. 2008). A quantity of 0.1 g OD powder of wood (40 mesh) was hydrolyzed with 72% H$_2$SO$_4$ for 2 hours. The hydrolyzed biomass was diluted to 3% H$_2$SO$_4$ and autoclaved at 120°C for 1.5 hrs. The hydrolysate was filtered and oven-dried to determine the insoluble solids, and the filtrate was collected for the determination of acid soluble lignin (UV-Vis (Lambda XLS, Perkin Elmer, Waltham, MA, USA) at 205 nm and 110 Abs coefficient) and carbohydrate content analysis using HPLC (filtered with 0.2 μm syringe filter).

4.3.2 Biomass pretreatment procedure

The unrefined hardwood sodium carbonate pulp (HWSC) was generated at an industrial facility with a six chamber Pandia digester. The fiber composition was 100% hardwood chips. The chips were charged at 6.5 kg of wet chips per L of Na$_2$CO$_3$ liquor. The
cook time in the chamber was approximately 5.5 minutes at 190°C. The pretreatment yield was 84.5% with an average final consistency of the stock leaving the digesters being ~30% before going through a chip defibrator (pump) and brown stock washers. The pulp mill utilizes on average 1250 tons of chips per day and produces an average of 650 oven dry tons of pulp per day. Pulp after brown stock washing (to remove the pulping chemicals and dissolved organics) was used in this study for lab and pilot plant refining. The pulp was stored at 4°C at about 30% consistency in closed bags until further analysis.

4.3.3 PFI refining

PFI refining (or milling) is a batch refining procedure where pulp is held against a bedplate using centrifugal force and the spinning refiner rotor is forced against the bedplate imparting a mechanical shearing action on the fibers (TAPPI 2008). The gap between refining bar and refiner bedplate is supposed to approach zero distance. Table 4-1 describes the refining condition for the PFI refiner, and provides images and comparisons of lab, pilot and industrial scale refining. A quantity of 30 g OD pulp was refined in a PFI mill (Norwegian Pulp and Paper Institute (PFI), Oslo, Norway) at 10% consistency at selected refining intensities between 2,000 and 10,000 revolutions, in increments of 2,000 revolutions. After refining, pulp was collected and stored in the refrigerator without washing. The PFI refining energy was estimated as 0.18 kWh/ton-rev (Kerekes 2005).
Table 4-1. Comparison of lab scale PFI refiner, pilot scale disk refiner, and industrial scale disk refiner.

<table>
<thead>
<tr>
<th></th>
<th>Lab Refiner</th>
<th>Pilot Refiner</th>
<th>Industrial Refiner</th>
</tr>
</thead>
<tbody>
<tr>
<td>Refiner Type</td>
<td>PFI Mill</td>
<td>Sprout-Waldron Disk</td>
<td>Beloit Double-Disk (1°)</td>
</tr>
<tr>
<td>Diameter</td>
<td>Not Applicable</td>
<td>12 inch</td>
<td>Spout-Bauer Twin Flow</td>
</tr>
<tr>
<td>Plate Style</td>
<td>Bar and bedplate</td>
<td>D2A-507</td>
<td>42 inch</td>
</tr>
<tr>
<td>Capacity</td>
<td>30 OD g per run</td>
<td>1-5 OD kg per run</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10-20 ton/hr</td>
</tr>
<tr>
<td>Image</td>
<td><img src="http://spectrum.andritz.com/twinflo_tf.pdf" alt="Image" /></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Image of example (not actual) Industrial-TwinFlow (http://spectrum.andritz.com/twinflo_tf.pdf)

4.3.4  **Pilot scale refining**

Pilot scale refining was performed using a 12” Sprout Waldron Thermo-Mechanical Pulping (TMP) unit (Koppers Company, Inc., Model 12-1CP, Muncy, PA), Table 4-1. The plates used were Sprout Waldron D2A-507 type plates for three zone fine refining. The refiner motor was a 60 HP and 1775 RPM Reliance Duty Master AC Motor (Cleveland, OH). The agitator, screw feeder and refiner speeds were all constant. The consistency was manually adjusted to approximately 20% with water prior to loading the pulp into the refiner feed vessel. A dilution water flow of 4 liters/minute produced a pulp consistency of about 5% for refining. The gap size was controlled by setting the zero gap (touching the plates together) and then backing off the plates to the desired gap width, 50.8 µm gap. The energy consumption was measured using a Schneider Electric (Power Logic, PM 1200, Rueil-Malmaison, France) energy meter and power integrator. The temperature was adjusted using
direct steam at the desired saturated steam pressure when needed. The time was recorded between when the screw feeder was started and when the feed vessel was empty, and the energy was integrated over that time and used to calculate the kWh/t for each refiner trial. This process gave almost 100% yield with respect to the starting material.

4.3.5 Industrial scale refining

The washed pulp was sent through a 42” Beloit Double-Disk primary refining step at 5.8% consistency and 67.0 kWh/t load, Table 4-1. The primary refiner used dammed high-intensity plates (~18km/rev) designed for shive reduction. There are minimal changes in freeness (CSF) or fiber drainage rate during this step. Then, the fiber was passed through the secondary refining step. The secondary refining targets a fibrillation action by using mid-intensity, dammed plates and a 42” Spout-Bauer twin flow refiner. The refining conditions were 5.7% consistency and 79.5 kWh/t load. The freeness drop was approximately 100 mL CSF over the secondary refiner. The refining gap is unknown, control of the refiner is based on energy input per ton of pulp.

4.3.6 Refining energy analysis on sugar conversion

Refining energy measurements and calculations can provide useful tools for analyzing the refining effects on the biomass substrate. It should first be noted that the magnitude and relative changes in refining energy at the different lab, pilot, and industrial scales are not equivalent (Kerekes 2005). In order to create similar refining action at smaller scales it is much less energy efficient compared to industrial scale pulp refining. The refining energy efficiency (η) and the refining energy efficiency improvement (ηi) were calculations
adapted from (Barakat et al. 2013), and used as tools to compare the effectiveness of the mechanical refining treatment with respect to the refining energy input. The pilot scale refining energy was interpolated to 50.8 µm gap based on energy measurements from the same refining conditions with a 25.4 and 127 µm gap.

Equation 4-1. Refining energy efficiency.

$$\eta = \frac{\text{sugar conversion}}{\text{total energy}}$$

Equation 4-2. Refining energy efficiency improvement.

$$\eta_i = \frac{(\text{sugar conversion refined} - \text{sugar conversion unrefined})}{\text{total energy}}$$

4.3.7 Enzymatic Hydrolysis

The conversion of cellulose and hemicelluloses into monomer sugars was performed using 1, 3, and 5 FPU/OD g of substrate Novozymes Cellic® CTec2 cellulase enzyme – a blend of endoglucanases, exoglucanases (celllobiohydrolases, CBHI works from the reducing end and CBHII works from the non-reducing end), beta-glucosidase, among others – and 1/9 v/v Cellic® HTec2 hemicellulase enzyme – primarily composed of endoxylanases (Novozymes NA, Franklinton, NC, USA). The activity of the CTec2 cellulase was determined to be 112 FPU/mL according to a standard method (Ghose 1987). The protein concentration of CTec2 was not measured but was provided a 210 mg/mL. The activity of the HTec2 hemicellulase was not known, and was charged based on supplier recommendation.
One FPU (Filter Paper Unit) is defined as the amount of enzyme (in mL) that releases 1 μmol of glucose equivalents from Whatman No. 1 filter paper per minute. Experiments were conducted on a 1 OD g basis in a 50 mL conical tube and a 20 mL working volume (5% w/v consistency). A 1 mM sodium acetate buffer solution (pH 4.8) with 0.1% sodium azide antibiotic was used for pH and microbial control. The rotating hybridization incubator (FinePCR COMBI-D24, Seoul, Korea) was controlled to a temperature of 50°C and 12 rpm. Samples were collected using 0.5 mL aliquots from the well-mixed hydrolysis at five different residence times (12, 24, 48, 96, and 144 hours after inoculation). The solids were separated via micro centrifugation for 10 minutes at 13,000 rpm, and then the supernatant was diluted with deionized water to 5x and filtered at 0.2 μm into a HPLC vial for sugar conversion analysis.

4.3.8 Sugar Analysis

Sugar analysis was carried out following a modified NREL Laboratory Analytical Procedure (Sluiter et al. 2008), via ligand-exchange chromatography (SP0810, Shodex, Kawasaki, Japan) on the high-performance liquid chromatography (HPLC) system (Agilent 1200, Agilent, Santa Clara, CA, USA) with an Agilent guard column, degasser, pump, and refractive index detector (RID). HPLC grade milliQ water was used as the mobile phase. Aliquots of Klason lignin filtrate and enzyme hydrolysate were used to determine the carbohydrate content for hardwood pulps and enzymatic hydrolysates, respectively. Samples were analyzed with a 20 μL injection volume and 0.5 mL/min flow rate at a column temperature of 80°C. The sugars detected were glucose and xylose, and solutions with
mixtures of known sugar concentrations were measured to generate a calibration curve for each sugar. The minor sugar concentrations of galactose, arabinose, and mannose were below the detectable limit and therefore not reported. The digestibility is reported as sugar conversion:

Equation 4-3. Determination of biomass digestibility.

\[
\text{sugar conversion (\%) = 100\% \times \frac{\text{total sugar released during enzyme hydrolysis (g)}}{\text{carbohydrate content after pretreatment (g)}}}
\]

4.3.9 Fiber properties

Water retention value (WRV) is defined as the water absorbed by the substrate relative to the oven-dried mass of the substrate (TAPPI 2011). For measurement of the WRV a quantity of approximately 0.30 OD g of sample was placed into centrifuge filtering tube (Amicon Centriplus, Millipore Corp., Bedford, MA, USA) with a glass frit (25mm, med porosity borosilicate fritted disc, Chemglass, Vineland, NJ, USA) and centrifuged for 30 minutes at 0.9 relative centrifugal force (\( \text{rcf} = rw^2/g \)), or the acceleration (radius times the square of the angular velocity) relative to gravity. The recorded weights of the wet centrifuged sample \((m_1)\) and the oven-dried at 105°C sample \((m_2)\) were used to calculate the water retention value (WRV). The basis weight of the final pads was approximately 700 g/m².

\[ \text{WRV (g/g)} = \frac{(m_1 - m_2)}{m_2} \]

The HiRes Fiber Quality Analyzer (FQA) from OpTest Equipment, Inc. (Hawkesbury, ON, Canada) was used to measure length weighted fiber length, fiber width, and percent fines (1999). Well-mixed dilute fiber suspensions (~1mg/L) of the pulp samples were prepared. The fiber length is the true contoured length of the fiber and is reported as the length weighted value average. Fines are defined as any particle less than 0.2 mm in size and are reported as a percentage of the total number of particles counted.

### 4.4 Results and Discussion

#### 4.4.1 Composition analysis of HWSC pulps

The compositions of the unrefined and different scale refined pulps are similar, Table 4-2. The total carbohydrate and lignin content is similar for the unrefined and all refined pulps. Extractive content and other minor sugars (which were not measured) should account for some of the remaining mass closure. It is observed that compared to the unrefined pulp, the glucan content is slightly higher for the pilot scale refined pulps and slightly lower for the industrially refined pulps. Also, the ash content is the lowest for the pilot scale refined pulps and the highest for the industrially refined pulps. The pilot scale refined pulp has the lowest acid soluble lignin (ASL), and the two industrially refined pulps have the highest. The differences between the pulps are minor and within the standard experimental error.
Table 4-2. Composition analyses of hardwood sodium carbonate (HWSC) pulps in weight percent. Minor sugars (arabinose, mannose, and galactose) not shown. Total lignin includes acid insoluble residue (AIR) and acid soluble lignin (ASL). The HWSC biomass treatments are (top-bottom): unrefined, lab scale PFI refining, pilot scale thermomechanical pulp (TMP) refining, industrial biomass samples taken after primary disk refining stage, and industrial biomass after secondary disk refining.

<table>
<thead>
<tr>
<th></th>
<th>Glucan ±</th>
<th>Xylan ±</th>
<th>Total Carb ±</th>
<th>ASL ±</th>
<th>Total Lignin ±</th>
<th>Avg Ash ±</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unrefined</td>
<td>45.91 ± 0.54</td>
<td>11.95 ± 0.28</td>
<td>58.51 ± 1.12</td>
<td>2.87 ± 0.05</td>
<td>27.78 ± 0.53</td>
<td>1.61 ± 0.06</td>
</tr>
<tr>
<td>Lab Scale (PFI)</td>
<td>44.87 ± 0.32</td>
<td>12.27 ± 0.38</td>
<td>57.50 ± 1.16</td>
<td>2.98 ± 0.04</td>
<td>28.45 ± 0.43</td>
<td>1.88 ± 0.08</td>
</tr>
<tr>
<td>Pilot Scale</td>
<td>47.81 ± 0.27</td>
<td>10.00 ± 0.22</td>
<td>58.33 ± 0.94</td>
<td>2.62 ± 0.09</td>
<td>26.24 ± 0.97</td>
<td>1.50 ± 0.04</td>
</tr>
<tr>
<td>Ind-Primary</td>
<td>41.59 ± 0.30</td>
<td>9.58 ± 0.11</td>
<td>51.78 ± 0.62</td>
<td>3.06 ± 0.01</td>
<td>27.88 ± 0.25</td>
<td>5.46 ± 0.04</td>
</tr>
<tr>
<td>Ind-Secondary</td>
<td>40.60 ± 0.30</td>
<td>10.13 ± 0.23</td>
<td>51.30 ± 0.80</td>
<td>3.04 ± 0.11</td>
<td>27.79 ± 0.20</td>
<td>4.99 ± 0.20</td>
</tr>
</tbody>
</table>

Table 4-3. Enzymatic hydrolysis and fiber property results for hardwood sodium carbonate (HWSC) unrefined, PFI lab refining, thermomechanical pulp (TMP) pilot scale refining, and industrial (Ind-) disk refining samples taken after the primary and secondary refining stage, compared to the mechanical refining energy and the subsequent refining energy efficiency ($\eta$) and the refining energy efficiency improvement ($\eta_i$).

<table>
<thead>
<tr>
<th></th>
<th>Total sugar-48hr average (%)</th>
<th>Total sugar-144hr average (%)</th>
<th>$L_w$ (mm)</th>
<th>FQA Width ($\mu$m)</th>
<th>Fines (wt %)</th>
<th>WRV g/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unrefined</td>
<td>26.30 ± 0.43</td>
<td>42.11 ± 1.49</td>
<td>1.106</td>
<td>21.3</td>
<td>12.26</td>
<td>1.104 ± 0.118</td>
</tr>
<tr>
<td>PFI-2k</td>
<td>36.87 ± 0.67</td>
<td>46.90 ± 0.96</td>
<td>1.034</td>
<td>21.1</td>
<td>8.75</td>
<td>1.919 ± 0.104</td>
</tr>
<tr>
<td>PFI-4k</td>
<td>41.57 ± 0.39</td>
<td>53.12 ± 1.27</td>
<td>0.925</td>
<td>21.0</td>
<td>11.00</td>
<td>2.026 ± 0.007</td>
</tr>
<tr>
<td>PFI-6k</td>
<td>39.45 ± 1.70</td>
<td>50.80 ± 2.05</td>
<td>0.919</td>
<td>21.2</td>
<td>10.09</td>
<td>2.306 ± 0.016</td>
</tr>
<tr>
<td>PFI-8k</td>
<td>40.05 ± 0.27</td>
<td>51.86 ± 0.21</td>
<td>0.903</td>
<td>21.1</td>
<td>10.39</td>
<td>2.210 ± 0.036</td>
</tr>
<tr>
<td>PFI-10k</td>
<td>38.42 ± 1.63</td>
<td>50.26 ± 1.55</td>
<td>0.898</td>
<td>20.9</td>
<td>9.84</td>
<td>2.334 ± 0.023</td>
</tr>
<tr>
<td>Pilot Scale</td>
<td>46.23 ± 0.52</td>
<td>69.51 ± 1.25</td>
<td>0.980</td>
<td>20.5</td>
<td>10.75</td>
<td>1.881 ± 0.106</td>
</tr>
<tr>
<td>Ind-Primary</td>
<td>43.94 ± 0.75</td>
<td>62.48 ± 3.49</td>
<td>1.117</td>
<td>21.6</td>
<td>9.35</td>
<td>1.543 ± 0.015</td>
</tr>
<tr>
<td>Ind-Secondary</td>
<td>50.13 ± 0.79</td>
<td>66.51 ± 2.16</td>
<td>0.961</td>
<td>20.9</td>
<td>9.64</td>
<td>1.708 ± 0.015</td>
</tr>
</tbody>
</table>
Table 4-3. Continued.

<table>
<thead>
<tr>
<th></th>
<th>Energy kWh/t</th>
<th>48hr</th>
<th>144hr</th>
<th>48hr</th>
<th>144hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unrefined</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PFI-2k</td>
<td>360</td>
<td>1.024</td>
<td>1.303</td>
<td>0.294</td>
<td>0.133</td>
</tr>
<tr>
<td>PFI-4k</td>
<td>720</td>
<td>0.577</td>
<td>0.738</td>
<td>0.212</td>
<td>0.153</td>
</tr>
<tr>
<td>PFI-6k</td>
<td>1080</td>
<td>0.365</td>
<td>0.470</td>
<td>0.122</td>
<td>0.080</td>
</tr>
<tr>
<td>PFI-8k</td>
<td>1440</td>
<td>0.278</td>
<td>0.360</td>
<td>0.095</td>
<td>0.068</td>
</tr>
<tr>
<td>PFI-10k</td>
<td>1800</td>
<td>0.213</td>
<td>0.279</td>
<td>0.067</td>
<td>0.045</td>
</tr>
<tr>
<td>Pilot Scale</td>
<td>698</td>
<td>0.662</td>
<td>0.996</td>
<td>0.286</td>
<td>0.392</td>
</tr>
<tr>
<td>Ind-Primary</td>
<td>67</td>
<td>6.555</td>
<td>9.320</td>
<td>1.970</td>
<td>2.400</td>
</tr>
<tr>
<td>Ind-Secondary</td>
<td>147</td>
<td>3.420</td>
<td>4.538</td>
<td>1.323</td>
<td>1.373</td>
</tr>
</tbody>
</table>

4.4.2 Effect of PFI refining on HWSC pulp

PFI refining was utilized to determine the effectiveness of refining on HWSC pulp fibers. Enzymatic hydrolysis results show that all levels of PFI refining improved the biomass digestibility, Table 4-3. A maximum in enzymatic hydrolysis improvement of refined pulps was achieved after 4,000 revolutions in the PFI mill, Figure 4-1. Note that the energy input using the PFI mill is very high, much higher than industrial refining. Further increases in refining intensity by increasing the number of revolutions in the PFI yielded no significant difference in the biomass digestibility. In fact, the total sugar conversion for all enzyme hydrolysis times decreases slightly after 4,000 revolutions.
Figure 4-1. Total sugar conversion of HWSC biomass versus PFI revolutions. Total energy (kWh/t) is estimated to be 0.18 kWh/t/rev*number of revolutions. Error bars show the 95% confidence interval of experiments performed in triplicate.

The average enzymatic hydrolysis improvement with refining at all levels of refining and all hydrolysis retention times was 11.7 percent. The total sugar conversions were very similar to the glucose conversions since cellulose is the major carbohydrate component in the biomass (~85% glucan with respect to total carbohydrate). Glucose and xylose conversions had similar responses with respect to increased PFI refining, both showing a maximum at 4,000 revolutions, but the xylose conversions were much higher and closer to the theoretical maximum compared to the glucose conversions, Figure 4-2.
Figure 4-2. Glucose (a) and xylose (b) sugar conversions of HWSC biomass versus PFI revolutions. Total energy (kWh/t) is 0.18 kWh/t/rev*number of revolutions. Error bars show the 95% confidence interval of experiments, done in triplicate.

Several fiber properties were measured to explore the cause of the improved sugar conversion and these properties are listed in Table 4-3. It was observed that the water retention value increases with refining whereas the fiber length decreases. The percent fines and the fiber width did not change significantly with refining.

Linear correlation coefficients between fiber properties and sugar conversions appear in Table 4-4. The percent sugar conversion versus WRV has higher linear correlations for short enzyme hydrolysis times, whereas length weighted fiber length has higher correlations at the longer enzyme hydrolysis times. It is of interest to note that energy has a low correlation since after 4,000 PFI revolutions no further enhancement and maybe even a decrease in sugar conversion is realized when more refining (energy) is expended. This is shown as a generally decreasing $\eta$ and $\eta_i$ for the 48 hours and 144 hours enzyme hydrolysis times, Table 4-3.
Table 4-4. Linear correlation coefficients ($R^2$) values of total sugar EH conversion at different PFI refining times with respect to measured refined fiber properties. Cells are shaded from lowest (red) to highest (green) linear correlations.

<table>
<thead>
<tr>
<th>EH Time (hr)</th>
<th>Fines</th>
<th>Width</th>
<th>Length</th>
<th>WRV</th>
<th>Energy</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>0.311</td>
<td>0.352</td>
<td>0.859</td>
<td>0.890</td>
<td>0.532</td>
</tr>
<tr>
<td>48</td>
<td>0.314</td>
<td>0.349</td>
<td>0.787</td>
<td>0.828</td>
<td>0.432</td>
</tr>
<tr>
<td>96</td>
<td>0.174</td>
<td>0.359</td>
<td>0.902</td>
<td>0.817</td>
<td>0.543</td>
</tr>
<tr>
<td>144</td>
<td>0.092</td>
<td>0.329</td>
<td>0.878</td>
<td>0.719</td>
<td>0.490</td>
</tr>
</tbody>
</table>

The use of these correlation coefficients, shows correlation but not cause and effect; there is no reason to believe that the relationships should be linear. However, it is somewhat informative to observe very low correlations with percent fines and fiber width which would rule out any significant correlation with sugar conversion. These correlations do suggest which variables may be used as indicators of improved enzyme hydrolysis via refining.

The refining energy efficiency, $\eta$, is shown in Table 4-3 to increase for a given refining level with increased enzymatic hydrolysis time, as in accordance with Figure 4-1 for 48 and 144 hours enzyme hydrolysis time and Figure 4-3. Note that the sugar conversion increases for enzymatic hydrolysis times from 0 to 144 hours. Unlike the $\eta$, the refining energy efficiency improvement, $\eta_i$, decreases at all levels of refining with increased enzyme hydrolysis time Table 4-3 and Figure 4-3. This is simply because the $\eta_i$ includes the sugar conversion of the unrefined pulp, which is greater for longer enzyme hydrolysis time. Since the refining energy is constantly increasing with refining time and the increases in sugar conversion relative to the unrefined biomass begin to be smaller or negative for higher levels
of refining, both the $\eta$ and the $\eta_i$, generally decrease with increased refining energy (Figure 4-3). These results reiterate that longer enzyme hydrolysis times minimize the benefit of refining on the biomass digestibility, in accordance with our previous research (Jones et al. 2013).

![Graph of refining energy efficiency and improvement](image)

Figure 4-3. Refining energy efficiency ($\eta$) and the refining energy efficiency improvement ($\eta_i$) versus the refining energy (kWh/t) for PFI refined pulp.

### 4.4.3 Pilot scale refining

Pilot plant scale disk refining was performed (12-inch refining plate, 5% consistency and 50.8 µm gap) on the unrefined industrial pulp. The pilot scale refining results had similar
or improved sugar conversions relative to the lab or industrial refining, Table 4-3 and Figure 4-4. The sugar conversions for pilot plant refining were the highest observed in this study.

Figure 4-4. Industrial, pilot and lab scale refining effect on the total sugar conversion at 5 FPU per gram of oven dry HWSC biomass. Error bars indicate a 95 percent confidence interval based on experiments performed in triplicate.

The energy input to the pulp was determined to be approximately 500 kWh/t, a considerably greater energy input than the industrial refining but within the laboratory PFI refining energy range, Table 4-3. There are many variables that affect the total mechanical refining energy including no load energy consumption, biomass consistency, plate gap width, and plate design among others. Pilot scale equipment is not often designed for energy
efficiency but rather for technical application purposes, and is limited in the total throughput capacity. The no load refining energy is very high for the pilot scale refiner (~250 kWh/t), compared to the industrial scale refiner (~25 kWh/t). These results show that pilot scale refining can be effective in evaluating the conditions of mechanical refining and their impact on subsequent sugar conversion. Future research will involve investigating the refining conditions (gap, consistency, temperature, plate design) using the pilot plant refiner.

Photomicrographs of the unrefined and refined fibers are shown in Figure 4-5. Refining is expected to cause fibrillation of the fiber surface and fiber cutting. These types of changes were observed for lab, pilot and industrial refining processes. In addition, internal delamination is expected from refining but not observable in these images. The number of kinks and extent of curl in the fibers was also observed to increase with refining. It was observed that non-surface fibrillated fibers existed in the PFI refined pulps at very long refining time. This suggests that there is a distribution of fiber responses to the refining action and that some fibers seem to be less-responsive to the refining. This may explain the detected plateau in the biomass digestibility with long refining times. Another explanation that has been proposed for decreased biomass digestibility for high refining action is that the fibers flatten since they are very flexible and may cause the fiber pore structure to collapse (Chen et al. 2013). The images herein do not assist in evaluating this hypothesis.
Figure 4-5. Photomicrographs of unrefined and refined hardwood sodium carbonate (HWSC) fibers. a) HWSC Unrefined, b) after industrial primary refiner, c) after industrial secondary refiner, d) pilot plant refining, e) lab scale refining: PFI 2,000 revolutions, f) lab scale refining: PFI 8,000 revolutions.
4.4.4 Industrial scale refining

Industrial sodium carbonate hardwood pulps with 28% lignin (almost no lignin removal, pulp yield of 85%) were collected after the primary and secondary refiners to evaluate the impact of industrial refining on enzyme digestibility. The primary refiner utilized 67.0 kWh/t and the secondary refiner utilized an additional 79.5 kWh/t, both were 46-inch diameter disk refiners operating on pulp at 5% consistency. These energy inputs are based on producing paper products but are not optimized for biofuel production. The plate gap is not known as the refiner is controlled based on the energy demand (kWh/t). Both the primary and secondary refined pulps had significantly higher total sugar conversions relative to the unrefined pulp (15.9 and 20.1 % average gains, respectively), Figure 4-4. However, much larger gains in sugar conversion occur after the primary refining than the secondary refining. This agrees with the PFI lab scale data that shows a lesser improvement and eventually a maximum in sugar conversion at higher refining energies. It should be mentioned that at relatively low enzyme charges (5 FPU/g) total sugar conversions of over 70% can be reached through the mechanical refining of a high yield (85% total biomass yield on pulping) hardwood sodium carbonate pretreated biomass. This corresponds to a ~60% sugar recovery based on wood assuming equivalent loss of components during pulping.

Despite a higher energy intensity, secondary refining did not significantly increase sugar conversions above the primary refined pulp for long enzymatic hydrolysis times, Figure 4-4. Thus, secondary refining and its associated energy utilization may not be required for practical application to increase enzyme hydrolysis, especially for longer enzyme
hydrolysis times. In fact, the $\eta$ and $\eta_i$ are very high for the primary industrial refiner and are decreased after the application of the secondary refiner to the pulp, Table 4-3. Economic considerations could be used to determine the tradeoff between increased power costs in refining versus capital costs in larger vessels for longer hydrolysis retention times. Similarly, there can be tradeoffs between the amount of mechanical refining and the amount of enzyme charged in the hydrolysis process.

4.4.5 Economic Consequence of Refining

To investigate the economics of incorporating mechanical refining at the industrial scale a specific case based on an economic analysis of hardwood green liquor pretreatment for bioethanol production (Gonzalez et al. 2011) was considered. For this case, the biorefinery consumed 450,000 BD ton/year and produced 322 L ethanol/BD ton of input biomass.

The installed capital cost for the mechanical refining equipment was estimated at $5 million (straight-line depreciation over 10 years; $0.00345/L or $0.0131/gal). The operating cost to run the refiner at 67.0 kWh/ton (note this is not an optimized refining energy for biofuel production) with a purchase price of electricity of $0.065/kWh would be $0.0115/L or $0.0435/gal. These costs are small compared to the total estimated bioethanol production cost of $0.838/L or $3.15/gal. Based on improved digestibility of the biomass due to refining, it is estimated that this would allow for a 15% reduction in enzyme use at the same conversion with a savings of $0.0219/L or $0.0825/gal. This is based on an estimated cost of enzymes at 5 FPU/gram of cellulose for unrefined biomass of $0.146/L or $0.55/gal. The net
savings by incorporating refining is estimated to be $0.00695/L ($0.0259/gal) or approximately one million dollars per year, suggesting that mechanical refining can reasonably improve the economics with minimal operating costs and capital expenditure. Other benefits of refining that could be exploited include higher sugar yields or lower pretreatment costs at a constant enzyme charge. Further research needs to be performed to optimize refining conditions and determine if further savings are possible.

4.4.6 Correlating sugar conversion and WRV

The sugar conversions in general increase with WRV for several different types of pulps analyzed in this laboratory with different lignin contents are shown in Figure 4-6. Previous research on refiners with different fiber cutting propensity has shown that biomass digestibility does not correlate as well with fiber length, and therefore WRV is used herein to compare the effect of refining on wood pulps with different pulp chemistries (Jones et al. 2013). In addition to the WRV, the chemistry and/or fiber morphology also plays a significant role in determining sugar conversion. Much of the scatter within fiber groups in Figure 4-6 are due to different lignin contents, a strong example is the hardwood Kraft data which is due to the lignin contents of these pulps ranging from 5-20%, a significant range.
Figure 4-6. Total sugar conversion at 5 FPU/g-OD biomass and 48 hours versus WRV for various types of pulps. Legend indicates: the fiber type-refining method, [lignin concentration in %]. For fiber type; HW: hardwood, SW: softwood, GL: pulped with green liquor at a total titratable alkali in % indicated, and SC: pulped with sodium carbonate. Each color represents different biomass pretreatments and therefore different pulp chemistries. Abbreviations for the refining method used; PFI: PFI lab refining, VB: Valley Beater lab refining, ind: industrial disk refining, pilot: pilot disk refining. The dotted trend line is a logarithmic fit of softwood pretreated biomass (SWGL20). The solid trend line is a fit of all of the results with hardwood biomass having an approximate lignin content of 20-30% (i.e. HWSC, HWGL16, and HWKraft-20%). The HWSC data was generated from this research. The HWGL16 and HWKraft data sets were collected from previous research (Jones et al., 2013). The dashed trend line is for Kraft pulps that were fully bleached (Chen et al., 2012a) or had 5% lignin or less (Jones et al., 2013).

As very well known, softwood is more recalcitrant than hardwood at the same lignin content. It is shown that the softwood pulp has much lower sugar conversion than the hardwood pulp both produced using the green liquor process even at the same WRV and lignin content. Also sugar conversion is higher for Kraft hardwood pulp than green liquor
hardwood pulp even at the same WRV. The Kraft hardwood pulps with high sugar conversions had lower lignin content than the green liquor hardwood pulp. When the Kraft hardwood pulps (around 20%) had similar lignin contents to the green liquor pulps (25%) at the same WRV, the sugar conversions were similar.

The solid trend line drawn in Figure 4-6 was a logarithmic fit of all hardwood pulps with lignin contents in the range of 20-25%. Data along this trend line for the different pulping processes overlap, indicating that these pulps all are responding to changes in WRV in a similar manner. Note that the lab scale refined (PFI) sodium carbonate pulps with very high refining energy depart from this trend line presumably due to some unspecified effect of over refining not captured by WRV.

The results in Figure 4-6 also summarize lab, pilot scale and industrial refining. All three of these refining methods result in data that are generally consistent with one another in the sugar conversion versus WRV map (for hardwood pulps with lignin contents in the range of 20-25%). This indicates that each refining method is impacting the sugar conversion through a similar mechanism which relates to changes in WRV. Thus, knowledge acquired with lab scale and pilot scale experiments can be used to assist in the development of improved industrial scale refining. In the case of pilot scale disk refiners, many variables of interest, such as disk speed, consistency, temperature, plate gap width and plate design can be probed more conveniently than at industrial scale.

It is of interest to note that the bleached Kraft pulps include hardwoods, softwood, and copy paper with a blend of hardwood and softwood. It appears that the extensive lignin
removal in Kraft pulping and bleaching has rendered the materials to be highly digestible and 
as such the sensitivity to refining as reflected by WRV is not as pronounced as for biomass 
with less delignification. However, it is maintained that increases in WRV due to refining 
still has some positive influence on digestibility under these conditions.

The plateau in enzymatic hydrolysis improvement at high refining levels has been 
previously reported and attributed to the creation of small pores that do not increase enzyme 
accessibility (Chen et al. 2013). WRV is an indicator of the total pore volume that can be 
occupied by water, and is related to the biomass cellulase accessibility and the Simon’s stain 
dye absorption ratio (Luo et al. 2011). Water is a very small molecule which can penetrate 
into almost all of the pores of the fiber, whereas cellulase enzymes, are much larger and can 
only reach the external surface and other large pores of the biomass substrate. This can 
explain the flattening of the total sugar conversion versus WRV response curve, Figure 4-6. 
Although the WRV is increasing with more severe refining, the mechanical refining action 
could be collapsing large pores or creating small inaccessible pores that would not benefit the 
sugar conversion at overly high refining intensities. This phenomenon can also be observed 
in the plateau of the kinetic curves for high severity lab scale refining, Figure 4-1. Further 
research in this area of biomass pore structure is needed to prove this effect as experienced 
during mechanical refining.
4.5 Conclusions

Refining increases the biomass digestibility for lignocellulosic materials from wood relative to the unrefined material. This was true for industrial refining of high yield pulps at an energy input of 67.0 kWh/t. There is a maximum in the sugar conversion with respect to the amount of refining energy. Sugar conversion strongly correlates with water retention value and fiber length. WRV is a good predictor of improvements in sugar conversion due to refining for a given fiber source/composition. Improvements in biomass digestibility with refining due to lab, pilot plant and industrial refining were similar with respect to WRV.

4.6 Acknowledgements

Special thanks to United States Department of Agriculture as a part of the National Needs Foundation for providing the funding for this research (Grant number 2010-38420-21828), to Georgia Pacific for providing the biomass, and Novozymes for providing the enzymes.
4.7 References


5 CHAPTER FIVE: OPTIMIZATION OF PILOT SCALE MECHANICAL DISK REFINING FOR IMPROVEMENTS IN ENZYMATIC DIGESTIBILITY OF PRETREATED HARDWOOD LIGNOCELLULOSICS

5.1 Abstract

Mechanical refining has potential application for overcoming lignocellulosic biomass recalcitrance to enzyme hydrolysis and improving biomass digestibility. This study highlighted the ability for a pilot scale disc refiner to improve the total carbohydrate conversion to sugars from 39% (unrefined hardwood sodium carbonate biomass) to 90% (0.13 mm gap, 20% consistency, ambient temperature) by optimizing the refining variables. The different biomass properties that changed with refining indicated the expected increase in sugar conversion. Controlling the refining parameters to narrower gaps and higher consistencies increased the resulting refined biomass hydrolysis. Positive correlations that increases in net specific energy (NSE) input and refining intensity (SEL) improved the enzymatic hydrolysis. In some severe cases, over-refining occurred when smaller gaps, higher consistencies, and more energy input reached a point of diminished return. The energy input in these scenarios, however, was much greater than realistically feasible for industrial application. Although well-established in the pulp and paper industry, gaps in understanding the fundamentals of refining remain. The observations and results herein provide the justification and opportunity for further mechanical refining optimization to maximize and
adapt the mechanical refining technology for maximum efficiency within the process of biochemical conversion to sugar.

5.2 Introduction

5.2.1 Biorefinery Concept

The biorefinery concept, defined as “the sustainable processing of biomass into a spectrum of marketable products and energy,” is an approach that can mitigate the negative environmental impacts of fuel and chemical production by providing alternatives to oil refining products (Cherubini 2010).

Lignocellulosic biomass is an extremely diverse class of material and has a complex structure composed of cellulose, hemicellulose, and lignin naturally constructed in a multifaceted matrix. Understanding the chemical and biochemical challenges based on lignocellulosic biomass recalcitrance, which can be defined as the natural resistance to biological deconstruction, remains one of the major technical barriers to the commercialization of second generation cellulosic biorefineries (Zhao et al. 2012). Embracing the challenge as an intentional practice of engineered biomass deconstruction allows for an opportunity to harvest the inherently useful properties of biomass for creating value-added products through the biorefinery concept.

Many innovative technologies have been developed to address this issue and improve the biomass hydrolysis yield. The issue with these technologies is that they often require expensive direct operational costs or they have not been evaluated at scale, which makes
these technologies inherently carry more risk and often become unfeasible to implement in commercial biorefineries. Mechanical refining is one technology that has been demonstrated to be reliable in the paper industry and has been shown in many laboratory and pilot scale cases to reduce the pretreatment severity, improve enzymatic hydrolysis, reduce the enzyme dose, and provide additional benefits like biomass homogeneity and decreased particle size with increased surface area. These all have the potential to provide notable cost and operational benefits for a biorefinery.

5.2.2 Industrial Pulp and Paper Refiner Processing Variables

This mechanical refining technology is well known in the pulp and paper production industry and can be applied before or after pulping to mechanical and chemical pulps. It is important to distinguish between these two types of applications of mechanical operations and their historical uses.

5.2.2.1 Mechanical size reduction

Many processes are involved in wood handling to prepare biomass for chemical pretreatment, such as debarking to strip the bark from the round wood and clean the logs, chipping to reduce the round wood into wood chips, and chip screening to separate out the oversize and fine wood pieces that can be reprocessed to create a chip with suitable thickness for optimized chemical impregnation properties (Smook 2016). Oversized chips can be pulverized using a hammermill or wood crushers to further reduce the particle size and increase the surface area. These unit operations are important upstream pre-processing
operations for process control in a pulp mill and in a biorefinery, but are not considered alone as mechanical pretreatment options in this study.

5.2.2.2 Mechanical pulping

Traditional mechanical pulping is performed on raw uncooked whole log biomass or biomass chips and utilizes high mechanical energy input to achieve individual fiber separation. Stone ground wood pulping (SGW), in which wood is pushed against a coarse stone to physically separate fibers, was one of the first inventions that combined with the Fourdrinier paper machine allowed the use of woody biomass for pulp and paper production. Pressurized ground wood (PGW), like SGW, can operate at even higher temperatures and pressures utilizing hydraulically loaded pockets. Refiner mechanical pulping (RMP) feeds biomass between rotating discs to break chips into individual fibers. When the mechanical pulping is combined with temperature and chemical impregnation it is called thermomechanical pulping (TMP) and chemical thermomechanical pulping (CTMP), respectively.

5.2.2.3 Mechanical refining

Conversely to mechanical pulping, fiber separation can also be achieved with chemical pulping (sometimes called pretreatment within the biorefinery process) using heat and chemicals to selectively solubilize lignin which binds lignocellulosic fibers together. Traditional chemical pulping in the pulp and paper industry is followed by three phases of mechanical refining;

1) blow-line or hot stock refining – breaks apart cooked chips into fiber bundles,
2) de-shive refining – further separates fiber bundles into fibers, and

3) stock preparation or paper machine refining – has been optimized for fiber development.

The mechanical refining during stock preparation is designed to balance the refining mechanisms of a) external fibrillation, b) internal delamination or swelling, and c) fiber cutting or shortening. Goals of refining are typically to increase fiber flexibility and fiber bonding surface areas to improve the strength and optical properties of the final paper product, while minimizing energy consumption and reducing fines production to avoid drainage issues on the paper machine. These characteristic goals of mechanical refiner operation and plate design for a pulp and paper mill contradict those desired in a biorefinery. This difference in technological design reveals a major gap and an opportunity to improve mechanical refining operation and design for biorefinery specific applications.

Some mechanical refining operating variables include refining consistency, temperature, plate design, and refiner speed. It has been shown that changing the refining consistency, from low 1% consistency refining to a medium consistency of 4%, has significant impacts on fiber water retention value (WRV%), refining energy consumption, and refining intensity (Olejnik 2013). Optimization of refiner plate design can reduce energy consumption, increase production rate, and improve pulp quality in a pulp refining process (Musselman et al. 2007). Plate design changes in combination with optimized rotational speed can control the fiber residence time in the refiner and impact important fiber properties for strength and smoothness (Kure et al. 2000). The study also demonstrated that high
intensity plate designs combined with high rotational speed could provide significant energy savings.

Other research in the pulp and paper industry has investigated the impacts of enzymatic treatments prior to mechanical refining applications. It has been shown that cellulase-assisted refining of dried eucalyptus pulp can overcome hornification effects, and in some cases increase the binding and strength properties of the paper compared with the never-dried eucalyptus pulp control (Garcia et al. 2002). The research also demonstrated that significant energy savings could be realized by adding cellulase enzymes prior to refining. Enzyme treatments of ~350 g/OD-metric ton wood have been shown to significantly reduce the refining energy (24% reduction compared to the double impregnation positive control) on alkaline pretreated mechanical pulp without negatively impacting the percent shives content, the bulk density, the opacity, and the tensile strength any more than a second impregnation positive control (Hart et al. 2009). These refining energy reductions were achieved without significant increase in soluble sugar concentrations in the hydrolysate. Another thermomechanical pulping evaluation with pectinase enzyme application when applied prior to mechanical refining realized enhanced fiber quality based on tensile index, tear index, and specific surface area, all with reduced total specific energy consumption (Sabourin et al. 2010). Other cellulase and beta-glucosidase biotechnology solutions have been shown to increase the drainage rate and degree of fiber hydration when combined with mechanical refining (Gil et al. 2009). These improvements might also impact fiber homogeneity, allowing for better pumpability and process control. Again, all of these biomass performance
metrics are not directly applicable to the biorefining industry, as the applications were optimized to reduce fiber degradation.

5.2.3 Benefits of Mechanical Refining in the Biorefinery Concept

5.2.3.1 Increased hydrolysis efficiency

Previous work on the use of mechanical refining to improve the hydrolysis efficiency of lignocellulosic biomass has shown promise for the technology’s use in the lignocellulosic biorefinery industry. The effectiveness of mechanical refining to improve enzymatic hydrolysis of industrially relevant hardwood sodium carbonate pretreated biomass has been shown (Jones et al. 2014). Its effectiveness suggests the opportunity for the optimization of mechanical refining conditions at the pilot scale to maximize the biomass digestibility.

Mechanical refining can be applied to untreated biomass alone or before pretreatment. This is oftentimes called mechanical pulping, or mechanical size reduction, or grinding, as discussed in the previous section, and this step generally requires higher energy input. When applied after the pretreatment and before enzymatic hydrolysis, the mechanical refining post-treatment step is called wet milling or disk refining, and it can be performed at high or low consistency. This post-treatment mechanical refining technology can provide benefits when used in combination with many different biomass substrates and pretreatment technologies, including; hot-water pretreatment (autohydrolysis), acid pretreatment (dilute sulfuric acid), alkaline pretreatment (AFEX, green liquor, kraft, SPORL), and steam explosion among many others (Zhu et al. 2012).
Steam explosion is a technology that has shown many promising applications for lignocellulosic biomass pretreatment (Jacquet et al. 2015). High pressure steam is used to control the relatively high reaction temperatures (165-205°C), sometimes catalyzed in combination with chemicals (0.5-4.5w/w% SO₂, or 0.7-2w/w% H₂SO₄), that in a relatively short time (1.5-7.5 min) prepares the biomass for fiber separation (Olsen et al. 2012). A rapid depressurization step at the end of the steam explosion pretreatment helps to achieve biomass fiber separation, reduced particle size, and increased surface area. These biomass property changes that impact its accessibility to cellulases are similar to those benefits in fiber characteristics achieved with mechanical refining (Lu et al. 2002). Steam explosion uses high potential energy transfer from pressurized steam, instead of the physical transfer of energy to the biomass with mechanical refining.

It should be noted that steam explosion pretreatment is an interesting alternative to the combination of pretreatment and mechanical refining, but like all other second generation biofuel pretreatment technologies it must be justified techno-economically at industrial scale. Each pretreatment has different operating conditions and energy requirements, along with varying benefits and challenges (i.e. inhibitors, carbohydrate yield, operating cost, process complexity, and capital requirements, etc.). It is expected that when applied in combination, mechanical refining can allow further optimization of the pretreatment operating conditions to extract the maximum benefit from each pretreatment technology. For example, pilot scale pressurized mechanical refining has been combined with continuous steam explosion to provide an overall sugar yield of 88% (Fang et al. 2011).
Woody biomass mechanical refining and size reduction applications have been evaluated without or prior to pretreatment, and these applications require more energy input compared with mechanically refining post-pretreatment of woody biomass (Zhu et al. 2010). This increased energy input is attributed to not benefiting from the softening that occurs during the chemical impregnation and solubilization of material in woody biomass pretreatment. Therefore, all discussions herein are related to mechanical refining as a post-treatment to traditional woody biomass pretreatment.

The use of mechanical refining in a lignocellulosic biorefinery has also been studied for many different biomass and pretreatment types (Park et al. 2016). Sulfur dioxide free steam pretreated hybrid poplar achieved a 32% improvement in hydrolysis efficiency after 30 min of low consistency valley beater refining, comparable to the high severity steam plus sulfur dioxide treatments (Dou et al. 2016). Used in a sodium lignosulfonate biorefinery concept, PFI milling (Papirindustriens Forskningsinstitut, PFI – the Norwegian Paper and Fibre Research Institute), and disk refining were both shown to boost the total sugar yields of modified alkaline pretreated corn stover, from 72% to 79% (Xu et al. 2015). Although increased sulfonation charges improved enzymatic hydrolysis, the refining improvement compared with the unrefined pretreated biomass was constant, relating to a constant pretreatment severity of 11 wt% sodium hydroxide cooked for 40 min at 120 °C. In another study, sugarcane bagasse was autohydrolyzed at different temperatures and pressures (Batalha et al. 2015). All treatments received the same level of lab scale PFI refining (6000 revolutions), with the greatest improvements (from 72% to 84% total sugar recovery) and
highest overall sugar recovery occurring at the lowest pretreatment severity investigated (180 °C, 20 min).

5.2.3.2 Enhanced process optimization

There is a trend that mechanical refining tends to have the greatest improvement at low to medium severity pretreatments, allowing for savings in operating costs from required pretreatment chemicals and hydrolysis enzyme loadings. These savings further provide the opportunity to optimize the mechanical refining conditions at pilot scale for multiple pilot scale pretreatment conditions. The goal for the treatment of lignocellulosic biomass in a biorefinery should be optimized mechanical refining conditions for biomass deconstruction and maximum biomass digestibility, with minimum energy consumption and improved biorefinery operation (reduced viscosity or improved pumpability, based on the inherent shear thinning non-Newtonian behavior of wood pulp fibers).

Biorefineries can realize major benefits of mechanical refining through improved process yield and sugar recovery, or, for the same carbohydrate conversion level, mechanical refining can decrease the necessary enzyme loading, reduce the pretreatment severity, or shorten the required hydrolysis time. All of those benefits create cost savings through reduced operating costs, less yield loss due to sugar degradation and inhibition, or lower tank volume and capital costs, respectively (Jones et al. 2014).

5.2.3.3 Improved process control

Another benefit of mechanical refining is that it provides a mechanism for consistent process control and homogenization of the biomass. Sometimes there can be variations in the
biomass properties of the incoming raw biomass and pretreated material, but the operating consistency throughout the biorefinery may be improved by monitoring certain parameters before and after mechanical refining. In addition to its ability to reach the maximum theoretical conversion with mechanical refining, the technology should be evaluated for its ability to consistently control and monitor the process conditions and biomass properties within the biorefinery. Xu et al. quantified the changes that occurred during the mechanical refining of alkaline pretreated corn stover, and they identified correlations between these changes and enzymatic hydrolysis conversion (Xu et al. 2014). Their work allowed for the prediction of the downstream hydrolysis efficiency and increased effective control of the biorefinery process, based on upstream real time mechanical refining targets.

5.2.4 Why Lab and Pilot Scale Are Different

It is well known in the engineering disciplines that there are considerable scale up implications when evaluating a technology at lab (bench) scale versus pilot (demonstration) scale and full industrial production scale. Once a technology concept has been identified and proven at the lab scale, it is important to extend those research learnings and begin optimization at a larger/pilot scale. At the pilot scale, a technology like mechanical refining can give more realistic representations of how the unit operation will function at production scale, which can significantly minimize the risks associated with technological failure of the full biorefinery concept. The information gathered during pilot plant optimization efforts can facilitate effective full scale implementation by identifying and avoiding potentially costly full scale issues.
This need for the evaluation of mechanical refining at a continuous commercial scale has been identified by other groups as well, using a mechanical refining treatment such as the Szego Mill. This is a continuous high consistency orbital milling device, developed at the University of Toronto, capable of continuous wet or dry grinding of material to reduce particle size with low power consumption (Trass et al. 1990). This device has been evaluated at the demonstration scale as a mechanical post-treatment refining for low severity pretreated corn stover. It has been shown that after just one pass through the Szego Mill, the 168-h enzymatic hydrolysis increased from approximately 82% to 91% glucose conversion compared with the unrefined control (Chen et al. 2013). After four passes, the glucose conversion increased to over 95% near the theoretical maximum. This successful trial with the compression-type Szego Mill refining was expounded upon and evaluated in a multistage deacetylation pretreated corn stover with high solids Szego milling (X. Chen et al. 2015). Combining the Szego Mill compression refining (128 kWh/t disk refiner, 68 kWh/t 10% Szego pass 1, 85 kWh/t 10% Szego pass 2; 281 kWh/t total refining energy) with a disk refiner achieved a glucose conversion of 90% from 69%, compared with the unrefined deacetylated corn stover control.

These results bring important and encouraging findings to design and the feasibility of mechanical refining applications at the pilot scale. They display the application of low severity pretreatment followed by mechanical refining to achieve near maximum theoretical carbohydrate conversions. This technology, however, has not yet been optimized at the pilot scale or evaluated for other types of pretreatment or refining technologies. It should be noted
that a multiple tube vibration mill woody biomass pulverizer required 1200 to 2600 kWh/t at
the lab scale, with an estimated minimum 280 kWh/t requirement for large-scale
pulverization performance (Kobayashi et al. 2015). This necessity demonstrates the energy
savings that can be realized at pilot and commercial scale, but reiterates the large energy
demand when mechanical refining is applied prior to biomass pretreatment.

5.3 Motivation and Objectives

In this work, many biomass properties of high kappa (lignin content) chemical pulp
were monitored as metrics for explaining the improvement due to refining on enzymatic
hydrolysis. The effects of pilot scale mechanical refining temperature, plate gap width, and
biomass consistency were evaluated for their effectiveness for optimizing engineered
biomass deconstruction and subsequent enzymatic hydrolysis digestibility. Changing the
operating parameters allowed for varying the mechanical refining intensity. The impact of
these operation variables on mechanical refining energy and biorefinery costs were also
explored. In addition to the implications of the primary optimization parameters, pilot plant
scale work can provide additional value by illuminating process control issues, by
experiencing process variations, and by forcing reliable standard operation procedures and
practices. These learnings are critical for the realization of mechanical refining in full scale
biorefinery applications. The objective of this study was to optimize the pilot scale
mechanical refining conditions and attempt to achieve the theoretical maximum in enzymatic
hydrolysis of industrially produced hardwood sodium carbonate (HWSC) pretreated biomass.
5.4 Experimental

5.4.1 Materials

The unrefined HWSC biomass used in this study was the same biomass as described in a previous study; see Table 5-1 (Jones et al. 2014).

Table 5-1. Carbohydrate and Klason Lignin Content of the Raw, Pretreated Unrefined, and Pretreated Biomass Used in This Study. 1) Minor wood carbohydrate contents not shown, 2) Acid Insoluble Residue, 3) Acid Soluble Lignin.

<table>
<thead>
<tr>
<th></th>
<th>Glucan</th>
<th>Xylan</th>
<th>Total Carb.¹</th>
<th>AIR²</th>
<th>ASL³</th>
<th>Total Lignin</th>
<th>Avg. Ash</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unrefined</td>
<td>43.0%</td>
<td>9.1%</td>
<td>52.1%</td>
<td>25.1%</td>
<td>3.4%</td>
<td>28.6%</td>
<td>2.4%</td>
</tr>
<tr>
<td>Pilot-150 °C</td>
<td>47.8%</td>
<td>10.0%</td>
<td>58.3%</td>
<td>23.6%</td>
<td>2.6%</td>
<td>26.2%</td>
<td>1.5%</td>
</tr>
<tr>
<td>Pilot-30 °C</td>
<td>47.4%</td>
<td>7.3%</td>
<td>54.7%</td>
<td>23.7%</td>
<td>2.8%</td>
<td>26.5%</td>
<td>1.7%</td>
</tr>
<tr>
<td>Ind-Primary</td>
<td>42.0%</td>
<td>7.6%</td>
<td>49.6%</td>
<td>25.6%</td>
<td>3.2%</td>
<td>28.8%</td>
<td>2.3%</td>
</tr>
<tr>
<td>Ind-Secondary</td>
<td>43.7%</td>
<td>8.1%</td>
<td>51.8%</td>
<td>26.7%</td>
<td>3.1%</td>
<td>29.7%</td>
<td>2.4%</td>
</tr>
</tbody>
</table>

The pretreatment or pulping yield was 84.5% with cooking conditions of 5.5 min at 190 °C. The unrefined HWSC biomass was used in 1-kg oven-dry (OD) batches for each pilot scale refining trial.

5.4.2 Methods

5.4.2.1 Mechanical Refining

Pilot scale refining was performed using a 12" Sprout Waldron Thermo-Mechanical Pulping (TMP) unit (Koppers Company, Inc., Model 12-1CP, Muncy, PA). Figure 5-1 shows the Sprout Waldron D2A-507-type plates that were used for three-zone fine refining in the optimization study. In general, these plates are regarded as low-severity refining plates. The D2A507 refiner plate (Andritz, Muncy, PA) is a 12” diameter, medium intensity plate with coarse interior defiberization zone and fine periphery refining zone. There are 14x8x3=336
bars in the periphery refining zone 1, and $6 \times 8 \times 3 = 72$ bars in the middle refining zone 2 of the refiner plate. The intensity of the plate is approximately 2.11 km/revolution. The refiner motor was a 60 HP and 1775 rpm Reliance Duty Master AC Motor (Cleveland, OH, USA). The agitator, screw feeder, and refiner plate speeds were all constant, at 18, 18, and 2950 rpm, respectively. The consistency was manually adjusted with water prior to loading the pulp into the refiner feed vessel. The gap size was controlled by setting the zero gap (touching/clashing the plates together) and then backing off the plates to the desired gap width. The gap width was calibrated by measuring the distance from the rotor plate to housing at 0, then backing off the plate in 100 unit increments (one full turn of plate gap adjustment wheel) and measuring the change in distance from the rotor plate to the housing (average measured 1 unit = 0.00108 in). Although there are uncertainties in the accuracy of this gap measurement based on plate wear and changes in plate properties during operation (i.e. plates expanding when heated), this method for adjusting the plate gap width is an acceptable standard for manual pilot refiner operation and allows for valid comparisons in plate gap width as more sophisticated and automated gap sensors and gap width control technologies have been developed (Alahautala et al. 2004; Eriksen et al. 2007).

The energy consumption was measured using a Schneider Electric energy meter and power integrator (Power Logic, PM 1200, Rueil-Malmaison, France). The no-load energy for the pilot scale refiner averaged 326 kWh/t, and it was measured by setting the refiner to the operating conditions (plate gap width and dilution water if applicable), then reading the integrated energy over a controlled time. In comparison, the no-load energy for an industrial
scale low consistency (normal operation ~5%) refiner is 25.4 kWh/t, based on industrially supplied PI System™ process information data sourced from the time when the pulp samples were collected from Georgia Pacific. Due to the natural instability in the pilot scale refining operation, which can impact the final biomass quality, each experiment was performed for a controlled time of 5 minutes to ensure that the entire batch had passed through the refiner. Because of substrate limitations, each experiment was conducted as an individual replicate for proof of concept purposes, and any conclusions made herein consider this replication limitation.

The temperature was adjusted using direct steam at the desired saturated steam pressure (6 psig and 55 psig, 110°C and 150°C respectively) when needed. The temperature was stabilized for 5 minutes prior to initiating the screw feeder and refiner. The time was recorded from when the screw feeder was started to when the feed vessel was empty, and the energy was integrated over that time and used to calculate the kWh/t for each refiner trial.

This process gave almost 100% yield with respect to the starting material based on experiments performed with agricultural biomass at similar conditions yielding 80 to 95% (Gonzalez et al. 2011); however, the biomass refining yield has not been validated for these trials. It is expected that pilot refining yield losses will occur from fines creation and losses and increased temperatures causing biomass degradation and small carbohydrate solubilization. These yield losses will be further reduced at industrial scale with continuous operation, no washing, or no solid-liquid separation prior to enzymatic hydrolysis.
Figure 5-1. Detailed diagram of the D2A-507 Ni-hardened plate geometry used during the optimization of mechanical refining for improved lignocellulosic enzymatic hydrolysis

It was observed that under some conditions there was more pulsing, and this caused challenges for refiner control. This was most common when no feed dilution water was used at higher temperature and higher refining consistency conditions. It is expected that this pulsing effect was caused by the combination of plates heating up over time and causing the metal of the refiner plates to expand. This expansion reduces the effective plate gap width compared to the starting gap and slows the flow of the biomass through the refiner. The pulsing effect is likely caused by biomass build-up at the refiner entry point from the biomass screw feeder reaching a critical pressure to periodically forces biomass through the refiner zone releasing the back pressure from the biomass screw feeder, then repeating the cycle.
5.4.2.2 Enzymatic hydrolysis and sugar analysis

Sugar analysis was carried out following a modified National Renewable Energy Laboratory Analytical Procedure, as described in previous works (Jones et al. 2013). Enzymatic hydrolysis of the unrefined and refined biomass was performed at 5 FPU/OD g of substrate Novozymes Cellic® CTec2 cellulase enzyme and 1/9 v/v Cellic® HTec2 hemicellulase enzyme (Novozymes NA, Franklinton, NC), as described by Jones et al. (2013). The detected carbohydrates were glucose and xylose, and solutions with mixtures of known sugar concentrations were measured to generate a calibration curve for each sugar. The minor sugar concentrations of galactose, arabinose, and mannose were below the detectable limit and therefore not reported. The biomass digestibility was reported as sugar conversion:

\[
\text{Sugar conversion (\%) = total sugar released (g) / carbohydrate content after pretreatment (g) \times 100%}
\]

5.4.2.3 Biomass properties and characterization

Water retention value (WRV) is defined as the water absorbed by the substrate relative to the oven-dried mass of the substrate (TAPPI 2011). For measurement of the WRV, a quantity of approximately 0.30 OD g of sample was placed into a centrifuge filtering tube (Amicon Centriplus, Millipore Corp., Bedford, MA) with a glass frit (25 mm, med porosity
borosilicate fritted disc, Chemglass, Vineland, NJ) and centrifuged for 30 min at 0.9 relative centrifugal force (RCF = rw^2/g), or the acceleration (radius times the square of the angular velocity) relative to gravity. The basis weight of the final pads was approximately 700 g/m^2. The recorded weights of the wet centrifuged sample (m_1) and the overnight oven-dried at 105 °C sample (m_2) were used to calculate the WRV:


\[
\text{WRV (g/g)} = \frac{(m_1-m_2)}{m_2}
\]

A HiRes Fiber Quality Analyzer (FQA) from OpTest Equipment Inc. (Hawkesbury, ON, Canada) was used to measure fiber length and percent fines (1999). Well-mixed dilute fiber suspensions (~1 mg/L) of the pulp samples were prepared. The fiber length was the true contoured length of the fiber and was reported as the length weighted average (L_w). The fines fraction had a de-emphasized effect on the L_w average. Fines are defined as any particle greater than 0.07 mm (default HiRes FQA detection limit) and less than 0.2 mm in length. The CCD detector camera has a resolution of 7 μm/pixel for width and 14 μm/pixel for length, and fines are reported as a length weighted average of the sum of the number of fine particles counted times the fiber class length, divided by the total fiber length. It is understood that the length is proportional to the weight for a given fiber coarseness (TAPPI T234 cm-02 2002). The fiber quality results are reported as follows:
Equation 5-3. Determination of biomass fiber length.

\[
\text{Mean Length-weighted Fiber Length} = L_w = \frac{\sum n_i L_i^2}{\sum n_i L_i}
\]

Equation 5-4. Determination of biomass fines content.

\[
\text{Percent Length-weighted Fines} = \%F_1 = 100\% \times \frac{\sum n_i L_i}{L_T}
\]

5.4.2.4 Refining energy calculations

The amount of energy input during mechanical refining is an important factor in economic viability, as well as a main parameter to estimate the amount of work done on the fibers. The energy transferred to the pulp is considered as “specific energy,” and it does not include the energy required to run the refiner. The net specific energy input, \(NSE\), can be calculated by the following equation,

\[
NSE, \left(\frac{\text{kWh}}{\text{t}}\right) = \frac{\text{Net load}}{\text{Dry mass of fibers per hour}} = \frac{\text{Refining Load–No Load}}{\text{Dry mass of fibers per hour}}
\]

where the mass of fibers can be calculated by multiplying the consistency of pulp by the volumetric flow rate of pulp into refiner. The applied energy is dependent on two parameters: refining intensity and number of bar impacts. Refining intensity is controlled by motor load during the process, and the load is a response variable to the width of the refiner plates gap. The “refining intensity” \(I\), also known as the “specific edge load” (SEL), can be
calculated based on the applied power divided by the product of rotating speed and edge length, which is shown in the following equation,

Equation 5-6. Mechanical refining intensity or specific edge load.

\[
SEL \text{ or } I, \left(\frac{W_s}{m}\right) = \frac{\text{Refining Load\textendash No Load}}{\text{RPM} \times \text{Bar Edge Length} \times \text{rpm} \times 60 \text{ s}}
\]

where the “Bar Edge Length” (BEL), sometimes referred to as the “Cutting Edge Length” (CEL), is a property of the specific plate design expressed in units [km/rev] and is the total length of bar edges the fibers will experience in one revolution. The revolutions per minute (rpm) is simply the refiner speed controlled by the motor. Additionally, a parameter independent of energy for refining can indicate the number of impacts (N) or deformations per unit mass, and can be calculated by the following equation:

Equation 5-7. Determination of the normalized number of mechanical refining impacts.

\[
N, \left(\frac{\text{km}}{\text{ton}}\right) = \frac{\text{RPM} \times \text{Bar Edge Length} \times 60 \text{ min} / \text{hr}}{\text{Dry mass of fibers per hour}}
\]

The refining energy (NSE) is related to the product of refining intensity (I) and the number of impacts (N):
Equation 5-8. Relationship between refining net specific energy, refining intensity, and number of impacts.

\[ NSE = I \times \left( \frac{1000m/km}{3600s/hr} \right) \times N \]

Figure 5-2. NCSU full detail pilot scale refining apparatus. The unrefined biomass was fed via the screw feeder into the refining zone, and the refined biomass was collected in the bottom vessel.
Plate design, consistency of pulp, and rotational speed of the motor are also process variables that affect the applied energy on the pulp. The number of bar impacts during refining is closely related to the flow rate of the pulp through the refiner, as previously mentioned. Again, plate design and rotational speed are also process variables affecting the number of bar impacts on the pulp. For the pilot scale refining system in this study, the plate design was the same for all experiments (D2A-507, 2.11 km/rev). The unrefined biomass was loaded into a hopper for mixing above the screw feeder, fed through the center of the stationary plate (stator) into the refining zone, and dropped down via gravity and centrifugal force into the collection vessel, Figure 5-2. The refining action was controlled by the gap distance of the rotating plate (rotor) from the stator.

Refining intensity can also be considered based on the normal force exerted on the biomass by the refiner, but differences in rheological behavior of pulp slurries at low and high consistency make it challenging to characterize the refining action (Kerekes 2011). During low consistency refining the refining operation is more continuous, and there is a much higher probability of fiber-to-bar impacts. This makes SEL a good estimate for refining intensity based on energy per mass per impact, and it can be even more accurately described with the C-factor derived from refining zone geometries (Kerekes 1990). This relationship becomes more complicated as the refining consistency increases, and in addition to fiber-to-bar interactions there, more fiber-to-fiber interactions are probable, which cause different fiber forces such as compression, bending, surface shear, and tension. High consistency
refining theory must consider the compressibility and coefficient of friction (viscosity) of heterogeneous pulp suspensions.

Simplifying the complex relationship of the refining action based on the force exerted on the biomass – with constant plate geometry and unrefined fiber characteristics – shows the normal force on fibers \( f_n \) is related to the biomass consistency \( C_s \), the biomass coefficient of friction \( \mu_E \), refining plate gap width \( T \), and refining intensity (SEL) by (Kerekes et al. 2006):

\[
f_n \propto \frac{C_s}{T} \left( \frac{\text{SEL}}{\mu_E} \right)^{0.7}
\]

Equation 5-9. Relationship of biomass properties and mechanical refining variables to the force exerted on the biomass during refining.

5.5 Results and Discussion

5.5.1 Effect of Refining Temperature on the Efficiency of Enzymatic Hydrolysis

Hardwood sodium carbonate pulp was refined using a 12-in pilot scale refiner at a constant gap (0.050 mm) and consistency (30% initial consistency with 4 L/min dilution, 4.3% final refining consistency) to understand how the pre-steaming temperature affected the refining improvement for enzymatic hydrolysis. All cases of pilot scale refining at different temperatures improved the total sugar conversion compared with the unrefined control Figure 5-3. The average total sugar conversion improvement (21.6%) was similar to the improvement shown by industrial scale refining. The overall biomass digestibility, however,
slightly decreased with increasing temperature. This indicated that lignin redistribution above the lignin glass transition temperature ($T_g$) reduced the enzymatic hydrolysis efficiency. The decrease in enzymatic hydrolysis was most pronounced at the highest temperature of 150 °C. It was hypothesized that the reduction in enzymatic hydrolysis efficiency between 110 and 150 °C was caused by the fluidization and redistribution of lignin throughout the biomass matrix. It was expected that this dynamic change in the state of the biomass during mechanical refining would alter the pores created during refining, inhibit the enzyme accessibility, and lower the enzymatic hydrolysis (Yu et al. 2014).

Figure 5-3. Effect of pilot refining temperature on HWSC digestibility, comparing “unrefined”, “pilot-temp”, and industrial scale “primary” and “secondary” refining. Unrefined biomass had no temperature control and the industrial scale refining temperature was unknown. Increasing temperature from ambient (30 °C) to 110 °C and to 150 °C showed a decrease in total sugar conversion, which was most pronounced at 150 °C.
This phenomenon was supported by the observation of a glass transition of isolated and dried steam pretreated hardwood lignin between 113 and 139 °C (Lora et al. 2002). Lignocellulosic material in the water saturated conditions will exhibit glass transition temperature depression, due to water acting as a plasticizer within the polymer matrix (Salmén 1984). Dynamic mechanical analysis of wild type and transgenic aspen indicates that the thermal softening of in situ lignin ($T_g=84^\circ$C) is significantly impacted by reducing the total lignin content ($T_g=72^\circ$C) and changing the lignin structure by increasing the S/G ratio ($T_g=80^\circ$C), which both change after pretreatment (Horvath et al. 2011). It has also been reported that, based on the Williams-Landry-Ferry (WLF) time correspondence equation of laboratory derived measurements, the glass transition for in situ Pinus radiata softwood lignin occurs between 100 and 170 °C with the optimal temperature range for thermomechanical pulping from 130 to 135 °C, based on a deformation frequency of $10^{3.5}$ to $10^{4.5}$ Hz (Irvine 1985). Light microscope images further supported this hypothesis, as deposits of what appeared to be lignin within the lumen were observed in the 150 °C refined biomass, and were not seen in any of the unrefined, or ambient refined samples Figure 5-4.

To further understand this effect, the surface lignin content of the lignocellulosic biomass was evaluated using a method adapted by (Gray et al. 2010) and (H. Chen et al. 2015). X-ray photoelectron spectroscopy (XPS) measurement of carbon and oxygen signals of handsheets made from the unrefined and refined biomass (Table 5-2) indicated that there was a slight decrease in the calculated surface lignin in the 150 °C refined biomass compared with the ambient temperature refining and the unrefined control. This again agreed with the
statement that operating a mechanical refiner above the glass transition temperature of lignin allows the network biopolymer to become flowable and move within the biomass structure. This observation might be convoluted by other wood extractive compounds deposited on the fiber surface, and should be confirmed by removing any non-lignin extractives, which can contribute to high surface oxygen/carbon (O/C) ratios, with an acetone wash or benzene: ethanol extraction (Saputra et al. 2004). Although this might be a desired effect when removing lignin from the lignocellulosic biomass during pretreatment, it allows for lignin to redistribute and block potential substrate hydrolysis sites when limited wash water is available to facilitate lignin dissolution.
Figure 5-4. Light microscope images hardwood sodium carbonate pretreated biomass, including a) unrefined, and b) 5% cons, 0.05-mm gap, 150 °C, c) 20% cons, 0.03-mm gap, 30 °C, and d) 20% cons, 0.03-mm gap, 30 °C mechanically refined biomass. The arrows in Fig. 4b reveal material deposits (presumably redistributed lignin) in the 150 °C sample that were not seen in any other condition, explaining the reduction in 150 °C refined biomass digestibility compared with the other refined conditions.
Table 5-2. Surface Lignin Content of Hardwood Sodium Carbonate Samples Measured by XPS

<table>
<thead>
<tr>
<th></th>
<th>Unrefined</th>
<th>Pilot-30 °C</th>
<th>Pilot-150 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon (C)</td>
<td>66.3</td>
<td>65.3</td>
<td>64.2</td>
</tr>
<tr>
<td>Oxygen (O)</td>
<td>33.7</td>
<td>34.7</td>
<td>35.8</td>
</tr>
<tr>
<td>((N_0/N_C)_m)*</td>
<td>0.508</td>
<td>0.531</td>
<td>0.558</td>
</tr>
<tr>
<td>((N_0/N_C)_c)**</td>
<td>0.466</td>
<td>0.487</td>
<td>0.510</td>
</tr>
<tr>
<td>Segment mole fraction of Lignin (S_L)</td>
<td>63%</td>
<td>58%</td>
<td>53%</td>
</tr>
<tr>
<td>Weight fraction of Lignin (W_L)</td>
<td>66%</td>
<td>61%</td>
<td>56%</td>
</tr>
</tbody>
</table>

\* \((N_0/N_C)_m\): measured mole ratio of oxygen and carbon
\** \((N_0/N_C)_c\): corrected mole ratio of oxygen and carbon

Other reasonable explanations for the decrease in enzymatic hydrolysis with increasing mechanical refining operating temperature include: inhibitor formation from lignin and carbohydrate decomposition; changes induced by direct-steaming of the biomass to control the refining temperature (contaminants in the steam/condensate, variations in refiner operation under pressure to control the steam temperature); or, at higher temperatures, the biomass exhibited a lower relative viscosity resulting in less work successfully applied to the fibers during refining. It is, however, believed that the decrease was primarily explained by the softening and relocation of lignin deposits throughout the biomass matrix. Because the ambient refining temperature showed the best improvement compared with increased refining temperatures, all other pilot scale refining optimization experiments were performed at ambient temperature without pre-steaming.

5.5.2 Pilot Scale Refining and the Effects of Changing the Plate Gap Width

The unrefined HWSC biomass was refined with the 12” pilot scale disc refiner at ambient conditions and 5% refining consistency (starting consistency 30% with 4 L/min...
dilution rate in 1-kg batches, 4.3% final refining consistency after dilution). As the refining plate gap width decreased, the refining severity increased and the resulting improvements, in enzymatic hydrolysis, compared with the unrefined enzymatic hydrolysis increased, Figure 5-5. The greatest increase in digestibility, compared with the unrefined biomass and the other refining conditions, was seen at the smallest set plate gap with of 0.03 mm. There was a noticeable difference between 0.03 and 0.13 mm plate gap width refining conditions.

It should be noted that the average fiber width (0.020 mm) was at the lower end of this range, and decreasing the plate gap width towards the limit of the fiber width maximized the number of fiber-bar interactions. It was this concept of increased interactions between the fiber and the refiner plate that explained why the refining improvement was observed at the smallest refiner plate gap width. In general, more fiber-bar interactions were associated with the refining mechanism of fiber cutting or shearing and the internal delamination of the secondary cell wall structures within the fiber. This mechanism can be explained as a rapid compression/decompression mode where the fiber was repeatedly trapped between two bars (compression) and released into the grooves (decompression).

Although only one plate design was used for the pilot refining experiments, changes in plate design could alter the fiber-bar interactions and change the refining intensity. Wider bars and grooves created a more turbulent atmosphere in the refining zone. Surface and subsurface refiner plate dams between the refiner bars increased the biomass hold up and allowed for more fiber-bar interactions as the fibers passed through the refining zone.
Figure 5-5. Effect of refining gap size on the HWSC digestibility; legend indicates refining plate gap width in mm

Various biomass properties were plotted versus their respective refining plate gap width, Figure 5-6. The unrefined biomass was arbitrarily plotted at 1.27 mm plate gap width, which was the average unrefined HWSC biomass fiber length, for comparison with the refined HWSC biomass conditions. As the refining plate gap width decreased, there were increasing signs of refining action; the refining energy input increased, the fiber fines percentage increased, the length weighted average fiber length ($L_w$) decreased, and the WRV increased. These are all metrics that can be used to measure the changes in biomass properties before and after refining. It is expected that these changes will correlate with the improvement in enzymatic hydrolysis with mechanical refining.
Figure 5-6. Changes in refining metrics of HWSC biomass with respect to plate gap width. Unrefined biomass properties are plotted at 1.27 mm, which was the average length of the biomass and the gap width (in theory) at which no further significant refining action would occur.

5.5.3  **Pilot Scale Mechanical Refining and the Effects of Changing the Refining Consistency**

**Consistency**

Increasing the refining consistency was shown to increase the refining energy for the same refiner plate gap width, Figure 5-7. The trend continued for all refining consistencies; as the plate gap width narrowed, the refining energy increased. By increasing the mechanical
refining consistency, the effective fiber-fiber interactions were increased for each plate gap width. It is thought that this type of refining action contributed to the external fibrillation, or “rubbing” of the primary cell wall of the fiber.

Figure 5-7. Changes in pilot mechanical refining net specific energy (NSE) and refining consistency (5%, 10%, and 20%) and refiner plate gap width changes for HWSC refined biomass. Power trendlines are fitted to the data to more clearly illustrate the differences in the net specific energy input as the refining consistency and plate gap width changes.

There are important issues that should be considered when discussing the scale up implications of the low consistency refining process (3 to 5%) and the high consistency refining process (over 20%). Although both types of mechanical refining were compared as a
post-treatment to chemical pulping in this study, there is not one industrial mechanical refiner that can run optimally at low and high consistency. When discussing the differences between low and high consistency refining, the compressibility of high consistency pulp suspensions should be considered. Compared to low consistency refining where the refining zone is continuously full of pulp due to its ease in pumpability, high consistency refining and the high consistency biomass heterogeneity generate non-uniform refining intensities (kJ/kg/impact) and variable distributions of fiber and forces on high consistency refiner bars (Kerekés 2015). Despite good correlations in the experiments with different refining consistencies, and similarities in low and high consistency refining operation, the heterogeneity and variation in pulp behavior at different consistencies can convolute the results, making it difficult to interpret the impact of specific refining mechanisms on biomass digestibility.

5.5.4 Correlations of Biomass Digestibility with Changes in Mechanical Refining Conditions and Biomass Properties

There was a positive correlation between 48-h total sugar conversion and pilot refining net energy input, Figure 5-8. It should be noted that there was a slight decrease in total sugar conversion with increased refining after 1500 kWh/ton pilot refining net energy input (the most severe mechanical refining condition). It was expected that this over-refining phenomenon, observed during these refining experiments and others (Chen et al. 2013; Jones et al. 2014), can be explained by a collapsed pore structure that reduced the relative biomass accessibility to enzyme proteins. It was difficult to adequately determine with limited data if
the industrial refining improvement also followed the same trend as the pilot scale refining conditions. Therefore, the over-refining outlier and the industrial refining conditions were not included in the calculated trend of 48-h total sugar conversion versus net refining energy.

Figure 5-8. Hardwood sodium carbonate biomass digestibility showed a positive correlation for samples with different consistency, and gaps existed versus net mechanical refining energy input (NSE; kWh/t). Linear trendline excluded the over-refining condition (20% cons, 0.03 mm gap). Unrefined: control; Pilot-Temp-5%: included ambient, 110 °C, and 150 °C refined conditions; Pilot: denoted % cons and different conditions described previously; Industrial: comparison from Jones et al. (2014), 5% cons.
Water retention value correlated positively with total sugar conversion improvement for all 5% consistency refining conditions, Figure 5-9. Pilot disc refining at 10% consistency showed a greater positive response to the increased WRV at 48 h total sugar conversion, which was indicated by steeper slopes compared with the 5% industrial, pilot, and lab scale refining conditions. Again, an over-refining effect was observed, for the most severe pilot refining at 20% consistency condition. Increase in WRV from increased refining intensity at extreme refining conditions actually showed a slight negative trend with respect to 48-h enzymatic hydrolysis, although all refining conditions performed much better than the unrefined control. It was a general trend that as fiber length decreased and refining action increased, the 48-h total sugar conversion increased, Figure 9-1. This trend was also seen with percent fines in that as the fines content increased with increasing refining action, the 48-h total sugar conversion increased, Figure 9-2.
Figure 5-9. The 48-h total sugar conversion plotted against the HWSC biomass WRV. Linear trendlines are grouped by different refining consistencies to visualize the differences in how the sugar conversion changed as the relative WRV increased.

The 48-h enzymatic hydrolysis sugar conversion showed relatively good correlations with most of the measured biomass property changes, but correlation does not always prove causation. The methods discussed within for measuring changes in biomass properties with refining can be used as effective tools to indicate that refining action occurred. These changes in biomass properties, however, cannot fully explain the observed improvement in enzymatic hydrolysis by mechanical refining. Additionally, when an optimum refining condition has been established for a specific biomass type and pretreatment condition, these
properties (energy input, length, fines, WRV) are all suitable metrics for monitoring and maintaining the optimum desired refining action. More research is necessary to fully understand the improvement of enzymatic hydrolysis with mechanical refining. It is expected that measurements of biomass accessibility to enzymes will strongly correlate to enzymatic hydrolysis kinetics; such measurement will bring a deeper insight into enzymatic hydrolysis and fundamentally explain the mechanical refining mechanisms.

The intensity (or specific edge load) of mechanical refining also showed a positive trend with respect to the improvement of total carbohydrate conversion, Figure 5-10. A maximum in sugar conversion with increasing refining intensity indicated that there may have been diminishing technical returns at the most extreme case of over-refining (Jones et al. 2013), but the majority of cases within the studied operating range showed that the more refining energy input into the biomass and the more intense conditions of refining, the better the efficiency of carbohydrate conversion. It was, however, expected that a financial tradeoff occurs between refining energy demand and total carbohydrate conversion. This tradeoff should be considered when optimizing mechanical refining conditions for other systems with different biomass types and pretreatments.
Figure 5-10. Total sugar conversion (48-h) in relation to the mechanical refining intensity (SEL).

It should also be noted that the magnitude of the pilot scale refining energy was much higher than would ever be considered economically attractive. The reported values provide a technical comparison of energy for the different conditions. Assuming a crude energy cost of $0.10/kWh, the operational cost of operating a mechanical refiner ranged from $4.16/t (Ind-Primary) to $146.40/t (Pilot-30 °C-20%K-0.25 mm). At a conversion of 77 gal per ton, this translated to a cost of $0.05/gal and $1.90/gal, respectively. It is understood that the pilot refiner is not as efficient as an industrial refiner, and as such the magnitude of the pilot scale refining energy would not be considered practical. It is expected that an optimized large-scale
disc refiner could achieve similar results with much lower energy inputs due to economies of scale-type considerations (larger refining zone, more refining time per pass or rotation, reduced bearings friction, better engineering for more efficient energy transfer or less energy loss, etc.). Compared to the total pretreatment energy consumption for select representative conditions of aqueous pretreatments with and without disk milling (433 to 1183 kWh/t), steam explosion (550 kWh/t), organosolv (348 to 448 kWh/t), and SPORL with size reduction (418 to 518 kWh/t); it is reasonable to consider that mechanical refining energy (67 to 121 kWh/t industrial; or 517 to 2484 kWh/t pilot total energy) can be added to or supplemented from the total energy consumption of these pretreatments in an industrial setting to achieve higher overall pretreatment energy efficiencies (Zhu et al. 2010).

It was also observed that conditions with higher consistency and lower plate gap widths produced steam, with the most severe conditions producing the most steam. In a consolidated biorefinery concept this steam could be captured and used to offset process steam and reduce the net energy required to operate the refiner. This phenomenon was not included in the economic analysis, but would only increase the benefit of using mechanical refining in a lignocellulosic biorefinery.

This work illuminates the great potential for optimizing mechanical refining operating conditions and process and plate designs for use in biorefinery applications. Further work is necessary to fully understand the enzymatic hydrolysis improvement with mechanical refining mechanism for other biomass types and multiple pretreatment severities. Future work should target refiner plate design that will achieve a higher SEL (intensity) with lower
energy input or more efficient energy transfer to the biomass. This would allow for higher carbohydrate conversion efficiencies to be achieved with lower energy demand. It is interesting that the best refining conditions for the biorefinery concept may be similar to those for corrugated boxboard medium fiber (Gustafsson et al. 2003). It can be explained that the same operation conditions required to generate a bulky but pliable fiber for corrugated medium sheet in a medium mill have similar goals to treat a lignocellulosic fiber for optimum hydrolysis and use in a biorefinery.

Although it was found that low temperature refining was better for low consistency refining, it is expected that higher temperature refining might be more effective for higher consistency refining and might allow for improved refiner flow biomass separation. Further work is necessary to fully understand the impacts of combined high temperature and high consistency refining. Table S1 details the refining conditions and their respective biomass properties and hydrolysis efficiencies. The no-load energy for the pilot scale refiner was calculated to be between 250 and 500 kWh/t, with an average measured value of 326 kWh/t.

5.6 Conclusions

The best condition for pilot scale mechanical refining for optimized hardwood sodium carbonate (HWSC) biomass digestibility was 20% refining consistency, at 30 °C with a plate gap width of 0.127 mm. This condition achieved a total carbohydrate conversion of 89.5% after 48 h, compared with the 39.2% of the unrefined control. In general, increased refining severity was achieved by decreasing the plate gap width and increasing the refining
consistency. The severity was expected to increase by utilizing higher intensity refiner plates. These changes in refining were monitored with increased energy input, WRV, fines content, and decreased fiber length. Changes in WRV, fines (%), and fiber length indicated that refining occurred and gave a general trend of the improvement in biomass digestibility, but none completely explained the enzymatic hydrolysis improvement with mechanical refining.

At industrially relevant 48 h and 96 h enzymatic hydrolysis times, increasing the refining temperature to 110 °C and 150 °C decreased the refining improvement relative to ambient refining. In this study, higher temperature refining was less favorable, likely due to lignin redistribution throughout the biomass matrix. Refining improved enzymatic hydrolysis conversion up to a maximum point, after which increased refining severity showed lower enzymatic hydrolysis conversions. Hardwood sodium carbonate biomass digestibility showed a positive linear correlation for samples with different consistency and gaps existed versus net mechanical refining energy input (NSE; kWh/t) up to the maximum point.

5.7 **Acknowledgements**

The authors are grateful for the support of the United States Department of Agriculture as a part of the National Needs Foundation for providing the funding for this research (Grant number 2010-38420-21828), to Georgia Pacific for providing the biomass, and to Novozymes for providing the enzymes.
5.8 References


6 CHAPTER SIX: ADSORPTION OF NON-HYDROLYTIC FUSION PROTEINS ON MODEL LIGNOCELLULOSIC ISOLATES FOR PREDICTION AND EXPLANATION OF BIOMASS DIGESTIBILITY

6.1 Abstract

A novel non-hydrolytic fluorescent recombinant protein system is applied to model biomass as well as unrefined and refined lignocellulosic biomass fractions to provide a fundamental explanation for how mechanical refining improves the biomass digestibility. The protein, a green fluorescent protein linked to a family-3 carbohydrate binding module (GC3), is successfully produced and scaled up for bioreactor production and purification. Langmuir isotherms on model substrates confirm the binding specificity of the GC3 protein, and confocal laser scanning microscopy with quantitative image analysis help show how the biomass accessibility to cellulase-type binding enzymes changes with mechanical refining. It was determined that more evenly distributed protein across the biomass fiber due to the mechanical refining action was associated with superior enzymatic hydrolysis kinetics.
6.2 Introduction

It has been well documented that mechanical refining treatment can enhance the biomass digestibility of many different pretreated biomass to assist in overcome the inherent biomass recalcitrance. This enhancement in enzymatic hydrolysis is credited to the combination of the major physical actions that occur during mechanical refining, 1) external fibrillation, 2) internal delamination, and 3) fiber cutting. However, limited research has been made to track the protein adsorption throughout the biomass matrix before and after mechanical refining to illuminate which mechanisms are most important and most effective for maximizing the biomass digestibility.

Cellulose accessibility limits have been described as one of the primary limiting factors for effective lignocellulosic biomass hydrolysis (Gourlay et al. 2012). It is hypothesized that for mechanical refining, it is not only the increase in protein adsorption that facilities improved enzymatic hydrolysis, but also more uniform distribution throughout the biomass matrix which will facilitate more rapid substrate amorphogenesis and greater instances of productive binding for the enzyme protein cellulase to the substrate cellulose.

Many different types of adsorption measurements have been used before to characterize the biomass before and after pretreatment or mechanical refining. The most elementary of these is water sorption, measured by the amount of water retained by the biomass suspension after centrifugation and reported as the water retention value (WRV g-water/g-biomass) (TAPPI 2011). In Chapter 0, the WRV is used as a reasonable model to predict the improvement in enzymatic hydrolysis for specific biomass types, Figure 4-6. It is
logical that the increased WRV reflects more fiber swelling and better cellulose accessibility to cellulase enzymes, leading to improved enzymatic hydrolysis. However, this model and this explanation breaks down as a maximum in mechanical refining benefit is often observed as refining severity and WRV continue to increase. A water molecule is much smaller and associates with the lignocellulosic substrates with much different binding mechanisms that a carbohydrate binding module of a cellulase enzyme.

Other methods involve drying the biomass to measure the accessibility by measure the biomass specific surface area (SSA), including pore size distribution measured by thermogravimetric analysis and differential scanning calorimetry (TGA-DSC) (Park et al. 2006), or the BET method (Zhang et al. 2004) which can provide valuable information about the surface structure, but can often be difficult to relate to the biomass digestibility due to differences in the conditions during SSA measurement and enzymatic hydrolysis. Simon’s stain dye adsorption ratios have also been shown to correlate well with the protein loading and predict the carbohydrate conversation (Arantes et al. 2011). Because of the difference in dye polymer sizes, this system can provide more details about the interior and exterior surface area of the cellulosic biomass than a monocomponent system. However, although the blue and orange Simon stain dyes theoretically bind more closely with cellulose than other polymers within the biomass matrix, the binding is still different than enzyme carbohydrate binding.

More recently, improved characterization of enzyme proteins and advancements in recombinant DNA technology have allowed for the selection and generation of fusion
proteins that have the same carbohydrate binding modules (CBM’s) as cellulases with fluorescent protein (Hong et al. 2007). These fusion proteins have been reconstructed to utilize the carbohydrate binding modules from a CBM family 3 cellulase and are chemically bound to a green fluorescent protein (GFP) molecule in place of the native cellulases catalytic core. Without the catalytic core, the proteins do not catalyze cellulose hydrolysis, but will still diffuse and bind to the substrate. This tagged fluorescent fusion protein allows the protein adsorption to be quantified and tracked when used in complex biomass systems.

It is believed that this work is the first of its kind to use non-hydrolytic fluorescent fusion protein adsorption on lignocellulosic biomass substrates to investigate and understand the fundamental impacts of enzyme accessibility before and after mechanical refining for improving the overall lignocellulosic biomass digestibility.

6.3 Experimental

6.3.1 Materials

Model substrates were used for Langmuir adsorption experiments and in confocal laser scanning microscopy. Cellulose-based model substrates included filter paper cellulose that was regenerated in 85% o-phosphoric acid to make regenerated amorphous (RAC), 11% suspension of cellulose nanocrystals (CNC) provided by University of Maine, and microcrystalline cellulose purchased from Fisher. Beechwood Xylan (Júnior et al. 2013) is a model substrate of hemicellulose to compare the carbohydrate-binding to the cellulose model substrates. Alkaline pretreated mixed Hardwood Kraft Lignin (HWKL) was washed and
isolated and used as the model substrate for lignin within the lignocellulosic biomass matrix ((Jiang et al. 2016)).

Lignocellulosic substrates were also used in the adsorption and imaging experiments. Hardwood Sodium Carbonate (HWSC) unrefined and pilot TMP refined biomass was provided by an industrial pulp and paper facility. To examine the differences in types of mechanical refining, southern bleached hardwood kraft market pulp (BHKP) was disintegrated to create the unrefined biomass for comparison to BHKP PFI mill (10,000 revolutions) and valley beater (90 min residence time).

6.3.2 Methods

6.3.2.1 Benchtop flask and bioreactor production of recombinant non-hydrolytic proteins

Two cultures of E. coli-BL21(DE3) host cells containing plasmids pNT02 (pNT01 (GFP)+CBM3) and pET20b-mCherry-CBM17 were supplied by the Biofuels and Carbohydrates Lab, Department of Biological and Systems Engineering at Virginia Polytechnic Institute and State University (Gao et al. 2014). These plasmids contained the genetic encoding for isolation via selective ampicillin resistance and for production of fusion proteins when induced that contain a carbohydrate binding module (CBM), a linker, a fluorescent protein for detection, and a His-tag (pNT02 only) for purification, Figure 6-1. GC3 denotes the protein expressed by the plasmid pNT02 that contains a green fluorescent protein (GFP), a carbohydrate-binding module from family 3, and a HP-TRX His-tag for purification. CC17 denotes the non-hydrolytic fusion protein expressed by the plasmid pET20b-mCherry-CBM17 that contains a red fluorescent protein called mono-Cherry, and a
The carbohydrate-binding module family 3 in GC3 selectively binds to both crystalline and amorphous cellulose via surface binding, and the carbohydrate-binding module family 17 in CC17 binds to only amorphous cellulose via chain-binding (Boraston et al. 2004).

Figure 6-1. Plasmid maps, recombination schemes and protein molecular structures. A) GC3, green fluorescent protein linked to a family 3 surface binding carbohydrate-binding module. B) CC17, mono-cherry fluorescent protein linked to a family 17 chain binding carbohydrate-binding module (Gao et al. 2014).
The purification process was modified from the original recombinant protein preparation method based on available chemicals and equipment (Hong et al. 2007). The *E. coli* BL21(DE3) cells containing the plasmids pNT02 and pET20b-mCherry-CBM17 for expression of GC3 and CC17, respectively, were aseptically transferred from the received micro slant, the streaked on LB agar plates with 100 µg/mL Ampicillin (CAS# 69-52-3, Life Technologies, Carlsbad, CA, Catalog # 11593027) and 40 µg/mL IPTG (isopropyl β-D-1-thiogalactopyranoside, Life Technologies, CAS# 367-93-1, Catalog# 15529019). The ampicillin ensures selective resistance for the target plasmid by inhibiting cell wall biosynthesis for bacteria without the plasmid. Also, by including IPTG during plate growth, which mimics allolactose – the metabolite that triggers DNA transcription of the lac operon within the target recombinant plasmid, the induction of the target fluorescent protein within all colonies can be confirmed.

A single colony was picked with a sterile loop and inoculated into autoclaved cell culture tubes with 4 mL of Luria-Bertani (LB), Miller broth base (Fisher BioReagents, catalog # BP9723-2; 10g/L peptone, 5g/L yeast extract, and 10g/L sodium chloride) and 4 µL of 100 mg/mL ampicillin stock after autoclaving to prevent antibiotic destruction. Cell seed cultures were incubated at 37 ºC and 300 rpm and grew aerobically overnight in an Infors-HT Multitron Standard shaker incubator. Overnight cell biomass optical density at 600 nm (OD$_{600}$) reached 3.93 for GC3 (*E. coli* BL21(DE3)-pNT02) and 4.81 for CC17 (*E. coli* BL21(DE3)-pET20b-mCherry-CBM17), measured by a Thermo Scientific GENESYS 20 visible spectrophotometer.
Overnight cultures were used to inoculate parallel 250 mL baffled culture flasks of 50 mL sterile LB media, with a starting target concentration of 0.1 OD_{600}. Cell cultures were incubated at 37 ºC and 300 rpm until the OD_{600} was ≥0.6 (approximately 2-3 hours), then 1 flask from each parallel incubation of GC3 and CC17 was induced with 9.5 µL of IPTG (200 mg/mL stock) for a final induction concentration of 40 µg IPTG/mL cell culture. The induced flasks were returned to the shaker incubator and reduced to 18 ºC and 250 rpm for overnight expression of their respective target proteins. After overnight expression, the flasks were removed from the incubator, final optical densities were measured to be 5.19 and 5.46 for GC3 and CC17, respectively, and cells were pelleted by centrifugation for 5 min at 3500 rpm (Eppendorf 5810R, swing-bucket rotor, 50 mL conical tube adapters, Hauppauge, NY, USA) and stored in the freezer (-20 ºC) prior to executing the protein purification process.

The other parallel flask was collected for cell banking in 50 mL centrifuge tubes, the culture was centrifuged for 5 min at 3500 rpm to pellet the cells the supernatant was discarded, and the cells were resuspended in 25 mL of sterile media (80% fresh LB broth, 10% glycerol, 10% ultrapure water) and distributed into 1 mL aliquot cryovials. The cryovials containing viable cells of *E. coli* BL21(DE3)-pNT02 and *E. coli* BL21(DE3)-pET20b-mCherry-CBM17 were stored at -80 ºC as a safety stock for backup purposes.

After successful production at the 50 mL flask scale, this entire production process was scaled up for production in 2L BIOSTAT® B plus bioreactors (Sartorius, Bohemia, NY, USA). A quantity of 30x volume increase (1.5 L working volume) was used to generate more recombinant protein at higher concentrations for optimized experimental purposes. All
component concentrations for initial seed culture, bioreactor inoculations and inductions were kept constant. Time to induction and final cell densities were similar at the bioreactor scale. After overnight protein expression at 18 °C, cells were collected into 1000 mL centrifuge bottles (750 mL per bottle), and centrifuged at 7000 x g for 10 min at 6 °C using a ProteomeLab XL-A analytical ultracentrifuge (Beckman Coulter, Indianapolis, IN, USA). The supernatant was discarded from the 1000 mL centrifuge bottle, and the pelleted cells were resuspended and washed with approximately 50 mL of 100 mM HEPES buffer (zwitterionic N-substituted aminosulfonic acid buffer, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid, CAS# 7365-45-9, Sigma, Catalog # 391340). The washed cells were collected in 50 mL centrifuge tubes, centrifuged at 3500 rpm for 15 minutes at 18°C, and stored in the freezer (-20 °C) after discarding the supernatant until ready to be purified.

6.3.2.2 Purification of recombinant non-hydrolytic fluorescent proteins

To ensure only the protein of interest (i.e. GC3 and CC17) was isolated for use in adsorption and imaging experiments, two types of affinity chromatography purifications were performed that take advantage of the polyhistidine-tag (or 6xHis-tag) vector that is linked to the N-terminus of the GC3 protein, and by utilizing the selective binding of the carbohydrate-binding module in the CC17 protein. Frozen pellets of overnight expressed target proteins were thawed and resuspended in 8 mL of 100 mM HEPES buffer (pH 7.3). The cells were lysed on ice to free the intracellular proteins using a sonicator equipped with a microtip and sound enclosure (Q700, Qsonica, Newton, CT, USA). Sonication was performed with 6 iterations of 10 second bursts with 10 seconds of cooling at 60% max
power for a total of 1 minute active sonication time. Viscous samples were drawn through an 18-gauge needle to complete the cell lysis. The total energy for all 250 mL flask cells were on average 1900 J, and an average of 5300 J for the more concentrated 2000 mL bioreactor cells. The lysate was centrifuge to pellet the cell debris (3500 rpm, 15 min for 50 mL; or 14000 x g, 20 min for microcentrifuge) and a portion of lysate was set aside for protein characterization. The remaining lysate in the supernatant was transferred to a fresh conical tube and stored cold or on ice (4 ºC) while the purification columns were prepared.

The GC3 protein, with a linked 6xHis-tag, could be purified using a nickel-charged affinity resin, 50-70% Ni-NTA agarose (nickel charged nitrilotriacetic acid, Qiagen, Valencia, CA, USA, Catalog # 30230) in 10-20% ethanol, as the chelating ligand in immobilized metal affinity chromatography (IMAC). The column resin was washed by suspending 1.5 mL of resin with 8 mL of ultrapure water. After allowing the Ni-NTA resin to settle, the wash water could drain through the column and discarded. The Ni-NTA resin with a 45-165 µm bead size remained in the working bed range of the column reservoir that has a 30 µm bed support to retain fine particles. The column was primed with 8 mL of 100 mM HEPES, resuspended gently on an orbital rocker, allowed the column resin to settle, and aspirated and discarded the supernatant without disturbing the resin. The priming step was repeated to ensure that the column was fully equilibrated.

After the column was fully prepared, 8 mL of the GC3 lysate was added to the chromatography column, resuspended for 10 minutes on an orbital rocker, and allowed to settle for 5 minutes before removing the cap and collecting the flow-through (FT) which
included all non-bound protein and other material from the lysate supernatant. Without disturbing the column, the IMAC system was washed 4 times with 2 mL of HEPES wash buffer while collecting the wash fractions. The wash buffer was spiked with a small amount of a 3M imidazole stock solution (Sigma, CAS# 288-32-4, Catalog # I5513) to a final concentration of 20 mM to wash away the undesired molecules and remove any molecules that were only slightly bound to the column. The column was then rinsed 4 times with 1 mL of HEPES elution buffer (250 mM imidazole). The imidazole competes with the target protein and its binding to the Ni-NTA resin, and as the imidazole preferentially binds to the resin, the target protein GC3 elutes from the column and is collected. All fractions were stored in the refrigerator (4 °C) and the resin was rinsed and restored with 20% ethanol.

The CC17 protein was purified using similar a similar procedure to the GC3 protein; however, instead of Ni-NTA affinity from the 6xHis-tag, regenerated amorphous cellulose was used as the binding medium in the affinity chromatography column. The regenerated amorphous cellulose (RAC) was produced from Whatman No.1 filter paper (Fisher, Catalog # 09-805H) by concentrated 85% o-phosphoric acid (Fisher, CAS# 7664-38-2, Catalog # S25470B) dissolution on ice following the procedure described by Zhang et al. (2006). The RAC was loaded to the column by adding 1 mL of 8 g/L RAC. After priming the RAC column with 100 mM HEPES, 8 mL of the CC17 cell lysate supernatant was added to selectively bind to the amorphous cellulose. The flow-through and wash steps were performed the same as the GC3 purification above, but after the final washing step, the column with fixed CC17 protein was resuspended with 8 mL of 100% ethylene glycol (EG,
Fisher, CAS# 107-21-1, Catalog# E178-1) at room temperature for 10 minutes on the orbital rocker. The RAC – CC17 – EG suspension was transferred from the column to a 15 mL centrifuge tube. The RAC was pelleted after centrifugation (3500 rpm, 5 minutes, 4 ºC) and the supernatant containing CC17 in ethylene glycol was collected in a fresh tube.

Membrane dialysis using 28.6 mm diameter regenerated cellulose dialysis tubing (Fisherbrand, Catalog # 21-152-14) was the final purification step to remove the imidazole or ethylene glycol and prepare the non-hydrolytic fluorescent proteins for experimentation. The ratio of dialysis buffer to target protein volume remained above 100x for all dialysis steps. A quantity of 3 fresh 10 mM HEPES buffer exchanges were made every 2 hours, and after the last exchange the dialysis setup was left overnight in a 4 ºC environmental incubation chamber. The purified protein was collected in centrifuge tubes and stored long-term covered in foil at 4 ºC.

6.3.2.3 SDS-PAGE and Western Blot of purified protein

For efficacy of the protein production and purification procedure, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed for each production step including positive and negative control of the lysate, column flow through, four column washes, and four column elution samples. 20 µL of each sample for each protein, GC3 and CC17, were mixed with 4 µL of 4x loading buffer (1 part 4x Laemmli sample buffer – 277.8mM Tris-HCl, pH 6.8, 44% (v/v) glycerol, 4.4% SDS, 0.02% bromophenol blue BIO-RAD catalog# 161-0747; 9 parts reducing agent: β-mercaptoethanol, BIO-RAD catalog# 161-0710, Hercules, CA, USA) then heated on a heating block at 95 ºC for 5 minutes. After
incubation, 20 µL of each prepared sample was loaded into BIO-RAD Ready Gel, 4-20%, Tris-HCl, 12-well Precast Gels (BIO-RAD catalog# 161-1177) and the gels were run using a BIO-RAD Mini-PROTEAN® Tetra Vertical Electrophoresis Cell at 300V for approximately 15 minutes. Fluorescence of the protein fractions obtained during purification and the protein bands in the SGS-PAGE gel were observed under a UV light board, Figure 6-2.

Figure 6-2. GC3 (A, C) and CC17 (B, D) protein purification fractions (A, B) and SDS-PAGE analysis (C, D) under UV light.

Western Blot of the finished gels was accomplished following a modified BIO-RAD blotting procedure. Gels were first placed in the 1x transfer buffer for 10-15 minutes, then assembling the transfer sandwich (blot on the cathode side, gel on the anode side) in the cassette, the proteins in the gel were transferred to the blot membrane at 100V for 30 min.
After a successful transfer to the blot, the blot was briefly rinsed with ultrapure water, then washed with 30 mL phosphate buffered saline with tween 20 (PBS-T, made from Thermo Scientific Pierce™ 20X PBS Tween 20 buffer, catalog# 28352) for 5 minutes on an orbital rocker. Approximately 25 mL of 3% bovine serum albumin (BSA) blocking buffer (from Blocker™ BSA (10X) in PBS, Thermo Scientific, catalog# 37525) was used to block unbound membrane sites for 45 min slowly mixed using the orbital rocker. Then the primary antibody was applied to the blot for 45 min on the orbital rocker, with 25 mL of antibody blocking reagent that was prepared with 10 µL of anti-His C-term HRP (horseradish peroxidase) Antibody (Invitrogen, catalog# R931-25 in 50 mL of blocking buffer). Excess primary Ab was washed away with PBS-T by rinsing 4 times for 5 minutes each. Then the blot was incubated with 20 mL of the developing reagent buffer for approximately 10 min until adequate colorimetric detection of the target proteins were observed. The developing reagent was made with 36 mL ultrapure water, 4 mL of the Opti-4CN diluent, and 800 µL optimized 4CN developing reagent (BIO-RAD Opti-4CN Substrate Kit, catalog# 1708235). The BIO-RAD Gel Doc™ EZ Gel Documentation System was used to capture images of the SDS-PAGE gel and the Western Blot membrane, Figure 6-3.
Figure 6-3. Images of Western Blot papers for GC3 (left) and CC17 (right). “MWM” = Molecular Weight Marker- 10 to 250 kDa with more intense bands at 25 and 75 kDa, “L-“= Lysate of culture without IPTG induction, “L+” = Lysate of culture with IPTG induction, “FT” = Flow-through, “W#” = Wash step number, “E#” = Elution step number.

6.3.2.4 Determination of protein concentration and characterization of other protein parameters

After the purified protein solutions were made, it was critical to determine the protein concentration for further experimentation. The Thermo Scientific Pierce BCA (bicinchoninic acid assay) Protein Assay Kit (Fisher, Catalog# 23225) was used for quantifying the protein concentration for GC3 and CC17 compared to the Bovine Serum Albumin (BSA) standard protein. The kit included 1000 mL of BCA Reagent A (containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide), 25 mL of BCA Reagent B (containing 4% cupric sulfate), and 1 mL ampules of the Albumin Standard (containing bovine serum albumin at 2 mg/mL in 0.9% saline and 0.05% sodium azide).

The standard preparation method was modified to contain 10 standard points with a range of 5 to 2000 µg/mL final BSA concentrations, or a 10 point factor 400 standard curve.
The working reagent is prepared by mixing BCA Reagent A:B at a ratio of 50:1. After 25 µL of each standard and unknown sample dilution replicate into a well on a UV applicable 96-well microtiter plate, 200 µL of the working reagent (sample to working reagent ratio = 1:8) was added to each well, and the plate was sealed with optical tape and mixed thoroughly for 30 seconds. The plate was covered to protect from light sensitivity and incubated at 37 ºC for 30 minutes in a Thermal Cycler (BIO-RAD C1000 Touch), then after cooling to room temperature, the plate was read at 562 nm on a Synergy 2 SLFA plate reader equipped with Gen5 analysis software (BioTek, Winooski, VT, USA). The total protein concentration in related to the colorimetric evolution of the sample from green to purple, Figure 6-4.

Figure 6-4. Image of BCA protein concentration plate of BSA standards and unknown GC3 and CC17 proteins after incubation.

The blank corrected absorbance at 562 nm (ABS:562-B) for the standards was plotted against the known Albumin Standard concentrations and fitted with a 4-parameter logistic curve (Rodbard model), Figure 6-5.
Equation 6-1. Rodbard equation for fitting standard curves for the determination of protein concentration in unknown samples via bicinchoninic acid method.

\[ Y = \frac{(A-D)}{(1+(X/C)^B)} + D \]

Where \( Y = \text{ABS:562-B} \), and \( X \) the known BSA standard concentrations.

Figure 6-5. BCA protein concentration assay curve fitting. A) The fit model of normalized BCA absorbance at 562 nm minus the blank (ABS:562-B) versus the known concentration of the BCA standard is shown by the black curve. B) The measured absorbance curve is shown in red in overlay with the 4P fit model in blue. C) Comparison of the Rodbard model predicted ABS:562-B versus the actual ABS:562-B measurements shows a very strong positive linear correlation suggesting a good fit. D) Table of the converged fit model parameters for the BCA standard curve.
Solving for X allows for calculation of the unknown protein concentration at the various dilutions (1, 2, 4, 8, and 16x).

Equation 6-2. Calculation of unknown non-hydrolytic fluorescent protein probe concentrations.

\[ \text{Eq. } X = C^*\left(\frac{(A-D)}{(Y-D)}-1\right)^{1/B} \]

The average value for the calculated protein concentration for GC3 was 1374 µg/mL (CV 5.7%), and for CC17 was 886 µg/mL (CV 5.9%). The purified protein was also subjected to an excitation and emission scan to identify the optimum excitation and emission profiles for the fluorescent protein adsorption experiments and images. The scans were performed using a Cytation 5 Cell Imaging Multi-Mode Microplate Fluorescent Reader (BioTek, Winooski, VT, USA) at 5 nm interval wavelengths – from 450-700 nm excitation wavelengths, and from 500-750 emission wavelengths – the optimized imaging parameters were compared to the reported spectral analysis obtained from the ThermoScientific Fluorescence SpectraViewer and matched very well, Figure 6-6. A comparison of all the protein characteristics are shown in Table 6-1.
Figure 6-6. Overlay of experimentally optimized fluorescence scan profiles (black) and from fluorescent protein fluorophore library (color). (ThermoFisher Scientific 2016)
Table 6-1. A comparison of the measured purified protein characteristics to those reported in previous works (Rollin et al. 2011; Gao et al. 2014). WB denotes measurements from Western Blot images, the protein concentration is reported in average µg/mL and µmol/L, and comparison of maximum Ex: excitation wavelengths and Em: emission wavelengths are shown.

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<th>(Jones 2017)</th>
<th>(Gao 2014), (Rollin 2011)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>kDa</td>
<td>AVG (µg/mL)</td>
</tr>
<tr>
<td>GC3</td>
<td>~60</td>
<td>1373.3</td>
</tr>
<tr>
<td>CC17</td>
<td>~50</td>
<td>885.5</td>
</tr>
<tr>
<td>CBHI</td>
<td></td>
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</tr>
</tbody>
</table>
6.3.2.5 Evaluation of Langmuir isotherm absorption of green fluorescent protein tagged CBM3 on biomass substrates

Adsorption experiments were performed by preparing 2 g/L biomass suspensions in 10mM HEPES, 200mM NaCl buffer of all biomass to create solutions that could be pipetted into 1.5 mL microcentrifuge tubes. With working volumes between 0.75 and 0.9 mL, the biomass stock solutions were first aliquoted to the appropriate tubes for final concentration of approximately 0.5g/L biomass, then additional HEPES buffer was added if necessary, and finally the non-hydrolytic green fluorescent protein (GC3) was added to the microcentrifuge tubes at initial protein charges of 1-20 µmol/g-biomass in duplicate. The microcentrifuge tubes were weighed on an analytical balance when empty and after each subsequent material addition step to ensure exact biomass and protein concentration calculations. The adsorption experiment tubes were sealed and incubated for at least 2 hours at 50°C. After reaching adsorption equilibrium, the samples were centrifuged (14,000 rpm for 2 min). A quantity of 450 µL of supernatant with unbound GC3 protein was collected from each tube and stored in a separate clean microcentrifuge tube at 4°C until ready for analysis.

The remaining biomass was resuspended in 900 µL of 10 mM HEPES buffer, then centrifuged for another solid-liquid separation. The supernatant was discarded by carefully aspirating 900 µL with a pipette, and the wash step was repeated. The biomass was returned to the starting concentration by adding 450 µL of 10 mM HEPES buffer and the samples were sealed and stored in their original microcentrifuge tubes in the refrigerator until ready to be plated and analyzed via confocal microscopy.
To measure the amount of protein that was adsorbed onto the biomass at equilibrium for each biomass, 200 µL of the supernatant was pipetted into a black 96 well microplate for measuring solution fluorescence. Some supernatant samples were diluted up to 5x to ensure that the fluorescence intensity was within the range of the 7-point factor 40 curve. Standards were diluted from the 21.8 µmol/L GC3 stock solution (dilution factors: 6, 8, 10, 15, 40, 120, 240) to a range of 0.1 to 3.6 µmol/L GC3 and 200 µL of each standard was added to each plate in at least duplicate, along with duplicate measurements of the background (no solution), and the blank (10 mM HEPES). Once the plates were prepared, the fluorescence was measured after 15 seconds of orbital shaking using various micro plate readers (TECAN SAFIRE, XFLUOR4 v4.22d software, Ex: 484, Em: 511nm; and BioTek Cytation5, Gen5 v2.09.1 software, Ex: 485, Em: 528nm) that had optimized conditions of excitation and emission wavelength, and probe height calibration for the maximum fluorescent intensity response, Figure 6-7. An overview of the standard curves and the residuals from the linear estimates are shown in Figure 6-8. Again, very strong linear correlations ($R^2 > 0.998$) of the protein concentration and RFU or blank corrected fluorescent intensity (FL-Blank) and negligible residuals, allow to accurately measure the free GC3 protein in suspension.
Figure 6-7. Microplate fluorescence reader optimization for GC3. Top, examples of the excitation and emission (FL GC3) wavelength optimization matrix (TECAN) displayed as a contour plot (left) and surface plot (right). Bottom, probe height calibration (BioTek) of relative fluorescent units (RFU) versus probe height (µm).
Figure 6-8. Selected GC3 7-point factor 40 standard curves and residual plot. Relative fluorescence units for experiments using a TECAN plate reader (GC3-1 and GC3-2) are shown on the primary y-axis, and BioTek Cytation plate reader (GC3-3) on the secondary y-axis.

Langmuir adsorption isotherms for the biomass samples were fit using the standard Langmuir equation adapted to describe the adsorption and binding of non-hydrolytic fusion protein GC3 to biomass samples.
Equation 6-3. Langmuir isotherm equation for modeling the non-hydrolytic fluorescent protein adsorption in the protein biomass systems.

\[ E_a = \frac{A_{\text{max}} K_p E_f}{1 + K_p E_f} \]

Where; \( E_a \) is the dependent variable of adsorbed GC3 (µmol/g). \( E_f \) is the free GC3 protein at equilibrium (µmol/L). \( A_{\text{max}} \) is the maximum theoretical GC3 adsorption (µmol/g). \( K_p \) is the protein adsorption rate constant (Hong et al. 2007).

6.3.2.6 Confocal laser scanning microscopy of fusion proteins in biomass matrix

Washed biomass from the Langmuir isotherm adsorption incubation experiments were collected and stored at 4 °C until ready for analysis using confocal fluorescent microscopy. The confocal microscope was a Zeiss 710 Laser Scanning Microscope workstation owned and maintained by the Cellular and Molecular Imaging Facility (CMIF) at North Carolina State University. The Zeiss 710 LSM was fitted with four Zeiss objectives, 10x (0.45 NA) dry, 20x (0.8 NA) dry, 40x (1.1 NA) water immersion, and 63x (1.4 NA) oil immersion. The confocal microscope was equipped with four laser lines for excitation, 405 nm diode laser (for DAPI, Alexa 350); 458 nm, 488 nm, 514 nm multiline argon laser; 561 nm diode pumped solid state laser; and 633 nm HeNe laser. The 488 nm argon laser line was used for green fluorescent protein excitation, and all objectives were used for specimen location and inspection; however, all quantitative image analyses were made using the LD C-Apochromat 40x, 1.1 Numerical Aperture (NA), W Korr M27, water immersion lens with a constant argon laser power level of 0.04% (700 detector gain, 1.0 amplifier gain, 10.0
amplifier offset). The bright field channel was illuminated by halogen lamp during eyepiece detection, and the transmitted light (T-PMT) was detected during quantitative image analysis with a photomultiplier tube (320 detector gain, 1.0 amplifier gain, 0.0 amplifier offset).

It is recognized that potential sources of error during quantitative confocal microscopy could include biomass autofluorescence, reflections from laser excitation causing high background or signal-to-noise ratio, high fluorescence intensity causing image saturation, microscope configuration, and fluorescent protein photobleaching, among others. Special care was made to ensure images were captured under identical conditions using the same equipment setup: objective, pinhole diameter, offset, amplifier gain, photomultiplier voltage, wavelength range, dichromatic mirrors for filtration, laser power, pixel dwell time, and specimen mounting conditions including sample suspension medium, microscope slides and coverslips, and immersion conditions, among others. These conditions were optimized by ensuring that all biomass concentrations remained constant. The optimum conditions for imaging was found by analyzing a biomass sample with maximum absorbed protein to ensure that no image was saturated with fluorescent intensity, and this condition was confirmed with samples containing the lowest bound protein to biomass ratios still maintaining adequate contrast for imaging.

Despite these sources of error during quantitative confocal microscopy, a simple imaging experiment was performed by imaging the GC3 solution on the microscope and recording the background fluorescence, to ensure that fluorescence from the protein in solution was negligible. A maximum in background fluorescence intensity of 13.15 RFU
gray levels for the 21.8 µmol/L stock was observed. Additionally, a protein adsorption experiment was performed with a serial dilution of the non-hydrolytic fusion protein GC3 (starting concentrations from 0.5 to 10 µmol/L GC3) on MCC at the same sample biomass concentration used for all Langmuir adsorption isotherm and confocal imaging experiments (0.5 g/L biomass). The average maximum fluorescence intensity observed and multiple cross sections of the MCC biomass was compared to the calculated protein adsorption (µmol/g-biomass), Figure 6-9. A linear approximation shows a positive trend and indicates that when all experimental conditions are consistent, the relative fluorescence intensity is comparable to the overall protein adsorption.

![Graph showing the relationship between GC3 protein adsorption concentration and average maximum fluorescence intensity](image)

Each error bar is constructed using 1 standard error from the mean.

Figure 6-9. Average maximum fluorescence (FL) intensity versus the GC3 protein adsorption concentration on micro crystalline cellulose (MCC) biomass. Linear approximation and confidence region for predicted values are shown. Each error bar is constructed using 1 standard error from the mean.
Profiles of the fluorescence intensity of the various biomass samples were made using ImageJ 1.51j8 software. All fibers were imaged in three dimensions in bright field and using the 488 nm argon laser, using the confocal z-stack slicing tool. For quantitative comparison of protein adsorption along the fiber cell wall, images were analyzed at or near the middle z-plane cross section of the fibers with perpendicular plot profile lines drawn from across the diameter of the biomass particle. Some examples of these plot profile lines are drawn on the green (GC3) channel images in yellow. To improve the statistical significance of the fluorescence intensity calculations, and to provide a meaningful representation of the biomass protein adsorption cross sections; multiple stained fibers were analyzed for each sample and at least 3 cross section plots were made for each particle in the viewing window.

6.4 Results and Discussion

6.4.1 Characterization of purified non-hydrolytic fluorescent proteins

All steps in the protein production protocol indicated that the target proteins were being selected for and expressed properly. Observation under UV light, Figure 6-2; SDS-PAGE and Western Blot, Figure 6-3; and fluorescent emission/excitation scanning, Figure 6-6 all confirmed the presence of the target proteins GC3 and CC17 in solution. The fit model of the BSA standard in the BCA protein concentration determination assay when compared to values in literature also confirmed that these target non-hydrolytic fusion proteins had been produced, Figure 6-5.
6.4.2 Non-hydrolytic fluorescent protein adsorption models

The Langmuir isotherms for the model substrates confirmed the binding selectivity of the GC3 protein to cellulose substrates, Table 6-2. The maximum protein adsorption was observed for CNC, and the next highest protein adsorption was observed for RAC. The calculated $A_{max}$ values for MCC and RAC, in Table 6-2, are very close to the reported theoretical maximum adsorption for bacterial micro crystalline cellulose (BMCC, 4.76 µmol/g) and comparable to the regenerated amorphous cellulose (RAC, 8.64 µmol/g), respectively (Hong et al. 2007; Gao et al. 2014). The protein adsorption was less for substrates containing lignin, and was negligible for the purified hardwood kraft lignin and xylan substrates.

Table 6-2. Calculated Langmuir adsorption isotherm theoretical maximum adsorptions for the GC3 protein. Protein adsorption was characterized on regenerated amorphous cellulose (RAC), cellulose nanocrystals (CNC), microcrystalline cellulose (MCC), beechwood xylan (Xylan), hardwood kraft lignin (HWKL), bleached hardwood kraft pulp (BHKP) and hardwood sodium carbonate pulp (HWSC). These biomass samples were either analyzed without further treatment or unrefined (-U), or refined with PFI milling (-PFI), valley beating (-VB), or with a pilot scale thermomechanical pulp refiner (-TMP) at 30 or 150 centigrade (-30, -150). The listed date is the day when the fluorescence of unabsorbed protein in the supernatant was analyzed on the micro-well plate reader, suggesting that enzyme degradation had occurred between March and June 2017.

<table>
<thead>
<tr>
<th>Biomass</th>
<th>$A_{max}$</th>
<th>Std. Err</th>
<th>Date</th>
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<tr>
<td>RAC</td>
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</tr>
<tr>
<td>CNC</td>
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<td>0.32</td>
<td>3/25/2017</td>
</tr>
<tr>
<td>MCC</td>
<td>3.13</td>
<td>1.08</td>
<td>3/25/2017</td>
</tr>
<tr>
<td>Xylan</td>
<td>0.77</td>
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<td>3/25/2017</td>
</tr>
<tr>
<td>HWKL</td>
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<td>0.89</td>
<td>3/25/2017</td>
</tr>
<tr>
<td>BHKP-U</td>
<td>3.15</td>
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<td>3/25/2017</td>
</tr>
<tr>
<td>BHKP-PFI</td>
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</tr>
<tr>
<td>BHKP-VB</td>
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<td>0.06</td>
<td>6/13/2017</td>
</tr>
<tr>
<td>HWSC-U</td>
<td>3.31</td>
<td>0.94</td>
<td>3/25/2017</td>
</tr>
<tr>
<td>HWSC-TMP-30</td>
<td>0.22</td>
<td>0.15</td>
<td>6/13/2017</td>
</tr>
<tr>
<td>HWSC-TMP-200</td>
<td>0.38</td>
<td>0.12</td>
<td>6/13/2017</td>
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Calculated $A_{max}$ values for the refined biomass substrates (BHKP-PFI, BHKP-VB, and HWSC-TMP) were much lower than expected, Table 6-2. These Langmuir isotherm experiments were performed separately from the model substrate adsorptions. An example of the Langmuir isotherm plots are shown for cellulosic substrate bleached hardwood kraft pulp with PFI refining (BHKP-PFI), Figure 6-10. The remaining Langmuir isotherms are shown in Appendix 9.2.

![Figure 6-10. Langmuir isotherm of BHKP-PFI biomass. Excluded results are shown in gray as they were seen to not have been adsorbed to the biomass as expected. $E_a$ is the dependent variable of adsorbed GC3 ($\mu$mol/g) and is plotted versus the $E_f$, the free GC3 protein at equilibrium ($\mu$mol/L).](image-url)
It was hypothesized that the GC3 protein adsorption would have been higher for refined biomass to explain the increased biomass digestibility after mechanical refining. A possible explanation for the apparent decrease in adsorption in the refined biomass compared to the unrefined samples is that the GC3 protein may have partially degraded before these biomass samples were analyzed and the protein may have been denatured and fragmented into its recombinant segments of the carbohydrate binding module which could still bind to the protein, and the GFP fluorescent protein portion which would stay in suspension. Evidence to support the unexpected protein adsorption results are shown in Figure 6-11.

Figure 6-11. Confocal micrographs of the unexpected protein adsorption behavior of non-hydrolytic fusion protein (GC3) on BHKP-PFI. Brightfield (left) and GC3 (right). High intensity (RFU) GC3 image indicates evenly dispersed fluorescent protein at the specific viewing plane near the microscope slide. It is expected that this is green fluorescent protein that has been separated from the carbohydrate binding module.
6.4.3 Non-hydrolytic fluorescent protein CLSM images and analysis.

The confocal images of the model substrates support the results of the Langmuir adsorption isotherms, Appendix 9.3. The max fluorescence intensity of the CLSM images was measured for each biomass type, Figure 6-12. The linear plot profiles of the biomass cell walls were captured. The gray level fluorescence (Y) was plotted versus the line distance in μm (X) Figure 6-16. The maximum fluorescent intensity for each plot profile was used to normalize all of the Y-axis fluorescence intensity data. Then all the fiber widths (X-axis) were normalized to the average of all the fiber widths investigated (32.4μm) and grouped into 0.5μm increments to help smooth the data. The X and Y normalized data was then averaged for all different refining types.

The BHKP unrefined biomass shows significant peaks in fluorescent intensity and protein concentration on the outside of the fiber wall and inside the lumen. Comparatively, the PFI 10k revolution refined BHKP biomass shows a much flatter protein cross section profile, but with still distinguishable plateaus across the cell wall and valleys of low concentration in the lumen. Valley beating refining shows an even flatter profile, indicating a much more even distribution of GC3 protein throughout the biomass matrix. These results indicate that refining alters the initial adsorption distribution in the cell wall, improving the penetration and adsorption of cellulase enzymes in the interior of the biomass cell wall matrix.
Figure 6-12. Average maximum fluorescence intensity of the CLSM images by biomass. Multiple images were analyzed to find the average maximum fluorescence intensity for each biomass samples adsorbed with non-hydrolytic fusion protein GC3. Samples are ordered from lowest to highest maximum fluorescence intensity. The Max FL (y-axis) was measured by analyzing multiple images and determining the maximum fluorescence intensity. These values were average for each biomass and error bars are constructed using one standard error from the mean. Biomass substrates (x-axis) include: Hardwood Kraft Lignin (HWKL), Beechwood Xylan (Xylan), Micro Crystalline Cellulose (MCC), Cellulose Nano-Crystals (CNC), Regenerated Amorphous Cellulose (RAC), Bleached Hardwood Kraft Pulp (BHKP), Hardwood Sodium Carbonate biomass (HWSC). The treatments include: Untreated Unrefined (-Unref), PFI refining 10,000 revolutions (-PFI), Valley Beating refining 90 minutes (-VB), Thermomechanical Pulping (TMP), fine fiber fraction between 200 and 100 mesh (-fines).
Figure 6-13. Fluorescence intensity on the y-axis (Relative Fluorescent Units, RFU) versus distance of the perpendicular cross-section of the biomass cell well on the x-axis (Distance, µm). Higher fluorescence intensity indicates more concentrated non-hydrolytic fusion protein at that location along the cross-section. Multiple center z-plane cross-sections of unrefined BHKP (red), PFI 10,000 revolution BHKP (blue), and VB 90 min BHKP (green) are shown.
Figure 6-14. \( Y_{\text{norm}} \) = Normalized Relative Fluorescence Units (RFU) versus BHKP biomass fiber cross-section. \( X=\) distance (µm). Data normalized by dividing the fluorescence at each location from Figure 6-13 by the maximum fluorescence intensity for each biomass type;
unrefined BHKP (red), PFI 10,000 revolution BHKP (blue), and VB 90 min BHKP (green).

Figure 6-15. Normalized cross-section distance and fluorescence intensities of BHKP biomass. $Y_{\text{norm}}$=Normalized RFU from Figure 6-14. $X_{\text{normR0.5}}$= cross sectional distance divided by the specific cross section inspection distance multiplied by the average BHKP fiber width (32.5µm), also blocked into 0.5µm groups for improved data visualization. Smooth curves through the average $Y_{\text{norm}}$ RFU are shown for unrefined BHKP (red), PFI 10,000 revolution BHKP (blue), and VB 90 min BHKP (green), which shown fluorescence
intensity trends across the different BHKP biomass fibers.

Figure 6-16. Summary of normalization steps of CLSM fluorescent profiles for BHKP unrefined (red), and PFI 10K (blue) and VB90min (blue) refined biomass. Data points show the average normalized RFU at each blocked 0.5µm distance across the fiber cell wall. Each error bar is constructed using 1 standard error from the mean. Vertical lines (black) show approximate location of external (solid) and internal (dotted) fiber cell walls for the unrefined BHKP biomass.

This is the first instance that fluorescent non-hydrolytic protein probes have been used to evaluate the changes in biomass before and after mechanical refining. The cross-section plots, Figure 6-16, indicate that the increased enzymatic hydrolysis efficiency observed during PFI milling and valley beating refining versus the unrefined bleached hardwood kraft pulp (BHKP) control can be explained by improved protein distribution and
penetration into the biomass cell wall. There are distinct peaks in relative fluorescence intensity in the unrefined BHKP (red), which become less pronounce after 10,000 revolutions of PFI mill refining of BHKP (blue), and the curve is further smoothed after the unrefined BHKP is valley beating refined for 90 minutes (green). The more uniform distribution observed in the BHKP-VB curve suggests the most even distribution of non-hydrolytic fusion protein (GC3) throughout the biomass matrix. This theory is further supported by the enzymatic hydrolysis comparison of hardwood green liquor (HWGL) unrefined and refined biomass, Figure 3-3. This figure shows that both VB and PFI refining improve the biomass digestibility versus the unrefined HWGL control, and at the same water retention value (WRV), the VB sugar conversion is significantly higher than PFI refined HWGL.

6.5 Conclusions

Although successful production of active CC17 protein was not achieved, the purification and application of GC3 proved to be very useful and influential multi-disciplinary approach in gaining insight towards a more fundamental understanding of mechanical refining and its applications within a lignocellulosic biorefinery. The selective binding of carbohydrate-binding module family 3 in GC3 was confirmed using model substrate Langmuir isotherm adsorption. Quantitative confocal laser scanning microscopy confirmed these observations with visual 3-dimensional reconstructions of Z-stack photomicrographs. Inconclusive results were seen for the adsorption of the non-hydrolytic fusion protein GC3 on lignocellulosic biomass unrefined and refined substrates. It is
expected that the poor protein-to-substrate binding was due to denaturation and dissociation of the fluorescent probe structural domains over time. However, valuable insights into the changes in appearance of the biomass before and after mechanical refining were achieved. Future research should continue to experiment with different biomass pretreatment conditions and chemistries as well as examining the impacts of other pilot and industrial scale refining conditions on the total adsorption and effective distribution of enzyme proteins within the lignocellulosic biomass matrix.

6.6 Acknowledgements

The authors acknowledge Dr. YH Percival Zhang and the Biofuels and Carbohydrates Lab, Department of Biological and Systems Engineering at Virginia Polytechnic Institute and State University for supplying the E. coli BL21 Star (DE3) host cells with recombinant fluorescent protein expression that were used for production and purification of the non-hydrolytic fusion proteins, Dr. Gisele Gurgel and Dr. Driss Elhanafi from the Golden LEAF Biomanufacturing Training and Education Center (BTEC), for the design and support of the protein production and purification process, and Dr. Eva Johannes from the Cellular and Molecular Imaging Facility (CMIF) at North Carolina State University, which is supported by the State of North Carolina and the National Science Foundation, for training and optimization of confocal imaging of the biomass substrates.
6.7 References


7 CHAPTER SEVEN: CONCLUSIONS

Mechanical refining has been confirmed in many situations to improve the biomass digestibility and overcome the biomass recalcitrance within the lignocellulosic biorefinery concept. The increased sugar conversion with respect to the addition of mechanical refining showed positive correlations with many biomass characteristics, including: decreased fiber length, increased fines content, and increased water retention values. WRV was found to be a strong indicator of sugar conversion improvement for a specific biomass type and biomass chemistry or lignin content after pretreatment. Sugar conversion also increased with reduced plate gap width (µm), increased refining consistency (w/w%), increased net specific energy input (kWh/t) and higher refining intensity (W-s/m) during mechanical refining. Although no single property or process condition adequately could predict the improvement in biomass digestibility compared to the unrefined control, once a biorefinery is optimized these trends could allow for real-time at-line process monitoring and control of the mechanical refining operation within the lignocellulosic biorefinery concept. Additional benefits include increased homogeneity which allows for better biorefinery process control.

This work has also illuminated the phenomenon of a maximum in mechanical refining improvement which allows the use of strategic engineered biomass deconstruction (EBD) for each specific combination of biomass and pretreatment technology. The observation of over-refining at the most severe mechanical refining conditions shows that extreme levels of mechanical refining which consume very high amounts of energy are not required. Optimizing the EBD conditions in combination with the pretreatment conditions
towards the maximum mechanical refining improvement, instead of simply adding mechanical refining to an already optimized lignocellulosic biomass pretreatment, facilitate reductions in pretreatment severity. In addition to consuming less pretreatment chemicals and energy, optimized mechanical refining and pretreatment scenarios can generate less downstream inhibitors that could negatively impact enzymatic hydrolysis, fermentation and other bioprocesses.

A multi-disciplinary approach using non-hydrolytic fluorescent fusion proteins was made to explain the improvements observed in enzymatic hydrolysis with mechanical refining. The carbohydrate binding module linked to a fluorescent protein was a strong representative model for the diffusion, adsorption and binding of hydrolytic cellulases used in enzymatic hydrolysis. Quantitative analysis of confocal laser scanning microscopy images of unrefined and refined biomass showed that conditions with higher digestibility also had improved substrate accessibility to the enzymes and more uniform protein distribution. Successful demonstration of the application of the biomass and non-hydrolytic fluorescent fusion protein system allows for future optimization of mechanical refiner operation and plate design for more targeted engineered biomass deconstruction.

Although certain market barriers still exist, mechanical refining has been shown to be an effective operation that can be optimized to achieve maximum yields within a lignocellulosic biorefinery.
8 CHAPTER EIGHT: SUGGESTED FUTURE RESEARCH

The first, and most logical extension of this research would be to install various disk refiner plates with a range of refining intensity properties. This would allow to compare how refining intensity and plate design impact the overall effectiveness of engineered biomass deconstruction. This same methodology of optimizing the biomass pretreatment conditions with the mechanical refining should be applied to a wider range of biomass types and different pretreatment technologies. Most of the research in this dissertation was performed on alkaline pretreated hardwood. There are still significant gaps in understanding the effectiveness of mechanical refining on softwood and non-wood biomass, and further combinations of biomass type and pretreatment chemistry should be evaluated.

Another area for future research should consider a different process scheme for implementation of mechanical refining. Instead of applying the total refining energy before enzymatic hydrolysis and dosing all the enzymes after mechanical refining, refining could be an intermediate step between a primary and secondary enzymatic hydrolysis. This would likely yield benefits to both operations. It is hypothesized that a primary enzymatic hydrolysis step would solubilize and separate the “easy-to-hydrolyze” carbohydrate portion of the lignocellulosic substrate and also would soften the biomass structure to reduce the overall energy requirement during mechanical refining. After the primary hydrolysis step, the total biomass flow through the refiner would be reduced and the mechanical action would be only used for the remaining “hard-to-hydrolyze” portion of the biomass. This even more targeted engineered biomass deconstruction (EBD) approach would increase the
effectiveness of the mechanical refining and would enable access to previously inaccessible areas within the lignocellulosic biomass matrix. Following the target EBD mechanical refining step, a secondary enzymatic hydrolysis would be executed and could be more effective at generate productive substrate binding and increased enzyme accessible surface area. In addition to optimizing pretreatment conditions and mechanical refining conditions together, primary and secondary hydrolysis enzyme doses must be optimized. For additional complexity and clarity, the monocomponent enzyme cocktail breakdown should be compared to determine which enzymes are more effective during primary and secondary hydrolysis.

There are many improvements that can be made to the non-hydrolytic fusion protein production and application system. During future production and purification of this type, it is recommended to avoid using cellulose derived dialysis membranes, as they could bind with some of the target protein and reduce the effective protein production yield. If possible, the purified proteins should be spray dried or lyophilized to increase the storability of the protein. Storing liquid concentrates of the protein at a lower concentration relative to purified solid proteins could limit the protein stability and the flexibility of protein adsorption ranges during experimentation.

As this type of adsorption tool advances, new fluorescent probes with different binding characteristics that would preferentially bind to different parts of the lignocellulosic biomass matrix or utilizing the autofluorescence of lignin would improve the understanding and visualization of protein adsorption during enzymatic hydrolysis. The CMIF at NC State recently secured a new Zeiss LSM 880 laser scanning confocal microscope with Airyscan
which has much greater resolution than the images analyzed within this dissertation. This
system also has functionality to perform in-situ time-resolved kinetic visualization
experiments. It would be very beneficial to use a probe with an active catalytic core to
visualize how the adsorption and distribution of enzymes throughout the biomass matrix
change during enzymatic hydrolysis.

The suggested future research experiments lead towards a better understanding of the
fundamentals of enzymatic hydrolysis of lignocellulosic biomass and help overcome the
biomass recalcitrance and facilitate the development and commercialization of mechanical
refining technology within the lignocellulosic biorefinery concept. Mechanical refining is a
mature technology within the pulp and paper industry, which makes in a lower technical risk;
but there is still a large knowledge gap for industrial scale operation of mechanical refining
as a part of the lignocellulosic biorefinery. Future demonstrations and industrial scale
operations of mechanical refining for biorefineries should ensure that the Engineered
Biomass Deconstruction (EBD) approaches are used to optimize pretreatment and
mechanical refining conditions together.
APPENDICES
9 CHAPTER NINE: APPENDICES

9.1 Sugar conversion correlations versus fiber length and fines

Figure 9-1. HWSC 48-h total sugar conversion versus length weighted fiber length; correlation of 48-h total sugar conversion and fiber length for all refining conditions; $R^2 = 0.555$. 
Figure 9-2. HWSC 48-h total sugar conversion *versus* length weighted fines percentage; correlation of 48-h total sugar conversion and fines content for all refining conditions; $R^2 = 0.423$. 
### 9.2 Langmuir Isotherms

![Langmuir Isotherms](image)

**Figure 9-3.** Langmuir isotherm overlay of all cellulosic and non-cellulosic model substrates.
9.3 Confocal laser scanning microscopy images

Figure 9-4. Confocal light scanning microscope images of model cellulosic substrates. The rows of the image matrix describe each substrate; RAC=regenerated amorphous cellulose, MCC=microcrystalline cellulose, CNC=cellulose nanocrystals. Each column is a different representation of the non-hydrolytic fusion binding: GC3) filtered CLSM channel showing the location of the green fluorescent protein bound to carbohydrate binding module family-3, Brightfield) image of biomass particle, Z-projection) is the overlay of GC3 stacked images with purple-to-yellow farthest-to-nearest the viewing plane respectively, FL profile) shows the changes in GC3 protein fluorescent intensity (y-axis) over the distance (x-axis) across the width of the biomass particle. The yellow inspection line, drawn perpendicular to the biomass cell wall when possible, is shown over the GC3 and Brightfield images for a specific z-plane.
Figure 9-5. Confocal light scanning microscope images of model non-cellulosic substrates. The rows of the image matrix describe each substrate; Xylan=beechwood xylan hemicellulose, HKWL=hardwood kraft. Each column is a different representation of the non-hydrolytic fusion binding: GC3) filtered CLSM channel showing the location of the green fluorescent protein bound to carbohydrate binding module family-3, Brightfield) image of biomass particle, Z-projection) is the overlay of GC3 stacked images with purple-to-yellow farthest-to-nearest the viewing plane respectively, FL profile) shows the changes in GC3 protein fluorescent intensity (y-axis) over the distance (x-axis) across the width of the biomass particle. The yellow inspection line, drawn perpendicular to the biomass cell wall when possible, is shown over the GC3 and Brightfield images for a specific z-plane.
Figure 9-6. Confocal light scanning microscope images of bleached hardwood kraft pulp substrates. The rows of the image matrix describe each substrate; BHKP-Unref=unrefined bleached hardwood kraft pulp, BHKP-VB90m=bleached hardwood kraft pulp with 90 minute valley beating, BHKP-PFI10k=bleached hardwood kraft pulp with 10,000 PFI milling revolutions. Each column is a different representation of the non-hydrolytic fusion binding: GC3) filtered CLSM channel showing the location of the green fluorescent protein bound to carbohydrate binding module family-3, Brightfield) image of biomass particle, Z-projection) is the overlay of GC3 stacked images with purple-to-yellow farthest-to-nearest the viewing plane respectively, FL profile) shows the changes in GC3 protein fluorescent intensity (y-axis) over the distance (x-axis) across the width of the biomass particle. The yellow inspection line, drawn perpendicular to the biomass cell wall when possible, is shown over the GC3 and Brightfield images for a specific z-plane.
Figure 9-7. Confocal light scanning microscope images of hardwood sodium carbonate lignocellulosic substrates. The rows of the image matrix describe each substrate; HWSC-Unref=unrefined hardwood sodium carbonate biomass, HWSC-TMP 150C=hardwood sodium carbonate biomass refined with a thermomechanical pulp refiner at 150°C. Each column is a different representation of the non-hydrolytic fusion binding: GC3) filtered CLSM channel showing the location of the green fluorescent protein bound to carbohydrate binding module family-3, Brightfield) image of biomass particle, Z-projection) is the overlay of GC3 stacked images with purple-to-yellow farthest-to-nearest the viewing plane respectively, FL profile) shows the changes in GC3 protein fluorescent intensity (y-axis) over the distance (x-axis) across the width of the biomass particle. The yellow inspection line, drawn perpendicular to the biomass cell wall when possible, is shown over the GC3 and Brightfield images for a specific z-plane.