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Effects of impregnation of softwood with sulfuric acid and sulfur dioxide on chemical and physical characteristics, enzymatic digestibility, and fermentability

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ABSTRACT

Hydrothermal pretreatment improves bioconversion of lignocellulose, but the effects of different acid catalysts are poorly understood. The effects of sulfuric acid (SA) and sulfur dioxide (SD) in continuous steam pretreatment of wood of Norway spruce were compared in the temperature range 195 °C–215 °C. The inhibitory effects of the pretreatment liquid on cellulosytic enzymes and Saccharomyces cerevisiae yeast were higher for SD- than for SA-pretreated material, and the inhibitory effects increased with increasing pretreatment temperature. However, the susceptibility to cellulosytic enzymes of wood pretreated with SD was 2.9–2.9 times higher than that of wood pretreated with SA at the same temperature. Data conclusively show that the superior convertibility of SD-pretreated material was not due to inhibition phenomena but rather to the greater capability of the SD pretreatment to reduce the particle size through partial delignification and cellulose degradation. Particle size was shown to be correlated with enzymatic digestibility (R² 0.97–0.98).

1. Introduction

Lignocellulosic biomass is an abundant bioresource that can be an ideal feedstock for biofuel production. Pretreatment is required for disrupting the resistant structure of lignocellulosic biomass to make the cellulose more accessible for biochemical conversion using cellulosytic enzymes (Mosier et al., 2005; Chandra et al., 2007; Yang and Wyman, 2008; Wyman et al., 2009; Sun et al., 2016). Steam explosion, which can be seen as a form of hydrothermal pretreatment, is a method that is highly relevant from an industrial point of view. It can be enforced by impregnation with acid catalysts, such as sulfuric acid (SA) or sulfur dioxide (SD), for handling of recalcitrant forms of lignocellulose such as softwood (Galbe and Zacchi, 2007; Hu and Ragauskas, 2012; Jönsson and Martín, 2016). Although batchwise steam pretreatment is most commonly studied, continuous steam pretreatment is an industrially relevant alternative.

Comparisons of steam pretreatment using SA and SD as impregnating agents have been made for softwood (Tengborg et al., 1998), hardwood (Eklund et al., 1995; Mackie et al., 1985), and agricultural residues (Martín et al., 2002; Shi et al., 2011). However, the reasons behind differences between pretreatment using sulfuric acid and sulfur dioxide are not well understood.

Different acidity of impregnating agents can affect the severity of the pretreatment, which affects the sugar yield. Determination of the combined severity (CS), which takes the temperature, the time period, and the acidity into account, is a way to compare different pretreatments (Chum et al., 1990). Pretreatment with different impregnating agents can also give different results with regard to formation of inhibitors of microorganisms and enzymes. Microbial inhibitors formed during pretreatment under acidic conditions include aliphatic acids, aliphatic aldehydes, benzoquinones, furanic compounds, and phenolic compounds (reviewed by Ko et al. (2015) and Jönsson and Martín (2016)). Lignin in the solid phase after pretreatment can cause catalytically non-productive binding of carbohydrate-degrading enzymes, but the enzymes are also inhibited by water-soluble substances in the liquid phase (Ko et al., 2015; Jönsson and Martín, 2016). Although the identity of these water-soluble enzyme inhibitors remains to be fully elucidated, they include sugars causing end-product inhibition and aromatic compounds, such as phenolics (Kim et al., 2011; Ko et al., 2015). Hydrophobilization of the aromatic inhibitors appears to be important to alleviate the inhibition (Cavka and Jönsson, 2013; Jönsson and Martín, 2016), which indicates that hydrophobic interactions between inhibitors and enzymes contribute to the problem. Recent findings show that among the sugars in steam-pretreated biomass the inhibitory effects of oligomeric sugars were small compared to inhibition caused by monosaccharide sugars (Zhai et al., 2016). If formation of
inhibitors is causing differences in bioconversion efficiency for different impregnating agents, it is evident that both microbial inhibition and enzyme inhibition need be taken into account.

To achieve a better understanding of the differences between using sulfuric acid and sulfur dioxide for impregnation, continuous steam pretreatment was performed at three temperatures (195 °C, 205 °C, and 215 °C) for the same time period using impregnation with sulfuric acid or sulfur dioxide. The pretreatments were designed to have a similar acidity to avoid trivial differences caused by different CS for treatments performed at the same temperature. The resulting slurries were compared using a novel analytical tool-box comprising physical, chemical, biochemical, and microbial assays. The analyses covered the physical structure and the chemical composition of the pretreated biomass, the enzymatic digestibility of the pretreated biomass, enzyme inhibition by components in the liquid phase, and microbial inhibition by components in the liquid phase. A better understanding of the mechanisms behind pretreatment reactions will facilitate industrial implementation of efficient biochemical conversion of lignocellulosic feedstocks.

2. Materials and methods

2.1. Materials

Steam-pretreated spruce was prepared in the Biorefinery Demo Plant (Örnsköldsvik, Sweden) operated by the SP Technical Research Institute of Sweden (now a part of RISE Research Institutes of Sweden). Chips of unbarked Norway spruce (size distribution: 1% < 3 mm; 51% 3–7 mm; 21% 7–13 mm; 27% > 13 mm) were steam-pretreated in continuous mode using a 30-L pretreatment reactor. The feeding rate was 39 kg (dry weight) wood chips/h. The wood chips were impregnated with either sulfuric acid (SA) [0.34–0.40 kg concentrated SA/h corresponding to around 0.4 kg concentrated SA/100 kg wood chips (wet weight)] or sulfur dioxide (SD) [0.9 kg SD/h corresponding to around 0.4 kg concentrated SA/100 kg wood chips (wet weight)]. For each catalyst, three different temperatures were used, viz. low (L) (~195 °C), medium (M) (~205 °C), and high (H) (~215 °C). The retention time was 7 min. This procedure generated six slurries of pretreated spruce wood, which, taking pretreatment catalyst and temperature into consideration, are hereafter referred to as: SA-L, SA-M, SA-H, SD-L, SD-M, and SD-H. The slurries, which had a pH of around 1.6, were stored at 4 °C until further use.

For further analysis, portions of each of the slurries were diluted with deionized water to a total solid (TS) content of 25% (w/w). The initial pH of the media was adjusted to 5.2 with a 10 M aqueous solution of sodium hydroxide. The pH was adjusted to 5.2 with a 10 M aqueous solution of sodium hydroxide. The liquid and solid phases were then separated by centrifugation for 20 min with 12,800×g. The washed material was freeze-dried behind a vacuum (Eclipse, Wirtz, USA) and stored at 4 °C until further use.

2.2. Effect of pretreatment liquid on enzymatic hydrolysis of cellulose

The effects of the SPL on enzymatic digestion of cellulose were determined using Avicel PH-101 (Sigma-Aldrich, St. Louis, MO, USA) as substrate. Two liquid enzyme preparations were used in the experiments: (A) a 1:1 (v/v) mixture of the conventional Celluclast 1.5 L and Novozyne 188 (both of which were obtained from Sigma-Aldrich), and (B) a state-of-the-art cellulolytic enzyme preparation from a leading manufacturer.

The analytical enzymatic hydrolysis was performed in 2 mL sealed microcentrifuge tubes in an orbital shaker set at 170 rpm and 45 °C (Ecoutron incubator shaker, Infors, Bottmingen, Switzerland) for 72 h. The reaction mixture contained: 50 mg Avicel PH-101, 950 μL SPL, and 10 μL enzyme preparation A or 5 μL enzyme preparation B. Seven different control reactions were included in the experiments: one with 50 mM sodium citrate buffer (pH 5.2) instead of SPL and six other with the same buffer containing monosaccharide mixtures (arabinose, galactose, glucose, mannose, xylose) corresponding to the monosaccharide contents of the six different SPLs. The inhibition caused by the SPL except the inhibition caused by monosaccharide sugars was determined by calculating the fraction (mg/mg) of the amount of glucose released in SPL medium divided by the amount of glucose released in the corresponding SPL sugar control. Each of the 13 reactions was performed in triplicate. The monosaccharide contents of the resulting 39 hydrolysates were determined using HPAEC (Section 2.11).

2.3. Effect of pretreatment liquid on yeast

Fermentation experiments were performed in 30-ml glass flasks containing 25 mL yeast culture. The flasks were incubated in an orbital shaker at 180 rpm and 30 °C. Diluted SPL corresponding to a TS of 12% was used, as undiluted SPL (corresponding to TS 25%) was too toxic to reveal differences between the pretreatment conditions. The experimental series contained reference fermentations based on a synthetic sugar solution (average concentration of hexose sugars in diluted SPL amounting to 2.62 g/L galactose, 9.92 g/L glucose, and 12.64 g/L mannose). Diluted and pH-adjusted SPL was mixed with 0.5 mL of a nutrient solution (150 g/L yeast extract, 7.5 g/L (NH₄)₂HPO₄, 3.75 g/L MgSO₄·7 H₂O, 238.2 g/L NaH₂PO₄·H₂O), and yeast inoculum [Red ethanol yeast (Fermits, Marcq en Baroeul, France) added to a final concentration of 2 g/L (dry weight)]. The initial pH of the media was 5.5. The flasks were flushed with nitrogen gas before the start of the fermentation and after taking samples to avoid excessive amounts of oxygen. Fermentations were performed in duplicate. Sugars were analyzed using HPLC (Section 2.11), and ethanol was analyzed using HPLC (Section 2.12).

2.4. Chemical analysis of composition of solid fraction

The lignin and carbohydrate contents of the six PSS samples were determined essentially according to the NREL-TP-510-42618 method (Sluiter et al., 2012), but with 100 mg sample size and using HPAEC to analyze the contents of monosaccharides (Section 2.11). The extraction step was skipped due to the steam pretreatment performed previously. The sulfur content of the PSS was analyzed by Bränselektoratet (Umeå, Sweden) using a combustion method (ISO 16994, 2016). The measurements were always performed in triplicate.

2.5. Pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS) analysis

Py-GC/MS was used to determine the lignin-carbohydrate fraction of the pretreated solids. The analysis was performed at the Plant Cell Wall and Carbohydrate Analytical Facility of the Umeå Plant Science Center (UPSC) (Umeå, Sweden) according to the method described in Gerber et al. (2012).
2.6. FTIR analysis

FTIR analyses were performed at VISP, the Vibrational Spectroscopy Platform of the Chemical-Biological Centre (KBC) (Umeå, Sweden), using potassium bromide (Spectrograde KBr, Fisher Scientific, Waltham, MA). The spectra were obtained on a Bruker IFS 66v/S FTIR spectrometer with a standard Deuterated Triglycine Sulfate detector, and fitted with a diffuse reflectance accessory (Bruker Corporation, Billerica, MA). The background and the measurements spectra were recorded at 256 scans and 4 cm⁻¹ resolution. Experiments were carried out in vacuo.

2.7. Digestibility of pretreated spruce in buffer

Analytical enzymatic hydrolysis of washed and freeze-dried PSS was performed for 72 h in 2-mL tubes in an orbital shaker set at 170 rpm and 45 °C. The reaction mixture contained 50 mg PSS (dry weight), 950 μL 50 mM sodium citrate buffer (pH 5.2), and 10 μL enzyme preparation A or 5 μL enzyme preparation B. Reactions were performed in triplicate. The monosaccharide contents after 72 h of incubation were analyzed using HPAEC.

2.8. Digestibility of pretreated spruce in pretreatment liquid

A series of PSS in original pretreatment liquid with two TS contents (12.5% and 25%) was prepared. The pH was adjusted to 5.2 with a 10 M solution of sodium hydroxide. Analytical enzymatic hydrolysis was performed in 2-mL tubes in an orbital shaker set at 200 rpm and 45 °C. The reaction mixtures contained one g of spruce slurry (125 mg PSS reaction and 250 mg PSS for the 125 mg PSS reaction and 250 mg PSS for the 25 mg PSS reaction), and enzyme preparation A (25 μL for the 125 mg PSS reaction and 50 μL for the 25 mg PSS reaction) or B (12.5 μL for the 125 mg PSS reaction and 25 μL for the 25 mg PSS reaction). Thus, a total of four sets of enzymatic reactions were performed (25% TS + A, 25% TS + B, 12.5% TS + A, and 12.5% TS + B), each set in triplicate. The reactions were finished when the glucose production levelled off as judged by glucometer measurements. For 12.5%TS + A, this occurred after 120 h, whereas the other reactions were terminated after 72 h. After the reactions, the residual solids were collected and washed with deionized water until no glucose was detected in the washing liquid using the glucometer. The washed solids were collected and washed with deionized water until no glucose production was detected in the washing liquid using the glucometer. The washed solids were collected and washed with deionized water until no glucose production was detected in the washing liquid using the glucometer. The washed solids were collected and washed with deionized water until no glucose production was detected in the washing liquid using the glucometer. The washed solids were collected and washed with deionized water until no glucose production was detected in the washing liquid using the glucometer. The washed solids were collected and washed with deionized water until no glucose production was detected in the washing liquid using the glucometer. The washed solids were collected and washed with deionized water until no glucose production was detected in the washing liquid using the glucometer. The washed solids were collected and washed with deionized water until no glucose production was detected in the washing liquid using the glucometer. The washed solids were collected and washed with deionized water until no glucose production was detected in the washing liquid using the glucometer.

2.9. Simons' stain assay

Simons' stain assay was performed using Direct Blue (DB, Pontamine Fast Sky Blue 6BX) and Direct Orange (DO, Pontamine Fast Sky Orange 6RN) dyes, which were obtained from Pylam Products (Garden City, NY, USA). The procedure developed by Chandra et al. (2008) was applied with a slight modification of the incubation temperature (65 °C was used instead of 70 °C). The assay was scaled down with a factor of two, as previously shown possible by Chandra and Saddler (2012). Duplicate assays were performed.

2.10. Size distribution assay

For measuring the size distribution, portions of 40 g of wet PSS were passed through a series of four sieves (800 μm, 500 μm, 100 μm, and 50 μm) using water. The mass of the solids retained by each sieve was measured.

2.11. HPAEC analysis of monosaccharides

All samples were diluted with ultra-pure water and filtered through a 0.2 μm nylon membrane (Millipore). The analysis was performed using an ICS-5000 instrument ( Dionex, Sunnyvale, CA, USA) equipped with an electrochemical detector, a CarboPac PA1 (4 × 250 mm) separation column, and a CarboPac PA1 (4 × 50 mm) guard column (all from Dionex). The temperature of the column oven was 30 °C. Prior to injection the column was regenerated with a solution of 260 mM sodium hydroxide (Sodium Hydroxide Solution for IC, Sigma-Aldrich) and 68 mM sodium acetate (Anhydrous Sodium Acetate for IC, Sigma-Aldrich) during 12 min followed by ultra-pure water during 2 min. Each sample was injected once, and elution was performed with ultra-pure water during 25 min. The flow rate was 1 mL min⁻¹. External calibration curves were established by using series of solutions [concentrations (mg L⁻¹): 0.5, 1, 5, 10, 20, and 40] of arabinose, galactose, glucose, mannose, and xylose [prepared from monosaccharide preparations (> 99%) obtained from Sigma-Aldrich].

2.12. HPLC analysis of ethanol

Ethanol analysis was performed by using high-performance liquid chromatography (HPLC). A Waters Ion Exclusion column (7.8 × 150 mm) was used in an Agilent 1260 Infinity series system (Agilent, Santa Clara, CA, USA) equipped with a 1260 series refractive index (RI) detector. Elution was performed with isocratic flow of a 0.005 M aqueous solution of sulfuric acid. The flow rate was 0.6 mL/min and the column temperature was set to 55 °C. Agilent software was used for data analysis.

3. Result and discussion

3.1. Chemical composition of PSS

Table 1 shows the chemical composition of the washed PSS. The sulfur content of the SA-pretreated material was about 0.02% (dry weight), whereas SD pretreatment resulted in a content of sulfur in SD-pretreated material than in SA-pretreated material. The result indicates that pretreatment with SA and SD differs with respect to the effects on lignin and that SD pretreatment shows a superficial resemblance to sulfite pulping. The SD-H sample exhibited the highest sulfur content (Table 1), which indicates that the sulfonation reaction proceeded faster at higher temperature.

In studies of aspenwood, Mackie et al. (1985) reported higher content of sulfur in SD-pretreated material than in SA-pretreated material. Clark et al. (1989) found that 96% of the sulfur in SD-pretreated Pinus radiata was bonded with lignin. Mackie et al. (1985) and Schwald et al. (1989) reported that delignification of aspen was increased by using SD as catalyst during steam pretreatment.

Two methods, compositional analysis and Py-GC/MS, were used to determine the contents of carbohydrate and lignin (Table 1). The chemical composition (% w/w) of untreated Norway spruce was: Klassen lignin 28.3 ± 0.8, ASL 38.3 ± 0.1, arabinan 1.0 ± 0.1, galactan 2.0 ± 0.1, glucon 43.2 ± 0.2, xylan 6.0 ± 0.1, and mannan 11.3 ± 0.1. With compositional analysis, the only detectable carbohydrates than glucan were below the detection level (i.e. below the lowest concentration of the external calibration curve, Section 2.11). The three constituents reported in Table 1 as the result of the compositional analysis (glucan, Klassen lignin, and ASL) account for 96.5–99.6% of the total mass, which again
indicates that the PSS contained very low amounts of other constituents. Within each series (SA and SD), the glucan content decreased slightly with increasing temperature (Table 1), most probably due to partial degradation of cellulose at higher temperatures. In agreement with that, within each series the content of Klasson lignin increased slightly with increasing temperature (Table 1). The Klasson lignin content was slightly higher for SD-pretreated wood than for the corresponding SA-pretreated wood (Table 1). Similarly, the acid-soluble lignin (ASL) content increased with increasing temperature and was higher in SD-pretreated material than in SA-pretreated material (Table 1). The higher content of ASL in SD-pretreated material may be due to sulfonation and fragmentation of lignin in a similar manner as in sulfite pulping.

The increase of the ASL content in SA-pretreated material with increasing temperature (Table 1) may be due to cleavage of \( \beta-O-4 \) ether bonds generating a more fragmented lignin. Pretreatment under acidic conditions has been reported to result in extensive cleavage of \( \beta-O-4 \) ether bonds in lignin of both softwood and hardwood (Zhang et al., 2016).

Analysis with Py-GC/MS gave partially different information compared to compositional analysis (Table 1). With SD pretreatment the PSS contained more carbohydrate and less lignin than with SA pretreatment (Table 1). As for the higher sulfur content of SD-pretreated material, this again points to a superficial resemblance between SD pretreatment and sulfite pulping, in which wood is delignified in reactions involving bisulfite.

The different results of the compositional analysis and the Py-GC/MS analysis could be attributed to the formation of pseudo-lignin during the pretreatment. Pseudo-lignin is a Klasson-positive (acid-insoluble) aromatic substance that is formed from biomass poly saccharides under both wet and dry thermal treatments (Sannigrahi et al., 2011; Normark et al., 2016). Lignin analysis with Py-GC/MS will cover both acid-insoluble lignin and ASL, but not pseudo-lignin. This is because pseudo-lignin consists of partially decomposed carbohydrates which are further decomposed to carbohydrate-degradation products during pyrolysis and will thus be measured as carbohydrate in analysis using Py-GC/MS. Py-GC/MS even distinguishes between different monomeric units of real lignin, such as p-hydroxyphenyl units, guaiacyl units, and syringyl units (Gerber et al., 2012). As pseudo-lignin does not contain the phenylpropanoid guaiacyl units that are characteristic of real softwood lignin, it will not contribute to the lignin estimation in an analysis by Py-GC/MS. Klasson lignin values will be the sum of the acid-insoluble fraction of the real lignin and the pseudo-lignin. A comparison of the data obtained from these two methods (Table 1) shows that the pseudo-lignin content of the PSS was higher after pretreatment with SD than after pretreatment with SA. SD-H had the lowest lignin content and the highest pseudo-lignin content.

FTIR spectra were acquired from 800 to 1800 cm\(^{-1}\) and were normalized with the band at 1510 cm\(^{-1}\) (aromatic skeletal vibrations) (Hergert, 1971). Compared with untreated spruce, the six PSS samples exhibited signs of removal of hemicelluloses in the region from 800 to 1600 cm\(^{-1}\) where signals from pretreated samples generally overlap. The main difference between pretreated samples was formation of a new band at 1714 cm\(^{-1}\) (Table 1, C=O stretching in unconjugated ketones), which is associated with formation of pseudo-lignin (Sannigrahi et al., 2011). Since the band at 1743 cm\(^{-1}\) (C=O stretching of acetyl groups in hemicellulose; Labbé et al., 2005) appeared in untreated spruce and disappeared in pretreated spruce, it can be assumed that the hemicellulose was the precursor of pseudo-lignin formation. The relative intensity of the band at 1714 cm\(^{-1}\) (1714/1510) (Table 1) indicated an increasing content of pseudo-lignin in the order SA-L < SA-M < SD-L < SD-M < SA-H < SD-H.

### Table 1

<table>
<thead>
<tr>
<th>Sulfur content</th>
<th>Compositional analysis(^a)</th>
<th>Py-GC/MS</th>
<th>FTIR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucan(^d)</td>
<td>Klasson(^d)</td>
<td>ASL(^d)</td>
</tr>
<tr>
<td>SA-L</td>
<td>0.023 ± 0.001</td>
<td>52.2 ± 1.1</td>
<td>40.9 ± 0.3</td>
</tr>
<tr>
<td>SA-M</td>
<td>0.018 ± 0.004</td>
<td>50.1 ± 0.6</td>
<td>43.4 ± 0.1</td>
</tr>
<tr>
<td>SA-H</td>
<td>0.100 ± 0.003</td>
<td>51.2 ± 0.3</td>
<td>41.2 ± 0.6</td>
</tr>
<tr>
<td>SD-L</td>
<td>0.103 ± 0.003</td>
<td>50.7 ± 1.2</td>
<td>42.4 ± 0.4</td>
</tr>
<tr>
<td>SD-M</td>
<td>0.118 ± 0.003</td>
<td>47.0 ± 0.6</td>
<td>45.9 ± 0.3</td>
</tr>
</tbody>
</table>

\(^a\) Values given in percent dry weight (except FTIR ratios).  
\(^b\) According to procedure described by Sluiter et al. (2012).  
\(^c\) Other carbohydrates (arabinan, galactan, xylan, and mannan) were not detectable.  
\(^d\) Klasson lignin, i.e. acid-insoluble lignin fraction.  
\(^e\) Acid-soluble lignin.  
\(^f\) Carbohydrate-related compounds (Py-GC/MS).  
\(^g\) Summary of guaiacyl, syringyl, p-hydroxyphenyl, and generic phenolics (Py-GC/MS).  
\(^h\) Relative intensity of 1714/1510.
That interpretation agrees with literature reports about increased crystallinity after pretreatment (reviewed by Hu and Ragauskas (2012)).

For each catalyst, the highest severity gave the lowest yield of hemicellulosic sugars (Table 2). For a given pretreatment temperature, the yield of hemicellulosic sugars was higher for SA than for SD (Table 2). These results indicate that the lower pretreatment temperatures were sufficient for optimal hemicellulose saccharification, and that high severity pretreatment and use of SD as catalyst tended to result in degradation of hemicellulosic sugars and therefore slightly lower yields.

Previous studies of softwood have indicated that increased severity in the range CS 1.2–3.2 results in higher glucose concentrations and that the concentrations of mannose decrease at CS values over 2.7 (Tengborg et al., 1998). It is well known that high severity pretreatment results in dehydration of monosaccharides to furan aldehydes and further on to carboxylic acids, such as levulinic acid (reviewed by Ko et al. (2015); Jönsson and Martín (2016)).

Formation of by-products during pretreatment is indicated in Table 2 as TAC and TCAC. TAC covers common inhibitory by-products such as phenolics and furan aldehydes, whereas TCAC covers acetic acid, formic acid, and other carboxylic acids. For each series (SA and SD), the contents of TAC and TCAC increased with increasing pretreatment temperature (Table 2). For a given pretreatment temperature, SD pretreatment resulted in higher contents of TAC and TCAC than SA pretreatment (Table 2). As many by-products of pretreatment are derived from hemicellulosic sugars (Ko et al., 2015; Jönsson and Martín, 2016), it is not surprising that high values for TAC and TCAC tend to correspond with low concentrations of hemicellulosic sugars (Table 2).

The intention of the pretreatment is hemicellulose degradation rather than cellulose degradation, as the enzymatic step following the pretreatment targets cellulose. Degradation of hemicellulosic sugars to furan aldehydes and carboxylic acids would in the end lead to lower total sugar yields and formation of inhibitors of the fermenting microorganism. From that point of view, SA-M, SA-L, and SD-L emerged as the most promising pretreatment procedures, whereas SD-H, SD-M, and SA-H gave low yields of hemicellulosic sugars (Table 2).

### 3.3. Effects of SPL on enzyme and yeast

#### Fig. 1

Fig. 1 shows the glucose yields from Avicel hydrolyzed with two enzyme preparations (A and B) using SPL as reaction medium. Except for SD-M, the SA pretreatment liquids had lower inhibitory effect than the SD pretreatment liquids (Fig. 1). Controls with the same monosaccharide contents as the SPL were included in the experimental series to estimate the parts of the inhibitory effects that were caused by sugars. The fractions of glucose released in SPLs and in the corresponding SPL sugar controls were calculated and are indicated above each set of bars. Error bars show standard deviation of triplicate reactions.

![Fig. 1. Glucose yield from Avicel with spruce pretreatment liquid as medium (A: with enzyme preparation A; B: with enzyme preparation B). The fractions of glucose released in SPLs and in the corresponding SPL sugar controls were calculated and are indicated above each set of bars. Error bars show standard deviation of triplicate reactions.](image)

### Table 2

Analysis of liquid phase of pretreated spruce.

<table>
<thead>
<tr>
<th></th>
<th>SA-L</th>
<th>SA-M</th>
<th>SA-H</th>
<th>SD-L</th>
<th>SD-M</th>
<th>SD-H</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS</td>
<td>2.1</td>
<td>2.2</td>
<td>2.5</td>
<td>2.1</td>
<td>2.2</td>
<td>2.3</td>
</tr>
<tr>
<td>pH</td>
<td>1.6</td>
<td>1.7</td>
<td>1.7</td>
<td>1.6</td>
<td>1.7</td>
<td>1.9</td>
</tr>
<tr>
<td>Conductivity (μS/cm)</td>
<td>3.98 ± 0.06</td>
<td>3.86 ± 0.02</td>
<td>3.94 ± 0.04</td>
<td>4.76 ± 0.08</td>
<td>4.95 ± 0.06</td>
<td>4.46 ± 0.04</td>
</tr>
<tr>
<td>Glc (g/L)</td>
<td>15.1 ± 0.3</td>
<td>16.6 ± 0.5</td>
<td>18.3 ± 0.3</td>
<td>20.5 ± 0.5</td>
<td>31.7 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>Man (g/L)</td>
<td>30.1 ± 1.0</td>
<td>31.1 ± 1.7</td>
<td>26.0 ± 0.4</td>
<td>30.3 ± 1.1</td>
<td>23.1 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>Xyl (g/L)</td>
<td>17.5 ± 0.6</td>
<td>17.5 ± 1.0</td>
<td>13.7 ± 0.3</td>
<td>16.5 ± 0.6</td>
<td>17.7 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Gal (g/L)</td>
<td>6.3 ± 0.1</td>
<td>6.3 ± 0.1</td>
<td>5.6 ± 0.1</td>
<td>5.8 ± 0.1</td>
<td>8.0 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Ara (g/L)</td>
<td>4.3 ± 0.1</td>
<td>4.1 ± 0.2</td>
<td>3.2 ± 0.1</td>
<td>3.7 ± 0.2</td>
<td>2.1 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Hemicellulosic sugars (g/L)</td>
<td>58.1 ± 1.8</td>
<td>58.9 ± 3.0</td>
<td>48.4 ± 0.9</td>
<td>56.3 ± 1.9</td>
<td>42.5 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>TACc</td>
<td>0.59 ± 0.01</td>
<td>0.65 ± 0.01</td>
<td>1.61 ± 0.01</td>
<td>1.13 ± 0.01</td>
<td>2.74 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>TCACd (mM)</td>
<td>146.1 ± 1.2</td>
<td>164.2 ± 0.9</td>
<td>193.5 ± 1.6</td>
<td>171.6 ± 2.8</td>
<td>178.9 ± 0.4</td>
<td></td>
</tr>
</tbody>
</table>

* Properties of slurries with 25% TS.

* Combined severity.

* Total Aromatic Content determined as absorption at 280 nm. Indicated values correspond to a dilution factor of 500.

* Total Carboxylic Acid Content determined by titration.
the SA pretreatment liquids and (ii) that for each series of sample (a certain enzyme preparation combined with a certain impregnating agent) the inhibitory effect always increased with increasing pretreatment temperature.

Phenolic substances inhibit cellulolytic enzymes (Ximenes et al., 2011), an effect that can be alleviated by hydrophilization of the inhibitors through sulfonation in reactions not involving monosaccharide sugars (Cavka and Jönsson, 2013). Recent results indicate that the inhibitory effect remaining after deduction of the monosaccharide inhibition could largely be attributed to aromatics, such as phenolics (Zhai et al., 2016).

Table 3 shows hexose (glucose, mannose, and galactose) consumption data and ethanol yield data for fermentation of the SPLs with yeast. Hexose consumption followed the order SD-H > SD-M > SD-L > SA-H > SA-M > SA-L > control. Thus, hexose consumption was always lower with SD pretreatment than with SA pretreatment, and, for each impregnating agent, hexose consumption decreased as the pretreatment temperature increased. The ethanol yields on consumed fermentable sugar were essentially the same for SA pretreatment liquids and (ii) that for each series of sample (a certain enzyme preparation combined with a certain impregnating agent) the inhibitory effect always increased with increasing pretreatment temperature.

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![Fig. 2. Adsorption of Simons' stain dyes on pretreated spruce. Error bars show standard deviation of duplicate assays.](image)

The result that SD pretreatment resulted in poorer fermentability than SA pretreatment is surprising considering previous reports in the literature about the opposite (Tengborg et al., 1998; Martín et al., 2002). It is noteworthy, however, that Tengborg et al. (1998) found a sharp decrease in fermentability of SA-pretreated spruce above a CS of 3, which is higher than the range we investigated (CS 2.1–2.5), and Martín et al. (2002) used harsher conditions (205 °C for 10 min) than we did and investigated sugarcane bagasse, which might be less recalcitrant than softwood. Furthermore, both studies (Tengborg et al., 1998; Martín et al., 2002) were based on conventional batch steam pretreatment equipment, whereas our study was based on an advanced continuous steam pretreatment process. Thus, there are fundamental differences between our investigation and previous work in the field (Tengborg et al., 1998; Martín et al., 2002), which may explain different findings with regard to fermentability.

### 3.4. Cellulose accessibility of PSS

The accessibility of the PSS to enzymes was investigated using the Simons' stain method (Yu et al., 1995; Arantes and Saddler, 2011). The results are presented in Fig. 2. Surprisingly, there were no obvious differences between SA and SD. Possibly, there was a tendency that pretreatments performed at the lowest temperature (195 °C) displayed greatest adsorption of total dyes and that pretreatments performed at the intermediate temperature (205 °C) displayed least adsorption (Fig. 2).

### 3.5. Particle size distribution of PSS

The effects of the pretreatments on particle size distribution were also determined and the results are shown in Fig. 3. Particles were divided into four size ranges: 50–100 μm, dust; 100–500 μm, fine particles, 500–800 μm, small particles, and > 800 μm, large particles. The result shows that the fraction with dust and fine particles increases in the order SA-L < SA-M < SA-H < SD-L < SD-M < SD-H. The increase was more significant from the intermediate to the high temperature than from the low to the intermediate temperature. For SD-H, the fraction of dust and fine particles was as high as 94%. Similarly, the fraction of large particles decreased correspondingly.

Delignification through SD pretreatment with concomitant hydrolysis of amorphous regions of cellulose would contribute to fractionation and increased formation of dust and fine particles in the PSS, with higher temperatures resulting in a faster process. Regression analysis points towards a positive correlation between the ASL content and the fractions of dust and fine particles (R² 0.98). There was also a positive correlation (R² 0.96) between the glucose content in the pretreatment liquids and the fractions of dust and fine particles.

### Table 3

Comparison of the inhibitory effect of the pretreatment liquids on fermentation with yeast.

<table>
<thead>
<tr>
<th>Main hexose fraction (galactose, glucose and mannose) (g/L)</th>
<th>Ethanol yield</th>
<th>BEY</th>
<th>Ethanol productivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Start After 12 h Consumed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sugar C</td>
<td>24.3 ± 0.4</td>
<td>1.6 ± 0.1</td>
<td>22.7 ± 0.3</td>
</tr>
<tr>
<td>SA-L</td>
<td>22.4 ± 0.7</td>
<td>4.5 ± 0.3</td>
<td>17.9 ± 0.3</td>
</tr>
<tr>
<td>SA-M</td>
<td>24.1 ± 0.4</td>
<td>8.1 ± 0.8</td>
<td>16.0 ± 0.5</td>
</tr>
<tr>
<td>SA-H</td>
<td>20.7 ± 0.4</td>
<td>12.7 ± 0.4</td>
<td>8.0 ± 0.1</td>
</tr>
<tr>
<td>SD-L</td>
<td>26.2 ± 0.5</td>
<td>18.8 ± 0.2</td>
<td>7.4 ± 0.3</td>
</tr>
<tr>
<td>SD-M</td>
<td>22.6 ± 0.7</td>
<td>21.2 ± 0.9</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>SD-H</td>
<td>24.5 ± 0.4</td>
<td>24.4 ± 0.3</td>
<td>0.1 ± 0.1</td>
</tr>
</tbody>
</table>

* The table shows the values at start and after 12 h of fermentation. Pretreatment liquids were diluted corresponding to a TS of 12%.

** Sugar control.

*** Balanced Ethanol Yield: g EtOH/g consumed galactose, glucose and mannose.

**** g EtOH × L⁻¹ × h⁻¹.
3.6. Enzymatic digestibility of PSS with buffer as medium

The digestibility of PSS was analyzed through analytical enzymatic saccharification, i.e. non-exhaustive saccharification of the biomass designed to reveal differences in susceptibility to cellulolytic enzymes. The results are presented in Fig. 4A.

For both enzyme preparations, A and B, the release of glucose from PSS followed the order SA-L < SA-M < SA-H < SD-L < SD-M < SD-H (Fig. 4A). For enzyme preparation A the glucan digestion (% g anhydrous glucose per 100 g glucan in PSS) increased from 14% for SA-L to 56% for SD-H. For enzyme preparation B the glucose yield increased in the same order, from 21% for SA-L to 99% for SD-H. For pretreatments performed at the same temperature, SD pretreatment resulted in 2.0–2.7 times higher glucose yield than SA pretreatment with enzyme preparation A, and 2.4–2.9 times higher with enzyme preparation B.

The increase in glucose yield for SA-M compared to SA-L (10% for enzyme preparation A and 24% for enzyme preparation B) was lower than the increase for SD-M compared to SD-L (31% for enzyme preparation A and 43% for enzyme preparation B). This difference was less apparent for higher pretreatment temperatures, as the glucose yield for SA-H compared to SA-M increased with 33% for enzyme preparation A and with 31% for enzyme preparation B, whereas the glucose yield for SD-H compared to SD-M increased with 52% for enzyme preparation A and 39% for enzyme preparation B. Thus, for a given enzyme preparation the increase in digestibility with increasing pretreatment temperature was higher for SD-pretreated than for SA-pretreated spruce.

The digestibility of the PSS (Fig. 4A) increased in the same order as the fraction of dust and fine particles (Fig. 3). Indeed, regression analysis showed a positive correlation with R² values of 0.98 for enzyme preparation A and 0.97 for enzyme preparation B. This can explain both the superior enzymatic digestibility of SD-pretreated compared to SA-pretreated spruce and the superior enzymatic digestibility of the material pretreated at higher temperatures.

Bisulfite formed from SD caused partial delignification of the spruce wood and the reaction proceeded faster at higher temperature. When more lignin was degraded, more ASL was formed and more cellulose was exposed, which led to increasing hydrolysis of amorphous regions of the cellulose under the acidic conditions of the pretreatment. Degradation of amorphous regions of cellulose would create smaller cellulotic fragments, and then the particle size would be reduced. Moreover, when smaller particles with shorter cellulose chains were formed, more cellulose end groups were created. Since these end groups serve as targets for cellulbiohydrolyases, enzymatic saccharification would be improved. Regression analysis of the correlation between variables indicative of this tentative chain of events (from ASL content to glucose concentration in pretreatment liquid, and further on to size fractions and enzymatic digestibility) resulted in R² values in the range 0.96–0.99.
3.7. Enzymatic digestibility of PSS with SPL, as medium

As pretreatment with SD as catalyst and pretreatment at higher temperature resulted in superior enzymatic digestibility but a pretreatment liquid that was more inhibitory to cellulosic enzymes, an additional digestibility study was performed to investigate both of these effects taken together. Pretreatment liquids corresponding to both 25% and 12.5% TS were studied. The lower concentration (12.5%) was included as efficient mixing of 25% TS with enzyme might be technically challenging and lead to decreased digestion for that reason. With 25% TS (Fig. 4B), the slurries from SA pretreatment showed similar glucon digestion regardless of the pretreatment temperature (27–28%) with enzyme preparation A and 38–42% with enzyme preparation B). SD pretreatment resulted in higher glucon digestibility than SA pretreatment and generally the digestibility increased with the pretreatment temperature (39–51% for enzyme preparation A and 57–70% for enzyme preparation B). The experimental series with 12.5% TS (Fig. 4C) showed a similar tendency. The SA-pretreated material exhibited similar glucon digestibility regardless of the pretreatment temperature (38–39% with enzyme preparation A and 46–51% with enzyme preparation B). The SD-pretreated material exhibited higher digestibility than the SA-pretreated material (51–71% with enzyme preparation A and 67–88% with enzyme preparation B). The higher glucon digestibility with 12.5% TS than with 25% TS can be attributed to superior enzymatic digestibility. Further research is needed into the use of SD and high pretreatment temperature caused more degradation of lignin and cellulose resulting in smaller particle size, which correlated well with enzymatic digestibility. Further research is needed to investigate the effects of different acid catalysts on formation of specific inhibitors and the potential economic benefits of using SD for pretreatment.

4. Conclusions

Using SD and higher temperatures in the pretreatment of spruce wood caused increased inhibition of both enzymatic and microbial biocatalysts, and increased formation of pseudo-lignin. Despite of that, spruce pretreated with SD at high temperatures exhibited superior enzymatic digestibility. The discrepancy was resolved by the finding that the use of SD and high pretreatment temperature caused more degradation of lignin and cellulose resulting in smaller particle size, which correlated well with enzymatic digestibility. Further research is needed to investigate the effects of different acid catalysts on formation of specific inhibitors and the potential economic benefits of using SD for pretreatment.

Declaration of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2017.09.081.

References


