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# Plant Maturity Effects on the Physicochemical Properties and Dilute Acid Hydrolysis of Switchgrass (*Panicum virgatum*, L.) Hemicelluloses

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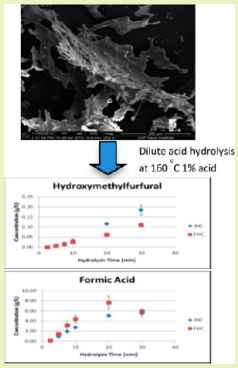
**ABSTRACT:** Hemicelluloses account for 20−35% of grass feedstocks and not only present a barrier to enzymes accessing cellulose during enzymatic hydrolysis but also contain sugars that can be utilized as substrates for fermentation. Elucidating the physicochemical properties of hemicelluloses could help maximize the release of monosaccharides, while minimizing the production of inhibitory byproducts during pretreatment. This work focused on characterizing alkali-extracted hemicelluloses from *Panicum virgatum*, L. (switchgrass), cultivar Alamo, harvested at midgrowing season (July) and weathered post-frost (February). The alkali-extracted hemicelluloses were analyzed for monosaccharide constituents, glycosyl linkages, and molecular size using acid hydrolysis, per-O-methylation analysis, and size exclusion chromatography, respectively. The results revealed that the July hemicelluloses contained 14% glucose, 67% xylose, and 19% arabinose, and the February hemicelluloses contained 5% glucose, 79% xylose, and 16% arabinose. Glycosyl linkage analysis revealed both hemicelluloses to have similar linkages but in different proportions. Size exclusion chromatography showed that the July hemicelluloses had an average molecular weight of 30,000 g mol$^{-1}$, and the February hemicelluloses had an average molecular weight of 28,000 g mol$^{-1}$. Extracted hemicelluloses were also subjected to dilute acid pretreatment at 160 °C using 1% (w/w) sulfuric acid. At maximum concentrations, the July hemicelluloses produced 162% and 73% more glucose and hydroxymethylfurfural, respectively, than the February hemicelluloses, and the February hemicelluloses formed 41% more formic acid than the July hemicelluloses.




**KEYWORDS:** *Switchgrass, Hemicelluloses, Biofuels, Pretreatment, Degradation products*

## INTRODUCTION

Switchgrass (*Panicum virgatum*, L.) is considered to be an important candidate as a dedicated bioenergy crop because it requires low inputs, produces high yields of biomass, provides good carbon sequestration, prevents erosion, and has a wide geographic distribution throughout North America.[1] The composition of switchgrass varies among cultivars, levels of plant maturity, and even within different regions of the plant, but is roughly 30−40% cellulose, 20−35% hemicelluloses, and 10−20% lignin, with the remaining mass being comprised of extractives, protein, and ash.[2−4] The cell wall matrix is a complex network of cellulose microfibrils partially linked, through hydrogen bonds, to hemicelluloses that are also covalently bound with lignin, thus forming a network that is recalcitrant when trying to breakdown biomass to its substituent molecules.[5−7] This recalcitrant nature requires that biomass must undergo a series of unit operations for effective conversion to fuels and chemicals.

For biochemical conversion, the biomass must first be pretreated to render the cellulose more accessible to enzymes for saccharification to fermentable sugars. After pretreatment, the cellulose is enzymatically hydrolyzed to glucose, before monomeric sugars from pretreatment and enzymatic hydrolysis are used for conversion to products.[8] The pretreatment scheme chosen will affect the mechanism for rendering the cellulose more enzymatically accessible. Some pretreatments, such as ammonia fiber explosion (AFEX) and other alkaline treatments, disrupt lignin structure and leave hemicelluloses and cellulose largely intact but with modifications to their structures.[8] Other pretreatments, such as dilute acid hydrolysis, hydrolyze hemicelluloses to monosaccharides and alter the lignin and cellulose structures.[8,9] The overall goal is to produce a maximum amount of fermentable substrates by releasing monomeric sugars from hemicelluloses and making cellulose most amenable to enzymatic hydrolysis, but there also has to be a balance in the pretreatment severity so as to not overproduce inhibitory compounds.

During dilute acid hydrolysis, the harsh environment of acidic media and high temperatures can degrade six-carbon sugars such as glucose into hydroxymethylfurfural (HMF), which can further degrade into levulinic acid, formic acid, and humin.[10] Similarly, five-carbon sugars, such as xylose and arabinose, can degrade into

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furfural and formic acid (either through degradation of furfural or directly from five-carbon sugars).[11,12] These degradation products are inhibitory to saccharifying enzymes and fermentation microorganisms.[13−17] However, all compounds are not equal in regards to strength of inhibition, and some even increase ethanol production when in dilute concentrations.[15] This complex nature of inhibitors requires an understanding of the starting material such that reaction conditions can be optimized for selective production of monosaccharides and enzyme- and microorganism-enhancing compounds.

No matter the pretreatment scheme chosen, elucidating the physicochemical properties of hemicelluloses would improve the understanding of the production of monosaccharides and degradation products formed during pretreatment so that the "sweet spot" of high monosaccharide and low inhibitor yields could be attained. Elucidating the physicochemical properties could also provide more insight into the physiological role of hemicelluloses. Thus, the objectives of this work were to characterize the physicochemical properties and investigate the dilute acid hydrolysis of hemicelluloses extracted from midgrowing season (July) and weathered, post-frost (February) switchgrass.

## MATERIALS AND METHODS

**Materials.** Alamo switchgrass plots were planted July 3, 2008 at the University of Arkansas Agricultural Research and Extension Center in Fayetteville, AR (36.0625° N, 94.1572° W). Plots were established by drilling seed cultivar Alamo in 18 cm wide rows into a prepared seedbed with a 12 row drill. Switchgrass samples were harvested on either July 4, 2009 (pre-anthesis) or February 18, 2010 (weathered, post-frost). From the 0.1 ha plots, approximately 10 kg of biomass were air dried at 55 °C; 100 g samples were ground to a size 20 mesh and stored in a 4 °C cold room until being used.

**Hemicelluloses Extraction.** An alkali extraction method, modified from Methacanon et al. and Bowman et al., was used to extract and purify switchgrass hemicelluloses.[18,19] First, extractives were removed by means of a water wash and Soxhlet extraction. Five grams of ground switchgrass were mixed with 100 mL of water and stirred for 2 h at ambient temperature. The water-washed switchgrass was then extracted using a Soxhlet apparatus with 180 mL of chloroform:methanol (2:1, V/V) for 4 h. The extracted switchgrass was then delignified by mixing the biomass with 100 mL of water and stirring at ambient temperature while adding 1 mL of glacial acetic acid and 2 g of sodium chlorite. After 1 h, an additional 1 mL of glacial acetic acid and 2 g of sodium chlorite were added. After 2 h, the mixture was filtered through four layers of cheesecloth. The holocellulose (remaining solids) was washed with water until near neutral pH, washed again with 50 mL of acetone, and air dried. Next, the cellulose and hemicelluloses were separated by mixing the holocellulose with 100 mL of 4 M KOH. The holocellulose−KOH mixture was stirred overnight at ambient temperature. The solution was then filtered through four layers of cheesecloth. The cellulose (remaining solids) was washed with 50 mL of 4 M KOH, followed by 50 mL of water. The filtrate (hemicelluloses) was adjusted to pH 5 with the addition of acetic acid and stirred at ambient temperature for 4 h. Then 1000 mL (4:1, V/V) of 100% ethanol was added and briefly stirred. The mixture was then stored in a 4 °C cold room overnight. Then the mixture was filtered using Miracloth (Calbiochem, La Jolla, CA), and the precipitate was dialyzed for 96 h in 18.2 MΩ water using 10,000 MWCO SpectraPor 7 (Spectrum Laboratories, Inc., Rancho Dominguez, CA.) dialysis tubing. The dialyzed precipitate was then lyophilized and stored in a −20 °C freezer until being used.

**Compositional Analysis.** Switchgrass and the hemicelluloses were characterized using the National Renewable Energy Laboratory's (NREL) suite of laboratory analytical procedures (LAP).[20−22] Moisture content was measured using an Ohaus infrared moisture analyzer (Nanikon, Switzerland). Ash content was determined by first igniting 2 g of switchgrass; the switchgrass was then loaded into a furnace (Thermolyne, Dubuque, IA) set at 575 °C and ashed to constant weight over 24 h. Extractives were quantified by successive water and ethanol Soxhlet extractions. First, 190 mL of water were refluxed through 5 g of switchgrass for 8 h. Next, 190 mL of 190 proof ethanol were refluxed through the material for 8 h. The difference between the initial weight of switchgrass and the weight of the extracted switchgrass was considered as extractives. Extractives-free switchgrass was then used to determine the structural carbohydrates and lignin in the biomass; hemicelluloses were also subjected to the structural carbohydrate and lignin analyses. One hundred milligrams of biomass were mixed with 1.0 mL of 72% (w/w) aqueous sulfuric acid and agitated at 100 rpm in a 30 °C water bath for 1 h. Mixtures were then diluted to 4% (w/w) aqueous sulfuric acid by addition of water. Samples were hydrolyzed at 121 °C for 1 h in an autoclave. An aliquot from each of the samples was then neutralized to pH 7 with calcium carbonate before being filtered through a 2-$\mu$m syringe filter and analyzed via high performance liquid chromatography (HPLC). Acid insoluble lignin (Klason lignin) was determined by recovering, drying, and weighing the solids remaining after hydrolysis. Klason lignin was corrected for ash by heating the recovered solids in the furnace at 575 °C after drying. Protein was determined by first determining combustible nitrogen using an Elementar Rapid N instrument (Mt. Laurel, NJ). Crude protein was then calculated as N × 6.25.[23]

**Linkage Analysis.** Extracted July and February hemicelluloses were permethylated, hydrolyzed to monomers, and derivatized to partially methylated alditol acetates before being analyzed using a Hewlett-Packard 5975C gas chromatogram (GC) equipped with a 30 m Supelco (St. Louis, MO) 2330 bonded phase fused silica capillary column and a 7890A mass selective detector operated in the electron impact ionization mode.[24,25]

**Scanning Electron Microscopy (SEM).** Switchgrass internode samples and extracted hemicelluloses were mounted on stubs and sputter coated with 1−2 nm of gold. Scanning electron micrographs were obtained using an FEI Nova Nanolab duo-beam SEM/FIB (Hillsboro, OR).

**Dilute Acid Hydrolysis.** Twenty milligrams of biomass were hydrolyzed in stainless steel reactors (4.9 cm in length, 0.56 cm ID, 0.79 cm OD, 1.21 mL capacity) using 1 mL of 1% (w/w) sulfuric acid at 160 °C in an industrial fluidized sand bath (Techne, Burlington, NJ). When the predetermined reaction time had elapsed, the reactors were cooled under running tap water for 1 min. The hydrolysate was then collected and separated into two aliquots. One aliquot was directly filtered and analyzed for degradation products via HPLC, and the other aliquot was neutralized, filtered, and analyzed for monomeric sugars via HPLC.

**HPLC Analysis for Monomeric Sugars.** HPLC analyses were carried out using a Waters 2695 Separations Module (Milford, MA) equipped with a Shodex (New York, NY) SP-G guard column and SP0810 column operated at 85 °C. Water was used as eluent at a flow rate of 0.2 mL min$^{-1}$. Compounds were monitored using a Waters 2414 refractive index detector, and monomers were quantified using calibration curves built using purchased reference standards.

**HPLC Analysis for Degradation Products.** HPLC analyses were carried out using a Waters 2695 Separations Module equipped with a Micro-Guard Cation H precolumn and Biorad Aminex HPX-87H (Hercules, CA) column operated at 55 °C. Eluent was 5 mM sulfuric acid at a flow rate of 0.6 mL min$^{-1}$. Compounds were monitored using a Waters 2996 photodiode array detector, and degradation products were quantified using calibration curves built using purchased reference standards.

**Molecular Weight analysis.** Extracted hemicelluloses were dissolved in dimethylsulfoxide (DMSO) and separated using Phenomenex Phenogel (Torrance, CA) $10^5$ Å and 100 Å columns in tandem with a Phenomenex Phenogel guard column. Eluent was 100% DMSO at a flow rate of 0.4 mL min$^{-1}$ provided by a Waters 515 HPLC pump. Eluted compounds were monitored using a Waters 2410 refractive index detector. Molecular weight was determined using a calibration curve built with dextran standards (Polymer Standards Service, Silver Spring, MD) and glucose (Sigma-Aldrich, St. Louis, MO).

**Statistical Analysis.** Dilute acid hydrolysis experiments were carried out in triplicate, and compositional analyses were carried out

in sextuplicate. Means were compared using a Student's *t* test in JMP 8 software. Means were considered significantly different when the p-value was less than 0.05.

## RESULTS AND DISCUSSION

**Hemicelluloses Extraction.** Extraction of February and July switchgrass yielded 25% and 22% (dry basis) hemicelluloses from starting biomass, respectively, corresponding to 85% and 79% of available hemicelluloses according to compositional analysis results of the switchgrass samples. These results are comparable with those obtained by Bowman et al., who obtained 27% hemicelluloses from the extraction of Alamo switchgrass.[19]

**Compositional Analyses.** Table 1 reports the composition of the switchgrass samples from which the hemicelluloses were extracted. Statistical analyses revealed significant differences in the extractives, ash, Klason lignin, and protein contents between the February and July harvest samples. No significant differences were observed among polysaccharide contents.

**Table 1. Switchgrass Composition by Percent Mass (dry basis)**

| component | July[a,b] | February[a,b] |
|---|---|---|
| cellulose | 37.01 ± 1.51A | 36.7 ± 1.34A |
| hemicelluloses | 28.10 ± 3.60A | 28.90 ± 1.50A |
| ash | 4.91 ± 0.17A | 2.60 ± 0.13B |
| extractives | 15.6 ± 0.15A | 12.2 ± 0.18B |
| klason lignin | 6.74 ± 2.14B | 13.6 ± 1.05A |
| protein | 5.38 ± 0.05A | 2.13 ± 0.04B |

[a]Numbers represent mean ± standard deviation. [b]Values in the same row with different letters are significantly different at the $\alpha = 0.05$ level.

The extracted switchgrass hemicelluloses were characterized in terms of their monomeric composition, which consisted of xylose, glucose, and arabinose as shown in Figure 1. The differences in xylose, glucose, and arabinose contents between July and February hemicelluloses were 11.4%, 8.7%, and 2.8%, respectively, which were significantly different at the $\alpha = 0.05$ level. Minor amounts of galactose were also detected in some samples; however, quantities detected were below the level of quantification of the HPLC system used.

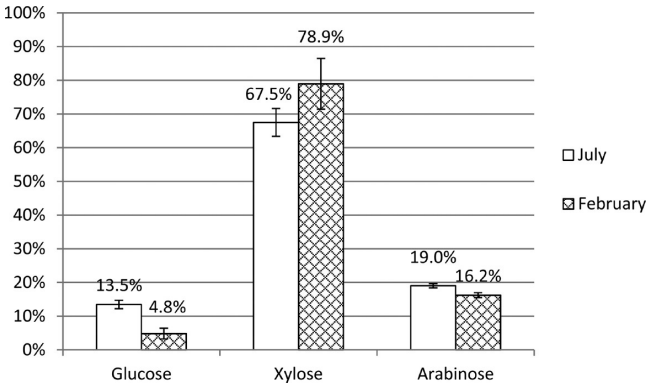


**Figure 1.** Carbohydrate composition of extracted switchgrass hemicelluloses. Differences were significantly different at the $\alpha = 0.05$ level.

**Linkage Analysis.** Linkage analysis data showed both hemicelluloses to contain structurally identical glycosyl residues (Table 2). The main residue in both samples was 1,4-linked xylose (53% for July and 67% for February), with additional 1,3,4-linked xylose residues accounting for 12% and 11% of July and February hemicelluloses, respectively. July hemicelluloses contained 8.3% more 1,4-linked glucose residues than February hemicelluloses. Terminally linked arabinose and 1,2-linked arabinose residues were also present in both samples. On the basis of these results, hemicelluloses are arabinoxylans, which are common to grasses, and mixed-linkage glucans, which are associated with cell wall growth.[26,27]

**Table 2. Glycosyl Linkages of July and February Switchgrass Hemicelluloses**

| | | peak area (%) | |
|---|---|---|---|
| glycosyl residue | linkages | July | February |
| 4 linked xylopyranose | →4)-Xyl*p*-(1→ | 52.7 | 66.7 |
| 3,4 linked xylopyranose | →3,4)-Xyl*p*-(1→ | 12.1 | 10.8 |
| terminally linked xylopyranose | Xyl*p*-(1→ | 3.6 | 3.7 |
| terminally linked arabinofuranose | Ara*f*-(1→ | 9.9 | 8.4 |
| 3 linked arabinofuranose | →3)-Ara*f*-(1→ | 0.5 | 0.3 |
| 2 linked arabinopyranose | →2)-Ara*p*-(1→ | 1.6 | 1.1 |
| 4 linked arabinopyranose or 5 linked arabinofuranose | →4)-Ara*p*-(1→ or →5)-Ara*f*-(1→ | 0.1 | 0.1 |
| 4 linked glucopyranose | →4)-Glc*p*-(1→ | 14.1 | 5.8 |
| 3 linked glucopyranose | →3)-Glc*p*-(1→ | 1.3 | 0.8 |
| terminally linked glucopyranose | Glc*p*-(1→ | 0.8 | 0.3 |

**Molecular Weight Analysis.** Figure 2 presents the results of size exclusion chromatography experiments. July hemicelluloses started eluting at 8.5 mL compared to February hemicelluloses starting elution at 9.75 mL, suggesting that July hemicelluloses contained a broader distribution of molecular weights than February hemicelluloses. Average molecular weights of 30,000 and 28,000 g $mol^{-1}$ were calculated for July and February hemicelluloses, respectively. On the basis of the compositional analysis results, these molecular weights correspond to average degrees of polymerization of 219 for July hemicelluloses and 205 for February hemicelluloses.

**Scanning Electron Microscopy.** Micrographs of the February and July harvested switchgrass internode samples and extracted hemicelluloses are shown in Figure 3. July internode samples appeared to have smoother fibers compared to those of the February samples, as noted by the numerous trichomes occurring along the internode area of the blade. No observable differences could be seen between the July and February hemicelluloses.

**Dilute Acid Hydrolysis.** Dilute acid hydrolysis showed similar profiles for the yield of monomers as a percent of initial glycosyl content released from July and February hemicelluloses (Figure 4). For February hemicelluloses, the maximum xylose, glucose, and arabinose concentrations were 13.5, 0.7, and 2.8 g $L^{-1}$ at 5, 2.5, and 5 min, respectively. July hemicelluloses exhibited a similar trend for maximum monomer concentrations. Xylose reached a maximum of 12.9 g $L^{-1}$ at 2.5 min. A maximum glucose concentration of 1.7 g $L^{-1}$ was reached at 7.5 min; and arabinose reached a maximum concentration of 2.6 g $L^{-1}$ at 7.5 min. These results show that, when not embedded in the cell wall, hemicelluloses depolymerize very quickly into monomers. In comparison, Morinelly et al.'s switchgrass hydrolysis experiments at 0.75% (w/w) sulfuric acid and 150 °C did not yield maximum xylose concentrations until after 50 min of hydrolysis.[28] Bowman et al. also conducted dilute acid hydrolysis experiments on extracted switchgrass hemicelluloses using 0.1 M TFA at 100 °C; however, the group did not track degradation products.[19]


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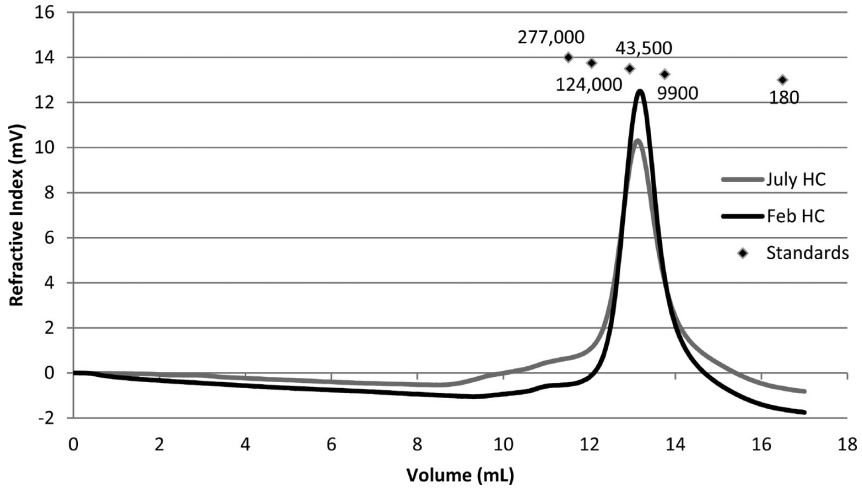


**Figure 2.** High performance size exclusion chromatograms of July (gray line) and February (black line) switchgrass hemicelluloses and dextran standards (diamonds).

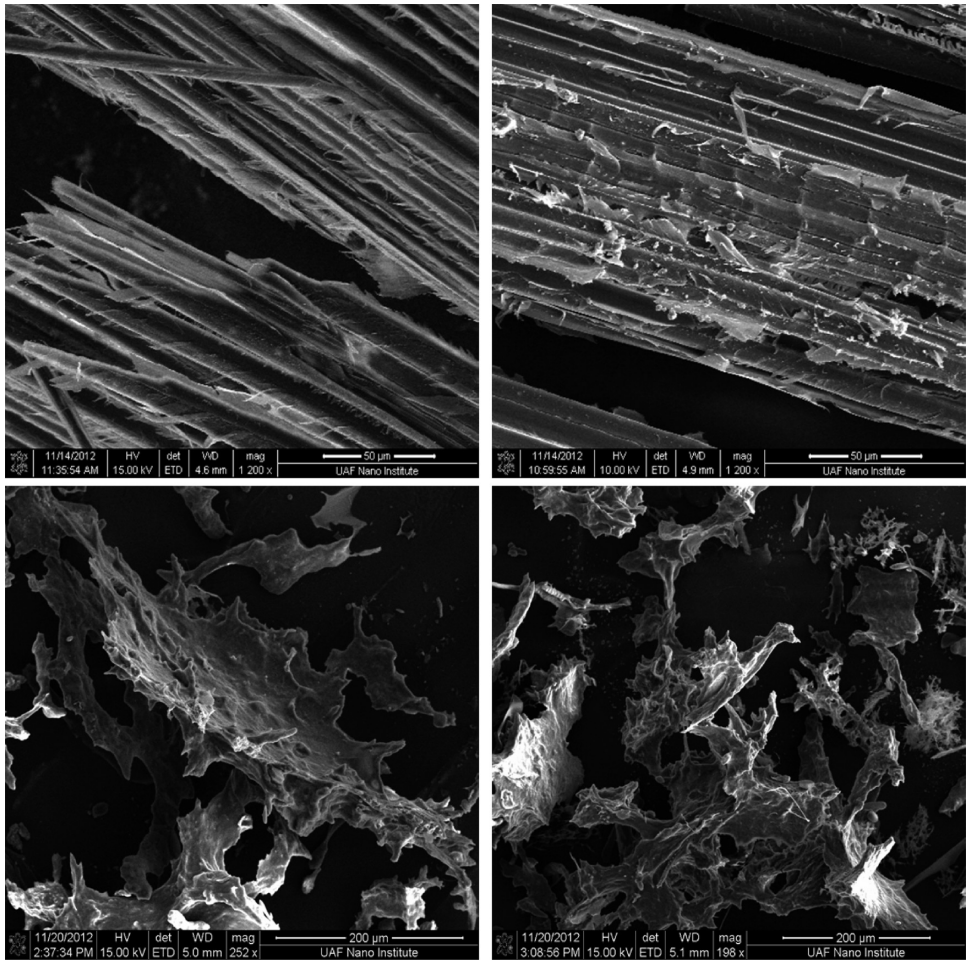


**Figure 3.** Scanning electron micrographs of July- (top left) and February-harvested (top right) switchgrass internode samples and extracted July- (bottom left) and February-harvested (bottom right) switchgrass hemicelluloses.

Furfural profiles were similar for both hemicelluloses. However, HMF concentrations released from July hemicelluloses were higher than those from February hemicelluloses after 10 min of hydrolysis (Figure 5), with July hemicelluloses reaching a maximum HMF concentration of 0.19 g $L^{-1}$ and February hemicelluloses reaching a maximum concentration of 0.11 g $L^{-1}$. Larsson et al. reported that HMF was metabolized much slower than furfural, with volumetric ethanol productivity being twice as low for *Saccharomyces cerevisiea* when in the presence similar concentrations of HMF versus furfural.[16] Thus, minor differences in HMF production could have severe implications in downstream processes. Maximum formic acid


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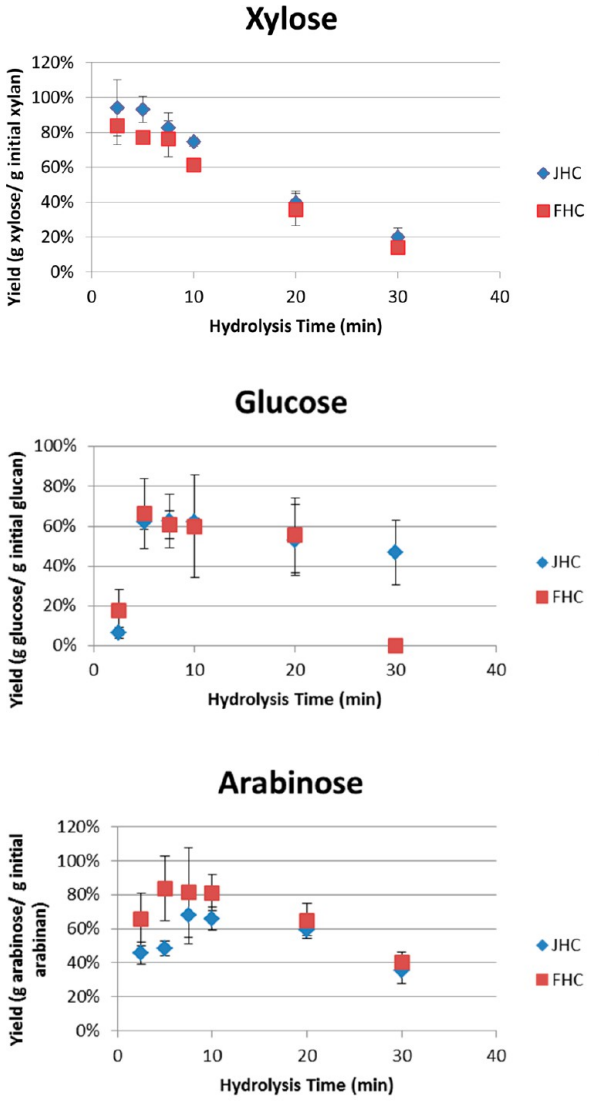


**Figure 4.** Percent monomeric sugars released during dilute acid hydrolysis at 160 °C and 1% (w/w) sulfuric acid of July (JHC, diamonds) and February (FHC, squares) hemicelluloses based on initial monomer content within hemicelluloses.

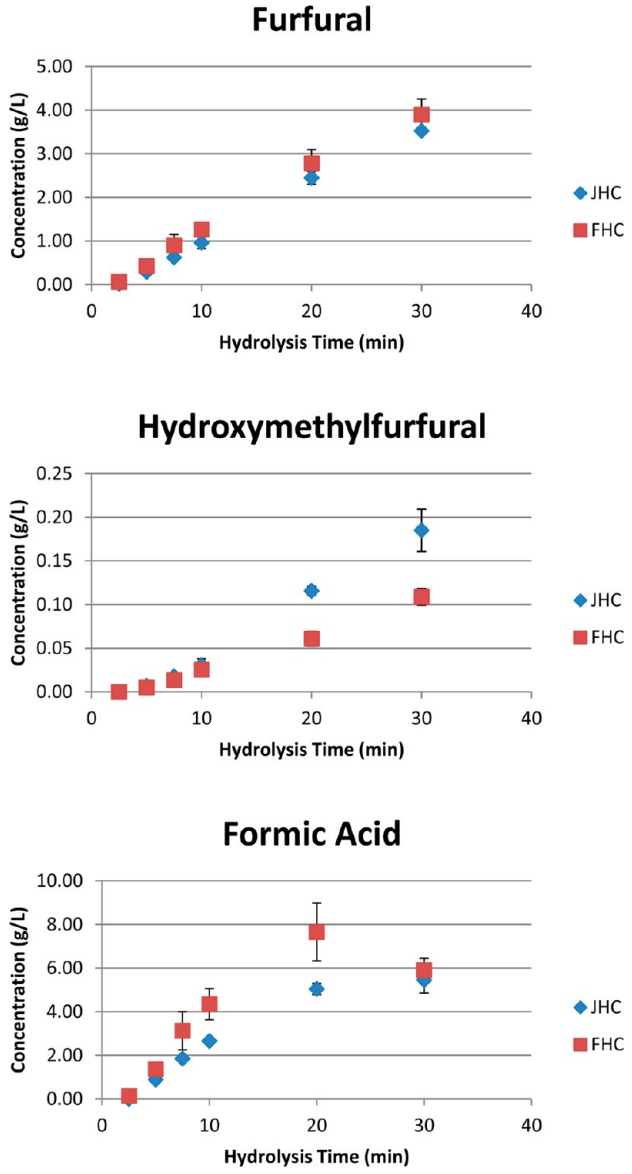


**Figure 5.** Concentration versus hydrolysis time plots of degradation products released during dilute acid hydrolysis at 160 °C and 1% (w/w) sulfuric acid of July (JHC, diamonds) and February (FHC, squares) hemicelluloses.

concentrations were 7.7 g $L^{-1}$ and 5.5 g $L^{-1}$ for February and July hemicelluloses, respectively, likely because of the increased five-carbon monomer content as compared to July hemicelluloses. Increased formic acid generation may prove to hinder enzymatic hydrolysis. Arora et al. showed that the addition of 5 and 10 g $L^{-1}$ formic acid to cellulose powder and Accelerase1500 enzymes decreased glucose recovery by 34% and 81%, respectively, in comparison to the control.[13] Using dilute acid pretreated poplar biomass and Accelerase1500 enzymes, Arora et al. reported that the addition of 5 g $L^{-1}$ formic acid decreased glucose recovery by 94%.[13] Formic acid may prove to be an inhibitor that needs to be minimized during pretreatment.

Dien et al. reported increased glucose yields from dilute acid hydrolysis when comparing anthesis switchgrass with post-frost switchgrass; however, this group determined that the glucose content of the post-frost switchgrass was higher than that of the anthesis switchgrass.[4] This could be because of negative interactions of the increased lignin content in the more mature biomass, but this effect could also be explained by an increase in the glucose content of the hemicelluloses, as found in this study. The glucose associated with hemicelluloses is likely more readily hydrolyzed than the glucose comprising cellulose.

## CONCLUSIONS

The physicochemical properties of switchgrass hemicelluloses from midgrowing season and weathered post-frost samples were determined and compared. Results showed that changes do occur in the composition, glycosyl linkages, and size of the hemicelluloses as the plant senesces. Dilute acid hydrolysis experiments also demonstrated differences between July and February hemicelluloses in terms of the amount of monosaccharides and degradation products released. These results have implications for converting biomass into fuels and chemicals as well as providing insight on the physiological role of hemicelluloses. Degradation kinetics of these polysaccharides should be investigated to further highlight any differences in behavior during pretreatment.


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**Notes**
The authors declare no competing financial interest.

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