Effect of Endosperm Hardness on an Ethanol Process Using a Granular Starch Hydrolyzing Enzyme

P. Wang, W. Liu, D. B. Johnston, K. D. Rausch, S. J. Schmidt, M. E. Tumbleson, V. Singh

Abstract. *Granular starch hydrolyzing enzymes (GSHE) can hydrolyze starch at low temperature (32˚C). The dry grind process using GSHE (GSH process) has fewer unit operations and no changes in process conditions (pH 4.0 and 32˚C) compared to the conventional process because it dispenses with the cooking and liquefaction step. In this study, the effects of endosperm hardness, protease, urea, and GSHE levels on GSH process were evaluated. Ground corn, soft endosperm, and hard endosperm were processed using two GSHE levels (0.1 and 0.4 mL per 100 g ground material) and four treatments of protease and urea addition. Soft and hard endosperm materials were obtained by grinding and sifting flaking grits from a dry milling pilot plant; classifications were confirmed using scanning electron microscopy. During 72 h of simultaneous granular starch hydrolysis and fermentation (GSHF), ethanol and glucose profiles were determined using HPLC. Soft endosperm resulted in higher final ethanol concentrations compared to ground corn or hard endosperm. Addition of urea increased final ethanol concentrations for soft and hard endosperm. Protease addition increased ethanol concentrations and fermentation rates for soft endosperm, hard endosperm, and ground corn. The effect of protease addition on ethanol concentrations and fermentation rates was most predominant for soft endosperm, less for hard endosperm, and least for ground corn. Samples (soft endosperm, hard endosperm, or corn) with protease resulted in higher (1.0% to 10.5% v/v) ethanol concentration compared to samples with urea. The GSH process with protease requires little or no urea addition. For fermentation of soft endosperm, GSHE dose can be reduced. Due to nutrients (lipids, minerals, and soluble proteins) present in corn that enhance yeast growth, ground corn fermented faster at the beginning than hard and soft endosperm.*

Keywords. *Corn, Dry grind process, Endosperm, Endosperm hardness, Ethanol, Granular starch hydrolyzing enzyme, Protease, Urea.*

Granular starch hydrolyzing enzymes (GSHE) can hydrolyze starch at low temperature (32˚C). Use of GSHE in the dry grind process can eliminate the high-temperature cooking and liquefaction steps required in the conventional process, can save energy, and can simplify the dry grind process (Wang et al., 2007). The GSH process (the dry grind process using GSHE) is an energy-conserving alternative to the conventional dry grind process (Robertson et al., 2006). In the conventional process, during cooking, Maillard reactions increase nonfermentable constituents and reduce ethanol yields (Galvez, 2005). The GSH process was comparable in ethanol concentration and rate of fermentation to the conventional dry grind process using the traditional enzymes (α-amylase and glucoamylase) (Wang et al., 2007). However, because the GSH process is a new process, the effects of endosperm hardness on the process are not known.

The corn kernel has two types of endosperm, hard and soft (Watson, 2003). Endosperm hardness is related to endosperm structure, composition and structure of starch granules, protein distribution, and amylose content (Robutti et al., 1974; Dombrink-Kurtzman and Bietz, 1993; Dombrink-Kurtzman and Knutson, 1997; Robutti et al., 1997; Landry et al., 2004). Hard endosperm starch granule surfaces are smooth and have few pores, whereas soft endosperm surfaces are rough and have more pores (Badi et al., 1976; Dombrink-Kurtzman and Knutson, 1997). Pores may be a site of initial enzymatic reaction. Starch granules in hard endosperm are compressed by a thick protein matrix into polygonal shapes and tightly packed with no spaces (Robutti et al., 1974; Badi et al., 1976). Starch granules in soft endosperm are round shaped and have spaces among them. When endosperm is ground, soft endosperm produces grits (pieces of endosperm) with rough surfaces and many exposed starch granules. However, hard endosperm produces grits with smooth surfaces and fewer exposed starch granules. Soft endosperm with more exposed starch granules and rough surfaces will create more surface

Submitted for review in October 2008 as manuscript number BE 7754; approved for publication by the Biological Engineering Division of ASABE in December 2009.

Mention of trade names or commercial products is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Energy, USDA, or University of Illinois at Urbana-Champaign.

The authors are **Ping Wang,** General Engineer, U.S. Department of Energy, National Energy Technology Laboratory, Morgantown, West Virginia; **Wei Liu,** Visiting Research Specialist, Department of Agricultural and Biological Engineering, University of Illinois, Urbana, Illinois; **David B. Johnston,** Food Technologist, USDA-ARS Eastern Regional Research Center, Wyndmoor, Pennsylvania; **Kent D. Rausch, ASABE Member Engineer,** Associate Professor, Department of Agricultural and Biological Engineering, University of Illinois, Urbana, Illinois; **Shelly J. Schmidt,** Professor, Department of Food Science and Human Nutrition, University of Illinois, Urbana, Illinois; **M. E. Tumbleson,** Professor Emeritus, and **Vijay Singh, ASABE Member Engineer,** Associate Professor, Department of Agricultural and Biological Engineering, University of Illinois, Urbana, Illinois. **Corresponding author:** Vijay Singh, Department of Agricultural and Biological Engineering, University of Illinois at Urbana-Champaign, 360G AESB, 1304 W. Pennsylvania Avenue, Urbana, IL 61801; phone: 217-333-9510; fax: 217-244-0323; e-mail: vsingh@illinois.edu.

area (compared to hard endosperm) and will benefit the solid phase hydrolysis as used in the GSH process.

Another important consideration in regard to the effect of endosperm structure is the role of proteases. Proteases are added to the GSH process to hydrolyze protein into free amino nitrogen (FAN), which can be used by the yeast as a nitrogen source (Lantero and Fish, 1993). Protease might also affect starch availability (Johnston and Singh, 2001); hard endosperm has twice (79%) the zein content of soft endosperm (44%) (Lawton and Wilson, 2003; Landry et al., 2004). Zeins are not hydrolyzed or are hydrolyzed at a slow rate by proteases (Spanheimer et al., 1972; Wolf and Khoo, 1975). Addition of proteases, therefore, might be an important factor in determining the influence of hard versus soft endosperm on the GSH process. Urea is used as a nitrogen source for fermentation in ethanol plants. In last three years, urea (45% to 46% nitrogen) cost has increased 66% (USDA, 2008). Use of proteases may reduce or replace use of urea as a nitrogen source; therefore, protease and urea were added as factors in this study.

The objective of this study was to evaluate the effects of endosperm hardness on the GSH process and investigate the use of protease and urea to improve fermentation of soft endosperm, hard endosperm, and ground corn in the GSH process.

Materials and Methods

Experimental Material

Yellow dent corn (34M78, Pioneer Hi-Bred International, Johnston, Iowa) was grown in 2007 at the Agricultural and Biological Engineering Research Farm, University of Illinois at Urbana-Champaign, and used for the study. Granular starch hydrolyzing enzyme (GSHE) (Stargen 001) and acid fungal protease (GC 106) were obtained from Genencor International (Palo Alto, Cal.). Stargen 001 is a mixture of alpha-amylase from *Aspergillus kawachi* and glucomylase from *Aspergillus* niger. *Saccharomyces cerevisiae* yeast, Ethanol Red, was from Fermentis (Lesaffre Yeast Corp., Milwaukee, Wisc.). Urea (>98%) was from Fisher Scientific (Fair Lawn, N.J.).

Fractionation to Obtain Soft and Hard Endosperm

Corn was cleaned to remove broken corn and foreign material (BCFM) using a seed cleaner (Vac-A-Way, J. W. Hence Manufacturing Co., Westerville, Ohio) with a 4 mesh (4.76 mm opening) screen. In a ribbon blender, cleaned corn was tempered for 25 min by adding water to increase the corn moisture from 13.6% to 21.6% (w.b.) to loosen the pericarp, soften the germ, and hydrate the endosperm. Tempered corn was milled using a degerminator (model No. 0, The Beall Improvements Co., Decatur, Ill.). During milling, the pericarp and germ were separated from the endosperm, and the endosperm was broken into smaller pieces. Larger pieces of endosperm (flaking grits) exited the end of the degerminator (tails). Pericarp, germ, and smaller pieces of endosperm (small grits) passed through perforations at the side of the degerminator (throughs). Tails were sifted using a box sifter with 3.5 and 7 mesh screens (5.66 and 2.83 mm openings, respectively) (model 130-U, Great Western Mfg. Co, Inc., Leavenworth, Kans.) for 1 min to remove whole kernels and small pieces of endosperm. Flaking grits through the 3.5 mesh and over the 7 mesh screen were aspirated (model 6DT4 Kice, Kice Metal Product Co., Wichita, Kans.) to remove pericarp and small pieces of germ. Flaking grits were dried in an oven at 49°C for 1 h. Additional germ in the flaking grits was removed manually.

Flaking grits were ground using a hammer mill (model MHM4, Glen Mills, Clifton, N.J.) at 500 rpm equipped with a 1.5 mm round hole sieve. Ground grits were sifted in a box sifter with 40 and 70 mesh screens (0.42 and 0.21 mm openings, respectively) (model 130-U, Great Western Mfg. Co, Inc., Leavenworth, Kans.) for 5 min. Material through the 70 mesh screen was classified as soft endosperm. Material over the 40 mesh screen was ground again using a hammer mill equipped with a 0.5 mm screen. Material from this second grind was sifted using the box sifter with 30 (0.59 mm openings) and 70 mesh (0.21 mm opening) screens. Material through the 30 mesh and over the 70 mesh screen was classified as hard endosperm. Classification of hard and soft endosperm was confirmed using scanning electron microscopy (SEM). Total starch contents of soft endosperm (93.2% d.b.), hard endosperm (91.9% d.b.), and ground corn (73.3% d.b.) were quantified (AOAC, 2007) by Midwest Laboratories, Inc. (Omaha, Neb.). Crude protein contents of soft endosperm, hard endosperm, and ground corn were 7.01%, 8.75%, and 9.70% (d.b.), respectively. Crude fat contents of soft endosperm, hard endosperm, and ground corn were 0.93%, 0.39%, and 4.5% (d.b.), respectively.

Scanning Electron Microscopy Images

Dry endosperm samples were mounted on aluminum specimen stubs using colloidal silver adhesive (Electron Microscopy Sciences, Ft. Washington, Pa.). After coating with a thin layer of gold by DC sputtering, samples were examined and imaged digitally using a model JSM 840A scanning electron microscope (JEOL USA, Peabody, Mass.) operated in the secondary electron imaging mode at an accelerating voltage of 10 kV and coupled to a model Imix-1 digital image workstation (Princeton Gamma-Tech, Princeton, N.J.). All images had 500× or 1000× magnification. Ten micrographs were taken of each soft endosperm and hard endosperm sample. Results were similar for all images of each sample.

Dry Grind Process Using a Granular Starch Hydrolyzing Enzyme (GSH Process)

In a 500 mL flask, 100 g soft endosperm, hard endosperm, or ground whole corn was mixed with water to obtain 30% dry solids content. Ground whole corn was obtained by grinding cleaned corn in a hammer mill (model MHM4, Glen Mills, Clifton, N.J.) at 500 rpm and using a 2 mm round hole sieve. Mash was adjusted to pH 4.0 by using 10N sulfuric acid. Yeast culture was prepared by dispersing 10 g yeast in 50 mL distilled water with agitation at 32°C for 20 min. Yeast culture had a cell count of 2.5×10^9 cells mL^{-1} using Petrifilm plate (3M Co., St. Paul, Minn.). One mL yeast culture and GSHE with or without protease or urea were added to the mash. The flask was placed in a shaking water bath (model SHKA 7000, Barnstead/Lab-Line, Melrose Park, Ill.) at 32°C with agitation at 130 rpm. Granular starch hydrolysis and fermentation (GSHF) were sampled (1 mL) at 3, 6, 12, 24, 30, 48, 54, and 72 h. Each sample was centrifuged at 13,362 × *g* for 3 min (model 5415D, Eppendorf AG, Hamburg, Germany). HPLC sample preparation and analyses were performed according to methods described by Wang et al. (2007).

Experiment Design

Based on a study of the effects of protease and urea on the GSH process for ethanol production (Wang et al., 2009), two levels of GSHE (0.1 and 0.4 mL per 100 g sample; protein concentration of 0.037% to 0.15% v/v, respectively), protease (0 and 0.2 mL per 100 g sample; protein concentration of 0.075% v/v) and urea (0 and 0.125 g per 100 g sample, or 460 mg L^{-1}) were selected. For each GSHE dosage, four treatments were investigated: both protease and urea, only protease, only urea, and no urea or protease addition (control). Soft endosperm, hard endosperm, and ground corn were used. There were 24 treatments (table 1); each treatment was repeated three times. Randomized complete block experimental design was used. Each replicate sample was analyzed in duplicate using HPLC.

Measuring the amount of ethanol produced over time is the most direct and useful way to measure the rate of yeast fermentation. Rates were calculated as slopes of a linear portion of the ethanol profile during 3 to 12 h during fermentation. Fermentation profiles (concentration vs. fermentation time) of ethanol and glucose were plotted. For each treatment (GSHE/endosperm or corn), fermentation rates, final ethanol concentration, and residual glucose concentrations were compared using analysis of variance (ANOVA) (SAS Institute, Inc., Cary, N.C.). The level to show statistical significance was 5% ($P \leq 0.05$).

Table 1. Mean fermentation rates and final ethanol (72 h) and residual glucose concentrations of soft endosperm, hard endosperm, and ground corn with GSHE, protease, and urea addition.[a]

GSHE (mL)	Treatment	Material	Fermentation Rate (% v/v/h)	Final Concentration	
				Ethanol (% v/v)	Glucose (% w/v)
0.1	Protease + Urea	Hard	0.014 a	13.4 s	0.63 x
		Soft	0.120 b	17.3 t	1.44 y
		Corn	0.495 c	13.8 s	0.11 x
	Protease	Hard	0.017 a	14.5 s	0.85 y
		Soft	0.097 b	16.9 t	1.76 z
		Corn	0.542 c	14.5 s	0.07 x
	Urea	Hard	0.019 a	9.8 s	0.08 x
		Soft	0.074 b	9.4 s	7.82 y
		Corn	0.456 c	12.9 t	0.08 x
	Control[b]	Hard	0.018 a	8.4 t	0.57 x
		Soft	0.045 a	5.7 s	12.38 y
		Corn	0.466 b	13.3 u	0.05 x
0.4	Protease + Urea	Hard	0.011 a	15.9 s	3.93 z
		Soft	0.079 b	17.7 t	2.78 y
		Corn	0.721 c	16.4 s	0.12 x
	Protease	Hard	0.011 a	15.4 s	4.33 y
		Soft	0.061 b	16.9 t	4.33 y
		Corn	0.682 c	16.7 t	0.07 x
	Urea	Hard	0.018 a	11.5 t	5.98 y
		Soft	0.038 a	6.4 s	15.23 z
		Corn	0.694 b	15.7 u	0.07 x
	Control[b]	Hard	0.020 a	7.1 t	8.72 y
		Soft	0.024 a	4.8 s	17.33 z
		Corn	0.585 b	15.4 u	0.01 x
LSD[c] (each column)			0.042	0.93	0.71

[a] Values are means of three observations. Values followed by the same letter within a treatment and material are not different ($P < 0.05$).
[b] Control was no addition of protease or urea.
[c] LSD = least significant difference.

Results and Discussion

Separation of Soft and Hard Endosperm

From scanning electron micrographs (SEMs) of soft endosperm samples, granules had a loosely packed appearance, thin protein matrix, and exposed starch granules (fig. 1). For hard endosperm samples, granules had a tightly packed appearance with adhering protein matrix and fewer exposed starch granules (fig. 2). SEMs of soft and hard endosperm samples were similar to micrographs described by Robutti et al. (1974) and Badi et al. (1976).

Effect of Endosperm Hardness on GSH Process

At the same percentage dry solids, total fermentable substrates (starch) in mash were higher for hard and soft endosperm fractions compared to ground corn; however, final ethanol concentrations were lower for the two endosperm

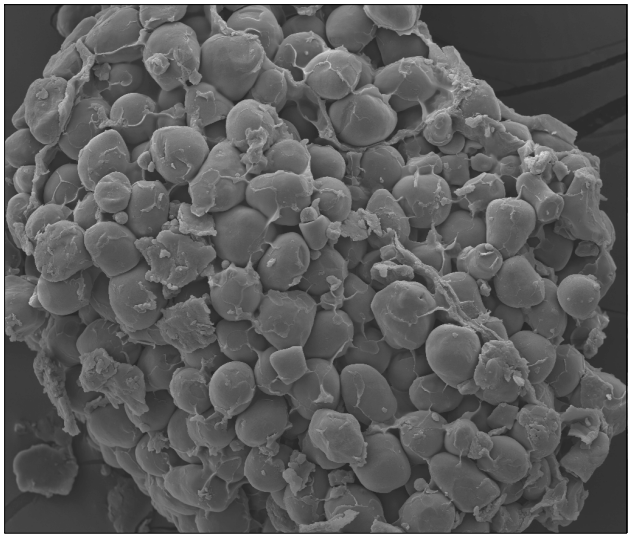

Figure 1. Representative SEM image of soft endosperm showing loosely packed starch granules and thin protein matrix. Image magnification or scale = 1000×.

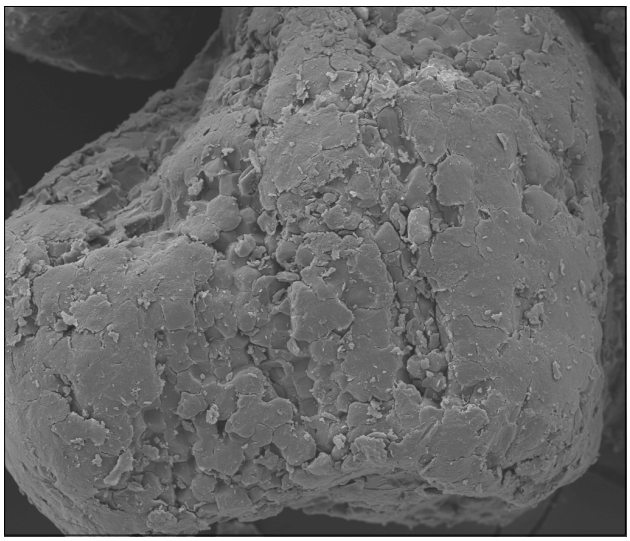

Figure 2. Representative SEM image of hard endosperm showing indentations left by polygonal-shaped starch granules, tightly packed starch granules with adhering protein matrix, and fewer exposed starch granules. Image magnification or scale = 500×.

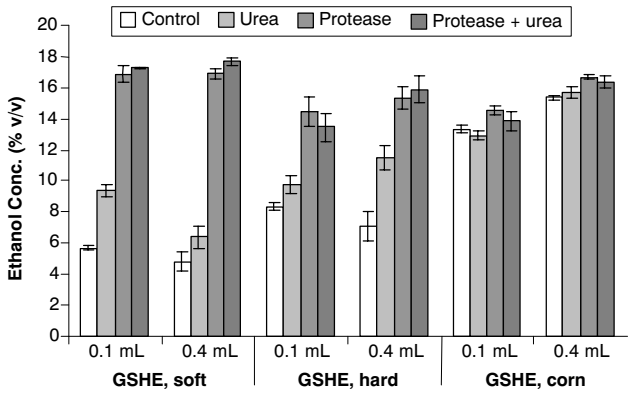

Figure 3. Final ethanol concentrations (72 h) of soft endosperm, hard endosperm, and corn with 0.1 or 0.4 mL GSHE additions and four treatments of protease and urea addition. Control = no protease or urea addition.

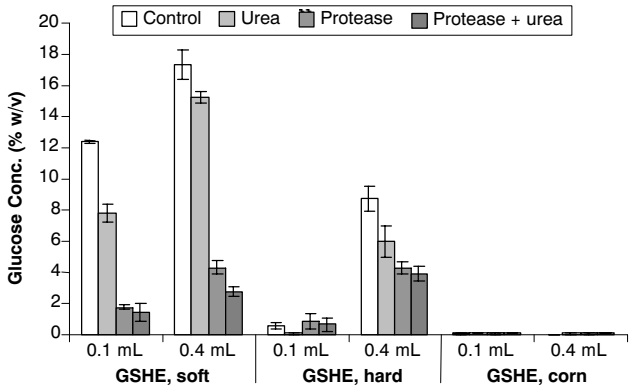

Figure 4. Residual glucose concentrations (72 h) of soft endosperm, hard endosperm, and corn with 0.1 or 0.4 mL GSHE additions and four treatments of protease and urea addition. Control = no protease or urea addition.

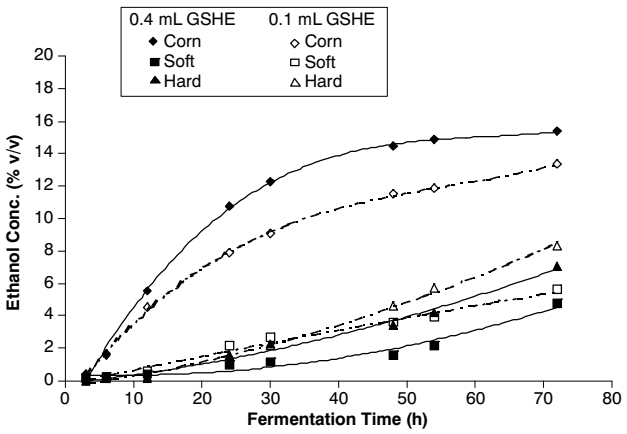

Figure 5. Ethanol concentrations of corn, soft endosperm, and hard endosperm with 0.1 or 0.4 mL GSHE and no protease or urea addition.

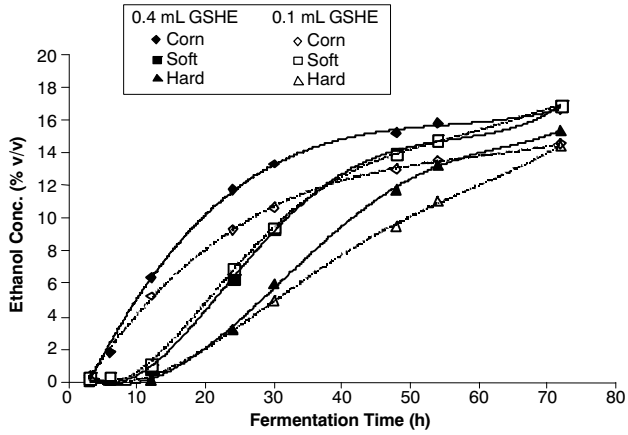

Figure 6. Ethanol concentrations of corn, soft endosperm, and hard endosperm with 0.1 or 0.4 mL GSHE and protease addition.

fractions for treatments with no urea and/or protease addition. At the 0.1 mL GSHE dose, final ethanol concentrations for hard endosperm, soft endosperm, and corn without addition of urea and protease were 8.4%, 5.7%, and 13.3% (v/v) (table 1, fig. 3). Glucose concentrations for hard and soft endosperm were higher compared to corn (fig. 4). Fermentation rates (up to 12 h fermentation time) were slower for hard and soft endosperm fractions compared to corn samples (fig. 5). At the higher GSHE dose (0.4 mL), similar results were observed for treatments with no urea and/or protease addition; soft and hard endosperm fractions had slower fermentation rates, lower final ethanol concentrations, and higher residual glucose concentrations. Slower fermentation rates and final ethanol concentrations for soft and hard endosperm compared to ground whole corn were due to reduced nutrients (lipids, minerals, and soluble proteins) needed for yeast as a result of removal of germ and fiber (Murthy et al., 2006).

Effect of Protease and Urea Addition on GSH Process

Urea addition increased final ethanol concentrations and fermentation rates for both endosperm (hard and soft) fractions but had no effect on the whole corn fraction. Compared to the control at 0.1 mL GSHE, urea addition increased final ethanol concentrations of hard and soft endosperm fractions from 8.4% to 9.8% (v/v) and from 5.7% to 9.4% (v/v), respectively (table 1). Residual glucose concentrations of hard and soft endosperm decreased from 0.57% to 0.08% (w/v) and from 12.38% to 7.82% (w/v), respectively, for treatments at 0.1 mL GSHE dose. Similarly, increases in final ethanol concentrations and decreases in glucose concentrations for soft and hard endosperm fractions were observed for treatments at 0.4 mL GSHE dose (table 1).

Protease addition increased final ethanol concentrations and fermentation rates for all three fractions (soft endosperm, hard endosperm, and whole corn) (table 1, fig. 6). Compared to the control, protease addition increased final ethanol concentrations of hard and soft endosperm fractions from 8.4% to 14.5% (v/v) and from 5.7% to 16.9% (v/v), respectively (at 0.1 mL GSHE dose) (table 1). Compared to the control at 0.1 mL GSHE, protease addition did not change residual glucose concentrations for hard endosperm (only for treatment at 0.1 mL GSHE) but decreased residual glucose concentrations for soft endosperm from 12.38% to 1.76% (w/v). Similar results (increased final ethanol concentrations and decreased residual glucose concentrations) were also observed for treatments with 0.4 mL GSHE addition (table 1). With protease addition, rates of fermentation for all three fractions increased during 12 to 30 h fermentation (figs. 5 and 6) due to FAN availability from corn protein hydrolysis.

Compared to urea, protease treatments had a larger effect on increasing final ethanol concentration and fermentation

rate as well as reducing residual glucose concentration. Compared to the control and depending on GSHE dose (0.1 or 0.4 mL), protease addition increased final ethanol concentrations for all fractions (soft endosperm, hard endosperm, and corn) by 8% to 251%; however, with urea addition, increase in final ethanol concentration was negligible to 66%. Larger increases in ethanol concentrations with protease addition compared to urea addition may be caused by protease hydrolysis of the protein matrix and allowing more starch to be available during fermentation, as well as providing nitrogen (amino acids and small peptides) for yeast growth and metabolism. Proteolytic action results in a mixture of amino acids from hydrolyzing proteins, which are more effective than a single nitrogen source (urea) for yeast production of ethanol (Russel, 2003).

With protease and urea together, final ethanol concentration of soft endosperm was higher than that of hard endosperm and corn (fig. 3). No effects of the addition of protease and urea together compared to protease alone were observed on final ethanol concentrations, fermentation rates, or residual glucose concentrations for all three fractions, except residual glucose concentrations for soft endosperm fraction (table 1, figs. 3 and 4). The GSH process with protease requires little or no urea addition. These results are consistent with results reported by Torija et al. (2003). They reported that fermentation rate was slower with a mixture of ammonium and amino acids than with amino acids alone.

Compared to soft and hard endosperm, ground corn was less affected by the addition of urea or protease or protease and urea together on final ethanol concentrations, fermentation rates, or residual glucose concentrations (table 1, figs. 3 and 4). The endosperm has fewer nutrients (lipids, minerals, and soluble proteins) for yeast growth compared to ground corn due to removal of germ and fiber. Germ and fiber have different fat and proteins cotents compared to endosperm. In this study, crude fat contents of soft endosperm, hard endosperm, and ground corn were 0.93%, 0.39%, and 4.5% (d.b.), respectively. Crude protein contents of soft endosperm, hard endosperm, and ground corn were 7.01%, 8.75%, and 9.70% (d.b.), respectively. Germ and fiber have mostly albumins and globulins proteins, and endosperm has mainly prolamins (zeins) and glutelins proteins (Wall and Paulis, 1978; Lawton and Wilson, 2003). Zeins are not hydrolyzed or are hydrolyzed at a slow rate by proteases.

As GSHE dose increased, the final ethanol concentrations and fermentation rates for hard endosperm and corn fractions increased, except for the control treatment for hard endosperm (table 1, figs. 3, 5, and 6). However, little or no effects were observed on soft endosperm fraction when protease and protease plus urea were added (table 1, figs. 3 and 6). Residual glucose concentrations of hard and soft endosperm increased as GSHE dose increased (table 1, fig. 4). Increase in GSHE dose increased the frequency of interactions between enzyme and starch, and resulted in faster starch hydrolysis. However, the lack of nutrients in hard and soft endosperm limited yeast growth, and fermentation was not complete (residual glucose in soft and hard endosperm at 0.4 mL GSHE was 8.72% and 17.33% w/v, respectively). Little or no effects of GSHE dose on soft endosperm may have been because the starch granules in soft endosperm have rough surfaces and more pores (Badi et al., 1976; Dombrink-Kurtzman and Knutson, 1997) and allow easier access for enzymatic reactions, even at low GSHE dose. Starch granules in hard endosperm have smooth surfaces and fewer pores; therefore, more enzyme is needed for hydrolysis. Grits produced from soft endosperm require less GSHE compared to grits from hard endosperm.

These results are relevant to dry fractionation processes being implemented on the front end of commercial dry grind ethanol processes. Yields of grits and floury starch produced from corn depend on endosperm hardness (Duensing et al., 2003). Use of proteases (without urea addition) and optimizing GSHE dose will increase final ethanol concentration and fermentation rate for these modified dry grind ethanol processes.

Conclusions

Using protease and urea to improve fermentation of soft endosperm, hard endosperm, and corn was investigated. A method to fractionate soft and hard endosperm from corn was developed and verified. For soft endosperm, GSHE can be used at lower doses. Addition of urea and protease improved soft and hard endosperm fermentation. The GSH process with protease resulted in higher final ethanol concentration than with urea. Ethanol concentrations with protease addition increased most for soft endosperm, less for hard endosperm, and least for corn. With protease addition, soft endosperm resulted in higher final ethanol concentrations than whole corn or hard endosperm. The GSH process with protease requires little or no urea addition. With increased GSHE level and protease addition, final ethanol concentrations of corn and hard endosperm increased, but final ethanol concentration of soft endosperm was not different. Whole corn fermented faster at the beginning than hard and soft endosperm. Use of proteases (without urea addition) and optimizing GSHE dose will increase the final ethanol concentrations and fermentation rates for dry grind ethanol processes using dry fractionation.

Acknowledgements

Special thanks to Larry Pruiett for help in the pilot plant dry milling process. Also thanks to Peter Cooke, USDA-ARS Eastern Regional Research Center, for scanning electron micrographs of the soft and hard endosperm.

References

AOAC. 2007. Method 979.10. In *Official Methods of Analysis*. 18th ed. Gaithersburg, Md.: Association of Official Analytical Chemists.

Badi, S. M., R. C. Hoseney, and A. J. Casady. 1976. Pearl millet: I. Characterization by SEM, amino acid analysis, lipid composition, and prolamine solubility. *Cereal Chem*. 53(4): 478-487.

Dombrink-Kurtzman, M. A., and J. A. Bietz. 1993. Zein composition in hard and soft endosperm of maize. *Cereal Chem*. 70(1): 105-108.

Dombrink-Kurtzman, M. A., and C. A. Knutson. 1997. A study of maize endosperm hardness in relation to amylose content and susceptibility to damage. *Cereal Chem*. 74(6): 776-780.

Duensing, W. J., A. B. Roskens, and R. J. Alexander. 2003. Corn dry milling: Processes, products, and applications. In *Corn: Chemistry and Technology*, 407-447. 2nd ed. P. J. White and L. A. Johnson, eds. St. Paul, Minn.: American Association of Cereal Chemists.

Galvez, A. 2005. Analyzing cold enzyme starch hydrolysis technology in new ethanol plant design. *Ethanol Producer Magazine* 11(1): 58-60.

Johnston, D. B., and V. Singh. 2001. Use of proteases to reduce steep time and SO_2 requirements in a corn wet-milling process. *Cereal Chem*. 78(4): 405-411.

Landry, L., S. Delhaye, and C. Damerval. 2004. Protein distribution pattern in floury and vitreous endosperm of maize grain. *Cereal Chem*. 81(2): 153-158.

Lantero, O. J., and J. J. Fish. 1993. Process for producing ethanol. U.S. Patent No. 5,231,017.

Lawton, J. W., and C. M. Wilson. 2003. Proteins of the kernel. In *Corn: Chemistry and Technology*, 313-354. 2nd ed. P. J. White and L. A. Johnson, eds. St. Paul, Minn.: American Association of Cereal Chemists.

Murthy, G. S., V. Singh, D. B. Johnston, K. D. Rausch, and M. E. Tumbleson. 2006. Evaluation and strategies to improve fermentation characteristics of modified dry grind corn processes. *Cereal Chem*. 83(5): 455-459.

Robertson, G. H., D. W. S. Wong, C. C. Lee, K. Wagschal, M. R. Smith, and W. J. Orts. 2006. Native or raw starch digestion: A key step in energy efficient biorefining of grain. *J. Agric. Food Chem*. 54(2): 353-365.

Robutti, J. L., R. C. Hoseney, and C. E. Wassom. 1974. Modified opaque-2 corn endosperms. II. Structure viewed with a scanning electron microscope. *Cereal Chem*. 51(2): 173-180.

Robutti, J. L., F. S. Borras, and G. H. Eyherabide. 1997. Zein compositions of mechanically separated coarse and fine portions of maize kernels. *Cereal Chem*. 74(1): 75-78.

Russel, I. 2003. Understanding yeast fundamentals. In *The Alcohol Textbook: A Reference for the Beverage, Fuel, and Industrial Alcohol Industries*, 89-117. 4th ed. K. A. Jacques, T. P. Lyons, and D. R. Kelsall, eds. Nottingham, U.K.: Nottingham University Press.

Spanheimer, J., J. E. Freeman, R. E. Heady, and V. E. Headley. 1972. Air classification of corn grits: I. Softening grits with enzymes and chemicals. *Cereal Chem*. 49(2): 131-141.

Torija, M. J., G. Beltran, M. Novo, M. Poblet, N. Rozes, A. Mas, and J. M. Guillamon. 2003. Effect of organic acids and nitrogen source on alcoholic fermentation: Study of their buffering capacity. *J. Agric. Food Chem*. 51(4): 916-922.

USDA. 2008. Table 7: Average U.S. farm prices of selected fertilizers, 1960-2008. Washington, D.C.: USDA. Available at: www.ers.usda.gov/Data/FertilizerUse.

Wall, J. S., and J. W. Paulis. 1978. Corn and sorghum grain protein. In *Advances in Cereal Science and Technology*, Vol. 2: 135-219. Y. Pomeranz, ed. St. Paul, Minn.: American Association of Cereal Chemists.

Wang, P., V. Singh, H. Xue, D. B. Johnston, K. D. Rausch, and M. E. Tumbleson. 2007. Comparison of raw starch hydrolyzing enzyme with conventional liquefaction and saccharification enzymes in dry grind corn processing. *Cereal Chem*. 84(1): 10-14.

Wang, P., D. B. Johnston, K. D. Rausch, S. J. Schmidt, M. E. Tumbleson, and V. Singh. 2009. Effects of protease and urea on a granular starch hydrolyzing process for corn ethanol production. *Cereal Chem.* 86(3):319-322.

Watson, S. A. 2003. Description, development, structure, and composition of the corn kernel. In *Corn: Chemistry and Technology*, 69-106. 2nd ed. P. J. White and L. A. Johnson, eds. St. Paul, Minn.: American Association of Cereal Chemists.

Wolf, M. J., and U. Khoo. 1975. Subcellular distribution and enzyme digestibility of endosperm proteins of amylomaize and normal corn. *Cereal Chem*. 52(6): 771-778.