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Effect of compositional variability of distillers' grains on cellulosic ethanol production

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ABSTRACT

In a dry grind ethanol plant, approximately 0.84 kg of dried distillers' grains with solubles (DDGS) is produced per liter of ethanol. The distillers' grains contain the unhydrolyzed and unprocessed cellulosic fraction of corn kernels, which could be further converted to ethanol or other valuable bioproducts by applying cellulose conversion technology. Its compositional variability is one of the factors that could affect the overall process design and economics. In this study, we present compositional variability of distillers' grains collected from four different dry grind ethanol plants and its effect on enzymatic digestibility and fermentability. We then selected two sources of distillers grains based on their distinctive compositional difference. These were pretreated by either controlled pH liquid hot water (LHW) or ammonia fiber expansion (AFEX) and subjected to enzymatic hydrolysis and fermentation. Fermentation of the pretreated distillers' grains using either industrial yeast or genetically engineered glucose and xylose co-fermenting yeast, yielded 70–80% of theoretical maximum ethanol concentration, which varied depending on the batch of distillers' grains used. Results show that cellulose conversion and ethanol fermentation yields are affected by the compositions of distillers' grains. Distillers' grains with a high extractives content exhibit a lower enzymatic digestibility but a higher fermentability.

1. Introduction

In response to the rapidly growing demand for fuel ethanol, US ethanol production capacity has achieved 10 billion gallons per year in 2009. Additional production capacity is currently under construction (RFA Annual Ethanol Industry Outlook, 2010). Most of the corn ethanol is produced via dry grind processes (approximately 80%) while the remainder is produced via wet milling. In a dry grind ethanol plant, corn is ground and the corn starch is hydrolyzed and fermented to produce ethanol. The unutilized, and unfermented components of the corn kernel, which are mostly hull (fiber), germ, and protein, are marketed as DDG (dried distillers' grains), DDGS (dried distillers' grains with soluble), WDG (wet distillers' grains, wet cake) and CDS (condensed distillers' soluble or syrup). Due to their high nutritional value, these co-products have been mainly utilized as supplementary livestock feed. Most studies, therefore, have focused on their application as an alternative protein and nutrients source in livestock diet (Klopfenstein et al., 2008; Koster, 2007; Lumpkins et al., 2004). As the production of ethanol grows, the amount of co-products from the biorefineries also increases. According to the US grains council and RFA, the production of DDGS, the major co-product of dry milling process, from the corn biorefineries reached 25 million metric tons in 2009, and is expected to climb further in the near future (RFA Annual Ethanol Industry Outlook, 2010). Considering the expected increase of traditional co-products output from the expanding corn-to-ethanol biorefineries, it is critical to identify and develop new value added co-products that will open up new markets for fermentation by-products.

While the production of fuel ethanol from corn is a mature technology, research is ongoing to further improve process economics and long-term competitiveness of the industry by developing higher quality hybrid corn seed varieties (Weller et al., 1988; Murthy et al., 2008), improving process efficiency for more gallon of ethanol per bushel of corn processed (Kim et al., 2008a,b; Dien et al., 2008; Lau et al., 2008; Sharma et al., 2008; Srinivasan et al., 2007; Bals et al., 2006; Bothast and Schlicher, 2005), and identifying new applications for current co-products (Sharma et al., 2008; Selling and Woods, 2008; Wang et al., 2008; West and Nemmers,

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2008; Tatara et al., 2008; Romero et al., 2007). Recently, significant collaborative efforts have been put forth to add value to distiller's grains by further processing them into fermentable sugars, ethanol, and a protein rich co-product. These studies were published in a special edition of Bioresource Technology journal (Ricke et al., 2008).

In processing cellulosic biomass to produce ethanol in a large scale, it is important to supply a feedstock of consistent composition and characteristics to maintain a constant productivity and yield of fuel ethanol. Composition of distiller's grains is expected to vary depending upon the composition of feedstock corn kernels, manufacturing plant process efficiency and design. If distillers' grains were to vary significantly depending upon its origin and dry grind facilities, this variability would complicate design and implementation of process modifications as well as estimates of its economics.

Compositional variability of distillers' grains was extensively studied by several researchers (Belyea et al., 2004; Spiehs et al., 2002). However, these studies were mainly focused on the composition of DDGS only and its nutritional value, as its main utilization has been as animal feed. An extensive compositional analysis of different types of dry grind co-products including DDGS, WDG and thin stillage as a source of additional fermentable sugars to increase overall bioethanol production has been completed by Kim et al. (2008a). Thin stillage is also an important dry grind co-product that has a significant role in cellulose conversion technology. It can be used as a recycle water stream for the pretreatment and hydrolysis stages to minimize the fresh water consumption in the overall process (Kim et al., 2008c). The study has shown that distillers' grains contain as much as 30–35% wt/wt carbohydrates, which are available for fermentation to ethanol. However, only one specific batch of each co-product obtained from a single dry grind facility was utilized.

In this study, we examine compositional variability of various dry milling co-products, such as DDGS, WDG, and thin stillage, collected from four different dry grind ethanol plants in the Midwestern area of US, all located in Corn Belt states. Two separate batches of distillers' grains samples with significant compositional differences were further studied and compared in terms of enzymatic digestibility and fermentability. The selection of the two different samples was based on rationale that differences in processing and fermentation yield would be most readily observed for distillers' grain with significant compositional differences. Enzymatic digestibility and fermentability of those selected distillers' grains were further examined by applying different types of pretreatment technologies (liquid hot water or ammonia fiber expansion), hydrolysis methods (low or high solids loadings; with or without supplementary xylanase enzymes), and yeast strains (industrial yeast or xylose-fermenting recombinant yeast) for fermentation in consideration of various conditions applicable in cellulosic ethanol production.

2. Methods

2.1. Materials

DDGS, wet distiller's grains (WDG or wet cake), and thin stillage were obtained from four different dry grind facilities, all located in the Midwestern US, and each owned and operated independently. Annual ethanol production capacity of the selected dry grind facilities ranges from 50 to 110 million gallon per year.

Spezyme CP (cellulase) and Multifect Pectinase PE were provided by Genencor International, Inc. (Rochester, NY) and Novozym 188 (β-glucosidase, Novo Nordisk, Novo Allé, Denmark) was purchased from Sigma (Cat. No. C6150). Depol 740L (feruloyl esterase) was provided by Biocatalysts Enzymes (Wales, UK). All other reagents and chemicals, unless otherwise noted, were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Compositional analysis

Composition of the collected DDGS, WDG, and stillage samples were analyzed by procedures (cellulosic biomass compositional analyses) described in Kim et al. (2008a). The components analyzed were extractives, crude protein, glucan, xylan, arabinan and ash. Each sample was analyzed for complete compositions by three different laboratories. All measurements were in triplicates. The values obtained were averaged and errors were calculated at the 95% confidence level using Microsoft Excel. The F test in single factor analysis of variance (ANOVA) was carried out in order to test the significance of the variability of the components. Statistical analysis was done by Data Analysis Tool pack in Microsoft Excel. The p values of <0.05 were considered as significant.

2.3. Pretreatment

Two different pretreatment technologies were applied: liquid hot water (LHW) pretreatment and ammonia fiber expansion (AFEX) on the selected distillers' grains with the most significant difference in compositions to examine the compositional variability on enzymatic digestibility. All pretreatment runs were in triplicates. Error bars represent 95% CI of a mean.

2.3.1. Liquid hot water pretreatment

The aqueous pretreatment of distillers' grains consisted of mixing the substrate with thin stillage at 15% solids loading (w/w, g dry solids per g total) and heating at 160 °C for 20 min under pressure in order to keep the water in a liquid state. Reactions were conducted in 1 in. OD × 0.083 in. (2.54 cm × 2.1 mm) wall thickness, 316 stainless steel tubing capped at either end with 1 in. (2.54 cm) Swagelok tube end fittings (Swagelok, Indianapolis, IN). Each tube was 4.5 in. (11.4 cm) in length and 45 mL in total volume. The sample volume was kept at 33.7 mL to give approximately 25% of head space for liquid expansion during heating to 160 °C (Kim et al., 2009). The reactor tube containing the slurry of distillers' grains and thin stillage was heated by placing it in a Tecam® SBL-1 fluidized sand bath (Cole-Parmer, Vernon Hills, IL) set to 160 °C for 24 min, which included a 4 min heat-up and 20 min reaction time. After pretreatment, each tube was cooled by quenching in water and transferring to an ice-water slurry. The pretreated material was enzymatically hydrolyzed and fermented as described in the following sections.

2.3.2. Ammonia fiber expansion (AFEX) treatment

The AFEX pretreatment process was performed in a 300 mL 316 stainless steel pressure vessel. For each batch, 40 g dry weight of distillers' grains at 60% moisture content (total weight basis) was added to the vessel. Glass spheres were added to minimize void space, thereby minimizing the amount of ammonia in the vapor phase within the reactor. The reactor was sealed and charged with ammonia by connecting a pressure cylinder containing 15.0 g of liquid anhydrous ammonia. Approximately 1 g of ammonia remained in the sample cylinder after charging, so that the total ammonia loading was 0.80:1 g/g dry biomass. The reactor was heated to 90 °C using a 400 W PARR heating mantle (Parr Instrument Company, Moline, IL), requiring between 14 and 18 min, and allowed to stand at a constant temperature (±2 °C) for 5 min. At these conditions, the final pressure of the reactor ranged from 350 to 430 psi. The pressure was explosively released by rapidly turning the exhaust valve. The treated biomass was removed and placed in a fume hood overnight to evaporate residual ammonia.

Multiple batches were combined and thoroughly mixed before being used for future experiments.

2.4. Enzymatic hydrolysis test

Enzymatic digestibility of the pretreated distillers' grains was examined at a very low solids loading (5% w/w) as well as at a higher dry solids loading (15%). The low solids hydrolysis was to measure digestibility of biomass with minimal end-product inhibition during the hydrolysis, while the higher solids hydrolysis was to measure saccharification yield at a more economically feasible level. All hydrolysis runs were in triplicates. Error bars represent 95% CI of a mean.

2.4.1. Low-solids enzymatic hydrolysis

Enzymatic saccharification of LHW or AFEX pretreated distillers' grains were done by following a modified LAP 009 procedure (Brown and Torget, 1996). The procedure was modified by scaling-up the masses and volumes by a factor of 10. The material was used as is without grinding. Enzyme loading for the hydrolysis was 15 FPU of Spezyme CP cellulase activity and 40 CBU of Novozym 188 β-glucosidase activity per g glucan (equivalent to 32 mg total protein per g glucan or 6.4 mg total protein per g dry substrate). Enzymes and the pretreated substrate at about 5% dry solids loading (w/w) were added to 250 mL Nalgene bottles and the bottles placed in a New Brunswick Scientific model G24 Environmental Incubator Shaker (Edison, NJ) set at 50 °C and an agitation rate of 200 rpm. The pretreated slurry was allowed to digest at 50 °C for up to 72 h. A 1.0 mL sample was removed at regular intervals for the analysis.

2.4.2. High-solids enzymatic hydrolysis

Distillers' grains, pretreated at a solids level of 15% dry solids w/w, was hydrolyzed by Spezyme CP at 15 FPU cellulase and Novozym 188 at 40 CBU β-glucosidase per g glucan. In some cases supplementary xylanses were added: Multifect Pectinase at 50 U xylanase and Depol 740L at 2 U feruloyl esterase per g dry solids. The entire slurry of the high dry solids pretreated distillers' grains was subjected to the hydrolysis without any further dilution. The pH of the LHW pretreated distillers' grains was about 4.5 which is already close to the optimal pH of the cellulase enzymes. Hydrolysis was carried out without adjusting the pH, at 50 °C and 200 rpm. Duration of the hydrolysis was varied depending on the experiment. Further details are presented in the Section 3.

2.5. Fermentability test

Fermentability of enzymatically hydrolyzed distillers' grains was examined using two different yeast strains: industrial *Saccharomyces cerevisiae* D5A and *Saccharomyces cerevisiae* 424A (LNH-ST). The latter is a genetically modified glucose–xylose co-fermenting yeast developed by Dr. Nancy Ho of Purdue University.

Distillers' grains, pretreated as described previously, was pre-hydrolyzed at 15% dry solids loading (w/w) by cellulase enzymes at a loading of 32 mg total protein per g glucan (15 FPU Spezyme CP and 40 CBU Novo 188 per g glucan) for 24 h. The hydrolysis conditions were as described in the previous section. After 24 h the entire hydrolysate (100 mL total volume) was transferred into side-arm flask, pH adjusted to 5.5–6.0 with ammonium hydroxide, then inoculated with either *S. cerevisiae* D5A or *S. cerevisiae* 424A (LNH-ST).

For inocula generation, 8 mL of seed culture were used to inoculate 100 mL YEPD (YEP plus 2% glucose) in a 500 mL baffled Erlenmeyer flask equipped with a side-arm. The inoculation cultures were incubated in a shaker at 28 °C and 200 rpm and grown aerobically for 24 h (final O.D. 500–550 KU). The yeast was harvested by centrifugation at 3000*g* for 5 min at room temperature. The supernatant was discarded and the cells were transferred into a 300 mL baffled Erlenmeyer flask containing the WDG hydrolysate. The flasks were subsequently sealed with Saran plastic wrap to allow fermentation to be carried out micro-aerobically. The SSF was carried out at 28 °C, 200 rpm for 72 h. At regular intervals 1 mL samples of the culture were removed for monitoring the fermentation. All fermentations were run in duplicate. Error bars represent 95% CI of a mean.

2.6. HPLC analysis

Measurements of sugars, ethanol, and fermentation by-products were performed using a HPLC system consisting of a Varian 9010 Solvent Delivery System, Waters 717plus Auto sampler, Aminex HPX-87H column (Biorad, Hercules, CA), Waters 2414Refractive Index Detector, Waters 2487 Dual λ Absorbance Detector, and a Hewlett Packard HP3396G Integrator. The mobile phase was 5 mM H_2SO_4 filtered through 0.2 μm nylon filter (Millipore) and degassed. The mobile phase flow rate was 0.6 mL/min and the column temperature was maintained at 60 °C by an Eppendorf CH-30 Column Heater controlled by an Eppendorf TC-50.

3. Results and discussion

3.1. Compositional variability of DDGS, WDG and thin stillage

Compositions of DDGS, WDG and thin stillage collected from four different dry grind ethanol plants are summarized in Table 1A. The values are the average of data provided by Purdue University, Michigan State University (MSU), and the National Center for Agricultural Research (NCAUR). DDGS were uniform in composition; the average coefficient of variation for all components was less than 5.7%. Ether extractives (mostly oil) content was on average 11.7 ± 0.7% and crude protein 30.1 ± 1.4%. Glucan content was 18.2–20.1% which was divided between cellulose (2/3) and residual starch (1/3). The hemicellulose fraction was 15.8–18.0%. If combined, this gives 35–39% of total carbohydrates.

The mass closure was excellent with over 95% of the mass accounted for the distillers' grains. The 110% mass closure on sample 4 is due to some of components in the water extractives being double-counted. The water extractives are mostly residual mono- and oligo-saccharides, organic acids, and fermentation by-products. Kim et al. (2008a,b,c) found that about 10% of the total protein and carbohydrates were recovered during the hot water extraction. This explains the mass balance of over 100% for all DDGS samples and WDG sample 4 in Table 1(A), all of which contain evaporated stillage.

As shown in the ANOVA test *P*-values in Table 1(C) and (D), glucan, xylan and ash contents in the four different DDGS samples were not statistically different while the major difference was in the extractives content among the samples. The ANOVA analysis between the samples indicated that the #4 DDGS sample's extractives content is significantly different from the other samples at the $p < 0.05$ level. Comparisons between WDG samples also showed that #4 WDG is statistically different from other samples in terms of xylan, ash, protein and extractives contents. With the exception of sample #4, all the other three WDG samples were found to have similar compositions. The WDG sample #4 contained greater amounts of water and ether extractives and less carbohydrate than other WDG samples. In particular water extractives content, which are mostly fermentation by-products, soluble sugars, soluble proteins, and organic acids, was more than three times greater than measured for the other WDG samples. In a conventional dry grind facility, evaporated stillage, which is generally referred as "syrup",

Table 1
Compositions of various dry grind co-products collected from four different dry grind plants. (A) Compositions of DDGS and WDG by% dry weight (B) thin stillage by concentration in g/L and (C) ANOVA test *p*-values of DDGS and WDG compositions comparisons at significance level of 0.05. Compositions numbers are average of Purdue, MSU and USDA-NCAUR results. Errors in 95% CI are less than 6% for all values.

(A)	DDGS				WDG (wet cake)			
	1	2	3	4	1	2	3	4
Dry matter (%)	90.3	90.7	90.0	90.4	36.9	36.1	36.2	51.7
Water extractives (%)	23.1	23.0	24.6	21.7	7.8	8.4	8.7	31.9
Ether extractives (%)	12.0	12.3	11.8	10.7	8.7	8.5	7.9	12.7
Crude protein (%)	28.0	31.1	30.4	30.9	33.3	35.6	35.5	28.7
Glucan (total) (%)	20.1	18.2	19.0	18.3	20.2	18.8	19.6	17.6
Starch (%)	6.1	4.7	6.1	5.9	5.5	4.2	4.7	5.8
Cellulose (%)	14.0	13.5	12.9	12.4	14.7	14.5	14.9	11.9
Xylan and arabinan (%)	18.0	16.4	15.8	16.7	23.1	21.4	21.0	14.9
Xylan (%)	11.9	10.7	10.4%	10.8	14.4	13.5	13.4	9.7
Arabinan (%)	6.2	5.7	5.5	5.8	8.8	7.8	7.7	5.2
Ash (%)	4.69	4.52	4.37	4.50	2.23	2.19	2.04	5.55
Total dry matter mass closure (%)	105.9	105.5	106.0	102.8	95.3	94.8	94.7	111.3

(B)	Thin stillage		
	1	2	3
Glucan (g/L)	15.3	11.4	11.3
Glucose (g/L)	0.2	0.1	3.7
Xylan (g/L)	3.1	1.8	3.2
Xylose (g/L)	0.9	1.0	0.0
Arabinan (g/L)	1.3	1.1	1.0
Arabinose (g/L)	0.2	0.2	0.2
Glycerol (g/L)	15.6	16.7	17.9
Acetic Acid (g/L)	0.9	0.9	0.9
Ethanol (g/L)	0.6	0.5	0.8

(C)	ANOVA test *p*-values					
DDGS	Glucan	Xylan	Protein	Ash	Water extractives	Ether extractives
1 vs. 2 vs. 3 vs. 4	0.310	0.306	***0.045***	0.393	***0.003***	***0.000***
1 vs. 2	>0.05	>0.05	***0.004***	>0.05	0.876	***0.011***
1 vs. 3	>0.05	>0.05	0.056	>0.05	0.069	0.635
1 vs. 4	>0.05	>0.05	0.060	>0.05	***0.001***	***0.003***
2 vs. 3	>0.05	>0.05	0.418	>0.05	0.084	***0.002***
2 vs. 4	>0.05	>0.05	0.830	>0.05	***0.020***	***0.001***
3 vs. 4	>0.05	>0.05	0.711	>0.05	***0.008***	***0.003***
WDG	Glucan	Xylan	Protein	Ash	Water extractives	Ether extractives
1 vs. 2 vs. 3 vs. 4	0.343	***0.036***	***0.012***	***3.2E-05***	***7.9E-15***	***6.4E-08***
1 vs. 2	>0.05	0.559	0.277	0.662	0.279	0.279
1 vs. 3	>0.05	0.499	0.291	0.318	***0.002***	0.481
1 vs. 4	>0.05	***0.026***	0.064	***0.001***	***3.6E-08***	***1.2E-04***
2 vs. 3	>0.05	0.886	0.933	0.422	***0.012***	***0.001***
2 vs. 4	>0.05	***0.021***	***0.014***	***0.001***	***2.0E-08***	***3.6E-06***
3 vs. 4	>0.05	***0.031***	***0.013***	***0.002***	***1.8E-08***	***1.2E-06***

is mixed with WDG prior to drying into DDGS. We believe that sample #4 was taken after DG was mixed with thin stillage. This particular sample was likely collected after mixing of the syrup and prior to drying step, as suggested from the significantly higher water extractives content. The thin stillage consisted mainly of soluble gluco-oligomers and glycerol (Table 1B). In summary, the compositional analysis showed that the WDG samples 1, 2, and 3 are similar in compositions, while sample 4 exhibited significantly different composition from the others.

Further studies were conducted using WDG samples #2 and #4 to study the effect of the compositional variability on enzymatic digestibility and fermentability. The #2 WDG sample was selected as a representative sample of a normal WDG. The #4 WDG sample was chosen as its composition was distinctively different from the other samples. The #4 WDG was of particular interest as it contains significant amount of components that are accumulated in the process and dissolved in the "syrup". Process water is commonly recycled in commercial ethanol plants and various fermentation by-products, including acetic acid and glycerol, accumulate (Galbe and Zacchi, 1992, 1994). As a result, these substances, which could be toxic to yeast fermentation, are present at high concentrations in the evaporated stillage (syrup). Their concentrations are expected to be even higher in a modified dry grind process that converts distillers' grains to ethanol (Kim et al., 2008c). Therefore, sample 4 with its higher concentrations of condensed substances, enables us to examine their effect and compare WDG with its lower extractives content.

3.2. Enzymatic digestibility test

Enzymatic digestibility of #2 and #4 WDG samples were tested at 5% dry solids loading (50 g dry solids/L or 10 g glucan/L) with commercial cellulase enzymes. The enzyme loading was 15 FPU cellulase (Spezyme CP) and 40 IU β-glucosidase (Novozym 188) per g glucan. A low solids concentration was selected to minimize inhibition effects due to end-products or other substances released during the hydrolysis, as well as mass transfer resistance due to mixing difficulties. With reduced inhibition effects at this low solids level, the yields were expected to reflect mainly intrinsic characteristics of the biomass, including those that limit access of the cellulase enzymes to cellulose, and whose effect is moderated by the pretreatment (Zeng et al., 2007).

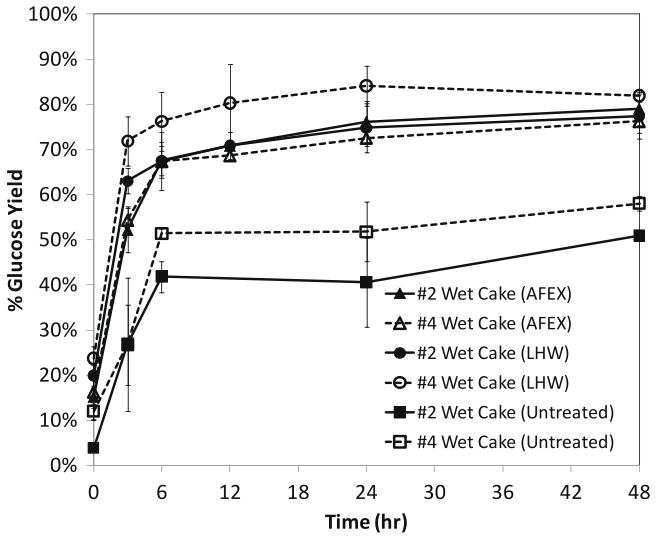

Fig. 1. Enzymatic digestibility of LHW pretreated and AFEX pretreated WDG (wet cake) samples. Hydrolysis conditions: modified LAP 009, 5% (w/w) dry solids loading, 15 FPU Spezyme CP and 40 IU Novozym 188 per g glucan (or 32 mg total protein/g glucan). About 50 °C, pH 4.8, 200 rpm, 48 h hydrolysis. Numbers in parenthesis are errors in 95% CI.

Yields of glucose from hydrolysis of WDG samples pretreated by either AFEX or LHW are compared in Fig. 1. Pretreated wet cake showed higher conversions than untreated wet cake. Hydrolysis reactions were 90% complete within the initial 6 h. The maximum glucose yields were 70–80% for both LHW and AFEX pretreated WDG samples. As for the LHW pretreated WDG, the #4 WDG resulted in approximately 10% higher average glucose yield than #2 WDG, and 4% higher for the AFEX pretreated WDG samples, as measured after 24 h which is when the yield reached a maximum. The ANOVA analysis between the samples indicated that the difference in digestibility between #4 WDG and #2 WDG was statistically significant at a 5% level, regardless of the pretreatment method applied. Yields for untreated WDG samples were lower than for pretreated samples: 40% glucose yield for #2 WDG and 52% for #4 WDG, as measured after 24 h. Again, the #4 WDG gave 10% higher average glucan conversion. The #4 WDG contains three times more water extractives than #2 WDG. These water extractives include fermentation by-products, soluble sugars and proteins. The slightly higher sugar yield from #4 WDG than from #2 WDG is thought to be due to soluble sugars that were already present in the water extractives and not from higher conversion efficiencies of cellulose to glucose. This could be confirmed by comparing the 0 h yields which show 5–10% conversion due to pretreatment. These were measured before adding cellulase, of untreated #2 and #4 WDG samples in Fig. 1.

Hydrolysis of WDG was also evaluated at a moderate dry solids loading of 15% or 150 g dry solids per kg total slurry, which is equivalent to 30 g glucan per kg total slurry. Yields of glucose and xylose from the hydrolysis of #2 and #4 pretreated WDGs are summarized in Table 2. The glucose yields were 55–60% and those for xylose only 12–23%. Contrary to the low solids digestibility tests, the average glucose yield was slightly higher for #2 WDG than for #4 WDG for both AFEX and LHW pretreatments. The difference in glucose yields of #2 and #4 LHW pretreated WDG was, however, statistically insignificant at 5% level, while it was statistically significant for AFEX pretreated WDG samples. The xylose yield of #4 WDG was statistically significantly higher than #2 WDG at 5% level for both pretreatments.

Another observation was that the yields were significantly lower than those measured from the 5% dry solids hydrolysis. This observation is consistent with Kim et al. (2008b) who reported that the sugar yields decreased as the% dry solids increased from 15% to 30%. Reduced yields of sugars for the hydrolysis of high solids slurry can be caused by various factors including inefficient mixing of the slurry due to its high viscosity, and local buildup of glucose and cellobiose that inhibit cellulase activity. The high solids slurry has lower water activity, which may affect the ability of the enzyme to diffuse and bind to the cellulose efficiently, and is more pronounced at low enzyme dosages. The concentration of other non-product inhibitors increases along with solids concentration, further reducing cellulase activity.

As presented in Table 2, the yield of xylose was low (12–23%) when pretreated WDG was hydrolyzed at 15% dry solids. Although almost 50% of the total xylan in the material was released as oligomers during the LHW pretreatment, the yield of xylose monomers was low due to missing enzyme activities required to cleave the heterogeneous linkages present in this complex arabinoxylan. The heteroxylan of corn fiber (corn bran) is known to be highly branched with ferulic acid ester-linked to arabinofuranosyl residues and the heteroxylan strands are cross-linked through diferulate ester bridges (Bunzel et al., 2005; Montgomery and Smith, 1957; Saulnier et al., 1999; Whistler and Corbette, 1955; Yadav et al., 2007). This is thought to be a way for the non-lignified cell walls of maize bran to resist exogenous enzyme attacks (Saulnier and Thibault, 1999). The ferulic acid groups can be saponified by using feruloyl esterase. Release of mono sugars from corn fiber has been shown to increase with release of ferulic acid by feruloyl esterase, indicating that breaking down heteroxylans of corn fiber by feruloyl esterase improves the extent of polysaccharides hydrolysis (Dien et al., 2008; Shin et al., 2006).

Dien et al. (2008) has shown the effect of supplementary enzymes, which contain high feruloyl esterase as well as xylanase activities, to facilitate the hydrolysis of heteroxylan in distillers' grains. The two enzymes, Multifect Pectinase PE and Depol 740L, were supplemented, in addition to Spezyme CP and Novo 188, at the loadings recommended by Dien et al. (2008). Yields of both glucose and xylose from hydrolysis of #2 and #4 WDG, pretreated by either LHW or AFEX, were improved by addition of the supplementary enzymes (Fig. 2). As expected, the yield of xylose was greatly improved (11–50%). Also, there was 10–15% increase in the glucose yield by the action of these supplementary enzymes.

The results were consistent with the previous observations shown in the studies by Dien et al. (2008) and Kim et al. (2008b) with the two different batches of WDG samples (#2 and #4) appeared to respond consistently. With addition of the supplementary enzymes, #2 WDG still exhibited a slightly higher or similar glucose yield but a lower xylose yield than #4 WDG. The difference of the mean values was statistically significant at 5% level. The

Table 2
Glucose and xylose yields from 24 hydrolysis of various pretreated WDG samples at 15% solid loadings. Numbers in parenthesis are errors in 95% CI.

WDG sample no.	LHW pretreated		AFEX pretreated	
	#2	#4	#2	#4
Glucose yield (%)	54.5% (±9.6%)	53.7% (±5.5%)	58.8% (±0.3%)	54.8% (±0.2%)
Xylose yield (%)	14.4% (±1.3%)	23.2% (±1.7%)	12.2% (±0.3%)	14.2% (±0.4%)

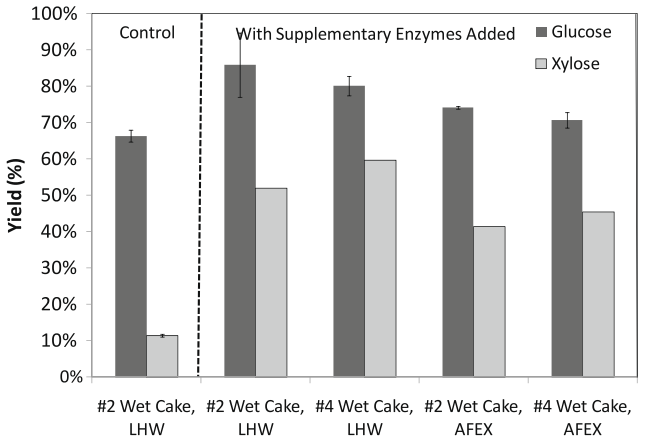

Fig. 2. Enzymatic digestibility of LHW pretreated and AFEX pretreated WDG (wet cake) with addition of supplementary enzymes (Multifect Pectinase PE and Depol 740L). Hydrolysis conditions: 15% (w/w) dry solids loading, 50 °C, pH 4.8, 200 rpm, 48 h hydrolysis. Control: 15 FPU Spezyme CP and 40 IU Novozym 188 per g glucan. Supplementary enzymes were added at 50 U/g dry solids xylanase (Multifect Pectinase PE) and 2 U/g dry solids feruloyl esterase (Depol 740). Numbers in parenthesis are errors in 95% CI.

hydrolysates of WDG were evaluated for fermentation to ethanol using a commercial *Saccharomyces* yeast strain and a recombinant xylose-co-fermenting yeast (424A (LNHST)), results from which are discussed in the next sections.

3.3. Fermentability tests

SSF of Pretreated WDG Hydrolysate using S. cerevisiae D5A: The WDG samples. #2 and #4, upon LHW or AFEX pretreatment, were enzymatically pre-hydrolyzed by adding 15 FPU Spezyme CP and 40 IU Novo 188 per g glucan at 15% dry solids loading and incubating at 50 °C for 24 h. The whole slurry was inoculated with *Saccharomyces cerevisiae* D5A and fermented for 72 h. The yields of sugars following the 24 h pre-hydrolysis are summarized in Table 2. The fermentation time courses are plotted in Fig. 3. The initial glucose concentration for WDG pretreated with LHW was higher than for AFEX treated due to the thin stillage added in for the LHW samples, which contained additional sugar oligomers. Xylose concentrations were below 5 g/L for all cases. Glucose was completely consumed within the first 12 h of fermentation. As measured at its highest point, the metabolic ethanol yield was 0.56–0.59 g/g (or 110–115% of the maximum ethanol theoretical yield) for the LHW pretreated WDG and 0.63–0.68 g/g (or 123–133%) for the AFEX treated WDG, implying that additional glucose was generated probably from cellulose and fermented during the SSF. AFEX treated hydrolysate contains higher nitrogen than LHW pretreated hydrolysate probably due to ammonia carried over from the pretreatment process (Kim et al., 2008b). The higher metabolic ethanol yield for the

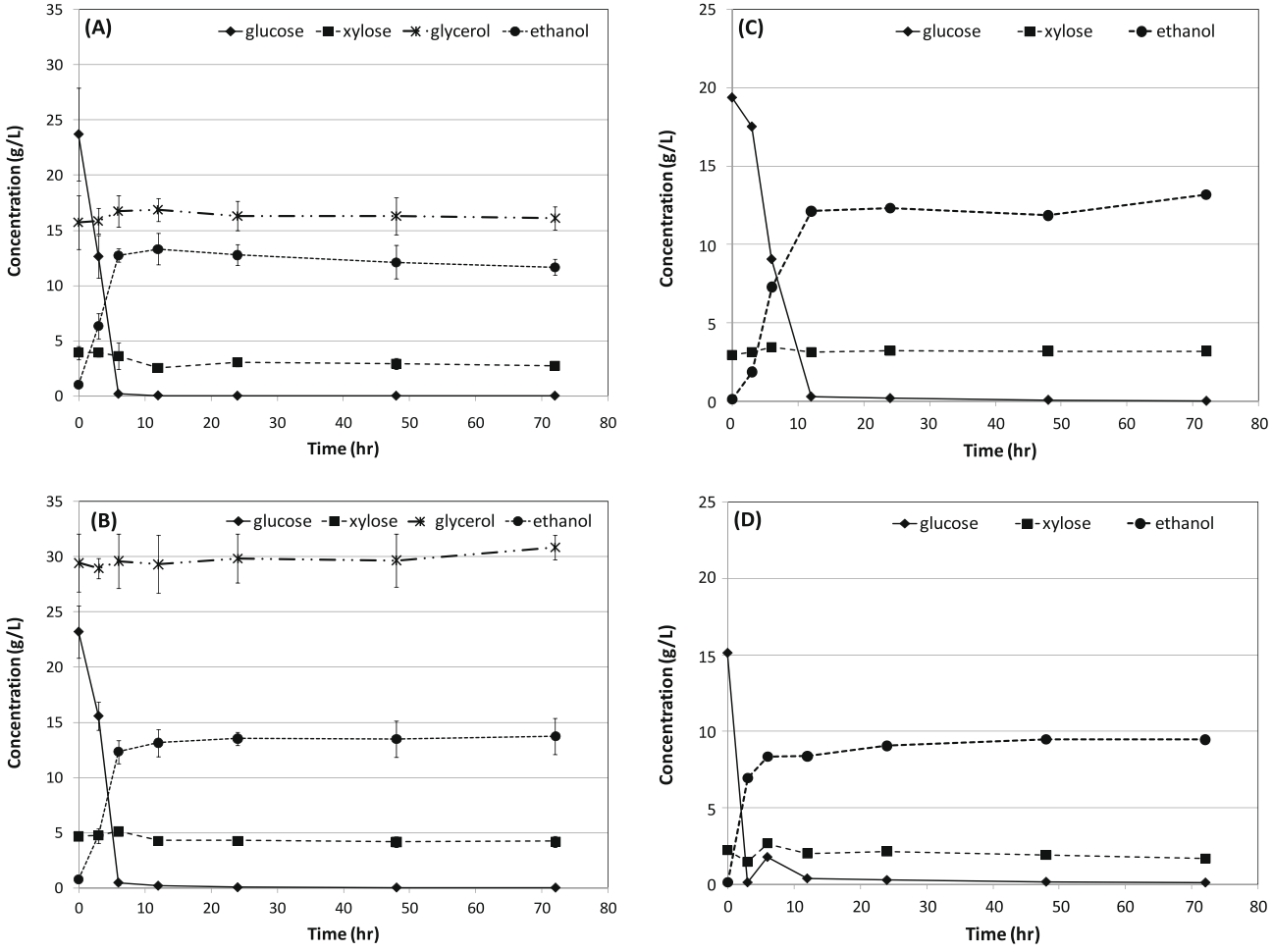

Fig. 3. Simultaneous saccharification and fermentation time course of LHW pretreated and AFEX pretreated WDG (wet cake) samples using *Saccharomyces cerevisiae* D5A. (A) #2 WDG, LHW; (B) #4 WDG, LHW; (C) #2 WDG, AFEX; (D) #4 WDG, AFEX. Pre-hydrolysis conditions: 15% (w/w) dry solids loading for 24 h using 15 FPU Spezyme CP and 40 IU Novozym 188 per g glucan, 50 °C, pH 4.8, 200 rpm. SSF conditions: entire pre-hydrolysate, *Saccharomyces cerevisiae* D5A, 28 °C, 100 rpm 72 h. Error bars represent 95% CI. Data without error bars are average of duplicate runs.

AFEX versus LHW treated WDG hydrolysate may suggest the beneficial effect this additional nitrogen has on yeast metabolism.

Ethanol production yields, calculated based on the maximum theoretical ethanol concentration, are summarized in Table 3 and calculated as below.

$$\text{Ethanol production yield}(\%) = \frac{\text{measured ethanol concentration}}{\text{theoretical max.sugar conc. based on raw biomass}(g/L) \times 0.511} \times 100$$

The ethanol production yield as measured at the highest ethanol concentration during the fermentation was 74% and 79% for #2 and #4 LHW pretreated WDG, respectively. Similar yields were observed for AFEX treated WDGs, 78% for #2, but a lower yield of 67% for #4 WDG. These results mean that at least 70–80% of glucan is hydrolyzed to glucose during the hydrolysis and fermentation, which neglects glucose metabolized for cell mass and production of side-products. The glucose yield after 24 h hydrolysis was between 55% and 60% (Table 2). Therefore at least 15–20% of additional glucans were hydrolyzed during the SSF.

Table 3
Ethanol production yield based on the maximum theoretical ethanol concentration during fermentation of various LHW or AFEX treated WDGs. Numbers in parenthesis represent 95% CI. Data without errors are average of duplicate runs.

WDG sample no.	Maximum theoretical yield			
	LHW		AFEX	
	#2	#4	#2	#4
Saccharomyces D5A(SSF)[a]	73.9% (±3.7%)	79% (±4.7%)	78.4% (±1.2%)	67.3% (±3.1%)
Xylose-co-fermenting *Saccharomyces* 424A (LNH-ST)[b] including contribution of xylose addition	95.3% (±7.2%)[c] 73.6% (±2.4%)	83.6% (±0.6%)[c] 79.4% (±4.5%)	80.80%[c] 71.0%	71.4% (±12%)[c] 80.9% (±3.9%)

[a] Conversion scheme: 24 h pre-hydrolysis (Spezyme CP + Novo188) + 72 h SSF.
[b] Conversion scheme: 48 hydrolysis (Spezyme CP + Novo188 + Multifect Pectinase + Depol 740), supplemented with xylose to give 60 g/L xylose concentration, and 72 h fermentation of liquid hydrolysate only.
[c] Yield calculated based on the maximum theoretical ethanol concentration from glucose only during the first 3 h of fermentation.

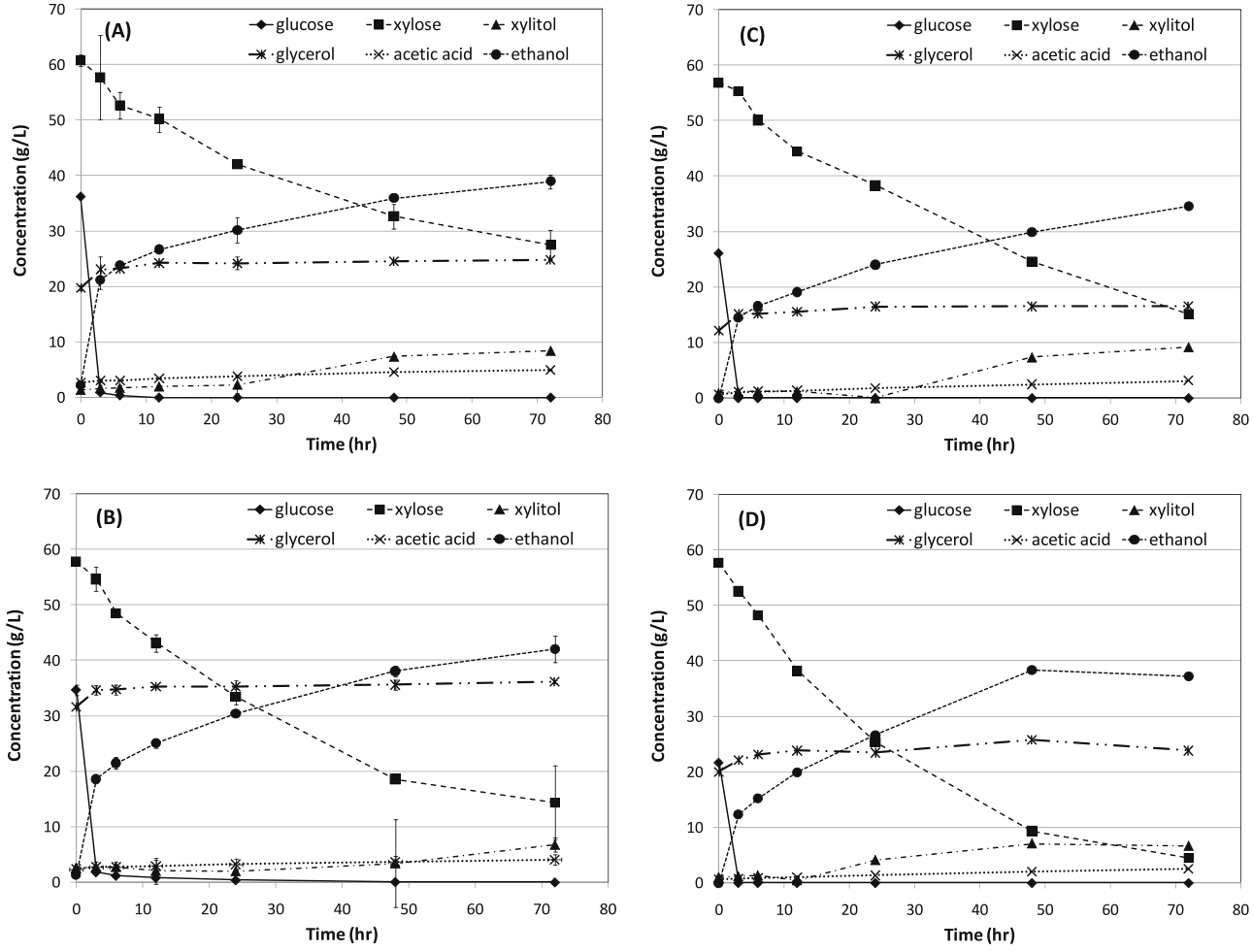

Fig. 4. Fermentation time course of LHW pretreated and AFEX pretreated WDG samples with added xylose using *Saccharomyces cerevisiae* 424A (LNH-ST). (A) #2 WDG, LHW; (B) #4 WDG, LHW; (C) #2 WDG, AFEX; (D) #4 WDG, AFEX. Hydrolysis conditions: 15% (w/w) dry solids loading for 48 h using 15 FPU Spezyme CP and 40 IU Novozym 188 per g glucan, Multifect Pectinase PE at 50 U xylanase/g dry solids and Depol 740L at 2 U feruloyl esterase/g dry solids. 50 °C, pH 4.8, 200 rpm. Fermentation conditions: liquid fraction of the hydrolysate, *Saccharomyces cerevisiae* 424A (LNH-ST), 28 °C, 100 rpm 72 h. Error bars represent 95% CI. Data without error bars are average of duplicate runs.

As shown in Fig. 3, the LHW pretreated WDG samples were fermented at a similar rate and, as a result, the ethanol production yields were also similar. However, the fermentation time course of AFEX pretreated #2 and #4 WDG were notably different from each other. The glucose from the AFEX treated #4 WDG was consumed at a faster rate than that from #2 WDG and the final ethanol production yield was significantly higher for #2 WDG than for #4 WDG. The lower ethanol production yield for the AFEX treated #4 WDG was expected as the sugar concentration at the end of the-pre-hydrolysis was lower than for #2 WDG. The faster sugar consumption rate of AFEX treated #4 WDG reflected a possible beneficial effect of the high extractives content during the fermentation of AFEX treated biomass.

Glucose/xylose co-fermentation of pretreated WDG hydrolysate: Fig. 4 shows the fermentation time course of WDG, pretreated and hydrolyzed for 48 h with the cocktail of enzymes listed previously. Unlike the previous experiment, solids (including residual cellulose) were removed prior to fermentation. Also, instead of using a native *Saccharomyces strain* only capable of fermenting hexoses, the cultures were inoculated with a recombinant *Saccharomyces cerevisiae* strain (424A (LNH-ST) engineered for xylose co-fermentation. This recombinant yeast was previously reported to efficiently co-ferment xylose and glucose in hydrolysate prepared from various lignocellulosic biomasses (Sedlak and Ho, 2004a,b). Since the initial xylose concentration in the hydrolysate of WDG was too low (10–15 g/L) to observe effectiveness of the xylose fermentation, reagent grade xylose was added to the hydrolysate until a final xylose concentration of 60 g/L was reached. The theoretical maximum xylose concentration for the solids loading used here is 20 g/L for #2 WDG and 25 g/L for #4 WDG. Therefore, 60 g/L xylose is beyond what can be achieved here. However, this concentration can be achieved by raising the solids loading to 30% (w/w) and increasing the hydrolysis efficiency to 100%, albeit achieving this could be challenging due to various factors described in the introduction.

The ethanol production yields calculated in Table 3 suggest that at least 74–80% of the fermentable sugars (glucose and xylose combined) present in the initial WDG and added xylose were converted to ethanol during the xylose co-fermentation. Metabolic ethanol yield (% of theoretical ethanol based on the consumed sugars) was 103–105% for all runs. Some oligomeric sugars, present in the liquid hydrolysate, may have been further hydrolyzed and converted by the yeast to ethanol during the fermentation.

As illustrated in Fig. 4, glucose was completely consumed within the initial 3 h of fermentation. Ethanol production yield in terms of maximum theoretical yield at 3 h, based only upon glucose, was 85% for #2 WDG and 77% for #4 WDG pretreated by LHW (Table 3). It was 81% and 71%, respectively, for AFEX treated #2 and #4 WDG. Xylose was also quickly consumed as the fermentation proceeded. Xylose was consumed at a faster rate for #4 WDG than for #2 WDG irrespective of pretreatment. Even when the fermentation was continued for more than 120 h, more residual xylose was present for #2 than for #4 WDG. As a result, ethanol production yield after 72 h fermentation, based on the maximum theoretical ethanol concentration from both glucose and xylose, was slightly higher for #4 WDG (80% for both LHW and AFEX treated) than for #2 (74% for LHW pretreated and 71% for AFEX treated), as presented in Table 3. The difference was statistically significant at 5% level.

As discussed earlier, the #4 WDG contains approximately four times more water extractives on a dry mass basis than the other WDG samples, which is probably due to presence of evaporated stillage on the wet cake. Water extractable compounds promote xylose metabolism by 424A (LNH ST). Hence syrup from condensed stillage, should be added as a nutrient supplement for the yeast fermentation, especially to improve xylose fermentation. Further buffering effect of the syrup maintained the pH at 5.5–5.9 during the fermentation. This reduced the toxic effect of fermentation inhibitors such as acetic acid, a well-known strong yeast inhibitor present ubiquitously in various cellulosic biomass. The beneficiary effect of the condensed stillage on the yeast fermentation deserves further study.

4. Summary and conclusions

By-product streams originating from four dry grind plants were analyzed for composition. DDGS compositions were uniform; varying by an average of 5.7%. With the exception of sample 4 (contained evaporated thin stillage) WDG compositions were also uniform and varied by an average of 5.4%. The mass closure for sets was excellent with 105% and 99.0% of the mass accounted for in DDGS and WDG respectively. The overall results show that ethanol yields per bushel of corn may be increased by 7–10% when wet cake is pretreated and hydrolyzed. The condensed stillage was found to be beneficial to xylose fermentation either because it added to the nutrient quality of the media or because it added buffering capacity, which putatively slowed the drop in pH during the fermentation.

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