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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 cellulolytic 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 cellulolytic enzymes of wood pretreated with SD was 2.0–2.9 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 (R^2 0.97–0.98).

1. Introduction

Lignocellulosic biomass is an abundant bioresource that can be an ideal feedstock for biorefineries. Pretreatment is required for disrupting the resistant structure of lignocellulosic biomass to make the cellulose more accessible for biochemical conversion using cellulolytic 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 phenylic 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). Hydrophilization 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

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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 1 kg SD/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 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 monosaccharide contents in the spruce pretreatment liquids (SPL) were determined by using high-performance anion-exchange chromatography (HPAEC) (Section 2.11). Degradation by-products were measured as Total Aromatic Content (TAC) and Total Carboxylic Acid Content (TCAC). TAC, which includes heteroaromatics, such as 5-hydroxymethylfurfural (HMF) and furfural, and aromatics, such as phenols, was measured as absorbance at 280 nm using a UV1800 spectrophotometer (Shimadzu, Kyoto, Japan). This wavelength was selected as the six pretreatment liquids showed absorption maxima at 279–282 nm, and as quantitatively important heteroaromatics and aromatics exhibit absorption maxima close to 280 nm: HMF, 284 nm; furfural, 278 nm; vanillin, 279 nm. TCAC was determined through titration from pH 2.8 to pH 7.0 using a 200 mM aqueous solution of sodium hydroxide. The conductivity was measured using a conductivity meter (CO 3100 L, VWR, USA). The pretreated spruce solids (PSS) were washed six times with deionized water until glucose was no longer detectable in the washing liquid using a glucometer (Accu-Chek Aviva, Roche Diagnostics GmbH). The washed material was freeze-dried before further analysis.

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 Novozyme 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 safe-seal microcentrifuge tubes in an orbital shaker set at 170 rpm and 45 °C (Ecotron 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, 75 g/L $(NH_4)_2HPO_4$, 3.75 g/L $MgSO_4 \cdot 7\ H_2O$, 238.2 g/L $NaH_2PO_4 \cdot H_2O$), and yeast inoculum [Ethanol Red yeast (Fermentis, 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 HPAEC (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 Bränslelaboratoriet (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).