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*Research article*

## Switchgrass storage effects on the recovery of carbohydrates after liquid hot water pretreatment and enzymatic hydrolysis

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**Abstract:** Perennial grasses that would be used for bioenergy and bioproducts production will need to be stored for various periods of time to ensure a continual feedstock supply to a bioprocessing facility. The effects of storage practices on grass composition and the response of grasses to subsequent bioprocesses such as pretreatment and enzymatic hydrolysis needs to be understood to develop the most efficient storage protocols. This study examined the effect of outdoor storage of round switchgrass bales on composition before and after liquid hot water pretreatment (LHW) and enzymatic hydrolysis. This study also examined the effect of washing LHW pretreated biomass prior to enzymatic hydrolysis. It was determined that switchgrass composition after baling was stable. As expected, glucan and lignin contents increased after LHW due to decreases in xylan and galactan. Washing biomass prior to enzymatic hydrolysis reduced saccharification, especially in samples from the interior of the bale, by at least 5%.

**Keywords:** bioenergy; bales; cellulose; sugars; biofuel; lignocellulose

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### 1. Introduction

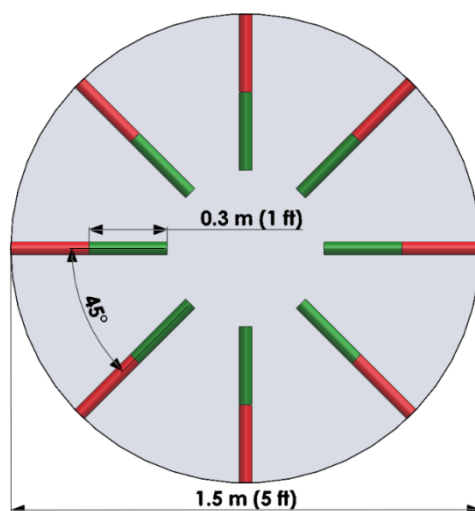
Perennial forage grasses, such as switchgrass (*Panicum virgatum* L), can be deconstructed into monomeric sugars, which can then be fermented into biofuels or various biobased products. Although biomass feedstock supply chains are not yet fully designed and developed, packaging

switchgrass in round bale format and storing the bales outside is a likely mode of biomass storage. Unfortunately, little is known about storage effects on feedstock deconstruction and ensuing saccharification. Generally, harvesting biomass while dry, usually below 18% moisture content, tends to result in high losses of biomass during cutting and baling, while harvesting while wet, usually greater than 60% moisture content, tends to promote biological growth, converting biomass into CO<sub>2</sub> [1]. Interestingly, higher feedstock moisture content can enhance delignification, resulting in higher saccharification yields [2]. On the other hand, weathered layers, located on bale surfaces, are subject to biomass losses of up to 23% over time, decreasing biomass availability [3].

Although there are numerous biomass storage studies that examine storage effects on quality for animal feed, few studies have examined the effects of storage in terms of biomass deconstruction [1]. Biomass storage methods most likely affect enzymatic hydrolysis yields, altering carbohydrate and byproduct profiles. One study reported on differences in hydrolyzate profiles of dilute acid pretreated switchgrass bales that were stored in protected or open field conditions [4]. Storage conditions affected xylose and organic acids profiles of switchgrass biomass [4], indicating that the combination of storage and compositional change has to be considered in any biofuel processing supply chain using stored biomass. The goal of this study was to examine the effects of outdoor, uncovered storage of switchgrass packaged as round bales on saccharification.

## 2. Materials and Methods

### 2.1. Switchgrass Establishment, Storage Treatments, Bale Composition, and Carbohydrate Determination



**Figure 1.** Sampling diagram for round switchgrass bales.

Switchgrass (*Panicum virgatum* var. Kanlow) was grown at the Oklahoma State University South Central Research Station in Chickasha, OK, USA (35.0383 N, 97.9461 W). Grass was cut and raked into windrows on December 18, 2013, from which 12 round bales, 1.5 m by 1.2 m (diameter by height), were made on December 19, 2013, using a John Deere 459 round baler (East Moline, IL, USA). The bales were transported from Chickasha, OK to Stillwater, OK (36.1157 N, 97.0586 W)

no more than two days after baling. Bales were stored on wooden pallets in an open field with 1 m open space around each bales in Stillwater, OK for 3, 5, 7, and 9 months. Biomass sampling was as follows: core samples were taken 0.3 m and 0.6 m deep into the bales using a hay corer that was 0.05 m in diameter and 0.9 m long and mounted on a handheld electric drill. Cores were taken 0.15 m from each edge and in the center of the bale in 8 different places (Figure 1). A total of 48 samples were collected from each bale. Moisture content for each sample was determined using an Ohaus MB45 Moisture Analyzer (Pine Brook, NJ, USA) both before and after liquid hot water pretreatments. Rainfall data was gathered at the Oklahoma Mesonet weather station in Stillwater, OK. All samples were stored at 4 °C until analyzed for composition.

Samples taken at 0.3 m were combined and samples taken at 0.6 m were combined to make two composite samples for composition analysis. The composite samples were ground and sifted through a 13 mm screen by a hammer mill (Model E9506, Bliss Industries, Ponca City, OK, USA). Compositional analysis of biomass before and after liquid hot water pretreatment was performed following the National Renewable Energy Laboratory (NREL) standard protocol for herbaceous crops [5]. Total biomass solids content was determined following [6], while the structural carbohydrates and lignin content were calculated using the methods in [7]. Extraction of the solid portion of the biomass was done using deionized water and 190 proof ethanol (Pharm CO-AAPER Brookfield, CT, USA) with an Accelerated Solvent Extractor, ASE® 300 system (Dionex Corporation, Sunnyvale, CA, USA). Biomass sugars and lignin contents were determined using an acid soluble lignin test (ASL). Monomer and oligomer sugar contents were determined using the methods in [8]. Analyses of hydrolyzates were performed using a wavelength of 205 nm with a UV-VIS spectrophotometer (Cary 50 Bio, Varian Inc, Palo Alto, CA, USA). Detection and quantification of the carbohydrates were performed with a refractive index detector (RID) and a Bio-Rad Aminex HPX-87 P column (Bio-Rad, Sunnyvale, CA, USA), using a high-pressure liquid chromatography (HPLC) instrument (Waters 2695, Milford, MA, USA) as previously described [9]. The HPLC eluent was deionized water flowing at a rate of 0.6 mL min<sup>-1</sup> at a temperature of 86 °C. The standards used for carbohydrate detection were glucose and xylose from Alfa-Aesar (Ward Hill, MA, USA).

Biomass digestibility was carried out using 30 ml of 72% sulfuric acid (EMD Millipore Bellerica, MA, USA) along with 1 g of solids, shaken in a 30 °C water bath. Quantification of the glucose content of the biomass was carried out using an YSI 2900 Biochemistry Analyzer (YSI Life Sciences Inc, Yellow Springs, OH, USA) with an immobilized enzyme membrane.

## 2.2. Liquid Hot Water Pretreatment and Enzymatic Hydrolysis

Pretreatment was carried out in a 1 L bench top stirred reaction vessel (Parr Series 4520, Parr Instrument Company, Moline, IL, USA) with a propeller agitator and a 1 kW electrical heater. The reactor volume was loaded with a mass switchgrass that contained 75 g dry solids and a mass of deionized water to bring the total mass to 500 g. For pretreatments carried out at 200 °C, a reaction time of 10 min was used, while a reaction time of 20 min was set when pretreating at 180 °C. For both pretreatment temperatures, agitation of the reactor was set at 300 RPM with manual agitation during cooling.

The severity of each pretreatment was calculated by equation 1 [10],

$$Severity = \text{Log}(t \cdot e^{\frac{T-100}{14.75}}) \quad (1)$$

where  $t$  was the pretreatment time and  $T$  was the pretreatment temperature in °C. The two distinct pretreatment conditions resulted in severities of 3.66 and 3.94 for the 180 F/20 min and 200 F/10 min pretreatments, respectively. The pretreatment hydrolyzate and solids were separated using a vacuum filter with a Buchner funnel and Whatman #1 filter paper (Whatman PLC, Brentford UK). For the washed samples, the solid portions were washed with 375 g of water. For the unwashed samples, the solids were hydrolyzed directly. All solids and hydrolyzates were stored at 4 °C until needed for enzymatic hydrolysis. Recoveries of glucan and xylan were calculated by equations 2 and 3,

$$Glucan \text{ recovery} = \frac{[Glucan]_{pretreated \ solids} + [Glucan]_{prehydrolyzate}}{[Glucan]_{stored \ samples}} \quad (2)$$

$$Xylan \text{ recovery} = \frac{[Xylan]_{pretreated \ solids} + [Xylan]_{prehydrolyzate}}{[Xylan]_{stored \ samples}} \quad (3)$$

where  $[Glucan, Xylan]_{pretreated \ solids}$  is the glucan or xylan content of pretreated solids (g),  $[Glucan, Xylan]_{prehydrolyzate}$  is the glucan or xylan content in prehydrolyzate after liquid and solid separation by filtration (g), and  $[Glucan, Xylan]_{stored \ samples}$  is the glucan or xylan content in the samples after storage (g).

### 2.3. Enzymatic Hydrolysis

Enzymatic hydrolysis of either washed or unwashed samples were carried out in 30 ml reaction vessels. All enzyme reactions were conducted at 50 °C in a 100 RPM shaking water bath (Thermo Electron Corporation, Winchester, VA). All enzyme reactions were performed in a 0.1 M sodium citrate buffer solution (EMD Millipore, Bellerica MA) adjusted to a pH of 4.8. Water was obtained from a Direct-Q system (EMD Millipore, Billerica, MA) that displayed 12.2 MΩ resistance. Accelerase® 1500 (Genencor, Cedar Rapids IA) loaded at 60 FPU/g cellulose was used for enzymatic hydrolysis experiments.

An amount of biomass containing 0.10 g of glucan was added to each hydrolysis tube. For the filter paper controls, 0.10 g of filter paper was added with the assumption that the filter paper was 100% glucan. It was further assumed that the quantity converted was equal to 100% of the recoverable glucan. The total reaction vessel volumes were adjusted with deionized water so that the final reaction volume was 10 ml. While each reaction vessel was loaded to 1% glucan loading, corresponding to 0.10 g glucan in 10 g water, the total biomass loading in each vessel was dependent on the biomass composition. Enzymatic hydrolysis was conducted for 24 h. Aliquots of hydrolyzate were saved and analyzed on HPLC and YSI and then stored at 4 °C.

### 2.4. Statistical Analysis

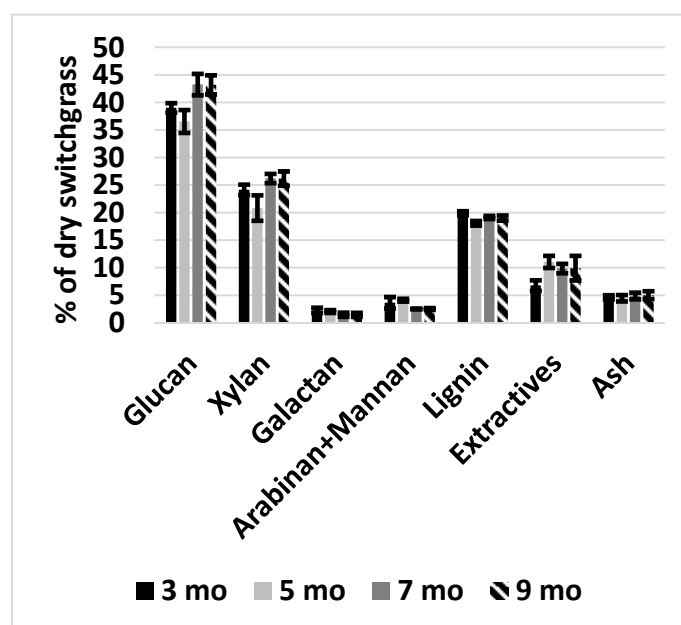
Analysis of variance (ANOVA) using the GLM procedure in SAS 9.4 (SAS, Cary NC) was conducted on composition data from samples before pretreatment, samples after pretreatment and glucose yields after hydrolysis of pretreated samples. The main effects for composition data before pretreatment were sampling time and sampling depth. The main effects for composition data after

pretreatment were sampling time, sampling depth and severity. The main effects for pretreated samples hydrolysis glucose yields were sampling time, sampling depth, severity and washing. Separation of means for significant main effects and interactions between main effects was done using Fisher's protected least significant difference method [11]. Graphs were constructed with Excel 2016 (Microsoft, Redmond WA, USA).

### 3. Results and Discussion

#### 3.1. Effect of Storage and Pretreatment on Composition of Switchgrass Biomass

Figure 2 presents the glucan, xylan, lignin, extractives, galactan, arabinan + mannan (arabinose and mannose were not separated by the HPLC column) and ash content of round switchgrass bales as a function of storage time.

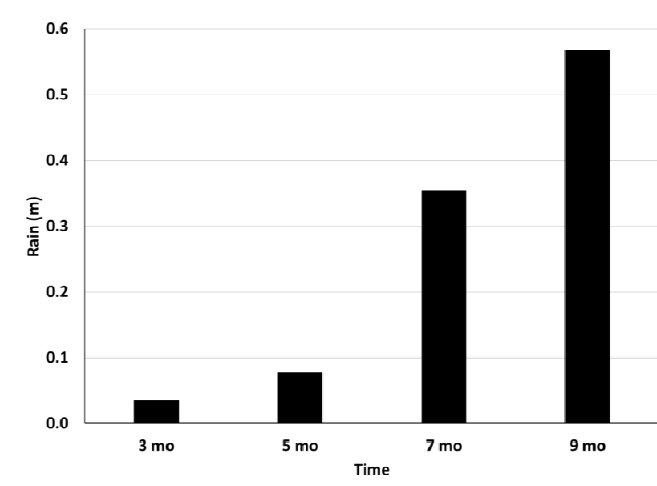


**Figure 2.** Mean glucan, xylan and lignin content of round bales after storage for 3, 5, 7 and 9 months. Error bars are  $\pm$  one standard deviation.

Contents are presented as % of dry switchgrass. Storage time affected ( $p < 0.05$ ) glucan, xylan, lignin, extractives, galactan and arabinan + mannan contents. Storage time had no effect on ash content. For glucan, 210 d (43.26%) and 270 d (43.20%) samples had similar contents, and 90 d (39.02%) and 150 d (36.56%) samples had similar contents. The mean glucan content for 210 and 270 d samples was 14.4% greater than the mean glucan content for 90 and 150 d samples. For xylan, 150 d (20.84%) samples had 18.3% less xylan than the mean of all other samples, which were similar. For lignin, 210 d (19.04%) and 270 d (19.02%) samples had similar lignin contents that were 5.2% less than 90 d samples (20.02%) and 5.3% greater than 150 d samples (18.08%). For extractives, 90 d samples (6.77%) had 34.2% less extractives than all other samples, which were similar. For galactan, 90 d samples (2.25%) had 61.7% more galactan than all other samples, which were similar. For arabinan + mannan, 90 d (4.11%) and 150 d (3.66%) had similar contents and 210 d (2.53%) and

270 d (2.53%) had similar contents. The mean arabinan + mannan content for 90 and 150 d samples was 53.6% greater than the mean arabinan + mannan content for 210 and 270 d samples. Sampling depth affected ( $p < 0.05$ ) only galactan content, with 0.3 m samples (1.77%) having 22.9% more galactan than 0.6 m samples (1.44%). There were no significant interactions ( $p > 0.05$ ) between the main effects of storage time and sampling depth for any component. It is important to note that composition percentages do not yield information with respect to total mass; however, they can provide information in terms of relative proportions of glucan, xylan and lignin throughout the storage study.

Bale composition changed significantly between 3 and 5 months, with xylan, lignin, and galactan decreasing, extractives increasing, and glucan and arabinan + mannan contents not changing significantly. The increase in extractives was evidence of products from xylan, lignin, and galactan degradation. Xylan and galactan are constituents of hemicellulose, which is more amenable to hydrolysis by microorganisms than cellulose (glucan). Hydrolysis of these components could lead to an increase in soluble sugars, which would be expressed as extractives in this analysis. Lignin decreased about 6% between 3 and 5 months. This could be due to biological deconstruction, whose products would also increase extractives. Glucan contents of bales stored 3 and 5 months were not significantly different; however, there was a decrease in glucan content and degradation products from glucan hydrolysis could have led to increased extractives. Between 5 and 7 months, glucan, xylan, lignin contents increased, arabinan + mannan content decreased, and extractives and galactan did not change significantly. The decrease in arabinan + mannan did not account for the increase in glucan, xylan and lignin. The compositional analysis only accounted for six sugars and ash content; thus, components, such as proteins, that were not measured may have been removed from the bales between 5 and 7 months, which may account for the increase in glucan, xylan and lignin. Rainfall of 0.28 m fell on the bales between 5 and 7 months (Figure 3), which could have promoted loss of components. Bale composition did not change significantly from 7 to 9 months.

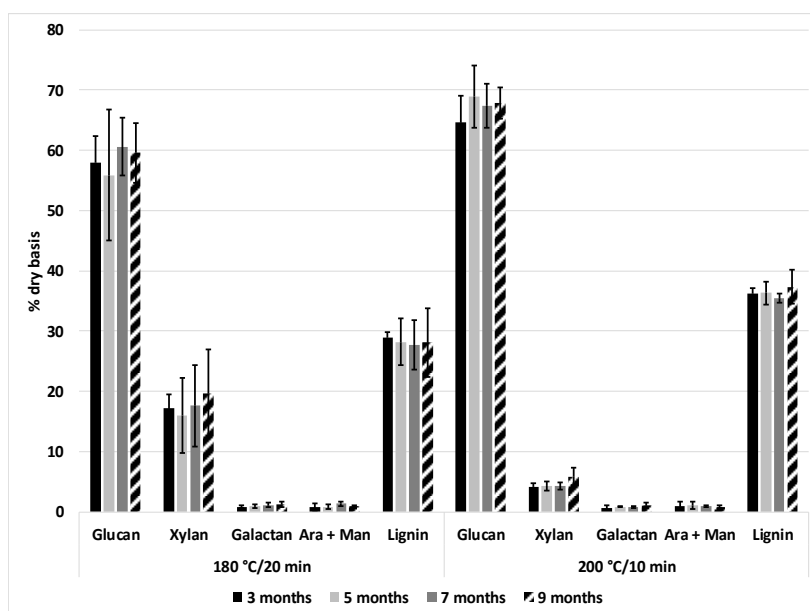


**Figure 3.** Cumulative rainfall at Oklahoma Mesonet Stillwater weather station.

Statistically significant changes happened during storage; however, the magnitude of these changes was not large, especially with regards to the three main components, glucan, xylan and lignin. The means  $\pm$  one standard deviation for the 12 bales sampled were: glucan, 40.51%  $\pm$  3.21%;

xylan,  $24.35\% \pm 2.51\%$ ; and lignin  $19.04 \pm 0.73\%$ . The standard deviations were 7.9% of the glucan mean, 10.3% of the xylan mean, and 3.8% of the lignin mean. Also, the mean bale glucan, xylan and lignin composition varied little from the harvested grass (before baling) dry basis composition of 39.03% glucan, 24.53% xylan, and 19.71% lignin. The similarity between the mean bale composition and the harvested grass composition along with the standard deviation of 10.3% or less indicates that the composition of the switchgrass after baling was stable. A previous study [12] also observed stable biomass composition (4% cellulose loss and 1.4% hemicellulose loss) in large round bales stored outdoors with no covering.

Figure 4 presents biomass composition after 180 °C/20 min and 200 °C/10 min liquid hot water (LHW) pretreatments. Ash and extractives are not shown as these components were removed from the biomass during pretreatment and not detected. Severity was 3.66 for the 180 °C/20 min pretreatment and 3.94 for the 200 °C/10 min pretreatment. Experiments with two different severities were conducted to determine if the combination of storage and pretreatment severity affected saccharification. Higher severity, such as for the 200 °C/10 min pretreatment, typically results in biomass that displays higher saccharification efficiencies [13–16].



**Figure 4.** Mean glucan, xylan and lignin content of switchgrass treated with liquid, hot water pretreatment after storage for 3, 5, 7 and 9 months. Error bars are  $\pm$  one standard deviation.

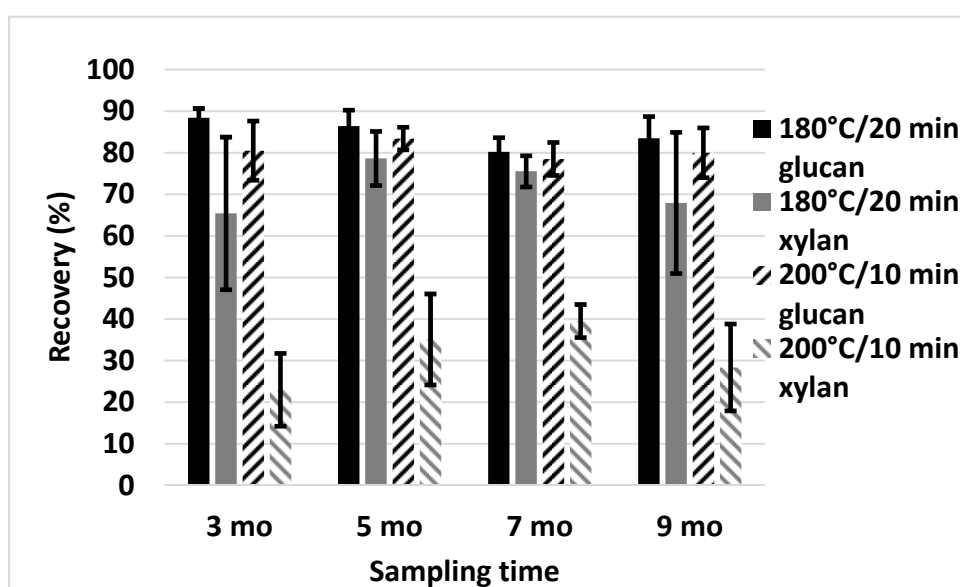
Sampling depth did not have an effect on any of the biomass components content. Severity had an effect on glucan, xylan, galactan and lignin content. Sampling time had an effect on xylan and galactan content. None of the main effects tested had an effect on arabinan + mannan content. There were no significant two-way or three-way interactions among main effects.

Mean glucan content in 200 °C/10 min samples (67.21%) was 14.9% greater than mean glucan content in 180 °C/20 min samples (58.49%). Mean xylan content in 200 °C/10 min samples (17.60%) was 73.9% less than mean xylan content in 180 °C/20 min samples (4.59%). Mean galactan content in 200 °C/10 min samples (0.84%) was 23.6% less than mean galactan content in



180 °C/20 min samples (1.10%). Mean lignin content in 200 °C/10 min samples (36.37%) was 27.7% greater than mean lignin content in 180 °C/20 min samples (28.47%). Mean xylan content in 9 month samples (12.72%) was 20.5% greater than mean xylan content in 3, 5, and 7 month samples (10.56%). Xylan content in 3, 5, and 7 month samples were similar. Mean galactan content in 9 month samples (1.23%) was 38.2% greater than mean galactan content in 3, 5, and 7 month samples (0.89%). Galactan content in 3, 5, and 7 month samples were similar.

The total recovery of glucan and xylan in the pretreated solids and prehydrolyzate was calculated as described above in the methods (Figure 5). Sampling depth and sampling time did not significantly affect glucan or xylan recovery. Severity did significantly affect glucan and xylan recovery. Glucan recovery for 180 °C/20 min samples (84.7%) was 5.1% greater than for 200 °C/10 min samples (80.6%), and xylan recovery for 180 °C/20 min samples (73.5%) was 145.8% greater than for 200 °C/10 min samples (29.9%).

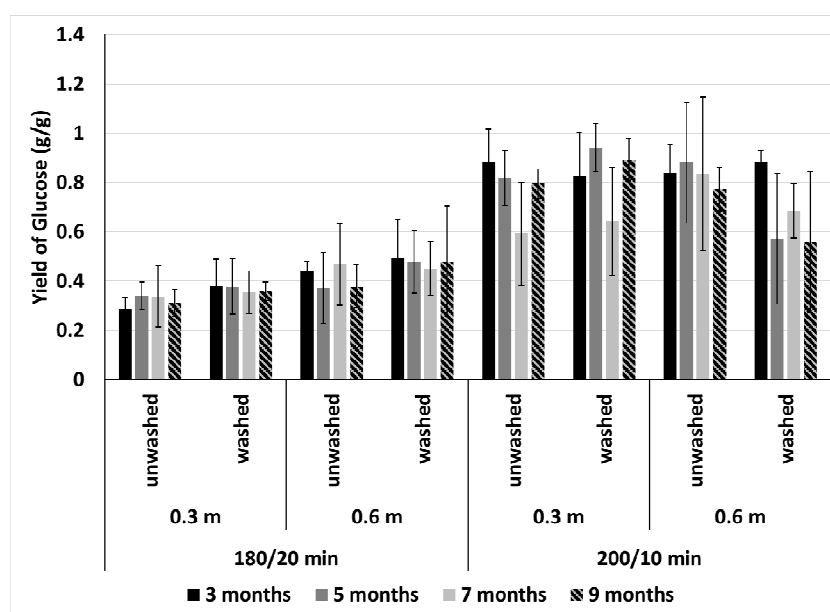


**Figure 5.** Recoveries of glucan and xylan for switchgrass treated with liquid, hot water pretreatment after storage for 3, 5, 7 and 9 months. Error bars are  $\pm$  one standard deviation.

The effects of severity on the composition of pretreated samples and the recovery of sugars were expected. Hemicellulose, of which xylan, galactan, arabinan and mannan were the primary components, is more heat labile than cellulose and is hydrolyzed at the high temperatures used in liquid hot water pretreatment [17]. As temperature and severity increased, more hemicellulose was hydrolyzed, xylan and galactan contents decreased. Glucan and lignin contents increased with increased severity due to decreases in xylan and galactan. Also, increased severity leads to degradation of sugars into furfural compounds and organic acids. Thus, greater severity reduces recoveries of glucan and xylan after pretreatment. Xylan recovery is affected more than glucose due to xylan's greater heat lability. The trend for xylan and galactan content versus sampling time was similar to the trend observed before pretreatment except that 7 month samples had less galactan and xylan than 9 month samples after pretreatment, but had similar xylan and galactan contents before pretreatment.

### 3.2. Effect Storage on Enzymatic Hydrolysis of Switchgrass Biomass

Enzymatic hydrolysis of washed and unwashed pretreated switchgrass was conducted. The yields of glucose from these hydrolyses are shown in Figure 6 as g glucan recovered over maximum available g of glucan present in the sample after pretreatment. The effects of sampling time, sampling depth, severity and washing on glucose yield after hydrolysis were evaluated. A significant two-way interaction between main effects indicates that the direction of one effect is different at different levels of the other effect. In this case, glucose yield for samples taken at a depth of 0.6 m (0.445) was 29.7% higher than for samples taken at a depth of 0.3 m (0.343) when 180 °C /20 min pretreatment was used, but there was no effect of sampling depth when 200 °C/20 min pretreatment was used. The reason behind this observation is not clear since sampling depth had no effect of the composition of pretreated switchgrass. However, the difference observed due to sampling depth for 180 °C/20 min was much less than difference observed due to severity. Glucose yields from 200 °C/10 min samples (0.776) were 97.0% greater than 180 °C/20 min samples (0.394). This was expected as the 200 °C/10 min pretreatment removed more xylan from biomass than did the 180 °C/20 min pretreatment. Removal of xylan allows cellulase enzymes used in hydrolysis to have better access to cellulose. Despite the slightly greater (5.1%) recovery of glucan using the 180 °C/20 min pretreatment as compared with the 200 °C/10 min pretreatment, the much greater glucose yield (97.0%) from 200 °C/10 min samples compared to 180 °C/20 min samples means that 200 °C/10 min pretreatment must be used to maximize glucose production from enzymatic hydrolysis.



**Figure 6.** Yield of glucose (g/g glucan) after enzymatic hydrolysis as a function of pretreatment temperature. Error bars are  $\pm$  one standard deviation.

It is interesting to note that washing did not affect hydrolysis glucose yield. In fact, washing biomass prior to enzymatic hydrolysis may deter saccharification as glucose yields were lower in 5, 7 and 9 month 0.6 m 200 °C/10 min LHW pretreated samples, as shown in Figure 6. This indicates that

enzyme inhibitors were not produced during pretreatment in concentrations substantial enough to affect hydrolysis. Elimination of washing during production of sugars from switchgrass would save a large amount of water and this could be useful information for biomass processors. However, it should be noted that the effect of washing on subsequent fermentation of the produced glucose was not tested, so inhibitors to fermentation microorganisms may still be present in the unwashed and hydrolyzed biomass. If present, these inhibitors could impede downstream processing; in that case, they would need to be removed through washing or other detoxification techniques.

#### 4. Conclusions

The composition of switchgrass in terms of structural carbohydrates and lignin was stable when stored as uncovered round bales for 9 months. Changes in glucan, xylan and lignin composition over time were in a range of  $\pm 10\%$  or less. When samples were removed from storage and pretreated using a LHW pretreatment, storage time affected xylan and galactan content after pretreatment, with samples stored for 9 months having more xylan and galactan than samples stored for shorter times. Greater pretreatment severity resulted in lower recoveries of glucan and xylan from the stored switchgrass after pretreatment, but much greater hydrolysis of glucan by cellulase enzyme. Therefore, the severity of the pretreatment cannot be reduced without greatly reducing the production of glucose from enzymatic hydrolysis. Washing did not affect enzymatic hydrolysis; thus, washing solids after pretreatment could be eliminated without affecting glucose yields from enzymatic hydrolysis. This would save a great deal of water.

#### Acknowledgements

The authors would like acknowledge that this material is based upon work that is supported by the National Institute of Food and Agriculture, U.S. Department of Agriculture, under award number 2010-38502-21836 through the South Central Sun Grant Program. The authors also would like to acknowledge the Arkansas Center for Plant Powered Production (P3), the Department of Biological and Agricultural Engineering and the Graduate School at the University of Arkansas, and the Oklahoma Agricultural Experiment Station for their funding and support. The P3 Center was funded through the RII: Arkansas ASSET Initiatives (AR EPSCoR) I (EPS-0701890) and II (EPS-1003970) by the National Science Foundation and the Arkansas Science and Technology Center.

#### Conflict of Interest

All authors declare no conflicts of interest in this paper.

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