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PII: S0960-8524(18)30265-7
DOI: https://doi.org/10.1016/j.biortech.2018.02.072
Reference: BITE 19581

To appear in: *Bioresource Technology*

Received Date: 15 December 2017
Revised Date: 13 February 2018
Accepted Date: 14 February 2018

Please cite this article as: Yang, M., Xu, M., Nan, Y., Kuittinen, S., Kamrul Hassan, Md., Vepsäläinen, J., Xin, D., Zhang, J., Pappinen, A., Influence of size reduction treatments on sugar recovery from Norway spruce for butanol production, *Bioresource Technology* (2018), doi: https://doi.org/10.1016/j.biortech.2018.02.072

Influence of size reduction treatments on sugar recovery from Norway spruce for butanol production

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Abstract:

This study investigated whether the effectiveness of pretreatment is limited by a size reduction of Norway spruce wood in biobutanol production. The spruce was milled, chipped, and mashed for hydrogen peroxide-acetic acid (HPAC) and dilute acid (DA) pretreatment. Sugar recoveries from chipped and mashed spruce after enzymatic hydrolysis were higher than from milled spruce, and the recoveries were not correlated with the spruce fiber length. HPAC pretreatment resulted in almost 100% glucose and 88% total reducing sugars recoveries from chipped spruce, which were apparently higher than DA pretreatment, demonstrating greater effectiveness of

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HPAC pretreatment on sugar production. The butanol and ABE yield from chipped spruce were 126.5 and 201.2 g/kg pretreated spruce, respectively. The yields decreased with decreasing particle size due to biomass loss in the pretreatment. The results suggested that Norway spruce chipped to a 20 mm length is applicable to the production of platform sugars for butanol fermentation.

Keywords: Norway spruce; size reduction; pretreatment; sugar recovery; biobutanol

1. Introduction

Butanol as a potential biofuel could be produced biochemically via acetone-butanol-ethanol (ABE) fermentation using the sugars extracted from lignocelluloses. Softwood such as Norway spruce is the dominant source of lignocellulosic materials in Nordic countries, and it would be the subject of great interest for biobutanol production. However, softwood is one of the hardest lignocellulosic feedstocks, which needs technological improvements and even new process configurations for conversion to fermentable sugars (Galbe and Zacchi, 2002).

Pretreatment could disrupt the heterogeneous structure of lignocellulose, remove hemicelluloses and/or lignin, and increase the surface area and porosity of biomass, thus enhancing the enzymatic hydrolysis of cellulose to sugars (Wyman et al., 2005; Mosier et al., 2005). To date, a wide range of pretreatment technologies for softwood has been developed by combining thermal treatments with chemicals, such as SO_2,

acid or ammonia (Galbe and Zacchi, 2002). However, only a few of them seem to be promising due to the extensive energy requirement, hemicelluloses loss, and formation of inhibitors. A newly developed hydrogen peroxide (H_2O_2)-acetic acid (CH_3COOH) (HPAC) pretreatment removes lignin from lignocellulosic cell walls, which results in enhanced enzymatic accessibility of the substrate and more efficient cellulose hydrolysis of multiple lignocellulosic materials, reducing enzyme loading and downstream enzymatic hydrolysis time. Moreover, this method does not need high temperatures or strong acid treatments (Wi et al., 2015). Therefore, it is hypothesized in this study that the HPAC pretreatment is a highly effective pretreatment to convert Nordic softwood such as Norway spruce to fermentable sugars for butanol fermentation.

Prior to pretreatment, a physical treatment of lignocellulosic materials such as grinding, milling or chipping is recommended for particle size reduction (Sun and Cheng, 2002). The size reduction can eliminate mass and heat transfer limitations during pretreatment and enzymatic hydrolysis (Schnell and Harwood, 1994) as well as reduce the crystallinity of the cellulose fibers in the biomass (Keshwani and Cheng, 2009). Nevertheless, size reduction of biomass is an energy-intensive and expensive process (Zhu et al., 2010). Thus, the development of efficient biomass processing technology with relatively larger particle sizes would be good for achieving high sugar conversion and low production cost (Zhu et al., 2009). In the last decade, the effect of particle size on the hydrolysis yield of agricultural residuals such as corn stover, rice straw, energy crops, and wheat straw has been extensively investigated,

and inconsistent results were reported (Bridgeman et al., 2007; Pedersen and Meyer, 2009; Khullar et al., 2013; Harun et al., 2013; Li et al., 2016). This might be attributed to the various biomass categories, particle size definition, sugar yield definition, biomass treatment procedure, and particle size (Zhang et al., 2013). In regard to Norway spruce, the particle size used for pretreatments varied from micrometers to millimeters (Zhu et al., 2009； Zhao et al., 2010; Shafiei et al., 2010; Shafiei et al., 2013; Frankó et al., 2015). It was reported that the glucose yield of steam-pretreated spruce after enzymatic hydrolysis was slightly higher for the smaller chips (Monavari et al., 2009). Shafiei et al. (2013) found that less glucose was released from the ionic liquid pretreated spruce wood chips compared to wood powder. However, the pretreatments improved the hydrolysis yield of wood chips much more than that of the powder.

To the best of our knowledge, there is no systematic research on evaluating the effects of different size reduction treatments on pretreatment performance on Norway spruce for sugar production in the context of biobutanol fermentation. The aim of this study is to understand whether the effectiveness of HPAC pretreatment is limited by the physical treated particle sizes of Norway spruce wood. The milled, chipped, and mashed spruce wood with different fiber lengths were used for HPAC pretreatment. A dilute sulfuric acid pretreatment was also performed for comparison. The sugar recovery from raw materials after enzymatic hydrolysis was used to evaluate the pretreatment efficiency, and the effect of fiber length of spruce wood on the sugar recovery was clarified. The mass balance around the sugar recovery for biobutanol

production was calculated. In addition, the HPAC pretreatment with a relatively larger particle size by using lower chemical dosages was also performed.

2. Materials and methods

2.1 Raw materials

The Norway spruce wood was harvested from Polvijärvi Teerivaara, Finland, in 2016. The wood was milled and sieved to 0.25 and 1 mm by using Ultra-Centrifugal Mill ZM 200 (Retsch GmbH, Germany). The wood was chipped to 6 and 20 mm in length by using a commercial chipping machine (HJ-260GT, Junkkari Oy, Finland). The wood lumber with the size of 50 mm x 22 mm x 3000 mm was mashed to a size of 20 mm in length by a custom made machine. In this machine, there are 4 pieces 125 mm diameter rotating blades which are 12 mm wide each and having six claws each of them. The blades were sharpened into 45 degree angles. There are two sets of blades which are interlocked each other making blade edge, and the feedstock will pass and get mashed. All the prepared samples were air-dried at 45 ℃ for 2 days and stored in paper bags in room temperature.

2.2 Pretreatment

The milled, chipped, and mashed spruce wood were pretreated with a mixture of hydrogen peroxide and acetic acid (volume ratio is 1:1, HPAC) and 0.15% (w/v) sulfuric acid, respectively. In the process of HPAC pretreatment, 5 g spruce wood was mixed with 50 mL HPAC solution and pretreated at 80 ℃ for 4 hours. In the process of sulfuric acid pretreatment, 10 g spruce was mixed with 100 mL sulfuric acid in a steel cylinder and pretreated at 190 ℃ for 10 minutes. The pretreatments with

different dosages of HPAC solution and resident times were also investigated for the pretreatment of chipped (20 mm) spruce wood. The dosages were designated as 1/5 HPAC, 1/3 HPAC, 1/2 HPAC, and 2/3 HPAC. The resident times were 4 h, 6 h, and 8 h. All pretreatments were performed in duplicate. After pretreatment, the slurry was cooled to room temperature and pressed through filter paper to separate the pretreated solid from the liquid. The pretreated solids were washed using 500 mL deionized water and air-dried for chemical composition analysis and enzymatic hydrolysis.

2.3 Composition analysis

The composition of raw and pretreated spruce wood was analyzed according to the standard protocol of the National Renewable Energy Laboratory (NREL) (Sluiter et al., 2008). 300 mg materials were weighted and treated with 72% H_2SO_4 at 30 °C for 1 hour in a 100 mL triangle-flask and then diluted to 4% H_2SO_4 with deionized water and autoclaved at 121 °C for one hour. The slurry was neutralized with solid $CaCO_3$ to pH 4-5 and centrifuged at 1200 rpm for 10 minutes. The supernatant was collected for sugar analysis by high performance liquid chromatography (HPLC).

2.4 Fiber length and width measurement

The raw and pretreated materials (0.5 g) were suspended in a mixture liquid of 50% acetic acid and 30% hydrogen peroxide and placed in the oven at 65 °C for two days. After that, the slurry was placed in a beaker containing 100 mL deionized water. The electric stirrer was applied for one minute. Then the slurry was pressed through filter paper to separate the liquid and solid. The solid was transferred to another beaker containing 200 mL deionized water and agitated with an electric stirrer again

for one minute. The beaker was then placed on the L&W fiber tester (model 912) in accordance with ISO standard 16065-2 to analyze the fiber length and width of the materials.

2.5 Enzymatic hydrolysis

The enzymes Celluclast 1.5L, Novozyme 188 and Viscozyme@L (Novozymes A/S, Bagsværd, Denmark) were used for enzymatic hydrolysis of pretreated solids. Polyethylene glycol 4000 (PEG) was used as an ionic surfactant for increasing the hydrolysis efficiency. Hydrolysis with 2% dry matter (DM) loading was performed in tubes with a 3 mL working volume in 50 mM sodium citrate buffer (pH 5.0). The hydrolysis was conducted in a shaker stirred at 200 rpm at 50 °C for 48 h. Prior to hydrolysis, 10 FPU/g biomass of Celluclast 1.5 L (50-70 FPU/mL), 400 nkat/g biomass of Novozyme 188 (6000 nkat/mL), and 20 FBG/g biomass of Viscozyme L (100 FBG/g) were added to the slurry for enzymatic hydrolysis. The dosage of PEG 4000 was 0.02 g/g DM. The hydrolysis of pretreated samples for fermentation was conducted with 5% DM loading in a 100 mL glass bottle with 50 mL working volume of sodium citrate buffer. After the hydrolysis, the samples were boiled for 10 min to stop the enzymatic hydrolysis and then centrifuged for 10 min at 12,000 rpm. The supernatants of each sample were collected for the analysis of sugars. All the hydrolysis experiments were performed in duplicate. The average data of two independent experiments and standard errors calculated based on the relative difference of duplicate experiments were presented.

2.6 Fermentation

Freeze-stored *Clostridium acetobutylicum* DSM 1731 culture was activated for 14-16 h at 37 °C in 50 mL Reinforced Clostridial Medium (tryptone 10 g/L; yeast extract 3 g/L; beef extract 10 g/L; glucose 5 g/L; Cys-HCl 0.5 g/L; NaCl 5 g/L; NaAc 5 g/L; soluble starch 1 g/L). Then, 1 mL of active growing cells was inoculated into 50 mL of sterilized pre-fermentation P2 media prepared in a 125 mL screw-capped bottle. The pre-fermentation P2 media contained glucose 30 g/L and yeast extract 1 g/L. Before inoculation, 0.5 mL each of the filter-sterilized stock solutions (buffer: KH_2PO_4 50 g/L, K_2HPO_4 50 g/L, ammonium acetate 220 g/L; mineral: $MgSO_4 \cdot 7H_2O$ 20 g/L, $MnSO_4 \cdot H_2O$ 1 g/L, $FeSO_4 \cdot 7H_2O$ 1 g/L, NaCl 1 g/L; and vitamin: para-aminobenzoic acid 0.1 g/L, thiamin 0.1 g/L, biotin 0.001 g/L) was added into the P2 media. The culture was allowed to grow for approximately 16 h at 37 °C before inoculation into the ABE production media. All experiments were conducted in duplicate.

ABE fermentation was conducted in a 125 mL screw-capped bottle containing 50 mL medium. The pH of the medium was adjusted to 6.5 with $Ca(OH)_2$. The medium was boiled for 10 minutes in the water bath and purged with N_2 for 2 minutes to keep anaerobic conditions and then was sterilized at 121°C for 20 minutes. Fermentation started at 37 °C when inoculated into the *C. acetobutylicum* DSM 1731 culture (10%, v/v). Prior to the inoculation, 0.5 mL each of the filter-sterilized stock solutions (buffer, mineral, and vitamin) was added to the hydrolysate medium.

2.7 Chemical analysis

The HPLC system (Hitachi L-2000, Hitachi Ltd., Japan) for compositional sugar analysis was equipped with a refractive index detector (Hitachi Ltd., Japan) and an autosampler (Hitachi Ltd., Japan). An ion-moderated partition chromatography column (Aminex column HPX-87H) with a Cation H micro-guard cartridge was used. The column was maintained at 45 °C with 5-mM H_2SO_4 as the eluent at a flow rate of 0.5 mL/min. Before injection, the samples were filtered through 0.22-μm MicroPES filters, and a volume of 20 μL was injected. Peaks were detected by refractive index and were identified and quantified by comparing the retention times of authenticated standards (D-glucose, D-xylose, and D-mannose). The xylose and mannose were quantified as hemicellulose because their peaks cannot be separated with chromatography column HPX-87H. Due to the presence of PEG 4000, which affects the sugar analysis by HPLC with column HPX-87H, the concentration of glucose in enzymatic hydrolysate was determined a by SBA-40 C biosensor analyzer (Institute of Biology, Shandong Academy of Sciences, China). The total reducing sugars in the enzymatic samples were analyzed with the 3,5-dinitrosalicylic acid method (Miller, 1959).

Fermentation samples were analyzed with Nuclear Magnetic Resonance (NMR). The NMR spectra for quantification of sugars, butanol, acetone and ethanol were recorded on a Bruker AVANCE 500 DRX NMR spectrometer equipped with a 5 mm QNP SB probe. The above-mentioned compounds were identified from routine two-dimensional proton-proton and proton-carbon correlated spectra. Quantitative 1H NMR spectra were collected with water presaturation (zgcppr) by using a 90° pulse

angle, 48 dB presaturation power, 40 s relaxation delay, and 16 scans at 300 K. Prior to the NMR measurements, 200 μL of sample liquid was transferred to a 5 mm NMR tube followed by the addition of deuterium oxide (D_2O, 275 μL) and 3-(trimethylsilyl)-propionic-d4 acid (25 μL, 20 mM) in D_2O as an internal standard of known concentration.

The sugar recoveries were calculated as follows:

$$\text{Glucose recovery}(\%) = \frac{\text{Glucose in enzymatic hydrolysate(g)} \times 0.9}{\text{Cellulose in raw material (g)}} \times 100 \quad (1)$$

$$\text{Total sugars recovery}(\%) = \frac{\text{Total reducing sugars in enzymatic hydrolysate(g)} \times 0.88}{\text{Cellulose and Hemicellulose in raw material (g)}} \times 100 \quad (2)$$

3. Results and discussion

3.1 Effect of size reduction treatments on the composition of spruce wood pretreated with HPAC and DA

The raw Norway spruce wood contained 43.0% cellulose, 20.6% hemicelluloses, and 26.9% lignin (Table 1). After HPAC pretreatment, 49.0–63.1% of the original biomass was recovered as a solid. The solid recoveries from milled (0.25 and 1 mm) materials were apparently lower than that from chipped (6 and 20 mm) and mashed materials. HPAC pretreatment removed 85.1–88.4% lignin from raw material, retaining a low lignin level in the solid. The contents of cellulose in pretreated solids were not changed much compared with the raw material, whereas the hemicelluloses were removed by 33.5–58.8%. This is in agreement with the result of Wi et al. (2015) that HPAC pretreatment was highly effective in removing lignin from lignocellulosic

cell walls, but it also caused the loss of hemicelluloses. The lignin blocks access of cellulase to cellulose during enzymatic hydrolysis, and binds non-productively with cellulases. The removal of lignin would enhance the enzymatic hydrolysis of cellulose.

After DA pretreatment, the solid recoveries increased from 55.7–67.9% with the increasing particle size from 0.25 mm to 20 mm in milled and chipped samples, and 61.1% of solid was recovered from mashed spruce. The contents of cellulose in pretreated solids decreased to 25.7–35.6% from of the original. The hemicelluloses were not found in the solid, and most of the lignin remained in the pretreated material. This is the typical characteristic of DA pretreatment. As reported earlier, DA pretreatment was highly effective for removing hemicelluloses but not lignin, which also resulted in the further degradation of sugars to inhibitory compounds such as furfural, 5-hydroxymethylfurfural (HMF), and organic acids for enzymatic hydrolysis and fermentation (Yang et al., 2015; Jönsson and Martín, 2016). In contrast, there were no such inhibitors detected after HPAC pretreatment, which is in agreement with the study of Wi et al. (2015). This would allow conducting the enzymatic hydrolysis and fermentation under severe pretreatment conditions.

The results outlined above showed that the delignification effects of HPAC and the removal of the hemicelluloses effects of DA pretreatments are both similar among different physically treated particle sizes of Norway spruce. However, the smaller particle size, the less solid was recovered, and the more carbohydrates loss was observed. This is in accordance with the study of Bridgeman et al. (2007) that

extensive size reduction causes significant carbohydrate losses during the pretreatment, which ultimately results in less reducing sugars in enzymatic hydrolysis and a reduction in ethanol yield. The reason was probably that the decreasing particle size caused the increase of surface area for the severe reaction with pretreatment chemicals, which would result in relatively more degradation of carbohydrates. As reported earlier, particle size may have an effect on mass and heat transfer during pretreatments (Zheng et al., 2007).

3.2 Effect of size reduction treatments on enzymatic hydrolysis of pretreated spruce wood

With HPAC pretreatment, glucose recoveries of 61.1–65.5% and total sugars recoveries of 46.3–50.3% were obtained from milled and chipped spruce, respectively (Fig. 1A). The glucose and total sugars recoveries have no significant differences between milled and chipped spruce. However, the highest glucose recovery (almost 100%) and the total sugars recovery of 67.5% were obtained from mashed spruce, which were apparently higher than that from other treated materials. By adding PEG 4000 during the hydrolysis, the glucose and total sugars recoveries from spruce were significantly increased (Fig. 1B). The glucose and total sugars recoveries from chipped (6 and 20 mm) spruce were somewhat higher than that from milled (0.25 and 1 mm) spruce and were comparable with that from mashed spruce. The effect of PEG 4000 on glucose recovery was not obvious, which is due to the cellulose being completely hydrolyzed even without PEG 4000; thus there was no potential for a further increase. After DA pretreatment, the glucose recoveries increased slightly with

the increasing particle sizes of milled and chipped spruce, and there were no increases for total sugars recoveries (Fig. 1C). The highest glucose recovery of 47.0% and total sugars recovery of 33.8% were obtained from mashed spruce.

The results showed that enzymatic hydrolysis of HPAC pretreated spruce yielded higher sugar recoveries than from DA pretreated spruce with all particle sizes. HPAC pretreatment was reported as a potential pretreatment method in enhancing enzymatic accessibility of the substrate and more efficient cellulose hydrolysis (Wi et al., 2015). The total sugars recoveries were always lower than glucose recoveries, which is due to the loss of hemicelluloses during the pretreatments. The xylose, xylooligosaccharides, and their degradation products such as furfural, HMF, and organic acids were probably formed (Jönsson and Martín, 2016). The promoting effect of PEG 4000 as an ionic surfactant for further increasing the sugar recoveries (sugar yields) has been extensively studied, and the mechanism has been described (Eriksson et al., 2002; Li et al., 2016; Yang et al., 2017). The surfactants could increase the cellulase activity and enzyme stability. The degree of increased free cellulase activity obtained by PEG addition is connected to the amount of phenolic hydroxyl groups in various substrates. The phenolic hydroxyl groups exposed on the lignin surface interact with PEG through hydrogen bonding, forming a layer of PEG on the lignin surface, which prevents unproductive binding of cellulases on lignin (Sipos et al., 2011). Interestingly, the mashed spruce showed the highest sugar recovery without the addition of PEG during the hydrolysis (Fig. 1A). This is probably due to the different structural characteristics that prevented the unproductive

adsorption of enzymes or promoted the accessibility of enzymes to substrates. In that sense, further study of the surface characteristics of pretreated mashed spruce would be interesting.

The sugar recoveries from milled spruce were slightly lower than that from chipped and mashed spruce, which is due to the bigger mass loss after the pretreatment of smaller particle sized spruce. The results suggested that it is not necessary to reduce the particle size to the micrometer scale for the efficient enzymatic hydrolysis following HPAC pretreatment. In the context of biofuels production from lignocellulosic materials, the utilization of larger biomass particles would be more desirable to achieve the high pretreatment efficiency and hence improve subsequent enzymatic hydrolysis performance compared to the smaller ones. This is in agreement with the study of Harun et al. (2013), where the larger cut rice straw particles (5 cm) treated using high severity AFEX conditions significantly demonstrated higher sugar conversion compared to small particles during enzymatic hydrolysis. It was reported that the specific surface area of pretreated corn stover significantly increased, and the crystallinity index apparently decreased with the increased particle size, which promoted the enzymatic hydrolysis (Liu et al., 2013). During the pretreatment of corn stover with the larger particle size, the high-pressure steam easily penetrated the interior of the pile of corn stover due to the higher porosity of the pile, which resulted in the efficient auto-hydrolysis and explosion (Liu et al., 2013). In this study, it seems that the HPAC did not result in the higher

pretreatment efficiency of chipped and mashed spruce. On the contrary, it caused the bigger biomass loss of milled spruce.

Nevertheless, an increasing trend in total sugar conversion of pretreated *Miscanthus* was observed with decreasing mean particle size (Khullar et al., 2013). The glucose release of both wet oxidized and not wet oxidized wheat straw increased with reduced particle size (53-149 mm compared to 2-4 cm). Liu et al. (2013) also reported that the highest sugar recovery reached 99.6% for glucan and 67.0% for xylan at the particle size of 1.0 and 0.5 cm, respectively, but the highest sugar conversion (100% for glucan and 83% for xylan) was observed at the particle size of 2.5 cm. The effect of particle size on the sugar yields not only depended on the feedstocks, pretreatment methods, and particle size level, but also the sugar yield definition. Zhang et al. (2013) summarized three different definitions of sugar yield (glucose/pre-hydrolysis cellulose ratio, glucose/pre-hydrolysis biomass ratio, and glucose/pre-pretreatment cellulose ratio), which were used in the reported studies. The positive relationship between particle size and sugar yield defined as glucose/pre-pretreatment cellulose ratio was found (Ballesteros et al., 2002). This is in good accordance with our study, in which sugar recovery (glucose/original cellulose ratio) was used. The sugar recovery would be a good index to evaluate the effect of particle size on pretreatment efficiency because it took the biomass loss into account. HPAC pretreatment of chipped and mashed spruce with a relatively larger particle size gave high sugar recoveries after enzymatic hydrolysis; thus it would be promising for its application in the process of biofuel production.

3.3 Effect of fiber length and distribution on sugar recovery

Gross fiber characteristics of biomass such as length, width, and size distribution have been shown to influence its enzymatic hydrolysis (Pan et al., 2008; Del Rio et al., 2010; Yeh et al., 2010). However, there are a few reports about the effect of fiber length on the pretreatment efficiency for sugar recovery. In this study, the fiber length distribution of the raw and pretreated spruce was measured with a fiber-quality analyzer. The fibers of milled spruce were the shortest, which contained 25% of 0.2-0.5 mm and 30% of 0.5-1.0 mm length fibers. The percentage of longer fibers (e.g., 1.0-2.0 mm length) in chipped and mashed spruce was higher than in milled spruce (Fig. 2). The average fiber length and width of the materials were presented in Table 2. After HPAC pretreatment, the average fiber length and width as well as the distribution of fiber length did not change (Fig. 2). However, after DA pretreatment, the average fiber length was considerably reduced (except for 0.25 mm materials), in which the fibers with 0.2-0.5 length accounted for 89–98%. The fiber length of 0.25 mm spruce was higher than its original particle size, which is probably due to the materials being too small for fiber length analysis.

The results indicated that HPAC pretreatment removed lignin without destroying the cellulose chain. The delignification exposed the cellulose fibrils in cell walls and resulted in enhanced enzymatic accessibility of the substrate, leading to more efficient cellulose hydrolysis (Wi et al., 2015). However, the fiber characteristics had no relationship with sugar recoveries from spruce after both pretreatments. The same as sugar recovery, the enzymatic hydrolysis yield of milled spruce was no higher than

chipped and mashed spruce even though it had a shorter fiber length after HPAC pretreatment (data not shown). It was reported that substrates with the lower average fiber size were hydrolyzed both at a faster rate and more completely due to the increased specific surface area of small and fine fibers (Mooney et al., 1999). However, Del Rio et al. (2012) reported that the fiber size (ranging in size from 0.2 mm to approximately 4.5 mm) had little influence on enzymatic hydrolysis, which is likely due to the similarities in the substrates' chemical composition, accessible surface area, cellulose crystallinity, and degree of polymerization. The relationships between fiber length and enzymatic hydrolysis probably depended on the pretreatment method. It is likely that the overall accessibility of cellulose to cellulases is the most important substrate characteristic rather than any single component such as fiber length.

3.4 Overall mass balance for butanol production

Due to low sugar recovery obtained with DA pretreatment and enzymatic hydrolysis, the fermentation using HPAC pretreated spruce as substrate was conducted. The fermentation of enzymatic hydrolysate derived from HPAC pretreated spruce chips (20 mm) produced 10.6 g/L ABE, in which 6.4 g/L was butanol. The butanol and ABE yields were 0.22 and 0.35 g/g sugars, respectively. The production yields were comparable to our previous study where barley straw was used as substrate (Yang et al., 2015). According to the sugar recoveries and the yields of ABE products, the mass balance of butanol production from HPAC pretreated spruce with different particle sizes was calculated and presented in Fig. 3. When 1 kg of 20 mm

particle size spruce wood was used as the substrate, 620 g solids were recovered by HPAC pretreatment. After enzymatic hydrolysis, 575 g fermentable sugars were obtained, which could produce 201.2 g ABE containing 126.5 g butanol. The ABE yield (201.2 g/kg pretreated spruce), calculated based on the raw material, was higher than that from dilute sulfuric acid pretreated barley straw (Yang et al., 2015). With decreasing particle sizes, the butanol and ABE yields decreased. The yields from mashed spruce were similar to that from 6 mm particle size spruce. However, it is worth emphasizing that the mashed spruce could be efficiently hydrolyzed without using PEG as the surfactant to increase the enzyme performance during the hydrolysis, which can reduce the butanol production cost. The results showed that larger particles could be utilized during the pretreatment more efficiently than smaller particles, as the pretreatment of smaller particles resulted in more biomass loss. From the biorefinery and economy point of view, the utilization of larger biomass particles producing more sugars and reducing the electric energy consumption during the biomass size reduction process would be preferred (Liu et al., 2013).

3.5 HPAC pretreatment with lower chemical dosages

The HPAC pretreatment resulted in high sugar recovery from chipped spruce (20 mm). Moreover, further investigation of the pretreatment with lower dosages of HPAC to reduce the chemicals cost would be of interest for its upscale application. Thus, different dosages of HPAC with a different pretreatment time were investigated (Fig. 4). For 4 h, with 1/5 and 1/3 dosage of HPAC (diluted by water), the glucose and total sugars recoveries were only about 22.8% and 17.9%, respectively (Fig. 4A).

When the dosage increased to 1/2 HPAC, the glucose and total sugars recoveries increased to 69.4% and 79.1%, respectively, and the 2/3 HPAC resulted in a sugar recovery of 100% from the following enzymatic hydrolysis. For 6 h, with 1/2 dosage of HPAC in pretreatment, the glucose and total sugars recoveries increased to 100% (Fig. 4B). There were no further increases for the sugar recoveries with longer pretreatment time because the complete hydrolysis was achieved (Fig. 4C). In the previous study, HPAC was used as pretreatment for rice straw, pine wood, and oak wood, and the optimized ratio of hydrogen peroxide/acetic acid (1:1) was obtained. However, the dosage of HPAC was not optimized (Wi et al., 2015). Tan et al. (2010) reported that being treated with 69.1% peroxide-HAc at 80 °C for 26.5 h, 97.1% of the lignin could be removed while keeping 68.2% of the hemicelluloses intact. Over 93.6% of carbohydrate in treated bagasse could be hydrolyzed with exoglucanase in a dosage of 138 FPU/g carbohydrates (Tan et al., 2010). Alkaline hydrogen peroxide pretreatment was also studied for the hydrolysis of sugarcane bagasse, but only a glucose recovery of 41.7% was obtained (Yu et al., 2015). In this study, 1/2 dosage of HPAC could be used for pretreatment of spruce in 6 h, which would be more environmental friendly and cost-effective. Compared with the results of using one dosage of HPAC, 1/2 HPAC achieved the higher total sugars recovery from enzymatic hydrolysis, which suggested the lower hemicelluloses loss during the pretreatment.

3.6 Comparison to other studies

Softwoods such as Norway spruce are generally more refractory than hardwoods or agricultural residues for hydrolysis to fermentable sugars. This is because softwoods contain more lignin. Also, the content of acetylated groups in softwoods is lower than in hardwoods, and autohydrolysis during pretreatment cannot occur to the same extent (Galbe and Zacchi, 2002). For the pretreatment of softwood, the most investigated method is steam explosion with and without addition of acid or SO_2 catalyst (Söderström et al., 2003; Söderström et al.,2004; Monavari et al., 2009; Wiman et al., 2012; Frankó et al., 2015). The results from steam-explosion pretreatment of softwood species are not encouraging. Pretreatments of spruce with alkaline, N-methylmorpholine-N-oxide, and sulfite were also investigated, and the range of sugar yield varied from 66–92% (Zhao et al., 2010; Shafiei et al., 2010; Shafiei et al., 2013).

In this study, an excellent sugar recovery was obtained by HPAC pretreatment. The previous study demonstrated the potential of HPAC pretreatment for multiple materials; therein the biomass was chipped into small pieces of 2 cm in length with a cutter and grounded with a wet-disk mill to a particle size of 0.7 cm (Wi et al., 2015). The spruce used in pretreatment are usually milled and sieved with a size from 2 to 10 mm (Söderström et al., 2003; Söderström et al., 2004; Monavari et al., 2009; Wiman et al., 2012; Frankó et al., 2015). Biomass particle size obviously impacts the design of handling, transportation, and conversion facilities (Obernberger and Thek, 2004). A suitable size reduction will significantly improve the efficiency of pretreatment due to the high efficient mass and heat transfer. The materials used for

pretreatment with an industrially relevant size would be cost-effective. The results of this study suggested that chipped spruce 20 mm in length could be used for HPAC pretreatment, and lower dosages of HPAC (2/3 HPAC for 4 h or 1/2 HPAC for 6 h) achieved high sugar recoveries.

In recent years, various lignocellulosic feedstocks have been investigated for butanol production via ABE fermentation (Jurgens et al., 2012; Bankar et al., 2013). The concentrations of ABE and the production yields differ with different feedstocks, pretreatments, hydrolysis methods, fermentation technologies, and Clostridium species. A typical butanol yield in ABE fermentation is 0.20 to 0.25 g/g sugars (Lu et al., 2013). The butanol production from spruce has not been studied previously. However, the yields were comparable with the studies of butanol fermentation from other lignocellulosic materials, for example, sugar maple wood, rice straw, and wheat straw (Sun and Liu, 2012; Gottumukkala et al., 2013; Bellido et al., 2014). Consequently, HPAC pretreatment for Norway spruce chipping to 20 mm in length would be promising for the application in biofuels production.

4. Conclusions

Norway spruce wood was milled (0.25 and 1 mm), chipped (6 and 20 mm), and mashed (20 mm) for HPAC and DA pretreatment and enzymatic hydrolysis. For all samples, enzymatic hydrolysis of HPAC pretreated spruce gave higher sugar recoveries compared to DA pretreated spruce, showing the greater effect of HPAC pretreatment on sugar production. After HPAC pretreatment, the sugar recoveries

from chipped and mashed spruce were higher than from milled spruce, which resulted in a higher butanol production yield. The results suggested that the effectiveness of pretreatment of Norway spruce is not limited by a size reduction in biobutanol production.

Acknowledgements

This work was supported by the Doctoral Start-up Fund of Northwest A&F University, China (Z109021612), the Fundamental Research Funds for the Central Universities of China (2452017098), the National Natural Science Foundation of China (31670598), and the Science Foundation for Distinguished Young Scholars of Northwest A&F University, China (2452015098). Financial support also came from the projects: "Sustainable production concepts in the integrated biorefining industry (SusBioRef)" funded by the Academy of Finland, "New value chains for biorefining (A71029)" funded by the European Regional Development Fund, and "Development of efficient biomass conversion routes for biofuel production and utilization (CONVER-B)" funded by INNO-INDIGO/Academy of Finland." The study was also partly financed by Niemi Foundation (Finland).

References

1. Ballesteros, I., Oliva, J.M., Negro, M.J., Manzanares, P., Ballesteros, M., 2002. Enzymatic hydrolysis of steam exploded herbaceous agricultural waste (*Brassica carinata*) at different particle sizes. Process Biochem. 38, 187-192.
2. Bankar, S.B., Survase, S.A., Ojamo, H., Granström, T., 2013. Biobutanol: the

outlook of an academic and industrialist. RSC Adv. 3, 24734-24757.

3. Bellido, C., Loureiro, P.M., Coca, M., González-Benito, G., García-Cubero, M.T., 2014. Acetone-butanol-ethanol (ABE) production by *Clostridium beijerinckii* from wheat straw hydrolysates: efficient use of penta and hexa carbohydrates. Bioresour. Technol. 167, 198-205.
4. Bridgeman, T.G., Darvell, L.L., Jones, J.M., Williams, P.T., Fahmi, R., Bridgewater, A.V., Barraclough, T., Shield, I., Yates, N., Thain, S.C., Donnison I.S., 2007. Influence of particle size on the analytical and chemical properties on two energy crops. Fuel 86(1–2), 60-72.
5. Del Rio, L.F., Chandra, R.P., Saddler, J.N., 2010. The effects of varying organosolv pretreatment chemicals on the physicochemical properties and cellulolytic hydrolysis of mountain pine beetle-killed lodgepole pine. Appl. Biochem. Biotechnol. 161, 1-21.
6. Eriksson, T., Börjesson, J., Tjerneld, F., 2002. Mechanism of surfactant effect in enzymatic hydrolysis of lignocellulose. Enzyme Microb. Technol. 31: 353-364.
7. Frankó, B., Galbe, M., Wallberg, O., 2015. Influence of bark on fuel ethanol production from steam-pretreated spruce. Biotechnol. Biofuels 8, 15.
8. Galbe, M., Zacchi, G., 2002. A review of the production of ethanol from softwood. Appl. Microbiol. Biotechnol. 59, 618-628.
9. Gottumukkala, L.D., Parameswaran, B., Valappil, S.K., Mathiyazhakan, K., Pandey, A., Sukumaran, R.K., 2013. Biobutanol production from rice straw by a non acetone producing *Clostridium sporogenes* BE01. Bioresour. Technol. 145,

182-187.

10. Harun, S., Balan, V., Takriff, M.S., Hassan, O., Jahim J., Dale B.E., 2013. Performance of AFEX™ pretreated rice straw as source of fermentable sugars: the influence of particle size. Biotechnol. Biofuels 6, 40.
11. Jönsson, L.J., Martín, C., 2016. Pretreatment of lignocellulose: formation of inhibitory by-products and strategies for minimizing their effects. Bioresour. Technol. 199, 103-112.
12. Jurgens, G., Survase, S., Berezina, O., Sklavounos, E., Linnekoski, J., Kurkijärvi, A., Väkevä, M., van Heiningen, A., Granström, T., 2012. Butanol production from lignocellulosics. Biotechnol. Lett. 34(8), 1415-1434.
13. Keshwani, D.R., Cheng, J.J., 2009. Switchgrass for bioethanol and other value added applications: a review. Bioresour. Technol. 2009, 100, 1515-1523.
14. Khullar, E., Dien, B.S., Rausch, K.D., Tumbleson, M.E., Singh, V., 2013. Effect of particle size on enzymatic hydrolysis of pretreated *Miscanthus*. Ind. Crop. Prod. 44(1), 11-17.
15. Li, K., Wan, J., Wang, X., Wang, J., Zhang, J., 2016. Comparison of dilute acid and alkali pretreatments in production of fermentable sugars from bamboo: effect of Tween 80. Ind. Crop. Prod. 83, 414-422.
16. Liu, Z.H., Qin. L., Pang. F., Jin, M.J., Li, B.Z., Kang, Y., Dale, B.E., Yuan, Y.J., 2013. Effects of biomass particle size on steam explosion pretreatment performance for improving the enzyme digestibility of corn stover. Ind. Crop. Prod. 44(2), 176-184.

17. Lu, C., Dong, J., Yang, S., 2013. Butanol production from wood pulping hydrolysate in an integrated fermentation–gas stripping process. Bioresour. Technol. 143, 467-475.

18. Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31, 426-428.

19. Monavari, S., Gable, M., Zacchi, G., 2009. The influence of solid/liquid separation techniques on the sugar yield in two-step dilute acid hydrolysis of softwood followed by enzymatic hydrolysis. Biotechnol. Biofuels 2, 1-9.

20. Mooney, C.A., Mansfield, S.D., Beatson, R.P., Saddler, J.N., 1999. The effect of fiber characteristics on hydrolysis and cellulase accessibility to softwood substrates. Enzyme Microb. Technol. 25 (9), 644-650.

21. Mosier, N., Wyman, C.E., Dale, B.E., Elander, R.T., Lee, Y.Y., Holtzapple, M., Ladisch, M., 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresour. Technol. 96, 673-686.

22. Obernberger, I., Thek, G., 2004. Physical characterisation and chemical composition of densified biomass fuels with regard to their combustion behavior Biomass Bioenerg. 27(6), 653-669.

23. Pan, X., Xie, D., Yu, R.W., Saddler, J.N., 2008. The bioconversion of mountain pine beetle-killed lodgepole pine to fuel ethanol using the organosolv process. Biotechnol. Bioeng. 101, 39-48.

24. Pedersen, M., Meyer, A.S., 2009. Influence of substrate particle size and wet oxidation on physical surface structures and enzymatic hydrolysis of wheat straw.

Biotechnol. Prog. 25 (2), 399-408.

25. Schnell, D.J., Harwood, C., 1994. Milling of lignocelluloses biomass-result of pilot scale testing. Appl. Biochem. Biotechnol. 45, 159-168.

26. Shafiei, M., Karimi, K., Taherzadeh, M.J., 2010. Pretreatment of spruce and oak by N-methylmorpholine-N-oxide (NMMO) for efficient conversion of their cellulose to ethanol. Bioresour. Technol. 101, 4914-4918.

27. Shafiei, M., Zilouei, H., Zamani, A., Taherzadeh, M.J., Karimi, K., 2013. Enhancement of ethanol production from spruce wood chips by ionic liquid pretreatment. Appl. Energy 102, 163-169.

28. Sipos, B., Szilágyi, M., Sebestyén, Z., Perazzini, R., Dienes, D., Jakab, E., Crestini, C., Réczey, K., 2011. Mechanism of the positive effect of poly (ethylene glycol) addition in enzymatic hydrolysis of steam pretreated lignocelluloses. C. R. Biol. 334(11), 812-823.

29. Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D., 2008. Determination of structural carbohydrates and lignin in Biomass National Renewable Energy Laboratory. National Renewable Energy Laboratory (NREL), Golden CO.

30. Söderström, J., Galbe, M., Zacchi, G., 2004. Effects of washing on yield in one- and two-step steam pretreatment of softwood for production of ethanol. Biotechnol. Prog. 20, 744-749.

31. Söderström, J., Pilcher, L., Galbe, M., Zacchi, G., 2003. Combined use of H_2SO_4 and SO_2 impregnation for steam pretreatment of spruce in ethanol production.

Appl. Biochem. Biotechnol. 105, 127-140.

32. Sun, Y., Cheng, J., 2002. Hydrolysis of lignocellulosic material for ethanol production: a review. Bioresour. Technol. 83, 1-11.

33. Sun, Z., Liu, S., 2012. Production of n-butanol from concentrated sugar maple hemicellulosic hydrolysate by *Clostridia acetobutylicum* ATCC 824. Biomass Bioenerg. 39, 39-47.

34. Tan, H., Yang, R., Sun, W., Wang, S., 2010. Peroxide-acetic acid pretreatment to remove bagasse lignin prior to enzymatic hydrolysis. Ind. Eng. Chem. Res. 49, 1473-1479.

35. Wi, S.G., Cho, E.J., Lee, D.S., Lee, S.J., Lee, Y.J., Bae, H.J., 2015. Lignocellulose conversion for biofuel: a new pretreatment greatly improves downstream biocatalytic hydrolysis of various lignocellulosic materials. Biotechnol. Biofuels 8, 228.

36. Wiman, M., Dienes, D., Hansen, M.A., van der Meulen, T., Zacchi, G., Lidén, G., 2012. Cellulose accessibility determines the rate of enzymatic hydrolysis of steam-pretreated spruce. Bioresour. Technol. 126, 208-215.

37. Wyman, C.E., Dale, B.E., Elander, R.T., Holtzapple, M., Ladisch, M.R., Lee, Y.Y., 2005. Coordinated development of leading biomass pretreatment technologies. Bioresour. Technol. 96, 1959-1966.

38. Yang, M., Kuittinen, S., Vepsäläinen, J., Zhang, J, Pappinen, A., 2017. Enhanced acetone-butanol-ethanol production from lignocellulosic hydrolysates by using starchy slurry as supplement. Bioresour. Technol. 243, 126-134.

39. Yang, M., Zhang, J., Kuittinen, S., Vepsäläinen, J., Soininen, P., Keinänen, M., Pappinen, A., 2015. Enhanced sugar production from pretreated barley straw by additive xylanase and surfactants in enzymatic hydrolysis for acetone-butanol-ethanol fermentation. Bioresour. Technol. 189, 131-137.

40. Yeh, A.I., Huang, Y.C., Chen, S.H., 2010. Effect of particle size on the rate of enzymatic hydrolysis of cellulose. Carbohydr. Polym. 79, 192-199.

41. Yu, H., You, Y., Lei, F., Liu, Z., Zhang, W., Jiang, J., 2015. Comparative study of alkaline hydrogen peroxide and organosolv pretreatments of sugarcane bagasse to improve the overall sugar yield. Bioresour. Technol. 187, 161-166.

42. Zhang, Q., Zhang, P.F, Pei, Z.J., Wang, D.H., 2013. Relationships between cellulosic biomass particle size and enzymatic hydrolysis sugar yield: Analysis of inconsistent reports in the literature. Renewable Energy, 60(4), 127-136.

43. Zhao,Y., Wang, Y., Zhu, J.Y. Ragauskas, A., Deng, Y., 2010. Enhanced enzymatic hydrolysis of spruce by alkaline pretreatment at low temperature. Biotechnol. Bioeng. 99(6), 1320-1328.

44. Zheng, M., Mosier, N.S., Huang, C.P., Sherman, D.M., and Ladisch, M.R. 2007.Microscopic examination of changes of plant cell structure in corn stover due to hot water pretreatment and enzymatic hydrolysis. Biotechnol. Bioeng. 97, 265-278.

45. Zhu, J.Y., Pan, X.J., 2010. Woody biomass pretreatment for cellulosic ethanol production: technology and energy consumption evaluation. Bioresour. Technol. 101, 4992-5002.

46. Zhu, J.Y., Pan, X.J., Wang, G.S., Gleisner, R., 2009. Sulfite pretreatment (SPORL) for robust enzymatic saccharification of spruce and red pine. Bioresour. Technol. 100, 2411-2418.

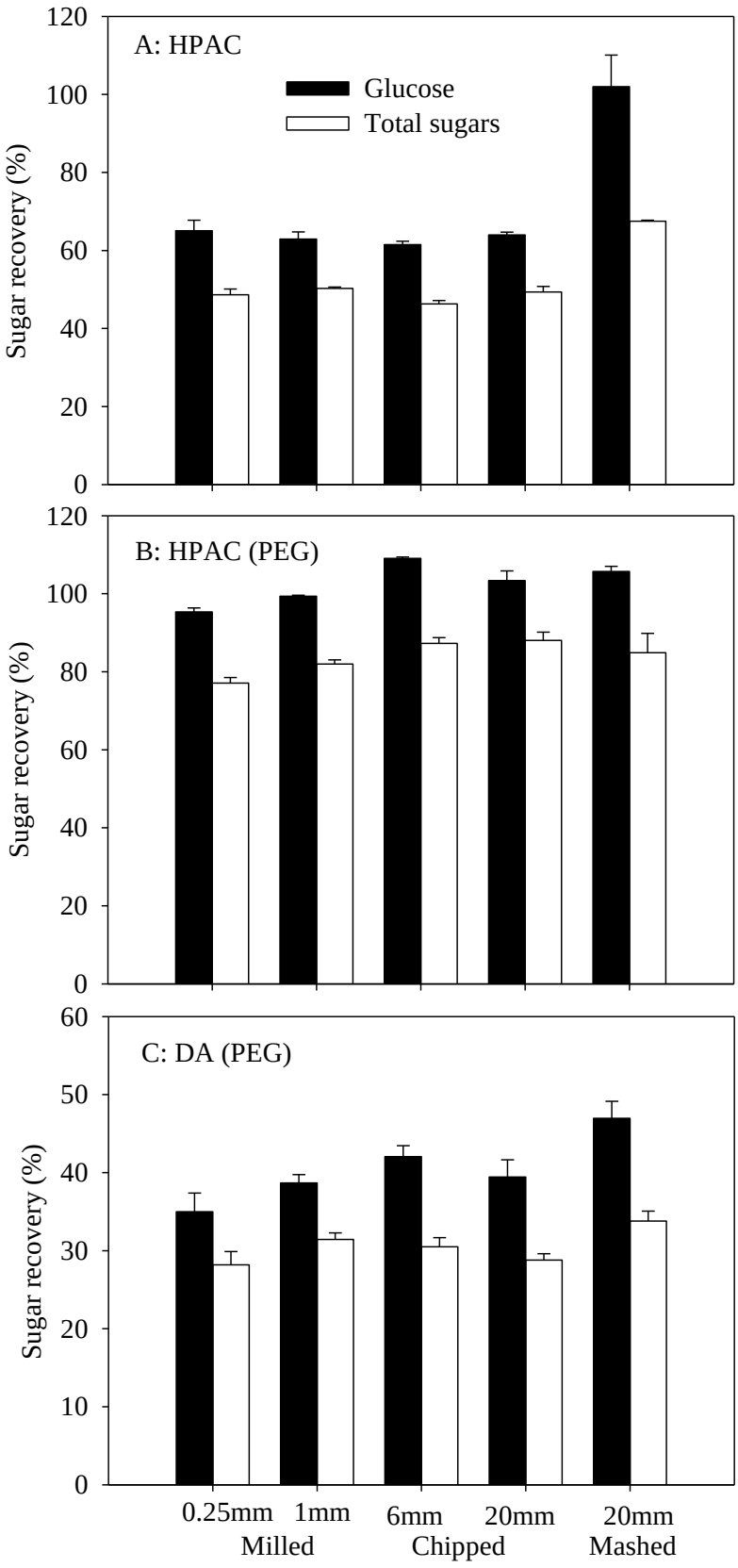
Figure captions

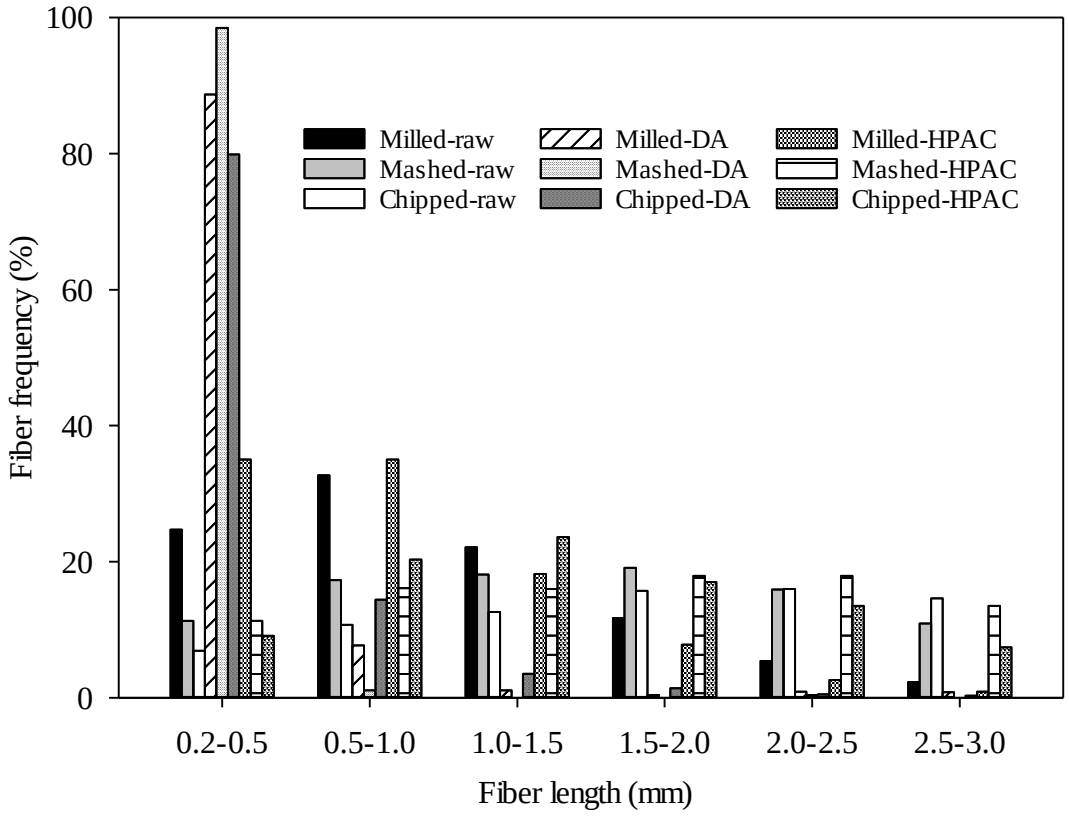
Figure 1. Glucose and total sugars recoveries from milled (0.25 mm and 1 mm), chipped (6 mm and 20 mm), and mashed (20 mm) spruce wood. A: The spruce was pretreated with HPAC and followed by enzymatic hydrolysis. B: The spruce was pretreated with HPAC and followed by enzymatic hydrolysis, in which PEG 4000 was added. C: The spruce was pretreated with DA and followed by enzymatic hydrolysis, in which PEG 4000 was added. HPAC: hydrogen peroxide-acetic acid; DA: dilute acid.

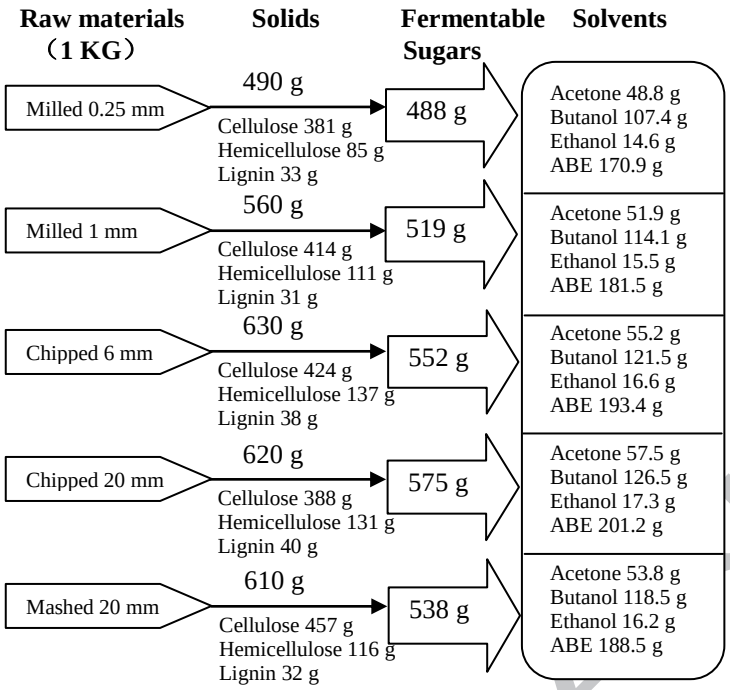
Figure 2. The distribution of fiber length in milled (1 mm), chipped (20 mm), and mashed (20 mm) spruce wood before and after HPAC and DA pretreatment. HPAC: hydrogen peroxide-acetic acid; DA: Dilute acid.

Figure 3. Mass balance for butanol production from milled (0.25 mm and 1 mm), chipped (6 mm and 20 mm), and mashed (20 mm) spruce wood pretreated with HPAC. HPAC: hydrogen peroxide-acetic acid.

Figure 4. Glucose and total sugars recoveries from chipped spruce wood (20 mm) pretreated using different dosages of HPAC and pretreatment times. HPAC: hydrogen peroxide-acetic acid.







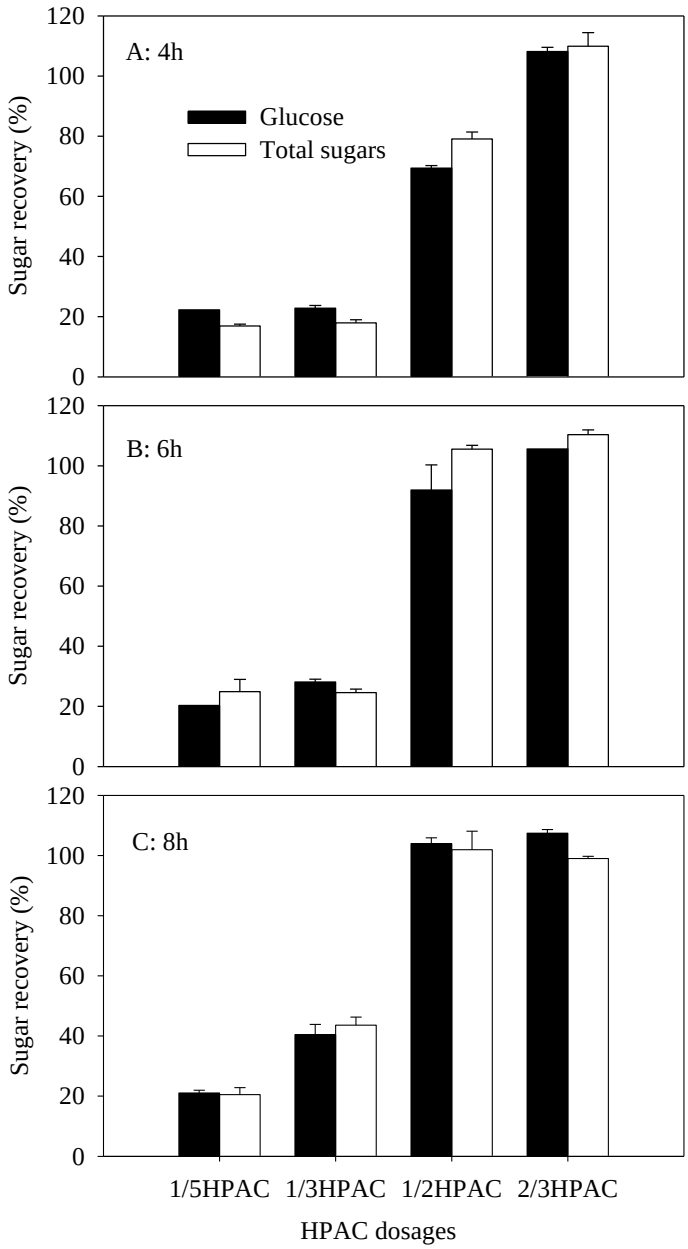


Table 1 The contents of cellulose, hemicellulose and lignin in milled, chipped, and mashed spruce wood before and after HPAC and DA pretreatment. HPAC: hydrogen peroxide-acetic acid; DA: dilute acid. Standard errors of two independent experiments were presented.

Physical treatment	Pretreatment	Particle size (mm)	Solid recovery (%)	Composition (% of original material)		
				Cellulose	Hemicellulose[a]	Lignin[b]
Chipping	Non pretreatment	20	100	43.0±0.3	20.6±0.1	26.9±0.1
Milling	HPAC	0.25	49.0	38.1±0.6	8.5±1.2	3.3±1.2
		1	56.1	41.4±0.7	11.1±1.6	3.1±0.3
Chipping		6	63.1	42.4±1.6	13.7±0.2	3.8±0.4
		20	62.0	38.8±1.8	13.1±1.2	4.0±0.1
Mashing		20	61.0	45.7±0.3	11.6±0.3	3.2±0.1
Milling	DA	0.25	55.7	25.7±0.9	0	27.7±0.5
		1	56.9	28.6±2.8	0	28.8±4.0
Chipping		6	62.7	34.6±0.0	0	30.6±1.2
		20	67.9	35.4±0.4	0	34.8±0.0
Mashing		20	61.1	26.2±1.0	0	23.8±0.2

[a] Hemicellulose contains xylan and mannan.

[b] Lignin contains acid soluble and insoluble lignin.

Table 2 Average fiber length and width in milled, chipped, and mashed spruce wood before and after HPAC and DA pretreatment. HPAC: hydrogen peroxide-acetic acid; DA: dilute acid.

Treatment	Particle size (mm)	Length (mm)			Width (μm)		
		Raw	HPAC	DA	Raw	HPAC	DA
Milling	0.25	0.4	0.4	1.5	35.3	33.4	32.8
	1.0	1.0	0.8	0.4	32.5	29.5	34.9
Chipping	6	1.8	1.6	0.3	30.9	30.1	31.3
	20	2.1	1.6	0.4	30.7	26.0	30.7
Mashing	20	1.6	1.7	0.3	31.5	30.5	34.0