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## Evaluation of physical structural features on influencing enzymatic hydrolysis efficiency of micronized wood

Jinxue Jiang,<sup>a</sup> Jinwu Wang,<sup>b</sup> Xiao Zhang<sup>c</sup> and Michael Wolcott<sup>\*a</sup>

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 micronized wood particles from mechanical milling pretreatment. We have also evaluated the energy efficiency of this processing method. Results indicate that the influence of processing variables on enzymatic hydrolysis of micronized wood relate mainly to the structural properties of particles. Reducing particle size down to ca. 30  $\mu\text{m}$  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  $\mu\text{m}$  further increases surface area and disrupts 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.

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### 1. Introduction

Due to increasing global energy demand and concerns on climatic issues from excessive utilization of fossil fuels, it is critical to identify alternative and renewable energy sources.<sup>1,2</sup> 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.<sup>3,4</sup> Close contact between the hydrolytic enzyme and lignocellulosic substrate is recognized as a primary step for initiating hydrolysis.<sup>5</sup> Efficient enzymatic hydrolysis of biomass is thus directly related to the structural features of the substrate.<sup>6</sup> 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.*<sup>7,8</sup>

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.<sup>9,10</sup> For example, when sawdust slurries with different initial particle sizes were subjected to enzymatic hydrolysis, the smallest particle size fraction (33–75  $\mu\text{m}$ ) was found to perform 50–55% more glucose yield than that from the largest particle size portion (590–850  $\mu\text{m}$ ).<sup>11</sup> 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.<sup>12</sup> 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 that was pretreated by organosolv. Ju *et al.*, also found that the fiber size had little effect on the substrate digestibility of delignified wood fiber.<sup>13</sup>

<sup>a</sup>Composite Materials and Engineering Center, Washington State University, Pullman, WA, 99164, USA. E-mail: wolcott@wsu.edu<sup>b</sup>Forest Products Laboratory, United States Department of Agriculture Forest Service, Orono, ME, 04469, USA<sup>c</sup>Voiland School of Chemical Engineering and Bioengineering, Washington State University, Richland, WA, 99354, USA

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.<sup>6,10</sup> 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.<sup>5,6</sup> In contrast, some studies were emphasized that cellulose crystallinity does not significantly affect overall cellulose hydrolysis for heterogeneous lignocellulosic substrates.<sup>6</sup> For example, Bali *et al.*, found that the enzymatic hydrolysis of alkaline pretreated *Populus* increased significantly, while the crystallinity change was negligible.<sup>14</sup>

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.<sup>15,16</sup> 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 physicochemical 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 the 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 strategy.

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.

## 2. Materials and methods

### 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.<sup>17</sup>

### 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. 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.

### 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 ( $\text{kJ kg}^{-1}$ );  $P_t$  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.

## 2.4. Characterization of wood powder

**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.<sup>18</sup> 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,<sup>19</sup> 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.

**2.4.2. Measurement of particle size.** 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.

**2.4.3. Aspect ratio analysis.** The aspect ratio analysis of milled particle was performed by imaging technique.<sup>18</sup> 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.<sup>20,21</sup> 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.

**2.4.4. 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.<sup>22</sup> 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.<sup>18</sup> The volumetric SSA based on ellipsoid model for the particle was estimated using following equation:

$$SSA^v = \frac{6 \left( \frac{2x^p + 1}{3} \right)^{\frac{1}{p}}}{xD}$$

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

## 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 K $\alpha$  ( $\lambda = 0.154$  nm) radiation source at 40 kV and 15 mA. The diffractogram was detected in the  $2\theta$  range of 10–40° at a scan rate of 1° per min. The crystallinity index (CrI), was estimated using an equation as described by Segal in the following form:<sup>23</sup>

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

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

## 2.6. Enzymatic hydrolysis

Enzymatic hydrolysis of cellulose and hemicellulose was performed using Cellic CTec2 cellulase with dosage 15 FPU g<sup>-1</sup> 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<sup>-1</sup> according to a standard method.<sup>24</sup> Experiments were conducted in duplicate according to the NREL Laboratory Analytical Procedure.<sup>25</sup> The enzymatic hydrolysis was conducted in a 20 mL glass scintillation vial and placed in an incubator with rotation speed of 180 rpm. The buffer was 0.1 M sodium citrate solution (pH 4.8), containing 100  $\mu$ L 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 0.2 g on oven-dry weight basis. The hydrolysis reaction was performed at 50 °C. The hydrolysis was carried out for 72 hours. The hydrolysate 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:

$$\begin{aligned} \text{Carbohydrate conversion (\%)} \\ = \frac{\text{released sugar amount in hydrolysate}}{\text{theoretic sugar amount in milled wood}} \times 100 \end{aligned}$$

## 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.<sup>26</sup> Briefly, a 300 mg sample and 3 mL of 72% H<sub>2</sub>SO<sub>4</sub> 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 (Dionex Corp., Bannockburn, IL, USA). Sugars were separated on a CarboPac PA 20 Guard (4  $\times$  50 mm) and analytical columns (4  $\times$  250 mm) at room temperature (25 °C). Then, 10  $\mu$ L 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<sup>-1</sup>. 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.

## 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. Results and discussion

## 3.1. Effect of mechanical milling conditions on enzymatic hydrolysis

One of the targets of this study was to evaluate the enzymatic hydrolysis efficiency of micronized wood, as affected by mechanical milling conditions. Fig. 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 hemicellulose) conversion was achieved with 12 minutes milling, suggesting that efficient saccharification of native softwood biomass is possible without any thermochemical pretreatments. These results concur with the previous observations on enzymatic hydrolysis of other milled woody or herbaceous biomass using different milling techniques.<sup>10,27</sup> 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.<sup>28</sup> 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.<sup>29</sup> 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 (Fig. 1A). This may be due to the distinctive structural alternations of micronized substrates produced in various milling conditions. Fig. 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.<sup>30</sup> This may be due to the similar structural properties of substrates after the milling process.

## 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.<sup>8,9,12</sup> Silva *et al.*, suggested that mechanical size reduction of biomass must overcome a limit (around 100  $\mu$ m) to significantly enhance enzymatic hydrolysis.<sup>31</sup> This size limit enables altering the cellular-scale structure of biomass, which significantly facilitates enzyme accessibility and the efficiency of micronized biomass.<sup>32</sup>

In present study, we effectively comminuted softwood feedstock at the cellular scale for particle size smaller than 100  $\mu$ m to reveal the relationship between enzymatic hydrolysis and particle size of micronized wood. Therefore, Fig. 2 shows the carbohydrate conversion in enzymatic hydrolysis with respect to median particle size of micronized wood produced from various milling conditions. Results indicate that carbohydrate conversion was improved as the particle size decreased, regardless of initial moisture content or feed size during the milling process. This reveals 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

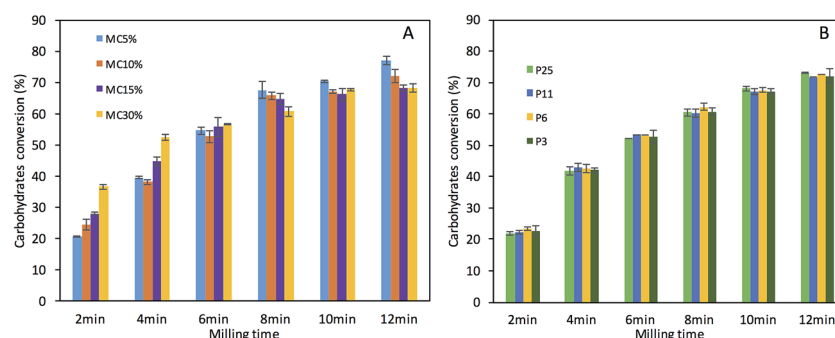


Fig. 1 Effect of initial moisture content (A) and feed size (B) during milling process on carbohydrate conversion of micronized wood.



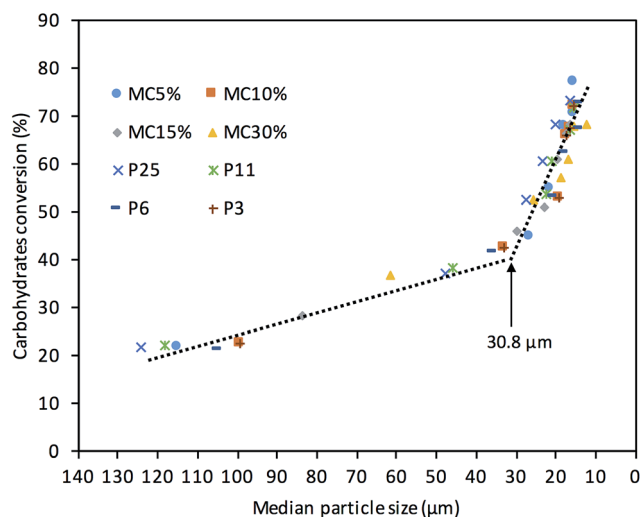


Fig. 2 Carbohydrate conversion of micronized wood as a function of median particle size for samples with various moisture content and feed size during mechanical pretreatment process.

in these size regions. The piecewise linear regression algorithm shows that the trend of carbohydrate conversion changes at a particle size of 30.8  $\mu\text{m}$  (Fig. 2), suggesting that two regimes affect carbohydrate conversion with respective of particle size. In the first regime, reducing particle size to around 30  $\mu\text{m}$  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 Fig. 3. Fiber bundles and single fibers still exist when the particle size is equal to or larger than 30  $\mu\text{m}$  (Fig. 3A 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 30  $\mu\text{m}$ , carbohydrate conversion can approach 77% depending on the specific milling condition, although the additional particle size reduction was slight in this range (Fig. 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  $\mu\text{m}$  (Fig. 3C–F). Mechanically disintegrating the fiber cell walls into micronized fragments smaller than 30  $\mu\text{m}$  may allow high accessible surface area for achieving significant carbohydrate conversion. In previous study, Zhu *et al.*,<sup>29</sup> 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  $\mu\text{m}$ .<sup>30</sup> However, holocellulose conversion increased greatly up to 70–90%, depending on the biomass type, when median particle sizes were smaller than 40  $\mu\text{m}$ . 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 Fig. 2). These results also suggest that particle size is not the only factor affecting enzymatic hydrolysis.

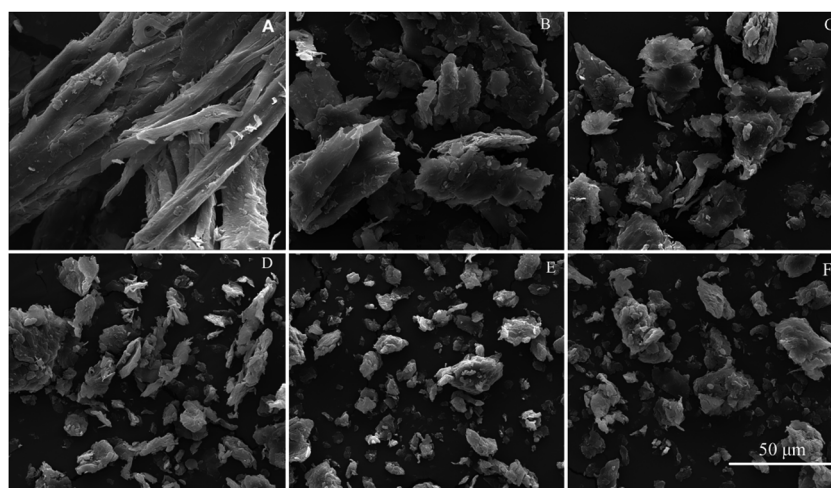


Fig. 3 SEM images show morphology variations of particles of different sizes. (A) Particles with median particle size bigger than 30  $\mu\text{m}$  and sample was milled with 2 minutes; (B) particles with median particle size around 30  $\mu\text{m}$ ; (C–F) particles with median particle size smaller than 30  $\mu\text{m}$ . Particles were milled from initial size P3 and moisture content of 5%.

### 3.3. Relation between crystallinity and enzymatic hydrolysis

Mechanically fragmenting lignocellulosic biomass into micronized particles also disrupts the cellulose crystalline structure.<sup>10</sup> 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.<sup>27,33</sup> Fig. 4 illustrates the relationship between the enzymatic carbohydrate conversion and the crystallinity index (CrI) of micronized wood. In this study, the correlation of carbohydrate conversion with CrI of micronized wood samples produced from various milling conditions was poor ( $R^2 = 0.65$ , data not shown here). The unsatisfied correlation result may due to the higher moisture content samples. This suggests that the influence of crystallinity on enzymatic hydrolysis of micronized wood is complicated. However, Fig. 4A 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 (Fig. 4A 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%.<sup>34</sup> 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%.<sup>30</sup> 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% (Fig. 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.<sup>29</sup> 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.<sup>35</sup> 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.

### 3.4. Empirical prediction of enzymatic hydrolysis

Mechanical milling pretreatment is known to disrupt cellular integrity of biomass with coincident structural alternations.<sup>10</sup> 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 particle morphological characteristics of micronized wood particles were firstly transformed into volumetric specific surface area (SSA) as described in the Material and methods section. The linear relation between carbohydrate conversion and volumetric SSA was evaluated (Fig. 5). The carbohydrate conversion increases linearly with increasing volumetric SSA of micronized wood ( $R^2 = 0.93$ ). Despite of the good correlation between carbohydrate conversion with SSA, CrI is also independently correlated with carbohydrate conversion as discussed above. In addition, there is no multicollinearity between SSA and CrI, indicating very low correlation of CrI with SSA. 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. The developed model in terms of coded variables is given in following equation:  $Y = 31.46 + 129.09X_1 - 0.242X_2$ , where  $X_1$ ,  $X_2$  and  $Y$  represent SSA, CrI and carbohydrate conversion of

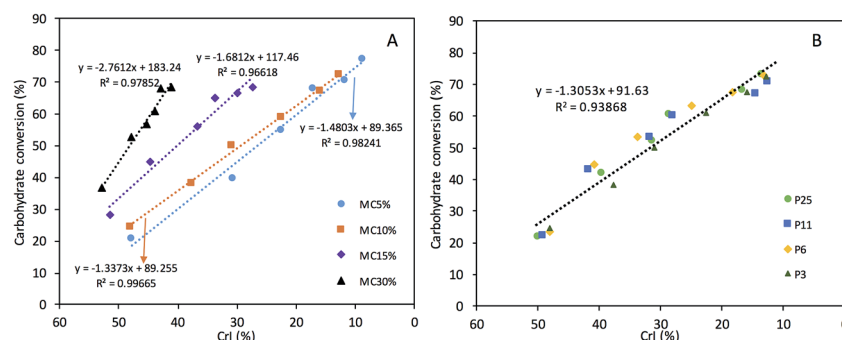


Fig. 4 Carbohydrate conversion of micronized wood as a function of crystallinity index (CrI) for samples produced from various (A) initial moisture contents (MC) and (B) feed sizes during mechanical milling pretreatment.

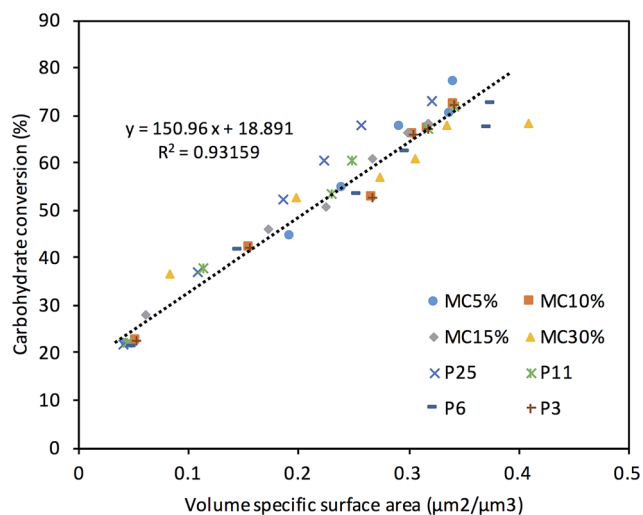


Fig. 5 Carbohydrate conversion of micronized wood as a function of volumetric specific surface area for samples produced from various moisture content and feed size during mechanical milling pretreatment.

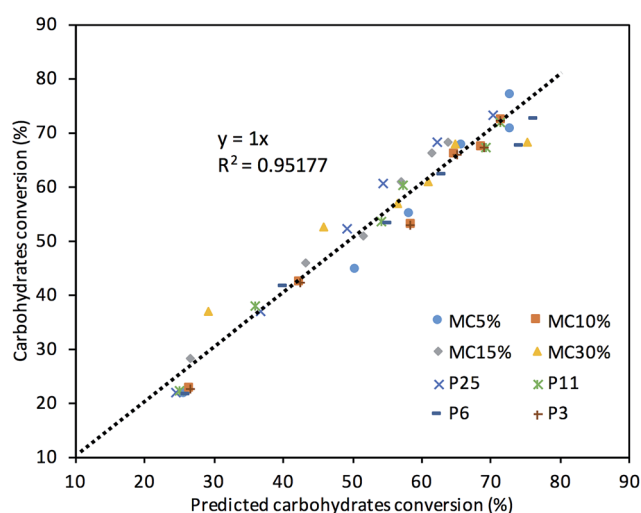


Fig. 6 Graph of experimentally measured carbohydrate conversion versus predicted values from multiple linear regression model.

milled wood, respectively. As demonstrated in Fig. 6, the model can explain the 95.2% variability of the response variable. Correlation parameters for slopes and intercept are summarized in Table 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 and aspect ratio) improves carbohydrate conversion. This is well

consistent 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.<sup>9,36</sup> Mechanically fragmenting the robust biological structure of biomass feedstock is an effective option for generating new surface area.<sup>10</sup> 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.<sup>37,38</sup>

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. Douglas-fir is an important softwood species in Northwest Pacific area in North America. Softwood is believed to be the most recalcitrant biomass feedstock, which requires more severe pretreatment than those other feedstock (*e.g.* hardwood, herbaceous biomass) for facilitating enzymatic hydrolysis. Mechanical milling pretreatment is a versatile approach in altering the physical structure of all biomass feedstock cell wall, resulting in reducing particle size and decreasing cellulose crystallinity.<sup>10,28,30,31</sup> The multiple linear model obtained in this study will also provide insights for mechanically overcoming biomass recalcitrance in other species for facilitating enzymatic hydrolysis of native lignocellulosic biomass.

### 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.<sup>39,40</sup> In this study, we used the energy efficiency (kg sugar per kW per h) to evaluate the performance of the mechanical milling process for producing simple sugars. The total amount of sugars recovered after enzymatic hydrolysis (kg sugar per kg wood) was divided by the total specific energy consumption for mechanical milling woody biomass ( $\text{kW h kg}^{-1}$  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

Table 1 Regression equation for carbohydrate conversion by enzymatic hydrolysis using calculated specific surface area (SSA) and crystallinity index (CrI) of micronized wood

| Carbohydrate conversion | Intercept | SSA ( $X_1$ ) | CrI ( $X_2$ ) | $R^2$  | Adjusted $R^2$ | $P$ -Value |
|-------------------------|-----------|---------------|---------------|--------|----------------|------------|
| Y                       | 31.46     | 129.09        | −0.242        | 0.9512 | 0.949          | <0.001     |

Table 2 Effects of mechanical milling on enzymatic hydrolysis and energy efficiency<sup>a</sup>

| Milling effect   |     | Glucan conversion (%) | Xyl/mannan conversion (%) | Total sugar yield (g kg <sup>-1</sup> wood) | Energy efficiency (kg sugar per kW per h) | Normalized energy efficiency <sup>b</sup> (kg sugar per kW per h) |
|------------------|-----|-----------------------|---------------------------|---------------------------------------------|-------------------------------------------|-------------------------------------------------------------------|
| Moisture content | 5%  | 89.81                 | 42.93                     | 526                                         | 1.06                                      | 0.77                                                              |
|                  | 10% | 84.1                  | 40.42                     | 493                                         | 0.57                                      | 0.49                                                              |
|                  | 15% | 77.14                 | 40.56                     | 459                                         | 0.32                                      | 0.29                                                              |
|                  | 30% | 73.5                  | 43.54                     | 446                                         | 0.23                                      | 0.23                                                              |
| Feed size        | P25 | 83.3                  | 46.06                     | 500                                         | 0.36                                      | 0.36                                                              |
|                  | P11 | 81.55                 | 41.95                     | 483                                         | 0.39                                      | 0.38                                                              |
|                  | P6  | 83.81                 | 42.8                      | 496                                         | 0.45                                      | 0.44                                                              |
|                  | P3  | 84.1                  | 40.42                     | 493                                         | 0.57                                      | 0.55                                                              |

<sup>a</sup> Samples were milled for 12 minutes using a ring and puck mill for each milling effect. <sup>b</sup> 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).

milling, as shown in Table 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.<sup>41</sup> 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 per kW per h 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 per kg wood, respectively. In contrast, for steam explosion pretreatment of spruce, Wingren *et al.* obtained a maximum energy efficiency 0.77 kg glucose per kW per h with a pretreatment temperature of 215 °C and no data on total sugar yield.<sup>42</sup> 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.<sup>43</sup> 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.

## 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.

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