Crude Aflatoxin B₁ Production Using Maize and Rice Substrates for Animal Research

Muhsin Al Anas¹, Lies Mira Yusiati¹, Cuk Tri Noviandi¹, and Ali Agus¹.*

¹ Department of Animal Nutrition and Feed Science, Faculty of Animal Science, Universitas Gadjah Mada, Yogyakarta, Indonesia 55821
*Corresponding author. Email: aliagus@ugm.ac.id

ABSTRACT
This study aimed to develop a simple method of crude aflatoxin (AF) B₁ production for animal research. A. flavus was planted on potato dextrose agar (PDA) medium as an isolate to be grown on maize and rice substrates at a concentration of 10⁷/mL. Maize was added to water until the water content reached 25%, while rice was soaked in water at a ratio of 1 mL of water for 2 g of rice for 1 hour. The rice and maize were sterilized using an autoclave. A total of 250 g of each substrate was inoculated with A. flavus FNCC 6122. Incubation was carried out for 6 days at 30°C at a humidity of 80-90%. On the second day of the incubation period, 1 mL of water was added to the substrates. At the end of the incubation period, crude AF samples were analyzed by enzyme-linked immunosorbent assay (ELISA). The results showed that level of AFB₁ on rice higher than maize (P<0.001). The AFB₁ production was 3229 µg/kg in rice and 1153 µg/kg in maize. Rice and maize can be used as a substrate source for crude AFB₁ production for animal research.

Keywords: Aflatoxin B₁, Production, Substrat, Maize dan Rice

1. INTRODUCTION

Aflatoxin is a major problem in the livestock industry and is produced by Aspergillus flavus and Aspergillus parasiticus. It causes low productivity and serious economic losses [1,2]. Aflatoxin contaminates many agricultural and food products [3,4,5]. In addition, aflatoxin can leave a residue in livestock products such as milk, meat and eggs [6,7,8,9]. Aflatoxin residue in foodstuffs is dangerous if consumed by humans [10,11].

Aflatoxin is classified as a carcinogenic agent by the International Agency for Research on Cancer [12]. Aflatoxin given to chickens at low doses does not cause death but causes a decrease in body weight and changes in feed conversion [13]. Increased levels of aflatoxin in feed can reduce body weight by 5% in broiler chickens [14]. The administration of aflatoxin at even a dose of 500-1000 µg/kg can cause liver damage and biochemical changes in the blood serum of broiler chickens [15,1,7]. Aflatoxin found in feed at levels from 50-100 mg/kg can leave residue in the liver and meat at levels from 0.40-1.02 µg/kg [16,7] that is dangerous if consumed by humans.

Aflatoxin is a global problem especially in tropical and subtropical regions with high temperature and humidity that suitable for fungal growth. Climate change increases the production of aflatoxin in various parts of the world, including Europe which is a subtropical country [17]. Based on the results of the BIOMIN [18] survey of mycotoxin contamination in agricultural commodities (corn, wheat, barley, including animal feed), North America and East Asia were the regions with the highest risk of contamination (80-92% of samples polluted), followed by Africa, South America, Central America, and Southeast Asia (the level of product contamination reaches 60-72%). The lowest risk of mycotoxin contamination is in the European region with a rate of contamination of 37-67%. In Southeast Asia, aflatoxin contamination in agricultural products reaches 81%.

Researchers have been investigating aflatoxin to find preventive and mitigating techniques for its negative effects. Research on the effects of aflatoxin on livestock has continued to develop from 1965 to the present [19,20,21]. However, the availability of aflatoxin is often a problem for animal research. Pure aflatoxin is expensive, so a simple method for aflatoxin production is needed to support the development of research, particularly that done in animals. This study aims to find
a simple method to produce crude aflatoxin that can be used for animal research.

2. MATERIALS AND METHOD

2.1. Experimental Site

The study was carried out at the Laboratory of Nutritional Biochemistry in the Department of Animal Nutrition and Feed Science (Faculty of Animal Science, Universitas Gadjah Mada, Yogyakarta, Indonesia). Analysis of AFB$_1$ was conducted in the Research Unit for Natural Product Technology (BPTBA) (Indonesia Institute of Science (LIPI), Yogyakarta, Indonesia).

2.2. Materials and Research Tools

Maize, rice, *A. Flavus* FNCC 6122 (Pusat Antar Universitas, Universitas Gadjah Mada), potato dextrose agar (Pronadisa, CONDA), an ELISA (*enzyme-linked immunosorbent assay*) kit (Romer Labs, Singapore), Tween 80 (Merck), methanol (Merck), a Model KT-2322 water bath (ALP Co., Ltd. Tokyo, Japan), and a Multiskan™ FC Microplate Photometer (Thermo Scientific™) were used for the experiments.

2.3. Research procedure

- Substrate preparation to reach 25% water content
- Sterilization of substrate using an autoclave (121°C, 105 atm)
- Inoculation substrate with *A. flavus* isolate, 250 g per plastic box
- Incubation at 30°C and 80-90% humidity for 6 days
- Day 2, addition of 1 mL of water to the substrate
- Sterilization of substrate using autoclave, and AFB$_1$ analysis at day 6

Figure 1. *A. flavus* growth procedure in maize and rice substrates for the production of crude aflatoxin B$_1$

*A. flavus* was grown on potato dextrose agar (PDA) medium as an initial culture for the production of AF on substrates. *A. flavus* was incubated for 5-7 days at 30°C. Fungal colonies were harvested using 8 mL of a sterile 2% Tween 80 solution and vortexed for 30 seconds. There were $10^6$-$10^7$ spores in the solution. Aflatoxin was produced using maize and rice as substrates. *A. flavus* was used to inoculate maize and rice in mash. Sterile distilled water was added until the water content of the maize was 30%, while the rice was soaked in 1 mL of water for every 2 g of rice. After their preparation, the maize and rice were sterilized using an autoclave for 15 minutes at 121°C and 105 atm. Two hundred fifty grams of each substrate were placed in a plastic jar, with 5 replicates prepared for each substrate. The substrates were inoculated using 1 mL of the *A. flavus* mixture dissolved in Tween 2% and then mixed. The substrates were incubated at 30°C for 5 days. On the second day, 1 mL of distilled water was added to the substrates, which were then mixed. Crude aflatoxin was harvested on day 6. The substrates were sterilized in an autoclave to stop spore growth. Before aflatoxin analysis, the substrates were dried in an oven at 105°C. The procedure for crude AFB$_1$ production is shown in Figure 1.

2.4. Parameters Measured

The samples were ground using a Willey mill 2 mm screen. A total of 5 g of each sample was put into a 50 ml test tube and extracted by adding 25 ml of 70% methanol (1:5, v:v). The tubes were shaken for 3 minutes using a vortex and then filtered using Whatman filter paper number 1. The extracted samples were ready to be used for testing by ELISA (*enzyme-linked immunosorbent assay*).

2.5. Statistical Analysis

Data were analyzed using a *t*-test to compare the levels of AFB$_1$ produced by *A. flavus* grown on rice and maize substrates.

3. RESULT AND DISCUSSION

3.1. Growth of *A. flavus* observed from days 1 to 6

The observed *A. flavus* growth on the substrates is shown in Figure 2. The growth of *A. flavus* on the substrate surfaces began with white spores on day 3. Spore growth started at the incubation sites at the bottom of the substrates. On day 4, the spores turned yellow. The *A. flavus* began to turn green on day 5, starting at the bottom of the substrates. All of the substrates were overgrown with green *A. flavus* on day 6. The first research on aflatoxin production was conducted by Shotwell *et al*., [26] using rice substrate inoculated with *A. flavus* strain NRRL 2999 obtained from Ugandan beans. Observations showed that the growth of *A. flavus* fungi began with white spores, which then became yellow, and ended with green growth.

The result shows that *A. flavus* grown on the rice substrate was greener than that grown on the maize substrate. *A. flavus* grown on the maize substrate had a brown/green color, while that grown on the rice substrate had a dark green color. Iheanacho *et al*., [23] and Ren *et al*., [24] also reported the same result showing that *Aspergillus flavus* grown on both substrates turned green between days 5 and 6.
AFB₁ production by *A. flavus* grown on the rice and maize substrates is shown in Figure 4. The production of aflatoxin by *A. flavus* grown on the rice substrate was higher than that by *A. flavus* grown on the maize substrate (P<0.001). The AFB₁ levels from *A. flavus* grown on the rice substrate were 3229 µg/kg, and the AFB₁ levels from *A. flavus* grown on the maize substrate were 1153 µg/kg. Aflatoxin production is mainly affected by temperature and humidity, and the optimal temperature for fungal growth is 25-30°C. Schmidt-Heydt et al., [26] showed that the expression of *afIR* increased at 37°C compared to that at other temperatures. In addition, aflatoxin growth is also influenced by light, carbon and nitrogen sources, pH, and plant metabolites [28].

Each *Aspergillus* strain has a different genetic ability to produce AF (toxigenic potential). Probst et al., [29] isolated 4469 *A. flavus* sect. Flavi isolates from 339 samples, and Khodavaisy et al., [30] isolated 143 *A. flavus* isolates; the results of these experiments showed that each strain of *A. flavus* had a different toxigenic potential. In general, the *afIR* and *afIS* genes play a role in AF production. The *afIR* gene is needed for the activation of many genes in the AF synthesis pathway. Moreover, the overexpression of *afIR* can increase transcription in the aflatoxin pathway. AF biosynthesis is also regulated by the *afIS* gene, although the role of *afIS* is not known in depth. The deletion of the *afIS* gene did not have a major impact on the expression of the aflatoxin pathway genes [31,32].

Many studies have used rice as a substrate to produce crude AFB₁ [33,34,35,36]. However, this depends on the level of AFB₁ production, and the low production of AFB₁ by *A. flavus* grown on rice substrate would cause problems for feed formulations used for research that requires high aflatoxin levels. Therefore, maize and rice can be used as alternative substrates to produce AFB₁.

### 3.2. Production of AFB₁ by *A. flavus* Grown on Different Substrates

The results of *A. flavus* growth on the maize and rice substrates are shown in Figure 3. On the rice substrate, *A. flavus* was greener in color than that grown on the maize substrate. *A. flavus* grown on the maize substrate had a brown/green color. Yu *et al.* [25]; Schmidt-Heydt *et al.*, [26]; Chen *et al.*, [27] described that the ability of *A. flavus* to produce AF is influenced by various factors that can be divided into two groups consisting of internal and external factors. Internal factors include genetics, which play a role in the production of AF. External factors consist of moisture, temperature, and the nutrient level of the substrate.
REFERENCES


