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NOTES

Production of Aflatoxin M in a Liquid Medium

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Aspergillus flavus NRRL 3251 grown on modified yeast extract-sucrose medium produced 1 mg of aflatoxin M_1 per 100 ml of medium.

Aflatoxin M has received much attention since its discovery in the milk of cows fed toxic ground nut meal (1). Several species of animals are able to metabolize aflatoxin B_1 to M_1. Aflatoxins B_1 and M_1 produce similar acute effects in rats (3) and ducklings (4). Reports on the effects of natural aflatoxin M_1 on animals are highly limited because of its scarce availability. Stubblefield et al. (8) have described the preparation of aflatoxins M_1 and M_2 by the fermentation of rice with *Aspergillus flavus* NRRL 3251. In view of the ease with which toxins can be extracted and purified from liquid media, the following method was developed to produce natural aflatoxin M_1 for our studies on animals.

Medium containing 200 g of sucrose, 20 g of yeast extract (Difco), 10 g of $MgSO_4 \cdot 7H_2O$, and 5 mg of $FeSO_4$ made up to 1 liter in distilled water was distributed in 100-ml quantities into 500-ml Erlenmeyer flasks, sterilized, and inoculated with 2 ml of aqueous spore suspension. The spores of *A. flavus* NRRL 3251 were grown for 4 to 5 days at 37 C on glucose-peptone agar slants. The flasks were incubated at 26 ± 1 C for 6 days. At the end of incubation, the media and mycelia were extracted with chloroform, dried, and concentrated in vacuo. Aliquots of this extract were chromatographed on silica gel plates and developed with chloroform-acetone (4:1). Aflatoxins B_1 and M_1 were eluted with acetone and estimated spectrophotometrically in chloroform solution (5, 7). Aflatoxins B_2 and M_2 were produced in very little quantities and hence were not estimated.

The production of aflatoxins on different days of growth is shown in Table 1. About 1 mg of aflatoxin M_1 and 14 mg of aflatoxin B_1 were obtained after 6 days of growth. The yield of these toxins increased with further incubation, but the resultant formation of pigments, which were difficult to separate from aflatoxin M, made it undesirable.

The chloroform extract containing aflatoxins B_1, B_2, M_1, and M_2 was chromatographed on partially neutralized basic alumina with benzene-acetone-ethanol (97:2:1) as the eluting solvent (6). When most of the B_1 was eluted, the remaining B_1, B_2, and pigments on the column were removed by washing with chloroform. Aflatoxins M_1 and M_2 were then eluted together with chloroform-methanol (85:15). Fractions (4 ml) were collected and monitored by thin-layer chromatography. Fractions containing M_1 and M_2 were concentrated and chromatographed on silica gel plates with chloroform-acetone (4:1) as the developing solvent. Bands corresponding to aflatoxin M_1 were scraped out and extracted with acetone. The extracts were combined, acetone was evaporated off, and the colorless residue was crystallized from chloroform-methanol.

Aflatoxin M_1 produced and purified by this method could be readily crystallized into colorless crystals. The purified aflatoxin M_1 moved

TABLE 1. *Production of aflatoxins by A. flavus in 100 ml of medium*[a]

Incubation period (days)	Aflatoxin (mg)[b]					
	Mycelium		Medium		Total	
	M_1	B_1	M_1	B_1	M_1	B_1
2	0.04	1.0	0.13	2.8	0.17	3.8
3	0.09	1.8	0.22	5.1	0.31	6.9
4	0.20	2.2	0.32	7.2	0.52	9.4
5	0.33	2.8	0.45	8.8	0.78	11.6
6	0.41	3.2	0.62	10.5	1.03	13.7

[a] The medium contained 20 g of sucrose, 2 g of yeast extract, 1 g of $MgSO_4 \cdot 7H_2O$, and 0.5 mg of $FeSO_4$.

[b] The values are the average of six separate experiments done in duplicate.

as a single spot in chloroform-acetone (4:1), chloroform-methanol (98:2), and 2-propanol-water-acetone-chloroform (1:1.5:12:88) and had the same chromatographic mobility as standard aflatoxin M_1. The purified toxin had a molar absorptivity of 19,300 in chloroform solution at 357 nm, well in agreement with the report of Purchase and Altenkirk (5).

The present report is the first of its kind for the production of natural aflatoxin M in a liquid medium. When the spores, germinated on glucose-peptone agar, were grown in YES medium (2) or in synthetic media where sporulation of mycelia was low, only traces of aflatoxin M were produced. The washed-spore suspension was able to convert pure aflatoxin B_1 to M_1 when incubated in a medium containing 50 mM phosphate buffer, pH 7.0, 10 mM $MgSO_4$, 0.03 mM $FeSO_4$, and 1 mM aflatoxin B_1 dissolved in 0.2 ml of dimethyl sulfoxide at 30 C for 6 h. The conversion of B_1 to M_1 varied from 5 to 8% with different batches of spores. This conversion was completely inhibited when the spore suspension was heated in a boiling water bath for 30 min. No such conversion could be observed under identical conditions with spore-free mycelium also. These observations indicate that aflatoxin M_1 is produced by the enzymatic hydroxylation of aflatoxin B_1 and that the hydroxylase is present in the spores of the fungus. However, the presence of aflatoxin B_1-4-hydroxylase cannot be confirmed until further studies have been made.

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