High Production of Kojic Acid Crystals by *Aspergillus parasiticus* UNBF A12 in Liquid Medium

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The crystalline compound produced in large quantity in liquid medium by *Aspergillus parasiticus* UNBF A12, a high aflatoxin-producing strain isolated from the air in the Federal District of Brazil, was identified as kojic acid. The effect of pH on the production of crystalline kojic acid and aflatoxins by the strain was studied. Fourteen single spore isolates were evaluated for their capacity to produce kojic acid crystals and aflatoxins.

In a study to determine the aflatoxin (AT)-producing ability of *Aspergillus* strains in our culture collection, a 1.0-ml spore suspension (10^6 spores) of each strain was inoculated into three 250-ml Erlenmeyer flasks containing 30 ml of a liquid YES medium (2) and incubated as standing culture at room temperature (24 to 26°C) in darkness for 7 days. The culture broth was filtered through two layers of cheesecloth into another flask and maintained under refrigeration (5 to 8°C) until further processing.

During this study, we observed that many yellowish-brown crystals were present in the flasks containing the culture filtrate of *Aspergillus parasiticus* UNBF A12 after 1 night of storage. Thin-layer chromatographic examination, performed on Silica Gel G plates using a solvent system of chloroform-methanol (9:1), revealed high levels of AT B₁, B₂, G₁, and G₂ in the filtrate but not in the crystals. The crystals were collected, dried at 80°C for 24 h, and weighed. An average of 0.5 g of crystals per flask was obtained. They were combined and purified by repeated crystallization from a mixture of water and acetone, yielding colorless long needles (melting point of 153 to 154°C). The compound gave wine-red color with ferric chloride solution and was recognized as kojic acid (KA) due to the identity of its nuclear magnetic resonance and infrared spectra with those already described in the literatures (1, 7).

KA has been reported to be produced by many species of *Penicillium* and *Aspergillus*, including strains of *A. parasiticus* (6, 7). But there are no reports that KA in the medium could reach high enough concentration to be precipitated out as crystals, as in the case of strain UNBF A12.

Two known high AT producers, *A. parasiticus* NRRL 2999 and *Aspergillus flavus* NRRL 3251, were compared with strain UNBF A12 in

relation to their ability to produce KA crystals and AT in YES medium. Under the conditions described above, no KA crystals were produced by the two NRRL strains, whereas UNBF A12 yielded an average of 15.2 mg of KA crystals per ml of culture filtrate in 7 days. Production of AT by UNBF A12 was higher than by the NRRL strains, as estimated by comparing the size and intensity of fluorescent AT spots on Silica Gel G plates developed with the chloroform-methanol solvent system.

The effect of pH on the production of KA crystals and AT by UNBF A12 was studied by adjusting the pH of the YES medium to the desired values with 0.1 N NaOH or 0.1 N HCl. The results are shown in Table 1. At pH 4.0 or 4.5, high levels of AT were produced. The pro-

TABLE 1. Effect of pH on the production of KA crystals and AT by *A. parasiticus* UNBF A12^a

pH	Mycelium (g/flask) ^b	KA (mg/flask) ^b	KA/mycelium (mg/g)	AT in broth ^c
3.0	0.877	337	384	+++
4.0	0.943	611	647	++++
4.5	0.933	697	747	++++
5.1	0.851	397	466	++
5.8	0.771	561	729	+
6.2	0.713	614	861	+
6.8	0.723	329	455	-
7.3	0.837	0	0	-
8.2	0.927	0	0	-

^a The test was performed in 250-ml flasks containing 30 ml of YES medium and incubated at room temperature for 7 days. The values shown are the average of three flasks.

^b Determined by gravimetric method after drying at 80°C for 24 h.

^c AT concentration was estimated relatively by comparing the size and intensity of fluorescent AT spots on a Silica Gel G plate after development in a mixture of CM (9:1).

duction began to decrease at pH 5.1 and no AT was detected at pH 6.8 or above. The decrease or disappearance of AT at high pH values was accompanied by the appearance of two new metabolites, one of which had an R_f of 0.39 on a thin-layer chromatographic plate (Silica Gel G developed with the chloroform-methanol solvent system) and was a yellow nonfluorescent pigment; the other compound (R_f , 0.89) was also a yellow pigment but showed red fluorescence under ultraviolet light on the thin-layer chromatographic plate. The identity of these two compounds is not known at the present time. They may be related to the biosynthetic pathway of AT. Averufin (5), a yellow pigment, as well as three other metabolites of *Aspergillus* spp. (3, 4, 8), have been shown to be involved in the biosynthesis of AT.

The production of KA by UNBF A12 at various pH values was measured by the amount of precipitated KA crystals, but not by the total amount of KA produced in the culture filtrate. The complex conditions in the culture filtrate may affect the crystallization of KA. Our data showed two optimal pH values for the production of KA crystals (4.5 and 6.2), whereas the reported optimal pH for KA formation appears to be 2 to 3, and small upward changes tend to reduce the production sharply (7).

Fourteen single spore isolates of strain UNBF A12 were obtained by the dilution plate method and were tested for their capacity to produce KA crystals and AT in YES medium. Differences in KA crystals production among the isolates were observed (Table 2). The yield of KA ranged from 11.17 to 20.93 mg/ml. However, no significant difference was noted in AT production except for isolate G, which produced slightly more AT than the other isolates.

A. parasiticus UNBF A12 was isolated from the air in the Federal District of Brazil. AT is a highly toxic compound and a potent carcinogen. KA is also toxic to animals (7). Since *Aspergillus* fungi can grow and produce toxins on a myriad of foodstuffs consumed by both humans and animals, the occurrence of this high AT- and KA-producer strain in the air poses a potentially dangerous health hazard.

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TABLE 2. Production of KA crystals and AT by single spore isolates of *A. parasiticus* UNBF A12^a

Isolate no.	KA (mg/ml)	AT (relative)
A	15.43	+
B	16.50	+
C	11.67	+
D	15.57	+
E	13.70	+
F	11.17	+
G	14.77	++
H	13.17	+
I	17.07	+
J	12.27	+
K	20.93	+
L	19.47	+
M	12.10	+
N	12.03	+

^a The test was carried out at room temperature for 7 days in YES medium. The values shown are the average of three flasks.

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