

Dry-Grind Processing of Corn with Endogenous Liquefaction Enzymes

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ABSTRACT

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An amylase corn has been developed that produces an α -amylase enzyme that is activated in the presence of water at elevated temperatures ($>70^{\circ}\text{C}$). Amylase corn in the dry-grind process was evaluated and compared with the performance of exogenous amylases used in dry-grind processing. Amylase corn (1–10% by weight) was added to dent corn (of the same genetic background as the amylase corn) as treatments and resulting samples were evaluated for dry-grind ethanol fermentation using 150-g and 3-kg laboratory procedures. Ethanol concentrations during fermentation were compared with the control treatment (0% amylase corn addition or 100% dent corn) which was processed with a conventional

amount of exogenous α -amylase enzymes used in the dry-grind corn process. The 1% amylase corn treatment (adding 1% amylase corn to dent corn) was sufficient to liquefy starch into dextrins. Following fermentation, ethanol concentrations from the 1% amylase corn treatment were similar to that of the control. Peak and breakdown viscosities of liquefied slurries for all amylase corn treatments were significantly higher than the control treatment. In contrast, final viscosities of liquefied slurries for all amylase corn treatments were lower than those of the control. Protein, fat, ash, and crude fiber contents of DDGS samples from the 3% amylase corn treatment and control were similar.

Production of ethanol in the United States has increased in the last several years. Most of the recent increase in ethanol capacity in the United States has come from construction or expansion of dry-grind corn processing plants. The increase in ethanol capacity from dry-grind corn plants is expected to continue for the next several years due to replacement of MTBE in motor fuels and the passage of the Energy Bill of 2005. In a conventional dry-grind process, corn is ground and mixed with water to produce slurry, which is cooked, liquefied, saccharified, and fermented to produce ethanol. Conventional liquefaction and saccharification of slurry uses exogenous α -amylase and glucoamylase enzymes, respectively. Liquefaction enzymes break down starch molecules into lower molecular weight dextrins; glucoamylase enzymes break down the dextrins into glucose. Remaining nonfermentables in corn (germ, fiber, and protein) are recovered at the end of the dry-grind process as a coproduct called distillers dried grains with solubles (DDGS).

New advances are being made to improve the dry-grind corn process and increase profitability of ethanol production. These include development of new and modified dry-grind processes (Hojilla-Evangelista et al 1992; Bryan 2005; Singh et al 2005), recovery of valuable coproducts (Singh and Eckhoff 1996; Singh et al 1999; Hojilla-Evangelista and Johnson 2003), better and lower cost enzymes (Anonymous 2005), and high-ethanol-yielding corn hybrids (Haeefele et al 2004; Singh and Graeber 2005). Recently, an amylase corn has been developed by transgenic technology that produces and stores α -amylase within the kernel (Lanahan 2003). These enzymes are activated in the presence of water at elevated temperatures ($>70^{\circ}\text{C}$). In conventional dry-grind corn processing, exogenous α -amylase enzymes are added during liquefaction and are one of the operating costs in dry-grind processing. Expression levels of the α -amylase in the amylase corn are high and, therefore, it is expected that only small amounts of amylase corn will need to be added to regular dent corn to achieve adequate liquefaction.

Performance of amylase corn in the dry-grind process and the requirements of amylase corn addition are not known. Use of amylase corn in dry-grind ethanol processing was evaluated and the

performance was compared with exogenous amylases currently used in the dry-grind process.

MATERIALS AND METHODS

Experimental Material and Design

Transgenic amylase corn and dent corn with the same genetic background were obtained from a commercial seed company (Syngenta Biotechnology, Inc., Research Triangle Park, NC). Corn samples were hand-cleaned to remove broken corn and foreign material, packaged in plastic bags, and stored at 4°C until processing. Whole kernel moisture content was measured using the 103°C convection oven method (Approved Method 44-15A, AACC International 2000). Starch content of amylase (71%) and dent (72%) corn was determined by near-infrared transmittance (GrainSpec, Foss North America, Minneapolis, MN) using Corn Refiners Association standard methods (CRA 1980).

Three experiments evaluated the performance of amylase corn in the dry-grind process. In the first experiment, four levels (0, 3, 5, and 10%) of amylase corn were added to the dent corn as treatments, and samples were evaluated in a 150-g laboratory dry-grind procedure. Zero percent amylase corn treatment (0% amylase corn addition or 100% dent corn) was the control treatment. No exogenous α -amylase enzyme was added, except for the control treatment in which normal amounts of exogenous α -amylase enzyme was added. A second experiment evaluated four amylase corn treatments (0, 1, 2, and 3%) using a 150-g dry-grind procedure. A third experiment was conducted in which a 3% amylase corn treatment was compared with a control treatment in a 3-kg dry-grind procedure.

150-g Dry-Grind Laboratory Process

Corn samples were ground in a laboratory hammer mill (model MHM4, Glen Mills, Clifton, NJ) equipped with a 2.0-mm sieve and operated at 500 rpm. Ground corn weighing 150 g was mixed with tap water (35°C) to obtain slurry containing 27% solids (db). All runs were done in 1,000-mL flasks in a shaking water bath (model DHOD-182, Bellco Glass, Vineland, NJ).

The first experiment used sequential saccharification and fermentation after liquefaction for the control treatment. Samples were liquefied by increasing the temperature of the slurry to $90 \pm 0.5^{\circ}\text{C}$. No α -amylase enzyme was added to 3, 5, and 10% amylase corn treatments. α -Amylase (0.2 mL, α -amylase solution *Bacillus licheniformis*, type XII-A saline solution 500–1,000 units/mg of protein, 1,4- α -D-glucan-glucohydrolase, 9000-85-5, Sigma-Aldrich, St. Louis, MO) was added to the control treatment (0% amylase corn or 100% dent corn). The slurry was held at 90°C for 90 min with continuous agitation at 150 rpm. After 90 min, slurry

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temperature was reduced to 60°C. The slurry was adjusted to 4.1–4.2 pH using 1*N* sulfuric acid solution. Samples were saccharified by adding 0.6 mL of glucoamylase (amyloglucosidase from *Aspergillus niger*, glucoamylase, 1,4- α -D-glucan glucohydrolase, exo-1,4- α -glucosidase, 9032-08-0, Sigma-Aldrich, St. Louis, MO) and incubating slurry at 60°C for 2 hr with constant agitation at 150 rpm. Before fermentation, saccharified mash was cooled to 30°C.

Yeast (Fleischmann's Yeast, Fenton, MO) inoculum was prepared using the procedure of Wang et al (1997) by dispersing active dry yeast (11 g) in 99 mL of distilled water and agitating at 38°C for 20 min. The inoculum had a viable cell count of $\approx 2 \times 10^8$ cells/mL. Yeast inoculum (2 mL) was added to the saccharified mash. Ammonium nitrogen (300 ppm) was provided by adding (NH₄)₂SO₄ as a yeast nutrient. Fermentation was for 72 hr at 30°C with continuous agitation at 50 rpm. Fermentation was monitored by withdrawing 5 mL of broth at 24-hr intervals and measuring sugar and ethanol concentrations using HPLC. Aliquots (5 mL) drawn from fermentation vessels were centrifuged (model Dura-fuge 100, Precision, Winchester, VA) at $1,476 \times g$ for 5 min. Supernatant (5.0 μ L) was filtered through a 0.2- μ m filter and injected into an ion-exclusion column (Aminex HPX-87H, Bio-Rad, Hercules, CA) maintained at 50°C. Sugars (glucose, fructose, maltose, and maltotriose), organic acids (lactic, succinic, and acetic acid) and alcohols (ethanol, methanol, and glycerol) were eluted from the column with HPLC-grade water containing 5 mM sulfuric acid. Separated components were detected with a refractive index detector (model 2414, Waters Corp., Milford, MA). The elution rate was 0.6 mL/min; a calibration standard was used before each batch run. Data were processed using HPLC software (Waters, Milford, MA).

For the second experiment, samples were processed as in the first experiment, except that simultaneous saccharification and fermentation was used. After liquefaction, slurry temperature was lowered to 30°C and the sample was adjusted to pH 4.1–4.2 with 1*N* sulfuric acid solution and glucoamylase enzyme was added, followed by addition of yeast inoculum. Fermentation was for 72 hr at 30°C with continuous agitation at 50 rpm. Sugars and ethanol concentrations were measured using HPLC as in the first experiment.

3-kg Dry-Grind Laboratory Process

Based on results using 3% amylase corn, an additional experiment at a larger (20 \times) scale compared processing results between a control and a 3% amylase corn treatment. The same corn, enzymes, and yeast were used as in the experiments using the 150-g dry-grind procedure. Corn samples were ground using a cross-beater mill (model Retsch SK 100, Glen Mills, Clifton, NJ) equipped with a 2.0-mm sieve. A feed rate of whole kernels to the hopper of the cross-beater mill was maintained by an electromagnetic vibratory feeder (model F-To, FMC Corporation, Homer City, PA) during mill operation. Ground corn weighing 3,000 g (as-is basis) was mixed with tap water (35°C) to obtain slurry with 25% solids (db) and an initial pH of 5.7 ± 0.1 . Liquefaction and simultaneous saccharification and fermentation steps were done in 20-L flasks equipped with overhead drives (model DHOD-182, Bellco Glass, Vineland, NJ) for agitation. Samples were liquefied by increasing slurry temperature to $90 \pm 1^\circ\text{C}$. No α -amylase enzyme was added to the 3% amylase corn treatment; 5 mL of α -amylase was added to the control. During liquefaction, slurry was held at 90°C for 90 min, with continuous agitation at 150 rpm. Remaining procedures were similar to the 150-g dry-grind procedure described above.

After fermentation, mash was heated to 85°C for 2 hr to evaporate ethanol. Stillage (material left after boiling) was poured into 5-L flat bottom, open aluminum pans and dried in a convection oven for 24 hr at 59°C. Dried stillage was called distillers dried grains with solubles (DDGS) and analyzed for crude protein (Method

990.03), crude fat (Method 920.3), ash (Method 942.05), and crude fiber (Method 962.09) (AOAC 2003). Moisture content of DDGS was determined using a two stage convection oven method (Approved Method 44-18, AACC International 2000).

Ground Corn Viscosity Profile

Viscosities of ground amylase corn mixtures and control treatments during liquefaction were determined using a Rapid Visco Analyser (RVA-4, Newport Scientific, Warriewood, Australia). Each sample of ground corn slurry (8 g db or 25% solids) was heated rapidly from room temperature to 50°C, heated from 50 to 95°C at 18°C/min, held at 95°C for 4 min, and cooled to 50°C at 15°C/min. Five amylase corn treatments (0, 1, 3, 5, and 10%) were evaluated in triplicate for viscosity profiles. Viscosity profiles from all the amylase corn treatments were compared with a control treatment (0% amylase corn) in which 13 μ L of exogenous α -amylase was added to the 100% dent corn sample (with the same genetic background as amylase corn). Pasting temperature, peak viscosity, breakdown, and final viscosity were recorded.

Residual Enzyme Activity in DDGS

Residual enzyme activities in DDGS samples were determined by the Ceralpha HR amylase assay kit (Megazyme International, Wicklow, Ireland) according to manufacturer's recommendations. Dried DDGS samples were ground and suspended in 10 \times weight of 50 mM CAPS buffer pH 10.0. The buffer was adjusted to pH 10 as needed. Slurries were mixed at 55°C for at least 1 hr, then centrifuged at $3,220 \times g$ for 15 min. Aliquots from the supernatants were diluted serially in assay buffer (250 mM MOPS buffer pH 7.0) to bring the enzymatic activity into the range of the Ceralpha HR assay. Enzyme assay (Ceralpha HR reagent, 50 μ L) was mixed with diluted enzyme (50 μ L) in 96-well PCR plates. An aliquot (25 μ L) was removed and mixed with Tris base (1*M* \times 175 μ L). The PCR plates were heated to 60°C for 20 min then cooled to 4°C. Heating and cooling were at the maximum rate of the PE 9700 thermocycler (PerkinElmer, Wellesley, MA). An additional 25- μ L aliquot was removed from each assay and mixed with Tris base as above. Quenched samples were arrayed in a 96-well spectrophotometer plate and an A₄₀₀ wavelength was read using a spectrophotometer. The pathlength correction feature of the spectrophotometer (Spectromax Plus, Molecular Devices) was used to correct to the absorbance of a 1-cm sample. Samples that yield an absorbance of 0.5–1.5 are in the linear range of the assay. Units of enzyme (μ mol of PNP/min) were determined using the known molar absorptivity of PNP (18,100 M⁻¹ cm⁻¹) according to the instructions of the manufacturer. Dilution of the extract and weight of extracted sample were factored in to yield a value of units of amylase per gram of dry weight.

Statistical Analysis

Dry-grind processing of all treatments used three replicates. Fermentation samples were analyzed using HPLC with at least two determinations. HPLC analyses for each replicate were averaged. Viscosity profiles were measured in triplicate. Analysis of variance (ANOVA) and Duncan's multiple range test (SAS Institute, Cary, NC) were used to determine significant differences in sugar profiles and ethanol concentrations. The level selected to show statistical significance was 5% ($P < 0.05$).

RESULTS AND DISCUSSION

Comparison of Sugar Profiles

No differences were observed from 0 to 72 hr of fermentation in glucose, maltose, maltotriose, and DP4+ concentrations among control (0% amylase corn or 100% dent corn) and 3, 5, and 10% amylase corn treatments (Table I).

These results suggest that the amylolytic action of the endogenous liquefaction enzyme in amylase corn is comparable to the

action of exogenous enzyme currently used in the liquefaction step of the dry-grind ethanol process. Final concentrations (at 72 hr of fermentation) of glucose, maltose, maltotriose, and DP4+ for all treatments were <0.9% (w/v), indicating that fermentations reached completion with negligible unconverted sugar in the fermentation broth.

Fermentation for 0, 3, 5, and 10% Amylase Treatments in the 150-g Procedure

No differences were observed in ethanol concentrations among the control and the three amylase corn treatments (Fig. 1). Ethanol concentrations of the control treatment were 0.0, 10.5, 15.4, and 16.7% (v/v) at 0, 24, 48, and 72 hr, respectively. A final ethanol concentration of 16.7% (v/v) for the control indicated ≈95% maximum theoretical conversion of starch into ethanol (based on initial starch content of the corn). Final ethanol concentrations for 3, 5, and 10% amylase treatments were comparable to the control and were at least 16.7% (v/v). The 3% amylase corn treatment was adequate for liquefaction of ground corn in dry-grind processing.

No differences were observed in ethanol concentrations among the control and 1, 2, and 3% amylase corn treatments (Fig. 2). Ethanol concentrations of the control were 0.0, 11.2, 15.4, and 17.0% (v/v) at 0, 24, 48, and 72 hr, respectively. The 1% amylase corn treatment was sufficient to liquefy corn solids. However, during liquefaction a visual difference in viscosity between the 1% amylase corn and control treatment was observed. Viscosity

of 1% amylase corn treatment was observed to be higher than the viscosity of control treatment. Based on these visual differences, another experiment was done to quantify viscosities of amylase corn mixtures and control treatments.

Viscosity Profiles

Significant differences in peak, breakdown, and final viscosities were observed among the control and 1, 3, 5, and 10% amylase corn treatments (Fig. 3). Peak and breakdown viscosities of the 1% amylase corn treatment were approximately five times higher than the control treatment. Comparisons among treatments showed viscosity values decreased as the amylase corn treatment increased from 1 to 10%. Peak viscosity values decreased from ≈11,000 to 6,000 cp as amylase corn treatment increased from 1 to 10%. The decrease in peak viscosity value was larger in the 1–3% amylase corn treatment than it was from the 3–5 or 10% amylase corn treatments (Fig. 3). Final viscosity of the 1% amylase corn treatment was comparable to that of the control treatment. However, final viscosities of the 3, 5, and 10% amylase corn treatments were significantly lower than those of the control sample. High peak and breakdown viscosities with 1% amylase corn treatment compared with the control or higher percent amylase corn treatments is probably due to low availability of endogenous liquefaction enzyme during the initial stages of liquefaction reaction. However, as liquefaction reaction proceeds, the enzyme is eventually able to break down starch into dextrans and reduces final viscosity comparable to that of the control sample. Because the final viscosity values

TABLE I

Comparison of Sugar Profile (% w/v) Among Control Treatment (0% Amylase Corn) and 3, 5, and 10% Amylase Corn Treatments (150-g Procedure)^a

Amylase Corn	Fermentation Time (hr)	Glucose	Maltose	Maltotriose	DP4+
0% (Control)	0	10.4 ± 2.9	5.3 ± 1.9	2.2 ± 1.3	9.9 ± 2.6
	24	8.6 ± 0.7	0.3 ± 0.0	0.2 ± 0.0	1.5 ± 0.3
	48	2.5 ± 0.9	0.3 ± 0.0	0.1 ± 0.0	0.5 ± 0.1
	72	0.9 ± 0.3	0.5 ± 0.1	0.1 ± 0.0	0.4 ± 0.0
3%	0	10.0 ± 3.7	6.5 ± 2.0	2.6 ± 1.0	8.5 ± 3.6
	24	7.8 ± 0.9	0.3 ± 0.0	1.9 ± 0.3	0.5 ± 0.1
	48	2.5 ± 0.8	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.0
	72	0.8 ± 0.2	0.4 ± 0.1	0.1 ± 0.0	0.4 ± 0.0
5%	0	10.0 ± 3.2	7.4 ± 1.8	2.9 ± 1.4	7.9 ± 2.6
	24	8.0 ± 0.8	0.3 ± 0.0	2.0 ± 0.2	0.5 ± 0.0
	48	2.5 ± 0.9	0.3 ± 0.1	0.3 ± 0.2	0.4 ± 0.0
	72	0.8 ± 0.4	0.4 ± 0.1	0.1 ± 0.0	0.4 ± 0.0
10%	0	10.4 ± 2.9	8.6 ± 1.6	2.8 ± 1.5	6.8 ± 2.2
	24	8.1 ± 0.9	0.3 ± 0.0	2.1 ± 0.2	0.5 ± 0.1
	48	2.6 ± 0.9	0.3 ± 0.1	0.3 ± 0.2	0.4 ± 0.0
	72	0.8 ± 0.3	0.4 ± 0.0	0.1 ± 0.0	0.4 ± 0.0

^a Average ± SD values in same column for the same fermentation time are not significantly different at 95% confidence level.

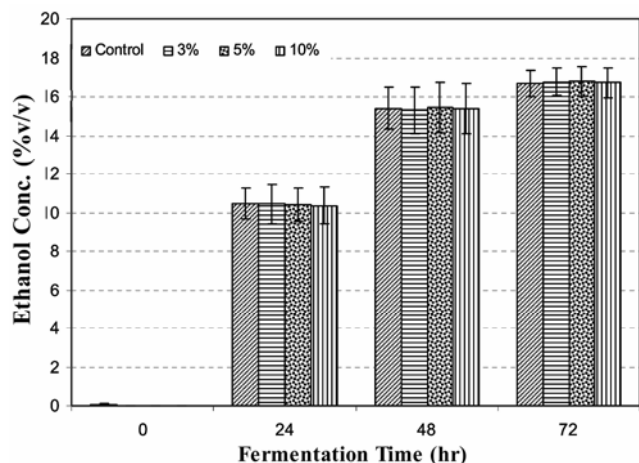


Fig. 1. Ethanol concentrations ± 1 SD for control (0% amylase corn), 3, 5, and 10% amylase corn treatments using a 150-g dry-grind procedure.

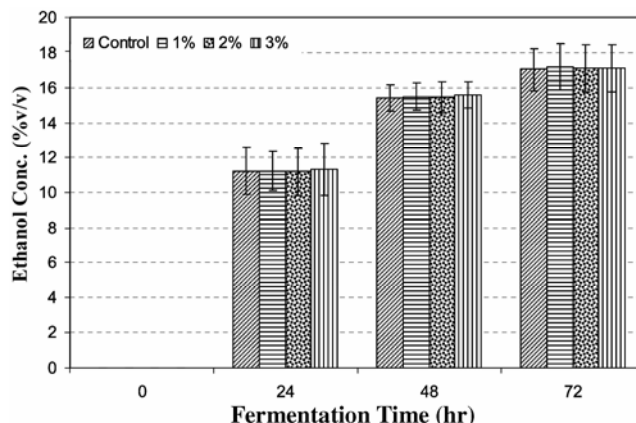


Fig. 2. Ethanol concentrations ± 1 SD for control (0% amylase corn), 1, 2, and 3% amylase corn treatment using a 150-g dry-grind procedure.

TABLE II
DDGS Composition (db) of Control Treatment (0% Amylase Corn)
and 3% Amylase Corn Treatment (3-kg Procedure)^a

Component	Amylase Corn	
	3%	0% (Control)
Crude protein (%)	26.18 ± 0.20	25.65 ± 0.17
Crude fat (%)	14.1 ± 0.13	13.6 ± 0.27
Crude fiber (%)	6.58 ± 0.18	6.8 ± 1.10
Ash (%)	3.78 ± 0.10	3.35 ± 0.09
Residual amylase content (U/g)	0.00	0.00

^a Mean ± SD.

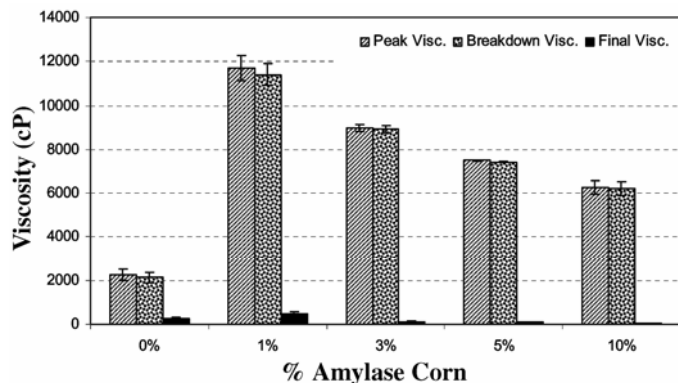


Fig. 3. Peak, breakdown, and final viscosities ± 1 SD of control (0% amylase corn), 1, 3, 5, and 10% amylase corn treatments.

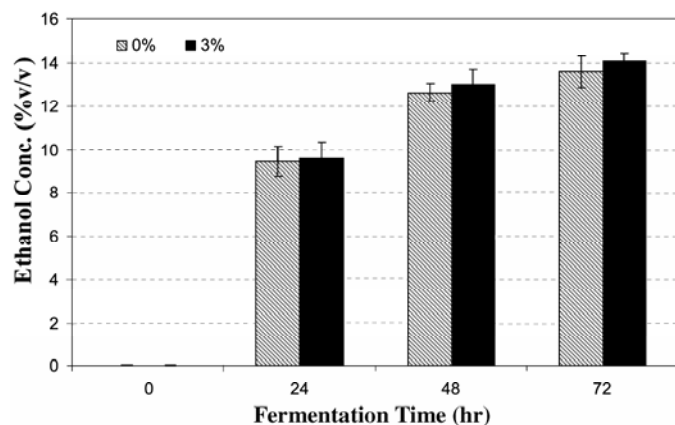


Fig. 4. Ethanol concentrations ± 1 SD for control (0% amylase corn) and 3% amylase corn treatments using a 3-kg dry-grind procedure.

for all amylase corn treatments were comparable to or lower than that of the control sample, there should be little or no effect on the mash viscosity as it enters the simultaneous saccharification and fermentation step in the dry-grind process.

Fermentation and DDGS Composition Using 0 and 3% Amylase Corn in the 3-kg Procedure

Based on comparable ethanol concentrations (Figs. 1 and 2) and lower final viscosity (Fig. 3) for the 3% amylase corn treatment compared with the control treatment, a scaled-up (20×) experiment was conducted to compare fermentation profiles between control and the 3% amylase corn treatment. No significant differences were observed in ethanol concentrations between the control and the 3% amylase corn treatment when tested with the 3-kg laboratory dry-grind procedure (Fig. 4). Ethanol concen-

trations of the control were 0.0, 9.5, 12.6, and 13.6% (v/v) at 0, 24, 48, and 72 hr, respectively. The DDGS compositions for control and the 3% amylase treatments (Table II) were in the range of compositional values reported in the literature for commercial DDGS samples (Belyea et al 2004). Compared with the control, the protein and fat contents of the 3% amylase corn treatment were ≈0.53 and 0.5% higher, respectively. No residual endogenous liquefaction enzyme activity was detected in the DDGS for the 3% amylase corn treatment.

CONCLUSIONS

Small amounts of amylase corn (≈3% of total corn per batch) can be used to replace the exogenous liquefaction enzyme currently used in the dry-grind process for ethanol production. Addition of amylase corn as low as 1% to dent corn was sufficient for liquefying starch in ground corn slurries. Final ethanol concentration for the 1% amylase corn treatment was similar to that of the control treatment. Compared with the control, amylase corn treatments produced sugar profiles similar to glucose, fructose, maltose, maltotriose, and DP4+ sugars. Peak and breakdown viscosities of liquefied slurries for amylase corn treatments were 3–5× higher than the control treatment, but final viscosities of liquefied slurries using amylase corn treatments were similar or lower than those of the control treatment. The DDGS compositions from the 3% amylase corn and control treatments were similar.

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