High Aflatoxin Production on a Chemically Defined Medium

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Aspergillus parasiticus ATCC 15517 produced 28 to 30 mg of aflatoxin per 100 ml of a medium containing sucrose, asparagine, and salts in stationary and shaken cultures. In the absence of asparagine in the medium, the toxin yields fell drastically, and the thin-layer chromatograms of the chloroform extracts of the cultures indicated the total absence of aflatoxin G₁ and the presence of new intense blue and green fluorescent bands having Rf values lower than aflatoxins. Initial pH was critical and had to be around 4.5 for good growth and high toxin production on this medium. Optimum concentrations of K₂HPO₄ and MgSO₄·7H₂O in the medium were much lower than those normally used in fungal growth media.

Materials and Methods

Culture conditions. A. parasiticus ATCC 15517 (formerly known as A. flavus) was used throughout the study and was maintained as a soil culture. Five-to-7 day-old spores, obtained from a bottle containing 50 ml of glucose-peptone-agar, were distributed equally to five 500-ml Erlenmeyer flasks containing 100 ml of sterile basal medium. These flasks were incubated at 26 ± 1°C on a rotary shaker or as stationary cultures for 8 days unless otherwise stated.

Growth media. The following media were used. Medium 1 was glucose-ammonium nitrate sulfate medium (AM medium; reference 1). Medium 2 was glucose-ammonium nitrate medium (GAN medium; reference 2). Medium 3 was synthetic high-salts medium (SH medium) containing 85 g of sucrose, 10 g of asparagine, 3.5 g of (NH₄)₂SO₄, 1 g of K₂HPO₄, 500 mg of MgSO₄·7H₂O, 200 mg of CaCl₂·2H₂O, 10 mg of ZnSO₄·7H₂O, 5 mg of MnCl₂·4H₂O, 2 mg of ammonium molybdate·4H₂O, 2 mg of Na₂B₄O₇·2H₂O, and 2 mg of FeSO₄·7H₂O made up to 1 liter with double-distilled water. The fourth medium, synthetic low-salts medium (SL medium), was similar to SH medium, except in the concentrations of K₂HPO₄, MgSO₄·7H₂O, and CaCl₂·2H₂O, which were reduced to 750, 350, and 75 mg per liter, respectively.

All of the chemicals used were of British Drug House Ltd., analytical reagent grade, and asparagine was obtained from Sigma Chemical Co. Unless specified otherwise, the initial reaction of all the media used was pH 4.5. Sodium hydroxide and distilled hydrochloric acid were used for readjusting the pH. In some experiments, the media were freed from trace-clement impurities by alumina treatment (10). The experiments were performed in duplicate, and the results are reported as averages. The difference in aflatoxin production between duplicate flasks was generally less than 10%.

Assay. The media and mycelia were separated, and wet and dry weights of the mycelium were determined. Aflatoxins were extracted with chloroform, separated by thin-layer chromatography on Silica Gel G by using toluene-isoamyl alcohol-methanol (90:32:3; reference 17), eluted with methanol, and estimated by measuring the absorption at 363 nm (15). Since the amounts of aflatoxins B₁ and G₂ were very low, aflatoxins B₁ and B₂, as well as G₁ and G₂, were generally measured together.

Results

The effect of removing trace impurities from AM and GAN media by treatment with alumina and the effect of supplementation with asparagine were studied. Either alumina treatment or the addition of asparagine increased the toxin yields on both media (Table 1). Higher yields
Table 1. Effect of alumina treatment and supplementation with asparagine on aflatoxin production by stationary cultures

| Mediuma | Asparagine | Alumina treatment | Dry weight of mycelium (g/100 ml) | Aflatoxin (mg/100 ml) |
|---------|------------|------------------|----------------------------------|-----------------------|
|         |            |                  | B | G | Total |
| AM      | –          | –                | 0.59 | 0.10 | 0.39 | 0.26 |
| AM      | +          | –                | 1.89 | 3.50 | 1.84 | 5.34 |
| AM      | –          | +                | 0.87 | 2.25 | 0.80 | 3.05 |
| AM      | +          | +                | 2.05 | 5.55 | 4.35 | 9.90 |
| GAN     | –          | –                | 1.65 | 1.52 | 0.42 | 1.94 |
| GAN     | +          | –                | 3.20 | 3.45 | 2.05 | 7.60 |
| GAN     | +          | +                | 1.60 | 1.95 | 0.76 | 2.71 |
| GAN     | +          | +                | 1.97 | 12.10 | 2.57 | 14.67 |

* AM and GAN were glucose-ammonium sulfate medium (1) and glucose-ammonium nitrate medium (2), respectively.

were obtained by the addition of asparagine to alumina-treated media.

In another set of experiments, a nutrient medium containing the components of SH medium and a number of amino acids and B group vitamins was used. This did not give high yields of aflatoxins (Table 2). However, as a result of chance observation, it was found that, on removal of the precipitate formed by dissolving the salts required for 1 liter of medium in 50 ml of double-distilled water, this medium yielded 26 mg of toxin per 100 ml. Asparagine was necessary for good growth and high toxin yields. The presence of other amino acids and vitamins was not obligatory for toxin production, and essentially the same results could be obtained with SH medium if the precipitated salts were removed.

SL medium which contained low concentrations of inorganic salts supported the formation of 28.5 mg of aflatoxin per 100 ml (Table 2). In the absence of asparagine, aflatoxin G1 was totally absent and aflatoxins B1, B2, and G2 were present in small amounts; in addition, intense blue and green bands having Rf values lower than aflatoxins were observed on thin-layer chromatograms. The bands were not noticed when asparagine was present in the medium. A detailed study of the nature of these fluorescent bands and the effect of other salts on their formation is under investigation.

The effect of initial pH of the medium on growth and aflatoxin production is shown in Table 3. An optimal initial pH of 4.5 assured good growth and high toxin production.

KH2PO4 and MgSO4·7H2O were essential for growth and aflatoxin formation. The optimal levels of these salts for toxin production were 750 and 350 mg per liter, respectively. An increase in their concentration to 10 and 2 g per liter, as in GAN or AM media, drastically reduced toxin yields (Table 2); calcium chloride at concentrations as high as 200 mg per liter did not have any effect on aflatoxin production in stationary cultures.

Maximum yields in SL medium were obtained by 8 days in the case of stationary cultures, and there was a continuous increase up to 10 days in the case of shaken cultures (Table 4).

Table 2. Aflatoxin production by stationary cultures on synthetic media SH and SL

| Mediuma | Removal of precipitate | Dry weight of mycelium (g/100 ml) | Aflatoxin (mg/100 ml) |
|---------|------------------------|----------------------------------|-----------------------|
|         |                        |                                  | B | G | Total |
| SH      | +                      | 3.20                             | 9.52 | 19.48 | 29.00 |
| SH plus supplements | +                     | 4.60                             | 6.70 | 19.30 | 26.00 |
| SH minus asparagine | +                    | 0.71                             | 2.20 | 0.68 | 2.88 |
| SH     | +                      | 2.80                             | 1.70 | 6.30 | 8.00 |
| SH plus supplements | ++                     | 2.70                             | 0.26 | 0.53 | 0.81 |
| SL     | +                      | 3.10                             | 9.40 | 19.10 | 28.50 |
| SL minus asparagine | ++                     | 0.72                             | 2.40 | 0.97 | 3.37 |
| SL     | +                      | 2.80                             | 0.64 | 1.11 | 1.75 |
| SL plus 9.25 g of KH2PO4 per liter | ++ | 2.70                             | 1.25 | 4.46 | 5.71 |
| SL plus 1.65 g of MgSO4·7H2O per liter | ++ |                                  |            |       |

* SH was synthetic high-salts medium; SL was synthetic low-salts medium.

* The precipitate formed on dissolving the salts required for 1 liter of medium in 50 ml of double-distilled water was removed, and then the other components of the medium were added.

* Precipitate was not removed; the salts were added to a sufficiently large volume of the medium so that no precipitate was formed.
TABLE 3. Effect of initial pH of the medium on aflatoxin production by stationary cultures on SL medium

| Initial pH | Dry weight of mycelium (g/100 ml) | Aflatoxin (mg/100 ml) |
|------------|-----------------------------------|----------------------|
|            | B | G | Total |
| 3.5        | 2.40 | 7.00 | 11.00 | 18.00 |
| 4.5        | 3.00 | 9.84 | 18.60 | 28.44 |
| 5.5        | 2.30 | 7.83 | 17.14 | 24.97 |
| 6.5        | 1.40 | 0.40 | 0.71  | 1.10  |
| 7.0        | 1.30 | 0.30 | 0.49  | 0.79  |

a SL medium was synthetic low-salts medium.

TABLE 4. Aflatoxin production by stationary and shake cultures on SL medium

| Growth period (days) | Stationary cultures | Shake cultures |
|----------------------|---------------------|----------------|
|                      | Dry weight of mycelium (g/100 ml) | Total aflatoxin (mg/100 ml) | Dry weight of mycelium (g/100 ml) | Total aflatoxin (mg/100 ml) |
| 2                    | 1.25               | 1.54            | —b                       | —              |
| 3                    | 2.00               | 7.43            | 2.23                     | 6.80 |
| 4                    | 2.71               | 10.85           | 2.52                     | 10.00 |
| 5                    | 2.78               | 16.00           | 2.86                     | 15.90 |
| 6                    | 2.80               | 22.00           | —                        | —              |
| 7                    | 2.80               | 26.24           | —                        | —              |
| 8                    | 2.88               | 28.30           | 2.80                     | 24.70 |
| 9                    | 2.88               | 28.10           | —                        | —              |
| 10                   | —                  | —               | 2.64                     | 27.16 |
| 11                   | 3.01               | 27.50           | —                        | —              |

a SL medium was synthetic low-salts medium. 
b Values were not determined.

DISCUSSION

The increase in yields obtained on alumina treatment of the media suggest the presence of inhibitory impurities in AM and GAN media (Table 1). At levels normally used in fungal growth media, KH₂PO₄ inhibited toxin production. A similar inhibition of streptomycin production by phosphate has been reported and has been ascribed to the inhibition by phosphate of some phosphatases involved in streptomycin biosynthesis (7, 14). Since the biosynthetic pathway of aflatoxin is not very well understood, it is not possible to state if a similar mechanism is responsible for the effect observed.

The effect of asparagine is not due simply to the presence of excessive concentrations of nitrogen, since replacing asparagine by an equimolar amount of nitrogen led to a considerable decrease in toxin yields. Preliminary experiments indicated that aspartic acid could replace asparagine effectively.

The initial pH of the medium is another important factor influencing aflatoxin production. Lie and Marth (13) reported that aflatoxin formation on a casein substrate was high at extreme acidic or alkaline pH values (pH 2 and 9.5, respectively). It has also been reported that aflatoxin yields on a Czapek-Dox medium increased several-fold when the pH was changed from 7.4 to 4.0 (11). With SL medium, toxin production was maximal at a pH 4.5 and was reduced at lower or higher pH values. These data are consistent with the observation of Detroy and Hesseltine (8) that incorporation of acetate-1-C into aflatoxin by resting cells of A. parasiticus was optimal in the pH range of 3 to 5 and decreased markedly above pH 5.

Several workers have reported that, under conditions in which large amounts of aflatoxins are formed, aflatoxin G is produced in much greater amounts than aflatoxin B (17, 18; S. R. Gupta et al., J. Gen. Microbiol., in press). The ratio of aflatoxin B to aflatoxin G varied between 0.3 and 0.5 on SL medium on stationary cultures in agreement with these findings. However, shaken cultures on the same medium gave three to four times higher amounts of aflatoxin B than aflatoxin G, although the total yields of aflatoxins were of the same order as on stationary cultures.

Most of the previous work on the factors affecting aflatoxin production on synthetic media, especially the effect of trace elements, has been carried out on media containing high levels of phosphate and Mg²⁺ and at near neutral pH, conditions which are obviously unfavorable for high yields of aflatoxin (1, 5, 12, 19).

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LITERATURE CITED

1. Adye, J., and R. I. Mateles. 1964. Incorporation of labelled compounds into aflatoxins. Biochim. Biophys. Acta 86:418–420.
2. Brian, P. W., A. W. Dawkins, J. F. Grove, H. G. Hemmings, D. Lowe, and G. L. F. Norris. 1961. Phytotoxic compounds produced by Fusarium equiseti. J. Exp. Bot. 121–12.
3. Ciegler, A., R. E. Peterson, A. A. Logoda, and H. H. Hall. 1966. Aflatoxin production and degradation by Aspergillus flavus in 20-liter fermentors. Appl. Microbiol. 14:826–833.
4. Davis, N. D., U. L. Diener, and V. P. Agrisbiri. 1967. Production of aflatoxins B₃ and G₃ in a chemically defined medium. Mycopathol. Mycol. Appl. 31:251–256.
5. Davis, N. D., U. L. Diener, and D. W. Eldridge. 1966. Production of aflatoxins B₃ and G₃ by Aspergillus flavus in a semisynthetic medium. Appl. Microbiol. 14:378–380.
6. de Jongh, H., K. K. Beerthuis, R. O. Vles, C. B. Barrett, and O. W. Ord. 1962. Investigation of the factor in groundnut meal responsible for "turkey X disease." Biochim. Biophys. Acta 65:548–551.
7. Demain, A. L., and H. N. Inamine. 1970. Biochemistry and regulation of streptomycin and mannosidastreptomycine (α-D-mannoside) formation. Bacteriol. Rev. 34:1–19.
8. Detroy, R. W., and C. W. Hesselinc. 1969. Net synthesis of 14C-labeled lipids and aflatoxins in resting cells of Aspergillus parasiticus. Develop. Ind. Microbiol. 10:127-135.

9. Diener, U. L., and N. D. Davis. 1969. Aflatoxin formation by Aspergillus flavus. p. 13-54. In L. A. Goldblatt (ed.), Aflatoxin, scientific background, control and implications. Academic Press Inc., New York.

10. Donald, C., I. B. Passey, and R. J. Swaby. 1952. A comparison of methods for removing trace metals from microbiological media. J. Gen. Microbiol. 7:211-220.

11. Joffe, A. Z., and N. Lisker. 1969. Effects of light, temperature, and pH value on aflatoxin production in vitro. Appl. Microbiol. 18:517-518.

12. Lee, E. G., P. M. Townsley, and C. C. Walden. 1966. Effect of bivalent metals on the production of aflatoxins in submerged cultures. J. Food Sci. 31:432-436.

13. Lie, J. L., and E. H. Marth. 1968. Aflatoxin formation by Aspergillus flavus and Aspergillus parasiticus in a casein substrate at different pH values. J. Dairy Sci. 51:1743-1747.

14. Miller, A. L., and J. B. Walker. 1970. Accumulation of streptomycin-phosphate in cultures of streptomycin producers grown on a high-phosphate medium. J. Bacteriol 104:8-12.

15. Nabney, J., and B. F. Nesbitt. 1965. A spectrophotometric method for determining the aflatoxins. Analyst 90:155-160.

16. Reddy, T. V., L. Viswanathan, and T. A. Venkitasubramanian. 1970. Thin layer chromatography of aflatoxins. Anal. Biochem. 38:568-571.

17. Schindler, A. F., J. G. Palmer, and W. V. Eisenberg. 1967. Aflatoxin production by Aspergillus flavus as related to various temperatures. Appl. Microbiol. 15:1006-1009.

18. Schroeder, H. W. 1966. Effect of corn steep liquor on mycelial growth and aflatoxin production in Aspergillus parasiticus. Appl. Microbiol. 14:381-385.

19. Tulpule, P. G. 1969. Aflatoxicosis. Indian J. Med. Res. 57:102-114.

20. Van der Zijden, A. S. M., W. A. A. B. Koelersamid, J. Bolding, C. B. Barrett, O. W. Ord, and J. Philp. 1962. Isolation in crystalline form of a toxin responsible for turkey X disease. Nature (London) 195:1060-1062.

21. Wildman, J. D., L. Stoloff, and R. Jacobs. 1967. Aflatoxin production by a potent Aspergillus flavus Link isolate. Biotechnol. Bioeng. 9:429-437.