ASSESSING MECHANICAL DECONSTRUCTION OF SOFTWOOD CELL WALL FOR CELLULOSIC BIOFUELS PRODUCTION

By

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A dissertation submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

WASHINGTON STATE UNIVERSITY
Materials Science and Engineering Program

DECEMBER 2016
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ACKNOWLEDGEMENTS

I would like to express my appreciation to many individuals, with whose generous help for completing this doctoral dissertation. First and foremost, I am strongly indebted to my advisor and mentor, Professor Michael P. Wolcott, for ideas, inspiration, support, guidance, encouragement and patience throughout my entire Ph.D. study. His mastery, expertise, dedication and leadership have guided me to the track of being a qualified researcher who possesses skills for conducting complex research, problem solving, comprehensive thinking, and communicating. I would also like to express my gratitude to Dr. Jinwu Wang for his gracious help, insightful discussions and guidance throughout the research period. Special thank you goes to Dr. Xiao Zhang for his valuable comments and suggestions to solving problems in my research and reviewing the scientific manuscripts. Special acknowledgement also goes to Dr. Jinwen Zhang for his interest in my research work and perceptive help throughout my study.

My acknowledgement is also extended to the staff in Composites Material and Engineering Center (CMEC): Bob Duncan, Scott Lewis, Suzanne D. Hamada, Wanda Terry, Jannet Duncan for their kind help and patience. Special thanks go to Dr. Daniel Mullendore, Dr. Christine Davitt, and Dr. Valerie Lynch-Holm at Franceschi Microscopy and Imaging Center for their help on microscopy experiments. I would also appreciate the help from fellows in CMEC: Rui Zhu, Hanwen Zhang, Yalan Liu, Junna Xin, Fang Chen, Yu Fu, Bon-jae Gu, Huinan Liu, Shuai Zhang, Lanxing Du, Tuhua Zhong, Lang Huang, Harris Handoko for their unreserved help on the guidance of lab instruments and experiments. My special appreciation is also extended to all fellow classmates and labmates, those whom made my enjoyable time at WSU.
I would also express my sincere appreciation for the never-ended love, and support from my mother and father. The completion of my degree and personal achievements would have been impossible without their unconditional love and encouragement throughout my life.

Finally, I wish to express my love to Shenwan (Sherry) Wang; your warm heart, support, devotion and love made this dissertation finally come through!

Jinxue Jiang

July, 2016
ASSESSING MECHANICAL DECONSTRUCTION OF SOFTWOOD CELL WALL FOR CELLULOSIC BIOFUELS PRODUCTION

Abstract

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Mechanical deconstruction offers a promising strategy to overcome biomass recalcitrance for facilitating enzymatic hydrolysis of pretreated substrates with zero chemicals input and presence of inhibitors. The goal of this dissertation research is to gain a more fundamental understanding on the impact of mechanical pretreatment on generating digestible micronized-wood and how the physicochemical characteristics influence the subsequent enzymatic hydrolysis of micronized wood.

The initial moisture content of feedstock was found to be the key factor affecting the development of physical features and enzymatic hydrolysis of micronized wood. Lower moisture content resulted in much rounder particles with lower crystallinity, while higher moisture content resulted in the milled particles with larger aspect ratio and crystallinity. The enzymatic hydrolysis of micronized wood was improved as collectively increasing surface area (i.e., reducing particle size and aspect ratio) and decreasing crystallinity during mechanical milling pretreatment. Energy efficiency analysis demonstrated that low-moisture content feedstock with multi-step milling
process would contribute to cost-effectiveness of mechanical pretreatment for achieving more than 70% of total sugars conversion.

In the early stage of mechanical pretreatment, the types of cell fractures were distinguished by the initial moisture contents of wood, leading to interwall fracture at the middle lamella region for low moisture content samples and intrawall fracture at the inner cell wall for high moisture content samples. The changes in cell wall fractures also resulted in difference in the distribution of surface chemical composition and energy required for milling process.

In an effort to exploit the underlying mechanism associated with the reduced recalcitrance in micronized wood, we reported the increased enzymatic sugar yield and correspondingly structural and accessible properties of micronized feedstock. Electronic microscopy analysis detailed the structural alternation of cell wall during mechanical process, including cell fracture and delamination, ultrastructure disintegration, and cell wall fragments amorphization, as coincident with the particle size reduction. It was confirmed with Simons’ staining that longer milling time resulted in increased substrate accessibility and porosity. The changes in cellulose molecular structure with respect to degree of polymerization (DP) and crystallinity index (CrI) also benefited to decreasing recalcitrance and facilitating enzymatic hydrolysis of micronized wood.
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CHAPTER 1 INTRODUCTION

1.1 Background

With great concerns about the potential depletion of feasible fossil reserves, as well as the serious environmental problems derived from excessive utilization of fossil resource around the world, there is urgent interest for search and development of alternative, sustainable and economically viable fuels and platform chemicals from natural resources [1, 2]. Compared to other sustainable energy options, such as solar, wind, hydro and nuclear, it is the lignocellulosic biomass that has great potential in providing renewable energy-dense fuels and industrial chemicals in a substantial and economic scale. In the United States, for example, goals to reduce fossil fuels dependence and reduce greenhouse gas emissions from the transportation sectors through increasing the supply of renewable liquid fuels to 36 billion gallons by 2022, have been outlined in the Energy Independence and Security Act of 2007 [3]. Similarly, the European Union aims to substitute 25% of transportation fuel consumption with biofuels converted from renewable biomass by 2030 [1]. Lignocellulosic biomass, referring to non-food natural feedstock, including agriculture residues, woody and herbaceous plant, as well as dedicated energy crops, has been considered as the most abundant renewable carbon source with estimated global production of around 10-50 billion dry tons each year [4].

Over the past decades, multiple conversion technology strategies have been developed to initiate a promising route for creating new bio-based industries process from biomass and providing potentially cost-competitive energy dense, fungible liquid transportation fuels as well as bioproducts and/or chemical intermediates [5, 6]. With great advances in biotechnology and bioengineering, biochemical conversion has emerged as one of the most prominent strategies to fulfill bio-based industries (i.e., biomass-to-bioethanol, etc.), using pretreatment and enzymatic
hydrolysis steps to convert carbohydrate portion of biomass cell wall (i.e. cellulose and hemicellulose) into intermediate sugars, which serve as building blocks for various liquid fuels production under fermentation process by appropriate microorganisms [1, 7]. It is reasonable that efficient liberation of fermentable sugars from biomass cell wall plays vital role in industrialization of lignocellulose biorefinery, although various fermentation conditions and microorganisms may be employed for producing different final products. However, lignocellulosic biomass polysaccharides are naturally resisting to enzymatic or microbial attack for deconstruction due to the evolved superb protection, which is called biomass recalcitrance [1, 8]. Pretreatment is believed to be the most essential step to make the cellulosic substrates much more susceptible and digestible for subsequent release of fermentable sugars in enzymatic hydrolysis process.

Pretreatment.

1. 2 Pretreatment as a prerequisite for bioconversion of biomass

The main constituents of lignocellulosic biomass are cellulose, hemicellulose and lignin, which are nowadays being considered as the most attractive feedstock for production of biofuels and value-added chemicals. Cellulose is the main constituent of plant cell wall, existing as linear polymer chain with various degree of β-D-glucopyranose moieties linked via β-(1-4) glycosidic bonds [5]. Hemicellulose has branches with short lateral chains consisting of hexoses (mannose, glucose, and galactose) and pentose (xylose and arabinose), while Lignin is an amorphous polymer linked with phenolic monomers [9]. The plant cell wall is indeed a complex nanocomposite in which cellulose microfibrils embed into amorphous matrix of hemicellulose and lignin to form tri-dimensional hierarchical architecture. The heterogeneity of cell wall chemistry and complexity of spatial organization of structural polymers contribute to the so-called biomass recalcitrance, which has been investigated a lot and summarized in previous review papers [1, 10, 11]. Efficient
enzymatic hydrolysis of polysaccharides in biomass cell wall is believed to the prior step for conversion of lignocellulosic biomass into monomer sugars for subsequent fermentation into liquid biofuels such as bioethanol or biobutanol [12, 13]. In order to maximize the generation of intermediate fermentable sugars from enzymatic hydrolysis of lignocellulosic biomass cell wall, a pretreatment has been considered as a prerequisite step, which aims to facilitate the polysaccharides more accessible and susceptible to efficient enzymatic attack through altering the ultrastructure of carbohydrate-lignin matrix and/or partially removing chemical compositions [14, 15]. The pretreatment is not only vital for its own process, but also has significant impact on overall process economics of whole biorefinery, since it will intensively influence the subsequent downstream steps. The effective pretreatment technologies will require in addition to maximizing the enzymatic hydrolysis of carbohydrates efficiency, an ideal pretreatment approach also prefers to facilitate the fractionation of cell wall constitutes for biomass potential valorization with both biofuels and high value co-products [16].

Research attentions have been focused intensively for several decades on exploring various effective pretreatment approaches to liberate sugars for bioconversion [14, 17, 17–22]. The current pretreatment technologies can be categorized as physical, chemical/physicochemical, and biological processes [23]. Biological pretreatment, as normally defined, involves lignin degradation by microorganisms attack such as white or brown rot fungi [24, 25]. Biological pretreatment shows the advantage of mild reaction conditions and reduced output of waste stream, however, the substantial carbohydrate degradation and long pretreatment period significantly limit the development of efficient industrial-scale biorefinery [17, 26]. Over the past decades, chemical/physicochemical pretreatment methods have been considered as the leading technologies for lignocellulosic biomass pretreatment, since they can lead to increase of biomass hydrolysability
by removing chemical composition and/or modifying cell wall structure. Nevertheless, intensive investigations demonstrated that chemical/physicochemical pretreatment require expensive corrosion resistant equipment, extensive washing of solid stream, and disposal of liquid waste, as well as introducing inhibitors (i.e., 5-hydroxymethylfurfural, furfural, levulinic acids, formic acids and organic acids, etc.), which significantly hurdle the downstream enzymatic hydrolysis and fermentation processes [8, 27, 9]. Therefore, to address the deficiencies and limitations of mentioned pretreatments and achieve high theoretic conversion rates of both cellulose and hemicellulose, mechanical pretreatment gain recent attention, due to its simple process and versatile to various feedstock [28–30]. The high-energy requirement for mechanical pretreatment is also a key factor for its wide application.

1.3 Mechanical pretreatment advantage and challenge

Enzymatic hydrolysis has been initially focused on the conversion of cellulose into glucose, while the research is nowadays also interest in utilizing both hexoses and pentose derived from hemicellulose in subsequent fermentation for the purpose of improving the profitability of biorefinery [12]. Although, no best option exists, mechanical pretreatment (mainly refers to milling and grinding) appears to be closest to commercialization, through further R&D (research and development) on technology and cost performance [28, 31]. As mentioned above, mechanical pretreatment has long been used to ensure all biomass feedstock enzyme digestible, with the features of avoiding chemical input, undesirable inhibitors production, low capital and labor force investment [32]. Particularly with softwood, which is believed to be the most recalcitrant and has high content of hexose in hemicellulose, mechanical pretreatment offers green process for maximizing fermentable hexoses generation. Moreover, the environmentally friendly mechanical pretreatment enables generating substrates with sufficient enzymatic hydrolysis of polysaccharides
in cell wall, in addition, offers very pure and chemically unmodified lignin for high value added co-product development [33–37]. Mechanical pretreatment allows dissociation of botanical tissues (i.e., epidermal, parenchymatous and vessel tissue) and further disintegration of structural plant cell wall, rendering inevitable particle size reduction and increase of dependent external surface of the particles. It has been suggested that substrate surface area plays vital importance on determining the effectiveness of enzymatic hydrolysis, as the intimate contact between cellulose and cellulase (e.g., endoglucanase) is a primary step for initiation of enzymatic hydrolysis [38]. In other words, interactions between specific enzymes and cellulosic substrates including hydrolytic enzyme penetration into cell wall, binding to specific cellulosic substrate and performing catalysis are responsible for efficient enzymatic hydrolysis [38]. In addition, intensive mechanical pretreatment also results in disruption of the highly ordered cellulose structure, which also benefits to enzymatic hydrolysis, since it has been suggested that highly crystalline cellulose is less accessible to cellulase attack as compared to amorphous cellulose [31].

Despite of many advantages, there are several technical issues that still need to be tackled when using mechanical pretreatment for lignocellulosic biomass conversion. Substantial energy requirement is the main issue associated with mechanical deconstruction of lignocellulosic biomass. The initial substrate properties (such as physical structure, chemical compositions, and moisture content, etc.) and final product features are important factors affecting energy requirement for mechanical size reduction of lignocellulose biomass, as indicated in previous studies [28, 39]. More investigation on energy consumption of mechanical pretreatment targeted to generate digestible biomass substrate is still required to ensure conversion in economically viable manner. On the other hand, the current leading pretreatment technologies have claimed that cell wall chemistry changes are responsible for enhancement of accessibility and digestibility of
lignocellulosic substrate after chemical/biological pretreatments [6, 22]. For example, solubilizing hemicellulose fraction of biomass with dilute acid pretreatment results in increased substrate pore size and volume (i.e., increasing accessible area of cellulose to cellulase), improving subsequent enzymatic digestibility [40, 41]. Modifying of lignin by approaches such as alkaline, ammonia fiber expansion, and brown rot fungi pretreatments enables relieving non-productive adsorption of enzyme, leading subsequent increase of enzymatic hydrolysis efficiency [4, 42]. Nevertheless, it is well recognized that mechanical pretreatment enables generation of susceptible and digestible biomass substrates with negligible cell wall bulk chemistry change. Such contradictive results make understanding biomass recalcitrance complicated and also require a comprehensive elucidation of physicochemical characteristics of mechanically pretreated substrates towards enhancing enzymatic hydrolysis efficiency.

1.4 Research objective

Despite decades of research and development, commercialization of an economically viable woody biomass conversion process to fuels and chemicals still remains as a significant challenge. A successful bioconversion of lignocellulosic biomass into biofuels and bioproducts is mainly dependent on the efficient liberation of fermentable sugars from enzymatic hydrolysis process, which is influenced by the substrate characteristics such as cell wall chemistry and structural features, etc. Mechanical deconstruction of wood by pulverization offers a promising manner for alternating most of the current severe thermo/chemical pretreatment technologies, which are associated with high capital cost, low product yield and presence of inhibitory compounds to subsequent process. However, comprehensive understanding of the mechanism of wood structure deconstruction towards generating wood cell wall disruption and producing substrates accessible to enzyme saccharification is still lacking. Moreover, understanding on
characteristics of micronized wood, energy consumption and enzymatic hydrolysis performance and their relationships will provide meaningful information and guidance for overcoming biomass recalcitrance in an economic manner with mechanical pretreatment.

The goal of this research is to gain fundamental understanding of the mechanism of softwood cell wall deconstruction by mechanical pretreatment for fermentable sugar production. Therefore, the specific objectives of this research are:

To evaluate the development of physical characteristics of micronized wood particles and related energy consumption during fine milling process.

To evaluate the enzymatic hydrolysis efficiency of micronized wood and develop property-hydrolysis relationship.

To investigate fracture features of wood cell wall at the early stage of mechanical pretreatment and its influence on enzymatic hydrolysis.

To assess effect of mechanical milling pretreatment on ultrastructural properties and cellulose chemistry of micronized wood that relate to the sugar release efficiency or recalcitrance properties of biomass.

1.5 Dissertation scope and organization

Mechanical pretreatment offers a versatile approach for producing enzyme susceptible substrates from all kinds of feedstock by deconstruction of cell wall. The aim of this dissertation is at understanding the fundamentals of wood cell wall deconstruction during mechanical pretreatment and induced changes for facilitating enzymatic digestibility. Background pertaining to the major focus of each chapter is outlined in the introduction section and conclusions for the work are presented at the end of all the chapters.
Chapter 2 deals with the effect of mechanical pretreatment factors (i.e. input moisture content, particle size of feedstock and milling time) on development of physical characteristics of mechanically milled wood particles. Semi-empiric relationship based on Rittinger’s model is established between specific energy consumption of milling and particle size characteristic of milled wood to evaluate the milling performance of specific wood material.

Chapter 3 investigates the effect of mechanical pretreatment factors on enzymatic saccharification performance of milled wood particles and analyzes relationships between characteristics of wood particles and their saccharification efficiency. It provides metrics for correlating the degree of milling with degree of relevant digestibility of milled product and guidance for scaling-up the mechanical pretreatment.

Chapter 4 describes softwood cell wall fractures distinguished by the initial moisture content under primary stage of mechanical pretreatment and its influence on enzymatic digestibility. The micro-morphological characteristics and surface chemistry variations are tracked with microscopy and probe dye adsorption technologies.

Chapter 5 is focusing on elucidating the changes in ultrastructure of wood cell wall and cellulose characteristics, which influence the cellulose accessibility for subsequent enzymatic hydrolysis.

Chapter 6 summarizes key conclusions and major findings of this research as well as recommendations for future work based on results from this research.
References


Abstract

Mechanical milling demonstrates potential in the pretreatment arena to valorize lignocellulosic biomass because it eliminates chemicals and simplifies processing. The development of physical properties and energy consumption for generating micronized particles are key factors affecting potential use. This study investigated the effect of input moisture content, and feedstock particle size on developing physical characteristics of micronized particles and the resultant specific energy consumption in the milling process. Wood particles with different sizes were effectively comminuted to less than 20-µm within several minutes using a vibratory ring and puck mill. Moisture content was found to be a key factor influencing the development of particle morphology and crystallinity. Lower moisture content resulted in much rounder particles with lower crystallinity, while higher moisture content resulted in the milled particles with larger aspect ratio and crystallinity. Crystallinity index and median aspect ratio of the micronized particles were linearly correlated. The particle size change during milling was highly correlated to the specific energy consumption of milling through the Rittinger’s model ($0.91 < R^2 < 0.96$). Input moisture content and feed size were found to affect the energy intensity of grinding woody biomass. The Rittinger’s constant was a good indicator of the material performance in this area. The results will provide a guidance for preferred milling conditions as well as designing scalable micronizing mills.

**Key words:** fine milling, woody biomass, morphology, crystallinity, specific energy consumption, particle size distribution, aspect ratio.
2.1 Introduction

The development of sustainable biofuels from woody biomass is becoming increasingly important for diversifying our transportation energy portfolio and mitigating the global greenhouse gas emission [1]. Both biochemical and thermochemical biorefinery processes have encountered challenges for commercial implementation, including the robust biomass recalcitrance and the need for an integrated feedstock supply. Depot-based technologies may be useful for transforming bulky biomass into an interchangeable feedstock for multiple applications; e.g. pretreated wood, or fermentable sugars [2]. Such an approach can potentially reduce risk and logistics costs of feedstock supply for centralized downstream processes. An appropriate pretreatment plays a key role for such depots by facilitating the potential distributed production of cellulosic sugars.

Mechanical deconstruction of biomass offers potential for streamlining processes and enabling supply chains that utilize existing forest products facilities, provide flexibility to operate at various scales, eliminate chemical input, and reduce or eliminating waste water treatment requirements [3,4]. Mechanical milling is a common method of altering physical properties of substrates (e.g., particle size, aspect ratio and cellulose crystallinity, etc.) and can significantly facilitate either biochemical or thermochemical conversions. Reduced particle size is beneficial to biomass conversion by improving particle reactivity and heat / mass transfer efficiency [3,5]. The aspect ratio of particles is a crucial factor for influencing biomass pyrolysis efficiency, though the relationship between particle shape and biochemical conversion efficiency remains elusive [6–8]. However, it is known that the cellulose crystallinity in lignocellulosic biomass influences the rate and extent of enzymatic digestion [3]. The physical properties of milled biomass can be controlled by milling conditions; e.g. the moisture content of feed material, and feedstock particle size. Kobayashi et al. [9] reported that wood powder pulverized with a vibratory mill at 3% moisture
content had a reduced crystallinity of 14.3%, while wood powder ground from 11% moisture content resulted in a crystallinity of 37.7%. Even though the exact fracture mechanism of particles in the milling chamber is unknown, Gil et al. [10] have shown that the feedstock particle size influenced the particle morphology of final products in hammer milling. According to Gil et al, single particle breakage in an impact milling is related to the particle volume and contact area upon impact; both of which are determined by the particle size. Thus, a larger particle size increases the probability of structure fragmentation, leading to rapid particle size reduction. Despite a significant amount of information obtained on the effect of milling conditions on the physical properties of coarsely milled particles from several types of mills [4,11], a systematic understanding of the effect of milling conditions on producing fine particle fractions (i.e., partial size below 100µm) and their ensuing physical properties is lacking. This finely milled biomass with an average particle size below 100µm is termed here as micronized biomass. These micronized particles exhibiting a significant level of cell wall disintegration, have shown to be susceptible to enzymatic hydrolysis [12,13]. Conventional milling technology like hammer milling is incapable of providing sufficient size reduction to produce a micronized particle [14]. In contrast, micronized wood is commonly prepared using vibratory milling [15,16].

It is apparent that a considerable amount of energy is consumed during size reduction of lignocellulosic biomass [17,18]. A number of previous studies have attempted to establish the relationship between energy consumption and particle size reduction during biomass milling. For example, a power-law relation was proposed to correlate energy consumption with product particle size (e.g., screen size) or the biomass comminution ratios [19,20]. Inspired by the milling theories proposed to delineate the specific relationship for milling ores, Temmerman et al. characterized the energy-size relationship with the Rittinger’s model for hammer milling wood chip and pellet.
Among these studies, the Rittinger’s model was recognized as the best approach to describe fine milling performance, as well as a good indicator for assessing a given comminution system [21–23]. However, predicting the energy consumption for micronized wood by mechanical milling with different operational conditions is still lacking. Therefore, modeling the energy consumption of fine wood milling with the size characteristics will be the first step to optimize the milling process for potential industrial application.

The main goal of this study is to delineate the physical property development of micronized particles under various milling conditions and correlate size reduction with energy consumption of the process. The experiments focus on exploring effects of moisture content and feed size for the starting material on changes in particle morphology, cellulose crystallinity, and specific energy consumption of the fine milling process. The knowledge gained in this study will aid in developing potential approaches and strategies for producing a micronized biomass substrate in an economical manner with mechanical pretreatment.

2.2 Materials and methods

2.2.1 Material

Clean, Douglas-fir (Pseudotsuga menziesii) wood chips were obtained locally (Vaagen Brothers Lumber Inc., Colville, WA). The as-received chips were passed through a vibrating screen with a 25.4-mm aperture and retained on a 4.75-mm screen, producing a chip with a geometric mean diameter of 10.36-mm. The screened wood chips were processed into particles by a hammer mill fitted with a 11.11-mm, 6.35-mm, and 3.18-mm, screen, respectively. These screened feedstock samples were labeled as Pxx (pass) with xx being the screen size (i.e., P25, P11, P6, and P3, respectively). The processed feedstock was subsequently stored in a temperature- and humidity-conditioned room with an equilibrium moisture content (EMC) of 15% and
subsequently conditioned to different target EMCs values. After final conditioning, all material was stored in sealed plastic bags and moisture content was validated using gravimetric methods according to standard protocol [24].

2.2.2 Mechanical milling process

Fine milling experiments were carried out with a high-energy vibratory ring and puck mill (Standard Ring milling, motor power 1.1-kw, Rocklab Pty Ltd, New Zealand). The milling chamber had an inner diameter of 128-mm and height of 43-mm. The grinding media were a ring (inner diameter 78-mm, outside diameter, 100-mm, height 41-mm) and a puck (diameter 52-mm and height 41-mm). Both milling chamber and grinding media were made of tungsten carbide.

The milling experiments were designed to investigate different milling variables; these include milling time, initial moisture content, and size of feedstock. The resulting micronized wood powder was then characterized for particle size distribution, aspect ratio, and crystallinity. Milling times were varied from 2 minutes to 12 minutes with 2-minutes interval. Four initial moisture contents (5, 10, 15, and 30% oven dried base) were tested, using P3 particles with charge quantities of 10-grams per grinding batch. For investigating effect of the feed size on milling performance, four different initial size levels (P25, P11, P6 and P3) were tested, using conditioned moisture content of 10% samples with charge quantities of 10-grams per milling batch.

2.2.3 Measurement of specific energy consumption

The milling energy consumption of the ring and puck mill was measured with a Fluke 1735 power logger (Fluke, USA). The active power, active energy, power factor, frequency, and time were acquired by computer. The specific grinding energy consumption was calculated using the following relation:
\[ E_p = \int_0^t (P_t - P_0) \, dt/m = \int_0^t \Delta P_t \, dt/m \]

where: \( E_p \) is the specific net energy consumption (kJ/kg); \( P_t \) is the power consumed at time \( t \); \( P_0 \) is the average power consumption under idle condition measured from an empty mill; and \( m \) is the mass charge in kg of wood to be pulverized.

### 2.2.4 Characterization of wood powder

#### 2.2.4.1 Preparation of wood powder for analyses

The milled wood samples were prepared for particle size distribution and aspect ratio analyses as follows. Each sample was suspended in distilled water to achieve a 0.5% solids mass concentration. For the aspect ratio measurement, a diluted powder suspension was dispersed by adding 1-mL of a dispersant solution (0.1% w/w sodium dodecyl sulfonate) per 100-mg wood [25]. The suspended samples were stirred for 1 hour with a magnetic stir rod, followed by sonication with 20% amplitude (Branson, Switzerland) for 2 minutes to ensure complete disintegration of particle agglomeration. The same procedure was following for measuring particle size distribution, except dispersant was not added.

#### 2.2.4.2 Particle size distribution

The volumetric particle size distribution of the wood powders was analyzed using a laser scattering particle size analyzer (Mastersizer 3000 with Hydro LV wet sample dispersion, Malvern instrument, UK). The median size was used to represent the particles size for analysis. The dimensionless parameter \( \Delta_d \) is used to represent the breadth of the particle size distribution, where:

\[ \Delta_d = \frac{d_{90} - d_{10}}{10 \mu m} \]

and \( d_{90} \) and \( d_{10} \) are particle sizes corresponding to the 90% and 10% percentiles as assessed from the cumulative size distribution, respectively.
2.2.4.3 Aspect ratio analysis

The solid suspension of samples prepared as described above, was distributed on a silica wafer mounted on a metal stab. This process involved using a pipette to form a droplet with a volume of ca. 200-µL. After allowing the water to evaporate at ambient conditions, samples were sputtered with gold to prepare examination in a Scanning Electron Microscopy (SEM). The measurement of aspect ratio was obtained from the analysis of SEM images using ImageJ (National Institutes of Health, USA) following the procedures described elsewhere [26,27]. For each sample, about 20 SEM images containing around 30000 particles were analyzed. The aspect ratio of each particle was calculated as the ratio of the major and minor axis of the fit ellipse for the selected particle. The minimum aspect ratio value of 1 was restricted and describes a perfect and filled circle. The number based cumulative aspect ratio distribution was used to obtain the median aspect ratio $AR_{50}$, which was the value of 50% cumulative aspect ratio distribution. The width of aspect ratio distribution $\Delta_{AR}$ was defined as the difference between the aspect ratios corresponding to 90% and 10% values in the cumulative distribution.

2.2.5 Cellulose crystallinity

X-ray diffractograms of the milled wood particles were obtained using a powder x-ray diffractometer (Rigaku, Miniflex 600, Japan) with a Cu Kα ($\lambda = 0.154$ nm) radiation source generated at 40-kV and 15-mA. The instrument scanning range $2\theta$ was from 10 to 40°. The relative degree of cellulose crystallinity, in terms of crystallinity index (CrI), was estimated using equation as described by Segal [28]:

$$CrI \,(\%) = \left[ (I_{002} - I_{am}) / I_{002} \right] \times 100$$

where $I_{002}$ is the intensity of main peak, and $I_{am}$ is the intensity due to amorphous portion evaluated as the minimum intensity between the main and secondary peaks.
2.2.6 Scanning electron microscopy (SEM)

The morphology of prepared samples as described above was analyzed by scanning electron microscopy. Images of samples were acquired typically at 20-kV accelerating voltage using FEI Quanta 200F, field emission gun with high vacuum ETD detectors (FEI Company, Hillsboro, Oregon, USA). Prior to imaging, the suspended wet sample was air dried and sputtered with gold for good conductivity.

2.3 Results and discussion

2.3.1 Effect of milling conditions on characteristics of particle morphology

Mechanically fragmenting the lignocellulosic biomass into the micronized size range has been recognized as an essential step to enhance the substrate digestibility of mechanically pretreated substrates by others [12,29]. In this study, effectively micronized softwood was achieved using ring and puck mill under various milling conditions. The change in median particle size $d_{50}$ as a function of milling time for feedstock with various initial moisture contents is presented in Figure 2.1A. At the early stage of milling (e.g., at the 2-minutes point), an elevated moisture content facilitates more rapid particle size reduction (Figure 2.1A), which is likely a result of the higher specific energy input for milling higher moisture content samples (e.g. MC30%). A previous study reported that a mechanical refining process with a higher energy input caused more severe breakage of wood fiber with shorter length and width [30]. Additionally, Karinkanta et al., demonstrated that smaller particles were obtained at conditions with higher impact energy input when Norway spruce was pulverized with an oscillatory ball mill [25]. We also observe that $d_{50}$ approached around 20µm at 6-minutes milling time under all tested moisture content conditions, while the changes in $d_{50}$ were slight for additional milling time to 12 minutes. These systematic changes in particle size with respect to milling time are in agreement with the literature reports,
which claimed that particle size could not infinitely decrease by prolonging milling time after attaining a critical size [18]. The width of particle size distribution $\Delta_d$ is increased as a function of $d_{50}$ for samples with different initial moisture contents (Figure 2.1B). The strong linear decrease in $\Delta_d$ with respect to $d_{50}$ suggests that the mechanical milling resulted in effective particle size reduction of micronized wood, yielding more uniform particles.

The median particle size $d_{50}$ of micronized wood produced from various feedstock sizes is depicted in Figure 2.1C. For different feed size samples, we successfully comminuted the particles from the millimeter to micrometer range in 12-minutes or less, using a highly versatile and energy-efficient ring and puck milling system. Micronizing woody biomass from a chip size (i.e., P25 in Figure 2.1C) can thus be effectively achieved in relatively a short milling time compared to the ball milling times of several hours or days reported by others [13,31,32]. At an early stage of the milling process, we notice that feedstock with various initial sizes were milled to similar size around 100-µm. This behavior was likely a result of a higher contact area upon impact for larger-sized samples during milling process. A previous study on impact milling of lignocellulosic biomass indicated that larger particles experienced a higher breakage probability, since the internal failure probability increases with volume as well as with the particle’s contact area upon impact [10]. We also observe that a rapid decrease of particles reaching ca. 20 µm for various initial sizes at 6-minutes milling time point, followed by a slight change of particle size after further milling to 12 minutes. The similar tendency of particle size evolution from different input sizes suggests that initial size has a negligible effect on development of micronized wood particle size, even though bigger size sample breaks up faster at early stage of milling process. The relationship between the width $\Delta_d$ and mean $d_{50}$ of the particle size distribution for samples with different feed sizes is presented in Figure 2.1D. The results also demonstrate effective size
reduction and generation of uniform particles where the $\Delta_{d}$ decreases linearly with $d_{50}$ (with high coefficient $R^2 = 0.98$).

![Figure 2.1](image)

**Figure 2.1** Effect of milling conditions on the median particle size and width of particle size distribution.

(A) median particle size changes of samples with different moisture contents; (B) width of particle size distribution of samples with different moisture contents; (C) median particle size changes of samples with different feed sizes; (D) width of particle size distribution of samples with different feed sizes.

The influence of milling conditions on changes to the shape of micronized particles, as represented by median aspect ratio $AR_{50}$ and width distribution of aspect ratio $\Delta_{AR}$ are shown in
Figure 2.2. The initial moisture content differentiated the shape development of micronized wood (Figure 2.2A). We observe that $AR_{50}$ decreased gradually with the increase of milling time for those samples with initial moisture content of 5%, 10% and 15%, whereas less change in aspect ratio for samples with initial moisture content of 30% is noticed. These observations indicate that lower moisture content contributes to much rounder particles, and higher moisture content results in particles with higher aspect ratio. The linear decrease of aspect ratio distribution width $\Delta AR$ with respect to mean $AR_{50}$ indicates that micronized particles possessed much more uniform shape after mechanical milling. The median aspect ratio $AR_{50}$ of micronized wood milled from various feedstock sizes is depicted in Figure 2.1C and D. We notice that the feed size has negligible influence on $AR_{50}$ to milling time relation for micronized wood produced by this type of milling (Figure 2.2C). All the samples evolved to similar particle shape during milling process, regardless of their initial size. In addition, the linear relation between the breadth $\Delta AR$ and mean $AR_{50}$ of the particle aspect ratio distribution also suggests uniform particle shape of micronized wood regardless of the feed sizes (Figure 2.2D).
2.3.2 Effect of milling conditions on cellulose crystallinity

Besides observing the morphology changes of micronized wood during the milling process, changes in cellulose crystallinity were also characterized. The crystallinity index (CrI) changes of micronized wood produced from various initial moisture contents are delineated in Figure 2.3A. Overall, CrI decreases significantly with the increase of milling time for those tested samples,
suggesting effective disruption of crystalline structure during ring and puck milling process. This is in agreement with other reports on crystallinity reduction of lignocellulosic biomass subjected to mechanical actions (e.g., impacting, and shearing, etc.) with various milling techniques [3,4]. More precisely, the CrI decreased rapidly to around 10% for micronized wood produced with relative low moisture contents (e.g., MC5% and MC10%), suggesting significant amorphization of crystalline cellulose. Nevertheless, the CrI of micronized samples that were milled with a relatively higher initial moisture content (e.g., MC30%) only decreased to 40% even after the longest milling time of 12-minutes, suggesting less changes in crystalline structure during milling process. These phenomena indicate that the initial moisture content significantly influenced the development of cellulose crystallinity. Previous research on milling pretreatment of sugarcane bagasse and wheat straw using dry ball and wet disk milling indicated that dry milling resulted in significant amorphization of these herbaceous biomass feedstock, whereas wet milling only slightly reduced the crystallinity [33]. Hoeger et al., also reported that mechanical fibrillation of softwood pulping fibers in wet state with stone grinding contributed to only 15-25% crystallinity reduction with fibrillation time of 6-12hours [29]. The CrI changes of micronized wood produced from various initial sizes are depicted in Figure 2.3B. There is gradual decrease of CrI to around 10% with respect to milling time for samples milled from different feed sizes. The changes in CrI show similar tendency for different feed size samples, suggesting negligible effect of feed size on crystallinity development of micronized wood. These results are consistent with previously reported observations that various woody and herbaceous biomass feedstock with different initial sizes were effectively milled to similar crystallinity using a tandem-ring mill [16].
2.3.3 Relations of structural characteristics of micronized wood

Mechanical milling conditions can generate micronized wood with distinctive particle morphology and crystallinity as discussed above. It is also interesting and essential to investigate the relationships among these structural characteristics of micronized wood for better understanding of sequential particle evolution during milling process. Thus, the changes of median aspect ratio $AR_{50}$ as a function of median size $d_{50}$ for those obtained samples under various milling conditions are shown in Figure 2.4. It is observed that $AR_{50}$ decreased slightly with the decline of $d_{50}$ for samples with initial moisture content of MC30%, whereas those from other samples showed drastic decrease. Such phenomena may suggest that the development of micronized wood is mainly related to fracture modes that are determined by initial moisture contents during milling process. It is interpreted that the fiber fragmentation mode is responsible for the development of micronized wood with high moisture content input (e.g., MC30%). That means the fiber fracture parallel to the fiber length occurred more frequently than it did.
perpendicular to the wood grain, with higher initial wood moisture content (i.e., 30%). This fracture behavior resulted in a decrease in particle size with a negligible change in aspect ratio. As elucidated by previous research on mechanical pulping, refining along the fiber length resulted in fibers peeling rather than fracturing across the fiber length [34,35]. During the milling process, the lower moisture content samples irrespective of their sizes might undergo chipping and abrasion, leading to shorter fibers along with particle size reduction, collectively.

On the other hand, the strength of wood fibers perpendicular to the cellulose fibrils is significantly higher when wood is below the fiber saturation point; ca. 30%. This behavior results from increased hydrogen bonding between cellulose chains that would otherwise be bridged by water molecules [36]. With relatively lower moisture contents, wood fibers possessed higher stiffness. Fracture would thus propagate in the direction perpendicular to the fiber length more frequently at low moisture content, reducing aspect ratio along with particle size. In addition, the increased probability of fracture perpendicular to the fiber length was also coincident with the disruption of crystalline structure, resulting in lower cellulose crystallinity for lower moisture content samples.

The difference of particle aspect ratios for milled wood at various moisture contents is also visually evident. SEM images of micronized particles produced at various moisture contents and a milling time of 12-minutes are shown in Figure 2.5. Fibrous particles were obtained from MC30% sample, while relatively low moisture content samples resulted in glabular and sperical particles.
Figure 2.4 Relation of median aspect ratio and median particle size of micronized wood from various milling conditions.
Figure 2.5 Morphology difference of micronized wood with different initial moisture contents (A: MC5%, B: MC10%, C: MC15%, D: MC30%) during milling process; Feed size: P3; Loading: 10 g; Milling time: 12 minutes.

The relations between cellulose crystallinity and particle morphology are depicted in Figure 2.6. The CrI decreases significantly with the decrease of median particle size $d_{50}$ for all milling conditions (Figure 2.6A). Nevertheless, there is less change of CrI for samples milled with relatively higher moisture content (e.g., MC30%) compared to all others. This decreased change in crystallinity is noted despite the fact that the resulting particles were smaller than those produced
at the lower moisture contents. This observation is in agreement with previous reports, which claimed that the moisture content of lignocellulosic substrates was a primary factor affecting the decrease in crystallinity during ball milling [9,37].

Moisture content is an important factor influencing the glass transition of amorphous polymers in wood cell wall matrix, and the glass transition temperature (Tg) shifts to lower temperature range when moisture content increases [38,39]. With the moisture content around 30%, hemicellulose has a Tg around -22°C, thus existing in its rubbery state with greatly reduced stiffness at ambient milling condition. The Tg of hemicellulose is about 30°C at approximately 10% moisture content [38]. That means the amorphous polymers were in their glass state during ambient milling of low moisture content samples. Wood fiber structure is also known to have microfibril bundles embedded within the amorphous polymers matrix [40]. Thus, during the milling process, the fracture of wood fibers occurring in higher moisture content samples would primarily include the breakage of rubbery state matrix and splitting apart of microfibril bundles without causing extensive disorder within the crystalline structure of cellulose. However, for the lower moisture content samples, the impact milling disrupted the brittle cell wall matrix and crystalline cellulose skeleton. This led to a collective decrease of particle size and cellulose crystallinity, which is in agreement with the previously discussed evolution of particle aspect ratio. Figure 2.6B illustrates the strong linear relationship between \( AR_{50} \) and CrI, regardless of the moisture content and initial size. The quality of the linear fit is characterized by high coefficients (\( R^2 = 0.90 \)). Here, larger aspect ratio values correspond to higher CrI.
2.3.4 Analysis of energy requirements for size reduction

Besides the physical properties of micronized particles, specific energy consumption for producing such particles is another important factor for evaluating fine milling performance of woody biomass. Rittinger’s model is an appropriate model to describe the fine milling performance, where the new surface area generated per unit mass of the particle is directly proportional to the specific energy consumption for size reduction [21–23]. The specific surface area is inversely proportional to the particle size. Therefore, the Rittinger’s model can be expressed as follows: 

\[ E = C \left( \frac{1}{d_p} - \frac{1}{d_f} \right) \]

where \( C \) is a constant characteristic of the material, \( d_p \) and \( d_f \) are the characteristic particle size of milled product and feed [41]. The advantage of applying this model lies in characterizing the grinding performance of woody biomass with one parameter, which can be determined from model constant \( C \). The collection of limited parameters such as specific energy consumption and particle size will benefit to scale-up processing of fine milling of woody biomass.
Linear relationships between specific energy consumption and particle size reduction parameter in Rittinger’s model \((1/d_p - 1/d_f)\) for samples with different initial moisture contents are shown in Figure 2.7. The quality of the linear fit is characterized by high coefficients \((0.91 < R^2 < 0.95)\). In previous study, Karinkanta et al. also reported linear relationship between the inverse value of \(d_{50}\) of finely ground wood powder and total available impact energy with oscillatory ball mill in accordance to Rittinger’s model [25]. The model constant C can be a good indicator for grindability of woody biomass, and for evaluating the energy efficiency of milling process when similar materials are used as a probe [42]. As discussed above, moisture content was the key factor influencing the development of morphology and crystallinity of micronized wood, and to a large extent, the specific energy consumption. Increasing the moisture content induced increase of the specific energy consumption during milling process, as indicated by model constant values. This phenomenon was in agreement with previous research on energy consumption for milling various wood species with different moisture contents conducted using laboratory-scale hammer mill [42].

As indicated above, moisture content greatly affects the toughness of woody biomass. At low initial moisture content (e.g., MC 5%), the brittle polymer components fractured easily with low energy consumption. Increasing the moisture content resulted in the amorphous polymers becoming tougher due to the moisture acting as a plasticizer, thus requiring more energy consumption on fatigue to cause rupturing.
Figure 2.7 Effect of moisture content on specific net energy consumption as a function of particle size reduction ($1/d_p - 1/d_f$).

The specific energy consumption as a function of size reduction parameter ($1/d_p - 1/d_f$) in Rittinger’s model for various initial size samples is shown in Figure 2.8. The experimental results are also fitted well to Rittinger’s model. The linear relations are identified with high coefficients ($0.91 < R^2 < 0.96$). The larger the feed size, the more energy was required for fine milling. Although ring and puck mill is capable of comminuting chip-sized feedstock to micrometer range within minutes, it is not economically viable from the energy perspective. Coarse milling of Douglas-fir with different fitted screen sizes as used in the current study through pilot-scale hammer mill had been carried out in our lab, and the results indicated that the energy consumption for milling wood chips was in the range of 59-141 kJ/kg depending on specific fitted screen size (Liu et al. in review). Several researchers also reported that fine milling of woody biomass was
much more energy consuming than coarse milling [4,17,42,43]. Therefore, multi-step comminution strategy with combination of coarse and fine milling seems much more economical than directly fine milling of large-sized feedstock. However, the multi-step comminution will inevitably increase the capital investments.

The difference in energy consumption for milling samples with various properties suggests that minimizing the grindability of woody biomass is necessary when using ring and puck mill for fine milling. Therefore, it is reasonable to standardize the milling variables using a reference standard (e.g. chemical composition, moisture content and size) to obtain the grindability of lab-scale test as reported in mineral and biomass industry [44–46].

![Figure 2.8](image)

**Figure 2.8** Effect of feed size on specific net energy consumption as a function of particle size reduction \((1/d_p - 1/d_f)\).
2.4 Conclusions

We effectively micronized softwood Douglas-fir using a vibratory ring and puck mill within several minutes. The effect of substrate properties (i.e., moisture content and initial size) on development of physical properties of micronized particles and specific energy consumption for producing such particles were investigated. Results indicated that moisture content was the most important factor influencing milling behavior in terms of particle morphology and cellulose crystallinity. The lower moisture content samples resulted in much rounder particles with relatively smaller crystallinity than their higher moisture counterparts. The feed size had negligible influence on development of particle physical properties, but only affected the specific energy consumption. A multi-step milling strategy is recommended for a more economical approach for fine milling of woody biomass. The crystallinity and aspect ratio of particles underwent similar development tendency. There was a linear relationship between crystallinity index and median aspect ratio of milled particles.

Rittinger’s model was ideal for describing the relationship between specific energy consumption, and particle size changes. The constant in this model was a good indicator of grindability of woody biomass with different properties. It is also recommended that standardizing milling test and parameters could benefit to evaluating the grindability of fibrous materials on a lab-scale, as such information would be useful for designing scale-up milling facilities of woody and herbaceous biomass feedstock.
References


CHAPTER 3 CORRELATING ENZYMATIC HYDROLYSIS EFFICIENCY WITH PHYSICAL STRUCTURAL FEATURES OF MICRONIZED WOOD

Abstract

Enzymatic hydrolysis of lignocellulosic biomass is highly dependent on the changes in structural features after pretreatment. Mechanical milling pretreatment is an effective approach to alter the physical structure of biomass and thus improve enzymatic hydrolysis. This study examined the influence of structural characteristics on the enzymatic hydrolysis of mechanically milled wood particles. We have also evaluated the energy efficiency of this processing method. Results indicate that the influence of processing variables on enzymatic hydrolysis of milled wood relate mainly to the structural properties of milled wood. Findings reveal that reducing particle size down to ca. 30-µm disintegrates fibers and fiber bundles, while improving the enzymatic hydrolysis of the milled wood to around 40% of theoretical yield. Mechanically disintegrating the fiber cell wall into micronized fragments smaller than 30-µm further increases surface area and crystalline structure of cellulose, facilitating significant carbohydrate conversion (over 70% of theoretical yield). Empirical prediction of carbohydrate conversion with structural characteristics using a multiple linear regression model indicated that the enzymatic hydrolysis of micronized wood improved as collectively increasing surface area (i.e., reducing particle size and aspect ratio) and decreasing crystallinity during mechanical milling pretreatment. Energy efficiency results demonstrate that using a low-moisture content of the starting material and a multi-step milling process decreases the energy required when producing simple sugars with a mechanical pretreatment. Findings from this study provide new insights for mechanically overcoming biomass
recalcitrance and developing cost-effective milling technologies for industrial scale applications in biorefinery.

**Key words:** Mechanical milling pretreatment, particle size, aspect ratio, crystallinity index, enzymatic hydrolysis, energy efficiency

### 3.1 Introduction

Due to increasing global energy demand and concerns on the climatic issues from excessive utilization of fossil fuels, it is critical to identify alternative and renewable energy sources [1,2]. Biochemical conversion of lignocellulosic biomass into liquid biofuels through enzymatic hydrolysis and microbial fermentation offers a prominent route for creation of a sustainable liquid energy source for substituting fossil derivatives [3,4]. Close contact between the hydrolytic enzyme and lignocellulosic substrate is recognized as a primary step for initiating hydrolysis [5]. Efficient enzymatic hydrolysis of biomass is thus directly related to the structural features of the substrate [6]. Several factors that have been identified to influence the sugar yields in enzymatic hydrolysis include chemical structural features such as cell wall compositions and physical structural features such as morphological characteristics of substrate, and cellulose crystallinity, etc. [7,8].

Among all substrate-related factors, particle size has a strong influence on enzymatic hydrolysis of pretreated biomass. Studies show that particle size reduction facilitates enzymatic hydrolysis of biomass [9,10]. For example, when sawdust slurries with different initial particle sizes were subjected to enzymatic hydrolysis, the smallest particle size fraction (33-75µm) was found to perform 50-55% more glucose yield than that from the largest particle size portion (590-850µm) [11]. Wang et al. investigated the dilute acid pretreatment of corn stover using three reactors with different configuration types and reported that the reduced particle size after
pretreatment was strongly correlated with the glucose release after enzymatic hydrolysis [12]. However, the precise role of substrate particle on enzymatic hydrolysis is a complex issue. Del Rio et al., found that fiber size does not appear to affect ease of enzymatic hydrolysis, since there was no difference in sugar yields for six portions of size-fractionated softwood pretreated by organosolv. Ju et al., also found that the fiber size had little effect on the substrate digestibility of delignified wood fiber [13].

Cellulose chains contain both crystalline and amorphous regions. Crystalline cellulose is believed to be more resistant to enzyme attack than amorphous cellulose; thereby negatively affecting cellulose digestibility [6,10]. This is evident in previous studies, showing that the hydrolysis rate and yield of amorphous cellulose were typically 3 to 30 times faster than that of high crystalline cellulose [5,6]. In contrast, some studies were emphasized that cellulose crystallinity does not significantly affect overall cellulose hydrolysis for heterogeneous lignocellulosic substrates [6]. For example, Bali et al., found that the enzymatic hydrolysis of alkaline pretreated *Populus* increased significantly, while the crystallinity change was negligible [14].

Generally, these physical structures affect the enzymatic hydrolysis of the substrates due to the changes of surface area. However, if the substrate already has sufficient or excess surface area resulted from modification or removal of chemical compositions, then the structural alternation would have less of an effect. Thus, changes in particle size and crystallinity are intricately linked to chemical composition and morphological features. However, previous work on this phenomenon has focused on chemically modified substrates [15,16]. The changes in structural features due to chemical modification can lead to inconclusive results.
Mechanical pretreatment can produce micronized substrates with various degrees of enzymatic digestibility. The micronized biomass has unique physico-chemical characteristics. Compared to thermochemical pretreatment methods, mechanical process prevents chemical modification of nearly all of the chemical constituents, while significantly disrupting the fiber cell wall structure. Identifying the relationship between structural features and enzymatic hydrolysis of micronized biomass will provide, at a minimum, perspective towards relieving structural recalcitrance for facilitating enzymatic hydrolysis. This knowledge also lays the groundwork for determining the technical feasibility of mechanical milling as an effective biomass deconstruction method.

On the other hand, intensive energy consumption of mechanical pretreatment is a limiting factor in scaling-up application for biorefinery. Maximizing the release of fermentable sugars from micronized biomass is essential in reducing the specific energy consumption. Evaluating how pretreatment conditions affect energy efficiency may help maximize sugar yields in economic manner and lead to potential development of cost-effective milling pretreatments for industrial biofuel conversion.

The objective of this study is to discern how the structural features of micronized wood affect its enzymatic hydrolysis efficiency. We evaluated the enzymatic hydrolysis performance of micronized wood with distinctive structural properties resulting from various milling variables. We also examined the effects of different milling variables on energy efficiency of the mechanical pretreatment process for producing fermentable sugars from native softwood feedstock. This study provides insight on new avenues to mechanically overcome structural recalcitrance in order to improve the enzymatic hydrolysis of native lignocellulosic biomass.
3.2 Materials and methods

3.2.1 Materials

The raw material used in this study was Douglas-fir (*Pseudotsuga menziesii*) wood chip obtained from a local company (Vaagen Brothers Lumber Inc., Colville, WA). The received chips were separated using a vibrating screen with 25.4-mm aperture size. The screened wood chips were then pre-ground into various particles using a hammer mill fitting with 11.11-mm, 6.35-mm, and 3.18-mm, screen, respectively. Thus, the four kinds of screened sample were labeled as Pxx (pass) with xx being the screen size (i.e., P25, P11, P6, and P3, respectively). These feedstock samples were subsequently stored in a temperature- and humidity-conditioned room with an equilibrium moisture content (EMC) of 15%. Before fine milling, the feedstock samples were subsequently conditioned to different target EMCs values in sealed plastic bags and the specific moisture content was validated using gravimetric methods according to standard protocol [17].

3.2.2 Mechanical milling pretreatment

Mechanical milling experiments were performed using a Standard Ring and Puck mill with motor power of 1.1-kw (Rocklab Pty Ltd, New Zealand). The milling chamber had an inner diameter of 128-mm and height of 43-mm. The grinding media consisted of a ring (inner diameter of 78-mm, outside diameter of 100-mm, height of 41-mm) and a puck (diameter of 52-mm and height of 41-mm). Both the milling chamber and grinding media were made of tungsten carbide.

In order to generate micronized wood with different structural features, the experimental setups for fine milling were conducted with various milling variables, including milling time, initial moisture content and the size of feedstock. The detailed information was provided elsewhere (Jiang, et al., under review). Milling times varied from 2 minutes to 12 minutes with 2-minute intervals. Four initial moisture contents (5, 10, 15, and 30% oven dried base) were tested, using
P3 particles with charge quantities of 10-grams per milling batch. Four different initial size levels (i.e. P25, P11, P6 and P3) were tested, using samples with conditioned moisture content of 10% and charge quantities of 10-grams per milling batch.

3.2.3 Measurement of specific energy consumption

The milling energy consumption of the ring and puck mill was measured with a Fluke 1735 power logger (Fluke, USA). The active power, active energy, power factor, frequency, and time were measured and recorded. The power required to run the empty milling was recorded to obtain baseline data. The specific grinding energy consumption was calculated by integrating the area under the power demand curve for the total time required for milling, as shown the following relation:

\[ E_p = \int_0^t (P_t - P_0) \, dt / m = \int_0^t \Delta P_t \, dt / m \]

where: \( E_p \) is the specific net energy consumption (kJ/kg); \( P_t \) is the power consumed at time \( t \); \( P_0 \) is the average power consumption under empty milling condition; and \( m \) is the mass charge in kg of wood to be pulverized.

3.2.4 Characterization of wood powder

3.2.4.1 Preparation of wood powder for analyses

The milled wood samples were prepared for particle size distribution and aspect ratio analyses as detailed elsewhere (Jiang et al., under review). In brief, each wood sample was suspended in distilled water with a 0.5% solids mass concentration. The diluted powder suspension was dispersed by adding 1-mL of a dispersant solution (0.1% w/w sodium dodecyl sulfonate) per 100-mg wood [18], when performing aspect ratio test. The suspended samples were stirred for 1 hour with a magnetic stir rod, followed by sonication with 20% amplitude (Branson, Switzerland).
for 2 minutes to disintegrate particle agglomeration. The same procedure was followed to measure the particle size distribution, except the dispersant was not added.

3.2.4.2 Particle size distribution

The volumetric particle size distribution of the wood powders was analyzed using laser diffraction (Mastersizer 3000 with Hydro LV wet sample dispersion, Malvern instrument, UK). The median size corresponding to the 50% percentiles of the cumulative size distribution was used to represent the particles size for analysis.

3.2.4.3 Aspect ratio analysis

The aspect ratio analysis of milled particle was performed by imaging technique as detailed elsewhere (Jiang, et al., under review). In brief, the solid suspension of samples prepared as described above, was distributed on a silica wafer mounted on a metal stab. After allowing the water to evaporate at ambient conditions, samples were sputtered with gold to prepare examination in a Scanning Electron Microscopy (SEM). Calculation of the aspect ratio was conducted by analyzing SEM images using ImageJ (National Institutes of Health, USA) following the procedures described elsewhere [19,20]. The aspect ratio of each particle was calculated as the ratio of the major and minor axis of the fit ellipse for the selected particle. The minimum aspect ratio value of one was restricted and describes a perfect and filled circle. The median aspect ratio corresponding to the value of 50% number-based cumulative aspect ratio distribution was obtained.

3.2.4.3 Calculation of surface area

Linear transformation of morphological characteristics (i.e. particle size and aspect ratio) of micronized particle into the volumetric specific surface area (SSA) was conducted according to
the procedures described elsewhere [21]. The milled wood particle was approximated as ellipsoid with a circular cross section instead of a sphere, since the aspect ratio was significantly larger than unity as reported in our previous study (Jiang, et al., under review). The volumetric SSA based on ellipsoid model for the particle was estimated using following equation:

\[
SSA' = \frac{6(\frac{2x^p + 1}{3})^p}{xD}
\]

where \(x\) is the aspect ratio of particle, \(D\) is the particle diameter, and \(p\) is the constant 1.6075.

### 3.2.5 Cellulose crystallinity

X-ray diffraction patterns of the milled wood particles were obtained using a Rigaku Miniflex 600 X-ray diffractometer (Tokyo, Japan) equipped with a Cu Kα (\(\lambda = 0.154\)-nm) radiation source at 40-kV and 15-mA. The diffractogram was detected in the 2θ range of 10-40° at a scan rate of 1°/min. The crystallinity index (CrI), was estimated using an equation as described by Segal in the following form [22]:

\[
CrI \% = \left[\frac{(I_{002} - I_{am})}{I_{002}}\right] \times 100
\]

where \(I_{002}\) is the intensity of main peak at a 2θ close to 22°, and \(I_{am}\) is the intensity due to amorphous portion evaluated as the minimum intensity between the main and secondary peaks.

### 3.2.6 Enzymatic hydrolysis

Enzymatic hydrolysis of cellulose and hemicellulose was performed using Cellic CTec2 cellulase with dosage 15-FPU/g of substrate and supplementary enzyme Cellic HTec2 hemicellulase (Novozymes NA, Franklinton, NC, USA). The weight ratio of cellulase and hemicellulase was 9:1 according to the supplier’s recommendation. The activity of the CTec2 cellulase was determined to be 150 FPU/mL according to a standard method [23]. Experiments were conducted in duplicate according to the NREL Laboratory Analytical Procedure [23]. The enzymatic hydrolysis was conducted in a 20-mL glass scintillation vial and placed in an incubator
with rotation speed of 180rpm. The buffer was 0.1-M sodium citrate solution (pH 4.8), containing 100-µL of 2% sodium azide solution. Sodium azide was added to prevent the growth of organisms during the hydrolysis. The total volume of the reaction solution was 10-mL and the amount of total wood sample loading was 0.2g on oven-dry weight basis. The hydrolysis reaction was performed at 50°C. The hydrolysis was carried out for 72 hours. The dydrolysate was then placed into boiling water for 15 minutes in order to deactivate enzymes. Enzymatic hydrolysis efficiency was represented by the obtained carbohydrate conversion and calculated as follows:

\[
\text{Carbohydrate conversion (\%)} = \frac{\text{Released sugar amount in hydrolysate}}{\text{Theoretic sugar amount in milled wood}} \times 100
\]

3.2.7 Analytical methods

The chemical composition analysis of the raw material was conducted according to the two-step acid hydrolysis procedure from the NREL protocol [25]. Briefly, a 300-mg sample and 3-mL of 72% H\textsubscript{2}SO\textsubscript{4} was added to a 100-mL pressure tube and incubated at 30°C for 1 hour, while stirring every 15 minutes. The sample was then diluted with 84-mL deionized water and autoclaved for 1 hour.

Detection of sugars before and after enzymatic hydrolysis was performed using high performance anion exchange chromatography (HPAEC) (ICS-3000, Dionex, Sunnyvale, California) with ED 50 electrochemical detector (Dionext Corp., Bannockburn, IL, USA). Sugars were separated on a CarboPac PA 20 Guard (4×50-mm) and analytical columns (4×250-mm) at room temperature (25°C). Then, 10-µL of sample solution were injected into the HPAEC system to quantify the content of monosugars. The mobile phases were deionized water and 50-mM aqueous sodium hydroxide solution at a flow rate of 0.5-mL/min. The detector was maintained at a pH of 10.4. An AS40 sampler (Dionext Corp.) was used for continuous running and Dionex PeakNet 5.1 chromatography software was used for analysis of results.
3.2.8 Statistical analysis

The nonlinear behavior of carbohydrate conversion in relation to particle size was analyzed using a piecewise regression. Multiple linear regression was conducted to predict the carbohydrate conversion in terms of surface area (linear transformation of particle size) and crystallinity. All analyses were performed with the SAS 9.0 statistical software package (SAS Institute Inc., Cary, NC).

3.3 Results and discussion

3.3.1 Effect of mechanical milling conditions on enzymatic hydrolysis

One of the targets of this study was to investigate the enzymatic hydrolysis efficiency of micronized wood, as affected by mechanical milling conditions. Figure 2.1 shows the enzymatic conversion of micronized wood produced from different moisture contents, feed sizes and milling times. It is evident that increasing the milling time improved the efficiency of the enzymatic hydrolysis. More than 70% of carbohydrate (i.e., cellulose and hemicellulos) conversion was achieved with 12-minutes milling, suggesting that efficient saccharification of native softwood biomass is possible without any thermo-chemical pretreatments. These results concur with the previous observations on enzymatic hydrolysis of other milled woody or herbaceous biomass using different milling techniques [10,26]. Sipponen found a gradual increase of total carbohydrate conversion to around 76% with ball-milled maize stem when the milling time increased to 6 hours [27]. Hoeger et al., also reported that more than 60% of cellulose saccharification efficiency of softwood pulp can be achieved by mechanically fibrillating with a SuperMassColloider for up to 12 hours [28]. Compared to these studies that employ typical ball milling and fibrillating for long treatment periods (i.e., several hours or days), our method of ring and puck milling shows superior efficiency for achieving high enzymatic hydrolysis of milled substrates. We have also observed
that initial moisture content of the feedstock influenced the carbohydrate conversion of milled substrates. Higher moisture content during the milling process induced more carbohydrate conversion for samples with short milling times, while the carbohydrate conversion was higher for samples milled from lower moisture content with longer milling times (Figure 2.1A). This may be due to the distinctive structural alternations of micronized substrates produced in various milling conditions. Figure 2.1B illustrates that the feed size during the milling process has little effect on the carbohydrate conversion of milled samples. Takahashi et al., also reported similar results for five kinds of milled biomass feedstock with various initial sizes using tandem ring milling [29]. This may be due to the similar structural properties of substrates after the milling process.

**Figure 3.1** Effect of initial moisture content (A) and feed size (B) during milling process on carbohydrate conversion of micronized wood

3.3.2 Relation between particle size and enzymatic hydrolysis

There is a strong consensus that particle size reduction plays an important role in enhancing the enzymatic hydrolysis of biomass, due to the dependent increase in both external surface area and heat / mass transfer efficiency. However, there is still debates on how to best reduce particle
size to facilitate enzymatic hydrolysis of substrates produced from chemical modification [8,9,12]. Silva et al., suggested that mechanical size reduction of biomass must overcome a limit (around 100-µm) to significantly enhance enzymatic hydrolysis [30]. This size limit enables altering the cellular-scale structure of biomass, which significantly facilitates enzyme accessibility and the efficiency of micronized biomass [31].

In present study, we effectively comminuted softwood feedstock at the cellular scale for particle size smaller than 100-µm to reveal the relationship between enzymatic hydrolysis and particle size of micronized wood. Therefore, Figure 3.2 shows the carbohydrate conversion in enzymatic hydrolysis with respect to median particle size of micronized wood produced from various milling conditions. Results show that carbohydrate conversion was improved as the particle size decreased, regardless of initial moisture content or feed size during the milling process. This indicates the beneficial effects of reducing particle size to facilitate enzymatic hydrolysis, however, the carbohydrate conversion of micronized wood clusters into different groups, possibly exhibiting different relationships between enzymatic hydrolysis and particle size in these size regions. The piecewise linear regression algorithm shows that the trend of carbohydrate conversion changes at a particle size of 30.8-µm (Figure 3.2), suggesting that two regimes affect carbohydrate conversion with respective of particle size. In the first regime, reducing particle size to around 30 µm increases enzymatic hydrolysis of milled substrate, but only achieves limited ca. 40% of theoretical carbohydrate conversion. It is likely that increasing the external surface area may facilitate enzymatic hydrolysis while still limiting maximum carbohydrate conversion. For example, the difference in particle morphology for various particle sizes is visually evident in Figure 2.3. Fiber bundles and single fibers still exist when the particle size is equal to or larger than 30-µm (Figure 3.2A and B), suggesting a change in external surface area during milling.
process. It is possible that the relatively intact fiber cell wall does not provide sufficient accessible surface area for achieving significant carbohydrate conversion (e.g., 50% or higher). After the particle size reached below 30µm, carbohydrate conversion can approach 77% depending on the specific milling condition, although the additional particle size reduction was slight in this range (Figure 3.2). This may be because completely fragmenting the fiber cell wall increased accessible surface area for enzymes. Microscopic observation also indicates that the fiber cell walls were completely fractured to become uniform fragments with size less than 30-µm (Figure 3.2C-F). Mechanically disintegrating the fiber cell walls into micronized fragments smaller than 30µm may allow high accessible surface area for achieving significant carbohydrate conversion. In previous study, Zhu et al. [28], reported a similar conclusion by investigating the enzymatic hydrolysis of wood pulping fibers after mechanical fibrillation with stone grinding. In their study, a Class I size reduction which referred to the partial breakage of fibers and fiber bundles by mechanical fibrillation, rendered an unsatisfactory ca. 30% of substrate enzymatic digestibility. Class II size reduction, with complete disintegration of the cell wall into microfibrils, could result in high substrate enzymatic digestibility of 60-90%. However, they did not examine the particle size of fibrillated substrate. Additionally, Takahashi et al., reported that various woody and herbaceous biomass after tandem-ring milling pretreatment could only reach around 40% of holocellulose conversion when the median particle size decreased to around 40-µm [29]. However, holocellulose conversion increased greatly up to 70-90%, depending on the biomass type, when median particle sizes were smaller than 40-µm. It is noteworthy that the carbohydrate conversion also varies among samples produced from different pretreatment conditions during the milling process, although the particle sizes are milled into a smaller range (e.g., MC30% in Figure 3.2). These results also suggest that particle size is not the only factor affecting enzymatic hydrolysis.
Figure 3.2 Carbohydrate conversion of micronized wood as a function of median particle size for samples with various moisture content and feed size during mechanical milling pretreatment.
Figure 3.3 SEM images show morphology variations of particles of different sizes. (A) Particles with median particle size bigger than 30-µm and sample was milled with 2 minutes; (B) Particles with median particle size around 30-µm; (C-F) Particles with median particle size smaller than 30-µm.

3.3.3 Relation between crystallinity and enzymatic hydrolysis

Mechanically fragmenting lignocellulosic biomass into micronized particles also disrupts the cellulose crystalline structure. The significant change in crystallinity of milled biomass is also a major motivation for developing relevant pretreatment technologies, since amorphous cellulose is more amenable to hydrolytic enzymes than crystalline cellulose [26,32]. Figure 3.4 illustrates the relationship between the enzymatic carbohydrate conversion and the crystallinity index (CrI) of micronized wood produced from various milling conditions. There exists correlation between carbohydrate conversion and CrI. We can also observe that the unsatisfied correlation results ($R^2 = 0.65$) may due to overlet of the higher moisture content samples (Figure 3.4). This suggests that the influence of crystallinity on enzymatic hydrolysis of micronized wood is complex. However, Figure 3.5A and B illustrate that the carbohydrate conversion is proportional increase to the decrease of CrI with high coefficient factors ($0.93 < R^2 < 0.99$), when micronized wood samples
are grouped by specific initial moisture content during milling process. It is likely that disrupting the tightly crystalline structure of native wood cellulose increases enzyme accessibility and subsequent carbohydrate conversion, although the degree of crystalline structure disruption varied depending on different initial moisture contents during the milling process. For the micronized samples produced from lower initial moisture contents during milling process, a significant decrease of crystallinity to around 10% (suggesting intensive amorphization of wood cellulose) resulted in over 70% of carbohydrate conversion (Figure 3.5A and B).

The beneficial effect of low crystallinity on improving enzymatic efficiency in the present study concurred with results from previous studies using other biomass feedstock subjected to dry ball milling pretreatment. Zakaria et al. reported that disrupting oil palm cellulose to a highly amorphous state (CrI = 9%) by planetary ball milling pretreatment resulted in high total sugar yields of 72% [33]. Takahashi et al. also reported that tandem-ring milling significantly reduced the CrI of five kinds of woody and herbaceous feedstock to around 10%, resulting in holocellulose conversion as high as 94% [29]. They also noted a linear relationship between holocellulose conversion and CrI in their study. For the micronized samples produced from higher initial moisture contents (e.g., MC30%) during the milling process, a close and linear relationship between the CrI and carbohydrate conversion was also achieved, despite a slight reduction in CrI from 52% to 40% (Figure 3.4A). This indicates that reducing crystallinity in a small range also improves enzymatic hydrolysis of milled wood. Previous research on mechanically fibrillating wood pulping fibers showed that a decrease in crystallinity of 15-25% after 6-12 hours of fibrillation resulted in 60-90% cellulose substrate enzymatic digestibility [34]. Similar results were reported by da Silva et. al, who evaluated the digestibility of sugarcane bagasse and straw subjected to wet disk milling and dry ball milling pretreatments [35]. Although a similar digestibility of
milled biomass with different methods was obtained, wet disk milling induced very little change in biomass crystallinity and dry ball milling led to significant decrease of crystallinity. In our study, samples milled with a moisture content of 30% were similar to samples from wet milling, as the wood fibers were in their moisture saturation state during the milling process.

Figure 3.4 Carbohydrate conversion of micronized wood as a function of crystallinity index (CrI) for samples produced from various milling conditions.
3.3.4 Empirical prediction of enzymatic hydrolysis

Mechanical milling pretreatment is known to disrupt cellular integrity of biomass with coincident structural alternations [10]. Carbohydrate conversion of micronized wood may be collectively affected by the structural features (i.e., morphology and crystallinity) as discussed above. In this section, the morphological characteristics of micronized particles were first transformed into volumetric specific surface area (SSA) as described earlier. The linear relation between the carbohydrate conversion and volumetric SSA was evaluated (Figure 3.6). The carbohydrate conversion increases linearly with increasing volumetric SSA of micronized wood ($R^2 = 0.93$). Despite of the good correlation between carbohydrate conversion and SSA, CrI is also independently correlated with carbohydrate conversion as discussed above. Therefore, a more accurate empirical prediction of enzymatic carbohydrate conversion was expected when considering both volumetric SSA and crystallinity using a multiple linear regression model. As demonstrated in Figure 3.7, the model can explain the 95.2% variability of the response variable.
Correlation parameters for slopes and intercept are summarized in Table 3.1. The two factors (i.e., SSA and CrI) significantly influence the carbohydrate conversion (p-value < 0.001, data not shown here). The positive coefficient for SSA indicates that increasing the surface area (i.e., decreasing particle size) improves carbohydrate conversion. This is well consisted with previous claims on increasing surface area using different pretreatment approaches to facilitate enzyme accessibility, as close contact between enzyme and cellulosic substrates is the primary step for initiating hydrolysis actions [9,36]. Mechanically fragmenting the robust biological structure of biomass feedstock is an effective option for generating new surface area [10]. The negative coefficient for CrI in the regression model suggests the importance of disrupting the crystalline structure of biomass on improving the carbohydrate conversion. Low crystallinity allows more space for enzyme contact within the cellulose fibrils. This is in good agreement with other reports on lowering crystallinity with phosphoric acid or ionic liquid swelling [37,38].

Findings from our study show that the collective decrease of crystallinity and increase of surface area (reducing particle size and aspect ratio) during mechanical milling process increase the subsequent enzymatic hydrolysis of micronized wood.
**Figure 3.6** Carbohydrate conversion of micronized wood as a function of volumetric specific surface area for samples produced from various moisture content and feed size during mechanical milling pretreatment.

**Table 3.1** Regression equation for carbohydrate conversion by enzymatic hydrolysis using calculated specific surface area (SSA) and crystallinity index (CrI) of micronized wood.

<table>
<thead>
<tr>
<th>Carbohydrate conversion</th>
<th>Intercept</th>
<th>SSA (X1)</th>
<th>CrI (X2)</th>
<th>( R^2 )</th>
<th>Adjusted ( R^2 )</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y</td>
<td>31.46</td>
<td>129.09</td>
<td>-0.242</td>
<td>0.9512</td>
<td>0.949</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Figure 3.7 Graph of experimentally measured carbohydrate conversion versus predicted values from multiple linear regression model.

3.3.5 Energy efficiency of mechanical milling pretreatment

Energy input for the pretreatment process is also an important aspect of assessing the viability of pretreatment methods [39,40]. In this study, we used the energy efficiency (kg sugar/kWh) to evaluate the performance of the mechanical milling process for producing simple sugars. The total amount of sugars recovered after enzymatic hydrolysis (kg sugar/kg wood) was divided by the total specific energy consumption for mechanical milling woody biomass (kWh/kg wood). The current definition delineates the sugars production with unit energy consumption in the pretreatment process. Therefore, the higher the value, the higher the energy efficiency of the pretreatment process. Normalized energy efficiency was calculated by including the drying energy requirement for various moisture content samples or energy consumed in coarsely milling various sizes of feedstock.
To evaluate the effect of milling conditions on the energy efficiency of milling pretreatment, we used experimental setups with maximum carbohydrate conversion with 12-minutes of milling, as shown in Table. 3.2. Results show that an increase of initial moisture content decreased glucan conversion and total sugar yield per unit energy. This is likely due to the intensive energy requirements for milling high-moisture content samples, a topic detailed in previous studies [41]. Feed size did not significantly influence the conversion of carbohydrate and total sugar yield, but led to various levels of energy efficiency. Larger feed size reduced energy efficiency, due to the extra energy consumption needed to break down the integrity of wood fiber structure with fine milling equipment such as the ring and puck mill. Therefore, coarse milling of large-size feedstock is necessary before fine milling for economically feasible mechanical milling pretreatment of woody biomass.

In this study, the highest energy efficiency was achieved with 1.06 kg sugar/kWh from sample with moisture content 5% and a feed size of P3. With this milling condition, the maximum glucan conversion and total sugar yield were obtained with 89.8% and 526 g sugar/kg wood, respectively. In contrast, for steam explosion pretreatment of spruce, Wingren et al. obtained a maximum energy efficiency 0.77 kg glucose/kWh with a pretreatment temperature of 215°C and no data on total sugar yield [42]. It is likely that utilizing both hexoses and pentoses in fermentation would potentially increase the theoretical yield and substantially improve the economics of the biofuel production [43]. Our study found that mechanical milling pretreatment retained the hemicellulose component for down-stream enzymatic hydrolysis and fermentation processes, thereby improving of energy efficiency. Therefore, this study demonstrated that mechanical milling pretreatment conducted with feedstock of a lower moisture content and smaller size is an economic way to produce simple sugars.
Table 3.2 Effects of mechanical milling on enzymatic hydrolysis and energy efficiency*

<table>
<thead>
<tr>
<th>Milling effect</th>
<th>Glucan conversion %</th>
<th>Xyl/mannan conversion %</th>
<th>Total sugar yield g/kg wood</th>
<th>Energy efficiency kg sugar / kWh</th>
<th>Normalized energy efficiency** kg sugar / kWh</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture content</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5%</td>
<td>89.81</td>
<td>42.93</td>
<td>526</td>
<td>1.06</td>
<td>0.77</td>
</tr>
<tr>
<td>10%</td>
<td>84.1</td>
<td>40.42</td>
<td>493</td>
<td>0.57</td>
<td>0.49</td>
</tr>
<tr>
<td>15%</td>
<td>77.14</td>
<td>40.56</td>
<td>459</td>
<td>0.32</td>
<td>0.29</td>
</tr>
<tr>
<td>30%</td>
<td>73.5</td>
<td>43.54</td>
<td>446</td>
<td>0.23</td>
<td>0.23</td>
</tr>
<tr>
<td>Feed size</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P25</td>
<td>83.3</td>
<td>46.06</td>
<td>500</td>
<td>0.36</td>
<td>0.36</td>
</tr>
<tr>
<td>P11</td>
<td>81.55</td>
<td>41.95</td>
<td>483</td>
<td>0.39</td>
<td>0.38</td>
</tr>
<tr>
<td>P6</td>
<td>83.81</td>
<td>42.8</td>
<td>496</td>
<td>0.45</td>
<td>0.44</td>
</tr>
<tr>
<td>P3</td>
<td>84.1</td>
<td>40.42</td>
<td>493</td>
<td>0.57</td>
<td>0.55</td>
</tr>
</tbody>
</table>

* Samples were milled for 12 minutes using a ring and puck mill for each milling effect
** For the moisture content effect, energy consumption was normalized to the same moisture content of 30% for comparison (i.e. including energy drying samples from 30% to various lower moisture contents); for the feed size effect, energy consumption was normalized to the same size level of P25 for comparison (i.e., including coarse milling energy from P25 size to various screen sizes).

3.4 Conclusions

This study demonstrated that mechanical milling pretreatment is an effective approach to alter the physical structure of biomass to improve enzymatic hydrolysis. The influence of milling variables on enzymatic hydrolysis of milled wood mainly relates to the structural properties of micronized wood. Findings show that mechanical particle size reduction that primarily break fibers or/and fiber bundles can improve enzymatic hydrolysis for milled wood; however, carbohydrate conversion is limited to around 40% of theoretical yield. Mechanically disintegrating the fiber cell wall into micronized fragments with a size smaller than 30-μm allows to achieve a carbohydrate conversion to 77% of theoretical yield. Crystallinity also plays important role in facilitating enzymatic hydrolysis of milled wood. The correlations between CrI and carbohydrate varied for samples with different initial moisture contents during the milling process. Empirical prediction
of carbohydrate conversion with structural characteristics using a multiple linear regression model indicated that the enzymatic hydrolysis of micronized wood improved as collectively increasing surface area (i.e., reducing particle size) and decreasing crystallinity during mechanical milling pretreatment. We recommend using a relatively low-moisture content starting material and a multi-step milling process of producing simple sugars through mechanical milling pretreatment.
References


CHAPTER 4 SOFTWOOD CELL WALL FRACTURE CHANGES IN THE EARLY STAGE OF MECHANICAL PRETREATMENT

Abstract

Mechanical pretreatment is an effective process for chemical or biochemical conversion of woody biomass. The deconstruction features of the wood cell wall play an important role in its chemical or biochemical processing. In this work, we evaluated the wood cell wall fracture in early stage of mechanical pretreatment process conducted at various initial moisture contents. Electronic microscopy (i.e., SEM and TEM) and confocal laser scanning microscopy (CLSM) were used to visualize the cellular structure changes due to cell wall fractures. Results reveal that the types of cell wall fractures after mechanical pretreatment were distinguished by the initial moisture contents of wood. In wood samples with lower moisture content, interwall fracture occurred predominantly at the middle lamella region, while intrawall fracture occurred primarily at inner cell wall layers, with sever breakage in wood fibers for high moisture content samples. Differences in the distribution of surface chemical composition also resulted from different cell wall fractures. Lignin preferentially covered the fracture surface of low-moisture content samples, while carbohydrates were more predominate in high-moisture content samples. Morphological and structural alternation were found to improve enzymatic digestibility of micronized wood 2-6 times over that of the raw material. Findings from this study demonstrate how mechanical pretreatment modifies the fracture features of wood cell wall for further chemical/biochemical reactions.

Key words: Mechanical pretreatment, cell wall fracture, morphology, structure, digestibility, energy efficiency
4.1 Introduction

Currently, there is great interest in utilizing lignocellulosic biomass as a global energy source to reduce reliance of modern societies on fossil resources and mitigates greenhouse gas emissions. However, complex macromolecular interaction networks among biopolymer components in the plant cell wall matrix create natural recalcitrance. This, in turn, technically and economically limits the cost-effective release of fermentable sugars for subsequent liquid biofuels production [1]. The effectiveness of enzymatic saccharification of biomass is intricately related to their inherent properties, such as structural and chemical characteristics. Significant particle size reduction after pretreatment has been found to improve enzyme accessibility and mass/heat transfer efficiency [1]. The distribution of chemical composition is also integral to subsequent digestibility or a post pretreatment if required. Zhu et al. found that wood fiber with surface exposure of cellulose after chemimechanical pretreatment was more effective than wood fiber with lignin covering the surface in terms of subsequent enzymatic hydrolysis [2]. Ju et al. found that, despite a similar bulk lignin content in wood fibers, the variation of surface lignin after chemical pulping pretreatments directly affected enzyme adsorption kinetics and hydrolysis rate [3].

From an anatomical viewpoint, the wood cell wall is composed of a hierarchical ultrastructure assemble ranging from the molecular level to micrometers cell wall level [4]. Adjacent cells are separated by the middle lamella, while the individual cell wall is typically composed of three layers (i.e., the middle lamella, primary cell wall, and secondary cell wall). The secondary cell wall can be further divided into sublayers (i.e., S1 outer, S2 middle, and S3 inner layer), with different self-assembly and hierarchical organization [4]. In the layered cell wall, semi-crystalline cellulose microfibrils are assembled as the reinforcement structure and coated with
amorphous hemicellulose-lignin matrix through hydrogen bonds or covalent bonds. Thus, it is conceivable that mechanical action on wood cell wall deconstruction would result in substrates with distinguished morphological and physicochemical characteristics.

In the mechanical pulping process, wood fiber fibrillation has been found to affect the characteristics of pulps, such as fiber length, aspect ratio and surface composition. These characteristics drastically impacted subsequent post treatment and final performance of paper [5,6]. Mechanical pretreatment aimed at overcoming the recalcitrant structure to facilitate enzymatic saccharification differs from traditional mechanical pulping processes because maintaining fiber integrity is not necessary for sugar production. Considerable research has focused on maximizing fermentable sugar release by disrupting biomass cell wall structure after mechanical pretreatment [7–9]. In fact, the fracture of fiber bundles and the fragmentation of individual fibers at specific positions during the early stage of mechanical pretreatment may significantly affect substrate properties and/or post-treatment requirements for improving enzymatic hydrolysis [2,3]. However, there is still a research gap in the literature addressing the fundamental characteristics of cell wall fracture and the corresponding influence on surface chemical composition and enzymatic hydrolysis.

In addition, mechanical pretreatment is generally considered to be energy intensive, which elicits particular attention for scaling bioconversion systems [6]. Studies show that energy consumption in mechanical wood pulping depends significantly on the mechanism of the wood fractured [10,11]. The energy input for the mechanical refining process also affects final morphological and structural properties of wood fibers, e.g., fiber length/width and fineness [12,13]. However, additional research is needed to elucidate the fundamentals of wood cell fracture and its corresponding energy consumption during mechanical pretreatment.
This study is aimed at obtaining a better understanding of the structural and morphological characteristics of cell wall fractures of wood in the early stage of mechanical pretreatment and the influence of these characteristics on enzymatic hydrolysis. The influence of moisture content on the type of cell wall fracture in micronized wood is examined with a series of characterization techniques. These included electronic microscopy to delineate the structural changes in wood cell wall and, as it turned out, to reveal surface morphology and ultrastructural features of fractured cell walls. Fluorescence microscopy was applied as a rapid, effective way to identify and classify the fracture surface chemical composition distribution of micronized wood after mechanical pretreatment. We also evaluated the enzymatic hydrolysis of micronized wood and energy consumption of the mechanical pretreatment process in an effort to assess the change in the recalcitrance corresponding to wood cell wall fracture. Together, these data were integrated to provide insight into overcoming woody biomass recalcitrance for producing digestible substrate with mechanical pretreatment, or a combination of a second chemical treatment.

4.2 Materials and methods

4.2.1 Materials

Douglas-fir (*Pseudotsuga menziesii*) wood chip was obtained locally (Vaagen Brothers Lumber Inc., Colville, WA). Prior to pretreatment, the received chips were passed through a vibrating screen with 25.4-mm aperture and then hammer-milled to pass a 3.18-mm screen. The pre-processed feedstock was subsequently conditioned to different equilibrium moisture content (EMC) values (i.e., 5-30%, dry weight). Before conducting fine milling pretreatment, all conditioned material was stored in sealed plastic bags and the moisture content was validated using gravimetric methods according to standard protocol [14].
4.2.2 Mechanical pretreatment process

Mechanical milling pretreatment of woody feedstock was performed using a high-energy vibratory Standard Ring and Puck mill with motor power of 1.1-kw (Rocklab Pty Ltd, New Zealand). The detailed parameters of the equipment were described elsewhere (Jiang, et al., under review). The samples (10-g, oven-dry base) with different moisture content were loaded to the milling chamber and milled for 2 minutes. The milling time was chosen based on preliminary test showing that the particle size of the milled substrate for 2-minutes milling was in the micrometer range with a discernable cell wall structure. Thus, the milled samples were also noted as micronized wood (or micronized particles) in this study.

4.2.3 Measurement of specific energy consumption

The specific energy consumed during mechanical milling process was measured using a Fluke 1735 power logger (Fluke, USA). The active power, active energy, power factor, frequency, and time were acquired by a computer. The specific energy consumption was calculated according to the following equation:

\[ E_p = \int_0^t (P_t - P_0) dt / m = \int_0^t \Delta P_t dt / m \]

where: \( E_p \) is the specific net energy consumption (kJ/kg); \( P_t \) is the power consumed at time \( t \); \( P_0 \) is the average power consumption under idle condition measured from an empty mill; and \( m \) is the mass charge in kg of wood to be pulverized. All measurements were performed in duplicate.

4.2.4 Particle size distribution

The volumetric particle size distribution of the micronized wood was determined using a laser scattering particle size analyzer Mastersizer 3000 with Hydro LV wet sample dispersion (Malvern instrument, UK). The median size \( (D_{50}) \) was used to represent the particle size for analysis.
4.2.5 Dye adsorption and cellulose specific surface area

Langmuir-type adsorption of a mono disperse dye DR28 (Congo red) was obtained on 1% wood substrate in 0.03-M phosphate buffer (pH 6) with 1.4-mM sodium chloride and an incubation temperature of 60 °C. Suspensions were prepared with a series of increasing dye concentrations and incubated for 24 hours at 180 rpm. After incubation, each suspension was centrifuged for 5 minutes at 740g and the absorbance of the supernatant was measured using UV-VIS spectrophotometer (Lambda 25, PerkinElmer) at a wavelength of 498 nm. The Langmuir maximum adsorption capacity was determined according to the equation described below [15]:

\[
\frac{[C]}{[A]} = \frac{1}{K_{ads}[A]_{max}} + \frac{[C]}{[A]_{max}}
\]

where \([C]\) (mg/mL) is the free dye concentration at equilibrium, \([A]\) (mg/g) is the amount of dye adsorbed by the substrate, \([A]_{max}\) is the maximum amount of dye adsorbed onto cellulose (mg/g), and \(K_{ads}\) is the adsorption equilibrium constant. The cellulose specific surface area (SSA) can be calculated from the following relation [16]:

\[
SSA = [A]_{max} \times N_A \times SA_{CR} / MW
\]

where \(N_A\) is Avogadro’s constant, \(SA_{CR}\) is the surface area of one molecule of Congo Red (1.73 nm\(^2\)), and \(MW\) is the molecular weight of Congo red (696.7 g/mol).

4.2.6 Composition analysis of the wood sample

The chemical composition of wood material was assessed according to the two-step acid hydrolysis procedure from the NREL standard protocol [17]. Briefly, a 300-mg sample and 3-mL of 72% H\(_2\)SO\(_4\) was added to a 100-mL pressure tube and incubated at 30 °C for 1 hour and stirred every 15 minutes. The sample was then diluted with 84 mL deionized water and autoclaved for 1 hour. Sugars were detected using a high-performance anion exchange chromatography (HPAEC) (Dionex, ICS-3000).
4.2.7 Enzymatic hydrolysis

Enzymatic hydrolysis was performed with 15 FPU/OD g of substrate Cellic CTec2 cellulase and cellic HTec2 hemicellulase (1/9 of the cellulase amount). Digestion was carried out in 125-mL flasks with a citrate buffer (pH 4.8) at a solid loading of 2%. The flasks were settled in an incubator with a rotation speed of 180 rpm at 50 °C. After digestion of 72 hours, the hydrolysate was analyzed by HPAEC. Glucan and xyl/mannan conversions were defined as the percentage of glucose and xyl/mannose released compared to the theoretical maximum.

4.2.8 Scanning electron microscopy (SEM)

SEM images of samples were acquired at 20-kV accelerating voltage using a FEI Quanta 200F, field emission gun with high vacuum ETD detectors (FEI Company, Hillsboro, Oregon, USA). Samples were mounted on aluminum stubs using carbon tape and sputter coated with 8 nm of gold for good conductivity prior to imaging.

4.2.9 Sample embedding and sectioning

Wood samples were prepared using microwave electronic microscopy processing. Samples were fixed in 3% glutaraldehyde, and buffered in 0.05-M Pipes buffer (Sigma, St Louis, MO) with a microwave on full power. Dehydration was conducted in graded ethanol series for 40 seconds under microwave at 30%, 50%, 60%, 70%, 80%, 90%, and 3×100% ethanol. Samples were infiltrated with Spurr’s low viscosity resin and incubated overnight at room temperature in a hood with increasing concentration of the resin (30%, 50%, 3×100% resin, diluted in isopropanol). Samples were then transferred to 1.5-mL micro-centrifuge tubes with fresh Spurr’s resin. The resin was then polymerized overnight in an oven with a temperature of 70 °C. Glass knives were used to trim the block surface, and then a Diatome diamond knife was used to obtain the sections with a Leica Reichert Ultracut R microtome (Leica, Wetzlar, Germany).
4.10 Confocal laser scanning microscopy (CLSM)

Semi-thin (200 - 500nm) sample sections were positioned on glass microscope slides and stained with saturated HPLC-grade acridine orange (AO; 3, 6-bis (dimethylamino) acridine hydrochloride, Sigma-Aldrich, St. Louis, MO) for 1 hour at room temperature. The images of stained samples were captured using a Leica TCS SP8 confocal scanning laser microscopy with a 40\times oil objective lens. A white laser at $\lambda=500$ nm was used as the excitation light source. Fluorescence emission between $\lambda=515$ and 540 nm were collected as the green channel and emissions above $\lambda=590$ nm were collected as the red channel. Image analysis was performed using LAS AF Lite imaging analysis software. The images that appeared green in color were rich in carbohydrate, and areas that were red in color were rich in lignin. When AO interacts with carbohydrates, it stays in a monomeric form, leading to fluorescence and emission in the green region of the visible light spectrum. However, when AO interacts with the aromatic $\pi$ electrons of lignin, causes aggregation of AO molecules occurs because of changes in the electron density of the molecule. This causes a fluorescence emission shift from the green to red light spectrum.

4.2.1 Transmission electron microscopy (TEM)

Ultrathin sections (70-120nm) were collected on Formvar coated copper slot grids (SPI Supplies, West Chester, PA). Grids were post-stained for 10 minutes with 1% aqueous KMnO$_4$ to selectively stain for lignin. Images were captured with a 4 megapixel Gatan UltraScan 4K Eagle camera (Gatan, Pleasanton, CA) on a FEI Tecnai G2 20 Twin 200kV LaB6 TEM (FEI, Hillsboro, OR).
4.3 Results and discussion

4.3.1 Digestibility of micronized wood

Enzymatic digestibility strongly relates to pretreatment effectiveness and is considered to be an excellent probe for assessing cellulose accessibility and susceptibility to depolymerization catalysts [18]. Micronized wood samples with different initial moisture contents during mechanical pretreatment were digested with commercial enzymes, and results are shown in Figure 4.1. The raw material without pretreatment showed fairly low digestibility, with a theoretical glucan conversion of 6.2% (Figure 4.1), demonstrating significant cell wall recalcitrance. In contrast, the glucan conversion of micronized wood was 2-6 times higher, depending on its initial moisture content during this early stage of milling process. Our previous study has also demonstrated that the glucan conversion of micronized wood with full mechanical processing (i.e. 12-minutes milling) could be as high as 90%, which was 14 times higher than that of the raw material (Jiang, et al., under review). The xyl/mannan conversion of micronized wood also increases 3-5 times compared to that of the raw material. Among the micronized wood samples, the enzymatic digestibility increases as the initial moisture content increases. It is worthy to note that there was not any significant change in crystallinity of wood samples milled in the early stage as reported in our previous study (Jiang, et al., under review). Thus, it may suggest that the cell wall fracture difference may contribute to the variance of micronized wood digestibility. Therefore, we examined the structural and morphological properties derived from cell wall fractures in order to find evidence for difference in digestibility.
4.3.2 Particle size of micronized wood samples

The volume-based particle size distribution (PSD) of micronized wood was measured using the laser diffraction technique, as shown in Figure 4.2. The PSD of samples after mechanical pretreatment shifted to a smaller particle size range than that of raw material (Figure 4.2A). Although there is obvious overlap in the PSD among the samples with an initial moisture content (MC) of 5-15%, the sample with a higher initial moisture content contained a larger fraction of small particles than the others. The distribution curves skewed to smaller size as moisture content increased (Figure 4.2A). For the MC30% sample, the reduction in particle size was substantially greater (Figure 4.2A). Overall, there was significant reduction in particle size for all samples after mechanical pretreatment compared to the raw material. The effects of initial moisture content on median particle size development of micronized wood particles is illustrated in Figure 4.2B. Here, the median particle size decreased from 754µm (raw material) to 115µm (MC5%), 95.9µm
(MC10%), 83.6µm (MC15%) and 61.5µm (MC30%), respectively. This result suggests that mechanical pretreatment was more destructive to the structural integrity of high-moisture wood than with the drier samples.

Mechanically fragmenting lignocellulosic biomass is a complex process that is often affected by the material physicochemical properties, fracture of cell walls, energy input, and/or interactions among these variables [5,6,19]. In the thermomechanical pulping (TMP) process, studies showed that secondary cell wall fibrillation with higher energy refining created more severe breakage in finer wood fibers (i.e., smaller particle size) than fibrillation occurring at the middle lamella /or primary cell wall with lower energy consumption [5,12]. In the following sections, we will describe features of the cell wall fracture in the early stage of producing micronized wood and the energy requirements for the mechanical fragmentation process.

Particle size is a key structural feature affecting enzymatic digestion of lignocellulosic biomass. Pretreatment involving decrease of particle size has been demonstrated to be a versatile means of ensuring cellulose digestible for all biomass feedstock [8,9,20]. Our results also indicate that particle size reduction improves the digestibility of softwood, arguably the most recalcitrant of biomass type.
Figure 4.2 (A) Volume-based particle size distribution of micronized wood samples from different initial moisture contents (MC); (B) effect of MC on median particle size development. Each data point is the average of three replicates. Enzymatic conversion of raw and micronized wood with various initial moisture content (MC).

4.3.3 Specific surface area of cellulose in micronized wood

The specific surface area (SSA) of cellulose is an important factor affecting the enzymatic digestion of lignocellulosic biomass, since close contact between cellulose and cellulase (e.g., endoglucanase) is an essential step to initiate hydrolysis reactions [21]. Lignocellulosic biomass is also known as a heterogeneous composite with cellulose chains embedded in the hemicellulose and lignin matrix [1]. The layer structure also results in a heterogeneous distribution of chemical compositions (i.e., cellulose, hemicellulose and lignin) at cellular level. Mechanically fragmenting the lignocellulosic biomass is a process that disintegrates the cellular integrity of heterogeneous composites by generating new surface area [22]. Thus, the SSA of cellulose is closely linked to cell wall fracture and resulting composition exposure on the newly generated surface.

DR28 (Congo red), a dye for specifically binding to cellulosic substrate, was used to estimate the cellulose SSA of samples with different initial moisture contents during the milling
process [15]. More dye adsorption indicates a more accessible cellulose surface area [23]. Correlation coefficients for a linear fit of the free dye concentration versus the free dye concentration/dye on substrate produced values of 0.99 for all samples. This suggests close adherence to Langmuir behavior [16]. Figure 4.3A represents the dye adsorption isotherms and demonstrates that the dye binding behavior can be reasonably described by Langmuir adsorption theory. Compared to the raw material, the micronized particles show an increased capacity of dye adsorption (Figure 4.3A). This indicates the increase of exposed surface cellulose after mechanical pretreatment. The maximum amount of dye adsorption was calculated according to Langmuir equation. The cellulose surface area of micronized wood was calculated by using the maximum amount of dye adsorbed onto the particles and the specific surface area of the Congo dye molecule [16], as presented in materials and methods section. The calculation of cellulose surface area is based on the assumption that the dye adsorbs parallel to the surface of the substrates.

In this study, the cellulose SSA increased from 20.18 m²/g (raw material) to 31.24 m²/g (MC5%), 36.17 m²/g (MC10%), 42 m²/g (MC15%) and 52.17 m²/g (MC30%), suggesting that the initial moisture content influenced the generation of accessible cellulose surface area during the mechanical milling process. Although this result may have been influenced by the particle size variation in the samples, another contribution may be the difference in cell wall fracture modes from the different moisture contents during the mechanical milling process.

Figure 4.4 illustrates the cellular fracture of wood during mechanical milling as imaged with confocal laser scanning microscopy (CLSM). With a low initial moisture contents (e.g., MC5% or 10%), the dislocation and delamination of individual fiber or fiber bundles occurred predominately at the middle lamella region, exposing this lignin rich area on the surface of micronized wood (note the red color on the fracture surface in Figure 4.4A-B). For samples with
higher moisture contents (e.g., MC30%), severe cellular fracture was observed, with splits in adjacent fibers at the inner cell wall layer. This fracture mode likely exposed the embedded cellulose microfibrils on the fracture surface (Figure 4.4D). These results indicate that particle size reduction with mechanical pretreatment may have been coincident with creating a difference in the surface composition distribution, influencing the accessible cellulose SSA. The difference in enzymatic hydrolysis efficiency highlights the important influence of cell wall fracture on the digestibility of micronized wood.

Figure 4.3 Langmuir isotherms of raw and micronized wood samples as a function of initial moisture content (MC).
Figure 4.4 Confocal laser scanning microscopy (CLSM) reveals surface chemical composition distribution of micronized wood with various initial moisture contents (MC) during the milling process. Colors in the images show lignin with red and polysaccharides with green. MC5% (A) and MC10% (B) samples indicate cell wall fracture at the middle lamella, with major lignin exposure due to fracture at the middle lamella area. MC15% (D) and MC30% (D) shows exposure of polysaccharides on the fracture surface due to severe cell wall layer fracture.
4.3.4 Fracture surface morphology of micronized wood

Surface morphology of the micronized wood produced with different initial moisture contents was investigated using SEM technique (Figure 4.5). Loosening fiber bundles while promoting fiber separation at the middle lamella regions was observed in the SEM micrograph for the low initial MC samples (Figures 4.5A1-A3). Mechanically fragmenting the middle lamella regions resulted in considerable debris (see the double-heads arrow in Figure 4.5A3). With an increase in the initial MC (i.e., MC10%), individual fiber and fiber bundles were the main products derived from intensive milling. Detailed investigation reveals that the cell wall fracture also preferentially occurred at the middle lamella and primary cell wall layer regions (see arrows as shown in Figures 4.5B2-B3). Observations of the MC15% samples indicate that fiber separation and internal fracture coincidently occurred (arrows in Figures 4.5C2-C3). The cracks in the S1 layer indicate increased breakage in the fiber cell wall compared to samples with a lower initial moisture content. When the initial moisture content of the sample was 30%, the micronized wood underwent the most severe fracture at the cell wall level, as shown in Figures 4.5D1-D3. By observing the microfibrill orientation, it is possible to distinguish that the breakage fractions also originated in the thicker inner S2 layer after intensive mechanical milling. The random split and fracture of fiber walls resulted in multi-directional breakage of structural cell walls. Qualitatively, the higher initial moisture content samples had a much rougher surface with severe breakage. This supports the above findings of increased cellulose SSA, suggesting that disruption of micronized wood surface may improve enzymatic digestibility. Mechanically fragmenting the inner S2 layer may also present benefits to the direct action of chemical pretreatments (e.g., disrupting cellulose structure with ionic liquid treatment), while fracture of middle lamella of the cell wall may facilitate delignification treatments.
Differences in the morphology of micronized wood surfaces may also reveal that different fractures govern the breakage of wood cell walls with different moisture contents during the early stage of mechanical pretreatment. Interwall fracture, or delamination and separation of fiber cell walls at the adjacent lamella regions, preferentially occurred for samples with low initial moisture content (i.e., MC 5% and 10%). Fracture transition from the outer to inner cell wall occurred for samples with MC15% during the milling process. For the substrate with a high initial MC (i.e., MC30%), intrawall fracture predominated the breakage of cell walls, leading to separation and delamination of structural cell wall layers.

The main chemical composition in the middle lamella and primary cell wall is lignin-hemicellulose composites, an amorphous polymer[24]. Moisture content is believed to be an important factor influencing the stiffness of wood polymer constitutions [25]. In the low moisture content state, the amorphous polymer becomes brittle, resulting in easy fracture propagation at the middle lamella region. An increase in moisture content leads to increase in toughness of the amorphous polymer. Thus, with intensive mechanical action, the fracture may occur preferentially at the brittle cell wall layer, due to the high content of crystalline cellulose.
Figure 4.5 Surface morphological features of wood fiber cell wall fracture with different moisture contents during the milling process; A1-A3 (MC5%) showing fiber bundle delamination at adjacent middle lamella; B1-B3 (MC 10%) showing fiber separation at primary wall and middle lamella; C1-C3 (MC15%) showing fiber separation and breakage at the S1 layer; D1-D3 (MC30%) showing fiber fracture at the S1 and S2 layers. Arrows show cell wall fractures. A1-D1 scale bars are 50 µm; A2-D2 scale bars are 25 µm; A3-D3 scale bars are 10 µm. Arrows show cell wall fracture, and double-head arrows show debris.
4.3.5 Differences in the ultrastructure of micronized wood cell walls

TEM analysis identified differences in the cell wall ultrastructure, distinguished by the initial moisture content after mechanical deconstruction, as shown in Figure 4.6. When the initial moisture content was 5%, dislocation of adjacent cell walls and delamination in the middle lamella caused individual fiber or fiber bundles to split (Figure 4.6A). Cracks also occurred in the cell wall corners, suggesting easy fracture of brittle components for samples with the low moisture content. Figure 4.6B indicates that delamination at the middle lamella/primary wall compound regions was the main breakage of MC10% samples, which also contributed to fiber separation. Total or partial removal of the middle lamella and cell wall corners generated considerable fragment debris, as shown in SEM micrographs (double-heads arrow in Figure 4.5A3, B3).

There was little difference in the fracture surfaces of samples with these two lowest moisture content levels. As discussed above, relatively low moisture contents rendered high stiffness of the amorphous polymers, leading to brittle fracture during the mechanical treatment process [25]. The typical secondary cell wall structure may still be discerned for sample MC15% (Figure 4.6C), but the middle lamella and primary cell wall interphase is split. It is hard to distinguish whether the outer surface is the S1 layer or S2 layer. With an increase in MC to 30%, crack propagation and microfibrills delamination of the secondary cell wall could be detected in addition to the obvious peeling of the middle lamella region (Figure 4.6D). TEM imaging revealed that the fracture features of cell wall ultrastructure was in good agreement with the results of surface morphology indicated by SEM imaging. Therefore, the particle surface morphology and ultrastructure changes indicate that structural disruptions at the cell wall level may contribute to the improved the enzymatic hydrolysis of micronized wood.
Figure 4.6 Ultrastructure variation of milled wood cell walls with different moisture contents (MC). A (MC5%), B (MC10%), C (MC15%), D (MC30%). Arrows show major fractures at specific positions of the cell wall; ccML = cell corner middle lamella, ML = middle lamella.

4.3.6 Energy efficiency of mechanical pretreatment
The energy intensity of pretreatment is generally considered to play a vital role for the economical production of biofuels [26,27]. Table 1 indicates that the specific energy consumption of mechanical pretreatment process is differentiated by the initial moisture content of the wood. Energy consumption values for mechanical pretreatment increase from 0.079 to 0.358 kWh kg\(^{-1}\) as the initial moisture content increases. The significant difference in energy requirements may be attributed to differences in fractures of wood cell walls during mechanical pretreatment process. It is well understood that energy consumption in mechanical pulping is highly depending on the location of cell wall separation and fibrillation [19,28]. The TMP process, in which wood fibers are fractured in the secondary cell wall (i.e., S1 or S2 layers) requires more energy than medium-density fiberboard (MDF) fiber fibrillation, in which fiber wall fracture and separation at the middle lamella region [6].

Moisture content is known to be an important factor influencing the stiffness of cell wall amorphous components such as hemicellulose and lignin [29,30]. At a low initial moisture content (e.g., MC 5%), the brittle middle lamella appears to fracture easily with low energy consumption. As the initial moisture content increases to 10%, energy is consumed to overcome the toughness of amorphous polymer before fracturing. This leads to greater energy consumption than was required by the MC5% sample. When the initial moisture content increases further, the toughness of the amorphous polymers increases, since moisture acts as plasticizer. This results in more energy consumption with less rupture. Previous research indicated that TMP pulping process with high refining energy could increase the internal fibrillation of pulp fibers with more exposure of the S2 layer and generate more fines, compared to the products created using TMP pulping with low energy input [12].
Table 4.1 Enzymatic hydrolysis of micronized wood and energy efficiency of mechanical pretreatment.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Glucan conversion %</th>
<th>Xyl/mannan conversion %</th>
<th>Glucose yield g kg(^{-1}) wood</th>
<th>Total sugar yield g kg(^{-1}) wood</th>
<th>Energy requirement for milling kWh kg(^{-1}) wood</th>
<th>Energy efficiency kg glucose kWh(^{-1})</th>
<th>Energy efficiency kg sugars kWh(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw</td>
<td>6.22</td>
<td>3.67</td>
<td>31</td>
<td>38</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MC5%</td>
<td>21.73</td>
<td>17.62</td>
<td>109</td>
<td>142</td>
<td>0.079</td>
<td>1.385</td>
<td>1.8</td>
</tr>
<tr>
<td>MC10%</td>
<td>25.69</td>
<td>21.22</td>
<td>129</td>
<td>169</td>
<td>0.151</td>
<td>0.852</td>
<td>1.114</td>
</tr>
<tr>
<td>MC15%</td>
<td>32.96</td>
<td>24.41</td>
<td>165</td>
<td>211</td>
<td>0.248</td>
<td>0.667</td>
<td>0.851</td>
</tr>
<tr>
<td>MC30%</td>
<td>40.36</td>
<td>27.02</td>
<td>203</td>
<td>253</td>
<td>0.358</td>
<td>0.565</td>
<td>0.707</td>
</tr>
</tbody>
</table>

In this present study, we evaluated the performance of mechanical pretreatment of woody biomass by calculating energy efficiency (kg glucose kWh\(^{-1}\)). The total amount of glucose obtained after enzymatic hydrolysis (kg glucose/kg wood) was divided by the specific energy consumption for mechanical pretreatment (kWh/kg wood). Therefore, the higher value the higher energy efficiency. Table 1 indicates that energy efficiency decreases as initial moisture content increases, even though samples with higher moisture content resulted in higher enzymatic hydrolysis efficiency. Thus, energy requirements for the pretreatment process are key factor related to processing conditions. On the other hand, utilization of hemicellulose can improve energy efficiency in the pretreatment process. This is evident in the higher energy efficiency values from the total sugar yield than from glucose yield (Table 1).

4.4 Conclusions

In this study, we evaluated the differences in cell wall fracture in the early stage of mechanical milling pretreatment for wood samples with different initial moisture contents. As initial moisture content increased, there was a transition in cell wall fracture from the middle lamella region to the inner cell wall. In the wood substrate with lower initial moisture contents, cell wall fractures occurred predominantly in the middle lamella region and the secondary cell wall.
layers, with severe breakage for wood fibers with high moisture contents. Difference in cell wall fractures led to difference in the chemical compositions of the fracture surface. Higher-moisture content samples were characterized by higher available surface cellulose exposure as measured by Congo dye adsorption. The CLSM technique confirmed that lignin domains occupied the fracture surface of low-moisture content samples, while carbohydrates mainly exposed on the fracture surface of high-moisture content samples. The enzymatic digestibility of micronized wood increased 2-6 times compared to that of raw material, due to the structural and morphological differences caused by the different types of cell wall fracture during mechanical process. The energy requirement for mechanical pretreatment of moisture-conditioned wood samples increase from 0.079 to 0.358 kWh kg$^{-1}$ as the initial moisture content increases. The structural differences in fractures of wood cell walls may result in significant difference in energy requirements for mechanical pretreatment process.
References


CHAPTER 5 EFFECT OF MECHANICAL PRETREATMENT ON
PHYSICOCHEMICAL CHANGES IN WOOD CELL WALL
AND ENZYMATIC HYDROLYSIS EFFICIENCY

Abstract

The hierarchical ultrastructure of wood cell walls is considered to impact significant impediment to enzymatic deconstruction for simple sugars. Earlier work has demonstrated reduced recalcitrance in mechanically pretreated wood. In an attempt to discern the underlying mechanism, we applied a variety of analytic techniques to delineate the ultrastructural changes and alternation of cellulose chemistry resulting from the mechanical deconstruction of the wood cell wall. Gel permeation chromatography (GPC) and X-ray diffraction (XRD) revealed that the molecular weight and crystallinity of the cellulose in micronized wood decrease with increased milling time. Electronic microscopy detailed the structural changes of cell wall from mechanical milling. Four distinct stages were observed to be coincident with the particle size reduction of the wood material: (1) intact cell wall (1) cell fracture and delamination, (3) cell wall disintegration, and (4) amorphization of cell wall fragments. It was confirmed with Simons’ staining that longer milling time resulted in increased substrate accessibility and porosity, likely due to the ultrastructural alternation of cell walls. As evidenced by fluorescence confocal laser scanning microscopy (CLSM), the constituent cell wall polymers are more evenly redistributed following the ultrastructural disruption from the mechanical process than that of the raw material. Findings indicated that changes in a combination of cell wall properties contribute to enhancing enzymatic sugar yields of micronized wood by 4-14 folds over that of intact cell walls, depending on degree of mechanical pretreatment.
Key words: Mechanical milling pretreatment, Cell wall ultrastructure, Cellulose structure, Accessibility

5.1 Introduction

Lignocellulosic biomass is a highly heterogeneous composite with cell walls composed of multiple biopolymers in varying proportions [1]. Considerable interest has emerged in converting lignocellulosic biomass into fossil fuel alternatives to create a diverse and economically viable renewable portfolio for fuels and chemicals. One feasible biomass conversion route is the depolymerization of cell wall polysaccharides with hydrolytic enzymes, followed by fermentation of intermediate sugars to alcohols or hydrocarbons by specific microorganisms [1,2]. However, plant cell walls have a hierarchical architecture resulted from complex interactions of these biopolymers, making the biomass difficult to deconstruct [2]. Therefore, pretreatment is recognized a necessary step to overcome the recalcitrance and make cell wall polysaccharides more susceptible and amendable to hydrolytic action by enzymes to maximize the release of intermediate sugars [2].

Pretreatment has been found to improve enzymatic hydrolysis of biomass by creating changes in substrate chemistry, structure and morphology. These changes include but are not limited to crystallinity, degree of polymerization, specific surface area, and lignin distribution, which result in increasing accessibility of the cell wall polysaccharides to hydrolytic enzymes [3,4]. Several thermochemical approaches (including acid, alkaline, acid sulfite pulping, organosolv pulping, steam explosion, and ionic liquid pretreatments, etc.), have made substantial progress on facilitating enzymatic hydrolysis through depolymerizing and/or partial removal of the cell wall non-cellulose constitutes [5–9]. However, these severe chemical processes often carry high capital cost, employ chemicals and solvents, and that require recovery or treatment may lead to formation
of inhibitors to down-stream cell metabolism to produce desired fuels or chemicals. These challenges pose barriers to commercializing an economically viable biomass conversion process to fuels and chemicals [10]. Mechanical milling pretreatment offers an attractive alternative to producing digestible biomass substrates with the advantages of eliminating chemicals/solvents and the formation of inhibitors typically originated from degrading and/or transforming non-cellulose components (i.e. hemicellulose and lignin) in chemical pretreatment processes [11].

The mechanical milling process is an effective strategy to break down the robust cell wall structure of woody biomass feedstock. It has long been used to deconstruct the native structure of plant cell wall for improving substrate accessibility, which enables providing the milled wood lignin [12–14]. The milling process profoundly imparts multiple length-scale structural alternations (e.g., plant, tissue, cellular and molecular levels) through disrupting supramolecular cross-links of cellulose-hemicellulose-lignin networks [15]. Numerous studies also demonstrated that mechanical milling facilitates enzymatic hydrolysis of various feedstock (i.e., herbaceous and woody biomass) [10,16–18]. These improvements may occur in tandem with decreases in particle size and cellulose crystallinity in milled substrates, which are associated with increased enzyme accessibility to cell wall polysaccharides. Our prior work demonstrated that partial breakage of wood fiber and/or fiber bundles improves the enzymatic hydrolysis of milled softwood, resulting in limited total sugar yields of around 40%. Mechanically disintegrating the fiber cell wall into micronized fragments significantly improves the enzymatic hydrolysis, with total sugar yields of over 70% (as presented in Chapter 3). Similarly, Ji et al. also found that mechanically fragmenting corncob samples at a cellular scale resulted in a 98.3% conversion yield of cellulose to glucose [15]. In addition, decreasing the cellulose crystallinity during the milling process also contributes to increasing accessibility to cellulose microfibrils [19]. Substantial research reveals a strong
correlation between increased enzymatic hydrolysis efficiency and decreasing crystallinity in various milled feedstock [10]. Despite the demonstrated efficiency of mechanical milling pretreatment for disrupting the cell wall structure and increasing enzymatic hydrolysis yields, a clear understanding of the ultrastructural alternation and cellulose characteristics that are presumably responsible for facilitating enzymatic digestion is still needed.

The objective of this study is to delineate the effects of mechanical milling pretreatment on the ultrastructural properties and cell wall chemistry of micronized wood as related to the sugar release efficiency or recalcitrance properties of biomass. Specifically, we have examined the physicochemical changes resulting from the mechanical milling pretreatment of softwood Douglas-fir (*Pseudotsuga menziesii*) feedstock by using combined wet chemistry techniques with other analytical and imaging techniques in new ways. Transmission electron microscopy (TEM) was used to investigate the ultrastructural disintegration of the wood cell wall and, as it turned out, to reveal the increased substrate accessibility and porosity within the cell wall fragments. Confocal laser scanning microscopy (CLSM) was employed to delineate the distribution of chemical composition related to breakdown of cell walls during the milling process. We also conducted enzymatic hydrolysis experiments on micronized wood in an effort to assess the change in the recalcitrance of milled substrates. Together, these data were integrated to provide insights into the mechanical deconstruction of wood cell wall as well as fundamental nature of recalcitrance related to enzymatic hydrolysis efficiency of lignocellulosic biomass.

### 5.2 Materials and methods

#### 5.2.1 Materials

Clean, Douglas-fir (*Pseudotsuga menziesii*) wood chips were obtained from a local company (Vaagen Brothers Lumber Inc., Colville, WA). The as-received chips were separated by
a vibrating screen with 25.4-mm aperture and the pre-ground into particles by a hammer mill fitted with a 3.18-mm screen. The pre-ground feedstock was subsequently conditioned to a target equilibrium moisture content of 5%. Before further mechanical pretreatment, the conditioned sample was stored in sealed plastic bag and the moisture content was validated using gravimetric methods according to standard protocol [20].

5.2.2 Mechanical pretreatment process

Mechanical pretreatment was performed using a high-energy vibratory ring and puck mill with motor power of 1.1-kw (Rocklab Pty Ltd, New Zealand). The sample (10-g, oven-dry base) was milled in a chamber with an inner diameter of 128-mm and height of 43-mm along with a ring (78-mm inner diameter, 100-mm outside diameter, 41-mm height) and a puck (52-mm diameter and 41-mm height) as the milling media. Both the milling chamber and grinding media were made of tungsten carbide. Milling was conducted for a total time of 2-12 minutes, with 2-minute intervals.

5.2.3 Composition analysis of the wood samples

The chemical composition analysis of wood material was conducted according to the two-step acid hydrolysis procedures from the NREL standard protocol [21]. Briefly, a 300-mg sample and 3-mL of 72% H₂SO₄ was added to a 100-mL pressure tube, and incubated at 30 °C for 1 hour and stirred every 15 minutes. The sample was then diluted with 84-mL deionized water and autoclaved for one hour. Detection of sugars was performed with high-performance anion exchange chromatography (HPAEC) (Dionex, ICS-3000).

5.2.4 Enzymatic hydrolysis

Enzymatic hydrolysis experiments were performed using Cellic CTec2 cellulase (15 FPU/OD g of substrate) and cellic HTec2 hemicellulase (1/9 of the cellulase amount). Digestion was carried out in 125-mL flasks with a citrate buffer (pH 4.8) at a solid loading of 2%. The flasks
were settled in an incubator with a rotation speed of 180 rpm at 50 °C. After digestion for 72 hours, the hydrolysate was analyzed by HPAEC. Glucan and xyl/mannan conversions were defined as the percentage of glucose and xyl/mannose that was released compared to the theoretical maximum.

5.2.5 Simons’ staining for measurement of substrate accessibility

The Simons’ staining procedure was adopted from the literature [22]. Direct Blue 1 (DB) (Pontamine Fast Sky Blue 6BX) and Direct Orange 15 (DO) (Pontamine Fast Orange 6RN) dyes were obtained from Pylam Products Co. Inc. (Garden City, NY). DB was used as received, while DO was separated by 10K membrane using the Amicon ultrafiltration apparatus (Amicon Inc., Beverly, MA) under pressure of 35-psi. The purpose of fractionation of high molecular weight DO is to ensure its absorption to represent enzyme. This is because only high molecular weight fraction of the DO dye is responsible for the increased affinity for cellulose. Wood samples (100-mg, oven dry base) were weighed into six centrifuge tubes with a 1.0-mL phosphate buffer saline solution (pH 6, 0.3-M PO4, 1.4-M NaCl). The same amount of 1% dye solution of DB and DO were added to each of the six tubes with increasing volumes (0.25, 0.5, 0.75, 1.0, 1.5, 2.0mL). Next, distilled water was added to each tube to bring the final volume to 10.0-mL. After incubation at 70 °C for 6 hours with shaking at 180rpm, the slurry was separated by centrifugation. The absorbance of the supernatant from sample was measured at wavelength of 455 nm and 624 nm with a UV-vis spectrophotometer (Lambda 25, PerkinElmer), and the amount of adsorbed dye was calculated. The maximum adsorption capacity of the two dyes were determined according to the Langmuir adsorption equation and the ratio between DO and DB adsorption capacities (DO/DB) was used as a measure of the large-to-small pore ratio of the material [22].

5.2.6 Gel permeation chromatography (GPC) analysis of cellulose

The molecular weight distribution of cellulose in micronized wood was determined by
GPC after benzylation of α-cellulose that enables cellulose to be dissolved in tetrahydrofuran (THF) [23,24]. Isolation of α-cellulose was obtained by delignifying wood samples with sodium chlorite/acetic acid, and then through alkaline extraction of holocellulose following the procedures elsewhere [25]. Benzylation of α-cellulose was also conducted according to previously established procedures [23]. In brief, 1-allyl-3-methylimidazolium chloride ([amim]Cl) (950-mg) was mixed with cellulose (50-mg) in a round-bottom flask, and then heated at 80°C until the solution was clear. Pyridine (400-µL) was added and the solution was vortexed until it was homogeneous, and then left to cool to room temperature. Benzoyl chloride (350-µL) was added to the above solution and vortexed until a homogeneous white paste formed. The sample was left at room temperature for 3 hours. An ethanol-water mixture (3:1 v/v) was added, and the mixture was vigorously shaken for 5 minutes, creating a pale, fluffy precipitate. The solid was filtered through a sintered glass filter (grade 3), washed with further ethanol, and trituted with methanol at room temperature for 18 hours. The solid was then filtered and dried under a vacuum, yielding a white to brown benzoylated product.

Prior to GPC analysis, benzyolated samples were dissolved in THF (1 mg/mL), filtered through a 0.45-µm membrane, and placed in a 2-mL auto-sampler vial. Size-exclusion separation was performed on a Viscotek gel permeation chromatograph system equipped with a GPCmax™ unit and a TDA 305 multidetector unit using THF as the mobile phase. One AM Gel 100/5 column (American Polymer Standards Corporation) and two MBHMW-3078 columns (300 × 7.8mm, Malvern) were used. The temperature of column oven was 30°C and the injection volume was 100µL. A calibration curve was constructed based on 8 polystyrene standards, each with narrow ranges in molecular weight from 2.2 to 3600 kDa. The number-average degree of polymerization (DPₙ) and weight-average degree of polymerization (DPₙ) were obtained by dividing Mₙ and Mₘ
respectively by 162 g/mol. All reported values were the mean average of duplicate samples.

5.2.7 Determination of cellulose reducing ends

The amount of cellulose reducing ends (µmoles/g wood) was determined by DNS assay. The DNS reagent was prepared by mixing 1416-mL distilled water, 10.6-g 3, 5 Dinitrosalicylic acid and 19.8-g sodium hydroxide, following the addition of 306-g Rochelle salts (sodium potassium tartrate), 7.6-mL phenol (melt at 50°C) and 8.3-g sodium metabisulfite, as noted in a technical report [26]. About 1.5-mL of sample slurry containing 50-mg wood in distilled water was added into the test tubes and followed by adding 3-mL freshly made DNS reagent. The tubes along with duplicate anhydrous glucose calibration standards (2-6.7 mg/mL) were boiled for 5 minutes in a vigorously boiling water bath. After boiling, tubes were transferred to a cold ice-water bath. Color formation of the supernatant was measured at 540 nm with a UV-vis spectrophotometer (Lambda 25, PerkinElmer), and the amount of cellulose reducing ends was determined from absorbance calibration plots generated for standards.

5.2.8 Sample embedding and sectioning

Wood samples were processed using microwave electronic microscopy processing methodology as described elsewhere [27]. Briefly, samples were fixed in 3% glutaraldehyde and buffered in 0.05-M Pipes buffer (Sigma, St Louis, MO) with a microwave on full power. The samples were dehydrated in graded ethanol series (i.e., 30%, 50%, 60%, 70%, 80%, 90%, and 3×100% ethanol) for 40 seconds in a microwave oven. The samples were then infiltrated with Spurr’s resin and incubated overnight at room temperature in a hood with increasing concentrations of the resin (i.e., 30%, 50%, 3×100% resin, diluted in isopropanol). The samples were transferred to micro-centrifuge tubes and the resin polymerized overnight at 70 °C. The embedded samples were sectioned to 300 nm for light microscopy and to approximately 100 nm
for TEM using a Diatome diamond knife on a Leica Reichert Ultracut R microtome (Leica, Wetzlar, Germany).

5.2.9 Determination of composition distribution in micronized wood

The composition distribution in micronized wood was characterized by using confocal laser scanning microscopy (CLSM) as detailed elsewhere [28,29]. Semi-thin (200-500nm) sectioned samples were positioned on glass microscope slides and stained with saturated HPLC-grade acridine orange (AO; 3, 6-bis (dimethylamino) acridine hydrochloride, Sigma-Aldrich, St. Louis, MO) for one hour at room temperature. After staining, the samples were washed three time with DI water. The imaging of stained samples was performed using a Leica TCS SP8 with a 40× oil objective lens. A white laser at wavelength of 500 nm was used as the excitation light source. Fluorescence emission in the 515-540 nm spectral region were acquired as the green channel, and emissions above 590 nm spectral region were collected as the red channel. Image analysis was performed using LAS AF Lite imaging analysis software. Color in green represented area rich in carbohydrates, while lignin was represented by red color. Furthermore, multiple line scans across cell walls and wall fragments at various mechanical milling time points were also analyzed to investigate the lignin/carbohydrates redistribution. The signal intensity represented as raw pixel intensity and distance as the pixel distance.

5.2.10 Transmission electron microscopy (TEM)

Ultrathin sections were placed on Formvar coated copper slot grids (SPI Supplies, West Chester, PA). Grids were post-stained for 10 minutes with 1% w/v KMnO₄ to selectively stain for lignin. TEM imaging were taken with a 4 megapixel Gatan UltraScan 4K Eagle camera (Gatan, Pleasanton, CA) on a FEI Tecnai G2 20 Twin 200kV LaB6 TEM (FEI, Hillsboro, OR).
5.2.11 Scanning electron microscopy (SEM)

Milled wood samples were mounted on aluminum stubs using carbon tape and sputter-coated with 8-nm of gold for good conductivity prior to imaging. Imaging by SEM was performed using an FEI Quanta 200F field emission gun with high vacuum ETD detectors (FEI Company, Hillsboro, Oregon, USA). Imaging was taken at beam accelerating voltage of 20-kV.

5.3 Results

5.3.1 Enzymatic hydrolysis of micronized wood

The main target of pretreatment is to overcome the recalcitrance of lignocellulosic substrates for efficient enzymatic hydrolysis of polysaccharides. Table 1 shows monosaccharide yield in enzymatic hydrolysis for micronized wood samples with various milling times. After mechanical pretreatment, the sugar yield of samples improved compared to the starting material. These results concurred well with the literatures, which claim that mechanical milling increases accessibility and digestibility of substrates for various herbaceous and woody biomass [16,18,30]. In our study, the monosaccharide yield after enzymatic hydrolysis also increased with increased milling time. The highest glucose yield was around 90%, approximately 14 times higher than the yield of the starting material. To examine the enhanced sugar release, the cellulose chemistry and cell wall ultrastructure of micronized wood samples were explored in depth.

5.3.2 Chemical composition of micronized wood

Typically, changes in chemical composition of biomass after thermo-chemical pretreatments contribute to increasing sugar yields from enzymatic hydrolysis of pretreated substrates [31]. However, for the mechanical pretreatment here, the bulk chemical composition remains unchanged regardless of mechanical milling times (Table 1). We note that others have found that ball milling for long periods (i.e., tens of hours or several weeks) may result in
degradation of components of biomass cell wall [23,32]. In our study, the ring and puck milling required much shorter milling time resulted in satisfactory enzymatic sugar release without degradation of chemical components. The difference in sugar release of micronized wood with different milling times may be related to the structural alternation of substrates.

Table 5.1 Chemical composition and theoretical enzymatic hydrolysis yield of micronized wood

<table>
<thead>
<tr>
<th>Milling time (min)</th>
<th>Chemical composition (mass percentage of the wood)</th>
<th>Enzymatic hydrolysis yield (% of theoretical yield)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucan</td>
<td>Xyl/mannan</td>
</tr>
<tr>
<td>0</td>
<td>45.18</td>
<td>16.53</td>
</tr>
<tr>
<td>2</td>
<td>45.18</td>
<td>16.53</td>
</tr>
<tr>
<td>4</td>
<td>44.47</td>
<td>16.45</td>
</tr>
<tr>
<td>6</td>
<td>44.85</td>
<td>16.60</td>
</tr>
<tr>
<td>8</td>
<td>44.40</td>
<td>16.35</td>
</tr>
<tr>
<td>10</td>
<td>44.63</td>
<td>16.57</td>
</tr>
<tr>
<td>12</td>
<td>44.62</td>
<td>16.51</td>
</tr>
</tbody>
</table>

Each amount is the average of duplicates.

5.3.3 Particle morphology of micronized wood

In this study, we have examined the changes in the particle morphology for milled wood with increasing milling times by using scanning electronic microscopy (Figure 5.1). Effectively micronized softwood was obtained using a ring and puck mill. A disrupted fiber structure was observed after 2-minutes of milling and is characterized by separated fibers/fiber bundles. The fiber fragments started to prevail after 4-minutes of milling. After 6 minutes, the fiber cell wall was almost entirely fragmented, resulting in sizes of ca. 20-μm. No further particle size reduction
was observed with additional milling times up to 12 minutes. However, a decreased aspect ratio is apparent, producing a more uniform particle shape.

![SEM micrographs of micronized wood samples for various mechanical pretreatment times.](image)

**Figure 5.1** SEM micrographs of micronized wood samples for various mechanical pretreatment times.

Mechanical pretreatment caused tissue fracture with cell wall separation at the early stage (A, arrows). Further milling resulted in chopping and breakage of individual fibers (B, arrows). There was no noticeable change of particle morphology after total disintegration of the cell wall (C-F) due to aggregation effect.

**5.3.4 Chemical composition distribution of micronized wood**

In this study, we applied confocal laser scanning microscopy (CLSM) with acridine orange-stained sections to explore the deconstruction phenomena of cell wall and redistribution of lignin in the cell wall. Acridine orange (AO) is used to stain the heterogeneous plant cell wall and its brightness has been reported to be proportional to lignin concentration [33,34]. When AO interacts with polysaccharides, it remains in a monomeric state, resulting in fluorescence emission
in the green light region. However, when AO interacts with the aromatic π electrons in lignin, the electron density of the molecules will cause aggregation of AO molecules, leading to a fluorescence emission shift to red light spectrum [28]. Line scans provide a more quantitative analysis of the changes in lignin distribution within the intact cell walls and variable cell wall fragments, as shown in Figure 5.2.

As anticipated, the raw material (Figure 5.2A) shows a very uneven distribution of lignin across the cell walls, with lignin rich regions in the cell wall corner and middle lamella. In contrast, a strong green signal is visibly evident in polysaccharides-rich secondary cell wall layers (Figure 5.2A). These observations are consistent with previous results reported by others [35,36]. Compared to the intact cell walls, lignin distribution changes dramatically along with the cell wall fracture. The densely packed middle lamella lignin becomes loosely distributed on the fracture surface as the fiber bundles are separated among the middle lamella of adjacent cell walls at a 2-minute milling time (Figure 5.2B). Figure 5.2C illustrate that additional milling produces remarkable fragmentation of the cell wall structure along with the continuous lignin redistribution in the micronized fragments. After 12 minutes of milling, the lignin that once coated the cellulosic fibers is evenly distributed across the cell wall fragments (Figure 5.2D). Figure 5.2D also indicates the most uniform signal intensity of lignin and polysaccharides, respectively, suggesting that the physical barrier of lignin has been eliminated.
Figure 5.2 Confocal laser scanning microscopy (CLSM) images of chemical composition distribution across intact cell wall and cell wall fragments with various mechanical milling times. Line scan profiles demonstrate that the distribution of lignin and polysaccharides changes along with the deconstruction of cell wall structure under intensive mechanical action. The red color indicates lignin and green indicates carbohydrates. ROI: region of interest.

5.3.5 Ultrastructure changes of micronized wood

TEM micrographs of particles exposed to increasing levels of mechanical pretreatment are shown in Figure 5.3. Interpretation of ultrastructure changes in these micrographs reveals several stages of mechanical deconstruction of wood cell wall as outlined below.

1) Intact Cell Walls – Representative cross and longitudinal sections are shown in Figure 5.3 A and B. In these sections, the secondary cell walls (S1, S2 and S3), compound middle lamella (CML) and cell wall corners (CC) are clearly evident. Coarse milling produces particles in this stage, likely due to mechanical action on plant tissue separation with maintaining intact cell wall.

2) Cell Fracture and Delamination – After 2-4 minutes of milling time (Figure 5.3 C and D) cell fractures in the CML and CC become prevalent. Fractures progress to larger scale
delamination and eventual cell wall fragmentation as evident in Figure 5.3 D. Combined, these processes serve mainly to separate adjacent cells and reduce particle size, while largely maintaining the original cell wall ultrastructure.

(3) Cell Wall Disintegration – Figure 5.3 E and F reveal that additional milling results in disintegration of cell wall ultrastructure, displaying a total collapse of orderly structural cell wall and randomly generating multiple cell wall fragments. These fragments are difficult to assign to specific cell wall locations. The longer the milling time, the higher the degree of ultrastructural disruption.

(4) Cell Fragments Structure Amorphization – The final milling stage is characterized as amorphization of cell wall fragments (Figure 5.3 G and H). This generates aggregates that are largely agglomeration to nanoscale particles with a much more amorphous structure than the samples with shorter milling times.

It is conceivable that the generating various cell wall fragments under mechanical action would also disorder and disorient the cellulose microfibrils. TEM images with higher magnification (Figure 5.4) of the micronized wood visualize the disruption of microfibrils network from these milling stages. Figure 5.4A shows the dense and solid cell wall with highly packed cellulose microfibrils network in the raw material. At the early milling stage, the densely ordered microfibrils is still visibly evident (Figure 5.4B), since fractures mainly occurred in the CML and CC regions. Samples that were milled for 6 minutes produce complete disintegration of the cell wall and show disorientation in the microfibrils of cell wall fragments (Figure 5.4C). Delamination of the microfibrils is also visibly evident in this sample, producing additional internal porosity (Figure 5.4C). Disintegrating the cellulose microfibrils with various nanoscale aggregates (Figure 5.4D) is clearly evident in the microfibrils network for samples milled for 12 minutes. Figure 5.4
also indicates that disrupting the cellulose microfibrils network resulted in various intra-particle voids (Figure 5.4D).

**Figure 5.3** TEM micrographs with low magnification display intact cell walls and structural cell wall deconstruction produced with different milling times.
Figure 5.4 TEM micrographs with higher magnification display disruption of microfibrils network along with structural cell wall deconstruction produced with different milling times. Arrows show orientation of cellulose microfibrils in intact cell wall and cell wall fragments. Double headed arrows show microfibrils delamination and intra voids. Arrow heads show microfibrils nanoscale aggregates.

5.3.6 Cellulose degree of polymerization (DP) and reducing ends

One of the important recalcitrant factors influencing enzymatic hydrolysis is related to the cellulose molecular structure, namely molecular weight. To determine the potential influence of
mechanical pretreatment on the cellulose structure, the average degree of polymerization of benzoyleate derivatized α-cellulose isolated from micronized wood as analyzed by GPC (Table 2). The data clearly shows that the molecular weight of micronized wood cellulose decreases as milling time increases. Compared to the starting material, the DP\textsubscript{n} of a sample milled for 2-minutes decreases by 17%, with additional milling, the decrease in DP\textsubscript{n} continues, reaching a 73% decrease in sample milled for 12-minutes. Liimatainen et al. previously reported similar results of decreasing DP for cellulose microfibers after wet-stirred media milling. In their study, DP values decreased by about 75% after 15- or 35-minutes milling, depending on the amounts of milling media used [37]. It is likely that mechanical action led to scission of cellulose molecular chains along with mechanical size reduction of lignocellulosic biomass. Breakage of the molecular chain collectively results in a decrease of particle size and cellulose DP. Ju et al. also reported significant difference in DP of kraft pulping fibers after fractionating in a Bauer-McNett fiber classifier fitted with different mesh screens [38].

Cellobiohydrolase I (Cel7A) plays vital important role in hydrolyzing cellulose, since it can attack the reducing ends to remove a molecular strand of cellulose with more internal sites exposure [39]. Therefore, it is commonly thought that reducing ends influence the rate and extent of enzymatic hydrolysis of cellulose. In this study, we examined the effect of mechanical milling on the total number of reducing ends using DNS assay, in which 2-hydroxy-3, 5-dinitrobenzoic acid which reacts with the reducing ends accompany color formation [40]. In Table 2, the number of reducing ends increases from 16.64 µmoles/g for a starting sample to 51 µmoles/g for the sample with 12 minutes of milling. A previous study also claimed that milled wood samples with various vibrational milling times had increased carbonyl groups, which were quantified by the copper number [41]. The increase in reducing ends agrees with the results discussed previously,
specifically the decrease in cellulose DP during mechanical milling, mainly due to fragmentation of cellulose chains.

Table 5.2 Molecular characterization of micronized wood samples.

<table>
<thead>
<tr>
<th>Milling time (min)</th>
<th>DP\textsubscript{n}</th>
<th>DP\textsubscript{w}</th>
<th>PDI (M\textsubscript{w}/M\textsubscript{n})</th>
<th>Reducing ends (µmoles/g)</th>
<th>CrI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1068</td>
<td>5460</td>
<td>5.1</td>
<td>16.64</td>
<td>52.3</td>
</tr>
<tr>
<td>2</td>
<td>891</td>
<td>3953</td>
<td>4.4</td>
<td>21.22</td>
<td>47.9</td>
</tr>
<tr>
<td>4</td>
<td>565</td>
<td>3450</td>
<td>6.1</td>
<td>31.48</td>
<td>30.8</td>
</tr>
<tr>
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<td>2484</td>
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<td>398</td>
<td>1705</td>
<td>4.3</td>
<td>45.22</td>
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</tr>
<tr>
<td>10</td>
<td>324</td>
<td>1228</td>
<td>3.8</td>
<td>50.11</td>
<td>11.9</td>
</tr>
<tr>
<td>12</td>
<td>293</td>
<td>1368</td>
<td>4.7</td>
<td>50.96</td>
<td>8.9</td>
</tr>
</tbody>
</table>

DP\textsubscript{w} = weight-average degree of polymerization, DP\textsubscript{n} = number-average degree of polymerization, PDI = polydispersity index, CrI = crystallinity index. Each result is the average of duplicate samples.

5.3.7 Cellulose crystallinity

The effects of mechanical milling pretreatment on the crystalline structure of wood cellulose as measured by WAXD are shown in Figure 5.5. The X-ray diffraction patterns show typical peaks of cellulose I, with a gradual decrease in crystalline ordering as milling time increased. Crystallinity index (CrI) values decreased from 52% for raw material to around 9% for micronized wood with 12 minutes of milling (Table 2). With the longest milling time, the sample showed a broad peak at approximately 2θ =21°, suggesting that the material was in a largely amorphous state. The decrease in CrI may be due to the fragmentation of crystalline grains, deformation of the crystalline structure, promoting an increase in amorphization during the mechanical pretreatment. Similar CrI changes as a function of milling time have been previously reported for diverse biomass feedstock [10]. Collectively, the XRD spectra and CrI values
confirmed the disruption of the crystalline structure, which facilitates accessibility for enzyme attack. In our study, there was no transformation of the cellulose I structure into the cellulose II allomorph, which has been previously found for ball milling of pure cellulose in wet media [42].

![X-ray diffraction patterns of micronized wood from various mechanical milling pretreatment times.](image)

**Figure 5.5** X-ray diffraction patterns of micronized wood from various mechanical milling pretreatment times.

### 5.3.8 Substrate accessibility as measured by Simons’ staining

The Simons’ staining technique has been shown to be a semi-quantitative method for characterizing the accessible features of lignocellulosic biomass with two color differential dye probes (i.e., Direct Orange 15 and Direct Blue 1) [22]. The molecular diameter of Direct Blue 1 (DB) is approximately 1 nm, while the molecular diameter of Direct Orange (DO) ranges 5 to 36 nm for the high molecular weight fraction (which has higher binding affinity to the cellulose hydroxyl group than does DB) [43]. Therefore, competing phenomena occur in the treatment of lignocellulosic substrates with a mixed dye solution of DO and DB molecules. In other words, the
DB probes enter the smaller pores (diameter around 1 nm), while DO molecules will occupy the larger substrate pores or surface. The ratio between the maximum adsorption of DO to DB dyes on the substrates is therefore a good indicator of large-to-small pore ratio [22]. Moreover, the hydraulic diameter of cellulase is approximately 7 nm, thus, DO adsorption has been proposed to assess the cellulose accessible surface area, and shows good correlation between enzyme accessibility and enzymatic hydrolysis of pretreated lignocellulosic biomass [22,44]. A modified Simons’ staining assay developed previously was used to explore the pore profile and accessibility of milled substrates.

As shown in Figure 5.6, the total dye adsorption (DO and DB) increases with milling time, from 60.6 mg/g for the starting material to 169.2 mg/g for the sample milled for 12 minutes. In addition, the orange dye showed a higher adsorption increase than the blue dye for the same milling time, resulting in an increase of O/B ratio, from 0.21(starting material) to 0.54 (sample milled for 12 minutes). The increased DO dye adsorption and ratio of O/B for milled wood substrates suggest a significant increase of the accessible cellulose surface area and porosity after intense mechanical pretreatment. Overall, as shown in Figure 5.7, strong correlations of sugar yields with cellulose accessibility measured by DO adsorption and porosity as measured by O/B indicate that increasing accessible surface area facilitates the enzymatic hydrolysis of micronized wood.
Figure 5.6 Simon’s staining results for the micronized wood accessible surface area represented by the amount of adsorbed dye (mg dye per g of dry wood) and the relative porosity represented by the ratio of the adsorbed large orange to small blue dye (O/B).
5.4 Discussion

Mechanical milling pretreatment is an effective approach to improve the enzymatic hydrolysis of softwood, which is known as the most recalcitrant biomass feedstock for enzymatic digestion [10,45]. Results from analysis of the chemical and physical structural properties of micronized wood explains the difference in digestibility of samples prepared with different milling times. Findings also provide better understanding on how to overcome the recalcitrant factors of native biomass to facilitate enzymatic hydrolysis.

The efficiency of enzymatic hydrolysis is directly related to hydrolytic enzyme activity and the biomass recalcitrance. This, in turn, relates to various substrate characteristics, including cell wall chemistry, cellulose crystallinity, cellulose DP, accessible surface area, and lignin distribution. Results demonstrates that mechanical milling pretreatment did not alter the chemical compositions of micronized wood with different milling times. In this study, mechanical fragmentation broke the β–glycosidic bonds in cellulose chains, since cellulose DP decreases significantly after milling.
pretreatment. The main function of decreasing cellulose DP is that it offers increased available reducing ends for endoglucanase attack, thereby weakening the networks to permit better accessibility, subsequently enhancing enzymatic hydrolysis rate and extent [6,46]. The increase in the values of reducing ends in micronized wood samples also confirmed the significant changes in the cellulose molecular structure. These findings collectively demonstrate the important role of fragmenting cellulose chains (i.e., decreasing cellulose DP) to relieve the recalcitrance of native biomass and improve enzymatic hydrolysis of micronized wood.

Results of CLSM imaging indicates that mechanically fragmenting the fiber cell wall eventually results in a even redistribution of lignin, which originally encapsulated the cellulose microfibrils in the hierarchical cell walls. The accessible surface area of cellulose was estimated with orange dye adsorption using Simons’ staining. This technique provides metrics for measuring the surface area of lignocellulosic feedstock in a dehydrated state, similar to the enzymatic hydrolysis environment. The increase in DO adsorption from 10.5 mg/g to 59.5 mg/g was dependent on milling times. This, indicates that redistributing the lignin plays a key role in improving the available/accessible cellulose surface area of micronized wood by degrading the physical barrier formed by this polymer. This phenomenon complements the increased surface area afforded by particle size reduction. In a previous study, Ji et al. found that redistribution of cellulose in the inner cell wall layers on the surface of particles after destroying the cellular structure of fibers significantly improved the surface area of accessible cellulose, as the O/C ratio revealed by XPS characterization increased significantly for this sample [15]. Several studies have found that lignin migrates and relocates within and/or out of the plant cell walls after thermochemical pretreatments (e.g., dilute acid and AFEX) [27,47]. The relocalization of lignin caused by phase transition during thermochemical pretreatment process is thought to play
important role in providing more accessible cellulose area and facilitating subsequent enzymatic hydrolysis efficiency of pretreated substrates. In this study, results from imaging and dye probing analyses demonstrated that mechanical milling pretreatment effectively relieves the physical barrier effects of lignin and increases the available cellulose surface area by destroying the highly ordered cell wall structure and coincidently causing redistribution of cell wall compositions.

Another important recalcitrance factor that impedes the efficient enzymatic hydrolysis of cellulose is related to the cell wall heterogenity and the polymer interaction forming a tightly packed structure. Such packing density physically limits enzyme accessibility to the cellulose molecules [48]. Removing the lignin and/or hemicellulose has been recognized as the leading factor for increasing substrate porosity with chemical or biological approaches [49]. Mechanical pretreatment is considered to be ineffective for removal of hemicellulose and lignin, which our study has confirmed. Particle size reduction was an important physical change caused by mechanical milling pretreatment. Decreasing the particle size of micronized wood increases external surface area, favoring enzyme adsorption. However, particle size reduction may be just one factor that improves enzymatic hydrolysis of micronized wood. This consideration is supported by the factor that there was less changes in particle morphology after 6 minutes of milling, however, hydrolysis efficiency enhances dramatically (as presented in Chapter 3). TEM imaging reveals a remarkable ultrastructural disruption, providing a substantial accessible surface area by fragmenting the cell wall into fragments and disrupting cellulose microfibrils network (Figure 5.3 and Figure 5.4). The degree of ultrastructure disruption increases with milling time, leading to substantial nanoscale microfibrils aggregates and intra voids in sample with 12-minutes milling (Figure 5.4D). The increase of total dye adsorption and O/B ratio from Simons’ staining technique confirmed that mechanical milling pretreatment caused a substantial increase in
accessible surface area and porosity, although the chemical components in the cell wall matrix did not change. These results also indicate that in addition to changes in cell wall chemistry, deconstruction of cell wall appears to increase porosity and cellulose accessible surface area, facilitating subsequent enzymatic hydrolysis (Figure 5.7). In a study, Wang et al. [19] found that the chemical composition of dilute acid pretreated corn stover was similar using three different reactors configurations, however, the enzymatic hydrolysis efficiency of these samples varied significantly. Simon’s stain and electronic microscopy analysis revealed that mechanical action in the reactors increased cellulose accessible surface area due to cell wall delamination. The difference in digestibility between pretreated biomass samples with the same chemistry indicates that treatments which maximize the substrate accessible surface area dominates over changes in cell wall chemistry.

5.5 Conclusions

This study provides key perspectives for understanding biomass recalcitrance towards altering cell wall structure and increasing cellulose accessibility arising from mechanical pretreatment. Mechanical milling pretreatment produced micronized wood with significantly different digestibility performance with negligible changes in chemical composition. TEM imaging showed that the disintegration of the ordered cell wall structure by mechanical action produces increased substrate accessibility and porosity. In tandem with particle size reduction, mechanical pretreatment causes cell wall fracture and delamination, ultrastructure disintegration and cell fragments structure amorphization, thereby, creating a highly porous structure with increased accessible surface area. A combination of imaging and dye probing analysis demonstrated that disintegrating the fiber cell wall into various fragments coincidently resulted in redistribution of cell wall components, maximizing the surface area of accessible cellulose.
Findings also show that mechanical pretreatment decreased the degree of polymerization of the cellulose, while simultaneously increasing the fraction of reducing ends. Both these factors have been correlated to increasing ratio of enzymatic hydrolysis. Results of this study seem to indicate that combination of decreased particle size, disintegration of cell wall ultrastructure, redistribution of lignin, increased accessible surface area and decreased cellulose molecular weight and crystallinity considerably facilitates enzymatic hydrolysis efficiency and overcomes biomass recalcitrance.
References


CHAPTER 6 SUMMARY AND CONCLUSIONS

Mechanical pretreatment offers an effective approach towards generating highly digestible micronized wood for subsequent enzymatic hydrolysis with features of no chemicals input and zero inhibitors presence. A better understanding of characteristics of micronized wood, energy consumption of process, enzymatic hydrolysis performance and their relationships will provide meaningful information and guidance for overcoming biomass recalcitrance in an economic manner by using mechanical pretreatment. The main motivation of this dissertation was to understand the effect of mechanical pretreatment on wood cell wall deconstruction as well as how features of micronized wood affect subsequent enzymatic hydrolysis. Information from such studies can customize mechanical pretreatment conditions for scale-up applications and provide fundamental insight to overcoming biomass recalcitrance.

The first study started with the idea of determining the effect of mechanical pretreatment conditions involving substrate properties (moisture content and feed size) and milling time on developing physical properties of micronized wood and energy requirement of mechanical pretreatment process using a vibratory ring and puck mill. The physical characteristics of micronized wood including particle size, aspect ratio and cellulose crystallinity were characterized. In general, moisture content was the most important factor milling behavior in terms of particle morphology and cellulose crystallinity. The relative lower moisture content resulted in much rounder particles with relative smaller crystallinity index than particles derived from higher moisture content samples. Within the same moisture content, the crystallinity and aspect ratio of particles underwent the similar evolution trend. The feed size had negligible influence on evolution of particle physical properties, but only affected the specific energy consumption. A multi-step
milling strategy is recommended for a more economical approach for fine milling of woody biomass. Rittinger’s model was successfully employed to correlate specific energy consumption of mechanical pretreatment with particle size changes. The Rittinger’s model constant was a good indicator of milling performance of woody biomass with different properties. It was also recommended that standardizing milling test and parameters could benefit to evaluating the milling performance of fibrous materials on a lab-scale, as such information would be useful designing scale-up woody biomass milling facilities of woody and herbaceous biomass feedstock.

The second part of this dissertation evaluated the effect of structural characteristics on enzymatic hydrolysis of mechanically pretreated woody biomass and energy efficiency of mechanical pretreatment towards high fermentable sugars yield. Micronized wood with distinguished structural features were obtained under various milling variables. This study revealed the beneficial effects of reducing particle size to facilitate enzymatic hydrolysis, however, exhibiting complex relationships between carbohydrate conversion and particle size in the examined size range. Generally speaking, mechanical size reduction down to ca. 30µm could contribute to 40% of theoretic carbohydrate conversion of milled wood, because mechanical pretreatment resulted in breaking up fibers and fiber bundles in this size range. Mechanical fragmenting fiber cell wall into micronized fragments with size smaller than 30µm further facilitated over 70% of theoretic carbohydrate conversion, mainly due to increasing surface area and decreasing cellulose crystallinity of micronized wood. A multiple linear regression model successfully provided empirical prediction of carbohydrate conversion of micronized wood with the structural characteristics. Energy efficiency results demonstrated that using a low-moisture content of the feedstock biomass and a multi-step milling process would benefit to decreasing the energy requirement for producing fermentable sugars with a mechanical pretreatment.
Inspired by the difference of physical features and enzymatic hydrolysis of micronized wood resulted from the difference in initial moisture content of the starting material, the third part of the study focused on understanding wood cell fracture performance in the early stage of mechanical pretreatment. By evaluating the morphological and structural characteristics of micronized wood, transition in cell wall fracture from middle lamella region to the inner cell wall was observed with increase of initial moisture content. Generally speaking, interwall fracture occurs predominantly at the middle lamella region for wood sample with lower initial moisture content, while the wood sample with high moisture contents preferentially displayed intrawall fracture type at the secondary cell wall layers with sever breakage in wood fibers. The different cell wall fracture types resulted in distinguished surface chemical components exposure. Quantitatively, higher moisture content sample had more available cellulose surface area than that of lower moisture content sample. CLSM technique confirmed that lignin domains occupied the fracture surface of low moisture content sample, while polysaccharides were the main surface chemical composition of high moisture content samples. The structural and morphological difference resulted from the cell wall fracture types might be responsible for the 2-6 times increase of enzymatic hydrolysis of micronized wood, comparing to that of the raw material. The structural differences in fractures of wood cell walls might be highly associated with the energy requirements for mechanical pretreatment process.

A comprehensive investigation regarding changes in structure of cell wall and cellulose chemistry in the last part provided deeper insights into evolution of wood cell wall deconstruction in Douglas-fir feedstock subjected to mechanical pretreatment and its influence on subsequent enzymatic hydrolysis. Collective with particle size reduction, mechanical deconstruction of cell wall resulted in cell fracture and delamination, ultrastructure disintegration and amorphization of
cell wall fragments, as revealed by electronic microscopy imaging. Dye adsorption from Simon’s staining confirmed that increasing mechanical milling time caused increased substrate accessible area and porosity of milled substrate, mainly due to the disrupting cell wall ultrastructure. In addition, the disintegration of cell wall structure also coincidently resulted in more even redistribution of cell wall compositions in micronized cell wall fragments than that of the intact raw cell wall, contributing to increasing cellulose accessibility. Mechanical pretreatment rendered significant changes in cellulose chemistry such as decreasing molecular weight and crystallinity. These alternations together overcame the biomass recalcitrance and facilitated 4-14 times enhancement of enzymatic hydrolysis of micronized wood cellulose.