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 by different pretreatments could impact performance.

Key words: hydrolysis, microscopy, pretreatment, switchgrass, yields

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., July 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 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 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 many fuel production facilities 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 though 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, relatively little 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 leaders in biomass pretreatment and hydrolysis in late 1999 in Dallas and early 2000 in Chicago to meet this need(Wyman et al., 2005). The goals of the team were to compare the effectiveness of leading pretreatments in recovering sugars from the coupled operations of pretreatment and enzymatic hydrolysis of cellulosic materials, gain insight into fundamentals to facilitate commercialization and lead to step change cost reductions, and 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 U.S. 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., 2005). Then, from 2004 to 2007, CAFI was selected through a competitive solicitation by the Office of the Biomass Program of the U.S. 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., 2008). 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. Materials and 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(1995; Wyman et al., 2005).

2.2 Switchgrass

Three different varieties of switchgrass, supplied by Ceres Corporation, 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.

As shown in Figure 1, no statistically significant difference in composition was found between the Alamo and Shawnee switchgrass, other than noticeable soluble carbohydrates and protein contents, despite the fact they are different ecotypes based on the latitude-of-origin. Dacotah which, like Shawnee, is an upland variety contained significantly more glucan and lignin but less water extractable sugars and protein compared to both Alamo and Shawnee. The higher lignocellulose concentration in Dacotah could be partly due to its morphological type. Dacotah is a northern-upland variety of which latitude-of-origin and harvest location was the

highest among the samples. In upland varieties, lignocellulose content generally increases with latitude, while the opposite is observed in lowland varieties. Alamo was grown at more northern latitude than its latitude-of-origin, which might have affected its compositions as well. The compositional variability seemed to more depend on the harvest times rather than genotype or ecotype of the switchgrass cultivars. Spring harvest of switchgrass exhibited a higher concentration of lignocellulose and a lower protein and water extractable soluble sugars than fall harvests.

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 *T. 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 gram of pre-washed switchgrass was presoaked overnight in 1 % wt/v dilute sulfuric acid solution at room temperature with a solids loading of 10% wt/wt on a dry basis. Pretreatments were conducted at 140°C for 40 min in a 1 L Parr reactor made of

Hastelloy C (Parr Instruments, Moline, IL) and heated in 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 that rotated to push the material downwards. The temperature inside the reactor was monitored using a K-type thermocouple. The heat up time for this system was about 2 min and 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 vacuum filtered immediately through a glass fiber filter (Whatman®, Grade GF/A, diam. 11.0 cm), with the temperature being always greater than 60 °C. The resulting solids were then washed with 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.

2.4.3 Liquid Hot Water (LHW)

Aqueous pretreatment of switchgrass consisted of mixing the substrate with DI water at 15% solids loading (w/w, g dry solids per g total) and heating at 200°C for a target reaction time under pressure in order to keep the water in a liquid state and minimize formation of degradation products(Ladisch et al., 1998; Weil et al., 1997; Weil, 1998). Pretreatments were conducted in 1 in. OD x 0.083 in. wall thickness (2.54 cm × 2.1 mm), 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(Kim, 2009; Kim, 2009). Each tube was 4.5 in. (11.4 cm) in length and 45 mL in total volume. The sample volume was kept at 33.7 mL to give approximately 25% of head space for liquid expansion during heating to 200°C. The reactor tube containing the switchgrass slurry was heated by placing in a Tecam® SBL-1 fluidized sand bath (Cole-Parmer, Vernon Hills, IL) set to 200°C for 18 min, which included a 8 min heat-up and 10 min reaction time. After pretreatment, each tube was cooled by quenching in water for 10 min. The pretreated slurry was vacuum filtered using Whatman® No 41 filter paper to remove the liquid fraction which was collected for further analysis. The retained solids on the filter paper were then washed with hot water as described previously(Kim, 2009). The pretreated solids and collected pretreatment liquid were stored in a freezer (-20 °C) until further analysis.

2.4.4 Ammonia Fiber Expansion (AFEX)

Four batches of AFEX pretreated switchgrass were prepared in a 300mL 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 H2O: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 minute 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"IDx6"L) followed by adding 90 mL of 15 % NH4OH. 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 conducted in a pair of 304 stainless steel pipe reactors (5" long, 1.5" I.D.) with 1.5" 304 stainless steel caps and sealed using Teflon tape. The reactors were each loaded with 8 g of dry switchgrass, excess calcium hydroxide (1 g $Ca(OH)_2$/g dry biomass), and water (15 g/g dry biomass). A constant 100 psi pressure of pure oxygen was supplied to a manifold through a flexible stainless steel hose attached to an oxygen tank. The reactors were connected to a swing arm to provide constant stirring and placed in a preheated temperature controlled oven at 120°C. After 4 hours, the reaction was quenched by removing the reactors from the oven and immediately placing them in an ice bath. Once cooled, the reactors were opened slowly to relieve pressure, and the contents were transferred to a 1L plastic centrifuge bottle using DI water. The slurry was neutralized using 5N HCl to a pH of approximately 4, then washed several times with DI water until the pH of the slurry rose to approximately 6. The final slurry was vacuum filtered, and the filtrate was collected for carbohydrate analysis. Moisture

content and final weight of the solids were recorded to obtain pretreatment yields, and the solids were stored in the freezer until compositional analysis and enzymatic hydrolysis were performed.

2.5 Material balances

Material/mass balances for the different pretreatments, hydrolysis, and fermentation are critical to meaningful comparison of the different pretreatment options as well as in judging process economics and operational feasibility, and Figure 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, 2008; Sluiter, 2008). 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 hr 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 1h, 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 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.

$$Y_{Glc} = \{[(Glc+GO)_{ST1P} + (Glc+GO)_{ST2EH}]/[(180/162)\ X\ (Gln)_{UTB} + (Glc)_{UTB}]\}x100\%$$

$$Y_{Xyl} = \{[(Xyl+XO)_{ST1P} + (Xyl+XO)_{ST2EH}]/[(150/132)\ X\ [(Xyn)_{UTB} + (Xyl)_{UTB}]\}x100\%$$

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, 2009; Kim, 2009; Wyman et al., 2008; Wyman et al., 2005; Zhu, 2010). In

Figure 2, a 100% mass closure results when the captured output streams compositions (5 to 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 to 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 20 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 SSA 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 was 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., 2008) but contrasts with the more uniform performance shown by the CAFI study on corn stover(Wyman et al., 2005).

3.2 Compositions of pretreated solids

Table 3 shows the different pretreatments resulted in distinct differences in lignin, glucan, xylan, arabinan, and acetyl contents in the pretreated solids. Pretreated solids under acidic conditions by dilute acid (DA), sulfur dioxide (SO_2), and liquid hot water (LHW) had very low xylan contents of 2.5-4.7% but high glucan contents of 60.1-62.4%. For pretreatments with alkaline chemicals, i.e., soaking in aqueous ammonia (SAA) and lime, the solids remaining after 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 to 55.6%. Ammonia fiber expansion (AFEX) pretreatment of switchgrass resulted in virtually no compositional change except for negligible xylan loss and minor acetyl removal. However, as shown in previous studies, improvements in digestibility by AFEX pretreatment are possibly due to relocation of lignin, decrystallization of cellulose, and a phase change in the crystal structure from cellulose I to cellulose III as a result of cellulose swelling(Chundawat, 2009; Dale, 1996).

Table 3 includes the effect of the pretreatments studied here on the removal of major biomass components. First, we can see that DA, SO_2, LHW, lime, and SAA solubilized about 35 to 40% of the switchgrass. Pretreatments at acidic conditions, i.e., DA, SO_2 and LHW, led to nearly complete (>90%) xylan and arabinan removal but only removed about 13.0-18.6% of the original lignin. However, SAA and lime pretreatments at alkaline conditions removed 55.1-59.3% of the original lignin plus 38.8-39.9% of the original xylan. In addition, alkali pretreatments removed carboxylic acid substitutions, e.g., acetyl groups and uronic acids, from hemicellulose in addition to some hemicellulose, with improved enzyme access to hemicellulose and cellulose(Kim, 2006; Kumar, 2009). In contrast, AFEX removed virtually nothing and resulted in nearly 100% recovery of solids with essentially the same composition as the raw

switchgrass. In summary, low pH pretreatments removed a major portion of hemicellulose, and high pH pretreatments, except AFEX, removed a large part of the lignin plus some xylan. However, the extent of these effects varied with substrate and pretreatment, in line with previous studies(Elander, 2009; Kumar, 2009; Mosier et al., 2005; Wyman et al., 2005).

3.3 Reaction conditions for best performance

Table 3 also summarizes the 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 the 4 hour pretreatment of lime, all other pretreatments were run for between 10 and 60 min, making it possible to pretreat biomass in modest sized vessels. Table 3 also lists chemical inputs. Because LHW relies on the carboxylic acid released during hydrothermal breakdown of the cell wall structure, it required no additional chemical catalyst. DA and SO_2 pretreatments used the lowest amounts of chemicals of all pretreatments, and alkaline pretreatments by AFEX, SAA and lime technologies used the largest amounts. Although recycle of the chemicals is possible, the additional capital and operating costs must be considered.

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, 2003). 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 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 with total protein numbers of 82 mg protein/mL and 67 mg protein/mL, respectively. 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.

The highest 72 h xylan digestibilities achieved for alkaline treated samples (SAA, AFEX and Lime) with Spezyme CP alone were 56%, 64%, and 84%, respectively, for an enzyme loading of 83.6 mg protein/g-glucan. Novozyme 188 supplementation at a combined total protein loading of 96.8 mg protein/g glucan improved digestibilities to 62%, 70%, and 93%, respectively. The increase in xylan digestibility with β-glucosidase supplementation probably results from β-xylosidase activity in Novozyme 188(Dien, 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.

Significant amounts of oligomers were formed as intermediates during hydrolysis of all pretreated solids. Glucose and xylose oligomers were as high as 32 % (SO_2) and 27% (AFEX) of initial glucan and xylan, respectively. Both glucose and xylose oligomers have been shown to substantially inhibit cellulase activity(Gupta, 2008; Kumar, 2008; Wilson, 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, 2008), 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 ratio) is employed.

Figure 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 [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 pure Spezyme CP 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. Figure 4 compares yields after hydrolysis for 168 hours 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 feedstock types 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 1X and 4 X magnification, bright field light microscopy using sectioned samples stained with toluidine blue at 600X magnification, epi-fluorescence microscopy at 600X magnification, scanning electron microscopy (SEM) of dehydrated, resin-impregnated, thin-sectioned samples at 1,000X and 20,000X magnification, and transmission electron microscopy (TEM) of stained and thin-sectioned samples at 2,500X and 7,000X

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 Figure 5. Representative SEM, TEM, and epi-fluorescence images from each pretreatment technique are also shown in Figure 5. This microscopic analysis reveals 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. When comparing the pretreated solids compositional data to the images generated for each pretreated sample, it is clear that observed structural changes upon pretreatment cannot be easily related to composition data. This phenomenon is especially evident for AFEX pretreated switchgrass, which shows virtually no gross change in composition upon pretreatment but displays significant structural changes at the cellular and cell wall ultra structural level. Some of the observed structural changes appear to be consistent with the AFEX compositional data, as there is strong evidence of lignin rearrangement (but not necessarily lignin removal). For the pretreatments that achieve significant hemicellulose removal (dilute acid and LHW pretreatment), significant cell wall delamination is evident, along with areas of lignin re-localization and globule formation that are typical of these pretreatments on other feedstocks (i.e., corn stover). Imaging results for pretreatments that remove substantial amounts of lignin (lime and SAA) show evidence of lower lignin intensity in the staining-based images, along with greater porosity and some cell wall swelling. It is evident that different types of changes to cell

wall ultra structure significantly improve enzymatic digestibility for the various pretreatments compared to untreated switchgrass,

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 remains largely intact. In these images, increased color intensity correlates to high local lignin concentration. AFEX pretreated samples show some regions of lignin concentration, indicative of some lignin re-arrangement and localization. On the other hand, dilute acid and LHW pretreated samples show an increased signal in the middle lamella and in cell corners, and SAA and lime pretreated samples both show loss of lignin as evidenced by decreased auto-fluorescence intensity. However, the pattern of loss is slightly different for the latter. Because the thicker-walled cells from lime pretreatment appear to retain most of their lignin and the signal becomes weaker in thinner-walled cells, lime pretreatment appears to have removed material from the middle lamella between cells, leaving detached cells with newly accessible surfaces. In the SAA samples, the fluorescence signal loss appears more uniform across the width of the cell wall and more uniform across the different cell types, with the middle lamella remaining largely intact.

Scanning electron microscopy images reveal significant surface disruption and irregular cell wall surfaces in AFEX pretreated solids. Dilute acid and LHW pretreated samples show evidence of lignin re-localization into lignin-rich globules that are especially apparent at high magnifications. In solids from lime pretreatment, the surface appears to be deeply etched with widespread and nearly complete removal of cell wall matrix, leaving behind exposed cellulose micro fibrils. This extensive erosion does not appear to penetrate very far into the cell wall but likely creates a highly accessible surface for initial cellulase binding. There appears to be a more

homogeneous surface texture in the SAA samples, indicative of a more uniform pattern of lignin relocation and removal.

Finally, transmission electron microscopy of AFEX pretreated solids showed the most dramatic evidence for lignin re-localization, with clearly evident globule formation. Dilute acid and LHW pretreated samples also displayed some lignin globules on cell wall surfaces. The dilute acid sample displayed delamination of cell wall lamella and some coalescence of lignin in the middle lamella, cell corners, and delamination zones while the LHW sample revealed a widespread increase in porosity across the width of even the thickest cell walls. In addition, the LHW sample showed a striking and extensive delamination in the cell walls. Some cell walls in the lime pretreatment sample appeared thinned, with enlarged spaces in the cell corners and some removal of material from the middle lamella region. The SAA pretreated sample displayed swollen cell walls and a uniform decrease in lignin staining across the layers of the cell wall. There was some indication of an increase in porosity and a general loosening of the cell wall structure, but no major delamination in the SAA -pretreated cell walls.

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, SO2, 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 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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References

1995. Chemical Analysis and Testing Standard Procedures. National Renewable Energy Laboratory, Golden, CO.

Balan, V., Bals, B, Chundawat, SP, Marshall, D, Dale, BE., 2009. Lignocelluloses' biomass pretreatment using AFEX. in: J.R. Mielenz (Ed.) *Methods Mol Biol.* Humana Press, Totawa, NJ, pp. 61-77.

Chundawat, S., 2009. Ultrastructural and physicochemical modifications within ammonia treated lignocellulosic cell walls and their influence on enzymatic digestibility, Chemical Engineering & Materials Science. Michigan State University, East Lansing, MI.

Dale, B.E., Leong, C.K., Pham, T.K., Esquivel, V.M., Rios, I., Latimer, V.M., 1996. Hydrolysis of lignocellulosics at low enzyme levels: Application of the AFEX process, *Bioresour. Technol.* , 56 111-116.

Dien, B.S., Ximenes, E. A, O'Bryan, P. J, Moniruzzaman, M, Li, X. L, Balan, V, Dale, B, Cotta, M. A., 2008. Enzyme characterization for hydrolysis of AFEX and liquid hot-water pretreated distillers grains and their conversion to ethanol, *Bioresource Technology*, 99, 5216-5225.

Elander, R.T., Dale, B.E., Holtzapple, M., Ladisch, M.R., Lee, Y.Y., Mitchinson, C., Saddler, J.N., Wyman, C.E., 2009. Summary of findings from the Biomass Refining Consortium for Applied Fundamentals and Innovation (CAFI): Corn stover pretreatment, *Cellulose* 16 649-659.

Gupta, R., Kim, T., Lee, Y.Y., 2008. Substrate dependency and effect of xylanase supplementation on enzymatic hydrolysis of ammonia-treated biomass, *Applied Biochemistry and Biotechnology*, **148** 59-70.

Kim, S., Holtzapple, M.T., 2006. Effect of structural features on enzyme digestibility of corn stover, *Bioresour. Technol.* , 97 583-591.

Kim, Y., Hendrickson, R.; Mosier, N.S., Ladisch, M.R., 2009. Biofuels: Methods and Protocols. in: J.R. Mielenz (Ed.) Methods in Molecular Biology. Humana Press, Totowa, NJ, pp. 93-102.

Kim, Y.M., Mosier, N.S., Ladisch, M.R., 2009. Enzymatic digestion of liquid hot water pretreated hybrid poplar. *Biotechnol. Progr*, 25, 340-348.

Kumar, R., Mago, G., Balan, V., Wyman, C.E., 2009. Physical and chemical characterizations of corn stover and poplar solids resulting from leading pretreatment technologies, *Bioresour. Technol.* , 100 3948-3962.

Kumar, R., Wyman, C. E., 2008. Effect of enzyme supplementation at moderate cellulase loadings on initial glucose and xylose release from corn stover solids pretreated by leading technologies, *Biotechnology and Bioengineering*, 102 457-467.

Ladisch, M.R., Kohlmann, K., Westgate, P., Weil, J., Yang, Y., 1998. Processes for Treating Cellulosic Material, United States.

Lau, M.W., Dale, B.E., 2009. Cellulosic Ethanol Production from AFEX-treated Corn Stover using *Saccharomyces cerevisiae* 424A(LNH-ST), *Proc. National Acad. Sci. USA*, 106, 1368-1373.

Lynd, L.R., Laser, M.S., Bransby, D., Dale, B.E., Davison, B.H., Hamilton, R., Himmel, M., Keller, M., McMillan, J.D., Sheehan, J., Wyman, C.E., 2008. How Biotech can Transform Biofuels. *Nature Biotechnology*, 26, 169-172.

Lynd, L.R., Wyman, C.E., Gerngross, T.U., 1999. Biocommodity engineering. *Biotechnology Progress*, 15, 777-793.

Merino, S.T., Cherry, J., 2007. Progress and challenges in enzyme development for biomass utilization. *Adv Biochem Eng Biotechnol*, 108, 95--120.

Mosier, N., Wyman, C.E., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M., Ladisch, M., 2005. Features of Promising Technologies for Pretreatment of Lignocellulosic Biomass. *Bioresource Technology*, 96, 673-686.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D., 2008. Determination of ash in biomass Laboratory Analytical Procedures (LAPs). National Renewable Energy Laboratory, Golden, CO.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D., 2008. Determination of structural carbohydrates and lignin in biomass, Laboratory Analytical Procedures (LAPs). National Renewable Energy Laboratory, Golden, CO.

Sun, Y., Cheng, J., 2003. Enzymatic hydrolysis of rye straw and bermuda grass using cellulases Supplemented with β-glucosidase, *ASAE*, 47, 343-349.

Weil, J., Sarikaya, A., Rau, S.L., Goetz, J., Ladisch, C.M., Brewer, M., Hendrickson, R., Ladisch, M.R., :, 1997. Pretreatment of yellow poplar sawdust by pressure cooking in water. *Applied Biochemistry Biotechnology*, 68, 21-40.

Weil, J.R., Sarikaya, A., Rau, S-L., Goetz, J., Ladisch, C.M., Brewer, M., Hendrickson, R. and Ladisch, M.R., 1998. Pretreatment of Corn Fiber By Pressure Cooking in Water. *Appl. Biochem. Biotechnol.*,, 73, 1-17.

Wilson, C.A., McCrae, S. I., Wood, T. M., 1994. Characterization of a β-glucosidase from the anaerobic rumen fungus Neocallimastix frontalis with particular reference to attack on cello-oligosaccharides, *Journal of Biotechnology*, 37, 217-227.

Wooley, R., Ruth, M., Sheehan, J., Ibsen, K., Majdeski, H., Galvez, A., July 1999. Lignocellulosic Biomass to Ethanol Process Design and Economics Utilizing Co-Current Dilute Acid Prehydrolysis and Enzymatic Hydrolysis: Current and Futuristic Scenarios. National Renewable Energy Laboratory, Golden CO NREL/TP-580-26157, pp. 128.

Wyman, C., Dale, B., Elander, R., Holtzapple, M., Ladisch, M., Lee, Y., 2005. Coordinated Development of Leading Biomass Pretreatment Technologies. *Bioresource Technology*, 96, 1959-1966.

Wyman, C., Dale, B., Elander, R., Holtzapple, M., Ladisch, M., Lee, Y., Mitchinson, C., Saddler, J., 2008. Comparative Sugar Recovery and Fermentation Data Following Pretreatment of Poplar Wood y Leading Technologies. *Biotechnology Progress*, **25**, 333-339.

Wyman, C.E., 1995. Economic fundamentals of ethanol production from lignocellulosic biomass. in: J.N. Saddler, M.H. Penner (Eds.), Enzymatic Degradation of Insoluble Carbohydrates. American Chemical Society, Washington, DC, pp. 272-290.

Wyman, C.E., 2007. What is (and is not) vital to advancing cellulosic ethanol. *Trends in Biotechnology*, 25, 153-157.

Wyman, C.E., Dale, B.E., Elander, R.T., Holtzapple, M., Ladisch, M.R., Lee, Y.Y., 2005. Comparative sugar recovery data from laboratory scale application of leading pretreatment technologies to corn stover. *Bioresource Technology*, 96, 2026-2032.

Wyman, C.E., Dale, B.E., Elander, R.T., Holtzapple, M., Ladisch, M.R., Lee, Y.Y., 2005. Coordinated development of leading biomass pretreatment technologies. *Bioresource Technology*, 96, 1959-1966.

Yang, B., Wyman, C.E., 2008. Pretreatment: The Key to Unlocking Low Cost Cellulosic Ethanol. *Biofuels, Bioproducts, and Biorefining* 2, 26-40.

Yang, B., Wyman, C.E., 2009. Dilute Acid and Autohydrolysis Pretreatment. in: J. Mielenz (Ed.) Biofuels Methods and Protocols Series, *Methods in Molecular Biology: Biofuels,* Humana Press, Totowa, NJ, pp. 103-114.

Zhu, J., Zhu, W, Obryan, P, Dien, BS, Tian, S, Gleisner, R, Pan, XJ, 2010. Ethanol production from SPORL-pretreated lodgepole pine: preliminary evaluation of mass balance and process energy efficiency, *Appl Microbiol Biotechnol.*, 86, 1355-65.

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

Figure 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.

Figure 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.

Table 1. Protein concentrations for CAFI enzymes.		
Product Name	Lot number	Total protein by nitrogen-analysis, TCA precipitated protein **mg Total Protein/mL**
Spezyme® CP	301-05330-206	82 (123*)
Multifect® Xylanase	301-04296-205	27(42*)
Beta-glucosidase		67

*Protein values in mg/ml estimated by BCA method

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 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	33.5/2.1	45.6	79.1/2.1
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	56.0/1.1	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	14.5/14.3	76.4/3.8	90.9/18.1
SAA	9.5/8.7	17.8/6.9	27.3/15.5	0.2/0.2	39.8/1.2	39.9/1.4	9.6/8.8	57.6/8.1	67.2/16.9
AFEX	11.1/11.1	25.6/3.0	36.7/14.2	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., in the same special volume submission).

	Pretreatment technology						
	None	***DA***	***SO_2***	***AFEX***	***LHW***	***SAA***	***Lime***
Pretreatment conditions							
Water/Solid ratio		9	9	2	5.6	9	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, %†							
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*)	21.1	29.4	27.6	24.4	30.6	13.9	14.6
Others**	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

*AIS- acid insoluble lignin;

** Others include proteins, ash, and uronic acids etc.

† Percent component in solids is based on the remaining solids after pretreatment, except for solids recovery after pretreatment, which is based on starting biomass.

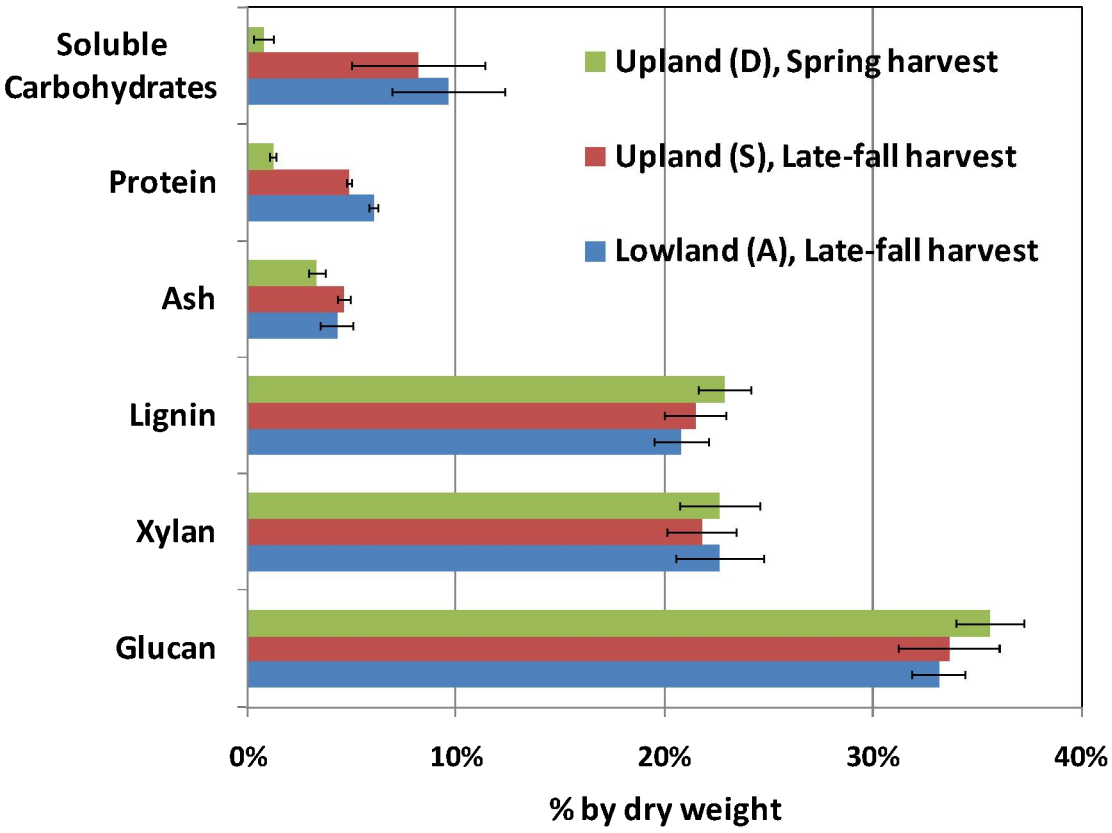

Figure 2. Compositions of different ecotypes and harvest season of switchgrass. Error bars represent 95% CI. A-Alamo 1; S-Shawnee; D-Dacotah

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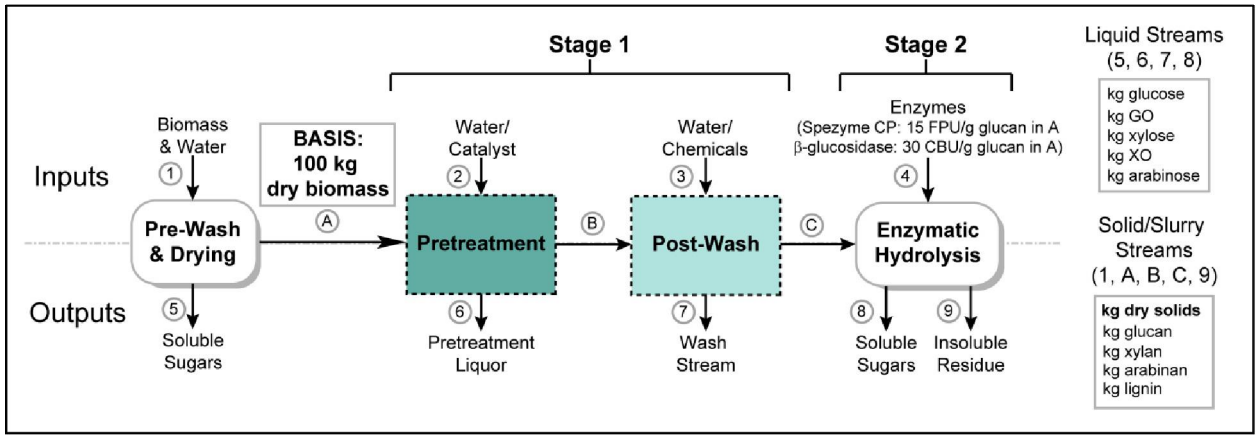

Figure 2. Flow diagram outlining the key steps and streams tracked by the material balance for pretreatment and enzyme hydrolysis. Here, Stage 1 includes pretreatment and post-wash, while Stage 2 is enzymatic hydrolysis. All flows are based on 100 kg of untreated, washed biomass, dry weight basis (DWB) (Stream A). The inputs and outputs to the process are indicated by numbered streams (1 to 9). Lettered streams (A to 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 (eg., glucan, xylan and arabinan). Lignin was not measured for the liquid streams. Cellulase enzymes are reported as kg protein/100 kg of pre-washed, dry biomass input to pretreatment.

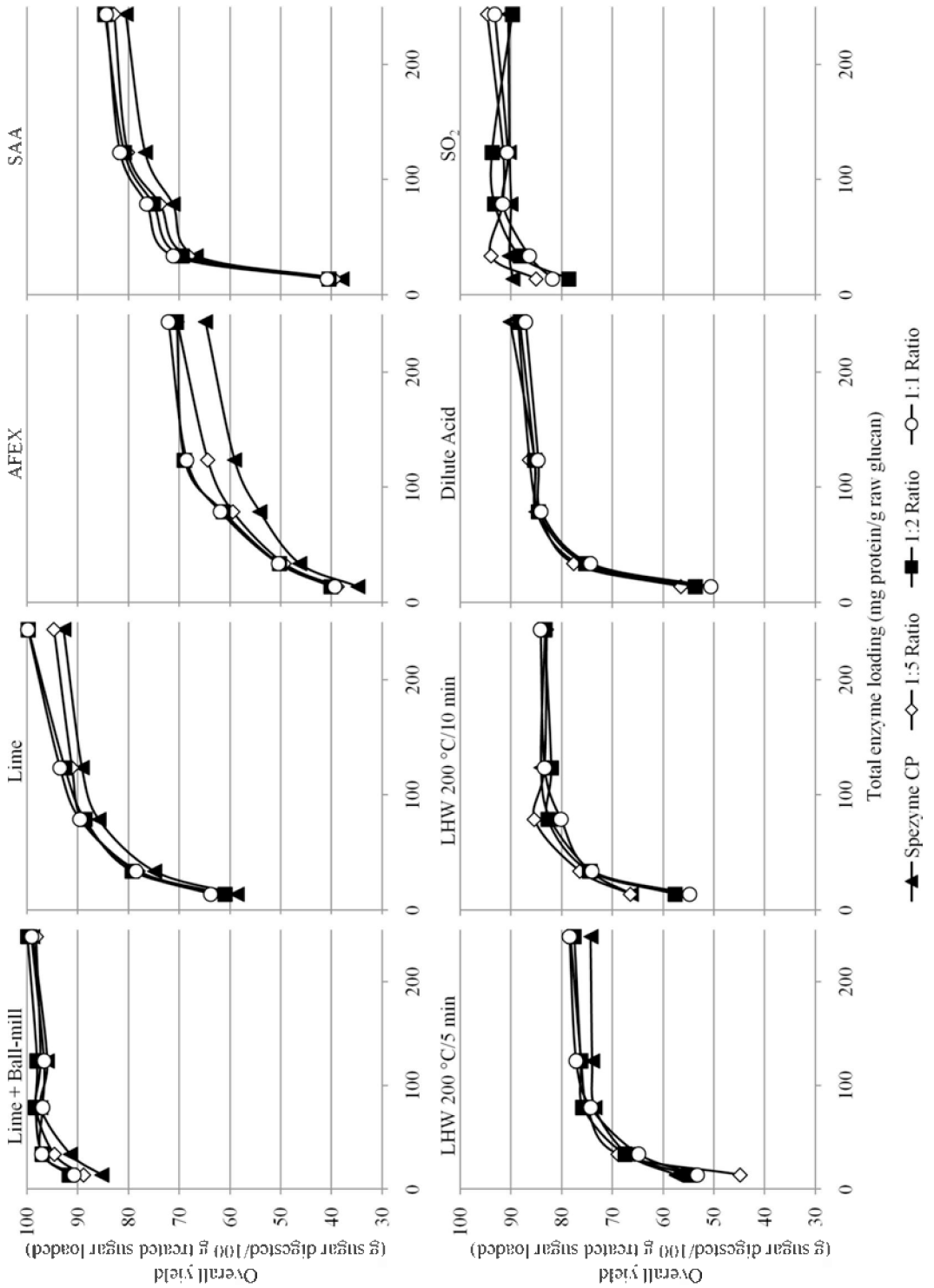

Figure 3. Total glucose plus xylose yields plotted against total enzyme protein loadings for different supplementation ratios of xylanase.

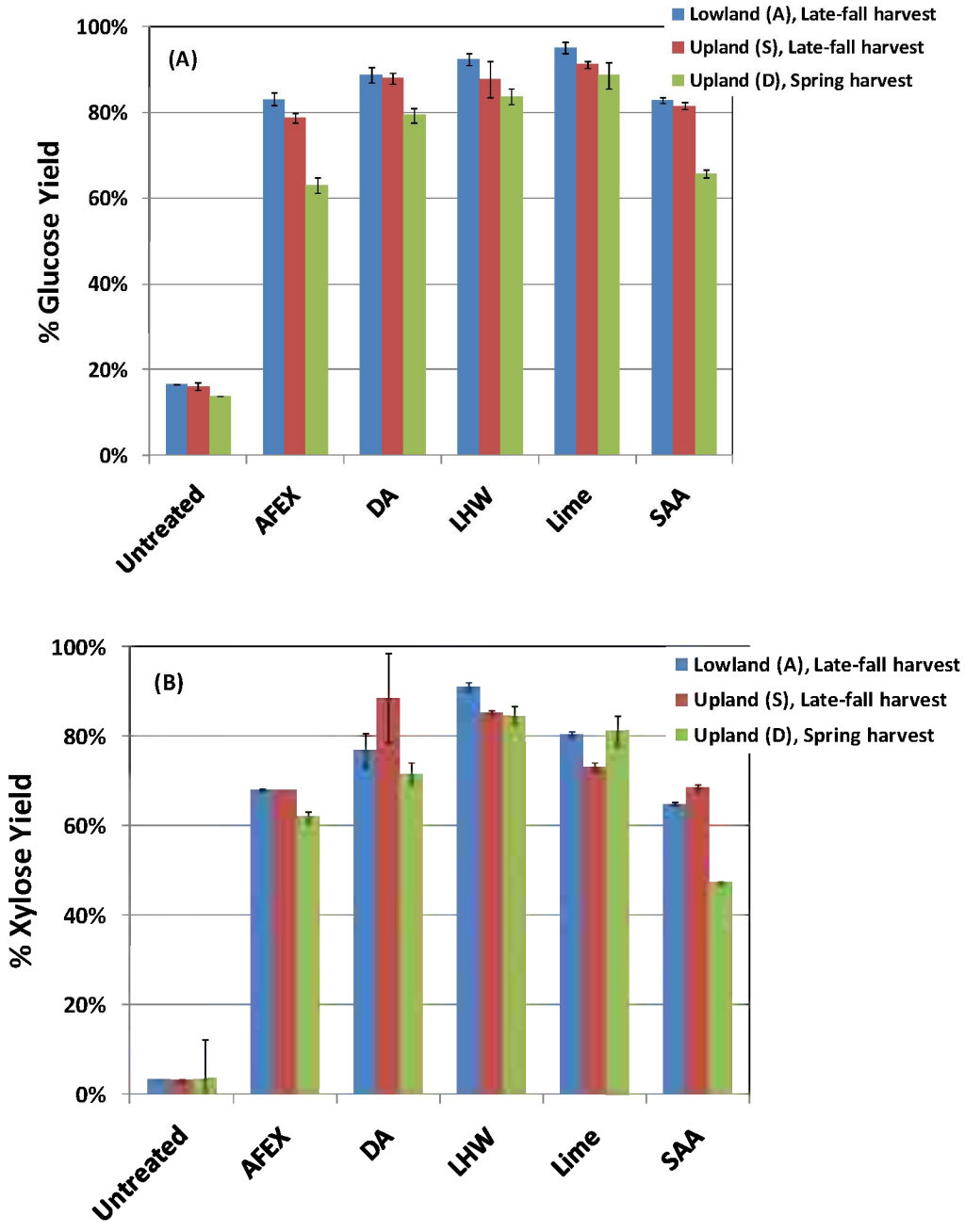

Figure 4. Glucose and xylose yields from enzymatic hydrolysis of different switchgrass varieties pretreated by CAFI pretreatment technologies. (A) glucose yield, (B) xylose yield. Yields based on glucan or xylan content in the pretreated/hot washed solids following each pretreatment except for AFEX.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

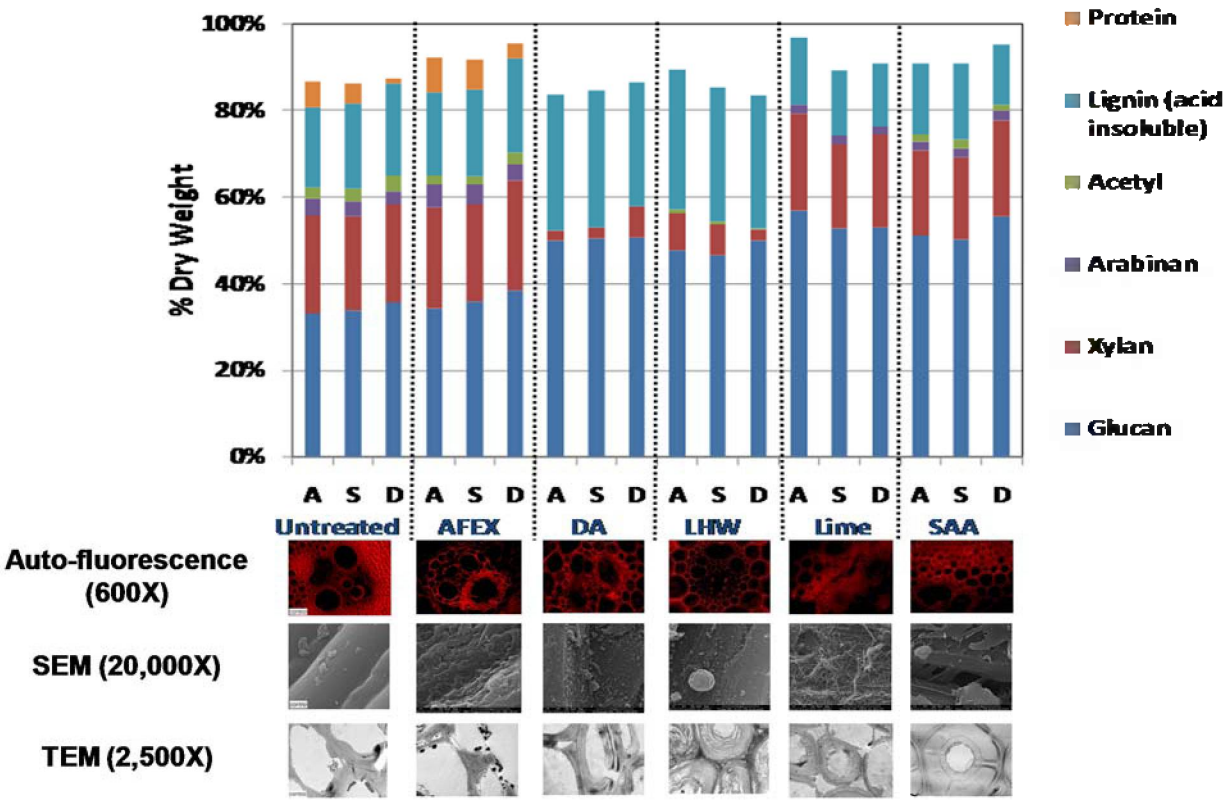

Figure 5. Compositional analysis of native switchgrass and of solids resulting from each CAFI pretreatment (compositional analysis performed by Purdue University). A refers to Alamo variety, S refers to Shawnee variety, D refers to Dacotah variety. In lower portion, auto-fluorescence light microscope,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