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Optimizing Harvest of Corn Stover Fractions Based on Overall Sugar Yields following AFEX Pretreatment and Enzymatic Hydrolysis

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Abstract

Corn stover composition changes considerably throughout the growing season and also varies between the various fractions of the plant. These differences can impact optimal pretreatment conditions, enzymatic digestibility, and maximum achievable sugar yields in the process of converting lignocellulosics to ethanol. The goal of this project was to determine which combination of corn stover fractions provides the most benefit to the biorefinery in terms of sugar yields, and to determine the preferential order in which fractions should be harvested. Ammonia Fiber Expansion (AFEX) pretreatment followed by enzymatic hydrolysis was performed on early and late harvest corn stover fractions (stem, leaf, husk and cob). Sugar yields were used to optimize scenarios for the selective harvest of

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corn stover assuming 70% or 30% collection of the total available stover. The optimal AFEX conditions for all stover fractions, regardless of harvest period, were: 1.5 (g NH_3 g^{-1} biomass), 60% moisture content (dwb), 90°C, and 5 min residence time. Enzymatic hydrolysis was conducted using cellulase, β-glucosidase, and xylanase at 31.3, 41.3, and 3.1 mg g^{-1} glucan, respectively. The optimal harvest order for selectively harvested corn stover (SHCS) was husk > leaf > stem > cob. This harvest scenario, combined with optimal AFEX pretreatment conditions, gave a theoretical ethanol yield of 1.62 t^{-1} ha and 0.72 t^{-1} ha for 70% and 30% corn stover collection, respectively. In the end, resources may be more effectively spent on improving sustainable harvesting and optimizing biomass processing rather than focusing on the selective harvest of specific corn stover fractions.

Keywords: AFEX; *Zea mays*; Corn stover; Enzymatic hydrolysis; Fractionation; Harvest scenario; Agricultural residue

1. Introduction

Corn stover, the aboveground, vegetative portion of maize (*Zea mays* L.), makes up roughly 80% of all agricultural residues produced in the U.S. [1]. Data on annual corn stover production in the U.S. are not readily available, so various sources have independently estimated that anywhere from 200 to 250 million dry tons of corn stover are produced per year [1-4]. Sustainably harvested corn stover could be used as a feedstock for a variety of applications including lignocellulosic ethanol production. It has been estimated that 35 GL of ethanol per year could be produced from U.S. corn stover assuming 40% of the stover is collected [5]. It is widely acknowledged that a percentage of the produced corn stover should be retained on the field following harvest in order to prevent soil erosion and maintain soil organic carbon (SOC) levels. The amount that can be sustainably harvested is highly debated and depends heavily on cropping practices, climate, topography, and soil type [4, 6-8]. Because of these factors, estimates on the amount of corn stover that can be sustainably harvested vary widely, anywhere from 20-80% [1, 5-6].

Lignocellulosic feedstocks, such as corn stover, derive their name from the three primary components of the plant cell wall: cellulose, hemicellulose and lignin. The complex polysaccharides, cellulose and hemicellulose, must be broken down into monomeric form (primarily glucose and xylose) prior to microbial fermentation into ethanol or other valuable products. High sugar yields require a two-step process: generally a chemical and/or physical pretreatment step followed by enzymatic hydrolysis of the polysaccharides. Previous work has shown that ammonia fiber expansion (AFEX) is a promising pretreatment that can be used in the process of converting corn stover polysaccharides into ethanol as a liquid fuel source [9-12]. AFEX pretreatment uses concentrated ammonia-water mixtures under moderate temperatures (60-180°C) and high pressures (200-1000 psi) to disrupt the cellular structure of the plant material by decrystallizing the cellulose, partially depolymerizing and solubilizing the hemicellulose, and altering the form, location and structure of lignin [9-10].

The structure and composition of the plant cell wall depends on a number of factors including: developmental stage at harvest, geographical origin, type of tissue, and other external factors including season of harvest as well as environmental conditions experienced during growth [13]. Corn stover, like most grasses, experiences considerable compositional changes throughout the yearly growth period as well as significant variation between the various fractions of the plant (i.e. leaf vs. stem) [14-16]. Largely because of these differences in composition, stover fractions have been shown to respond differently to pretreatment and enzymatic hydrolysis, resulting in different sugar yields [17-19]. It is reasonable to assume that differences in composition, due largely to differences in morphology and cell and tissue organization, could cause different stover fractions to have different optimal pretreatment conditions for maximizing sugar yields. For example, wheat straw leaves, when treated with dilute NaOH, required less severe pretreatment conditions to optimize glucan yields than stem internodes and nodes [20], so the same might be true for corn stover pretreated with ammonia (or AFEX). Maximum sugar yields from individual fractions would be one criterion for determining which fractions should be left on the field following harvest. Assuming no other constraining factors, it would be most logical to harvest the least recalcitrant biomass and leave the remainder for erosion control and soil organic carbon maintenance [21]. Crofcheck and Montross recommended, based on glucose yields from fractionated corn stover, a roughly 30% corn stover harvest scenario where the selectively harvested corn stover (SHCS) was composed of all of the available cobs and 74% of the leaves and husks, leaving the most recalcitrant stalks on the field [17].

For our experiment, AFEX followed by enzymatic hydrolysis was performed on four different corn stover fractions (stem, leaf, husk and cob) from September (early) and November (late) harvests. The objectives of this project were: 1) to determine whether individual stover fractions have different optimal AFEX conditions and whether this is different from previously optimized values for homogeneously milled corn stover [9-10], 2) to discover which fractions give the highest glucose and xylose yields at optimal pretreatment conditions, and 3) to model optimal harvest scenarios, assuming 30% and 70% collection of total available dry corn stover, based on the maximum monomeric glucose and xylose yields from each fraction.

2. Materials and methods

2.1 Harvest and milling

Corn stover, from a variety intended for grain production, was manually harvested from the Michigan State University Agronomy Center in East Lansing, MI in September (early harvest) and November (late harvest) of 2006. The early and late stover harvests were separately hand-sorted into four individual fractions: stems, leaves with leaf sheaths, cobs and husks. The early husk and early cob fractions were not used due to spoilage of the material prior to use. All other fractions were air-dried, with stems split lengthwise to increase the drying rate. Fractions were then milled using a Fitzpatrick JT-6 Homoloid mill (Continental

Process Systems, Inc., Westmont, IL), with leaf, husk, and cob fractions passing through a 4.763mm (3/16”) mesh screen and stem fractions passing through a 3.175mm (1/8”) mesh screen.

2.2 Composition Analysis

Biomass moisture content was determined using a moisture analyzer (A&D, Model MF-50; San Jose, CA). The composition of each corn stover fraction (ash, lignin, glucan and xylan content) was determined using the NREL standard protocols [22]: Ash analysis, (LAP 005); Removal of Extractives (LAP 010); and Structural Carbohydrates & Lignin (LAP 002, 003, 004, 007, 019). The acid insoluble lignin analysis method was modified to use 47mm, 0.22μm 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, could not be dried in the vacuum oven and so were dried overnight in a desiccator prior to weighing. Soluble sugars could not be quantified following the extraction due to difficulties in resolving distinct peaks using the HPLC, and were not included in the composition.

2.3 AFEX Treatment

A small-scale benchtop reactor system, consisting of four separate 22 mL stainless steel (#316) reaction vessels, was used for the pretreatment process. Prior to biomass loading, the biomass was adjusted to the appropriate moisture content with deionized water, after which 3.0 g (dwb) of biomass was added to each reaction vessel. A metal screen was placed over the biomass inside of each vessel, to prevent escape of biomass during venting. The loaded reactor units were weighed and then attached to the reactor manifold, and any air within the reactor vessels was then removed using a rotary vacuum pump. Liquid anhydrous ammonia was dispensed into the manifold via Swagelok screw valves (Swagelok Co., Solon, OH) and then added to the reactor vessels. The reactors were weighed to determine the amount of ammonia added and then were vented slightly to reach the appropriate ammonia loading. A heating mantle was used to raise the reactors to the desired temperature and maintain it for the set residence time. At the completion of the residence time, the reactor pressure was explosively released via a stainless steel (#316) ball valve, and the reactor simultaneously cooled. The pretreated biomass was removed from the vessel and left in the fume hood overnight to allow the residual ammonia to evaporate.

2.4 Enzymatic Hydrolysis

NREL protocol (LAP 009) [22] was followed for the enzymatic hydrolysis of pretreated and untreated (control) samples. All samples were hydrolyzed in 20mL screw-cap vials at 1% glucan loading and a total volume of 15mL. Samples were adjusted to a pH of 4.8 by 1M citrate buffer solution. Spezyme® CP (Genencor Division of Danisco US, Inc.) cellulase at 15 FPU g^{-1} glucan (31.3 mg protein g^{-1} glucan) and β-glucosidase (Novozyme® 188, Novozymes Corp.) at 64 p-NPGU g^{-1} glucan (41.3 mg protein g^{-1} glucan) were added to each vial with a total protein content of 72.6 mg protein g^{-1} glucan. In addition, certain samples were also hydrolyzed using xylanase (Multifect® Xylanase, Genencor Division of Danisco US Inc.) at 10% of total cellulase protein (3.1 mg protein g^{-1} glucan), giving a total protein content of 75.7 mg protein g^{-1} glucan. Samples were placed in a New Brunswick Scientific (Edison, NJ) incubator shaker and hydrolyzed at 50°C and 150 rpm for 72 hr. The hydrolysates were sampled at 24 hr and 72 hr, following which samples were heated at 90°C for 15 minutes, cooled, and centrifuged at 15K for 5 minutes. The supernatant was filtered into HPLC shell vials using a 25mm, 0.2 μm polyethersulfone syringe filter (Whatman Inc. Florham Park, NJ) after which samples were stored at -20°C until further sugar analysis.

2.5 Sugar Analysis

A high-pressure liquid-chromatography (HPLC) system was used to determine the sample monomeric glucose and xylose concentrations following enzymatic hydrolysis. The HPLC system consisted of a Waters (Milford, MA) pump, auto-sampler and Waters 410 refractive index detector, equipped with a Bio-Rad (Hercules, CA) Aminex HPX-87P carbohydrate analysis column with attached deashing guard column. Degassed HPLC grade water was used as the mobile phase, at 0.6 mL/min, with the column temperature set at 85°C. Injection volume was 10 μL with a run time of 20 min per sample. Mixed sugar standards were used to quantify the amount of monomeric glucose and xylose in each hydrolysate sample.

2.6 Statistical Analysis

Monomeric glucose and xylose yields following enzymatic hydrolysis were analyzed using multivariate analysis of variance (MANOVA) in Minitab15 Statistical Software (Minitab Inc., 2006). The interactive effects plot of harvest period and stover fraction between each other, the four AFEX pretreatment parameters (moisture content, ammonia loading, temperature and residence time), and xylanase addition, was also constructed using Minitab.

3. Results

3.1 Composition Analysis

The composition of each of the corn stover fractions from each harvest are listed in Table 1. The value of the “other” column was determined by difference from 100% and the standard deviation is representative of three replicates. Statistically, the early and late stem and the late leaves and husk had the highest glucan content, while the early leaves and late cob had the lowest glucan content. The xylan content of the late fractions was significantly higher than their early counterparts and tended to decrease from late cob > late husk > late stem > late leaves > early stem > early leaves. The acid-insoluble lignin content was similar for all fractions, except for the cob, which had the highest lignin content, and the late husk which had statistically less lignin than the late stem. The ash content of all fractions were statistically different and decreased from early leaves > late leaves > early stem > late stem > late husk > late cob.

3.2 AFEX Pretreatment and Hydrolysis

Pretreatment conditions for AFEX-treated corn stover have been previously optimized at 1.0 (g ammonia g^{-1} dry biomass), 60% moisture content (dwb), 90°C and 5 min. residence time [9-10]. These conditions were treated as the "base case" for analysis of pretreatment conditions. The effect of pretreatment conditions on monomeric glucose and xylose yields, following hydrolysis, was tested by varying one process parameter (temperature, ammonia loading, moisture content or residence time) at a time (*e.g.* raising the temperature from 90°C to 100°C). Once the preliminary data had been gathered, the untreated control, base case, and best case were supplemented with xylanase during hydrolysis to observe the effect on sugar yields.

Figure 1 shows the monomeric glucose and xylose yields for a variety of conditions with particular comparisons between untreated and AFEX treated materials at a range of ammonia loadings. The effect of xylanase addition to the enzyme cocktail can also be observed in this figure. Error bars in all figures represent the mean ± 1 standard deviation. From the figure, it can be seen that AFEX substantially improves both glucose and xylose monomeric sugar yields for all harvest periods and corn stover fractions when compared to untreated materials. The xylose yields for the late fractions are especially improved by AFEX pretreatment, even at low ammonia loadings such as 0.5 (g ammonia g^{-1} biomass).

The increase in ammonia loading from 0.5 to 1.5 (g ammonia g^{-1} biomass) had different effects on early harvest and late harvest corn stover fractions. For the early harvest stover, without xylanase addition, glucose yields peak at 1.0 (g ammonia g^{-1} biomass). This optimum is similar to what has been seen previously with AFEX-treated corn stover [9-10], which may indicate that that material was from an earlier harvest. The xylose yields are relatively unaffected by any further increase above 1.0 (g ammonia g^{-1} biomass). However, when performing the same experiment with the late harvest corn stover there is an increase in both glucose and xylose yields for all fractions when increasing from 1.0 to 1.5 (g ammonia g^{-1} biomass).

Xylanase addition had little to no effect on increasing either glucose or xylose sugar yields in untreated corn stover fractions. For AFEX-treated early harvest fractions, the addition of xylanase at 1.0 (g ammonia g^{-1} biomass) had no effect on monomeric xylose yields, and it slightly lowered glucose yields. At 1.5 (g ammonia g^{-1} biomass), all fractions and harvests experienced an increase in both the monomeric xylose and glucose yields with the addition of xylanase.

The leaf and stem, for both early and late harvests, have similar glucose yields for 1.5 (g ammonia g^{-1} biomass) ammonia loading, however the leaf glucan is more digestible as seen by the greater yield (percent of maximum theoretical glucan available). The late harvest husk approaches theoretical glucose yields at the optimal condition of 1.5 (g ammonia g^{-1} biomass). Because of this, the addition of xylanase for this pretreatment condition increases husk xylose yields slightly, but not glucose yields as is seen for the other fractions. With the addition of xylanase at 1.5 (g ammonia g^{-1} biomass), the cob and leaf also approach near theoretical glucose yields.

Figure 2 shows the effect of pretreatment temperature on glucose and xylose yields from corn stover fractions. Altering the temperature by 10°C from the base case had little effect on glucose and xylose yields. There is a definite peak in glucose yields at 90°C for the early harvest but the late harvest has no apparent difference in yields for 80, 90 or 100°C. In previous work [10], raising the temperature above 90°C had a negative impact on ethanol yields from simultaneous saccharification and fermentation (SSF).

Decreasing the moisture content to 40% (dwb) and eliminating the residence time (the time for which the reactor was held at the set temperature following heat-up) each had a negative impact on glucose and xylose yields for all fractions (Figure 3). For all stover fractions, except the late husk, it was more detrimental in terms of sugar yields to decrease the residence time rather than the moisture content.

3.3 Statistical Analysis

Multivariate analysis of variance (MANOVA) was conducted to determine the significance of harvest date, corn stover fraction, AFEX parameters and xylanase addition on both the 24 hour and 72 hour monomeric glucose and xylose yields. Interactive effects were also examined between harvest date and stover fraction and each of the other parameters. Because conclusions regarding significance were the same for 24 hour and 72 hour yields for both glucose and xylose (Table 2), only the 72 hour yields were used for the interactive effects plot (Figure 4).

Glucose yields were significantly affected by three of the AFEX pretreatment conditions: ammonia loading, moisture content, and residence time, but not by temperature. Glucose yields were also dependent on corn stover fraction and whether xylanase was added to the hydrolysis cocktail. Of the interactive effects analyzed, only harvest date X ammonia loading had any significant effect on monomeric glucose yields.

Xylose yields were significantly affected by all four AFEX pretreatment conditions, including temperature. Unlike the case for glucose yields, xylose yields were not significantly affected by corn stover fraction, but they were affected by both the harvest date and the addition of xylanase to the hydrolysis cocktail. There were also interactive effects on xylose yields from harvest date X ammonia loading and corn stover fraction X xylanase addition.

When analyzing the interactive effects plot, significant interactive effects will have very different slopes for the different lines in that portion of the graph. For example, when observing the interactive effect of harvest X ammonia on xylose yields, the slope of the early and late harvest lines are roughly the same when increasing the ammonia loading from 0.5 to 1.0 (g ammonia g^{-1} biomass). However, when increasing the ammonia loading from 1.0 to 1.5 (g ammonia g^{-1} biomass), the slope of the late harvest line is significantly steeper than the slope of the early harvest line. This difference in slope signifies that most of the impact of ammonia loading on this interaction is due to the second increase, not the first. This implies that the higher ammonia loading has a greater effect on the later harvest than the early harvest.

3.4 Optimization of Harvest Scenarios

Crofcheck and Montross [17] found that the weighted sum of the glucose yields from individual pretreated fractions was not statistically different from the glucose yield from whole pretreated corn stover. This means that glucose yields for selectively harvested corn stover (SHCS) could be predicted using glucose yields from individual fractions. Our estimate of the late harvest dry matter distribution of corn stover (Figure 5), was based on published data from four sources [15-17, 23], and is similar to standard estimates of corn stover dry matter distribution near corn harvest [24]. Corn stover dry matter yields, particularly of the husk and leaf, tend to decrease rapidly due to weathering over the course of the grain harvest season [15-16, 21, 23, 25-27]. This estimate attempts to account for both the effects of the late harvest date as well as our inclusion of the leaf sheath with the leaf fraction instead of the stem fraction, as is often the case [15-16].

Because of the wide range of opinions on how much corn stover can be sustainably harvested and because the amount will likely change for a given field depending on environmental conditions and agricultural practices [4, 6-8], we have modeled a number of corn stover harvest scenarios for both a liberal harvest estimate (70% dwb) and a conservative harvest estimate (30% dwb). The goal was to determine which combination of fractions provides the most benefit to the biorefinery in terms of sugar yields, and to determine the preferential order in which fractions should harvested from the field. For this analysis, the sugar yields used were from 72 hour hydrolysis of AFEX-treated late corn stover. The option of an early harvest was not analyzed because of the lack of data for husk and cob fractions. Scenarios were analyzed with regard to the effect of increasing ammonia loading from 1.0 to 1.5 (g ammonia g^{-1} biomass) and for the maximized sugar yield, either glucose or xylose. This gave four potential scenarios (1.0 + glucose, 1.5 + glucose, 1.0 + xylose and 1.5 + xylose). All other AFEX and hydrolysis conditions were held constant (60% dwb moisture, 90°C, 5 min residence time + 10% xylanase addition). Because glucose yields were consistently higher than xylose yields, the harvest conditions to obtain maximum glucose yields for all of the scenarios also corresponded with the maximum total sugar yields. The conditions selected resulted in three scenarios for selectively harvesting corn stover (Table 3), because the harvest scenario to maximize glucose yields was the same for both ammonia loadings. The relative amounts of harvested fractions for each scenario are represented in Figure 5 for both the 70% and 30% harvests.

The estimated whole corn stover dry matter distribution was used to predict monomeric glucose and xylose yields from the three different harvest scenarios for both 70% (Table 4) and 30% (Table 5) harvest of on-field corn stover using weighted averaging of individual fraction sugar yields. It is important to note that values given in these tables do not attempt to take into account the ability or inability to harvest the specific fractions or any losses due to inefficiencies in harvest, transport, and storage of corn stover, which can be significant depending on the methods used. A recent study found that the maximum amount of corn stover was available at grain physiological maturity (15.6 t ha^{-1}) and steadily decreased over the harvest period to a minimum of 8.6 t ha^{-1} [27]. Because this value takes into account the late season of harvest and because it is in the range of most literature estimates of corn stover yields (7.8 – 8.8 t ha^{-1}) [1-2, 28], 8.6 t ha^{-1} of available corn stover was chosen to estimate the total sugars that could be produced per hectare for the given harvest scenario. The standard value of 0.51 (theoretical g ethanol produced g^{-1} sugar consumed) was used to determine the theoretical ethanol production from a hectare of SHCS and does not take into account inefficiencies in fermentation.

4. Discussion

4.1 Composition Analysis

Fractions from the late harvest tended to have a slightly higher percentage of cell wall components (although not always significant) and slightly lower percentage of ash compared to their early harvest counterparts. For corn stover, the increase in lignin and cellulose, and the decrease in ash have been observed elsewhere [23, 25] as well as a general increase in all cell wall components with a decrease in non-structural carbohydrates [26], and an increase in lignin and xylan with a drop in soluble solids [15] with increasing maturity. This observed increase in cellulose (glucan), hemicellulose (glucan and xylan) and lignin content is due to the secondary thickening of the plant cell wall that continues to occur for as long as the plant matures. During this same time there is also a decrease in ash content [14]. However, while there is continual change in the dry matter composition until late in the season, this tends to be very small during the grain harvest period [2, 15], the time during which our samples were harvested.

4.2 AFEX Pretreatment

Based on the final total sugar yields, the optimal AFEX pretreatment conditions were observed to be consistent for all fractions for both early and late harvest corn stover: 1.5:1 (g NH_3 g^{-1} biomass), 60% moisture content (dwb), 90°C, 5 min residence time; with 10% xylanase addition (mg xylanase protein mg^{-1} cellulase protein) in addition to the standard enzyme mixture used during enzymatic hydrolysis.

For AFEX-treated early harvest fractions, the addition of xylanase at 1.0 (g ammonia g^{-1} biomass) had no effect on monomeric xylose yields, and it slightly lowered glucose yields. This drop in glucose yields could be due to competition for binding sites on the cellulose chains between enzymes in the xylanase and cellulase mixtures. The fact that there is no increase in xylose yields with the addition of xylanase supports this conclusion.

The higher optimal ammonia loading for the late harvest fractions compared with the early harvest could be due to a number of reasons. AFEX, by ammoniation of the active methoxyl sites of lignin [29], may be preventing lignin from binding to the hydrolysis enzymes. It is likely that this mechanism is operating, and it may be one of the main reasons for the increase seen from 0.5 to 1.0 (g ammonia g^{-1} biomass). However, if this were the reason for the difference in optimum ammonia loading between the early and late harvests, then the lignin content of the later harvest should be greater. However, this is not the case; statistically the lignin contents of the early and late fractions are identical. The difference in optimal ammonia loading is more likely due to the increase in xylan content and possibly increased cross-linking between hemicellulose and lignin from the early to late harvest. Ferulate cross-linking occurs between lignin and arabinoxylan in the plant cell wall, with the ferulates ether-linked to lignin and ester-linked to the arabinoxylan [30]. Ammonolysis of the ferulate ester linkages to arabinoxylan side-chains is believed to be one major reactions occurring during the AFEX process [31]. These mechanisms may be opening up the cell wall ultrastructure more effectively at the higher ammonia loading, allowing the enzymes greater access to cellulose, hence the optimum at 1.5 (g ammonia g^{-1} biomass). Also, by allowing greater access to a larger amount of xylan, the xylanase enzymes may be competing less with the cellulases for binding sites, hence the increase in glucose yields with xylanase addition for the early harvests at 1.5 (g ammonia g^{-1} biomass).

It is worth noting that the husk, the material with the lowest lignin content, while having the second-highest xylan content, is least affected by the combination of increased ammonia loading and xylanase addition. At 1.5 (g ammonia g^{-1} biomass), the xylose yield only increases by 6.1% with the addition of xylanase to the hydrolysis cocktail. The late cob, which has a significantly higher lignin and xylan content than all of the other materials, experiences the largest impact on xylose yields due to the combination of increased ammonia loading and addition of xylanase, a 22.5% increase at 1.5 (g ammonia g^{-1} biomass) with the addition of xylanase. The higher ammonia loading would cleave more linkages between the hemicellulose and lignin, solubilizing more oligomeric and monomeric xylose and perhaps some lignin as well. These exposed, solubilized sugars would be much easier to hydrolyze with the xylanase. It might be possible, given the very high xylan content of the cob, that more xylanase would be needed to achieve near complete monomeric xylose yields. Because the xylanase loading was based on a percentage of the cellulase loading (and therefore the glucan content), and because the g glucan g^{-1} xylan ratio for the cob (0.85 g glucan g^{-1} xylan) is much lower than the other fractions (1.47 – 1.85 g glucan g^{-1} xylan), the xylanase loading in terms of the xylan content (mg xylanase g^{-1} xylan) is much lower for the cob fraction (Table 6). This may be one reason for the much lower xylose yield relative to the maximum theoretical xylose yield of the late cob fraction.

4.3 Statistical Analysis

All AFEX conditions had significant impacts on sugar yields, except for temperature which had no effect on glucose yield. Based on least squares means analysis (data not shown), the temperature effect on xylose yield is likely due to a greater yield increase as the temperature is raised from 80 to 90°C rather than the decrease in yield when temperature is raised from 90 to 100°C. For the range of conditions tested, optimizing the ammonia loading, moisture content and residence time are more important for maximizing sugar yields from corn stover. However, this conclusion may change for a different range of temperatures and should not be extrapolated to other conditions.

Harvest date had a significant impact on xylose yields but not on glucose yields. This is largely due to the fact that the xylan content of the late fractions was greater than the xylan content of the early fractions, whereas the glucan content was not significantly different between harvests. The corn stover fractions tested had a significant effect on glucose yields but not on xylose yields. The late stem, husk, leaf and early stem fractions had no significant statistical difference in their glucan contents, so their relative recalcitrance, in terms of glucose yields, can be inferred from Figure 4. Because the husk has the highest glucose yield, it can be considered the least recalcitrant followed by the leaf and then the stem. Inferences cannot be made regarding the cob because its glucan content is statistically lower than the other three fractions. However, because the cob approaches theoretical glucan yields at optimal conditions while the stem does not (Figure 1), it may be less recalcitrant in terms of the conversion of glucan.

For interactive effects, only two were significant: harvest date X ammonia loading and corn stover fraction X xylanase addition. The harvest X ammonia interaction was significant for both glucose and xylose yields. The increase in ammonia loading from 1.0 to 1.5 (g ammonia g^{-1} biomass) appears to have a greater effect on the late harvest than the early harvest, but this may be due to the lack of data for the early harvest cob. Because the cob is the fraction most affected by the increase in ammonia loading, particularly for xylose yield, the lack of early cob data may lead to an apparent difference in effects between harvests, that is not actually present.

The second significant interactive effect was for corn stover fraction X xylanase addition, but only for xylose yields. The main reason for this effect, as can be observed from Figure 4, is due to the cob fraction which was much more strongly affected by the addition of xylanase than all of the other fractions, whose responses were fairly similar. This conclusion is supported by the fact that when the data for the late cob was removed from the analysis, the fraction X xylanase interaction became non-significant (data not shown). Taken together, these results indicate that of all the corn stover components, the cob reacts the most differently during enzymatic hydrolysis. As mentioned previously, the cob may require a much higher xylanase loading than the other fractions in order to release xylose remaining in the biomass or to convert the AFEX solubilized xylo-oligomers.

4.4 Empirical Modeling of Harvest Scenarios

Harvest scenario A, which selectively harvests the husk followed by the leaf, stem and lastly cob, obtained the highest sugar and ethanol yields of all the scenarios, and as a result was chosen as the optimal harvest scenario for AFEX-treated corn stover. Harvest scenario A was also preferable to scenarios B and C for a number of other reasons. First, optimizing collection for maximum glucose yields is preferable because most current, relevant microbial strains selectively utilize hexoses over pentoses as a carbon source during ethanolic fermentation [11, 32]. Second, harvest scenario A selectively leaves behind the more lignified fractions on the field which may prove more valuable for improving SOC levels due to the longer half-life of lignin compared to cellulose and hemicellulose [4, 33]. Lastly, selectively leaving the least desirable cob and/or lower portion of the stem behind may be feasible with current stover harvesting systems, but the downside is, that depending on the method, complete collection of the leaves may not be possible [34-35].

Crofcheck and Montross [17] recommended, based on glucose yields from fractionated corn stover, a roughly 30% corn stover harvest scenario where the selectively harvested corn stover (SHCS) was composed of all of the available cobs and 74% of the leaves and husks, leaving the most recalcitrant stalks on the field. The difference between their optimal harvest scenario and ours is most likely due to their experimental methods for pretreatment and the subsequent analysis. Pretreatment of lignocellulosic biomass using dilute sodium hydroxide, solubilizes much of the lignin and some of the hemicellulose into the liquid pretreatment stream [36, 37], so it is unlikely that glucan content of the pretreated corn stover corresponds to glucan content of the untreated corn stover. For similar pretreatment conditions of corn stover, Varga et al. found a 41.9% mass loss from the untreated dry corn stover to the pretreated solids, and the composition of the pretreated material shifted in favor of a higher glucan content [36]. The cob has a significantly higher xylan and lignin content than the other fractions of the corn plant, and so it is reasonable to assume that it will lose a greater proportion of its mass following dilute alkali pretreatment. Because this mass loss wasn’t taken into account [17], the amount of glucan that could be obtained on a mass basis from the untreated fractions was over-exaggerated, particularly from the xylan- and lignin-rich cob. If the mass loss had been taken into account, it is likely that their choice of optimal fractions for harvest would have been different. Because AFEX is a dry-to-dry process with little mass loss during pretreatment, the glucan content of the untreated material can be used as the glucan content of the pretreated material [9].

A comparison of Table 4 and Table 5 reveals that the amount of corn stover harvested has the largest impact on total sugars ha^{-1} and theoretical ethanol yield ha^{-1}. Going from a 70% collection scenario to a 30% collection scenario cut the ethanol yield by ~55% for all scenarios. Dropping the ammonia loading from 1.5 to 1.0 (g ammonia g^{-1} biomass) caused a ~15-20% decrease, while switching desired sugars from glucose to xylose (*i.e.* changing harvest scenarios but keeping stover collection and AFEX and enzymatic hydrolyisis conditions constant) caused a ~2-4% decrease. To determine the sensitivity of changing the harvest scenario, the model was run assuming the "worst case scenario", where the biomass was harvested in the manner that would give the worst possible sugar yields (data not shown). The worst case scenario led to a decrease in the total sugar yields and theoretical ethanol yields per hectare ranging from ~4 – 18.5%. The drop was more pronounced for the 30% stover harvest because the SHCS dry matter distribution will be completely different for the best and worst case harvests, while the 70% harvest will have some similarity in dry matter distribution between the best and worst cases. As expected, the theoretical ethanol yield was substantially lower (~70-85%), when comparing untreated corn stover to the AFEX-treated cases (data not shown).

Shinners et al. [35] analyzed the effect of cut height of corn stover on predicted ethanol yields and found that the amount of ethanol produced was only ~3% greater (L Mg^{-1} DM) for the low cut vs. the high cut. Because the cut height is basically a harvest scenario that leaves a portion of the lower stem and leaves behind, when basing the analysis on dry matter harvested, these results are comparable to our results for the effect of harvested fraction type on ethanol yields. Similarly to our results, they found that the proportion of fractions harvested had little impact on predicted ethanol yields. However, when analyzed based on the ethanol yield per hectare, the increase in total dry matter harvested with the lower cut height increased the predicted ethanol yield by 52% compared to the higher cut [35], which was similar to our results for increased dry matter collection.

Based on these results, optimizing the fractions collected during harvest has a much smaller impact on potential yields than optimizing pretreatment and hydrolysis conditions. The amount of stover harvested has the greatest impact on theoretical ethanol production per hectare. It will be very important, in terms of maximizing ethanol production, to develop methods to efficiently maximize harvest of corn stover, while still maintaining soil productivity and preventing erosion.

5. Conclusions

Based on monomeric glucose and xylose yields, the optimal AFEX conditions, for all stover fractions (leaf, stem, husk and cob) regardless of harvest period, were found to be 1.5 (g NH_3 g^{-1} biomass), 60% moisture content (dwb), 90°C, and 5 min residence time; with enzyme loading during hydrolysis of 31.3 mg of cellulase (Spezyme® CP), 41.3 mg of β-glucosidase (Novozyme® 188) and 3.1 mg xylanase, g^{-1} glucan. These conditions are different from those presented in previous analyses [9, 10], largely due to the inclusion of xylanase in the hydrolysis cocktail. The addition of xylanase was necessary to achieve high xylose yields at moderate cellulase loadings and moderate AFEX conditions, particularly with respect to the more recalcitrant cob and stem fractions.

The optimal harvest scenario for the collection of selectively harvested corn stover (SHCS) would harvest the husk followed by the leaves, then the stem, and lastly the cob. This harvest scenario was independent of ammonia loading during AFEX pretreatment and maximized glucose and ethanol yield from SHCS. This scenario, combined with the optimal AFEX pretreatment conditions for SHCS gave a theoretical ethanol yield of 1.62 t^{-1} ha for the 70% dry matter harvest and 0.72 t^{-1} ha for the 30% dry matter harvest. Decreasing the stover collection from 70% to 30% dropped the ethanol yield per hectare by ~55%. Maximizing stover collection while protecting soil health will be the most important factor for maximizing ethanol yields from corn stover.

Optimizing collection of corn stover fractions has little impact on the theoretical ethanol yield per hectare (~2-4%), especially compared to the moderate effect of optimizing pretreatment and hydrolysis conditions (~15-20%). The dry matter distribution of collected corn stover fractions is generally much less important than the optimization of the ethanol production process, however it is still something that needs to be taken into account because harvesting the worst fractions can also decrease ethanol yields, particularly for a more conservative harvest.

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Figure Legends

Figure 1: Effect of AFEX pretreatment ammonia loading and addition of xylanase during enzymatic hydrolysis on glucose (A) and xylose (B) monomeric sugar yields. All AFEX runs were kept at constant moisture content (60% dwb), temperature (90°C) and residence time (5 min). Yields are in terms of untreated dry biomass.

Figure 2: Effect of AFEX pretreatment temperature on glucose and xylose monomeric sugar yields following enzymatic hydrolysis. All AFEX runs were kept at a constant moisture content (60% dwb), ammonia loading (1.0 g NH_3 g^{-1} dry biomass) and residence time (5 min). Yields are in terms of untreated dry biomass. Glu = glucose, Xyl = xylose, MTSY = maximum theoretical sugar yield.

Figure 3: Effect of changes to AFEX moisture content and residence time on monomeric glucose and xylose yields following enzymatic hydrolysis. Base AFEX conditions: moisture content (60% dwb), ammonia loading (1.0 g NH_3 g^{-1} dry biomass), temperature (90°C) and residence time (5 min). Yields are in terms of untreated dry biomass. MC = moisture content, RT = residence time, Glu = glucose, Xyl = xylose, MTSY = maximum theoretical sugar yield.

Figure 4: Interaction effect plot of key parameter interaction with corn stover fraction and harvest period on monomeric glucose (A) and xylose (B) yields following 72 hours of enzymatic hydrolysis. Yields are in terms of untreated dry biomass. MC = moisture content, RT = residence time, DWB = dry weight basis, N = no xylanase added, Y = xylanase added (10% of total cellulase protein).

Figure 5: Estimated dry matter distribution for late harvest corn stover and harvest scenarios based on 70% and 30% dry weight corn stover harvests. Percentages of individual fraction harvested are based on the total amount of each fraction available.

Tables

Table 1: Corn stover composition for early and late harvest stover fractions. Values with different superscripts in an individual column were statistically different using Tukey's pairwise comparison with α = 0.05. [The 'other' column determined by difference from 100%]

		Corn Stover Fraction Composition (% dry biomass)				
Corn Stover Fraction		**Glucan**	**Xylan**	**Acid-Insoluble Lignin**	**Ash**	**Other**
Early	**Leaves**	27.5^{b} ± 3.2	17.8^{e} ± 1.7	13.2^{bc} ± 0.7	7.3^{a} ± 0.13	34.2
	Stem	35.1^{a} ± 2.6	19.0^{de} ± 1.1	14.9^{bc} ± 0.2	3.4^{c} ± 0.10	27.6
Late	**Leaves**	35.3^{a} ± 1.2	21.8^{cd} ± 0.6	13.6^{bc} ± 1.7	6.0^{b} ± 0.25	23.3
	Stem	37.8^{a} ± 0.9	23.6^{bc} ± 0.4	16.9^{b} ± 0.5	2.4^{d} ± 0.08	19.3
	Husk	39.0^{a} ± 2.2	26.5^{b} ± 1.5	11.6^{c} ± 0.3	2.1^{e} ± 0.11	20.8
	Cob	27.5^{b} ± 1.1	32.3^{a} ± 1.3	25.8^{a} ± 2.6	1.1^{f} ± 0.02	13.3

Table 2: Analysis of Variance for Factors influencing Sugar Yields. *significant at α = 0.05

Factor	**P-value**			
	24 hr Glucose	**72 hr Glucose**	**24 hr Xylose**	**72 hr Xylose**
Harvest Date	0.775	0.437	0.000*	0.000*
Corn Stover Fraction	0.000*	0.006*	0.526	0.528
Ammonia Loading	0.000*	0.000*	0.000*	0.000*
Temperature	0.082	0.161	0.000*	0.022*
Moisture Content	0.018*	0.002*	0.000*	0.000*
Residence Time	0.001*	0.000*	0.003*	0.000*
Xylanase Addition	0.001*	0.002*	0.000*	0.000*
Harvest x Ammonia	0.007*	0.001*	0.001*	0.002*
Harvest x Temperature	0.918	0.932	0.824	0.392
Harvest x Moisture	0.687	0.762	0.943	0.424
Harvest x Res. Time	0.829	0.719	0.377	0.317
Harvest x Xylanase	0.919	0.760	0.111	0.063
Fraction x Ammonia	0.288	0.080	0.416	0.152
Fraction x Temperature	0.746	0.684	0.588	0.400
Fraction x Moisture	0.278	0.075	0.163	0.109
Fraction x Res. Time	0.916	0.859	0.715	0.542
Fraction x Xylanase	0.711	0.300	0.008*	0.030*

Table 3: Optimized harvest scenarios based on desired sugar and AFEX ammonia loading

Harvest Scenario	**A**	**B**	**C**
Optimized Sugar	**Glucose/Total**	**Xylose**	**Xylose**
Ammonia Loading ($g\ NH_3\ g^{-1}$ dry SHCS)	**1.0, 1.5**	**1.0**	**1.5**
Best Fraction	Husk	Husk	Cob
	Leaf	Stem	Husk
	Stem	Cob	Stem
Worst Fraction	Cob	Leaf	Leaf

Table 4: Estimated yields for 70% collection of selectively harvested corn stover (SHCS) following AFEX, enzymatic hydrolysis and fermentation.

		1.0 g NH_3 g^{-1} dry SHCS		**1.5 g NH_3 g^{-1} dry SHCS**	
		Harvest Scenario A	**Harvest Scenario B**	**Harvest Scenario A**	**Harvest Scenario C**
Sugar Yield	**Glucose**	273.7	254.2	331.5	310.8
g sugar kg^{-1} dry SHCS	**Xylose**	150.0	153.1	195.7	206.8
	Total	423.6	407.3	527.2	517.5
Hectare Yields *t ha^{-1}*	**Total Sugars**	2.55	2.45	3.17	3.12
	Theoretical Ethanol	1.30	1.25	1.62	1.59

Table 5: Estimated yields for 30% collection of selectively harvested corn stover (SHCS) following AFEX, enzymatic hydrolysis and fermentation.

		1.0 g NH_3 g^{-1} dry SHCS		1.5 g NH_3 g^{-1} dry SHCS	
		Harvest Scenario A	**Harvest Scenario B**	**Harvest Scenario A**	**Harvest Scenario C**
Sugar Yield *g sugar kg^{-1} dry SHCS*	**Glucose**	305.1	278.2	354.4	311.3
	Xylose	151.8	161.3	192.7	215.4
	Total	456.9	439.5	547.1	526.7
Hectare Yields *t ha^{-1}*	**Total Sugars**	1.18	1.13	1.41	1.36
	Theoretical Ethanol	0.60	0.58	0.72	0.69

Table 6: Enzymatic hydrolysis xylanase loading in terms of xylan content of each fraction

Corn Stover Fraction		Xylanase Loading *mg xylanase g^{-1} xylan*
Early	**Leaves**	4.78
	Stem	5.73
Late	**Leaves**	5.03
	Stem	4.97
	Husk	4.57
	Cob	2.64

Figure 1

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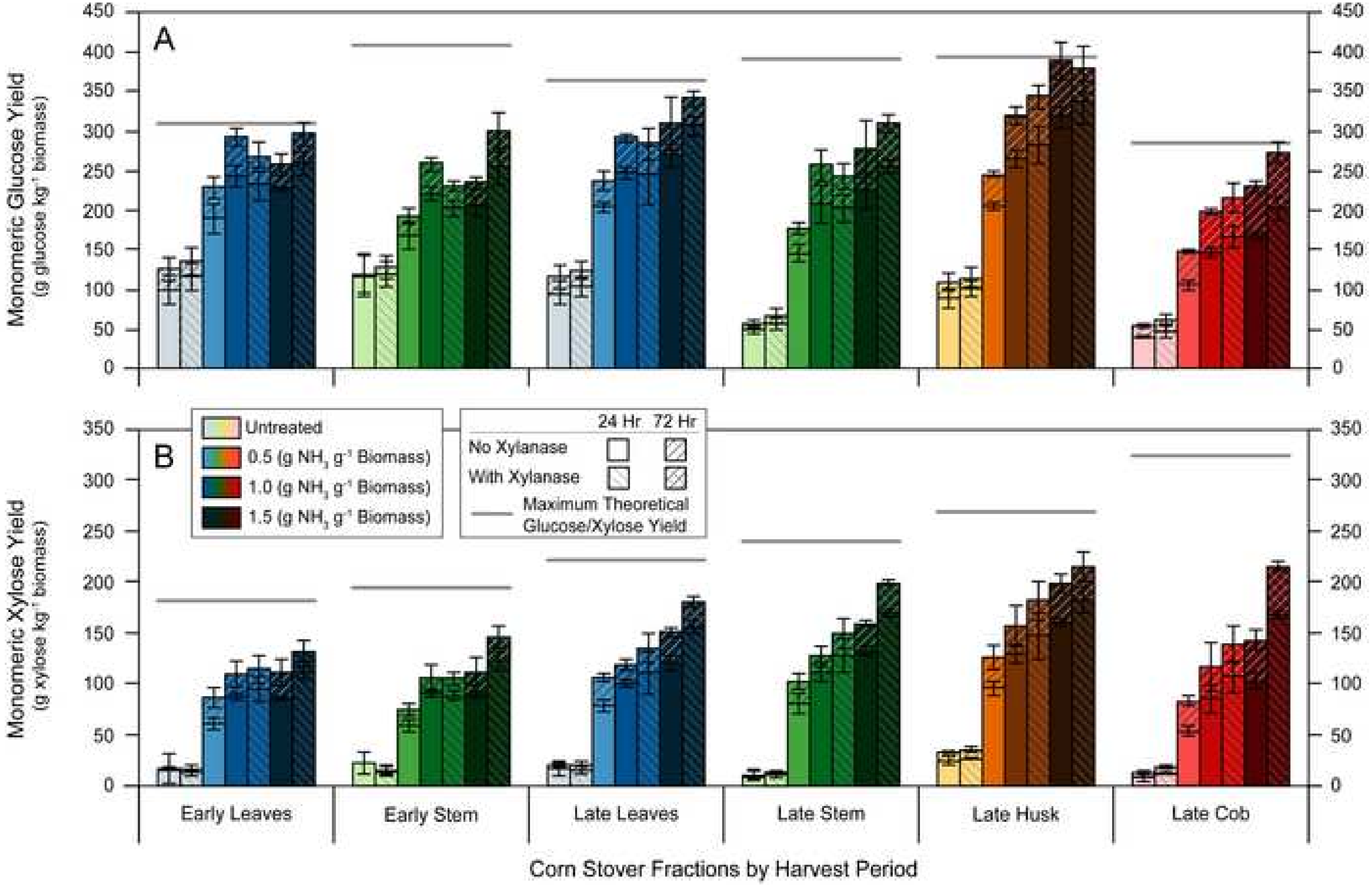

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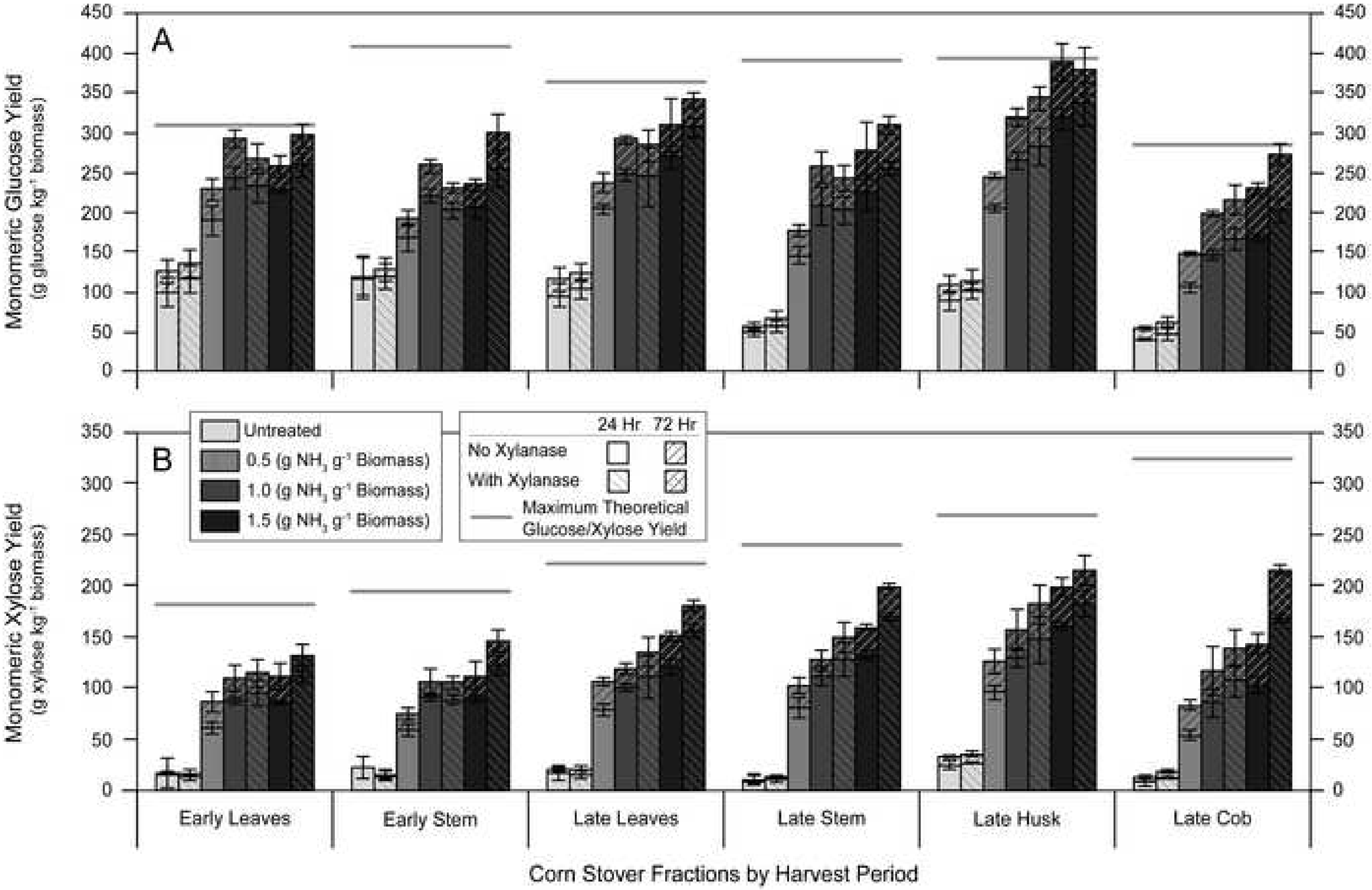

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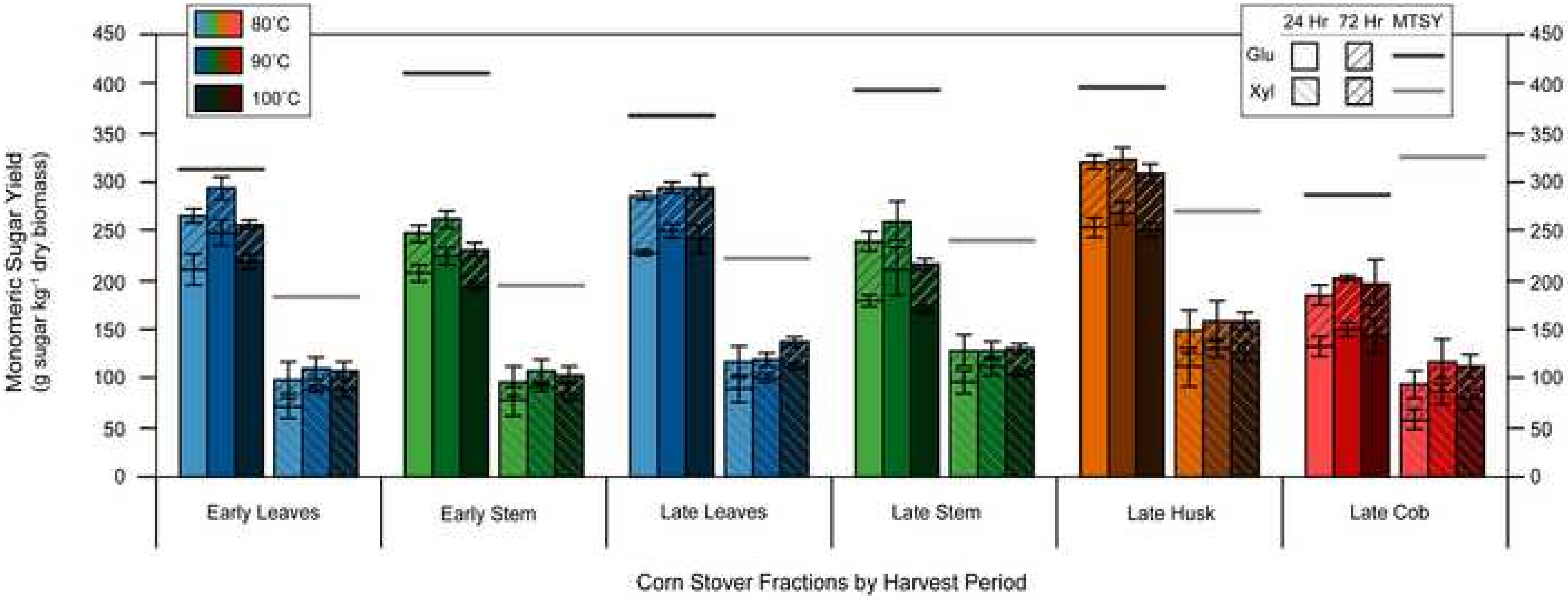

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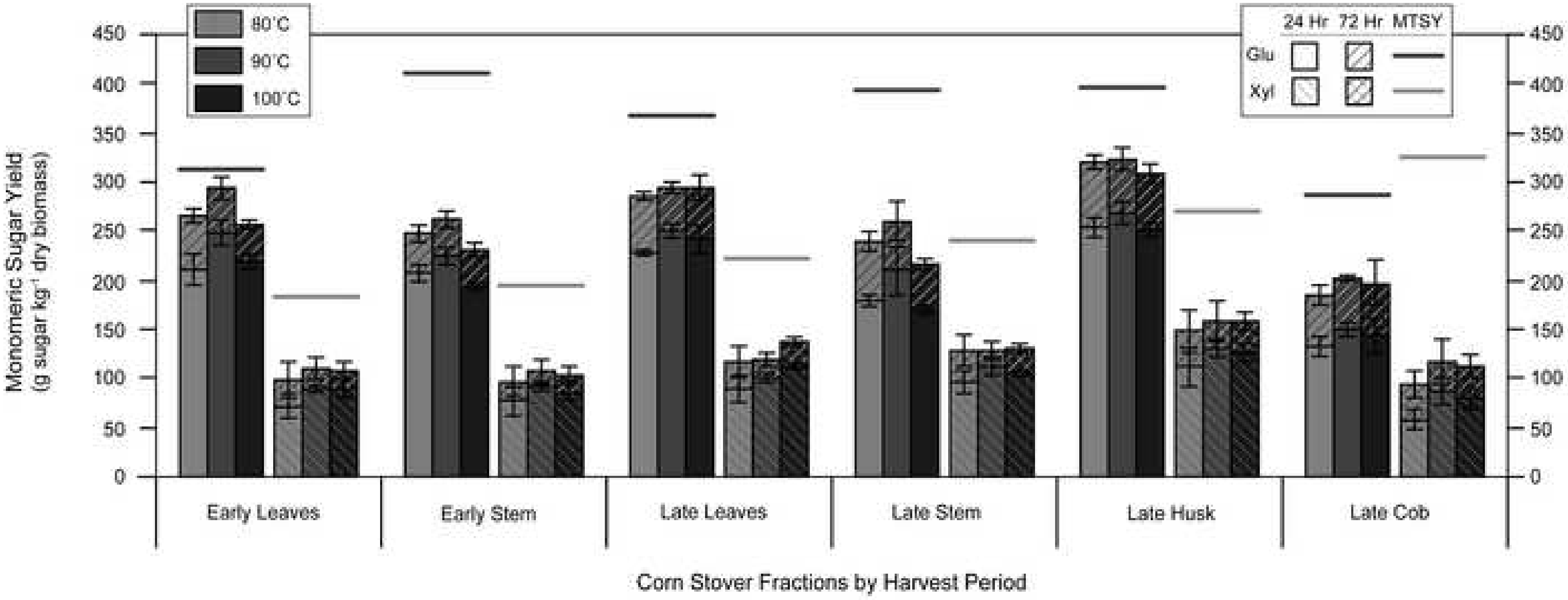

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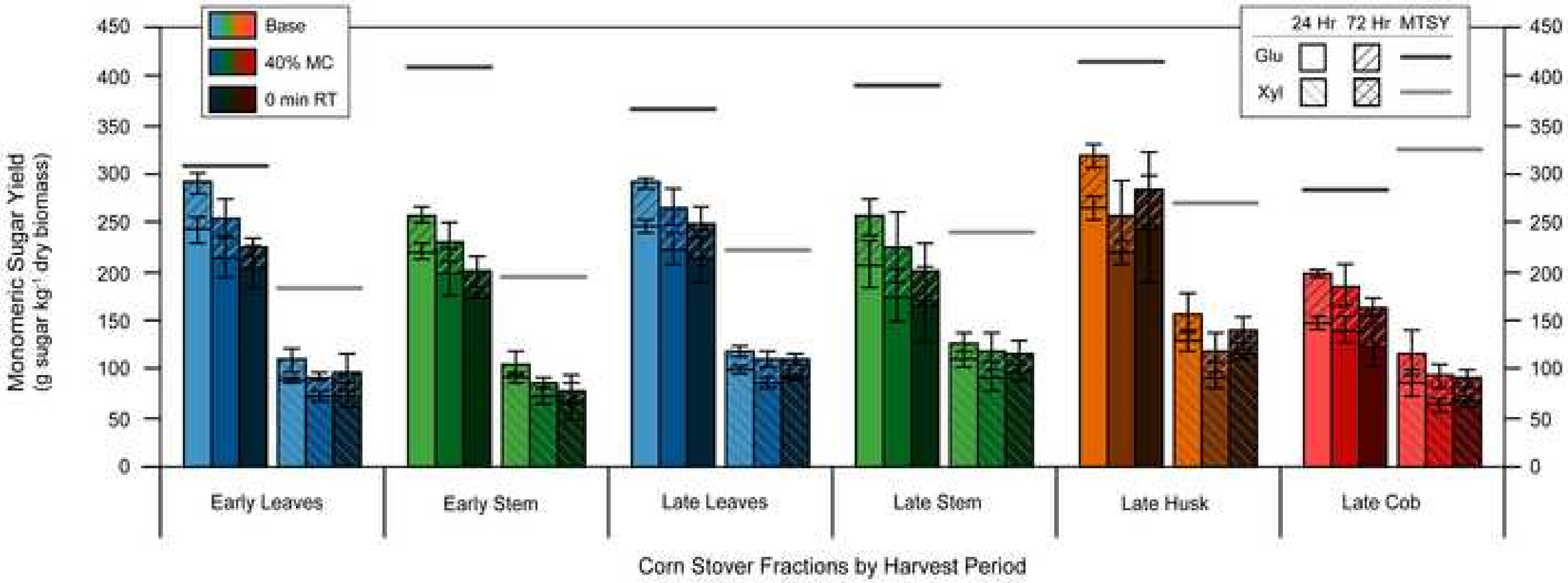

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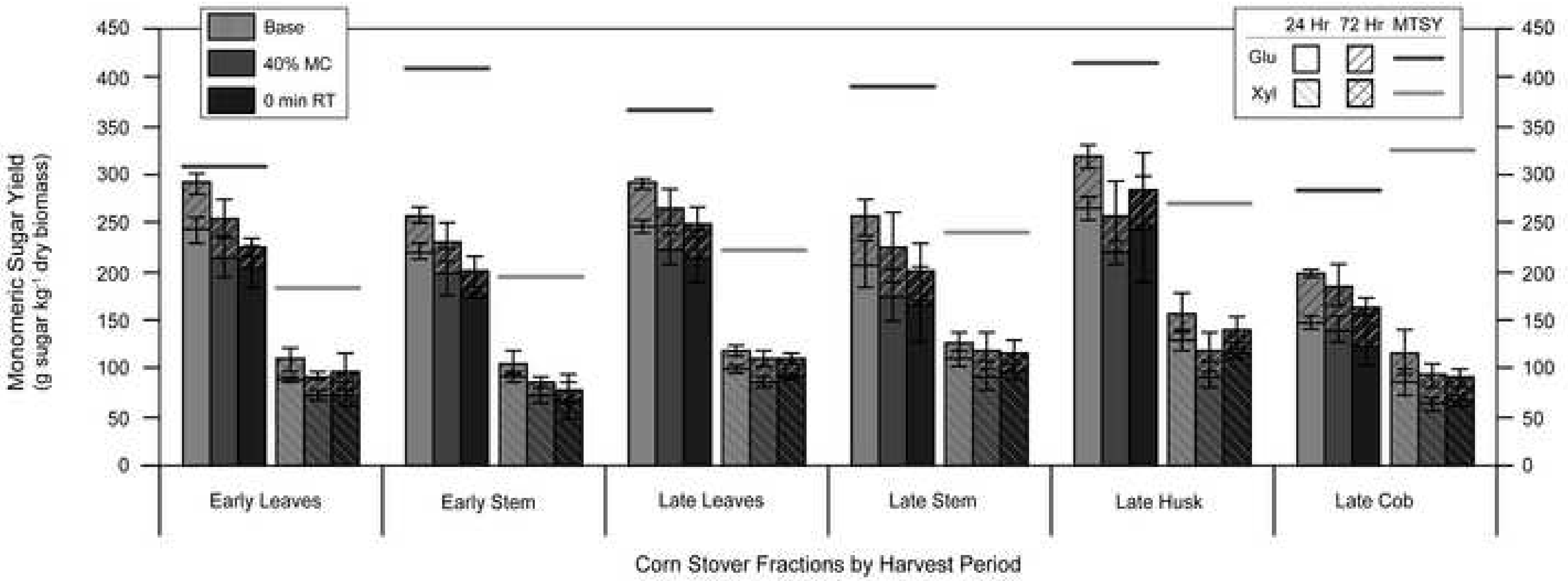

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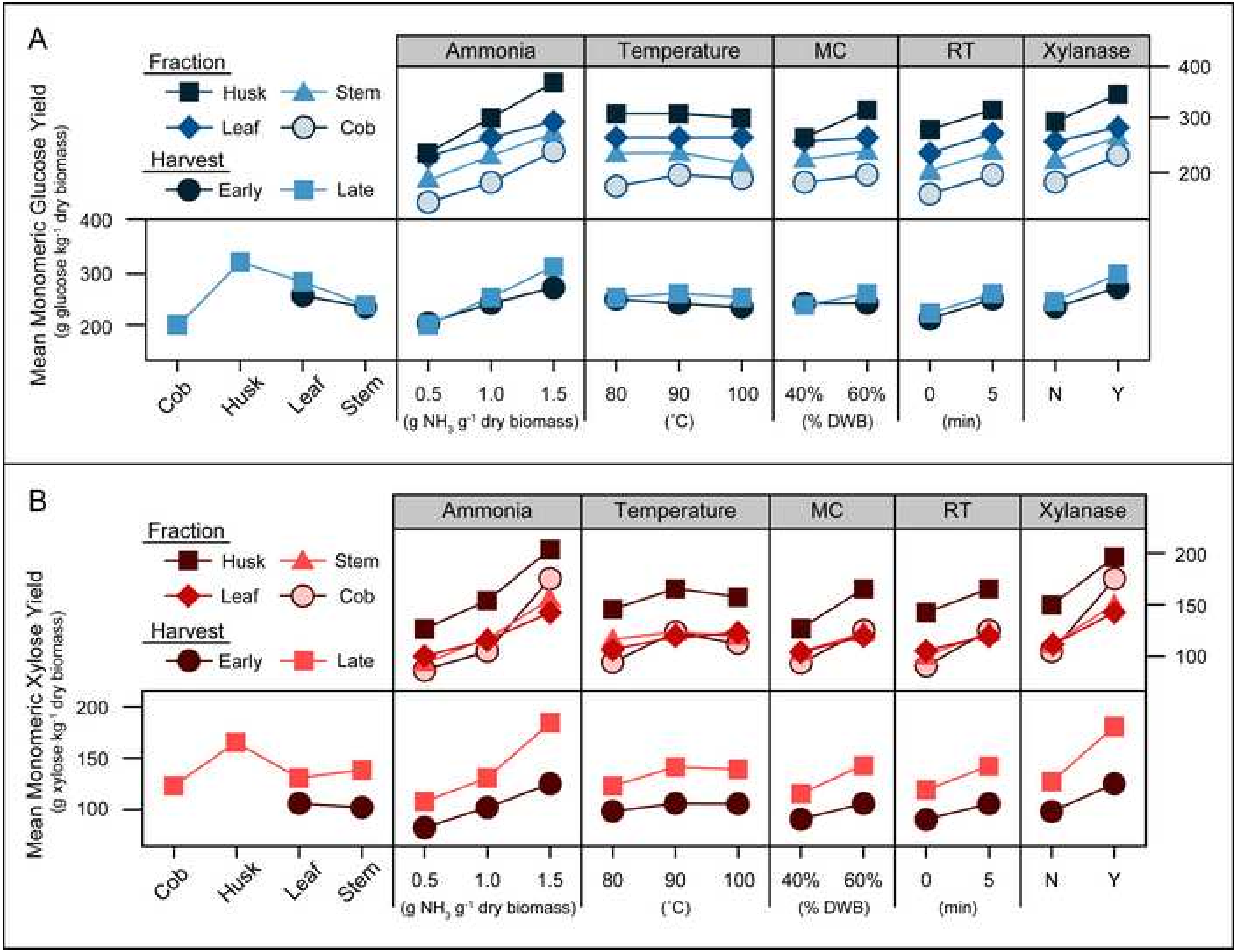

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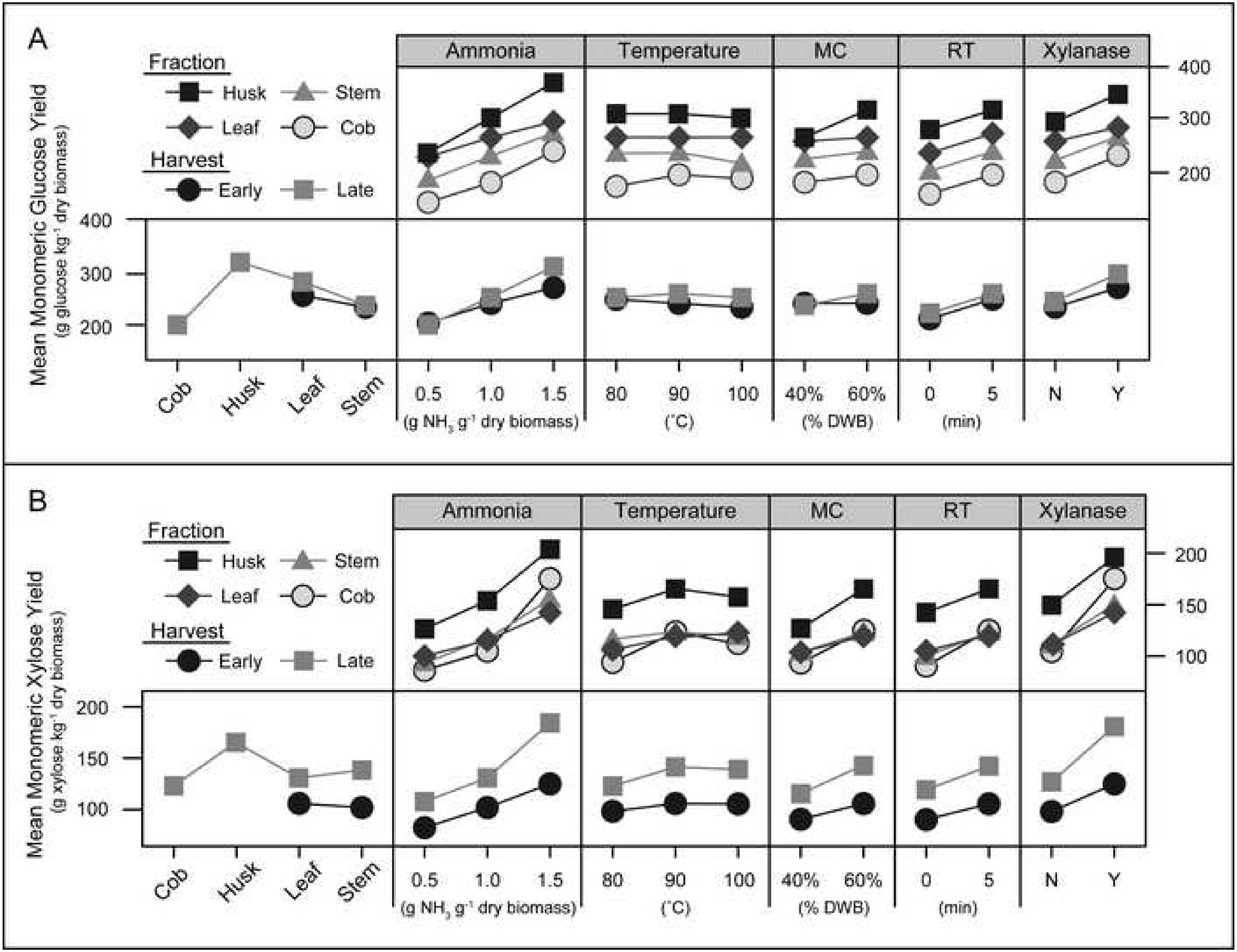

Figure 5

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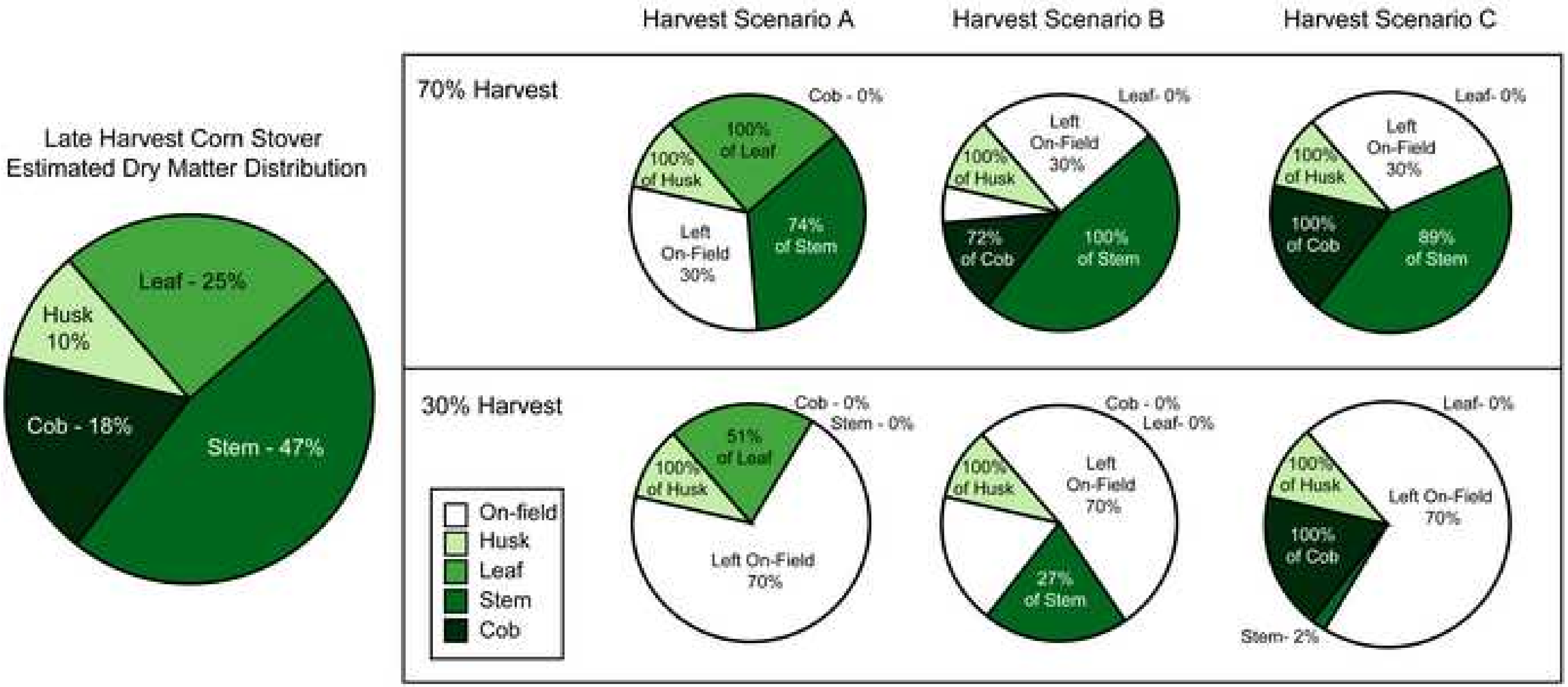

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