Acetone–Butanol–Ethanol Fermentation of Non-detoxified Dilute Acid extracted Hemicellulosic Hydrolysate from the Short-rotation Coppice *Salix schwerinii* E. Wolf

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The dilute acid-catalyzed extraction of hemicellulosic monosaccharides from the short-rotation coppice *Salix schwerinii* E. Wolf was optimized to find a balance between the yields of fermentable monosaccharides and sugar degradation products for non-detoxified acetone-butanol-ethanol fermentation with *Clostridium acetobutylicum*. At the same time, the cellulosic and lignin fractions were kept as intact as possible for further refining. The highest xylose and glucose yields in the liquid prehydrolysate were 65% and 9.45% of their respective original content at a combined severity of 2.29. Increased concentrations of glucose and sugar degradation products in the liquid prehydrolysates were detected with an increasing pretreatment temperature and combined severity, while the acid-insoluble lignin remained stable. During the fermentation of the non-detoxified liquid prehydrolysate with grain starch as the supplement, 66% of the xylose was utilized, and the butanol and acetone-butanol-ethanol yields were 0.22 g/g and 0.35 g/g monosaccharides, respectively, in the fermentation medium. The results suggested that the hemicellulose in *S. schwerinii* can be separately extracted as a side stream and utilized for butanol fermentation with starch-based materials without a separate detoxification stage.

Keywords: Short rotation coppice; Salix schwerinii; Pretreatment; Acetone-butanol-ethanol fermentation; Clostridium acetobutylicum; Biorefining

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INTRODUCTION

The implementation of bioeconomy strategies has created a growing demand for renewable products, and lignocellulosic biomass can be a vast resource in the bioeconomy. However, growing demand has raised the question of the sufficiency of naturally occurring feedstocks. Short-rotation coppicing (SRC) of *Salix* and *Poplar* species has been presented as one of the most effective ways to produce lignocellulosic biomass with a limited amount of land (Makechin 1999). The effective vegetative propagation from cuttings, high ability to resprout, wide genetic reserve, ease of breeding, and potential to produce high yields of biomass over short time periods in wide climatic conditions are characteristics that support the use of *Salix* species for SRC (Volk

et al. 2004).

During SRC, *Salix* can produce woody lignocellulosic biomass in three-year harvesting cycles (Grigoras *et al.* 2017), which makes *Salix* a potential species for improving the sufficiency of lignocellulosic biomass for different industrial applications in the modern bioeconomy. *Salix viminalis* has been widely used in research on biomass production potential, biomass quality, and clonal breeding (Mleczec *et al.* 2010; Larsen *et al.* 2014; Liu *et al.* 2016).

However, *S. schwerinii*, a species genetically very close to *S. viminalis* (Berlin *et al.* 2011), has been proven to be more suitable for short-rotation wood biomass production in northern European climates (Tahvanainen and Rytkönen 1999). *Salix schwerinii* is an interesting material for biorefining research and has been used in clonal breeding with other *Salix* species (Larsen *et al.* 2014).

Traditionally, *Salix* species have received much attention in bioenergy production research. Also, studies have been conducted on pulp production from *Salix* biomass (Sassner *et al.* 2008; Ai and Tschirner 2010; Lavoie *et al.* 2010; Pesonen *et al.* 2014; Pinto *et al.* 2015). Still, the cellulose in wood materials is the main fraction utilized in chemical pulping processes, while most of the hemicellulose, lignin, and cooking inorganics end up in energy production. However, the heating value of hemicellulose is only 13.6 MJ/kg, which is half that of lignin, and conversion of hemicellulosic sugars, such as pentoses, to more valuable products is in that sense highly desirable (Van Heiningen 2006; Shokri and Adibkia 2013).

An example of a product refined from hemicellulosic sugars is biobutanol, which is a platform chemical, but can also be directly utilized as a transportation fuel. When biobutanol is used as a fuel, no modification of a car engine is needed (Dürre 2007). Butanol is also far less corrosive than ethanol. Biochemically, butanol is produced mainly by *Clostridium* bacteria *via* acetone-butanol-ethanol (ABE) fermentation (Zhang *et al.* 2012), and sugar- and starch-based materials are traditionally used as substrates (Madihah *et al.* 2001; Ezeji *et al.* 2007; Li *et al.* 2014). The price of the sugar- or starch-based substrate for biobutanol fermentation is a major factor when determining the profitability of butanol production (Napoli *et al.* 2010). Consequently, much of the economic feasibility research that has been conducted on biobutanol production has focused on identifying renewable and low-price substrates for biobutanol production from agricultural waste, food, and food industry waste (Qureshi *et al.* 2007; Dwidar *et. al.* 2012; Raganati *et al.* 2013; Yang *et al.* 2013; Gao 2016). Lignocellulosic sugars, such as xylose from residual process streams, can provide a cost-efficient option for butanol production.

In addition to the substrate price, another factor that highly affects the feasibility of biochemical butanol production is the pretreatment stage, which is necessary for lignocellulosic biomass and influences the yield of fermentable sugars from hemicellulose. The purpose of the pretreatment stage or prehydrolysis is to alter the structure of the lignocellulosic biomass and enhance the efficiency of the subsequent biorefining process stages (*e.g.*, pulping or enzymatic saccharification of cellulose).

There are several pretreatment methods, each of which has advantages and disadvantages (Alvira *et al.* 2010; Brodeur *et al.* 2011). Chiaramonti *et al.* (2012) estimated that the cost of pretreatment accounts for approximately 20% of the total biorefining process cost, and thus it has a considerable influence on the economic feasibility of the process.

During pretreatment, depending on the methods and conditions used,

lignocellulose is converted to a variety of products, such as hemicellulosic and cellulosic sugars and lignin-derived compounds (Borrega *et al.* 2013). For example, water prehydrolysis is used during dissolving pulp production to remove most of the hemicellulose, as well as in mechanical pulping to soften the wood structure and save energy during the pulping process. Hot water or steam creates acidic conditions in the lignocellulosic material *via* water autohydrolysis, and the water functions as a pretreatment before pulping, facilitating the formation of refinable intermediate chemicals (*e.g.*, sugars). To increase the pretreatment effect of water, different acids or alkali can be added to the pretreatment liquid. The advantage of the acid-catalyzed pretreatment process is the effective hydrolysis and solubilization of hemicellulose, but the disadvantage is the partial degradation of hemicellulosic monosaccharides (Brodeur *et al.* 2011). Degradation products, such as carboxylic acids (acetic acid and formic acid), furfural, and hydroxymethylfurfural (HMF), and phenolic compounds inhibit the growth of fermentative microorganisms (Ezeji *et al.* 2007).

To overcome this challenge, various different chemical, biological, and physical methods have been tested or used for detoxifying of prehydrolysates (Jönsson *et al.* 2013; Jönsson and Martín 2016). High concentrations of inhibitors in prehydrolysates lead to complicated detoxification and conditioning stages during the biorefining process and cause additional costs (Pienkos and Zhang 2009).

Challenges in inhibition during microbial biorefining processes are likely to become more and more important as processes based on high dry-matter content, high product concentrations, and processes with recirculation of process water are developed (Jönsson *et al.* 2013). Even small changes in the pretreatment conditions, such as the pH and temperature, can noticeably affect especially the hemicellulose solubilization from the solid material to the liquid prehydrolysate and the formation of harmful degradation products (Yang *et al.* 2013, Kuittinen *et al.* 2016). Additionally, changes in the pretreatment conditions affect the quality of the remaining solid fraction and in that sense the applicability of the pretreated material. When the utilization of the lignocellulosic biomass is well-designed, different techniques can be used at the same biorefinery according the idea of Kamm and Kamm (2004) to produce a variety of products from the same material.

Therefore, the emphasis of this work is 1) optimization of hemicellulosic monosaccharides (mainly xylan as monomeric xylose form) extraction using dilute sulfuric acid pretreatment with very low concentrations of acid to avoid serious degradation of the monosaccharides, 2) conserving the cellulosic and lignin fractions in the remaining solid fraction after pretreatment to enable further refining or modification, and 3) testing the fermentability of the non-detoxified hemicellulosic prehydrolysate to butanol *via* ABE fermentation using *C. acetobutylicum*.

EXPERIMENTAL

S. schwerinii Material and Pretreatment Procedure

The experimental plot of *S. schwerinii* (0.4 ha) was located in Siikasalmi, North Karelia, Finland. The material was harvested at the age of six years, debarked, chipped, transported to the laboratory, air-dried (30 °C), milled to a particle size of less than 1 mm, and stored in paper bags. The chemical composition of the *S. schwerinii* material is presented in Table 1.

The air-dried *S. schwerinii* material (10 g dry weight) and pretreatment solution (H_2O, 0.05% (w/v) H_2SO_4, 0.1% (w/v) H_2SO_4, and 0.15% (w/v) H_2SO_4) were mixed at a ratio of 1:10 in a steel cylinder and heated to 170 °C or 200 °C under a corresponding pressure. After cooling to room temperature, the mixture was separated by filtering (Whatman 589/1, Schleicher and Schuell, Little Chalfont, UK), and the liquid prehydrolysate was stored at -18 °C until the carbohydrates and degradation products were analyzed. The solids from the cylinder and filtrate were washed with water and stored at -18 °C until enzymatic hydrolysis and analysis of the residual carbohydrates, acid-insoluble lignin, and ASL were conducted.

Table 1. Chemical Composition of the *S. schwerinii* Biomass

Component	*Salix schwerinii* (% dry basis)
Xylan	17.57
Glucan	43.99
Arabinan	0.29
Galactan	0.68
Mannan	1.56
Rhamnan	0.39
Klason lignin	21.26
Acid soluble lignin	2.48
Ash	0.52
Extractives	3.42

Enzymatic Hydrolysis

Enzymatic hydrolysis was performed using a commercial cellulase mixture (Celluclast 1.5L, Sigma-Aldrich, Saint Louis, MO, USA) (10 filter paper units (FPUs)/g of dry matter) and β-glucosidase (Novozyme 188, Sigma-Aldrich) (200 nkat/g of dry matter) after 48 h of incubation at 45 °C. The hydrolysis samples consisted of a solid prehydrolysate (1% of dry matter) in 50 mM sodium citrate buffer (pH = 5.0). The samples were prepared in triplicate with substrate blanks.

Microorganism Culture Conditions and ABE Fermentation

Freeze-stored *C. acetobutylicum* (DSM 1731, DSMZ, Braunschweig, Germany) was activated in RCM media for 14 h to 16 h (Hirsch and Grinsted 1954). Then, active growing cells (1 mL) were inoculated in 50 mL of sterilized pre-fermentation P2 media prepared in a 125-mL screw-capped bottle. The P2 media contained 30 g/L glucose and 1 g/L yeast extract. Before inoculation, each filter-sterilized stock solution (buffer: 50 g/L KH_2PO_4, 50 g/L K_2HPO_4, and 220 g/L ammonium acetate; minerals: 20 g/L $MgSO_4 \cdot 7H_2O$, 1 g/L $MnSO_4 \cdot H_2O$, 1 g/L $FeSO_4 \cdot 7H_2O$, and 1 g/L NaCl; and vitamins: 0.1 g/L para-aminobenzoic acid, 0.1 g/L thiamin, and 0.001 g/L biotin) was added to the P2 media. The culture was allowed to grow for approximately 16 h at 37 °C before inoculation in the ABE production media.

The liquid prehydrolysate (30 mL) was mixed in 125-mL screw-capped bottles with 20 mL of heat-treated (121 °C for 20 min) barley grain slurry that contained starch, and the mixture was used as the ABE production media after a pH adjustment to 6.5 with 10 M NaOH before fermentation. The pure heat-treated barley grain slurry and glucose media were used as control fermentations. The media were purged with N_2 for 10 min to

maintain anaerobic conditions and then were sterilized (121 °C for 20 min).

Fermentation began at 37 °C when the *C. acetobutylicum* DSM 1731 culture (10%, v/v) was inoculated. Fermentation samples were collected after 0 h, 24 h, 48 h, 72 h, 96 h, 120 h, and 144 h of incubation. The ABE fermentation was conducted in duplicate.

Chemical Analyses

The ash, extractives, lignin, and sugar contents from the solid materials were determined according to Hayes (2012). For the analysis of the extractives from the original *S. schwerinii* material, ethanol and water were used. For determining the Klason acid-insoluble lignin and ASL contents, the TAPPI UM 250 (1991) method was used. The efficiency of the enzymatic hydrolysis of the solid prehydrolysates was estimated by the reducing sugar yield (RSY), which was measured with the DNS method using a spectrophotometer at a wavelength of 540 nm (Miller 1959).

During the hydrolysis of the original *S. schwerinii* material and solid prehydrolysate to their carbohydrate and lignin components, 3 mL of 72% H_2SO_4 was added to a 300-mg sample, which was followed by incubation for 1 h at 30 °C. The mixture was stirred every 5 min. After incubation, the mixture was diluted to 4% H_2SO_4 by adding water and autoclaved at 121 °C for 60 min. Standard samples with 10 mL of a known sugar solution and 348 µL of 72% H_2SO_4 were prepared and autoclaved to determine the sugar loss during autoclaving.

The autoclaved samples and standard mixtures were vacuum filtered through filter crucibles with a known weight, which was followed by the analysis of the sugar composition. The ASL was analyzed on a DIONEX ICS-3000 ion chromatography system (California, USA) consisting of an electrochemical detector (using pulsed amperometric detection), gradient pump, temperature-controlled column, and detector enclosure with an AS50 autosampler that had an injection volume of 10 µL (Hayes 2012).

Hydrogen nuclear magnetic resonance (^{1}H NMR) spectroscopy was used to quantify the carbohydrates and their derivatives in the liquid prehydrolysate, the fermentation products of ethanol, acetone, butanol, acetic acid, and butyric acid, and the residual sugars glucose and xylose in the fermentation media (Yang *et al.* 2015). A Bruker AVANCE 500 DRX NMR spectrometer (Birmingham, UK) equipped with a 5-mm QNP SB probe was used to perform the ^{1}H NMR spectroscopy. The ^{1}H NMR spectra were collected with water presaturation (zgcppr) using a 90° pulse angle, 48-dB presaturation power, 20-s relaxation delay, and with 16 scans at 300 K. Before the NMR measurements, 200 µL of the sample liquid was transferred to a 5-mm NMR tube, which was followed by the addition of D_2O (275 µL) and 3-(trimethylsilyl)-propionic-d_4 acid (25 µL, 20 mM) in D_2O as an internal standard with a known concentration.

Calculations

The hemicellulose sugar yields from the liquid prehydrolysate were calculated as mg polysaccharide/g original dry *S. schwerinii* material and as percentages (%) of their individual contents in the original *S. schwerinii* material. The combined severity (*CS*) factors under the various pretreatment conditions were calculated using Eq. 1,

$$CS = log\{t \times exp[(T_H - T_R)/14.75]\} - pH \quad (1)$$

where t is the reaction time (min), T_H is the reaction temperature (°C), and T_R is the

reference temperature (100 °C).

RESULTS AND DISCUSSION

Hemicellulosic Xylose Extraction from *S. schwerinii* Biomass with Dilute Sulfuric Acid-catalyzed Pretreatment

Xylan was the main hemicellulosic polysaccharide in the original *S. schwerinii* material. Thus, monosaccharidic xylose was taken as an example to present the hemicellulose extraction from *S. schwerinii* (Table 2). Results of sugars are calculated as polysaccharidic form (mg/g original dry matter) to make the comparison between original materials and liquid prehydrolysate more clear.

Table 2. Pretreatment Conditions and Composition of the Liquid and Solid Prehydrolysates for the Dilute Acid Experiments of the *S. schwerinii* Biomass

Experiment	1	2	3	4	5	6	7	8
Pretreatment conditions								
Temperature (°C)	170	170	170	170	200	200	200	200
H_2SO_4 concentration (%)	0	0.05	0.1	0.15	0	0.05	0.1	0.15
Pretreatment time (min)	44	47	50	51	38	34	33	36
CS	0.37	1.11	1.59	1.77	1.23	1.87	2.29	2.51
Sugars in liquid prehydrolysate								
Xylan*	0	20.39	88.66	109.21	30.54	95.12	114.66	96.35
Glucan*	0	10.57	22.89	22.59	10.98	30.87	42.17	75.83
Total sugars*	0	30.96	111.55	131.80	41.52	125.99	156.83	172.18
Furans and acids in liquid prehydrolysate								
Furfural	0	0.06	0.21	0.26	0.60	1.15	1.75	2.87
HMF	0	0.01	0.02	0.03	0.05	0.14	0.25	0.42
Acetic acid	0.35	1.01	3.32	4.13	2.08	3.70	5.34	5.79
Formic acid	0.09	0.11	0.16	0.13	0.29	0.25	0.17	0.28
Levulinic acid	0	0	0	0	0	0	0	0.10
Sugars in solid prehydrolysate								
Xylan	130.26	62.09	41.25	33.48	29.65	18.46	7.42	2.43
Arabinan	0.37	0.22	0.14	0.07	0.14	0.13	0.06	n.d.
Glucan	473.79	404.30	434.79	438.03	438.45	413.79	410.07	374.99
Total sugars	604.42	466.61	476.18	471.58	468.24	432.39	417.55	377.42
Lignin in solid prehydrolysate								
Klason	23.66	21.46	21.59	22.13	23.45	23.05	22.62	22.36
ASL	1.35	1.1	0.95	1.03	1.02	0.79	0.81	0.86

*results of sugars are calculated as polysaccharidic form mg/g original dry matter basis to make the comparison between original materials and liquid prehydrolysate easier; Furans and acids are presented as mg/mL; Klason = acid-insoluble lignin and ASL = acid-soluble lignin, both are presented as % of original dry matter; n.d. = not determined

The monosaccharide xylose yield in the liquid prehydrolysate was highest at *CS* values of 1.59 and 2.29 (Table 2). Calculated as polysaccharidic xylan, the yields were 62% and 65%, respectively. In general, the amount of extracted xylan increased when the *CS* value increased from 0.37 to 2.29; this was also shown by the residual amount of xylan in the solid prehydrolysate decreasing at the same time. Pretreatment at 170 °C with H_2O (experiment 1) resulted in no monosaccharide xylose in the liquid prehydrolysate. However, when the pretreated solid material was analyzed, the xylan content had decreased from 100% to 74.2% of the original (130.3 mg/g). This result showed that pretreatment with H_2O at 170 °C had extracted the *S. schwerinii* hemicellulosic xylan from the polysaccharide form to the oligosaccharide and smaller polysaccharide forms. During previous hot water extractions, a considerable portion of xylan extracted from hardwood materials has been reported to remain in a higher molecular form than monosaccharides, either as smaller polysaccharides or oligosaccharides (Borrega *et al.* 2011).

Similar to the xylan extraction, the concentrations of furfural and HMF in the liquid prehydrolysate increased with an increasing pretreatment severity (Fig. 1A). However, the concentrations of these degradation products were higher after the pretreatment at 200 °C, even at the same *CS* levels (Fig. 1). For example, at a *CS* of 2.29, the xylan extraction to the liquid prehydrolysate was close to that at a *CS* of 1.77, but the xylan content in the solid pretreated materials differed (7.420 mg/g and 33.48 mg/g xylan for a *CS* of 2.29 and 1.77, respectively). This result showed the effect of the higher pretreatment temperature on the *S. schwerinii* biomass. The liberated monosaccharide xylose was further degraded to furfural, as the furfural concentration (formed by the loss of chemically bound water from pentose carbohydrates) after the pretreatment at a *CS* of 2.29 was 1.75 mg/mL, which was at least six times higher than the concentration after the pretreatment at a *CS* of 1.77 (0.26 mg/mL). The highest furfural (2.87 mg/mL) and HMF (0.42 mg/mL) concentrations were detected at a *CS* of 2.51 (experiment 8). At the same time, a decrease in the concentration of xylan extracted to the liquid prehydrolysate was observed. The concentration of acetic acid increased from 0.35 mg/mL to 5.79 mg/mL for experiments 1 to 8 (Table 2). The concentration of acetic acid clearly increased with an increase in the *CS*, while the amount of formic acid remained low in all of the experiments (Fig. 1B). The lowest formic acid concentration was 0.09 mg/mL, and the highest concentration was 0.29 mg/mL. In a previous study, a detrimental effect was found on the fermentation processes with *C. acetobutylicum* when the formic acid concentration reached 0.4 g/L (Cho *et al.* 2012).

The amount of residual carbohydrates in the pretreated solid materials is an important factor if the solid fraction after pretreatment is considered for utilization, such as in pharmaceutical applications or dissolving pulp. When the solid *S. schwerinii* materials were pretreated at a *CS* of 2.29 and 2.51, only 4.22% and 1.39% of the original xylan content (7.42 mg/g and 2.43 mg/g) were detected, which meant that nearly all of the hemicellulose xylan was extracted from the material at those pretreatment conditions (Table 2). According to Sixta (2006), the highest-purity dissolving pulps (acetate-grade pulps) should contain less than 2% residual xylan and at least 97% cellulose. Thus, the pretreatment with optimal conditions for hemicellulosic sugar extraction and a minimal effect on the cellulose fraction enables the efficient use of both fractions in industrial applications. From this point of view, the *S. schwerinii* biomass after acid pretreatment at a *CS* of 2.29 could have provided pentose-free natural fiber material for further testing in different applications.

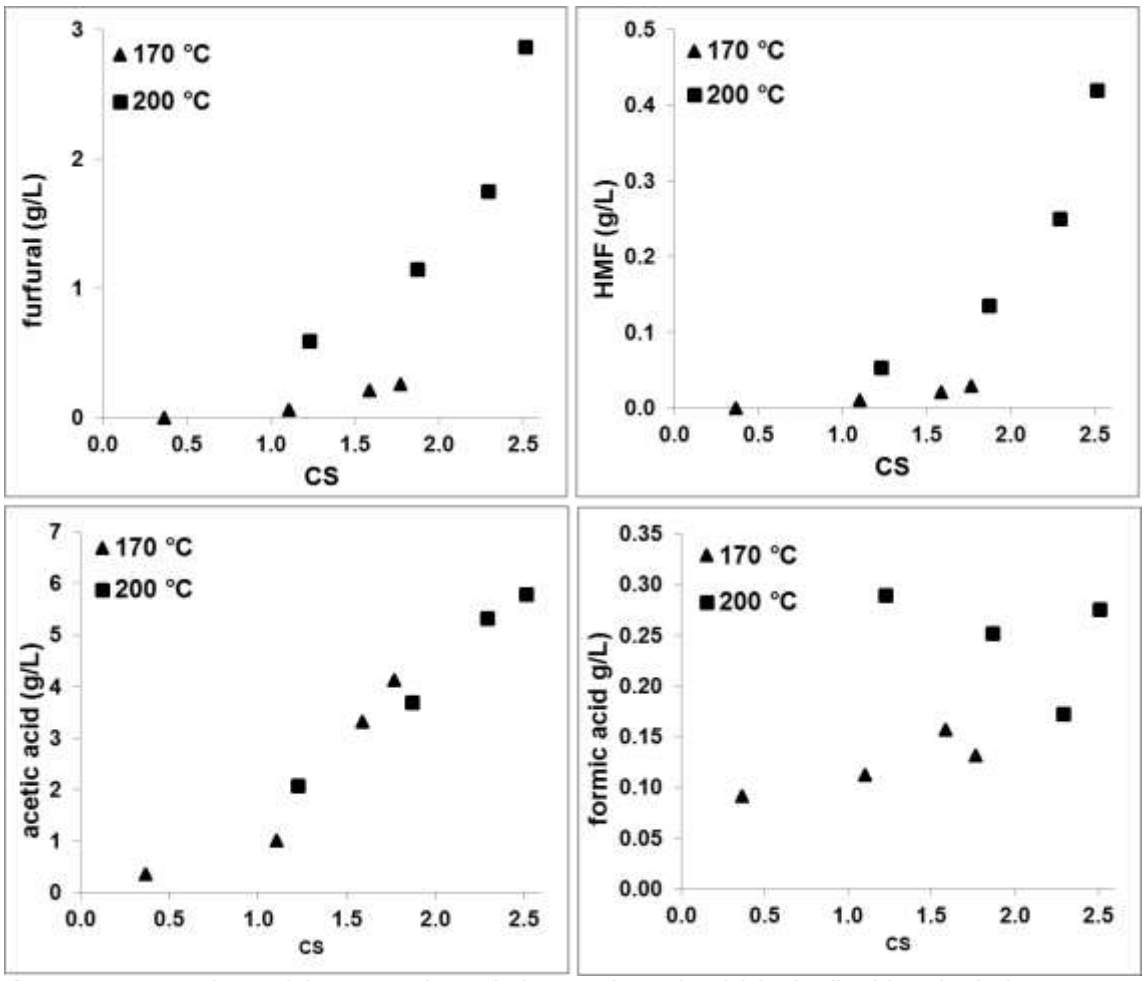

Fig. 1. Concentrations of the sugar degradation products (mg/L) in the liquid prehydrolysate at different *CS* values: A) furfural, B) HMF, C) acetic acid, and D) formic acid

Glucan Solubility during Pretreatment and Enzymatic Hydrolysis of the Solid Prehydrolysate

In contrast to the *S. schwerinii* xylan content, the pretreatment effect on the *S. schwerinii* glucan content was minor (Table 2). With the highest yields of xylan extracted to the liquid prehydrolysate at *CS* values of 1.77 and 2.29, the liberation of glucan to the liquid prehydrolysate was 5.13% and 9.45% of the original glucan content, respectively. This result showed that the conditions utilized in this study fractionated the hemicellulose, but kept the cellulose nearly intact. In general, cellulose needs more severe pretreatment conditions for degradation because of the higher thermal stability of cellulose caused by the higher polymerization stage and crystalline regions (Borrega *et al.* 2011; Ji *et al.* 2017). Additionally, during hot water extraction of silver birch in the study by Borrega *et al.* (2011), the glucan remained mostly stable up to 180 °C. Moreover, with hardwood and softwood materials pretreated with hot water extraction, the degradation of cellulosic glucan is reported to begin at 230 °C (Ando *et al.* 2000). In this study, the amount of glucan in the solid prehydrolysates was more than 400 mg/g, which was greater than 90% of the original material glucan, except at a *CS* of 2.51 (experiment 8), where the amount of residual glucose in the solid prehydrolysate was 375 mg/g (85%).

After pretreatment at 200 °C, the *S. schwerinii* pretreated solid material became more accessible to the enzymes (Fig. 2). Lim and Lee (2013) reported that the reaction temperature increased the cellulose hydrolyzation when sulfuric acid was used as a pretreatment catalyst. In the present study, the highest RSY from enzymatic hydrolysis was after experiment 7 when 70% of the solid prehydrolysate glucan (281.4 mg/g of the original *S. schwerinii* material) was released. In general, the enzymatic hydrolysis yields were low, which demonstrated the better usability of the *S. schwerinii* cellulosic fraction after dilute sulfuric acid pretreatment for bioprocesses other than enzymatic hydrolysis to fermentable sugars. The lignin most probably influenced the efficiency of the enzymatic hydrolysis of the dilute acid-pretreated *S. schwerinii*. This is because lignin in pretreated wood biomass material has been shown to hinder enzymatic hydrolysis, for example, with steam-pretreated poplar wood chips (Panagiotopoulos *et al.* 2013). In this study, in addition to the lignin content, the difference in the enzyme hydrolysis efficiency was partly caused by the residual xylan and degree of polymerization of the cellulose. Bura *et al.* (2009) showed the strong effect of residual xylan on the enzymatic hydrolysis efficiency with pretreated hybrid poplar. Interestingly, in the present study, the amounts of residual xylan in the solid prehydrolysates after experiments 4 and 5 were similar (33.48 mg/g and 29.65 mg/g, respectively), and enzymatic hydrolysis was more efficient after experiment 5 (*CS* = 1.23; temperature = 200 °C) with a RSY of 55.9% of the pretreated material glucan (Fig. 2). The RSY after experiment 4 (*CS* = 1.77; temperature = 170 °C) was 33.3% of the pretreated material glucan. Thus, further optimization of enzymatic hydrolysis is needed if the cellulosic fraction of dilute acid-pretreated *S. schwerinii* is to be utilized as fermentable sugars.

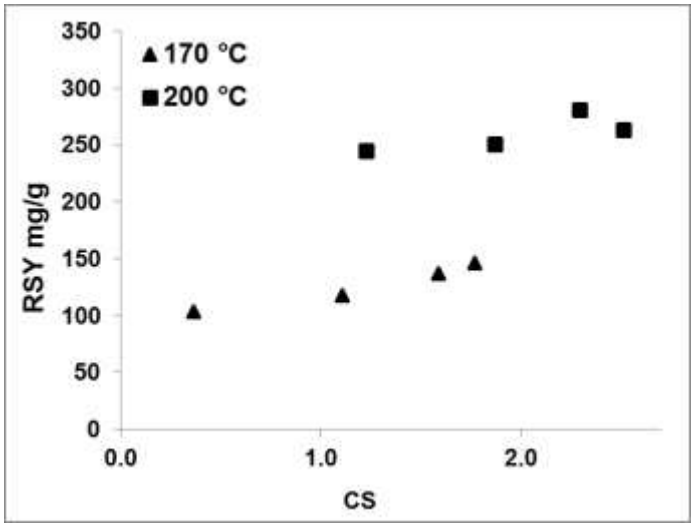

Fig. 2. Enzymatic hydrolysis of the pretreated *S. schwerinii* materials in milligrams of reducing sugars per gram of the original *S. schwerinii* biomass; ▲: Enzymatic hydrolysis with the material pretreated at 170 °C; ■: Enzymatic hydrolysis with the material pretreated at 200 °C

Fermentability of the Liquid Prehydrolysate from *S. Schwerinii* Using *C. acetobutylicum*

The liquid prehydrolysate after experiment 4 was chosen to test the ABE fermentability because of its high monosaccharide xylose yield and low degradation of cellulose to liquid prehydrolysate. Another reason for using the liquid prehydrolysate

obtained after pretreatment at a *CS* of 1.77, 170 °C, and with 0.15% H_2SO_4 was to balance the fermentable sugar concentrations with the carbohydrate degradation products (furfural, HMF, formic acid, acetic acid, and levulinic acid) that inhibit microorganism growth and therefore, minimize the need for detoxification before the fermentation process.

Along with sugar degradation products (especially formic acid), dissolved and degraded lignin products (ferulic acid and syringaldehyde) have an inhibitory effect on ABE fermentation (Ezeji *et al.* 2007; Wang and Chen 2011). That is why in the utilization and downstream processing of liquid prehydrolysate it is necessary to have a dissolution of lignin that is as low as possible during pretreatment (Gütch *et al.* 2012). In the present study, the effect of the pretreatment on the ASL content was apparent because it decreased constantly in the pretreated solid material with an increasing *CS* (Table 2). The lowest ASL content was 0.79% of the original dry biomass. According to Gütch *et al.* (2012), ASL is the main lignin fraction dissolved in dilute acid environments. In their work, which used *Eucalyptus globulus*, the amount of ASL decreased from 4.8% to less than 0.8%.

In the present study, during the fermentation of liquid prehydrolysate supplemented with barley grain starch, 10.1 g/L ABE was produced after 144 h, of which 6.3 g/L was butanol (Fig. 3). The aim of the supplementation of fermentation media with starchy material was to increase the level of fermentable sugars in the media. However, in a previous work, the authors found that the addition of starch also accelerates *C. acetobutylicum* xylose utilization (Yang *et al.* 2015). In the present study, when liquid prehydrolysate from *Salix schwerinii* was fermented with barley grain starch, 98% of the starch, 66% of the xylose, and 95% of the glucose were utilized during fermentation with *C. acetobutylicum* (Table 3). This showed the preferential use of glucose during the co-fermentation, although a distinct xylose utilization was observed. The corresponding ABE and butanol yields were 0.35 g/g and 0.22 g/g monosaccharides in the fermentation medium, respectively. The butanol and ABE yields were in agreement with those of the control experiments that used barley grain slurry and pure glucose as substrates (Table 3). When the *Salix* hydrolysate after acid hydrolysis was fermented with *C. beijerinckii*, Han *et al.* (2013) reported a butanol yield of 0.12 g/g sugar with 72% xylose consumption from the fermentation liquid.

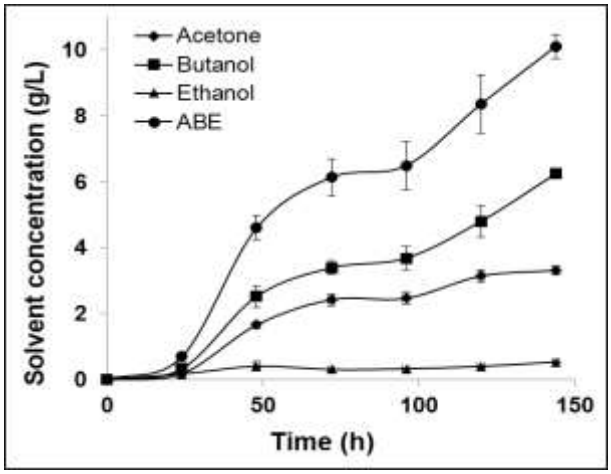

Fig. 3. ABE fermentation with the prehydrolysates of *Salix* supplemented with barley grain slurry

Table 3. Concentrations of the Sugars and Solvents Present in the Media Before and After Fermentation, and Production Yields of Butanol and ABE

Substrate	Before Fermentation (g/L)			After Fermentation (g/L)			BuOH (g/L)	BuOH yield g/g	ABE (g/L)	ABE yield g/g
	Glu (Starch)	Glu	Xyl	Glu (Starch)	Glu	Xyl				
Pre-170 °C	17.6	6.6	7.9	0.4	0.3	2.7	6.3	0.22	10.1	0.35
Barley grain	36.0	12.4	0.0	1.2	11.6	0.0	8.1	0.22	12.3	0.34
Glucose	0.0	30	0.0	0.0	1.8	0.0	6.7	0.24	9.3	0.33

Pre-170 °C: prehydrolysate from experiment 4 amended with barley grain starch; Glu: Glucose; Xyl: xylose; and BuOH yield: g/g monosaccharide sugars in the fermentation medium

Han *et al.* (2013) found that furfural, HMF, and formic acid were present in the hydrolysates at concentrations of 0.09 g/L, 0.02 g/L, and 0.4 g/L, respectively. These potential butanol fermentation inhibitors in the acid hydrolysates and extractives from the wood biomass were assumed to hinder the microbes switching from the acid-producing pathway to the solvent-producing pathway (Han *et al.* 2013). In the study by Sun and Liu (2012), detoxification through the overliming treatment of sugar maple hemicellulose hydrolysate increased butanol production from 0.8 g/L to 7.0 g/L, which corresponded to a butanol yield of 0.14 g/g xylose. Recently, kraft black liquor from the pulp industry was hydrolyzed, detoxified, and fermented with *C. acetobutylicum* ATCC 824, which resulted in a butanol production rate of 0.1 g/g and xylose consumption of 93% to 95% (Kudahettige-Nilsson *et al.* 2015). The fermentability of the hydrolysate was also assumed to decrease because of the presence of inhibitory compounds in the detoxified hydrolysates.

In the fermentation test conducted in this study, the dilute acid prehydrolysate of *S. schwerinii* showed good fermentability during ABE production without detoxification when grain starch was added as supplement to the fermentation media. Grain starch was added to the fermentation medium to increase the concentration of fermentable sugars, but it also decreased the concentration of sugar degradation products, which is favorable for fermentation. After the fermentation process, it was detected that furfural and HMF had been consumed in the medium during fermentation (Table 4). Researchers have shown that furfural and HMF at concentrations below 1.0 g/L are not toxic for butanol fermentation and even stimulate ABE fermentation (Ezeji *et al.* 2007). Although formic acid is a critical fermentation inhibitor of *C. acetobutylicum*, formic acid does not affect ABE production at a concentration below 0.4 g/L (Cho *et al.* 2012).

Table 4. Concentrations of the Potential Inhibitors Before and After Fermentation of the Prehydrolysates

Substrate	Before Fermentation (g/L)				After Fermentation (g/L)			
	Furfural	HMF	Formic acid	Acetic acid	Furfural	HMF	Formic acid	Acetic acid
Pre-170 °C	0.4	0.1	0.2	4.9	0.0	0.0	0.1	5.6

Pre-170 °C: prehydrolysate from experiment 4 amended with barley grain starch

The efficient utilization of hemicellulosic biomass could contribute to profitable biorefining of the short-rotation crop *S. schwerinii*, and the addition of starch could mean

possibly combining side streams from different industrial sources, such as liquid prehydrolysates from the pulping industry and starch-containing liquids from the food industry. Research on ABE fermentation and butanol production with *Clostridium* using a combination of industrial residues, both lignocellulosic and sugar- and starch-based substrates, has increased (Survase *et al.* 2013; Mechmech *et al.* 2016). Although the fermentation test showed the promising possibility of producing biobutanol from the liquid prehydrolysate of dilute acid-pretreated *S. schwerinii* without a detoxification process, optimization of the fermentation process requires more study.

CONCLUSIONS

1. The ABE fermentation of the *Salix schwerinii* hemicellulosic prehydrolysate supplemented with starch was successfully performed without detoxification and resulted in butanol and ABE yields of 0.22 g/g and 0.35 g/g monosaccharides in the fermentation medium, respectively.
2. During the pretreatment of *S. schwerinii*, 65% of the hemicellulosic xylan was easily hydrolyzed to a monosaccharide xylose form at 170 °C, which resulted in low concentrations of the degradation products (furfural and formic acid) and left most of the cellulosic fraction in the solid prehydrolysate for further refining.
3. The *S. schwerinii* hemicellulosic fraction extracted with the dilute acid pretreatment presents a valuable source of fermentable sugars for ABE fermentation together with industrial waste fractions that contain starch or glucose.

ACKNOWLEDGMENTS

The results of this study were part of the projects "Sustainable production concepts in the integrated biorefining industry (SusBioRef)" funded by the Academy of Finland, "Biomass value chains" funded by the European Regional Development Fund, and "Sustainable production and use of short rotation coppice *Salix*" funded by the European Agricultural Fund for Rural Development.

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Article submitted: January 23, 2018; Peer review completed: April 8, 2018; Revised version received: May 9, 2018; Accepted: May 11, 2018; Published: May 18, 2018.
DOI: 10.15376/biores.13.3.5225-5240