Hydrolysis of Tifton 85 bermudagrass in a pressurized batch hot water reactor

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Abstract

BACKGROUND: Ethanol production from grass is desirable due to the large amount of biomass it produces. However, a pretreatment is necessary before fermentation to increase ethanol yield. Tifton 85 bermudagrass was treated with a newly designed pressurized batch hot water reactor. Multiple temperatures, pressures, and reaction times were evaluated, and reducing sugars liberated during enzymatic hydrolysis were determined.

RESULTS: Pressure had a negligible effect on digestibility of the grass, and a reaction temperature of 230 °C for 2 min was the most effective in releasing reducing sugars. Fermentations were conducted with untreated grass and with grass treated for 2 min at 200 °C or 230 °C to confirm that the increase in reducing sugar concentration resulted in an increased ethanol yield. Following hydrolysis with 2 filter paper units (FPU) of a mixed cellulase enzyme cocktail per gram dry weight of grass, fermentations were performed with engineered *Escherichia coli* strain LY01. Grass treated at 230 °C produced 14.7 g L^{-1} of ethanol, which was significantly higher than 200 °C treated grass (11.0 g L^{-1}) and untreated grass (9.0 g L^{-1}). Ferulic and *para*-coumaric acids were also released during the fermentations.

CONCLUSION: Pressurized batch hot water reactor pretreatment is effective in increasing ethanol yield of grass in fermentations.

Keywords: pressurized batch hot water; lignocellulosic biomass; ethanol; pretreatment; bermudagrass

INTRODUCTION

Renewed interest in alternatives to petroleum products, especially for liquid transportation fuels, has increased demand for ethanol. Producing fuel from renewable resources such as grasses is desirable because of the large quantities of biomass available.[1] Established forage grass crops, such as switchgrass, bermudagrass, and napiergrass, initially were bred for increased biomass production as animal feedstocks, but this characteristic is amenable to ethanol production. Bermudagrass (*Cynodon dactylon*) is grown on 10–15 million acres in the southern USA. Tifton 85 (T85) is a hybrid between Tifton 68 and PI 290 884 from South Africa. This grass is hardy and produces significantly more dry matter than other bermudagrass cultivars.[2]

Efficient conversion of plant material to ethanol requires a pretreatment prior to enzymatic hydrolysis, making the substrate more available for enzymatic action. Once the hemicellulose and cellulose are converted to monomeric sugars by enzymatic hydrolysis, these sugars can be fermented by microorganisms to produce ethanol. In addition to lignified cell walls, grasses have concentrations of low molecular weight phenolic acids ester-linked to arabinose.[3] These compounds also occur in grasses in non-lignified parts of the cell walls.[4] Treatments designed to separate the fermentable sugars from the aromatic constituents could enhance fermentation yields and provide a valuable co-product.

Liquid hot water (LHW) extraction of biomass provides an effective way to pretreat cellulosic material by beginning disruption of hemicellulose prior to enzymatic hydrolysis. This treatment consists of exposing biomass to highly pressurized water at high temperatures. Liquid water at 220 °C has a pH of approximately 5.5 as a result of an ion product of 10^{-11}.[5] Exposure of biomass to LHW causes liberation of acetyl groups from hemicellulose and increased depolymerization. These reactions decrease the pH of the solution further, mimicking very dilute acid hydrolysis (DAH), a common technique which uses low concentrations of acid in hot water to break down hemicellulose.[6] When LHW was applied to sugarcane

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bagasse and leaves, all hemicellulose and more than 60% of the lignin was hydrolyzed with little loss of cellulose.[5] A similar study with alfalfa fiber resulted in hydrolysis of almost 90% of hemicellulose, 24% of cellulose, and 6% of lignin.[7] In this paper, we examine the effects of a high-temperature pressurized water reactor for hydrolysis of T85 bermudagrass and the subsequent effect of this pretreatment on ethanol production.

MATERIALS AND METHODS

Pressurized batch hot water (PBHW) hydrolysis reaction

Tifton 85 bermudagrass obtained from the USDA-ARS Coastal Plain Experiment Station (Tifton, GA, USA) was used for all hydrolysis studies. The bermudagrass was harvested at 4 weeks and dried in the field in bales for an additional week. The moisture content of the grass was determined to be 6.5% by drying at 110 °C for 1 h.

PBHW hydrolysis was examined in a 2 L pressure vessel (Model 4600, Parr Instrument Co., Moline, IL, USA) surrounded by retractable ceramic heaters (Fig. 1). Approximately 15 g of unprocessed grass was placed in a 500 μm (35 mesh) stainless steel basket and then immersed in 1450 mL of deionized water in the vessel for a final solids concentration of 1% w/v. Prior to reaction cycles, the headplate was secured and the headspace purged with nitrogen via two ports. The vessel was filled with nitrogen at room temperature to achieve a target pressure at the set point temperature. Heating, release of vessel contents, and collection of time, temperature and pressure data were measured via a datalogger and associated software (Model 21X Micrologger, Campbell Scientific, Inc., Logan, UT, USA). The reaction cycle began by heating the vessel to a set point temperature. The *reaction time* was the time set to elapse from the moment the contents of the reactor first reached the set point temperature to the moment the outlet valve automatically opened. The *reaction temperature* and *reaction pressure* were calculated as the mean of each variable recorded at 15 s intervals during the reaction time. After the reaction time elapsed at this set point temperature, an 80 psi pneumatically actuated ball valve released the liquid hydrolyzate from the pressure vessel to a partially evacuated condenser cooled by tap water. The hot liquid was cooled to less than 50 °C and the system depressurized to less than 40 psi in roughly 10 s. The hydrolyzed solids (wet but no longer pressurized) remained in the basket to cool. As a safety precaution, a low-pressure switch at the water inlet required a minimum pressure of 10 psi to actuate the pneumatic valve and to allow the ball valve to release the hydrolyzate into the condenser. A manual ball valve to release the condensate and a 50 psi pressure relief valve were located at the outlet of the condenser. The hydrolyzed solids were then removed from the vessel and dried at 40 °C for 90 min using a fluidized bed dryer (Endecott FBD2000, London, UK). Liquid and dried samples were stored (at −20 °C and 4 °C, respectively) for subsequent enzyme and fermentation studies.

Temperature was monitored inside the vessel using two 1.5 mm platinum resistance temperature detectors (RTDs, Model PR11, Omega Engineering, Inc., Stamford, CT, USA). One RTD was connected to the process controller (CN8200 Series, Omega Engineering) for the system. The remaining RTD was connected to the datalogger. A pressure transducer (PX02 Series, Omegadyne, Inc., Sunbury, OH, USA) occupied a port on the reactor head plate. The vessel, valves, and sensors were designed to withstand operating conditions of 350 °C and 1000 psi, and the maximum operating conditions used in this study were 230 °C and 700 psi (5 MPa).

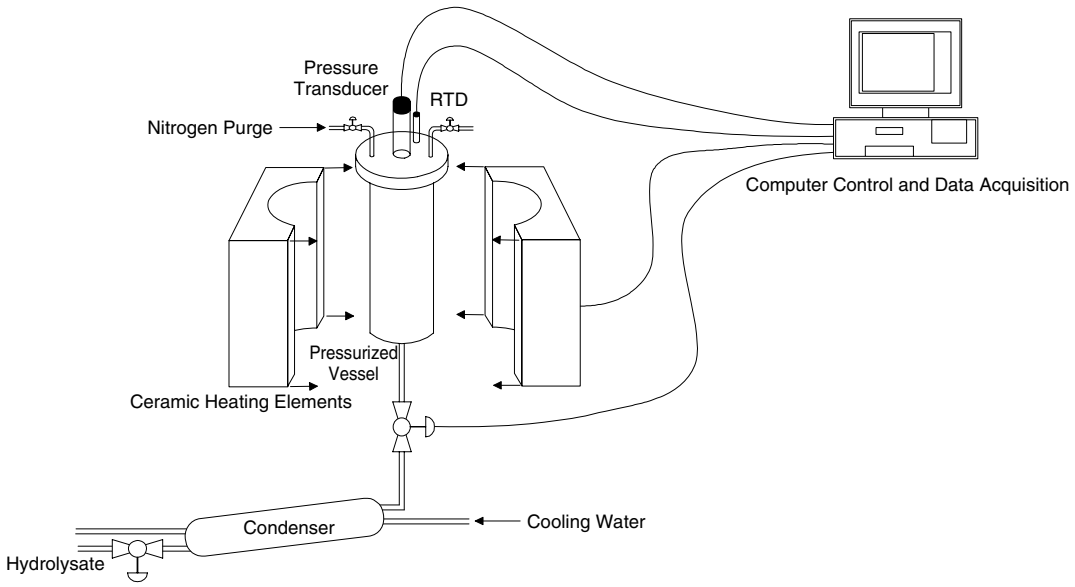

Figure 1. Schematic diagram of pressurized hot water hydrolysis system.

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Post-PBHW enzymatic hydrolysis

After PBHW pretreatment, dried bermudagrass samples were ground in a mill (Cyclotech Model Sample mill, Foss, Tecator, AB Hognas, Sweden) and further hydrolyzed enzymatically for later statistical analysis. The enzyme reaction was conducted for 48 h at 40 °C in a 0.05 mol L^{-1} citrate buffer solution, pH 4.5, with a 5% (w/v) solids load. Sodium azide was added at 0.15% (w/v) to inhibit microbial contamination. Celluclast 1.5 FG containing approximately 102 filter paper units (FPU) mL^{-1} and Novozyme 431 containing approximately 250 cellobiase units (CBU) mL^{-1} (both from Novozymes, Franklinton, NC, USA) were loaded at a rate of 4.5 FPU and 44.3 CBU per gram of dry weight of bermudagrass. Samples were boiled for 15 min to terminate enzymatic hydrolysis.[8]

Statistical analysis of enzymatic hydrolysis

Reducing sugar concentrations of the hot water hydrolyzate and the enzyme hydrolyzate were measured using the dinitrosalicylic acid assay with glucose as the standard.[9] Glucose and xylose concentrations of the hot water hydrolyzate were determined by high-performance liquid chromatography (HPLC).[10] These values were then applied to a Box–Behnken response surface statistical design (Design-Expert software, Stat-Ease, Inc., Minneapolis, MN, USA) to evaluate the performance of the pressurized water vessel and the effect of temperature (targeted as 200–230 °C), pressure (targeted to be in the range 315–700 psia) and reaction time (2–8 min) on four hydrolysis dependent variables: glucose dissolution, xylose dissolution, total reducing sugars dissolution, and the enzymatic digestibility of the remaining solid material. The actual recorded reaction temperature and pressure, rather than the target values, were used in the statistical analysis. The saturated model was fitted for each of these variables according to the constraints of the design (the model with linear terms, two-way interaction effects plus quadratic effects), and eliminated the non-significant terms to yield a reduced model. The values for the pressure were constrained by the vapor pressure of water at the reaction temperature (minimum) and by the permissible pressure in the condenser (maximum); therefore additional values for this parameter were used in the statistical analysis.

PBHW hydrolysis for fermentation

T85 bermudagrass was treated by PBHW at 200 °C and 230 °C at 1% w/v solids to generate enough material for two fermentations of each treatment. The hydrolyzate collected was analyzed for sugars, furfural, 5-hydroxymethylfurfural (5-HMF), *p*-coumaric acid, and ferulic acid using HPLC. Treated grass was dried as described previously. Following drying, grass samples with the same treatment were combined, ground twice in a Fritsch Pulverisette 25 (6.0 grill) (Laval Labs, Laval (Quebec), Canada), and ground in a coffee grinder (IDS77 Mr. Coffee, Inc., Bedford Heights, OH, USA). Final particle size varied between 0.1 mm and 3 mm. Percent moisture was determined by drying a sample of each condition overnight in a drying oven at 100 °C. Grass samples were then analyzed for neutral detergent fiber (NDF), acid detergent fiber (ADF), lignin (ADL), and protein NIR at the Feed and Environmental Water Lab (FEW-AESL, University of Georgia, Athens, GA, USA), according to standard protocols.[11]

Fermentations

Fermentations were conducted at 10% w/v solids. Grass and dH_2O were added to equal 100 mL and autoclaved. Subsequently, 90 mL of 2× Luria Bertani medium (LB, Fisher, Fair Lawn, NJ, USA) was added. Novozymes (Franklinton, NC, USA) Batch NS50012 (23 FPU mL^{-1}, 443 IU mL^{-1} xylanase, and 3497 polygalacturonase units (PGU) mL^{-1}) and Batch NS50013 (57 FPU mL^{-1}, 4049 IU mL^{-1} xylanase, and 12 PGU mL^{-1}) were filter sterilized, added to 2× LB, and then added to the fermentors for a final concentration of 2 FPU g^{-1} dry weight substrate. The pH was adjusted to 4.5 with 2 mol L^{-1} HCl. These mixtures were incubated in a 45 °C circulating water bath with stirring for 22 h.

Escherichia coli strain LY01[12,13] was inoculated from glycerol stocks and incubated at 37 °C for 18 h in LB containing 50 g glucose and 40 mg chloramphenicol. Fermentors were inoculated for a starting OD_{550} of 1. The pH was adjusted to 5.5 with KOH, and water temperature bath decreased to 35 °C. Samples were taken every 24 h for 120 h. Samples were filtered (Corning Spin-X® Centrifuge Tube Filter 0.22 μm, Sigma-Aldrich, St Louis, MO, USA), stored in O-ring microfuge tubes and frozen at −80 °C. Reducing sugars were determined as described previously.[9] Filtered samples were analyzed for ethanol by gas chromatography (GC) (Shimadzu GC-8A, Columbia, MD, USA) as previously described,[14] using a flame ionization detector and the following parameters: injector/detector temperature of 250 °C, column temperature of 65 °C, 0.53 mm i.d. × 30 m column with 3 μm film. They were also analyzed for phenolic acids by HPLC and for sugars by GC.

Analysis of soluble carbohydrates

25 μL of filtered liquid sample was blown to dryness by nitrogen after adding 50 μL of MeOH containing 91 μg of phenyl glucose as the internal standard. One to two drops of acetonitrile were also added to dried samples and then blown to dryness again. Silylation was performed by adding 50 μL of both trimethylsilane (TMS) and *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) to dried samples followed by incubation at 75 °C for 30 min. Arabinose, xylose, and glucose, both α and β conformations, were determined for 1 μL aliquots of silylated sugar derivatives by GC (model 5890, Hewlett Packard Inc., Atlanta, GA, USA) using J&W DB-5 capillary column (30 m × 0.25 mm i.d.) (Agilent, Wilmington, DE, USA). The temperature

program started at 155 °C and increased to 215 °C at a rate of 1.3 °C min^{-1}. The temperature then increased to a final temperature of 320 °C at a rate of 5 °C min^{-1}. Injector temperature was 250 °C and detector temperature was 350 °C.

Phenolic quantification

This procedure was adapted from a chlorogenic acid quantification protocol.[15] 100 μL of sample was diluted with 100 μL dH_2O. 50 μL of MeOH containing 0.0403 mg of chrysin was added as an internal standard. 3-(4-Hydroxy-3-methoxy-phenyl)prop-2-enoic acid (ferulic) and 3-(4-hydroxyphenyl)-2-propenoic (*p*-coumaric) acid concentrations were determined for 20 μL aliquots of the solution by reverse-phase HPLC (model 1050, Hewlett Packard) using an H_2O/MeOH linear gradient from 10% to 100% MeOH in 35 min and a flow rate of 1 mL min^{-1}. The column was a 250 mm × 4.6 mm i.d., 5 μm Ultrasphere C18 (Beckmann Instruments Inc., Norcross, GA, USA). The detector was a diode array system and 340 nm was used for further analysis. Each solvent contained 0.1% H_3PO_4. Response factors were determined with pure authentic compounds (Sigma-Aldrich Co., St Louis, MO, USA). Quantification of ferulic and *p*-coumaric acid was based on the internal standard (chrysin) and peak identification was based on co-chromatography (spiking) and spectral analysis.

RESULTS

A series of 25 experiments were performed using three variables: reaction time (2 min, 5 min, and 8 min), reaction temperature (200 °C, 215 °C, 230 °C) and reaction pressure (range of 315–700 psia). Table 1 depicts both the set values for temperature and pressure and the actual values obtained during the reactor runs. Figure 2 shows the temperature and pressure profile during a typical run. The vessel was heated from ambient to 100 °C in 8 min, at which point data collection began, and from 100 °C to a set point (e.g., 200 °C) in another 15 min. The temperature increased linearly to the set point, and then commonly exceeded the set point by 2–3 °C, before decreasing slightly during the reaction time. The release of the hot water reduced the pressure immediately to less than 50 psia, but the temperature of the grass remaining in the vessel typically decreased only 10–15 °C immediately, and then slowly over 20–30 min to 100 °C. In general, the actual temperature deviated less than 5 °C from the targeted temperature (mean deviation was 2.6 °C), while the actual pressure generally deviated less than 40 psia from the targeted pressure (19 psia mean deviation). The rapid heating (6.67 °C min^{-1} average) and cooling (4 °C min^{-1} average) of the reactor justifies using the 2 min temperature plateau region as the reaction time.

Of the four hydrolysis dependent variables studied, three quantified the effect of the physical parameters of the reactor (time, temperature, and pressure) on the dissolution of simple sugars in liquid hydrolysate. Pressure did not significantly affect any of the four measured variables. The mass of glucose dissolved over the range of temperature and time studied did not correlate with either of these two factors. However, the mass of xylose and the total mass of reducing sugar both correlated linearly with the time and temperature, increasing as either variable increased, but with the temperature having a slightly greater effect. The mass of xylose dissolved was determined

Table 1. The effect of reaction time, temperature, and pressure on hydrolysis of Tifton 85 bermudagrass in a pressurized batch hot water reactor

Set temperature (°C)	Real temperature values (°C)	Time (min)	Initial pressure (psig)	Set pressure (psig)	Real pressure values (psig)
200	205.4	2	35	315	352.7
200	205.2	2	80	400	436.5
200	201.7	2	140	525	519.6
200	206.1	5	35	315	342
200	201.8	5	140	525	532.9
200	202.3	8	35	315	310.9
200	202.1	8	80	400	415.4
200	201.1	8	140	525	522
215	215.8	2	37	400	372.5
215	216.3	2	140	625	620.8
215	215.7	5	105	550	548.9
215	224.9	5	105	550	610.9
215	216.9	5	105	550	548.4
215	215.4	5	105	550	544.4
215	216.1	5	105	550	534
215	212.1	8	37	400	378.4
215	215.5	8	140	625	627.8
230	231.1	2	0	415	418.2
230	231.7	2	57	550	560
230	230	2	120	700	696.8
230	231.2	5	0	415	409.5
230	230.2	5	120	700	644.7
230	229.1	8	0	415	408.5
230	230.4	8	57	550	526.8
230	230.5	8	120	700	682.2

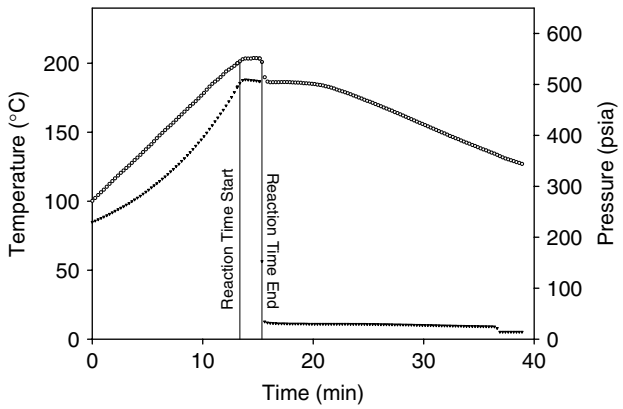

Figure 2. Temperature and pressure profile for a typical hydrolysis experiment. For this particular experiment the reaction time was 2 min and the set point temperature was 200 °C.

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as described by the following model (33 degrees of freedom, F-test = 29.67, $R^2 = 0.657$, $P < 0.0001$):

$$\text{Xylose dissolved (mg)} = 62.6 + 25.63 \times \text{time} + 50.76 \times \text{temperature}$$

In this model, both the time ($-1 = 2$ min, $0 = 5$ min, $+1 = 8$ min) and temperature ($-1 = 200\,^{\circ}$C, $0 = 215\,^{\circ}$C; $+1 = 230\,^{\circ}$C) are represented as coded variables. Similarly, the mass of reducing sugar was described by the following coded model (35 degrees of freedom, F-test = 26.96, $R^2 = 0.620$, $P < 0.0001$):

$$\text{Reducing sugar dissolved (mg)} = 1225.0 + 312.6 \times \text{time} + 418.3 \times \text{temperature}$$

The other variable studied was the digestibility of the solid grass, which was calculated by determining the sugar yield, defined as the mass of reducing sugar hydrolyzed in the enzymatic reaction after hot water treatment per mass of sample. Both temperature and time significantly affected this sugar yield. Specifically, the sugar yield was described by a (coded) model which included a quadratic term and an interaction term (24 degrees of freedom, F-test = 27.81, $R^2 = 0.848$, $P < 0.0001$):

$$\text{Sugar yield (mg/mg)} = 0.4074 + 0.0304 \times \text{time} + 0.0896 \times \text{temperature} - 0.0448 \times \text{temperature}^2 - 0.0442 \times \text{temperature} \times \text{time}$$

Although sugar yield increased linearly with both time and temperature, the presence of the negative interaction term caused the optimal time to be shorter the higher the temperature. Moreover, the maximum sugar yield within the range studied occurred at the highest temperature (230 °C) and shortest time (2 min), while the minimum occurred at the lowest temperature (200 °C) and shortest time (2 min). This phenomenon for a 2 min hydrolysis time is shown in Fig. 3.

By combining net weight loss data with the NIR data, the percent dissolution of cellulose, hemicellulose and lignin were estimated. There is a significant increase in the dissolution of hemicellulose for the 230 °C pretreatment (54%) over the 200 °C pretreatment (21%). A modest increase in cellulose dissolution from 6% at 200 °C to 11% at 230 °C was observed. Lignin dissolution increased from 0% to 5% at the higher temperature.

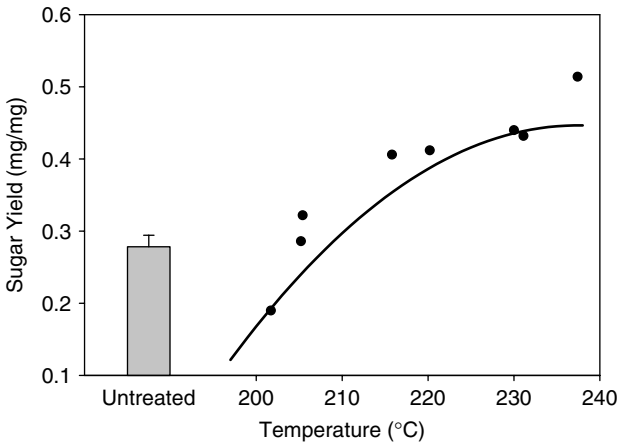

Figure 3. Enzyme digestibility of bermudagrass following 2 min hydrolysis. The curve depicts the model prediction of sugar yield resulting from statistical analysis and the data points represent observed sugar yields as a function of reaction temperature (at a variety of reaction pressures). Values are corrected for contribution of DNS-reactive stabilizers in the enzyme mixtures. The bar represents the sugar yield of a sample of untreated bermudagrass.

To confirm that increase in digestibility would correlate in increased ethanol yield, a series of partial saccharification and co-fermentation experiments (PSCF) were conducted using three conditions: untreated T85, 200 °C (2 min) treated T85, and 230 °C (2 min) treated T85. There was no furfural or 5-HMF present in the hydrolysate following the pretreatments (data not shown). Table 2 outlines the profile of sugars released by the PBHW pretreatments as well as by the 24 h enzymatic hydrolysis. Minimal sugars were released by the PBHW pretreatment alone. More arabinose and xylose were released from the 230 °C treated solids than the 200 °C or untreated solids, which corresponded well with the increased dissolution of hemicellulose that occurred in the 230 °C treated grass (Table 2). At time zero there is more glucose liberated in the untreated grass, perhaps because autoclaving liberated the easily released sugars and these sugars had already been released in the PBHW-pretreated samples. However, after 24 h of enzymatic hydrolysis, the glucose released from either pretreatment of the grass solids is very similar and higher than the untreated grass, presumably due to enhanced accessibility of the cellulose. Ethanol production and reducing sugar levels over the course of the fermentations are shown in Fig. 4. As expected from the preliminary reducing sugar analyses, the 230 °C pretreated grass resulted in an increase in ethanol production of roughly 4.5 g L^{-1} over the

Table 2. Sugars released from treated and untreated Tifton 85 bermudagrass by 24 h enzymatic hydrolysis

	Arabinose (mg g^{-1} grass)			Xylose (mg g^{-1} grass)			Glucose (mg g^{-1} grass)		
Treatment	Hydrolysate	0 h	24 h	Hydrolysate	0 h	24 h	Hydrolysate	0 h	24 h
Untreated	n/a	48.91	60.45	n/a	14.79	29.88	n/a	371.69	541.63
200 °C Treated	0	56.28	69.23	0	60.03	133.15	0.69	244.09	640.34
230 °C Treated	0.25	79.39	76.75	0	129.49	282.36	0.31	209.5	635.32

200 °C pretreated grass. Untreated grass produced the least amount of ethanol of the experiment (9 g L^{-1}).

Phenolic acids, *p*-coumaric acid and ferulic acid, were also released during the fermentations. There were small amounts of these compounds in the hydrolysate from each of the pretreatment conditions. Following enzyme addition and inoculation of the fermentations, both *p*-coumaric and ferulic acid levels increased over the 120 h. Of the three conditions, the levels of both compounds were highest in the untreated grass (data not shown). Hydrolysate samples from the PBHW pretreatment were also analyzed for furfural and 5-hydroxymethylfurfural (5-HMF), neither of which was present.

DISCUSSION

PBHW pretreatment is a promising option for grass biomass. Our non-flow-through PBHW reactor is reliable and effective; pressure and temperature were held constant over the reaction time and significant dissolution of complex carbohydrates occurred as measured by enzymatic hydrolysis. The first objective was to evaluate the effectiveness of enzymatic hydrolysis of PBHW-pretreated T85 bermudagrass compared to untreated grass samples. Cellulase and cellobiase enzymes used for this aspect of our studies have been used previously to determine the effectiveness of cellulose degradation from pretreated biomass.[8,16,17] The later enzymatic hydrolysis reactions and subsequent fermentations were conducted in order to correlate digestibility with fermentability of PBHW-pretreated bermudagrass. Several different commercial enzyme combinations were compared for their ability to liberate sugars from cellulose and hemicellulose in the course of this study. Although all performed well, the Novozyme batch preparations used during the fermentation study performed the best for our current protocol (data not shown). We reduced the FPU enzyme load in the fermentations in order to better observe differences in the pretreatment conditions. Future studies will optimize the enzyme loading for maximum ethanol production. The ethanologen LY01 was selected as the biocatalyst because it is more resistant, than many other ethanol-producing organisms, to potential fermentation inhibitors such as furfural, HMF, and phenolic compounds.[18–20]

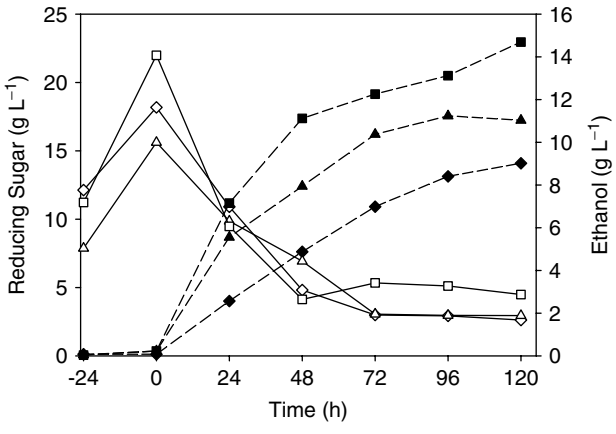

Figure 4. Average reducing sugar concentration and ethanol production over the course of fermentations of untreated, 200 °C, and 230 °C treated T85. Solid lines correspond to sugar concentrations and dotted lines correspond to ethanol concentrations. Symbols are as follows: diamond, untreated; triangle, 200 °C treated; square, 230 °C treated. Fermentations were conducted at 35 °C in an immersion circulator for 5 days at 10% solids. The −24 h time point corresponds to the beginning of the 24 h enzymatic hydrolysis (at 45 °C). The actual fermentation began (bacterial inoculation) at time 0 h. Reducing sugars are removed at the same rate that ethanol is produced.

Pressure had a negligible affect on sugar yield from T85, which agrees with prior research on other cellulosic materials.[5,21,22] Even though the heating and cooling took longer than the specified reaction time, the rates were rapid enough that the plateau region is justified as the actual reaction time (Fig. 2). After determining the sugar yield of the reactions in Table 1, the data were fit to a graph (Fig. 3). Based on the data presented in Fig. 3, we determined the optimal reaction time of 2 min and temperature of 230 °C, which agrees with previous findings of other groups.[5,7,21,23] We conducted the pretreatment at 1% w/v solids concentration since low solids concentrations may also be critical to effective pressurized batch hot water pretreatment.[23]

An advantage of this pretreatment is the absence of a required strong base or acid, used respectively in ammonia fiber explosion (AFEX) and DAH pretreatments.[24] Not only does this remove the additional cost of these reagents, but it also eliminates the expense for their subsequent safe removal and disposal. van Walsum and colleagues compared DAH, steam explosion and LHW pretreatments for effectiveness based on several criteria, from fiber reactivity to construction materials. Though they are all effective pretreatments, LWH resulted in high pentosan recovery and was less costly than the more researched DAH, which also requires particle size reduction, unlike LHW.[22]

Grass particle size and enzyme loading were not optimized for fermentation in these experiments. Mosier and colleagues determined that biomass undergoing LHW does not need to have particle size reduction owing to the physical properties of the treatment.[24] This conclusion should apply to our PBHW pretreatment; however, this was not investigated as our reactor design does not currently permit processing of very small particles. In order to standardize the enzymatic hydrolysis of the pretreated grasses, particle size was reduced prior to enzymatic digestion. Even with low enzyme loading, the 230 °C pretreatment was effective in making the grass more available for enzymatic attack.

Inhibitors are often produced by biomass degradation during pretreatment and hydrolysis steps, and include phenolics from lignin degradation and furfural

and 5-HMF produced when monomeric sugars are degraded into aldehydes or reactive acids. One study found that these are produced by LHW pretreatment when *O*-acetyl and uronic groups from hemicellulose are cleaved and become reactive acids.[24] The high temperatures and pressures in LHW pretreatments accelerate this acid-catalyzed degradation of monomeric sugars by decreasing the pH as organic acids are formed. This is a result of the pretreatment as well as the substrate that is being treated. Weil and colleagues found that controlling the pH of yellow poplar wood sawdust, which reached a pH between 2.8 and 3, during an LHW pretreatment by adding base prevented the formation of inhibitors.[25] The pH of the liquid hydrolyzate from our reactor ranged from 4.2 to 4.8 and may not have been low enough to promote significant formation of inhibitors. The short residence time of the pretreatment likely prevented the formation of inhibitors as well. The absence of these compounds in this study is promising for future applications of our PBHW system specifically for bermudagrass.

Samples after pretreatment, at the beginning, and at the end of the fermentations were also analyzed for phenolic acids, specifically *p*-coumaric and ferulic. These compounds are released from grasses during hydrolysis and are inhibitory to fermentations.[26] Ferulic acid and its related compounds possess potent antioxidant properties and may have applications in disease prevention and treatment.[27] Extraction of these compounds prior to fermentation could be pursued further and may serve as a potential source of value-added by-product from ethanol production in addition to increasing ethanol yields.

PBHW is an effective and gentle pretreatment resulting in greater enzymatic digestibility of T85 bermudagrass. For our reactor, 230 °C is the most efficient temperature for increasing the digestibility without producing detrimental concentrations of inhibitors. The increased digestibility directly resulted in an increased ethanol yield from fermentations using *E. coli* LY01. The results of this study warrant further research to determine the efficacy of PBHW pretreatment for other biomass sources and possibly use on a larger scale.

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