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Production of Aflatoxin on Rice

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ABSTRACT

SHOTWELL, ODETTE L., (Northern Regional Research Laboratory, Peoria, Ill.), C. W. HESSELTINE, R. D. STUBBLEFIELD, AND W. G. SORENSON. Production of aflatoxin on rice. Appl. Microbiol. 14:425–428. 1966.—A method has been developed for the production of aflatoxin by growing *Aspergillus flavus* strain NRRL 2999 on the solid substrate rice. Optimal yields, more than 1 mg of aflatoxin B_1 per g of starting material, were obtained in 5 days at 28 C. A crude product containing aflatoxins was isolated by chloroform extraction and precipitation with hexane from concentrated solutions. The crude product consisted of 50% aflatoxin in the following ratio: B_1–B_2–G_1–G_2, 100:0.15:0.22:0.02. Aflatoxin B_1 was separated from almost all the impurities and from the other aflatoxins by chromatography on silica gel with 1% ethyl alcohol in chloroform. Analytically pure aflatoxin B_1 was recrystallized from chloroform-hexane mixtures.

Aflatoxins (B_1, B_2, G_1, and G_2) are hepatotoxic metabolites produced by *Aspergillus flavus* species on a number of agricultural commodities. Since the initial discoveries that the deaths of a hundred thousand turkey poults in 1960 (2) and the hepatomas in trout (1) were caused by feeds containing aflatoxins, there has been much effort to determine the effect of aflatoxin on other animals. To conduct the necessary feeding trials, large quantities of toxin are necessary. Aflatoxin has been produced on peanuts (5), crushed wheat (4), and corn meal (9). A method for producing the substance in submerged culture has been developed by Mateles and Adye (8). Hesseltine et al. (Bacteriol. Rev., *in press*) of this Laboratory investigated the production of aflatoxins on the agricultural commodities, rice, wheat, corn, soybeans, and sorghum, and found that rice was the best substrate. This paper describes a method for production of the toxin on rice; it is an adaptation of the techniques used in making Ang kak (6).

MATERIALS AND METHODS

Culture and inoculum. A. flavus strain NRRL 2999 was used to produce aflatoxins because it was one of the best found in a survey of *A. flavus* strains (Hesseltine et al., *in press*). This strain was isolated from Ugandan peanuts in 1961 by P. K. C. Austwick, Weybridge, England, who designated it as V.3734/10 and deposited it in the Commonwealth Mycological Institute where it was assigned IMI 91019b. This strain is very stable and consistently yields high levels of aflatoxin, especially B_1, even after many transfers.

Inoculum was prepared by inoculating tubes (1.5 by 15 cm) of potato-dextrose-agar with spores of NRRL 2999. The potato-dextrose-agar was prepared as follows.

Flask 1 contained: distilled water, 100.0 ml; dextrose, 20.0 g; $CaCO_3$, 0.2 g; and $MgSO_4 \cdot 7H_2O$, 0.2 g. Flask 2 contained 400.0 ml of distilled water and 15.0 g of agar. Flask 3 contained 200.0 g of potatoes (peeled and sliced) and 500.0 ml of distilled water. Contents of flask 3 were brought momentarily to 121 C in an autoclave and filtered through cheesecloth. The solution was brought up to original volume. Simultaneously, the agar in flask 2 was melted and the solution in flask 1 was heated to boiling. Contents of the three flasks were mixed, but the *p*H was not adjusted.

Inoculated slants were incubated for 7 to 21 days at 28 C. By 7 days, the cultures had a heavy crop of green conidia, removed by adding 3.0 ml of 0.005% Triton X-100 per slant. Spores were scraped loose with a loop, the slant was shaken to give a uniform suspension of spores, and the spore suspension (0.5 ml) was used to inoculate each 50 g of substrate.

Fermentation. Fermentations were carried out in either 300-ml Erlenmeyer flasks containing 50 g of polished rice (Sunnyfield fancy long grain) or 2.8-liter Fernbach flasks containing 300 g of rice. Indented Fernbachs did not increase yields of aflatoxin.

After tap water (25 ml) was added to the rice (50 g) in the Erlenmeyer flasks, the mixture was allowed to stand for 2 hr with frequent shaking. The flasks were autoclaved at 15 psi for 15 min and cooled. They were then inoculated and placed on a

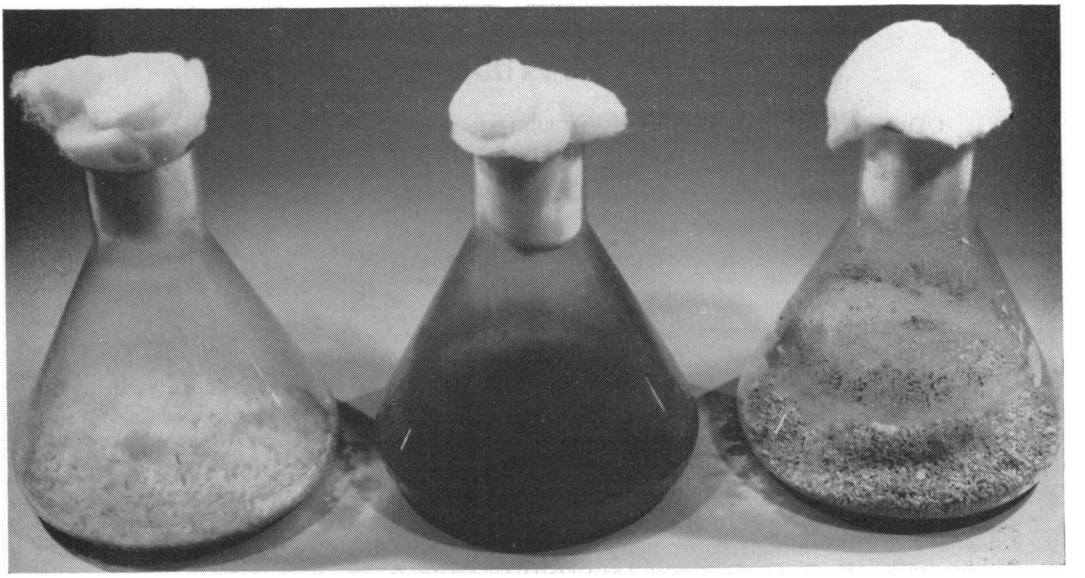

FIG. 1. *(left) Flask of uninoculated rice; (center) flask of fermented rice allowed to stand for 6 days with shaking by hand once a day; and (right) flask of fermented rice incubated for 6 days on a rotary shaker with continuous agitation.*

New Brunswick shaker set at 28 C and 188 rev/min. Comparable yields could be obtained on a Gump shaker, but not on a reciprocal shaker. Sterile water was added at 24 and 45 hr, but not enough to cause the individual rice kernels to adhere to one another. If the rice did pack in clumps, the flasks were removed from the shaker and the material was loosened by shaking each flask vigorously. It is extremely important that the rice does not remain as a compact mass with the mold mycelium binding the kernels together. The technique was adapted from the Ang kak fermentation, which involves the growth of *Monascus* on rice at low moisture levels to produce red rice. In the Ang kak fermentation, each rice kernel must be kept free from its neighbors (6).

At 48 hr after inoculation, the rice kernels should show small white areas at the sites where the mold has begun to grow. Shortly afterwards, the rice assumes a bright yellowish color which darkens to a dull light brown about the color of wheat. In all successful fermentations, these color changes occur. Sporulation does not occur except perhaps on the wall of the flask above the fermenting rice. If rice is inoculated in flasks and allowed to stand with daily shaking by hand, some aflatoxin is formed but not nearly as much as with continuous shaking. When rice is allowed to stand, it does not go through the color changes described, but the mold sporulates to give a large crop of green conidia (Fig. 1). When such a flask was opened, a cloud of spores was released. At the end of the fermentation (5 to 6 days), the flasks were briefly steamed to destroy the fungus.

Assay procedure. Moldy rice (25 g) was blended in a Waring Blendor for 5 min with water (250 ml) and blended another 5 min after chloroform (250 ml) was added (7). The mixture was centrifuged for 15 min at 3,000 rev/min and was filtered through cheesecloth into a separatory funnel. The chloroform layer was treated with anhydrous sodium sulfate (20 to 25 g) to remove water. Sodium sulfate was collected on a Büchner funnel, and the clarified filtrate was concentrated in vacuo on a rotary evaporator. The residue was made up to 10 ml with chloroform for chromatography on thin-layer plates.

Thin-layer chromatoplates were prepared by the method of Pons and Goldblatt (10). Plates were coated by mixing 30 g of Silica Gel G-HR (Brinkmann Instruments, Inc., Westbury, N.Y.) with 64 ml of water in a Waring Blendor for 30 sec and spreading to 0.250-mm thickness. The solvent was 3% methanol in chloroform (v/v). The plates were inspected after development in a Chromato-Viewer (Ultra-Violet Products, Inc., San Gabriel, Calif.) to locate zones of fluorescence caused by the four aflatoxins. Amounts of aflatoxin present were determined by visual comparisons of fluorescing zones with known quantities of standard aflatoxins spotted on the same plate. The thin-layer chromatoplate assay is accurate within 20%.

Isolation. The product obtained by growing *A. flavus* NRRL 2999 on rice [1,050 g in 21 Erlenmeyer (300-ml) flasks] was extracted by refluxing (4 hr) three times with chloroform (1,600 ml each time; 3). The third extraction recovered only 6% of the total aflatoxin B_1 extracted. Extracts were filtered through cheesecloth, pooled, concentrated in vacuo to 100 to 150 ml, and dried over anhydrous sodium sulfate (50 g). Sodium sulfate was removed by filtration, and the clarified filtrate was concentrated in vacuo. The concentrate (10 to 15 ml) was added to 10 volumes of hexane to precipitate crude aflatoxin. The precipitate was dried in vacuo. Aflatoxin could also be extracted

TABLE 1. *Production of aflatoxin on rice by Aspergillus flavus NRRL 2999 in 300-ml Erlenmeyer flasks**

Days	Aflatoxin (μg/g of substrate)				
	B_1	B_2	G_1	G_2	Total
2	184	20	64	10	278
4	760	167	458	56	1,441
5	950	143	356	62	1,511
6	634	100	160	20	914
7	760	111	180	25	1,076
9	476	100	80	25	681
10	634	125	183	22	964
11	476	100	142	56	774
12	762	166	160	25	1,113

* Incubated on a New Brunswick rotary shaker, 188 rev/min.

by either soaking rice in chloroform overnight at room temperature or stirring rice for 4 hr at room temperature. Three extractions removed aflatoxin.

Column chromatography. Columns for chromatography were prepared by packing silicic acid (Mallinckrodt's analytical reagent; 100 mesh) as a slurry in 1% ethyl alcohol in chloroform (v/v) into columns (final dimensions were 2.4 by 31 cm). A pressure of 3 psi was used to pack columns and to elute product. Crude aflatoxin (1.51 g) isolated from moldy rice was dissolved in a minimal volume of 1% ethyl alcohol in chloroform (8 to 10 ml), placed on the chromatographic column, and washed into it with small portions of the same solvent (total wash was 30 ml). The solvent used for both development and elution was 1% ethyl alcohol in chloroform (11). As eluting solvent was added, 20-ml fractions were collected and were chromatographed on thin-layer plates to determine their aflatoxin composition. Appropriate fractions were combined, concentrated, and precipitated in hexane. Precipitates were dried in vacuo and analyzed by thin-layer chromatography.

Crystallization. Aflatoxin B_1 (116 mg) from the silicic acid column was crystallized by dissolving in chloroform (6.6 ml) and filtering the solution to remove insoluble impurities The filter was washed with chloroform (2 ml) and the wash was added to chloroform solution. Addition of hexane (16.5 ml) to this solution caused the appearance of crystals. Crystals (68 mg; mp, 261 to 264 C) were collected after the mixture stood overnight at −20 C. Recrystallization with the same solvent system did not change the melting point of aflatoxin B_1 (37 mg) markedly (mp, 261 to 263 C). Recrystallization from pentane-chloroform gave crystals (29 mg) of mp 260.5 to 262 C (Found: C, 65.2; H, 4.09. Calculated: C, 65.4; H, 3.96).

RESULTS

A typical fermentation of rice in 300-ml flasks with *A. flavus* strain NRRL 2999 is shown in Table 1. Many of the fluctuations reported in amounts of aflatoxins on rice samples are caused by expected variations in the assay rather than in actual quantities present. Peak yields were obtained in 5 days. Aflatoxin B_1 predominated over other aflatoxins in the product. Data obtained with indented Fernbach flasks containing 300 g of rice and incubated on a Gump shaker at 28 C are shown in Table 2.

Eight fermentations on rice in 300-ml Erlenmeyer flasks gave the following yields in milligrams per gram of substrate: 1.00, 0.16, 0.80, 1.11, 1.10, 0.74, 1.19, and 0.88. A biological assay with ducklings of the moldy rice, conducted at the Western Regional Research Laboratory, confirmed the results of our chemical analysis. The advantage of producing aflatoxin in a New Brunswick shaker-incubator and on Gump shakers over producing it in unshaken flasks is that larger quantities of substrate can be added to a given flask without decrease in per cent yield. The crude isolated product was about 50% pure in terms of total aflatoxins with a distribution of individual factors as indicated by the ratio B_1–B_2–G_1–G_2; 1.00:0.15:0.22:0.02. The yield in Fernbach flasks was lower (0.68 mg of aflatoxin B_1 per g of substrate).

To save time, moldy rice can be used in animal feeding tests without extraction. It should be easier to prepare a feed homogeneously contaminated with ground rice containing aflatoxin than with the extracted product. Aflatoxins are produced when *A. flavus* is grown on a number of agricultural substrates (Hesseltine et al., *in press*), any one of which could be used to prepare feeds containing levels of toxin desired for animal trials.

The crude product precipitated from chloroform extracts was partially purified on silicic acid columns to yield several fractions containing about one-half of the total aflatoxin B_1 uncontaminated by other aflatoxins. Fractions that followed contained mixtures of B_1, B_2, G_1, and G_2. Composition of products obtained from a typical column was as follows: product from

TABLE 2. *Production of aflatoxin B_1 on rice by Aspergillus flavus NRRL 2999 in Fernbach flasks**

Days	Aflatoxin B_1 (μg/g of substrate)	Wt of rice at time of harvest
		g
4	428	416
5	724	402
6	1,020–1,120	389
7	735–785	383

* Incubated on a Gump rotary shaker, 200 rev/min.

fractions 8 to 10, 100% aflatoxin, 334 mg of aflatoxin B_1; product from fractions 11 and 12, 100% aflatoxin, 209 mg of B_1, 60 mg of B_2, and 5 mg of G_1; product from fractions 13 to 22, 75% aflatoxin, 66 mg of B_1, 51 mg of B_2, 71 mg of G_1, and 13 mg of G_2. Recoveries of 80% can be expected from silicic acid columns. Rechromatography with the same conditions of fractions containing mixtures of aflatoxins yielded more aflatoxin B_1.

Three recrystallizations of the product obtained from silicic acid columns resulted in pure B_1.

A convenient method for the production of quantities of moldy rice containing aflatoxins, crude aflatoxins, or pure aflatoxin B_1 has been developed for animal feeding trials.

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