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The Effect of Varying Organosolv Pretreatment Chemicals on the Physicochemical Properties and Cellulolytic Hydrolysis of Mountain Pine Beetle-Killed Lodgepole Pine

Luis F. Del Rio · Richard P. Chandra · Jack N. Saddler

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Abstract Mountain pine beetle-killed lodgepole pine (*Pinus contorta*) chips were pretreated using the organosolv process, and their ease of subsequent enzymatic hydrolysis was assessed. The effect of varying pretreatment chemicals and solvents on the substrate's physicochemical characteristics was also investigated. The chemicals employed were $MgCl_2$, H_2SO_4, SO_2, and NaOH, and the solvents were ethanol and butanol. It was apparent that the different pretreatments resulted in variations in both the chemical composition of the solid and liquid fractions as well in the extent of cellulolytic hydrolysis (ranging from 21% to 82% hydrolysis after 12 h). Pretreatment under acidic conditions resulted in substrates that were readily hydrolyzed despite the apparent contradiction that pretreatment under alkaline conditions resulted in increased delignification (approximately 7% and 10% residual lignin for alkaline conditions versus 17% to 19% for acidic conditions). Acidic pretreatments also resulted in lower cellulose degree of polymerization, shorter fiber lengths, and increased substrate porosity. The substrates generated when butanol/water mixtures were used as the pretreatment solvent were also hydrolyzed more readily than those generated with ethanol/water. This was likely due to the limited miscibility of the solvents resulting in an increased concentration of pretreatment chemicals in the aqueous layer and thus a higher pretreatment severity.

Keywords Enzymatic hydrolysis · Organosolv · Cellulose · Ethanol · Substrate characteristics

Introduction

Lodgepole pine (*Pinus contorta,* LPP) is one of the most prevalent tree species in the Pacific Northwest, especially in the province of British Columbia (BC) Canada, covering

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L. F. Del Rio · R. P. Chandra · J. N. Saddler (✉)
Forest Products Biotechnology, Faculty of Forestry, University of British Columbia, 2424 Main Mall, Vancouver, BC V6T 1Z4, Canada
e-mail: Jack.Saddler@ubc.ca

approximately 25% of forested land [1]. The current mountain pine beetle (*Dendroctonus ponderosae*) epidemic has resulted in the infestation of approximately half the LPP population. It has been estimated that over 80% of the LPP population will be infected by the middle of the next decade [1] (an area equivalent to more than 15 million hectares). Although the mountain pine beetle does not have a direct effect on the physical properties of the wood, the fungi associated with the infection produces melanin, which causes a blue/black discoloration of the sapwood, consequently reducing its commercial value [2]. In addition, beetle-killed trees are susceptible to colonization by decay fungi, which result in drying of the wood, reducing its quality and commercial value, and increasing the risk of wildfire [3]. It is estimated that the current timeframe where beetle-killed LPP retains its commercial value for lumber applications is in the 5–10-year range [1]. In response to this, there has been a significant increase in harvest activity of this wood, generating significant wood residue opportunities from sawdust, etc. In the longer term, it has been estimated that the ratio of rate of beetle killed LPP to the current harvesting rate will be 23:1 [1]. Thus, the remaining LPP tree stands will possess limited value for commercial applications. Therefore, it is imperative to develop new applications for beetle-killed LPP such as bioconversion to ethanol. Bioconversion of lignocellulosic materials to ethanol, from both agricultural and wood residues, has increased as a significant area of research because of both the volatility of oil prices and environmental concerns.

The bioconversion of lignocellulosics to ethanol is composed of three major steps, which include pretreatment, to recover the lignin and hemicellulose in a useful form and to increase accessibility of the cellulose to hydrolytic enzymes, enzymatic hydrolysis of cellulose, and subsequent fermentation of the resulting monosaccharide's from the cellulose and hemicellulose fractions to ethanol. The pretreatment and hydrolysis steps have been identified as major economic barriers toward the commercialization of ethanol derived from lignocellulosic biomass [4]. Significant developments with regard to hydrolytic enzymes have resulted in cost reductions. However, it is also recognized that an increase in the efficiency of hydrolytic enzymes can also be achieved through improvements in pretreatment.

One of the pretreatment methods currently being assessed for its ability to produce a substrate amenable to hydrolysis by cellulases, while maximizing lignin values and sugar recovery, is the organosolv process. The advantages of this process include a clean separation of the lignin and carbohydrate components for potential use in co-product applications, the use of an organic solvent that can be recycled, and the ability to pretreat recalcitrant substrates such as softwoods [5]. Early work initially looked at the chemical fractionation of lignocellulosics using organic solvents [6] and was later modified and assessed by the pulp and paper industry as an alternative to Kraft pulping of hardwoods [7]. Briefly, this process involved treating wood chips with a mixture of water, an organic solvent, and a catalyst at approximately 200 °C and 400 psi resulting in delignification of the lignocellulosic biomass through the cleavage of lignin–carbohydrate bonds, lignin–lignin bonds, and the solubilization of the lignin in the organic solvent [8, 9]. Although most of these earlier investigations relate to the pulp and paper applications of the organosolv process [10], the need for alternatives to fossil-based transportation fuels has renewed interest in its application as a pretreatment that produces a lignocellulosic substrate, which is readily hydrolyzed by cellulases [11]. Previous research in our laboratory has shown that the hydrolysis of organosolv pretreated LPP [5] resulted in greater amounts of glucose conversion than did steam pretreated LPP at equivalent enzyme activity loadings (20 filter paper units or FPU per gram cellulose) [12]. The organosolv process also has the potential for the recovery of components such as hemicellulosic sugars, furfural, and high-quality lignin from the liquor stream, which can also be used for the development of commodity chemicals such as binding agents, dispersants, dyes, and pigments and food additives and possibly antioxidants [13, 14].

Previous work [5, 11, 15–17] has shown that, by varying process parameters such as catalyst concentration, solvent concentration, reaction temperature, and reaction time, it was possible to create a range of substrates with varying susceptibilities to enzymatic hydrolysis (from approximately 35% to 100% conversion within 48 h) and extents of delignification (ranging from 4.98% to 22.88% Klason lignin in the substrate). However, it was also found that the conditions required to obtain a substrate highly susceptible to enzymatic hydrolysis also resulted in lower sugar recoveries, most likely due to excessive carbohydrate degradation to furfural, hydroxymethyl furfural (HMF), levulinic, and formic acids [5]. It was also evident that the residual lignin content of a given substrate was not always related to that substrate's ease of enzymatic hydrolysis. It should be noted that these previous studies on the organosolv pretreatment of LPP were all performed using H_2SO_4 as the pretreatment catalyst and ethanol as the solvent, and the hydrolysis reactions were performed using relatively high enzyme loadings (20–40 FPU per gram cellulose).

It is possible that pretreatment conditions other than acidic [5] (i.e., alkaline and neutral) and the use of a solvent other than ethanol, may result in the generation of substrates with varying chemical compositions (i.e., different fractionation of the cellulose, hemicellulose, and lignin components) and possibly varying degrees of ease of enzymatic hydrolysis. For example, acidic pretreatments result in extensive hemicellulose removal and moderate delignification [4, 18, 19]. Alternatively, pretreatment under alkaline conditions, such as ammonia fiber explosion and lime pretreatment, results in extensive delignification while preserving a greater amount of hemicellulose in the solid fraction, which may require the addition of hemicellulosic enzymes [4, 18]. Moreover, it may also be advantageous to consider the use of neutral alkaline earth metal (NAEM) salt catalysts, such as $AlCl_2$ and $MgCl_2$, as previous studies [20, 21] have shown that organosolv pulping using $MgCl_2$ resulted in the partial preservation of hemicellulose in the solid fraction and comparable delignification when compared to the use of an acid catalyst such as H_2SO_4. Although studies with alternative catalysts have focused mostly on the optimization of pulps for papermaking, they may also be useful for creating substrates with varying characteristics and in limiting the formation of sugar degradation products such as furfurals and organic acids [22].

In addition to changing pretreatment chemicals, it may also be advantageous to utilize a different solvent rather than ethanol. Previous work studying the organosolv delignification of lignocellulosic residues with a variety of aliphatic and aromatic alcohols [23] showed that butanol was the most effective delignification solvent due to its increased hydrophobicity. By taking advantage of the fact that butanol has limited miscibility in water, it may be possible to concentrate the hemicellulosic sugars in the aqueous layer resulting in a more efficient separation of the cellulose-rich fraction (solid stream), lignin-rich fraction (butanol layer of the liquid stream), and hemicellulose-rich fraction (aqueous layer of the liquid stream) [24].

In the work reported here, we hypothesize that variations in solvent and pretreatment chemicals during organosolv pretreatment will result in the formation of substrates with unique chemical and physical properties, which will consequently result in varying degrees of ease of hydrolysis at low enzyme loadings (2.5–10 FPU per gram cellulose). By varying the pretreatment solvents and chemicals, we have also been able to elucidate the substrate characteristics that affect their susceptibility to subsequent enzymatic hydrolysis.

Materials and Methods

Feedstock Preparation

Lodgepole pine trees infested by mountain pine beetle (MPB-LPP) at the gray phase (dead tree) were harvested and prepared as described previously [5].

Organosolv Pretreatment

The laboratory-scale organosolv process was a modification of the one described by Pan et al. [11]. Briefly, MPB-LPP chips were pretreated in a mixture containing water, solvent (either ethanol or butanol), and a catalyst using a custom-built, four-vessel (2 L each) rotating digester made by Aurora Products Ltd. (Savona, BC, Canada). A 200-g (oven-dried weight) batch of chips was pretreated in each vessel. Vessels were opened after being cooled to room temperature in a water bath. The substrate and spent liquor were separated using a nylon cloth. The substrate was washed three times with a 300-ml mixture of water and alcohol at the same concentration as the pretreatment liquor. The washes were combined with the spent liquor, which was then sampled immediately for determination of furfural and 5-(hydroxymethyl) furfural (HMF) content. The washed substrates were homogenized in a standard British disintegrator for 5 min and passed through a laboratory flat screen with 0.203-mm slits (Voith, Inc., Appleton, WI) to remove rejects. The yield of screened substrate and rejects were determined, and the screened substrate was stored at 4 °C for analysis and hydrolysis. The pretreatment conditions and yields are summarized in Table 1.

Chemical Analysis

Oven-dried weights were determined by drying to constant weight at 105 °C in a convection oven. The Klason lignin content of the organosolv substrates was determined according to Technical Association of the Pulp and Paper Industry (TAPPI) standard method T-222 om-98. The hydrolysate was retained for the determination of monosaccha-

Table 1 Summary of pretreatment conditions used for the generation of beetle-killed lodgepole pine organosolv substrates.

Substrate	Conditions	Pulp yield (%)
NAEM60 EtOH	200 °C, 60 min, 0.025 M $MgCl_2$, 78% EtOH	45.1
NAEM30 EtOH	205 °C, 30 min, 0.025 M $MgCl_2$, 78% EtOH	49.1
$H2SO_4$ EtOH	170 °C, 60 min, 1.1% H_2SO_4, 65% EtOH	43.5
SO_2 EtOH	170 °C, 60 min, 1.1% SO_2, 65% EtOH	43.9
NaOH EtOH	170 °C, 60 min, 20% NaOH, 65% EtOH	45.3
NAEM60 BuOH	200 °C, 60 min, 0.025 M $MgCl_2$, 78% BuOH	46.2
NAEM30 BuOH	205 °C, 30 min, 0.025 M $MgCl_2$, 78% BuOH	47.4
H_2SO_4 BuOH	170 °C, 60 min, 1.1% H_2SO_4, 65% BuOH	45.0
SO_2 BuOH	170 °C, 60 min, 1.1% SO_2, 65% BuOH	44.5
NaOH BuOH	170 °C, 60 min, 20% NaOH, 65% BuOH	44.6

EtOH ethanol, *BuOH* butanol, *NAEM* neutral alkaline earth metal ride composition and acid-soluble lignin content. Acid-soluble lignin was determined by UV absorption at 205 nm as described by Dence [25]. Monosaccharides were determined using a DX-3000 high-performance liquid chromatography (HPLC) system (Dionex, Sunnyvale, CA), equipped with an anion exchange column (Dionex CarboPac PA1), and fucose as the internal standard. The column was eluted with deionized water at a flow rate of 1 ml/min. Aliquots (20 µl) were injected after being passed through a 0.45-µm nylon syringe filter (Chromatographic Specialties Inc., Brockville, ON, Canada). Baseline stability and detector sensitivity were optimized by postcolumn addition of 0.2 M NaOH at a flow rate of 0.5 ml/min using a Dionex AXP pump. The column was reconditioned using 1 M NaOH after each analysis. Monosaccharides in the substrates were quantified with reference to standards. The sugar standards were autoclaved at 121 °C for 1.5 h prior to analysis to compensate for possible decomposition caused by heating during Klason lignin determination. Furfural and HMF were determined using a Dionex Summit HPLC system equipped with a P680 pump, an ASI-100 autosampler, and a PDA100 photodiode array detector. A LiChrospher 5RP18 column (Varian, Palo Alto, CA) was used at 60 °C with an eluent flow rate of 0.5 ml/min. A gradient of 7.4 mM H_3PO_4 (A), acetonitrile (B), and a mixture of 7.4 mM H_3PO_4, methanol, and acetonitrile (4:3:3, *v*/*v*/*v*) (C) was used as follows: 0–20 min from 95% A and 5% C to 50% A and 50% C; 20–24 min from 50% A and 50% C to 100% C; 24–25 min, 100% C; 25–26 min from 100% C to 100% B; 26–27 min, 100% B; 27–28 min from 100% B to 95% A and 5%C; and 28–38 min, 95% A and 5% C. Diluted aliquots (20 µl) were injected after being passed through a 0.45-µm PTFE filter (Chromatographic Specialties Inc.). Furfural and HMF were determined by absorbance at 280 nm.

Characterization of Organosolv Substrates

The molecular weight distribution of the organosolv substrates was determined by gel permeation chromatography (GPC) analysis of their tricarbanyl derivatives. The substrates were carefully delignified prior to tricarbanylation using sodium chlorite according to the Pulp and Paper Technical Association of Canada's useful method G.10U. Tricarbanylation was carried out as described by Schroeder and Haigh [26]. Briefly, approximately 20 mg of oven-dried substrate was placed in glass tubes. The samples were then resuspended in 7 ml anhydrous pyridine followed by the addition of 3 ml phenylisocyanate. The flasks were then sealed with Teflon stoppers and incubated at 80 °C for 48 h at which time the reaction was stopped by the addition of 1 ml methanol. The tricarbanylated cellulose was recovered by precipitation in 4 vol. of methanol followed by centrifugation at 5,000 rpm. The precipitated cellulose tricarbanylate was washed three times with methanol to remove traces of pyridine and air-dried. The samples were resuspended overnight in tetrahydrofuran (THF, final concentration 2 mg/ml). The GPC analysis of the tricarbanylated samples was carried out in an Agilent 1100 HPLC system (Palo Alto, Ca) equipped with two styragel columns (HR5E and HR1 purchased from Waters, Milford, MA) in tandem. We used THF as the mobile phase at a flow rate of 1 ml/min. The GPC calibration curve was generated from the elution profile of polystyrene standards. The tricarbanylated samples and polystyrene standards were detected by a refractive index detector. The degree of polymerization (DP) of cellulose was obtained by dividing the molecular weight of the tricarbanylated polymer by the molecular weight of tricarbanylated anhydroglucose (DP=MW/519) as described by Mansfield et al. [27]. Both the number average (DP_N) and molecular weight average (DP_W) were determined.

Fiber size and distribution of the substrates were measured using a Fiber Quality Analyzer (LDA02, OpTest Equipment, Inc., Hawkesbury, ON, Canada). Briefly, a dilute suspension of fibers with a fiber frequency of 25–40 events per second was transported through a sheath flow cell where the fibers are oriented and positioned. The images of the fibers were detected by a built-in CCD camera, and the length of the fibers was measured by circular polarized light. The experiment was conducted according to the procedure described by Robertson et al. [28]. All samples were run in triplicate. Water retention values (WRVs) of the organosolv substrates were determined according to TAPPI useful method UM 256.

Simon's stain was performed according to the modified procedure by Chandra et al. [29]. Briefly, direct orange (DO) (Pontamine Fast Orange 6RN) and direct blue (DB) (Pontamine Fast Sky Blue 6BX) were obtained from Pylam Products Co. Inc. (Garden City, NY). Fractionation of the orange dye was performed according to Esteghlalian et al. [30]. For each substrate, approximately 100 mg (OD wt) of pulp samples were weighed into six 15 ml Corning Inc. polypropylene centrifuge tubes. Each tube received 1.0 ml of phosphate-buffered saline solution at pH 6. The DO solution (10 mg/ml) was added in a series of increasing volumes (0.25, 0.50, 0.75, 1.0, 1.5, and 2.0 ml) to the six tubes containing pulp sample and PBS. The DB solution (10 mg/ml) was also added to each tube using the same series of increasing volumes, resulting in each set of tubes containing a 1:1 mixture of DO and DB dyes at increasing concentrations. Distilled water was added to increase the final volume of the samples to 10.0 ml. The tubes were then incubated overnight at 70 °C with shaking at 200 rpm. After the incubation period, the tubes were centrifuged at 5,000 rpm for 5 min, and a sample of the supernatant was placed in a cuvette and the absorbance read on a Cary 50 UV-Vis spectrophotometer at 624 and 455 nm. The amount of dye adsorbed onto the fiber was determined using the difference in the concentration of the initial added dye and the concentration of the dye in the supernatant according to the Beer–Lambert law [30]. The extinction coefficients were calculated by preparing standard curves of each dye and measuring the slope of their absorbance at 455 and 624 nm. The values calculated and used in this study were $\varepsilon_{\mathrm{O}/455}=35.62$, $\varepsilon_{\mathrm{B}/455}=2.59$, $\varepsilon_{\mathrm{O}/624}=0.19$, $\varepsilon_{\mathrm{B}/624}=15.62\ \mathrm{L\ g^{-1}\ cm^{-1}}$.

Surface Chemistry of Organosolv Substrates Using Electron Spectroscopy for Chemical Analysis (ESCA)

Pulp handsheets (80 g $\mathrm{m^{-2}}$) for the ten organosolv substrates were prepared using distilled water. The pulp handsheets were soxhlet extracted with acetone for 8 h at a rate of six cycles per hour prior to analysis. The ESCA measurements were carried out using a Leybold Max 200 x-ray photoelectron spectrometer (Cologne, Germany) with a monochromated Al K_α X-ray source. The detector position was at an angle of 90° relative to the sample surface. The samples for analysis (1 $\mathrm{cm^2}$) were cut from the extracted handsheets, attached to the sample holder and evacuated in a prechamber for 12 h. The high-resolution spectra were charge corrected using the C–C component of the C1s signal at 285 eV as an internal standard. A Gaussian curve fitting program (XPSPEAK 4.1) with the Shirley background was used to deconvolute the C1s signal. The following binding energies relative to the C–C position were used: 1.7±0.2 eV for C–O, 3.1±0.2 for C=O, or O–C–O, and 4.2±0.3 for O=C–O [31]. The theoretical surface lignin coverage (TSLC) was calculated from the O/C ratios as described by Laine et al. [31] according to the following equation:

$$\Phi_{\mathrm{lig}} = \left(\mathrm{O/C_{(sample)}} - \mathrm{O/C_{(cellulose)}}\right) / \left(\mathrm{O/C_{(lignin)}} - \mathrm{O/C_{(cellulose)}}\right)$$

where $\mathrm{O/C_{(sample)}}$ is the O/C ratio of the analyzed sample, and $\mathrm{O/C_{(cellulose)}}$ and $\mathrm{O/C_{(lignin)}}$ are the theoretical O/C ratios of pure cellulose and lignin.

Enzymatic Hydrolysis

Commercial cellulase (Spezyme CP) and β-glucosidase (Novozym 188) were provided by Genencor International Inc. (Rochester, NY) and Novozymes (Franklington, NC), respectively. Cellulase activity was determined using the filter paper assay developed by Ghose [23] and is expressed in terms of FPU. β-Glucosidase activity was determined using *p*-nitrophenyl-β-D-glucoside as the substrate as described by Wood and Bhat [and is expressed in terms of international units (IUs)].

Batch hydrolysis was conducted at 2% consistency (solids, *w*/*v*) in 50 mM acetate buffer, pH 4.8, with 0.004% tetracycline and 0.003% cycloheximide, to prevent microbial contamination. Cellulase was used at loadings of 2.5, 5, and 10 FPU per gram of cellulose in the substrate with a supplementation of β-glucosidase at loadings of (5, 10, and 20 IU) to avoid inhibition by cellobiose accumulation. The reaction mixture (50 ml) was incubated at 150 rpm, 50 °C, in a rotary shaker and sampled periodically for glucose determination. Glucose was quantified by HPLC as described above, with the exception that the saccharide standards were not autoclaved. Hydrolysis data are averages of triplicate experiments.

Results and Discussion

Previous research in our laboratory, which used MPB-LPP [5] as a feedstock for organosolv pretreatment, showed that varying pretreatment variables such as reaction time, solvent concentration, and catalyst concentration affected the resulting substrates' chemical composition and susceptibility to enzymatic hydrolysis. Subsequent studies by Pan et al. [16, 17] indicated that, by varying organosolv process variables, significant effects on the physical substrates characteristics including fiber size, DP of cellulose, and crystallinity index were observed. Although our previous work provided valuable information with regard to process conditions, it should be noted that these studies only focused on the application of a single solvent (ethanol) and catalyst (H_2SO_4). Therefore, this study was undertaken with the objective of varying both the chemicals and solvent utilized during organosolv pretreatment of MPB-LPP. We hypothesized that by varying both the pretreatment solvent and chemical additives, this would result in diverse chemical and physical properties of the solid and liquid fractions and a range of susceptibilities to enzymatic hydrolysis. We also thought it might be possible to utilize the variations in both the physical and chemical substrate properties to assess different substrate properties and correlate them to their susceptibility to enzymatic hydrolysis. Organosolv pretreatments were performed as described previously [5, 20, 32, 33], utilizing both ethanol and butanol as solvents and using acidic, neutral, and alkaline chemical additives. Initial compositional analysis indicated that the different pretreatments resulted in substrates with varying cellulose and hemicellulose contents (Table 2). For example, the amount of glucan ranged from 64.00% (NaOH EtOH) to 77.30% (NAEM30 BuOH) and xylan ranges from 0.90% (NAEM60 BuOH) to 7.20% (NaOH EtOH), while the total hemicellulose content ranged from 1.6% to 15.4%. However, the amount of lignin in the solid fraction was quite similar in the majority of the substrates (approximately 17%) with the exception of substrates generated under alkaline conditions (NaOH EtOH, and NaOH BuOH), which resulted in 10.93% and 7.55% lignin content, respectively. This was expected since the hydrolysis of glycosidic bonds proceeds at a much slower rate than delignification reactions under alkaline condition compared to neutral and acidic conditions [34].

Table 2 Chemical composition (%) of mountain pine beetle-killed lodgepole pine solid substrates generated by organosolv pretreatment.

Substrate	Glucan	Xylan	Arabinan	Mannan	Galactan	AIL	ASL
NAEM60 EtOH	72.45 (0.71)	2.51 (0.10)	0.07 (0.00)	2.83 (0.14)	0.11 (0.00)	17.71 (1.56)	0.41 (0.02)
NAEM30 EtOH	67.42 (1.19)	1.39 (0.05)	0.06 (0.00)	2.77 (0.11)	0.10 (0.00)	19.16 (3.71)	0.41 (0.01)
H_2SO_4 EtOH	74.80 (1.08)	1.61 (0.03)	0.08 (0.00)	1.81 (0.08)	0.08 (0.00)	17.27 (0.67)	0.32 (0.03)
SO_2 EtOH	73.83 (0.32)	1.75 (0.02)	0.08 (0.00)	1.94 (0.04)	0.09 (0.00)	18.30 (0.23)	0.34 (0.01)
NaOH EtOH	64.00 (2.81)	7.20 (0.39)	0.74 (0.03)	6.85 (0.28)	0.62 (0.04)	10.04 (0.89)	0.89 (0.03)
NAEM60 BuOH	75.02 (0.48)	0.90 (0.02)	0.06 (0.01)	0.98 (0.01)	0.07 (0.01)	18.02 (1.68)	0.45 (0.01)
NAEM30 BuOH	77.30 (1.31)	1.20 (0.06)	0.06 (0.01)	1.12 (0.20)	0.06 (0.00)	17.30 (0.36)	0.46 (0.04)
H_2SO_4 BuOH	74.64 (1.41)	1.01 (0.01)	0.08 (0.00)	1.18 (0.07)	0.08 (0.00)	18.01 (1.25)	0.24 (0.02)
SO_2 BuOH	77.30 (1.44)	0.58 (0.02)	0.05 (0.01)	0.91 (0.06)	0.06 (0.00)	16.93 (0.49)	0.53 (0.02)
NaOH BuOH	70.01 (0.72)	5.44 (0.04)	0.40 (0.01)	7.55 (0.04)	0.50 (0.01)	6.94 (1.49)	0.61 (0.03)

Numbers in parentheses indicate the standard deviation ($n=3$)

AIL acid insoluble lignin, *ASL* acid soluble lignin.

Effect of Pretreatment Chemicals on the Water-Soluble Fractions

It was expected that the variation shown in the chemical composition of the solid fraction would be reflected in that of the liquid fraction (Tables 1, 3 and 4). Similar to a previous work, the water-soluble fraction was obtained following the precipitation of lignin from the spent pulping liquor [15]. The water-soluble fractions from ethanol organosolv pulping of hardwoods have been shown to be composed of mainly cellulose and hemicellulose-derived sugars as mono- and oligosaccharide form, sugar degradation product such as HMF and furfural, and low MW (i.e., water soluble) lignin fragments [35]. Our results were similar to previous work in that pretreatment under acidic conditions led to an increase in the

Table 3 Oligomeric sugar and sugar degradation products (furfural and HMF) composition (g l^{-1}) of the water-soluble fractions obtained from organosolv pretreatment of mountain pine beetle-killed lodgepole pine wood chips.

Substrate	Glucan	Xylan	Arabinan	Mannan	Galactan	Furfural	HMF
NAEM60 EtOH	bdl	bdl	0.02 (0.00)	0.36 (0.02)	0.02 (0.00)	2.24 (0.07)	0.66 (0.02)
NAEM30 EtOH	bdl	0.03 (0.00)	0.02 (0.00)	0.41 (0.02)	0.02 (0.00)	2.54 (0.10)	0.76 (0.03)
H_2SO_4 EtOH	0.30 (0.02)	1.12 (0.05)	0.35 (0.01)	2.03 (0.06)	0.68 (0.01)	1.26 (0.04)	0.97 (0.03)
SO_2 EtOH	0.87 (0.03)	0.96 (0.04)	0.27 (0.00)	1.27 (0.01)	0.50 (0.00)	0.82 (0.06)	0.77 (0.09)
NaOH EtOH	bdl	0.05 (0.01)	0.28 (0.01)	0.04 (0.00)	0.59 (0.01)	bdl	bdl
NAEM60 BuOH	0.33 (0.02)	0.14 (0.01)	0.02 (0.00)	1.05 (0.01)	0.18 (0.01)	1.34 (0.04)	0.26 (0.02)
NAEM30 BuOH	bdl	0.08 (0.01)	0.02 (0.00)	0.72 (0.04)	0.13 (0.00)	1.61 (0.06)	0.28 (0.01)
H_2SO_4 BuOH	7.37 (0.05)	5.00 (0.02)	1.59 (0.14)	10.11 (0.07)	3.50 (0.01)	1.04 (0.08)	0.32 (0.04)
SO_2 BuOH	7.84 (0.04)	1.70 (0.01)	0.72 (0.00)	4.67 (0.03)	2.03 (0.01)	1.78 (0.05)	0.54 (0.03)
NaOH BuOH	bdl	1.00 (0.03)	0.77 (0.02)	0.15 (0.01)	1.65 (0.02)	bdl	bdl

Numbers in parentheses indicate the standard deviation of three replicate samples

bdl below detection limit.

Table 4 Monomeric sugar composition (g l^{-1}) of the water soluble fractions obtained from organosolv pretreatment of mountain pine beetle-killed lodgepole pine wood chips.

Substrate	Glucose	Xylose	Arabinose	Mannose	Galactose
NAEM60 EtOH	bdl	0.06 (0.01)	bdl	0.18 (0.02)	0.04 (0.00)
NAEM30 EtOH	bdl	0.08 (0.01)	bdl	0.23 (0.02)	0.05 (0.00)
H_2SO_4 EtOH	1.12 (0.03)	1.39 (0.03)	0.41 (0.01)	2.09 (0.05)	0.67 (0.02)
SO_2 EtOH	1.77 (0.10)	0.91 (0.06)	0.28 (0.01)	1.32 (0.08)	0.51 (0.02)
NaOH EtOH	bdl	bdl	bdl	0.01 (0.00)	bdl
NAEM60 BuOH	0.32 (0.01)	0.15 (0.01)	0.03 (0.01)	0.37 (0.01)	0.06 (0.01)
NAEM30 BuOH	0.12 (0.01)	0.08 (0.00)	bdl	0.23 (0.01)	0.04 (0.00)
H_2SO_4 BuOH	6.60 (0.23)	5.25 (0.21)	1.58 (0.06)	8.09 (0.34)	2.93 (0.10)
SO_2 BuOH	6.98 (0.30)	2.67 (0.12)	0.77 (0.03)	3.95 (0.16)	1.71 (0.07)
NaOH BuOH	bdl	bdl	bdl	0.01 (0.00)	bdl

Numbers in parentheses indicate the standard deviation of three replicate samples

bdl below detection limit.

concentration of mono- and oligosaccharides in the liquid fraction resulting in a cellulose-rich solid fraction. In contrast, the sugar concentration in the liquid fraction of substrates generated under alkaline conditions was significantly lower (Tables 3 and 4). This was expected as acidic and neutral pretreatments result in the hydrolysis of the hemicellulosic component [19]. It was apparent that, for the most part, more than half the sugars in the water-soluble fraction were oligomeric. This is of significance as hexose monomers (glucose, galactose, and mannose) are readily fermentable and can be used for ethanol production [36]. Oligosaccharides need to be further hydrolyzed to their monomeric components for further ethanol production [37] or could be used to generate higher value co-products such as animal feed additives [38, 39]. It should also be noted that the pretreatments that used butanol yielded higher concentrations of sugars in the aqueous fractions. This was also expected since the volume of the aqueous layer in the butanol pretreatments was approximately 20% of the volume of the ethanol pretreated substrates due to the limited miscibility between butanol and water. Therefore, especially in the case of the pretreatments with H_2SO_4, the utilization of butanol resulted in relegation of the sugars to the limited aqueous layer resulting in their higher concentration. Since previous work showed that the H_2SO_4 catalyzed ethanol pretreatment of LPP resulted in significant sugar degradation, we next measured the formation of both furfural and HMF.

Furfural and HMF are generated by the thermal decomposition of pentoses and hexoses under acidic and neutral conditions [34]. The furfural and HMF concentrations were quite variable ranging from 0.26 to 0.97 mg ml^{-1} and 0.82 to 2.54 mg ml^{-1} for HMF and furfural, respectively (Table 3). It was apparent that pretreatment combining the NAEM salts with the ethanol solvent (NAEM60 EtOH and NAEM30 EtOH) resulted in a considerably higher concentration of furfural (2.24 and 2.54 g l^{-1}, respectively) and a low level of pentoses in the liquid fraction. The increased level of furfural and HMF during the NAEM60 EtOH and NAEM30 EtOH pretreatments was most likely caused by the higher temperatures required for NAEM pretreatment (200 and 205 °C for NAEM60 and NAEM30, respectively) compared to the 170 °C utilized for the other pretreatments and the fact that pretreatment with NAEM salts is actually acidic (pH~4.2). However, pretreatment using NAEM salts with butanol resulted in lower furfural concentrations compared to their ethanol counterparts (1.34 and 1.61 gl^{-1} for NAEM60 BuOH and NAEM30 BuOH, respectively) despite being carried out at the same temperatures. This was most likely because of the ability of butanol to absorb a greater amount of heat than ethanol as evidenced by the higher boiling point of butanol (78.4 vs. 117.7 °C for ethanol and butanol, respectively). In general, with the exception of the substrate generated with SO_2, all of the pretreatments that utilized butanol resulted in lower HMF concentrations than those generated with ethanol, indicating less hexose degradation. The behavior of the substrate generated with SO_2 and the butanol solvent (SO_2 BuOH) may be due to the ability of the gaseous SO_2 to penetrate the wood chips [40] especially since these samples were impregnated 12 h prior to pretreatment [12]. This is supported by Eklund et al. [40] who showed that impregnation of Willow chips with H_2SO_4 resulted in higher xylose recovery, whereas impregnation with SO_2 resulted in higher glucose yields during hydrolysis. Since it was apparent that the changes in the pretreatment solvent and chemical additive had a significant effect on the chemical composition of the liquid stream and the solid fraction, we next assessed whether the pretreatment parameters affected both physical properties and consequently the ease of saccharification of the resulting substrate by cellulases.

Effect of Pretreatment Chemicals on Physicochemical Properties and Enzymatic Hydrolysis of the Solid Fraction

The enzymatic hydrolysis of the ten organosolv substrates was carried out at 2% solids (*w*/*v*), using lower enzyme loadings (2.5, 5, and 10 FPU per gram cellulose). The reaction was monitored over 72 h (Fig. 1). It was hoped that by employing minimal enzyme loadings (2.5 and 5 FPU per gram cellulose) differences in the substrate's ease of hydrolysis would be apparent, since higher dosages may mask differences between the substrates by saturating the substrate with enzyme. Furthermore, as enzymatic hydrolysis is one of the economic barriers for the commercialization of the bioconversion process [4], it would be advantageous to identify substrates that hydrolyze at low enzyme dosages.

The hydrolytic potential for these substrates was assessed by measuring the amount of cellulose hydrolyzed to glucose during the first 12 h and is shown in Fig. 1a. This time point was chosen from the hydrolytic profiles because the differences in the extent of glucose conversion were the most noticeable at this point in the hydrolysis compared to 72 h (Fig. 1b) and 12 h was within the linear portion of the hydrolysis curve when using enzyme loadings of 10 FPU per gram cellulose (data not shown). Significant differences were observed in the extent of hydrolysis (at 10 FPU per gram cellulose) after 12 h ranging from 21% (NaOH EtOH) to 82% (SO_2 BuOH) (Fig. 1a). Substrates pretreated employing butanol as the solvent hydrolyzed at a faster rate and to a greater extent than substrates pretreated with ethanol. However, the differences in hydrolysis yields were less pronounced at 5 FPU per gram cellulose and indistinguishable at 2.5 FPU per gram cellulose. A possible explanation for the lack of effectiveness at the lower enzyme loadings may be due to the non-productive binding of the cellulases to the lignin component of the substrate. Previous work has shown that cellulases bind to lignin via hydrophobic interactions or interactions between phenolic groups or a combination of both [41, 42]. However, it should be pointed out that, in the current study, the substrates that possessed the lowest amount of lignin (NaOH EtOH and NaOH BuOH, 10.93% and 7.55%, respectively) exhibited limited extents of glucose conversion after 12 h (21% and 34%, respectively, Fig. 2a). This is in agreement with previous studies [43–45], which showed that partial delignification of steam-pretreated softwoods by alkaline post-treatment resulted in decreased hydrolysis yields. This decrease in hydrolysis yield has been attributed to redeposition of lignin on

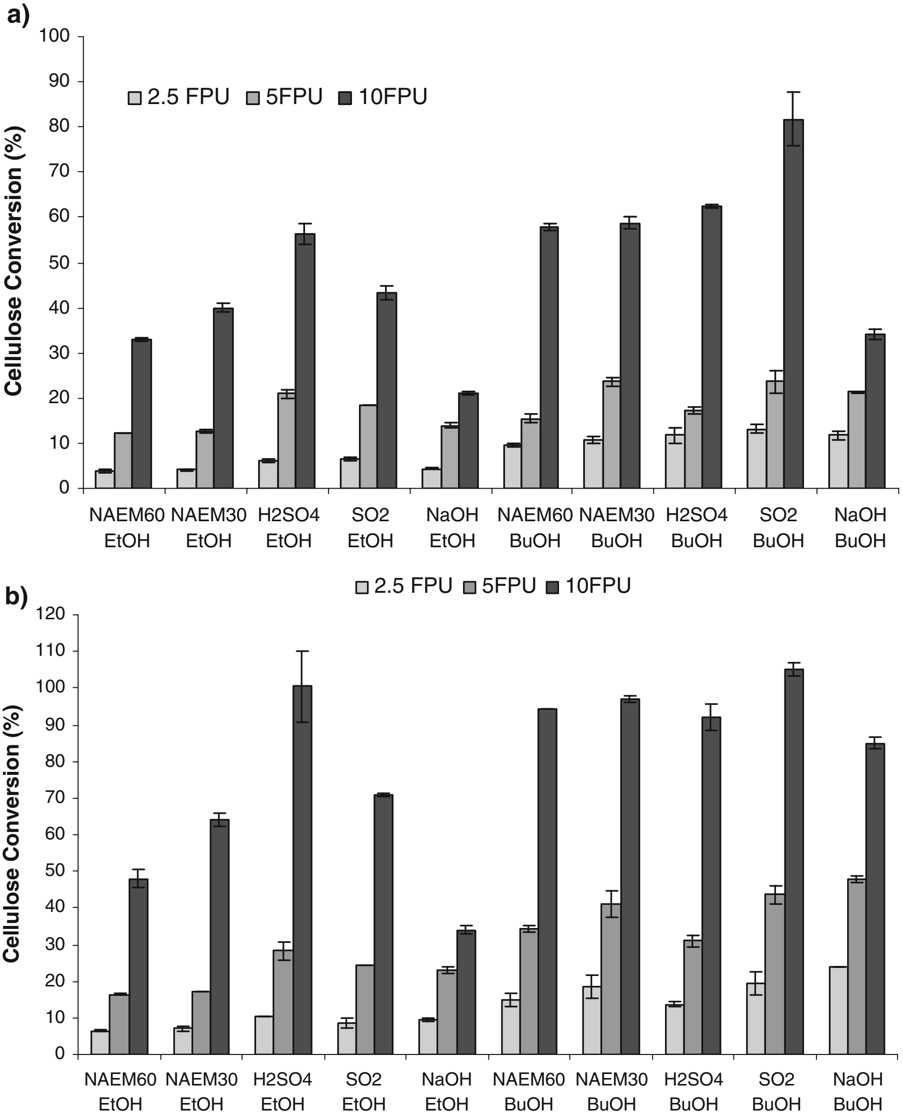

Fig. 1 Extent of conversion of cellulose to glucose during cellulolytic hydrolysis at 2% consistency and 2.5, 5, and 10 FPU per gram cellulose of organosolv pretreated lodgepole pine substrates after **a** 12 h of incubation and **b** 72 h of incubation

enzyme-accessible pores and cellulose surfaces [44, 45]. In addition, Ishizawa et al. [46] recently showed that complete delignification and removal of xylan from acid-pretreated corn stover also results in decreased hydrolysis yields. That study however attributed the decrease in cellulose digestibility to aggregation of cellulose microfibrils, resulting in decreased cellulase accessibility.

In addition to the effects of lignin content on hydrolysis, we also investigated the influence of the substrates' surface composition on enzymatic hydrolysis using ESCA,

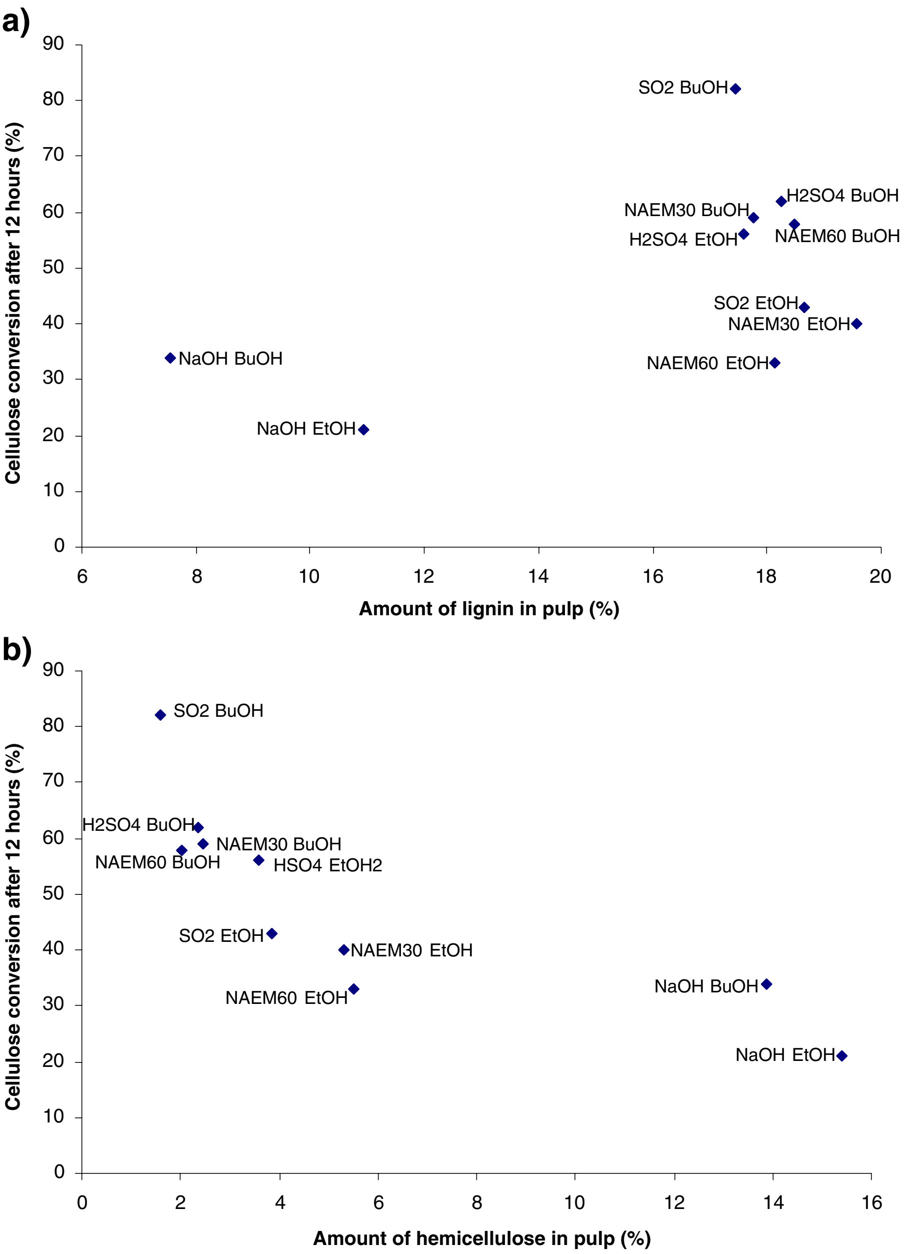

Fig. 2 Relationship between chemical composition of lodgepole pine organosolv substrates and ease of hydrolysis determined as the cellulose conversion after 12 h of incubation with 10 FPU per gram cellulose at 2% consistency. **a** Relationship between lignin content in pulp and ease of hydrolysis and, **b** relationship between hemicellulose content in pulp and ease of hydrolysis

which enables the analysis of the substrate surface to a depth of approximately 5–10 nm. Early studies on surface analysis of paper and wood samples with this technique showed that their spectrum was mainly composed of carbon and oxygen peaks [47] and that the carbon peak was actually composed of four peaks representing the four oxidation states of carbon. Using the oxygen/carbon ratio of the substrate surface, ESCA can be readily used to estimate the amount of lignin and carbohydrates on the substrate's surface [31, 47]. In addition, quantification of carboxylic carbons can be used as an indicator of a substrate's hydrophilicity and swelling [48], both of which have been implicated in a lignocellulosic substrate's susceptibility to cellulolytic hydrolysis [29]. The ESCA results show that the O/C ratio ranges from 0.33 to 0.50, suggesting that all the substrates' surfaces are very rich in lignin as the theoretical O/C ratios are 0.33 for pure lignin and 0.83 for pure cellulose [31] (Table 5). The TSLC was also shown to range from 66% to 99%. These results are in agreement with those presented by Palonen et al. [49] who used ESCA to determine the surface composition of a steam-pretreated softwood mixture before and after treatment with cellulases and a laccase–cellulase mixture. That study showed that the proportion of lignin at the substrate surface was significantly higher than the total lignin content (66% and 51% TSLC and total lignin, respectively). We were unable to determine a relationship between enzymatic hydrolysis and either the O/C ratio or TSLC, though our results indicate that in accordance with their compositional data (Table 2), the NaOH EtOH and NaOH BuOH substrates exhibited the lowest amount of surface lignin (O/C ratio 0.48% and 0.50% and TSLC 66% and 70%, respectively). We were also unable to detect any surface carboxylic groups using this technique, suggesting that substrates generated by organosolv pretreatment at the applied conditions did not result in the formation of detectable carboxylic acid groups at the surface. Although the differences observed in the results of enzymatic hydrolysis did not seem to be related to the surface/total lignin content, it is possible that this was due to the amount of lignin in the ten substrates being quite similar

Table 5 Proportion of different carbons (C1–C4,), oxygen to carbon ratios (O/C), and theoretical surface lignin coverage (TSLC) in mountain pine beetle-killed lodgepole pine substrates generated by organosolv pretreatment as measured using ESCA.

Substrate	C1 (%)	C2 (%)	C3 (%)	C4 (%)	O/C	TSLC (%)
Pure cellulose[a]	–	83	17	ND	0.83	0
Pure lignin[a]	49	49	2	ND	0.33	100
NAEM60 EtOH	66	31	3	ND	0.40	86
NAEM30 EtOH	68	29	3	ND	0.41	84
H_2SO_4 EtOH	69	28	3	ND	0.40	85
SO_2 EtOH	63	33	4	ND	0.40	87
NaOH EtOH	67	28	5	ND	0.48	70
NAEM60 BuOH	68	30	2	ND	0.35	95
NAEM30 BuOH	71	27	2	ND	0.34	97
H_2SO_4 BuOH	66	31	3	ND	0.38	91
SO_2 BuOH	70	28	2	ND	0.33	99
NaOH BuOH	57	36	7	ND	0.50	66

C1 unoxidized carbon (C–C, C–H), *C2* carbon with one oxygen bond (C–O), *C3* carbon with two oxygen bonds (O–C–O or C=O), *C4* carbon with three oxygen bonds (O=C–O), *ND* not detected

[a] Theoretical values from Laine et al. [31].

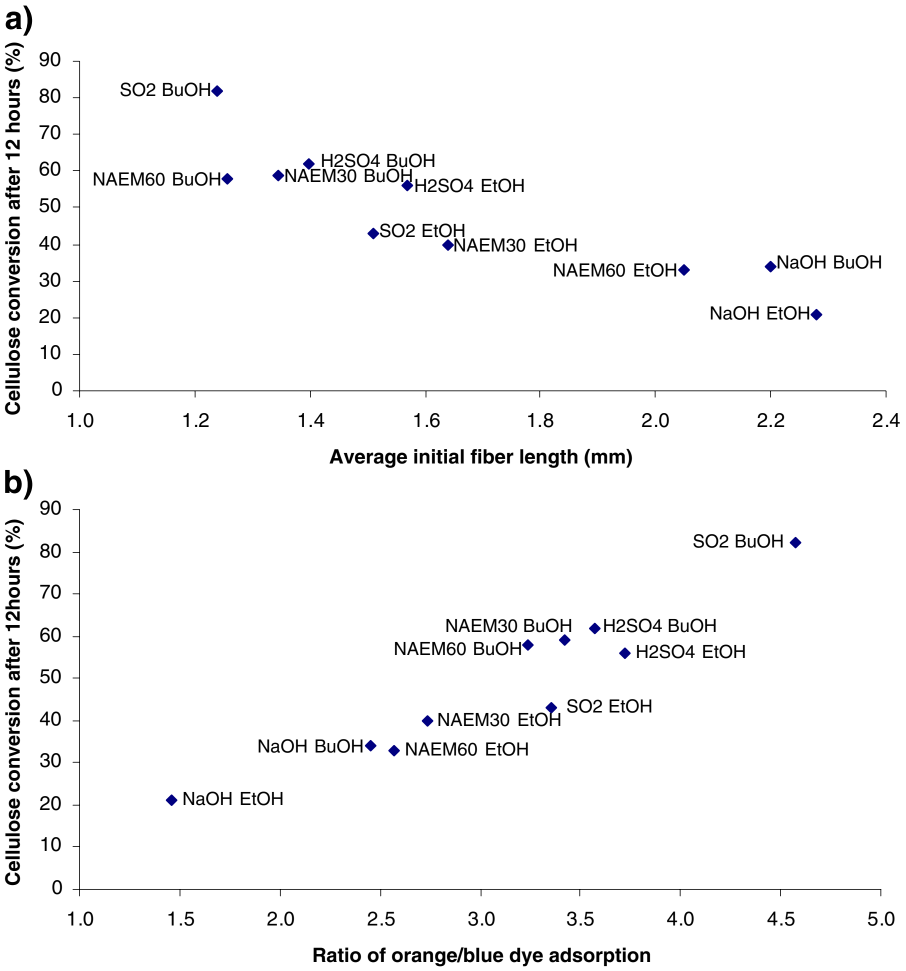

Fig. 3 Relationship between surface area of lodgepole pine organosolv substrates and ease of hydrolysis determined as the cellulose conversion after 12 h of incubation with 10 FPU per gram cellulose at 2% consistency. **a** Relationship between initial fiber length (exterior surface area) and ease of hydrolysis and. **b** Relationship between distribution of large and small pores (combination interior/exterior surface area) determined by the Simon's stain method and ease of hydrolysis

with the exception of the substrates generated under alkaline conditions. Thus, it is possible that the observed differences in hydrolysis yields were due to variations in other physicochemical properties, such as hemicellulose content, enzyme-accessible surface area, and DP.

Similar to lignin, it has been shown that hemicellulose acts as a physical barrier preventing cellulases from accessing cellulose [50, 51]. Our results indicate an inverse relationship between enzymatic hydrolysis after 12 h and the amount of hemicellulose present in the solid fraction (Fig. 2b). This is in agreement with previous studies, which showed that the removal of hemicellulose during steam pretreatment resulted in an increase in the ease of hydrolysis [52, 53]. Moreover, work by Grethlein et al. [54] showed that an increase in enzymatic hydrolysis rates was likely caused by increased (substrate) pore volume as a result of hemicellulose removal from acid pretreated softwoods. A recent study by Mussatto et al. [55] also showed that removing 86.5% of the hemicellulose and only 14% of the lignin present in brewer's spent grain resulted in a 3.5-fold increase in the rate of cellulose hydrolysis likely caused by increased accessibility of cellulase to cellulose. In contrast, Kumar and Wyman [56] did not find a significant correlation between cellulose accessibility and lignin/xylan removal. Unlike lignin, studies evaluating the effect of hemicellulose on enzymatic hydrolysis have been less frequent [22]. This is likely due to the fact that hemicellulose is highly sensitive to pretreatment conditions and varies widely in content and composition between different feedstocks. Recognizing the effects of hemicellulose, Berlin et al. [57] supplemented cellulase preparations with a variety of hemicellulose-degrading enzymes resulting in an increase in the rate of hydrolysis of acid-pretreated corn stover and a twofold reduction in the amount of protein required to hydrolyze cellulose and xylan. However, in this study, supplementation of the cellulase mixture with 50 U xylanase per gram xylan (Multifect xylanase) did not result in a significant increase in the rate or extent of hydrolysis of the NaOH EtOH substrate (data not shown).

In addition to the chemical composition, physical properties, such as surface area, DP, and swelling, have all been implicated in the substrate's inherent recalcitrance to enzymatic hydrolysis [58]. The surface area of pulp fibers can be further separated into the exterior surface area, which is determined by fiber dimensions such as length and width and interior surface area determined by pore volume, fissures, and micro-cracks [22]. Our results show an inverse relationship between initial fiber length and hydrolysis after 12 h using 10 FPU per gram cellulose (Fig. 3a). It should also be noted that, in general, the substrates generated under alkaline conditions (NaOH EtOH and NaOH BuOH) exhibited much larger fiber lengths (2.28 and 2.2 mm, respectively) than those generated under acidic conditions. The increase in fiber lengths obtained during alkaline pretreatment was most likely due to a cleaner separation of fibers due to greater efficiency of lignin removal and limited cellulose hydrolysis during the alkaline pretreatment process, which are two of the reasons alkaline processes are used in producing pulps for papermaking. In addition, similar to the effects of the solvent on hemicellulose content, the average fiber length of the substrates generated with butanol was shorter than those generated with ethanol. This was likely caused by the limited miscibility of butanol and water resulting in the catalysts being allocated to the aqueous layer, thus increasing their active concentration and resulting in an increased hydrolysis of the cellulose. Similarly, Pan et al. [16] showed that increasing the severity of the pretreatment conditions by increasing catalyst dosage during organosolv pretreatment of poplar chips led to a decrease in the average fiber size, which was attributed to possible chemical cutting of the fibers as shown using electron microscopy.

The relationship between initial fiber length and hydrolysis was expected since smaller particles possess greater surface area [59, 60], thus the smaller particles were anticipated to hydrolyze at a faster rate. Mooney et al. [61] also showed that the larger fibers isolated from Douglas-Fir kraft pulp were hydrolyzed at a slower rate and to a lesser extent than the whole pulp containing the "fines" and small fibers likely due to the increased surface area of the whole pulp provided by the fines and small fibers. Although exterior surface area plays an important role in the enzymatic hydrolysis of lignocellulosic substrates, the information obtained from these measurements is limited, as it does not account for the substrate's topology and porosity (interior surface area). Consequently, further information may be obtained from the measurement of interior surface area available to cellulases [29].

In this study, we used the Simon's stain (SS) method as modified by Chandra et al. [62] to evaluate the distribution of large and small pores in our substrates. Simon's stain was originally developed as a lignocellulose stain for the microscopic evaluation of mechanical damage undergone by pulp fibers during beating [63]. The SS technique is based on the competitive adsorption of two dyes (blue and orange) in an aqueous environment. The orange dye has a higher molecular weight and affinity for cellulose (via hydrogen bonding to hydroxyl groups), thus it is able to penetrate and displace the smaller blue dye from the larger pores. Due to the differences in size and affinities of the two dyes for cellulose, the ratio of adsorbed orange to blue dye can be used as a measure of the distribution of large and small pores [64].

Esteghlalian et al. [30] used SS to correlate the extent of hydrolysis of Kraft pulp samples dried to different degrees (never dried, oven dried, air dried, and freeze dried). The SS dye measurements and ratios were directly proportional to the extent of cellulolytic hydrolysis of the substrates [30, 62]. More recently, Chandra and coworkers [62], modified this technique to increase throughput, decrease time required for analysis, and increase reproducibility. This allowed them to show a direct correlation between the results of this technique and the ease of hydrolysis of lignocellulosic substrates pretreated by a variety of methods.

The SS measurements of the organosolv pulps indicated that the ratio of orange to blue dye adsorption was quite diverse, ranging from 1.46 in the case of the substrate generated with NaOH and ethanol (NaOH EtOH) to 4.60 for the substrate pretreated using the combination of SO_2 and butanol (SO_2 BuOH), suggesting that the distribution of large and small pores varied between pretreatments. It is evident that the ratio of DO/DB adsorption by each substrate was a strong indicator of its susceptibility to enzymatic hydrolysis (Fig. 3b). The results suggest that the ease of enzymatic hydrolysis is heavily dependent on the accessibility of the enzyme to the substrate (i.e., amount of large pores) as has been hypothesized previously with various substrates [37, 45, 46, 53, 58, 65]. It has also been established that the threshold pore size for effective hydrolysis of lignocellulosic substrates is 5.1 nm [54]. On the other hand, it should be pointed out that Mooney et al. [66] showed that increased substrate porosity of Douglas-Fir mechanical pulps only resulted in enhanced hydrolysis when accompanied by a delignification step (from 27.3% to 8.2%). In addition, Ishizawa et al. [67] failed to find a significant correlation between substrate porosity and ease of hydrolysis of dilute acid-pretreated corn stover generated under varying pretreatment severities. This suggests that, in addition to substrate porosity, other factors such as location of lignin and hemicellulose also play a role in enzymatic hydrolysis of lignocellulosic substrates. Of note, the two substrates containing the highest amount of hemicellulose and the lowest amount of lignin (NaOH EtOH and NaOH BuOH, 15.4% and 13.9% hemicellulose, and 10.9% and 7.5% lignin, respectively) also showed low ratios of orange to blue dye adsorption (1.46 and 2.45, respectively), implying that hemicellulose may act as a physical barrier preventing the orange dye (and by extrapolation cellulases) from accessing the cellulose component of the substrate. Indeed, it was apparent that the ratio of orange to blue dye adsorption was inversely proportional to the amount of hemicellulose present (Fig. 4). Furthermore, the substrates generated by pretreatment with butanol exhibited a larger orange to blue dye adsorption ratio than those generated with ethanol possibly due to the enhanced hemicellulose removal during the butanol pretreatment.

In addition to the distribution of large and small pores, other physical properties such as swelling of lignocellulosic substrates in aqueous solutions and cellulose DP have been shown to have an effect on cellulolytic hydrolysis [22, 29]. Swelling is largely influenced by several factors, including charged groups in the interior and exterior of the fibers, pH of the medium, and presence of electrolytes [66]. A widespread method used to estimate swelling of lignocellulosic substrates is the centrifugal water retention value, (WRV) [29].

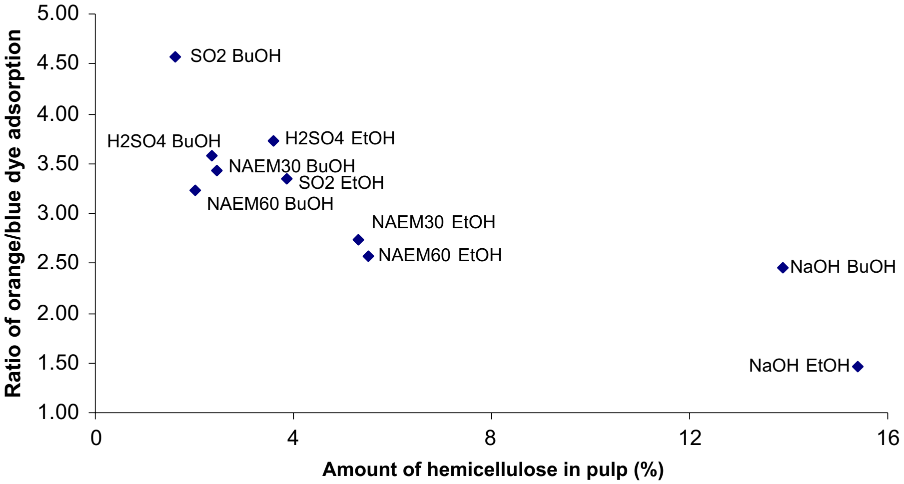

Fig. 4 Relationship between distribution of large and small pores in the substrate (combination interior/exterior surface area) determined by the Simon's stain method and amount of hemicellulose in pulp

Previous work by Bendzalova et al. [68] as well as Ogiwara and Arai [69, 70] attributed increased cellulase performance to increased porosity caused by swelling of cellulosic fibers and found linear correlations between initial hydrolysis rates and WRV when comparing sulfite, sulfate, and semi-chemical pulps. From the results of WRV measurements of the organosolv pulps, it was evident that, although differences in WRV were not significant when comparing substrates generated with the same solvent, the substrates generated with butanol had a greater WRV than those generated with ethanol (Table 6). However, a notable exception is the NaOH EtOH substrate, which possessed a WRV comparable to that of the butanol substrates. This is likely because NaOH can be used to

Table 6 Effect of pretreatment conditions on the substrate's initial degree of polymerization, fiber size and water retention value.

Substrate	DP_W	Average Fiber Length (mm)	WRV
NAEM60 EtOH	1,440 (31)	2.05 (0.02)	2.80 (0.27)
NAEM30 EtOH	1,790 (21)	1.64 (0.06)	2.69 (0.02)
H_2SO_4 EtOH	1,062 (46)	1.57 (0.04)	2.89 (0.40)
SO_2 EtOH	1,200 (23)	1.51 (0.04)	2.88 (0.28)
NaOH EtOH	1,512 (23)	2.28 (0.17)	3.19 (0.01)
NAEM60 BuOH	848 (22)	1.33 (0.01)	3.19 (0.23)
NAEM30 BuOH	820 (30)	0.97 (0.05)	3.11 (0.22)
H_2SO_4 BuOH	1,060 (56)	1.51 (0.02)	3.17 (0.20)
SO_2 BuOH	769 (20)	1.24 (0.02)	3.45 (0.14)
NaOH BuOH	2,159 (87)	2.23 (0.01)	3.12 (0.01)

Numbers in parentheses indicate the standard deviation of three replicate samples

DP_W weight average degree of polymerization, *WRV* water retention value

enhance the swelling of lignocellulosics [69, 70]. Although we were unable to find a correlation between hydrolysis and WRV, it is possible that the greater swelling of the substrates generated with butanol contributes to their enhanced hydrolysis compared to their ethanol counterparts.

As noted earlier, cellulose DP has also been shown to have an effect on cellulolytic hydrolysis. However, the role of DP on cellulolytic hydrolysis is somewhat unclear, as some studies have not found a clear relationship between ease of hydrolysis and initial DP [5, 71], while other studies have shown that substrates with low initial DP hydrolyzed more quickly and to a greater extent than those with high DP [17, 72, 73]. In the current study, the initial DP was measured by cellulose derivatization by the tricarbanylation method with subsequent molecular weight measurement employing GPC. The average molecular weight distribution for the ten substrates was highly diverse ranging from 769 (SO_2 BuOH) to 2,159 (NaOH BuOH) (Table 6). Substrates generated under acidic conditions exhibited significantly lower initial DP than those generated under alkaline conditions (Table 6). This is likely caused by the acid-catalyzed hydrolysis of the cellulose chains [34]. Furthermore, the substrates generated with butanol using acidic conditions and NAEM salts showed a significantly lower initial DP than did their ethanol counterparts. As mentioned earlier, the butanol pretreatments were likely more severe due to the limited miscibility of butanol and water increasing the effective concentration of in the aqueous layer. This is in agreement with previous studies in our laboratory [5, 16, 17], which showed that increases in organosolv pretreatment severity led to significant decreases in cellulose DP as well as increased hydrolysis yields. It was apparent that the substrates that had a lower cellulose DP tended to hydrolyze to a greater extent after 12 h (Fig. 5), suggesting that the substrates' initial DP is an important factor in the cellulolytic hydrolysis of organosolv pretreated MPB-LPP.

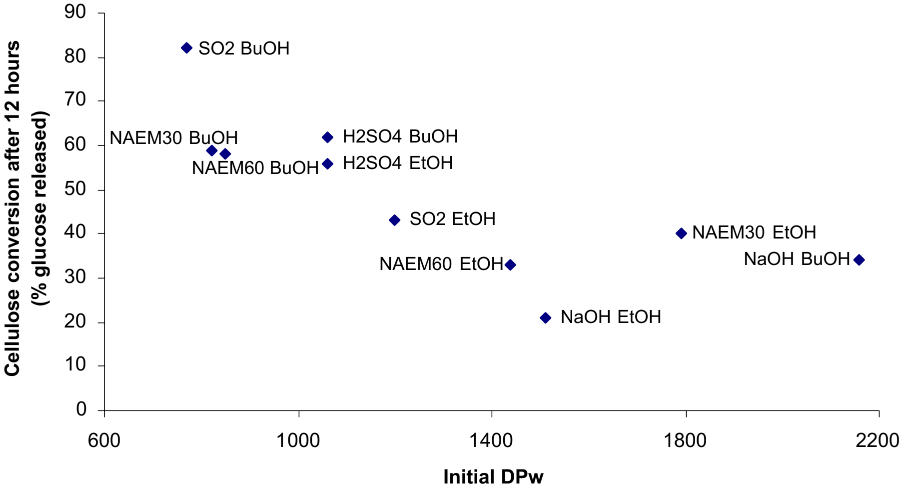

Fig. 5 Relationship between initial cellulose chain length (degree of polymerization DP_w) and ease of hydrolysis determined as the cellulose conversion after 12 h of incubation with 10 FPU per gram cellulose at 2% consistency

Conclusions

Variations in the organosolv pretreatment conditions of MPB-LPP resulted in the generation of substrates with a range of chemical compositions. Substrates generated under acidic conditions were readily hydrolyzed despite more selective delignification resulting after alkaline pretreatment. This was most likely due to a combination of hemicellulose removal and cellulose depolymerization. The use of butanol produced substrates that were more readily hydrolyzed compared to when ethanol was used as the solvent. This is likely caused by the higher severity encountered during butanol pretreatments due to the limited miscibility of butanol and water. This increased severity resulted in the generation of substrates with less hemicellulose, smaller initial fiber length, increased ratio of large vs. small pores, lower initial DP, and increased swelling. In addition to creating a substrate more amenable to subsequent hydrolysis, butanol pretreatment appears to minimize sugar degradation. These results strongly suggest that the chemical composition of the substrate should not be considered in isolation but that factors such as enzyme accessible surface area in the form of porosity or initial fiber length, the greater number of exposed cellulose chain ends as demonstrated by a lower initial cellulose DP, etc. all have a significant effect on the susceptibility of the organosolv pretreated substrates to enzymatic hydrolysis.

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