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Local strains *Aspergillus oryzae* KKB4 and *Rhizopus oryzae* KP1R1 as a reducing and detoxifying agents for deoxynivalenol

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ABSTRACT

Aims: Deoxynivalenol is a type B trichothecene produced by *Fusarium graminearum* that can cause serious health problems in human and livestock. The present study aimed to reduce and detoxify deoxynivalenol using a local strain *Aspergillus oryzae* KKB4 and *Rhizopus oryzae* KP1R1.
Methodology and results: Corn as solid substrate artificially inoculated with *F. graminearum* bio 163252 to produce deoxynivalenol. Deoxynivalenol contaminated corn then inoculated with *A. oryzae* KKB4 and *R. oryzae* KP1R1. During fermentation, a decrease in deoxynivalenol levels is analyzed including loss of dry matter and glucosamine content. Deoxynivalenol was extracted from the substrate by solid phase extraction and quantified using high-performance liquid chromatography. The reduction of deoxynivalenol by *A. oryzae* KKB4 and *R. oryzae* KP1R1 were 65.91% and 56.82%, respectively after ten days of fermentation. Toxicity analysis revealed that residues of deoxynivalenol were not toxic to growth of *Saccharomyces cerevisiae* cells.
Conclusion, significance and impact of study: Local strains *A. oryzae* KKB4 and *R. oryzae* KP1R1 were able to reduce and detoxify deoxynivalenol in solid substrates. This study provides supporting data to control mycotoxin that is critical for food and feed safety.

Keywords: Deoxynivalenol, *Aspergillus oryzae* KKB4, *Rhizopus oryzae* KP1R1, solid state fermentation

INTRODUCTION

Aspergillus, *Penicillium,* and *Fusarium* are three genera of fungus that generally contaminates plants during pre-, post-harvest, and storage. Among this group, *Fusarium* sp. is a major contaminant in cereal crops such as barley, wheat and maize (Turner *et al.*, 2009; Awad *et al.*, 2010; Reddy *et al.*, 2010). According to JECFA (2002), *F. graminearum* is mainly causing plants diseases and producing deoxynivalenol (DON). DON also known as the group of trichothecenes mycotoxin and most common toxin in cereals group. Although the toxicity level of DON is lower than other trichothecenes, its presence can be an indicator of other toxic trichothecenes (Lindblad *et al*., 2013; Kos *et al*., 2016).

The occurrence of DON contamination influenced by several factors such as substrate condition, the presence of fungal and another competitor microorganism, and the environmental condition (Bhat *et al*., 2010). *Fusarium graminearum* grow optimally at a temperature between 24.5 to 25.5 °C with a_w0.977-0.995 (Neagu and Borda, 2013). Indonesia has a warm and humid climate that suitable for fungal infection. The first case of DON contamination in Indonesian maize occurred at concentration 21 and 32 ng/g (Ali *et al*., 1998). Moreover, studies by Setyabudi *et al*. (2012) showed a higher concentration for contamination in maize at 47.5-205.7 µg/kg.

Since mycotoxins become important objective worldwide due to potential toxicity on human and animals, several decontamination strategies are needed to reduce the prevalence of mycotoxin contamination. The biological method is one of the promising approaches to reduce mycotoxin in food and feed using microorganisms. Several studies have demonstrated the ability of filamentous fungi to degrade mycotoxin. *Aspergillus oryzae* KKB4 can degrade aflatoxin B1 in a liquid medium (Sardjono *et al*., 2004a; 2004b) or in a solid substrate (Sardjono, 2008; Djunaidi *et al.*, 2017). Similarly, *R. oryzae* can reduce aflatoxin in agar medium (Hackbart *et al.*, 2014).

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Previous study has used bacteria and yeast to metabolize and detoxify DON (Fucsh *et al*., 2000). Meanwhile, Garda-Buffon and Badiale-Furlong (2010) describe the ability of *A. oryzae* and *R. oryzae* to degrade DON in the liquid media. However, not much literature shows the degradation of DON by fungi in a solid substrate. Solid substrate fermentation (SSF) has been widely published about considerable advantages compared to liquid fermentation, such as easy aeration, low energy requirements, higher metabolite production and easy product purification (Romano *et al.*, 2007).

Residual levels of deoxynivalenol may cause long-term chronic toxicity due to accumulation in target organs. Therefore, analysis for assessing the toxicity of DON reduction product is essential, using a yeast *Saccharomyces cerevisiae* (Madhyastha *et al.*, 1994; Binder, 1999; Tanaka *et al.*, 2013). This study, henceforth, was to reduce and detoxify DON using a local strain of *A. oryzae K*KB4 and *R. oryzae* KP1R1 through solid state fermentation that produced fermented corn grains with no toxic effect.

MATERIALS AND METHODS

Microorganisms and preparation of starter culture

Fusarium graminearum bio 163252 was isolated from tomato by SEAMEO BIOTROP Bogor. Meanwhile, *A. oryzae* KKB4 (isolated from koji Kebumen area) and *R. oryzae* KP1R1 (isolated from koji Purwokerto area) by Prof. Sardjono and collected at Laboratory of Biotechnology, Faculty of Agricultural Technology, Universitas Gadjah Mada, Yogyakarta. Medium for starter culture was rice grain sterilized at 121 °C for 20 min. Then inoculated with spore suspension from 7 days agar slant using 0.05% tween 80. The incubation of starter was done at room temperature for 7 days, and later dried then ground into rough powder, so the starter powder was ready to be used for inoculation (Sardjono *et al*., 2004[b]; Sardjono 2008; Shi *et al.*, 2014).

Production of DON in corn grains

About 5 kg of yellow corn grains was sterilized by a dry heating method in an oven at 170 °C for 2 h. Sterile corn was moistened with sterile water (2:1) and then inoculated with 0.1% starter *F. graminearum* bio 163252. The inoculated corn was distributed over perforated tray and placed in a closed rack fermentation. The corn was then incubated for seven days at room temperature. After seven days, the fungal growth was stopped by dry sterilization at a temperature of 170 °C for 2 h (Gupta *et al.*, 2011).

Reduction of DON by *A. oryzae* KKB4 and *R. oryzae* KP1R1

Sterile distilled water is added to contaminated corn grains in ratio 1:2 and each of 0.1% starter *A. oryzae* KKB4 and *R. oryzae* KP1R1 inoculated separately to contaminated wet corn grains. Incubation was conducted in the incubator rack equipped with a reserve of water in the bottom part to achieve a relative humidity above 90% during fermentation. Incubation is carried out at room temperature for ten days.

Loss of dry matter

Loss of dry matter was calculated from the weight of corn grains in day 0, 2, 4, 6, 8 and 10 fermentation. Approximately 1 g sample was dried at 105 °C until a constant weight was achieved (Smits *et al.*, 1996).

Glucosamine content

Estimation of fungal mass was determined by hydrolysis of chitin contained in the cell wall into the N-acetyl glucosamine. Approximately 0.2 g dry sample was added 5 mL of HCl 6 M. The mixture was then put in an autoclave at 121 °C for 8 min. The mixture is cooled and filtered. Then the volume is adjusted to 5 mL using aquabidest. The glucosamine content was measured based on the methods as described Souza *et al.* (2011). The absorbance was measured at 530 nm using a spectrophotometer. Glucosamine hydrochloride (Sigma) was used as a standard.

Extraction, purification, and determination of DON

A total of 25 g samples was homogenized with 100 mL of a mixture of acetonitrile: Water (80:20) in a blender for 3 min. The mixture was filtered using a Whatman paper No 4.4 mL of the obtained solutions purified by passage on a solid phase extraction (SPE). Two millilitre of the obtained eluate was dried using a nitrogen evaporator, and then reconstituted with 500 µL acetonitrile: Water (10:90) and subsequently analyzed for levels of Deoxynivalenol (Klotzel and Lauber, 2017).

The analysis was carried out using High-Pressure Liquid Chromatography (HPLC) with reverse phase C18 column (5 µm particle size, 150× 4.6 i.d, Shimpack), detector ultraviolet, wavelength 218 nm, mobile phase acetonitrile: Water (10:90) and the flow rate 0.6 mL/min. To calculate the DON sample concentration, the standard DON range (Supelco) is 25.00-3200.00 ng/mL which is used to create the DON standard curve (Kotal and Radova, 2002).

Toxicity assay

This analysis to confirm toxicity of the DON reduction products by using a yeast *S. cerevisiae*. Toxicity was determined by measuring optical density at 600 nm and viability of yeast cells after incubation at 30 °C for 48 h (modified from Binder, 1999; Sardjono *et al.*, 2004b). The filtered solution that had been already prepared from DON analysis were used for this assay. Precisely 10 mL solution was evaporated using nitrogen evaporator in 15 mL test tube; then residues were dissolved in 4 mL Peptone Glucose Yeast broth and later inoculated with

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0.4 mL suspension of *S. cerevisiae* (cell viability 5.5 × 10^6 CFU/mL).

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

Aspergillus oryzae KKB4 and _R. oryzae_ KP1R1 growth and metabolic activity during solid-state fermentation

During fermentation, the growth of fungus can be observed using glucosamine content analysis (Smits *et al*., 1996; Terebiznik and Pilosof, 1999). Figure 1 shows the accumulated pattern of increase in glucosamine as an indicator of fungal biomass during fermentation by *A. oryzae* KKB4 and *R. oryzae* KP1R1. In the figure shows the ability of *A. oryzae* KKB4 and *R. oryzae* KP1R1 to growing well on corn substrates. During the fermentation, there was an increase of glucosamine accumulation and on the 10th day reached 6.528 ± 5.752 mg/g and 1.079 ± 0.556 mg/g by *A. oryzae* KKB4 and *R. oryzae* KP1R1 respectively.

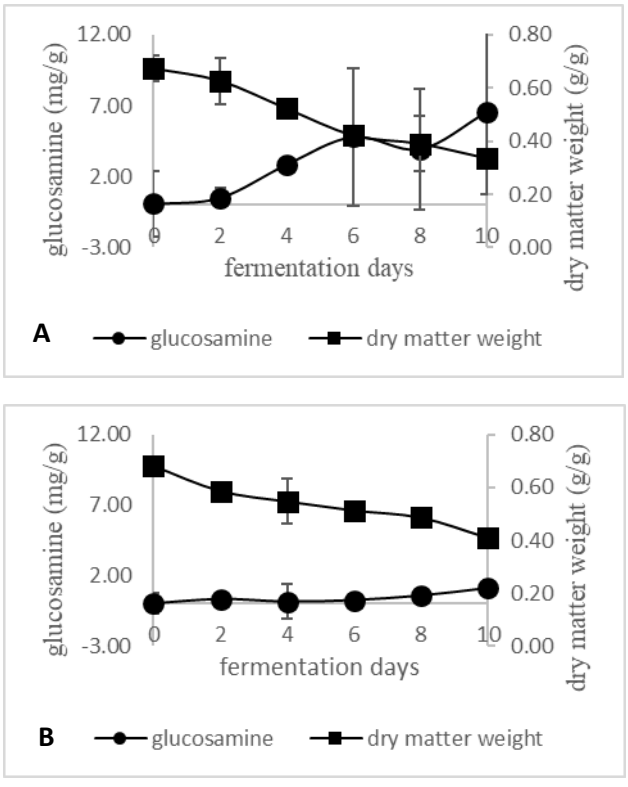

Figure 1: The pattern of growth profile; (A) *Aspergillus oryzae* KKB4 and (B) *Rhizopus oryzae* KP1R1.

Terebiznik and Pilosof (1999) and Sardjono (2008) revealed that the growth of fungal in SSF system has a good correlation with the loss of dry matter weight. Substrate changes characterized by high water loss, loss of dry matter and increased CO_2 production indicate metabolic activity of the fungal. During fermentation, the substrate will lose weight because it is used for growth and production of metabolites. The use of corn substrate by *A. oryzae* KKB4 and *R. oryzae* KP1R1 each caused dry matter loss of 0.336 ± 0.032 g/g and 0.409 ± 0.004 g/g on the 10th day of fermentation.

The pattern of glucosamine change and changes in the weight of the dry matter during fermentation by *A. oryzae* KKB4 and *R. oryzae* KP1R1 can be seen in Figure 1. The image shows an increase in glucosamine in line with dry matter loss which is an indicator of metabolic activity. The difference in the rise of glucosamine between *A. oryzae* KKB4 with *R. oryzae* KP1R1 is thought to be due to *A. oryzae* KKB4 using corn substrate dominant for biomass growth characterized by high glucosamine rise. While *R. oryzae* KP1R1 uses corn substrate more to produce metabolite compared to biomass growth so that glucosamine increase is not too high.

DON reduction by _A. oryzae_ KKB4 and _R. oryzae_ KP1R1

Fermentation by *A. oryzae* KKB4 and *R. oryzae* KPR1 has an exciting result to decreased levels of deoxynivalenol on corn, correlated between fermentation periods and fungal biomass as described in Figure 2. *Aspergillus oryzae* KKB4 can decrease the DON level from 397.49 ng/g on the first day to 135.52 ng/g at the end of fermentation. While *R. oryzae* KP1R1 can reduce the DON level from 399.41 ng/g to 172.46 ng/g. In the fermentation process, it is suspected to be degraded by a mechanism such as adsorption of mycotoxin by fungi (Garda-Buffon and Badiale-Furlong, 2010) and or extracellular enzyme activity which can reduce mycotoxin (Sardjono *et al*., 2004a; Garda-Buffon *et al*., 2011).

Figure 2 also illustrates the relationship between biomass growth and decreased levels of DON by *A. oryzae* KKB4 and *R. oryzae* KP1R1. During fermentation, the biomass production of *A. oryzae* KKB4 is high enough and the increase in biomass followed by a decrease in DON levels. It is suspected that the growing mycelia of *A. oryzae* KKB4 can produce an enzyme that degrade DON. While *R. oryzae* KP1R1 has a biomass production that is not too high but shows a constant decrease in DON levels until the end of fermentation. This difference is estimated because *R. oryzae* KP1R1 uses a substrate to produce DON degrading enzymes more dominant than for biomass growth. Thus either *A. oryzae* KKB4 and *R. oryzae* KP1R1 could degrade DON.

During the fermentation of solid substrate, the fungus will degrade the substrate to take its nutrients (Gowthaman *et al*., 2001). Corn is one of the food commodities that have essential nutrients for *A. oryzae* KKB4 and *R. oryzae* KP1R1. After getting the nutrients, they can grow well, and growth can be observed through

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the glucosamine content. They also simultaneously release primary or secondary metabolites that have the potential to degrade and detoxify DON. The biologicalreduction of mycotoxins by solid substrate fermentation is influenced by several factors such as starter spores, fermentation process conditions, substrate composition and initial concentrations of mycotoxins (Gowthaman *et al*., 2001; Hackbart *et al*., 2014).

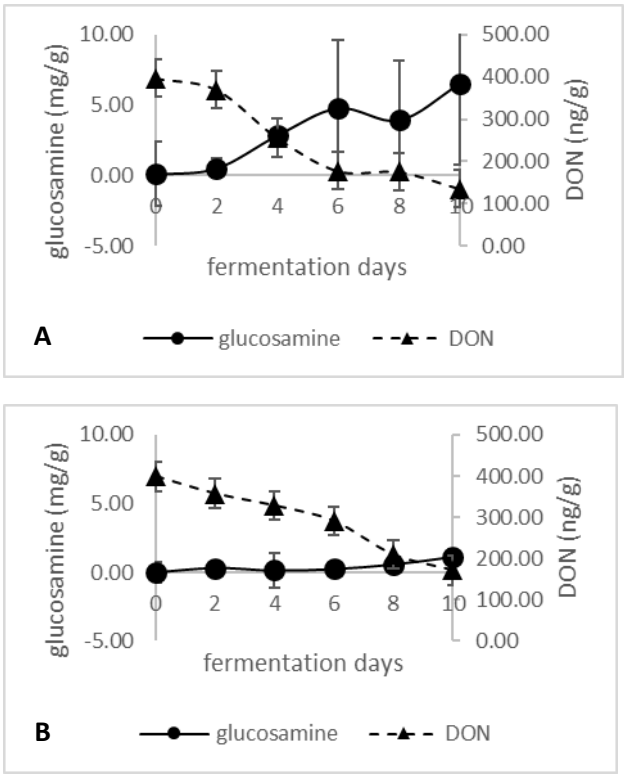

Figure 2: The correlation between fungal biomass and levels of DON; (A) *Aspergillus oryzae* KKB4 and (B) *Rhizopus oryzae* KP1R1.

DON detoxification by *A. oryzae* KKB4 and *R. oryzae* KP1R1

Several previous studies just focused on reducing DON levels without considering the safety of the degradation products. In this research, toxicity test of fermentation products by *A. oryzae* KKB4 and *R. oryzae* KP1R1 was conducted using yeast *S. cerevisiae*. Trichothecenes are potent inhibitors of protein biosynthesis in eukaryotic cells due to receptor like binding sites on 80S ribosomes, leading to an inhibition of *peptidyl transferase* (Binder *et al.,* 1997; Binder, 1999). Thus, another application for testing toxicity of trichothecenes is by inhibition of yeast cell growth.

The growth of *S. cerevisiae* cells was inhibited by the pure standard of DON, as identified in Figure 3. It was clearly that there decreased optical density and cell viability values in 500 ng/mL DON. Consequently, 500 ng/mL DON was an initial toxicity limit in disrupting cell growth.

The successful DON detoxification by *A. oryzae* KKB4 and *R. oryzae* KP1R1 in 10 days fermentation has been exhibited in Figure 4. The medium containing 500 ng/mL DON standard had adverse effects on the cell growth. Meanwhile, a medium that containing fermentation samples by *A. oryzae* KKB4 and *R. oryzae* KP1R1 after being concentrated to reach the same concentration as the initial toxic concentrations of DON did not give a side effect in disruption to cell growth. As a result, the cells had still grown normally. Although, its sample had a half DON concentration with the maximum limit of DON at 1000 ng/g; the DON reduction products by *A. oryzae* KKB4 and *R. orzyae* KP1R1 had no toxic effects on *S. cerevisiae* cells. It might be happened since DON reduction by *A. oryzae* KKB4, and *R. oryzae* KP1R1 was enzymatic and resulting in the changing of DON structure (data not shown), such as disrupting the double bond in the A-ring at C9, disrupting the R5 keto functional group at C8 and modification at C3 (Foroud *et al.*, 2016). Studies by Borzekowski *et al.* (2018) showed that genera *Aspergillus* and *Rhizopus* can reduce the toxicity by glycosylation at C3 in trichothecenes.

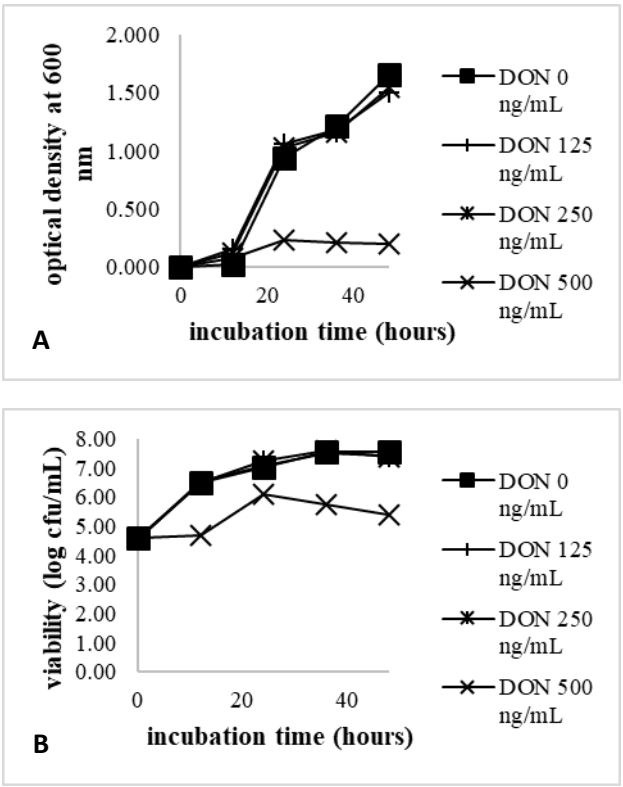

Figure 3: The growth of *S. cerevisiae* cell in *PGY broth* mixed with DON standard solution (A) optical density at 600 nm and (B) cell viability.

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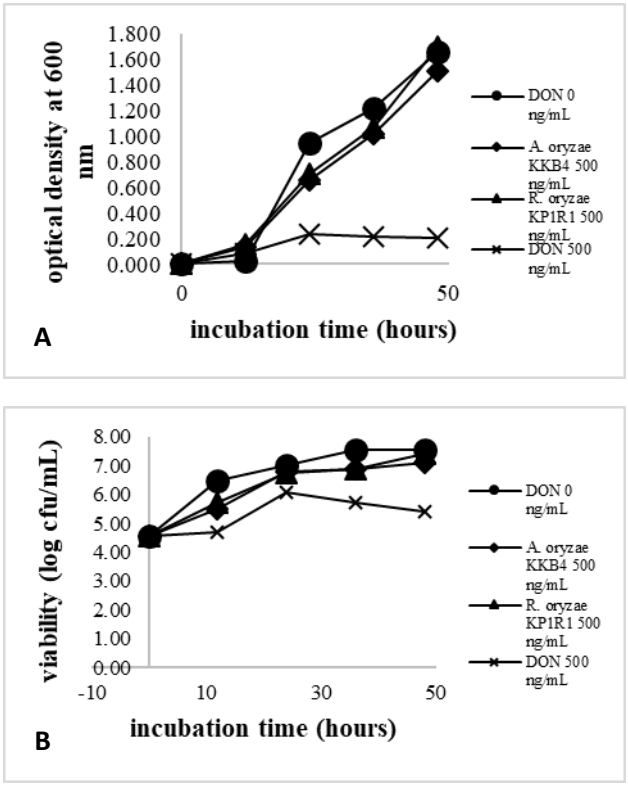

Figure 4: Toxicity of DON degradation products; (A) optical density at 600 nm and (B) cell viability.

CONCLUSION

Based on the results, *A. oryzae* KKB4 and *R. oryzae* KP1R1 were able to reduce DON concentration in contaminated corn through solid-state fermentation. The toxicity results in this bioassay revealed that *A. oryzae* KKB4 and *R. orzyae* KP1R1 could also detoxify and produced fermented corn which has no toxic effects to *Saccharomyces cerevisiae.*

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