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INVESTING IN FOOD QUALITY, SAFETY & NUTRITION

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Roles of Consumers

Improving Food Safety & Quality

Improving Competitiveness of Traditional Foods

Improving Food Security

Improving Nutrition

Inhibition of *Aspergillus parasiticus* Growth and Reduction of Aflatoxin by Yeast Isolated from *Ragi*, an Indonesian Traditional Culture Starter

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Abstract

Aflatoxins are secondary metabolites of *Aspergillus flavus* and *A. parasiticus* frequently found to contaminate both food and feed. The toxins have been found to be carcinogenic and their presence in food or feed is strictly regulated. Indonesia's climate with high humidity, temperature and amount of rainfall are supportive of mold growth and hence aflatoxin production. Control of aflatoxin production can be achieved by using competitor microorganisms such as mold and yeast. The objective of this research was to isolate yeasts capable of reducing aflatoxin from *ragi*, an Indonesian traditional culture starter and to evaluate the yeast or its metabolite's activity in inhibiting *A. parasiticus* growth, aflatoxin synthesis and degradation of preformed aflatoxin.

The research showed that *all* isolates reduced aflatoxin production by *A. parasiticus*. *Saccharomyces* sp. of *ragi* NKL gave the highest reduction of aflatoxin (AF) production, i.e. 95.9% for aflatoxin B1 (AFB1); 97.1% for aflatoxin B2 (AFB2); 89.4% for aflatoxin G1(AFG1); 99.1% for AFG2 and 98.1% for total aflatoxin. The metabolites of the *Saccharomyces sp.* also decreased aflatoxin production by *A. parasiticus*. The yeast also showed ability to degrade preformed AFB1, AFB2, AFG1, AFG2 by 36.4 %; 55.6 %; 37.8 %; and 46.7 %, respectively.

Introduction

Aflatoxins are secondary metabolites produced by mold such as *Aspergillus flavus* and *A. parasiticus*. The molds are frequently found to contaminate both food and feed and may produce the toxin to a harmful level.

The toxins have been reported as class 1 carcinogens and their presence in food or feed is strictly regulated.

Indonesia's tropical climate with high humidity, temperature and amount of rainfall are supportive of mold growth and hence aflatoxin production. The mold could grow during the plantation and may also enter during uncontrolled storage of some cereals and nuts. Several studies had reported high prevalence of aflatoxin in peanut (Dharmaputra *et al.*,1991) and maize (Roedjito *et al.*, 1994) in Indonesia.

Control of aflatoxins in food can be carried out using physical, chemical and biological approach. Due to their resistant to heat, heating is not an effective mean of reducing the toxins. Chemical treatment to reduced aflatoxin using ammonium has been reported by yielded unacceptable food by sensory evaluation. Biological control of aflatoxin using molds or yeast that competes with aflatoxin producer has been reported, such as the use of "afla-guard" in peanut plant (Gardener, 2005).

In Indonesia, several traditional culture starters, called *ragi*, are commonly used in producing various fermented food. Such *ragi* is usually composed of mixtures of molds and yeasts. These microorganisms are potentially capable of competing with *A. flavus* or *A. parasiticus* thus can be used for controlling aflatoxin production in food. Since *ragi* has been used as food ingredient, the mold and or yeasts contained in ragi are generally non toxigenic.

Objectives

The objectives of this research were to isolate yeasts capable of reducing aflatoxin production by *A. parasiticus* from *ragi*, evaluate yeast and its metabolites'ability to inhibit *A. parasiticus* growth and aflatoxin synthesis as well as determine its ability to reduce preformed aflatoxin.

Materials and Methods

Material used for obtaining the yeast isolates was *ragi*, an Indonesian traditional dry starter culture to make *tape* (fermented glutinous rice). Various brands of *ragi* were obtained from traditional markets in several cities in Java, i.e. Jakarta, Bandung, Semarang, Rembang, Yogyakarta and Madiun. A toxigenic mold, *Aspergillus parasiticus* F 010 was obtained from Balitvet Culture Collection (BCC), Research Institute for Veterinary Science (Balitvet), Ministry of Agriculture, Bogor). Media used for this study were Potato Dextrose Agar (PDA, Difco), Acidified Potato Dextrose Agar (APDA), Potato Dextrose Broth (PDB, Difco), Malt Extract Broth (MEB, Difco), Peptone (Difco), and Yeast Nitrogen Base, Corn Meal Agar (Difco), Czapek Yeast Extract Agar, Malt Extract Agar dan 25 Glycerol Nitrate Agar.

The research consisted of the following steps: (1) isolation and identification of yeasts isolated from ragi, (2) selection of a yeast isolate based on its ability in reducing aflatoxin production by A. parasiticus, (3) evaluation of the effect of selected yeast on *A. parasiticus* growth, (4) evaluation of the effect of the yeast metabolites on *A. parasiticus* growth and aflatoxin production by *A. parasiticus*, (4) evaluation of effect of the yeast isolate on preformed aflatoxin. Figure 1 describes the outline of the research.

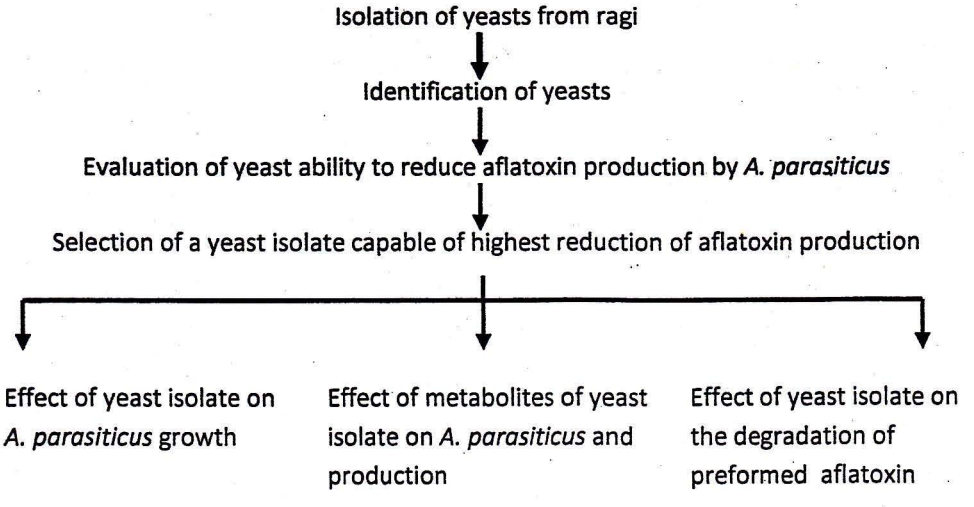


Figure 1. Outline of the research

Isolation of Yeasts from Ragi

Isolation of yeasts was carried out according to Daulay (1989), in which 5 g of ragi is plated onto APDA.

Identification of Yeast

Yeast identification consisted of morphological examination, physiological testings including fermentation and assimilation of seven types of sugar growth at 37°C and their ability to produce extracellular polysaccharide. Results of morphological observation and physioolgical testings were matched with yeast characteristics according Lodder (1974) and Barnett *et al.* (2000).

Yeast Culture Preparation

Yeast isolates were grown in PDA slants for 2 day, and cell suspension was made by addition of sterile distilled water. Cell number was enumerated with hemasitometer and its concentration wass adjusted to ca. 10^6 CFU/g.

Selection of Yeast Isolates for Reduction of Aflatoxin Produced by A. parasiticus (Sardjono *et al.* 1992 with some modification)

A half mililiter of *A. parasiticus spores* (ca 10^6 spores/ml) was mixed with 0.5 ml of each yeast isolate suspension (10^6 cells/ml). The mixture was then inoculated onto 100 ml of PDB and incubated for 10 days at room temperature. As a control, a half mililiter of *A. parasiticus* spores (10^6 spores/ml) was inoculated onto PDB medium and incubated for the same period. The amount of aflatoxin produced by the mold was analysed using thin layer chromatography (TLC). The amount of aflatoxin produced by *A. parasiticus* in the presence of yeast was then compared to that of *A. parasiticus* only. Yeast isolate causeing the most reduction of aflatoxin produced was selected for further studies.

Effect of Yeast Isolate on *A. parasiticus* Growth (modified from Gourama and Bullerman 1995)

Approximately 0.5 ml of spore suspension of *A. parasiticus* (10^6 spores/ml) was mixed with 0.5 ml of yeast cultures (10^6 CFU/ml) and then inoculated into 100 ml of MEB. The mold and yeast mixtures in MEB was

incubated at room temperature.As a control, 0.5 ml of spore suspensions of *A. parasiticus* (10^6 spores/ml) or 0.5 ml yeast suspension (10^6 CFU/ml) is independently inoculated into MEB medium, and was also incubated at room temperature. Samples of the mixtures and indiviual mold or yeast were taken and samples were plated on PDA at days 0, 3, 6 and 9. The number of colonies of *A. parasiticus* dan yeast was enumerated. At day 9, slide cultures were made and direct interaction between the yeast and the mold was observed using light microscope.

Direct Interaction of Yeast and *A. parasiticus* (modified from Chan dan Tian 2005)

Onto a Petridish containing solidified PDA, place agar (diameter of 5 mm) containing 3 days old mycellia of *A. parasiticus* and then incubated for 72 h at room temperature. A drop (50µl) of yeast suspension (10^8 cells/ml) was then placed onto the edge of the mycellium, and incubated for 48 h. Yeast colonies growing on the Petri dished was washed with running water for 2 min and the were then observed using light microscope.

Effect of Yeast Metabolites on *A. parasiticus* Growth and Aflatoxin Synthesis (modified from Sardjono *et al.* 1992)

Yeast metabolites was prepared by growing the yeast isolate in PDB for 7 d at room temperature. Yeast cells were separated by centrifugation at 4000 rpm for 15 min, and supernatant containing metabolites was passed through a filter with pore size of 0.2 µm in diameter. Yeast metabolites was then inoculated with 0.5 ml spore suspension of *A. parasiticus* (10^6 spores/ml) and was incubated at room temperature for 12 d. As a control, 0.5 ml of *A. parasiticus* spore (10^6 spore/ml) was inoculated into MEB in the absence of yeast metabolite. Aflatoxin concentration and the dry weight of the mycellium were measured after 3, 6, 9 and 12 days of incubation at room temperature.

Effect of Yeast Isolates on the Degradation of Preformed Aflatoxin (modified from Sardjono *et al.* 1992)

Aflatoxin of *A. parasiticus* was produced by growing 7 days old of *A. parasiticus* in PDB and incubate it for 7 days at room temperature. Supernatant was separated from the mycellium and then passed through a filter with pore

size of 0.2 μm in diameter. Supernatant containing aflatoxin was mixed with MEB medium (1:1) and 0.5 ml of yeast cell suspension was inoculated into the aflatoxin-containing MEB. The mddium was then incubated for 12 d at room temperature. As a control, a mixture of supernatant containing aflatoxin and MEB medium without addition of yeast cell suspension was incubated at the same incubation time and temperature. Aflatoxin concentration was measured at day-0, 3, 6, 9 and 12.

Aflatoxin Extraction (Heathcot 1984)

Ten mililiters of supernatant was added with 10 ml of chloroform and then homogenized in a blender for 2 min and shaken for 30 min. The mixture was placed in a separation bottle such that two layers were formed. The lower layer containing aflatoxin was filtered through anhydrous sodium sulphate, and the extract was evaporated using a rotavapor. The remaining residue was solubilized in chloroform and was used for aflatoxin analysis.

Aflatoxin Analysis (AOAC 1995)

Analysis of aflatoxin was carried out with one dimension Thin Layer Chromatography (TLC) using chloroform : acetone (9 : 1) as the mobile phase.

The TLC plate used was Silica Gel 60 (Merck). Identification step was done by spotting sample and standard solutions on the TLC plate. The plate was placed in a chamber containing mobile phase and when the spot has stopped moving, the plate was then air dried. Results of the elution was observed under uv light at 365 nm. The Rf (*Rate of Flow*) values of samples and standards were compared and aflatoxin was determined as positif when the Rf of the sample was similar to that of the standard. The aflatoxin concentration was enumerated by comparing fluoresence intensity of samples with those of aflatoxin standards using the following formula :

$$\text{Aflatoxin (ppb)} = \frac{S \times Y \times V \times Fp}{W \times Z}$$

S : Volume of aflatoxin standard (μl) giving fluoresence equivalent to Z μl of sample

Y : Aflatoxin standard concentration (µg/ml)
Z : Volume of sample extract that gives fluoresence equivalent to S µl aflatoxin concentration
V : Volume of solvent used to solubilize final sample extract (µl)
W : Volume of sampel (ml)
Fp : Dilution factor

Measurement of Dry Weight of Mold Mycellium (Gourama dan Bullerman 1995).

The mold mycellium was filtered using vacuum filtration, washed twice in distilled water and dried with oven at 105°C until constant weight was obtained.

Results and Discussions

Isolation of Yeasts from *Ragi* and Effect of the Yeasts on Aflatoxin Reduction

Three morphologically different yeasts were isolated from samples of *ragi*. Identification step showed that they belonged to the Genera *Saccharomycopsis* and *Saccharomyces*. The ability of isolates in reducing aflatoxin production by *A. parasiticus* varies and is shown in Table 1.

Saccharomyces sp. isolated from Ragi NKL has the highest aflatoxin reduction capacity. The isolate decreased aflatoxin production by *A. parasiticus* by 95.9%, 97.1%, 99.1% and 98.1% for AFB1, AFB2, AFG1 and AFG2, respectively. *Saccharomyces* of ragi NKL was then selected for further studies to determine the mechanism of aflatoxin reduction. .

Table 1. Aflatoxin reduction by yeasts isolated from *ragi*

Yeast	Brand of *Ragi*	Aflatoxin reduction (%)				
		AFB1	AFB2	AFG1	AFG2	Total
Saccharomycopsis sp.	Berlian	71.6	85.9	89.4	89.7	86.2
Saccharomycopsis sp.	Gedang	77.1	77.5	68.7	64.3	72.4
Saccharomyces sp.	NKL	95.9	97.1	89.4	99.1	98.1

Effect of Saccharomyces on *A. parasiticus* Growth

Figure 2 showed the effect of the yeast isolate on the growth of *A. parasiticus*. *Saccharomyces* sp. caused three logs cycle reduction of *A. parasiticus* growth at three days of incubation. Inhibition of the mold growth was likely caused by nutritive competition between *Saccharomyces* sp. and *A. parasiticus*, since *Saccharomyces* sp. had shorter generation time than that of *A. parasiticus*. Dharmaputra *et al* (2003) stated that fungi with faster growth than *A. flavus* would be potential to control the growth of *Aspergillus flavus*.

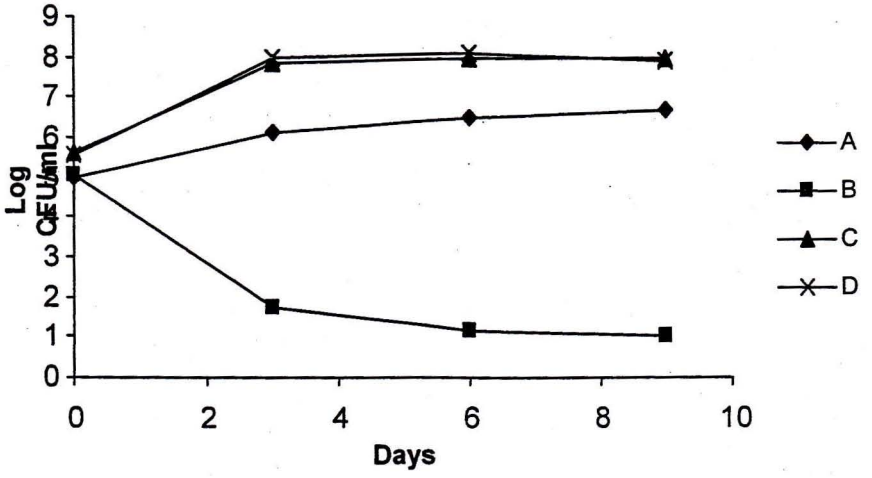
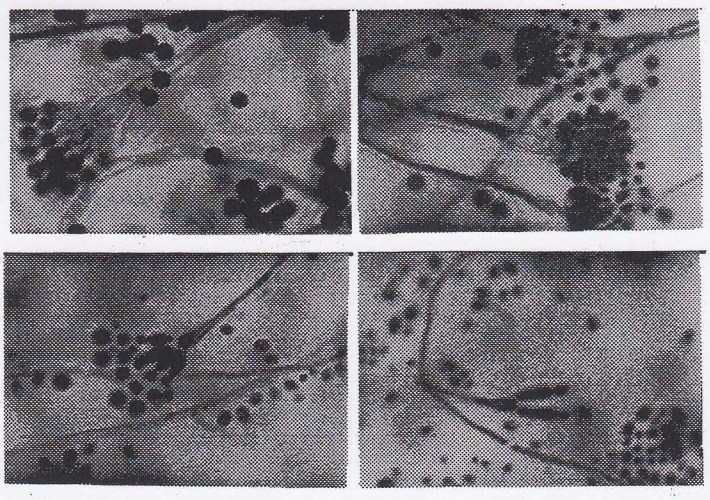


Figure 2. Inhibition of *A. parasiticus* growth by *Saccharomyces* sp. ; **A.** Number of *A. parasiticus* in the absence of competitor; **B** Number of *A. parasiticus* in the presence of ; **C** Number of *Saccharomyces* sp. in the presence of *A. parasiticus* ; **D** Number of *Saccharomyces* sp. in the absence of mold.

The yeast isolated from ragi also influence the physical appearance of the aflatoxin producing mold. Observation of the morphology of *A. parasiticus* growing in the presence of *Saccharomyces* sp. using light microscope showed that the vesicle size of the mold was smaller and the number of phyalide were less than those of control) (Figure 3). The observation was made at the end incubation (ninth day).



(1)

(2)

Figure 3. Effect of *Saccharomyces* sp. on *A. parasiticus* morphology after 9 days incubation (1) *A. parasiticus* (2) *A. parasiticus* together with *Saccharomyces* sp. (magnification1000x)

β-gluconase enzyme produced by *Saccharomyces* sp. has been reported to destroy cell wall of *A. parasiticus*. This could be accounted for the morphological changes of *A. parasiticus* when it was grown in the presence of the yeast. The microscopic observation also showed that *Saccharomyces* sp. adhered to *A. parasiticus* hypha as presented in Figure 4.

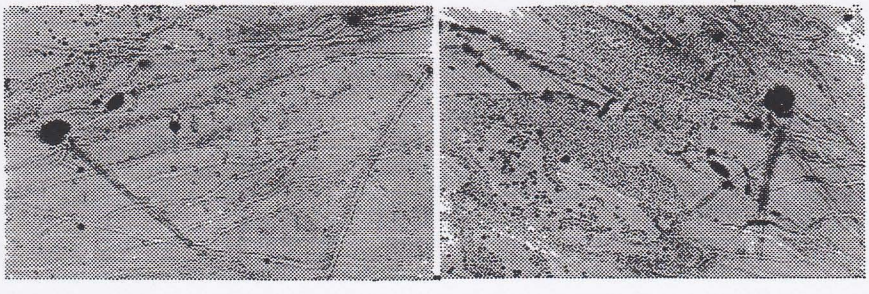


Figure 4. Microscopic observation showing direct interaction between *Saccharomyces* sp. and *A. parasiticus*

Effect of Yeast Metabolites on *A. parasiticus* growth and Aflatoxin Biosynthesis

The metabolites of *Saccharomyces* sp. isolated from ragi had inhibition activity on *A. parasiticus* growth. This research showed that *A. parasiticus*

grown in a medium containing yeast metabolite yieded a lower mass as indicated by the lower weight of the mycelium as compared to that grown alone in a medium (Figure 5).

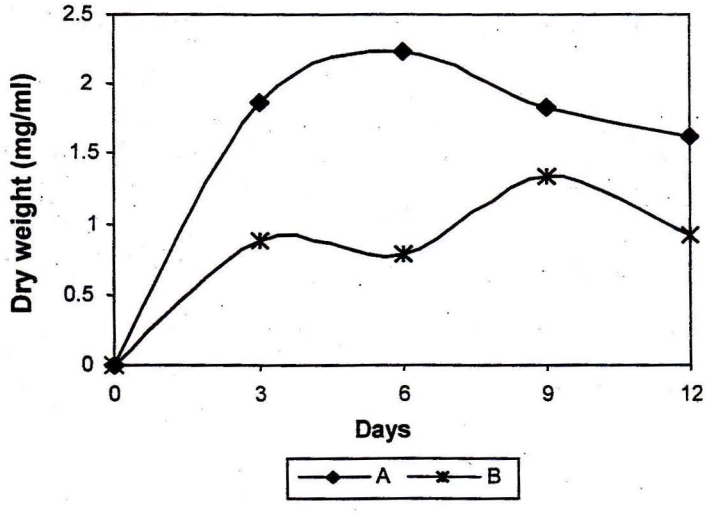


Figure 5. Effect of *Saccharomyces* sp. metabolites on *A. parasiticus* growth; **A** *A. parasiticus* control; **B** *A. parasiticus* growing in medium supplemented with *Saccharomyces* sp. metabolites

In addition, *Saccharomyces* sp. metabolites also inhibited aflatoxin biosynthesis by *A. parasiticus*. The yeast isolated from *ragi* inhibited all types of aflatoxins, i.e. aflatoxin B1, B2, G1 and G2 (Figure 6). This observation was similar to Purwijantiningsih (2005) who reported that *Candida* sp. metabolites inhibited *A. flavus* growth and aflatoxin biosynthesis by the mold.

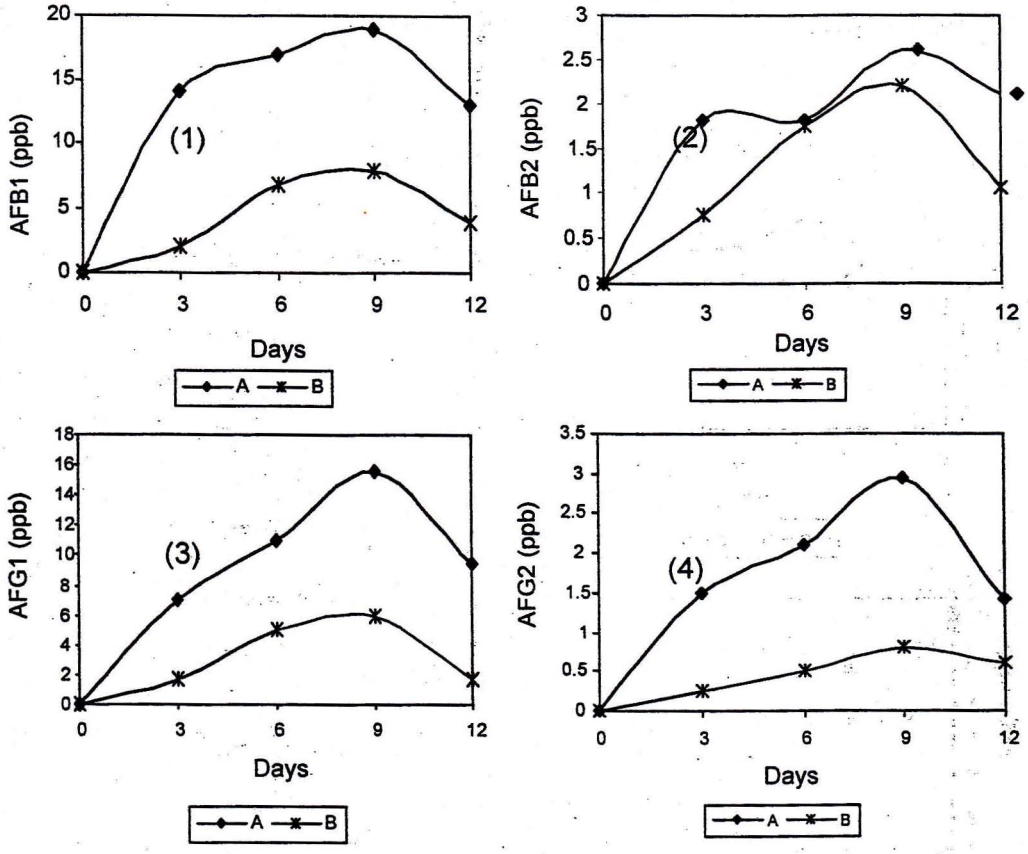


Figure 6. Effect of *Saccharomyces* sp. metabolite on biosynthesis of aflatoxin (1) B1 (2) B2 (3) G1 (4) G2: **A** *A. parasiticus* growing in the absence of yeast metabolites; **B** *A. parasiticus* growing in medium supplemented with yeast metabolites

Effect of *Saccharomyces* sp. on the Degradation of Preformed Aflatoxin

Degradation of aflatoxin was studied by inoculating *Saccharomyces* sp. into medium containing preformed aflatoxin. The results showed that *Saccharomyces* sp. decreased the amount of preformed aflatoxin by 44.88% (Figure 7).

Degradation of aflatoxin could be caused by aflatoxin binding by other microorganisms or their components.. Mannan and glucan of the cell wall of *S. cerevisiae* cell have been reported to bind and reduce several mycotoxins, such as aflatoxin B1, zearalenon, fumonisin and deoxynivalenol by 95%, 77%, 59%

and 12 % consecutively (Galvano *et al* 2001). Aflatoxin reduction by other fungi has been reported by Nakazato et al. (1990).

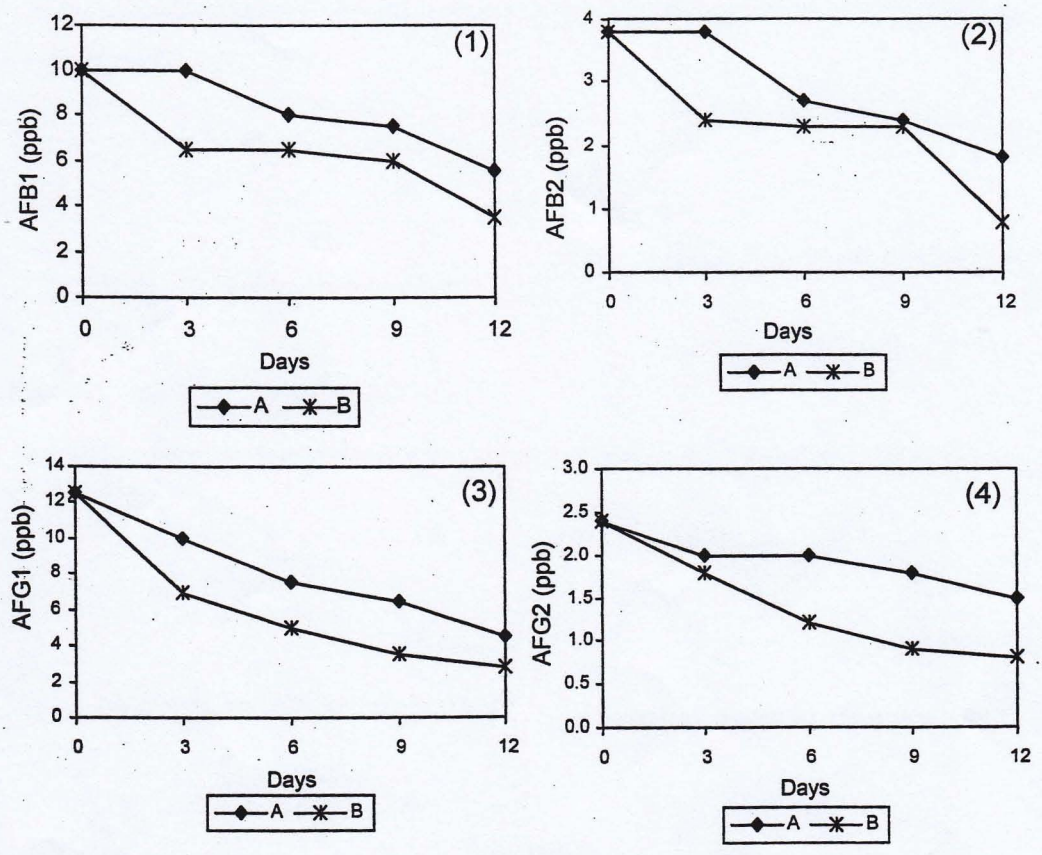


Figure 7. Degradation of preformed aflatoxin (1) B1 (2) B2 (3) G1 (4) G2 by *Saccharomyces* sp.

Conclusions

Several yeasts belonging to Genera of *Saccharomyces* and *Saccharomycopsis* isolated from *ragi* possesed ability to reduce aflatoxin produced by *A. parasiticus*. *Saccharomyces* sp. from *ragi* NKL was able to reduce aflatoxins of *A. parasiticus* by 98,1%. The isolated yeast also inhibited *A. parasiticus* growth, as shown by the decrease in mold growth, smaller vesicles and lower number of phyallids. Reduction of aflatoxin by *Saccharomyces* was also due to its metabolites. The yeast metabolites were able to inhibit *A.*

parasiticus growth and aflatoxin biosynthesis. *Saccharomyces* sp. also capable of degradation of preformed aflatoxin and reduced preformed aflatoxin by 44.88%.

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