Fate of Fumonisin B_1 in Naturally Contaminated Corn during Ethanol Fermentation

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Two lots of corn naturally contaminated with fumonisin B_1 (15 and 36 ppm) and a control lot (no fumonisin B_1 detected) were used as substrates for ethanol production in replicate 8.5-liter yeast fermentations. Ethanol yields were 8.8% for both the control and low-fumonisin corn, while the high-fumonisin corn contained less starch and produced 7.2% ethanol. Little degradation of fumonisin occurred during fermentation, and most was recovered in the distillers' grains, thin stillage, and distillers' solubles fractions. No toxin was detected in the distilled alcohol or centrifuge solids. Ethanol fermentation of fumonisin-contaminated corn coupled with effective detoxification of distillers' grains and aqueous stillage is suggested as a practical process strategy for salvaging contaminated corn.

A number of studies have been carried out on the fate of mycotoxins in contaminated grains used as substrates for the fermentative production of ethanol (1, 5, 6, 10, 11, 15, 16). Results common to these studies are (i) little degradation of toxin during fermentation, (ii) no toxin in the distilled alcohol, and (iii) toxin accumulation in the spent grains. This last result presents a serious problem because spent grains are often used for animal feed. Consequently, practical detoxification procedures are essential. Lillehoj et al. (11) detoxified aflatoxin B_1 in wet postfermentation stillage with sodium hydroxide, ammonium hydroxide, sodium hypochlorite, and hydrogen peroxide. Bennett et al. (1) reduced zearalenone in fermentation solids with formaldehyde. Subsequently, an integrated process (4) was developed for ammonia inactivation of aflatoxin-contaminated corn and ethanol fermentation.

In 1988, a new group of water-soluble toxins was discovered that is produced by certain strains of *Fusarium moniliforme* Sheldon, one of the most prevalent fungal contaminants of corn (13), and these toxins were named fumonisins (2). Subsequently, the fumonisins have been linked to cancer-promoting activity (7), and equine leukoencephalomalacia has been reproduced by administration of the toxin to a horse (12). Wilson et al. (20) described an epizootic of equine leukoencephalomalacia and the association of fumonisin B_1 in the diet of the affected animals. Fumonisin B_1 has also been associated with a fatal disease of swine in which the animals had pulmonary edema and pancreatic and liver lesions (8).

Recently, *Fusarium proliferatum*, originally isolated from corn, was shown to produce fumonisins B_1 and B_2 in corn culture (17). Similar results were obtained with a newly described species, *Fusarium nygami* (19). Bennett et al. (1a) and Nelson et al. (14) demonstrated the production of fumonisins by additional *Fusarium* species, which increases the likelihood of the occurrence of these toxins in commodities that are used for food, feed, and fuel. A practical strategy for salvaging fumonisin-contaminated grain and screenings, both of which tend to contain high levels of fumonisins (18), is production of ethanol fuel. Since fumonisins are water soluble (2), they could differ from other mycotoxins in their effect on alcohol yield and in their distribution in stillage fractions. Consequently, the current study was undertaken to determine the fate of fumonisins in the fermentation of naturally contaminated corn to ethanol.

MATERIALS AND METHODS

Preparation and fermentation of corn. The procedures used in the fermentation of standard no. 2 yellow dent corn and two lots of naturally fumonisin-contaminated substrates are described below.

First, the corn was ground into a fine meal to pass through a 10-mesh (2-mm-pore-size) screen. The fumonisin content and composition of each lot are shown in Table 1. The fermentation medium was prepared in duplicate to approximate 20% glucose (2,356 g of ground corn per 8.5-liter run). Ground corn was initially dispersed in 5,000 ml of tap water in 20-liter, stainless-steel, temperature-controlled, jacketed fermentors equipped with stirrers. The pH of the dispersed corn was adjusted to 6.2, and 6 ml of Solvay Taka-Therm L-340 α-amylase was added. The temperature was elevated and maintained at 90°C for 1 h with stirring until the starch gelatinized and degraded to soluble dextrins. Tap water (1,560 ml) was then added, the fermentor was cooled to 60°C, and the pH was adjusted to 4.0. Solvay Diazyme L-200 glucoamylase (18 ml) was then added to hydrolyze the dextrins to glucose during a 2-h incubation period.

The mash was cooled to 30°C and adjusted to pH 4.5, and 1,000 ml of yeast inoculum was added. The yeast inoculum consisted of 0.3% yeast extract, 0.5% peptone, 1.0% glucose, and 18 g of Fermivin dry yeast (G. B. Industries) in 1,000 ml of tap water. The inoculum culture was incubated for 24 h at 30°C and shaken at 200 rpm prior to use. Samples of mash were taken at 0, 24, 48, and 72 h after inoculation and assayed for glucose and ethanol. The fermentation was halted at 72 h.

Separation of products. After fermentation, 1 liter of whole stillage was removed from the fermentor. Ethanol was distilled from the sample in a fractional still that simulated the type of distillation column used in the spirits industry. Distilled ethanol (77 to 78°C) was concentrated 10-fold (from 50 to 5 ml) and assayed for fumonisin B_1. The remaining

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TABLE 1. Fumonisin content and composition
of corn after grinding

Type of corn	Fumonisin content (ppm)	Moisture (%)	Protein (%)	Ash (%)	Starch (%)
Control	ND[a]	11.3	8.9	1.1	64.1
Low fumonisin (1989 corn)	15	11.2	8.9	1.0	64.0
High fumonisin (1990 corn screenings)	36	9.5	8.9	2.1	61.4

[a] ND, none detected.

whole stillage was fractionated by the process diagrammed in Fig. 1.

Analyses. Glucose and ethanol levels in the fermentation media were determined by high-performance liquid chromatography (HPLC) by use of a Fam-Pak ion-exchange column with a Spectra Physics SP8880 autosampler, a Waters 590 programmable pump, a Waters 410 differential refractometer, a Spectra Physics SP 4270 integrator, a Waters column heater, and a Guard-Pak precolumn. The column was at 40°C, and 1.5 mM phosphoric acid was used as the mobile phase.

Fumonisin B_1 was determined by the following method. Twenty-five grams of ground, blended corn or distillers' dried grains was extracted with 100 ml of methanol-water (80:20) by being shaken on a wrist-action shaker for 30 min. Extracts were filtered through rapid-flow filter paper, and 2.0 ml of filtered extract was transferred to amber vials containing 4.0 ml of distilled, deionized water. Diluted sample extracts were partially purified on solid-phase extraction columns as follows. The cleanup columns (Mega Bondelute C_{18}, 2 g of packing) were preconditioned by aspirating 6.0 ml of methanol (HPLC quality) through the column with a vacuum. The column was washed with 6.0 ml of methanol-water (20:80). Care was taken to prevent drying of the column. Diluted samples were added to the column and washed with 4.0 ml of methanol-water (20:80). A vacuum was applied for 15 min to dry the column. The dried column was washed with 4.0 ml of acetone-ethyl acetate (1:1), and then fumonisin B_1 was eluted with 10.0 ml of chloroform-methanol-acetic acid (60:40:10). The solvent was removed either with a Rotovapor or under nitrogen (<60°C). The resulting residue was saved for derivatization.

Aqueous fermentation products were analyzed by diluting

Whole stillage

Through cheese cloth
under vacuum

On cloth   Filtrate

Distillers grains   Thin stillage

Centrifuged on
Sharples Model L1
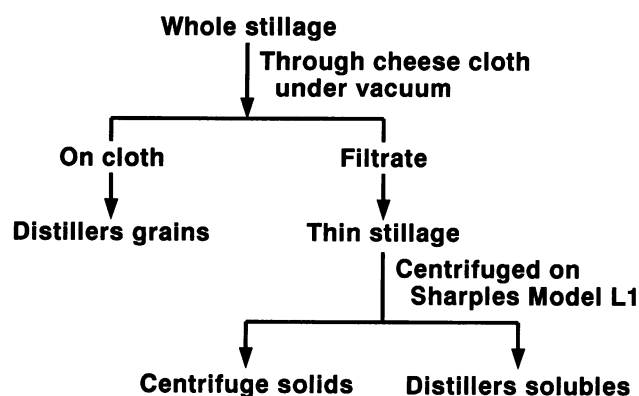
Centrifuge solids   Distillers solubles

FIG. 1. Procedure to fractionate fermented corn mash.

2.0 ml of supernatant from the various stillage fractions with 4.0 ml of distilled, deionized water. The diluted extract was then cleaned up on a solid-phase extraction column as described above.

Preparation of the naphthalene-2,3-dicarboxyaldehyde derivative (19a) was accomplished by dissolving the residues in 1.0 ml of methanol and diluting them with 1.0 ml of 0.05 M sodium borate buffer (pH 9.5). To this solution was added 0.5 ml of a sodium cyanide (0.13 mg/ml) (catalyst) solution, and then 0.5 ml of a naphthalene-2,3-dicarboxyaldehyde solution (0.5 mg/ml) was added. The vials, protected from light by being wrapped in aluminum foil, were tightly capped and heated for 15 min at 60°C on a heating block. Each sample was cooled and diluted with 7.0 ml of 0.05 M phosphate buffer (pH 7.0)-acetonitrile (40:60) and assayed by HPLC. Standard fumonisin B_1 derivative (5 and 10 µg) was prepared in an identical manner. Fumonisin B_1 used for the standard was obtained from preparative HPLC of extracts from rice cultures of a fumonisin-producing isolate, strain NRRL 13616. The purity of the standard material was established by gas chromatography-mass spectroscopy of a trimethylsilane derivative of hydrolyzed fumonisin B_1 (9).

HPLC analyses and quantitation of fumonisin B_1 were accomplished as follows. The flow rate of the mobile phase (acetonitrile-water-acetic acid [60:40:1]) was set at 1.0 ml/min, and a C_{18} reverse-phase column (25 cm by 4.6 mm) was used. A 10- to 50-µl derivatized sample was injected, and fumonisin B_1 was detected with a fluorescence detector set at 250-nm excitation and a 418-nm emission cutoff filter. A range setting of 1.0 or 0.5 was used, depending on the sensitivity of the detector and the concentration of fumonisin B_1. The attenuation on the integrator was adjusted so that a 20-ng standard gave ca. 80% full-scale peaks. The retention time of fumonisin B_1 in this mobile phase was 7.7 to 7.8 min. Standards were run each day to verify retention times. A standard curve was constructed by plotting peak height (in millimeters) versus nanograms of fumonisin. The linear range was from 1 to 25 ng. Samples were diluted with 0.05 M phosphate buffer-acetonitrile (40:60) so that the amount of fumonisin B_1 injected fell within this range. The amount of fumonisin B_1 in a sample was then calculated by the following formula:

$$ppm = \frac{\left(\dfrac{\text{peak height of sample}}{\text{peak height of standard}}\right) \times \text{nanograms of standard injected}}{\text{weight equivalent (milligrams) of sample injected}}$$

The peak height of the sample should be near the peak height of the standard.

RESULTS AND DISCUSSION

Two lots of corn naturally contaminated with fumonisin B_1 (15 and 36 ppm) plus a control lot (no fumonisin detected) were used as substrates for replicate 8.5-liter ethanol fermentations. The control and low-fumonisin lots were similar in moisture, protein, ash, and starch contents, while the high-fumonisin lot was lower in moisture and starch, contained screenings, and was visibly darker (Table 1). The final ethanol concentration in the fermented mash of both the control and low-fumonisin corn was 8.8% (wt/vol), while the high-fumonisin lot produced 7.2% (wt/vol) ethanol. These yields represent conversion efficiencies of 88 and 75%, respectively. These differences reflect variations in compo-

TABLE 2. Distribution of fumonisin B$_1$ in products from ethanol fermentations

Sample	Fumonisin B$_1$ (ppm)^a					
	Control corn		Low-fumonisin 1989 corn		High-fumonisin 1990 corn screenings	
	1^b	2	3	4	5	6
Original corn	ND^c	ND	15	15	36	36
Fermented mash (beer)						
0 h	ND	ND	1.3	1.4	3.4	4.3
72 h	0.4	ND	1.8	2.7	5.5	4.7
Distillers' dried grains	4.0	5.0	19.2	25.3	48.5	65.0
Thin stillage	0.6	ND	1.5	1.7	4.9	5.8
Centrifuge solids	ND	ND	ND	ND	ND	ND
Distillers' solubles	0.2	ND	1.3	1.7	5.2	4.2
Distilled ethanol	ND	ND	NA^d	ND	NA	ND
Fermented mash minus ethanol	0.3	0.4	NA	3.2	NA	4.7

^{a} ppm, micrograms per milliliter or micrograms per gram where appropriate (average of duplicate assays).
^{b} Run number.
^{c} ND, none detected (less than 0.2 ppm).
^{d} NA, not available.

sition and amount of starch saccharified rather than a detrimental effect of low fumonisin B$_1$ levels (<6 ppm) in the liquid mash.

Assays on toxin-free corn and liquid substrates spiked with fumonisin B$_1$ at 5 and 10 ppm were done by the procedure described above. Average recoveries of duplicate assays were 88 and 92% for solid and liquid substrates, respectively. The levels of fumonisin B$_1$ on distillers' dried grains reported in this article were confirmed by HPLC of the o-phthaldehyde derivative (16a), with a reliable detection limit of 0.20 ppm.

The fate of fumonisin B$_1$ during fermentation of naturally contaminated and control corn to ethanol is reported in Table 2. Only a limited amount of fumonisin B$_1$ was degraded by the process, and most was recovered in the distillers' dried grains, thin stillage, and distillers' solubles. Of distillers' dried grains, 487 and 517 g were recovered from the low-contamination corn (15 ppm) and high-contamination corn screenings (36 ppm), respectively, and these samples contained 19.2 to 25.3 and 48.5 to 65.0 ppm of fumonisin B$_1$, respectively. This accounts for 31% (low) and 35% (high) of the total fumonisin B$_1$ in the starting corn. The recovered distillers' dried grains account for 21 and 22% of the weight of the original corn. Facilitated by solubility in water, 54% (low-fumonisin corn) and 51% (high-fumonisin corn) of total fumonisin B$_1$ in the starting sample were extracted into the whole stillage of the two naturally contaminated substrates. Material balance calculations, based on the initial toxin levels and levels in the solid and liquid fractions obtained from the fermentations, indicate that 85% of the fumonisin B$_1$ in the starting corn was recovered. No toxin was recovered in the distilled ethanol or centrifuge solids. Similar to other toxins such as aflatoxin and zearalenone during the ethanol fermentation process (1, 4, 11, 16), fumonisin B$_1$ was also concentrated in the spent grains but to a lesser extent. Interestingly, fumonisin B$_1$ was also detected in distillers' dried grains (4 and 5 ppm) from both fermentations of control corn, although none was detected in the original corn. One explanation of this phenomenon, supported by preliminary

experiments, is that the fermentation process caused the fumonisin B$_1$ to be more readily extracted with the solvent used. Exhaustive extraction studies on this toxin are currently under way.

In the past, physical, chemical, and biological processes have been explored to dispose of or salvage contaminated agricultural commodities (3) for animal feed. However, restrictions such as (i) economic feasibility, (ii) reduction of toxin concentration to safe levels, (iii) freedom from toxic residues, and (iv) retention of nutritional value have narrowed the types of agents likely to succeed in a commercial-scale detoxification process to chemicals such as oxidizing agents, acids, and bases. Agents such as ammonia, formaldehyde, sodium hypochlorite, and aqueous wash will be evaluated in a subsequent study for detoxifying spent grains and stillage containing fumonisin B$_1$.

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