DESTRUCTION OF AFLATOXINS DURING FERMENTATION AND BY-PRODUCT ISOLATION FROM ARTIFICIALLY CONTAMINATED GRAINS[1]

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ABSTRACT

Aflatoxins B_1, B_2, G_1, and G_2 were added to corn, wheat, and corn-milo fermentation mixtures at levels of 1000 μg each per 100 g grain mixture. The degree to which aflatoxins would persist during fermentation, isolation of distiller's protein concentrate, and by-products such as residue, syrup, and whey were then determined. The data indicated a loss after fermentation of slightly greater than 60%. Further process treatments involved in the isolation of distiller's protein concentrate led to a destruction of total aflatoxins in excess of 90%.

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Based on the anticipated demands for quality protein and industrial alcohol, both producible from grains, a series of studies was conducted to determine if the two products could be obtained from a continuous fermentation process (1). When an alkaline extraction procedure was used, a protein concentrate fit for human consumption could be obtained from corn and wheat distiller's by-products. In addition, this extraction procedure yielded new forms of residue, syrup, and whey fractions for animal feeds (1).

Damaged grains that might be contaminated with *Aspergillus flavus* and/or *A. parasiticus* molds are sometimes used in commercial distillation. Aflatoxins produced by these molds have been found in corn, wheat, and milo (2,3).

The degree of aflatoxin contamination in grains has been the subject of a number of surveys. In general, the incidence and degree of contamination in corn have been relatively low (3–5). The highest level for 1600 samples (representing all grades) in these studies was only 25 ppb. This is slightly above the permissible level of 20 ppb. However, moisture content and temperature during harvest, storage, or transport can effect a dramatic change (6,7). In a survey of corn (7) obtained from the South, 2 out of 10 (20%) No. 2 grade samples contained aflatoxin at a level of 62 ppb, while one sample of No. 5 corn contained 308 ppb aflatoxin. In a study of white corn from southeast Missouri, stored under loans from the Commodity Credit Corporation, 14% of 1283 truckloads contained more than 20 ppb aflatoxin. Approximately 2% had greater than 100 ppb aflatoxin (8). Most mammals (including man), birds, and fish, all potential consumers of protein isolates or by-products derived from fermentation media, could be affected by these toxins. Aflatoxins were therefore added to corn, wheat, and corn-milo fermentation mixtures to determine the degree to which they would persist during fermentation and isolation of distiller's protein concentrate (DPC) or distiller's by-products.

MATERIALS AND METHODS

Laboratory Fermentation Procedure

A laboratory procedure was developed for the fermentation of hammer-milled

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wheat, corn, or corn:milo mixtures. This procedure was designed to simulate the U.S. industrial fermentation procedure as closely as possible, with the following exceptions: 1) the industrial procedure involves high-temperature-pressure continuous cooking, but in the laboratory a batch-type cooking was performed; and 2) the industrial procedure uses a direct heat distillation for the beer still, whereas the laboratory used steam distillation for the beer still.

The detailed diagram in Fig. 1 shows the entire fermentation procedure for the various grains. All enzymes used were obtained from Marschall Division of Miles Laboratories, Inc., Elkhart, IN 46514.

Protein Extraction Procedure

Figure 2 shows the entire extraction procedure for the various grains that had gone through the fermentation described in Fig. 1. Because of the different chemical composition of the grains, each required differing amounts of NaOH to titrate the fermented wet solids to pH 12.2.

A second alkaline extraction of the wet solids was routinely performed after a preliminary investigation indicated it would increase final product yield by approximately 25%.

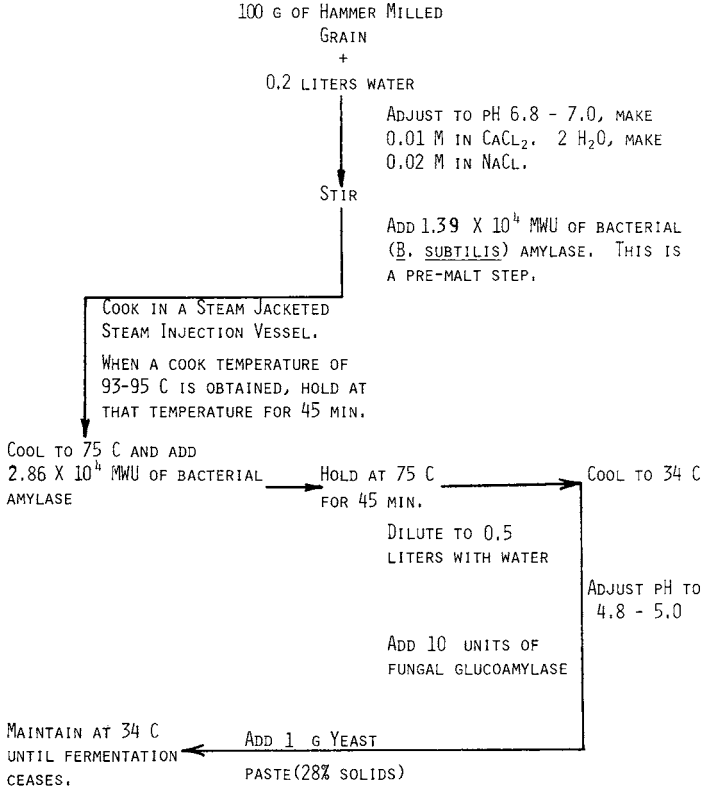

Fig. 1. The laboratory procedure used for the fermentation of corn, corn:milo, and wheat.

All glass vessels used in the fermentation, extraction, and freeze-dry procedures were covered with aluminum foil to protect aflatoxins against destruction by light.

Aflatoxins

Approximately 1 mg of each of the purified aflatoxins, in chloroform, was added to small duplicate samples (100 g) of corn, wheat, or corn:milo. These high levels were deliberately chosen in order to be able to trace destruction through the various process steps described above.

Aflatoxins B_1, B_2, G_1, and G_2 used for addition to the fermentation mixtures were purchased from Calbiochem, La Jolla, Calif. Primary standards containing aflatoxins B_1, B_2, G_1, G_2, and B_1-G_1 in solution were supplied through the courtesy of W. A. Pons, Southern Regional Research Center, ARS, USDA, New Orleans, La.

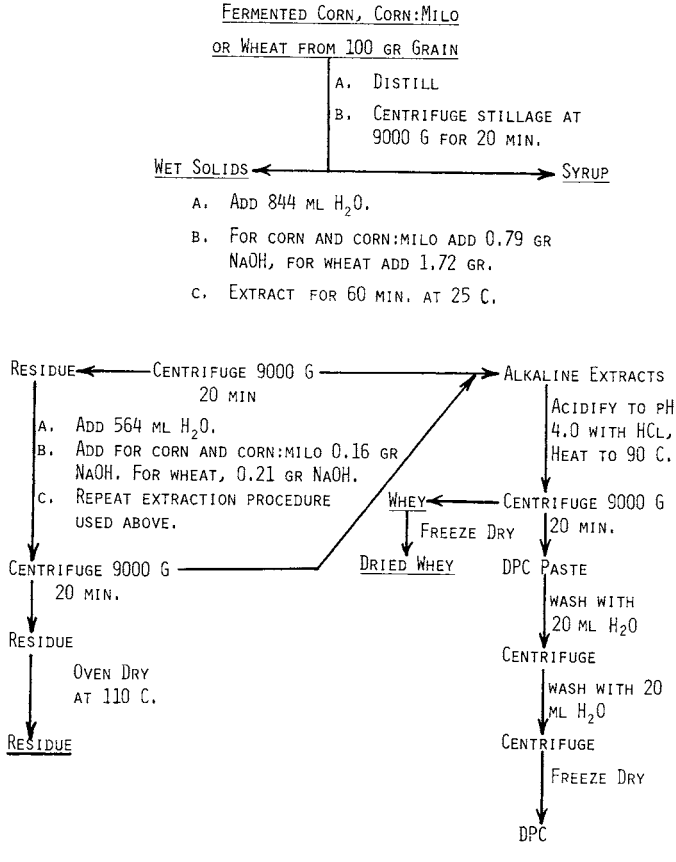

Fig. 2. The laboratory procedure for the alkaline extraction of fermented corn, corn:milo, and wheat to obtain a distiller's protein concentrate.

Aflatoxin Extraction Procedures

The 10-ml sample of the fermentation medium, taken after aflatoxin addition, was extracted by adding 30 ml of chloroform:acetonitrile:methanol (3:2:1), and 2 g diatomaceous earth, and a shaking for 30 min. The supernatant was decanted through a filter into a round-bottomed flask. An additional 10 ml of chloroform:acetonitrile (3:3) was added to the sample and after 15-min shaking, the entire contents were filtered into the same flask. A Carborundum chip was added to the flask and the combined phases from the extraction were evaporated under vacuum on a rotary evaporator. The sample was dissolved in chloroform, transferred to a vial, and dried with anhydrous sodium sulfate.

All other samples were extracted by the procedure of Shannon *et al.* (9). This procedure was modified to include an additional extraction of the sample with acetonitrile:water (80:20) and rinse of the flask with the same material. The combined acetonitrile:water fractions were further treated according to their procedure with the exception that the final filtrate was reextracted with an additional portion of benzene. All extractions were carried out in a darkened room.

Recovery studies were conducted by adding known amounts of aflatoxin to various fractions. Recoveries ranged from 75 to 90% with a mean value of 85%.

High-Pressure Liquid Chromatography

Samples taken after inoculation with aflatoxin were analyzed by high-pressure liquid chromatography. The packing material chosen was VYDAC Adsorbent (Applied Science Labs, Inc.). Initial studies with a 2-mm i.d. × 100-cm column of this material showed excellent separation and quantitation of standard. When isocratic elution was used, the optimum conditions for the separation were obtained with hexane:chloroform:acetonitrile (75:23.5:1 v/v/v), at a flow of 1.5 ml/min, 260 psi, and at ambient temperature. The compounds were eluted from the column, monitored at 365 nm, recorded, and integrated using an ISCO Model UA5 Absorbance Monitor equipped with a Type 6 Optical Unit and Model 950 Integrator. The retention times for aflatoxins B_1, B_2, G_1, and G_2 were 8.5, 11.1, 16.2, and 22.2 min, respectively, under these conditions. The minimum quantity necessary for accurate integration was approximately 50 nanograms (ng).

Following analysis of the first set of samples, the column became contaminated and resolution was eventually completely lost. Removal of the adsorbent from the column revealed that the residual pigments in the corn and corn:milo samples were being retained on the column. This effect eventually led to the inability of the column to resolve the aflatoxins. While separation was reproducible for a particular batch of adsorbent, considerable variation was obtained with different lots.

Thin-Layer Chromatography

For all other samples, quantitation was made by a fluorodensitometric method (10) using a Turner fluorimeter equipped with a plate scanning attachment (G. K. Turner Associates, Palo Alto, Calif.). Peak areas from the recorded scans were determined by a planimeter. Plates were coated with a 0.25-mm thickness of Adsorbosil-1 (Applied Science Labs, Inc.), activated at 110°C

for 30 min, and developed in a chloroform:acetone:water (88:12:1.5 v/v/v) solvent system.

RESULTS

The data for aflatoxin recoveries from the various fractions are summarized in Table I. The values represent the mean of six fermentations. No differences in recovery of aflatoxins were detected resulting from type of grain used in the fermentation medium.

Immediately after inoculation and mixing, samples were taken from the fermentation mixtures to determine their actual aflatoxin content for comparison with later samples. The data in Table I were obtained using the method of high-pressure liquid chromatography. Recoveries were very good. The mean value indicated a recovery of 95% of the anticipated value of 4000 μg of added aflatoxins.

The total aflatoxin content remaining in the samples after fermentation was 35% of the initial value, indicating significant destruction of aflatoxins. The total recoveries ranged from a minimum of 20% of the initial value to a maximum of 56% for the six samples. The mean recovery of aflatoxins B_1 and G_1 was in each case approximately twice that of B_2 or G_2.

Following fermentation, alcohol was removed by distillation at 76°–78° C for 15–20 min. The material remaining after distillation was centrifuged and yielded two fractions, syrup and wet solids. Water was removed from the syrup fraction by freeze-drying.

The mean total recovery of aflatoxins in the syrup fraction represents 2.2% of the initial level. The aflatoxins were present in relatively equal amounts with the exception of aflatoxin B_1, which was consistently present in about one-half the amount of the other aflatoxins.

The wet solids were extracted twice with NaOH at pH 12.2 and ambient temperature for a total time of 2 hr. The supernatants were acidified to pH 4.0, heated to 90° C, and then centrifuged, resulting in a distiller's protein concentrate (DPC) fraction and a whey fraction. The DPC fraction was further washed with water.

The mean total aflatoxin recovery from the combined DPC isolation was 58 μg (Table I) which represents 1.4% of the initial value. Aflatoxin B_1 persisted to a greater extent, approximately twofold, than any of the other aflatoxins. No G_1 and G_2 aflatoxins were detected in two of the samples.

The mean total recovery from the whey fractions (Table I) was 136 μg, or 3.5% of the initial recovery value. Aflatoxin B_1 was again most predominant.

The final residue fraction, remaining after alkaline extraction of the wet solids, contains very small amounts of aflatoxin. The total mean value for recovery was only 0.04% of the original.

DISCUSSION

The data indicate that considerable destruction of aflatoxins occurred during fermentation. The losses for B_1 were similar to those reported during fermentation for the brewing process (11). It was doubtful that this degradation resulted from the action of the yeast (12). In the present study, the samples were

TABLE I
Summary of Aflatoxin Recovery from Fermentation Media and Fractions

Isolation Step	Mean Recovery Values[a]					
	B_1	B_2	G_1	G_2	Total aflatoxin	
	μg Total				μg Total	% Recovery
Inoculation recovery[b]	963 ± 36	973 ± 27	981 ± 36	881 ± 42	3799 ± 114	95
After fermentation	515 ± 85	236 ± 40	462 ± 54	197 ± 22	1411 ± 183	35(41)[c]
First and second whey[d]	61 ± 18	34 ± 10	29 ± 8	12 ± 3	136 ± 36.8	3.4(4)
First and second DPC[d]	32 ± 5	15 ± 2	5 ± 2	5 ± 2	58 ± 11	1.4(1.6)
Syrup	11 ± 1	25 ± 3	27 ± 3	26 ± 3	89 ± 9.5	2.2(2.6)
Residue	0.6 ± 0.2	0.3 ± 0.1	0.5 ± 0.2	0.2 ± 0.1	1.6 ± 0.4	0.04(0.05)

[a]Mean ± standard deviation of mean. Initial addition of 1000 μg each aflatoxin to fermentation medium.
[b]Determined by high-pressure liquid chromatography. All other values determined by fluorodensitometric procedure.
[c]Values in parentheses are % recovery corrected for 85% recovery in extraction procedure.
[d]Combined totals of first and second extractions.

held at temperatures of 95° and 75°C, each for 45 min, as part of the malting process prior to addition of yeast. The water content of the medium was approximately 70%. Destruction of aflatoxin probably occurred during this process. Heating cottonseed meals, containing 30% moisture, at 100°C for 60 min has been shown to result in a 60% reduction in aflatoxin B_1 (13,14). Supposedly, aflatoxins G_1 and G_2 are much more susceptible to destruction by heating in the presence of moisture. The finding of as much aflatoxin G as B at this stage was unexpected and remains unexplained.

In view of low recovery of aflatoxin in the syrup fraction, the amount of aflatoxin associated with the wet solids fraction would be expected to be very high (>1000 μg). Since the wet solids were not analyzed, the actual partition in amounts of aflatoxin at this point was not determined. However, the amounts recovered in the whey, DPC, and residue fractions suggest considerable destruction of aflatoxin had taken place during their isolation. It is well established that alkaline treatments of aflatoxins lead to their destruction (14,15). The residue was dried at 110°C at this alkaline pH. This additional heat treatment would also contribute to aflatoxin destruction.

One of the major concerns of the distiller's industry has been to determine if aflatoxins would go into the distillate. A number of in-house research studies have shown that it does not[4].

A second concern of the industry is that of aflatoxin contamination of the by-products. In the normal distillation process, the solids content of the starting material is reduced to approximately 30% of the original value. Thus, if destruction did not occur during the process, aflatoxins would actually be concentrated in the by-products. In the present study, using artificially contaminated grains, only 35% of the total initial aflatoxin remained in the stillage. Additional treatments reduced the total recovery in all fractions to 7% of the original value.

In the usual industrial process, the stillage is separated into syrup and wet-solids. The syrup is concentrated and added back to the wet-solids. The combined mixture is then dried to produce distiller's dried grains plus solubles. Undoubtedly, additional destruction of aflatoxins remaining after fermentation would occur during these steps in the industrial process.

Should any small amounts of aflatoxin present in the residue from the industrial process still prove detrimental, additional heat or alkali treatment procedures would reduce them even further. All of these procedures would require additional time and expense. The process of isolating DPC offers a mechanism for incorporating these procedures and using highly contaminated grains.

Before any of these modifications could be accepted on a commercial basis, further studies would need to be conducted using naturally contaminated grains. In addition to chemical analyses, these studies would need to involve bioassay of the various fractions.

[4]Personal communication, Dr. Lawrence E. Carpenter, Executive Director, Distillers Feed Research Council, Cincinnati, Ohio.

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