

Effect of Aflatoxin B1 on Dry-Grind Ethanol Process

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ABSTRACT

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Aflatoxins, like all mycotoxins, are toxic fungal metabolites that can have adverse health effects on animals and human beings. Aflatoxins are a major concern for the dry-grind corn processing industry as it is believed that aflatoxins affect yeast and reduce its efficacy in producing ethanol. In the present study, aflatoxin B1 (100, 200, 350, or 775 ppb) was added to mycotoxin-free corn and laboratory-scale fermentations

were conducted. No effect of aflatoxin B1 was observed on the fermentation rates or final ethanol concentrations. Mean ethanol concentration in the fermenter was 14.01–14.51% (v/v) at 60 hr for all the treatments. In the dry-grind ethanol process, 55% of aflatoxin B1 was detected in wet grains and 45% in thin stillage.

Aflatoxins, like all mycotoxins, are metabolites produced by many fungi under certain conditions. Factors including plant stress, physical damage, nutrition deficiencies, and insect infestation affect fungal growth. The proliferation of these mold toxins does not necessarily correlate with the abundance of fungal growth. Aflatoxins are not produced in the normal course of growth by fungi but are produced only under specific conditions. Hence, presence of fungi is not a confirmatory test for presence of aflatoxins (Smith and Moss 1985).

The dry-grind ethanol industry is one of the fastest growing industries in the United States (MacDonald et al 2003). The process produces ethanol and distiller dried grains with solubles (DDGS). DDGS are used in ruminant animal diets. In dry-grind ethanol plants, incoming corn samples are screened for the presence of *Aspergillus* sp. using UV fluorescence (black light) or commercial test kits. Samples that test positive are rejected or blended with clean corn.

Mycotoxins in corn processed by dry-grind ethanol plants can present two problems. First, mycotoxins could end up in DDGS sold as animal foodstuffs. Second, it is believed that mycotoxins stress the yeast during fermentation, resulting in lower ethanol yields (Kelsall and Lyons 2003).

Knowledge about distribution of aflatoxins in wet grains and thin stillage can help in developing strategies to reduce final aflatoxin concentrations in DDGS. The objective was to determine the effect of aflatoxin B1 on the fermentation process and its distribution in wet grains and thin stillage.

MATERIALS AND METHODS

Samples and Replicates

Hand-cleaned yellow dent corn with no detectable levels of mycotoxins (aflatoxin B1, B2, G1, G2 with detection limit of 2 ppb; fumonisin B1, B2 with detection limit of 2 ppm; ochratoxin A with detection limit of 50 ppb; zearalenone with detection limit of 0.2 ppm, and deoxynivalenol with detection limit of 0.25 ppm) was used for the study. Moisture content of the corn was determined using a convection oven (AACC International 2002). Four

sets of replicate fermentations were conducted for five levels (control, 100, 200, 350, and 775 ppb) of aflatoxin B1 tested, of which three sets were selected for aflatoxin analyses.

Aflatoxin B1 Inoculation

From aflatoxin B1 stock solution (Sigma Chemical Co., St. Louis, MO; Calbiochem-Behring Corp., La Jolla, CA) of 0.322 g/L concentration, appropriate volumes were used to obtain the desired concentration for each replicate. The ethanol in each vial was evaporated with nitrogen at 30°C. Vials were washed three times with 10 mL of deionized water. Each wash was vortexed before being added to the corn flour.

Size Reduction, Liquefaction, and Saccharification

Samples were milled in a hammer mill (model MHM4, Glen Mills, Clifton, NJ) at 500 rpm using a 2-mm sieve with round holes. Each 500-g sample of ground corn was mixed with tap water at 60°C to form a 25% solids slurry. Aflatoxin B1 was inoculated into the slurry. The slurry sample was drawn and dried at 48°C to 10% moisture content. The slurry was liquefied using 2.8 mL of α -amylase for 90 min in a water bath maintained at 90°C, and then was cooled to 60°C and adjusted to pH 4.2 using 1N sulfuric acid. This slurry was saccharified by adding 2.8 mL of glucoamylase and holding at 60°C for 2 hr with a constant agitator speed of 150 rpm. Using HPLC, sugar (glucose, fructose, maltose, and maltotriose) profiles were measured after saccharification.

Fermentation, Evaporation, and Filtration

Saccharified mash was cooled to 30°C and inoculated with 11 g of active dry yeast (*Saccharomyces cerevisiae*, Fleischmann's Yeast, Fenton, MO, USA). Free amino nitrogen (300 ppm) was added to yeast with 1 g of (NH₄)₂SO₄. Fermentation was conducted at 30°C for 60 hr with continuous agitation at 50 rpm. Fermentation was monitored by withdrawing 5-mL samples of fermentation broth at 12-hr intervals and measuring sugar and ethanol concentrations using HPLC. Fermented mash was heated to 85°C and held for 2 hr to evaporate ethanol. Resulting whole stillage was filtered through a U.S. 200-mm sieve to obtain filtrate called thin stillage and a semisolid fraction called wet distillers grains (WDG). WDG was dried at 48°C to 10% mc. After drying the initial and wet grain samples at 48°C for 60 hr, aflatoxin B1 analyses were performed using HPLC. Thin stillage samples were analyzed by measuring the total volume of thin stillage and determining aflatoxin B1 in a 50-mL representative sample using HPLC.

HPLC Analyses

The 5-mL samples drawn from fermentation vessels were centrifuged (model Durafuge 100, Precision, Winchester, VA) at 1,476

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$\times g$ for 5 min to obtain the supernatant liquid that was filtered through a 0.2- μm 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 Corporation, 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).

Mycotoxin Analyses

Aflatoxin B1 analyses were done on three samples (initial sample, thin stillage, and WDG) recovered in the dry-grind ethanol process (Fig. 1). Initial and WDG samples were extracted using 50 g of sample and 100 mL of solvent consisting of 90% acetonitrile and 10% of KCl (4% solution in water) solution. The mixture was placed on an orbital shaker (Lab-Line, Melrose Park, IL) for 30 min and passed through a Fisher brand P8 fluted (coarse porosity) filter paper. Thin stillage was not extracted but centrifuged at 2,000 $\times g$ for 10 min. Supernatant (1 mL) was placed in a Type I shell vial (11.9 \times 31 mm) and evaporated with nitrogen at 60°C. Extracted product (1 mL) from the initial and WDG samples were evaporated with nitrogen at 60°C. Extracted

aflatoxin B1 was derivatized (Truskess 1992) using 400 μL of trifluoroacetic acid (TFA) derivatizing agent (20% TFA, 70% deionized water, and 10% acetic acid) and allowed to stand at room temperature for 30 min. After incubation, 100 μL of acetonitrile (HPLC-grade) was added. The whole volume was transferred to HPLC vials. Derivatized aflatoxin B1 was analyzed on a prodigy 150 \times 4.60 5 micron C-18 column (Phenomenex, Torrance, CA) on an HPLC 1100 with a HP1046A programmable fluorescence detector (Agilent, Colorado Springs, CO). Each sample (30 μL) was injected into the column. The gradient for the HPLC started at an 80:20 ratio of water and acetonitrile for 2 min. At 14 min, the gradient was 60:40, and at 16 min the gradient was 55:45 water and acetonitrile. The temperature was maintained at 25°C. Each sample and standard was chromatographed for 25 min. The detector excitation wavelength was 365 nm and the emission wavelength was 440 nm. All data were collected using HP Chem-Station software. The retention time of aflatoxin B1 was 14.1 min.

Statistical Analyses

Four sets of replicate fermentations were conducted. All four sets were considered for fermentation performance analyses. Three of the replicates were considered for aflatoxin B1 analyses. Fermentation samples from all experiments were analyzed using HPLC with two replicates. Mean values of two HPLC analyses were used for each replicate.

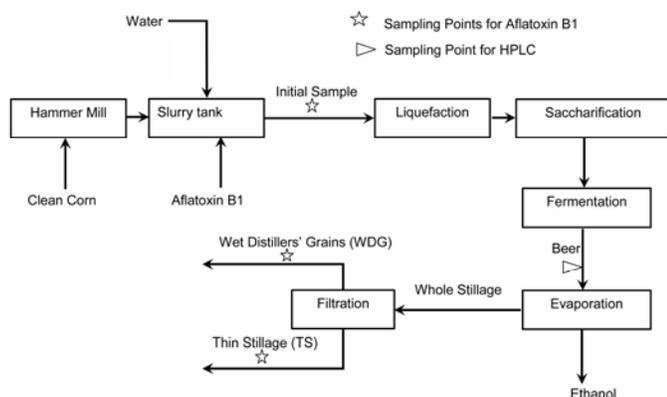


Fig. 1. Process flow diagram and sampling locations.

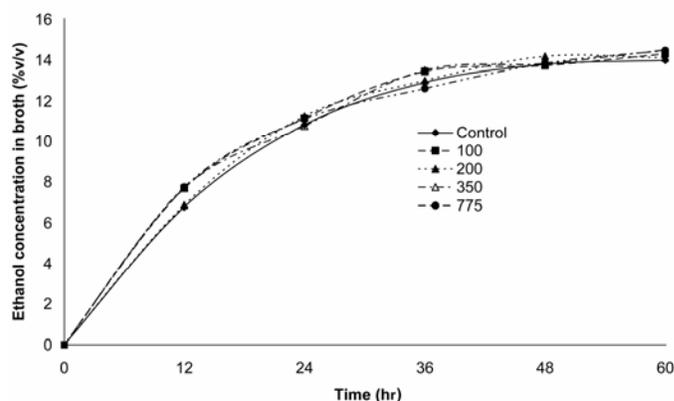


Fig. 2. Mean ethanol concentration (% v/v) in fermenter.

TABLE I
Mean Ethanol Concentration (% v/v) in Fermentation Broth^a

Aflatoxin B1 (ppb)	Time (hr)					
	0	12	24	36	48	60
Control	0	6.79	10.84	12.94	13.81	14.01
100	0	7.70	11.18	13.47	13.75	14.33
200	0	6.88	11.27	13.02	14.20	14.12
350	0	7.71	10.78	13.53	13.85	14.51
775	0	7.76	11.13	12.64	13.86	14.48

^a Values in the same column are not significantly different at 95% confidence levels.

TABLE II
Mean Glucose Concentration (% v/v) in Fermentation Broth^a

Aflatoxin B1 (ppb)	Time (hr)					
	0	12	24	36	48	60
Control	17.03	9.11	3.88	1.20	0.20	0.12
100	17.26	7.49	3.09	0.88	0.27	0.08
200	17.25	8.54	3.11	0.69	0.12	0.06
350	17.16	7.89	3.63	0.74	0.09	0.04
775	15.74	7.08	3.08	0.67	0.06	0.03

^a Values in the same column are not significantly different at 95% confidence levels.

RESULTS AND DISCUSSION

No significant effect of aflatoxin B1 (up to 775 ppb) was observed on ethanol concentration during the course of fermentation in the dry-grind corn process (Table I and Fig. 2). The ethanol profile (ethanol concentration vs. fermentation time) of the control corn sample (no aflatoxin B1 added) was the same as the profile obtained with corn samples inoculated with different aflatoxin B1 levels (100–775 ppb) (Fig. 2). Ethanol concentrations obtained for the control sample were comparable to concentrations from the dry-grind corn process. Final ethanol concentrations for all aflatoxin B1 treatments (100, 200, 350, and 775 ppb) were comparable to the control sample and higher than 14.01% (v/v). No significant differences were observed in the final ethanol concentrations among different aflatoxin treatments (Table I).

Comparison of sugar (glucose) utilization rates (glucose concentration vs. fermentation time) were comparable among different treatments (Table II). Final glucose concentrations of <0.12% (w/v) (at 60 hr) indicates complete fermentation with negligible glucose remaining unconverted.

Glycerol is an indicator of yeast stress. A small amount of glycerol (≈ 1.2 – 1.5%) is produced in all dry-grind ethanol fermentations (Russel 2003). Final concentrations of glycerol as an indicator of yeast stress were consistently low (<1.38%, w/v) and comparable among all the treatments, indicating that yeast was not under stress due to the presence of aflatoxin B1 and the observed results were not due to problems in fermentation (Table III). High concentrations of lactic acid indicate bacterial infections during dry-grind ethanol fermentation (Kelsall and Lyons 2003). Concentrations of lactic acid >0.2–0.8% (w/v) stress the yeast and result in lower growth rates and ethanol production rates (Narendranath et al 2001). Final lactic acid concentrations (not reported) were low (<0.02%, w/v) among all samples, indicating the absence of bacterial infections and associated yeast stress.

TABLE III
Mean Glycerol Concentrations (% w/v) in Fermentation Broth

Aflatoxin B1 (ppb)	Glycerol			
	Replicate 1	Replicate 2	Replicate 3	Replicate 4
Control	1.25	1.17	1.21	1.22
100	1.23	1.18	1.23	1.21
200	1.21	1.18	1.22	1.25
350	1.21	1.17	1.25	1.23
775	1.21	1.12	1.29	1.22

TABLE IV
Treatment Levels, Percentage Accounted and Distribution of Aflatoxin B1

Intended Treatment Level (ppb)	Actual Treatment Level (ppb)	Accounted ^a (%)	Wet Grains (%)	Thin Stillage (%)
Control	0
125	100 ± 17	80.5	55.2	44.8
200	204 ± 37	97.4	47.2	52.8
350	343 ± 54	93.4	58.1	41.9
775	772 ± 198	93.0	74.4	25.6

^a Average accounted percentage was 92.9% (80.5–97.4%).

In the dry-grind ethanol process, nonfermentables in corn are concentrated approximately threefold in the DDGS (due to conversion of starch into ethanol). Aflatoxin B1 is not destroyed during the dry-grind ethanol process and gets concentrated in DDGS. For all experiments in this study, initial mash samples were analyzed for aflatoxin B1 before any further processing. In this study, 80.5–97.4% and an average 92.9% of the aflatoxin B1 added to the initial samples was recovered (Table IV). Contrary to the report of Wright (1995), practically all of aflatoxin B1 survives chemical and unit operations in the conventional dry-grind ethanol process. These results suggest that aflatoxin B1 will get concentrated (approximately threefold) in the DDGS in the dry-grind ethanol process if it is present in the incoming corn.

In the dry-grind ethanol process, during downstream processing (after ethanol recovery) the whole stillage is centrifuged into thin stillage (6–10% solids) and WDG (30–35% solids). Determining the distribution of aflatoxins can help in developing strategies to reduce final mycotoxin concentrations in DDGS. Mass balance for the aflatoxin B1 was performed. Depending on the level of treatment, 47–74% aflatoxin B1 was found in WDG and 53–25% in thin stillage. On average (across aflatoxin B1 levels), 55% of the recovered aflatoxin B1 was removed in WDG and the remaining 45% was removed with thin stillage.

CONCLUSIONS

Aflatoxin B1 did not affect ethanol yields in the dry-grind ethanol process. Yeast performance, as inferred by fermentation rate, was not affected by aflatoxin B1 up to a concentration of 775 ppb. In the downstream dry-grind ethanol process, 45–55% of the aflatoxin B1 was found in wet grains. Future work could involve repeating the experiment using corn naturally contaminated with aflatoxin. The lower starch content of naturally contaminated corn should be considered while analyzing the results.

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