

Fate of Bt Protein and Influence of Corn Hybrid on Ethanol Production

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ABSTRACT

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Corn hybrids were compared to determine the fate of recombinant Bt protein (CRY1Ab from *Bacillus thuringiensis*) in coproducts from dry grind and wet-milled corn during production of fuel ethanol. Two pairs of Bt and non-Bt hybrids were wet milled, and each fraction was examined for the presence of the Bt protein. Bt protein was found in the germ, gluten, and fiber fractions of Bt hybrids. In addition, one set of Bt and non-Bt hybrids were treated by the dry-grind ethanol process and Bt protein was monitored during each step of the process. The Bt protein

was not detected after liquefaction. Subsequent experiments determined that the Bt protein is rapidly denatured at liquefaction temperatures. Finally, five hybrids were compared for ethanol yield after dry grinding. Analysis of fermentation data with an *F*-test revealed the percent of total starch available for conversion into ethanol varied significantly among the hybrids ($P < 0.002$), indicating ethanol yield is not exclusively dependent on starch content. No difference, however, was observed between Bt and non-Bt corn hybrids for either ethanol productivity or yield.

The United States produced 1.8 billion gallons of fuel ethanol last year; $\approx 95\%$ of it was produced using corn (Renewable Fuels Association 2001). Ethanol production has expanded dramatically since 1980 and today utilizes 5% (>600 million/bu) of the U.S. corn harvest (Corn Refiners Association 2001). Ethanol production is expected to continue to increase as states phase out the use of methyl tertiary butyl ether (MTBE) as a fuel oxygenate. The California Energy Commission has estimated that production capacity will be at more than 4 billion gals/yr by 2006 (MacDonald et al 2001). Despite the increased use of corn for production of ethanol, seed companies have not marketed corn cultivars that have been specifically tailored for ethanol production. Nor has the effect of the recombinant Bt protein in corn been determined on ethanol production, despite genetically modified (GMO) corn accounting for $\approx 20\%$ of the corn harvested in 2001 (USDA Economic Research Service 2002).

Corn is processed to ethanol by either dry or wet milling. Last year, for the first time, dry milling or dry-grind production (55%) exceeded that of wet milling. The growth of dry mill capacity has exceeded that of wet mills because dry mills have lower capital costs. Most newly constructed ethanol plants have been built for farmer-owned cooperatives that have limited amounts of capital available from private sources and beneficial state subsidy programs. While wet mills have not been constructed in recent years, ethanol production gains have been realized by expanding existing facilities. Each process depends on the sale of coproducts to remain profitable. The corn wet millers produce corn oil, gluten meal, and corn gluten feed. Corn oil is used for food, gluten meal is marketed as a high protein ($\geq 60\%$ protein) feed primarily for poultry, and corn gluten feed is marketed as a low protein (18–22%) cattle feed. Some wet millers also recover the yeast (*Saccharomyces cerevisiae*) produced during the fermentation and sell it as dried distillers yeast. The dry-grind ethanol producers market dried distillers grains and solubles (DDGS, 30% protein) as a feed. Several wet and dry millers also collect the CO_2 produced during fermentation.

Dry and wet millers market their coproducts to the international market. Over 5 million metric tons (mt) of corn gluten feed, 750,000

mt of gluten meal, and 502,000 mt brewers and distiller dried grains with solubles were exported last year (USDA Foreign Agricultural Service [www.fas.usda.gov/ustrade]). Many of these feed products were shipped to Europe, where there is considerable concern regarding GMO crops and labeling of products produced with them. Currently, the European Union is drafting the so-called Novel Feed Regulation, which would require the labeling of “all feedstuffs derived from GMO plants if the GMO content exceeds a certain threshold, most likely 1%” (Schumacher 2001). Therefore, even though the Yieldgard type of Bt corn varieties used in this study, is validated for use in food, many customers of corn-derived coproducts demand a product that is non-Bt because of public perception. In this study, the impact of Bt hybrids has been evaluated by monitoring the fate of active CRY1Ab (Yieldgard protein) throughout the ethanol production process and in end products. This study is also of use to millers in determining the applicability of the more common immunological assay (e.g., lateral flow strips) to monitoring Bt protein within their processes.

We also sought to determine how hybrids vary in dry grind ethanol yield. Normally, it has been assumed that ethanol yield is only related to starch content. However, for wet-milled corn, starch is harder to extract from some hybrids than others (Zehr 1995). This observation has led seed companies to market particular hybrids as superior for wet milling. In this study, five hybrids were compared to determine whether starch in specific hybrids are more readily hydrolyzed and fermented to ethanol than the others.

MATERIALS AND METHODS

Materials

Corn cultivars (36K50; 3335x33G26) were grown in central Illinois. Hybrid 3335x33G26 was developed for enhanced starch content. The other corn cultivars (Table I) were supplied by the University of Illinois and grown in their field plots. Enzymes and the *S. cerevisiae* strain used for fermentation were supplied by Alltech Inc. (Lexington, KY).

Corn Dry and Wet Milling

Corn was wet milled as described by Eckhoff et al (1993). Corn was dry milled using a modified protocol published by VanCauwen-

TABLE I
Corn Hybrids Used for This Study

Corn Hybrid	Bt Hybrid	Wet Milled	Dry Grind
Pioneer 33A13	–	+	+
Pioneer 33A14	+	+	+
Pioneer 33P66	–	+	–
Pioneer 33P67	+	+	–
Pioneer 36K50	–	–	+
Pioneer 3335x33G26	–	–	+

¹ Fermentation Biotechnology Research Unit, National Center for Agricultural Utilization Research, Peoria, IL 61604. Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

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berge et al (1982). Corn was ground in a disk stacked mill (model 4E grinding mill, Straub Co., Hatboro, PA) to pass through a 10-mesh screen. A corn mash was prepared by adding 3 g water/1 g of ground corn. It was adjusted to pH 5.5 with Ca(OH)₂. The mash was liquefied by adding α -amylase (10 μ L/1 g of corn; High T 2x) and incubating the mash with stirring at 90°C for 90 min. It was adjusted to pH 4.5 by addition of HCl. The mash was saccharified by incubating for 2 hr at 60°C with glucoamylase (15 μ L/1 g of corn; Allchololase II Liquid 300). Samples taken for Bt analysis were stored at -20°C until analyzed for Bt protein by enzyme-linked immunosorbent assay (ELISA). The corn mash was prepared for fermentation by adding (NH₄)₂SO₄ to a final concentration of 240 mg/L.

Fermentation

Dry active *S. cerevisiae* (Super Starter Yeast) was inoculated (5% w/v) into yeast extract (10 g/L, peptone 20 g/L, and glucose 20 g/L). Flasks were sized so to have a medium to flask volume ratio of 1:5. The inoculum was incubated for 24 hr at 30°C and agitated at 250 rpm. The corn mash was inoculated at 5% (v/v) and incubated at 30°C until the fermentation ended (\approx 72 hr). Fermentations were run in 300-mL flasks or 2.5-L Biostat B bioreactors, or a 30-L Biostat D bioreactor (B. Braun Biotech International, Melsungen, Germany). Flask fermentations were capped with a fermentation lock. The exhaust gas was bubbled through a concentrated sulfuric acid trap to trap organic volatiles.

Analytical Procedures

Protein CRY1Ab was measured using quantitative sandwich-type enzyme-linked immunosorbent assay (Catalog No. AP003; Enviroligix Inc., Portland, ME). A polyclonal antibody mixture is used for direct binding to the CRY1Ab. To determine starch, ground corn was diluted in distilled water and boiled for 15 min. Glucoamylase was added in a sodium acetate buffer and the starch hydrolyzed for 120 min. Glucose was analyzed by the glucose oxidase-peroxidase method (Trinder 1969). Moisture was determined by AOAC method 935.32. Weight loss of the fermentation flask was measured using a Sartorius balance (BP3100 S, Edgewood, NY). Ethanol concentrations in flasks were determined by weight loss, which was caused by evolution of CO₂ (equal moles of CO₂ and ethanol were produced by fermentation). Glucose and ethanol concentrations in the bioreactor, as well as final concentrations for the flasks, were determined by HPLC using an Aminex HPX-87H column

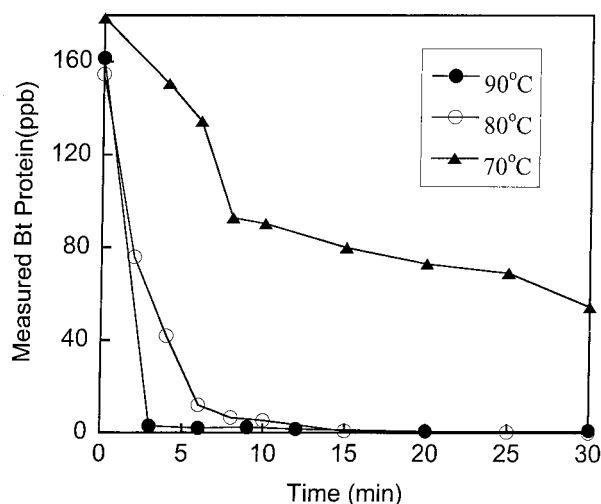


Fig. 1. Ground genetically modified (GMO) corn samples were diluted in water and incubated at 70, 80, and 90°C. Samples were subsequently freeze-dried and analyzed for the presence of Bt protein. Reported results are average of duplicate samples.

(Bio-Rad, Richmond, CA) and refractive index detector. Samples were analyzed at 65°C and eluted at 0.6 mL/min with 5 mM sulfuric acid. Ethanol yields for all fermentations were determined using HPLC results. Comparisons of ethanol yields were done using a statistical software package (v. 6, SAS Institute Inc., Cary, NC).

RESULTS

Fate of CRY1Ab in Wet-Milled Fractions

Two sets of corn hybrids were processed using a laboratory-scale wet-mill process that models the industrial process (Table I). Each set contained a Bt hybrid and a non-Bt hybrid that was otherwise genetically equivalent. Wet-milled fractions were collected from starch, steep liquor, germ, gluten, and fiber. The background detection limit for CRY1Ab was 3.8 ± 6.5 ppb (data not shown), as determined from the control hybrids. The CRY1Ab protein for the Bt hybrids was detected in whole corn, gluten, germ, and fiber fractions, but not in the starch or steep liquor fractions (Table II). The amount of CRY1Ab detected in corn samples was 170-453 ppb. The Bt protein was concentrated relative to the whole corn in the germ and gluten fractions.

Only 28-40% of the Bt protein was accounted for by the wet mill coproducts (Table II). Probable causes for loss include denaturation due to sulfite, denaturation due to endogenous proteases, or thermal inactivation (24 hr at 52°C). Endogenous proteases are found in low levels in corn (Kerpisci 1988).

Fate of CRY1Ab in Dry-Milled Fractions

One set of hybrids (33A13 and 33A14) was wet milled and also dry milled and fermented into ethanol (Table I). The corn was dry milled using a laboratory-scale process and fermented in Erlenmeyer flasks. The corn mash was tested for the presence of CRY1Ab after liquefaction, saccharification, and fermentation. As expected, CRY1Ab was not detected in the non-Bt ground corn or any of its dry-milled fractions. CRY1Ab was detected in the ground Bt corn hybrid (196 ± 1 ppb), but not in any of the corn mash samples. Therefore, loss of the CRY1Ab protein (as detected by ELISA) occurred during liquefaction. It was suspected that incubating the corn mash at 95°C denatured the protein. To test this theory, time samples were taken from corn mash incubated at 70, 80, 90°C (Fig. 1). No α -amylase was added to these samples of corn. Even for the sample incubated at 80°C, the protein was nondetectable after <15 min of heating.

Effect of CRY1Ab and Hybrid on Ethanol Fermentation

We were also interested to see whether CRY1Ab affected the fermentation of the saccharified corn mash. Three replicates of the 33A13 and 33A14 hybrids were fermented to ethanol using *S. cerevisiae*. The fermentation was monitored by weight loss assuming

TABLE II
Detection of Bt Protein in Wet-Milled Bt Corn Fractions

Corn Hybrid	Milled Fraction	Bt Protein ^a (ppb)	Bt Protein Yield ^b (ng/g)
33P67	Whole corn	201 \pm 34	201
	Starch	nd ^c	0
	Steep liquor	nd	0
	Germ	226 \pm 26	44
	Gluten	453 \pm 35	19
	Fiber	186 \pm 6	17
33A14	Whole corn	231 \pm 4	231
	Starch	nd	0
	Steep liquor	nd	0
	Germ	233 \pm 98	32
	Gluten	354 \pm 73	17
	Fiber	170 \pm 18	16

^a Values shown are mean of triplicates (db).

^b Total Bt protein (ng) recovered/g of corn (db).

^c Not determined.

0.97 g of CO₂ is produced per 1.00 g of ethanol. The results from all six samples are plotted in Fig. 2. No difference in productivities could be detected between the Bt and non-Bt hybrids. The ethanol yields were also similar (data not shown).

Next, we compared a collection of five hybrids to determine whether ethanol yield varied among the hybrids. The corn hybrids were fermented in flasks, laboratory-scale bioreactors, and a pilot-scale bioreactor. Apart from the scale, the corn was processed exactly the same in each case. Results from each of these fermentations were averaged together and are shown in Table III. Each result is the average of three flask and two bioreactor trials.

All hybrids had a starch content of 68–72% (db), within the expected range for #2 yellow dent corn. The ethanol yield was 84–95 g/L, which suggested a yield of 2.51–2.99 gal of ethanol per bushel of corn. The maximum yield possible can be calculated from the starch content for each hybrid and was 2.93–3.12 gal/bu. The effectiveness factor (e.g., how much of the starch was recovered as ethanol) was 87–96%. *F*-test analysis of the data revealed the corn hybrids varied significantly in efficiencies ($P < 0.002$) (data not shown). A Duncan's multiple range test was applied to the efficiencies data and determined that corn hybrids 33A13 and 33A14 fermented at higher efficiencies compared with hybrids 36K50 and

3335x33G26 ($P < 0.05$). A plot of the efficiencies versus hybrid with 95% confidence intervals shows the same result graphically (Fig. 3).

DISCUSSION

Fate of CRY1Ab Protein During Corn Milling

In this study, the fate of the CRY1Ab protein was monitored for corn that was either wet or dry milled. The hybrids tested were all Pioneer hybrids with YieldGard (Event MON180, Monsanto). The inserted vector contains a synthetic *cry1Ab* gene that includes the *cry1Ab* gene from *Bacillus thuringiensis* subsp. *kurstaki* strain HD-1, the 35S promoter region of the cauliflower mosaic virus that is needed for expression in maize, an intron from the maize *hsp70* gene for increased expression, and the 3' untranslated region from *Agrobacterium tumefaciens* (NOS3'), which provides a mRNA polyadenylation signal. The latter is required for stability of mRNA. CRY1Ab protein is also expressed in KnockOut (Novartis), NaturGard (Mycogen), YieldGard (Novartis), and Bt-Xtra (DeKalb) corn lines, though the genetic elements used for plant expression (promoter) varies in each. It is important to note that Yieldgard corn hybrids have approval for use in food in the United States and other countries. However, Europe requires labeling of food that contains GMO corn at concentrations >1%, w/w (Lipp et al 2001).

An immunological assay was used to monitor the fate of the Bt protein throughout the corn milling process. The most common methods used for detecting the presence of Bt corn are immunological and PCR-based methods. The PCR-based method detects heterologous DNA, most often using primers specific for the 35S promoter region (Hubner 1999). This method is especially suited for detecting the presence of GMO corn in a mixture of corn hybrids because of its high sensitivity (0.01–0.1%, w/w). Immunological methods have several advantages over the PCR-based method. They are much less expensive, require little in the way of sample preparation, and are available as commercial kits in either a qualitative (lateral flow strips) or quantitative (ELISA) format. Lateral flow strips are the most common method for detecting GMO corn at corn mills. We used ELISA to follow CRY1Ab in the corn milling samples.

For Bt corn samples processed by wet milling, CRY1Ab was detected in the gluten, germ, and fiber fractions. The gluten is processed directly into gluten meal and the oil-extracted germ and fiber are blended into corn gluten feed. Therefore, Bt protein was found in the ingredients for gluten meal and corn gluten feed. Bt protein was not detected in the starch fraction, which is converted into ethanol. Bt protein's absence from starch is anticipated because starch is repeatedly washed to remove protein (gluten). Nor was Bt detected in the steep, which also ends up in the corn gluten feed. In general, Bt protein was detected in those fractions of the corn expected to contain maize-derived protein. It is significant that Bt protein can be readily detected in the wet-milled fractions because a previous study concluded that steeping degraded maize DNA and, therefore, PCR-based detection might not be suitable (Gawienowski et al 1999). It is also significant that Bt protein became concentrated in the germ and gluten fraction compared with the whole Bt corn. This suggests that if a mixture of Bt and non-Bt corn were proces-

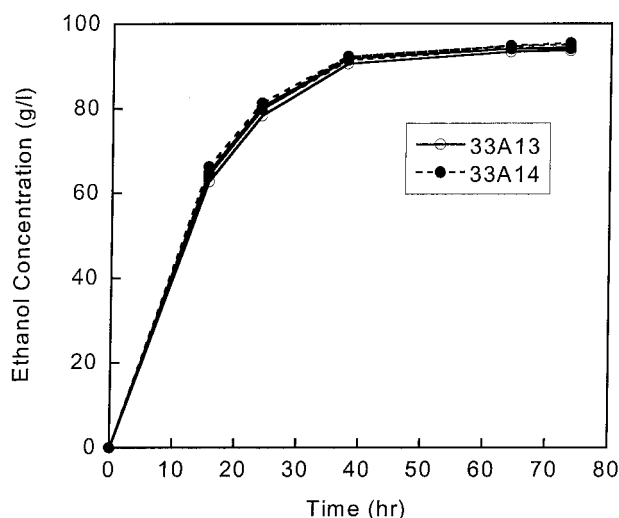


Fig. 2. Ethanol fermentation of dry-milled corn samples: 33A13 (genetically modified [GMO]) and 33A14 (non-GMO). Ethanol production was monitored by measuring weight loss from production of CO₂. Each sample was dry milled and fermented in triplicate.

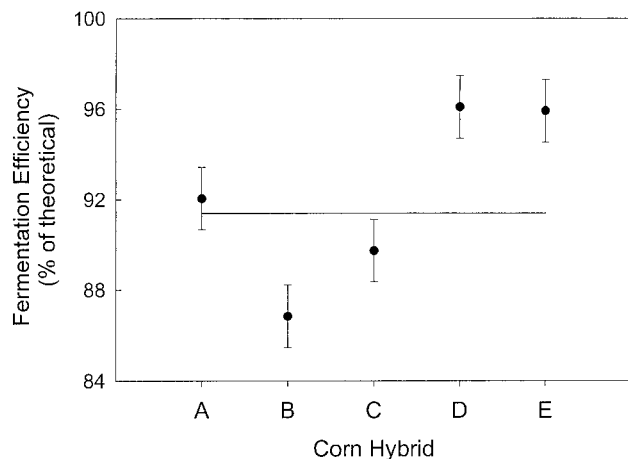


Fig. 3. Mean fermentation efficiency for corn hybrids with 95% confidence interval shown as error bars. Line is mean fermentation efficiency for all samples. Efficiency calculated as % of maximum possible ethanol.

TABLE III
Comparison of Fermentations of Different Corn Hybrids

Corn Hybrid	Bt Present	Starch Content ^a (% db)	Max. Ethanol (g/L)	Conversion Efficiency (%)
A (Unknown) ^b	–	68.5	87 ± 2.2	92 ± 2.3
B (36K50) ^b	–	69.3	84 ± 4.7	87 ± 4.8
C (3335x33G26) ^b	–	72.6	90 ± 2.4	90 ± 2.4
D (33A13) ^c	–	71.9	94 ± 0.4	96 ± 0.6
E (33A14) ^c	+	70.9	95 ± 0.6	96 ± 0.6

^a Standard deviation ±1% (db).

^b Mean values of one 30 L, two 1.6 L, and three 0.1 L fermentations.

^c Mean values of three 0.1 L fermentations.

sed, the germ, gluten, and fiber fractions could test positive even though the ground corn tested negative.

These results do not necessarily mean that active CRY1Ab is exiting wet-milling plants in the corn oil or feed products. First, the germ was not extracted in this study and, therefore, it is not possible to determine whether any detectable Bt protein would be extracted with the oil. Second, except for corn gluten feed sold locally, the feed products are dried in gas-fired evaporators. The absence of CRY1Ab in steep liquor and starch indicates the recombinant protein is not present in the fermentation nor its end products (except as blended into the corn gluten feed).

Active Bt protein did not survive the dry-milling process as measured by ELISA. Bt protein was not detected after liquefaction, the first enzymatic step in dry milling. Further experimentation demonstrated Bt protein was rapidly denatured at the temperature (95°C) used for liquefaction. Even at 80°C, the Bt protein was denatured within 15 min. Therefore, active CRY1Ab would not be expected to survive the dry-milling process.

The results indicate that Bt protein can be detected by using an ELISA assay in wet-milling operations in the gluten, unextracted germ, and fiber before heating, but not in the starch and steep fractions. An immuno-type assay may not be appropriate for detecting dry-milled coproducts that originated from Bt hybrids because liquefaction rendered the protein undetectable with the ELISA kit used in this study. For dry milling, DNA detection methods might be an effective alternative for downstream sampling. PCR-based methods have been used to detect *cry1Ab* DNA in heated corn mash samples used to make polenta (Hupfer et al 1999; Lipp et al 2000).

Variation in Ethanol Yields Among Hybrids

The dry-milled Bt and non-Bt hybrids were compared for ethanol yield and productivity. Ethanol production rates and yields were similar for fermentations using either Bt or non-Bt corn. Similar results can be expected for an industrial operation because CRY1Ab is inactivated before fermentation. Furthermore, even if some active CRY1Ab does make it to the fermentation, it is doubtful the protein would have bioactivity against yeast because the toxin is specific for lepidopteran insects and only becomes active in the gut of the insects (Schnepf et al 1998). The possibility still remains that expression of Bt protein could indirectly improve fermentation results because the GMO kernels have less insect damage and lower levels of mycotoxin (Dowd 2001).

Further fermentations were run on a larger collection of corn hybrids. The purpose was to see whether ethanol yields were affected by factors other than starch content. In other words, starch would be equally available from all hybrids. This hypothesis was tested by fermenting corn samples of five different hybrids. Five fermentations were run for each hybrid. The starch content for each hybrid was measured, and the maximum ethanol yields were calculated assuming that all starch was converted to ethanol. By comparing the actual and maximum possible ethanol yields, the efficiency of the fermentation could be determined. If ethanol yield only varied with starch content, it would be expected that efficiency should be the same for all the hybrids. In actuality, the efficiency varied between 87 and 96% among hybrids (Fig. 3). Furthermore, a Duncan's multiple range test indicated that hybrids 33A13 and 33A14 had significantly higher efficiencies ($P < 0.05$) than the other hybrids tested in this study. The conclusion that can be drawn from these results is that ethanol yield is not exclusively dependent on starch content.

Other genetic factors or perhaps environmental factors would also seem to affect the final ethanol concentration. Examples of putative genetic traits that could affect starch availability are the chemical structures of the starch and the starch-protein (gluten) matrix. Hybrids identified as superior for dry milling should be readily

adopted by the ethanol industry because many dry mills are run as farmer cooperatives. Therefore, farmers benefit directly from any steps they take to increase ethanol yield. However, a thorough study will be needed to determine the optimal hybrids for dry milling and the economic worth of switching to these hybrids.

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