Aflatoxin Production and Degradation by *Aspergillus flavus* in 20-Liter Fermentors

A. CIEGLER, R. E. PETERSON, A. A. LAGODA, AND H. H. HALL

Northern Regional Research Laboratory, Northern Utilization Research and Development Division, U.S. Department of Agriculture, Peoria, Illinois

Received for publication 12 May 1966

**ABSTRACT**

CIEGLER, A. (Northern Regional Research Laboratory, Peoria, Ill.), R. E. PETERSON, A. A. LAGODA, AND H. H. HALL. Aflatoxin production and degradation by *Aspergillus flavus* in 20-liter fermentors. Appl. Microbiol. 14:826–833. 1966.—Yields of from 200 to 300 mg per liter of aflatoxins B₁ and G₁ were produced by two strains of *Aspergillus flavus* in 20-liter fermentors under proper conditions of inoculum (well-dispersed growth) and aeration (0.5 volume per volume per min of air, 300 rev/min, 30 psi back pressure, baffles). Peak yields were usually attained in 72 hr, after which the aflatoxin concentration declined rapidly. Degradation of aflatoxin depended primarily on mycelial lysis and high-aeration conditions. Cultures previously reported not to degrade aflatoxin could be induced to do so under these conditions. The percentage and rate of toxin degradation were independent of toxin concentration, and appeared to be nonenzymatic and nonspecific. Degradation simulating that occurring in the fermentor was achieved by reacting aflatoxin with peroxidized methyl esters of vegetable oil; initial degradation was rapid and appeared to involve a complex series of reactions.

Aflatoxin, a mixture of at least four toxic and carcinogenic metabolites, is known to be produced by only a few fungi, *Aspergillus flavus* (18), *A. parasiticus* (5), and *Penicillium puberulum* (13). Although various methods for the biological production of aflatoxins have been reported (1, 4, 5), none of these processes has proved satisfactory for synthesis of large amounts of toxin; a maximal yield of only 30 to 50 mg per liter was reported in 5-liter fermentors by Mateles and Adye (16). We investigated aflatoxin production in 20-liter fermentors in conjunction with studies being carried out at the Northern Regional Research Laboratory on degradation of mycotoxins. These studies required larger amounts of toxin than could be produced by known methods.

Several investigators (2, 19; Hesseltine et al., Appl. Microbiol., in press) have noted a decline in aflatoxin concentration after peak yields in culture were attained. As part of our investigation, we attempted to determine the factors involved in this decline.

**MATERIALS AND METHODS**

A detailed description of the 20-liter stainless-steel fermentors and of their operation has been published (10). Because of the potential biological hazard of aflatoxins, however, we modified the fermentors to ensure a safe operation. The air effluent from the tanks was first exhausted through a stainless-steel filter packed with glass wool, then scrubbed through a Clorox bath, and finally vented into a drain that was continually flushed with water. A plastic housing was built around the exposed portion of the impeller shaft where it emerged from the fermentor, and a beaker of ammonium hydroxide was placed inside to give a saturated atmosphere of ammonia. At the end of an experiment, the fermentors, the attached stainless-steel lines, and the filters were decontaminated by flooding with Clorox until no fluorescence could be detected by ultraviolet monitoring.

Aflatoxin was recovered from the fermentation by addition of approximately 5 liters of chloroform plus 1 liter of methanol to each tank (10 liters of medium per tank) followed by vigorous agitation for about 30 min. After the chloroform layer was permitted to separate over a period of several hours, the chloroform extract was recovered by a closed-line system into carboys equipped with glass-wool filters. Extracts were concentrated to dryness with a continuous flash evaporator, and a crude preparation of aflatoxin was recovered by redissolving the dried solids in fresh chloroform.

The following strains of *A. flavus* used in our experiments were obtained from the Agricultural Research Service Culture Collection: *A. flavus* NRRL 2999, 3000, A-13570, and A-13367; the last strain has been reported in the literature as M 001 of the Tropical Products Institute. Inocula were produced in 500-ml
Erlenmeyer flasks containing 150 ml of MY broth (12). Flasks were inoculated with conidia from potato-dextrose-agar slants and incubated at 28 C for 2 days on a rotary shaker operating at 230 rev/min. From this culture, 10-ml samples were aseptically transferred to Fernbach flasks containing 700 ml of the following medium: cottonseed embryo meal, 5%; whole corn ground to a flour, 2.5%; and tap water to volume. The medium was then sterilized for 45 min at 121 C. The Fernbach flasks were incubated for 3 days at 25 C on a rotary shaker operating at 180 rev/min. Fermentors were inoculated with the contents of one Fernbach flask per tank unless otherwise noted.

In most experiments, the medium used in the fermentors was based on data obtained from the nutritional experiments of Mateles and Adye (16). Its composition was: NZ-Amine (Sheffield Chemical, Norwich, N.Y.), 0.5%; sucrose, 5%; MgSO4·7H2O, 0.1%; KH2PO4, 1%; ZnSO4·7H2O, 1.76 mg/100 ml; and tap water to 10 liters; the sterilization time was 10 min at 121 C. A silicone antifoam agent was added automatically to the fermentors on demand.

Approximately 200 ml was removed daily from the fermentors, and from these samples 25 ml was taken for analysis for aflatoxins B1 and G1. The reduced forms of these two toxins, B2 and G2, were not assayed because they constitute only a few per cent of the total toxin present. Each 25-ml portion was added to 150 ml of chloroform-methanol (7:3) in separatory funnels and sonically treated for several minutes. The chloroform extract was removed, and the remaining material was sonically treated twice more with 100 ml of chloroform. All extracts were combined and evaporated to dryness in a flash evaporator; then the residue was redissolved in a given volume of chloroform. Aflatoxin concentration was determined by thin-layer chromatography on silica gel G-HR (Brinkman Instruments, Westbury, N.Y.) with chloroform-methanol (95:5) used as developer. The fluorescence of the aflatoxin spots was compared with standards at 366 nm to obtain the degree of concentration.

Analysis for sugar was by the anthrone method and for total nitrogen by the micro-Kjeldahl method. Kojic acid was tentatively identified by spraying the thin-layer plates with a 1% solution of FeCl3·6H2O in a 0.12 n HCl.

RESULTS

Aflatoxin production. Preliminary experiments with Aspergillus flavus NRRL 3000 suggested that the type of growth in the inoculum considerably influenced fermentor yields; growth in the form of large pellets gave low toxin yields, whereas a well-dispersed, finely pelleted growth usually resulted in appreciable toxin production (Fig. 1). To achieve small pellet formation, a heavy inoculum of spores was added to the first-stage flask, and only 10 ml of the resulting growth was then added to the high-viscosity medium in the Fernbach flasks constituting the second stage; use of too heavy an inoculum in the second stage caused large pellets to form. The ratio of aflatoxin B1 to G1 was about 1:1, with B1 concentrations usually slightly higher.

The volume of inoculum added to each fermentor did not greatly influence total toxin yields, although peak production was attained a day earlier, i.e., by 48 hr, by use of a heavy charge (Fig. 2).

Preliminary studies suggested that aeration is important for good toxin synthesis. Adding baffles to the fermentors resulted in yields higher than those obtained in nonbaffled units (Fig. 3). Increasing the agitation rate to a maximum of 300 rev/min also increased aflatoxin production (Fig. 4). Increasing oxygen absorption by increasing back pressure in the tanks from .5 to 30 psi also led to higher aflatoxin yields.

The temperature optima for maximal toxin production by several strains of A. flavus were
CIEGLER ET AL.
APPL. MICROBIOL.

Fig. 2. Effect of inoculum volume on aflatoxin production. Fermentor conditions as in Fig. 1.

Fig. 3. Effect of baffles on aflatoxin production. Fermentor conditions as in Fig. 1.

Fig. 4. Aflatoxin production as a function of agitation rate and pressure. Fermentor conditions as in Fig. 1, except agitation rate was varied.

Fig. 5. Aflatoxin production and degradation by strain NRRL 3000 at various temperatures. Fermentor conditions as in Fig. 1, except the temperature varied.

amounts of toxin, whereas strain NRRL 3000 still synthesized a considerable quantity (60 mg per liter). Lower temperatures favored the production of aflatoxin G (Table 1). At 30 and narrow (Fig. 5 and 6), although strains differed in response to various temperatures. For all strains, a marked decline in production occurred at temperatures above or below 25 C. At 30 C, A. flavus A-13570 produced barely detectable
35°C, A-13570, after 24 hr of incubation, produced tremendous numbers of conidia that caused the medium to turn a deep green; at 30°C, NRRL 3000 did not produce significant quantities of conidia until after 3 days of incubation.

Several investigators have noted that there appears to be a concomitant synthesis of aflatoxin and a yellow water-soluble pigment, with the intensity of the pigment being roughly proportional to the concentration of toxin. Using *A. flavus* NRRL 3000, we found that this observation generally pertained but that pigment production increased with temperature, reaching a maximal intensity at 35°C, a temperature at which little aflatoxin could be detected. In addition, in several experiments good toxin production was achieved but color was scarcely detectable. Thus, there appears to be no metabolic connection between toxin and pigment synthesis.

Aflatoxin degradation. Peak yields of toxin and maximal culture growth are generally attained within the same period, usually 72 hr. After this length of time, often a period of variable decline in toxin concentration follows (Fig. 2, 3, 5, and 6). The variable loss from 15 to 55% suggests that the toxin was being degraded by some nonspecific mechanism; no relationship could be established between toxin concentration and percentage or rate of loss. Sonic extracts of 72-hr-old mycelia did not degrade toxin.

Strains of *A. flavus* NRRL 2999 and M 001 previously reported as nondegraders (16; Hessel-tine et al., *in press*.) actually were capable of degrading aflatoxin (both B₁ and G₁) if shear force sufficient to lyse the mycelium was developed in the fermentor (Fig. 7). Microscopic examination of the medium, after increasing impeller speed, showed primarily fragmented mycelia.

Loss of toxin occurred concomitantly with the following: a rapid rise in pH from a low of 3 to 3.9 to about 5.4 to 5.7 (the initial pH of the fermentation); a sharp increase in supernatant nitrogen, which again declined in concentration after 4 to 5 days; an initiation of mycelial lysis as determined by microscopy; a near exhaustion of available sucrose; and, occasionally, rapid production of conidia (Fig. 8).

Slow feeding of an additional 2% sucrose begun just prior to the period of toxin decline and

![Graph showing aflatoxin production and degradation by strain A-13570 at various temperatures.](http://aem.asm.org/)

**FIG. 6. Aflatoxin production and degradation by strain A-13570 at various temperatures. Fermentor conditions as in Fig. 1, except for the culture strain and temperature.**

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>15°C Amt of toxin (mg/liter)</th>
<th>20°C Amt of toxin (mg/liter)</th>
<th>25°C Amt of toxin (mg/liter)</th>
<th>30°C Amt of toxin (mg/liter)</th>
<th>Ratio G₁/B₁</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G₁</td>
<td>B₁</td>
<td>G₁</td>
<td>B₁</td>
<td>G₁</td>
</tr>
<tr>
<td>24</td>
<td></td>
<td>4</td>
<td>1</td>
<td>28</td>
<td>38</td>
</tr>
<tr>
<td>48</td>
<td></td>
<td>46</td>
<td>27</td>
<td>46</td>
<td>81</td>
</tr>
<tr>
<td>72</td>
<td></td>
<td>64</td>
<td>22</td>
<td>46</td>
<td>108</td>
</tr>
<tr>
<td>96</td>
<td></td>
<td>64</td>
<td>43</td>
<td>46</td>
<td>108</td>
</tr>
<tr>
<td>168</td>
<td></td>
<td>14</td>
<td>11</td>
<td>23</td>
<td>67</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>15°C</th>
<th>20°C</th>
<th>25°C</th>
<th>30°C</th>
<th>Ratio G₁/B₁</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>48</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>72</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>96</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>168</td>
<td>1.3</td>
<td>1.5</td>
<td>.34</td>
<td>.50</td>
<td></td>
</tr>
</tbody>
</table>

*Data based on information in Fig. 5.*

*No toxins detected for the first 72 hr of fermentation.*
FIG. 7. Aflatoxin degradation by Aspergillus flavus NRRL 2999 and A-13367 (M 001). Fermentor conditions as in Fig. 1; except for the agitation conditions.

FIG. 8. Kinetics of aflatoxin synthesis and degradation by Aspergillus flavus NRRL 3000. The same fermentor conditions as in Fig. 1, except an additional 2% sucrose was added by slow feeding where noted.

continued over a 48-hr period did not prevent loss of toxin. Nor was the loss averted by preventing the sharp rise of pH by using automatic addition of 0.1 N HCl (Fig. 9).

Rapid production of conidia during the degradation period suggested that incorporation of toxin into the spores during their formation might account for the loss of toxin. Repeated sonic treatment of washed spores recovered from the fermentors followed by solvent extraction, however, gave barely detectable amounts of toxin. The amount recovered was so small as to suggest that the toxin may have been merely a contaminating carry-over on the surface of the spores.

Several model experiments were run to determine whether aflatoxin degradation resulted from a nonspecific, coupled, enzymatic reaction. Aflatoxin B$_1$ (1 mg in 50 ml of 0.067 M phosphate buffer, pH 7.0) was incubated with the following enzyme systems: (i) glucose, glucose oxidase, and catalase (14); (ii) hydrogen peroxide and catalase (14); (iii) papain and NZ-Amine, Type B (Sheffield Chemical, Norwich, N.Y.); (iv) lipoxidase and the methyl esters of safflower oil (7); and (v) peroxidized methyl esters of soybean oil. (Enzymes were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.) Systems i through iv did not degrade any toxin, but considerable degradation was attained by system v. After preliminary experiments, system v was prepared in a Fernbach flask as follows: 500 ml of distilled water, 10 mg of aflatoxin B$_1$, 2.5 ml of peroxidized methyl esters of soybean oil, 0.5 ml of a 1:4 solution of Triton X-100 (Rohm &
Haas Co., Philadelphia, Pa.). The system was incubated at 28°C at 200 rev/min on a rotary shaker, and samples were withdrawn periodically. Within the first minute of the reaction, 70% of the toxin disappeared; thereafter, the toxin concentration increased and then decreased again with time until 28 hr, when most of the toxin had disappeared. At 24 hr, additional pale-blue fluorescing spots were observed with an $R_{Af} B_1$ of 0, 0.12, 0.25, 0.39, 0.60, and 0.78. Also, after 6 hr of incubation, a pale-yellow color appeared, which intensified with time.

**DISCUSSION**

**Aflatoxin production.** Difficulties encountered by earlier workers in producing aflatoxin in fermentors may have arisen from insufficient aeration. Present data indicate that desirable toxin yields depend not only on a well-dispersed mycelial growth, preferably in the form of very small pellets, in both inoculum and fermentor, but also on a comparatively high agitation and aeration rate, coupled with the use of baffles and high back pressure to increase oxygen absorption. It is also necessary to avoid certain conditions that favor conidia formation, i.e., excessively high agitation rates and temperatures of 30°C or higher. Once a culture is committed to conidia production, little or no toxin is synthesized. The low pH (3 to 3.9) that occurs during aflatoxin production by some strains of *A. flavus*, possibly as a result of kojic acid synthesis which we detected in all of our strains, is not correlated with toxin production; high toxin yields were attained by A-13570 even though the pH did not drop below 5.2 and remained at 5.7 for most of the fermentation.

**Aflatoxin degradation.** Degradation of aflatoxin during the course of a fermentation appears to be a complex process involving several parameters. Some authors have suggested that toxin is utilized as an energy source after available carbohydrate is exhausted. This usage appears unlikely, because adding an excess of carbohydrate after peak toxin yields were reached did not prevent degradation. Mycelial lysis does appear to be necessary for aflatoxin degradation to occur. Strains of *A. flavus* that previously did not degrade toxin could be induced to do so by fragmenting their mycelia; conversely, degraders could be prevented from destroying toxin by conducting the fermentation at either a low temperature or a low agitation rate, or both. Apparently, under these conditions the mycelium is less susceptible to lysis.

The necessity for fragmenting the mycelia to effect degradation might imply the release of a hypothetical "aflatoxinase." There are several objections, however, to the hypothesis of a specific aflatoxin-degrading enzyme: (i) aflatoxin is present in the mycelium, as well as in the supernatant fluid, but no degradation occurs unless the mycelium is fragmented; (ii) there is no correlation between concentration of toxin and rate of degradation; (iii) degradation normally occurs only over a brief time span; and (iv) sonically treated mycelium does not degrade toxin. The lack of action by sonified mycelia would appear to rule out the hypothesis that a repressed enzyme was present which was derepressed on mycelial lysis.

The frequently rapid, but brief, period of toxin loss might imply the occurrence of a coupled reaction between aflatoxin and some primary oxidizing enzyme system, as reported by Keilin and Hartree (14) for the secondary oxidation of ethyl alcohol. Although we did not obtain this reaction in vitro, its occurrence in vivo is not precluded.

Lillehoj et al. (Bacteriol. Proc., p. 5, 1966)
reported that cell walls of Flavobacterium aurantiacum actively take up aflatoxin, but in our experiments sonically treated mycelia did not remove aflatoxin on incubation. We did note a nonspecific attachment of aflatoxin B₁ to various fungal spores (unpublished data), but toxin could readily be recovered by sonic treatment of the spore-toxin complex in a solvent.

Roubal and Tappel (17) demonstrated that "free radical intermediates of polyunsaturated fatty acids undergoing peroxidation can react with proteins and enzymes to greatly modify their properties." Although incubating aflatoxin with lipoxidase and esterified fatty acids did not give a coupled oxidation, the rapid interaction of peroxidized fatty acids with aflatoxin, together with the need for a high aeration rate to achieve degradation in the fermentor, suggests that a similar nonspecific reaction may occur in the lysed mycelium. Whether this reaction involves the intermediate formation of free radicals is unknown and requires further investigation. The five blue-fluorescing compounds and the yellow color that appear with time on reacting the system are similar to the results reported by Butler and Clifford (3) for degradation of aflatoxin in the rat and by De Iongh et al. (6) in the cow. Whether a similar series of reactions occurs in these animals as noted in our in vitro experiments is unknown.

The plot obtained from the reaction of aflatoxin B₁ with the peroxidized methyl esters of the fatty acids suggests that some unstable non-fluorescing intermediate is first formed. The appearance of intensely fluorescing spots after 8 hr of incubation may result from the conversion of this unstable intermediate into a new compound or series of compounds, which, in turn, are mostly degraded after 28 hr of incubation.

Four- and five-membered lactone rings have been reported to act as alkylating agents reacting particularly with the SH group of cysteine (9). Cysteine has also been shown by Hauschka et al. (11) to react with parasorbic acid, a six-membered lactone. In agreement with the finding of Dickens (8), we noted little reaction of cysteine with aflatoxin B₁. This reaction was of particular interest, since cell rupture would release proteases that could, in turn, produce amino acids. In addition to cysteine, Zaugg (20) reported that β-lactones are capable of reacting with amine groups. The reactivity of complex lactones such as aflatoxin with amino groups, however, is unknown; we detected no reaction of aflatoxin B₁ on incubation with papain and NZ-Amine. Hence, the speculation that aflatoxin acts as a carcinogen because it is a potent alkylating agent (15) may be an unwarranted extrapolation derived from the alkylating ability of four- and five-membered lactones (8).

**Literature Cited**


