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Isolation and characterization of aflatoxigenic *Aspergillus* spp. from maize of livestock feed from Bogor

D Sukmawati [1]*, A Setyaningsih[1], T Handayani K[1], S Rahayu[1], Y Rustam[1], M Moersilah[2], P Wahyudi[3] and S N A Husna[4]

[1] Department of Biology, Faculty of Mathematics and Sciences, State University of Jakarta, Jakarta, Indonesia
[2] Department of Chemistry, Faculty of Mathematics and Sciences, State University of Jakarta, Jakarta, Indonesia
[3] BPPT, Jakarta, Indonesia
[4] Department of Microbiology, School of Life Sciences and Technology, Institut Teknologi Bandung, Bandung, Indonesia

*sukmawatidalia@gmail.com

Abstract. Aflatoxin is a naturally mutagenic and carcinogenic mycotoxin found in feed and food. Aflatoxin contamination on maize can affect productivity of feed and food manufacture. The purpose of this study was to obtain isolates and to understand the characteristics of aflatoxigenic *Aspergillus flavus* from maize on livestock feed. The study was divided into two stages: isolation and molecular identification of fungal ITS rDNA region in livestock feed obtained from Bogor, West Java. Isolation was conducted by enrichment and direct method using Dichloran-Glycerol (DG18) medium, while aflatoxin test and fungal characterization were done by CAM (Coconut Agar Medium) and selective medium of Aspergillus flavus and parasiticus (AFPA), respectively. The result showed that 9 of isolates are identified as molds (P2, P3, P4, P5, P7, P8, are green sporulated, while P1, P6, P9 are black sporulated). Aflatoxin detection on P3 and P8 isolates did not produce blue fluorescence fluid in CAM and did not form a beige ring on the back of petri. This indicated P3 and P8 did not produce aflatoxin on CAM media. Molecular identification results that P3 and P8 isolates have 100% and 99% homology with A. *flavus*, respectively.

1. Introduction

Aflatoxin is classified as highly toxic chemicals found on agricultural crops, feed and food sources, especially in chicken feed. Aflatoxin can cause mutagenic, carcinogenic, teratology, and immunosuppressive [1] ; [2]. Aflatoxin-producing mold, grouped into the genus of *Aspergillus* [3]. Aflatoxin is a type of mycotoxin which are mostly produced by group of Aspergillus spp., such as *Aspergillus flavus*, *Aspergillus parasiticus* [4] ;[5], *Aspergillus nomius* [6], and *Aspergillus pseudotamarii*.

Fungi is an eukaryotic organism that can be found in various substrates, such as in soil, plants, and food products [7];[8]. *A. flavus* and *A. parasiticus* are mostly found in seeds, nuts and corn. Contamination in feeds are usually found in poultry ration, such as local maize [9]. toxins in grains such as corn occur at 4˚C - 40˚C with optimum growth at 25˚C - 32˚C, as well as at water content 18% and relative humidity 85% [3].

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Certain fungal growth can cause physical and chemical damage to the feed. Physical damage can be seen by the changing of colors, shapes, and ingredients of the substrate because of the fungal growth. Chemical damage occurs by the contamination of aflatoxin produced by the fungi itself. Recent research showed that storage is the key problem of livestock rations contamination, since keeping the humidity in the area for a long time can stimulate the fungal growth. It is known that mycotoxins result in declining health and livestock productivity. The objective of this research is to detect the presence of *A. flavus* on corn pig feed. The detection are done using Coconut Agar Medium (CAM) and molecular identification using Internal Transcribed Spacer (ITS) rDNA region. This research is expected to present suggestions for the local farmers to avoid aflatoxin contamination in animal feed ingredients.

2. Methods

2.1. Fungal Isolation and morphological identification

Animal feed samples were obtained from livestock feed shop located in Bogor, West Java, Indonesia. 10 grams of decayed corn were obtained randomly by purposive sampling method.

Fungal isolation was done by dilution and direct method. For dilution sampel was incubated in medium Potato Dextrosa Agar (PDA) at 28˚C 2 days [10]. Fungal isolation by direct method was conducted using DG18 medium and incubated at 28˚C for 3 days. The activated fungal culture was inoculated into an AFPA selective medium and further incubated for 2-3 days to obtain the isolates. Positive isolate of *A. flavus* is characterized morphologically by the presence of yellow pigmentation on the reverse colony. Macroscopic and microscopic morphological characters are using Malt medium Agar extract (MEA) [11;12; 13].

2.2. Molecular identification of isolates

Fungal isolates are grown in PDB at room temperature for 2 days. For the purpose of DNA isolation, a total of 1 ounce mycelium is used. DNA isolation was using *Gneaid* kit, according to the manufacturer's instructions. PCR reaction was performed using *Go Taq Mastermix* (Promega) with final volume of 25 µl of each reaction contained 9 µl of *Nuclease Free Water* (*NFW*), 12.5 µl of *Gotaq Green Mastermix* (promega), 0.5 µl of primary ITS 4 (5'-TCCTCCGCTTATTGATATGC-3 '), 0.5 µl of primary ITS 5 (5'-GGAAGTAAAAGTCGTAACAA-3 ') and 0.5 µl DNA template. PCR reaction was done after 40 cycles and was set into optimum condition, consisting of pre-denaturation stage 95˚C for 4 minutes, denaturation 92˚C for 1 minute, annealing 51,3˚C for 1 minute, 72˚C amplification for 2 minutes and final extension 72˚C for 5 minutes. The DNA sequences are then analyzed using general approach Basic Local Alignment Search Tool Algorithm (BLAST) program which can be accessed at GenBank (www.ncbi.nlm.nih.gov). The DNA sequences are then compared with the existing database for further analysis using MEGA 6.0 program to constract phylogenetic tree based on the Neighbour Joining (NJ) method.

2.3. Detection of aflatoxin production from isolates

Fungal isolates identified as *A. flavus* were inoculated into CAM and were incubated at 26 ° C for 5-7 days [14]. Aflatoxin detection in CAM was obtained by fluorescence method under 365nm UV light. Aflatoxin production can be detected in the presence of blue fluorescence when exposed to UV rays and beige rings on the back of Petri [15]; [6]. It is common method to detect aflatoxin in Aspergillus spp. using CAM.

3. Result and disccusion

3.1. Fungal isolates

Corn kernel samples are obtained from a cattle feed store located in Bogor, West Java, Indonesia. The isolation was carried out using Dichloran Glycerol-18 (DG18) medium using enrichment method and followed by 10^{-1} and 10^{-2} dilution with duplication. The isolation was also performed by direct method

in which the direct maize samples were grown on medium DG18. DG18 medium can be used as a standard medium for fungal isolation in samples having low *aw* values [16]. DG18 medium has an adequate nutrient for the isolation and calculation of the amount of fungi produced from dried foods, such as fruits, spices, cereals, nuts, meat, and fish products [17].

DG18 medium has the main composition of 18% glycerol and dichloran. The use of 18% glycerol aims to decrease the water activity level in the sample to increase the growth of fungal colonies. The use of dichloran is as an antifungal agent that limits the distribution and size of the fungal colonies obtained [4]. The isolate culture are then purified using PDA medium. 9 fungal isolates were obtained with different sporulation characteristics, for instance green sporulation and black sporulation (table 1 and figure 2). that fungal contamination such as *Aspergillus* spp. and *Penicillium* spp. can be found in feed and feed ingredients such as corn [18].

The purified isolates were further inoculated and grown on selective medium for *A. flavus* and *A. parasiticus* using AFPA medium. *A. flavus* from Seameo-Biotrop collection was used as positive control. Fungal isolate and positive control of *A. flavus* were grown on AFPA medium for 3 days at 28˚C. AFPA medium is a selective medium for isolates of *A. flavus* and *A. parasiticus* where both fungi are classified into aflatoxin-producing fungi [19].

Table 1. Fungal isolates from corn kernel after purification in PDA medium, incubated at 28°C for 7 days.

Isolate	UNJCC Number	Morphology colony of Molds	Number of isolate
P2, P3, P4, P5, P7 and P8	F-8, F-9, F-10, F-11, F-12, and F-13, respectively	Green-sporulated granules	6
P1, P6, and P9	F-14, F-15, F-16, respectively	Black-sporulated granules	3

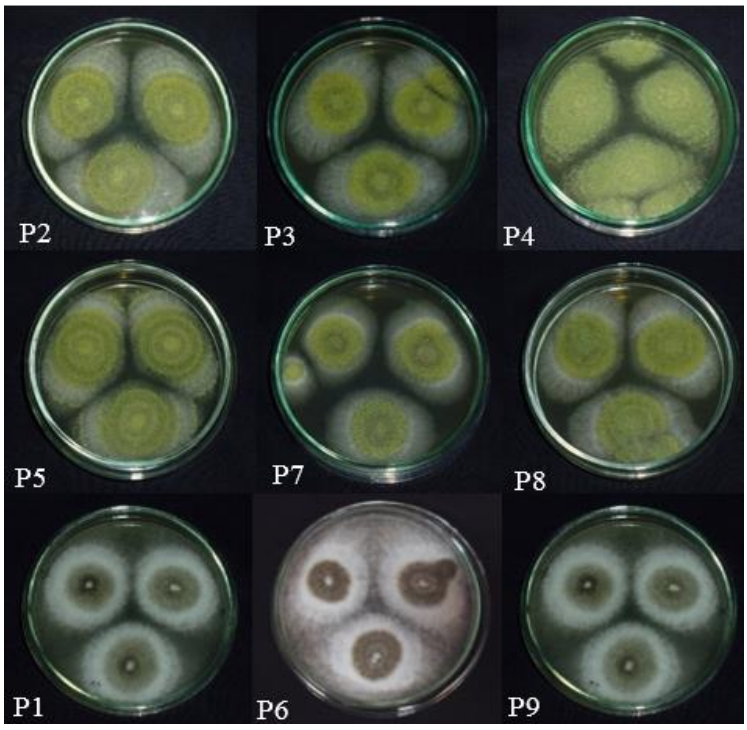

Figure 1. Fungal isolation from maize on PDA medium incubation under condition of 28°C for 7 days. Isolates of green sporulated: P2, P3, P4, P5, P7, and P8; as well as black sporulated isolates: P1, P6, and P9.

Positive results on the AFPA medium shows the yellow pigmentation color of the reverse on the colony. The negative results on the AFPA medium shows white on the colony's inverse. That the yellow pigmentation is produced from the reaction of Fe with aspergilic acid. The resulting Fe content comes from the compound contained in the AFPA medium, Ferric Ammonium Sulfate. Aspergilic acid is the result of metabolites released by *A. flavus.*

Detection of Aflatoxin in the isolates using AFPA selective medium resulted on two isolates verging *A. flavus* morphology, which are P3 and P8 isolate. These two isolates have reversed yellow pigmentation of the colony and the radial furrow as shown in positive control. Negative results were found in isolates that had black sporulation, one of which are in the P1 isolate (figure 1).

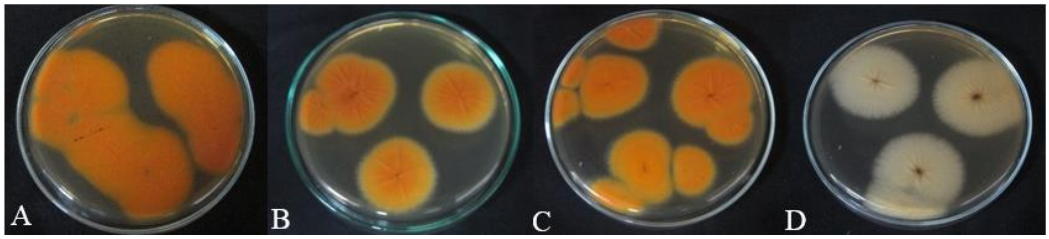

Figure 2. Detection of *A. flavus* using AFPA selective medium resulted in pink yellow pigmentation at (A) isolate of *A.flavus control*, (B) P3 isolate, (C) P8 isolate and (D) P1 isolate.

3.2. Fungal identification on corn kernel feed

3.2.1. Morphological identification on fungal isolates. Morphological identification on isolates was performed by observing the isolates characteristics using a 7 day aged MEA medium and incubated at 28˚C [13]. A macroscopic observation of 6 isolates of green spherical isolates (P2, P3, P4, P5, P7 and P8) contained two isolates characterized by A. flavus. P3 and P8 isolates have similar macroscopic characteristics to A. flavus. The macroscopic morphological observation of P3 showed a green colored colonies with white mycelium at the edges with an average colony diameter of 40.77 mm. The texture of the granular colony, has a growing zone, and has no exudate drops and radial furrow (figure 4). Macroscopic morphological observations of P8 isolate showed grass green colored colonies in which both had white mycelium on the edges of colonies and an average colony diameter of 37.13 mm. Texture of its granular colony has a glowing zone and has no exudate drops and radial furrow (figure 4).

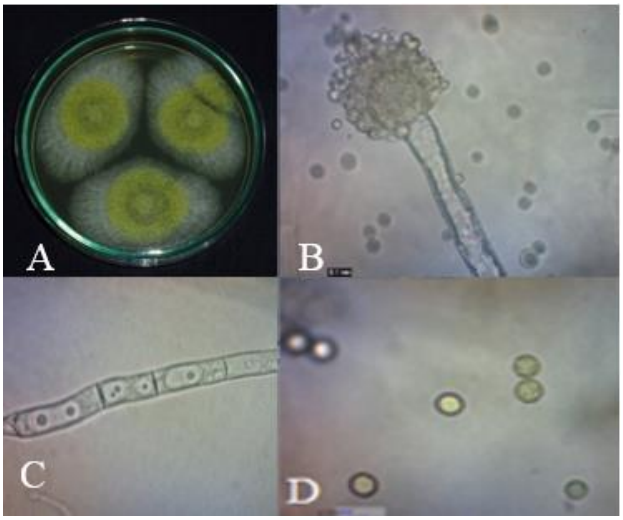

Figure 3. Isolate P3 on MEA medium, incubated at 28°C for 7 days. (A) P3 isolate, (B) 1000x magnification conidiophore, (C) hyphe (D) Spore.

A. flavus has morphology with dark green in colony, white mycelium, floccose colony texture and reverse colony color is not pale yellow [11]. These characteristics are consistent with the isolates P3 and P8. Microscopic morphological observations on P3 showed conidial heads in radiate form, and at the tip of conidiophore there is a uniseriate type vesicle with a width of 25.49 μm (figure 3). Isolate P3 is contained fialid, has a coarse conidiophor texture and has a hyphenated hyphae. Microscopic morphological observations on P8 isolate showed conidial heads radiate, and at the end of conidiophor are found uniseriate vesicles with 29.07 μm width (figure 4).

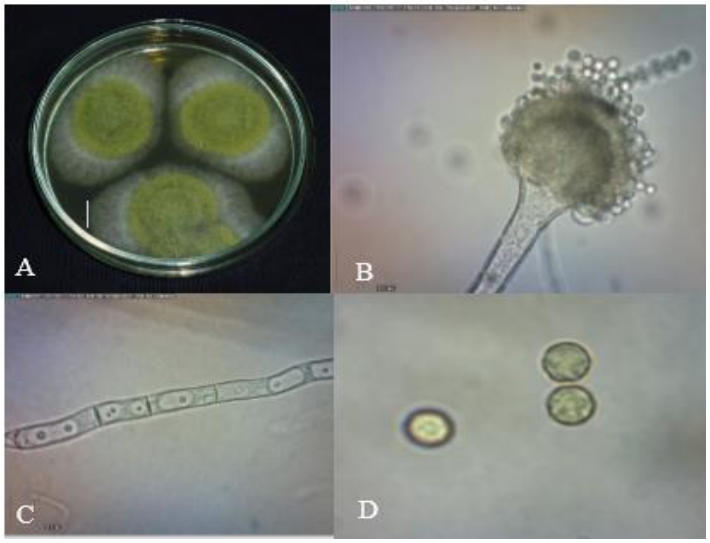

Figure 4. Isolate P8 on MEA medium, incubating 28°C for 7 days. (A) a macroscopic morphology,(B) a 1000x magnification conidiofor, (C) a hyphen hypha, (D) a conidia of 400x magnification.

The P8 mold isolate is fialid, has a coarse conidiophor texture and has a hyphenated hyphae. Microscopic morphological observations of P3 and P8 isolates are similar to Klich and Pitt (1988) and Afzal et al., (2013) that *A. flavus* has a type of biseriate vesicles on CYA medium and often found uniseriate type on MEA medium. In uniseriate vesicles only fialid for mis found, whereas in biseriate type there are metula and fialid. Head of conidia is radiate to columnar, has fialid and metula in on the vesicles, has a coarse texture in conidiophore, and the vesicle width ranges from 12-85 μm.

3.3. Molecular identification of Fungal Isolates using ITS rDNA region

Isolate of P3 and P8 are used for further identification. This selection is based on detection results by AFPA selective medium and molecular identification. Extracted DNA from isolate P3 and P8 were amplified using ITS 4 and 5, and then were sequenced in one direction using ITS 4 primers. That primary ITS 4 produces 468bp base in length. The sequencing result using primer ITS 4 on P3 isolate and P8 isolate resulted nucleotide base sequence 472 bp and 507bp respectively. ITS rDNA of Ascomycota group has 500bp of nucleotides in length [20]. The sequence results of P3 and P8 isolate are then compared to the nucleotide base sequence stored in the National Center for Biotechnology Information (NCBI) genotype by using the Basic Local Alignment Search Tool (BLAST) program to identify closely related species of P3 and P8 isolates.

The sequence alignment showed that P3 isolate has the highest sequence homology with *A. flavus* var. *flavus* ATCC 16883 with the sequence similarity of 100%. The BLAST results of P8 isolate indicated the highest sequence homology with *A. flavus* var isolates. *flavus* and *A. flavus* ATCC 16883 with the sequence similarity level of 99% (Table 2). Based on BLAST results of ITS sequence, P3 and P8 isolates have the same homology level with *A. flavus* var. *flavus* ATCC 16883. The result of alignment sequences of P3 isolate with *A. flavus* var sequence. *flavus* ATCC 16883 using muscle

program in MEGA 7 software indicates the absence of gaps in the nucleotide base sequence, while in the P8 isolate has the presence of gaps with *A. flavus* var sequence. *flavus* ATCC 16883 about 0.39%.

Based on the result of ITS DNA sequence analysis by constructing phylogenetic tree (figure 5), both of isolate P3 and P8 are in the same clade of *A. flavus*. The isolates of P3 and P8 are classified into monophyletic clade, together with the other fungal sequences from Aspergillus group, consisting of *A. flavus* ATCC strain 16883; *A. fasciculatus* strain CBS 110.55; *A. oryzae* NRRL 447; *A. minisclerotigenes* strain CBS 117635; *A. flavus* var *flavus* ATCC strain 16883; *A. flavus*; *A. kamberensis* CBS 542.69; and *A. parvisclerotigenus* CBS strain 121.62 with a bootstrap value of 53%. *A. flavus, A. oryzae, A. minisclerotigenes* and *A. parvisclerotigenus* are together in the same group with *A. flavus* in the flavi section [21].

The isolates of P3 and P8 were in the same clade as *A. kambarensis* CBS 542.69, *A. flavus* var. *flavus* ATCC 16883 and *A. flavus* with a bootstrap value of 79%. The DNA length of isolate P3 and P8 has different number of base pair with *A. flavus* ATCC 16883, while it has no differences with *Aspergillus fasciculatus, Aspergillus oryzae, A. minisclerotigenes, A. flavus var. flavus* and *A. flavus*. That sequence data using multilocus analysis showed that *A. flavus* var. *columnaris, A. kambarensis, A. fasciculatus, A. thomii*, and *A. subolivaceus* are the synonyms of A. flavus grouped in the section of *flava*.

The result of molecular identification on P3 and P8 isolate can be identified as *A. flavus* var. *flavus* because it has 100% and 99% homology. Analysis of the rDNA ITS area can be used in the fungal identification since the ITS region has a high sequence variability among fungal group up to the species level [22].

3.3.1. Detection of aflatoxin in isolates from corn kernel feed. Aflatoxin detection was performed based on the fluorescence method under 365 nm UV rays by inoculating the fungal isolates on Coconut Agar Medium (CAM). Only 2 selected isolates were used in this detection (P3 and P8), based on selective media isolation, morphological identification and molecular approach. The presence of blue fluorescence and beige rings on the reverse colony are used as parameters in the detection using 365nm UV rays.

Table 2. BLAST results of P3 and P8 isolates based on ITS regional sequence analysis.

Kode isolat	Takson terdekat hasil BLAST di NCBI	Max score	Query (%)	E-value	Accession number	Similarity (%)	Gaps
P3	*A. flavus* var. *flavus* ATCC 16883	874	100	0.0	KU729026.1	100	0/472 (0%)
P8	*A. flavus* var. *flavus* ATCC 16883	998	96	0.0	KU729026.1	99	2/507 (0,39%)

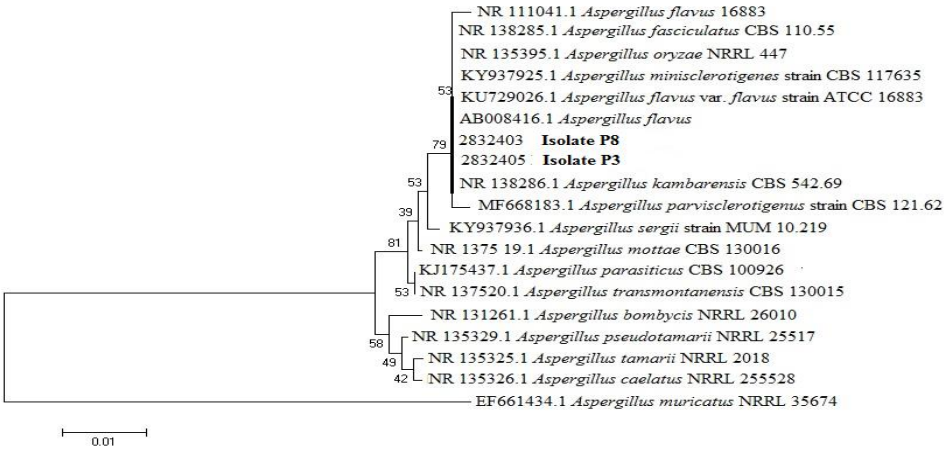

Figure 5. Phylogenetic tree based on ITS DNA analysis using Neighbour Joining Method (1000x Bootstrap).

That blue fluorescence is a method used to develop a qualitative method for detecting aflatoxigenic Aspergillus species grown in the specific medium [23].

The results showed no blue fluorescence and beige rings both in the P3 and P8 isolates. That toxigenic *A. flavus* can produce blue fluorescence in 2 days incubation in CAM media [9], the same test requiring 7 days incubation time to generate the same results [24].

Dyer and McCammon (1994) tested aflatoxin detection using coconut-based medium such as CAM, Coconut Milk Agar (CMA), coconut cream, and coconut milk powder. The test results revealed that the use of coconut milk powder medium with a concentration of 40% generate a stronger fluorescence rather than using coconut milk medium. aflatoxin detection test using CAM and Coconut Milk Agar (CMA) medium, and the results showed that CMA medium is more effective in producing blue fluorescence than using CAM medium [9].

The use of coconut media to detect the presence or absence of aflatoxin content is considered ineffective due to the high sensitivity of Aspergillus to the composition of coconut medium. Constituent materials of coconut-based medium can affect the production of fluorescence pigments. In this case, further identification are needed for isolates P3 and P8, since it could not be indicated either both isolates are classified as nontoxigenic *Aspergillus flavus* or do not produce aflatoxin.

4. Conclusion

Nine fungal isolates are successfully isolated from corn kernel (*Zea mays* L.) with the label P1, P2, P3, P4, P5, P6, P7, P8, and P9. Based on the morphological characteristics and ITS rDNA analysis, the sequences of fungal isolates P3 and P8 were identified as *A. flavus* var *flavus* with homology 100% and 99% respectively, showing no aflatoxin production in both.

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