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Production of Aflatoxin by an *Aspergillus flavus* Isolate Cultured Under a Limited Oxygen Supply

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In a previous experiment on the preservation of hay of high moisture content with formic acid, among other agents, aflatoxin was formed in the hay, and aflatoxin-forming strains of *Aspergillus flavus* were isolated from this hay after incubation in air as well as in an anaerobic jar. One isolate from the anaerobic jar was cultivated in a chemostat (Bioflo model C 30; New Brunswick Scientific Co.) in a defined medium with added B vitamins, yeast extract, or formic acid, with or without gas flow (air or nitrogen). In all cases where spore germination occurred, aflatoxin was formed in the cultures with gas flow, and small quantities of aflatoxins B_1 and B_2 occurred even in an atmosphere of nitrogen. Addition of B vitamins and supply of traces of air gave an approximately 15-fold increase in the amount of aflatoxin in 2 days. Carbon dioxide enrichment hindered aflatoxin formation on the defined medium even in the presence of B vitamins, but when formic acid was added, small quantities (5 to 15 μg/liter) were formed, and this low level remained constant until the gas flow was started.

In an earlier investigation (3) of the fungicidal effects of common salt, formic acid, and propionic acid in high-density bales of incompletely dried hay, a vigorous outgrowth of *Aspergillus flavus* Link was observed in all bales, especially in those treated with formic acid, in which formation of aflatoxin was also demonstrated. *A. flavus*, as well as some other fungi, proved to have the capacity of maturing in 3 days in an anaerobic jar. Isolation of fungi from hay material as well as from agar plates incubated in an anaerobic jar showed extensive occurrence of aflatoxin-forming strains of *A. flavus*.

Important methods for storing agricultural products are based on the principle of limited oxygen supply, and formic acid is a common preservative for coarse fodder and moist grain. From this viewpoint it is accordingly of great practical importance to determine whether *A. flavus* can grow and form toxin at low oxygen tension and whether formic acid contributes to increased toxin formation.

An isolate of *A. flavus* from an anaerobic jar was therefore cultivated on a defined medium in a Bioflo tank with the aim of examining its capacity for aflatoxin formation in a limited supply of oxygen and in a substrate containing formic acid.

MATERIALS AND METHODS

Microorganism and inoculation culture. The experimental organism used has the designation *A. flavus* 79-II:9 (3). It is a stable aflatoxin former. After a series of 10 reinoculations at intervals of 1 to 2 weeks, it gave a fluorescence reaction and formed aflatoxin to the same extent as on isolation.

The inoculum culture consisted of a standardized spore suspension (optical density at 540 nm = 0.2), stored at −20°C; 400 ml of medium was inoculated with 5 ml of this suspension.

Culture media. The culture medium consisted of a synthetic low-salts medium (13), here called SLM, with added vitamin B solution, yeast extract, or formic acid as stated.

B vitamins necessary for fungal growth under anaerobic conditions (1, 5) were added, partly as 0.4% yeast extract and partly as vitamin B solution, according to Walsh (17). Formic acid was added to give a 30 mM solution, and the medium was retitrated with 25% ammonia.

Before inoculation, the medium was deaerated with nitrogen for 2 h. It was then aerated to approximately 100% dissolved oxygen. Experiments were also performed with aeration to 15% dissolved oxygen and, in the complete absence of an air supply, with a flow of nitrogen.

Cultivation. The fungus was cultivated in a chemostat, Bioflo model C 30 (New Brunswick Scientific Co.). The culture tank has a capacity of about 500 ml and was loaded with 400 ml of medium. It is equipped with a stirrer, the speed of which can be varied, and the temperature can be kept constant. The gas flow can be regulated, and the percent oxygen content of the solution as well as the pH can be read off, the values also being registered automatically on a recorder.

All cultivations were performed at 28°C, with nor-

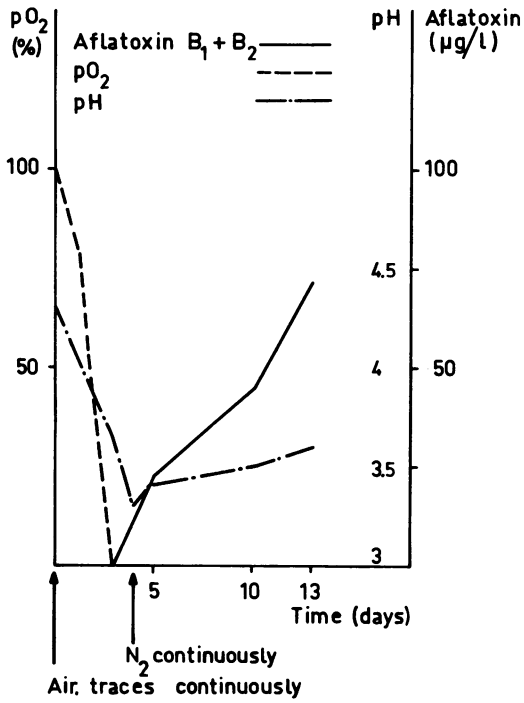

FIG. 1. *A. flavus* 79-II:9 aflatoxin (B_1 and B_2) formation in culture on SLM in a chemostat at 28°C and 200 rpm, with traces of air supplied over 4 days and nitrogen gas supplied during the succeeding 9 days.

mal diurnal variation for light and darkness and with stirring.

In general, air was supplied continuously as trace amounts, and this level is below the measuring capacity of the air flow meter. The minimum detectable amount is 100 ml/min, and the amount used was about 10% of this.

The nitrogen gas used contained <100 ppm (<100 µl/liter) of oxygen.

The stirring speed was kept at 200 rpm, but in the case of vigorous mycelial growth, it was raised to 500 rpm.

Sampling. Samples of 10 ml were withdrawn for aflatoxin analysis and replaced by sterile medium. Samples were autoclaved at 105°C for 5 min to prevent spread of the fungus.

About 10 ml of air was admitted during sampling. The oxygen tension in the solution rose 2 to 3% over 4 to 5 min, subsequently reverting to its initial value.

Carbon dioxide production was demonstrated qualitatively with saturated barium hydroxide solution.

The sugar content was measured, when appropriate, by means of Clinistix (Ames Laboratories).

Aflatoxin analyses were subsequently performed according to Clevström et al. (3) for all samples.

RESULTS

Inoculum size and growth characteristics. A standardized inoculum culture is necessary since inoculum size affects the amount of aflatoxin formed. When the inoculum culture was diluted to an optical density at 540 nm of 0.1 and the amount of inoculum was reduced to half, the amount of aflatoxin in the flask culture increased 8 to 10 times over 5 days (G. Clevström and H. Ljunggren, unpublished data). This agrees with the findings of Sharma and co-workers (14), who found that the amount of aflatoxin produced by strains of *A. flavus* and *A. parasiticus* was doubled if the inoculum size was reduced by a power of 4 to 5.

In flask culture on a shaking machine at 100 rpm, the *A. flavus* isolate grew as pellets, with somewhat lower aflatoxin formation than in stationary culture; with propeller stirring at various speeds in a fermentor, however, the pellet form of growth did not appear. The fungus then grew in the form of flakes and filaments on the surface, freely in the medium, and also on the stirrer and baffles. In the case of vigorous mycelial growth, a flocculent mass was formed, and conidial formation occurred irregularly in the surface and submersed.

Aflatoxin formation was accompanied by increasing yellow to yellowish-brown pigmentation of the medium. On prolonged incubation, with carbon dioxide accumulation, the solution

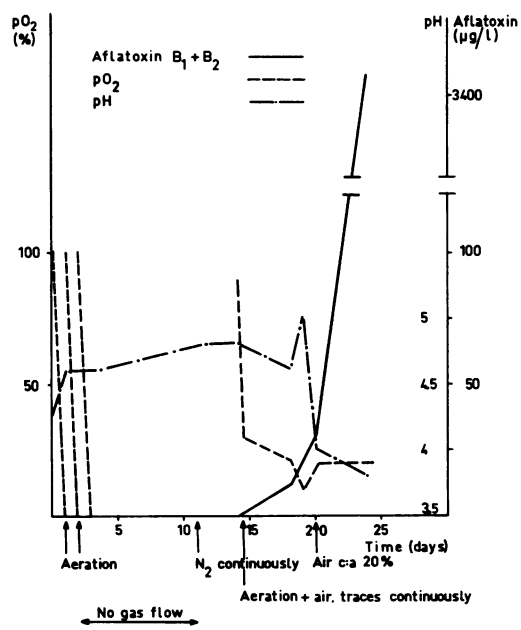

FIG. 2. *A. flavus* 79-II:9 aflatoxin (B_1 and B_2) formation in culture on SLM (addition of vitamin B solution according to Walsh [17] after 8 days) in a chemostat at 28°C and 200 rpm, raised to 500 rpm at the end of the culture period and with a varied oxygen supply. Samples for aflatoxin analysis were withdrawn twice a week.

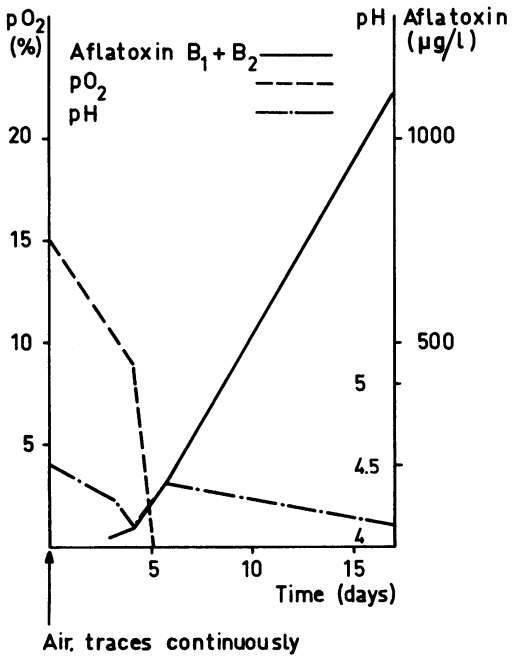

FIG. 3. *A. flavus* 79-II:9 aflatoxin (B_1 and B_2) formation in culture on SLM with added 0.4% yeast extract in a chemostat at 28°C and 200 rpm, with traces of air supplied over 17 days.

tended to become turbid and the mycelium fragmented.

Aflatoxin formation in reduced air supply and in the presence of B vitamins. Cultivation of *A. flavus* on SLM with traces of air for 3 days, followed by a flow of nitrogen, led to the formation of only small amounts of aflatoxin (B_1 and B_2), <100 µg/liter after 2 weeks, but toxin formation showed a tendency to increase with a steeper rise at the end of cultivation (Fig. 1).

In cultures with aeration to 100% dissolved oxygen once every 24 h for 2 days, mycelial growth was vigorous, with rapid consumption of the oxygen supplied during intervals of nonaeration and an overpressure of carbon dioxide (Fig. 2). B vitamins were added, but there was no stable aflatoxin formation until a flow of nitrogen was begun. Traces of air caused the aflatoxin content to rise slowly. Increasing the amount of air and attempts at stabilizing the dissolved oxygen at about 20% led to a dramatic increase in the amount of aflatoxins (B_1 and B_2) produced.

The pH rose after the first day of the experiment and then increased slightly over a 2-week period without gas flow through the system. With a flow of nitrogen, the rise in pH ceased, whereas traces of air led to a slight fall and 20% dissolved oxygen produced a very sharp fall in pH.

In cultivation with the addition of 0.4% yeast extract, with aeration to 15% dissolved oxygen before inoculation and with traces of air supplied throughout the cultivation period (Fig. 3), the measurable amount of dissolved oxygen rapidly fell to <0.2% (minimum detectable amount). After 1 week the aflatoxin content was about 200 µg/liter, and at about 3 weeks it was more than 1,000 µg/liter (B_1 and B_2).

The formation of aflatoxins (B_1 and B_2) was slower when the yeast extract was replaced with a vitamin B solution and traces of air were admitted over a 3-week period (Fig. 4). The sugar content of the medium was consumed in about 2 weeks, and supplementary medium was added. One week later, the air supply was replaced with a nitrogen flow, and cultivation was continued for another 3 weeks to show any tendency on the part of the aflatoxins to break down. The last sample after 6 weeks again showed the absence of sugar and a slight increase in aflatoxin content.

Aflatoxin formation is preceded by a fall in pH, most clearly marked in the culture with pure B vitamins added and performed with traces of air.

Experiments carried out over a 2-week period, completely without air supply but with a flow of nitrogen and in a culture medium containing B vitamins, did not result in any outgrowth of fungal mycelium.

Aflatoxin formation in the presence of formic acid. In cultures of the *A. flavus* isolate on medium containing 30 mM formic acid and held

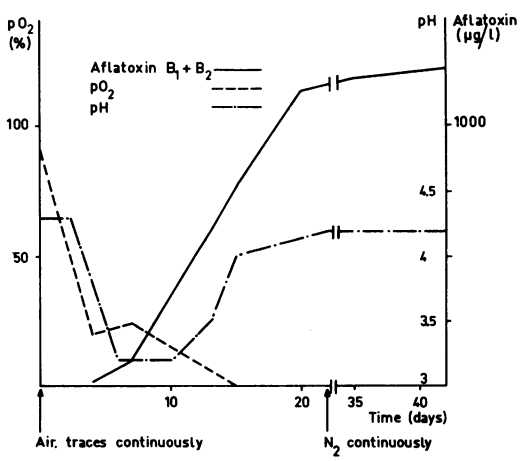

FIG. 4. *A. flavus* 79-II:9 aflatoxin (B_1 and B_2) formation in culture on SLM with added vitamin B solution in a Bioflo tank at 28°C and 200 rpm, raised after 20 days to 500 rpm. Traces of air were supplied for 22 days, and nitrogen was supplied during the remainder of the cultivation period; 100 ml of substrate was added after 15 days.

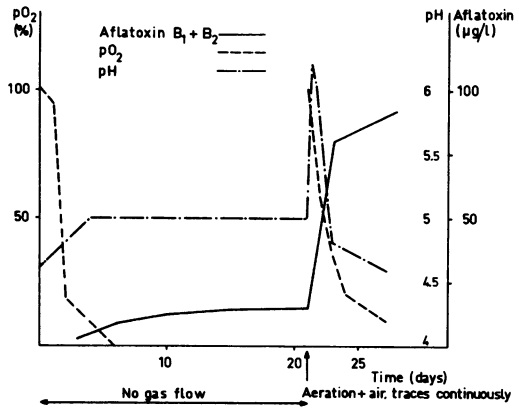

FIG. 5. *A. flavus* 79-II:9 aflatoxin (B_1 and B_2) formation in culture on SLM with added 30 mM formic acid, titrated with ammonia to pH 4.6, in a chemostat at 28°C and 200 rpm, raised after 20 days to 500 rpm. Aeration was performed before the start of culture but no air was supplied until after 21 days, when aeration was performed, and traces of air were supplied during the remainder of the cultivation period.

for 3 weeks without an air supply or subjected to any other interventions, aflatoxins (B_1 and B_2) were formed but remained at a low level (Fig. 5). The rate of toxin formation rose rapidly on aeration but slowed down on subsequent supply of traces of air only.

Like the aflatoxin level, the pH, after an initial upturn, remained constant without gas flow. Supply of air led to a rapid rise of about 1 pH unit, followed by an equally dramatic fall to below the initial pH of the medium.

In identical cultivations but with aeration replaced with nitrogen and vitamin B solution added (Fig. 6), aflatoxin was noted after a few days, but the amount of toxin remained unchanged until the flow of nitrogen was started. The aflatoxin content then increased, but only very slightly.

In cultivation with a gas flow, aflatoxins B_1 and B_2 were both formed, with B_2 in a much smaller amount (approximately 5 to 10% of B_1). Carbon dioxide repressed the aflatoxin formation, especially of B_2.

DISCUSSION

In Swedish products, aflatoxin has been reported only twice, in oats (12) and in experimental hay (3), both after formic acid treatment of moist materials. Recently, another case has occurred in grain treated with formic acid (A. T. Holmberg, R. Grossman, N. G. Nilsson, H. Pettersson, and B. Göransson, manuscript in preparation), which caused illness among animals and several deaths.

From bales of hay treated with formic acid, strains of *A. flavus* forming large quantities of aflatoxin and having a high capacity for withstanding low oxygen tension were isolated (3). The isolates of *A. flavus* were more tolerant than some other fungi to formic acid and were able to create apparently pure cultures in hay bales and in laboratory cultures. In stationary culture on SLM in Fernbach flasks some isolates increased the aflatoxin content by 45% in the presence of formic acid retitrated with ammonia.

Formic acid as a preservative of moist grain and as addition to silage is widely used in Sweden and so is storage under anaerobic conditions. This urged an investigation on the effects of lack of oxygen, presence of formic acid, and carbon dioxide supplied by *A. flavus* itself on the formation of aflatoxin.

Fungi are aerobic but may display anaerobic metabolism. This is well known in yeasts as alcoholic fermentation but even molds such as *Aspergillus, Fusarium*, and *Mucor* can produce ethanol, and lactic acid fermentation can occur in some fungi (9). In East Asia certain foods are prepared by fermentation with species of *Aspergillus, Mucor*, and *Rhizopus*, and the Japanese alcohol sake is produced by yeasts and strains of *A. oryzae*, a member of the *A. flavus* group (11).

A number of fungal species can grow under anaerobic conditions, the growth occurring best if B vitamins are supplied (5) and carbon dioxide is not allowed to accumulate (15). Fermentation may occur at isolated spots in a majority of

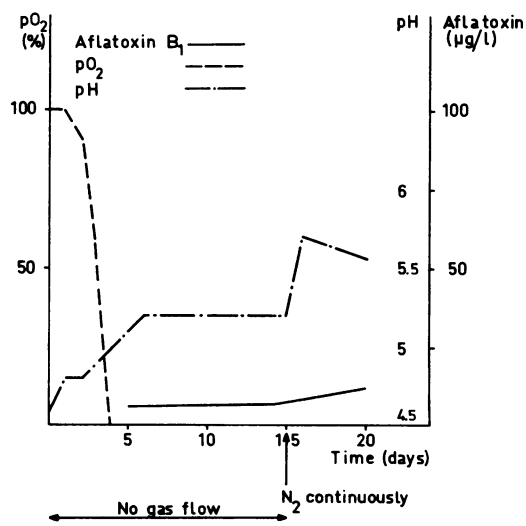

FIG. 6. *A. flavus* 79-II:9 aflatoxin (B_1) formation in culture on SLM-formic acid medium, pH 4.6, in a chemostat at 28°C and 200 rpm, with aeration before the start of culture but without a supply of gas for 15 days and, subsequently, nitrogen for 8 days. Vitamin B solution was added after 9 days.

fungi, but not all of them can utilize this type of metabolism as the only energy source (5). Vollbrecht and El Nawawy (16) consider fermentative growth and secretion of metabolites to be the method used by microorganisms to respond to limitations in the oxygen supply, even if they are wholly aerobic.

The isolate of *A. flavus* used proved capable of forming mycelia and aflatoxin under very low oxygen tension (Fig. 1). Traces of air supplied could not keep up with the metabolic requirements of the fungus, and the oxygen tension fell rapidly to <0.2%. In the presence of B vitamins, aflatoxin formation increased (Fig. 4), but it did so more rapidly when the medium contained yeast extract (Fig. 3). Some natural vitamin-containing products, such as yeast extract and potato extract, have a greater stimulating effect on growth under anaerobic conditions than do pure vitamin solutions (8). Higher amounts of aflatoxins and a faster rate of formation in the presence of yeast extract than in a synthetic medium are also found for *A. parasiticus* (6).

Substantial aflatoxin formation occurred in cultures with limited supply of oxygen in the presence of B vitamins. Even with a flow of nitrogen in a medium free of B vitamins, lower but still considerable aflatoxin formation occurred (Fig. 1).

When nitrogen was supplied, there was a period of adaptation before aflatoxin formation started, but when air was supplied, the aflatoxin formation began immediately or rose quickly, and in the absence of gas flow no toxin was formed (Fig. 2).

From Fig. 4 it is evident that the aflatoxin concentration did not decrease under conditions of low air supply even when all sugar was consumed. Decomposition can occur, however, with vigorous aeration and high stirring speed (2).

Large quantities of air supplied at the beginning of cultivation led to the formation of a large amount of mycelia with a high oxygen demand (Fig. 2) and high production of aflatoxin. At 20% dissolved oxygen, aflatoxin formation occurred to about the same extent as in stationary cultures on SLM in Fernbach flasks.

The lack of growth in the cultures carried out in the complete absence of an air supply is due to the fact that spore germination is an aerobic process in most fungi (9).

Formic acid retitrated with ammonia increases the aflatoxin content in stationary cultures (3). Figures 2 and 5 show that the amount of aflatoxin after aeration, followed by a continuous supply of traces of air for 2 days, was about 15 times higher in SLM with ammonium formate than in SLM with B vitamins.

If formic acid is not retitrated with ammonia, the pH falls about 1 pH unit, growth of *A. flavus* is very slow, and there is little aflatoxin formation, despite the fact that the fungus itself can give rise to low pH and still grow well (Fig. 4).

When carbon dioxide was allowed to accumulate, formic acid caused production of only small quantities of aflatoxins, which remained constant, and when air or nitrogen was used to remove the carbon dioxide the amount of aflatoxin increased, although it did so faster when air was supplied (Fig. 5 and 6). It is true that the increase in toxin formation with a nitrogen flow was small, but the amount doubled, and the pH reaction confirmed that there really was an upturn of the sequences leading to aflatoxin formation.

Under practical storage conditions an atmosphere of nitrogen or carbon dioxide with low oxygen content cannot prevent aflatoxin formation but can afford protection against high toxin contents, even if the product is highly infected with a potent aflatoxin producer (4, 7).

In cultures with gas flow, the pH showed a rapid fall with a minimum value 1 to 2 days after aflatoxin became detectable, a fact also observed previously (10). In cultures without gas flow, the pH rose at the start of cultivation, and this occurred even in formic acid-containing medium despite the formation of a certain amount of toxin. When the gas flow was started and the amount of aflatoxin increased, the pH first rose and then fell quickly. This was noted most clearly in the case where air was supplied to formic acid-containing medium (Fig. 5), but a pH reaction also occurred with nitrogen, albeit much less clearly (Fig. 6).

A pleasant aromatic odor instead of a moldy smell developed in cultures supplied with nitrogen and traces of air. In experiments with maize, inoculated with *A. flavus* and incubated in a controlled atmosphere containing <1% oxygen, an aromatic odor was reported together with small amounts of aflatoxin by Wilson and Jay (18). As agricultural products are frequently exposed to fungi, an aromatic odor can be misleading, since deterioration in appearance and a moldy smell are alarm signals to the farmer.

The nitrogen gas used in our experiments contained small quantities of oxygen, which were not removed. Even if the small amounts of oxygen which occur as an impurity in the nitrogen, are admitted during sampling, or enter through a leak in the apparatus can contribute to aflatoxin formation, it is clear that the minute amounts required for fungal growth cannot be avoided under practical storage conditions. A low oxygen tension cannot prevent aflatoxin formation if the substrate is suitable, nor can a

low pH, which can be produced by *A. flavus* itself when growing.

An atmosphere of carbon dioxide seems to counteract the lowering of pH and retain aflatoxin formation in a synthetic substrate and also to prevent high aflatoxin content in formic acid-containing medium.

It must be kept in mind that the SLM medium has been developed to produce high amounts of aflatoxin, which is true also of the strain of *A. flavus* used in the present investigation. The *A. flavus* isolate, though isolated from hay, can produce aflatoxin in rice (3) but not in a hay extract medium, not even in the presence of formic acid or ammonium formate (Clevström and Ljunggren, unpublished data).

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