1 Post-treatment mechanical refining as a method to improve overall sugar recovery of
2 steam pretreated hybrid poplar
3

4 Chang Dou ^a, Shannon Ewanick ^a, Renata Bura ^{a,*}, Rick Gustafson ^a
5

6 ^a Biofuels and Bioproducts Laboratory, School of Environmental and Forest Sciences,
7 University of Washington, Seattle, WA, 98115, USA.
8
9
10
11
12
13
14
15
16
17

18 * Corresponding author:

19 School of Environmental and Forest Sciences

20 University of Washington

21 Box 352100, Seattle, WA, 98195-2100, USA

22 Phone: 206-616-2179

23 Fax: 206-685-3091

24 E-mail address: renatab@u.washington.edu (R. Bura)

Abstract

This study investigates the effect of mechanical refining to improve the sugar yield from biomass processed under a wide range of steam pretreatment conditions. Hybrid poplar chips were steam pretreated using six different conditions with or without SO₂. The resulting water insoluble fractions were subjected to mechanical refining. After refining, poplar pretreated at 205 °C for 10 minutes without SO₂ obtained a 32% improvement in enzymatic hydrolysis and achieved similar overall monomeric sugar recovery (539 kg/tonne) to samples pretreated with SO₂. Refining did not improve hydrolyzability of samples pretreated at more severe conditions, nor did it improve the overall sugar recovery. By maximizing overall sugar recovery, refining could partially decouple the pretreatment from other unit operations, and enable the use of low temperature, non-sulfur pretreatment conditions. The study demonstrates the possibility of using post-treatment refining to accommodate potential pretreatment process upsets without sacrificing sugar yields.

Keywords

Steam explosion pretreatment; Mechanical refining; Particle size; Enzymatic hydrolysis; Overall sugar recovery

1. Introduction

Significant progress has been made towards the development of an economically feasible biorefinery. Pioneer commercial biorefineries are now operating all over the world. Most previous research **has** investigated scenarios where the feedstock to the biorefinery was of consistent quality and the bioconversion processes were optimized for that feedstock. In reality, biorefineries will have to deal with feedstock variability (Kenney et al., 2013) and process upsets that are inevitable in large scale biomass processing facilities – both of which will significantly impact the final product yields.

Biorefineries require a stable, year-round, and large scale supply of feedstock (Humbird et al., 2011; Kenney et al., 2013). To meet **these** requirements, biorefineries **must** use feedstock with variable compositions and properties. This can be a challenge, however, as different feedstocks have unique characteristics which affect their utilization. As the initial step of bioconversion process, pretreatment determines the sugar recovery and **subsequent** fuel/chemical yield of the biorefinery. Steam explosion pretreatment has been adopted by many biorefineries because of its robust nature and ability to **process** a variety of feedstocks (De Bari et al., 2007). By manipulating temperature, time and pH, steam pretreatment can efficiently fractionate lignocellulosic materials with short reaction time, limited or no use of chemicals, and low energy consumption (Chandra et al., 2007). High severity steam pretreatment generally fractionates lignocellulose more effectively and improves the enzymatic digestibility of pretreated biomass. However, more severe pretreatment conditions result in greater sugar and lignin degradation, forming more furans and phenolic compounds which inhibit fermentation. Less severe pretreatment conditions have limited sugar degradation, but may not open the lignocellulosic matrix sufficiently for enzymatic hydrolysis (Ramos, 2003).

1 Previous research has focused on determining unique steam pretreatment conditions
2 for individual types of biomass to efficiently fractionate the biomass and recover the
3 sugars. No single pretreatment condition is suitable for all feedstocks. The optimal
4 pretreatment is generally a compromise between enzymatic hydrolysis efficiency,
5 overall sugar recovery, and fermentation yield (Bura et al., 2009; Chandra et al., 2007).
6 Agricultural residuals typically have lower optimal pretreatment severity compared to
7 woody feedstocks (Bura et al., 2009; Sassner et al., 2005). Instead of continuously
8 varying processing conditions to accommodate variable feedstocks, a robust and stable
9 pretreatment would be preferable for future biorefineries.

10 Sulfuric acid or sulfur dioxide (SO₂) is routinely applied prior to steam pretreatment,
11 particularly to more recalcitrant woody biomass. The use of acid catalysts enables
12 shorter reaction time and lower pretreatment temperature (Sassner et al., 2005).
13 Nevertheless, acid catalysts do have three major drawbacks: Strongly acidic
14 environments can lead to equipment corrosion and increased maintenance costs (Alvira
15 et al., 2010); Acid catalyzed pretreated substrates require additional neutralization prior
16 to enzymatic hydrolysis and fermentation, meaning greater expense for neutralizing
17 reagents and subsequent wastewater treatment (Humbird et al., 2011); Finally, sulfur is
18 poisonous in many chemical catalytic reactions (Bartholomew et al., 1982; Dunleavy,
19 2006; Oudar, 1980). Since some biorefineries may also produce chemicals, such as
20 glycols, by catalysis, the presence of sulfur may prohibit production of higher value co-
21 products. For these three reasons, it would be desirable for future biorefineries to have
22 the flexibility to operate under sulfur free conditions.

23 Consistently maintaining optimal process conditions year round may prove difficult
24 for an industrial scale biorefinery. Pretreatment, like most chemical reactions, is
25 sensitive to process upsets. Three variables control steam pretreatment performance:

1 temperature, residence time, and pH (controlled by the addition of acid catalyst).
2 Process disturbances may move any of these critical variables off their optimal
3 setpoints. Disturbances in steam supply can impact temperature control. Variability in
4 biomass packing density can affect residence time depending on the pretreatment
5 reactor configuration. pH control can be particularly challenging due to variable
6 chemical uptake by different biomass feedstocks (De Bari et al., 2007). Instability in
7 any of these parameters may alter the final yield. The presence of a unit operation, after
8 pretreatment but before the expensive hydrolysis and fermentation processes, that could
9 mitigate the impact of these disturbances would significantly improve the operability of
10 the biorefinery and enable a more stable operation.

11 Mechanical refining is one potential unit operation that could serve to mitigate such
12 disturbances in the pretreatment process. As a mature technology in the papermaking
13 industry, refining is commonly applied to develop pulp fiber qualities and enhance
14 paper properties (Paulapuro, 2000). The main goal of refining for papermaking is to
15 improve the bonding among fibers by exposing internal and external microfibrils.
16 Refining also severs fibers and shortens the overall average fiber length (Paulapuro,
17 2000).

18 Previous research demonstrated that mechanical comminution by milling or
19 grinding of raw materials before pretreatment is energy-intensive and not economically
20 feasible (Alvira et al., 2010). Post-treatment refining, however, has been proposed to
21 improve the enzymatic hydrolysis efficiency, allowing reduced enzyme dosage and
22 higher solids consistency (Jones et al., 2013; Koo et al., 2011), and has been reported to
23 be cost-effective by offsetting energy consumption (Tao et al., 2012; Zhu and Pan,
24 2010). A number of studies in mechanical refining at laboratory, demonstration and
25 commercial scales have shown comparable outcomes in improving enzymatic

1 hydrolysis of feedstocks pretreated with different methods (Chen et al., 2013; Jones et
2 al., 2014; Zhu et al., 2010). In addition, considerable work has been done in the last 5
3 years on the mechanisms whereby refining enhances pretreatment effectiveness (Chen
4 et al., 2013; Hoeger et al., 2013; Jones et al., 2013). Previous work, however, only
5 focused on the impact of refining on digestibility of the water insoluble fraction, and did
6 not assess how refining could improve the overall pretreatment performance by
7 allowing milder pretreatment conditions and eliminating the need for sulfur based acid
8 catalysts. A more holistic approach that measures total sugar recovery is needed to
9 completely assess the benefits of mechanical refining.

10 This research investigates the effect of refining on the hydrolyzability of the water
11 insoluble fraction of steam pretreated biomass. In addition, the effects of refining on
12 overall sugar recovery are explored to better assess the use of mechanical refining to
13 decouple pretreatment from enzymatic hydrolysis and fermentation. The specific
14 objectives of this work are threefold. First, to determine whether refining changes the
15 chemical composition and physical properties of steam pretreated hybrid poplar.
16 Second, to study the influence of refining on cellulose to glucose conversion in
17 enzymatic hydrolysis for hybrid poplar steam pretreated at a range of conditions.
18 Finally, to investigate the effect of refining on the overall sugar recovery of hybrid
19 poplar steam pretreated at different conditions.

21 2. Materials and Methods

22 Hybrid poplar chips were steam pretreated using 6 different conditions with or without
23 SO₂ catalysis, shown in Fig. 1. After each pretreatment, the water soluble fraction
24 (WSF) was analyzed to determine chemical composition. Half of the water insoluble
25 fractions (WIF) were refined in a valley beater refiner for 30 min. All 12 samples,

including refined and unrefined solids, were enzymatically hydrolyzed at 5% consistency with 10 FPU/g cellulose enzyme loading. The fiber length and width for all the refined and unrefined solids were measured. The overall sugar recovery, based on monomeric glucose and xylose, was determined after steam pretreatment and enzymatic hydrolysis. Since arabinose, galactose, and mannose made up only a minor contribution to total sugars, only the sugar recovery of glucose (glucan) and xylose (xylan) were calculated.

2.1 Feedstock

Hybrid poplar chips used in this research were from fresh 18-year-old hybrid poplar obtained from Forest Concepts (Auburn, WA). Trees were debarked, chipped and screened into 1.0 cm × 2.0 cm at 5 mm thickness. Chips (50% moisture content) were stored at -20 °C until use. This poplar was composed of 0.3% arabinan, 1.1% galactan, 46.2% glucan, 15.1% xylan, 2.0% mannan, 22.6% lignin, 3.4% acetate, and 0.5% ash.

2.2 Pretreatment and processing conditions

In experiments utilizing SO₂ catalyst, samples of 800 g oven-dried weight hybrid poplar were pre-impregnated overnight with anhydrous SO₂ in plastic bags at atmospheric pressure. The amount of SO₂ added to the bag corresponded to 3% (w/w) loading of the samples, and was determined by weighing the bag before and after the addition of gas.

Steam pretreatment was performed in a 2.7 liter batch reactor (Aurora Technical, Savona, BC, Canada). Briefly, samples were subdivided into 400 g oven-dried weight batches and loaded into the preheated reactor. As shown in Fig. 1, samples were successively heated at temperatures ranging from 190 - 212 °C for 5 - 15 min periods. At the end of reaction time, the pneumatic valve was opened to explode and discharge the biomass into a collection container.

After steam explosion of two 400g OD batches, the pretreated biomass slurry was collected and then separated into WSF and WIF using vacuum filtration. To remove residual sugars, the WIF was washed with a volume of deionized water equivalent to 20 times the dry weight of the sample.

For each sample, pretreatment severity was calculated using the combined severity (CS) factor (Chum et al., 1990) based on time (t , min), reaction temperature (T_H , °C), reference temperature (T_R , 100 °C) and pH (measured from the WSF following pretreatment) (Eq.1 & Eq. 2). The combined pretreatment severity (CS) ranged from 0.6 to 2.1 (Table 1).

$$R_0 = t \times \exp[(T_H - T_R)/14.75] \quad (\text{Eq. 1})$$

$$CS = \log R_0 - pH \quad (\text{Eq. 2})$$

2.3 Mechanical refining

A 23 liter laboratory scale valley beater refiner was used to refine the washed WIF using adapted TAPPI Standard Method T-200 (TAPPI Test Methods, 2001a) with 30 kg of weight on bedplate lever arm. Suspended in water, WIF cycled in a loop and was beaten between the roll bars and bedplate at high mechanical shearing force. Each 360g sample was refined in a single batch at 1.57% consistency for 30 min at room temperature. After refining, solids were collected and separated by filtration and stored for later analysis.

2.4 Enzymatic hydrolysis

All 12 WIF samples, including 6 refined and 6 unrefined, were hydrolyzed at 5% (w/v) consistency in a total volume of 50 ml in 125 ml Erlenmeyer flasks. 50 mM citrate buffer was used to maintain the pH at 4.8 and the flasks were incubated at 50 °C and 175 rpm in an orbital shaker (New Brunswick). Cellulase (Celluclast 1.5 L, Sigma) at 10 FPU/g cellulose and β -glucosidase (Novozym 188, Sigma) at 20 CBU/g cellulose

were added to each flask. Additionally, tetracycline (40 µg/ml) and cycloheximide (30 µg/ml) were used to inhibit microbial contamination. 1 ml samples were taken periodically, boiled for 10 min to denature enzymes, and stored at -20 °C after filtration through a 0.22 µm syringe filter (Restek Corp., Bellefonte, PA).

2.5 High Pressure Liquid Chromatography (HPLC) analysis

Monomeric sugars (glucose and xylose) were measured on a Dionex (Sunnyvale, CA) HPLC (ICS-3000) system equipped with ED electrochemical detector and anion exchange column (Dionex, CarboPac PA1). Deionized water at 1 ml/min was used as an eluent, and post-column addition of 0.2 M NaOH at a flow rate of 0.5 ml/min ensured optimization of baseline stability and detector sensitivity. After each analysis, the column was reconditioned with 0.25 M NaOH. 20 µl of each sample were injected. Standards were prepared to encompass the same range of concentrations as the samples. Fucose (0.2 g/L) was added to all samples and standards as an internal standard.

Acetic acid, furfural and 5-hydroxymethylfurfural (5-HMF) were measured using refractive index detection on a Shimadzu Prominence LC. Separation of these compounds was achieved by an anion exchange column (REZEX-RHM Monosaccharide H⁺ (8%), Phenomenex, Inc., Torrance, CA) with an isocratic mobile phase that consisted of 5 mM H₂SO₄ at a flow rate of 0.6 ml/min. The column oven temperature was maintained at a constant temperature of 63 °C. Standards were prepared and used to quantify the unknown samples.

2.6 Compositional analysis

2.6.1 Water soluble fraction carbohydrates and degradation products

Monomeric and oligomeric soluble carbohydrates and degradation products were determined using NREL LAP TP-510-42623 (Sluiter et al., 2008). Briefly, 0.7 ml of 72% H₂SO₄ was added to 15 ml of the liquid samples, and the total volume was made

up to 20 ml with water. Samples were autoclaved at 121 °C for 60 min and analyzed by HPLC as described above. Monomeric sugar content was determined by analyzing the original samples without acid hydrolysis. Oligomeric sugar content was calculated by subtracting monomeric sugar content from total sugar content determined after acid hydrolysis.

Degradation products, such as acetic acid, furfural and 5-HMF were determined using HPLC by analyzing the original samples without acid hydrolysis. Total phenolic concentration in the WSF was assayed by the Folin-Ciocalteu method (Singleton et al., 1998) using a spectrophotometer (Shimadzu, Tokyo, Japan). Gallic acid was used as calibration standard.

2.6.2 Water insoluble fraction carbohydrates and lignin

The chemical compositions of refined and unrefined solids were determined as previously described (Bura et al., 2009) according to a modified method derived from TAPPI Standard Method T-222 om-98 (TAPPI Test Methods, 2001b). Briefly, 0.2 g of finely ground oven dried sample was treated with 3 ml 72% H₂SO₄ for 120 min at room temperature, then diluted into 120 ml total volume and autoclaved at 121 °C for 60 min. After filtration through tared sintered-glass crucibles, the acid insoluble lignin was determined by weighing the oven-dried crucibles. Acid soluble lignin in the filtrate was analyzed by ultraviolet at 205 nm. Carbohydrate composition of the filtrate was analyzed by HPLC.

2.7 Particle size analysis

Pretreated particle length and width distribution were measured using a Fiber Quality Analyzer (FQA) (OpTest Equipment, Inc., Hawkesbury, ON, Canada). As described in the FQA operation manual (OpTest Equipment, 2012), all samples were diluted with

deionized water to 0.75 mg/L until a fiber frequency around 20 - 40 events per second was achieved.

Length weighted mean length (L_w) was measured and reported in this work to represent fiber length (Eq. 3). L_w is the preferred parameter to compare fiber length in the pulp and paper industry. L_w places more weight on the fibers and is not so sensitive to the fines content of the pulp (OpTest Equipment, 2012). The particle width reported by FQA was the arithmetic mean width based on the pulp and paper industry standards (OpTest Equipment, 2012).

$$L_w = \sum_i n_i l_i^2 / \sum_i n_i \quad (\text{Eq. 3})$$

(n_i is the number of fibers in the specified length class l_i)

2.8 Sugar recovery calculation

A complete mass balance was calculated by measuring the composition and total mass of each WSF and WIF leaving pretreatment and enzymatic hydrolysis and converting this data to amount of sugars (Wyman et al., 2009). Recoveries were then calculated based on glucose and xylose available in the raw material fed to the systems. Ideally, when 1000 mass units of baseline hybrid poplar is entirely hydrolyzed, a maximum of about 514 mass units of glucose and 168 mass units of xylose could be produced, for a total maximum sugar potential of 682 units per 1000 units of dry poplar. Herein, monomeric glucose and xylose recoveries were expressed in terms of kg per tonne of raw hybrid poplar, with their summation representing overall sugar recovery.

2.9 Statistical analysis

The results were subjected to one way analysis of variance (ANOVA) followed by a Tukey test. Chemical composition (glucan and xylan), physical characteristics (length and width) and sugar recovery following enzymatic hydrolysis were analyzed based on 5% alpha level (95% confidence interval). Statistical differences in chemical

composition, physical characteristics and sugar yield were determined with p-values ($p < 0.05$). Data were analyzed using R (version 3.0.1) software.

3. Results and Discussion

In this study, hybrid poplar chips were steam pretreated at temperatures from 190 - 212 °C for 5 - 15 min with or without SO₂. The combined severity factor (CS) for each pretreatment was calculated by incorporating the pH of the WSF after pretreatment; the CS of SO₂ catalyzed samples (S4, S5 and S6) were higher than those of the uncatalyzed samples (S1, S2 and S3) (Table 1).

The overall sugar recovery (kg/tonne of raw biomass) following steam pretreatment and enzymatic hydrolysis was used to measure the influence of refining on the hydrolyzable and fermentable sugar yield of hybrid poplar pretreated at a range of conditions. Carbohydrates and inhibitors in the WSF after pretreatment and carbohydrates in the WIF were also measured after pretreatment and refining.

3.1 Chemical composition of water soluble fraction (WSF) after pretreatment

The effects of pretreatment conditions on the WSF composition are shown in Tables 2 and 3. For hybrid poplar pretreated without SO₂ (S1, S2, S3), the glucose yield increased from 16 to 21 kg/tonne and xylose yield decreased from 112 to 25 kg/tonne as the CS increased from 0.6 to 1.3. Similar trends happened in hybrid poplar pretreated with SO₂ (S4, S5, S6): glucose yields increased from 65 to 116 kg/tonne and xylose yields decreased from 140 to 109 kg/tonne as CS increased from 1.9 to 2.1. The decrease in xylose yield was caused by conversion of xylose into furfural at elevated temperature and time. For pretreatment without SO₂ in particular, when temperature rose from 205 to 212 °C, xylose yield decreased by 40% (S1 to S2). When pretreated for 5 minutes longer at 212 °C, xylose yield decreased by 60% (S2 to S3). In the absence of

SO₂, no more than 128 kg/tonne sugars were recovered and less than 40% were in monomeric form. The addition of SO₂ in samples S4-S6 led to more than 205 kg/tonne sugar recovery and up to 91% in monomeric form. S1 yielded the highest amount of sugars in WSF (128 kg/tonne) among all the uncatalyzed pretreatments, while sugar yields for all the catalyzed pretreatments were similar, ranging from 205 to 225 kg/tonne.

As pretreatment severity increased, formation of the carbohydrate degradation products furfural and 5-HMF increased. Furfural increased from 6.7 to 20.2 kg/tonne in uncatalyzed samples and from 8.7 to 17.2 kg/tonne in SO₂-catalyzed samples; 5-HMF increased from 1.5 to 5.5 kg/tonne in uncatalyzed samples, and from 2.3 to 4.0 kg/tonne in SO₂-catalyzed samples (Table 3). Acetic acid and phenolics, derivatives of hemicellulose and lignin, increased from 15.9 to 34.5 kg/tonne and from 7.8 to 10.6 kg/tonne respectively when uncatalyzed pretreatment CS increased from 0.6 to 1.3 (S1 to S3). At higher CS with the addition of SO₂, both acetic acid and phenolics plateaued at 36-38 kg/tonne and 14-15 kg/tonne, respectively. Among all WSF, S1 was pretreated at the lowest severity and accordingly formed the least amount of inhibitors.

3.2 Chemical composition of water insoluble fraction (WIF) after pretreatment and refining

Table 4 shows the recoveries of glucan and xylan in the water insoluble fractions. It can be seen that the glucan and xylan content of steam pretreated WIF ranged from 61.3% to 65.6% and from 0.2% to 2.6%, respectively. Consistent with previous work on steam pretreatment of hardwood, less WIF dry mass was recovered as pretreatment severity increased (De Bari et al., 2007; Schütt et al., 2013) as a result of more carbohydrate and lignin dissolution and/or degradation into the WSF. After steam pretreatment, no statistically significant differences in glucan and xylan content were found among S1,

S2 and S3 or among S4, S5 and S6. The xylan content in the uncatalyzed pretreated WIF (S4, S5 and S6) was significantly higher than in the catalyzed pretreated WIF (S1, S2 and S3). SO₂ was mainly responsible for the difference. When applied as an acidic catalyst, SO₂ hydrolyzes more hemicellulose during pretreatment (Ramos, 2003; Sassner et al., 2005). In the case of uncatalyzed pretreatment, acetic acid cleaved from acetyl groups in hemicellulose functions as an acid catalyst to hydrolyze hemicellulose – the so called autohydrolysis mechanism (Ramos, 2003). Autohydrolysis has a reduced acidification because of limited acetic acid formation and relative weakness of the acid, resulting in a pretreatment that is mild and slow compared with acid catalyzed pretreatment (Schütt et al., 2013). As shown in Table 3, the lower pH of SO₂ catalyzed pretreatment explains the lower xylan content of SO₂-pretreated WIF compared to those pretreated without SO₂.

Mechanical refining, as described in section 2.3, was applied to all the WIF samples in a valley beater. The sugar composition of the refined WIF was compared to that of unrefined samples. No statistically significant difference was observed in glucan or xylan content before and after refining (Table 4), which confirmed that refining did not affect the chemical composition of the substrates.

3.3 Water insoluble fraction (WIF) particle size analysis

The length and width of the pretreated solids particles before and after refining were measured by fiber quality analysis (Fig. 2). For unrefined samples, the particle length and width decreased from 0.72 mm to 0.19 mm and from 25 µm to 21 µm, respectively, as the pretreatment severity increased. The uncatalyzed pretreated particles were longer and wider than the catalyzed pretreated samples. SO₂ addition lowered the pretreatment pH, and facilitated the fractionation of the lignocellulosic complex, leading to a larger particle size reduction.

Consistent with previous research that refining reduces particle size of pretreated biomass (Chen et al., 2013; Mooney et al., 1999), particle lengths were shortened in S1-S5 after valley beater refining (Fig. 2a). Sample S1 was notable with a 57% length reduction (from 0.72 mm to 0.31 mm). Particle shortening was also statistically significant for S2 to S5, displaying length reductions from 46% to 26%. All the sample widths slightly decreased after refining (Fig. 2b). However, only the width reduction of S1 was statistically significant. Refining reduced both particle length and width such that there was an overall size decrease of all samples except S6. S1 had the most significant size reduction because it was not effectively pretreated and had the greatest potential in size reduction.

3.4 Enzymatic hydrolysis of water insoluble fraction (WIF)

Following pretreatment and refining, the enzymatic digestibility of all 12 samples was evaluated. The extent of cellulose to glucose conversion highlighted the variation in WIF hydrolyzability between catalyzed and uncatalyzed and refined and unrefined samples (Fig. 3). The cellulose conversion of unrefined samples ranged from 48% to 80% after 96 hours of enzymatic hydrolysis. In uncatalyzed samples (S1-S3), glucose yield improved from 48% to 80% as CS increased, while in catalyzed samples (S4-S6), all glucose yields were close to 75% conversion. Consistent with earlier work on steam pretreated hardwood (Sassner et al., 2005), the enzymatic release of glucose was found to improve with more severe pretreatment, but only to a certain extent. This result can be partially attributed to both particle size reduction (Fig. 2) and xylan dissolution during pretreatment (Table 4); each promotes enzymatic hydrolyzability by increasing cellulose accessibility.

Refining enhanced the cellulose to glucose conversion for most of the samples, resulting in cellulose conversions of 74 - 82% after 96 hours. Among all samples, S1

had the largest improvement in enzymatic hydrolysis, 32%, which corresponded to the most significant particle size reduction (Fig. 2). Although other samples achieved significant particle size reduction after refining, they showed far lower sugar yield improvements (no more than 5%) and none of the differences were statistically significant. Comparable phenomena reported by Dibble (2011) and Jones (2013) suggests that mechanical process such as refining reduces the particle size but does not ensure hydrolyzability improvement. In particular, refining showed little impact on enzymatic hydrolysis of high severity pretreated biomass. As mentioned above, mechanical refining **also** fibrillates the cell wall, which may potentially improve enzyme accessibility. Mechanical size reduction alters other properties including porosity, **specific surface area**, crystallinity and degree of polymerization which may also influence the hydrolyzability (Barakat et al., 2013; Mooney et al., 1999), but **were** not measured **in this study**.

3.5 Total sugar recovery after steam pretreatment, refining and enzymatic hydrolysis

Table 5 summarizes overall sugar recovery – combined monomeric glucose and xylose – in the WSF and enzymatic hydrolyzed WIF before and after mechanical refining. In unrefined samples, the overall glucose recovery increased from 295 to 469 kg/tonne with elevating severity as a result of the increased WSF monomeric glucose yields and WIF cellulose conversion. The total xylose recovery followed the WSF yield trends, where xylan was mostly solubilized during pretreatment. Monomeric xylose recovery was at least doubled in samples where SO₂ was applied in pretreatment. The overall monomeric glucose and xylose recovery was enhanced from 330 to 544 kg/tonne as a result of increasing pretreatment CS from 0.6 to 2.1. Consistent with previous studies (Schütt et al., 2013, 2011), addition of SO₂ improved the overall sugar recovery.

Glucose yield in the enzymatically hydrolyzed WIF has a much larger contribution to overall glucose recovery than glucose in the WSF since the majority of the glucan remained in the WIF. As a result, refining improved the total glucose recovery of S1 to S4 by 11 to 209 kg/tonne. As expected, the largest glucose recovery improvement among all the samples, 41%, was observed for S1. Refining had little impact on total xylose recovery due to the already limited xylan content in the WIF.

Fig. 4 illustrates the relationship between the pretreatment CS and overall sugar recoveries with and without refining. The goodness of fit of the unrefined regression line, $R^2 = 0.96$, indicates that the overall sugar recovery was highly correlated with pretreatment CS in the range from 0.6 to 2.1. As discussed above, the improvement of overall sugar recovery is a result of higher WIF hydrolyzability (Fig. 3) and increased monomeric sugar solubilization (Table 2) at higher CS with SO₂ impregnation. The highest overall sugar recovery of the unrefined samples was 544 kg/tonne, obtained following pretreatment at 200 °C for 5 min with 3% SO₂ impregnation, with a CS value of 2.1. Although higher sugar recovery was achieved at higher CS, more inhibitors were formed (Table 3), potentially leading to lower fermentation yields. Considering fermentation, S6 may not be the optimal pretreatment condition.

After refining, no correlation ($R^2 = 0.13$) between overall sugar recovery and CS was observed. S1 had the maximum (64%) overall sugar recovery improvement due to refining, whereas there were small or no improvements for the rest of samples. S2, S3, S4 and S6 only increased by 6%, 3%, 2% and 1%, respectively, whereas S5 didn't change. Although refining slightly improved the WIF hydrolyzability for S2 and S3 (Fig. 3), the low monomeric sugar yield in the WSF limited their overall sugar recovery (Table 2). Similarly, due to the lack of improvement in WIF hydrolyzability, the overall sugar recovery plateaued for refined S4, S5 and S6 compared to the unrefined samples.

Refining increased the overall sugar recovery for hybrid poplar pretreated only within a certain range of conditions: the more severe pretreatment, the less effective refining in increasing the overall sugar recovery (Fig. 4). Refining did have a greater effect on mildly pretreated biomass as evidenced by the fact that the overall sugar recovery of non-SO₂ pretreated S1 was increased to as high as that of SO₂-catalyzed S3, S4 and S5.

This work has shown that refining allows the use of low severity, catalyst-free pretreatment conditions to achieve high sugar recovery in hydrolyzable and fermentable form. However, refining cannot improve the overall sugar recovery when samples are pretreated at more severe conditions such as temperature higher than 205 °C and reaction time longer than 5 min. At higher severities, the sugar loss is too great and digestibility of the WIF is not improved by refining.

With lower severity pretreatment, however, these results show that there is a possibility of having unacceptably low process yields if there is no post pretreatment refining. The economic benefits of refining are to mitigate these upsets to maintain reasonable conversion yields. Nevertheless, such benefits will be site specific and are difficult to quantify without actual operating data. The potential to eliminate disturbances that result in yield losses up to 39% (S1) is compelling and warrants preliminary economic analysis. To assess the economic benefits it is possible to calculate the average increase in conversion yield required to justify the purchase and operation of a refiner. Using economic data for refiners provided by Jones et al. (2014) for a 450,000 tonne/yr biomass feed rate and assuming a payback period of 5 years and an annual refiner maintenance cost of 10% of the installed equipment cost, the annual savings needed to justify purchasing and operating the refiner can be calculated. The required annual savings will be the summation of yearly payback, electricity and maintenance cost. Jones (2014) presents a refiner cost of \$5 million with energy

consumption at 67 kWh/tonne and electricity cost of \$0.065/kWh. Based on the amount of recovered solids from S1, 88.4%, (Table 4), the annual electricity operating cost would be \$1.73 million. With these values, the required annual savings were calculated according to equation below:

$$\text{Required annual savings (million\$)} = \frac{\$5}{5 \text{ yrs}} + \$1.73 + 10\% \times \$5 = \$3.23$$

If poplar costs \$105/tonne (Huang et al., 2009) then the amount of purchased biomass would have to be reduced from 450,000 tonnes/year to 419,000 tonnes/year – a reduction of 7% – to justify the refiner. In other words, refining would have to result in an average yield increase of 7% to produce a reasonable payback period. It was shown in the present research yield losses up to 39% were possible with sub-optimal biomass and pretreatment conditions. It is not difficult to envision how these could accumulate in significant yield losses over the course of a year. In addition, the use of refiner may result in enzyme savings (Jones et al., 2014) and in other operational savings associated with having a more consistent biomass entering hydrolysis and fermentation.

Optimal pretreatment conditions are generally considered a compromise of pretreatment severity so as to balance the enzymatic hydrolysis efficiency, overall sugar recovery, and fermentation yield. Higher sugar recovery usually requires high severity pretreatment condition and consequently results in more inhibitors. By applying mechanical refining, it is possible to partially decouple the pretreatment condition from enzymatic hydrolysis and fermentation. Post-treatment refining allows the use of low severity, SO₂-free conditions to maximize the sugar recovery and lower inhibitor formation while still producing a readily hydrolyzable WIF. The study suggests that mechanical refining allows a biorefinery to achieve good sugar yields with a wide range of pretreatment conditions even in the absence of acid catalyst.

Current estimated costs of second generation biofuel production are two to three times more expensive than that of petroleum fuels on an energy equivalent basis (Balan, 2014). Biofuel and bio-based chemical production processes must become more efficient to be competitive with the equivalent processes using fossil fuels. Processes to produce fuels and chemicals from petroleum have a wealth of online control systems that permits them to operate at or near capacity with optimal process yields. This hyper-efficiency is a necessary condition for profitability in producing high volume, narrow-profit margin products such as fuels and commodity chemicals.

A majority of the current research focuses on reducing operating costs of biofuels and biochemical production by finding cheaper feedstocks, developing efficient and robust microorganisms, and co-product utilization (Balan, 2014). There has been relatively little emphasis on development of operation units that could mitigate the impact of process disturbances and enable a more stable operation. The need for process efficiency – and hence the need for unit operations, such as refining, that can mitigate disturbances – is especially acute in biomass fed refineries due to the process and feedstock complexity. Process improvements (feedstock pretreatment, enzymes, microorganisms, etc.) will likely reduce costs, but even with these improvements the use of refining to mitigate disturbances in the pretreatment process will have a great effect on overall process economics.

4. Conclusions

Post-treatment refining did not change the chemical composition of pretreated biomass, but reduced the particle size. For hybrid poplar pretreated at 205 °C, 10 min without SO₂, refining improved the cellulose to glucose conversion by 32%. The overall monomeric sugar recovery after pretreatment and enzymatic hydrolysis of S1 was

1 improved by 64% after refining, achieving similar sugar yields to samples pretreated
2 with SO₂. Refining **demonstrated** a method to partially decouple the pretreatment with
3 enzymatic hydrolysis and fermentation. These findings **illustrate** the potential of refining
4 to minimize the impact of pretreatment process fluctuations and to **increase the stability**
5 **of** future biorefineries.

7 **Acknowledgements**

8 This research was supported by the Denman Professorship in Bioresource Science and
9 Engineering at the University of Washington and by the Agriculture and Food Research
10 Initiative Competitive Grant no. 2011-68005-30407 from the USDA National Institute
11 of Food and Agriculture. We would like to thank Dr. Rodrigo Morales Vera for his help
12 with the steam explosion experiments and techno economic analysis.

Reference

- Alvira, P., Tomás-Pejó, E., Ballesteros, M., Negro, M.J., 2010. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. *Bioresour. Technol.* 101, 4851–4861.
- Balan, V., 2014. Current challenges in commercially producing biofuels from lignocellulosic biomass. *ISRN Biotechnol.* 2014, 463074–46107.
- Barakat, A., de Vries, H., Rouau, X., 2013. Dry fractionation process as an important step in current and future lignocellulose biorefineries: a review. *Bioresour. Technol.* 134, 362–373.
- Bartholomew, C.H., Agrawal, P.K., Katzer, J.R., 1982. Sulfur poisoning of metals. *Adv. Catal.* 31, 135–242.
- Bura, R., Chandra, R., Saddler, J., 2009. Influence of xylan on the enzymatic hydrolysis of steam-pretreated corn stover and hybrid poplar. *Biotechnol. Prog.* 25, 315–322.
- Chandra, R.P., Bura, R., Mabee, W.E., Berlin, A., Pan, X., Saddler, J.N., 2007. Substrate pretreatment: the key to effective enzymatic hydrolysis of lignocellulosics?, in: *Biofuels*. pp. 67–93.
- Chen, X., Kuhn, E., Wang, W., Park, S., Flanagan, K., Trass, O., Tenlep, L., Tao, L., Tucker, M., 2013. Comparison of different mechanical refining technologies on the enzymatic digestibility of low severity acid pretreated corn stover. *Bioresour. Technol.* 147, 401–408.
- Chum, H.L., Johnson, D.K., Black, S.K., Overend, R.P., 1990. Pretreatment-Catalyst effects and the combined severity parameter. *Appl. Biochem. Biotechnol.* 24-25, 1–14.
- De Bari, I., Nanna, F., Braccio, G., 2007. SO₂ -Catalyzed Steam Fractionation of Aspen Chips for Bioethanol Production: Optimization of the Catalyst Impregnation. *Ind. Eng. Chem. Res.* 46, 7711–7720.
- Dibble, C.J., Shatova, T.A., Jorgenson, J.L., Stickel, J.J., 2011. Particle morphology characterization and manipulation in biomass slurries and the effect on rheological properties and enzymatic conversion. *Biotechnol. Prog.* 27, 1751–1759.
- Dunleavy, J.K., 2006. Sulfur as a catalyst poison. *Platin. Met. Rev.* 50, 110–110.

- 1 Hoeger, I.C., Nair, S.S., Ragauskas, A.J., Deng, Y., Rojas, O.J., Zhu, J.Y., 2013.
2 Mechanical deconstruction of lignocellulose cell walls and their enzymatic
3 saccharification. *Cellulose* 20, 807–818.
- 4 Huang, H.-J., Ramaswamy, S., Al-Dajani, W., Tschirner, U., Cairncross, R.A., 2009.
5 Effect of biomass species and plant size on cellulosic ethanol: A comparative
6 process and economic analysis. *Biomass and Bioenergy* 33, 234–246.
- 7 Humbird, D., Davis, R., Tao, L., Kinchin, C., Hsu, D., Aden, A., Schoen, P., Lukas, J.,
8 Olthof, B., Worley, M., Sexton, D., Harris, D.D., Pretreatment, D., 2011. *Process*
9 *Design and Economics for Biochemical Conversion of Lignocellulosic Biomass to*
10 *Ethanol*. Golden, CO.
- 11 Jones, B.W., Venditti, R., Park, S., Jameel, H., 2014. Comparison of lab, pilot, and
12 industrial scale low consistency mechanical refining for improvements in
13 enzymatic digestibility of pretreated hardwood. *Bioresour. Technol.* 167, 514–520.
- 14 Jones, B.W., Venditti, R., Park, S., Jameel, H., Koo, B., 2013. Enhancement in
15 enzymatic hydrolysis by mechanical refining for pretreated hardwood
16 lignocellulosics. *Bioresour. Technol.* 147, 353–360.
- 17 Kenney, K.L., Smith, W.A., Gresham, G.L., Westover, T.L., 2013. Understanding
18 biomass feedstock variability. *Biofuels* 4, 111–127.
- 19 Koo, B.-W., Treasure, T.H., Jameel, H., Phillips, R.B., Chang, H.-M., Park, S., 2011.
20 Reduction of enzyme dosage by oxygen delignification and mechanical refining for
21 enzymatic hydrolysis of green liquor-pretreated hardwood. *Appl. Biochem.*
22 *Biotechnol.* 165, 832–844.
- 23 Mooney, C.A., Mansfield, S.D., Beatson, R.P., Saddler, J.N., 1999. The effect of fiber
24 characteristics on hydrolysis and cellulase accessibility to softwood substrates.
25 *Enzyme Microb. Technol.* 25, 644–650.
- 26 OpTest Equipment, 2012. *Fiber quality analyzer FQA-360 LDA12 operation manual*.
27 Nawkesbury, ON Canada.
- 28 Oudar, J., 1980. Sulfur Adsorption and Poisoning of Metallic Catalysts. *Catal. Rev.* 22,
29 171–195.
- 30 Paulapuro, H., 2000. *Book 8: Papermaking part 1: stock preparation and wet end*.

Helsinki: Fapet Oy.

Ramos, L.P., 2003. The chemistry involved in the steam treatment of lignocellulosic materials. *Quim. Nova* 26, 863–871.

Sassner, P., Galbe, M., Zacchi, G., 2005. Steam pretreatment of *Salix* with and without SO₂ impregnation for production of bioethanol. *Appl. Biochem. Biotechnol.* 124, 1101–1117.

Schütt, F., Haas, N.P., Dehne, L., Koch, G., Janzon, R., Saake, B., 2013. Steam pretreatment for enzymatic hydrolysis of poplar wood: comparison of optimal conditions with and without SO₂ impregnation. *Holzforschung* 67, 9–17.

Schütt, F., Puls, J., Saake, B., 2011. Optimization of steam pretreatment conditions for enzymatic hydrolysis of poplar wood. *Holzforschung* 65, 453–459.

Singleton, V.L., Orthofer, R., Lamuela-Raventós, R.M., 1998. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. *Methods Enzymol.* 299, 152–178.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., 2008. Determination of Sugars, Byproducts, and Degradation Products in Liquid Fraction Process Samples. Golden, CO.

Tao, L., Chen, X., Aden, A., Kuhn, E., Himmel, M.E., Tucker, M., Franden, M.A.A., Zhang, M., Johnson, D.K., Dowe, N., Elander, R.T., 2012. Improved ethanol yield and reduced minimum ethanol selling price (MESP) by modifying low severity dilute acid pretreatment with deacetylation and mechanical refining: 2) Techno-economic analysis. *Biotechnol. Biofuels* 5, 1–11.

TAPPI Test Methods, 2001a. Laboratory beating of pulp (Valley beater method) T 200 sp-01. Atlanta, GA.

TAPPI Test Methods, 2001b. Acid-insoluble lignin in wood and pulp, Test Method T 222 om-11. Atlanta, GA.

Wyman, C.E., Dale, B.E., Elander, R.T., Holtzapple, M., Ladisch, M.R., Lee, Y.Y., Mitchinson, C., Saddler, J.N., 2009. Comparative sugar recovery and fermentation data following pretreatment of poplar wood by leading technologies. *Biotechnol. Prog.* 25, 333–9.

- 1 Zhu, J.Y., Pan, X.J., 2010. Woody biomass pretreatment for cellulosic ethanol
2 production: Technology and energy consumption evaluation. *Bioresour. Technol.*
3 101, 4992–5002.
- 4 Zhu, W., Zhu, J.Y., Gleisner, R., Pan, X.J., 2010. On energy consumption for size-
5 reduction and yields from subsequent enzymatic saccharification of pretreated
6 lodgepole pine. *Bioresour. Technol.* 101, 2782–2792.
- 7

Figure Captions

Figure 1 Process flow diagram for bioconversion of hybrid poplar into carbohydrates.

Figure 2 Particle length (a) and width (b) of refined and unrefined steam pretreated

hybrid poplar with and without SO₂. Error bars indicate standard deviation.

Figure 3 96 hours cellulose to glucose conversion of refined and unrefined steam

pretreated hybrid poplar with cellulase at 10 FPU/g cellulose and 5% (w/v) solids

consistency. Error bars indicate standard deviation.

Figure 4 Overall monomeric glucose and xylose recovery of refined and unrefined

steam pretreated hybrid poplar (expressed as kg/tonne raw biomass). Regression lines

are shown for refined and unrefined samples respectively. Error bars indicate standard

deviation.

1 **Table 1** Steam pretreatment conditions for hybrid poplar.

Sample	Temperature (°C)	Time (minutes)	SO ₂ (% w/w)	Combined severity
S1	205	10	0	0.6
S2	212	10	0	1.0
S3	212	15	0	1.3
S4	190	5	3	1.9
S5	195	5	3	2.0
S6	200	5	3	2.1

2

1 **Table 2** Sugar composition yield in water soluble fraction of pretreated hybrid poplar
2 (expressed as kg/tonne raw biomass). All data are represented as the mean of duplicates
3 with standard deviation.

Sample	Glucose (kg/tonne)		Xylose (kg/tonne)		Glucose and Xylose (kg/tonne)	
	Total	% mon ^a	Total	% mon ^a	Total	% mon ^a
S1	16 ± 1	20	112 ± 3	19	128 ± 3	19
S2	19 ± 1	29	65 ± 2	38	84 ± 2	36
S3	21 ± 1	38	25 ± 1	41	45 ± 1	40
S4	65 ± 2	87	140 ± 6	93	205 ± 8	91
S5	93 ± 5	82	129 ± 7	87	223 ± 13	85
S6	116 ± 4	82	109 ± 2	91	225 ± 6	87

4 ^a monomeric sugar

- 1 **Table 3** Furfural, 5-HMF, acetic acid, and phenolics yield in water soluble fraction of
 2 pretreated hybrid poplar (expressed as kg/tonne of raw biomass) ^a.

Sample	pH	CS	Furfural	5-HMF	Acetic acid	Phenolics
S1	3.5	0.6	6.7	1.5	15.9	7.8
S2	3.3	1.0	12.2	2.9	26.2	9.5
S3	3.2	1.3	20.2	5.5	34.5	10.6
S4	1.4	1.9	8.7	2.3	36.1	14.4
S5	1.5	2.0	12.8	2.9	36.8	14.1
S6	1.5	2.1	17.2	4.0	37.7	14.6

- 3 ^a The reported results are the average of triplicate studies with a deviation of $\leq 2\%$

Table 4 Dry mass recoveries as kg/tonne of raw biomass and chemical composition of refined and unrefined water insoluble fraction (as percentages of the solid weight). All data are represented as the mean of triplicates with standard deviation.

Sample	Dry WIF (kg/tonne)	Refining Conditions	Glucan (%)	Xylan (%)
S1	884	Unrefined	62.2 ± 0.1	2.4 ± 0.1
		Refined	63.5 ± 0.2	2.6 ± 0.1
S2	802	Unrefined	62.2 ± 0.6	2.0 ± 0.1
		Refined	62.9 ± 0.8	2.1 ± 0.1
S3	740	Unrefined	61.4 ± 1.0	1.7 ± 0.1
		Refined	61.3 ± 0.3	1.7 ± 0.1
S4	722	Unrefined	65.3 ± 0.1	0.5 ± 0.1
		Refined	65.2 ± 0.2	0.5 ± 0.1
S5	739	Unrefined	65.6 ± 0.2	0.3 ± 0.1
		Refined	65.3 ± 0.2	0.3 ± 0.1
S6	738	Unrefined	64.3 ± 0.4	0.2 ± 0.1
		Refined	64.6 ± 0.4	0.2 ± 0.1

Table 5 Overall sugar recovery (glucose and xylose) after steam pretreatment and 96 hours enzymatic hydrolysis in water soluble fraction and water insoluble fraction with and without refining (expressed as kg of monomeric sugars per tonne of raw biomass). All data are represented as the mean of triplicates with standard deviation.

Sample	Refining Conditions	Glucose	Xylose	Overall sugar recovery
S1	Unrefined	295 ± 6	34 ± 1	330 ± 6
	Refined	500 ± 14	38 ± 1	539 ± 15
S2	Unrefined	392 ± 5	35 ± 1	427 ± 6
	Refined	416 ± 8	36 ± 1	452 ± 9
S3	Unrefined	402 ± 10	20 ± 1	422 ± 10
	Refined	413 ± 7	20 ± 1	433 ± 7
S4	Unrefined	433 ± 1	100 ± 1	533 ± 1
	Refined	445 ± 3	100 ± 1	544 ± 4
S5	Unrefined	451 ± 1	86 ± 1	537 ± 1
	Refined	451 ± 15	86 ± 1	537 ± 15
S6	Unrefined	469 ± 10	75 ± 1	544 ± 11
	Refined	476 ± 4	75 ± 1	551 ± 5

1

2 **Fig. 1.** Process flow diagram for bioconversion of hybrid poplar into carbohydrates.

3

Fig. 2. Particle length (a) and width (b) of refined and unrefined steam pretreated hybrid poplar with and without SO_2 . Error bars indicate standard deviation.

Fig. 3. 96 hours cellulose to glucose conversion of refined and unrefined steam pretreated hybrid poplar with cellulase at 10 FPU/g cellulose and 5% (w/v) solids consistency. Error bars indicate standard deviation.

Fig. 4. Overall monomeric glucose and xylose recovery of refined and unrefined steam pretreated hybrid poplar (expressed as kg/tonne raw biomass). Regression lines are shown for refined and unrefined samples respectively. Error bars indicate standard deviation.