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THE EFFECT OF PHYTIC ACID, ZINC AND SOYBEAN EXTRACT ON THE GROWTH AND AFLATOXIN B1 PRODUCTION BY *Aspergillus flavus*

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ABSTRACT

It has been reported that aflatoxin contamination in soybean was relatively low, but it was not guaranteed that soybean products is free from aflatoxin contamination. Naturally, soybean containing phytic acid and it bound zinc and protein. Zinc (Zn) is an important mineral for aflatoxin biosynthesis. Previous research indicated that some soybean products such as kecap was contaminated by aflatoxin. It might be Aspergillus flavus involved during kecap fermentation and it produced phytase for phytic acid degradation. Zinc will be released and available for aflatoxin biosynthesis. The aim of this research was to evaluate the effect of phytic acid, Zn and soybean extract on the growth and aflatoxin B1 (AFB1) production by Aspergillus flavus. Five kind of medium were used in the experiment, Glucose Ammonium Nitrate (GAN) medium, a special medium for aflatoxin production, GAN without Zn, GAN supplemented with phytic acid, GAN supplemented with soybean extract instead of glucose and soybean extract supplemented with phytic acid. Two and a half milliliter of spore suspension (10^7spores/ml) was inoculated into 250 ml of each medium in 1 liter flask. Incubation was done in shaker incubator at room temperature. The growth of mold and AFB1 production were analyzed on 3 and 6 days incubation time. The result indicated that phytic acid lowering the growth of mold in the early 3 days, but not at all after 6 days incubation. It seems that phytic acid delays the aflatoxin production. Lack of Zn in the medium brought about the lowering of aflatoxin production. Even glucose concentration in soybean extract medium was lower than in GAN medium, the growth of the mold was not inhibited but lower on glucose affect on decreasing of AFB1 production.

Keywords: *Phytic acid, zinc, soybean extract, aflatoxin B1*

INTRODUCTION

Kecap (Indonesian soy sauce) is a traditional fermented food that is widely produced and consumed in Indonesia. It is generally produced from black soybean through two step of fermentation. First, by mold fermentation, principaly by *Aspergillus oryzae* and *Aspergillus sojae,* and it is called koji fermentation. The second fermentation was brine fermentation, in which salt tolerant lactic acid bacteria and yeast were involved during the fermentation. Almost all traditional kecap fermentations were conducted under uncontrolled condition, and the probability of aflatoxin producing mold involved during koji fermentation was relatively high. Previous research showed that about 47 % commercial kecap was contaminated by aflatoxin (Sardjono *et al*., 1995). It was indicated that *Aspergillus flavus* involved in koji fermentation.

In general, zinc, magnesium, asparagines, proline, and high sucrose concentration stimulate aflatoxin production, whereas higher levels of inorganic nitrogen and phosphate inhibit it (Dutton, 1988). Zinc is considered to favor polyketide (intermediate product for aflatoxin) biosynthesis (Bhatnager et al., 1986). Other researcher also reported that Zn was important as a trigger for aflatoxin production (Eldridge, 1964; Mateles and Adye, 1965). Soybean contains phytic acid, which it able to bind Zn, and it should inhibit the aflatoxin production, but in fact, contamination of aflatoxin in soybean products such as kecap is still high. It might be because of the degradation of phytic acid during preparation of soybean as well as during fermentation.

Phytic acid is a phosphorus containing compound that is derivative of inositol, cyclohexanehexol. Phytic acid is generally found in the seed of plants, and the amount found in legumes is higher than that in cereals. The phytic acid decreases during fermentation, presumably, due to microbial phytase. Phytates are excellent chelators, and their importance in soybean products is linked with their binding of mineral. This binding may change the availability of some mineral. Zinc is mineral that seems to be the most affected by the presence of phytic acid from soybean (Snyder and Kwon, 1987).

There are several step of processing in kecap production before mold fermentation, i.e. soaking and boiling. During soaking, phytic acid decreased about 18 % (Setyono, 1987; Noor, 1992), and boiling or steaming really decreased the phytic acid in soybean (Lease *et al*., 1960). *Aspergillus flavus* produce phytase as an extracellular enzymes, that can degrade phytic acid during its growth. The degradation of phytic acid might be the important thing for aflatoxin production in soybean.

The aim of this research is to evaluate the effect of phytic acid, Zn, and soybean extract for the growth and aflatoxin production of *Aspergillus flavus*.

MATERIALS AND METHOD

Microorganism

Aspergillus flavus 0561 from Depertment of Fermentation Technology, Osaka University, was used for the experiment. Culture was maintain in Potato Dextrose Agar (PDA) medium.

Medium

Five different media was prepared:

1. Glucose Ammonium Nitrate (GAN) consist of 50 g glucose, 2.4 g NH_2NO_3, 10 g KH_2PO_4 and 2 g $MgSO_4.7H_2O$ per liter. Every 1 liter medium was added 1.3 ml A solution consist of 20 g $ZnSO_4.7H_2O$, 2 g $CuSO_4.5H_2O$ and 1 g of $Co(NO_3)_2.H_2O$ per liter and 1.3 ml B solution, consist of 50 g $CaCl_2$ per liter.
2. GAN medium without $ZnSO_4$&H_2O (GAN-Zn)
3. GAN medium added by phytic acid (GAN+ft). The amount of phytic acid was 0.5% in medium, similar with phytic acid in soybean
4. Glucose in GAN medium was substituted by 50 g soybean extract. The extract was prepared by water extraction of 1 part of soybean with 2 part of water and than filtered. The extract was used instead of glucose as carbon source (EAN)
5. EAN medium + phytic acid (EAN+ft)

Inoculum Preparation and Cultivation

Pure culture of *Aspergillus flavus* 0561was grown in PDA for 7 days. Spores was harvested by addition of 10 ml 0.05 % of Tween 80 solution, centrifuged at 5,000 rpm for 15 minutes. Spore was suspended for the concentration of 10^7/ml. Every 250 ml of each medium in 1 liter flask were inoculated by 2.5 ml spore suspension and incubated at room temperature in shaker incubator for 6 days. Experiment was carried out in duplicate. Sample was taken every day for analysis of mycelium dry weight and sugar, and at the third day and 6th day for AFB1 analysis.

Analysis

The growth of mold was measured by mycelium dry weight. Mycelium was separate from liquid medium by filtration on pre weight filter paper, wash and oven dried at 100 ºC for 20 jam, and constant weight of dried mycelium was used for growth evaluation.

Reducing sugar was analyzed by using Nelson Somogyi (Sudarmadji *et al*., 1984) and AFB1 in the growth medium was analyzed by extracting the medium using chloroform, and the extract was dryness, the extract was redisolved in 1 ml methanol. After clean up, AFB1 was quantitatively analyzed using HPLC. Octadecyl silica gel reverse phase column (250X4.6 mm), particle size 5 µm and Waters scanning fluorescens detector was used. The extension and emission wavelength were set at 365 nm and 435 nm, respectively. Methanol was used for mobile phase at 0.5 ml/min flow rate. Integrator Chrompact C-R3A Shimadzu Japan was used for data processing.

RESULT AND DISCUSSION

Mycelium Growth

It was shown in Figure 1, that Zn not only important for AFB1 biosynthesis but it is also important for mycelium growth. In the medium of GAN-Zn, after 3 days incubation, the mycelium dry weight almost did not increase, and it was similar in medium of soybean extract. Presumably, in soybean extract contained phytic acid that it can bind Zn, so Zn was not available for the growth. Addition of phytic acid in medium affect on lower growth of mycelium, but after 3 days, the growth was not inhibited. Presumably, phytase enzyme of *Aspergillus flavus* degrades the phytic acid and after 3 days, Zn was available for the growth of mold. The elements iron, calcium, manganese and zinc are required as cofactors for enzymes and other functional protein. The absent of these element of course affect the growth of mycelium (Carlie *et al*., 2001).

Reducing Sugar

Decreasing reducing sugar in medium containing soybean extract was small and relatively constant (Fig. 2). Even reducing sugar concentration of medium was lower than other medium, the growth of mold was not affected. Medium EAN+ phytic acid produced highest mycelium dry weight at the end of fermentation (Fig. 1). It might be in soybean extract contain many nutrient such as amino acids, other nitrogen compound and phosphorus compound that it can be used

by the mold for its growth. But the present of phytic acid in the medium slightly inhibit the growth, it was shown by the lower of mycelium dry weight during the 3 days earlier, presumably it was similar reasons that phytic acid binding Zn, and the mineral was not available for the growth of mold. Decreasing reducing sugar in GAN medium was faster after 3 days incubation, compare to the day before. It might be needed for AFB1 biosinthesis, shown by the highest AFB1 in this medium (Fig.3).

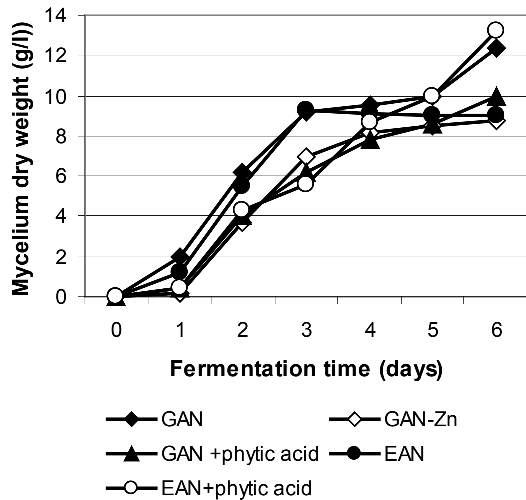

Figure 1 Mycelium growth of Aspergillus flavus in different media

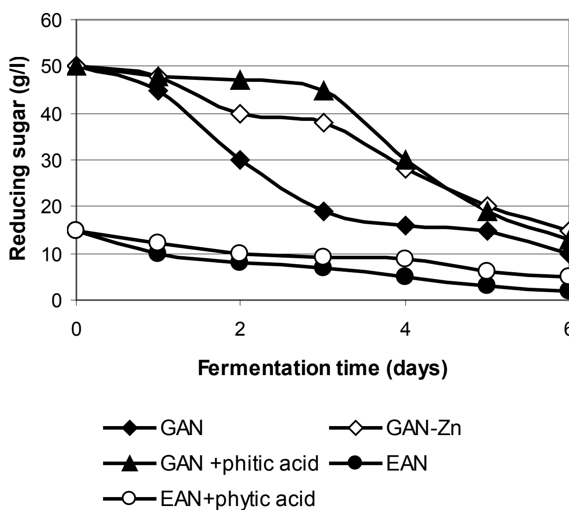

Figure 2 Concentration of reducing sugar during the growth of mold

Aflatoxin B1 Production

It was very clear that Zn is important for aflatoxin biosynthesis. In medium without Zn, mold produced the lowest concentration of AFB1. In general, Zinc, magnesium, asparagines, praline, and high sucrose concentration stimulate aflatoxin production, whereas higher levels of inorganic nitrogen

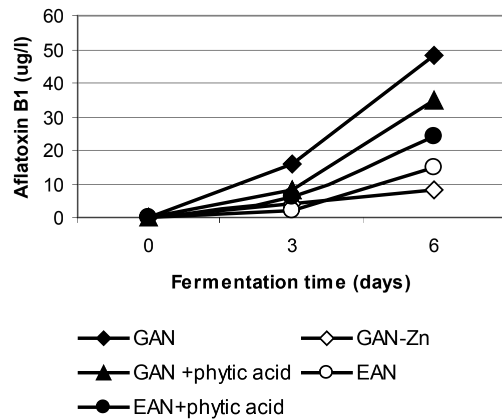

Figure 3 Production of aflatoxin B1during the growth of mold

and phosphate inhibit it (Dutton, 1988). Zinc is considered to favor polyketide (intermediate product for aflatoxin) biosynthesis (Bhatnager, *et al*., 1986). Phytic acid seems to inhibit the AFB1 production during the early 3 days incubation, but it was not shown after 3 days incubation period. Presumably in the beginning fermentation, phytic acid in the medium bound with Zn and there was no Zn available for aflatoxin biosynthesis, but after 3 days incubation, extracellular phytase enzyme produced by *Aspergillus flavus* degrades the phytic acid and finally Zn was released and available for aflatoxin biosynthesis. In medium containing soybean extract showed lower AFB1 production. It is because of lowering sugar in medium that it is necessary for inducing AFB1 biosynthesis (Dutton, 1984) and phytic acid found in soybean extract.

The Ability of AFB1 Production

It is defined as the amount of AFB1 produced every amount of mycelium (Fig. 4). It was clear that Zn was contribute on the ability of mold for AFB1 production shown by the lowest value if it was grown in the medium of (GAN-Zn). Glucose Ammonium Nitrate medium produce the highest ability of mold to produce AFB1, because this medium was designed for aflatoxin biosynthesis. The ability of mold for AFB1 biosynthesis was decrease when phytic acid was present in the growth medium, but did not inhibit the AFB1 production. Utilization of Soybean extract give the similar result, because in the soybean extract also found phytic acid

From this research it was clear that phytic acid did not inhibit the AFB1 production, but lowering the potency of mold for AFB1 production. It seem that phytic acid able to delay the AFB1 production. Presumably during the growth of mold, the phytase enzyme degrades the phytic acid, and finally Zn was available for AFB1 biosynthesis. It was also clear that Zn was very important for the growth and AFB1 biosynthesis.

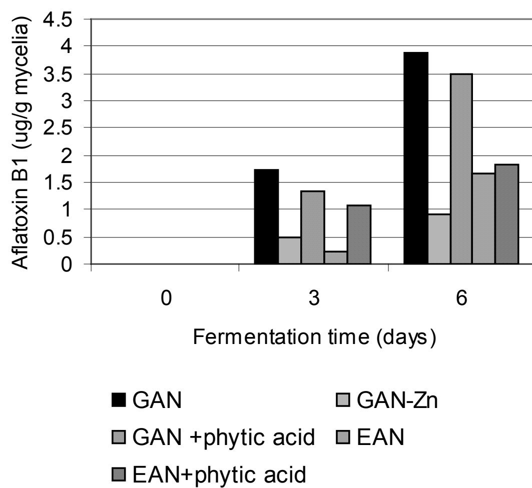

Figure 4. The ability of AFB1 production by mold on different media

CONCLUSSION

Phytic acid did not inhibit AFB1 biosynthesis, but delayed the production of AFB1 by *Aspergillus flavus*. Element Zn is very important for AFB1 biosynthesis. The growth of *Aspergillus flavus* in soybean extract medium produced the lower production of AFB1. This result indicated that soybean is not good medium for aflatoxin biosynthesis, as long as phytic acid in soybean is not degraded.

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