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Production of Aflatoxins B_1 and G_1 by *Aspergillus flavus* in a Semisynthetic Medium

N. D. DAVIS, U. L. DIENER, AND D. W. ELDRIDGE

Department of Botany and Plant Pathology, Auburn University Agricultural Experiment Station, Auburn, Alabama

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ABSTRACT

DAVIS, N. D. (Auburn University, Auburn, Ala.), U. L. DIENER, AND D. W. ELDRIDGE. Production of aflatoxins B_1 and G_1 by *Aspergillus flavus* in a semisynthetic medium. Appl. Microbiol. **14**:378–380. 1966.—Isolates of *Aspergillus flavus* produced 0.2 to 63 mg of aflatoxins B_1 and G_1 per 100 ml in a nutrient solution consisting of 20% sucrose and 2% yeast extract. Various factors influencing the fermentation were studied. The maximal amount of toxin was produced by ATCC culture 15548 in 1-liter flasks containing 100 ml of medium incubated as stationary cultures for 6 days at 25 C.

Research on mycotoxins has been extensively reviewed (9) since the first reports of "turkey X" disease (6) caused by a toxin produced by *Aspergillus flavus* Link ex Fries growing on peanuts. Also, since 1961, numerous papers on aflatoxin have been published (7). However, few papers have presented quantitative data on in vitro production of aflatoxins. From 100 to 200 mg per liter of total aflatoxins have reportedly been produced in submerged culture in a medium containing corn steep liquor (1). Data on individual aflatoxins B and G were not given. The influence of temperature on in vitro production of aflatoxin has been studied (5), but quantitative data were not presented. Factors influencing production of up to 100 mg per liter of aflatoxin by *A. flavus* in submerged culture have been reported (Adye and Mateles, Abstr. Meeting Am. Chem. Soc., 1964), and the influence of carbohydrate source, nitrogen source, and various trace elements has been studied (3). A preliminary report of factors influencing aflatoxin production in vitro in a medium containing yeast extract has been published (2) and nutritional factors influencing production of aflatoxin B_1 have been reported (Eldridge, M.S. Thesis, Auburn Univ., Auburn, Ala. 1964; Eldridge et al., Phytopathology **55**:498, 1965). Results reported have been with different isolates of *A. flavus* growing in different media, and are not directly comparable.

This paper reports cultural conditions influencing the production of aflatoxins B_1 and G_1 by *A. flavus* growing in a sucrose yeast extract liquid medium as stationary cultures and compares the toxin-producing ability of several isolates of *A. flavus* growing in that medium.

MATERIALS AND METHODS

Organism. *A. flavus* isolate 2 was a culture variant of British isolate 3734/10. Isolates 6 and 8 were obtained from Alabama-grown peanuts. Other isolates were obtained from the American Type Culture Collection (ATCC) and from U.S. Department of Agriculture (NURDD), Peoria, Ill. Test-tube cultures of *A. flavus* were maintained on Czapek solution agar with 20% sucrose (8) modified with 7 g per liter of Difco yeast extract.

Medium. The basal medium (YES) contained 2% yeast extract (Difco) and 20% sucrose. Demineralized water was used throughout the study.

Culture. Flasks (1-liter) containing 100 ml of medium per flask were stoppered with foam plugs and autoclaved for 15 min at 20 psi. Media were inoculated with spores from 1- to 3-week-old cultures of *A. flavus* and incubated 6 to 8 days at 25 C as stationary cultures. Experiments were replicated three times and results were reported as averages.

Assays. Cultures were filtered and the dry weight of mycelium was determined after drying the mycelial mats for 12 to 24 hr at 70 C. Aflatoxins were extracted from filtrates *in toto* by refluxing for 1 to 2 hr with chloroform. Where high yields of toxin were expected, 1 or 2 ml of medium were shaken vigorously with 25 ml of chloroform in a separatory funnel. The lower chloroform layers were recovered from separatory funnels and either concentrated on a steam bath or diluted with additional chloroform to a concentration suitable for assay.

Aflatoxin assays were by thin-layer chromatography procedures (4) except that development was on 0.4-mm silica gel plates with 2.5% methanol in chloroform. Plates were examined under high-intensity ultraviolet light, and the aflatoxins were determined quantitatively by visual comparison with external and internal aflatoxin standards.

TABLE 1. *Influence of sucrose concentration on aflatoxin production by Aspergillus flavus growing in 2% yeast extract medium*

Sucrose	Mycelial dry wt	Aflatoxin (mg/100 ml)		
		B_1	G_1	Total (B + G)
%	*g/100 ml*			
0	0.3	0.1	0.1	0.2
1	1.0	0.5	0.7	1.2
5	1.6	0.7	0.9	1.6
10	3.0	1.4	1.7	3.1
15	3.0	2.7	3.5	6.2
20	2.8	2.8	3.6	6.4
30	3.2	2.7	2.3	5.0
50	3.2	2.6	2.0	4.6

TABLE 2. *Influence of yeast extract concentration on aflatoxin production by Aspergillus flavus growing in 20% sucrose medium*

Yeast extract	Mycelial dry wt	Aflatoxin (mg/100 ml)		
		B_1	G_1	Total (B + G)
%	*g/100 ml*			
None	0	0	0	0
0.7	3.3	2.4	3.8	6.2
2.0	4.1	3.6	4.3	7.9
3.0	4.2	4.3	3.2	7.5
5.0	5.2	3.0	2.7	5.7

TABLE 3. *Influence of various additives on aflatoxin production by Aspergillus flavus growing in YES medium*

Additive	Amt	Mycelial dry wt	Aflatoxin (mg/100 ml)		
			B_1	G_1	Total (B + G)
	g/liter	*g/100 ml*			
None, YES only		2.8	3.2	2.6	5.8
$ZnSO_4$	0.01	2.9	2.1	2.6	4.7
$ZnSO_4$	0.1	2.9	1.0	2.6	3.6
$MgSO_4$	1.0	2.9	2.5	2.1	4.6
KNO_3	2.0	3.0	3.1	2.1	5.2
KH_2PO_4	2.0	2.7	2.0	1.7	3.7
K_2HPO_4	2.0	2.1	2.0	1.7	3.7
K_3PO_4	2.0	2.3	1.0	0.8	1.8
Glutamate	2.0	3.9	3.1	2.6	5.7

RESULTS

The influence of sucrose concentration on aflatoxin production by *A. flavus* is presented in Table 1. The fungus produced 6.2 and 6.4 mg/100 ml of aflatoxins, respectively, at the 15 and 20% sucrose levels. Substantially less aflatoxin was produced with 10% sucrose or less and with 30% sucrose or more. Growth in terms of mycelial dry weight increased markedly with increased sucrose concentration up to 10%. There was little or no difference in growth or toxin production between the 15 and 20% levels of sucrose.

TABLE 4. *Influence of pH on aflatoxin production by Aspergillus flavus growing in YES medium*

Initial *p*H*	Final *p*H	Mycelial dry wt	Aflatoxin (mg/100 ml)		
			B_1	G_1	Total (B + G)
		g/100 ml			
3.0	3.4	2.1	3.2	2.6	5.8
3.8	3.9	3.4	2.1	2.6	4.7
4.8	4.0	3.0	2.1	2.6	4.7
5.9	4.1	2.8	3.2	2.6	5.8
6.4†	4.1	2.9	3.2	2.6	5.8

* Adjusted with 1 N HCl where necessary.
† Unadjusted initial *p*H of YES medium.

TABLE 5. *Influence of time on aflatoxin production by Aspergillus flavus growing in YES medium*

Incubation period	Mycelial dry wt	Aflatoxin (mg/100 ml)		
		B_1	G_1	Total (B + G)
days	*g/100 ml*			
2	0.9	0.1	0.1	0.2
3	2.1	0.4	1.0	1.4
5	3.8	2.0	5.3	7.3
7	3.5	2.0	5.3	7.3
12	4.2	2.0	5.3	7.3
15	3.8	1.8	4.8	6.6
18	4.1	1.6	4.2	5.8

TABLE 6. *Production of aflatoxin by selected isolates of Aspergillus flavus growing in YES medium*

Isolate	Mycelial dry wt	Aflatoxin (mg/100 ml)		
		B_1	G_1	Total (B + G)
	g/100 ml			
2	2.6	3.8	3.2	7.0
6	4.6	17.1	14.4	31.5
8	3.7	15.2	1.4	16.6
NRRL 2999	4.3	24.7	20.8	45.5
ATCC 15517	5.3	28.5	24.0	52.5
ATCC 15548	6.5	34.2	28.8	63.0
ATCC 15547	2.1	0.1	0.1	0.2

The effect of yeast extract concentration on aflatoxin production and growth is shown in Table 2. Maximal growth was 5.2 g/100 ml of mycelium produced at the 5% yeast extract level. The maximal aflatoxin yield was 7.9 mg/100 ml of medium obtained with 2% yeast extract.

The influence of various additives to YES medium is indicated in Table 3. In no case did any of the additives increase significantly the yield of either aflatoxin or mycelium, except for glutamate, which markedly stimulated growth. The addition of zinc sulfate or one of the three phosphates markedly decreased production of toxin, and decreased growth to some extent. Addition of organic or inorganic nitrogen sources had little or no effect on toxin production.

The initial *p*H of the medium (Table 4) did not influence significantly either toxin production or growth. Regardless of initial *p*H, the final *p*H was generally about 4.0, except where the initial *p*H was 3.0; in this case, growth was restricted somewhat and the final *p*H of the medium was 3.4.

Influence of time on aflatoxin production is presented in Table 5. Maximal yields of 7.3 mg/100 ml were obtained in 5 to 12 days, after which lower yields were harvested.

Table 6 lists yields of mycelium and aflatoxin produced by six isolates of *A. flavus* growing in YES medium. *A. flavus* ATCC 15548, produced 6.5 g/100 ml of mycelium and 63 mg/100 ml of total aflatoxin, the highest yields obtained. Isolate 8 was notable in that it was the only one of the six which produced predominantly aflatoxin B_1 and relatively little G_1. The other five produced approximately equal amounts of the two aflatoxins.

Discussion

YES medium of 20% sucrose and 2% yeast extract apparently provided all necessary ingredients for the production of high levels of aflatoxin. None of the various ingredients studied increased toxin production when added to the YES medium. Similarly, adjustment of the initial *p*H did not influence toxin production, although production of high levels of aflatoxin was almost invariably accompanied by a final *p*H of about 4.0.

Different isolates of *A. flavus* produced widely differing amounts of aflatoxins B_1 and G_1 when grown in YES medium. With the exception of isolate 8, the several isolates produced approximately equal amounts of aflatoxin B_1 and G_1. Isolate 8 was notable in that it produced approximately 10 times as much B_1 as G_1. Parent cultures of isolate 8 have been noted to produce aflatoxin B_1 exclusively.

The YES medium is easy to prepare, relatively inexpensive, and is suitable for production of higher levels of aflatoxin than those reported for other media. For these reasons, YES medium appears to be suitable for both production of aflatoxin and for screening fungi for their ability to produce aflatoxins.

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