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# High temperature dilute acid pretreatment of coastal Bermuda grass for enzymatic hydrolysis

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## Abstract

Dilute sulfuric acid was used to pretreat coastal Bermuda grass at high temperature prior to enzymatic hydrolysis. After both pretreatment and enzymatic hydrolysis processes, the highest yield of total sugars (combined xylose and glucose) was 97% of the theoretical value. The prehydrolyzate liquor was analyzed for inhibitory compounds (furfural, hydroxymethylfurfural (HMF)) in order to assess potential risk for inhibition during the following fermentation. Accounting for the formation of the inhibitory compounds, a pretreatment with 1.2% acid at 140 °C for 30 min with a total sugar yield of 94% of the theoretical value may be more favorable for fermentation. From this study, it can be concluded that dilute sulfuric acid pretreatment can be successfully applied to coastal Bermuda grass to achieve high yields of monomeric glucose and xylose with acceptable levels of inhibitory compound formation.

**Keywords:** coastal Bermuda grass, dilute sulfuric acid pretreatment, enzymatic hydrolysis, furfural, hydroxymethylfurfural

## 1. Introduction

Due to a lack of resource sustainability of fossil fuels as well as negative environmental effects from emissions, research is being done to find a liquid fuel for use as a gasoline replacement. In the United States (US) ethanol appears to be a good near-term option. Ethanol is already mixed with gasoline at a level of 10% and support from vehicle manufacturers has resulted in vehicles that can use up to an 85% ethanol-15% gasoline mixture. Currently, ethanol is mainly produced from corn starch in the US, but competition with corn for food and limitations on arable land for expanding the cultivation of corn place a ceiling on the ethanol producible lower than what is required to replace gasoline (Sun and Cheng, 2002). This has spurred research into cellulosic feedstocks for ethanol production. Lignocellulosic materials are abundant nearly everywhere opening the potential for different geographic regions to take advantage of locally abundant cellulosic feedstocks for ethanol production. While corn ethanol simply requires a hot water pretreatment followed by enzymatic hydrolysis using inexpensive amylases, lignocellulosic materials require a harsher pretreatment step followed by hydrolysis using more costly cellulases (Solomon et al., 2007). Currently, pretreatment, enzymatic hydrolysis, and fermentation are the key areas being researched to make lignocellulosic feedstocks competitive with corn for the production of ethanol and this study focuses on the pretreatment step. Of the many pretreatment

technologies covered by a number of review papers, dilute acid pretreatment stands out having been already examined in a large number of studies as well as appearing more economically feasible at larger scale than other current pretreatment technologies (Sun and Cheng, 2002; Moiser et al., 2005; Egge-man and Elander, 2005).

In the southeastern US, coastal Bermuda grass (CBG) (*Cynodon dactylon* L.) is grown by hog producers as part of required nutrient management practices to remove nitrogen and phosphorus from hog waste. With a yield of 6–10 dry tons per acre each year and an active cropping system in place, CBG is potentially a viable lignocellulosic feedstock option for ethanol production in the southeastern US (Holtzapple et al., 1994). Sun and Cheng (2005) investigated the dilute sulfuric acid pretreatment of coastal Bermuda grass at 121 °C over a range of acid concentrations (0.6–1.5% w/w) and residence times (30–90 min). Optimal pretreatment conditions were found to be 1.2% acid for 60 min yielding about 70% of the total sugars from the biomass. Other studies using dilute acid pretreatment with different feedstocks have shown that increasing temperature increases sugar yields and can decrease the residence time and acid concentration required to generate the same yields at lower temperatures and longer residence times (Lloyd and Wyman, 2005; Saha et al., 2005, 2008). Based on results from Sun and Cheng (2005) and the review of research done on other feedstocks using dilute acid at temperatures higher than 121 °C, this study was performed to examine

if higher sugar yields from CBG could be generated at higher temperatures, to identify the effects of temperature, concentration, residence time, and the interactions between these factors on sugar yields, and to assess what pretreatment conditions would be the best in the pretreatment of CBG.

## 2. Methods

### 2.1. Biomass handling and storage

CBG was obtained in 2007 from Central Crops Research Station located in Clayton, NC, courtesy of Dr. Joe Burns of the Crop Science Department at North Carolina State University. The collected CBG was stored in loose bale at ambient room temperature in a large plastic bag. Before used for pretreatment, the CBG was ground to particle sizes no greater than 2 mm using a Wiley Laboratory Mill (Model No. 4, Thomas, Philadelphia, PA, USA) fitted with a 2 mm screen. The resulting ground biomass was stored in sealed bags at ambient room temperature in the lab until used.

### 2.2. Composition analysis of raw biomass

Prior to any pretreatment, the CBG was analyzed for its composition. The moisture content was determined using a 105 °C oven to dry the biomass according to the procedure outlined in National Renewable Energy Laboratory (NREL) laboratory analytical procedure (LAP) "Determination of Total Solids in Biomass" (Sluiter, 2005). Extractive components like waxes, chlorophyll, non-structural sugars, and other minor components were quantified using a Soxhlet extractor, a 2:1 toluene:ethanol mixture, and 24 h reflux time (Silverstein, 2004; Han and Rowell, 1997). The extractive-free biomass was then examined for the structural carbohydrates, i.e. glucan, xylan, arabinan, and galactan as well as acid insoluble lignin (AIL) and acid soluble lignin (ASL) using the NREL LAP "Determination of Structural Carbohydrates and Lignin in Biomass" (Sluiter, 2006a). The ash content of the biomass was determined using a benchtop muffle furnace in accordance with NREL LAP "Determination of Ash in Biomass" (Sluiter, 2005).

### 2.3. Pretreatment

A factorial experimental design was developed to examine the effect of the pretreatment conditions, i.e. reaction temperature, acid concentration and residence time, on the sugar yields in both the prehydrolyzate and the hydrolyzate liquors as well as the generation of sugar degradation products in the prehydrolyzate liquor. Factor levels of the experimental design are based on literature review and previous work on the dilute acid pretreatment of CBG done by Sun and Cheng (2005). Sulfuric acid concentrations of 0.3%, 0.6%, 0.9%, and 1.2% (w/w) were examined at temperatures of 120, 140, 160, and 180 °C and residence times of 5, 15, 30, and 60 min.

Dilute sulfuric acid pretreatment was performed in vessels constructed from threaded 4 in. long by 0.75 in. inner diameter 306 stainless steel pipe nipples capped on both ends with threaded 306 stainless steel pipe caps. The parts used to construct the vessels were purchased from McMaster Carr (Santa Fe Springs, CA, USA). Sulfuric acid was prepared on a w/w basis from a 96% stock solution beforehand and stored in a reagent bottle for continued use. Six vessels (two treatments in triplicate) were loaded at a ratio of 10 parts dilute acid to one part dry raw biomass (w/w) (30 ml acid and 3 g biomass). The 7th vessel was loaded with de-ionized (DI) water instead of dilute acid in the same 10% solids loading as the other vessels and sealed with a cap containing a plug k-type thermocouple

(Model TC-K-NPT-G-72-SMP, Omega, Stamford, CT, USA), served as a temperature monitor for the approximate internal temperature of the other reaction vessels.

A Fisher Scientific High-Temp Bath (Model 160A, Fisher Scientific, Dubuque, Iowa, USA) was used to indirectly heat the vessels with silicone oil (Poly(dimethylsiloxane),  $d = 0.96$ ) as a heat transfer fluid. A k-type thermocouple temperature probe was submerged in the oil in order to monitor the oil temperature and both this probe and the probe in the vessel were connected to an Extech Easyview Thermometer (Model 11A, Extech, Waltham, MA, USA) which displayed the temperature readings. The oil was heated to 20 °C higher than the desired set-point for the reaction to shorten preheat time. Once the oil reached the desired temperature, the seven vessels were added to the bath and the time of this addition was recorded. The blank vessel's internal temperature was then monitored along with the temperature of the oil until both reached the desired reaction temperature at which time it was inferred that all of the vessels were at the desired temperature. Heating was manually controlled using the dial on the oil bath to turn the heating element on and off in order to keep the time it took to heat the vessels to the desired temperature to 12 min plus or minus 2 min. Once the proper internal temperature in the vessels was reached, the residence time began and upon completion of the residence time, the vessels were quickly removed from the oil bath and placed in a bin of cool water where they were cooled to less than 40 °C within 5 min.

After pretreatment, the vessels were opened up for the filtration step. A Welch Duo-Seal vacuum pump (Model 1HAB-25B-M100X, GAST Manufacturing, Inc., Benton Harbor, MI, USA) was used to pull a vacuum across a Whatman P8 filter paper in a standard vacuum flask and buchner filter funnel setup. The contents of each vessel were emptied onto a pre-weighed filter paper associated with that particular sample. Sixty milliliters of de-ionized (DI) water at room temperature was used to rinse the biomass and ensure the collection of all the sugars hydrolyzed in the pretreatment step. The total amount of filtrate and wash water was measured in a graduated cylinder and a portion was stored in labeled 15 ml tubes and frozen at -20 °C. The solids on the filter paper were rinsed with an additional 140 ml of room temperature DI water and then placed into an appropriately labeled bag for storage at 4 °C. These bags were weighed before and after the addition of biomass for use in calculating solid loss later. The filter paper was dried for 24 h at 105 °C and then weighed to account for biomass remaining on the filter paper.

### 2.4. Enzymatic hydrolysis

Enzymatic hydrolysis was preformed in 50 ml centrifuge tubes maintained at 55 °C and 155 rpm agitation for 72 h by an automated shaking water bath (Model C76, New Brunswick Scientific, Edison, NJ, USA). Each tube was first loaded with the retained rinsed pretreated biomass weighed to account for moisture so that each tube received the equivalent of 0.5 g of dry biomass. Novozymes North America (Franklinton, NC, USA) provided the cellulase, NS-50013, and the cellobiase, NS-50010, which were determined using an assay procedure derived from Ghose (1987) to have activities of 76.44 FPU/ml (filter paper units, where one unit equates the amount of enzyme required to produce 1  $\mu$ mol of glucose per minute from filter paper) and 283.1 CBU/ml (cellobiase units, where one unit equates the amount of enzyme required to generate 2  $\mu$ mol of glucose per minute from cellobiose), respectively. The enzymes were loaded in excess at 40 FPU of cellulase per gram of dry biomass and 70 CBU of cellobiase per gram of dry biomass to avoid any limitation in monomeric sugar production caused by enzyme deficiency. Sodium azide, 0.3% (w/v),

was added to inhibit microbial growth. Sodium azide at 0.3% (w/v) was chosen according to the previous relevant studies in the literature (Kim and Holtzapfle, 1987; Sun and Cheng, 2005). Accounting for the amount of moisture in the biomass, the sodium azide, and the enzymes, the total amount of liquid in each tube was brought up to 15 ml using a 0.05 M sodium citrate buffer to maintain a pH of 4.8 during the enzymatic hydrolysis step. The primary purpose of the sodium citrate buffer was to maintain the pH at 4.8 which is an ideal pH for enzyme activity during the enzymatic hydrolysis (Ghose, 1987; Kim and Holtzapfle, 1987; Sun and Cheng, 2005).

### 2.5. Sugar analysis

The hydrolyzate and prehydrolyzate were analyzed using a high performance liquid chromatography (HPLC) system to quantify the sugar monomers. The HPLC was also used to quantify prehydrolyzate sugar degradation products. The NREL LAP "Determination of Sugars, Byproducts, and Degradation Products in Liquid Fraction Process Samples" was the foundation for the HPLC sample preparation and analysis procedure used in this study (Sluiter, 2006b). HPLC parameters used in this study which differ from those outlined in the NREL LAP were developed by Novozymes who ran the samples for this study on their HPLC units. An Aminex HPX-87P column coupled with a refractive index detector was used to distinguish amounts of glucose, xylose, galactose, arabinose in the hydrolyzate samples. Samples were prepared from the pH 4.8 hydrolyzate, diluted by four times, and filtered through a 2  $\mu$ m syringe filter. The column was run at 80 °C with a flow rate of 0.6 ml/min using HPLC grade water as a mobile phase. Samples injections were 10  $\mu$ L and the runtime was 35 min. A 25 min post-runtime was included to clear late-eluting compounds from the column. For the prehydrolyzate samples, an Aminex HPX-87H column was used to quantify the levels of glucose, xylose, furfural, 5-hydroxyfuranmethal (HMF), formic acid, and levulinic acid. Samples were prepared from the prehydrolyzate which had a pH of less than two and filtered through a 2  $\mu$ m syringe filter. This column was run at 65 °C with a flow rate of 0.7 mL/min. Sample injections were run for 51 min. Total sugars, monomeric sugars, and degradation products were calculated on a per gram of raw biomass basis.

### 2.6. Lignin analysis of pretreated biomass

The retained solid biomass was analyzed for acid insoluble lignin and acid soluble lignin composition in the same manner as the raw biomass was analyzed using the NREL LAP "Determination of Structural Carbohydrates and Lignin in Biomass" (Sluiter, 2006a).

### 2.7. Statistical analysis

Although triplicates were collected, the data had a number of outliers which appeared to be due to unequal heating during pretreatment, enzymatic hydrolysis error, or analysis error. As a result many triplicates had two sets of measured values very close and one set with a clear difference. To remedy this, one sample was removed systematically from each triplicate. The remaining data was run through the GLM procedure in SAS 9.1.3 (SAS Institute Inc., Cary, NC, USA) to perform an analysis of variance (ANOVA) to identify statistically significant effects from each of the factors as well as interactions of the factors. The interaction effect combinations included two-way interactions between temperature and concentration, temperature and time, concentration and time, and the three-way interaction among temperature, concentration, and time. The data was adjusted using Tukey's adjustment and significant

differences were evaluated where  $p < 0.01$  ( $\alpha = 0.01$ ). MATLAB 7.6.0.324 R2008a (The MathWorks, Natick, MA, USA) was used to generate contour plots for visual analysis of the data.

## 3. Results and discussion

### 3.1. Composition of coastal Bermuda grass

The CBG was examined for carbohydrate, lignin, extractive, and ash composition according to Wang et al. (2010) and Sun and Cheng (2005) with the resulting dry weight percentages shown in Table 1. The two sets of composition data are different from the set from Sun and Cheng (2005) reporting a greater amount of sugar with 6.77% more glucan and 3.49% more xylan than the set reported by Wang et al. (2010). This difference can likely be attributed to the difference in harvest time, crop inputs, or the fact that Wang et al. (2010) did the analysis on extractive-free biomass rather than the raw biomass. In removing the extractives, nonstructural carbohydrates could have also been removed. This study was run in parallel with the study done by Wang et al. (2010) and used the same CBG stock and composition data.

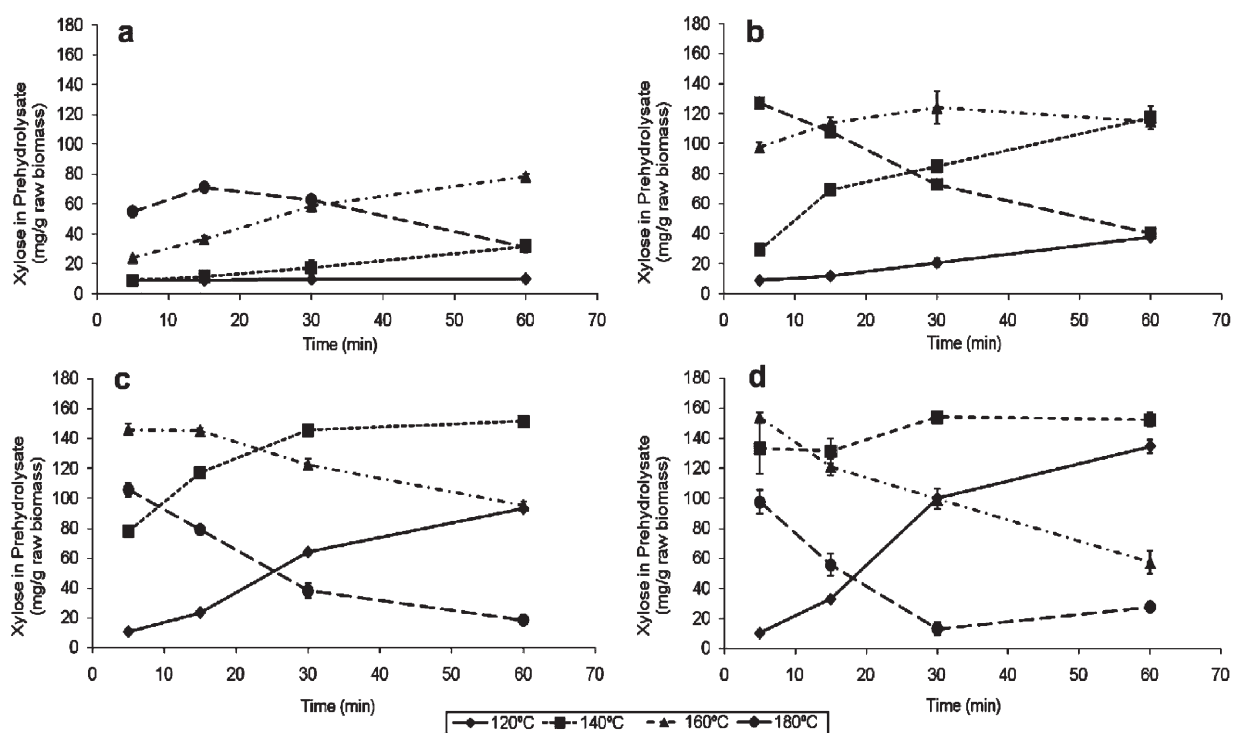
Glucan, xylan, arabinan, and galactan can be converted respectively to glucose, xylose, arabinose, and galactose using anhydro corrections of 0.90 for six-carbon sugars and 0.88 for five-carbon sugars which accounts for the removal of a water molecule. In this study arabinose and galactose concentrations in both prehydrolyzate and hydrolyzate are negligible. References to 'total sugars' will only include the sum of glucose and xylose for simplification. The theoretical maximum release of glucose is 284.3 mg/g of raw biomass (dry). The theoretical maximum for xylose is 180.5 mg/g of raw biomass (dry). The theoretical maximum for total sugars is therefore 464.8 mg/g of raw biomass. Further yield calculations will be based upon these theoretical values.

### 3.2. Dilute acid pretreatment

The pretreatment step had two major outputs, the pretreated CBG solids and the prehydrolyzate liquor. The solids were analyzed for both acid insoluble lignin and acid soluble lignin. The prehydrolyzate liquor was analyzed for carbohydrates and carbohydrate degradation products.

#### 3.2.1. Lignin reduction

Dilute acid pretreatment works primarily by hydrolyzing hemicellulose into sugars (mostly xylose) from the biomass structure (Moiser et al., 2005). However, lignin also undergoes simultaneous degradation and accumulation reactions during an acid pretreatment (Pearl, 1967). Yang and Wyman (2004) showed that the net prevailing reaction in a batch reactor appeared to correspond to the severity of the acid pretreatment. While intermediate pretreatment severities tended to correspond with higher measured lignin removal (20–30%), there was also data showing little or no lignin removal. Additionally, both lower and higher severity acid pretreatments tended to show either no lignin removal or apparent lignin accumulation above that measured in the initial biomass composition analysis. Yang and Wyman (2004) suggested that these unclear lignin measurements were a complication of using a batch reactor. They showed that using a flow-through reactor where solubilized lignin was removed from the system as the pretreatment progressed resulted in measured lignin removal of up to 85% that was clearly positively linearly correlated with the pretreatment severity. Since batch reactors were used in this study, the lignin values measured after pretreatments follow the general trend presented by Yang and Wyman (2004)



**Figure 1.** Monomeric xylose measured in the prehydrolyzate after pretreatment as a function of time and temperature for (a) 0.3%, (b) 0.6%, (c) 0.9%, and (d) 1.2% sulfuric acid concentration (w/w).

**Table 1.** Composition of coastal Bermuda grass.

| Component             | % Weight of dry biomass<br>(Wang et al. (2010)) | % Weight of dry biomass<br>(Sun and Cheng (2005)) |
|-----------------------|---|---|
| Glucan                | 25.59   | 32.36   |
| Xylan                 | 15.88   | 19.37   |
| Arabinan              | 1.95  | 4.33  |
| Galactan              | 1.46  | 1.09  |
| Acid insoluble lignin | 15.37   | 20.33   |
| Acid soluble lignin   | 3.96  | ND  |
| Extractives           | 4.17  | ND  |
| Ash                   | 6.6   | 4.17  |

for batch reactors, which made it difficult to elucidate the specific effects of the pretreatment on the lignin portion of the biomass. As a result, acid soluble lignin and acid insoluble lignin data have not been reported in the body of this paper, however, it can be inferred that acid pretreatment in a batch reactor may disrupt the structure of lignin.

### 3.2.2. Acid hydrolysis of biomass

Xylose is the major carbohydrate in the prehydrolyzate liquor and the largest component of hemicellulose. The highest yield of xylose measured in the prehydrolyzate, 83%, was generated after a pretreatment at 1.2% acid, 140 °C, and 30 min residence time. However, xylose yields at 140 °C for 30 and 60 min and 160 °C for 5 and 15 min at concentration of 0.9% acid as well as 140 °C for 60 min and 160 °C for 5 min at concentration of 1.2% acid were found to be statistically similar to the highest yield. Figure 1 contains four graphs corresponding to each of the four acid concentration levels. Each graph shows the monomeric xylose measured in the prehydrolyzate for each combination of temperature and residence time. An examination of the graphs shows indications of interaction effects that could explain why several sets of pretreatment combinations generate similarly high xylose yields. Table 2 shows the ANOVA output for several of the major response variables considered in this study, in-

cluding xylose. The ANOVA output for xylose confirms the significance of each of the factors as well as each of the interaction terms and shows that temperature and acid concentration offer the greatest influence, about 25% each, in the variance of the xylose yield. In general, the importance of temperature, acid concentration, time and interactions between these factors is not too surprising because the relationship has already been investigated using modified Arrhenius equations in other studies that have proposed these equations as rate constants in kinetic models to describe the acid hydrolysis of hemicellulose into xylose (Carrasco and Roy, 1992; Baugh and McCarty, 1988).

Figure 1 also highlights the loss of xylose under certain pretreatment conditions. This loss can most likely be attributed to the degradation of xylose into furfural as a result of harsh pretreatment conditions. The major degradation products will be addressed later in more detail. The loss of xylose is first evident at 0.3% acid and 180 °C after 15 min. At 0.6% acid and 160 °C after 30 min there is a slow decrease in xylose levels as well as a much more rapid decrease at 180 °C after 5 min. At both 0.9% and 1.2% acid the rapid degradation of xylose is clear at both 160 °C and 180 °C after 5 min.

The prehydrolyzate can also contain glucose. Table 3 shows the measured glucose monomers liberated from the CBG during the pretreatment averaged across the residence times for each acid concentration and temperature combination. The time variable is omitted because it does not influence glucose yield as strongly as the other factors (ANOVA results not shown), but the standard deviation is included so that the general effect of time on glucose yield in the prehydrolyzate can still be inferred. A clear trend is visible, showing that the increase of temperature or acid concentration results in the increase of glucose in the prehydrolyzate. This follows the logic that as the pretreatment conditions become harsher, the hemicellulose is hydrolyzed faster and more completely, exposing the cellulose to acid hydrolysis as well. Similar trends are reported in a study on the dilute acid pretreatment of corn stover by Lloyd and Wyman (2005).

**Table 2.** ANOVA responses for xylose, furfural, and hydroxymethylfurfural (HMF) in the prehydrolyzate, glucose in the hydrolyzate, and total reducing sugars (sum of glucose and xylose) during the pretreatment and enzymatic hydrolysis of coastal Bermuda grass.

| Source       | DF  | Type III sum of squares  |                            |                       |                        |                       | P-Value |          |        |         |                       |
|--------------|-----|--------------------------|----------------------------|-----------------------|------------------------|-----------------------|---------|----------|--------|---------|-----------------------|
|              |     | Xylose in prehydrolysate | Furfural in prehydrolysate | HMF in prehydrolysate | Glucose in hydrolysate | Total reducing sugars | Xylose  | Furfural | HMF    | Glucose | Total reducing sugars |
| T*           | 3   | 76499.71                 | 27467.55                   | 1026.81               | 184369.60              | 448978.77             | <.0001  | <.0001   | <.0001 | <.0001  | <.0001                |
| C**          | 3   | 72045.48                 | 2779.12                    | 151.33                | 52055.52               | 277733.29             | <.0001  | <.0001   | <.0001 | <.0001  | <.0001                |
| T × C        | 9   | 792.68                   | 1579.07                    | 178.09                | 14867.76               | 21775.18              | <.0001  | <.0001   | <.0001 | <.0001  | <.0001                |
| Time         | 3   | 55443.03                 | 4388.32                    | 93.62                 | 41856.66               | 172873.57             | <.0001  | <.0001   | <.0001 | <.0001  | <.0001                |
| T × time     | 9   | 52388.85                 | 2750.29                    | 100.43                | 20023.35               | 166082.95             | <.0001  | <.0001   | <.0001 | <.0001  | <.0001                |
| C × time     | 9   | 1840.37                  | 278.40                     | 25.75                 | 4730.23                | 21412.97              | <.0001  | <.0001   | 0.0294 | <.0001  | <.0001                |
| T × C × time | 27  | 36623.21                 | 1110.38                    | 61.57                 | 10667.38               | 83095.35              | <.0001  | <.0001   | 0.0288 | <.0001  | <.0001                |
| Error        | 64  | 1104.53                  | 238.82                     | 81.29                 | 2075.85                | 21183.18              |         |          |        |         |                       |
| Total        | 127 | 296737.85                | 40591.96                   | 1718.89               | 330646.35              | 1213135.26            |         |          |        |         |                       |

\* T = temperature in pretreatment. \*\* C = sulfuric acid concentration in pretreatment.

**Table 3.** Glucose in the prehydrolyzate after the dilute sulfuric acid pretreatment of coastal Bermuda grass.

| Sulfuric acid concentration % (w/w) | Temperature (°C) | Average glucose in pre-hydrolyzate mg/g raw biomass |
|-------------------------------------|------------------|---|
| 0.3                                 | 120              | 5.93 ± 0.25   |
| 0.3                                 | 140              | 6.47 ± 0.74   |
| 0.3                                 | 160              | 9.09 ± 2.72   |
| 0.3                                 | 180              | 13.30 ± 4.10  |
| 0.6                                 | 120              | 6.44 ± 0.90   |
| 0.6                                 | 140              | 10.71 ± 3.79  |
| 0.6                                 | 160              | 21.67 ± 6.48  |
| 0.6                                 | 180              | 24.05 ± 10.91                                       |
| 0.9                                 | 120              | 8.08 ± 2.14   |
| 0.9                                 | 140              | 20.22 ± 9.13  |
| 0.9                                 | 160              | 32.15 ± 5.16  |
| 0.9                                 | 180              | 34.18 ± 3.14  |
| 1.2                                 | 120              | 10.55 ± 4.90  |
| 1.2                                 | 140              | 28.30 ± 6.48  |
| 1.2                                 | 160              | 37.29 ± 2.17  |
| 1.2                                 | 180              | 43.43 ± 15.93                                       |

3.2.3. Degradation of carbohydrates

Under harsh conditions, carbohydrates are degraded into furfural and HMF which in turn are degraded into levulinic acid and formic acid, respectively. In this study, even the harshest conditions in the factorial did not generate levulinic acid and formic acid levels in the prehydrolyzate close to reported inhibitory levels (>200 mM) while furfural and HMF were measured well beyond reported inhibitory levels (>1.0 g/L) (Navarro, 1994; Delgenes et al., 1996; Taherzadeh et al., 1997; Palmqvist and Hahn-Hagerdah, 2000; Almeida et al., 2009). For this reason, Figure 2 shows only the furfural and HMF levels. There is one graph for each acid concentration and one bar for each combination of time and temperature. For ease of comparison, the individual amounts of furfural and HMF have been summed as a single bar in each graph of Figure 2 where 10 mg of furfural or HMF each corresponds to 1 g/L of that compound for the 10% solid loading used in this study. As inferred previously, the degradation product generation inversely reflects the xylose losses seen in Figure 1 which also helps explain why much more furfural than HMF is generated. The general trend seen in each of the graphs is supported by the ANOVA output for furfural and HMF in Table 2, which shows that temperature is the dominating significant effect for both response variables.

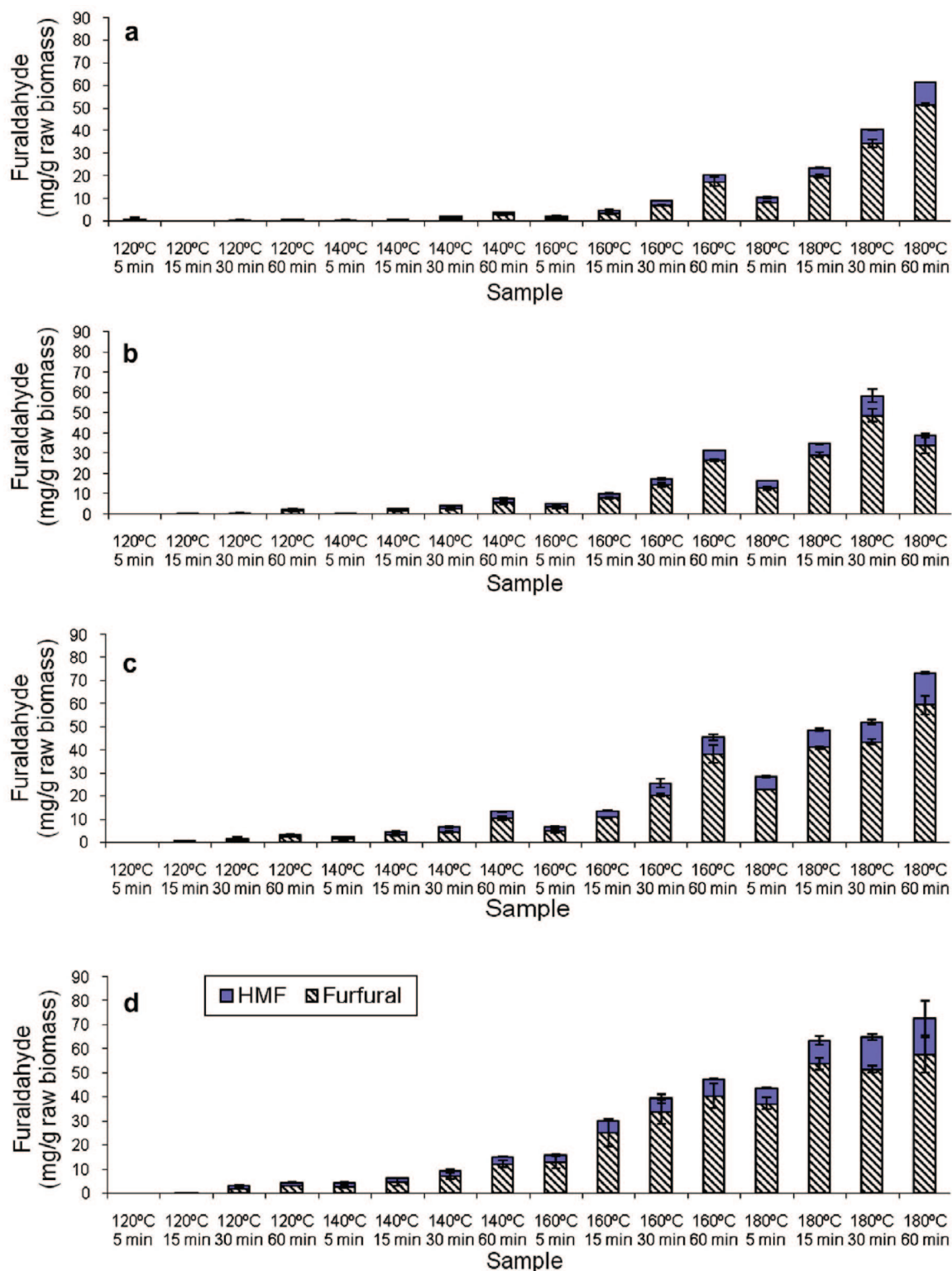
All of the factors and the interaction terms are significant for furfural and most are significant for HMF except for the in-

teraction between concentration and time and the three-way interaction term.

3.3. Enzymatic hydrolysis

After the enzymatic hydrolysis, the hydrolyzate was analyzed for glucose and xylose monomers. Figure 3 shows the levels of monomeric glucose measured in the hydrolyzate for each combination of the factors, temperature, acid concentration, and time. The highest yield of glucose measured in the hydrolyzate was 95% of the theoretical maximum and was generated after a pretreatment with 0.6% sulfuric acid at 160 °C for 30 min. Examining both Figure 3 and the output of a statistical multiple comparison of the entire data set (not shown), there are 18 other pretreatment condition sets that produce glucose yields that are similar to the 95% yield. As with the xylose generation in the prehydrolyzate, there are interaction effects contributing to the multiple pretreatment combinations with similar results, however, this is only part of the explanation. The number of pretreatment combinations that give high glucose yields is also increased by the fact that hemicellulose removal during the pretreatment in the form of either xylose or furfural generation can lead to high glucose yield in the hydrolyzate after the enzymatic hydrolysis. This can be observed upon a review of Figures 1 and 2 in comparison with Figure 3, and this makes sense because the success of the acid pretreatment is based around the concept of hemicellulose removal in order to maximize glucose yield after enzymatic hydrolysis. Since furfural levels are substantially influenced by temperature and xylose levels are influenced by temperature and acid concentration, it would be expected that glucose in the hydrolyzate would be influenced most substantially by temperature followed by acid concentration. The ANOVA output in Table 2 supports this claim. All of the factors and interaction terms significantly contribute to the glucose yield in the hydrolyzate, however, the temperature and concentration terms provide the most influence over the variance making up 55% and 15%, respectively. Figure 2 shows a loss in glucose yield under a few acid concentrations and residence times, but only for 180 °C. The fact that this apparent degradation happens under harsher conditions than xylose degradation and only at the highest temperature used in this study is reasonable because glucose is a more stable carbohydrate than xylose (Baugh and McCarty, 1988).

Table 4 shows the xylose found in the hydrolyzate. Like the glucose levels in Table 3, the xylose levels in Table 4 have been averaged across the residence times for each acid concentration and temperature combination. The standard deviation is



**Figure 2.** Combined furfural and hydroxymethylfurfural measured in the prehydrolyzate after pretreatment as a function of time and temperature for (a) 0.3%, (b) 0.6%, (c) 0.9%, and (d) 1.2% sulfuric acid concentration (w/w).

included so that the general effect of time on xylose levels in the hydrolyzate can still be inferred. A clear trend is visible, showing that the increase of temperature or acid concentration results in the decrease of xylose in the hydrolyzate. Only 0.3% acid at 120 °C does not appear to follow this trend and this is most likely explained by a weak acid hydrolysis during the pretreatment that did not adequately disrupt the bio-

mass. Generally, incomplete acid hydrolysis is what leaves hemicellulose in the solids. Under harsher pretreatments more hemicellulose is removed leaving less in the solids. This correlates to the removal of hemicellulose from the biomass as represented in Figures 1 and 2. Similar trends are reported in a study on the dilute acid pretreatment of corn stover by Lloyd and Wyman (2005).

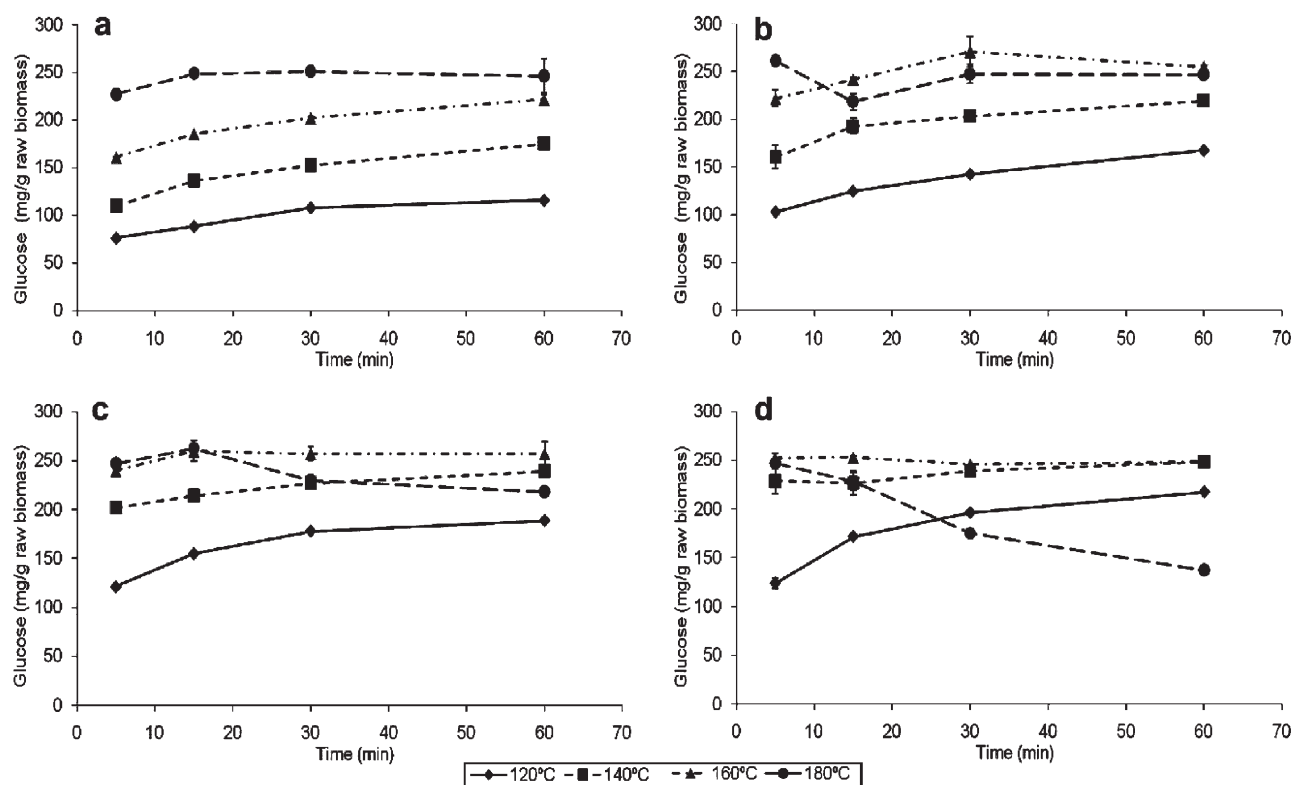


Figure 3. Monomeric glucose measured in the hydrolyzate after enzymatic hydrolysis as a function of time and temperature for (a) 0.3%, (b) 0.6%, (c) 0.9%, and (d) 1.2% sulfuric acid concentration (w/w) applied in the pretreatment.

Table 4. Xylose released in the hydrolyzate after enzymatic hydrolysis of coastal Bermuda grass under different pretreatment conditions.

| Pretreatment conditions             |                  | Average xylose in hydrolyzate mg/g raw biomass |
|-------------------------------------|------------------|--|
| Sulfuric acid concentration % (w/w) | Temperature (°C) |  |
| 0.3                                 | 120              | 18.67 ± 7.41                                   |
| 0.3                                 | 140              | 33.61 ± 8.17                                   |
| 0.3                                 | 160              | 28.57 ± 8.07                                   |
| 0.3                                 | 180              | 14.45 ± 5.99                                   |
| 0.6                                 | 120              | 31.14 ± 7.19                                   |
| 0.6                                 | 140              | 27.25 ± 5.85                                   |
| 0.6                                 | 160              | 17.76 ± 4.54                                   |
| 0.6                                 | 180              | 9.14 ± 3.66                                    |
| 0.9                                 | 120              | 30.79 ± 5.32                                   |
| 0.9                                 | 140              | 18.77 ± 4.87                                   |
| 0.9                                 | 160              | 10.50 ± 4.15                                   |
| 0.9                                 | 180              | 3.22 ± 1.64                                    |
| 1.2                                 | 120              | 29.11 ± 6.51                                   |
| 1.2                                 | 140              | 15.37 ± 3.68                                   |
| 1.2                                 | 160              | 6.36 ± 2.42                                    |
| 1.2                                 | 180              | 0.84 ± 0.97                                    |

The data in this study follow the trends of the data presented in other similar studies looking at the dilute acid pretreatment of herbaceous biomass or crop residues (Sun and Cheng, 2005; Guo et al., 2008). In general, as pretreatment severity increases, hemicellulose is more completely hydrolyzed from the solid biomass. The more complete hydrolysis of hemicellulose appears to correlate with the higher glucose yields after enzymatic hydrolysis even with higher generation of furfurals. Temperature significantly drives the degradation of xylose into furfural and therefore at higher temperatures there is faster xylose degradation. Additionally, the glucose found in the prehydrolyzate increases and the xylose found in the solid biomass decreases as pretreatment severity increases.

### 3.4. Total sugars

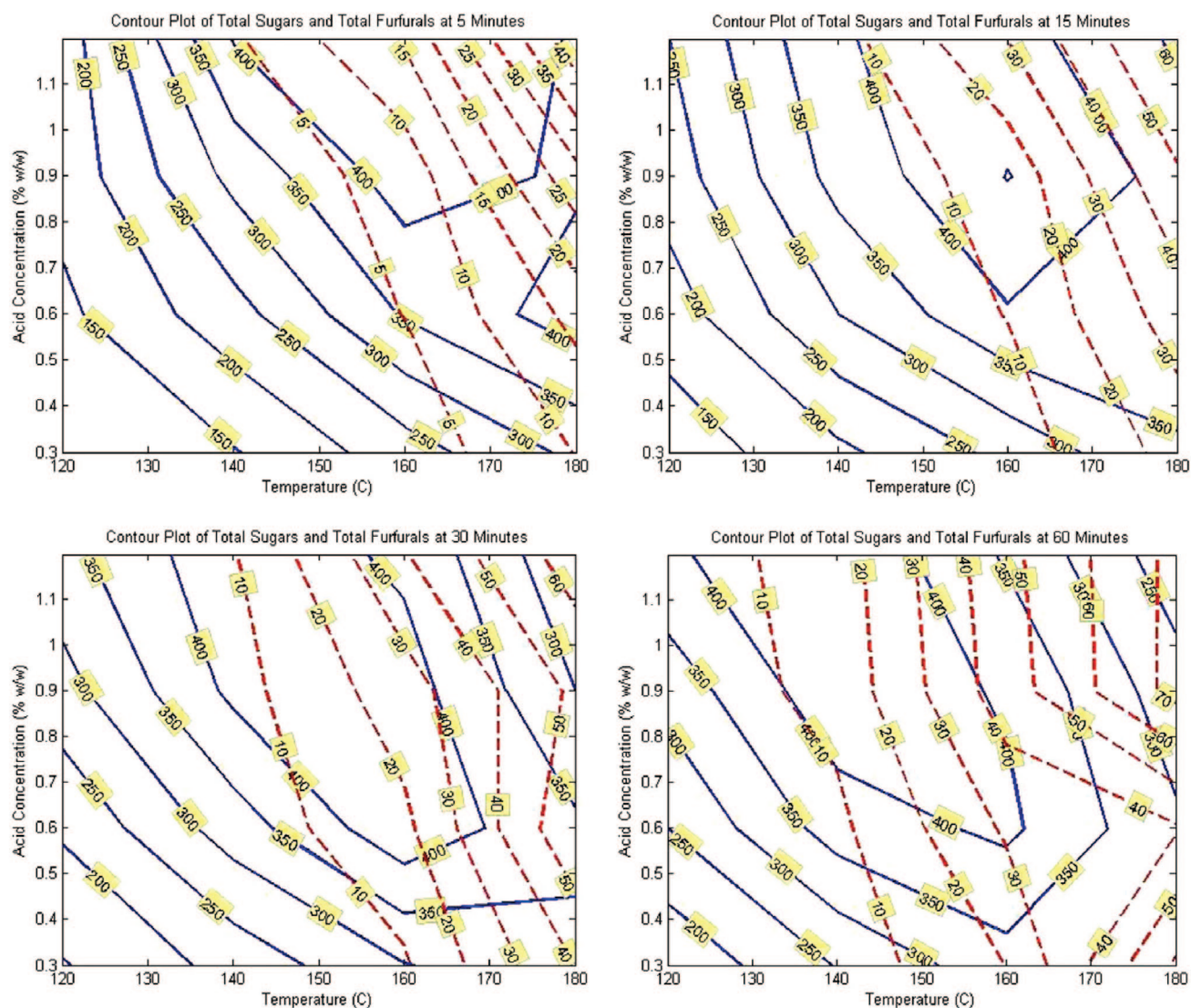
All of the available sugars from the biomass must be considered for fermentation into ethanol in order to make cellulosic biomass a feasible choice for ethanol production (Sun and Cheng, 2002). In this study, the total sugars available for fermentation from CBG are the summation of the xylose and glucose monomers found in both the prehydrolyzate and the hydrolyzate. The overall highest yield of total sugars is 97% of the theoretic maximum, which is obtained with the pretreatment conditions of 0.9% acid at 160 °C for 15 min and 1.2% acid at 160 °C for 5 min. Examining all of the pretreatment conditions with total sugar yields of 80% and greater (20 entries) highlights that while sugar production among these pretreatment conditions remains relatively close, the generation of furfurals varies from 3 mg/g raw biomass to 37 mg/g raw biomass (equivalent to furfural concentrations of 0.3–3.7 g/L, respectively). If the threshold concentration of furfurals where yeast inhibition may begin is assumed to be 1.0 g/L, based on the other studies cited previously, then this list can be narrowed from 20 entries to 7. In this shorter list, the pretreatment condition combination of 1.2% acid at 140 °C for 30 min appears to be the best choice based on total sugar yield.

### 3.5. Fermentation simulation

To help verify the best pretreatment parameters, a fermentation simulation step was used to approximate ethanol production relative to changes in ethanol yield by the organism and inhibition caused by furfurals. This step examined the co-fermentation of the sugars from the prehydrolyzate and enzymatic hydrolysis streams together.

All ethanol conversion values were taken from the study done by Krishnan et al. (1999) which examined the co-fermentation characteristics of *Saccharomyces* 1400 (pLNH33) for etha-





**Figure 4.** The overlay of contour plots for total sugar yield and total furfural generation as a function of sulfuric acid concentration and temperature at each time level. (Dashed line = furfurals, mg/g raw biomass; solid line = total sugars, mg/g raw biomass.)

**Table 5** The effect of dilute sulfuric acid pretreatment conditions on total sugar production and ethanol yield from coast Bermuda grass.

| Pretreatment conditions |                                     |            |                                    |                               |                |                       |  |
|-------------------------|-------------------------------------|------------|------------------------------------|-------------------------------|----------------|-----------------------|--|
| Temperature (°C)        | Sulfuric acid concentration % (w/w) | Time (min) | Average furfural, mg/g raw biomass | Average HMF, mg/g raw biomass | EtOH yield (%) | Total sugar yield (%) |  |
| 140                     | 0.6                                 | 60         |                                    | 0.8                           | 71             | 80                    |  |
| 160                     | 0.6                                 | 15         | 8.15                               | 2.15                          | 74             | 85                    |  |
| 160                     | 0.6                                 | 30         | 14.49                              | 2.91                          | 52             | 94                    |  |
| 180                     | 0.6                                 | 5          | 12.9                               | 3.55                          | 57             | 91                    |  |
| 140                     | 0.9                                 | 30         | 4.35                               | 1.99                          | 79             | 89                    |  |
| 140                     | 0.9                                 | 60         | 10.28                              | 2.95                          | 74             | 94                    |  |
| 160                     | 0.9                                 | 5          | 4.76                               | 1.85                          | 81             | 91                    |  |
| 160                     | 0.9                                 | 15         | 10.61                              | 2.86                          | 76             | 97                    |  |
| 120                     | 1.2                                 | 60         | 3.01                               | 1.38                          | 75             | 84                    |  |
| 140                     | 1.2                                 | 5          | 2.78                               | 1.56                          | 77             | 87                    |  |
| 140                     | 1.2                                 | 15         | 4.46                               | 1.81                          | 76             | 85                    |  |
| 140                     | 1.2                                 | 30         | 6.98                               | 2.3                           | 83             | 94                    |  |
| 140                     | 1.2                                 | 60         | 12.13                              | 2.89                          | 69             | 96                    |  |
| 160                     | 1.2                                 | 5          | 12.67                              | 3.14                          | 65             | 97                    |  |

nol production from varying mixtures of xylose and glucose in the media. The ethanol conversion of this particular organism was found to vary from 0.40 to 0.48 g of ethanol per gram of

sugar depending on the ratio of xylose to glucose in the media. A greater ratio of xylose to glucose resulted in a lower conversion by the organism.

A specific relationship between the combined level of furfural and HMF and the rate of ethanol production with *Saccharomyces cerevisiae* was identified in a study done by Taherzadeh et al. (1997). This study supported the same trend outlined in the other studies mentioned previously by showing the inhibition of the yeast beyond the furfural and HMF concentration of 1 g/L. An equation for this relationship was approximated from a diagram in the publication using Microsoft Excel to generate a 3rd-order polynomial regression trend line. For the simulation, it was assumed that the recombinant yeast from Krishnan et al. (1999) would be inhibited by the combined furfural and HMF to the same degree as the natural yeast.

Table 5 shows the output of the fermentation simulation with the approximated ethanol yields, exhibiting the expected relationship with combined furfural levels. The combined furfural levels under 10 mg/g (1 g/L) combined with the sugar yields over 90% give ethanol yields greater than 80%. This confirms that a pretreatment with 1.2% acid at 140 °C for 30 min is an optimal option as well as 0.9% acid at 160 °C for 5 min. The simulation also supports the use of 1 g/L as the threshold for furfural production when assessing the data for optimal pretreatment conditions.

### 3.6. Operating region for optimal pretreatments

The data presented in this study has identified two optimal pretreatment parameter options. However, if a contour plot for the total sugar data is overlaid on a contour plot of the furfurals data, we can find regions containing pretreatment condition combinations with the potential for high total sugar production and furfural production around 1 g/L that are not covered experimentally in this study. Figure 4 shows four contour plots with total sugar yields represented by the solid lines and total furfurals generated represented by the dashed lines. Each plot is at one level of the time factor. Favorable operating regions in between 10 mg furfurals/g raw biomass and 400 mg total sugars/g raw biomass can be found on each plot. These regions do not extend below 0.7% acid, above 165 °C, or below 125 °C on any of the plots within the range of the data investigated. The trend of the plots suggests that at higher acid concentrations than examined in this study it may be possible for the favorable region to extend to temperatures lower than 125 °C and at lower residence times than examined in this study, the favorable region might extend to higher temperatures than 165 °C. It would also appear that 0.7% acid is the minimum concentration to still be able to operate in the favorable region and operating between 0.9% and 1.2% acid gives the most options for different temperature and time combinations.

The experimental data suggest both statistically through the significance of interaction effects and visually in contour plots, that there are other non-experimentally found pretreatment combination options that would yield high total sugars. These options could be useful for the future economic analysis of CBG as a feedstock for fuel ethanol production because there may be several different operating points which share similar yields and costs and this could give some flexibility to the design of a process.

## 4. Conclusion

Dilute sulfuric acid pretreatment of coastal Bermuda grass at high temperatures is a technically feasible way to disrupt the biomass for enzymatic hydrolysis and can result in overall yields over 90% total sugars of the theoretical maximum. In the determination of the optimal pretreatment conditions, i.e. sulfuric acid concentration, temperature, and treatment time,

the formation of furfural and HMF (sugar degradation products) during the pretreatment needs to be accounted because of their inhibition to the subsequent fermentation process.

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