Malaysian Journal of Microbiology, Vol 13(2) June 2017, pp. 117-123

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

The role of *Aspergillus oryzae* KKB4 in reducing and detoxifying aflatoxin B1 applied in moist-heated corn

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Received 8 August 2016; Received in revised form 7 September 2016; Accepted 3 November 2016

ABSTRACT

Aims: The growth and metabolic activity of *Aspergillus oryzae* KKB4 in AFB1-contaminated corn and later coincided with AFB1 reduction and detoxification were investigated.
Methodology and results: The decreasing of AFB1 amount by *A. oryzae* KKB4 could be clearly observed if the initial AFB1 concentration in corn was increased. Thus, moist-heated corn was artificially inoculated with *Aspergillus flavus* FNCC 62C7 to increase AFB1 content. AFB1-contaminated corn was applied as solid substrate and then inoculated with *A. oryzae* KKB4. During fermentation periods, the growth, metabolic activity, and AFB1 decline were investigated by glucosamine content, water content loss, and AFB1 concentration, respectively. The maximum growth was occurred in 4thday at 1.499 ± 0.028 g glucosamine/ 100 g dry matter. The metabolic activity was going on up to the end of fermentation days, as shown as water content loss at 0.175 ± 0.007 g/g dry matter. In accordance with the growth and metabolic activity, the amount of AFB1 reduction was 37.04 ng AFB1/g dry matter during 5 days fermentation. According to toxicity analysis, it was found that the residues of AFB1 were not toxic to *Bacillus megaterium* cells.
Conclusion, significance and impact of study: *A. oryzae* KKB4 is able to be applied in solid substrates as AFB1 reduction and detoxification agent. These lucrative effects are also important in relation with food and feed safety.

Keywords: Aflatoxin B1, Detoxification, Solid Substrate, *Aspergillus oryzae* KKB4

INTRODUCTION

Aflatoxin is a carcinogenic mycotoxins produced by *Aspergillus* mold, such as *Aspergillus flavus*, *A. parasiticus* (Iheanacho *et al.*, 2014), *A. tamarii* (Tam *et al.*, 2014), *A. nomius* and *A. pseudonomius* (Massi *et al.*, 2014). Among them, *A. flavus* is a common producer of aflatoxin B, causing pre- and post-harvest agricultural stuff contamination (Klich, 2007). The contamination is mainly dominated by aflatoxin B1 (AFB1) (Noviandi *et al.*, 2001), and thus AFB1 would be the center concern in animal and human public health.

One of the promising strategies for managing AFB1 in foods is biological detoxification method with the microorganism employment. Fungal filamentous is one such microorganism known as the agents for fermented foods production, such as *Rhizopus oligosporus*, *Amylomyces rouxi*, *Aspergillus wentii*, *A. sojae*, *A. oryzae* (Liang *et al.*, 2016). *Aspergillus oryzae* KKB4 is an indigenous proteolytic fungus that has ability of AFB1 degradation (Sardjono *et al.*, 2004b). Previous study explored the ability of its extracellular enzymes to degrade and detoxify AFB1 in liquid synthetic medium using pure AFB1 standard (Sardjono *et al.*, 2004a). In contrast with the previous research, solid-state fermentation (SSF) has a bigger opportunity to be developed, compared to submerged and liquid fermentation, because of its apparent simplicity, economical factor, and wide applicability (Subramaniyam and Vimala, 2012; Li *et al.*, 2013). In addition to the opportunity of SSF, *A. oryzae* is able to produce the amount of enzymes which is necessary to maintain its life on the surface of solid substrates, where initially lacking of sugars and amino acids (Vishwanatha *et al.*, 2010).

The potentially solid substrate used for fungal fermentation is corn, since it has been the second main food crop in Indonesia, yet it is susceptible to AFB1 contamination during pre- and post-harvest (Ali *et al.*, 1998). The hazard risk of AFB1 contamination in corn could be minimized by *A. oryzae* KKB4 in the period of SSF. During SSF period, *A. oryzae* KKB4 could produce extracellular enzymes, closely collaborated with its growth (Sardjono, 2008). The observation of its growth could be done through glucosamine content as the course of

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 ISSN (print): 1823-8262, ISSN (online): 2231-7538

fungal biomass changing within the fermentation (Sitanggang *et al.*, 2012), because monomers of glucosamine and their acetylated form (N-acetyl glucosamine) compose building units of fungal chitin and chitosan (Mohammadi *et al.*, 2012).

Measuring AFB1 concentration is not enough to assure the safety of AFB1 reduction products. Since AFB1 toxicity affects have well known, analysis for assessing the toxicity of AFB1 reduction products is important, using a sensitive indicator, *Bacillus megaterium* (Sardjono *et al*., 2004a). This study, therefore, was mainly concerned in the ability of *A. oryzae* KKB4 for AFB1 reduction and detoxification through SSF that produced fermented corn which do not have toxic potential.

MATERIALS AND METHODS

Microorganisms and starter powder preparation

Aspergillus flavus FNCC 62C7 was obtained from Food and Nutrition Culture Collection (FNCC) at Universitas Gadjah Mada, Indonesia. Meanwhile, *A. oryzae* KKB4 was attained from Laboratory of Biotechnology, Faculty of Agricultural Technology (Sardjono *et al.*, 2004). Medium for starter culture was rice grain sterilized at 121 °C for 20 min. It was inoculated with spore suspension of *A. flavus* (10^8 spores/mL) or *A. oryzae* (4×10^8 spores/mL), which was attained by harvesting 7-day old fungi on potato dextrose agar (PDA) slant with 0.05% Tween 80. The incubation of starter was done at 30 °C for 7 days, and later dried then grinded into rough powder, so the starter powder was ready to be used for inoculation.

Substrate inoculation by *Aspergillus flavus*, and AFB1 Production

About 5 kg of yellow corn grains, obtained from Kulon Progo, Yogyakarta, were added by 35% distilled water (w/v) and sterilized by autoclaving at 121 °C for 20 min. This substrate was inoculated with 0.1% (w/v) *A. flavus* starter powder. The inoculated corn grains were distributed over perforated tray (45 cm × 30 cm × 6 cm), containing ± 2 kg grains of which the thickness was 2 cm. The tray was placed in fermentation rack which has 3 shelves stacked inside, as seen on Figure 1. The incubation was done for 5 days at room temperature and relative humidity (RH) 90-91%. The growth of *A. flavus* was stopped with moist sterilization using autoclave (EA-632 vertical autoclave, Trident Medical Corp., Taipei, Taiwan), by adding 35% distilled water (w/v) into solid substrate, at 121 °C for 20 min. Then samples were analyzed using HPLC as described below, in regard to detect the indirectly effect of moist sterilization toward the AFB1 concentration. The grains were washed with 0.05 % Tween 80 at a ratio of 2:1 (w/v) to dispose the residue of *A. flavus* spores and mycelia, according to Oluwafemi *et al*. (2010) modified method. Moreover, the glucosamine content of its sample was stated as "the *beginning* glucosamine level", for calculating "the *final* glucosamine content in the initial day (day-0)" as explained below.

Figure 1: The tray fermentation system; T, perforated tray; D&W, dry and wet bulb thermometer; P, plastic covered the rack fermentation; TW, bottom tray filled with water; P and TW were used to keep the desirable humidity and temperature.

In order to make sure that the growth of *A. flavus* had already been stopped, this sample was analyzed using AFPA (*Aspergillus flavus* and *parasiticus* Agar). AFPA is a selective identification medium for the rapidly detection of *A. flavus* and *A. parasiticus* (Pitt *et al*., 1983). Based on the observation, the formation of bright orange-yellow reverse pigment had not been happened (observation results were not shown).

Inoculation by *A. oryzae* KKB4 and incubation

To reduce AFB1 by biological method, 0.5% (w/v) *A. oryzae* starter powder was inoculated into the corn grains, which had already contaminated by *A. flavus*. The glucosamine content of its substrate was stated as "the *measured* glucosamine content in the initial day (day-0)". The *measured* glucosamine content in the initial day minus the *beginning* glucosamine level was resulted as "the *final* glucosamine content in day-0". This calculation

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

was used to assure that the glucosamine content in day-0 belong to *A.oryzae* KKB4.

The fermentation system of *A. oryzae* KKB4 was similar with *A. flavus* incubation as described earlier. The incubation was done for 5 days at room temperature and RH 91%. During the fermentation periods, the growth of *A. oryzae* KKB4 was measured by glucosamine content, meanwhile the metabolic activity of *A. oryzae* KKB4 was observed by measuring the water content loss. The increasing glucosamine content also indicated the increasing biomass of *A. oryzae* up to 5 days fermentation.

Glucosamine

The fungal biomass in the fermented corn was determined by estimating the presence of glucosamine in the cell wall of the culture, modified from Hackbart *et al.* (2014). Before entering the main glucosamine determining procedures, samples were treated as the modifying of Smits *et al.* (1996a). Corn grains from each sample were initially dried in an oven at 105 °C for 24 h, for determining the dry matter weight. The grains were grounded, and then dried again at 105 °C for 24 h. After those pretreatments, glucosamine was extracted by acid hydrolysis method. The second step was aimed for measuring the glucosamine content using colorimetric method. The absorbance was measured at 550 nm using a spectrophotometer (UV mini-1240, Shimadzu). Meanwhile, glucosamine hydrochloride (Sigma) was utilized as standard with different concentrations between 0 µg/mL up to 50 µg/mL. The fungal biomass is expressed as gram glucosamine per 100 gram dry matter.

Water content loss

Water content loss during fermentation periods was caused by the metabolic activity, and released heat, CO_2 and H_2O. Water content was determined by measuring the change of weight of approximately 2 g substrates after 24 h drying at 105 °C (modified from Mendez-Albores *et al.*, 2005). Water content loss was calculated from the total weight of remaining substrate and water content per gram of dry substrate (Smits *et al.*, 1996b).

Aflatoxin B1 extraction, purification and determination

To observe the amount of AFB1 reduction caused by physical treatment, i.e. moist sterilization, and biological treatment, i.e. solid-state fermentation, AFB1 extraction from each sample based on *Association of Official Analytic Chemists* (AOAC) 991.3 was modified from Suarez-Bonnet *et al.* (2013). The purification was executed by immunochemical method using AflaPrep immunoaffinity columns (R-Biopharm Rhone Ltd., Glasgow, Scotland). The eluate from the column was evaporated, and then 0.15 mL mixture of acetonitrile: methanol: $KBrHNO_3$ (11: 22: 67) was added. This solution was analyzed by reverse phase HPLC.

The separation and detection processes were modified from Mateo *et al.* (2011). Detection method for AFB1 used fluorescence-based detection in collaboration with a separation process, high-performance liquid chromatography (HPLC). The chromatography system had a fluorescence detector at maximum excitation wavelength ($\lambda_{Excit\ max}$) 360 nm and maximum emission wavelength ($\lambda_{Emis\ max}$) 440 nm. The mobile phase comprehended acetonitrile: methanol: $KBrHNO_3$ (11:22:67), was flowed at the rate of 1 mL/min. The separation was attained with a reverse phase C_{18} column (5 µm particle size, 250 × 4.6 mm i.d., Inertsil). To calculate the AFB1 concentration of samples, the range of AF standards (Sigma) was 1.00-100.00 ng/mL used for creating AFB1 standard curve. AFB1 concentration of the samples was quantified in ng AFB1/ g of dry matter.

Toxicity analysis

This analysis is important to confirm the safety of the AFB1 reduction products, related with the AFB1 toxic effects towards the sensitive bacteria, *Bacillus megaterium* FNCC 0083. This analysis was modified from Sardjono *et al.* (2004b). The solution after filtered through a Whatman no 4 (Dassel, Germany) paper filter that had been already prepared from AFB1 concentration analysis, were used for this bioassay. The solution (0.5 mL) was evaporated using nitrogen gas in 10 mL test tube. The dried samples were added by the amount of 5 mL nutrient broth, and later inoculated with 0.5 mL starter of *B. megaterium*. Those toxicities could be investigated after incubated at 30 °C for 16 h.

Statistical analysis

All experiments were conducted in triplicate. Data were analyzed using the Excel program (Microsoft), and the averaged results were expressed as the mean ± standard deviation.

RESULTS AND DISCUSSION

The growth and metabolic activity of *Aspergillus oryzae* KKB4 during solid-state fermentation

A popular method to measure fungal biomass in solid substrate is assessing the glucosamine content. Although this parameter was not able to describe the differentiation between active and inactive fungal biomass; it tended to increase during fermentation periods and correlated with the total amount of fungal biomass increasing (Hackbart *et al.*, 2014). According to Figure 2, the result of the glucosamine content was depended on the fermentation days. The raising of the glucosamine up to 4^{th} day fermentation at 1.499 ± 0.028 g/100 g dry matter, as seen on Figure 2, was caused by the fungal ability to compose mycelium. Bartnicki-Garcia (2015) supported this result by describing that filamentous fungi grew through the hyphae elongating and branching, and the mycelium used for

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

migrating over the substrate surface (Siripatrawan and Makino, 2015).

Moreover, this growth could also affect the changing of water content. The water content declined (0.175 ± 0.007 g/g dry matter) during 5 days fermentation, as described on Figure 2; possibly as a changing effect of water-binding capacity of the fermented corn (Smits *et al.*, 1996b). Furthermore, water content loss was followed by the increasing of the fungal growth, which was clearly described by the raising of glucosamine content in Figure 1. Similar findings have been explained by Zambare (2010), there was a reduction of substrate moisture content during fermentation because of fungal metabolic activities and evaporation. Smits *et al.* (1996b) supported that beside water content; fiber, starch, and total free sugar also decreased as the result of substrate utilization during the growth of filamentous fungi.

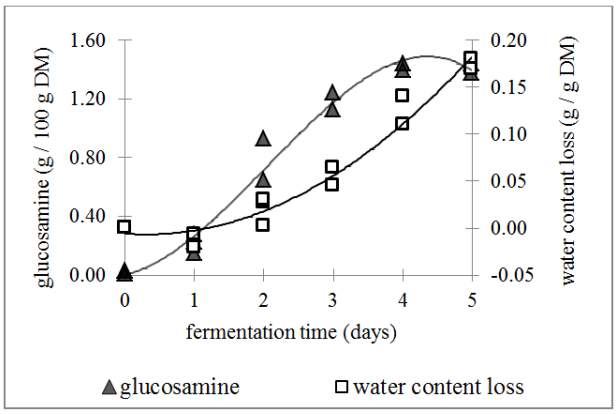

Figure 2: The experimentally fungal biomass (glucosamine) and metabolic activity (water content loss) within 5 days fermentation.

Reduction of AFB1 by *Aspergillus oryzae* KKB4

AFB1 formed by *A. flavus* was 60.54 ± 10.46 ng AFB1/g dry matter in 5 days incubation of *A. flavus*. Moist sterilization was then applied toward AFB1-contaminated corn, causing indirectly AFB1 reduction in the amount of 10.16 ± 4.91 ng AFB1/ g dry matter. AFB1 concentration could be reduced by this treatment because heating the water content was the requirement for hydrolyzing lactone ring of AFB1 structure (Lee *et al.*, 2015). However the water content (35-45%) of AFB1-contaminated corn and the heating temperature (121 °C for 20 min), did not properly give satisfying AFB1 reduction, since the residue concentration of AFB1 in corn was 56.31 ± 2.24 ng AFB1/g dry matter. According to the results obtained by Hwang and Lee (2006), increasing the water content in foodstuffs was able to increase the reduction of AFB1 concentration. In addition to water content increasing, temperature also held the main affect in reducing AFB1 significantly, if increased above 190 °C (Zhang *et al.*, 2011). Unfortunately, increasing moisture content and heating temperature above 190 °C could decrease the nutritional content and sensory quality of the food products (Saalia and Phillips, 2011).

Fermentation by *A. oryzae* KKB4 has an interesting result to reduce AFB1 in moist-heated corn, correlated with the increasing of fermentation periods and fungal biomass, as described at Figure 3. In accordance with the fermentation periods, the AFB1 reduction was 37.04 ng AFB1/ g dry matter in 5 days fermentation. The reduction of AFB1 was depended on the ability of *A. oryzae* KKB4 in producing extracellular enzymes, as reported by Sardjono *et al.* (2004b). Sardjono *et al.* (2004a) also supported that there were 5 protein fractions of extracellular enzyme produced by *A. oryzae* KKB4 in liquid synthetic medium. Moreover, the references have explained varied production and activity of AFB1 degrading enzymes, depending on type of substrate, moisture content, fermentation system, and environmental controlling (Guan *et al.*, 2008; Hackbart *et al.*, 2014).

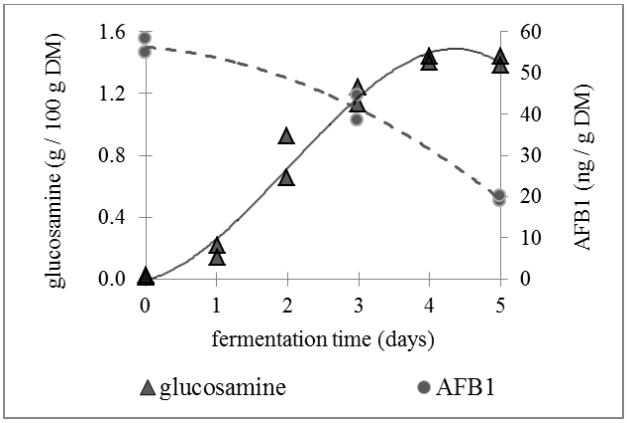

Figure 3: The relevance of *A. oryzae* KKB4 glucosamine (—) and fermentation time with regard to decline of AFB1 concentration (- - -).

The AFB1 reduction is rarely correlated with the fungal biomass. It was found that increasing fungal biomass is not always followed by reducing rate of AFB1, as shown in Figure 3. During 5 days fermentation, the amount of AFB1 reduction in the last 2 days (3.73 µg AFB1/g glucosamine/day) was higher than in the first 3 days (0.44 µg AFB1/g glucosamine/day). It might be possibly that the extracellular enzymes of AFB1 reduction were excreted from the beginning fermentation periods. When the 4th day of fermentation was attained, the glucosamine content remained constant, but the reduction of AFB1 still occurred. This result indicated that the fungal biomass was not produced, but extracellular enzymes of AFB1 reduction were still active up to 5 days fermentation.

Detoxification of AFB1 by *Aspergillus oryzae* KKB4

Preliminary publications have reported that the AFB1 reduction products were not completely enough identified through measuring the AFB1 concentration based on

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

chemical analysis (Park and Kim, 2006). Chromophore compound of AFB1 is able to be detected by fluorescence-based detection method. However the toxic moieties of AFB1 do not have fluorescence properties (Lee *et al.*, 1981). Therefore, AFB1 reduction product had been also confirmed with bio-analysis related to its safety.

The pure standards of AFB1 disrupted the division of *B. megaterium* cells, as identified in Figure 4. It was clearly represented from figure 4C that there were cells elongation in 20 ng AFB1/mL. Consequently, 20 ng AFB1/ mL was an initial toxicity limit in disturbing cells division.

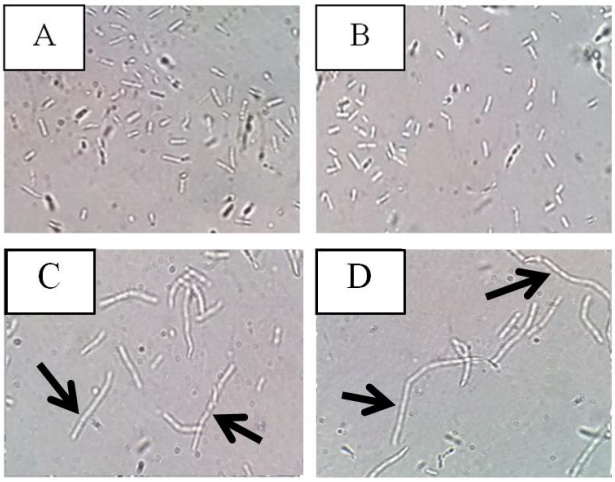

Figure 4: The growth of *Bacillus megaterium* cells in nutrient broth medium mixed with AFB1 pure standard solution: A, 0 ng/mL (normal growth); B, 10 ng/mL (still grown normally); C, 20 ng/mL (cells start to have division impairments); D, 40 ng/mL (cells become longer); the arrows point to cell division failure (magnification ×1000).

The successful AFB1 detoxification by *A. oryzae* KKB4 in 5 days fermentation has been exhibited on Figure 5. The sample before *A. oryzae* KKB4 fermentation, containing 38.51 ng AFB1/ g dry matter (quantitated by HPLC), had adverse effects on the cell division failure. Meanwhile, the sample after *A. oryzae* KKB4 fermentation, comprehending 20.02 ng AFB1/g dry matter (quantitated by HPLC), had not given side effects in the disturbance on cells division. As a result, the cells had still grown normally. Although, its sample had almost similar AFB1 concentration with the initial toxicity limit of AFB1 pure standard at 20 ng AFB1/mL; the AFB1 reduction products by *A. oryzae* KKB4 had no toxic effects on *B. megaterium* cells. It might be happened since AFB1 reduction by *A. oryzae* KKB4 was enzymatic and resulting the changing of AFB1 structure, such as bisfuranoid changing, lactone ring opening and cyclopentanone reducing (Sardjono *et al.*, 2004a).

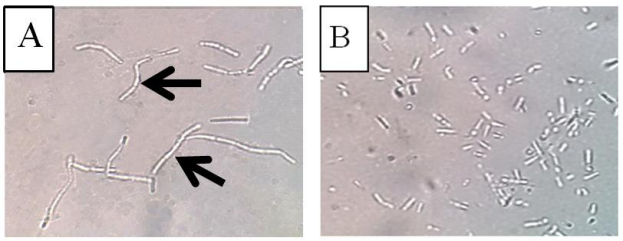

Figure 5: Toxicity of AFB1 reduction products, shown by *Bacillus megaterium* cells growth in medium containing sample of: A, Before AFB1 reduction (38.51 ng AFB1/ g dry matter); B, After AFB1 reduction (20.02 ng AFB1/ g dry matter); the arrows appoint towards cells division failure (magnification ×1000).

CONCLUSION

Based on the results, it was known that *A. oryzae* KKB4 had been able to reduce AFB1 concentration in moist-heated corn through solid-state fermentation attractively. The toxicity results in this bioassay revealed that *A. oryzae* KKB4 could also detoxify AFB1, and so made fermented corn which had no toxic effects.

ACKNOWLEDGEMENT

This research was supported by State Ministry of Research and Technology, Indonesia. Carolina also thanks to Mr. Purwito and Anang Juni Yastanto for the hospitality and assistance during whole periods of the research in Laboratory of Biotechnology.

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