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Influence of variable species composition on the saccharification of AFEX™ pretreated biomass from unmanaged fields in comparison to corn stover

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ABSTRACT

From the perspective of the biorefinery, the inherent heterogeneity of mixed-species feedstocks increases the apparent risk associated with their use due to the difficulty in predicting processing characteristics, potential yields and digestibility. These materials often contain species from different botanical classifications, which could affect pretreatment efficiency and Saccharification yields. For this study, we evaluated the impact of ammonia fiber expansion (AFEX™[1]) pretreatment conditions on hydrolysis sugar yields for five early successional old-field treatment replicates, each comprised of a different mixture of annual forb and grass species. Yields from these samples were also compared to a late successional old field sample and to corn stover that had been pretreated at the same conditions. Relatively high sugar yields were obtained for four of the five early successional feedstocks when pretreated at the same conditions: 2.0 g NH_3:g DM; 0.5 g H_2O:g DM; 100 °C; 30 min. It was found that glucan digestibility was strongly inversely correlated to the total lignin content; however this was not true of the xylan digestibility. The mixed-species feedstocks that had a higher grass content tended to also have a higher structural sugar content and were more digestible than forb-dominated feedstocks, thereby resulting in higher saccharification yields. Also, the grass-dominated mixed-species feedstocks were as digestible as corn stover (~80% total sugar yields). However, due to its higher structural sugar content, corn stover had higher total glucose and xylose yields (580 g/kg biomass) compared to the mixed-species feedstocks, which ranged from 290 to 470 g/kg biomass.

Abbreviations: AFEX™, ammonia fiber expansion pretreatment; CS, corn stover; DM, dry matter; E7, E21, E60, E83 and E87, early successional old field samples; GLBRC, Great Lakes Bioenergy Research Center; KBS, W.K. Kellogg Biological Station; NDF, neutral detergent fiber; LSF, late successional old field combined sample; LTER, Long-Term Ecological Research; R1-R5, GLBRC intensive site old field replicates; SF1-SF3, LTER late-successional old field treatments.

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1. Introduction

Feedstock cost is predicted to be the largest contributor to the overall cost of cellulosic ethanol production [1], and the relative importance of the feedstock will only increase as the liquid biofuel industry matures. The success of the cellulosic biofuel industry will be highly dependent on the availability of diverse sources of inexpensive, highly digestible plant materials. Potential biofuel feedstocks can be categorized in terms of their energy and chemical inputs, and diversity, ranging from high-input, low-diversity (conventional monoculture crops e.g. corn & soybeans) to low-input, high-diversity (native prairie/mixed-species grasslands) [2]. In addition to native prairie, old fields are another type of low-input natural mixed species ecosystem. Old fields are defined as agricultural fields that have been abandoned and no longer undergo reseeding or maintenance. First year production from these abandoned fields is comprised primarily of mixed-species annual weeds, which in later years typically succeeds into perennial grasses, composites and legumes, and eventually into shrubs and trees [3]. Mixed-species ecosystems such as native prairie, and to some extent old fields, provide higher value ecosystem services compared to conventional monocultures, including wildlife habitat and pollination services [4–6], water quality maintenance [7], nitrogen-fixation in fields containing legumes [8,9], improved soil carbon fixation/lower carbon debt [9–11], and decreased life cycle global warming potential and release of fine particulate matter [11–13]. However, from the perspective of the biorefinery the inherent heterogeneity of polycultures increases the apparent risk associated with these materials. Because the processing characteristics, potential yields and digestibility cannot currently be predicted or controlled, this could intensify the challenges associated with determining feedstock value and appropriate processing conditions compared to monoculture feedstocks. Additionally, because of high harvest costs associated with low predicted biomass yields, at the farm scale mixed-species fields are not considered to be economically competitive with other bioenergy cropping systems [14]. As a result, polycultures are often believed to be undesirable feedstocks.

But in spite of these issues, energy generation from mixed-species feedstocks has been examined experimentally using a number of different methods including biogas production [15], supercritical gasification [16], liquefaction [17] and co-combustion with coal [18]. To date there has been no experimental research on ethanol production via pretreatment and saccharification of mixed-species feedstocks, although two studies have reported theoretical ethanol yields [19,20]. Tilman et al. [19], in their paper on low-input high-diversity grasslands, used a generic ethanol yield (0.255 L/kg DM) that was based on a reported value for corn stover [21]. Adler et al. [20] estimated ethanol yields from conservation grasslands based on composition data with a set conversion rate and determined fermentability using *in vitro* gas production. However predicting ethanol yields solely from composition data and then drawing comparisons between feedstocks gives no indication of differences in digestibility, which can be considerable.

Because enzymatic sugar yields directly impact subsequent ethanol yields, it is important to determine whether there are general characteristics of mixed-species feedstocks that impact yields and subsequent profitability for the biorefinery. One characteristic that is unique to mixed-species feedstocks is the combination of species from different botanical classifications. The simplest classification that is often used for ecology and forage research includes the grasses and relatives (graminoids) and forbs (herbaceous non-graminoids) [22]. There is already evidence that there are distinct differences in effectiveness of chemical pretreatments and saccharification efficiency between species from these two groups [23,24]. By comparing feedstocks that contain varying distributions of the different plant classifications, it should be possible to observe whether there are classification effects on pretreatment efficiency and saccharification yields. This information might then be used to better manage a mixed-species ecosystem to maintain most of the ecological benefits of an unmanaged system while preserving most of the downstream economic benefits of an intensively managed grass monoculture.

For this study, we evaluated the sugar yields following ammonia fiber expansion (AFEX™) pretreatment and enzymatic hydrolysis of biomass harvested from five newly abandoned alfalfa fields (e.g. early succession old fields), each of which varied in its mixture of annual forb and grass species. This study was then broadened to include a single mixed-species sample that was prepared by mixing biomass harvested from three fields that had been abandoned for at least 45 years (e.g. late succession old fields) and, as a control, a separate sample of corn stover. Samples were pretreated using AFEX™ and sugar yields were measured following enzymatic saccharification. As animal feed is one possible co-product for a biorefinery, the AFEX™-treated samples were also evaluated for their forage quality as measured by a standard *in vitro* digestibility assay.

2. Methods

2.1. Sample harvest and preparation

Corn stover including cobs (CS) was provided by the Great Lakes Bioenergy Research Center (GLBRC) at the University of Wisconsin – Madison. This material was harvested on September 3rd, 2008. The corn stover was milled through a 2 mm screen using a Retsch centrifugal mill prior to composition analysis and enzymatic hydrolysis. Old field samples were provided from the five replicates (R1 – R5, treatment G9) of the GLBRC intensive experiment site at W.K. Kellogg Biological Station (KBS) of Michigan State University (MSU). During the previous year (2007) this site had been planted with alfalfa. Individual plots were 40 × 28 m and had received no maintenance or inputs in 2008 other than initial disking in May. Three quadrats of 2.0 × 0.5 m were harvested within each plot on August 20–21st, 2008. A sixth sample from the Long-Term Ecological Research (LTER) project was also provided from KBS and consisted of a mixed sample from three late-successional old fields (SF1 – SF3) that had been

abandoned and unmanaged since 1964, 1948 and 1963 respectively [25]. Five quadrats of 2.0 × 0.5 m were harvested on August 13–18th, 2008. For the GLBRC and the LTER plots, all plants rooted in each quadrat were clipped at ground level, bagged and dried at 60 °C for a minimum of 48 h. The dry weights were determined for each species, following which the quadrats for each plot were combined and milled through a 2 mm screen. The species composition of these plots is listed in the supporting information in Table A.1 (GLBRC) and Table A.2 (LTER). The biomass yield (g/m^2) for each plot was calculated from the combined quadrat yields (GLBRC: g/3 m^2 or LTER: g/5 m^2).

2.2. Composition analysis

Biomass moisture content was determined using a moisture analyzer (A&D, Model MF-50; San Jose, CA). The dry matter (DM) composition of each sample (extractives, ash, lignin, glucan and xylan content) was determined based on the NREL standard protocols [26]. The acid insoluble lignin analysis method was modified to use 47 mm, 0.22 μm pore-size, mixed-cellulose ester filter discs (Millipore Corp.; Bedford, MA) during the filtration step instead of fritted crucibles. Due to problems with burning, these discs with the filtered lignin residue were dried overnight in a desiccator prior to weighing rather than in a vacuum oven. The nitrogen contents of the extracted and unextracted samples were determined via the combustion method for nitrogen determination [27] using a Skalar Primacs SN Total Nitrogen Analyzer (Breda, The Netherlands). Nitrogen values were multiplied by 6.25 to determine the crude protein content. This conversion factor assumes that 16% of the protein is nitrogen and that there is negligible non-protein nitrogen present in the biomass. This factor varies with different types of plant samples due to differences in protein structure, however accurate determination of this factor requires a complete amino acid analysis [28]. As protein content does not directly impact our results and conclusions, we assume that the standard factor of 6.25 allows for a reasonable approximation. The protein that was removed during extraction steps was subtracted from the total extractives content.

2.3. Effect of pretreatment conditions on hydrolysis yields from early successional samples

AFEX™ pretreatment of the early successional old field samples was conducted using 3.0 g DM of sample in 22 mL reaction vessels as outlined by Bals et al. [29]. AFEX™ conditions for the pretreatment optimization experiments were chosen using a three level Box–Behnken statistical design and the parameters ranged from 0.5 to 2.0 g NH_3:g dry matter (DM), 0.5 to 2.0 g H_2O:g DM, 90 to 180 °C, and 5 to 30 min residence time. The central design point was conducted in triplicate to give a total of 24 different conditions and 27 experiments for each sample. In an attempt to improve the fit of the statistical models, three additional experimental points were tested (Table A.3). Enzymatic hydrolysis was then conducted on the samples as described in Section 2.5 and the enzymatic hydrolysis total sugar conversions (g oligomeric and monomeric glucose and xylose released per g sugar theoretically present in the untreated, dry biomass) were used as the metric of pretreatment efficacy.

The following polynomial quadratic equation of the AFEX™ pretreatment conditions was fitted to the enzymatic hydrolysis total sugar yield data (combined monomeric and oligomeric glucose and xylose in terms of the theoretical maximum) using Minitab15 Statistical Software (2006 Minitab Inc, Pennsylvania, USA):

$$Y = a_0 + \sum_{i=1}^{n} a_i x_i + \sum_{i=1}^{n} a_{ii} x_i^2 + \sum_{\substack{i,j=1 \\ i \neq j}}^{n} a_{ij} x_i x_j \quad (1)$$

where Y is the sugar yield; a_0 is the regression constant; a_i is the linear regression coefficient for the ith parameter; a_{ii} is the quadratic regression coefficient for the ith parameter; a_{ij} is the interaction coefficient for the ith and jth parameters; x_i and x_j are the values of the *ith* and *j*th parameters; and *n* is the number of factors, which in this case is 4. Coefficients with $p > 0.1$ were removed stepwise from the model, beginning with the largest *p*-value. The resulting coefficients and the adjusted and predictive R^2 values for each sample are reported in Table A.4.

An attempt was made to determine the optimum pretreatment conditions for each early successional sample using the polynomial models. However, the predictive capability of the models was very poor, as indicated by the low predictive R^2 values (Table A.4) and we were unable to determine the optimum pretreatment conditions. So for the later experiments, the same pretreatment condition was chosen for all of the feedstocks: 2.0 g NH_3:g DM; 0.5 g H_2O:g DM; 90 °C; 30 min. This set of conditions was chosen as it resulted in comparatively high sugar yields for four of the five feedstocks (the highest sugar yields for E7 and E87 and among the top five highest yields for E60 and E83) (Table A.3, Fig. A.1). As the last sample, E21, performed better when pretreated at slightly higher temperatures, we raised the selected temperature slightly to 100 °C.

2.4. AFEX™ pretreatment for comparison of early successional old field replicates with late successional old field and corn stover samples

AFEX™ was performed on the early successional samples, the late successional sample (LSF) and corn stover (CS) in a 300 mL stainless steel (#316) Parr reactor according to the method detailed in Bals et al. [30]. The conditions were chosen because of the relatively high hydrolysis yields for the early successional feedstocks as determined in the previous section: 2.0 g NH_3:g DM, 0.5 g H_2O:g DM, 100 °C, 30 min.

2.5. Enzymatic hydrolysis

Samples for pretreatment characterization were hydrolyzed in 20 mL screw-cap vials at 1.5% total sugar (glucan + xylan) loading and a total volume of 15 mL. For the feedstock comparison experiments, samples were hydrolyzed in 20 mL screw-cap vials at 3% solids loading and a total volume of 10 mL. All samples were adjusted to a pH of 4.8 using 1 M citrate buffer solution. To prevent fungal and bacterial

contamination during enzymatic hydrolysis, cycloheximide and tetracycline were loaded at a final concentration of 30 μg/mL and 40 μg/mL, respectively.

Accelerase®1000, Multifect® Xylanase, and Multifect® Pectinase (Genencor Division of Danisco US, Inc.) were used for all of the hydrolysis experiments. The protein content of each of the enzymes are as follows: Accelerase®1000 (84 mg protein/mL), Multifect® Xylanase (32 mg protein/mL), and Multifect® Pectinase (61 mg protein/mL). Enzyme protein content was determined from total N analysis using the Dumas method for combustion of nitrogen to NO_x [27] following trichloroacetic acid (TCA) precipitation to remove non-protein nitrogen [31].

For the pretreatment characterization experiments, Accelerase®1000 was added at 26.8 mg protein/g glucan in the untreated biomass, and Multifect® Xylanase and Multifect® Pectinase were each added at 7.5 mg protein/g xylan in the untreated biomass. For the feedstock comparison experiments, enzymes were loaded at 6.0 mg total protein/g DM sample and in the following relative percentages by protein mass, which were similar to those found effective for Alamo switchgrass (unpublished data): Accelerase®1000 (42%), Multifect® Xylanase (24%), and Multifect® Pectinase (34%).

Enzymatic hydrolysis for all experiments was conducted in a New Brunswick Scientific (Edison, NJ) shaking incubator at 50 °C and 200 rpm. Samples were taken at 72 h of enzymatic hydrolysis for both monomeric and oligomeric sugar analysis, as detailed below. Samples taken for monomeric sugar analysis were heated at 100 °C for 15–20 min, cooled in the freezer, and then centrifuged at 15,000× *g* for 5 min. The supernatant was filtered into HPLC shell vials using a 25 mm, 0.2 μm polyethersulfone syringe filter (Whatman Inc. Florham Park, NJ) then stored at −20 °C until further sugar analysis.

2.6. Oligomeric sugar analysis

Oligomeric sugar analysis of the pre-wash liquid and hydrolysate was conducted using a scaled-down version of the standard NREL method for oligomeric sugar determination of liquid streams [32]. The modified method was identical except that it was scaled down to use 2 mL of sample and run in duplicate in 10 mL screw-cap culture tubes. Instead of being autoclaved, the tubes were incubated in a 121 °C bench-top hot plate for 1 h, cooled on ice, and the liquid was filtered into HPLC vials. The oligomeric sugar concentration was determined by subtracting the monomeric sugar concentration of the non-hydrolyzed samples from the total sugar concentration of the acid hydrolyzed samples.

2.7. HPLC analysis

Sugar content of all composition analysis, wash liquid, and hydrolysate samples were determined using a Bio-Rad (Hercules, California, USA) Aminex HPX-87H column equipped with appropriate guard columns. Degassed 5 mM aqueous H_2SO_4 was used as the mobile phase and the column temperature was held at 60 °C. The reported total sugar (glucose or xylose) concentration was recalculated as the sum of the average monomeric and the average oligomeric sugar concentration for each sample. Because of the presence of soluble sugars in the biomass, the glucose percent conversions were calculated using the following equation, where 0.9 corrects for addition of the water molecule upon hydrolysis of glucan to glucose:

$$\text{Glucose Conversion}(\%) = \frac{\text{Glu}_{HY}}{\text{Gln}/0.9 + \text{Glu} + \text{Suc}^{*}(180.2/342.3)} \quad (2)$$

Glu_{HY} = hydrolysate glucose mass yield (g/kg dry biomass), Gln = biomass glucan content (g/kg dry biomass), Glu = biomass soluble glucose content (g/kg dry biomass), Suc = biomass soluble sucrose content (g/kg dry biomass), 180.2/342.3 = correction for glucose contribution by sucrose (molecular weight of glucose/molecular weight of sucrose).

2.8. In vitro *rumen digestibility and neutral detergent fiber determination*

In vitro rumen digestibility was performed in triplicate based on the method reported in Tilley and Terry [33] using rumen fluid obtained from a fistulated dairy cow. Neutral detergent fiber (NDF) concentration (without amylase digestion) was determined for each sample (treated and untreated) after 0 h and 48 h of incubation. The amount of NDF digested after 48 h was determined as the difference between these two values.

2.9. Statistical analysis

All statistical analyses, except for Pearson's correlation coefficients, which were calculated using Excel (Microsoft® Office Excel® 2007), were performed using Minitab15 Statistical Software (2006 Minitab Inc, Pennsylvania, USA). This included the response surface optimization (as described previously in Section 2.3), linear regressions, and Tukey's pairwise comparisons.

3. Results

3.1. Feedstock characteristics and plot yields

The species mass composition of the early successional old field samples (E7, E21, E60, E83, E87) consisted almost entirely of annual forbs and grasses (Table A.1). These samples have been labeled for ease of analysis in terms of their % grass content on a mass basis. There were 7 to 14 species in each replicate (Table 1), however five main species contributed > 95% of the total mass for all five replicates. The late successional old field sample (LSF) was composed of three different replicate plots with 20 to 45 species in each replicate (Table 1). Seven of these species comprised 86% of the combined sample mass (Table A.2). There was no distinct trend between plant classification and biomass yield (g/m^3) for the five early successional replicates. The late successional replicate that contained mostly grass had a much higher biomass yield than the other two samples that were composed predominantly of woody species.

The dry matter composition of each sample is shown in Table 2. The early successional replicates that had lower grass contents (E7 and E21) also had lower structural sugar contents

Table 1 – Species composition, biomass yield and distribution for the early successional old field replicates and late successional old field replicates.

Expt. Name	Field Name	# of species				Biomass yield (g/m^3)				Mass distribution (%)		
		Grass	Forb	Woody	Total	Grass	Forb	Woody	Total	Grass	Forb	Woody
Early Successional Old Field Treatment Replicates												
E7	R1	4	10	0	14	54	673	–	727	7%	93%	–
E21	R4	1	6	0	7	210	799	–	1009	21%	79%	–
E60	R5	4	4	0	8	516	347	–	863	60%	40%	–
E83	R3	3	8	0	11	753	158	–	911	83%	17%	–
E87	R2	4	4	0	8	576	88	–	664	87%	13%	–
Late Successional Old Field Replicates												
LSF	SF1	6	14	0	20	213	47	–	260	82%	18%	–
	SF2	5	10	13	28	7	1	23	31	21%	4%	75%
	SF3	3	25	17	45	<1	17	47	64	0%	27%	73%

compared to the samples that had higher grass contents (E60, E83, E87, and LSF). Corn stover had a significantly higher structural sugar content compared to all other samples (65.5% of the total dry mass). The Klason lignin content of the different samples ranged from 13.6% to 18.3% of the total dry biomass and was inversely correlated with the sample grass content ($r = -0.95$, $p = 0.01$, $n = 7$) when measured on a cell wall basis (generalized as the sum of the composition structural carbohydrates and Klason lignin). Ash content and soluble glucose and sucrose content were higher for the early successional samples compared to the late successional and corn stover samples.

3.2. *Relationship of AFEX™ pretreatment conditions to hydrolysis yields from early successional samples*

Each of the five early successional replicates was pretreated with AFEX™ using various ammonia and water loadings, temperatures, and residence times. Each sample was tested using 30 different sets of pretreatment conditions, except for E87 where one result was omitted from the analysis due to a high residual value that corresponded with abnormally low sugar yields. Information on the specific conditions examined and the resulting total sugar yields are reported in the supplemental information (Table A.3). The sugar conversion varied significantly for each feedstock across the pretreatment conditions (Fig. 1) and trends appeared to be associated in part with forb vs. grass-dominated samples. E7 had the lowest conversions of monomeric and oligomeric sugars (glucose: 46–67%; xylose: 46–82%), followed by E21 (glucose: 54–80%; xylose: 57–80%). E60, E83, and E87 all had similar ranges for xylose release (E60: 69–102%; E83: 67–98%; E87: 65–102%). The yields that are slightly over 100% are likely due to small errors that occurred during compositional analysis. Glucose release tended to increase from E87 to E83 to E60 and is more evident from Fig. 1 than from the range of glucose yields (E60: 65–91%; E83: 59–84%; E87: 56–82%). Pretreatment improved sugar yields in all cases compared to the untreated feedstock, for which sugar yields ranged from 32.2% for E7 to 46.3% for E83 (data not shown).

Each feedstock showed a significant linear correlation ($p < 0.05$) between glucose yield and xylose yield (Fig. 1). However, there was a much larger spread for the data points for the two feedstocks that had the lowest grass content (<20% grass), E7 and E21 ($r \sim 0.45$). The correlation between glucose and xylose release was both highly linear ($r > 0.90$) and highly significant ($p < 0.001$) for the feedstocks with the higher grass content (>60% grass), E60, E83 and E87.

Due to high error associated with the constructed response surface models (Table A.4), the optimum pretreatment conditions could not be accurately determined for the feedstocks. Because of this, the raw data were used to select a pretreatment condition that gave reasonably high yields for most of the feedstocks and could be used for further experiments. The pretreatment condition chosen was 2.0 g NH_3:g DM, 0.5 g H_2O:g DM; 100 °C and 30 min residence time. This condition was chosen as a similar pretreatment condition at 90 °C resulted in the highest sugar yields for E7 and E87 and among the top five sugar yields for E60 and E83 (Table A.3, Fig. A.1). Of those materials examined, only E21 did not obtain high yields when operated at these conditions (lower than the optimum condition by 50 g sugars released per kg untreated dry biomass), and it tended to require slightly higher temperatures compared to the other feedstocks. This difference appears unrelated to the differences between forbs and grasses. For example, sample E7, the other forb-dominated feedstock, has the same optimum as E87. The difference may instead be related to the specific species that were present in the mixture. E21 contained a much larger percentage of *Amaranthus retroflexus* L. (redroot pigweed), and it may be that this forb is more indigestible than the forb species present in the E7 sample.

3.3. In vitro *digestibility*

The sale of pretreated biomass as an animal feed co-product has the potential to improve the process economics in a biorefinery [34], so the *in vitro* rumen digestibility was determined for the untreated and the pretreated old field replicates (Table 3). In all cases, AFEX™ treatment decreased neutral detergent fiber (NDF) content compared to untreated controls, a difference of 60–104 g NDF/kg DM, indicating an increase in digestibility of pretreated materials. Except for the E60 sample, where almost 26% more was digested for the AFEX™-treated sample, there was very little difference between the untreated and AFEX™-treated samples in the amount of NDF

Table 2 – Composition analysis data as % of total dry matter (DM). The standard error is reported in parenthesis and represents three replicates. LSF = late successional old field sample.

Feedstock		Structural carbohydrates							Water Extractives[h]					
		Glucan	Xylan	Arabinan	Total	Klason Lignin	Ash	Crude Protein[g]	Acetyl	Glucose	Sucrose	Other	Ethanol Extractives[h]	Total mass
Early Successional Old Field	E7	23.0^{d} (0.1)	13.9^{f} (0.1)	2.1^{c} (0.04)	39.0^{e} (0.2)	18.3^{a} (0.1)	9.7^{a} (0.2)	11.2^{a} (0.1)	1.9^{b} (0.1)	1.8^{c} (0.2)	1.8^{d} (0.4)	14.2 (0.6)	3.4 (0.01)	101.3 (0.8)
	E21	26.4^{e} (0.7)	15.1^{e} (0.1)	2.1^{c} (0.1)	43.7^{d} (0.7)	16.3^{b} (0.3)	8.8^{bc} (0.04)	5.5^{d} (0.1)	2.0^{b} (0.1)	3.2^{a} (0.3)	3.3^{c} (0.7)	11.8 (1.0)	4.8 (0.3)	99.8 (1.5)
	E60	29.1^{b} (0.8)	17.2^{d} (0.6)	2.5^{b} (0.2)	48.9^{bc} (1.0)	16.9^{b} (0.3)	9.0^{bc} (0.1)	4.6^{f} (0.1)	1.7^{c} (0.1)	2.5^{b} (0.1)	3.1^{c} (0.1)	13.5 (0.3)	2.8 (0.2)	103.1 (1.1)
	E83	29.6^{b} (0.9)	18.7^{b} (0.5)	2.1^{c} (0.2)	50.5^{b} (1.0)	14.0^{d} (0.1)	8.5^{c} (0.1)	4.9^{e} (0.02)	1.8^{bc} (0.1)	2.4^{b} (0.05)	5.8^{a} (0.1)	11.2 (0.5)	2.9 (0.2)	102.0 (1.2)
	E87	30.7^{b} (0.7)	17.8^{cd} (1.4)	2.5^{b} (0.1)	50.9^{b} (1.6)	13.6^{d} (0.5)	9.2^{ab} (0.1)	6.2^{c} (0.2)	1.6^{cd} (0.1)	2.2^{bc} (0.05)	4.9^{b} (0.2)	13.3 (2.0)	3.6 (1.4)	105.5 (2.9)
LSF		26.5^{c} (0.7)	18.2^{bc} (0.4)	2.6^{b} (0.1)	47.3^{c} (0.8)	14.9^{c} (0.1)	5.7^{d} (0.1)	8.5^{b} (0.1)	1.5^{d} (0.1)	1.2^{d} (0.1)	0.2^{e} (0.03)	21.6 (0.4)	ND	100.9 (0.9)
Corn Stover		36.8^{a} (0.5)	25.0^{a} (0.2)	3.5^{a} (0.1)	65.5^{a} (0.6)	14.9^{c} (0.1)	4.6^{e} (0.5)	NM	2.5^{a} (0.1)	0.4^{e} (0.02)	0.3^{e} (0.04)	8.0 (0.3)	2.6 (0.1)	98.6 (0.8)

a–f Means with different superscripts in each column are significantly different at the 95% confidence level using Tukey's pairwise comparison.
g Crude protein content was not determined for the corn stover sample.
h The ethanol extraction was performed, but not quantified for the LSF sample. "Other Water Extractives" also includes the amount of ethanol extractives.

digested by the rumen microbes. The percent of digested NDF for the AFEX™-treated samples, from largest to smallest was E87 > E83 > E60 > E21 > E7, which corresponded with decreasing grass content in the samples. In all cases, AFEX™ improved overall NDF digestion compared to untreated samples. The materials with the lowest grass content had the largest improvement in digestibility due to AFEX™ pretreatment. One issue with NDF determination is that pectins, which are present in much larger amounts in forbs, are digested by the NDF process, resulting in a lower estimate of NDF value than is actually the case [35]. As pectins can be utilized by ruminants, this may result in underestimating the value of the forb-dominated samples as an animal feed.

3.4. *Comparison of early successional old field replicates with late successional old field (LSF) and corn stover (CS) samples*

The glucose, xylose and total sugar hydrolysis yields (g/kg dry biomass) are reported in Fig. 2. All of the total sugar yields were significantly different except for E60, E83 and E87, and can be arranged in decreasing order from CS > (E87, E83, E60) > LSF > E21 > E7. Both LSF and CS had a large amount of xylo-oligomers remaining, indicating that for these samples the enzyme combination used was not adequate to convert all of the xylo-oligomers to xylose. None of the samples reached the theoretical maximum sugar yield, except for the LSF xylose yield. The grass-enriched samples (E60, E83, and E87) had higher sugar mass yields and conversion efficiencies compared to the forb-enriched samples (E7 and E21). Based on Tukey's test ($p < 0.05$), the different feedstocks can be divided into two statistically different categories based on the total sugar conversion efficiencies: the low grass content samples (<20% grass): E7 and E21; and the corn stover and high grass content samples (>60% grass): E60, E83, E87, LSF and CS.

When the total (monomeric + oligomeric) sugar yields were plotted against the lignin content on a cell wall basis, there was some correlation between xylose release and lignin content (Fig. 3), but it was not statistically significant. However, there was a stronger and more significant correlation between glucose release and lignin content, which increased considerably when sample E60 was removed from the analysis.

4. Discussion

Old field mixed-species samples are considered unsuitable for biofuel production due to low predicted yields per hectare that cause the cost to the farmer to become prohibitively expensive [14]. Additionally, as harvestable yield per unit area decreases, the collection radius for the biorefinery increases, leading to significantly higher transportation costs. The large-scale biomass yields from the old field samples can be extrapolated from the small-scale sample data to estimate the biomass yields for a larger harvested area; however these numbers should be viewed with caution due to the potential for over- or under-estimation of yields. The early successional old field replicates had rather high extrapolated yields (6.6–10.1 Mg/ha) that are promising but would probably not be

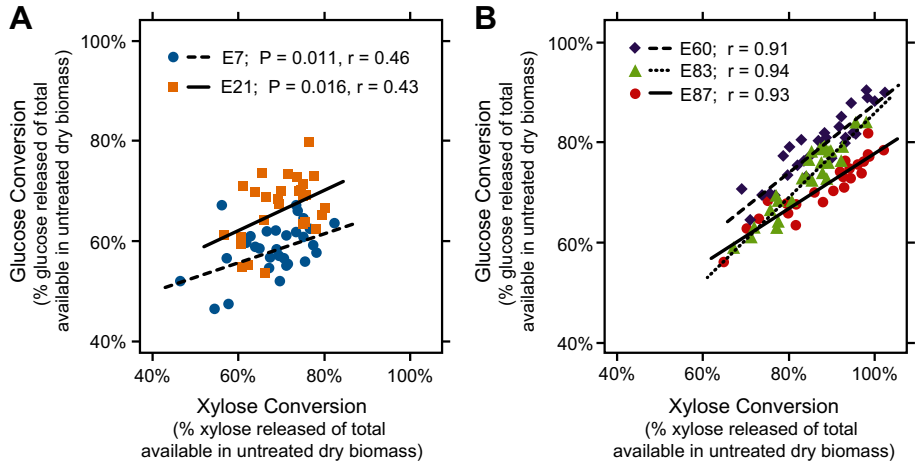

Fig. 1 – Relationship between enzymatic hydrolysis glucose and xylose yields for the GLBRC old field replicates. A: Forb-dominated samples (E7 and E21). B: Grass-dominated samples (E60, E83, and E87). Yields are calculated as the total monomeric and oligomeric sugar solubilized based on the total sugar theoretically available in the untreated dry biomass. For the E60, E83, and E87 regressions, ($p = 0.000$). Each data point represents one of 30 different pretreatment conditions (except for E87, which only has data for 29 conditions due to one significant outlier).

Table 3 – *In vitro* rumen digestibility of untreated and AFEX™-treated early successional old field samples.

	Untreated		AFEX™-treated			
	Average	SEM[a]	Average	SEM[a]	Diff[b]	% Inc[c]
Initial NDF (g NDF/kg DM)						
E7	528	6.6	442	4.5	86	–
E21	574	3.0	475	4.0	99	–
E60	586	1.9	526	5.9	60	–
E83	597	4.9	509	5.8	87	–
E87	595	2.7	491	0.9	104	–
Digested (g NDF/kg DM)[d]						
E7	130	9.7	133	11.8	3	2%
E21	205	7.9	212	4.6	7	4%
E60	260	2.4	327	6.5	67	26%
E83	290	15.2	313	8.2	23	8%
E87	313	6.6	323	2.8	10	3%

	Average	SEM[a]	Diff[b]	% Inc[c]	% Digested[f]
Total Digested (g NDF/kg DM)[e]					
E7	218	12.7	89	68%	41%
E21	311	3.7	107	52%	54%
E60	387	3.3	127	49%	66%
E83	400	7.6	111	38%	67%
E87	427	3.8	114	37%	72%

a Standard error of the mean based on three replicates.
b Difference between AFEX™-treated and untreated samples.
c Percent Increase in treated sample over untreated sample.
d NDF digested during 48 h *in vitro* rumen digestion.
e Total NDF removed for AFEX™-treated samples, including both the amount removed due to AFEX™ and the amount digested during *in vitro* rumen digestion.
f Total amount of NDF removed due to AFEX and during *in vitro* rumen digestion as a percentage of untreated NDF.

sustainable over multiple years without additional inputs. Additionally, the yields may be high because the GLBRC intensive plot, while unfertilized, was previously planted to alfalfa, and there may have been residual nitrogen remaining in the soil due to nitrogen fixation. The key species that grew in the old field plots, particularly *A. retroflexus* L. (redroot pigweed) and *Chenopodium album* L. (common lambsquarters) are extremely responsive to soil nitrogen levels and are highly efficient in nitrogen uptake [36]. Any fixed nitrogen remaining from the alfalfa would have been readily utilized by these plants, increasing production of biomass, assuming the presence of adequate amounts of any other potentially limiting nutrients. The late successional (SF) replicates, harvested from sites that have been abandoned and unmanaged for ~50 years, may give a more realistic estimate of long term yields from fields that contain very few nitrogen-fixing species (0.3–2.6 Mg/ha, *extrapolated*). The biomass yields from all three late successional (SF) replicates have continually decreased since 1993, particularly SF2 and SF3 that have become dominated by woody species (>70% of the total biomass) [25].

For mixed-species feedstocks, the relationship between glucose and xylose yields for different pretreatment conditions, the sugar conversion efficiencies (a measure of feedstock digestibility), and the *in vitro* digestibility were related to the grass content of the sample. Based on the results shown, the old field replicates can be categorized into either the grass-dominated samples (≥60% grass species on a mass basis): E60, E83 and E87; or the forb-dominated samples (≤20% grass species on a mass basis): E7 and E21. The differences observed between the two groups could be explained by the fact that grasses and their relatives (commelinids) and forbs (non-commelinids) have very different cell wall chemistries, including the type, structure, and localization of hemicellulose as well as lignin monomer content and composition [37–42]. Release of xylose from the cell wall of grasses [29] or

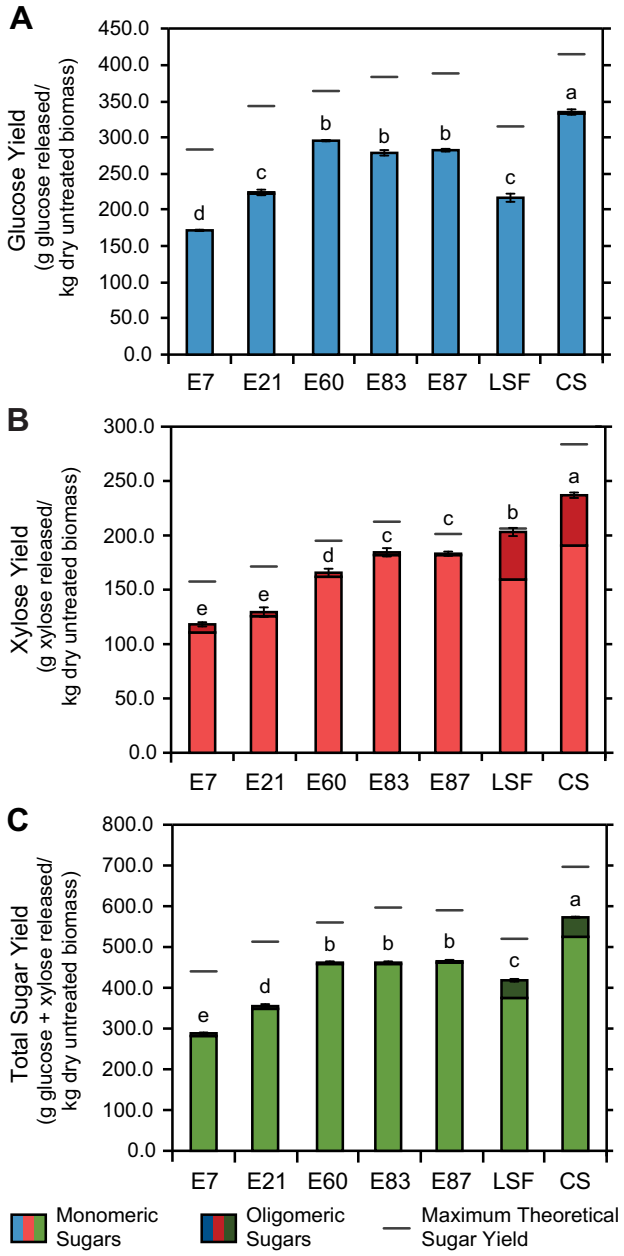

Fig. 2 – Comparative monomeric and oligomeric sugar yields. A: Glucose yields. B: Xylose yields. C: Total sugar (glucose + xylose) yields. Oligomeric sugars are reported in monomeric equivalents. The maximum theoretical sugar yield is the maximum amount of glucose, xylose or total sugars that could be released from the untreated dry biomass. Total (monomeric and oligomeric) sugar yields with different letters are statistically different based on Tukey's test ($p < 0.05$). E7, E21, E60, E83, E87 = early successional old field replicates from the GLBRC intensive site; LSF = LTER late-successional old field combined sample; CS = corn stover. Each sample was subjected to the same pretreatment and enzymatic hydrolysis conditions.

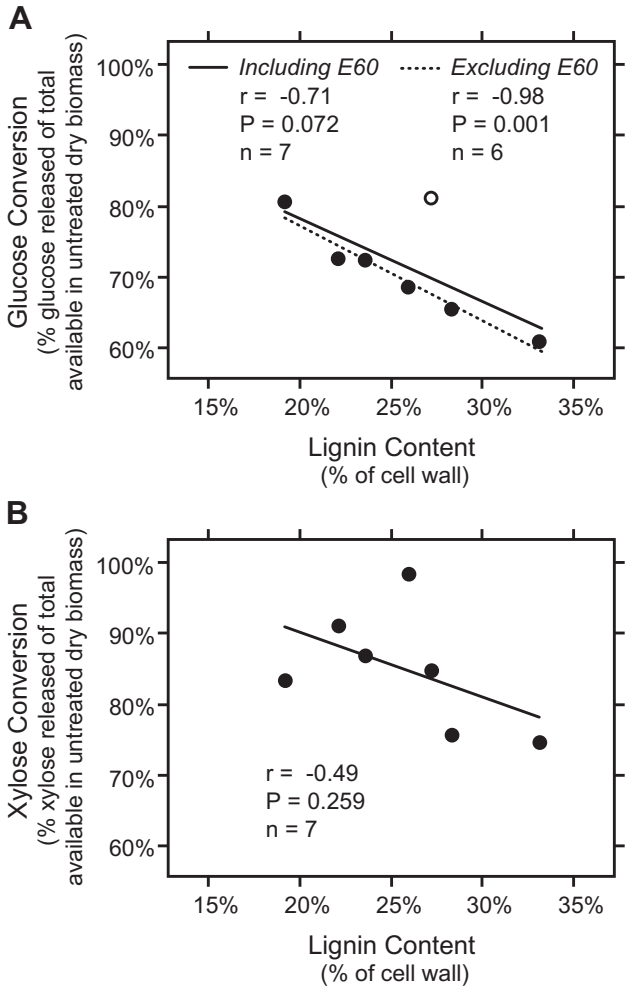

Fig. 3 – Correlation of glucose (A) and xylose (B) percent conversion (g sugar released/g theoretically available in untreated dry biomass) to Klason lignin content on a cell wall basis for the early successional old field replicates, late-successional old field sample, and corn stover. The solid line represents the correlation when the old field replicate E60 (represented by an open circle) is included in the analysis and the dashed line indicates the correlation when sample E60 is removed from the analysis.

grass-dominated samples is strongly correlated to increases in glucose yields. However, the relationship was not as strong for the forb-dominated samples. This may indicate that compared to the grass cell wall, the release of glucose from the forb cell wall depends more on other factors than on the presence or absence of xylan. This is supported by research on dilute acid pretreatment, which selectively removes xylan from the cell wall [43]. Dien et al. [23] observed that a dilute acid pretreated forb (alfalfa stems) had a lower glucan conversion efficiency that was not dependent on the release of non-glucan sugars, compared to two pretreated grasses. They hypothesized that the stem cellulose that was not digested may be more closely associated with lignin, which, unlike in the case of commelinids, is not evenly distributed within the non-commelinid cell wall [44,45].

It is well documented that total lignin content is negatively correlated with both rumen degradability [46,47] and enzymatic saccharification [48,49]. However, it is important to note that lignin content, while correlated with digestibility, is in

itself not a sufficient indicator for biomass digestibility, particularly if one is interested in total sugars. Based on our results, the cell wall lignin content was strongly negatively correlated with glucose digestibility, but not xylose digestibility, and the correlation was stronger when E60 was not included in the analysis. This is interesting because there are indications that there is something different about E60 compared to the other samples that were tested. E60, which is the most digestible sample and contains nearly 60% grass, and E21, which is significantly less digestible and contains only 21% grass, have statistically identical Klason lignin contents (Table 1). The large difference in digestibility between these samples could be attributed to differences between forbs and grasses, such as lignin distribution within the cell wall, or there might be some unique characteristic about E60 that makes it more responsive to pretreatment compared to the other samples. E60 had the largest increase in glucose release between the untreated and the optimally pretreated materials (45% increase versus 34–39% for the other old field replicates). E60 is also the only sample that experienced a large increase in rumen digestibility because of AFEX™ pretreatment (Table 3). Another possibility is that there are different types and quantities of covalent linkages between cellulose and lignin in the different plants. Recent evidence indicates the presence of oxygen-containing linkages (ether or ester) between lignin and cellulose in corn leaves [50]. Ester linkages are known to be more readily cleaved under alkaline conditions [51], so if more of these were present linking the cellulose and lignin in the E60 sample, then this might partially explain an increase in digestibility.

At the same pretreatment conditions and enzyme loading, the high grass content samples, CS, E87, E83, E60 and LSF, had statistically identical sugar conversions (g sugar released/g sugar theoretically available), approximately 80% of the total sugars. Woody species have previously been shown to result in lower yields compared to cereal residues [52], but their presence in the LSF sample did not reduce the digestibility. This was largely because a high xylose yield compensated for a lower glucose yield (Fig. 2). Glucose yields for the mixed-species samples ranged from 170 to 300 g/kg biomass and total sugars ranged from 290 to 470 g/kg biomass. This is in comparison to the glucose and total sugars released from corn stover: 340 and 580 g/kg biomass, respectively. While the corn stover had the highest sugar yields, this was not because it was significantly more digestible than the other materials, but rather because it had significantly higher cell wall sugar content. This may be partly related to the relative maturity of the samples. Even though they were harvested around the same time of the year (albeit in different locations), the corn stover may have been more mature than the old field replicates, because the cell wall characteristics (high glucan, xylan and lignin with low ash and soluble sugars) were typical of a more mature plant [42]. If the old field samples had been allowed to mature further and harvested in October or November, it is possible that the structural sugar content and subsequent sugar mass yields would have been higher.

AFEX™ pretreatment has been examined as a possible treatment for forages due to its similarity to the ammonia treatment that has been used by farmers for decades to increase digestibility of ruminant feeds [30]. The increase in digestibility we observed due to AFEX™ pretreatment of the mixed-species feedstocks is quite similar to that observed by Bals et al. for the more readily digestible forages [30]. As they concluded, it seems unlikely that the increase in digestibility observed for these types of materials is large enough to warrant the use of the pretreatment. It may be that if the mixed-species feedstocks we examined were allowed to mature further and harvested in late October or November, the recalcitrance would increase and there would be more of an effect by pretreatment on digestibility which would warrant the use of the pretreatment.

From the biorefinery standpoint, grass-dominated feedstocks will likely be more profitable because of their higher mass sugar yields that are directly related to a higher cell wall sugar content and greater digestibility. C4 grasses have previously been predicted to produce the greatest amount of ethanol per hectare [20]. It might be possible to manage mixed-species sites to have a majority of grass species, which could have a beneficial impact on the biomass yield, but this would likely come with some cost to the species diversity [20]. Additionally, although a farmer may not wish to replace their corn crop with a mixed-species feedstock based on the economic considerations, it may be worthwhile to harvest one of their abandoned fields or convert it to a mixed-species stand, such as native prairie or mixed native grasses, for use as a biofuel feedstock. The addition of a small amount of fertilizer to the fields and/or the incorporation of legumes in the species mixture may also increase yields to the extent that the fields become more profitable for the farmer, while still limiting the cost associated with inputs and fertilizer-related environmental impacts.

Mixed-species feedstocks have been considered unsuitable for biofuel production because of their inherently heterogeneous nature. However, no lignocellulosic feedstock will be homogenous, even monocultures of intensively selected varieties. This is because the feedstock characteristics and digestibility are highly dependent on the fraction of the plant considered (leaf, stem, etc.), maturity, location, and environmental conditions experienced during growth [29,53,54]. Large biorefineries, because of transportation costs and supply limitations, will need to accept a wide variety of feedstocks and have a method to quickly determine their value. However, it may be feasible to process most feedstocks at similar conditions to obtain the highest yields. Four of the five feedstocks gave fairly high yields at the chosen operating condition. It is very likely, given the example of E21, that there will be some feedstocks that do not process as well at the chosen condition. The operator will need to decide which is more cost effective: changing operating conditions for different materials to maximize yields, operating at the same conditions for all feedstocks and potentially losing yields for some of them, or perhaps blending feedstocks to make up for deficiencies in low-yielding materials and improve overall process stability.

While dedicated, managed monocultures will likely give higher yields and be more reliable once established, polycultures can be equally digestible, and could be used as a supplemental feedstock. By implementing this approach, the biorefinery can diversify their feedstock supply and increase source security, while simultaneously decreasing the collection area and reducing transportation costs. At the same

time, mixed-species feedstocks provide more valuable ecological services compared to monocultures and have much vto offer the lignocellulosic biorefinery and surrounding communities and landscapes.

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Appendix A. Supporting information

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.biombioe.2011.12.036.

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