ANTAGONISTIC EFFECT OF YEAST, ACETIC ACID BACTERIA AND MANGOSTEEN RIND EXTRACT ON AFLATOXIGENIC *Aspergillus flavus* IN UNFERMENTED COCOA BEANS

NIJMA NURFADILA[1,2], SUTRISNO[2], USMAN AHMAD[2] AND SAMSUDIN[3]

[1]*SEAMEO BIOTROP, Jalan Raya Tajur Km. 6, Bogor 16134, Indonesia*
[2]*Study Program of Postharvest Technology, Faculty of Agricultural Technology, Institut Pertanian Bogor, Bogor 16680, Indonesia*
[3]*Balai Penelitian Tanaman Industri dan Penyegar (BALITTRI), Parungkuda, Sukabumi, Indonesia 43357*

Received 22 May 2020 / Accepted 6 January 2021

ABSTRACT

Yeasts and bacteria are two of common biocontrol agents to control mycotoxigenic fungi. Meanwhile, the mangosteen rind extract contains xanthone and gartanin compounds for antioxidant, antiproliferation, antiinflammation, antimicrobial, and anticancer. The objectives of this research were to test the effects of yeasts, acetic acid bacteria (AAB), and mangosteen rind extract on the aflatoxigenic *Aspergillus flavus* growth and aflatoxin production in unfermented cocoa beans. Four yeast isolates, i.e., *Issatchenkia orientalis (Io)* BIO 211291, 286 and 288, and *Endomyces fibuliger (Ef)* BIO 132219; one bacteria isolate of *Acetobacter aceti* (*Aa*) FNCC0016; and mangosteen rind extract (MRE) were tested for their capabilities in inhibiting an aflatoxigenic *A. flavus* (*Af*) BIO 3361/747 growth using the well method (*in vitro*). Two types of yeast (*Io* BIO 211291 and 288) were combined with *Aa* and MRE in cocoa beans (*in vivo*). Aflatoxin production was analyzed using *Thin Layer Chromathography* (TLC). The results showed that interaction of *Io* BIO 211291 and 288, and *Ef* BIO 132219 on aflatoxigenic *Af* were interaction with inhibition zone ≥ 2 mm (type D), while the interaction type of *Io* BIO 211286 on *Af* were mutual intermingling growth, where both fungi grew into each other without any macroscopic sign of interaction (type A). The best treatment in agar media (*in vitro*) was *Io* BIO 211288 + *Aa* on Potato Dextrose Agar + 12 g/L MRE. The highest *Io* population was 5.88 log cfu/g on cocoa beans inoculated by *Io* BIO 211291 + MRE in 1 day after inoculation, while the highest *A. aceti* population was 4.74 log cfu/g on cocoa beans with *Io* BIO 211291 + BIO 211288 + *Aa* in 3 days after inoculation. Two best treatments were *Io* BIO 211288 + *Aa* + MRE and *Io* BIO 211291 + BIO 211288 + *Aa* + MRE, because there were no *A. flavus* population since 3 until 11 days after inoculation. Aflatoxin in all samples treatment was lower than limit detection B_1 (< 2.20 ppb), B_2 (< 3.50 ppb), (G_1 < 0.54 ppb), dan (G_2 < 1.00 ppb).

Keywords: aflatoxin, antagonistic, *Aspergillus flavus*, mangosteen rind, yeasts

INTRODUCTION

Indonesia is the 3rd cocoa beans exporter countries after Ivory Coast and Ghana (Dickson 2018). Agricultural Department (2010) reported as much as 93% of cocoa beans in Indonesia was processed without fermentation (only sun-dried) conducted by farmers, while the remaining 7% of cocoa beans was processed by fermentation. Thompson *et al.* (2013) explained that cocoa beans fermentation process involves some microorganisms, i.e., yeasts, acetic acid bacteria (AAB), lactic acid bacteria (LAB), *Bacillus* and several other bacteria, as well as filamentous fungi. According to Nurhansyah (2011) some importer countries such as Malaysia and United States of America reduced the amount of cocoa beans import from Indonesia, because the physicochemical quality of Indonesian cocoa beans were lower than those from Africa. The decreasing of cocoa beans quality was caused by unappropriate handling during the harvesting, fermentation, drying, storing, and packaging processes that facilitates fungal contamination.

*Corresponding author, email: n.nurfadila@yahoo.co.id; nijma@biotrop.org

According to Asrul (2009) unfermented cocoa beans and mycotoxigenic fungi contamination, especially aflatoxigenic *Aspergillus flavus,* were problems in Indonesia. Some fungi were isolated from cocoa beans in Central Sulawesi, i.e., *Aspergillus flavus*, *A. niger*, *A. fumigatus*, *Penicillium* sp., *Fusarium* sp., *Trichoderma* sp., *T. viride*, *Rhizopus* sp., *Mucor* sp., *Verticillium* sp., and *Geotrichum* sp. *Aspergillus flavus* was isolated in cocoa beans after being harvested by farmers (7.2×10^8 cfu/mL), collector (4.5×10^5 cfu/mL), and exporter levels (4.1×10^3 cfu/mL). Aflatoxin B_1 content in cocoa beans after being harvested by farmers (104.80 ppb), collectors (61.31 ppb), and exporters (47.74 ppb). Copetti *et al.* (2011) reported the occurrence of aflatoxigenic fungi and the presence of aflatoxin in 226 cocoa samples collected in Brazilian farms. The aflatoxigenic fungi isolated in cocoa beans were *A. flavus*, *A. parasiticus* and *A. nomius*. A considerable increase in numbers of these species was observed during drying and storage processes. Pires *et al.* (2019) explained that the total aflatoxins in two cocoa bean samples from their study, from Bahia, from Pará and from Rondônia were 13.2, 16.3, 11.7, and 30 µg/kg, respectively.

According to Basappa (2009) aflatoxin is a kind of toxins produced by *A. flavus* and *A. parasiticus* that causes liver cancer in human and animals. The types of aflatoxins found in foodstuffs and processed products are B_1, B_2, G_1, and G_2, but the most dangerous for human health is aflatoxin B_1 (AFB1).

One of the problems in unfermented cocoa beans is aflatoxigenic fungi contamination. Therefore, formulating and concocting a combination treatment to inhibit aflatoxigenic fungi is necessary. Combinations of yeast, acetic acid bacteria, and mangosteen rind extract were used for testing the effectiveness in decreasing aflatoxigenic *A. flavus* population and aflatoxin production. Dharmaputra *et al.* (2018a) reported that *Issactchenkia orientalis* could inhibit 100% of ochratoxigenic *A. ochraceus*. It means that *I. orientalis* can be used as biocontrol agent, eventhough there is no one research explains that *I. orientalis* can inhibit aflatoxigenic *A. flavus*. Sabahannur and Ralle (2018) reported not only yeast, but *Acetobacter aceti* also supposed to maintain shelf life of food stuff. Meanwhile Yatman (2012) explained that mangosteen can be used as medicine, because it contains xanthone for antioxidant, antiproliferation, antiimflammatory, antitumor, and anticancer. Research conducted by Akao *et al.* (2008) showed that xanthone α-mangostin compound of mangosteen rind exract could inhibit 50% of colon cancer cells growth. The inhibitive nature of the compound is similar with commercially available anticancer drugs, i.e., 5-FU, actinomycin D, and campotechin. Aisha *et al.* (2012) reported that xanthone extract, α-mangostin, and γ-mangostin inhibited 50% of cancer cell in 6.5 ± 1.0 mg/mL, 5.1 ± 0.2 µg/mL, and 7.2 ± 0.4 µg/mL. Therefore, combinations between yeast, acetic acid bacteria (AAB) and mangosteen rind extract (MRE) is expected to produce biocontrol agent on aflatoxigenic *A. flavus* growth and decrease aflatoxin production in unfermented cocoa beans to be safe for next processing of chocolate products.

The objectives of this research were to test the effects of yeasts, acetic acid bacteria (AAB), and mangosteen rind extract on the aflatoxigenic *A. flavus* growth and aflatoxin production in unfermented cocoa beans. It is expected that the research result would show a potential combination of yeast, acetic acid bacteria (AAB), and mangosteen rind extract (MRE) as biocontrol agent to inhibit aflatoxigenic *A. flavus* to improve food safety in unfermented cocoa beans.

MATERIALS AND METHODS

Yeast Isolates, Acetic Acid Bacteria, Aflatoxigenic Fungus, Unfermented Cocoa Beans, and Mangosteen Rind Extract

As many as four yeast isolates were used in interaction types and antagonistic test, i.e., 1 isolate of *Endomyces fibuliger* (*Ef*) BIO 132219 and three isolates of *Issatchenkia orientalis* (*Io*) BIO 211286, BIO 211288, and BIO 211291; only two yeast isolate were used in cocoa beans (*in vivo*) that showed the highest percentage of inhibition on *A. flavus in vitro*, i.e., *I. orientalis* BIO 211291 and BIO 211288. Aflatoxigenic *Aspergillus flavus* BIO 3361/747 were obtained from Phytopathology Laboratory Culture Collections,

SEAMEO BIOTROP; and 1 acetic acid bacteria isolate (*Acetobacter aceti* FNCC0016) was obtained from Food and Nutrition Study Centre, Universitas Gadjah Mada, Yogyakarta. As much as 62 kg of unfermented cocoa beans were obtained from Sumedang Regency, West Java Province. As much as 2 kg of mangosteen rind extracts was obtained from *RJ Herbal*, Surabaya, East Java Province.

Interaction Types Test Between Yeast and Aflatoxigenic *A. flavus*

Four yeast isolates were tested on aflatoxigenic *A. flavus* BIO 3361/747 using direct opposition method (Dharmaputra *et al.* 2018b) (Fig. 1). This method was used to determine the interaction types between the yeasts and aflatoxigenic *A. flavus*. Aflatoxigenic *Aspergillus flavus* BIO 3361/747 was inoculated after 4 days of each yeast inoculation in the middle of Potato Dextrose Agar (PDA) media in petri dishes (a diameter of 9 cm) with a distance of 3 cm between each other. The petri dishes with each fungal and yeast were then incubated at room temperature (27 ± 2 °C) for 7 days. Five replicates were used for each isolates. The observation on the interaction types was conducted macroscopically between the yeast and aflatoxigenic *A. flavus* (Wheeler and Hocking 1993). The interaction types were shown in Table 1.

The mathematical equation for calculating the percentage of inhibition between the yeast and aflatoxigenic *A. flavus* is as follows:

$$H = \frac{J_1 - J_2}{J_1} \times 100 \quad (1)$$

Notes: % I = percentage of inhibition, J_1 = diameter of *A. flavus* near to petridish, J_2 = diameter of *A. flavus* near to yeast.

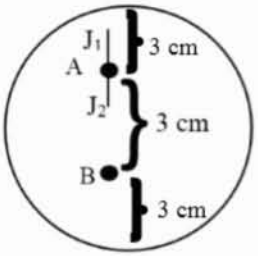
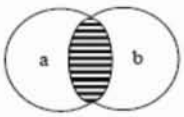
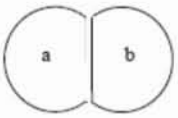
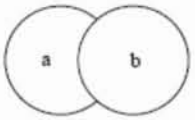
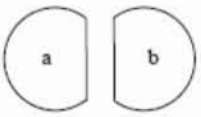
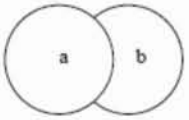


Figure 1 Scheme of antagonisms test between yeast isolate and toxigenic *A. flavus;* A= toxigenic *A. flavus,* B = yeast isolate, J_1 = diameter of *A. flavus* near to petridish, J_2 = diameter of *A. flavus* near to yeast

Table 1 Interaction types between two fungal colonies (Wheeler & Hocking 1993)

Type of interaction	Description of classification	
A	Mutual intermingling growth, where both fungi grew into each other without any macroscopic signs of interaction	
B	Mutual inhibition on contact or space between colonies small (< 2mm)	
C	Inhibition of one species on contact, the inhibited species continued to grow at a significantly reduced rate, while the inhibitor species grew at a slightly reduced rate or unchanged	
D	Mutual inhibition at a distance (> 2 mm)	
E	Inhibition of one species on contact, the inhibitor species continuing to grow at a reduced rate through the inhibited colony	
F	Inhibition of one species on contact or at a distance, the inhibitor species then continuing to grow at an unchanged rate through or over the inhibited colony	

Notes: a: inhibitor fungi, b: inhibited species.

Obtaining of Unfermented Cocoa Beans and Mangosteen Rind Extract

Ripe lindak cocoa (bulk cocoa) fruits with yellow color were harvested using sterile scissors from the trees. The cocoa beans were were then cut open using a knife and separated into parts of pods and cocoa beans with pulp. The next process were washing of cocoa beans from pulp and drying using sun-drying for 1 day (9 hours) to become unfermented cocoa beans. Obtaining of mangosteen rind extract is shown in Figure 2.

As much as 12 g/L of mangosteen rind extract (MRE) was used in *in vitro* stage, while 12 g for 500 g of cocoa beans used in *in vivo* stage. According to Kusumaputri (2011), Dr. Berna Eliya as a phytochemist in Universitas Indonesia, explained that many people consume a glass of boiled water containing mangosteen rind extract. They usually use 60 g of fresh mangosteen rind equal to 12 g of mangosteen rind extract for 1 day. The doses of mangosteen rind extract (MRE) was obtained based on consumer's doses in MRE capsule product, as follows:

$$DE = \frac{JK \times BE}{BK} \quad (2)$$

$$DE = \frac{60\ capsules \times 400\ mg}{2\ capsules}$$

$$DE = 12{,}000\ mg = 12\ g$$

Notes: DE = doses of mangosteen rind extract (g), JK = number of capsules in 1 package of mangosteen rind extract product, BE = weight of mangosteen rind extract in 1 capsule (mg), and BK = number of capsules to be consumed in 1 day.

Antagonistic Test of Yeast, *Acetobacter aceti,* and Mangosteen Rind Extract Combinations on Aflatoxigenic *A. flavus in Vitro*

Antagonistic test between yeast, *Acetobacter aceti* and mangosteen rind extract on aflatoxigenic *A. flavus* BIO 3361/747 was conducted using the well test method (Dharmaputra *et al.* 2016 with modification). *Acetobacter aceti* FNCC0016, *Issatchenkia orientalis* BIO 211291, BIO 211288, BIO 211286, and *Endomyces fibuliger* BIO 132219) were cultured on Malt Extract Agar (MEA) media and incubated for 7 days, while the *Acetobacter aceti* FNCC0016 was cultured on Peptone Glucose Yeast Extract Agar (PGYA) media and incubated for 3 days at room temperature (27 ± 2 °C). Aflatoxigenic *Aspergillus flavus* BIO 3361/747 was cultured on Potato Dextrose Agar (PDA) media and incubated for 7 days at room temperature (27 ± 2 °C). Five pieces (in 5 mm diameter) of pure culture of each yeast were placed into 25 mL of Nutrient Yeast Dextrose Broth (NYDB) media in an erlenmeyer flask (100 mL volume), while the *Acetobacter aceti* isolate was placed into 25 mL of Nutrient Broth (NB) media. They were then incubated at 27 ± 2 °C for 7 days, and were shaken for 1 hour every 24 hours for 5 days. The conidia cells of aflatoxigenic *A. flavus* (5 x 10^6 cells/mL) were obtained by adding 15 mL of distilled water, then the *A. flavus* was scratched on the upper surface using sterile inoculation needle. The conidia cells were filtered by sterile gauze on the funnel of erlenmeyer flask (100 mL volume).

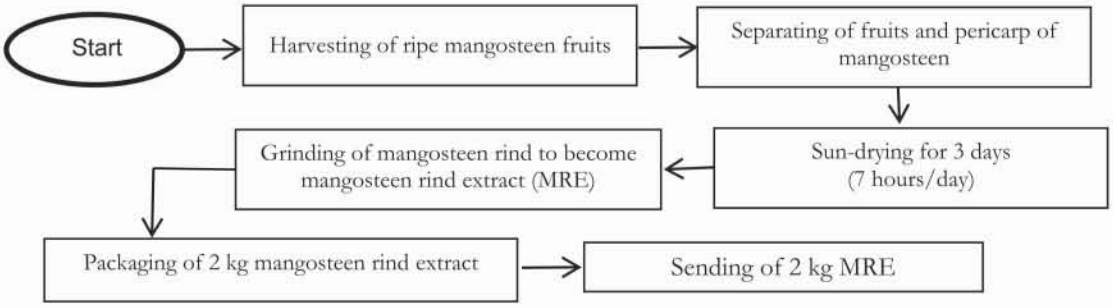


Figure 2 Stages of obtaining of mangosteen rind extract (MRE)

The yeast, *Acetobacter aceti*, and *A. flavus* cells were precipitated by centrifugation using a centrifuge at 7,000 rpm fixed angle rotor for 15 minutes and rinsed using twice sterile distilled water, and they were then resuspended in sterile distilledwater until the concentration reached 5 x 10^8 cells/mL (yeast cells) and 5 x 10^6 cells/mL (*Acetobacter aceti* and aflatoxigenic *A. flavus).* Yeasts, *Acetobacter aceti* and *Aspergillus flavus* cells were counted using a hemocytometer.

A well (5 mm diameter) was prepared using a cork borer in the center of Potato Dextrose Agar (PDA) media containing 15% cocoa beans juice and PDA media containing 15% cocoa beans juice and 12 g/L of mangosteen rind extract depending on the treatments into a petri dish (9 cm diameter). As much as 20 µL of $5x10^8$ cells/mL yeast cells suspension were placed into the well. The petri dishes were left for 30 minutes to allow penetration of cells suspension into the well. Next, as much as 20 µL of 5×10^6 cells/ml *Acetobacter aceti* was inoculated, then *A. flavus* was also inoculated into the well after 1 hour. Each yeast control was only inoculated with yeast cells suspension. Each treatment and controls were made in 3 replicates (= 5 petri dishes), which were then incubated at room temperature (27 ± 2 °C). The growth of the aflatoxigenic *A. flavus*, yeasts, and *Acetobacter aceti* in each petri dish was observed after 7 days of incubation. Total unit experiment for *in vitro* process was (4 yeast isolates x 2 within or without *Acetobacter aceti* x 2 kinds of media x 3 replicates) + ((4 yeast control + 1 *A. flavus* control) x 3 replicates) = 63. The radius of aflatoxigenic *A. flavus* colony in each petri dish was measured before the colony reached to petri dish. Mathematical equation for the percentage of fermentor inhibition to aflatoxigenic *A. flavus* is:

$$\% I = \frac{D_1 - D_2}{D_1} \times 100 \quad (3)$$

Notes: % I = percentage of inhibition, D_1 = diameter of *A. flavus* control (mm), D_2 = diameter of *A. flavus* in each treatment (mm).

Testing of Combination Treatments to Inhibit Aflatoxigenic *Aspergillus flavus in Vivo*

As many as two yeast isolates with the highest percentage of inhibition *in vitro* stage, *Acetobacter aceti* FNCC0016, and *Aspergillus flavus* BIO 3361/747 were used *in vivo* stage. The steps in the testing of the combination treatments to inhibit aflatoxigenic *A. flavus in vivo* were similar to *in vitro.* Each treatments included positive and negative controls using 500 g of unfermented cocoa beans for each replicates. As much as 10 mL of 10^8 cells/mL yeast cells suspension were placed into 500 g of unfermented cocoa beans depending on the treatments. Each samples was then inoculated or not inoculated by 10 mL of 10^6 cells/mL *A. aceti* (depend on the treatments) after 30 min, then as much as 10 mL of 10^6 cells/mL aflatoxigenic *A. flavus* was inoculated into the samples depending on the treatments after 1 hour. Each cells inoculation was conducted sequentially to allow the cells penetration. Total unit experiment for *in vivo* process was 12 x 4 x 2 = 96 (12 = types of combination including positive and negative controls; 4 = days after inoculation (1,3, 6 and 11); 2 = replication).

Determination of *Issatchenkia orientalis*, *Acetobacter aceti,* and Aflatoxigenic *Aspergillus flavus* Populations, and Aflatoxin Production

Yeast, *Acetobacter aceti*, and *A. flavus* were isolated using serial dilution method (10^{-1} up to 10^{-5}), followed by pour plate method on Potato Dextrose Agar (PDA) and incubated for 7 days incubation at 27 ± 2⌙ (INS 2008). Aflatoxin contents were determined using Thin Layer Chromatoghraphy (TLC) (Baiton *et al.* 2006).

RESULTS AND DISCUSSION

Interaction Types Between Yeast and Aflatoxigenic *A. flavus*

As many as four yeasts (one isolate of *Endomyces fibuliger* BIO 132219 and three isolates of *Issatchenkia orientalis* BIO 211286, BIO 211288, and BIO 211291) were used in testing the mechanisms of antagonism on aflatoxigenic *A. flavus* BIO 3361/747 using direct opposition method. The interaction type of antagonism mechanism between yeast and *I. orientalis* BIO 211291 (49.58%) and BIO 211288 (35.79%), and *E. fibuliger* 132219 (49.29%) with aflatoxigenic *A. flavus* was D (Fig. 3a, 3c, 3d; Table 1). This interaction type showed that there

was a mutual inhibition with the inhibition zone of ≥ 2 mm similar with Wheeler and Hocking (1993). The interaction type of *I. orientalis* BIO 211286 with *A. flavus* BIO 3361/747 was A (Fig. 3b) where the percentage of each inhibition was 15.63%, respectively (Table 1). This interaction showed mutual intermingling growth, where both fungi grew into each other without any macroscopic signs of interaction (Fig. 3; Table 1).

Based on the percentage of the inhibition of *A. flavus* growth using direct opposition method, three isolates (*I. orientalis* BIO 211291, *E. fibuliger* 13219, and *I. orientalis* BIO 211288) were higher than *I. orientalis* BIO 211286. It means that *I. orientalis* BIO 211291 and BIO 211288, and *E. fibuliger* BIO 13219 were prefer as potential component inhibitor for aflatoxigenic *A. flavus* in cocoa beans. Nevertheless, the three yeast isolates should be combined with additional materials to increase the ability of inhibition on aflatoxigenic *A. flavus* in unfermented cocoa beans. However, this result was strengthened in the next step using the well *in vitro* method.

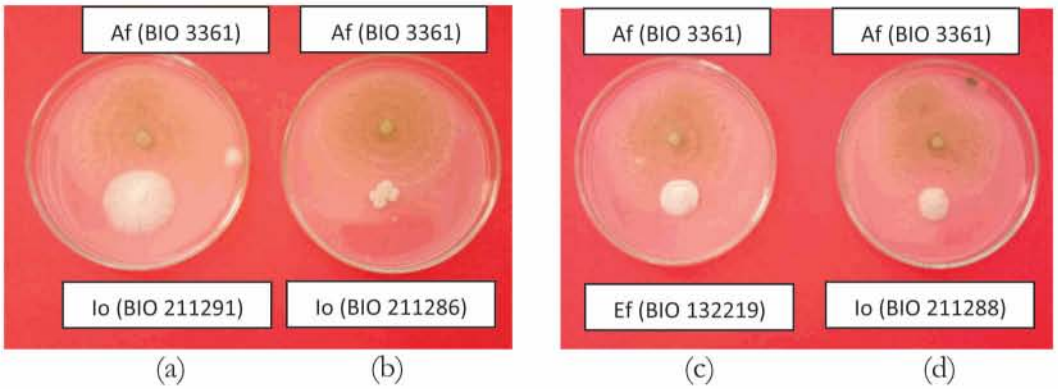
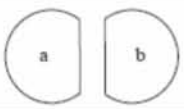
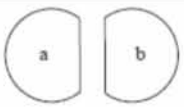
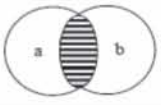
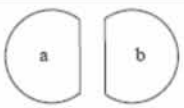


Figure 3 Mechanism of interaction between four yeast isolates (*Issatchenkia orientalis*): (a) BIO 211291; (b) BIO 211286; (c) *Endomyces fibuliger* BIO 133219; and (d) *I. orientalis* BIO 211288); aflatoxigenic *Aspergillus flavus* on Potato Dextrose Agar (PDA) media after 7 days of incubation at room temperature (28 ± 2 °C)

Table 1 Interaction types and the percentage of inhibition between yeast on aflatoxigenic *Aspergillus flavus* based on direct opposition method

Isolate	Mean of radius (mm)		% Inhibition	Interaction type	Interaction figure
	J_1	J_2			
Issatchenkia orientalis BIO 211291 vs *A. flavus*	23.8	12	49.58 a	D	
I. orientalis BIO 211288 vs *A. flavus*	23.75	15.25	35.79 b	D	
I. orientalis BIO 211286 vs *A. flavus*	24	20.25	15.63 c	A	
Endomyces fibuliger BIO 132219 vs *A. flavus*	28	14.2	49.29 a	D	

Antagonistic Test in Combination of Yeast, *Acetobacter aceti* and Mangosteen Rind Extract on Aflatoxigenic *Aspergillus flavus In Vitro*

According to Richard and Prusky (2002) yeast has some unique charactheristics such as fast growth, the ability to colonize surface of fruit, and the ability to join in nutrition competition with a pathogen, therefore it can be a biocontrol agent. Yeast also has an important role in fermentation, because it can convert glucose and maltose through anaerobic respiration. The well method is a method for determining on the percentage of inhibition between yeast and pathogenic fungi. All treatments except yeast controls were not inoculated by aflatoxigenic *A. flavus*.

Widiyanto *et al.* (2013) reported that unfermented cocoa beans has no any glucose, because the pulp has been removed. It means that additional material is needed to maintain beneficial microorganisms survival in unfermented cocoa beans. According to Maligan *et al.* (2018) mangosteen rind extract (MRE) contents in 100 g are 82.50% carbohydrate, 6.45% fats, 3.02% proteins, 5.87% water, and 2.10% total glucose. The nutritional content of MRE may increase the yeast survival. MRE also has xanthone for anticancer, antihyperglicemic, and antioxidant for human health. Other research, *Acetobacter* is one of bacteria that can oxidize glucose to gluconic and other organic acid in the same time to maintain the shelf life of foodstuff (Simanjuntak *et al.* 2016). Therefore, in this research, each of four yeast isolates were combined with acetic acid bacteria and MRE *in vitro* stage to test the effectiveness of those treatment in different media (PDA + 15% cocoa beans juice with and without 12 g/L MRE).

The highest percentage of inhibition on aflatoxigenic *A. flavus* BIO 3361/747 was 100%, in treatment with *Issatchenkia orientalis* BIO 211288 + *Acetobacter aceti* FNCC0016 on Potato Dextrose Agar (PDA) + 15% cocoa beans juice + 12 g/L mangosteen rind extract (MRE) media (Fig. 4a; Table 2). It means those treatment more effective than other treatments to against the aflatoxigenic *A. flavus in vitro*. The 2nd highest percentage of inhibition was 51.98%, in treatment *I. orientalis* BIO 211291 on PDA + 15% cocoa beans juice + 12/g MRE (Fig. 4b; Table 2). Hafsari (2011) explained that the differences in fungal diameter indicated that yeast growth is faster than that of fungi and it also obtained more nutrition than pathogenic fungi. The stunting growth of fungi was shown from the diameter of colony that was lower than the control (+). According to Janisiewicz and Korsen (2002) the mechanism of space and nutrition competitions could happened if the yeast's effort was higher than pathogenic fungi to get nutrition and space. The similar research from Golubev (2006) explained that the capability of yeast antagonism would increase on other microorganism from different habitat, because the fungi are a new competitor that should be defeated to become a dominant in the available space and nutrition.

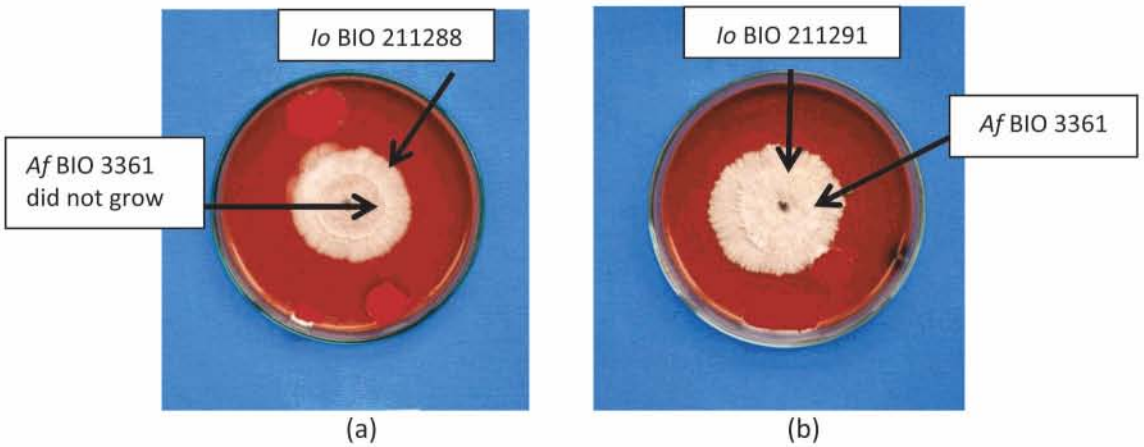


Figure 4 (a) *Issatchenkia orientalis* BIO 211288 and (b) *I. orientalis* BIO 211291 vs toxigenic *A. flavus* BIO 3361/747 with *Acetobacter aceti* on Potato Dextrose Agar + 15% cocoa beans juice + 12 g MRE at room temperature (27 ± 2 °C) after 7 days of incubation

Table 2 Effect of different combination and media treatments on the percentage of inhibition and growth of yeast, acetic acid bacteria, and aflatoxigenic *A. flavus*

Treatment		Diameter (mm)			Inhibition (%)
Combination	Media	Yeast	*Acetobacter aceti*	*Aspergillus flavus*	
Issatchenkia orientalis 291 + *A. flavus*	PDA + 15% cocoa beans juice	15.45	-	37.20	20.00
I. orientalis 288 + *A. flavus*		27.35	-	30.15	25.56
I. orientalis 286 + *A. flavus*		ND	-	40.00	9.03
Endomyces fibuliger 219 + *A. flavus*		ND	-	42.10	9.46
I. orientalis 291 + *A. flavus*	PDA + 15% cocoa beans juice + 12 g/L mangosteen rind extract	25.50	-	34.17	26.52
I. orientalis 288 + *A. flavus*		34.47	-	25.23	45.74
I. orientalis 286 + *A. flavus*		ND	-	39.79	14.43
E. fibuliger 219 + *A. flavus*		ND	-	38.13	18.00
I. orientalis 291 +*Acetobacter aceti* + *Aspergillus flavus*	PDA + 15% cocoa beans juice	11.21	ND	30.60	34.19
I. orientalis 288 +*A. aceti* + *A. flavus*		30.01	ND	24.57	47.16
I. orientalis 286 +*A. aceti* + *A. flavus*		ND	ND	37.92	18.45
E. fibuliger 219 + *A. aceti* + *A. flavus*		ND	ND	39.44	15.18
I. orientalis 291 +*A. aceti* + *A. flavus*	PDA + 15% cocoa beans juice + 12 g/L mangosteen rind extract	29.98	5.26	22.33	51.98
I. orientalis 288 +*A. aceti* + *A. flavus*		34.45	18.62	ND	100.00
I. orientalis 286 +*A. aceti* + *A. flavus*		ND	ND	40.87	12.11
E. fibuliger 219 + *A. aceti* + *A. flavus*		ND	ND	39.77	14.47
I. orientalis 291 (control)	PDA + 15% cocoa beans juice	35	-	-	0
I. orientalis 288 (control)		40	-	-	0
I. orientalis 286 (control)		15	-	-	0
E.fibuliger 219 (control)		11	-	-	0
A.flavus (control+)		-	-	46.50	0

Notes: (-) = not inoculated; ND = not detected; 0 = no inhibition (control).

Based on different yeast isolates, *I. orientalis* BIO 211291 and *I. orientalis* BIO 211288 were potential as a component inhibitor for *A. flavus* BIO 3361/747 *in vitro*. Based on different agar media, PDA + 15% cocoa beans juice + 12 g/L MRE media was more effective than PDA + 15% cocoa beans juice media to inhibit aflatoxigenic *A. flavus in vitro*. It means that MRE influenced on decreasing of aflatoxigenic *A. flavus* growth *in vitro*. Yatman (2012) reported that xanthone of mangosteen rind extract can be used as antimicrobial, antioxidant, antifungi, and anticancer.

Populations of *Issatchenkia orientalis*, *Acetobacter aceti*, and *Aspergillus flavus In Vivo*

Two yeast isolates with the highest percentage of inhibition on aflatoxigenic *A. flavus in vitro* were *Issatchenkia orientalis* BIO 211291 and BIO 211288. The yeast isolates were used *in vivo* stage. The highest population of yeast *I. orientalis* of cocoa beans in 1 day after inoculation was 5.88 log cfu/g, found in samples inoculated by with *I. orientalis* BIO 211291 + mangosteen rind extract (MRE) + *A. flavus*. The highest *I. orientalis* population in 3 and 6 days after inoculation were 4.67 and 3.75 log cfu/g, found in samples inoculated by *I. orientalis* BIO 211291 + BIO 211288 + *A. aceti* + MRE + *A. flavus*. The highest *I. orientalis* population in 11 days after inoculation was 2.82 log cfu/g, found in samples inoculated by *I. orientalis* BIO 211291 or BIO 211288 + MRE + *A. flavus* (Table 3). The negative and positive controls, sample with *Acetobacter aceti* + mangosteen rind extract + *A. flavus* were not inoculated by *I. orientalis*. Based on different days after inoculation, commonly *I. orientalis* population in 3 day after inoculation was higher than 1, 6 and 11 days after inoculation. Yeast population in all samples with MRE were not significant different with samples without MRE.

Jamili *et al.* (2016) reported the dominant yeasts were found in cocoa beans during fermentation, i.e., 1 isolate *Candida krusei*, 3 isolates *C. tropicalis*, 1 isolate *Saccharomycopsis fibuligera*, 1 isolate *Kloeckera* sp., and 1 isolate *Saccharomyces cerevisiae*. According to Ren *et al.* (2020) many microorganisms including bacteria, non-toxigenic fungi, and yeast strains have been investigated as potential biocontrol agents against to aflatoxigenic fungi. Lee *et al.*(2008) reported that peroxisomal *3-ketoacyl-CoA thiolase*

(ScFox3) in *Saccharomyces cerevisiae* inhibited pathogenic fungi such as *Botrytis cinerea, Didymella bryoniae, Fusarium moniliforme, F. solani, Penicillium verrucosum, Rhizoctonia solani*, and *T. harzianum*. Other research, *I. orientalis* also has *3-ketoacyl-CoA, 3-hydroxyacyl-CoA,* and *trans-2-enol-CoA* (WIPO-PCT 2019). It means that *I. orientalis* also can be used as a component of biocontrol agents to inhibit aflatoxigenic *A. flavus*.

As many as 7 of the 12 cocoa beans were inoculated by *A. aceti*. The highest *A. aceti* population of cocoa beans in 1 and 11 day after inoculation were 4.22 and 3.12 log cfu/g, found in sample inoculated by *I. orientalis* BIO 2111291 + BIO 211288 + *A. aceti* + mangosteen rind extract (MRE) + *A. flavus*. Cocoa beans were inoculated by *I. orientalis* BIO 2111291 + BIO 211288 + *A. aceti* + *A. flavus* had the highest *A. aceti* population in 3 and 6 days after inoculation, i.e. 4.75 and 3.30 log cfu/g (Table 3). Based on different days after inoculation, commonly the population of those in 3 days after inoculation was higher than 1, 6, and 11 days after inoculation. *Acetobacter aceti* population in all samples with MRE were higher than samples without MRE, although the populations were not significant differences.

The activity of acetic acid bacteria took place after converting of glucose into alcohol and lactic acid. Ardhana and Fleet (2003) reported that the population of acetic acid bacteria (*A. pasteurianus* and *A. aceti*) were isolated in cocoa beans as much as 10^5 - 10^6 cfu/g. According to Simanjuntak *et al.* (2016) oxidation of glucose into gluconic acid and other organic acid in *Acetobacter*'s activity aims to maintain the shelf life of foodstuff.

The highest population of *A. flavus* in 1 day after inoculation was 3.75 log cfu/g in samples inoculated by *I. orientalis* BIO 211291 + BIO 211288 + *A. aceti* + MRE + *A. flavus*, meanwhile the lowest population of those was 0.48 log cfu/g in three samples (samples inoculated by *I. orientalis* BIO 211291 + BIO 211288 + *A. aceti* + MRE + *A. flavus* and samples inoculated by *I. orientalis* BIO 211288 + *A. aceti* + *A. flavus* with and without MRE). The highest population of *A. flavus* in 3 days after inoculation was 3.56 log cfu/g in positive control, meanwhile the population was not found in samples inoculated by *I. orientalis* BIO 211291 + *I. orientalis* BIO 211288 + *Acetobcter aceti* + MRE + *A. flavus* and *I. orientalis* BIO 211291 or BIO 211288 + *A. aceti* + mangosteen rind extract + *A. flavus*. The highest population of *A. flavus* in 6 days after inoculation was 4.43 log cfu/g in positive control, meanwhile the population was not found in samples with *I. orientalis* BIO 211291 + *I. orientalis* BIO 211288 + *A. aceti* + MRE + *A. flavus*, *I. orientalis* BIO 211288 + *A. aceti* + MRE + *A. flavus,* and *A. aceti* + MRE + *A. flavus*. The highest population of *A. flavus* in 11 days after inoculation was 3.97 log cfu/g in negative control, meanwhile the population was not found in samples inoculated by *I. orientalis* BIO 211291 + *I. orientalis* BIO 211288 + *A. aceti* + *A. flavus* with and without MRE, *I. orientalis* BIO 211288 + *A. aceti* + mangosteen rind extract + *A. flavus* (Table 3).

Based on different days after inoculation, *A. flavus* population of cocoa beans in 1 day after inoculation was higher than 3, 6, and 11 days after inoculation. *Aspergillus flavus* population in all samples with MRE were lower than samples without MRE. It means that combination treatment with MRE addition is more appropriate to inhibit aflatoxigenic *A. flavus* population. Two best combination treatments to against aflatoxigenic *A. flavus* in unfermented cocoa beans were *I. orientalis* BIO 211291 + *I. orientalis* BIO 211288 + *A. aceti* + MRE and samples inoculated by *I. orientalis* BIO 2112288 + *A. aceti* + MRE, because *A. flavus* population of those treatment was not found in 3 up to 11 days after inoculation (Table 3). According to Aisha *et al.* (2012) mangoteen rind extract compunds are xanthone extract, α-mangostin, and γ-mangostin. Each compound inhibited 50% of cancer cell in 6.5 ± 1.0 mg/mL, 5.1 ± 0.2 μg/mL, and 7.2 ± 0.4 μg/mL. Rubiyanti *et al.* (2017) also reported that α-mangostin and gartanin infulenced in biological activity such as antifungi and anticancer.

Foodstuff could be infested by insects, microorganisms, mites and rats during storage. Among microorganisms, fungi are the most important cause of deterioration of stored grains or seeds. Fungal infection in grains cause discolouration, decreases in physical quality and nutritional contents, and mycotoxin contamination (Sauer *et al.* 1992). According to Waliyar *et al.* (2015) the main factors that

influence the ability of *A. flavus* growth during storage are temperature, relative humidity, and moisture content. Norlia *et al.* (2019) also reported that relative humidity and water activity (a_w) in foods are interrelated to each other and could be used to determine the ability of fungal growth. Not only temperature, relative humidity and water activity, but also CO_2 levels influence the fungal growth (Giorni *et al.* 2018).

Table 3 *Issatchenkia orientalis*, *Acetobacter aceti* and *Aspergillus flavus* populations in unfermented cocoa beans with combination treatments since 1 until 11 days after inoculation

Treatment	*Issatchenkia orientalis* population (log cfu/g)				*Acetobacter aceti* population (log cfu/g)				*Aspergillus flavus* population (log cfu/g)			
	Day after inoculation				Day after inoculation				Day after inoculation			
	1	3	6	11	1	3	6	11	1	3	6	11
Negative control (without inoculation of microorganisms)	-	-	-	-	-	-	-	-	-	-	-	3.97
I. orientalis 291 + *I. orientalis* 288 + MRE +*A. flavus*	2.52	3.80	2.90	2.73	-	-	-	-	2.52	2.48	0.48	0.48
I. orientalis 288 + MRE + *A. flavus*	4.38	4.30	3.20	2.82	-	-	-	-	1.51	0.48	0.48	0.48
I. orientalis 291 + MRE + *A. flavus*	5.88	3.75	2.82	2.82	-	-	-	-	3.23	2.12	1.83	1.89
* *I. orientalis* 291 + *I. orientalis* 288 + *Acetobacter aceti* + MRE +*A. flavus*	3.12	4.67	3.75	2.52	4.22	3.30	2.75	3.12	3.75	ND	ND	ND
I. orientalis 291 + *I. orientalis* 288 + *Acetobacter aceti* + *A. flavus*	3.43	4.12	2.78	1.94	3.52	4.75	3.30	2.14	0.48	1.48	1.23	ND
I. orientalis 288 + *Acetobacter aceti* + *A. flavus*	2.00	4.12	2.43	ND	2.43	2.48	2.88	2.64	0.48	0.48	3.08	2.22
I. orientalis 291 + *Acetobacter aceti* + MRE +*A. flavus*	3.07	4.64	2.12	ND	3.26	4.73	2.37	2.37	2.74	ND	1.00	1.52
* *I. orientalis* 288 + *Acetobacter aceti* + MRE +*A. flavus*	3.22	4.12	2.12	ND	3.75	3.56	2.12	2.00	0.48	ND	ND	ND
I. orientalis 291 + *Acetobacter aceti* + *A. flavus*	3.56	3.60	2.67	ND	2.12	2.48	2.87	2.80	2.75	2.00	2.94	3.48
A. aceti + MRE + *A. flavus*	-	-	-	-	4.10	3.37	2.78	2.11	1.83	1.51	ND	1.72
Positive control (*A. flavus*)	-	-	-	-	-	-	-	-	3.00	3.56	4.43	3.85

Notes: MRE = mangosteen rind extract; ND = not detected; (-) = not inoculated.

Table 4 Aflatoxins production in cocoa beans with various combination treatments since 1 up to 11 days after inoculation

Treatment	Aflatoxin production (ppb)															
	B_1				B_2				G_1				G_2			
	Day after inoculation				Day after inoculation				Day after inoculation				Day after inoculation			
	1	3	6	11	1	3	6	11	1	3	6	11	1	3	6	11
Negative control (without inoculation of microorganisms)	0	0	0	0	0	0	0	0	0	0	0	0	< 1.00	< 1.00	< 1.00	< 1.00
I. orientalis 291+ *I. orientalis* 288 + MRE +*A. flavus*	< 2.20	< 2.20	< 2.20	< 2.20	< 3.50	< 3.50	< 3.50	< 3.50	< 0.54	< 0.54	< 0.54	< 0.54	< 1.00	< 1.00	< 1.00	< 1.00
I. orientalis 288 + MRE + *A. flavus*	< 2.20	< 2.20	< 2.20	< 2.20	< 3.50	< 3.50	< 3.50	< 3.50	< 0.54	< 0.54	< 0.54	< 0.54	< 1.00	< 1.00	< 1.00	< 1.00
I. orientalis 291 + MRE + *A. flavus*	< 2.20	< 2.20	< 2.20	< 2.20	< 3.50	< 3.50	< 3.50	< 3.50	< 0.54	< 0.54	< 0.54	< 0.54	< 1.00	< 1.00	< 1.00	< 1.00
I. orientalis 291 + *I. orientalis* 288 + *Acetobacter aceti* + MRE +*A. flavus*	< 2.20	< 2.20	< 2.20	< 2.20	< 3.50	< 3.50	< 3.50	< 3.50	< 0.54	< 0.54	< 0.54	< 0.54	< 1.00	< 1.00	< 1.00	< 1.00
I. orientalis 291 + *I. orientalis* 288 + *Acetobacter aceti* + *A. flavus*	< 2.20	< 2.20	< 2.20	< 2.20	< 3.50	< 3.50	< 3.50	< 3.50	< 0.54	< 0.54	< 0.54	< 0.54	< 1.00	< 1.00	< 1.00	< 1.00
I. orientalis 288 + *Acetobacter aceti* + *A. flavus*	< 2.20	< 2.20	< 2.20	< 2.20	< 3.50	< 3.50	< 3.50	< 3.50	< 0.54	< 0.54	< 0.54	< 0.54	< 1.00	< 1.00	< 1.00	< 1.00
I. orientalis 291 + *Acetobacter aceti* + MRE +*A. flavus*	< 2.20	< 2.20	< 2.20	< 2.20	< 3.50	< 3.50	< 3.50	< 3.50	< 0.54	< 0.54	< 0.54	< 0.54	< 1.00	< 1.00	< 1.00	< 1.00
I. orientalis 288 + *Acetobacter aceti* + MRE +*A. flavus*	< 2.20	< 2.20	< 2.20	< 2.20	< 3.50	< 3.50	< 3.50	< 3.50	< 0.54	< 0.54	< 0.54	< 0.54	< 1.00	< 1.00	< 1.00	< 1.00
I. orientalis 291 + *Acetobacter aceti* +*A. flavus*	< 2.20	< 2.20	< 2.20	< 2.20	< 3.50	< 3.50	< 3.50	< 3.50	< 0.54	< 0.54	< 0.54	< 0.54	< 1.00	< 1.00	< 1.00	< 1.00
A. aceti + MRE + *A. flavus*	< 2.20	< 2.20	< 2.20	< 2.20	< 3.50	< 3.50	< 3.50	< 3.50	< 0.54	< 0.54	< 0.54	< 0.54	< 1.00	< 1.00	< 1.00	< 1.00
Positive control (*A. flavus*)	74.01	73.94	74.02	74.06	< 3.50	< 3.50	< 3.50	< 3.50	53.85	54.05	54.00	54.30	< 1.00	< 1.00	< 1.00	< 1.00

Notes: MRE = Mangosteen Rind Extract; Limit of Detection (LOD) for B_1 = 2.20 ppb; B_2 = 3.50 ppb; G_1 = 0.54 ppb; and G_2 = 1.00 ppb.

Range of temperature and relative humidity in the storage room after cocoa beans inoculation with various treatments were 27 - 28 °C dan 78 - 80%. Water activity and CO_2 levels were not determined in this research. Suttajit (2014) reported that the optimum temperature for fungal growth was 25 - 40 °C.

Aflatoxins content in positive control were aflatoxin B_1 (74.01 ppb), G_1 (54.05 ppb), and total aflatoxin (128.06 ppb), while all sample treatments included negative control contained aflatoxin B_1 were lower than the limit detection, i.e., B_1 (< 2.20 ppb), B_2 (< 3.50 ppb), G_1 (< 0.54 ppb), and G_2 (< 1.00 ppb) (Table 4). There were no significant differences between combination treatments on aflatoxin production.

It means that all unfermented cocoa beans with combination treatments within or without mangosteen rind extract could be processed into chocolate products, because the aflatoxins were relatively safe. According to Mazumder and Sasmal (2001) the maximum tolerable limit for aflatoxin in cocoa beans, cocoa butter, and cocoa powder in Bulgaria are 5 ppb, while in Uruguay and Malaysia the limits are 10 ppb.

According to Scott and Pryzbylski (2020) the range of aflatoxins in raw cocoa beans from Trinidad and Ghana was 8 - 35 µg/kg. Maciel *et al.* (2018) reported as much as 38% cocoa beans in southern region of Bahia, Brazil were contaminated by aflatoxin in the range < LOD-17.795 µg/kg, 25% and 18% of total samples were contaminated by AFB1 and ochratoxin A in the range of <LOD- 274.90 µg/kg.

CONCLUSION

There were 2 kinds of interaction types obtained from the antagonisms test between 4 yeasts (*I. orientalis* BIO 211291, BIO 211288, BIO 211286, and *Endomyces fibuliger* BIO 132219) and aflatoxigenic *A. flavus*, i.e., type D and A. The best combination treatment *in vitro* was found in unfermented cocoa beans with *I. orientalis* BIO 211288 + *Acetobacter aceti* + *A. flavus* on Potato Dextrose Agar with 15% cocoa beans juice and 12 g/L mangosteen rind extract media. Two best treatments in unfermented cocoa beans *in vivo* were found in samples inoculated by *I. orientalis* BIO 211291 + BIO 211288 + *A. aceti* + mangosteen rind extract and *I. orientalis* BIO 211288 + *A. aceti* + mangosteen rind extract, because *A. flavus* was not grown in the samples for 3 until 11 days after inoculation. Based on aflatoxins content, all combination treatments were relatively safe to be processed, because its very low.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the financial support through *Daftar Isian Pelaksanaan Anggaran* (DIPA) 2019 and SEAMEO BIOTROP. Thanks are due to Director of SEAMEO BIOTROP and Research Laboratory Supervisor for giving permission on using of laboratory facilities, to Ms. Okky Setyawati Dharmaputra and to Mrs. Ina Retnowati for her suggestion, Mrs. Ratnaningsih and Mrs. Syifa Fauzia for their assistance.

REFERENCES

Agricultural Department [Internet]. 2010. Pencanangan gerakan nasional fermentasi kakao untuk mendukung industri dalam negeri (*The declaration of the national movement for cacao fermentation to support the domestic industry*); [updated 2010; cited 2018 Nov 1]. Available from: www.deptan.go.id.

Aisha AFA, Abu-Salah KM, Ismail Z, Majid AMSA. 2012. In vitro and in vivo anti-colon cancer effects of *Garcinia mangostana* xanthones extract. Compl and Alternative Med 12:1-10.

Akao Y, Nakagawa Y, Linuma M, Nozawa Y. 2008. Anti-cancer effects of xanthones from pericarps of mangosteen. Int J of Mol Sci 9:355-70.

Ardhana M, Fleet G. 2003. The microbial ecology of cocoa bean fermentations in Indonesia. Int J of Food Microbiol 86(1):87-99.

Asrul. 2009. Populasi jamur mikotoksigenik dan kandungan aflatoksin pada beberapa contoh biji kakao (*Theobromae cocoa*) (*Mycotoxigenic fungi population and aflatoxin content in several examples of cacao beans*) (*Theobromae cocoa*). J Agroland 16(3):258-67.

Baiton SJ, Coker RD, Jones BD, Morley EM, Nagler MJ, Turner RL. 2006. *Mycotoxin Training Manual.* London (GB): Tropical Products Institute.

Basappa SC. 2009. *Aflatoxins; Formation, Analysis and Control.* New Delhi (IN): Narosa Publishing House.

Copetti MV, Iamanaka BT, Pereira JL, Fungaro MH, Taniwaki MH. 2011. Aflatoxigenic fungi and aflatoxin in cocoa. Int J of Food Microbiol 148(2):141-4.

Dharmaputra OS, Retnowati I, Nurfadila N. 2018a. Potency of yeast as biocontrol agent of ochratoxin A producing fungi and its effect on the taste of Arabica coffee. Research Report. Bogor (ID): SEAMEO BIOTROP.

Dharmaputra OS, Sudirman LI, Misnawati MM. 2016. Potensi khamir sebagai agen pengendalian hayati *Colletotrichum capsici*, cendawan penyebab antraknosa pada buah cabai (*Potency of yeast as biological control agent of Colletotrichum capsici; fungal causing anthracnose in chilli*). J Hort Indonesia 7(2):89-100.

Dharmaputra OS, Sudirman I, Ratnaningsih EA. 2018b. Mikobiota pada buah pisang kultivar Lampung untuk pengendalian hayati *Fusarium semitectum* (*Mycobiota on banana fruit Lampung cultivar for biological control of Fusarium semitectum*). JFI 14(1):30-8.

Dickson [Internet]. 2017. Sepuluh negara penghasil kakao terbesar di dunia (*The biggest of ten producing cocoa beans countries in the world*). [updated 2017; cited 2018 Mar 14]. Available from: https://ilmupengetahuanumum.com/10-negara-penghasil-kakao-terbesar-di-dunia/.

Giorni P, Battilani P, Pietri A, Magan N. 2008. Effect of water activity and CO_2 levels on *Aspergillus flavus* growth and aflatoxin production in high moisture maize postharvest. *Int Journal of Food Microbiol.* 122(1):109-13.

Golubev WI. 2006. Antagonistic Anteractions among Yeast. Germany (DE): Springer. p 197-219.

Hafsari AR, Ariyanti O, Andi S, Wellyzar S. 2011. Pengujian kemampuan antagonistik khamir *Rhodotorula* spp. asal Kebun Raya Cibodas terhadap kapang dari tanaman tomat terinfeksi dengan co-culture (*Testing the antagonistic potency of Rhodotorula spp yeast from Cibodas Botanical Garden against fungi from tomato plant infected with co-culture*). Researchgate 5(1):149-60.

Indonesian National Standard. 2008. Biji Kakao SNI 2323:2008 (*Cocoa beans SNI 2323:2008*). Jakarta (ID): National Standardization Company.

Jamili, Yanti NA, Susilowati PE. 2016. Diversity and the role of yeast spontaneous cocoa bean fermentation from Southeast Sulawesi, Indonesia. Biodiversitas 17(1):90-5.

Janisiewicz WJ, Korsen L. 2002. Biological control of postharvest diseases of fruits. Annu Rev Phytopathol 40:11-441.

Kusumaputri S. 2011. Dosis aman ratu buah (*Queen of fruit safe dosage*). [updated 2017; cited 2018 Apr 11]. Available from: https//www.trubus-online.co.id/dosis-aman-ratu-buah/

Lee JR, Kim SY, Chae HB, Jung HJ, Lee SY. 2008. Antifungal activity of *Saccharomyces cerevisiae* peroxisomal 3-ketoacyl-CoA thiolase. BMB reports p.281-5.

Maciel LF, Felicio ALSM, Miranda LCR, Pires TC, Bispo ES, Hirooka EY. 2018. Aflatoxins and ochratoxin A in different cocoa clones (*Theobromae cacao* L.) developed in the southern region of Bahia, Brazil. Food Additives and Contaminants: Part A 35(1):134-43.

Maligan JM, Chairunnisa F,Wulan SN. 2018. Peran xanthon kulit buah manggis (*Garcinia mangostana* L.) sebagai agen antihiperglikemik (*The role of mangosteen's skin* (*Garcinia mangostana* L.) *xanthone as antihyperglicemic agent.* Jurnal Ilmu Pangan dan Hasil Pertanian 2(2):99-106.

Mazumder PM, Sasmal D. 2001. Mycotoxins-limit and regulations. Ancient Sci of Life 20(1):1-19.

Norlia M, Jinap S, Nor-Khaizura MAR, Radu A, Samsudin NIP, Azri FA. 2019. *Aspergillus* section *Flavi* and aflatoxins: occurrence, detection, and identification in raw peanuts and peanut-based products along the supply chain. Frontiers in Microbiology 10(1):1-17.

Nurhansyah V [Internet]. 2011. Biji kakao Indonesia ditinggalkan oleh pembeli (*Indonesian cacao beans abandoned by buyers*). [updated 2011; cited 2018 Sept 9]. Available from: http://industri.kontan.co.id/news/biji-kakao-indonesia-ditinggalkan-para-pembeli.

Pires PN, Vargas EA,Gomes MB, Vieira CBM, dos Santos EA, Bicalho AAC, Silva SC, Rezende RP, De Oliveira IS, Martins ED, Luz N, Uetanabaro APT. 2019. Aflatoxins and ochratoxin A : occurrence and contamination levels in cocoa beans from Brazil. Food Additives and Contaminants: Part A 36(5):1-10.

Richard WJ, Prusky D. 2002. Expression of an antifungal peptide in *Saccharomyces*: a new approach for biological control of the postharvest disease caused by *Colletotrichum coccodes*. Phytopathol 92(1):33-7.

Ren X, Zhang Q, Zhang W, Mao J, Li P. 2020. Control of aflatoxigenic molds by antagonistic microorganisms: inhibitory behaviors, bioactive compounds, related mechanisms and influencing factors. Toxins 12(24):1-21.

Rubiyanti R, Susilawati Y, Muchtaridi M. 2017. Potensi ekonomi dan manfaat kandungan α- mangostin serta gartanin dalam kulit buah manggis (*Garcinia mangostana* Linn.) (*Economic and benefits potential a-mangostin and gartanin mangosteen rind* (*Garcinia mangostana* Linn.). Farmaka 15(1):15-25.

Sabahannur St, Ralle. 2018. Peningkatan kadar alkohol, asam dan polifenol limbah cairan pulp biji kakao dengan penambahan sukrosa dan ragi (*Increasing of alcohol, acid and polyphenol levels in the liquid waste of cocoa beans pulp with the addition of sucrose and yeast*). Jurnal Industri Hasil Perkebunan 13(1):53-61.

Sauer DB, Meronuck RA, Christensen CM. 1992. Microflora. *Dalam* Sauer DB (editor). *Storage of Cereal Grains and Their Product.* 4th ed. Minnesota:

American Association of Cereal Chemist. pp. 313-40.

Scott PM, Pryzbylski W. 2020. Collaborative study of a method for the analysis of cocoa beans for aflatoxins. Journal of Association of Official Analytical Chemists 54(3):540-4.

Simanjuntak DH, Herpandi, Lestari SD. 2016. Karakteristik kimia dan aktivitas antioksidan kombucha dari tumbuhan Apu-apu (*Pistia stratiotes*) selama fermentasi. Jurnal Hasil Teknologi Perikanan 5(2):123-33.

Suttajit M [Internet]. 2014. Prevention and control of mycotoxins FAO Corporate Document Repository. [uupdated 2018; cited 2019 Jul 11]. Available from: http://www.fao.org/docrep/x5036e/x5036EOq.htm.

Thompson SS, Miller KB, Lopez A, Camu N. 2013. Cocoa and coffee. In: Doyle MP, Buchanan RL, editor. *Food Microbiology: Fundamentals and Frontiers*. Washington DC (US): ASM Press. *Ed.4*. p.881-9.

Waliyar F, Osiru M, Ntare BR, Kumar KVK, Sudini H, Traore A. 2015. Postharvest management of aflatoxin contamination in groundnut. World Mycotoxin Journal 8:245-52.

Wheeler KA, Hocking AD. 1993. Interactions among xerophilic fungi associated with dried salted fish. J Applied Bacteriol 74:164-9.

Widiyanto D, Pramita AD, Wedhastari S. 2013. Perbaikan proses fermentasi biji kakao kering dengan penambahan tetes tebu, khamir, dan bakteri asam asetat (*Improvement of the fermentation process for dry cocoa beans by adding molasses, yeast, and acetic acid bacteria*). Jurnal Tekno Sains 3(1):38-44.

WIPO-PCT [World Intelectual Property Organization-Patent Cooperation Treaty]. 2019. WO2019/036202 A1. Minnesota (US): Patent Cooperation Treaty.

Yatman E. 2012. Kulit buah manggis mengandung xanton yang berkhasiat tinggi (*Mangosteen rind contains xanthone, which is highly beneficial*). Wawasan 29(324):1-9.