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Comparative data on effects of leading pretreatments and enzyme loadings and formulations on sugar yields from different switchgrass sources

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ABSTRACT

Dilute sulfuric acid (DA), sulfur dioxide (SO_2), liquid hot water (LHW), soaking in aqueous ammonia (SAA), ammonia fiber expansion (AFEX), and lime pretreatments were applied to Alamo, Dacotah, and Shawnee switchgrass. Application of the same analytical methods and material balance approaches facilitated meaningful comparisons of glucose and xylose yields from combined pretreatment and enzymatic hydrolysis. Use of a common supply of cellulase, beta-glucosidase, and xylanase also eased comparisons. All pretreatments enhanced sugar recovery from pretreatment and subsequent enzymatic hydrolysis substantially compared to untreated switchgrass. Adding beta-glucosidase was effective early in enzymatic hydrolysis while cellobiose levels were high but had limited effect on longer term yields at the enzyme loadings applied. Adding xylanase improved yields most for higher pH pretreatments where more xylan was left in the solids. Harvest time had more impact on performance than switchgrass variety, and microscopy showed changes in different features could impact performance by different pretreatments.

1. Introduction

The world is faced with dwindling fossil fuel reserves, and the most heavily used resource, petroleum, has the lowest supplies that are nearing a point of reduced production. The high world consumption of fossil energy also drives up accumulation of carbon dioxide, a powerful greenhouse gas that feeds global climate change. Biomass is the only sustainable resource that can practically be converted into liquid fuels that now are derived from petroleum, with cellulosic materials such as wood, grasses, and organic wastes essential to large scale production at costs that are potentially competitive with those for making fossil fuels (Lynd et al., 1999). Such biofuels can offer significant environmental, economic, and strategic advantages when implemented properly, and biological conversion has been of particular interest because of its ability to realize the high yields vital to economic competitiveness (Lynd et al., 2008). However, high yields are only realizable if cellulosic biomass is first pretreated to reduce its natural recalcitrance to sugar release, and pretreatment costs are among the highest in the overall conversion process (Lynd et al., 2008; Wooley et al., 1999). In addition, pretreatment has pervasive impacts on the costs and selection of all other operations from feedstock choices to product recovery and waste utilization (Wyman, 2007; Yang and Wyman, 2008). Because of the high cost of enzymes, interactions between pretreatment and enzymatic hydrolysis are particularly deterministic of overall processing costs (Yang and Wyman, 2008). A breakthrough in pretreatment could have tremendous impact in dramatically cutting enzyme loadings, the largest cost now, reducing hydrolysis times from days to hours, increasing sugar and therefore ethanol concentrations, and possibly transforming the overall process to resemble corn ethanol production. Thus, significant attention must be given to developing pretreatment systems

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and integrating them into the overall process to realize high yields with low enzyme loadings if we hope to realize low cost conversion of cellulosic biomass to liquid fuels for transportation.

However, pretreatment faces significant cost and performance constraints to be cost effective. First, high total sugar yields are critical to distribute both operating and capital costs over as much product as possible and thereby achieve the lowest possible unit costs. Operating costs must be kept low to provide a margin for return on capital, with the result that we can afford to spend little for chemicals, energy inputs, and labor. Furthermore, to compete with fuel production facilities such as for making gasoline that are already paid for (i.e., cash cows), the overall cash costs including those for pretreatment must be less than for the competition. On top of all this, the capital costs must be low enough to provide an acceptable rate of return, and low capital costs are essential to minimize exposure to market instabilities. Low capital costs in turn translate into the need for low cost containment through keeping vessel sizes small, pressure and temperature as low as possible, and materials of construction costs reasonable. Capital costs are also kept down by focusing on simple processes with the fewest possible operations (Wyman, 1995). Despite its importance and challenges, insufficient attention has been given to understanding or advancing pretreatment technologies over the years.

The Biomass Refining Consortium for Applied Fundamentals and Innovation (CAFI) was formed as a partnership among many leaders in biomass pretreatment and hydrolysis in late 1999 in Dallas and early 2000 in Chicago to meet this need (Wyman et al., 2005b). The goals of the team were to (1) compare the effectiveness of leading pretreatments in recovering sugars from the coupled operations of pretreatment and enzymatic hydrolysis of cellulosic materials, (2) gain insight into fundamentals to facilitate commercialization and lead to step change cost reductions, and (3) train and educate students in biomass conversion technologies. To accomplish these goals, the CAFI team recognized the importance of developing data on leading pretreatments using common feedstocks, shared enzymes, identical analytical methods, the same material and energy balance methods, and the same costing models. Because lack of data clouds decisions, a key focus of CAFI was to provide transparent information to help industry select technologies for applications and not to "downselect" pretreatments as it is vital to provide data on as many promising options as possible so others can decide which to employ. In addition to providing this technology base to facilitate commercial use, the CAFI team believed that it is important to understand mechanisms that influence performance and differentiate pretreatments to support optimization of pretreatment technologies, facilitate their integration into the overall process, and suggest promising paths to advance pretreatment technologies. An Agricultural and Industrial Advisory Board of about 25 members was given the opportunity to meet with the CAFI Team at 6 months intervals and provided valuable reviews and guidance to the team during those meetings.

The CAFI team was supported from 2000 to 2003 through a competitive solicitation by the Initiative for Future Agriculture and Food Systems Program of the US Department of Agriculture to focus on developing comparative data on pretreatment of corn stover by leading technologies followed by enzymatic hydrolysis; we now term this project as CAFI 1 (Wyman et al., 2005a). Then, from 2004 to 2007, CAFI was selected through a competitive solicitation by the Office of the Biomass Program of the US Department of Energy to develop comparative information on application of leading pretreatments followed by enzymatic hydrolysis and fermentation for poplar wood, now known as CAFI 2 (Wyman et al., 2009). Finally, from 2007 to now, the Office of the Biomass Program funded CAFI again, but this time to compare how switchgrass responds to leading pretreatments in a project we call CAFI 3. This paper provides an introduction and summary of key results for the CAFI 3 project on switchgrass; more details are provided in other papers by the CAFI Team in this volume of *Bioresource Technology* as well as in other publications.

2. Methods

2.1. Analytical

Unless noted otherwise in the CAFI papers, all analytical methods followed the Standard Analytical Procedures (SAP) of the National Renewable Energy Laboratory (NREL) and have been described previously (NREL, 1995; Wyman et al., 2005b).

2.2. Switchgrass

Three different varieties of switchgrass, supplied by Ceres Corporation (Thousand Oaks, CA), were used in this study: Alamo, Dacotah, and Shawnee. The Alamo employed was a lowland plant with thick stems that was planted in Ardmore, OK in June 2005 and harvested in the fall of 2006. The Dacotah switchgrass was an upland species with thin stems that was planted in northeast South Dakota in December 1999 and harvested in late winter in 2008. The Shawnee switchgrass was also an upland plant with thin stems planted in Stillwater, OK in June 2005 and harvested in the fall of 2006.

The cell wall chemical compositions of Alamo and Shawnee switchgrass were not found to be statistically different (Fig. 1) even though they represent different ecotypes. However, non-structural components (soluble carbohydrates and protein) were significantly different. Dacotah switchgrass, an upland ecotype, contained considerably more glucan and lignin and less non-structural sugars and protein than either Alamo or Shawnee. The Dacotah switchgrass represents a high-latitude variety of switchgrass, was grown the furthest north of any samples tested, and was allowed to stand in the field over winter before harvest. Thus, the compositional difference appears to depend more strongly on harvest time than on variety or ecotype. Late harvest correlates to a higher mass fraction associated with cell wall components (cellulose, hemicelluloses, and lignin) and a lower mass fraction associated with non-cell wall components such as residual monosaccharides and protein.

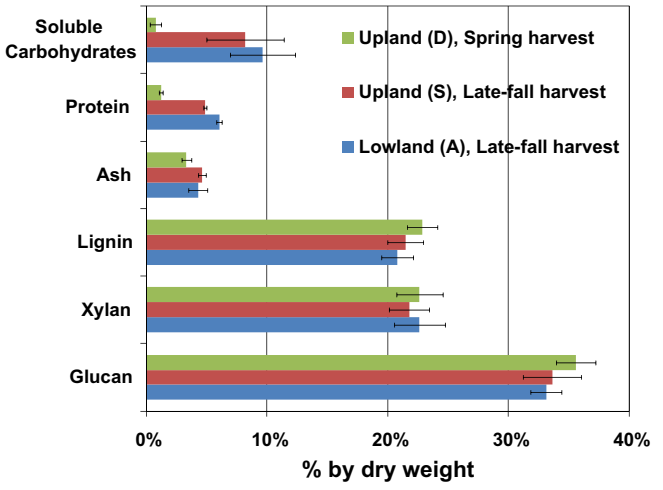

Fig. 1. Compositions of different ecotypes and harvest season of switchgrass. Error bars represent 95% CI. A-Alamo 1; S-Shawnee; D-Dacotah.

2.3. Enzymes

Genencor, a Danisco Division, provided enzymes to each member of the consortium, with each enzyme sample from a single same lot reserved for use, in a similar fashion to the approach for the prior two CAFI projects with corn stover (CAFI 1) and poplar (CAFI 2). A list of the enzymes is given in Table 1. Common protein numbers were agreed upon in order to have a uniform dosing basis across each lab in the consortium. Nitrogen analysis was performed with a LECO® TruSpec® nitrogen analyzer after a trichloroacetic acid (TCA) precipitation step to account for non-protein nitrogen. Spezyme® CP is a *Trichoderma reesei* whole cellulase product, and Multifect® Xylanase was provided for improved hemicellulose conversion. In addition to Genencor enzymes, Novozymes product Novozyme®188 was also used for exogenous beta-glucosidase supplementation.

2.4. Pretreatments

2.4.1. Dilute sulfuric acid (DA)

For dilute acid pretreatment, 50 g of each pre-washed switchgrass was presoaked overnight in 1 wt.% dilute sulfuric acid solution at room temperature with a solids loading of 10 wt.% on a dry basis. The presoaked biomass slurries were transferred to a 1 L Parr reactor made of Hastelloy C (Parr Instruments, Moline, IL) equipped with two 40 mm diameter stacked pitched blade impellers for stirring (200 rpm) and a K-type thermocouple for monitoring the temperature in the reactor. The reactor was heated in a 4-kW fluidized sand bath (model SBL-2D, Techne Co., Princeton, NJ), with the heat up time for this system of about 2 min not included in the stated reaction time. After pretreatment at the optimum conditions, presented by Shi et al. in this volume, of 140 °C for 40 min, the reactor was quenched in a room temperature water bath until the temperature dropped to 80 °C. The slurry was vacuum filtered immediately through a glass fiber filter (Whatman®, Grade GF/A, diam. 11.0 cm), and the filtrate was collected for sugar analysis. The resulting solids were then washed four times each with 500 mL room temperature deionized water until the filtrate pH was above 6 (Yang and Wyman, 2009).

2.4.2. Sulfur-dioxide impregnated steam explosion (SO_2)

Prewashed switchgrass was pressed to a moisture level of about 65 wt.% with a hydraulic press and then impregnated overnight with 5% wt/wt (or 0.05 gram SO_2 per gram dry grass) gaseous sulfur dioxide (SO_2, >99% pure, Matheson Tri-Gas, Newark, CA) at room temperature in a sealed heavy duty Ziploc bag. Prior to pretreatment, pre-impregnated Dacotah switchgrass was carefully transferred to a 1 L Parr reactor made of Hastelloy C (Parr Instruments, Moline, IL) and mixed with deionized water to a solids loading of 10% wt/wt on dry basis. Pretreatments were run at 180 °C for 10 min, with heat provided by a 4-kW fluidized sand bath (model SBL-2D, Techne Co., Princeton, NJ). The biomass slurries were stirred at 200 rpm with two 40 mm diameter stacked pitched blade impellers rotating to push the material downwards. The temperature inside the reactor was monitored using a K-type thermocouple, and the approximate 2 min heat up time was not included in the stated reaction time. After pretreatment, the reactor was quenched in a room temperature water bath until the temperature dropped to 80 °C. The slurry was immediately vacuum filtered through a glass fiber filter (Whatman®, Grade GF/A, diam. 11.0 cm) with the temperature being always higher than 60 °C. The resulting solids were then washed with room temperature deionized water until the filtrate pH was above 6.

Table 1
Protein concentrations for CAFI enzymes.

Product name	Lot number	Total protein by nitrogen-analysis, TCA precipitated protein mg Total Protein/mL	Enzyme activity
Spezyme® CP	301-05330-206	82 (123[a])	59 FPU/mL
Multifect® Xylanase	301-04296-205	27(42[a])	–
Novozyme188		67	600 CBU/mL

[a] Protein values in mg/ml estimated by BCA method; FPU- filter paper unit; CBU- cellobiosidase unit.

2.4.3. Liquid hot water (LHW)

Liquid hot water pretreatment was carried out on slurries of switchgrass and de-ionized water corresponding to 150 g of dry biomass per kg (15% w/w) heated to 200 °C under pressure to prevent significant water vaporization (Ladisch et al., 1998; Weil et al., 1997, 1998). Laboratory scale pretreatment reactors were constructed of 1 in. OD × 0.083 in. wall thickness (2.54 cm × 2.1 mm) × 4.5 in. (11.4 cm) long, 316 stainless steel tubing capped at both ends with 1 in. (2.54 cm) Swagelok tube end fittings (Swagelok, Indianapolis, IN), as described by Kim et al. (2009a). Reactors had a total volume of 45 mL and a working volume of 33.7 mL, allowing 25% of the volume for liquid expansion during heating. Reactor heating was achieved using a Tecam® SBL-1 fluidized sand bath (Cole-Parmer, Vernon Hills, IL) set to 200 °C. Heat up time was measured as 8 min, thus a total of 18 min were required for a standard 10 min reaction. Cool down was achieved by quenching the reactor in room-temperature water for 10 min. Liquid–solid separation of the pretreated slurry utilized Whatman® No 41 filter paper in a Büchner funnel with water aspiration. The liquid fraction was retained for further analysis. The retained solids were then washed with hot water as described previously (Kim et al., 2009b). Separated pretreated solids and pretreatment liquor were stored frozen (−20 °C) until analyzed.

2.4.4. Ammonia fiber expansion (AFEX)

Four batches of AFEX pretreated switchgrass were prepared in a 300 mL 316 stainless steel Parr reactor. Washed Dacotah switchgrass (15.0–16.5 g DM) was mixed with the appropriate amount of deionized water (2.0 g H_2O:g DM), and the reactor was preheated to 140 °C. Biomass was then loaded into the reactor, which was sealed and evacuated with a rotary vacuum pump. Meanwhile, the required amount of ammonia was loaded into a separate pressure vessel and heated until the pressure reached 640–780 psi. At that point, the ammonia was dispensed into the reactor (1.5 ± 0.1 g NH_3:g DM) to mark the beginning of the 30 min residence time. Temperature was maintained within ±10 °C of the target value for the duration of the residence time, at which point the reactor was vented into a hood. The reactor was quenched in a cold water bath for five minutes, and the biomass was unloaded. Residual ammonia was allowed to evaporate overnight in a fume hood, and the total weight (g DM) of the pretreated biomass was determined. Prior to performing the ammonia addition determination, all four batches were combined to reduce batch variation effects. For the post-wash, AFEX-treated switchgrass was washed with hot (100 °C) distilled water using an Accelerated Solvent Extractor (ASE 200, Dionex Corp., Sunnyvale, California, USA) at an average ratio of 11.6 ± 0.3 mL water: g DM. The liquid was extracted through glass fiber filters, the liquid volume was determined, and the sample was retained for further analysis of oligomeric and monomeric sugars. The dry mass of the solids for all five replicates was determined, the weight loss due to washing was calculated for each replicate, and then all five replicates were combined for enzymatic hydrolysis, without further water removal. Although

AFEX-treated biomass can be fermented without washing or inhibitor removal (Lau and Dale, 2009), the pretreated solids were washed here to be consistent with other pretreatments that employ washing. In addition, AFEX does not generate a separate liquid stream as other pretreatments considered here but rather is a "dry to dry" process with no free liquid stream.

2.4.5. Soaking in aqueous ammonia (SAA)

Batch reactors were used for the SAA pretreatment of switchgrass. First, 10 dry grams of switchgrass was soaked in a stainless steel reactor (1.375″ ID × 6″ L) followed by adding 90 mL of 15% NH_4OH. The reactor was sealed and kept in a preheated temperature controlled oven at 160 °C for a 60 min soaking time. The approximately 20 min time to reach 160 °C was not included in the stated reaction time. After pretreatment, the reactor was immediately removed from the oven and quenched to room temperature in a water bath. The cooled slurry was vacuum filtered immediately through filter paper (Whatman®, Grade 802 Fluted, size 32.0 cm), and the vacuum filtered wet solids were washed further with deionized water until the pH reached approximately 6.

2.4.6. Lime

Lime pretreatment was performed in a 304 stainless steel pipe reactor (5-in. long, 1.5-in. ID). Switchgrass (8 g) and excess calcium hydroxide (8 g) were thoroughly mixed and then transferred into the reactor. Water (15 g/g dry biomass) was added, and the reactor was sealed using Teflon tape and a 1.5-in 304 stainless steel cap. The reactor was pressurized with pure oxygen (6.89 bar) through a flexible stainless steel hose attached to an oxygen tank. The reactor was connected to a swing arm located in a preheated temperature controlled oven (120 °C). The shaking mechanism was activated and the reaction proceeded for 4 h. Once the target pretreatment time was reached, the reaction was quenched by quickly placing the reactor in an ice bath. Once cooled, the reactor was slowly opened to relieve pressure. The reactor contents were quantitatively transferred to a 1-L plastic centrifuge bottle, neutralized using 5 N HCl to a pH of 4.0, and then washed several times with distilled water until the pH increased to 6.0. After vacuum filtration, moisture content and final weight of the pretreated solids were recorded, and the solids were stored in the freezer for later analysis. The filtrate was collected for carbohydrate analysis.

2.5. Material balances

Material/mass balances for the different pretreatments, hydrolysis, and fermentations are critical to meaningful comparison of the different pretreatment options as well as in judging process economics and operational feasibility, and Fig. 2 outlines the steps and streams tracked by material balances around pretreatment and enzymatic hydrolysis. The water, solid biomass, and catalyst loadings and the reaction conditions were recorded for each pretreatment. Structural carbohydrates (glucan, xylan, arabinan), Klason lignin, and ash contents were determined for each stream using the National Renewable Energy Laboratory protocol mentioned above (Sluiter et al., 2008a,b). Because switchgrass can contain a substantial amount of free sugars (0.4–2.0 kg/100 kg) whose degradation during pretreatment would confuse the results, soluble sugars were removed using a water wash prior to all pretreatments. The pretreatments evaluated here other than AFEX generated a separate liquid stream rich in hemicelluloses sugars and lignin, and monomeric sugar equivalents in this stream were determined. Many pretreated solids contained residual bound catalyst that needed to be washed out prior to enzymatic hydrolysis, and the carbohydrate and lignin content of the pretreated solids (washed if necessary) were analyzed. All material balances were adjusted to a basis of 100 kg of dry untreated biomass to facilitate following the results and comparing pretreatments.

Enzymatic hydrolysis was performed at a solids loading equal to 1% glucan to minimize the impact of sugar inhibition of enzymes on determining pretreatment effectiveness. Enzymes were loaded based on glucan present in the prewashed, untreated biomass and reported in the mass balance as kg of protein per 100 kg of dry biomass input to pretreatment. After 72 h of hydrolysis at 50 °C and ~200 rpm, the solids were separated by centrifugation, and the monomeric sugars present in the hydrolyzate were determined using HPLC equipped with Aminex HPX -87H/87P columns (Biorad, Hercules, CA). Soluble oligosaccharides (e.g. gluco- and xylo-oligosaccharides) present in the hydrolyzates were further hydrolyzed to monomers by post hydrolysis with 4% sulfuric acid at 121 °C for 1 h, and the monomeric sugars generated were quantified by HPLC. The difference between the sugar levels after this post hydrolysis step and that prior to its use was taken as the amount of oligomers. The gluco- and xylo-oligosaccharides are reported as their respective monomeric sugar equivalents. Material

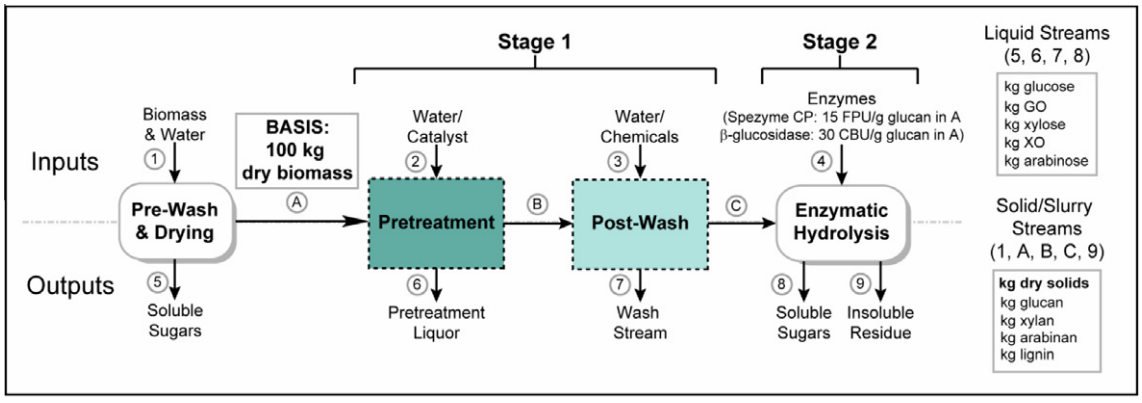

Fig. 2. Flow diagram outlining the key steps and streams tracked by the material balance for pretreatment and enzymatic hydrolysis. Here, Stage 1 includes pretreatment and post-wash, while Stage 2 is enzymatic hydrolysis. All flows are based on 100 kg of untreated, washed biomass, dry weight basis (DWB) (Stream A). The inputs and outputs to the process are indicated by numbered streams (1–9). Lettered streams (A–C) indicate internal streams for the given step. The washing and enzymatic hydrolysis steps were common to all pretreatments. The output streams 5, 6, 7, and 8 are presented on the basis of monomeric sugars (glucose, xylose and arabinose), with oligomers reported separately as their monomeric equivalents (GO = gluco-oligosaccharides, XO = xylo-oligosaccharides), while the slurry and solid streams 1, A, B, C, and 9 are presented on the basis of polymeric sugars (e.g., glucan, xylan and arabinan). Lignin was not measured for the liquid streams. Cellulase enzymes are reported as kg protein/100 kg of prewashed, dry biomass input to pretreatment.

balances around enzymatic hydrolysis were adjusted to a basis of 100 kg of dry untreated biomass, consistent with above.

Because of the high variability in pre-washing compositions, the mass balance did not include the pre-washing step, and all calculations were based on the pre-washed untreated biomass (kg/100 kg DBM) composition.

$$Y_{Glc} = \{[(Glc + GO)_{ST1P} + (Glc + GO)_{ST2EH}]/ [(180/162) \times (Gln)_{UTB} + (Glc)_{UTB}]\} \times 100\% \quad (1)$$

$$Y_{Xyl} = \{[(Xyl + XO)_{ST1P} + (Xyl + XO)_{ST2EH}]/ [(150/132) \times (Xyn)_{UTB} + (Xyl)_{UTB}]\} \times 100\% \quad (2)$$

in which Gln means glucan, Glc means glucose, GO means gluco-oligosaccharides (as monomeric equivalents), Xyn means xylan, Xyl means xylose, XO represents xylo-oligosaccharides (as monomeric equivalents), ST1P is Stage 1 pretreatment, ST2EH is Stage 2 enzymatic hydrolysis, UTB is untreated, pre-washed dry biomass, (180/162) is the correction coefficient between molecular weights of glucan and glucose, and (150/132) is the correction coefficient between molecular weights of xylan and xylose. A key to this work was to report material balances, in line with some published reports for both pretreatment and enzymatic hydrolysis (Balan et al., 2009; Kim et al., 2009b; Wyman et al., 2005a, 2009; Zhu et al., 2010). In Fig. 2, a 100% mass closure results when the captured output streams compositions (5–9) added together are equivalent to input stream 1, and departure from mass closure depends mostly on experimental error during the capture of output streams (5–9) as well as losses to compounds not being analyzed for. Other important parameters needed to characterize different pretreatment processes include: (1) pretreatment temperature, (2) residence time (total reaction time from start to finish), (3) amount of catalyst used (kg/kg of DBM), (4) amount of catalyst recycled, and (5) the amount of water used (L/100 kg DBM) during the pretreatment process and post-wash steps. Process energy balances are likewise important but are beyond the scope of this comparative work.

3. Results and discussion

3.1. Glucose and xylose yields

Table 2 summarizes glucose yields from pretreatment (Stage 1) and enzymatic hydrolysis of pretreated solids (Stage 2) at a cellulase loading of 30 mg/g glucan in prewashed Dacotah switchgrass. As for prior CAFI projects, yields are reported based on a maximum possible total glucose contribution of 60.6% and a maximum possible xylose contribution of 39.4% from the Dacotah feedstock, with yields from other switchgrass varieties adjusted according to their composition. Pretreatment technologies are listed in order of increasing pH, and all of the pretreatments resulted in a small fraction of the total glucose being released in Stage 1, with most solubilized in Stage 2. Lower pH pretreatments, i.e., DA, SO_2, and LHW, solubilized slightly higher levels of glucose in Stage 1 than higher pH pretreatments, i.e., AFEX, SAA, and lime. All pretreatments significantly increased total glucose yields compared to untreated switchgrass even at lower cellulase enzyme loadings, indicating that all pretreatments were effective in making cellulose accessible to enzymes. However, only lime and SO_2 pretreatments gave glucose yields close to the maximum possible of 60.6%, with the lowest glucose yield of only 39.9% being for SAA.

Table 2 also documents xylose yields from hemicellulose during pretreatment (Stage 1) and enzymatic hydrolysis of pretreated solids (Stage 2) at an enzyme loading of about 30 mg/g glucan in prewashed Dacotah switchgrass. Reasonably high xylose yields were achieved for all systems. Most of the xylose was released in pretreatment, Stage 1, for dilute acid, SO_2, and liquid hot water (LHW) pretreatments. Furthermore, most of the xylose was released as monomers for just the dilute acid system, with LHW giving high levels of xylose oligomers. On the other hand, the high-pH pretreatments by SAA and lime released more xylose sugars in the second stage, with about half being solubilized in the second stage for SAA and two thirds for lime. Surprisingly, about one third of xylose sugars, mostly as oligomers, were released by post-wash of AFEX pretreated switchgrass, suggesting that a large amount of hemicelluloses were solubilized during AFEX pretreatment, even though compositional analysis of the AFEX pretreated solids indicated virtually no changes.

Total glucose plus xylose sugar yields are also shown in Table 2. Most of the pretreatments realized overall sugar yields of around 80% at an enzyme loading of 30 mg/g glucan in pre-washed Dacotah switchgrass. However, although yields for SAA pretreatment were lower, the yields were similar when cellulase loadings were increased substantially to protein loadings corresponding to about 60 FPU/g. This yield variation is somewhat similar to what the CAFI team found for poplar wood (Wyman et al., 2009) but contrasts with the more uniform performance shown by the CAFI study on corn stover (Wyman et al., 2005a).

3.2. Reaction conditions for best performance

The top of Table 3 summarizes conditions employed to achieve the yields reported in Table 2. Most of the temperatures were in the range of 120–200 °C. Furthermore, with the exception of a 4 h pretreatment with lime, all pretreatments were applied for between 10 and 60 min, making it possible to pretreat biomass in

Table 2
Yields of glucose and xylose for each pretreatment (Stage 1) followed by enzymatic hydrolysis (Stage 2) with an enzyme protein loading of 30 mg/g glucan in the pre-washed untreated Dacotah switchgrass.

Pretreatment	Xylose yields			Glucose yields			Total sugars		
	Stage 1	Stage 2	Total xylose	Stage 1	Stage 2	Total glucose	Stage 1	Stage 2	Combined total
UT	N/A	1.9	1.9	N/A	8.4	8.4	N/A	10.3	10.3
Max possible			39.4			60.6			100.0
DA	29.3/1.7	3.4	32.6/1.7	4.3/0.5	42.2	46.5/0.5	33.6/2.2	45.6	79.2/2.2
SO_2	28.7/1.5	3.2	31.9/1.5	3.0/1.5	48.3	51.4/1.5	31.7/3.0	51.5	83.2/3.0
LHW	25.9/17.2	5.3/1.1	31.3/18.3	4.1/3.8	47.3	51.4/3.8	30.0/21.0	52.6/1.1	82.6/22.1
Lime	13.6/13.6	22.4/0.8	36.0/14.3	0.9/0.8	54.0/3.0	54.9/3.8	14.5/14.4	76.4/3.8	90.9/18.2
SAA	9.5/8.7	17.8/6.9	27.3/15.5	0.2/0.2	39.8/1.2	40.0/1.4	9.7/8.9	57.6/8.1	67.3/17.0
AFEX	11.1/11.1	25.6/3.0	36.7/14.1	0.8/0.8	47.1	47.9/0.8	11.9/11.9	72.7/3.0	84.6/14.9

Stage 1 refers to pretreatment and Stage 2 refers to the enzymatic digestion of the solids produced in pretreatment. The first value reported in each column is for total sugars released into solution, and the second is for just the oligomers released. A single value indicates release of only monomers. Yields are defined based on the maximum potential sugars released from the pre-washed Dacotah switchgrass used of 65.6 g per 100 g of dry solids with the maximum potential xylose being 39.4% and the maximum potential yield of glucose being 60.6% on this basis. ND = not determined.

Table 3
Pretreatment conditions, corresponding solids compositions, and component removals following pretreatment of Dacotah switchgrass by leading technologies (Shi et al., this issue, in the same issue).

	Pretreatment technology						
	None	DA	SO_2	AFEX	LHW	SAA	Lime
Pretreatment conditions							
Water/Solid ratio		8.91	8.95	2	5.6	7.65	15
Temperature, °C		140	180	140	200	160	120
Chemical loading		1.0 wt.% H_2SO_4 solution	0.05 g SO_2 per g biomass	1.5:1.0 NH_3	None	15% NH_4OH	1 g $Ca(OH)_2$ per g biomass + 100 psi O_2
Reaction time, minutes		40	10	30	10	60	240
Component in solids, %[c]							
Solids recovery after pretreatment	100.0	60.4	62.4	100.0	60.1	62.1	65.2
Glucan	35.6	50.3	53.9	35.9	50.1	55.6	53.0
Xylan	22.6	4.5	2.7	22.5	2.5	21.9	21.5
Arabinan	3.1	0.5	0.7	3.4	0.0	2.4	1.7
Acetyl	3.6	0.3	0.5	2.4	0.3	1.5	0.0
Lignin (AIS[a])	21.1	29.4	27.6	24.4	30.6	13.9	14.6
Others[b]	13.9	15.0	14.6	11.4	16.6	4.7	9.2
Component removal							
% Lignin removal	–	16.0	18.6	–	13.0	59.3	55.1
% Xylan removal	–	88.0	92.6	0.7	93.4	39.9	38.0
% Arabinan removal		90.3	85.9	Negligible	100	52	64
%Acetyl removal	–	94.9	91.3	32.7	95.6	74.9	100.0

[a] AIS – acid insoluble lignin.
[b] Others include proteins, ash, and uronic acids etc.
[c] Percent component in solids is based on the remaining solids after pretreatment, except for solids recovery after pretreatment, which is based on starting biomass.

reasonably sized vessels. Table 3 also lists chemical inputs. Because LHW relies on carboxylic acid released during hydrothermal breakdown of the cell wall structure and the drop in pH at higher temperatures, no additional chemical catalyst was needed. Dilute acid and SO_2 pretreatments used the lowest mass of chemicals of all other pretreatments, and alkaline pretreatments by AFEX, SAA, and lime technologies used the largest amounts. Chemical recycle is possible, but the additional capital and operating costs must be considered.

3.3. Compositions of pretreated solids

Because of variations in reaction mechanism, the pretreatment options tested produced very different effects on switchgrass solids. As shown in the lower portion of Table 3, lignin, glucan, xylan, arabinan, and acetyl contents in the pretreated solids were distinctive for the different pretreatments. Table 3 also includes the effect of the pretreatments studied here on the removal of major biomass components. In general, the major composition change for pretreatment at lower pH conditions, i.e., dilute acid, SO_2, and LHW, was nearly complete (>90%) removal of xylan and arabinan and about 13.0–18.6% removal of original lignin. As a result, solids resulting from pretreatment at acidic conditions had very low xylan contents of 2.5–4.7%, much lower than for the untreated material. However, for alkaline pretreatments, i.e., SAA and lime, substantial portions (55.1–59.3%) of the original lignin were removed while xylan removal was moderate (38.8–39.9% of the original). As a result, the solids remaining after SAA and lime pretreatment had lignin contents of only about 14%, xylan levels close to that in the feedstock of about 22%, and enriched glucan contents of 53.0–55.6%. In addition, both acid and alkali pretreatments removed almost all of the carboxylic acid substitutions, e.g., acetyl groups and uronic acids, from hemicellulose in addition to some hemicellulose, with an expected improvement in enzyme access to hemicellulose and cellulose (Kim and Holtzapple, 2006; Kumar et al., 2009). AFEX stood in stark contrast to the other pretreatments in that virtually no compositional change was evident for the switchgrass other than negligible xylan loss and minor acetyl removal. Thus, the improvements in digestibility by AFEX pretreatment could possibly be attributed to hemicellulose depolymerization, lignin relocation, cellulose decrystallization, phase change of cellulose as a result of cellulose swelling, and/or increases in the size and number of micropores in the cell wall, as shown in previous studies (Chundawat, 2009; Dale et al., 1996).

In summary, a major portion of hemicellulose was removed during low pH pretreatments, and a large part of the lignin plus some xylan were removed during high pH pretreatments, except AFEX. However, the extent of compositional changes varied with the type of biomass and pretreatment, in line with previous CAFI studies on pretreatment of corn stover and poplar (Elander et al., 2009; Kumar et al., 2009; Mosier et al., 2005; Wyman et al., 2005b,2009).

3.4. Yields vs. cellulase and beta glucosidase loadings

Because biomass is naturally resistant to breakdown into sugars, a key purpose of pretreatment is to make high yields by enzymatic hydrolysis possible in reasonable times. In addition, it is desirable for pretreatment to increase the efficiency of hydrolytic enzyme action, thus reducing the enzyme loadings required to achieve high yields in biomass hydrolysis. Because of the multitude of different enzyme activities in the cellulase system and the different roles they play in the reaction path, enzyme efficiency is strongly affected by biomass composition and structure. Literature information on saccharification yields for pretreated biomass collectively indicates that the cellulase loading required to attain acceptable sugar yields is so high that it becomes a major cost item in the overall bioconversion process (Merino and Cherry, 2007; Sun and Cheng, 2004). One way to improve the efficiency of overall enzymatic hydrolysis is to adjust the enzyme formulation. Supplementation with external β-glucosidase is often applied for this purpose to reduce inhibition by cellobiose, a reaction intermediate. Thus, an important task to the collaborative CAFI research was to delineate the effect of enzyme loadings and supplementation with β-glucosidase on sugar yields and to identify and analyze factors that influence sugar release during enzymatic hydrolysis.

Consistent with this direction, hydrolysis experiments were performed using solids left after application of the range of CAFI

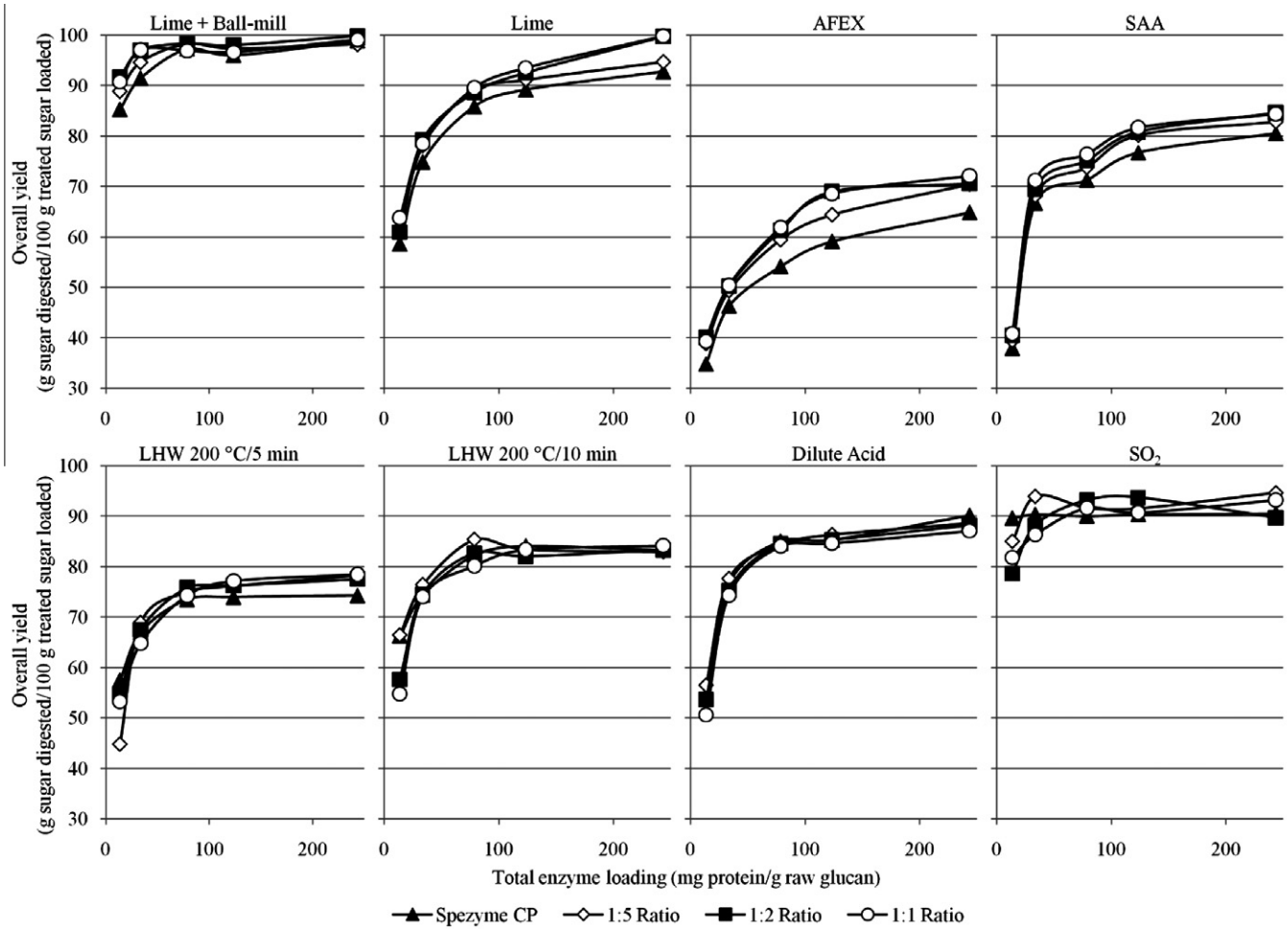

Fig. 3. Total glucose plus xylose yields plotted against total enzyme protein loadings for different supplementation ratios of xylanase.

pretreatments to switchgrass at conditions judged to maximize xylose plus glucose sugar recovery. Profiles of end products (monomers) and reaction intermediates (cellobiose and oligomers) were observed over the entire course of enzymatic hydrolysis by Spezyme CP and Novozyme 188. These Novozyme 188 and Spezyme CP enzymes were added in quantities to give a constant CBU/FPU ratio of 2.0 for all experiments. Four Spezyme CP loadings were applied: 4.2, 20.8, 41.7, or 83.4 mg protein/g-glucan, with the glucan amounts reflecting those in switchgrass prior to each pretreatment. After supplementation with Novozyme 188, the total enzyme loading increased to 4.9, 24.2, 48.4, or 96.8 mg total protein/g-glucan. Hydrolyzate samples were centrifuged to separate liquid from undigested solids and insoluble lignin, and monomeric glucose and xylose in the liquid were measured directly by HPLC. In addition, oligomers in the liquid were post hydrolyzed to monomers, and the difference in sugar amounts between the original sample and that measured after post hydrolysis was attributed to oligomers.

The highest 72 h glucan digestibilities of solids from AFEX, SAA, SO_2, DA, LHW, and Lime pretreatment of Dacotah switchgrass using Spezyme CP alone at 83.6 mg protein/g-glucan in untreated biomass were 57%, 76%, 83%, 83%, 87%, and 93%, respectively. However, supplementation with Novozyme 188 to give a combined total protein loading 96.8 mg protein/g glucan increased glucan digestibilities to 62%, 82%, 85%, 90%, 87%, and 94%, respectively. Thus, addition of Novozyme 188 to Spezyme CP had a limited effect on 72 h glucan digestibility at these total protein loadings, likely because yields were already so high as to leave little room for improvement. However, the benefits of beta-glucosidase supplementation were more apparent after 24 and 48 h of enzymatic hydrolysis for a given total enzyme loading. This outcome is likely related to the fact that cellobiose, a strong inhibitor for cellulase, is present in greater concentrations during the early phases of hydrolysis, and supplementation with Novozyme 188 reduced cellobiose concentrations significantly. Because the amount of cellobiose released during early phases of hydrolysis varied widely with pretreatment method, the benefits of beta-glucosidase addition also varied considerably with pretreatment choice.

With alkaline-treated samples, the xylan digestibilities of 56%, 64%, and 84%, were achieved for SAA, AFEX and Lime, respectively, when Spezyme CP was applied alone at the level of 83.6 mg protein/g-glucan. Supplementation of Spezyme CP (72.1 mg) with Novozyme 188 (11.5 mg) increased xylan digestibilities to 61%, 68%, and 91%, although the total protein loading was kept at the same level. The increase was due to β-xylosidase activity in Novozyme-188 (Dien et al., 2008).

For the samples treated by acidic methods (DA, SO_2), near maximum digestibility was attained with 24.8 mg protein/g-glucan, with limited impact of higher enzyme loadings. For alkaline (SAA, AFEX, and Lime) and LHW pretreatments, digestibility continued to increase gradually beyond 24.8 mg protein/g-glucan. For a given enzyme loading, DA, SO_2, LHW, and Lime pretreatments exhibited substantially higher 72 h glucan digestibility of pretreated switchgrass solids than for SAA and AFEX.

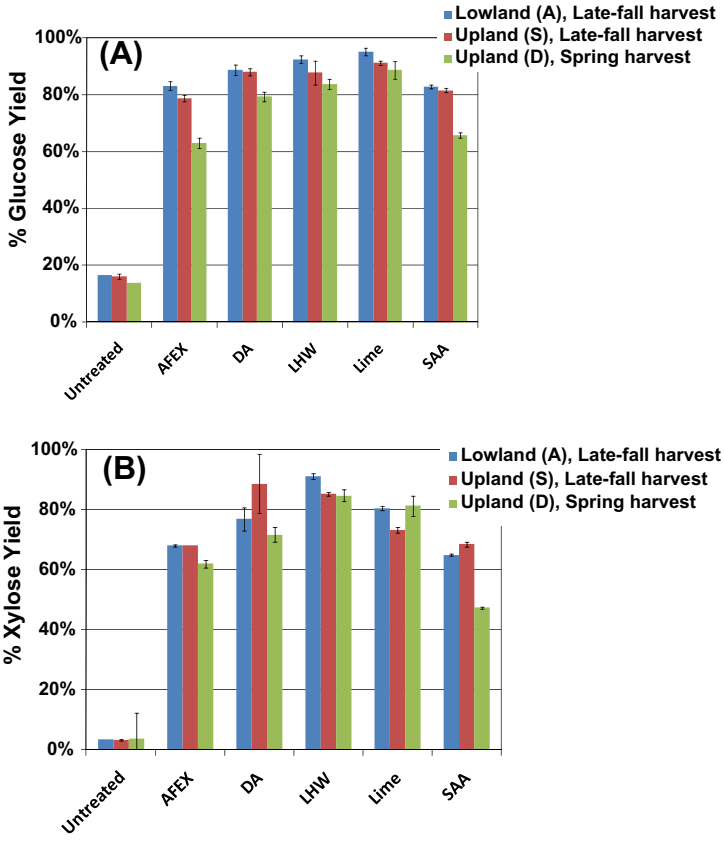

Fig. 4. Glucose and xylose yields from enzymatic hydrolysis of different switchgrass varieties pretreated by CAFI pretreatment technologies. (A) glucose yield, (B) xylose yield. Yields based on glucan or xylan content in the pretreated/hot washed solids following each pretreatment except for AFEX. A-Alamo 1; S-Shawnee; D-Dacotah. Error bars represent 95% CI.

For all pretreated samples, significant amounts of oligomers were formed as intermediate products of enzymatic hydrolysis. The highest glucose oligomers observed was 32% on the basis of initial glucan and it occurred with SO_2 treated samples at the early phase of the reaction (6 h). The highest observed xylose oligomers were 27% of initial xylan, and it occurred with AFEX samples. Both glucose and xylose oligomers have been shown to substantially inhibit cellulase activity (Gupta and Lee, 2009; Kumar and Wyman, 2009; Wilson et al., 1994), and addition of β-glucosidase enhanced the gross activity of cellulase by decreasing their concentrations.

3.5. Effects of xylanase loadings on yields

Because hemicellulose left in the solids can restrict access of enzymes to cellulose and xylooligomers have been recently shown to be a substantial inhibitors of cellulase action (Kumar and Wyman, 2009), this project also evaluated the impact of adding xylanase on sugar yields. In this case, a constant Novozyme 188 β-glucosidase loading of 3.4 mg protein/g raw glucan was employed in combination with xylanase:cellulase ratios of 0:1, 1:5, 1:2, and 1:1 with varying total enzyme loadings of 13.4, 33.4, 78.4, 123.4, and 243.4 mg protein/g raw glucan. As noted above, Spezyme CP was used as the cellulase, and Multifect Xylanase as the hemicellulase. As before, the Standard CAFI 3 Dacotah variety switchgrass was employed for development of the baseline data reported here. Each CAFI institution applied their respective pretreatment to the switchgrass, measured the composition of the pretreated material, carried out enzymatic hydrolysis, and calculated yields. Glucan, xylan, and overall yields are reported as the amount of that component released into solution as sugars per the amount of that component available initially. All results reported here are based on a total enzyme loading of 78.4 mg protein/g raw glucan, and sugar release is compared to that from the same pretreated solids when only Spezyme CP (0:1 xylanase:cellulase ratio) is employed.

Fig. 3 shows how the overall yield (g sugar released/100 g treated sugar loaded) was altered by varying the enzyme ratio at each total enzyme loading. The ammonia fiber expansion (AFEX) sample had a pretreated composition of 35.9% glucan and 22.5% xylan. At the lowest xylanase loading, the xylan yield increased by 6.3%, and the glucan yield increased by 4.6%. Increasing xylanase addition improved xylan yields by 8.1% (1:2) and 9.1% (1:1), and the highest overall yield of 61.9% was observed at the 1:1 ratio.

Soaking in aqueous ammonia (SAA) resulted in slightly lower xylan content than for AFEX of 13.6%. However, xylanase addition dramatically increased the xylan yield by 8.4% (1:5), 13.1% (1:2), and 17.9% (1:1). Adding xylanase moderately improved glucan yields from 73.8% (0:1) to 77.1% (1:1). A maximum overall yield of 76.5% was achieved at the 1:1 loading.

For lime pretreatment, xylanase addition was slightly less beneficial than for the AFEX or SAA cases. In this case, the pretreated solids composition was 53.0% glucan and 21.5% xylan, and xylanase additions improved xylan yields by 4.9% (1:5), 5.4% (1:2), and 7.1% (1:1). However, xylanase addition only slightly improved glucan and overall yields. The maximum overall yield for lime pretreatment was 89.6% (1:1). Supplementing lime pretreatment with mechanical pretreatment (ball milling) improved overall digestibility to 98.3% (1:2) but diminished the benefit of xylanase addition.

Liquid hot water pretreatment (LHW) for both 5 and 10 min produced a solid composition of 36.5% glucan and 22.7% xylan

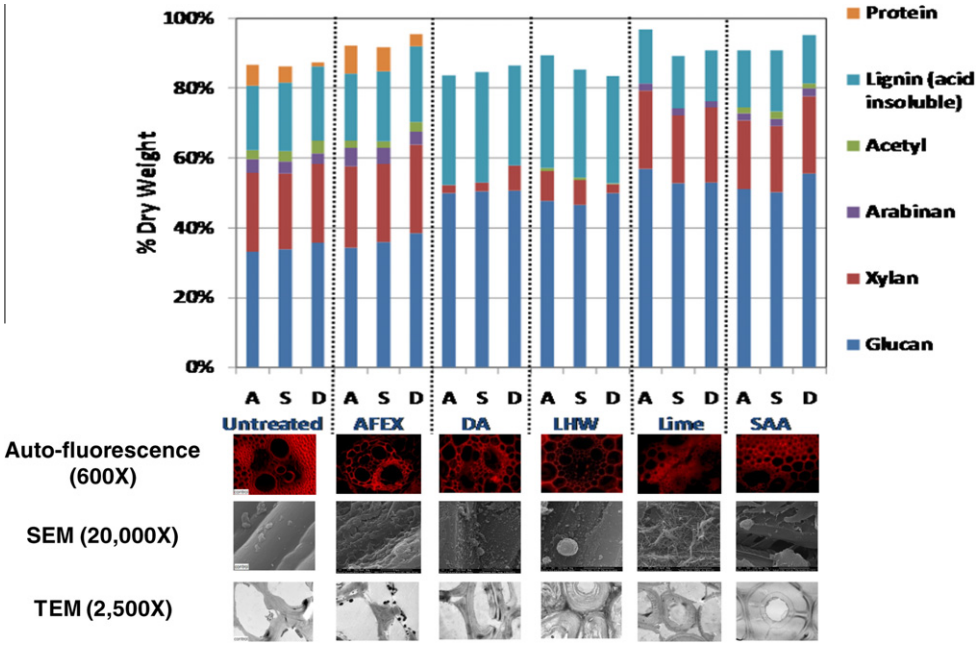

Fig. 5. Compositional analysis of native switchgrass and of solids resulting from each CAFI pretreatment (compositional analysis performed by Purdue University). A refers to Alamo variety, S refers to Shawnee variety, D refers to Dacotah variety. In lower portion, auto-fluorescence light microscope, scanning electron microscope, and transmission electron microscope images of each corresponding CAFI sample are displayed.

(This sample did not receive post-pretreatment hot washing which resulted in a different solids composition than reported above). Thus, xylan yields for the 5 min sample only increased by 1.8% (1:5), 2.2% (1:2), and 1.5% (1:1), and only the 1:5 ratio improved yields for the 10 min sample. The highest overall yield for the 5 min sample was 75.8% (1:2), with the 10-min sample achieving a maximum overall yield of 85.4% (1:5).

The acidic pretreatments, sulfur dioxide (SO_2) and dilute-acid (DA), both produced solids with very low xylan contents of 4.5% and 7.3%, respectively. As expected for these low levels, xylanase addition had negligible effect on xylan yields. SO_2 pretreatment showed a maximum overall yield of 93.2% (1:2). The dilute-acid pretreatment achieved its highest overall yield of 91.2% using just Spezyme CP. Thus, the high yields at the enzyme loadings applied also left little room for improvement by adding xylanase.

Overall, xylanase addition improved sugar yields from all pretreatments except dilute-acid at the total enzyme loading used for this portion of the project (78.4 mg protein/g raw glucan). Alkaline pretreatments produced solids with high xylan contents, and a 1:1 xylanase:cellulase ratio gave the highest yields for these materials. On the other hand, solids from the acidic pretreatments had low xylan contents, and low xylanase addition or even Spezyme CP by itself was sufficient to maximize sugar yields. This outcome demonstrated that optimal xylanase addition depends highly on the composition of the pretreated solids.

It is important to note that none of the enzyme formulations studied in this project is likely to be truly "optimal" for any given biomass or pretreatment, given the complexity of the different chemical bonds in biomass and the probable effects of the different pretreatment chemistries on these bonds. However, a thorough optimization of enzyme compositions in combination with pretreatment conditions was beyond the scope of this study.

3.6. Change in performance with switchgrass source

Although baseline CAFI data was developed with Dacotah switchgrass, the team also employed Alamo and Shawnee switchgrass, as noted in the Materials and Methods section. Fig. 4 compares yields after hydrolysis for 168 h at 50 °C of these different switchgrass varieties pretreated by the CAFI pretreatment technologies. All the CAFI pretreatments released fermentable sugars efficiently from the various cultivars and harvests of switchgrass at a considerably increased rate and yield compared to that possible without pretreatment. More importantly, the differences in enzymatic digestibility between the fall-harvest of lowland Alamo and upland Shawnee were less than the differences found between the Shawnee and Dacotah, the latter two being upland ecotypes with different harvest seasons. Furthermore, the sugar yields were lower from the later harvest than from early harvests. Thus, these results suggest that harvest time was a more important factor than ecological or morphological type of switchgrass in determining the quality of switchgrass for biofuels production. Late harvest is generally regarded as desirable to allow mineral nutrients time to translocate into the roots to be available to support the next growth cycle for these perennial grasses. As a result, important tradeoffs may be needed between harvest time and process sugar yields that would be an interesting topic for future research beyond the scope of this study.

These differences in pretreatment performance may be attributed to more severe conditions being required to overcome the combination of greater recalcitrance of upland cultivars coupled with field storage of switchgrass (leaving over winter until harvest). In addition, although the extent of the correlation varied, the relationship of saccharification yields to harvest season was similar regardless of the pretreatment applied. However, evaluation of a larger sample set is needed to confirm the generality of this trend.

3.7. Characterization of pretreated materials

In addition to generating comparative pretreatment and enzymatic hydrolysis data, the CAFI team also sought to gain insights into biomass deconstruction that will lead to improvements in process yields and economics that in turn facilitate commercialization of cellulosic conversion technologies. Consistent with this goal, the

NREL Biomass Surface Characterization Laboratory (BSCL) was applied to analyze surface and ultra structural features of the switchgrass feedstocks and pretreated solids generated by the CAFI 3 project participants to help explain how pretreatments that have such large differences in effects on composition can still result in good yield performance and also reveal distinctive features that could explain performance differences with pretreatment.

Because of its relatively high resolution and ability to image whole, intact biomass particles, scanning electron microscopy (SEM) has been commonly used to characterize the effect of biomass processes on the fine structure of biomass. These direct observations of changes in cell wall architecture have led to new insights into observed phenomena such as surface erosion and re-localization of cell wall matrix components that can cause increased enzyme accessibility and improved enzymatic digestion. In this study, a correlative microscopy approach including multiple light and electron imaging modes was applied to investigate the impact of pretreatment on disrupting biomass tissue, cellular structures, and cell wall architecture. Imaging methods used included stereomicroscopy at 1× and 4× magnification, bright field light microscopy using sectioned samples stained with toluidine blue at 600× magnification, epi-fluorescence microscopy at 600× magnification, scanning electron microscopy (SEM) of dehydrated, resin-impregnated, thin-sectioned samples at 1000× and 20,000× magnification, and transmission electron microscopy (TEM) of stained and thin-sectioned samples at 2500× and 7000× magnification. Observed features from the imaging analysis on raw switchgrass and representative pretreated switchgrass from each of the CAFI pretreatment processes were related to feedstock composition, pretreated solids composition, and performance of variously pretreated solids upon subsequent enzymatic hydrolysis.

Pretreated Dacotah switchgrass solids generated at the conditions identified in Table 3 were used in the various imaging techniques, with the compositional analysis of each CAFI pretreatment sample listed in Table 3 and shown in Fig. 5. Representative SEM, TEM, and epi-fluorescence images from each pretreatment technique are also shown in Fig. 5. Larger, annotated images showing greater detail of the structural features of these pretreated switchgrass samples are available electronically as Supplementary Data to a companion article in this special issue that focuses on surface and ultrastructural characterization of pretreated CAFI 3 switchgrass samples (Donohoe et al., this issue). This microscopic analysis revealed the various types of architectural changes in cell walls that may result from specific pretreatment processes and identifies features that are indicative of resulting compositional changes. Generally, these structural changes could not be directly related to changes in chemical composition that occurred for the various pretreatment processes. For instance, AFEX-pretreated switchgrass exhibited some very apparent structural changes in both the cellular and cell wall ultra structure, but there was virtually no change in AFEX-pretreated switchgrass composition as compared to unpretreated switchgrass. While there was strong evidence of lignin rearrangement for AFEX pretreatment of switchgrass, it was not clearly evident that such rearrangement resulted in lower overall lignin content. Dilute acid and LHW pretreatments produced pretreated residues for which much of the original hemicellulose content had been solubilized, which may be reflected by observed cell well delamination and thinning. Lime and SAA pretreatments resulted in significant lignin removal, which could be related to reduction in lignin signal intensity in staining-based image analysis, along with cell wall swelling and evidence of increased porosity. As all of the pretreatment approaches resulted in improved enzymatic hydrolysis performance, it is clear that very different compositional and ultra structural changes were responsible for the enzymatic digestibility performance seen for each pretreatment approach.

Light microscopy images of untreated and pretreated switchgrass by epi-fluorescence imaging confirmed that while various pretreatments can dramatically change chemical composition, the general cellular and tissue structure remained largely intact. In these images, increased color intensity correlated to high local lignin concentration. AFEX pretreated samples showed some regions of lignin concentration, indicative of some lignin re-arrangement and localization. On the other hand, dilute acid and LHW pretreated samples showed an increased signal in the middle lamella and in cell corners, and SAA and lime pretreated samples both showed loss of lignin as evidenced by decreased auto-fluorescence intensity, although there appeared to be different areas of lignin loss for these two pretreatment methods. Lime pretreatment resulted in greater removal of lignin from middle lamella regions, leaving detached cells that provided additional accessible surface, with much lignin remaining in the thicker cell walls. SAA pretreatment appeared to achieve a more uniform loss of lignin across the cell wall without significant disruption of middle lamella regions.

Scanning electron microscopy images revealed significant surface disruption and irregular cell wall surfaces in AFEX pretreated solids. Dilute acid and LHW pretreated samples showed evidence of lignin re-localization into globules likely rich in lignin that were especially apparent at high magnifications. In lime pretreated samples, there was significant disruption of the cell wall matrix, with broad exposure of cellulose microfibrils. Although this erosion could create accessible surface for enzyme binding, it did not appear to deeply penetrate into the cell wall. In SAA-pretreated samples, a more uniform lignin removal and relocation pattern resulted in a fairly homogeneous surface.

Finally, transmission electron microscopy of AFEX pretreated solids showed the most dramatic evidence for lignin re-localization, with clearly evident globule formation. Dilute acid pretreatment resulted in lignin coalescence in the middle lamella and in delaminated areas while LHW pretreatment caused increased porosity and extensive delamination, with apparent formation of some lignin globules by both of these pretreatment methods. Lime pretreatment resulted in some lignin removal from the middle lamella, enlarged spaces in cell corners, and thinning of some cell walls. SAA pretreatment appeared to cause an increase in porosity and a looser overall cell wall structure, along with a general decrease in lignin-staining intensity and some cell wall swelling, although showing no areas of significant cell wall delamination.

4. Conclusions

Material balances allowed proper sugar yield comparisons among different pretreatments. Enzyme loadings for a digestibility of 70% or more were substantially lower for DA, SO_2, and LHW pretreatments than for SAA or AFEX, but optimizing enzyme formulations could improve the latter. β-glucosidase supplementation only improved enzymatic digestibility early in hydrolysis and for low enzyme loadings when cellobiose and oligomer accumulation were significant. Adding xylanase improved yields from all but DA, with the benefit greater for substrates high in residual xylan. Imaging of pretreated solids showed that different pretreatments impact features differently but left much of the basic structure intact.

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