Comparison of Raw Starch Hydrolyzing Enzyme with Conventional Liquefaction and Saccharification Enzymes in Dry-Grind Corn Processing

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ABSTRACT

In a conventional dry-grind corn process, starch is converted into dextrins using liquefaction enzymes at high temperatures (90–120°C) during a liquefaction step. Dextrins are hydrolyzed into sugars using saccharification enzymes during a simultaneous saccharification and fermentation (SSF) step. Recently, a raw starch hydrolyzing enzyme (RSH), Stargen 001, was developed that converts starch into dextrins at low temperatures (<48°C) and hydrolyzes dextrins into sugars during SSF. In this study, a dry-grind corn process using RSH enzyme was compared with two combinations (DG1 and DG2) of commercial liquefaction and saccharification enzymes. Dry-grind corn processes for all enzyme treatments were performed at the same process conditions except for the liquefaction step. For RSH and DG1 and DG2 treatments, ethanol concentrations at 72 hr of fermentation were 14.1–14.2% (v/v). All three enzyme treatments resulted in comparable ethanol conversion efficiencies, ethanol yields, and DDGS yields. Sugar profiles for the RSH treatment were different from DG1 and DG2 treatments, especially for glucose. During SSF, the highest glucose concentration for RSH treatment was 7% (w/v), whereas for DG1 and DG2 treatments, glucose concentrations had maximum of 19% (w/v). Glycerol concentrations were 0.5% (w/v) for RSH treatment and 0.8% (w/v) for DG1 and DG2 treatments.

In the United States, ethanol from corn is produced primarily by dry-grind and wet-milling processes. In 2005, dry-grind corn plants produced 79% of U.S. ethanol (RFA 2006). The energy balance of corn to ethanol production is a major concern. Fuel ethanol yields 77% more energy than is required to produce it using the dry-grind process, including growing corn, harvesting, transporting, converting, and distributing (Shapouri et al 2004). Farrell et al (2006) evaluated six representative analyses of fuel ethanol (including Shapouri et al 2004) and reported that ethanol and coproducts produced from corn yielded a positive net energy (energy produced from a gallon of ethanol minus the energy used in making a gallon of ethanol) of 4–9 MJ/L. Further decreases in energy usage in corn to ethanol production will make ethanol a more attractive fuel.

In a dry-grind plant, energy is used in jet cooking, liquefaction, distilling, dehydrating, and drying operations. Ground corn is cooked and liquefied to dextrins at ≥90°C for 1–2 hr using liquefaction enzymes (Kelsall and Lyons 2003). Dextrins are hydrolyzed into fermentable sugars using saccharification enzymes during simultaneous saccharification and fermentation (SSF). Recently, a raw starch hydrolyzing (RSH) enzyme (Stargen 001, Genencor International, Palo Alto, CA) was developed. Stargen 001 enzyme has high raw starch hydrolyzing activity and can convert starch into dextrins at ≤48°C as well as hydrolyze dextrins into fermentable sugars during SSF. Use of RSH enzymes in the dry-grind process does not require high temperatures during cooking and liquefaction. Therefore, the RSH enzyme potentially reduces energy requirements and improves the net energy. Robertson et al (2006) reviewed RSH enzymes and estimated the reduction in energy usage achieved by using RSH enzymes in ethanol production is 10–20%. Another benefit of using RSH enzymes in the dry-grind corn process is that it replaces two types of enzymes (liquefaction and saccharification) with one enzyme.

Wang et al (2005) used Stargen 001 enzyme to improve enzymatic dry-grind process (a modified conventional dry-grind corn process). In the enzymatic dry-grind corn process, germ, pericarp fiber, and endosperm fiber are recovered as coproducts before fermentation. Germ and pericarp fiber are recovered by floatation due to specific gravity differences. Use of RSH enzymes helped to break down raw starch and increase specific gravity of the slurry, which helped in floating germ and pericarp fiber. Wang et al (2005) compared the enzymatic dry-grind process using RSH enzymes with the conventional dry-grind process also using RSH enzymes. The objective of this study was to compare dry-grind ethanol production using a RSH enzyme treatment with two liquefaction and saccharification enzyme treatments.

MATERIALS AND METHODS

Experimental Material

Yellow dent corn (33A14 Pioneer Hi-Bred International, Johnston, IA) grown in 2004 at the Agricultural and Biological Engineering Research Farm, University of Illinois at Urbana-Champaign, was used for the study. Corn was sieved over a 4.8 mm (12/64") round-holes screen to remove broken corn and foreign material. RSH (Stargen 001), protease (GC106), α-amylase (Spezyme Fred) and glucoamylase (Fermenzyme L-400) enzymes were obtained from Genencor International (Palo Alto, CA). α-Amylase (Termamyl 120L, Novozymes NA, Franklinton, NC) and amyloglucosidase (AMG 300L, Novozymes) were obtained from Sigma (St. Louis, MO).

Dry-Grind Corn Process

Cleaned corn samples were ground in a hammer mill (model MHM4, Glen Mills, Clifton, NJ) at 500 rpm using a 2-mm sieve with round holes. Particle size analysis (Standard Method S319.3, ASABE 2003) was performed in triplicate using a sieve shaker (model RX-86, W. S. Tyler, Cleveland, OH) equipped with four sieves (U.S. standard sieve No. 20, 30, 40, and 50) and pan. Particle size distributions of ground flour were 24.9, 13.4, 18.2, and 8.8% on No. 20, 30, 40, and 50 screens, respectively, and 33.7% on pan. Approximately 60.7% ground corn went through a No. 30 screen (openings 595 µm in diameter). Ground corn samples were packed in plastic bags and stored at 4°C. Before the dry-grind process, corn was acclimated at room temperature. Corn flour moisture content was measured using a 135°C convection oven method in triplicate (Approved Method 44-19, AACC International 2000).

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A flow diagram of the dry-grind corn process is given in Fig. 1. Three enzyme treatments (RSH, DG1, and DG2) were conducted using dry-grind corn process. The RSH treatment used Stargen 001 enzyme, which contains α-amylase from *Aspergillus kawachi* and a glucoamylase from *A. niger* and had activity of ≥456 GSHU/g (where GSHU = granular starch hydrolyzing units). The DG1 enzyme treatment included α-amylase and amyloglucosidase. The α-amylase is from *Bacillus licheniformis* and had activity of 930 KNU/g (where KNU = kilo novo α-amylase units). Amyloglucosidase is from *A. niger* and had activity of ≥300 NU/mL (where NU = novo units). The DG2 enzyme treatment included Spezyme Fred and Fermenzyme L-400. Spezyme Fred (endo-amylase) is from *B. licheniformis* and had activity of ≥17,400 LU/g (where LU = liquefon units). Fermenzyme L-400 (exo-glucoamylase) is from *A. niger* and has activity of ≥350 GAU/g (where GAU = glucoamylase units). Detailed assays for enzyme activities are available from enzyme manufacturers.

The ground corn was mixed with water (700 g corn/1,748 mL of water) to obtain a mash with 25% dry solids content. Using 10*N* sulfuric acid, mash was adjusted to pH 4.2 for RSH treatment. Liquefactions were conducted by adding 2 mL of enzyme for 2 hr with agitation (50 rpm) at 48°C for RSH and at 90°C for DG1 and DG2 treatments (Table I). Liquefaction (pretreatment before SSF) for RSH treatment was not required but recommended by the enzyme manufacturer. However, in this study, liquefaction for RSH treatment was conducted to allow comparison with other treatments (DG1 and DG2). The liquefaction temperature of 48°C for RSH treatment was selected based on recommendations of the enzyme manufacturer. For SSF, mash was cooled to 30°C and adjusted to pH 4.0 using 10*N* sulfuric acid solution; 35 mL of *Saccharomyces* yeast culture, 2 mL of saccharification enzyme, 0.5 g of $(NH_4)_2SO_4$ and 0.5 mL of acid fungal protease (GC 106) were added. Addition of acid fungal protease GC106 helps the rate of fermentation by hydrolyzing protein into free amino nitrogen (Lantero and Fish 1993). Protease (GC106) was added during SSF for RSH and DG1 enzyme treatments. For the DG2 enzyme treatment, no protease was added because Fermenzyme L-400 enzyme contains GC106. Because the objective of this study was not to optimize, but to compare performance of enzymes in the dry-grind corn process, enzyme amounts added for all three treatments were in excess of the manufacturer recommended dosages.

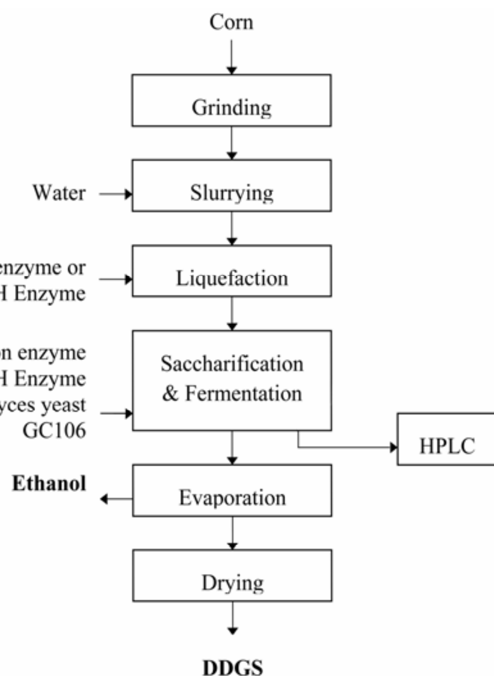

Fig. 1. Laboratory dry-grind corn process using a raw starch hydrolyzing (RSH) enzyme as well as two conventional liquefaction and saccharification enzyme treatments.

Saccharomyces yeast culture was prepared by dispersing 11 g of active dry yeast (Fleischmann's Yeast, Fenton, MO) and 1 g of yeast malt broth (Sigma, St. Louis, MO) in 89 mL of distilled water and agitated at 50 rpm and 30°C for 20 min (C24 Incubator Shaker, New Brunswick, NJ). *Saccharomyces* yeast culture had a viable cell count of 1.8×10^8 cells/mL using Petrifilm plates (3M, St. Paul, MN). The SSF process was performed using a 3-L flask with an overhead drive (model DHOD-182, Bellco Glass, Vineland, NJ) for agitation at 50 rpm, 30°C, and 72 hr.

Fermentation was monitored by taking 3-mL samples from the fermentation mash at 0, 2, 4, 6, 8, 10, 12, 18, 24, 30, 36, 48, and 72 hr. Using HPLC, each sample was analyzed to determine concentrations of ethanol, glucose, fructose, maltose, maltotriose, DP4+, glycerol, lactic acid, and acetic acid. From each 3-mL sample, clear supernatant liquid was obtained by centrifuging the sample at 1,789 × *g* for 5 min (Centra CL3, Thermo IEC, Needham Heights, MA). Supernatant was passed through a 0.2-μm syringe filter into 1-mL vials. Filtered liquid was injected into an ion-exclusion column (Aminex HPX-87H, Bio-Rad, Hercules, CA) maintained at 50°C. Sugars, organic acids, and alcohols were eluted from the column with HPLC-grade water containing 5 m*M* sulfuric acid. Elution rate was 0.6 mL/min. Separated components were detected with a refractive index detector (model 2414, Waters Corporation, Milford, MA). Data were processed using HPLC software (Waters Corporation). The HPLC was calibrated with standards containing all above components of interest at known concentrations at the beginning of each batch of samples. Calibration was verified with a secondary standard after every 10 samples and at the end of the batch. Each sample was injected twice for analysis. After fermentation, the mash was heated at 90°C for 3 hr to evaporate ethanol. To recover DDGS, the remaining materials were dried in a convection oven at 49°C for 72 hr. DDGS moisture content was determined using a 135°C convection oven method in triplicate (Approved Method 44-19, AACC International 2000).

Data Analysis

Each treatment (RSH, DG1, DG2) was replicated three times. Each sample was analyzed by HPLC in duplicate. Fermentation profiles (concentration vs. fermentation time) of ethanol, glucose, fructose, maltose, maltotriose, DP4+, glycerol, lactic acid, and acetic acid were plotted. Fermentation rates were expressed as the

TABLE I
Process Parameters of Dry-Grind Corn Processes Using RSH, DG1, and DG2 Enzyme Treatments

	RSH	DG1	DG2
Slurrying			
Solid content % (db)	25	25	25
Corn flour weight (g)	700	700	700
Water (mL)	1,748	1,748	1,748
Liquefaction			
Enzyme	Stargen 001	α-Amylase	Spezyme Fred
Enzyme usage (mL)	2	2	2
pH	4.2	5.5	5.5
Temperature (°C)	48	90	90
Time (hr)	2	2	2
Simultaneous saccharification and fermentation			
Enzyme	Stargen 001	Amylo glucosidase	Fermenzyme L-400
Enzyme usage (mL)	2	2	2
pH	4	4	4
Dry yeast (g)	4.2	4.2	4.2
Ammonium sulfate (g)	0.5	0.5	0.5
GC106 (mL)	0.5	0.5	None added

ratio of ethanol concentration at a specific time over ethanol concentration at 72 hr of fermentation. Theoretical ethanol yields (L/kg and gal/bu) were calculated based on corn test weight of 56 lb/bu and total starch content of 73.2 ± 0.3% (db) was determined using whole grain near-infrared transmittance (NIT) (Omeg analyzer G, Dickey-john, Springfield, IL). Actual ethanol yields (L/kg and gal/bu) were calculated based on final ethanol concentrations. Ethanol conversion efficiencies were calculated as the ratio of actual ethanol yield over theoretical ethanol yield. DDGS coproduct yields were calculated based on initial ground corn (db) used. For each enzyme treatment, final ethanol concentration, ethanol yield, ethanol conversion efficiency, and DDGS yield were compared using analysis of variance (ANOVA) (SAS Institute, Cary, NC). The level to show statistical significance was 5% ($P < 0.05$).

RESULTS AND DISCUSSION

Ethanol Profiles

Minor differences were observed in ethanol profiles among treatments (RSH, DG1, and DG2) (Fig. 2). During the first 18 hr, ethanol concentrations for the RSH treatment were higher than DG1 and DG2 treatments. At 24 hr, ethanol concentration of DG1 treatment was comparable to RSH treatment and higher than DG2 treatment. From 24 to 36 hr, ethanol concentrations of DG1 were higher compared with RSH and DG2. After 48 hr, ethanol concentrations for all treatments were similar. Final ethanol concentrations (at 72 hr) for RSH, DG1, and DG2 treatments were 14.1 ± 0.03, 14.1 ± 0.04, and 14.2 ± 0.09% (v/v), respectively; no differences ($P < 0.05$) in final ethanol concentrations were observed among treatments.

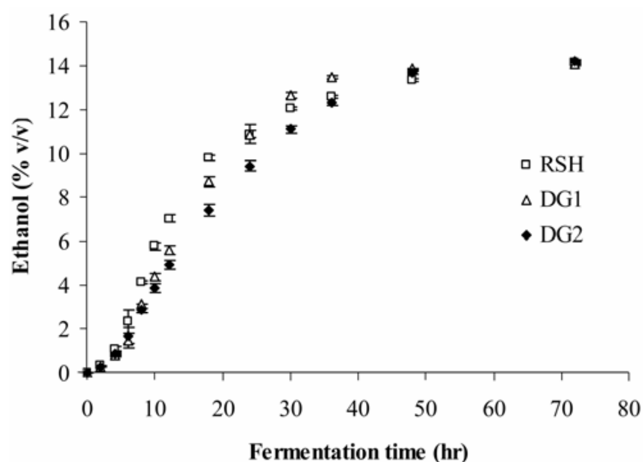

Fig. 2. Concentrations of ethanol during fermentation. Error bars are ± one standard deviation about the mean for each time period.

Glucose Sugar Profiles

Enzyme treatments DG1 and DG2 had similar glucose profiles, but were different from glucose profiles of RSH treatment (Fig. 3). During SSF, initial glucose concentration for the RSH treatment was 5.9% (w/v), which increased to 6.6% (w/v) at 2 hr, then exponentially decreased to negligible amounts by 24 hr. Initial glucose concentrations of DG1 and DG2 treatments were 18.7 and 19.3% (w/v), respectively, then exponentially decreased to negligible by 36 hr for DG1 treatment and 48 hr for DG2 treatment. Initial glucose concentration for the RSH treatment was lower than DG1 and DG2 treatments. This would suggest that enzymatic action for the Stargen 001 enzyme is different than action of commercial liquefaction enzymes.

Fructose, Maltose, Maltotriose and DP4+ Glucose Sugar Profiles

Saccharomyces yeast shows a distinct pattern of sugar utilization. After glucose consumption, fructose is used, followed by maltose, and then maltotriose (D'Amore et al 1989). Higher sugars (DP4+) can not be metabolized by *Saccharomyces* yeast. For all treatments, fructose, maltose, and maltotriose concentrations in SSF were low (<1.2%, w/v, data not shown). Initial fructose concentrations of RSH, DG1, and DG2 treatments were 0.6% (w/v). For RSH treatment, fructose concentration decreased to 0.07% (w/v) during the first 8 hr of SSF. For DG1 treatment, fructose concentration held constant at 0.6% (w/v) during the initial 6 hr of SSF and then decreased to 0.05% (w/v) at 36 hr. For DG2 treatment, fructose concentration increased to 0.7% (w/v) during the initial 2 hr, then decreased to 0.07% (w/v) at 48 hr.

Sugar profiles of DG1 and DG2 treatments for maltose, maltotriose, and DP4+ were similar but different from sugar profiles of RSH treatment. For the RSH treatment, maltose, maltotriose, and DP4+ were lower than concentrations of DG1 and DG2 treatments. For RSH treatment, initial DP4+ concentration was 0.4% (w/v) and held constant throughout SSF step (Fig. 4). For DG1 and DG2 treatments, initial DP4+ concentrations were 2.2 and 3.8% (w/v), respectively, during the first 6 hr, then decreased to 0.5 and 0.4% (w/v), respectively, at 30 hr and were constant for the rest of the process (Fig. 4). Overall, lower amounts of sugars (glucose, fructose, maltose, maltotriose, and DP4+) were present during SSF for RSH treatment than for treatments using conventional enzymes. Lower sugar concentrations during SSF using *Saccharomyces* yeast

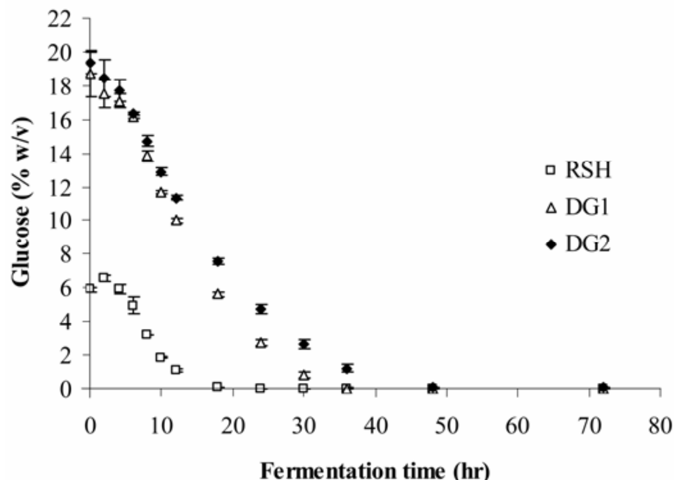

Fig. 3. Concentrations of glucose during fermentation. Error bars are ± one standard deviation about the mean for each time period.

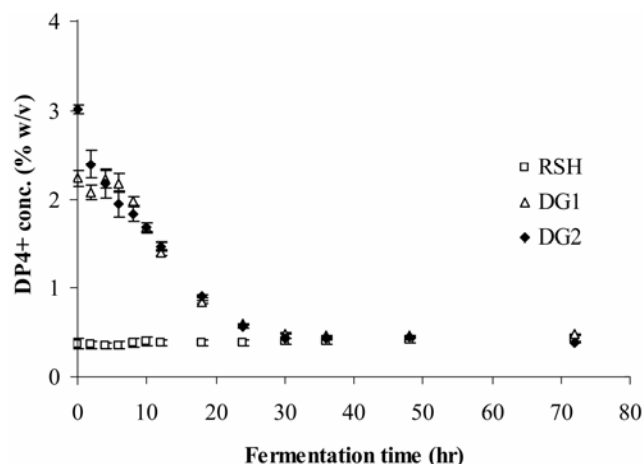

Fig. 4. Concentrations of DP4+ during fermentation. Error bars are ± one standard deviation about the mean for each time period.

is preferred because less osmotic stress is exerted on the yeast and because it retards growth of competing microorganisms that need to compete with the yeast for available glucose.

Glycerol Profile

Slightly higher amounts of glycerol were produced for DG1 and DG2 compared with RSH. For RSH treatment, glycerol concentration reached 0.5% (w/v) at 24 hr and was constant for the rest of SSF. For DG1 and DG2 treatments, glycerol concentrations reached 0.8% (w/v) at 36 and 48 hr, respectively, and were constant for the rest of SSF. Glycerol is a by-product of ethanol fermentation by *Saccharomyces* yeast. The yeast produces glycerol to help maintain intracellular redox balance (Nordström 1966) and as a response to osmotic stress (Hohmann 2002). Excessive glycerol production is an indicator of yeast stress. Glycerol production is undesirable because it lowers ethanol yield. Typical glycerol concentration is 1.2% for conventional dry-grind ethanol fermentation (Russel 2003).

Organic Acid Profiles

Final lactic acid concentrations were 0.03% (w/v) for RSH treatment and 0.02% (w/v) for DG1 and DG2 treatments. Acetic acid was not detected during SSF in any of the treatments. Concentrations of 0.2–0.8% (w/v) lactic acid and 0.05–0.1% (w/v) acetic acid stress *Saccharomyces* yeast (Narendranath et al 2001). Contaminating bacteria such as *Lactobacilli* convert glucose to lactic acid and acetic acid and result in lower ethanol yields. Low lactic acid concentrations and no acetic acid in the slurry suggests that there were no infections during fermentation. Plating the beer broth would be needed to measure actual infections.

TABLE II
Fermentation Rates for RSH, DG1, and DG2 Enzyme Treatments[a]

Fermenation Time (hr)	% Fermentation Completed		
	RSH	DG1	DG2
0	0	0	0
2	2.2	2.0	2.0
4	7.7	5.7	5.8
6	16.4	10.5	10.3
8	29.2	22.4	19.4
10	40.8	31.1	26.5
12	49.7	39.7	33.1
18	69.2	61.9	50.5
24	77.1	77.4	65.2
30	85.3	90.1	77.5
36	89.0	95.9	86.2
48	94.3	98.5	96.4
72	100.0	100.0	100.0

[a] Ratio of ethanol concentration at specific time over final ethanol concentration at 72 hr.

TABLE III
Final Ethanol Concentrations, Ethanol Yields, Ethanol Conversion Efficiencies, and DDGS Yields for Dry-Grind Corn Processes for RSH, DG1, and DG2 Enzyme Treatments[a]

	RSH	DG1	DG2[b]
Final ethanol concentration (% v/v)	14.1 ± 0.03	14.1 ± 0.04	14.2 ± 0.09
Ethanol yield (L/kg)	0.404 ± 0.001	0.399 ± 0.001	0.404 ± 0.004
Ethanol yield (gal/bu)	2.71 ± 0.01	2.68 ± 0.01	2.71 ± 0.03
Ethanol conversion efficiency (%)	88.4 ± 0.30	87.3 ± 0.30	88.4 ± 1.00
DDGS yield (% db)	30.3 ± 0.79	29.9 ± 0.66	30.1 ± 0.29

[a] Mean ± standard deviation of three observations.
[b] No differences for final ethanol concentrations, ethanol yields, ethanol conversion efficiencies, and DDGS yields of RSH, DG1, and DG2 were detected.

Fermentation Rate

During the first 18 hr of SSF, RSH treatment had higher ethanol productivity than either the DG1 or DD2 treatments (Table II). At 24 hr, fermentation rates of RSH and DG1 treatments were comparable (77.3% of maximum) and higher than the fermentation rate of DG2 treatment (66.4% of maximum). At 48 hr, DG1 treatment had the highest fermentation rate (97.9% of maximum) followed by the DG2 treatment (96.5% of maximum) and the RSH treatment (94.3% of maximum).

Ethanol Yields and Ethanol Conversion Efficiencies

Ethanol yields for RSH, DG1, and DG2 enzyme treatments were 0.404 ± 0.001, 0.399 ± 0.001, and 0.404 ± 0.004 L/kg (2.71 ± 0.01, 2.68 ± 0.01, and 2.71 ± 0.03 gal/bu), respectively (Table III). Theoretical ethanol yield was 0.457 L/kg (3.07 gal/bu) based on corn test weight of 56 lb/bu and total starch content of 73.2% (db). Ethanol conversion efficiencies for RSH, DG1, and DG2 treatments were 88.4 ± 0.30, 87.3 ± 0.30, and 88.4 ± 1.00%, respectively (Table III). Ethanol yields and conversion efficiencies for three enzyme treatments were not different ($P < 0.05$). RSH treatment for dry-grind corn process gave ethanol yield and ethanol conversion efficiencies similar to traditional enzymes.

DDGS Yields

For enzyme treatments RSH, DG1, and DG2, DDGS yields were 30.3 ± 0.79, 29.9 ± 0.66, and 30.1 ± 0.29% (db), respectively (Table III). DDDS yields for three enzyme treatments were not different ($P < 0.05$). For RSH treatment, liquefaction temperature was 48°C, which was lower than corn starch thermal swelling and gelatinization temperature of 55–65°C (Robertson et al 2006). However, for DG1 and DG2 treatments, the liquefaction temperature was 90°C. Low liquefaction temperature could have an effect on DDGS nutritional characteristics.

CONCLUSIONS

The dry-grind corn process using RSH enzyme was compared with dry-grind processes using two combinations of conventional liquefaction and saccharification enzymes. During SSF, glucose concentrations with RSH treatment were lower than those in conventional enzyme treatments. Final ethanol concentrations, ethanol yields, ethanol conversion efficiencies, and DDGS yields of the processes with RSH treatment and traditional enzyme treatments were similar. The dry-grind corn process using raw starch hydrolyzing enzyme is expected to reduce energy requirements during cooking and liquefaction as well as to simplify the operation.

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