Liquid chromatography–mass spectrometry investigation of enzyme-resistant xylooligosaccharide structures of switchgrass associated with ammonia pretreatment, enzymatic saccharification, and fermentation

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Switchgrass is a potential source of renewable biomass for conversion to liquid biofuels. Efficient conversion requires effective strategies for pretreatment and enzymatic saccharification to produce fermentable sugars. Standard analysis of fermentation liquids includes detection of monosaccharides and ethanol to determine efficiency of conversion. Larger components, specifically oligosaccharides, are typically not measured due to the structural complexity of the products; however, as oligosaccharides they represent carbon available in biomass that is not converted to liquid fuels. In this study, ammonia-pretreated switchgrass was enzymatically depolymerized either independently or under simultaneous saccharification and fermentation conditions. Residual oligosaccharides were reducing end-labeled followed by hydrophilic interaction liquid chromatography mass spectrometry/mass spectrometry analysis. These data reveal 20 oligosaccharide peaks with distinct retention times and tandem mass spectrometry fragmentation patterns representing 13 different oligosaccharide compositions. All measured compositions were smaller than a chain length of six and were neither linear xylooligosaccharides nor modified with phenolic esters. This work represents a robust method to monitor and identify unhydrolyzed oligosaccharides from fermentations, thereby permitting the screening of targeted enzymatic activities to promote the complete hydrolysis of xylan.

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1. Introduction

Ongoing concerns about climate change and the reliance on foreign sources of petroleum-based fuel have led to strong interest in the use of biomass as a renewable energy source. Presently the vast majority of bioethanol is produced from grains and sugarcane. Expanding biofuels production to lignocellulosic sources of biomass, such as agricultural residues and energy crops, could greatly expand the supply of biomass and add value to underutilized resources (US DOE, 2011). Life cycle analysis also predicts that lignocellulose-derived biofuels are efficient at lowering overall greenhouse gas emissions (Schmer et al., 2008).

Switchgrass (SG) is a native perennial grass that is being developed as a bioenergy crop because of its favorable agronomic traits and high productivity (Walsh et al., 2003). Switchgrass is converted to ethanol in a four step process that includes milling/pretreatment, enzymatic saccharification, fermentation, and product recovery. Pretreatment includes thermochemical and physical processes that are needed to increase enzymatic conversion of cellulose; native biomass treated with enzymes has glucose yields of 20% or less. Alkaline and, more specifically, ammonia-based pretreatment processes have been found to be effective on grasses such as SG for enzymatic conversion of cellulose (Aita et al., 2011; Dien et al., 2011; Salvi et al., 2010; Sills and Gossett, 2011); however, full conversion of the hemicellulose portion remains problematic.

Hemicellulose originating from warm season grasses is predominantly arabinoxylan, which is linked to core lignin groups via arabinose feruloyl ester bridges (Saha, 2003). Alkaline pretreatments saponify these ester linkages, separating the lignin and hemicellulose, removing acetyl side-groups from xylan, and partially depolymerizing xylan polymers. Xylanases and auxiliary enzymes are needed to complete xylan hydrolysis. As xylan represents 30–40% of the carbohydrates present in SG and other grasses, commercial feasibility necessitates its efficient conversion to xylose as a prerequisite for fermentation (Dien et al., 2006). Further hydrolysis of xylan also promotes cellulose conversion because insoluble xylan can bar access to cellulose fibers and xylan
oligosaccharides are potent inhibitors of cellulases (Kumar and Wyman, 2009; Qing et al., 2010).

For these reasons, complete depolymerization of arabinoxylan is highly desirable, preferably at the lowest possible enzyme loading. Most studies rely on an approach whereby either commercial enzyme preparations or, more recently, purified enzymes are blended in various combinations and the favored mixture selected based upon glucose and xylose yields. A more direct structural approach, in which the residual xylooligosaccharides following enzymatic processing are characterized to identify recalcitrant bonds, is proposed herein. Presumably this will suggest specific enzyme additions for increased xylose yields.

A major technical barrier to studying arabinoxylan is its chemical complexity. Xylan chains have a backbone of repeating 1,4-linked xylose residues with the potential for α-arabinofuranose or acetyl substitutions at either the 2-O or 3-O positions or both; 2-O substitution with α-glucuronic acid, where 4-methylthylation is a common modification; and added complexity from substitutions (hexose, pentose, and/or phenolics) on the arabinose side chains (Falk, 2010). Published analyses of oligosaccharide fermentation residues from any biomass source are limited. A study where wheat-derived arabino-xylooligosaccharide (AXO)-rich fermentation effluent was characterized by a combination of matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF-MS), electrospray ion-trap mass spectrometry, and GC–MS linkage analysis (Matamoros Fernández et al., 2007) highlights the difficulty of analyzing non-hydrolyzed glycosidic bonds recovered from fermentation broths. In a recent report, corn fiber (a byproduct of corn starch to ethanol conversion) was acid-pretreated and subjected to simultaneous saccharification and fermentation (SSF) conditions. Approximately 50% of the xylan component was determined to exist as oligomers, by a combination of MALDI-TOF-MS analysis of fractionated residual materials and analysis of the monosaccharide composition of oligosaccharide fractions (Appeldoorn et al., 2010). However, without in-line chromatography, the MALDI-TOF data revealed only compositions of oligosaccharides without information about isomers or structural information from additional fragmentation of the ions (i.e., MS² or MSⁿ). While both of these reports identified some of the possible complex oligosaccharide products generated by the enzymatic hydrolysis of hydrolyzing xylan, both studies used pericarp-derived xylan as a source (e.g., seed coats). It is likely that SG cell wall xylan (e.g., stalks and leaves), and the majority of other lignocellulosic biomass, will have different structures and properties.

Liquid chromatography mass spectrometry (LC–MS) has been shown to separate isomeric components from oligosaccharide-containing samples using a variety of chromatographic matrices (Ruhak et al., 2009; Westphal et al., 2010; Wuhrer et al., 2009). Specifically, hydrophilic interaction liquid chromatography mass spectrometry/mass spectrometry (HILIC–LC/MS/MS) of reducing end-labeled AXOs has been applied successfully to several types of extracted xylan samples (Bowman et al., 2011; Maslen et al., 2007; Ridlova et al., 2008). Here this technique was expanded to directly characterize enzyme-resistant structures of hemicellulose from plant cell wall material. Specifically, a HILIC–LC/MS/MS method, and its isomeric separation capability, was used to identify anthranilic acid reducing end-labeled oligosaccharides that were collected following enzymatic hydrolysis and ethanol fermentations.

For this study, SG was pretreated with dilute ammonium hydroxide. Ammonia was removed by evaporation. Pretreated SG samples were mixed with a commercial carbohydrolyase preparation and Saccharomyces cerevisiae yeast. Addition of the enzymes and yeast together allows for SSF, which typically has higher ethanol yields at lower enzyme loadings because it avoids glucose inhibition of the cellulases. Here an engineered S. cerevisiae capable of metabolizing xylose (Hector et al., 2011) was used; whereas native yeast only ferments hexoses. Two separate experiments were conducted using the parental and engineered yeast strains. Residual sugars were recovered from both experiments, and used as a source of xylooligosaccharides. A third experiment was also conducted in which pretreated SG was hydrolyzed with enzymes in the absence of yeast (i.e., without fermentation).

2. Methods

2.1. Materials

Sodium hydroxide (50% w/v), sodium acetate anhydrous, trifluoroacetic acid (TFA), acetonitrile, acetic acid, dimethylsulfoxide (DMSO), ammonium hydroxide, sodium cyanoborohydride, sodium borodeuteride, methyl iodide, sodium hydroxide powder, anthranilic acid, glucose, galactose, xylose, arabinose, fructose, xylitol, sorbitol, p-coumaric acid and ferulic acid were research grade and purchased from Sigma–Aldrich Company (St. Louis, MO). Purified xylose-xylohexose standards were purchased from Megazyme International Ireland Ltd., (Wicklow, Ireland). Formic acid was purchased from Fluka Chemical (Buchs, Switzerland). SG (Cave-in-rock) material was harvested from field-grown plants at the post anthesis stages (MPV-2) (Dien et al., 2006). GC220 cellulase (Genencor, Rochester, NY), Novo188 (Novozymes, purchased from Sigma Chemical Co., St. Louis, MO), and Multipectinase (Genencor, Rochester, NY) were used for saccharification. These enzyme preparations are all commercial grade. Cellulose packed Ultra-Micro SpinColumns were purchased from Harvard Apparatus (Holliston, MA).

2.2. SG pretreatment

Four replicates of MPV2 switchgrass (75 g) in 425 ml of 8% w/v ammonia/water were treated at 180 °C for 20 min using 316 stainless steel vessels (Dien et al., 2011). At the completion of the chemical pretreatment, samples were dried at 100 °C and milled to 4 mm.

2.3. Carbohydrate analysis

2.3.1. Determination of total carbohydrates by HPLC

The sugar profile of untreated SG was determined by two-stage acid hydrolysis (NREL procedure LAP002) (Sluiter et al., 2005). Monosaccharides were measured using a SpectraSYSTEM liquid chromatography system (Thermo Electron Corporation, CA) equipped with an automatic sampler, column heater, isocratic pump, refractive index detector (RID), and computer based integrator running Chromquest ver. 2.5 (Thermo Electron Corporation, CA). Samples were injected (20 µl) onto a sugar analysis column (Aminex HPX-87H Column, 300 × 7.8 mm, Bio Rad Laboratories, Inc., Hercules, CA) and eluted with 5 mM sulfuric acid at 0.6 ml/min and 65 °C. Monosaccharides were identified and quantified by comparison to authentic standards using a five-point calibration curve.

2.3.2. Enzyme-only treatment

For each of the four replicate pretreatments, duplicate 1.5 g samples of pretreated SG were prepared. To each was added: 50 ml citrate buffer (50 mM, pH 4.8); 0.05% w/v thymol; 15 FPU of GC220/g glucan; 40 U Novo 188/g glucan; and 50 U Multifect pectinase/g SG. The digestion was performed at 50 °C for 72 h. Soluble monosaccharides were analyzed by HPLC–RID as described above.
2.4. Fermentation

YP medium (10 g/l yeast extract, 20 g/l bacto-peptone) was autoclaved without carbohydrate. Sterile glucose (20 g/l final concentration) was added separately for precultures. For each of the four replicate pretreatments, 10 g of pretreated SG material (dry basis) was combined with 85 ml YP. To each was added: 15 FPU GC220/g glucan, 40 U of Novo188/g glucan, and 50 U Multifect pectinase/g SG. Each of the four pretreatment replicates were then inoculated with S. cerevisiae DS5A and a second set of replicates was inoculated with S. cerevisiae YRH400 (xylose-utilizing strain) (Hector et al., 2011) at an OD600 of 1.0, from a concentrated overnight culture that had been resuspended in 5 ml of YP. Fermentations were agitated at 100 rpm for 72 h at 35 °C. Conversion to ethanol was continuously monitored in a wireless gas production measurement system (Ankom Technologies; Macedon, NY). The wireless system monitors gas production indirectly by measuring cumulative gas pressure; CO2 production is calculated using the ideal gas law. The system was set to vent when the overhead pressure achieved 1 psi and to monitor pressure every 15 min. After 72 h of fermentation, each sample was centrifuged (20 min at 4000 g) to separate particulate matter and yeast from broth. An aliquot of fermentation broth or 66.6% of solid material were stored at −20 °C until needed.

2.5. Xylooligosaccharide labeling and LC–mass spectrometry

Oligosaccharides released by enzymatic saccharification were analyzed as follows:

2.5.1. Reducing end labeling

Either 20 μl of fermentation broth or 66.6 μl of enzyme-only digestion (due to different substrate loadings) aliquots were dried in vacuo. The samples were then derivatized with anthranilic acid (2-aminozobonic acid, 2-AA), as previously described (Bigge et al., 1995). Briefly, a dried sample was dissolved in 100 μl of a reaction reagent containing 2-AA in DMSO/glacial acetic acid (7:3) and 1.0 M sodium cyanoborohydride. The solutions were then centrifuged for 3 min and incubated at 65 °C for 3 h. Excess reagent was removed with cellulose Ultra-Micro SpinColumns. The derivatized glycan was then eluted with two 100 μl volumes of water and dried. Samples were resuspended in 20 μl distilled water. Acetonitrile and water were added to a concentration of 80:20 acetonitrile:water in 1 ml prior to HILIC LC–MS analysis.

2.5.2. LC–MS/MS

Mass spectrometry samples (20 μl injection) were analyzed by LC–MS (Thermo Surveyor HPLC, equipped with an autosampler and photodiode array detector) through an amide-80 (2.0 × 150 mm) column ( Tosoh Biosciences, King of Prussia, PA) HILIC column running a gradient elution of 20% A:80% B (buffer A 50 mM ammonium formate (pH 3.5), buffer B 100% acetonitrile) to 50% A:50% B over 45 min at a flow rate of 200 μl/min while maintaining a constant column temperature of 30 °C and UV monitoring at 310 nm. Electrospray negative mode ionization data were collected with a quadrupole ion trap mass spectrometer (ThermoFinniganDecaXPMS) under Xcalibur 2.0.1 control. The instrument was tuned with an anthranilic acid-labeled linear xylooligosaccharide (degree of polymerization (d.p.) 5, X5-AA, m/z 798 [M–H]+) introduced by infusion to optimize the signal of labeled xylooligosaccharide analytes. The data dependent analysis function was used to isolate and fragment the singly charged ions ([M–H]+) corresponding to AA-labeled arabinonoxylooligosaccharides, and hexose, acetyl-, or methyl-hexuronic acids containing xylooligosaccharides using a 2 dalton isolation window to encompass the isotopic window of each isolated ion. The collision energy was set to 35%, as this provided informative fragmentation from X5-AA and a d.p. 5-AA derived from wheat arabinoxylan as a model branched oligomer. MS/MS was decoded into graphical form using Microsoft Excel, where peak areas from extracted ion chromatograms (EICs) using a 1 m/z window for each composition are reported. Normalization to relative ion abundances was avoided due to the presence of a large AA-hexose peak (8.45 × 106 ± 0.66 × 106 area counts) in the enzyme-only sample set that was not present in the SSF samples; therefore Hex-AA was not included in Fig. 1. Standard deviations were calculated from values using three injections of each of the four replicates (36 total injections). MS2 data is reported as percent ion abundances of glycosidic fragment ions for each peak detected.

2.5.3. Purification of native soluble residual oligosaccharides

Excess fermentation residues (salts, organic acids, and phenolics) were removed by resuspending 50 ml of lyophilized material in 5 ml milliQ water followed by size-exclusion chromatography (P2 resin, 50 × 1000 mm column, 1.0 ml/min flow of milliQ water, FPLC Amersham-Biosciences, Äkta system 900) where the eluent was monitored for conductance as well as absorbance at wavelengths 254 and 190 nm. Early eluting fractions with absorbance at 190 nm were determined to contain oligosaccharide materials by HPAEC-PAD (Dionex ACS 3000, Sunnyvale, CA) utilizing a PA-100 column (Dionex) using previously reported conditions (Rantanen et al., 2007). AXO containing samples were pooled, lyophilized, and resuspended in 5 ml water. Pooled fractions were purified by HPAEC-PAD utilizing a PA-100 column (22 × 30 mm, Dionex) running 100% A isocratically for 15 min followed by a linear increase to 12% B over 40 min (A:100 mM NaOH; B:100 mM NaOH/1 M Sodium Acetate) followed by 15 min of reequilibration in 100% A, at a flow rate of 8 ml/min. Fraction collection (Foxy Jr., Isco, Lincoln, NE) was automated via Chromleon software. Collected fractions containing desired products were desalted using graphite solid-phase extraction cartridges (Alltech, Deerfield, IL) according to the manufacturer's protocol. The purity of two isolated oligosaccharides was confirmed by analytical HPAEC-PAD, with a portion reserved for anthranilic acid labeling (Sections 2.5.1–2.5.2) for comparison with samples directly labeled from SSF broth fermentation peak retention times and fragmentation patterns, and a portion reserved for permethylation analysis by multi-stage mass spectrometry and linkage analysis (Sections 2.5.4–2.5.8).

2.5.4. Permethylation

After drying, samples were reduced with sodium borodeuteride (500 mM) in 50 mM NaOH for 1 h at 45 °C. Permethylated (PM) was performed according to the method of Ciucanu and Kerek (Ciucanu and Kerek, 1984). A portion of each permethylated (E-PM and F-PM) sample was used for nanospray tandem mass spectrometry analysis, a second portion was carried through for linkage analysis.

2.5.5. Linkage analysis

Partially methylated alditol acetates (PMAAs) for linkage analysis were generated according to a previously reported method (Harris et al., 1984).

2.5.6. Gas chromatography–mass spectrometry

For gas chromatography–mass spectrometry (GC–MS) analysis, 4 μl of sample was injected on a SHRX5LB column ( Shimadzu Scientific Instruments, 30 m × 0.25 μm, Shimadzu GC–MS 2010 Plus equipped with A020i autosampler) with a 1:20 split ratio. After an initial 2 min of isothermal elution at 150 °C, elution was accomplished by use of a thermal gradient from 150 to 250 °C at a rate of
4 °C/min, followed by a 15 °C/min ramp to 300 °C. Electron impact positive mode mass spectra were collected from 35–450 m/z. The inlet temperature was set to 250 °C. Linkages were identified by GC–MS retention times and fragmentation patterns of PMAAs.

2.5.7. Positive mode nanospray MS²
Permethylated samples were dissolved in 7:3 methanol:water and analyzed by infusion tandem mass spectrometry in the positive mode (~40 nL/min) in a quadrupole orthogonal time-of-flight (Q-oTOF) mass spectrometer (Applied Biosystems/MDS Scienq Qstar Elite) using a nanospray source. Fragmentation energies of 65 V were used to maintain approximately 80% of the isolated parent ion peak intensity to minimize over-fragmentation that can occur in beam-type mass spectrometers. The instrument was tuned to a xylopentaose standard that had been reduced and permethylated as described above (X₅-PM, m/z 886.3 [M+Na][²⁺]).

2.5.8. Positive mode nanospray MSⁿ
Permethylated samples were dissolved in 7:3 methanol:water and analyzed by nanospray tandem mass spectrometry in the po-
sitive mode (40-40 nL/min) in a trap mass spectrometer (Thermo-FinneganDecaXPplus) under Xcalibur 2.0.1 control using a nanospray source made in-house. Fragmentation energies of 35% were used. The instrument was tuned to X$_2$-PM (m/z 886.3 [M+Na]$^+$). A fragmentation tree was used based on the MS$^n$ patterns for arabinoxyllooligosaccharides (Correia et al., 2011; Matamoros Fernández et al., 2003, 2004, 2007).

2.6. Residual ferulate ester determination

Sodium hydroxide (2 M) or water (1 ml) was added to 100 mg of ammonia-pretreated SG and incubated for 2 h at room temperature. The samples were acidified by the addition of 6 M hydrochloric acid. The solution was extracted with 1 ml of ethyl acetate thrice (Sarah et al., 2007). The combined ethyl acetate fractions were dried under nitrogen and reconstituted in 1 ml water. Residual insoluble material was dissolved in 500 µl methanol followed by the addition of 500 µl water prior to HPLC analysis. Recoveries were determined by comparison of the summed peak areas of both aqueous and methanol fractions determined by HPLC (Shimadzu Scientific Instruments equipped with: an SIL-20 AHT autosampler, a LC20AD pump, and SPD20A dual wavelength UV detector, under control of EZStart chromatography software) detection at 280 nm on a C$_4$ reverse-phase separation (3 x 30 mm column, Vydac, Deerfield, IL), using a gradient of 5– 75% 0.1% formic acid in water and acetonitrile over 45 min at a flow of 500 µl/min). Retention times and identities were compared to authentic standards and confirmed by LC–MS. Data is presented in area counts where error bars represent the standard deviation of three experimental replicates.

3. Results and discussion

3.1. Fermentation data

Ammonia pretreatment of SG and other grasses has been demonstrated to be particularly effective for biochemical conversion processes (Aita et al., 2011; Dien et al., 2011; Salvi et al., 2010) while avoiding the problem of fermentation inhibition associated with other types of pretreatment (e.g. dilute acid) (Chung and Lee, 1985; Larsson et al., 1999; Luo et al., 2002). The combination of saccharifying enzymes used here is based on previously described cocktails that possess auxiliary activities for improved digestion of biomass (Dien et al., 2008; Sills and Gossett, 2011). Quadruplicate samples of ammonia-pretreated SG were run under three conditions: enzyme-only digestion; SSF with a standard Saccharomyces cerevisiae (D5A), and SSF with a xylose-utilizing strain of S. cerevisiae (YRH400). Enzyme-only and SSF sample supernatants were quantified by standard HPLC-RID methods to determine monosaccharide release and, for the SSF experiments, ethanol yields (Table 1). Enzyme-only digestion showed a 48.6 ± 1.4% release of total glucose and 69.8 ± 0.6% release of monomeric xylose. For SSF conditions, released glucose is directly fermented to ethanol by the yeast present in the culture. Based upon the ethanol yield, glucose conversions were higher than observed for the enzyme only saccharifications (77 ± 4% D5A; 83 ± 3% YRH400); the theoretical yield for glucose to ethanol is 0.51 g/g. These results show that SSF conditions improve monosaccharide glucose release over enzyme-only conditions; further, in SSF glucose does not become concentrated enough to inhibit cellulase activities. Fermentation and enzyme-only experiments lasted 72 h, which is a typical duration for these types of experiments (Dien, 2010).

Xyloolitol formation was observed for SSF experiments using either YRH400 (recombinant S. cerevisiae engineered for xylose fermentation) or D5A (parental control strain). Xylose is reduced to xylitol via an endogenous xylose reductase and is an early intermediate in xylose metabolism. An increase in xylan digestion was also observed for the SSFs versus enzyme-only results based on the increase in xylene + xylitol for strain D5A. For the DSA SSF, 75 ± 5% of the available xylan was converted to xylose and xylitol. For the YRH400 SSF, only 68 ± 3% of the available xylan could be accounted for as xylose and xylitol. However, YRH400 also converts xylose to ethanol, which is confounded with ethanol produced from glucose.

3.2. HILIC–MS and compositional data

Sample supernatants were dried and AA-labeled under well-documented, mild reductive amination conditions (Bigge et al., 1995) to introduce a reducing end label that has been documented to be compatible for HILIC–MS/MS analysis, with specific cases of arabinino-xylosaccharide analysis (Bowman et al., 2011; Maslen et al., 2007). Analysis by HILIC–LC–MS of reducing end-labeled residual soluble oligosaccharides from each of the 2 SSF conditions revealed 20 mono- or oligosaccharide chromatographically-resolved extracted ion peaks corresponding to singly charged ions ([M–H]$^-$). These signals include 10 pentose-only compositions ranging from pentose d.p. 1–AA to d.p. 6–AA ([Pent]$_1$–AA, m/z 270, RT 4.1 min; [Pent]$_2$–AA, m/z 402, RT 5.3; [Pent]$_3$–AA, m/z 534, RT 6.5 min; [Pent]$_4$–AA, m/z 788, RT 7.8 min; [Pent]$_5$–AA, m/z 966, RT 9.8 min; [Pent]$_6$–AA, m/z 1152, RT 13.7 min; [Pent]$_7$–AA, m/z 798, RT 15.8 min; [Pent]$_8$–AA, m/z 974, RT 16.9 min; [Pent]$_9$–AA, m/z 930, RT 22.3 min) (Fig. 1A). Additionally, present were extracted ion chromatogram peaks corresponding to: 4 pentose-containing oligosaccharide linked to a hexose ([Hex]–[Pent]$_1$–AA, m/z 342, RT 6.6 min, [Hex]–[Pent]$_2$–AA, m/z 564, RT 5.7 min, [Pent]$_3$–[Pent]$_1$–AA, m/z 696, RT 8.9 min, 10.3 min), two oligosaccharide compositions containing acetyl groups ([Ac]–[Pent]$_1$–AA, m/z 576, RT 13.2 min, [Pent]$_1$[Ac]$_2$–AA, m/z 750, RT 18.4 min), and two oligosaccharide compositions containing methyl-glucuronic acid ([MeGlcA]–[Pent]$_2$MeGlcA$_1$–[Pent]$_1$–AA, m/z 460, RT 8.5 min, [Pent]$_2$MeGlcA$_1$–[Pent]$_1$–AA, m/z 592, RT 11.2 min) (Fig. 1B). The dominant compositions detected from SSF conditions were: (Pent)–AA, (Pent)$_2$–AA, (Pent)$_3$–AA, (Pent)$_4$–AA, and (Pent)$_5$–AA.

Enzyme-only samples contained the same pentose-based oligomers, as well as the Hex$_1$–[Pent]$_1$–AA (m/z 300, RT 4.7 min; m/z 462,RT

<table>
<thead>
<tr>
<th>Glucose</th>
<th>Xylose (m/z 270)</th>
<th>Xylitol (m/z 199)</th>
<th>Xylitol + Xylose (m/z 276)</th>
<th>Arabinose (m/z 260)</th>
<th>Ethanol Conversion Efficiency$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme Only</td>
<td>48.6 ± 1.4</td>
<td>69.8 ± 0.6</td>
<td>35.3 ± 4.5</td>
<td>75.2 ± 5.3</td>
<td>93.1 ± 1.2</td>
</tr>
<tr>
<td>DSA SSF</td>
<td>0$^b$</td>
<td>39.9 ± 0.7</td>
<td>69.7 ± 3.8</td>
<td>87.7 ± 7.9</td>
<td>83.0 ± 3.4</td>
</tr>
<tr>
<td>YRH400 SSF</td>
<td>0$^c$</td>
<td>27.5 ± 2.0</td>
<td>42.1 ± 1.8</td>
<td>77.0 ± 4.0</td>
<td>7.0 ± 4.0</td>
</tr>
</tbody>
</table>

$^a$ Percent of maximum based on glucose content.

$^b$ Released glucose was consumed during SSF conditions.

$^c$ DSA contains endogenous xylose reductase activity resulting in xylitol production.

$^d$ Released xylose is partially fermented to ethanol by the xylose-utilizing strain YRH400.
of the expected analytes. This is important to preclude the presence of artifacts arising from the complex mixtures that have the same nominal masses as the labeled oligomers. Therefore, in this study all assigned compositions were verified to be pentose-containing oligomers by analysis of on-line tandem mass spectral data. Typically in tandem mass spectrometry oligosaccharide ion fragment, in some intensity, at the glycosidic bonds between monosaccharide units. This can permit sequence determination of linear oligosaccharides; however, branched oligosaccharides can generate ambiguous structural assignments if two fragmentations occur simultaneously, especially in cases where the substituents have the same mass (e.g. arabinose and xylose). Fragment ions (ArH, Ca, Cb, X, YA, ZA) are labeled using Donom and Costello nomenclature (Donom and Costello, 1988). Fragmentation energies were optimized using X-AA ([M–H]−, m/z 798) to permit sufficient backbone fragmentation, thus providing more structurally informative fragment ions. This setting was used for all isolated ions where small oligosaccharide ions were expected to fragment to a greater extent than larger oligosaccharide ions.

Singly-charged ions of anthranilic acid-labeled xylooligosaccharides have negative mode fragmentation patterns containing dominant Y- and Z-type ions of pentose oligosaccharides (i.e. AA label-containing Y-type glycosidic bond fragments m/z: 270, 402, 534, 666, 798 and Z-type m/z: 252, 384, 516, 648, 780) due to the localization of the single negative charge on the carboxylic acid-containing reducing end label. The ketone-containing oligomer pattern is formed from the neutral losses of pentoses (loss of 132 da/unit), hexoses (loss of 162 da/unit), or methyl-hexuronic acids (loss of 190/unit) residues. The presence of these specific Y- and Z-type ions was, therefore, considered diagnostic for AA-labeled xylo-derivatized oligosaccharides. On-line tandem MS could allow for differentiation of the isomeric forms of oligosaccharides by their distinct fragmentation patterns (Fig 2A) in particular, in differing product ion abundances. Tandem mass spectrometric data of reducing end-labeled oligosaccharides show primarily glycosidic bond cleavage, whereas the Y-ion (d.p.,) dominates the spectrum. The on-line tandem MS data provides some information about the structural features of the observed oligosaccharide peaks.

The fragmentation spectrum of PentHx,AA (m/z 432, peak A-AA) has a Y1 ion (300 m/z) corresponding to an AA-labeled hexose leading to an assignment of a pentose attached to a hexose with a free reducing end; however, the position of the pentose substitution cannot be determined from this data due to the lack of structurally informative fragment ions. Similarly, structural determination cannot be determined from the fragmentation pattern of peak B-AA (d.p. 3-AA, m/z 534) as the product ion (m/z 402) could result from the loss of a pentose from either a branch position (i.e. ara) or the terminal chain position (i.e. xyl). The fragmentation pattern for peak C-AA (d.p. 4-AA, m/z 666) lacks Z- and Y-ions at m/z 384 and 402, indicative of substitution at the second pentose unit from the reducing end. In contrast, the fragmentation spectrum of peak D-AA (d.p. 4-AA, m/z 666) provides inconclusive structural information, as the Y- and Z-type ions in the fragmentation pattern are fully represented, consistent with a linear structure. Similarly, the mass spectrum of peak E-AA (d.p. 5-AA, m/z 798) has fragment ions representing each glycosidic cleavage event, indicating a linear structure (Fig. 2B and C); however,
X5-AA has a retention time of 20.6 min, demonstrating that Peak E-AA is not a linear xylose-only oligomer. Two possible structures, based on typical grass AXO characteristics (Faik, 2010), could generate the apparently linear fragmentation pattern of E-AA (Fig. 2B), where the substituents occur at the non-reducing end of the chain (i.e. not a 1,4 linkage). The tandem mass spectrum for peak F-AA (d.p. 5-AA, \( m/z \) 798) lacks a Y-type fragment ion at \( m/z \) 402, indicating that the second pentose unit of the oligosaccharide contains a branching pentose substitution, where a double fragmentation would be required to generate an \( m/z \) 402 ion (Fig. 3B and C). Peak G-AA (d.p. 6-AA, \( m/z \) 930) lacks Z- and Y-ions at \( m/z \) 516 and 534, indicative of substitution at the third pentose unit from the reducing end. While these fragmentation patterns allow for putative structural assignments of the residual oligosaccharides, further characterization to confirm the assigned structures and to remove any structural uncertainty due to the isobaric nature of branching arabinose and backbone xylose present in arabino-xylooligosaccharides is beneficial. The lack of informative cross-ring cleavages

Fig. 2. (A) Negative mode LC–MS/MS spectra of singly-charged anthranilic acid-labeled SG SSF residual pentose oligosaccharides (peaks A-AA to G-AA) present in fermentation broth following SSF with YRH400. Tandem mass spectra are labeled according to the retention time windows of Fig 1. (B) Three potential structures of the 798 ion based on MS/MS data of AA-labeled oligosaccharides. Fragment ions are labeled using the nomenclature of Domon and Costello (1988). (C) Graphical representation of the averaged fragment ions from \( m/z \) 798 (Pent5-AA) for E-AA and F-AA for all SSF and enzyme-only digestion runs.

Fig. 3. Peak areas from C4-RP-HPLC analysis of peaks corresponding to ferulic acid, \( p \)-coumaric acid, and their aminated byproducts from ammonia-pretreated SG extracted with either 2 N NaOH or water.
Fig. 4. Positive mode nanospray tandem mass spectra of borodeuteride-reduced, permethylated (PM) (Pent), oligosaccharides ([M+Na]+, m/z 886.3). (A) X5-PM; (B) Peak E-PM; (C) Peak F-PM. Fragment ions are magnified in comparison to parent ion for clarity and labeled using the Domon and Costello nomenclature (Domon and Costello, 1988).
makes linkage determination difficult. In the case of internally branched AXOs, the fragment ions provide the location of the modification by maintaining the branching saccharide thus generating an identifiable ion pattern in the tandem mass spectra. Structural information about Me-GlcA and acetyl-containing compositions could not be determined due to the facile losses of these groups, making the assignment of a single structure ambiguous. The distinct patterns of fragmentation allows for putative structural assignments to be made; however, additional confirmation of these structural assignments will demonstrate the potential for structural assignments from a straight-forward method of reducing end labeling followed by HILIC–MS/MS for the study of arabino-xylooligosaccharides.

### 3.4. Ferulate ester determination

A comparison of the observed oligosaccharide compositions from ammonia-pretreated SG SSF to dilute acid-pretreated corn fiber SSF residue (Appeldoorn et al., 2010) analysis shows a marked difference in the observation of esterified oligomers present in the mass spectra of acid-treated corn fiber versus this study with ammonia-pretreated SG. Acid-treated corn fiber has over 20 components containing feruloyl esters for oligomers of d.p. 7 or less (Appeldoorn et al., 2010); whereas in ammonia-pretreated SG feruloyl ester oligomers were absent and acetylations were present in small quantities. There are several potential explanations for this observation. The first is the inherent differences in the structural elements of corn fiber and SG, where corn fiber has more phenolic substitutions. The second is the different pretreatments used. Acid pretreatment is not able to cleave ester moieties, whereas the nucleophilic nature of the basic ammonia pretreatment can either hydrolyze esters to the free acid or convert the esters to their corresponding amides (e.g. feruloyl esters to ferulamide), thereby removing them from the polysaccharide backbone, as observed in ammonia fiber expansion treatment of corn stover (Chundawat et al., 2010). A third possible cause is that the enzyme cocktails used here possess additional esterase enzyme activities (Dien et al., 2008) that remove any phenolic esters remaining after pretreatment.

To verify the second possibility, a set of ammonia-pretreated SG samples was treated with 2 M NaOH (Sarath et al., 2007) or water to determine if any additional release of ferulic and coumaric acids occurred. Fig. 3 shows that nearly equivalent amounts of ferulamide and p-coumaramide were measured from both control and 2 M NaOH treated samples, which demonstrates that the majority of phenolic esters were modified in pretreatment and are not expected to be observed on residual oligosaccharides. No ferulic acid was observed from either the control or 2 M NaOH samples, showing that the amination of SG feruloyl esters is complete during ammonia pretreatment. In contrast, there was an increase in p-coumaric acid liberated from the NaOH-treated samples, which indicates that coumaroyl esters were not completely hydrolyzed by ammonia pretreatment; however, no masses corresponding to a p-coumaric acid containing xylooligomer were observed in this analysis. This is likely due to the auxiliary phenolic esterase activities of Multifect Pectinase (Dien et al., 2008).

### 3.5. Characterization of d.p. 5 oligomers

The complete structural characterization of all residual oligosaccharides from pretreated biomass undergoing SSF is impractical; therefore, additional analyses were aimed at identifying the largest and most prevalent oligosaccharides remaining post-fermentation. Alternate characterization of the d.p. 5 oligosaccharides could serve as a validation of the putative structures proposed from the HILIC–MS/MS analysis. To identify the most prevalent oligosaccharides in their unlabeled form, a large aliquot of fermenta-

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*Abbreviations used in table: xyl, xylopyranose; Me, methylation; Araf, arabinofuranose; PM, borodeuteride reduced and permethylated; Std, standard; d.p., degree of polymerization; parenthetic values are expected values.

b Peak E is >85% purity based on HPAGE-PAD analysis.

To verify the second possibility, a set of ammonia-pretreated SG SSF to dilute acid-pretreated corn fiber SSF residue (Appeldoorn et al., 2010) analysis shows a marked difference in the observation of esterified oligomers present in the mass spectra of acid-treated corn fiber versus this study with ammonia-pretreated SG. Acid-treated corn fiber has over 20 components containing feruloyl esters for oligomers of d.p. 7 or less (Appeldoorn et al., 2010); whereas in ammonia-pretreated SG feruloyl ester oligomers were absent and acetylations were present in small quantities. There are several potential explanations for this observation. The first is the inherent differences in the structural elements of corn fiber and SG, where corn fiber has more phenolic substitutions. The second is the different pretreatments used. Acid pretreatment is not able to cleave ester moieties, whereas the nucleophilic nature of the basic ammonia pretreatment can either hydrolyze esters to the free acid or convert the esters to their corresponding amides (e.g. feruloyl esters to ferulamide), thereby removing them from the polysaccharide backbone, as observed in ammonia fiber expansion treatment of corn stover (Chundawat et al., 2010). A third possible cause is that the enzyme cocktails used here possess additional esterase enzyme activities (Dien et al., 2008) that remove any phenolic esters remaining after pretreatment.

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treatment for maximal, rapid depolymerization of these structures. It is hypothesized that identification of an enzyme and/or pretreatment to target the dominant residual sequences can contain cross-reactivity with shorter oligomers containing similar structural motifs that will also be depolymerized.

The similarity of the fragmentation patterns of peaks A-AA, B-AA, C-AA, D-AA, and G-AA to the structurally identified components E-AA and F-AA, leads to putative structural assignments (designated by brackets in Fig. 5) for these compositions. The tentative assignments were assumed to be 1,3-linked, as this is the dominant linkage in grass xylans (Faik, 2010), and all are shown as α-1,3 linkages as previously reported in SG (Mazumder and York, 2010), despite the inability of MS to determine the anomericity of the linkages.
4. Conclusions

SG was pretreated with dilute ammonia followed by enzymatic digestion orSSF. Application of a reducing end labeling and HILIC–MS/MS strategy allowed for rapid analysis of residual xylooligosaccharides. The 20 detected oligosaccharides were ≤d.p.6 and structures were putatively assigned. All oligosaccharides were substituted, suggesting that xylanase activity was not limiting. Phenolic esters were successfully removed by the combination of pretreatment and enzymatic activity. Two native d.p. 5 AXOs were purified and putative structural assignments were confirmed. The use of HILIC–MS/MS to monitor isomeric recalcitrant xylooligosaccharides that might reflect differential susceptibility to saccharification by enzyme blends has been demonstrated.

Acknowledgements

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References


