AFLATOXIN PRODUCED BY *ASPERGILLUS PARASITICUS* WHEN INCUBATED IN THE PRESENCE OF DIFFERENT GASES[1]

C. N. Shih and E. H. Marth
Department of Food Science and the Food Research Institute
University of Wisconsin-Madison, Madison, Wisconsin 53706

(Received for publication March 16, 1973)

Abstract

The influence of atmospheric gases on aflatoxin formation by *Aspergillus parasiticus* was investigated using a chemically defined medium and controlled environments in a fermenter. Maximal yield of aflatoxin was obtained under fermentation conditions lacking agitation or sparging of air. Increasing the rate of aeration enhanced glucose utilization and acid formation, but reduced toxin production. Replacement of air by various mixtures of O_2 and CO_2, or O_2 and N_2 suppressed toxin formation. Increasing proportions of CO_2 or N_2 in the atmosphere enhanced their inhibitory effect on aflatoxin formation, and complete inhibition of toxin synthesis occurred in atmospheres of 100% CO_2 or 100% N_2. Synthesis of toxin was suppressed more by a high concentration of CO_2 than of N_2. The optimal condition for toxin formation was quiescent incubation. Suppression of toxin formation can be achieved by introducing high concentrations of CO_2 or N_2 into the environment.

Molds in the genus *Aspergillus*, more specifically *Aspergillus flavus* and *Aspergillus parasiticus*, can produce aflatoxin which is acutely toxic or carcinogenic to experimental animals. Because of its biological effects and its occasional appearance as a contaminant of agricultural commodities, including foods, this fungal metabolite has been widely investigated *(11, 21)*.

Growth of storage fungi such as the aspergilli in natural substrates is dependent not only on availability of moisture and favorable temperature, but also on atmospheric conditions surrounding the substrate *(15)*. The influence of atmospheric gases on germination, growth, and metabolic activities of molds was discussed by Tabak and Cooke *(18)*. Use of controlled atmospheric environments to retard microbial activities has been applied practically to store some feed and food products. Bampton *(1)* indicated that storage conditions which limit growth of *A. flavus* should keep a food-stuff aflatoxin-free.

Several reports have appeared on the effects of various atmospheric conditions on aflatoxin formation in agricultural commodities *(6, 9, 14)*. However, most such studies have not been done in a chemically defined liquid medium and hence have been subject to variable effects caused by the different substrates that were used. It was believed that atmospheric conditions could be more effectively controlled by using a liquid medium in a fermenter than when a solid substrate was used in which the gas or gases might not be exchanged uniformly. In addition, use of a liquid medium in a fermenter provides an opportunity to study toxin formation by the resting mycelium under conditions unfavorable for germination of spores and growth of the mold.

In this study a toxin-producing strain of *A. parasiticus* was cultured in a liquid medium in a fermenter with air (with and without sparging) and with other controlled gaseous environments. Effects of these conditions on aflatoxin synthesis were measured to determine *(a)* how to obtain a high yield of aflatoxin, and *(b)* if a controlled gaseous atmosphere could be employed to reduce production of aflatoxin. Results of the experiments are reported in this communication.

Materials and Methods

Organism

Aspergillus parasiticus NRRL 2999, a toxigenic strain, was obtained from the Northern Regional Research Laboratory, Peoria, Illinois. The mold was grown on mycological agar (Difco) slants at 28 C for 6-8 days before use.

Medium

A glucose-salts-amino acids broth formulated in our laboratory was used as the fermentation medium. The medium was composed on the basis of reports by Mateles and Adye *(12)*, Davis et al. *(5)*, and Lee et al. *(10)* and contained (per liter) 50 g glucose, 6 g $(NH_4)SO_4$, 5 g KH_2PO_4, 6.4 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g HCl, 2 g glycine, 2 g glutamic acid, 10 mg $FeSO_4 \cdot 7H_2O$, 5 mg $ZnSO_4 \cdot 7H_2O$, and 1 mg $MnSO_4 \cdot H_2O$. The medium was prepared by aseptically adding the sterile glucose solution to the sterile salts-amino acids solution in the fermenter after both solutions were cooled. The pH of the medium was 6.4 to 6.5.

Cultural conditions and fermentations

Fermentations using various gaseous environments were done as follows. A 1-liter Mini-Ferm fermenter equipped with an automatic temperature controller and a magnetic stirrer (model M-1000, Fermentation Design, Inc., Allentown, Pennsylvania) was used for the experiments. Each experiment was conducted with 500 ml of medium. The glucose-free salts-amino acids solution (450 ml) was placed in the fermenter jar and then jar and solution were sterilized in an autoclave. Glucose (50% solution) was autoclaved separately and then aseptically added to the salts-amino acids

[1]Research supported by the College of Agricultural and Life Sciences, University of Wisconsin-Madison and by Public Health Service Grant FD00143 from the Food and Drug Administration.

Downloaded from http://meridian.allenpress.com/jfp/article-pdf/36/8/421/2399484/0022-2747-36_8_421.pdf by guest on 18 August 2022

solution to provide a final concentration of 5% sugar. Five milliliters of a spore suspension (7-8 × 10^6 spores/ml) were added to the medium and the fermentation was conducted at 28 C for up to 6 days. The following gaseous environments were tested for their effects on aflatoxin formation when the spore suspension served as inoculum: air with and without sparging, mixtures of O_2 and CO_2, and mixtures of O_2 and N_2.

In other experiments, the mycelium of *A. parasiticus* served as the inoculum. The mycelium was prepared by incubating 2 ml of the spore suspension with 100 ml glucose-salts-amino acids medium in a 500-ml Erlenmeyer flask at 28 C for 3 days. After the mycelium was harvested from broth by filtration, it was washed with cold sterile distilled water. Two and one-half grams of mycelium (wet weight) served as inoculum for each experiment and incubation was at 28 C for 3 days. When the mycelium served as inoculum, the following gaseous environments were used: air without sparging, 100% CO_2, 100% N_2, and mixtures of O_2 and N_2.

Each atmosphere was maintained by sparging air (2 different rates), a pure gas, or a mixture of gases through a filter into the culture medium at a rate of 0.5 ml gas/ml of medium/min, except for the static culture. One experiment utilized 1.0 ml air/ml of medium/min. The gaseous effluent from the fermenter was first exhausted through a filter packed with glass wool, and then was bubbled into a 0.01% $CaOCl_2$ solution. The stirrer rate was 120 rev/min, except when static incubation was used.

Analysis of fermentation broth

Approximately 30 ml of medium were removed from the fermenter after 3, 5, and 6 days of incubation for determination of pH, residual glucose content, and aflatoxin concentration. Glucose was determined by the anthrone test *(19)*. Aflatoxin was measured as described below.

Aflatoxin determination

Twenty milliliters of broth were extracted with 40 ml chloroform; the extraction was repeated three times using a separatory funnel. The mycelium (Table 4) was harvested from broth by filtration and then was washed with cold distilled water. The mycelial mat was weighed to determine mold growth and then was blended with 50 ml chloroform, 100 ml methanol, and 40 ml water in a Waring blendor for a few minutes. Fifty milliliters of chloroform and 50 ml of water were then added to the mixture and it was blended again. After filtration, chloroform was separated from the mixture in a separatory funnel. The methanol-water mixture was then extracted twice with chloroform *(17)*. Chloroform extracts from either broth or mycelium then were concentrated for aflatoxin analysis. Concentration of aflatoxin was determined by procedures described by Shih and Marth *(16)*. Thin layer chromatographic plates were developed with chloroform:methanol:water (98:1:1, v/v/v).

RESULTS

Rate of aeration and aflatoxin formation

The influence of various rates of aeration on aflatoxin formation by *A. parasiticus* is shown in Table 1. The greatest concentration of aflatoxin was produced by the culture incubated in the fermenter without agitation or sparging of air through the medium. Aflatoxin production decreased when the culture was aerated, although the amount of toxin after 6 days was slightly higher with a aeration rate of 1.0 ml air/ml/min than with one of 0.5 ml air/ml/min. Increasing the rate of aeration increased glucose utilization and acid production. These data do not clarify why more toxin was formed initially when the lower rates of aeration were used and after 6 days with the higher rate of aeration.

Various levels of O_2 and CO_2 and aflatoxin production

The highest concentration of toxin produced with variable amounts of O_2 and CO_2 was present in the broth when the fermentation was conducted using an atmosphere composed of 9 parts O_2 and 1 part CO_2 (Table 2). Less aflatoxin was produced when the proportion of CO_2 to O_2 was increased. Negligible amounts of toxin appeared when the mold grew in an environment of 90% CO_2 and 10% O_2. It is evident that increasing the concentration of CO_2 diminished production of aflatoxin. Acid production and glucose uptake also were reduced as the proportion of CO_2 in the atmosphere increased.

Various levels of O_2 and N_2 and aflatoxin production

Increasing the N_2 and decreasing the O_2 content of the atmosphere reduced aflatoxin formation, acid production, and glucose utilization (Table 3). Although the effect of N_2 on these activities followed a pattern similar to that observed for CO_2, it was less profound than that of CO_2. It was also noted that various levels of O_2 and N_2 were similar in their inhibition of toxin formation, whereas some differences existed when similar concentrations of O_2 and CO_2 were used.

Formation of aflatoxin in broth inoculated with mycelium

To eliminate the problem of inhibiting or retarding spore germination in the absence of O_2 *(19)*, 3-day old mycelium also was used to study formation of aflatoxin in various gaseous environments including an atmosphere unfavorable for spore germination and mold growth. Results are in Table 4. The stationary culture produced the greatest concentration of aflatoxin, whereas mycelium incubated in atmospheres of 100% CO_2 or 100% N_2 essentially failed to form aflatoxin. Addition of O_2 to a N_2 (1:9 v/v) environment prompted the mycelium to resume toxin formation, and a further increase in the amount of O_2 (9:1 v/v; O_2:N_2) further enhanced toxin formation.

The extent of mycelial growth depended on the concentration of O_2 present; no growth occurred when the mycelium was incubated in atmospheres of 100% CO_2 or 100% N_2. These results demonstrate further that maximal aflatoxin formation is associated neither with maximal concentrations of O_2 present nor with maximal mycelial growth. Formation of greatest concentrations of aflatoxin in the static culture sug-

Downloaded from http://meridian.allenpress.com/jfp/article-pdf/36/8/421/2399484/0022-2747-36_8_421.pdf by guest on 18 August 2022

TABLE 1. AFLATOXIN FORMATION IN A LIQUID MEDIUM INOCULATED WITH SPORES OF *Aspergillus parasiticus* AND AERATED AT DIFFERENT RATES

	Static culture, incubated for days			Aerated cultures, incubated for days					
				0.5 ml air/ml[a]/min			1.0 ml air/ml[a]/min		
	3	5	6	3	5	6	3	5	6
Aflatoxins[b]	(μg/500 ml)								
B_1	12	1200	4113	123	385	368	53	120	488
B_2	5	75	325	14	63	65	10	10	75
G_1	9	925	3000	97	325	340	103	150	336
G_2	3	63	225	8	40	48	8	20	43
Total	29	2263	7663	242	813	821	174	300	942
pH	5.8	4.8	4.1	5.4	4.5	3.9	4.9	3.8	3.6
Residual glucose (%)	3.10	2.47	1.92	3.70	2.84	2.36	2.46	1.70	1.00

[a]Medium volume.
[b]Aflatoxins in broth.

TABLE 2. AFLATOXIN FORMATION IN A LIQUID MEDIUM INOCULATED WITH SPORES OF *Aspergillus parasiticus* AND SPARGED WITH DIFFERENT MIXTURES OF OXYGEN AND CARBON DIOXIDE[a]

	O_2 : CO_2 (9:1, v/v)			O_2 : CO_2 (1:1, v/v)			O_2 : CO_2 (1:9, v/v)		
	3 days	5 days	6 days	3 days	5 days	6 days	3 days	5 days	6 days
Aflatoxins[b]	(μg/500 ml)								
B_1	6	129	135	8	19	25	2	2	2
B_2	—[c]	13	16	2	2	3	—	—	1
G_1	12	103	126	8	30	34	2	3	3
G_2	—	—	11	—	3	4	—	—	—
Total	18	245	288	18	54	66	4	5	6
pH	5.8	3.9	3.5	5.8	5.4	4.2	6.2	5.8	5.10
Residual glucose (%)	3.85	3.28	1.70	3.90	3.70	3.30	4.75	4.05	3.50

[a]Flow rate of mixed gas = 0.5 ml gas/ml/min.
[b]Aflatoxins in broth.
[c]Not detected.

TABLE 3. AFLATOXIN FORMATION IN A LIQUID MEDIUM INOCULATED WITH SPORES OF *Aspergillus parasiticus* AND SPARGED WITH DIFFERENT MIXTURES OF OXYGEN AND NITROGEN[a]

	O_2 : N_2 (9:1, v/v)			O_2 : N_2 (1:1, v/v)			O_2 : N_2 (1:9, v/v)		
	3 days	5 days	6 days	3 days	5 days	6 days	3 days	5 days	6 days
Aflatoxins[b]	(μg/500 ml)								
B_1	135	210	225	19	80	85	8	74	110
B_2	5	40	55	3	20	20	—[c]	11	20
G_1	175	280	285	45	67	67	6	35	52
G_2	20	30	35	4	15	7	—	8	7
Total	335	560	600	71	182	179	14	128	189
pH	5.8	4.8	4.2	5.8	4.4	4.3	6.1	4.5	4.4
Residual glucose (%)	3.66	3.56	3.40	3.60	3.06	3.20	3.94	3.88	3.38

[a]Flow rate of mixed gas = 0.5 ml gas/ml/min.
[b]Aflatoxins in broth
[c]Not detected.

gests that biosynthesis of the toxin occurred when available oxygen was somewhat limited. However, absence of O_2 or an excess of O_2 both inhibited aflatoxin formation.

Downloaded from http://meridian.allenpress.com/jfp/article-pdf/36/8/421/2399484/0022-2747-36_8_421.pdf by guest on 18 August 2022

DISCUSSION

Results of this study indicate that static incubation was best for maximal production of aflatoxin, although it was not optimal for mycelial growth. Increasing the rate of aeration reduced formation of toxin (Tables 1 and 4). Production of higher concentrations of toxin with reduced aeration also has been reported in the literature. Codner et al. *(3)* obtained 100-200 mg of aflatoxin per liter of medium in 250-ml flasks incubated on a rotary shaker, whereas the same strain of fungus failed to produce any aflatoxin in 3- and 20-liter stirred aerated fermenters. Davis et al. *(4)* indicated that one strain of *A. flavus* produced up to 63 mg of aflatoxin per 100 ml of YES medium when grown as a stationary culture in 1-liter flasks. However, this culture produced only 21 mg of toxin per 100 ml of medium in submerged culture with stirring and aeration *(8)*. These results suggest that although O_2 is essential for growth and toxin formation, excessive amounts of O_2 reduce production of toxin.

Another factor which may cause the difference in production of aflatoxin between static and aerated cultures is the concentration of CO_2. It has been reported that low concentrations of CO_2 stimulate germination, growth, and metabolic activities of fungi, whereas high concentrations (above 20%) of CO_2 retard them *(2, 7, 13, 18, 20)*. Barinova *(2)* studied the importance of CO_2 to the vital activity of fungi, and found that the gas stimulated growth of *Aspergillus niger* when grown in a CO_2-free medium. The effect of CO_2 tension on production of citric acid was studied by Vakil and Bhattacharyya *(20)* who indicated that withdrawal of the gas from the atmosphere surrounding growing *Aspergillus niger* cultures decreased the rate of citrate synthesis and hence the yield of citrate. Atmospheric CO_2 in small amounts accelerated growth of the mold and of citrate synthesis; the effect disappeared with an increasing concentration of CO_2.

According to Tabak and Cooke *(19)*, low concentrations of CO_2 are involved in fungal metabolism leading to synthesis (fixation) of proteins, nucleic acids, and intermediates of the tricarboxylic acid cycle. Furthermore, CO_2 serves to catalyze the malonyl-coenzyme A system, which is involved in synthesis of fatty acids and of other natural products, including certain aromatic compounds. Hence, production of less aflatoxin in the aerated fermenter might have resulted because the concentration of CO_2 which was produced during fungal respiration was reduced, and thus it was not available for synthetic purposes.

Aflatoxin formation in an atmosphere of 90% O_2 plus 10% of N_2 was lower than that observed in an aerated (normal air) or static fermentation (Tables 1 and 3). The difference in toxin formation could have resulted from complete exclusion of CO_2 from the atmosphere during the fermentation. Similar results also were obtained when the mycelium was incubated quiescently and in an atmosphere of 90% O_2 and 10% N_2 (Table 4). These observations provide some evidence to indicate that low concentrations of CO_2 might have stimulated toxin formation.

When the environment contained more than 10% CO_2 (Tables 1 and 2), aflatoxin formation was markedly suppressed. Formation of aflatoxin was neg-

TABLE 4. AFLATOXIN FORMATION IN A LIQUID MEDIUM INOCULATED WITH MYCELIUM[a] OF *Aspergillus parasiticus* AND HELD UNDER DIFFERENT ATMOSPHERIC CONDITIONS[b] FOR 3 DAYS

	Mycelium (Inoculum)	Static culture	100% CO_2	100% N_2	O_2:N_2 (1:9, v/v)	O_2:N_2 (9:1, v/v)
Aflatoxins[c]			(μg/500 ml)			
B_1	21.5	1766	20.1	21.0	105	1442
B_2	11.6	464	8.5	9.5	31	289
G_1	6.0	1370	4.5	5.0	69	977
G_2	2.3	179	2.0	2.5	9	134
Total	41.4	3779	35.1	38.0	204	2842
Mycelium wt.[d] (g)	2.5	6.2	2.5	2.5	4.6	12.4
Aflatoxin produced μg/g mycelium	17.0	610.0	14.0	15.0	44.0	229.0
pH		3.9	5.4	5.2	6.0	4.8
Glucose residue (%)		1.6	3.2	3.2	3.2	2.4

[a] 3-Day old mycelium was washed before inoculation; 2.5 g (wet weight) used for each experiment in the fermenter.
[b] The conditions were created by passing pure or mixed gas continuously through the fermenter except for the static culture, flow rate = 0.5 ml gas/ml/min.
[c] Toxins in mycelium and broth.
[d] Wet weight, mycelium was prepared by removing water through vacuum filtration.

Downloaded from http://meridian.allenpress.com/jfp/article-pdf/36/8/421/2399484/0022-2747-36_8_421.pdf by guest on 18 August 2022

ligible when the atmosphere was composed of 90% CO_2 and 10% O_2, and complete inhibition of aflatoxin synthesis occurred when the mycelium was incubated in an atmosphere of 100% CO_2 (Table 4). Inhibition by CO_2 of aflatoxin formation on peanuts was previously demonstrated by Landers et al. (9). They also showed that toxin formation was reduced by 70% when the CO_2 concentration was increased from 0.03% (air) to 20%. Toxin formation continued to be suppressed by higher concentrations of CO_2 (40-80%), and no aflatoxin was formed in an atmosphere of 100% CO_2.

The effect of mixtures of O_2 and N_2 on aflatoxin formation was similar to that observed with mixtures of O_2 and CO_2. However, there was less inhibition of toxin production by N_2 than by CO_2 (Tables 2 and 3). Incubation of the mycelium in an atmosphere of 100% N_2 completely suppressed aflatoxin formation. Introduction of O_2 into an atmosphere of N_2 caused resumption of toxin formation (Table 4). Results of this study show that the atmospheric condition favoring maximal growth of molds does not favor formation of the greatest amount of aflatoxin. Production of less toxin in an aerated culture suggests that O_2 and CO_2 may be involved in regulating aflatoxin formation. Further study is needed to completely explain these relationships. Furthermore, these results indicate that the optimal condition for aflatoxin production is quiescent incubation, and that suppression of toxin formation can be achieved by introducing high concentrations of CO_2 or N_2 into the environment. This information may be useful when packaging techniques are selected for use with foods that are likely to support mold growth and hence become contaminated with mycotoxins.

REFERENCES

1. Bampton, S. S. 1963. Growth of *Aspergillus flavus* and production of aflatoxin in groundnuts. Trop. Sci. 5 (2):74.

2. Barinova, S. A. 1960. Growth stimulation of molds by carbon dioxide and effect of mesotertanic acid on this process. Mikrobiologiya 29:125-126.

3. Codner, R. C., K. Sargeant, and R. Yeo. 1963. Production of aflatoxin by the culture of strains of *Aspergillus flavus-oryzae* on sterilized peanuts. Biotechnol. Bioeng. 5: 185-192.

4. Davis, N. D., U. L. Diener, and D. W. Eldridge. 1966. Production of aflatoxins B_1 and G_1 by *Aspergillus flavus* in a semisynthetic medium. Appl. Microbiol. 14:378-380.

5. Davis, N. D., U. L. Diener, and V. P. Agnihotri. 1967. Production of aflatoxins B_1 and G_1 in chemically defined medium. Mycopathol. Mycol. Appl. 31:251-256.

6. Epstein, E., M. P. Steinberg, A. I. Nelson, and L. S. Wei. 1970. Aflatoxin production as affected by environmental conditions. J. Food Sci. 35:389-391.

7. Golding, N. S. 1940. The gas requirements of molds. II. The oxygen requirements of *Penicillium roqueforti* in the presence of nitrogen and the absence of carbon dioxide. J. Dairy Sci. 23:879-889.

8. Hayes, A. W., N. D. Davis, and U. L. Diener. 1966. Effect of aeration on growth and aflatoxin production by *Aspergillus flavus* in submerged culture. Appl. Microbiol. 14: 1019-1021.

9. Landers, D. E., N. D. Davis, and U. L. Diener. 1967. Influence of atmospheric gases on aflatoxin production by *Aspergillus flavus* in peanuts. Phytopathol. 57:1086-1090.

10. Lee, E. G. H., 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.

11. Marth, E. H. 1967. Aflatoxins and other mycotoxins in agricultural products. J. Milk Food Technol. 30:192-198.

12. Mateles, R. I., and J. C. Adye. 1965. Production of aflatoxins in submerged culture Appl. Microbiol. 13:208-211.

13. Rippel, A., and F. Heilman. 1930. Action of carbon dioxide on heterotrophs. Arch. Mikrobiol. 1:119-136.

14. Sanders, T. H., N. D. Davis, and U. L. Diener. 1968. Effect of carbon dioxide, temperature, and relative humidity on production of aflatoxin in peanuts. J. Amer. Oil Chem. Soc. 45:683-685.

15. Semeniuk, G. 1954. Microflora. Monograph II. pp. 77-151. *In* J. A. Anderson, and A. W. Alcock (eds.) Storage of cereal grains and their products. Amer. Ass. Cereal Chem., St. Paul, Minnesota.

16. Shih, C. N., and E. H. Marth. 1969. Improved procedure for measurement of aflatoxins with thin-layer chromatography and fluorometry. J. Milk Food Technol. 32:213-217.

17. Shih, C. N., and E. H. Marth. 1971. A procedure for rapid recovery of aflatoxins from cheese and other foods. J. Milk Food Technol. 34:119-123.

18. Tabak, H. H., and W. B. Cooke. 1968. The effect of gaseous environments on the growth and metabolism of fungi. Bot. Rev. 34:124-252.

19. Umbreit, W. W., and R. H. Burris. 1964. Method for glucose and other sugars. pp. 209-210. *In* W. W. Umbreit, R. H. Burris, and J. F. Stauffer (eds.) Manometric techniques. 4th ed. Burgess Publ. Co. Minneapolis, Minnesota.

20. Vakil, J. R., and P. K. Bhattacharyya. 1962. Role of carbon dioxide in citric acid fermentation. II. Effect of carbon dioxide tension on the production of citric acid. J. Sci. Ind. Res. 21C:202-206.

21. Wogan, G. N. 1966. Chemical nature and biological effects of the aflatoxins. Bacteriol. Rev. 30:460-470.

Downloaded from http://meridian.allenpress.com/jfp/article-pdf/36/8/421/2399484/0022-2747-36_8_421.pdf by guest on 18 August 2022