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Ethanol production from poplar wood through enzymatic saccharification and fermentation by dilute acid and SPORL pretreatments ☆

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ABSTRACT

Dilute acid (DA) and Sulfite Pretreatment to Overcome Recalcitrance of Lignocelluloses (SPORL) pretreatments were directly applied to wood chips of four poplar wood samples of different genotypes (hereafter referred to as poplars; *Populus tremuloides* Michx. 'native aspen collection'; *Populus deltoides* Bartr. ex Marsh × *Populus nigra* L. 'NE222' and 'DN5'; *P. nigra* × *Populus maximowiczii* A. Henry 'NM6') to evaluate their bioconversion potential. Plant biomass recalcitrance (PBR) was defined to quantitatively determine the recalcitrance of the poplars. Using DA pretreatment, NM6 produced the lowest bioconversion efficiency with a total monomeric sugar yield of 18% theoretical and an ethanol yield of 0.07 L kg^{-1} of wood compared with an aspen sugar yield of 47% theoretical and an ethanol yield of 0.17 L kg^{-1} of wood. Similar comparisons following SPORL pretreatment were 43% versus 55% and 0.11 versus 0.20 L kg^{-1} of wood for NM6 and aspen, respectively. Bioconversion performance of NE222 and DN5 fell between that of aspen and NM6. While substrate lignin content and lignin removal by pretreatments did not affect substrate enzymatic digestibility, the wood lignin content was found to negatively affect xylan or hemicellulose removal using both DA and SPORL pretreatments. The ability of lignin protecting hemicellulose removal dictates PBR through affecting disk milling energy for size reduction of pretreated wood chips, substrate enzymatic digestibility. The SPORL pretreatment not only improved sugar and ethanol yields over DA for all four poplars, but also better dealt with the differences among them, suggesting better tolerance to feedstock variability.

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1. Introduction

Approximately 368 million tons of woody biomass can be sustainably produced annually in the United States [1], forming the basis of many natural resource based industries (e.g., energy and fiber) that depend upon cellulosic feedstocks for development and expansion. Considering the renewable energy supply chain, woody biomass is derived from forestlands and intensively managed plantations [2]. Both feedstock sources have associated environmental, social, and economic advantages and disadvantages, and a substantial amount of research has been and is currently being conducted to address these issues [3–5].

Purpose-grown short rotation woody crops are a sustainable feedstock option for reducing pressure on native forests and managing the remaining landscape for non-production benefits [6,7]. These dedicated energy crops can offer an immense opportunity for woody biomass production in most regions of the United States [8]. Trees being considered for commercial use primarily belong to the following four genera: *Populus* (cottonwoods, poplars, aspens, and hybrids thereof), *Salix* (willows), *Pinus* (pines), and *Eucalyptus* (eucalypts) [9]. Among these options, intensively-grown *Populus* (hereafter referred to as poplars) has gained substantial attention in the midwestern United States. Its productivity can be up to eight times greater than native aspen (*Populus tremuloides* Michx.; *Populus grandidentata* L.) in the region [10,11]. In fact, aboveground productivity of nearly 9 Mg ha^{-1} yr^{-1} is common, and selection of genotypes adapted to site and climatic conditions has resulted in nearly 2.5 times as much growth [10–12].

Traits such as productivity and cold/drought tolerance are important factors in selecting poplar clones for fiber and environmental benefits. However, for biochemical conversion, wood recalcitrance is important for evaluating processing energy and economics [13,14]. Therefore, quantitatively evaluating genotypic recalcitrance and its effect on sugar and ethanol production is critical. A recent study tested the effect of lignin content on sugar release from over 1000 poplars and concluded that lignin content

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has a negative effect on sugar release [15]. However, the study did not decouple the effects of carbohydrate content and saccharification efficiency on sugar release and did not provide the information about the wood samples used. Furthermore, the energy input for pretreatment, such as energy consumption for wood size reduction, was not reported. Fundamentally, it is important to understand the genetic and physiological effects and associated clonal variability on carbohydrate content and wood saccharification efficiency. From a practical standpoint, information about how the energy efficiency for sugar production directly affects overall energy production economics from energy crops is also important for advancing these technologies. Therefore, a comprehensive evaluation that includes clonal variability in sugar yield and energy efficiencies for sugar and ethanol production is required.

The purpose of the present study was to acquire baseline information that is important for evaluating the potential of poplar wood for sugar and ethanol production. The four wood samples studied were from four different genotypes with contrasting yield potential, growth phenologies, and recalcitrance levels [11,13]. The genotypes were: native aspen (*Populus tremuloides* Michx.), NE222 and DN5 (*Populus deltoides* Bartr. ex Marsh × *Populus nigra* L.), and NM6 (*P. nigra* × *Populus maximowiczii* A. Henry). Recognizing the inverse relationship between scope of inference and precision in similar bioconversion studies, coupled with the common approach of testing at a very small scale using milled wood and specially-designed high throughput devices for a large numbers of samples [16], the conversion yields of the four wood samples were compared without drawing conclusions about the specific genotypes. This supported a direct use of the wood chips that is more representative of overall biomass for chemical pretreatment, as well as the collection of energy data for post chemical pretreatment wood chip size reduction. These energy data are vital because of the unique strong physical integrity of woody biomass and because such information is an integral component of evaluating bioconversion potential of woody biomass [17,18]. Mild dilute sulfuric acid (DA) and Sulfite Pretreatment to Overcome Recalcitrance of Lignocelluloses (SPORL) pretreatments were directly applied to wood chips to save energy in wood size reduction [18]. Enzymatic hydrolysis and simultaneous enzymatic saccharification and fermentation (SSF) using commercial enzymes and a conventional *Saccharomyces cerevisiae* were conducted using the pretreated solid fraction after disk milling. All process energy and yield data were recorded. Wood recalcitrance was quantitatively determined based on saccharification conversion and process energy data. The overarching goal of the study was to provide objective and practical bioconversion information to researchers, landowners, and policy makers for the production of poplars as a bioenergy crop.

2. Materials and methods

2.1. Materials

Celluclast 1.5 L and Novozyme 188 (β-glucosidase) were generously provided by Novozymes North America (Franklinton, NC). Sodium acetate, sulfuric acid, and sodium bisulfite were acquired from Sigma–Aldrich (St. Louis, MO), while all other chemicals, including culture media ingredients, were received from Fisher Scientific (Hanover Park, IL). All chemicals were of analytical quality.

Wood logs of native aspen were obtained from natural stands growing in northern Wisconsin, USA. Aspen was tested because it is a native species with very low recalcitrance shown by our previous work [19,20]. In addition, fourteen-year-old trees of poplar clones NE222, DN5, and NM6 were harvested from the US Forest Service, Hugo Sauer Nursery in Rhinelander, WI. Specific trees were selected based on size, tree form, and overall health (e.g., lack of disease). The genotypes were selected because they belonged to the most common genomic groups (*P. deltoides* × *P. nigra*; *P. nigra* × *P. maximowiczii*) utilized in the northern Lake States region, and because they have exhibited broad variation in yield potential, growth phenologies, and recalcitrance levels. All logs were hand-debarked and then chipped at the US Forest Service, Forest Products Laboratory (Madison, WI) using a laboratory chipper. The wood chips were then screened to remove all particles greater than 38 mm and less than 6 mm in length. The thickness of the accepted chips ranged from 1 to 5 mm. The chips were kept frozen at a temperature of −16 °C until used.

2.2. Microorganism and culture

S. cerevisiae FPL-450 (ATCC® Number 9080) was grown at 30 °C for 2 days on YPD-agar plates containing 10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} glucose, and 20 g L^{-1} agar. A colony from the plate was then transferred by loop to a liquid YP medium supplemented with 30 g L^{-1} glucose in a flask. The *S. cerevisiae* FPL-450 seed was grown overnight at 30 °C with agitation at 90 rpm on a shaking bed until the biomass concentration reached 2 g L^{-1} as monitored by optical density at 600 nm measurements (Agilent 8453, UV–visible spectroscopy system, Agilent Technologies, Santa Clara, CA).

2.3. Pretreatment and substrate production

Wood chips were directly pretreated by dilute acid (DA) and Sulfite Pretreatment to Overcome Recalcitrance of Lignocelluloses (SPORL) [21,22]. These two processes were applied because DA is the most widely studied and SPORL has demonstrated robust performance for woody biomass conversion [19] even when applied to softwood species [23,24]. The pretreated wood chips were disk milled after the separation of solids from the pretreatment hydrolysate (spent liquor) as shown in Fig. 1. All pretreatments were conducted using 150 g wood chips in oven-dried (od) weight in a 1-L reactor with a liquor to wood ratio of 3:1 at 170 °C for 20 min. This set of mild conditions was used to avoid over pretreatment so that the differences in the recalcitrance among the poplars could be shown. The pretreatment liquor was made of sulfuric acid and sodium bisulfite. Acid concentration was 0.2% (v/v) or 1.1% on od wood for DA pretreatment. Sodium bisulfite of 2% on od wood was applied in addition to the application of the same amount of sulfuric acid in SPORL pretreatment. Three, 1-L vessels were mounted inside of a 23-L wood pulping digester (pressure vessel) in an autoclave configuration as described elsewhere [21]. Therefore, DA or SPORL pretreatments of the three poplar samples were conducted in the same run. The 1-L pressure vessels were heated externally using steam while the wood pulping digester was rotating at the speed of 2 rpm for mixing. The temperature increased to170 °C in approximately 7 min. Aspen pretreatment was conducted separately, with aspen substrates produced in a previous study using two sodium bisulfite dosages of 1.5% and 3.0% on wood for SPORL pretreatment [20].

Solid substrates were produced from the pretreated wood chips by disk milling (Fig. 1) under atmospheric conditions after the separation of the solids from the pretreatment hydrolysate using a screen. The pretreatment hydrolysates were saved for chemical composition analysis. The disk mill was equipped with plates of pattern D2-B505 (Andritz Sprout-Bauer Atmospheric Refiner, Springfield, OH). The disk plate gap was set at 1.02 mm. Water was added during disk milling, which resulted in a solids discharge consistency of approximately 10%. The energy consumption for disk milling was recorded as described elsewhere [18,25]. The size-reduced solids were directly dewatered by pressing using a canvas bag to a solids content of approximately 30%, without a

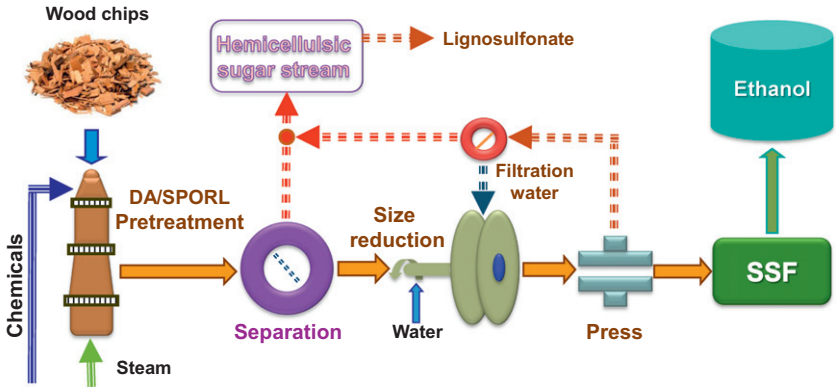

Fig. 1. Schematic flow diagram describing the experimental processes. Subprocesses connected by dashed lines were not conducted.

separate washing step. The yield of solid substrate in the form of fibers or fiber bundles was then determined from the weight and moisture content of the collected substrate. The moisture content was determined gravimetrically by drying the collected solids in an oven at 105 °C overnight.

2.4. Enzymatic hydrolysis

Enzymatic hydrolysis of the pretreated solid substrates was conducted. The substrate enzymatic digestibility (SED), defined as the percentage of glucan in the substrate converted to glucose, and the enzymatic hydrolysis glucose yield (EHGY) in kg ton^{-1} untreated wood were determined. Enzymatic hydrolysis was carried out at 2% substrate solids (w/v) in 100 mL of sodium acetate buffer (pH 4.8, concentration 100 mM) on a shaker/incubator (Thermo Fisher Scientific, Model 4450, Waltham, MA) at 50 °C and 200 rpm. Commercial enzymes of Celluclast 1.5 L at 7.5 FPU g^{-1} glucan and Novozyme 188 (β-glucosidase) at 11.25 CBU g^{-1} glucan were used. This relatively low enzyme dosage was chosen for detecting differences among the genotypes. Hydrolysate was sampled periodically for glucose concentration. Each data point is the average of duplicates. The average relative standard deviation was about 2%.

2.5. Quasi-simultaneous enzymatic saccharification and fermentation (SSF)

Quasi-SSFs were carried out in 250-mL Erlenmeyer flasks using a shaker incubator (Thermo Fisher Scientific, Model 4450, Waltham, MA) at 35 °C and 90 rpm with 10% solid substrate (water insoluble). The enzyme loadings were the same as for enzymatic hydrolysis described above. The solid substrate was first liquefied at 50 °C and 200 rpm for 120 min on the shaker incubator before adding the yeast *S. cerevisiae* FPL-450. Initial cell concentration for all SSF experiments was 0.4 mg dry cell g^{-1} substrate. No nutrients were added for all fermentation experiments. Samples of the fermentation broth were taken every 24 h and centrifuged at 10,000 g for 5 min and stored at −4 °C until analyzed for sugar and ethanol. Reported results are the average of duplicates with an average relative standard deviation of about 4%.

2.6. Analytical methods

The chemical compositions of the original and pretreated biomass were analyzed by the Analytical and Microscopy Laboratory of the Forest Products Laboratory [24,26]. Ethanol analysis in the cellulosic substrate fermentation broth was carried out using a gas chromatograph (GC, model 7890, Agilent Technologies, Palo Alto, CA) through direct sample injection using an external standard for calibration. The sample was centrifuged and the supernatant was filtered before injection to the GC column. The GC is equipped with a flame ionization detector and Agilent DB Wax column of 30 m with an ID 0.32 mm. A universal guard column was used to reduce column contamination. Inhibitor concentrations in the pretreatment hydrolysates were measured using an HPLC equipped with an Econosphere^{TM} C18 column (5-μm particle size, 250 mm × 4.6 mm, Alltech, Deerfield, IL) and a UV1000 ultraviolet detector (277 nm; Thermo Finnigan, San Jose, CA). Samples were run at ambient temperature and eluted at 0.8 mL min^{-1} with a linear gradient of 50–100% acidified methanol (containing 0.25% acetic acid) over 15 min. All analyses were carried out in duplicates at a minimum. The average data were reported. The standard deviations were calculated as measurement errors. For fast analysis, glucose in the enzymatic hydrolysates from pure enzymatic hydrolysis experiments was measured in duplicate using a commercial glucose analyzer (YSI 2700S, YSI, Yellow Springs, OH).

2.7. Definitions and calculations

The following definitions are used to quantitatively evaluate the four poplar wood samples for bioconversion to sugar and ethanol. The discussion of biomass recalcitrance has been qualitative in the literature [27]. We use a quantitative definition, Plant Biomass Recalcitrance (PBR), proposed in our previous study [13] to compare the recalcitrance of the four samples examined in this study,

$$\mathrm{PBR}\left(\frac{\mathrm{MJ}}{\mathrm{kg\ wood}}\right) = \frac{10000}{\mathrm{SED}(\%)} \cdot \frac{\text{Total energy input for pretreatment}\left(\frac{\mathrm{MJ}}{\mathrm{kg\ wood}}\right)}{\text{Total monomeric sugar recovery}(\%)} \quad (1)$$

where substrate enzymatic digestibility, SED, is defined as the percentage of glucan in the substrate converted to glucose enzymatically. Total energy input to pretreatment included wood log chipping estimated at 0.18 GJ ton^{-1} wood [28] and the measured energy consumption for disk milling of the pretreated solid substrate. Pretreatment thermal energy was based on thermodynamic calculations of enthalpy of the pulp suspension at 25% solids consistency (L/W = 3) at the pretreatment temperature of 170 °C, i.e., 1.25 GJ ton^{-1}, with consideration of 50% thermal energy recovery.

Component yields from the solid fraction were determined based on the measured chemical composition of the substrate and the yield of the solid fraction (Table 1) for mass balance analysis. The enzymatic hydrolysis glucose yield, EHGY (kg ton^{-1} wood), is calculated based on the measured glucose concentration in the

Table 1
Chemical composition of untreated wood samples and pretreated solid substrates along with pretreatment solids yields (%wt/wt).

Sample label[a]	Klason lignin	Arabinan	Galactan	Glucan	Xylan	Mannan	Solid substrate yield[c]
Native wood samples							
AS (Aspen)	20.2	0.35	0.46	45.61	16.35	1.41	100
NE2 (NE222)	23.5	0.30	0.67	40.75	16.42	3.79	100
DN5	22.9	0.35	0.68	39.11	17.30	3.94	100
NM6	25.2	0.39	0.73	39.39	15.81	3.70	100
Pretreated samples							
ASA2B0	29.6	0.84	Nd	64.70	2.80	0.10	66.50
NE2A2B0	29.6	nd	Nd	46.64	5.61	1.62	78.74
DN5A2B0	28.9	nd	Nd	45.28	7.04	1.94	77.31
NM6A2B0	30.4	nd	Nd	43.10	8.61	2.37	77.46
ASA2B1.5	26.4	0.85	Nd	68.22	2.06	0.29	62.00
ASA2B3	24.8	0.91	Nd	68.78	2.27	0.20	64.70
ASA2B2[b]	26.1	0.86		68.33	2.10	0.27	62.54
NE2A2B2	28.0	nd	Nd	48.21	4.38	1.48	75.25
DN5A2B2	28.1	nd	Nd	48.45	5.15	1.68	73.73
NM6A2B2	29.5	nd	Nd	47.72	6.17	2.00	72.69
Standard deviation	0.2	0.1	0.2	0.5	0.3	0.5	

[a] AS and NE2 stands for Aspen and NE222, respectively. A# and B# are percent of sulfuric acid and sodium bisulfite charge on od wood, respectively.
[b] Hypothetical sample and data from linear interpolation of ASA2B1.5 and ASA2B3.
[c] Defined as percent of starting materials recovered as insoluble solids.

Table 2
Lists of energy consumption for pretreatment, monomeric sugar yields from enzymatic and pretreatment hydrolysates, along with calculated plant biomass recalcitrance (PBR) of the four poplar samples.

Run label	Wood (untreated) Klason lignin content (%)	Pretreated wood chip milling energy (MJ kg^{-1})	Total pretreatment energy input (MJ kg^{-1})[a]	SED @ 72 h (%)	EHGY @ 72 h[c] (g kg^{-1} wood)	Pretreatment hydrolysate (spent liquor)			Total sugar recovery[d] (%)	PBR (MJ kg^{-1} wood)
						Glucose yield (g kg^{-1} wood)	Xylose yield (g kg^{-1} wood)	Mannose yield (g kg^{-1} wood)		
ASA2B0	20.2	0.343	1.430	49	212.7	9.8	99.3	10.6	46.93	6.9
NE2A2B0	23.5	0.609	2.037	44	180.9	8.2	84.8	11.2	41.82	11.0
DN5A2B0	22.9	0.647	2.075	38	149.1	6.4	73.2	8.8	35.19	15.4
NM6A2B0	25.2	0.763	2.191	21	76.2	3.7	34.0	4.3	17.94	59.4
ASA2B1.5		0.169	1.599	52	244.9	9.1	114.6	12.6		
ASA2B3		0.079	1.509	54	265.9	7.4	124.8	13.0		
ASA2B2[b]	20.2	0.139	1.569	53	251.9	8.5	118.0	12.7	55.22	5.4
NE2A2B2	23.5	0.572	2.000	47	201.0	8.3	101.9	11.9	47.41	8.5
DN5A2B2	22.9	0.399	1.827	44	186.6	6.8	87.6	12.6	43.48	8.9
NM6A2B2	25.2	0.652	2.080	42	170.1	7.0	92.1	10.9	42.53	11.0

[a] Including estimated wood chipping energy of 0.18 GJ ton^{-1} wood and thermal energy of 1.25 GJ ton^{-1} wood for pretreatment at 170 °C with liquid to solid ratio of 3 determined by thermal dynamic calculations with 50% thermal energy recovery.
[b] Hypothetical sample and data of sugar yield and energy from linear interpolation of ASA2B1.5 and ASA2B3.
[c] Enzymatic hydrolysis glucose yield.
[d] Total sugars recovered from pretreatment hydrolysate (spent liquor) and enzymatic hydrolysate.

enzymatic hydrolysate, the hydrolysis solid consistency of 2%, and the pretreatment solid substrate yield. Component yields (kg ton^{-1} wood) in the pretreatment hydrolysates (spent liquors) were based on the measured component concentrations (Table 2) and the pretreatment liquor to wood ratio of L/W = 3. Ethanol yield through simultaneous enzymatic saccharification and fermentation (SSF) can also be expressed as a percentage of theoretical yield that can be calculated by the amount of ethanol produced (from solid substrate only in this study) divided by the theoretical maximal amount of ethanol based on the amount of the saccharide in the untreated wood (only glucan in the present study), i.e.,

$$Y_{\text{Ethanol}}(\%) = \frac{\text{Ethanol from substrate}\left(\frac{L}{\text{kg substrate}}\right) \times \text{Solid substrate yield}\left(\frac{\text{kg substrate}}{\text{ton wood}}\right)}{\left(\frac{0.511}{0.9\times0.789}\right) \times \text{glucan content of wood}\left(\frac{\text{kg}}{\text{ton wood}}\right)} \quad (2)$$

Where 0.511 g/g is theoretical ethanol yield form glucose through yeast fermentation. A term of fermentation efficiency, $\eta_{\text{Fermentation}}$, is used to quantify the amount of ethanol produced (from solids substrate only in this study) as a percentage of the theoretical maximal amount of ethanol based on the amount of saccharide in the substrate (only glucan in the present study), i.e.,

$$\eta_{\text{Fermentation}} = \frac{\text{Ethanol from substrate}\left(\frac{L}{\text{kg substrate}}\right)}{\left(\frac{0.511}{0.9\times0.789}\right) \times \text{glucan content of substrate}\left(\frac{\text{kg}}{\text{kg substrate}}\right)} \quad (3)$$

It should be pointed out that fermentation efficiency is different from ethanol yield in percentage of theoretical maximal amount.

3. Results

3.1. Cell wall chemical structures before and after pretreatments

The cell wall chemical composition of aspen was very different from the other three poplars (Table 1). Aspen had a high glucan content of 45.6% and low lignin content of approximately 20%. The other three poplar samples had similar yet lower glucan content of approximately 40%. The lignin content was approximately 23% for NE222 and DN5 and greater than 25% for NM6.

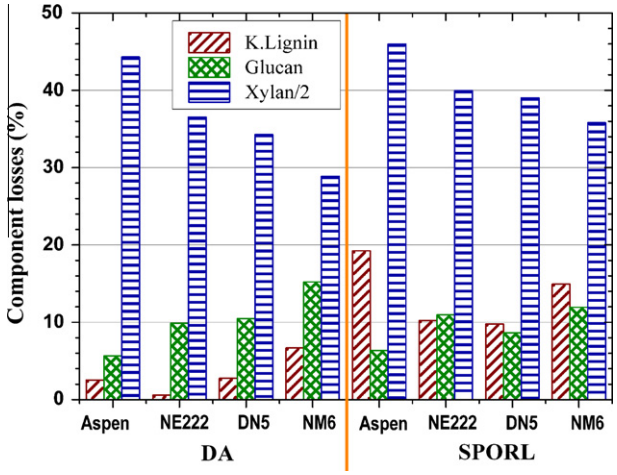

Fig. 2. Comparisons of losses of key components among four poplar wood samples by DA and SPORL pretreatments at 170 °C for 20 min with sulfuric acid charge on wood = 1.1% and additional sodium bisulfite charge on wood = 2.0% in SPORL.

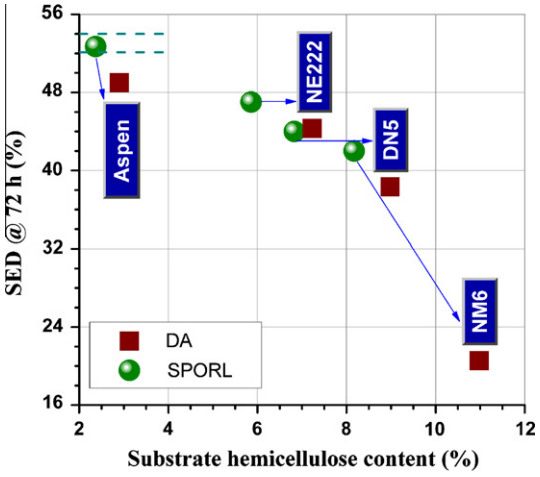

Fig. 3. Correlation between substrate enzymatic digestibilites (SEDs) and hemicellulose contents of pretreated solid substrate by DA and SPORL pretreatments. Cellulase loading = 7.5 FPU g^{-1} glucan with β-glucosidase loading = 11.2 CBU g^{-1} glucan.

The difference in xylan content among the four poplars was not significant, with DN5 exhibiting a slightly higher xylan content of 17.3%. However, the mannan content (~3.8%) of the three poplar clones was much higher than that of aspen (1.4%). The pretreated substrates by both DA and SPORL have enriched glucan and lignin content due primarily to the removal of hemicelluloses (Table 1). However glucan enrichment is much more pronounced for aspen (>64%) relative to the other poplar samples (43–49%). The glucan enrichment is more pronounced after SPORL pretreatment, while lignin enrichment is more pronounced after DA pretreatment due to lignin dissolution to lignosulfonate in SPORL pretreatment. The content of hemicelluloses of the pretreatment substrates ranged from less than 3% for aspen to approximately 8% (SPORL) and 11% (DA) for NM6. The solid substrate yields (>70%) of the three poplars were significantly higher than aspen (~65%). The dissolution of lignin by SPORL resulted in a lower solid substrate yield of between 2% and 4% than those of the corresponding DA substrate.

The removal of key components, i.e., lignin, glucan, and xylan, are calculated to illustrate the differences among the poplars when subjected to pretreatments (Fig. 2). The difference in xylan removal is obvious, with the highest for aspen at approximately 90% and lowest for NM6 at 71.6% (SPORL) and 57.8% (DA). The xylan removal for NE222 and DN5 ranged from 62% to 80%. The SPORL pretreatment removed slightly more xylan and lignin than DA did for all of the four samples studied. Except for NM6, glucan losses were all within 10% for both pretreatments.

3.2. Substrate enzymatic digestibility (SED) and sugar recovery

The cell wall structure, e.g., hemicellulose content, of the pretreated substrate affected substrate enzymatic hydrolysis as evidenced from the SEDs (Fig. 3). The aspen SPORL SED shown was interpolated from the two pretreated aspen SPORL substrates (ASA2B1.5 and ASA2B3). The difference in the SEDs of these two substrates was very small as shown by the two dashed lines in Fig. 3. Based on both the DA and SPORL pretreatment data, the rank of digestibility from greatest to least was: aspen, NE222, DN5, and NM6. The digestibility of DA-pretreated NM6 was very poor with SED of only 20%. The results also indicated that SPORL improved SED, especially for NM6. The results in Fig. 3 were corroborated by literature that showed substrate enzymatic digestibility was correlated with xylan contents (the major hemicelluloses) of substrates produced from the same feedstock but using different pretreatment conditions [29,30]. The SPORL substrates had both lower lignin and hemicellulose contents than their corresponding DA substrates, which resulted in improved substrate cellulose accessibility and therefore SED [31]. Because of the low cellulase loading of 7.5 FPU g^{-1} glucan, the SEDs of the four substrates were all below 60%.

The enzymatic hydrolysis glucose yield (EHGY) depends on wood cellulose content, cellulose dissolution through pretreatment, and SED. Except for NM6, the cellulose dissolution was lower than 10% for both DA and SPORL pretreatments (Fig. 2). The high cellulose content and high SED of aspen resulted in a significantly higher EHGY of 213 g kg^{-1} wood (42% theoretical yield) and 252 g kg^{-1} wood (50% theoretical yield) for DA and SPORL, respectively, compared to those of the other poplars (Table 2). Because the cellulose content of the three poplars was approximately the same, the low SED (Fig. 3) and high cellulose dissolution (Fig. 2) resulted in NM6 exhibiting the lowest EHGY (Table 2). The SPORL pretreatment produced higher EHGY than DA for each sample studied given the improved SED.

The glucan and hemicelluloses removed by pretreatments were partially hydrolyzed to fermentable sugars (Table 2). The following discussion is focused on xylose as it is the major monomeric sugar in the pretreatment spent liquor (hydrolysate). Xylose yields from aspen were significantly higher than those from the rest of the poplars for both DA (99 g kg^{-1} wood; 53% theoretical yield) and SPORL (118 g kg^{-1} wood; 63% theoretical yield) pretreatments. Xylose yield from NM6 after DA was the lowest (34 g kg^{-1} wood; 18% theoretical yield), and that from NE222 the second highest for both DA (84.8 g kg^{-1} wood; 47% theoretical yield) and SPORL (102 g kg^{-1} wood; 57% theoretical yield) pretreatments. In general, xylose yields are proportional to the amount of xylan removed through pretreatments (Fig. 2). Furthermore, SPORL pretreatment resulted in higher xylose yields than DA due to increased xylan dissolution by sulfite (Fig. 2) and relatively low degradation of xylose into furfural (Table 3). The incomplete removal of xylan was mainly responsible for the relatively low xylose yields of the three poplars.

3.3. Energy consumption for size reduction of pretreated wood chips

Pretreatment loosen wood structure by removed key wood components which can significantly reduce energy consumption for size reduction of pretreated wood chips [18]. Pretreatment also

Table 3
Lists of ethanol titers and yields from simultaneous enzymatic saccharification and fermentation (SSF) of solid substrates and compositions of the pretreatment hydrolysates.

Sample label	SSF @ 10% Substrate Solids				Pretreatment hydrolysate @ L/W = 3:1				
	SSF efficiency @ 120 h (%)	Ethanol titer @ 120 h (g L^{-1})	Ethanol yield @ 120 h (L kg^{-1} wood)	Glucose (g L^{-1})	Xylose (g L^{-1})	Mannose (g L^{-1})	Acetic acid (g L^{-1})	HMF (g L^{-1})	Furfural (g L^{-1})
ASA2B0	44.9	19.9	0.168	3.3	33.1	3.5	14.1	0.4	3.3
NE2A2B0	46.2	12.0	0.120	2.7	28.3	3.7	8.8	0.2	1.7
DN5A2B0	35.5	11.9	0.117	2.1	24.4	2.9	6.8	0.2	1.4
NM6A2B0	57.8	7.0	0.069	1.2	11.3	1.4	4.1	0.3	0.9
ASA2B1.5	60.0	23.3	0.183	3.0	38.2	4.2	17.6	0.4	3.8
ASA2B3	76.3	29.7	0.244	2.5	37.4	3.8	22.5	0.4	3.0
ASA2B2[a]	65.4	25.4	0.202	2.9	38.0	4.1	18.6	0.4	3.6
NE2A2B2	62.3	17.0	0.162	2.8	34.0	4.0	10.7	0.2	2.0
DN5A2B2	58.7	16.2	0.151	2.3	29.2	4.2	9.4	0.2	1.9
NM6A2B2	43.4	11.8	0.109	2.3	30.7	3.6	9.6	0.2	1.9

[a] Hypothetical sample and data from linear interpolation of ASA2B1.5 and ASA2B3.

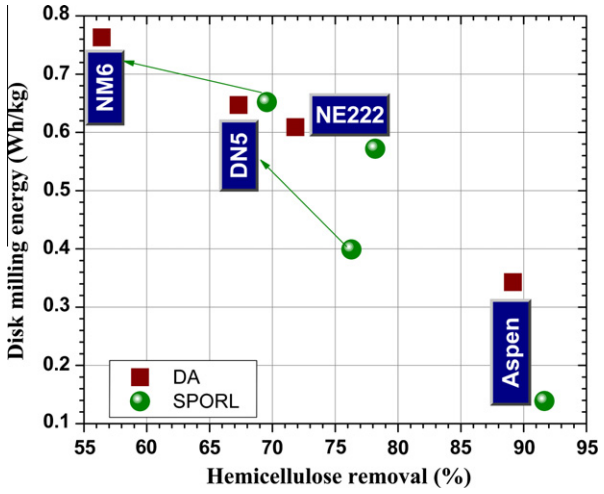

Fig. 4. Correlation between hemicellulose removal and energy consumption for size reduction of pretreated wood chips.

reduce solids yield which reduces energy consumption on untreated wood base. It was found that the energy consumption for wood size reduction correlate to hemicellulose (or xylan) removal very well (Fig. 4). Furthermore, it seems that the disk milling energy data fall to single curve for all of the four samples by both DA and SPORL pretreatments except for the DN5 SPORL run which most likely due to experimental error.

The disk milled solid substrate morphology can illustrate the overall physical integrity of the pretreated wood chips. Substrates produced from DA pretreatment will be used to illustrate this. The Aspen substrate (Fig. 5a) is much finer than the poplar substrates (Fig. 5b–d) even though the disk milling conditions were identical. The NM6 substrate consists of primary fiber bundles (Fig. 5d) while the NE222 and DN5 substrates consists of some individually separated fibers (Fig. 5b and c).

3.4. Plant biomass recalcitrance (PBR)

The plant biomass recalcitrance (PBR) was calculated using Eq. (1). The total energy inputs for pretreatment and total sugar recoveries are listed in Table 2. The total energy inputs included energy for wood log chipping, pretreatment thermal energy, and energy for disk milling of pretreated wood chips. The total sugar included EHGY, glucose, xylose, and mannose recovered in the pretreatment hydrolysate. Overall, PBR ranged from 6.9 (aspen) to 59.4 (NM6), based on DA pretreatment at 170 °C for 20 min. Recommendations from previous research were that PBR should be calculated based on data from a mild pretreatment at low to moderate enzyme dosages to differentiate among various feedstocks [13]. This was supported in the current study by the reduced range (5.4 for aspen to 11.0 for NM6) in PBR among the four poplars using the data from SPORL pretreatment (Table 2). The SPORL pretreatment conditions were the same as those for PBR determination in our previous study [13]. However, cellulase loading was reduced from 7.5 FPU g^{-1} substrate to 7.5 FPU g^{-1} glucan in the present study, equivalent to 32% reduction based on glucan content of 68% for the SPORL aspen substrate (ASA2B2 in Table 1). As a result, SED as well as EHGY were reduced and produced a higher PBR of 5.4 than the value of 2.2 reported previously [13]. Despite that PBR determined by Eq. (1) is affected by pretreatment and enzymatic hydrolysis conditions, the results shown in Table 2 indicated that it can be used to determine the relative variability in recalcitrance among different feedstocks.

3.5. Quasi-simultaneous enzymatic saccharification and fermentation (SSF) of solid substrate

The terminal ethanol concentrations after 120 h SSF along with ethanol yields from both the DA and SPORL solid substrates at 10% solid consistency are listed in Table 3. Due to the variations in the glucan content among the substrates, direct comparisons of the terminal ethanol concentrations have limited value. We calculated the SSF efficiency as described in the Materials and Methods section and found that NM6 had the lowest SSF efficiency among the four samples for both DA (36%) and SPORL (44%) pretreatments (Fig. 6a and b), which reflected the strong recalcitrance of NM6 (Table 2). It is noticed that these terminal SSF efficiencies were higher than the terminal SEDs (Fig. 2) because the termination time was 120 h for SSF, which was much longer than the 72 h for enzymatic hydrolysis. Saccharification continued after 72 h as evidenced by the time-dependent hydrolysis rate (not shown). The SPORL pretreatment improved SSF efficiency over DA for all of the four poplars by removing the feedstock recalcitrance. As a result, the differences in SSF among aspen, NE222, and DN5 became less distinct (Fig. 6b). The DA pretreatment resulted in aspen exhibiting the highest SSF efficiency, while NE222 and DN5 had similar SSF efficiencies that were lower than aspen but greater than NM6 (Fig. 6a). This order was consistent with the PBR reported previously (Table 2).

We also calculated the final ethanol yields from SSF (Table 3). The ethanol yields after 120 h SSF varied from 0.07 to 0.17 L kg^{-1} wood and 0.11 to 0.20 L kg^{-1} wood among the four poplars for DA and SPORL pretreatment, respectively. The rank order (from

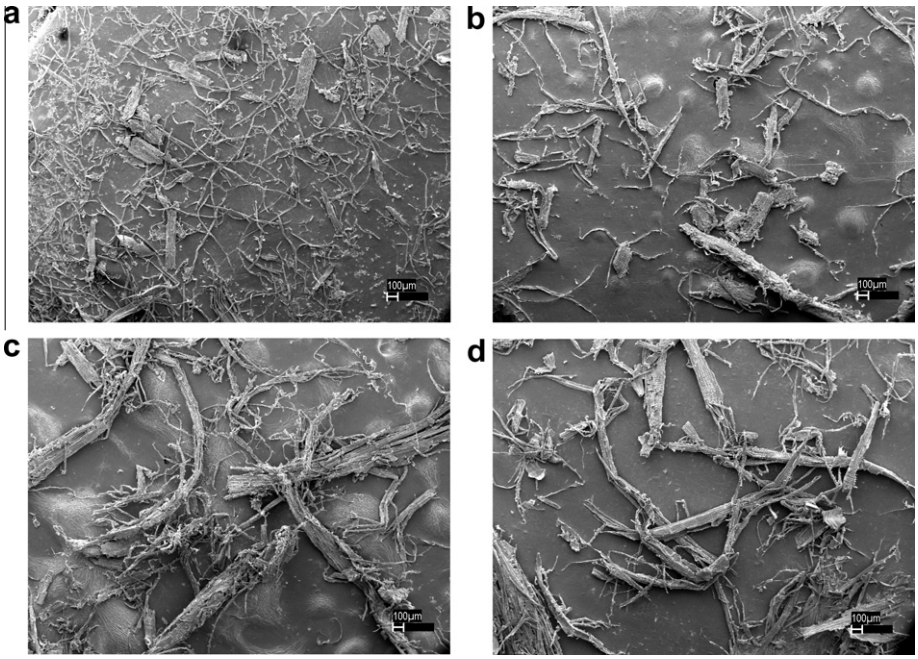

Fig. 5. SEM images of the DA pretreated substrates of the four poplar samples: (a) Aspen, (b) NE222, (c) DN5, and (d) NM6.

most to least) is consistent with SSF fermentation efficiency discussed above: aspen, NE222, DN5, and NM6.

Fermentation of pretreatment hydrolysates was not conducted due to lack of xylose fermenting strains. The monomeric sugar concentrations in the hydrolysate showed a similar order of rank as ethanol yields from the solid substrates. Xylose concentrations varied from 11.3 to 33.1 g L^{-1} for DA pretreatment. The variation in xylose concentrations was much narrower for the SPORL pretreatment. Both HMF and furfural concentrations were low in all the pretreatment hydrolysates. The greatest furfural concentration was 3.6 g L^{-1} found in the SPORL aspen hydrolysate. Acetic acid concentrations were high (especially in SPORL samples), ranging from 4.1 to 18.6 g L^{-1}. The production of acetic acid was correlated with SSF ethanol yield from solid substrate or xylan removal (Fig. 2), suggesting that deacetylation reactions were unavoidable when significant amount of xylan removal was required to improve cellulose conversion.

4. Discussion

The PBR of the four poplar samples correlates well with the lignin content of the untreated wood (Table 2). The measurement uncertainty may cause the problem in resolving the differences in lignin content between NE222 and DN5, noting that these genotypes are from the same parents and may exhibit similar wood properties. Furthermore, the key contributing factors to PBR, such as SED, sugar recovery, and energy consumption for size reduction of pretreated wood chips, also individually correlate well with wood lignin content (Table 2). A recent study reported that glucose yield correlated with wood lignin content along with lignin structure [15]. However, the lignin contents of the pretreated substrates produced from the same pretreatment process were approximately the same among the four samples (Table 1). It was highly probable that the pretreated SUBSTRATE lignin content does not significantly affect SED and sugar yield. Furthermore, the SED was found to decrease with the increase in lignin removal in general (not shown, based on data in Table 1). This does not mean that removing lignin reduces SED, but rather that increasing lignin removal is achieved at the expense of reduced hemicellulose removal using acid based pretreatments, which resulted in reduced SED. As discussed previously, SED is inversely proportional to substrate hemicellulose content (Fig. 3) or removal. However, reducing hemicellulose content by increasing pretreatment severity to improve hemicellulose removal usually resulted in reduced lignin removal due to lignin condensation in acid based pretreatments. This implies that removal of hemicelluloses is more important for increasing cellulose accessibility or removal of recalcitrance than lignin removal for the poplar wood samples studied using acid–based pretreatments. We further hypothesize that wood lignin content affects SED and sugar recovery by means of its effect on hemicellulose or xylan removal, which is corroborated by the fact that the amount of xylan and mannan removal was inversely proportional to the wood lignin content for both DA and SPORL pretreatments (Fig. 7). In other words, the ability of lignin protecting carbohydrate from depolymerization or hydrolysis dictates PBR. This will be further supported by the discussion of wood physical integrity in the next paragraph. Of course it is not simply the quantity of wood lignin, but also the type of lignin [15] and the distribution of lignin within the cell wall play a significant role in affecting xylan removal, cellulose accessibility to cellulase, and ultimately SED. These issues warrant further investigation.

Wood lignin also plays major role in the physical integrity of the pretreated wood chips. This is evidenced by the fact that the disk milling energy of the pretreated wood chips was inversely correlated to wood lignin content (not shown). It was found that the correlation between solids loss and disk milling energy was poor (not shown), however, the correlation between hemicellulose removal and disk milling energy was excellent (Fig. 4). This suggests that the removal of hemicelluloses is more directly related to wood structure changes than total solids loss by pretreatments. This is probably because that the majority of wood cell wall is made of a composite of primary cellulose and hemicelluloses in the *S* layers. Lignin is mainly located in the middle lamella that is thin and only occupies a small part of the cell wall. Consequently, the middle lamella may be not so important to the physical integrity of the cell wall. However, lignin protects the physical integrity of the cell wall by means of protecting carbohydrate loss by pretreatment, e.g., the NM6 sample with higher lignin content tends to loss less hemicelluloses, than aspen or NE222 both have lower lignin content. As a result, the disk milling energy for the pretreated NM6 was higher

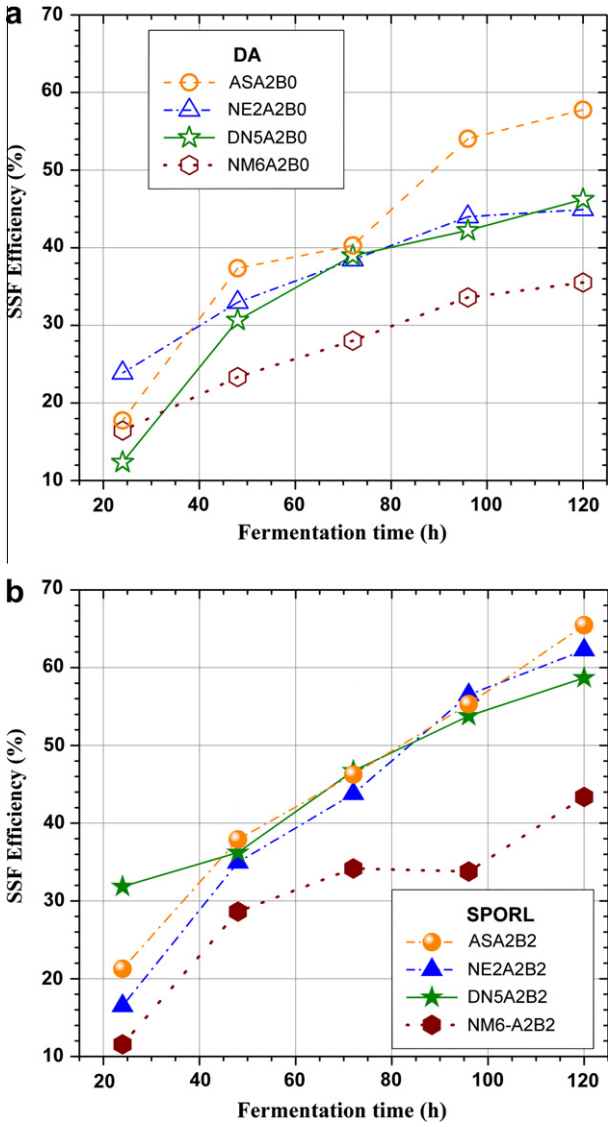

Fig. 6. Time-dependent simultaneous enzymatic saccharification and fermentation (SSF) efficiencies of four poplar samples at the same enzyme loadings as in Fig. 3. (a) Substrates pretreated by DA; (b) substrates pretreated by SPORL.

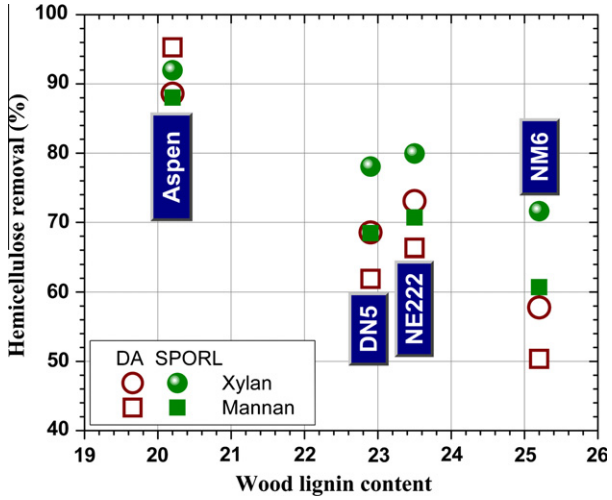

Fig. 7. Effect of wood lignin content on xylan and mannan removals by DA and SPORL pretreatments for the four poplar samples.

than those for the pretreated aspen and NE222. This is in agreement with the discussion in the previous paragraph.

Although statistical tree sampling was not conducted, we would like to point out that the four wood samples are from different genotypes. The variation in the ability of lignin protecting carbohydrate from deconstruction, or lignocellulose recalcitrance, among the studied samples may be partially attributed to the differences in their allometric traits. For example, both NE222 and DN5 belong to the *P. deltoides* × *P. nigra* genomic group and the magnitude of variability between these clones for their susceptibility to both DA and SPORL pretreatments was much less among themselves than those in the other genomic groups. It is also likely that the chemical composition of their cell wall is similar given that both parents (*P. deltoides*, *P. nigra*) belong to the taxonomic section *Aigeiros*, while the aspen belongs to the *Populus* section and the male parent of NM6 (*P. maximowiczii*) belongs to the *Tacamahaca* section. Nevertheless, despite the fact that the component removal for both pretreatments varied substantially among the four wood samples, the SPORL pretreatment was much less susceptible to feedstock variability.

5. Conclusions

DA and SPORL pretreatments were applied to four poplar wood samples of native aspen, NE222, DN5, and NM6. The aspen and NM6 showed significant differences for biochemical conversion in terms of substrate enzymatic digestibility, monomeric sugar yields, SSF efficiency, and ethanol yield. These results reflect the differences in wood recalcitrance and are consistent with comparison of a quantitative measure, plant biomass recalcitrance (PBR), which ranged from 6.9 MJ kg^{-1} wood (aspen) to 59 MJ kg^{-1} wood (NM6). Bioconversion results for the NE222 and DN5 samples were similar, and, in general, were between those of aspen and NM6. While both substrate lignin content and lignin removal by pretreatments did not affect substrate enzymatic digestibility, the wood lignin content was found to negatively affect xylan or hemicellulose removal, consequently disk milling energy for size reduction of pretreated wood chips, substrate enzymatic digestibility, and overall bioconversion efficiency in terms of sugar and ethanol yields. This indicates that the difference among different wood samples can be reflected from the difference in the ability of lignin protecting carbohydrate from depolymerization. More importantly, SPORL pretreatment not only improved sugar and ethanol yields over DA for all wood samples tested, but also reduced the magnitude of differences among them. This suggests that SPORL can better tolerate feedstock variability and is more effective in removing biomass recalcitrance, which has substantial practical advantages for bioconversion applications.

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