Crude Aflatoxin B_1 Production Using Maize and Rice Substrates for Animal Research

Muhsin Al Anas[1], Lies Mira Yusiati[1], Cuk Tri Noviandi[1], and Ali Agus[1,*]

[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) B_1 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 AFB_1 on rice higher than maize ($P<0.001$). The AFB_1 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 AFB_1 production for animal research.

Keywords: *Aflatoxin B_1, Production, Substrat, Maize dan Rice*

1. INTRODUCTION

Aflatoxin is a major problem in the livestock industry and is produced by *Aspergillus flavus* and A*spergillus 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.

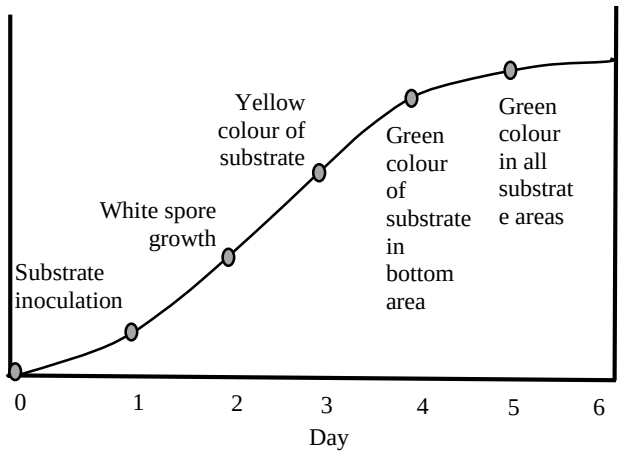

Figure 2. Qualitative measurement of fugal growth on substrates

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.

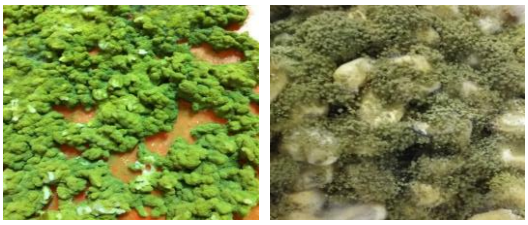

Figure 3. Final growth of *A. flavus* at day 6 onward (A) maize and (B) rice

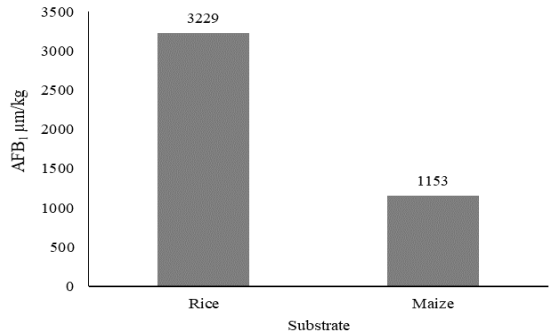

Figure 4. Aflatoxin B_1 production by *A. flavus* grown on rice and maize substrates

AFB_1 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 AFB_1 levels from *A. flavus* grown on the rice substrate were 3229 µm/kg, and the AFB_1 levels from *A. flavus* grown on the maize substrate were 1153 µm/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 *afl*S 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 *afl*R and *afl*S genes play a role in AF production. The *afl*R gene is needed for the activation of many genes in the AF synthesis pathway. Moreover, the overexpression of *afl*R can increase transcription in the aflatoxin pathway. AF biosynthesis is also regulated by the *afl*S gene, although the role of *afl*S is not known in depth. The deletion of the *afl*S 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 AFB_1 [33,34,35,36]. However, this depends on the level of AFB_1 production, and the low *production* of AFB_1 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 AFB_1.

4. CONCLUSION

Aflatoxin B_1 production was 3229 µg/kg by *A. flavus* grown on rice substrate and 1153 µg/kg by *A. flavus* grown on maize substrate, so rice and maize can be used as substrate sources for the production of crude AFB_1. Rice has great potential to produce crude AF, but it is necessary to consider its effect on the nutrient content of animal feed.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the Graduate Program of the Faculty of Animal Science at Universitas Gadjah Mada, which provided a research grant for graduate students in 2017.

REFERENCES

[1] Yang J., Bai F., Zhang K., Bai S., Peng X., Ding X., Li Y., Zhang J., and Zhao L. Effects of feeding corn naturally contaminated with aflatoxin B_1 and B2 on hepatic functions of broilers. Poult. Sci. 91, (2012)

[2] Bryden W.L. Mycotoxin Contamination of the Feed Supply Chain. In: Implications for animal productivity and feed security. Anim. Feed Sci. Tech. 173, 1-2 (2012)

[3] Razzazi-Fazeli E., Noviandi C.T., Porasuphatana S., Agus A., and Böhm J. A survey of aflatoxin B_1 and total aflatoxin contamination in baby food, peanut and corn products sold at retail in Indonesia analysed by ELISA and HPLC. Mycotoxin Res. 20, 2 (2004)

[4] Noviandi C.T., Razzazi-Fazeli E., Agus A., Böhm J., Hulan H.W., Wedhastri S., Maryudhani Y.B., Nuryono., Sardjono., and Leibetseder J. Natural occurrence of aflatoxin B_1 in some Indonesian food and feed products in Yogyakarta in year 1998-1999. Mycotoxin Res. 17, 2 (2001)

[5] Oliveira M.S., Rocha A., Sulyok M., Krska R., and Mallmann C.A. Natural mycotoxin contamination of maize (Zea mays L.) in the South region of Brazil. Food Control. 73, (2017)

[6] Nuryono N., Agus A., Wedhastri S., Maryudani Y.B., Sigit Setyabudi F.M.C., Bohm J., and Razzazi-Fazeli E. A limited survey of aflatoxin M1 in milk from Indonesia by ELISA. Food Control. 20, (2009)

[7] Magnoli A.P., Monge M.P., Miazzo R.D., Cavaglieri L.R., Magnoli C.E., Merkis C.I., Cristofolini A.L., Dalcero A.M., and Chiacchiera S.M. Effect of low levels of aflatoxin B_1 on performance, biochemical parameters, and aflatoxin B_1 in broiler liver tissues in the presence of monensin and sodium bentonite. Poult. Sci. 90, (2011)

[8] Herzallah S.M. (2013). Aflatoxin B_1 residues in eggs and flesh of laying hens fed aflatoxin B_1 contaminated diet. Am. J. Agric. Biol. Sci. 8, 2 (2013)

[9] Herzallah S.M., Al-Ameiri N.S., Al-Dmoor H., Masoud S., and Shawabkeh K. Meat and organs quality of broiler chickens fed diet contaminated with B_1 aflatoxin. Glob. Vet. 12, (2014)

[10] Partanen H.A., El-Nezami H.S., Leppanen J.M., Myllynen P.K., Woodhouse H.J., and Vakangas K.H. Aflatoxin B_1 transfer and metabolism in human placenta. Toxicol.Sci.. 113, 1 (2010)

[11] Harutyunyan T., Hovhannisyan G., Babayan N., Othman M.A., Liehr T., and Aroutiounian R. Influence of aflatoxin B_1 on copy number variants in human leukocytes in vitro. Molecular Cytogenetics. 8, 25 (2015)

[12] International Agency for Research on Cancer (IARC). Some traditional herbal medicines, some mycotoxins, naphthalene and styrene. Summary of data reported and evaluation. IARC Monogr. Eval. Carcinog. Risks Hum. 82, (2002).

[13] Denli M., Blandon J.C., Guynot M.E., Salado S., and Perez J.F. Effects of dietary AflaDetox on performance, serum biochemistry, histopathological changes, and aflatoxin residues in broilers exposed to aflatoxin B_1. Poult. Sci. 88, 7 (2009)

[14] Dersjant-Li Y., Verstegen M.W., and Gerrits W.J. The impact of low concentrations of aflatoxin, deoxynivalenol or fumonisins in diets on growing pigs and poultry. Nutr. Res. Rev. 16, (2003)

[15] Eraslan G., Essiz D., Akdogan M., Karaoz E., Oncu M., and Ozyildiz Z. Efficacy of dietary sodium bentonite against sub- chronic exposure to dietary aflatoxin in broilers. Bull Vet. Inst. Pulawy. 50, (2006)

[16] Bintvihok A., and Kositcharoenkul S. Effect of dietary calcium propionate on performance, hepatic enzyme activities and aflatoxin residues in broilers fed a diet containing low levels of aflatoxin B_1. Toxicon. 47, 1 (2006)

[17] Battilani P., Toscano P., Van der Fels-Klerx H.J., Moretti A., Leggieri M.C., Brera C., Rortais A., Goumperis T., and Robinson T. Aflatoxin B_1 contamination in maize in Europe increases due to climate change. Nature. 6, (2016)

[18] Biomin. World mycotoxin survey 2017. Annual report No. 14, (2017)

[19] Sargeant K., Sheridan J., O'Kelly J., and Carnaghan R.B.A. Toxicity associated with certain samples of groundnuts. Nature. 192, (1961)

[20] Kensler T.W., Roebuck B.D., Wogan G.N., and Groopman J.D. Aflatoxin: A 50-year odyssey of mechanistic and translational toxicology. Toxicol. Sci. 120, (S1) (2011)

[21] Mauro A., Garcia-Cela E., Pietri A., Cotty P.J., and Battilani P. Biological control products for aflatoxin prevention in Italy: Commercial field evaluation of atoxigenic Aspergillus flavus active ingredients. Toxins. 10, 30 (2018)

[22] Shotwell O.L., Hesseltine C.W., Stubblefield R.D., and Sorenson W.G. Production of aflatoxin on rice. J. Appl. Microbiol. 14, 3 (1966)

[23] Iheanacho H.E., Njobeh P.B., Dutton F.M., Steenkamp P.A., Steenkamp L., Mthombeni J.Q., Daru B.H., and Makun A.H. Morphological and molecular identification of filamentous Aspergillus flavus and Aspergillus parasiticus isolated from compound feeds in South Africa. Food Microbiol. 44, (2014)

[24] Ren S., Yang M., Li Y., Zhang F., Chen Z., Zhang J., Yang G., Yue Y., Li S., Ge F., and Wang S. Global phosphoproteomic analysis reveals the involvement of phosphorylation in aflatoxins biosynthesis in the pathogenic fungus Aspergillus flavus. Scientific Reports. 6, (2016)

[25] Yu J., Chang P.K., Cary J.W., Wright M., Bhatnagar D., Cleveland T.E., Payne G.A., and Linz J.E. Comparative Mapping of Aflatoxin Pathway Gene Clusters in Aspergillus parasiticus and Aspergillus flavus. Appl. Environ. Microbiol. 61, 6 (1995)

[26] Schmidt-Heydt M., Rüfer C.E., Abdel-Hadi A., Magan N., and Geisen R. The production of aflatoxin B_1 or G1 by Aspergillus parasiticus at various combinations of temperature and water activity is related to the ratio of aflS to aflR expression. Mycotoxin Res. 26, 4 (2010)

[27] Chen Z-Y., Rajasekaran K., Brown R.L., Sayler R.J., and Bhatnagar D. Discovery and confirmation of genes/proteins associated with maize aflatoxin resistance. World Myco. J. 8, 2 (2015)

[28] Calvo A.M., and Dhingra S. Aflatoxins Biochemistry and Molecular Biology. In: Conserved Regulatory Mechanisms Controlling Aflatoxin and Sterigmatocystin Biosynthesis, Aflatoxins Ramón Gerardo Guevara-González. IntechOpen. 4, (2011)

[29] Probst C., Bandyopadhyay R., and Cotty P.J. Diversity of aflatoxin-producing fungi and their impact on food safety in sub-Saharan Africa. Int. J. Food Microbiol. 174, (2014)

[30] Khodavaisy S., Badali H., Rezaie S., Nabili M., Moghadam K.G., Afhami S., Hagen F., Aala F., Hashemi S., and Meis J.F. Genotyping of clinical and environmental Aspergillus flavus isolates from Iran using microsatellites. Mycoses. 59, 4 (2016)

[31] Meyers D.M., Obrian G., Du W.L., Bhatnagar D., and Payne G.A. Characterization of aflJ, a gene required for conversion of pathway intermediates to aflatoxin. Appl. Environ. Microbiol. 64, 10 (1998)

[32] Chang P.K. The Aspergillus parasiticus protein AFLJ interacts with the aflatoxin pathway-specific regulator AFLR. Mol. Genet. Genom. 268, 6, (2003)

[33] Hussain S., Khan M.Z., Khan A., Javed I., and Asi M.R. Toxico-pathological effects in rats induced by concurrent exposure to aflatoxin and cypermethrin. Toxicon. 53, 1 (2009)

[34] Magnoli A.P., Monge M.P., Nazar F.N., Magnoli C.E., Cavaglieri L.R., Bagnis G., Dalcero A.M., and Marin R.H. Combined effects of aflatoxin B_1 and corticosterone treatment on selected performance indices and liver histopathology in Japanese quail. Poult. Sci. 91, 2, (2012)

[35] Cravens R.L., Goss G.R., Chi F., De Boer E.D., Davis S.W., Hendrix S.M., Richardson J.A., and Johnston S.L. The effects of necrotic enteritis, aflatoxin B_1, and virginiamycin on growth performance, necrotic enteritis lesion scores, and mortality in young broilers. Poult. Sci. 92, 8 (2013)

[36] Neeff D.V., Ledoux D.R., Rottinghaus G.E., Bermudez A.J., Dakovic A., Murarolli R.A., and Oliveira C.A. In vitro and in vivo efficacy of a hydrated sodium calcium aluminosilicate to bind and reduce aflatoxin residues in tissues of broiler chicks fed aflatoxin B_1. Poult. Sci. 92, 1 (2013)