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Alkali Pretreatment of Cereal Crop Residues for Second-Generation Biofuels

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ABSTRACT: Mild alkali cooking of lignocellulosic biomass is an effective pretreatment method, which improves enzymatic hydrolysis. Here, we report the use of dilute alkali (NaOH) pretreatment followed by enzyme saccharification of cereal residues for their potential to serve as feedstock in the production of next-generation biofuels in Australia. After pretreatment, both solids and lignin content were found to be inversely proportional to treatment severity. We also found that higher temperatures and alkali strength were quintessential for maximizing sugar recoveries from enzyme saccharifications. Generally, pretreatment conditions at elevated temperatures led to highly digestible material enriched in both cellulose and hemicellulose components. Increasing cellulase loadings and tailoring enzyme activities with additional β-glucosidases and xylanases delivered greater rates of monosaccharide sugar release and yields throughout enzyme hydrolysis. Considering their abundance, high sugar potential, and apparent ease of conversion, cereal crop residues are an excellent feedstock for the production of second-generation fuels and/or biorefineries.

1. INTRODUCTION

Interest in commercial-scale production of alternative transportation fuels chiefly emanates from issues relating to the use, impacts, and rising demand of traditional fossil fuels. Growing dependency upon oil and the inability to protect supply lines from global political intrigues, projected declines in worldwide petroleum reserves, and record crude oil prices afford major incentives for pursuing the development of alternative fuels. Global petroleum demands have steadily increased from 57×10^6 barrels/day in 1973 to 82×10^6 barrels/day in 2004 and is anticipated to rise another 50% by 2025.[1] Allowing for current rates of production and existing reserves, we will soon approach Hubbert's predicted "peak oil" levels.[2]

Biofuels, fuels derived from plant biomass, are currently the only sustainable class of liquid fuels.[3] First-generation biofuels, such as ethanol, are produced from plants rich in carbohydrates (sugar and starch). However, it does not significantly diminish greenhouse gas (GHG) emissions.[4] These shortcomings can be addressed by producing ethanol from lignocellulosic material (second-generation or next-generation biofuels), such as agricultural and forest waste residues.

Australia has approximately 500 000 km^2 of arable land for the production of drought-tolerant biomass.[5] Conservative estimates place agricultural biomass residues at about 65 million dry tons per year,[6] of which 25% could be converted into ethanol after accounting for soil management practices and livestock feed.[7] Sorghum and wheat straw are of particular interest because of their sizable cultivation in winter and summer, respectively.[8]

Lignocellulose forms the structural framework of plant cell walls and comprises cellulose, hemicellulose, and lignin, in proportions varying with the source of material.[9] Efficient use of lignocellulose requires pretreatment to liberate cellulose from its lignin seal and disrupt its structure before effective enzymatic hydrolysis to simple sugars can take place.[10] A range of chemical, physical, and biological processes to release these sugars have been configured, yet all face challenges of cost, technological breakthroughs, and infrastructure needs.[11,12] Research and development areas that influence lignocellulosics to ethanol conversion are pretreatment, enzymatic hydrolysis, and fermentation processes. All three stages must be fine-tuned and optimized for a particular feedstock. In recent years, alkali-based processes have become prominent in pretreatment of straw and stover-type residues, mainly because they operate under lower temperatures, pressures, and residence times compared to other pretreatment technologies.

Despite a plethora of studies reporting the use of dilute alkali as an effective lignocellulosic pretreatment option, few have reported using Australian biomass as a feedstock. Moreover, apart from our studies, no one has previously reported using grain sorghum residues in alkaline chemistries. This study reports sugar yields and profiles from post-grain-harvested sorghum and wheat straw residues using mild alkali process parameters and low enzyme dose saccharifications. Specifically, we examine and describe three characteristic phases: (1) the function of key pretreatment parameters (alkali strength, temperature, and residence time) and their impact on sugar solubilization, lignin reduction, and solid losses, (2) enzymatic hydrolysis efficacy of pretreated solid residues and variations in sugar composition with respect to pretreatment parameters, and (3) the role of individual and combined enzyme activities and their impact on the rates and yields of sugar release. Because of the impact of phenolic compounds on downstream processes, we also discuss their release during pretreatment and

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Table 1. Specific Activities of the Commercial Enzymes Used in Saccharification

	specific activity (units/mg of protein)[a]		
enzymes	NS50013	NS50010	NS50030
endoglucanase	14.20	0.11	0.02
exoglucanase	1.51	0.07	0.05
xylanase	7.05	75.00	129.50
β-glucosidase	1.07	10.08	0.04
pectinase	0.03	0.4	ND[b]
cellulase[c]	70.00	ND	ND
protein (mg/mL)[d]	135	150	33

[a] At pH 5.0 and 50 °C. [b] ND = not determined. [c] Measured as FPU/g of protein. [d] Concentration of Novozymes preparations.

saccharification. Understanding these key elements will enable further process optimization of wheat residues and assist in determining the efficacy of the conversion strategy.

2. MATERIALS AND METHODS

2.1. Preparation of Biomass. Post-grain-harvested sorghum straw (*Sorghum bicolour* var. MR Buster) and wheat straw (*Triticum aestivum*) were sourced from local farms of the Liverpool plains, northern New South Wales, Australia. Straws were dried at 50 °C for 48 h, ground in a rotary mill, and passed through a 1.5 mm screen. All chemicals used were of reagent or analytical grade and purchased from Sigma Chemical Co.

2.2. Pretreatment. To evaluate the effect of pretreatment parameters, a 2 × 3 × 4 factorial design was applied to individual biomass samples. Sodium hydroxide (NaOH) at concentrations of 0, 0.75, 1.0, and 2.0% (w/v) was used to pretreat milled samples at a solid loading of 10% (w/v). Treatments were performed in triplicate at two temperatures of 60 °C (water bath) and 121 °C (Labec autoclave, Australia), with residence times of 30, 60, and 90 min. The ramping and cooling time of the autoclave was approximately 10 min. The pretreated material was separated into solid and liquor (prehydrolysate) fractions using a Buchner funnel fitted with glass fiber filters (GF-A, Whatman). Pretreated solids were washed with water until the filtrate registered a neutral pH, sealed in plastic bags to retain moisture, and stored at −20 °C.

2.3. Enzyme Assays and Saccharifications. Cellulase (NS50013), β-glucosidase (NS50010), and xylanase (NS50030) preparations were kindly supplied by Novozyme. Enzyme activities as described by the supplier are 70 filter paper units (FPU)/g, 250 cellobiase units (CBU)/g, and 500 fungal xylanase units (FXU)/g, respectively. FXU is measured relative to a Novozymes fungal enzyme standard. The activity of the reference standard, *Humicola insolens*, xylanase batch number 17-1194, is defined to have an enzymatic activity of 3550 FXU/g at pH 6.0 and 50 °C in 30 min of reaction time of color release from the remazol−xylan substrate (from Novozymes biomass kit for conversion of lignocellulosic materials). Total cellulase activity of NS50013 was confirmed using the filter paper assay as described by the National Renewable Energy Laboratory (NREL) laboratory procedure LAP006.[13]

The protein content of liquid enzyme preparations was determined using a commercial bicinchoninic acid (BCA) protein assay reagent kit (Pierce Products, Rockford, IL) and reported in Table 1. Endoglucanase, exoglucanase, xylanase, and pectinase activities were individually determined in reaction mixtures (10 mL) containing 1% (w/v) carboxymethyl cellulose (CMC), 0.5% (w/v) Avicel, 0.5% (w/v) oat spelt xylan, and 0.5% (w/v) citrus pectin, respectively, in 50 mM citrate buffer (pH 5.2) and appropriately diluted enzyme solutions.[14−16] After 30 min of incubation at 50 °C, the reducing sugar liberated in the reaction mixture was measured by the dinitrosalicylic acid (DNS) method. A total of 1 unit of enzyme activity is defined as the amount of enzyme that produces 1 μmol of reducing sugar as glucose (xylose for xylanase) or galacturonic acid (for pectinase) in the reaction mixture per minute per milligram of protein under the above specified conditions (Table 1).

β-Glucosidase activities were assayed in reaction mixtures (1 mL) containing 4 mM *p*-nitrophenyl-β-D-glucoside, 50 mM acetate buffer (pH 5.0), and appropriately diluted enzyme solutions.[17] After incubation at 50 °C for 30 min, the reaction was stopped by adding 100 μL of ice-cold 100 mM NaOH and the resulting color change (*p*-nitrophenol) measured at 405 nm. A unit of each enzyme activity is defined as the amount of enzyme that releases 1 μmol of *p*-nitrophenol per minute per milligram of protein in the reaction mixture under these assay conditions. β-Glucosidase activities present in commercial enzyme preparations are reported in Table 1.

Pretreated materials were enzymatically saccharified by resuspending solid residues at a 5% (w/v) loading with 50 mM citrate buffer (pH 5.2) and appropriately diluted enzymes followed by incubation at 50 °C for up to 72 h. Reactions also contained 10 mM sodium azide to prevent microbial growth. Samples were withdrawn at time points specified and stored at −20 °C until sugars were analyzed.

2.4. Analytical Methods. Neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL), and acid-insoluble ash (AIA) were determined for untreated wheat straw by Diagnostic and Analytical Services of Industry and Investment NSW (Wagga Wagga, Australia) using the ANKOM Technology Methods.[18−20] The difference between NDF and ADF estimates detergent hemicellulose. Detergent cellulose was calculated by subtracting the values for ADL + AIA from ADF. Carbohydrate contents of the untreated material were also determined by measuring the hemicellulose- (xylan and araban) and cellulose- (glucan) derived sugars in supernatants following concentrated acid hydrolysis, as described by NREL.[21] Likewise, water and ethanol-soluble material were extracted from untreated wheat straw and quantified according to NREL methods[22]

Sugar composition of prehydrolysate and enzymatic saccharification liquors were determined using high-performance liquid chromatography (HPLC). The separation system consisted of a solvent delivery system (Controller 600 Waters) equipped with an auto sampler (717, Waters) and a refractive index detector (410 differential refractometer, Waters) managed by the Waters Empower software program. Sugars, organic acids, and alcohols were analyzed using either a Sugar-Pak 1 (6.5 × 300 mm, Waters) or an IC-Pak Ion-Exclusion 50 Å, 7 μm (7.8 × 300 mm, Waters) column, both fitted with the IC-Pak Ion-Exclusion Guard-Pak (Waters). The Sugar-Pak 1 column was maintained at 70 °C, and compounds eluted with a mobile phase consisting of degassed Milli-Q filtered water containing 50 mg/L Ca−EDTA at a constant (or isocratic) flow rate of 0.5 mL/min. The IC-Pak Ion-Exclusion column was maintained at 60 °C, and compounds eluted with a mobile phase consisting of degassed Milli-Q filtered water containing 2 mM H_2SO_4 at a constant (or isocratic) flow rate of 0.8 mL/min. The refractive index detector was maintained at 50 °C for all applications.

Peaks detected by the refractive index detector were identified and quantified by a comparison to retention times (RT) of analytical standards (glucose, xylose, galactose, arabinose, mannose, fructose, sucrose, cellobiose, formic acid, levulinic acid, and acetic acid). For comparative purposes, total reducing sugars were determined with dinitrosalicylic acid (DNS) according to NREL methodology.[13]

2.5. Xylan Extraction. The method was performed according to the procedure described by Hespell.[23] The liquid fractions following pretreatment were separated from solids using a Buchner funnel and then centrifuged (10000*g* for 10 min) to pellet any particulates. The decanted liquor was adjusted to pH ≤ 4.0 with 6 N HCl. After 10 min of continual stirring, precipitates were sedimented by centrifugation. Three volumes of cold ethanol were added while stirring for 15 min, and the precipitate (xylan) was collected by centrifugation. The isolated xylan was dried and determined gravimetrically. In addition, isolated xylan

Table 2. Composition of Untreated Straws

component	straw residue[a]	
	sorghum	wheat
neutral detergent fiber	63.0	69.0
acid detergent fiber	36.0	43.0
acid detergent lignin	2.9	5.9
acid-insoluble ash	0.7	0.9
cellulose	32.4	36.0
hemicellulose	27.0	26.0
acid-insoluble lignin	7.0	7.6
water extractives	21.1	13.0
ethanol extractives	8.7	5.5

[a] Composition percentages are on a dry weight basis.

fractions were enzyme-saccharified by resuspending with 50 mM citrate buffer (pH 5.2) containing 2% (v/v) xylanase (NS50030) and cellulase (NS50013) followed by incubation at 50 °C for up to 72 h. Sugars were quantified according to analytical methods described above.

2.6. Acid-Insoluble Lignin Extraction. The method was adapted from procedures described by Lin.[24] NaOH pretreatment hydrolysates were separated from remaining solids using a Buchner funnel and glass fiber filters (GF-A, Whatman) and were centrifuged (10000*g* at 20 °C for 10 min) to pellet particulates. The hydrolysate was heated ≥60 °C and adjusted to pH ≈ 2.0 with concentrated H_2SO_4 with rapid stirring. After 5 min of continual stirring, the samples were cooled to ambient temperature and the precipitate was sedimented by centrifugation as before. The acid-insoluble lignin precipitates were thoroughly washed with acidified water (pH 2.0) by gently inversion, collected by centrifuged as before, dried, and determined gravimetrically.

2.7. Total Phenolic Determination. The enzymatic method described by Ma and Cheung[25] was used to determine total phenolic content of hydrolysates. Samples to be analyzed were centrifuged and filtered (0.45 μm) prior to assaying. Aliquots (25 μL) of appropriately diluted phenolic sample were mixed with 225 μL of enzyme−reagent working solution into 96-well microtiter plates (clear F-bottom, Greiner Bio-one). The enzyme−reagent working solution was freshly prepared with 0.1 M potassium phosphate buffer solution (pH 8.0) containing 30 mM 4-aminoantipyrine (4-AP), 20 mM hydrogen peroxide (H_2O_2), and 6.6 μM horseradish peroxidase (HRP). After 15 min at room temperature, the absorbance was read at 540 nm, using a Fluorostar (BMG Lab Technologies, GmbH) plate reader. Vanillic acid standards (0−500 ng/mL) were subjected to the same assay conditions as the samples. Total phenolics were reported as vanillic acid equivalents.

2.8. Statistical Methods. Each set of observations was modeled as a response to the classifying factors generated by the experimental design. The data was analyzed using analysis of variance, which enabled partitioning of total variation in the data into components because of temperature, time, alkaline strength, and interactions between those terms.

The modeling process enabled prediction of the expected (average) response at each combination of the experimental factors and a measure of the experimental error. Estimated experimental error was used to calculate the "least significant difference" (LSD; $p = 0.05$) between three averages, required to indicate a statistically important effect. Statistical analysis and graphical presentation were conducted using software provided by the R Development Core Team.[26]

3. RESULTS AND DISCUSSION

3.1. Compositional Analysis of Straw Residues. The chemical composition of wheat and sorghum straw (presented in Table 2) is generally attributed to and reflects a number of factors, such as cultivar type, farming practices, geographical location, seasonal conditions, stage of harvest, and analytical procedures. The hybrid sorghum variety (MR Buster) used in this study is principally a grain variety as opposed to forage hybrids, which have been selected for high sugar and decreased lignin contents. The structural carbohydrate composition of the two straws are similar, with holocellulose fractions totalling 59.4 and 62% of dry sorghum and wheat straw biomass, respectively, with cellulose being the major component at 32.4 and 36%, while the remaining 27 and 26% derived from hemicelluloses. Both acid detergent and acid-insoluble lignin levels for sorghum and wheat straw were 2.9 and 5.9% and 7.0 and 7.6%, respectively.

Key differences between wheat and sorghum compositions are noted particularly in the extractives fractions, where water extractive compounds accounted for approximately 210 and 130 mg/g of dry sorghum and wheat straw, respectively. Within these water extractive fractions, sorghum straw contained 140 ± 6 mg of the storage disaccharide sucrose, whereas wheat straw contained 35 ± 2 mg of a higher oligosaccharide (glucose-plus 3 or more fructose). Although sucrose accumulation is typical of sorghum varieties (particularly forages), storage sugars are rarely reported in wheat straws. This may be due to the fact that storage oligosaccharides (found in stems and leaves) may or may not be available in all wheat straws because its accumulation and mobilization varies greatly between cultivars and in response to seasonal conditions.[27] Neither oligosaccharide was detected post-alkali treatment. Further solvent extraction of sorghum and wheat straws with ethanol resulted in 87 and 55 mg of material, respectively, presumably composed of oils, pigments, and waxes. The profile and size of individual wheat and sorghum straw components are comparable to reported values in the literature.[28,29]

3.2. Evaluating Enzymatic Hydrolysis of Alkali-Treated Straws. In a series of preliminary experiments, both wheat and sorghum straw were pretreated and subject to enzyme dose−response saccharifications. These experiments were initially aimed at evaluating pretreatment regimes and were particularly useful in defining the boundaries of enzyme trials. The results provided further information on the appropriate enzyme loads and activities for use in enzyme iso-dosing experiments, which were intended to assess the effectiveness of pretreatment parameters.

For any individual biomass feedstock and pretreatment strategy, it is essential to tailor the saccharification process (enzyme mixture and conditions) to maximize sugar yields.[30] Others reasons for optimization is to compensate for imbalances and/or shortfalls in commercially available enzyme preparations. Commercial cellulase mixtures maybe abundant in β-endoglucanase and cellobiohydrolyase but they are generally low in β-glucosidase and xylanase activity. They have been shown to be particularly inadequate for efficient monomeric sugar release from substrates containing higher amounts of arabinoxylan.[31] As shown in Table 1, the Novozymes cellulase preparation (NS50013) has 10- and 18-fold less β-glucosidase and xylanase activities, respectively, than NS50010 and NS50030 enzyme preparations; hence, enzyme blending may be necessary. The rate and extent of saccharification in response to differing enzyme combinations and dosages from pretreated wheat straw (2.0% NaOH for 90 min at 121 °C) and sorghum straw (1.0% NaOH for 60 min at 121 °C) were examined, and the data were plotted in panels a and b of Figure 1. The pretreatment regimes were employed to evaluate pretreated material that has been substantially delignified yet retained most of its xylan fraction.

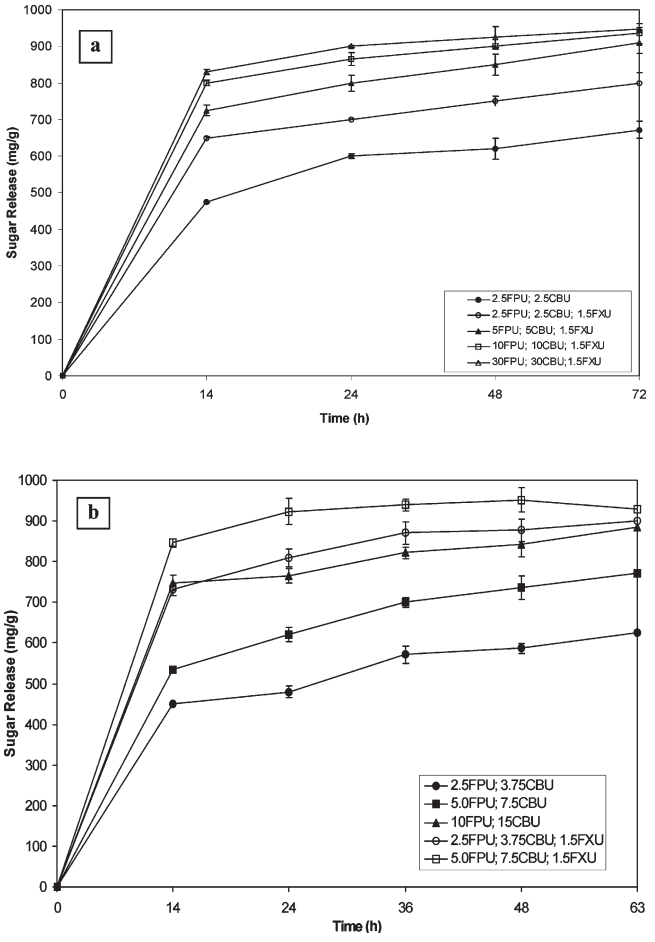

Figure 1. Time course of sugar release by enzymatic saccharification (50 °C and pH 5.2) of alkaline-pretreated (2.0% NaOH for 90 min at 121 °C) (a) wheat straw and (1.0% NaOH for 60 min at 121 °C) (b) sorghum straw using five enzyme combinations. Enzyme combinations and dosage were expressed as units of activity per gram of pretreated material, with cellulase, β-glucosidase, and xylanase activity measured in FPU, CBU, and FXU, respectively. Sugar yields are presented as milligrams per gram of pretreated material. Data represent averages of three separate experiments.

First, a combination of cellulase with β-glucosidase substantially promoted sugar release and was greater than the individual preparations. In supplementary experiments (unreported data), the addition of NS50010 to NS50013 at increasing ratios up to 4:1 lead to a corresponding rise in saccharification. However, beyond the ratio of 1:1, the gains were neither statistically significant nor cost-effective for cellulose conversion and this ratio was subsequently used in the following enzyme iso-dose trials. Tengborg and co-workers[32] also described similar benefits and limitations of β-glucosidases in enzymatic saccharifications of lignocellulosics in their work on softwoods.

Rates of sugar release and total sugar yield improved as the cellulase/β-glucosidase dosage was raised 4- and 12-fold for sorghum and wheat straw, respectively. Up to 1.4-fold gains were observed. However, final total sugar yields from these pretreated materials were lower than anticipated, considering the level of enzyme dosing. We assumed the xylanase activity in NS50010 (Table 1) would be adequate for hydrolyzing the remnant hemicellulose fractions. Hemicellulose (xylan) is known to act as a substrate-specific barrier, restricting cellulase access to cellulose fibers.[33,34] The hydrolytic efficiency (rate and yield) was improved by supplementing the cellulase/β-glucosidase mixture with xylanase (NS50030). For pretreated wheat (Figure 1a) and sorghum straw (Figure 1b), final sugar yields increased by 20 and 45%, following the addition of 1.5 FXU xylanase to a 2.5 FPU cellulase enzyme cocktail. For both straws, we also observe that doubling the load of the cellulase/glucosidase mixture while maintaining xylanase at 1.5 FXU resulted in a lesser total sugar gain. Responses to the addition of these non-cellulolytic enzymes (i.e., xylanase/pectinase) appear varied in the literature and are principally due to the nature of the substrates.[29,35,36] A further dosage increase in xylanases (3.0 FXU) failed to promote or deliver greater sugar gains (data not shown).

Finally, with the addition of xylanase in the saccharification mixture, rates of sugar release increased (>90% completion within 24 h) and a reduction in the cellulase loadings approached 4-fold.

3.3. Physical Effects of Alkaline Pretreatment. Pretreatment of the cereal straws with dilute NaOH resulted in dark-colored liquor containing insoluble material. We found that the color intensity of the liquor generally increased with pretreatment severity. Conversely, mass and color of remaining solids in prehydrolysate liquors decreased with severity. Others have reported similar reductions in solids during alkali pretreatment and attribute the degree of solubilization with the severity in temperature, residence time, and alkali concentrations. Under mild pretreatment conditions (1% NaOH for 60 min at 60 °C), solid losses were 25% (w/w) compared to 63% when pretreated at harsher conditions (2% NaOH at 121 °C). Although each variable under study contributed to solid loss, we found that the temperature had the greatest impact followed by alkalinity and then residence time. Comparable solid losses and treatment parameter trends have been reported in related studies on wheat straws.[37] However, a survey of the literature reveals some disparity in alkali pretreatment susceptibility between different crop residues.[37−39] These solid losses represent solubilization of the hemicellulose fraction and other components into prehydrolysate liquors. Aside from lignin (discussed later), several studies have reported hydrolysis of hemicellulose and release of oligoxylans (polyoses) of mixed molecular weights following exposure to alkali-based chemicals during the pretreatment process.[40,41] Once considered a drawback of alkaline chemistry (i.e., reduction in total fermentable sugar yield), current biorefinery platforms are exploiting alkali-based processes for recovery of valuable high-molecular-weight oligoxylans/arabinoxylans.[42−44]

We initially attempted to quantify liberated pentose sugars (xylose and arabinose), to determine the extent of hemicellulose solubilization. However, analysis of prehydrolysate liquors revealed a complex profile of poorly resolved compounds, including monosaccharides and high-molecular-weight oligosaccharides, levels of which were found to be proportional to the strength of alkali pretreatment, particularly at 121 °C. Isolation and characterization of the crude xylan from prehydrolysate liquors reveals that pretreating sorghum and wheat straw at 121 °C for 30 min in a 0.75% NaOH solution resulted in solubilization of 18.5 and 20%, respectively, of the hemicellulose component. Further increases in the NaOH concentration to 2.0% pushed the hemicellulose solubilization to approximately 30% for both sorghum and wheat straw. In addition to alkaline strength, variation of temperature and time may also impact the yield of isolated arabinoxylans.[44]

3.4. Effect of Alkaline Pretreatment on Enzymatic Saccharification. Hydrolysis of both cellulose and hemicellulose in

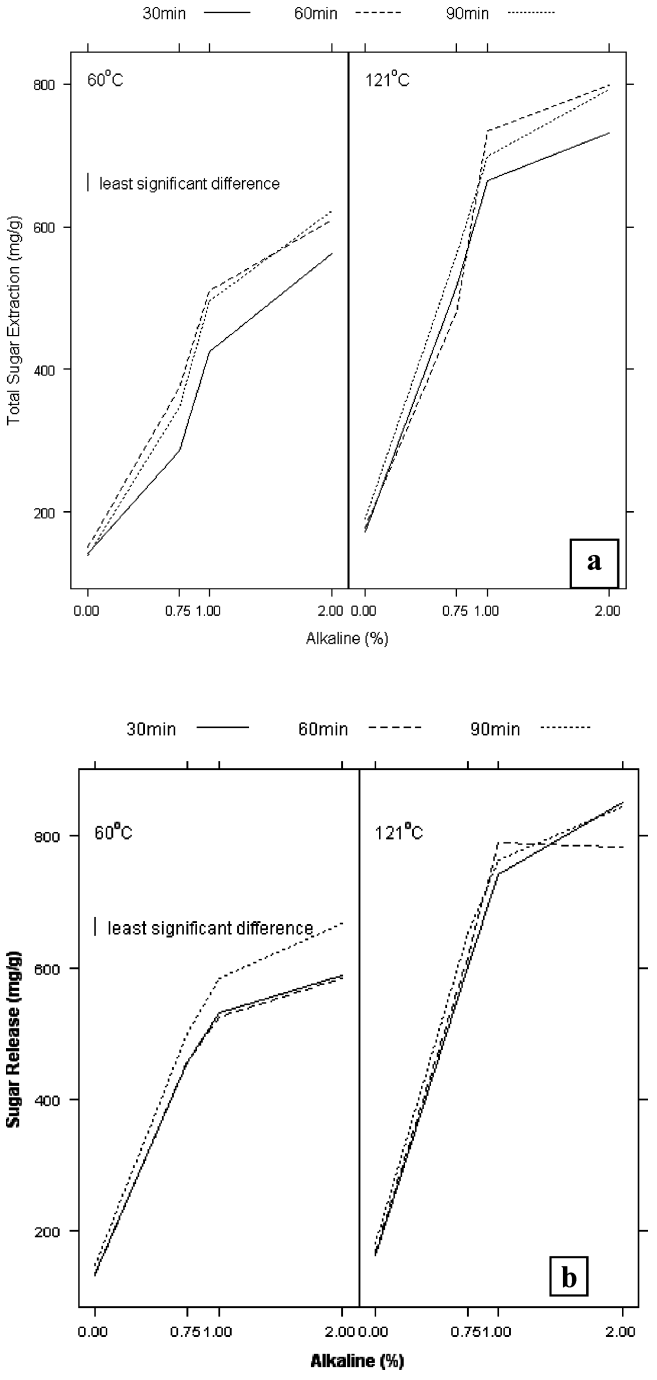

Figure 2. Total sugar release from NaOH-pretreated (10%, w/v) and enzyme-saccharified (50 °C and pH 5.2 for 48 h) (a) sorghum and (b) wheat straw presented as a function of the alkaline strength, temperature, and residence time. The enzyme dose was 2.5 FPU cellulase, 2.5 CBU β-glucosidase, and 1.5 FXU xylanase (per gram of pretreated material). Sugar yields are expressed as milligrams per gram of pretreated material. Data represent averages of three independent experiments. The average LSD (p = 0.05) = 24.4 and 25.0 for sorghum and wheat straw, respectively.

pretreated lignocellulosics via enzymatic action is critical in releasing monomeric sugars for fermentation to bioethanol. The rate and extent of enzymatic saccharification of the polysaccharide provides a measure of the effectiveness of the pretreatment. This section of work reports on the optimization of enzyme saccharification of both wheat and sorghum straw through a process of empirical testing of pretreatment variables. Specifically, we examine whether a relationship between pretreatment severity and enzyme saccharification of the pretreated material exists and, if so, attempt to describe the key variables. A total of 24 pretreatment combinations derived from varying test parameters, such as alkaline concentration (4 levels), time (3 levels), and temperature (2 levels), were trialed in triplicate on wheat and sorghum straw.

To draw out those variables(s) critical to the success of the pretreatment process, a relatively low enzyme iso-dose was applied, thereby averting large rapid sugar release. Pretreated solids were subject to enzymatic saccharification using the following set of conditions: 5% (w/v) substrate load in citrate buffer (pH 5.0) at 50 °C for 48 h. The enzyme iso-dose mixture consisted of 2.5 FPU cellulase, 2.5 CBU β-glucosidase, and 1.5 FXU xylanase per gram of pretreated solids. Sugar yields were quantified by HPLC analysis, and total sugar release was modeled as a response to pretreatment parameters and expressed as a function of the alkaline strength, temperature, and residence time (panels a and b of Figure 2).

The data in Figure 2 demonstrate that increases in pretreatment temperature, residence time, and alkali concentration improved enzymatic saccharification efficiency of the test material. In both cases, the temperature had the greatest ($p < 0.05$) impact on enzyme saccharification, above alkaline strength and/or time. That is, pretreatment at 121 °C was more acquiescent to enzymatic hydrolysis than at 60 °C. Within the 121 °C trials, increasing alkaline strength from 0 to 2% significantly ($p < 0.05$) improved saccharifications and led to a 4.6- and 5.2-fold improvement in total sugar release in sorghum and wheat, respectively. Pretreating wheat and sorghum straw with 2% NaOH for 30 and 60 min, respectively, at 121 °C followed by enzyme saccharification yielded the highest recorded total sugar release of 850 and 799 mg/g of pretreated material. Raising reaction times to 90 min under the same conditions failed to liberate further monomeric sugars; however, reducing treatment time to 30 min in the case of sorghum straw only slightly diminished sugar yields. Hu and Wen[45] and Wang et al.[31] reported similar sugar yields in response to comparable temperature and alkaline concentrations; albeit, they also describe switchgrass and coastal bermudagrass responding unfavorably (significantly less total recoverable sugars) to NaOH loading above 10 and 1%, respectively.

At the lower pretreatment temperatures of 60 °C, sugar release was found to generally rise with an increasing NaOH concentration. A maximum yield of 667 and 621 mg/g for wheat and sorghum straw, respectively, was attained in 2% NaOH followed by saccharification. Under these conditions, statistically similar ($p < 0.05$) yields were obtained from solid material exposed to elevated temperature and reduced hydroxide combinations (e.g., 121 °C and 0.75% NaOH). This raises the possibility that, under mild alkaline conditions, the optimal pretreatment temperature may be lower than 121 °C, offering potential power and cost savings in an industrial process. No discernible differences between the 30 and 60 min treatments were observed at 60 °C; however, extending the time to 90 min improved total sugar yields for all combinations of alkalinity. In the absence of NaOH, increasing time did not influence sugar yields but raising the temperature to 121 °C slightly improved saccharification.

3.5. Sugar Compositions of Hydrolysates. The effectiveness of enzymatic saccharification on pretreated material is principally evaluated by the degree of conversion of cellulose to glucose monomers. For a mild alkaline process, which only

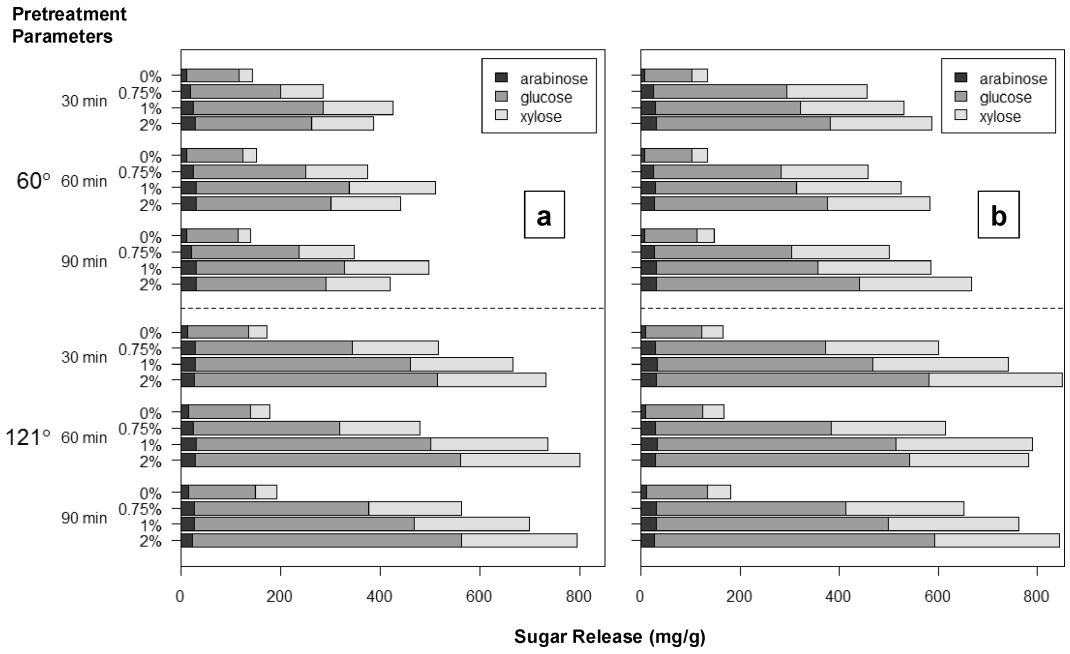

Figure 3. Sugar profile of NaOH-pretreated (10%, w/v) and enzyme-saccharified (50 °C and pH 5.2 for 48 h) (a) sorghum and (b) wheat straw. The enzyme dose was 2.5 FPU cellulase, 2.5 CBU β-glucosidase, and 1.5 FXU xylanase (per gram of pretreated material). Data represent averages of three independent experiments. LSD ($p = 0.05$) are 15.2 and 13.2 (glucose), 10.7 and 11.1 (xylose), and 3.6 and 2.7 (arabinose) for sorghum and wheat straw, respectively.

partially solubilizes hemicellulose fractions, assessing effectiveness should include measuring released monomeric pentoses (xylose and arabinose).[31,38,40] Quantifying individual sugar components not only assists in determining the best possible conversion strategy but also permits a rapid appraisal of its fermentation potential. Constituent monosaccharides of sorghum and wheat straw enzyme-saccharified hydrolysates were quantified and expressed as a function of alkaline strength, temperature, and residence time in panels a and b of Figure 3, respectively.

Generally, enzymatic hydrolysis of cellulose correspondingly increased with the pretreatment temperature, residence time, and alkali concentration. The temperature had the greatest significant ($p < 0.05$) impact, with 121 °C delivering greater cellulose saccharification than 60 °C. Maximum glucose yields were recorded (540 and 567 mg/g) for sorghum and wheat straw, respectively, when samples were pretreated at 121 °C for 90 min in 2% NaOH. Comparable glucose yields were observed with a pretreatment time of 60 and 30 min for sorghum (532 mg) and wheat straw (552 mg), respectively. Within the 121 °C treatments, elevating alkaline strengths resulted in a significant ($p < 0.05$) increase in glucose recovery for all pretreatment times. Similar trends were noted among samples treated at the lower temperature. Glucose release from sorghum (390 mg/g) and wheat (410 mg/g) straw exposed to 2% NaOH at 60 °C for 90 min was found to rival and surpass glucose levels resulting from straws treated with 0.75% NaOH at 121 °C. This suggests that increasing alkaline strength may potentially act as a trade-off to reducing reaction temperatures.

Increasing pretreatment severity also improved hemicellulose saccharification and subsequent xylose release. The temperature had a significant ($p < 0.05$) effect, with 121 °C producing greater xylose release than 60 °C. Maximum xylose yields were attained when sorghum and wheat straw samples were exposed to 2 and 1% NaOH at 121 °C, giving a peak yield of up to 235 and 275 mg, respectively, after 60 min of treatment time. As observed for glucose yields, reducing alkali strength (0.75%) for both samples resulted in significantly ($p > 0.05$) lower xylose release, implying modest exposure of the lignocellulosic structure. When both straw types were pretreated at conditions optimal for glucose recovery (i.e., 2% NaOH at 121 °C for 90 min), significantly lower xylose yields were obtained. Others have reported similar declines in xylose yield, which incidentally correlates with elevated xylan levels in prehydrolysate liquors and pretreatment settings.[37,39,44] In the control samples, xylose release was very small (25−45 mg/g), irrespective of temperature settings.

Lowering the pretreatment temperature to 60 °C led to a reduction in the maximum xylose yield for both sorghum (205 mg/g) and wheat straw (227 mg/g). However, we noted that xylose levels from sorghum straw exposed to 1−2% NaOH at 60 °C for extended times exceeded xylose release from enzyme-digested solids pretreated with 0.75% NaOH at 121 °C. Xylose concentrations from comparable wheat straw samples were found to be similar. Inadequate hemicellulose hydrolysis at this lower temperature has probably physically constrained and impeded cellulase attack. Supplementing the enzyme mixture with additional hemicellulase/xylanase activity should improve hydrolysis of mildly treated substrates containing higher amounts of xylan.[46] Pretreatment conditions for maximum arabinose sugar release correlated with those observed for xylose sugars at both temperatures. Maximum yields of up to 33 mg/g of pretreated material were attained under these conditions. Arabinose yields from solids pretreated in 2% NaOH at 121 °C were also significantly ($p < 0.05$) reduced. Glucose and xylose yields from controls (water-treated materials) were approximately 4- and 6-fold, respectively, lower than yields resulting from alkali-catalyzed pretreatment, confirming the need for an alkali catalyst.

3.6. Delignification during Mild Alkali Pretreatment. The degree of delignification reflects the effectiveness of the alkaline pretreatment process. Moreover, it is critical in improving enzymatic

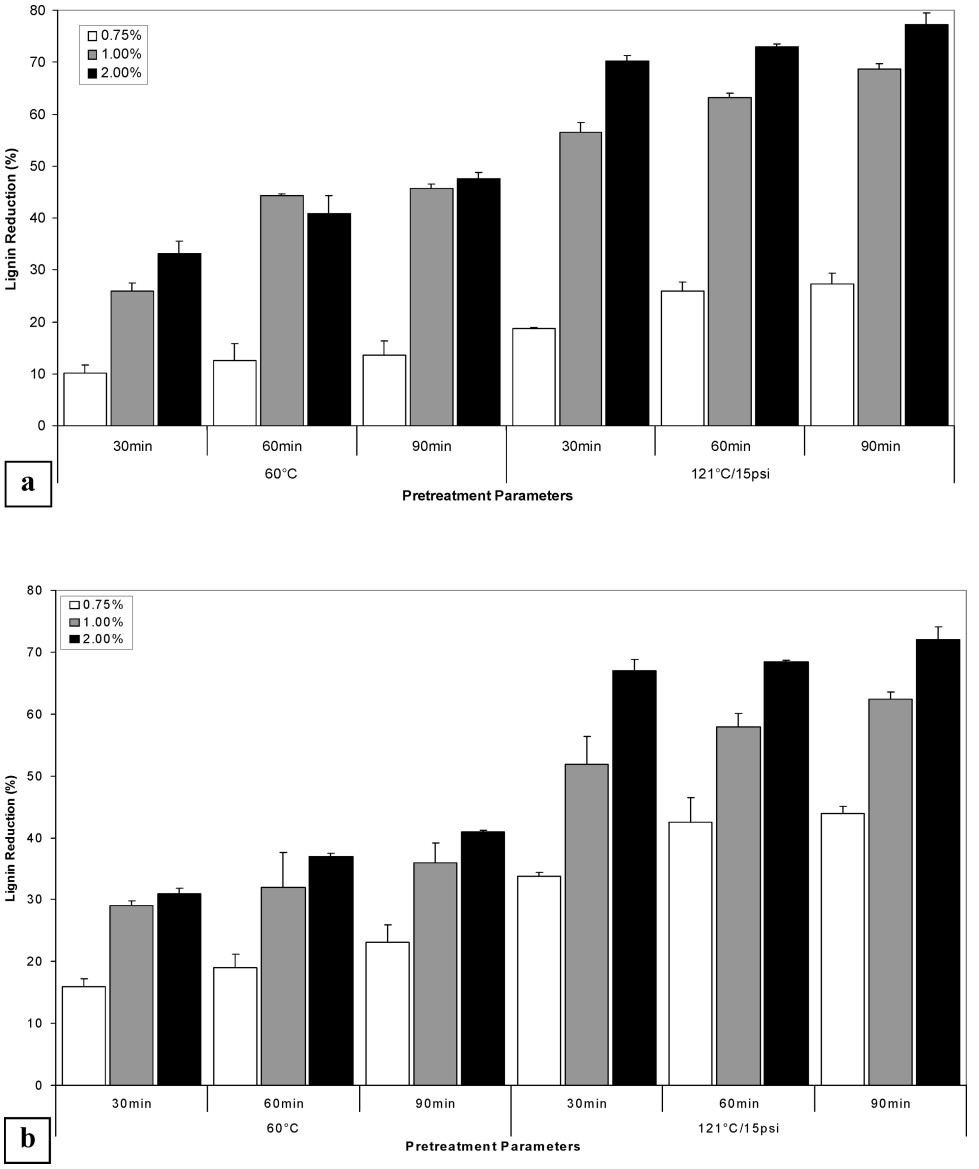

Figure 4. Reduction of acid-insoluble lignin in (a) sorghum and (b) wheat straw pretreated in 0.75% (white) 1.0% (gray), and 2.0% (black) NaOH expressed as a function of the temperature and residence time. The data presented are averages of three separate experiments. The average LSD (p = 0.05) = 3.44 and 4.77 for sorghum and wheat straw, respectively.

degradation of lignocellulosics and is ultimately influenced by pretreatment severity.[47] The effect of NaOH pretreatment on the delignification of sorghum and wheat straw were quantified by determining the reduction of acid-insoluble lignin in pretreated solids and is presented in panels a and b of Figure 4. Of all parameters tested, temperature had the most significant ($p < 0.05$) impact on delignification. At 121 °C, delignification of sorghum straw ranged from 18% (0.75% NaOH for 30 min at 121 °C) to a maximum of 77.3% (2.0% NaOH for 90 min at 121 °C). For wheat straw, delignification extended from 33% (0.75% NaOH for 30 min at 121 °C) to a maximum of 72% (2.0% NaOH for 90 min at 121 °C). In the presence of 2% NaOH, pretreatment times can be reduced to just 30 min and still achieve high lignin reductions of ∼70%. For both straws, we observe that, particularly at the elevated temperature (121 °C), increasing alkaline strengths significantly ($p < 0.05$) improved delignification, whereas the responses to time were substantially less pronounced. Only at lower alkaline concentrations (0.75%) was the response to increasing time significant ($p < 0.05$). Furthermore, at 0.75% NaOH, we observe a 1.6−1.8-fold improvement in delignification of wheat straw over sorghum straws. Contrary to lignin compositions observed in Table 2, wheat straw showed superior delignification compared to sorghum under all test conditions. Under similar temperature and time conditions, Varga et al.[39] reported that almost complete delignification (>95%) was achievable when alkaline concentrations were raised to 10%, although recoverable total carbohydrate levels had drastically diminished.

At 60 °C, delignification was substantially reduced and ranged from 10.2% (0.75% NaOH for 30 min) to 45% (1.0% NaOH for 60 min) for sorghum straw. There were no significant ($p < 0.05$) gains in delignification by raising the NaOH to 2% and residence time to 90 min. Similarly, delignification was substantially reduced at the lower temperature extending from 15% (0.75% NaOH for 30 min) to 42% (2.0% NaOH for 90 min) for wheat straw. Alkaline treatment at 0.75% NaOH failed to effectively

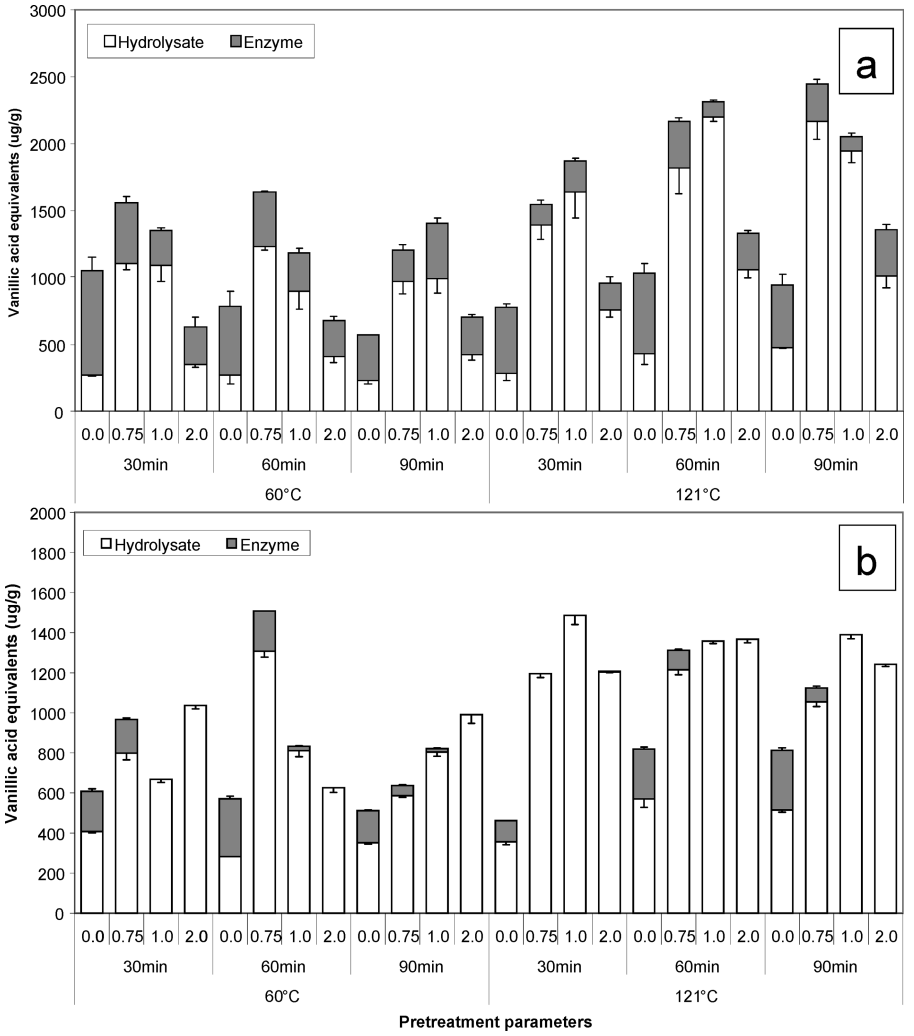

Figure 5. Total phenolics present in pretreated liquors and enzyme-saccharified hydrolysates of (a) sorghum and (b) wheat straw samples as a function of the alkaline strength, temperature, and residence time. Results are presented as micrograms per gram of vanillic acid equivalents. The data presented are averages of three separate experiments.

delignify either straw sample, irrespective of the reaction temperature and time. Incidentally, this coincided with reduced sugar yields in saccharified hydrolysates.

These findings imply a correlation between delignification and enhanced enzyme saccharification of both pretreated straws. Several studies have demonstrated strong negative correlations between lignin content and sugar released by enzymatic hydrolysis.[48,49] This is owing to lignin acting as a physical barrier, restricting cellulase access to cellulose and, thereby, reducing the activity of the enzyme through non-productive binding.[33]

3.7. Release of Phenolics into Prehydrolysate Liquors and Saccharified Hydrolysates. Phenols, furans, carboxylic acids, and inorganic salts formed or released during pretreatment of lignocellulosic materials are known to have an inhibitory effect on downstream processes.[50] Sorghum and wheat, similar to most monocotyledons, are typically rich in phenolic acid esters associated with hemicellulose and lignin.[51] Total phenolics from both pretreatment and enzyme-saccharified hydrolysates were quantified, and the data were presented as a function of changes in the treatment temperature, residence time, and alkaline strength in panels a and b of Figure 5.

Excluding the control samples, approximately 5- and 7.4-fold more total phenolics were found in pretreated sorghum and wheat hydrolysates (2200 and 1486 μg/g), respectively, compared to enzyme saccharification mixtures (450 and 200 μg/g). With respect to sorghum prehydrolysates, the temperature had a larger impact on phenolic levels than the reaction time or alkalinity (Figure 5a). Samples treated at 121 °C released almost double the amount (2200 μg/g) of phenolics than those pretreated at 60 °C (1200 μg/g). At 121 °C, increasing pretreatment reaction time and alkaline strength enhanced phenolic release from sorghum samples. However, at 2% NaOH, the detection of total phenolic levels diminished, because of either product decomposition or alteration. That is, sorghum produces pigmented phenolic compounds (e.g., anthocyanins), which are reportedly susceptibility to degradation and/or changes to their oxidative state at elevated pH values.[52] The decline in phenolic release was more pronounced at the lower (60 °C) pretreatment temperature and/or residency time. With respect to wheat straw samples, increasing pretreatment reaction time and alkaline strength > 0.75% at 121 °C did not generally enhance phenolic release (Figure 5b). At 60 °C, response to changes in time and

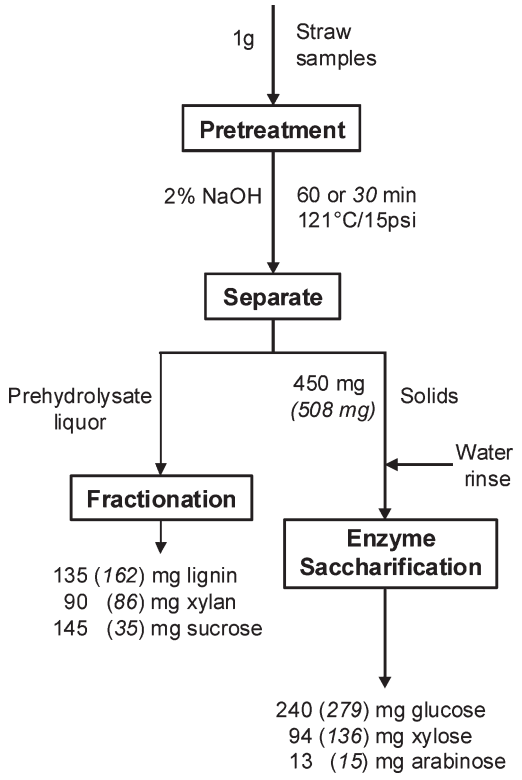

Figure 6. Mass balance of pretreatment and enzymatic hydrolysis process steps.

alkalinity were varied, although a net decrease in total yields was observed. Overall, wheat straw hydrolysates contained substantially less phenolics.

Total phenolic content in enzyme-saccharified hydrolysates were substantially lower, especially following higher temperature treatments. Most of the phenolics were recovered in prehydrolysate liquors. Conversely, saccharification mixtures of samples pretreated at 60 °C and all of the water controls contained higher phenolic content; total phenolics in their respective hydrolysates were comparatively low. Overall, these results suggest that harsher pretreatment conditions should provide saccharified hydrolysates with reduced phenolic content and greater fermentation potential.

3.8. Mass Balances. An overall mass balance diagram describing the process stages from pretreatment to enzymatic hydrolysis was undertaken and is presented in Figure 6. Both sorghum and wheat straw at a solid loading of 10% (w/v) were pretreated under conditions optimized for maximum sugar recovery (2% NaOH at 121 °C for 60 and 30 min for sorghum and wheat, respectively). The remaining insoluble fraction was separated from the pretreatment hydrolysate prior to enzymatic saccharification. The amount of recovered material corresponded to ≈45 and 51% (w/w) of the original (sorghum and wheat straw, respectively) starting material and was subjected to saccharification.

Enzyme saccharification was achieved using low dosages of cellulase (2.5 FPU), β-glucosidase (2.5 CBU), and (1.5 FXU) xylanase (per gram of pretreated solids) and incubated at 50 °C. Sugar yields were recorded at 240 and 279 mg of glucose, 94 and 136 mg of xylose, and 13 and 15 mg of arabinose per gram of original starting material (sorghum and wheat straw, respectively). Recovered prehydrolysate liquors were further fractionated through titration with 6 N H_2SO_4. At pH 4.0, 135 and 162.6 mg/g of acid-insoluble lignin was recovered as a precipitate from sorghum and wheat straw, respectively. The addition of 3 volumes of cold ethanol to the aqueous phase led to the precipitation of 90 and 86 mg/g of crude xylan from sorghum and wheat straw, respectively. Prehydrolysate liquors also contained approximately 145 and 35 mg/g of water extractive storage carbohydrate and other unquantified polysaccharides, phenolics, and degradation compounds from sorghum and wheat straw, respectively. Finally, combining mild pretreatment conditions with low enzyme doses has allowed for the recovery of approximately 73 and 83% of the theoretical sugar potential of sorghum and wheat straw, respectively.

4. CONCLUSION

In closing, we find that the dilute alkali pretreatment studies described above satisfy certain important requisites for an effective pretreatment process, namely, an excellent delignification, cellulose-enriched fraction that is responsive to enzyme digestion with high and rapid sugar release and low phenolic levels. Our study also showed that there are opportunities for further process optimization, such as the pretreatment temperature and/or enzyme combinations and dosages. Using alkaline pretreatment to extract oligoxylans and lignin while simultaneously improving cellulose hydrolysis can be a means of consolidating the economic viability of a biorefinery. However, selecting an appropriate pretreatment regime requires a degree of compromise between maximizing glucose yield and minimizing the creation of inhibitors. Considering its abundance and high sugar potential, sorghum and wheat straw are an excellent feedstock for biorefineries, particularly in producing ethanol.

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