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POTENCY OF YEAST AS BIOCONTROL AGENT OF OCHRATOXIN A PRODUCING FUNGI AND ITS EFFECT ON THE TASTE OF WET AND SEMI-WET PROCESSED ARABICA COFFEE

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ABSTRACT

Biological control using antagonistic microorganisms has been an efficient alternative control for controlling OTA-producing fungi. The use of biological control agents such as yeast to prevent the growth of OTA-producing fungi and increase the taste of coffee needs to be developed. The objectives of this research was to select yeast isolates that can inhibit the growth of ochratoxin A (OTA) producing fungi and increase the taste of wet and semi-wet processed Arabica coffee. Screening for antagonistic yeast on OTA producing fungi in vitro (3 isolates of *Aspergillus niger* and 4 isolates of *A.ochraceus* which showed the highest fluoresence) were conducted using well (dip) test method. Biocontrol agent of OTA producing fungi using yeasts *in vivo* was conducted by inoculating yeast into coffee beans. Moreover, the antagonism mechanism between yeast antagonists and OTA-producing fungi, and the taste of coffee in of inoculated coffee beans with yeast antagonists were also observed. As much as 3 isolates of yeasts (K287, K288 and K289) were used in biocontrol agent of OTA producing fungi using yeasts in vivo. The yeasts inhibited 3 isolates of *A. ochraceus* (BIO 37128, BIO 37310 and BIO 37311). The yeasts were *Issatchenkia orientalis.* The highest yeast population was found in coffee beans processed by semi-wet method inoculated with yeast isolates of K287 (36 333 ± 14 000 cfu / g) and K288 (46 222 ± 9576 cfu / g). Yeast isolates of K287, K288 and K289 can grow well either in coffee beans by wet and semi-wet processing methods inoculated with *A. ochraceus*. Interaction between the yeast isolates and the fungus resulted E type interaction, because the fungus was not able to grow anymore, while the yeast isolates grew further. Total score specialty grades of coffee beans inoculated with the yeasts were higher than those uninoculated and inoculated with commercial yeast.

1. INTRODUCTION

1.1. Background

Indonesia is the second ranking of coffee beans producing countries in Southeast Asia after Vietnam. Two kinds of coffee beans cultivated in Indonesia, i.e. Robusta coffee (*Coffea canephora*) and Arabica coffee (*C. arabica*). Composition of Robusta coffee is about 83% of total coffee production, while that Arabica coffee is about 17%.

In Indonesia, coffee fruits are fermented after harvesting. There are three methods of coffee bean processing, i.e. dry, wet and semi-wet processing. Dharmaputra *et al.* (2017) reported that **dry** processing is processing method by harvesting of ripe wet cherry beans, they were then dried using sun-drying for 14 days until the moisture content attained ±10% (dried cherries), their husk and hull were then shelled. **Wet** processing method is a processing of coffee beans by shelling of ripe wet cherry beans, therefore they became wet green beans with hull, they were then fermented for one night and washed to eliminate their mucus. Wet green beans were then dried using sun-drying for 7 days until their moisture contents attained ± 10% (dried green beans), hull of the beans were then shelled (green beans). **Semi-wet** processing method is processing method by shelling of ripe wet cherry beans, then wet green beans were fermented for one night and washed. Wet green beans were then dried using sun-drying for 1 day until the moisture content attained ± 40%, the hull were then shelled. After shelling, the beans were further dried using sun-drying for 5 days until the moisture content attained ± 10%.

During storage coffee beans could be infested by insects, microorganisms, mites and rats. Among microorganisms, fungi are the most important cause of deterioration of stored grains. Fungal infection in grains can cause discolouration, decreases in physical quality and nutritional contents, and mycotoxin contamination (Sauer *et al*. 1992).

Ochratoxin A (OTA) contamination in coffee beans have been becoming an important issue recently since some consuming countries applying its Maximum Tolerable Limits (MTL) of the presence of OTA in some coffee products (Ismayadi *et al*. 2003). OTA is a potent nephrotoxic mycotoxin that has been linked to kidney problems in both livestock and human populations (Clark and Snedeker 2006). Ismayadi *et al.* (2005) reported that OTA content is one of the criteria in determining quality standard in the world. SNI (Indonesian National Standard) (2009) has determined MTL of OTA in *kopi sangrai* (roasted coffee) and instant coffee 5 and 10 ppb, respectively. According to

Raghuramulu and Naidu (2001) Italia has determined maximum tolerable limit (MTL) of OTA in coffee beans and their processed products 8 and 4 ppb, respectively.

In tropical regions OTA is mainly produced by *Aspergillus carbonarius*, *A. niger* and *A. ochraceus* (Pitt *et al*. 2000). According to Martin *et al.* (2003) as much as 91.7% of 60 coffee bean samples collected from Brazil were contaminated by fungi. The dominant fungi were *Aspergillus niger* (83.3%), *A. ochraceus* (53.3%) and *A. flavus* (25%). The occurrence of *Cladosporium* sp. (16.6%) and *Penicillium* sp. (10%) were lower than Aspergilli section. As much as 20 samples (33.3%) of 60 samples were contaminated by ochratoxin with range and mean 0.2 – 7.3 ppb and 2.38 ppb, respectively.

Leong *et al*. (2007) reported that *A. carbonarius*, *A. niger* and yellow Aspergilli (*A. ochraceus* and related species in section *Circumdati*) were isolated by direct plating of surface-disinfected Robusta (65 samples) and Arabica (11 samples) coffee beans from southern and central Vietnam. *Aspergillus niger* infected 89% of Robusta beans, whereas *A. carbonarius* and yellow Aspergilli each infected 12-14% of beans. The maximum OTA observed in samples severely infected with toxigenic species was 1.8 ppb, however, no relationship between extent of infection and OTA contamination was observed.

According to Noonim *et al*. (2008) 32 Thai dried coffee bean (*C. arabica*) samples were collected from two growing sites of Chiang Mai Province The samples from North had an average of 78% incidence of colonization with *Aspergillus* of section *Circumdati* with *Aspergillus westerdijkiae* and *A. melleus* as the predominant species. *Aspergillus* spp. of section *Nigri* were found in 75% of samples, whereas *A. ochraceus* was not detected.

Nogaim and Gowri (2013) reported that 70 samples of *C. arabica* were collected from local markets in some Yemeni Governorates during 2010/2011. The samples were infected by *A. niger*, *A. ochraceus*, *A. fumigatus*, *A. flavus*, *A. parasiticus*, *A. ochraceus* and *A. candidus*. The percentage of samples infected by the fungi were 27.5, 17.5, 15, 12,5, 10 and 5%, respectively. The range and mean of ochratoxin A contents were 0.314-3.443 ng/g and 1.211ng/g, respectively.

de Fatima *et al*. (2013) reported a number of fungi were isolated from 30 samples of Arabica coffee. As much as 20 samples of coffee beans collected from conventional cultivation system, while 10 samples were collected from organic cultivation system. As much as 480 filamentous fungal species were isolated. They were belong to genus *Aspergillus*, group *Circumdati* and *Nigri*. Ochratoxin producing fungi isolated were *A. auricoumus*, *A. ochraceus*, *A. ostianus*, *A. niger* and agregate of *A. niger*. The most

frequent species which produced ochratoxin A was *A. ochraceus* corresponding to 89.55% of samples.

Dharmaputra *et al.* (2016) reported related on postharvest handling of coffee beans in Tana Toraja Regency, North Toraja, and Makassar City. Distribution chain of coffee beans are from farmers, collectors, big traders, and exporters. that *A. niger* and *A. ochraceus* have been found in some samples of Arabica coffee beans collected at certain trader and exporter levels in South Sulawesi Province. Coffee beans are processed using semi-wet processing. Research results showed that at farmer levels, 19% of 27 coffee beans were infected by *A. ochraceus* (1.4 x 10^2 cfu/g) , 4% were infected by *A. niger* (6.3 x 10 cfu/g) and 11% were contaminated by OTA (range 1.6 - 17.7 ppb, mean 7.2 ppb). Postharvest process of coffee beans were done by farmers, i.e collecting of ripe coffee beans, shelling of cherry and husk, washing and fermentation of coffee beans with hull until drying of coffee beans with hull. Therefore, fungal infection and OTA contamination were in coffee beans processing methods. At big trader levels, 46% of 13 coffee beans were infected by *A. ochraceus* (7.4 x 10^2 cfu/g), 46% were infected by *A. niger* (4.8 x 10^2 cfu/g), and 23% samples were contaminated by OTA (range 1.5 - 11.3 ppb, mean 6.6 ppb), while at exporter levels 44% of 9 samples were infected by *A. ochraceus* (2 x 10 cfu/g), 78% of samples were infected by *A. niger* (3.1 x 10^2cfu/g), and 33% of samples were contaminated by OTA (range 2.8 - 14.7 ppb, mean 8.7 ppb). Collectors distribute coffee beans with hull from farmers to traders and exporters. Shelling of hull, drying, and sortation of coffee beans are conducted by big traders and exporters.

Biological control using antagonistic microorganisms has been an emergent alternative to efficiently manage storage fungi and mycotoxins production and hence, reducing the use of chemical compounds (Janisiewics and Korsten 2002). According to Korsten (2006) there are a variety of microorganisms which may be used as biocontrol agents against mycotoxigenic fungi that include diggerent species of yeasts, fungi, and bacteria. Due to the positive findings regarding the use of these microbial antagonists, biocontrol agents have been gaining popularity worlwide.

Yeasts inherent characteristics such as fast growth, fruit surface colonization and deprive nutrients from pathogens (through competition) have placed these organisms as one of the most suitable biocontrol agents (Richard and Prusky 2002). Masoud *et al.* (2004) and Vilela *et al.* (2010) also reported that *Pichia kluyveri, P. anomala, Hanseniaspora uvarum, Saccharomyces cerevisiae, Debaryomyces hansenii* and *Torulaspora delbrueckii* were the most yeast species found during coffee processing.

Pereira *et al.* (2014) reported as much as 144 yeasts were collected from fermented coffee beans naturally, i.e. *Pichia fermentans, P. kluyveri, P. guilliermondii, P. caribbica, Candida glabrata, C. quercitrusa, Saccharomyces* sp., and *Hanseniaspora opuntiae.*

Using biocontrol agent such as yeast is useful to inhibit toxigenic fungal growth, because it is important for human healthy. This research will be one of new inovation researchs which has some benefits, especially for food security. Biocontrol agent can be obtained from the antagonist yeast which can not cause allergic cells or toxins.

1.2. Objective

To select yeast isolates that can inhibit the growth of ochratoxin A (OTA) producing fungi and increase the taste of wet and semi-wet processed Arabica coffee.

1.3. Expected Output

It is expected, that the research result could obtain a potential yeast isolate as biocontrol agent of ochratoxin A (OTA) producing fungi and as starter cultures used for wet and semi-wet processed Arabica coffee beans.

2. BENEFIT AND IMPORTANCE OF RESEARCH

The research is useful to be conducted, because :

- The use of biological control agents such as yeast to prevent the growth of OTA-producing fungi needs to be developed, as an alternative to the use of chemical controls that have a negative effect on public health.
- This research will be a new research finding that has several advantages, especially for food security. Biological control agents can be obtained from antagonist yeast that cannot cause allergic cells and toxic.

3. METHODOLOGY

3.1. Time and location of research

Preparation and research activity were conducted from April up to December 2018 at SEAMEO BIOTROP, Bogor.

3.2. Yeast isolates and OTA producing fungi

Twenty two yeast isolates were used as biocontrol agents (Appendix 1). Twenty nine *Aspergillus niger* isolates, and 16 isolates of *A. ochraceus* were used as producing OTA fungi. The isolates were obtained from SEAMEO BIOTROP Culture Collection, Bogor.

3.3. Selection of producing OTA fungi

Twenty nine isolates of *A. niger* and 16 isolates of *A. ochraceus* were selected based on OTA production, using UV light with 365 nm of wave length (Zhang *et al.* 2016). The isolates were cultured on media santan agar 50% for 14 days at 25ºC. Green flurorescence from each isolate was detected using UV light. Fluoring isolate showed as a potential OTA producing fungi.

3.4. Screening for antagonistic yeast on OTA producing fungi

Screening for antagonistic yeast on OTA producing fungi (3 isolates of *Aspergillus niger* BIO 35410/4 isolates of *A.ochraceus* BIO 37411) were conducted using well (dip) test method (Dan *et al.* 2003). Five pieces (in 5 mm diameter) of pure culture of each yeast isolate was cultured on 25 mL of Nutrient Yeast Dextrose Broth (NYDB) media in an erlenmeyer flask (volume 100 mL), they were then incubated at 28 ± 2 °C for 7 days, and shaked using shaker Kottermann 4020 for 1 hour each 24 hours. The yeast cells were precipitated by centrifugation using a microcentrifuge 200 R (kind of centrifuge rotor is fixed-angle rotor) at 7,000 rpm for 15 minutes and rinsed twice using sterile distilled water, they were then resuspended in sterile distilled water until the concentration reaches 5 x 10^8 cell mL^{-1}. The yeast cell was calculated using a haemacytometer.

A well (6 mm diameter) was prepared using a cork borer in the center of Potato Dextrose Agar (PDA) media containing 15% Arabica coffee bean juice in a Petri dish (9 cm in diameter). As much as 20 µL of 5x10^8 cell mL^{-1} of yeast cell suspension was placed in the well. Petri dishes were left for 2 hours to allow the cell suspension to diffuse into the well. Further, each 20 µL of 5×10^6 conidial cell mL^{-1} of the OTA-producing fungi was placed in the well. As a control, the well was not inoculated with yeast cell suspension. Each treatment (each isolated yeast) and control was made 3 replicates (= 3 petri dishes). The petri dishes were incubated at room temperature (28 ± 2ºC) under dark conditions. The growth of the OTA-producing fungi in each petri dish was observed in the presence of colonies after 7 days of incubation. The yeast isolate which inhibit the total growth of the

OTA-producing fungi after 7 days of incubation was used in the biocontrol assay of OTA producing fungi *in vivo*.

3.5. Biocontrol agent of OTA producing fungi using yeasts *in vivo*

Five hundred grams of Arabica coffee beans with hull (derived from coffee after harvest, shelled and washed) were placed respectively in a tray (30 x 40 x 5 cm). They were inoculated with 10 mL suspension of the yeast (5 x 10^6 cell mL^{-1}) (Evangelista *et al.* 2014) and left for 12 hours. Afterwards the coffee beans were inoculated with 10 mL (5 x 10^4 conidia mL^{-1}) of OTA-producing fungi. As a control, coffee beans with hull were not inoculated with the yeast suspension. After inoculation, coffee beans were placed on porous tray. Each treatment (included control) was used 3 replication (=3 trays). Coffee beans conducted by wet processing were dried using sun-drying until the moisture content attained 9 - 10% (5 days), their hulls were then shelled. In semi wet processed coffee, the coffee were sun-dried for 2 days after the inoculation of the yeast (m.c 15.0-24.5%), then their hull were shelled and further sun-dried until moisture content attained (m.c 9.6-11.8%). Determination of moisture content in coffee beans used Delmhorst moisture tester. Moisture content in coffee beans processed by wet and semi-wet processing can be seen in Appendix 2.

Population of the yeast and OTA producing fungi from each treatment was determined using dilution method, followed by pour method on Yeast Malt Extract Agar (YMEA) and PDA media, respectively.

3.6. Mechanism of antagonisms between the potential yeast isolate with *A. niger* and *A. ochraceus*

Dual culture method was conducted on Potato Dextrose Agar (PDA) media in a petri dish (9 cm in diameter) to obtain information about the mechanism of antagonism between the most potential yeast isolate and *A. niger* and *A. ochraceus* (Skidmore and Dickinson 1976) (Figure 1).

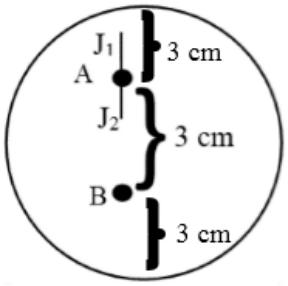

Figure 1. Scheme antagonism test between the most potential yeast isolate and *A. niger* / *A. ochraceus*; A = *A. niger* /*A. ochraceus*, B = Yeast isolate

Aspergillus niger and *A.ochraceus* was grown with yeast in the middle of PDA media in petri dish (diameter 9 cm) with distance 3 cm. *Aspergillus niger* and *A. ochraceus* were inoculated after 3 days of yeast inoculation. Each treatment was used 3 replications (= 3 petri dishes). All petri dishes were incubated at room temperature (±28 ^{o}C) for 7 days. The observation on the mechanism of antagonism was conducted macroscopically, i.e. by observing the type of interaction between the yeast and *A. niger* and *A. ochraceus* (Wheeler and Hocking 1993).

3.7. Cupping test

Cupping test of the samples were conducted Standard Cupping Protocol given by Coffee Quality Institute and Specialty Coffee Association of America (2015). The panelists have a certificate from CQI Q grader. Cupping test was conducted at Cupping Test of Coffee Laboratory PT Kemenady Industri Mandiri, Bogor.

3.8. Statistical analyses

The data of biocontrol assay of OTA producing fungi using yeast *in vivo* was analyzed using Completely Randomized Factorial with 2 factors (method of processing and yeast treatment, including control) (Mattjik and Sumertajaya 2002).

4. RESULT AND DISCUSSIONS

4.1. Selection of OTA producing fungi

Three of 20 isolates of *A. niger* (BIO 35117, BIO 35407, and BIO 35409) and 4 of 16 isolates of *A. ochraceus* (BIO 37128, BIO 37310, BIO 37311, and BIO 37312) showed

high fluorescence (Table 1 and Figure 2). The isolate with the highest fluorescence was used for controlling OTA producing fungi.

4.2. Screening for antagonistic yeast on OTA producing fungi

As much as 22 yeast isolates were used as antagonist yeast, i.e. KA, KA2, KB, KB2, KC, KD, K215, K216, K217, K218, K219, K220, K285, K286, K287, K288, K289, K290, K291, K363, K364, and K365 isolates. Each yeast isolates and OTA producing fungi were inoculated on PDA + 15% Arabica coffee beans extract using well (dip) test method. Diameter of each OTA producing fungi and yeast in antagonism testing could be seen in Appendix 3.

There was no yeast isolate that could inhibit the growth of 3 *A. niger* isolates totally (Figure 2), but some yeast isolates could inhibit the growth of 4 *A. ochraceus* isolates totally (Figure 3). Yeast isolates of KB2, K217, K220, K285, K287, K288, K289 and K290 could inhibit the growth of *A. ochraceus* BIO 37128 totally. Yeast isolates of K215, K286, K287, K288 and K289 could inhibit the growth of *A. ochraceus* BIO 37310 totally. Yeast isolates of K287 and K288 could inhibit the growth of *A. ochraceus* BIO 37311 totally, while yeast isolates of K217 and K286 could inhibit the growth of *A. ochraceus* BIO 37312 totally.

According to Pereira *et al.* (2015) microorganisms (especially yeasts and lactic acid bacteria) may also contribute beverage's sensory characteristics and other qualities in fermentation process. Yeasts are among the most frequently isolated microorganisms from fermented coffee beans. *P. anomala* CCMA0148 and *Saccharomyces cerevisiae* CCMA0159 inhibited fungal growth with high inhibitory. It means that the yeasts can be a potential biocontrol agent in controlled condition (de Souza *et al.* 2017).

4.3. Biocontrol test of OTA producing fungi using yeasts *in vivo*

Three yeast isolates (K287, K288 and K289) and 1 isolate of OTA producing fungi (*A. ochraceus* BIO 37310) were used in biocontrol test of OTA producing fungi using yeasts in vivo, because the yeasts could inhibit 3 isolates of *A. ochraceus* (BIO 37128, BIO 37310 and BIO 37311).

Aspergillus ochraceus BIO 37310 could not grow in coffee beans in all treatments of processing methods (wet and semi-wet) and yeast inoculation including control (coffee beans were inoculated by *A. ochraceus* without yeast). It was caused by the concentration of *A. ochraceus* conidium inoculated in coffee beans was lower (5×10^4 konidium mL^{-1}).

Interaction between processing methods and yeast inoculation gave significant effect on yeast population (Appendix 4).

Yeasts isolates of K287 and K288 grew well in coffee beans processed by semi-wet method. The highest yeast populations in coffee beans processed by semi-wet method that were inoculated by yeasts of K287 (36 333 ± 14 000 cfu/g) and K288 (46 222 ± 9576 cfu/g) (Table 2). Yeasts isolates of K287, K288, K289 grew well in coffee beans processed by wet and semi-wet inoculated by *A. ochraceus*. It showed that yeast isolates of K287, K288, and K289 could control *A. ochraceus* growth. Population of yeast isolate of K287 in coffee beans with semi-wet processing (32 444 ± 6159 cfu/g) inoculated by *A. ochraceus* and yeast K287 was higher than coffee beans processed by wet method (12 445 ±13 184 cfu/g). Yeast population of coffee beans inoculated by commercial yeast and without yeast either processed by wet or semi-wet methods were relative low.

According to Velmourougane *et al.* (2011) the dip treatment containing a yeast suspension (*Saccharomyces cerevisiae*) was used in coffee postharvest, during its processing, and resulted in a significant reduction of total mould incidence (*Aspergillus niger*, *A. ochraceus*) and ochratoxin A contamination without affecting the cup quality. According to Masoud *et al.* (2005) three yeasts (*Pichia anomala, P. kluyveri,* and *Hanseniaspora uvarum* inhibited fungal growth. The effect of their combination was more effective than each fungal species to inhibit fungal growth. *Aspergillus ochraceus* was inhibited by them with the highest inhibitory (53%).

4.4. Mechanism of antagonisms between the most potential yeast isolate and OTA producing fungi (*Aspergillus ochraceus*)

As much as 3 potential yeast isolates (BIO 211287, BIO 211288, and BIO 211289) inhibited OTA producing fungi (*A. ochraceus*) were used to the mechanism of antagonisms test on *A. ochraceus* BIO 37310 using direct oposition method.

Interaction between yeast isolates of BIO 211287, BIO 211288, and BIO 211289 with *A. ochraceus* BIO 37310 resulted E type interaction, because the fungus was not able to grow anymore, while yeast isolates grew further. Based on visual observation, colony of 3 yeasts and *A. ochraceus* BIO 37310 had mutual contact without inhibitory zone (Figure 5). It was assumed, that the competition of space and nutrients were antagonistic mechanism among the yeasts and the fungus. They could be seen in well (dip) method, yeast isolate colonized fastly used space and nutrients on PDA containing 15% Arabica coffee beans extract juice, so that it inhibited *A. ochraceus* BIO 37310.

This is appropriate with Chalutz *et al.* (1998) reported that competition for space and nutrients were basic mechanism on inhibition of pathogen conducted by yeast. The other mechanisms of antagonism were chitinase production, adhering of fungal cell wall, peroxidase activity, endurance induction (El Gouth *et al.* 2003), and produced secretion that inhibited pathogen growth (Guetsky *et al.* 2002). Type interactions between two fungal colonies according to Wheeler and Hocking (1993) adapted from Magan and Lacey (1984) was showed in Appendix 5.

4.5. Cupping test

According to Specialty Coffee Association of America (SCAA), specialty coffee bean was not accepted if the cupping score was lower than 80.

According to Atmawinata (2002) in general coffee was not consumed because of its nutritional value, but it was caused by the value of flavor and physiological influences that caused people to stay awake, add freshness, reduce fatigue and create a feeling more excited. Saepudin (2005) reported that the value of coffee beans was not only determined by physical quality, but more determined by the value of flavor so that some coffee importing countries determined the quality of coffee by cupping test.

According to SCAA (2015) qualification of coffee beans based on final score was divided into 4 categories, i.e. outstanding (90-100), excellent (85-89.99), very good (80-84.99), dan below specialty quality (below 80). Coffee bean with outstanding, excellent and very good categories or had final score (80-100) into specialty qualification and coffee bean with below specialty category into not specialty qualification but it may still consumed. According to panelist, coffee bean could not be consumed if the final score was ≤ 30.

The flavor of coffee beans in three processing methods and packed in polyethylene hermetic bag and polypropylene bag for 4 months of storage were still above the total score specialty grade ≥ 80 (Table 3). The total score of coffee beans inoculated by 3 potential yeasts to inhibit *A. ochraceus* BIO 37310 was higher than those uninoculated and inoculated by commercial yeast. The highest total score was coffee beans inoculated by yeast isolate of K287 (82.75). The result was the same with the result of biocontrol agent test of *A. ochraceus* using 3 yeast isolates. The population of the yeasts were higher than those without yeast and inoculated by commercial yeast (Table 2).

5. CONCLUSIONS

This study opens new possibilities for using yeast isolates as biocontrol agents of OTA producing fungus (*Aspergillus ochraceus*) and to increase the sensorial quality of coffee beverages. In vitro three yeast isolates of *Issatchenkia orientalis* were able to inhibit the growth of the fungus totally. Interaction between the yeast isolates and the fungus resulted E type interaction, because the fungus was not able to grow anymore, while the yeast isolates grew further. It was assumed, that the competition of space and nutrients were antagonistic mechanism among the yeasts and the fungus. In vivo the yeast isolates were able to grow in coffee beans inoculated with the fungus, either they were processed using wet or semi-wet methods, but yeast populations were higher in coffee beans processed using semi-wet method. Total score specialty grades of coffee beans inoculated with the yeasts were higher than those uninoculated and inoculated with commercial yeast. The result of this study gave an information that the three yeast isolates could be used as biocontrol agents of the fungus and increased the sensorial quality of coffee beverages.

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APPENDICES

Appendix 1. The code of yeast isolates used as biocontrol agent and their origin

No	Yeast	Isolate code	Name of isolate	Substrate
1	KA	KA	Not identified	Coffee beans
2	KA2	KA2	Not identified	Coffee beans
3	KB	KB	Not identified	Coffee beans
4	KB2	KB2	Not identified	Coffee beans
5	KC	KC	Not identified	Coffee beans
6	KD	KD	Not identified	Coffee beans
7	K215	BIO 131215	*Endomyces decipiens*	Tomato
8	K216	BIO 132216	*Endomyces fibuliger*	Paddy grain
9	K217	BIO 132217	*Endomyces fibuliger*	Nutmeg
10	K218	BIO 132218	*Endomyces fibuliger*	Peanut
11	K219	BIO 132219	*Endomyces fibuliger*	Cocoa beans
12	K220	BIO 132220	*Endomyces fibuliger*	Milled rice
13	K285	BIO 211285	*Issatchenkia orientalis*	Chilli fruit
14	K286	BIO 211286	*Issatchenkia orientalis*	Tomato
15	K287	BIO 211287	*Issatchenkia orientalis*	Chilli fruit
16	K288	BIO 211288	*Issatchenkia orientalis*	Eggplant
17	K289	BIO 211289	*Issatchenkia orientalis*	Chilli fruit
18	K290	BIO 211290	*Issatchenkia orientalis*	Chilli fruit
19	K291	BIO 211291	*Issatchenkia orientalis*	Banana
20	K363	BIO 341363	*Saccharomyces cerevisiae*	Cocoa beans
21	K364	BIO 341364	*Saccharomyces cerevisiae*	Bread
22	K365	BIO 341365	*Saccharomyces cerevisiae*	Bread

Appendix 2. Moisture content of coffee beans in all inoculation treatments using wet and semi-wet processing

No	Inoculation treatment	Moisture content (%)		
		Semi-wet		Wet
		First drying	Second drying	
1	Control (without inoculation)	15.0	10.0	9.7
2	Commercial yeast	22.1	9.9	9.7
3	K287	17.5	9.7	9.5
4	K288	21.7	10.0	9.4
5	K289	18.1	10.9	10.0
6	An 117	21.1	9.6	9.3
7	An 117-K287	21.4	10.8	9.1
8	An 117-K288	23.2	11.5	9.8
9	An 117-K289	21.0	11.5	9.7
10	Ao 310	24.5	10.7	9.4
11	Ao 310-K287	21.4	11.8	9.6
12	Ao 310-K288	21.2	11.0	9.2
13	Ao 310-K289	23.2	11.0	9.2

Notes :

K = Yeast (isolate codes 287-289)
An = *Aspergillus niger* (isolate code BIO 35117)
Ao = *A. ochraceus* (isolate code BIO 37310)

Appendix 3. Colony diameter of *A. niger* and yeast isolates on test of antagonism between *A. niger* BIO 35117 and 22 yeast isolates

No.	Isolate code	Diameter of colony (mm)	
		A. niger	Yeast
1.	An 117-KA	79.5	0
2.	An 117-KA2	90	0
3.	An117-KB	90	0
4.	An117-KB2	68.5	0
5.	An117-KC	90	0
6.	An117-KD	45	0
7.	An117-K215	83.5	0
8.	An117-K216	87	0
9.	An117-K217	69.25	0
10.	An 117-K218	70.75	0
11.	An 117-K219	87	0
12.	An 117-K220	72.75	0
13.	An 117-K285	73.25	0
14.	An 117-K286	59.25	0
15.	An 117-K287	32.5 (43% of 57)	50.5
16.	An 117-K288	72.25	0
17.	An 117-K289	53.25	0
18.	An 117-K290	66.5	0
19.	An 117-K291	81	0
20.	An 117-K363	83.75	0
21.	An 117-K364	74.25	0
22.	An 117-K365	83	0

Notes:

An = *Aspergillus niger*
K = Yeast

Appendix 3. Colony diameter of *A. niger* and yeast isolates on test of antagonism between *A.niger* BIO 35407 and 22 yeast isolates (Continued)

No.	Isolate code	Diameter of colony (mm)	
		A. niger	Yeast
1.	An 407-KA	90	0
2.	An 407-KA2	82	0
3.	An 407-KB	90	0
4.	An 407-KB2	69.25	0
5.	An 407-KC	86.25	0
6.	An 407-KD	90	0
7.	An 407-K215	90	0
8.	An 407-K216	88.5	0
9.	An 407-K217	82	0
10.	An 407-K218	90	0
11.	An 407-K219	54.25	0
12.	An 407-K220	87	0
13.	An 407-K285	86	0
14.	An 407-K286	82.25	0
15.	An 407-K287	63.25	0
16.	An 407-K288	73.75	0
17.	An 407-K289	59.5	0
18.	An 407-K290	74.5	0
19.	An 407-K291	81.75	0
20.	An 407-K363	80.25	0
21.	An 407-K364	84	0
22.	An 407-K365	90	0

Notes:

An = *Aspergillus niger*
K = Yeast

Appendix 3. Colony diameter of *A. niger* and yeast isolates on test of antagonism between *A.niger* BIO 35409 and 22 yeast isolates (Continued)

No.	Isolate code	Diameter of colony (mm)	
		A. niger	Yeast
1.	An 409-KA	77.7	0
2.	An 409-KA2	49.5	0
3.	An 409-KB	69	0
4.	An 409-KB2	37.5	0
5.	An 409-KC	57.5	0
6.	An 409-KD	62.75	0
7.	An 409-K215	49.75	0
8.	An 409-K216	67.25	0
9.	An 409-K217	63.5	0
10.	An 409-K218	42	0
11.	An 409-K219	46.5	0
12.	An 409-K220	42.5	20.25
13.	An 409-K285	38	28
14.	An 409-K286	57	0
15.	An 409-K287	23.25 (21% of 29.25)	48.5
16.	An 409-K288	21 (28% of 29.25)	10
17.	An 409-K289	15.75 (46% of 29.25)	50
18.	An 409-K290	55.75	0
19.	An 409-K291	46.5	0
20.	An 409-K363	48.75	0
21.	An 409-K364	60	0
22.	An 409-K365	54.25	0

Notes :

An = *Aspergillus niger*
K = Yeast

Appendix 3. Colony diameter of *A. ochraceus* and yeast isolates on test of antagonism between *A. ochraceus* BIO 37128 and 22 yeast isolates

No.	Isolate code	Diameter of colony (mm)	
		A. ochraceus	Yeast
1.	Ao 128-KA	25.5	0
2.	Ao 128-KA2	30	0
3.	Ao 128-KB	31.25	0
4.	Ao 128-KB2	0	16.75
5.	Ao 128-KC	22.5	13.25
6.	Ao 128-KD	24.5	8.25
7.	Ao 128-K 215	10	24.75
8.	Ao 128-K 216	21.25	0
9.	Ao 128-K 217	0	42.25
10.	Ao 128-K 218	14	24.25
11.	Ao 128-K 219	Contamination	Contamination
12.	Ao 128-K 220	0	34
13.	Ao 128-K 285	0	35
14.	Ao 128-K 286	19	16
15.	Ao 128-K 287	0	39
16.	Ao 128-K 288	0	49.25
17.	Ao 128-K 289	0	33.75
18.	Ao 128-K 290	0	25
19.	Ao 128-K 291	17	0
20.	Ao 128-K 363	28	0
21.	Ao 128-K 364	16.5	0
22.	Ao 128-K 365	23	13

Notes :

Ao = *Aspergillus ochraceus*
K = Yeast

Appendix 3. Colony diameter of *A. ochraceus* and yeast isolates on test of antagonism between *A. ochraceus* BIO 37310 and 22 yeast isolates (Continued)

No.	Isolate code	Diameter of colony (mm)	
		A. ochraceus	Yeast
1.	Ao 310-KA	34	0
2.	Ao 310-KA2	26.75	0
3.	Ao 310-KB	31.5	0
4.	Ao 310-KB2	17.25	3.25
5.	Ao 310-KC	21.75	13.25
6.	Ao 310-KD	37.25	0
7.	Ao 310-K 215	0	36.75
8.	Ao 310-K 216	28.5	0
9.	Ao 310-K 217	Contamination	47.5
10.	Ao 310-K 218	34.5	0
11.	Ao 310-K 219	42.5	0
12.	Ao 310-K 220	10.75	10
13.	Ao 310-K 285	18.75	36.25
14.	Ao 310-K 286	0	22.5
15.	Ao 310-K 287	0	46.75
16.	Ao 310-K 288	0	63
17.	Ao 310-K 289	0	42.75
18.	Ao 310-K 290	21.5	6.75
19.	Ao 310-K 291	37	0
20.	Ao 310-K 363	33.25	0
21.	Ao 310-K 364	Contamination	Contamination
22.	Ao 310-K 365	37.25	0

Notes :

Ao = *Aspergillus ochraceus*
K = Yeast

Appendix 3. Colony diameter of *A. ochraceus* and yeast isolates on test of antagonism between *A. ochraceus* BIO 37311 and 22 yeast isolates (Continued)

No.	Isolate code	Diameter of colony (mm)	
		A. ochraceus	Yeast
1	Ao 311-KA	35.25	0
2	Ao 311-KA2	40.5	0
3	Ao 311-KB	33.5	0
4	Ao 311-KB2	27.75	0
5	Ao 311-KC	28.25	0
6	Ao 311-KD	36.25	0
7	Ao 311-K 215	32	0
8	Ao 311-K 216	36.5	0
9	Ao 311-K 217	41.5	0
10	Ao 311-K 218	38.25	0
11	Ao 311-K 219	26.5	0
12	Ao 311-K 220	Contamination	Contamination
13	Ao 311-K 285	11.75	25.5
14	Ao 311-K 286	15.5	7.5
15	Ao 311-K 287	0	39.5
16	Ao 311-K 288	0	43
17	Ao 311-K 289	20	42.25
18	Ao 311-K 290	31.25	11
19	Ao 311-K 291	31	0
20	Ao 311-K 363	44.5	0
21	Ao 311-K 364	Contamination	Contamination
22	Ao 311-K 365	23.75	7

Notes :

Ao = *Aspergillus ochraceus*
K = Yeast

Appendix 3. Colony diameter of *A. ochraceus* and yeast isolates on test of antagonism between *A. ochraceus* BIO 37312 and 22 yeast isolates (Continued)

No.	Isolate code	Diameter of colony (mm)	
		A. ochraceus	Yeast
1	Ao 312-KA	37.5	0
2	Ao 312-KA2	32.25	0
3	Ao 312-KB	37.25	0
4	Ao 312-KB2	18.5	6.75
5	Ao 312-KC	39.75	0
6	Ao 312-KD	50.75	0
7	Ao 312-K 215	17.5	16.5
8	Ao 312-K 216	27.75	10.25
9	Ao 312-K 217	0	37.5
10	Ao 312-K 218	37	0
11	Ao 312-K 219	18.5	7.5
12	Ao 312-K 220	38.25	10.75
13	Ao 312-K 285	11.75	25.5
14	Ao 312-K 286	0	22.5
15	Ao 312-K 287	10	31
16	Ao 312-K 288	0	28
17	Ao 312-K 289	0	40
18	Ao 312-K 290	21.25	16
19	Ao 312-K 291	38.25	0
20	Ao 312-K 363	Contamination	Contamination
21	Ao 312-K 364	Contamination	Contamination
22	Ao 312-K 365	20.25	14.25

Notes :

Ao = *Aspergillus ochraceus*
K = Yeast

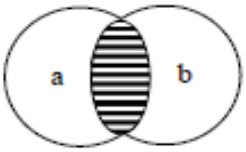
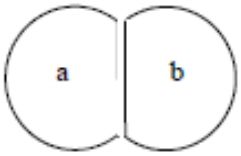
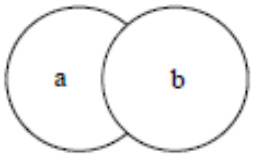
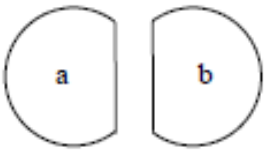
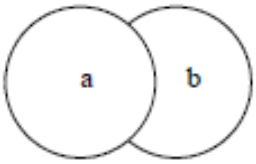
Appendix 4. Analysis of variance on the effect of processing method, yeast and/ or *A. ochraceus* inoculation on yeast population

Source of variance	df	SS	MS	F-value
Processed method (A)	1	1831616398	1831616398	17.06**
Yeast and/or OTA producing fungi (B) treatment	7	4736862286	676694612	6.30**
AB	7	1762700395	251814342	2.35**
Galat	32	3435612218	107362882	

* Significantly different at 95% confidence level

** Very significantly different at 99% confidence level

Appendix 5. Type of interactions between two fungal colonies (Wheeler dan Hocking 1993, adapted from Magan dan Lacey 1984)

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	

a: inhibitor fungi, b: inhibited species

Appendix 6. Explanation of attributes criteria in cupping test of coffee beans

No.	Attribute	Explanation
1	Fragrance	The aromatic aspects include Fragrance (defined as the smell of the ground coffee when still dry) and Aroma (the smell of the coffee when infused with hot water). One can evaluate this at three distinct steps in the cupping process: (1) sniffing the grounds placed into the cup before pouring water onto the coffee; (2) sniffing the aromas released while breaking the crust; and (3) sniffing the aromas released as the coffee steeps. Specific aromas can be noted under "qualities" and the intensity of the dry, break, and wet aroma aspects noted on the 5-point vertical scales. The score finally given should reflect the preference of all three aspects of a sample's Fragrance/Aroma.
2	Flavor	Flavor represents the coffee's principal character, the "mid-range" notes, in between the first impressions given by the coffee's first aroma and acidity to its final aftertaste. It is a combined impression of all the gustatory (taste bud) sensations and retro-nasal aromas that go from the mouth to nose. The score given for Flavor should account for the intensity, quality and complexity of its combined taste and aroma, experienced when the coffee is slurped into the mouth vigorously so as to involve the entire palate in the evaluation.
3	Aftertaste	Aftertaste is defined as the length of positive flavor (taste and aroma) qualities emanating from the back of the palate and remaining after the coffee is expectorated or swallowed. If the aftertaste were short or unpleasant, a lower score would be given.

Appendix 6. Explanation of attributes criteria in cupping test of coffee beans (Continued)

No.	Attribute	Explanation
4	Acidity	Acidity is often described as "brightness" when favorable or "sour" when unfavorable. At its best, acidity contributes to a coffee's liveliness, sweetness, and fresh- fruit character and is almost immediately experienced and evaluated when the coffee is first slurped into the mouth. Acidity that is overly intense or dominating may be unpleasant, however, and excessive acidity may not be appropriate to the flavor profile of the sample. The final score marked on the horizontal tick-mark scale should reflect the panelist's perceived quality for the Acidity relative to the expected flavor profile based on origin characteristics and/or other factors (degree of roast, intended use, etc.). Coffees expected to be high in Acidity, such as a Kenya coffee, or coffees expected to be low in Acidity, such as a Sumatra coffee, can receive equally high preference scores although their intensity rankings will be quite different.
5	Balance	How all the various aspects of Flavor, Aftertaste, Acidity and Body of the sample work together and complement or contrast to each other is Balance. If the sample is lacking in certain aroma or taste attributes or if some attributes are overpowering, the Balance score would be reduced.
6	Body	The quality of Body is based upon the tactile feeling of the liquid in the mouth, especially as perceived between the tongue and roof of the mouth. Most samples with heavy Body may also receive a high score in terms of quality due to the presence of brew colloids and sucrose. Some samples with lighter Body may also have a pleasant feeling in the mouth, however. Coffees expected to be high in Body, such as a Sumatra coffee, or coffees expected to be low in Body, such as a Mexican coffee, can receive equally high preference scores although their intensity rankings will be quite different.

Appendix 6. Explanation of attributes criteria in cupping test of coffee beans (Continued)

No.	Attribute	Explanation
7	Uniformity	Uniformity refers to consistency of flavor of the different cups of the sample tasted. If the cups taste different, the rating of this aspect would not be as high. 2 points are awarded for each cup displaying this attribute, with a maximum of 10 points if all 5 cups are the same.
8	Clean Cup	Clean Cup refers to a lack of interfering negative impressions from first ingestion to final aftertaste, a "transparency" of cup. In evaluating this attribute, notice the total flavor experience from the time of the initial ingestion to final swallowing or expectoration. Any non-coffee like tastes or aromas will disqualify an individual cup. 2 points are awarded for each cup displaying the attribute of Clean Cup.
9	Sweetness	Sweetness refers to a pleasing fullness of flavor as well as any obvious sweetness and its perception is the result of the presence of certain carbohydrates. The opposite of sweetness in this context is sour, astringency or "green" flavors. This quality may not be directly perceived as in sucrose-laden products such as soft drinks, but will affect other flavor attributes. 2 points are awarded for each cup displaying this attribute for a maximum score of 10 points.
10	Overall	The "overall" scoring aspect is meant to reflect the holistically integrated rating of the sample as perceived by the individual panelist. A sample with many highly pleasant aspects, but not quite "measuring up" would receive a lower rating. A coffee that met expectations as to its character and reflected particular origin flavor qualities would receive a high score. An exemplary example of preferred characteristics not fully reflected in the individual score of the individual attributes might receive an even higher score. This is the step where the panelists make their personal appraisal.

TABLES

Table 1 Intensity of fluorescence OTA producing fungi isolates using UV light (345 nm wave length)

No	Fungal species	Isolate code	Substrate	Intensity of fluorescence
1	*Aspergillus niger*	An BIO 35102	Nutmeg	_
2		An BIO 35105	Nutmeg	_
3		An BIO 35107	Peanut	_
4		An BIO 35109	Coffee bean	_
5		An BIO 35 110	Coffee bean	_
6		An BIO 35111	Maize	_
7		An BIO 35114	Coffee bean	+
8		An BIO 35115	Coffee bean	+
9		An BIO 35116	Coffee bean	+
10		An BIO 35117	Coffee bean	++
11		An BIO 35118	Coffee bean	_
12		An BIO 35120	Peanuts	_
13		An BIO 35121	Coffee bean	_
14		An BIO 35122	Coffee bean	_
15		An BIO 35307	Coffee bean	_
16		An BIO35308	Coffee bean	_
17		An BIO 35405	Cocoa bean	_
18		An BIO 35407	Coffee bean	++
19		An BIO 35409	Nutmeg	++
20		An 1R1	Coffee bean	+
21	*A. ochraceus*	Ao BIO 37126	Nutmeg	+
22		Ao BIO 37128	Peanut	++
23		Ao BIO 37129	Nutmeg	_
24		Ao BIO 37130	Smoked salai patin	_
25		Ao BIO 37133	Nutmeg	+
26		Ao BIO 37134	Nutmeg	+
27		Ao BIO 37135	Coffee bean	+
28		Ao BIO 37310	Coffee bean	++
29		Ao BIO 37311	Coffee bean	++

No	Fungal species	Isolate code	Substrate	Intensity of fluorescence
30	*A. ochraceus*	Ao BIO 37312	Coffee bean	++
31		Ao BIO 37313	Coffee bean	_
32		Ao BIO 37314	Coffee bean	+
33		Ao BIO 37315	Coffee bean	_
34		Ao BIO 37162	Nutmeg	+
35		Ao BIO 37163	Peanut	_
36		Ao BIO 37406	Coffee bean	+

Notes :

(-) No fluorescence
(+) Medium fluorescence
(++) High fluorescence

Table 2. Yeast population on coffee beans using wet and semi-wet processing methods and inoculated with yeast or *A. ochraceus* BIO 37310

Inoculation treatment	Drying method	
	Semi-wet (cfu/g)	Wet (cfu/g)
Control (without inoculation)	71 ± 31 a	2411 ± 548 ag
Inoculated by commercial yeast	4267 ± 416 afg	2422 ± 1496 ag
Inoculated by yeast of K287	36 333 ± 14 000 bh	12 333 ± 6333 adefg
Inoculated by yeast of K288	46 222 ± 9576 h	13 667 ± 9207 acdefg
Inoculated by yeast of K289	21 667 ± 2729 bcdefg	6889 ± 1678 aefg
Inoculated by *A. ochraceus* vs yeast of K287	32 444 ± 6159 bch	12 445 ±13 184 adefg
Inoculated by *A. ochraceus* vs yeast of K288	23 444 ± 2365 bcdef	26 556 ± 17 024 bcde
Inoculated by *A. ochraceus* vs yeast of K289	28 889 ± 26 943 bcdh	17 778 ± 7691 abcdefg

Table 3. Result of cupping test of coffee beans with inoculation treatments

Attribute*)	*Score**				
	Yeast K287	Yeast K288	Yeast K289	Ciragi	Without yeast
Fragrance	7.5	7.5	7.5	7.5	7.5
Flavor	7.5	7.5	7.5	7.25	7.5
Aftertaste	7.75	7.5	7.5	7.25	7.25
Acidity	7.5	7.5	7.5	7.5	7.5
Body	7.5	7.5	7.5	7.5	7.5
Balance	7.5	7.5	7.5	7.5	7.5
Uniformity	10	10	10	10	10
Clean Cup	10	10	10	10	10
Sweetness	10	10	10	10	10
Overall	7.5	7.5	7.5	7.5	7.5
Total score	82.75	82.50	82.50	82.00	82.25
Defect	0	0	0	0	0
Final score	82.75	82.50	82.50	82.00	82.25

Laboratory of cupping test : *Coffee Laboratory* PT Kemenady Industri Mandiri, Bogor
* keterangan skor : 0= Not present, 1= Unacceptable, 2= Verry Poor, 3= Poor, 4=Fair, 5= Average, 6= good, 7= Verry good, 8= Excellent, 9= Outstanding, 10= Exceptional
** Specialty grade ≥ 80
*) The explanation is presented in Appendix 6

FIGURES

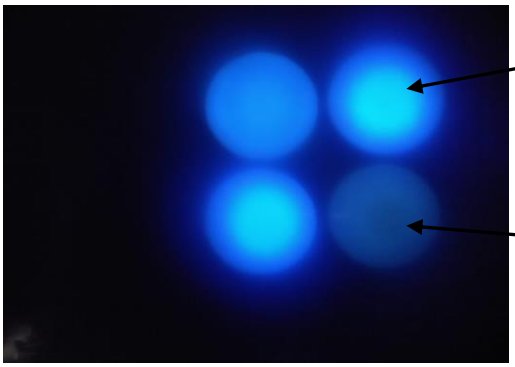


Figure 2. *Aspergillus ochraceus* flurorescence on coconut cream agar media 50% using UV light (λ 365 nm) showed as OTA producing fungi

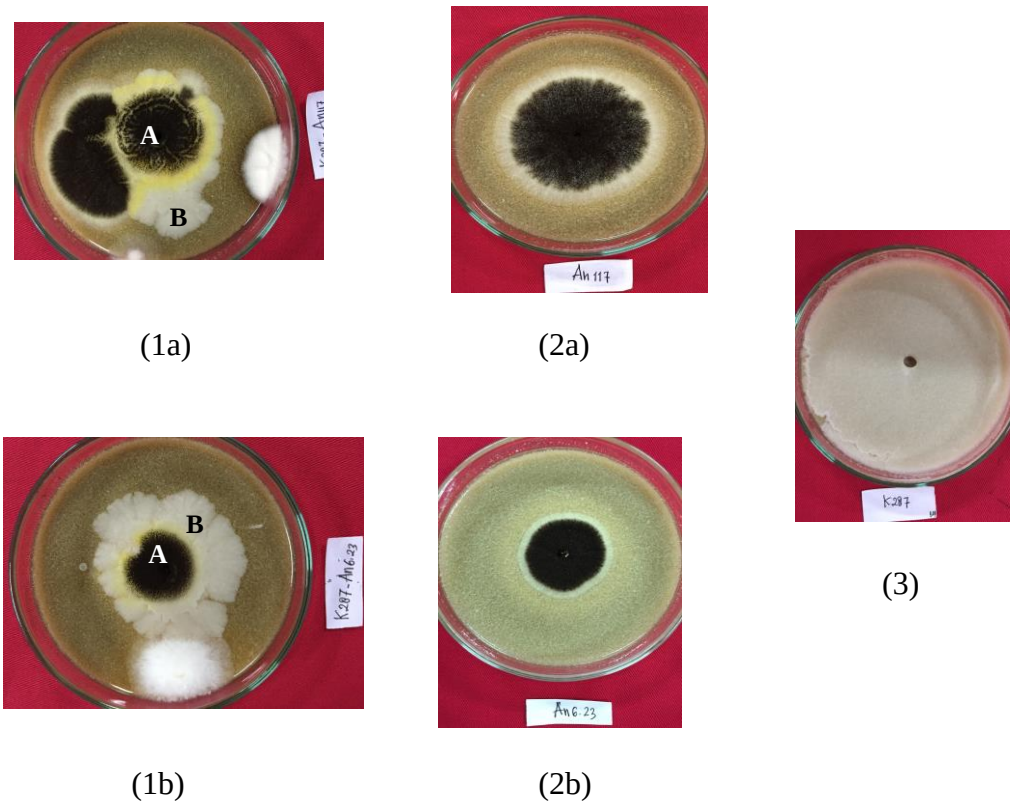
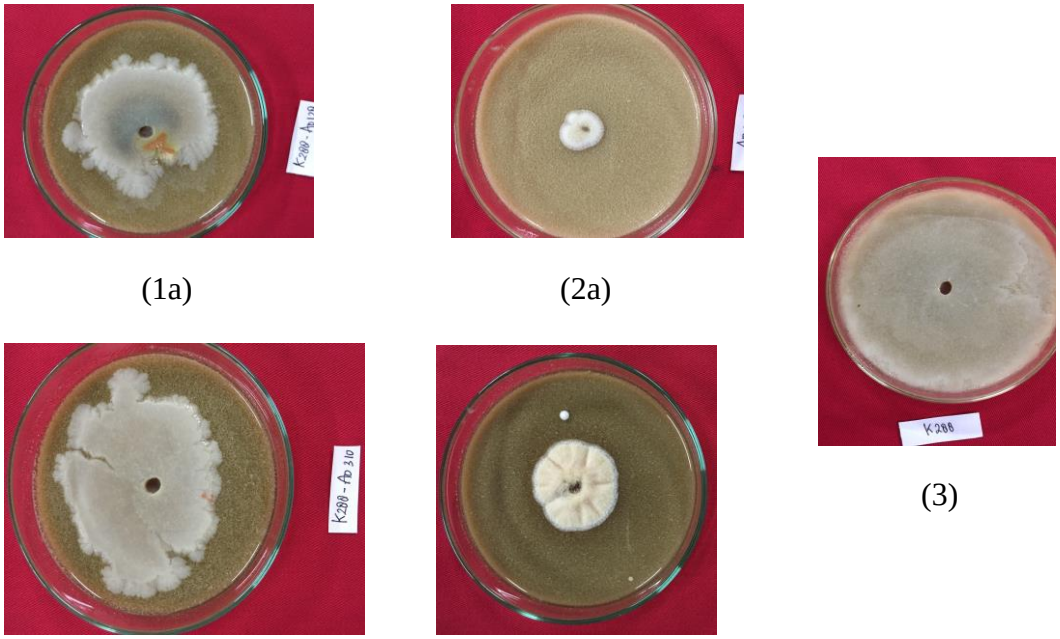


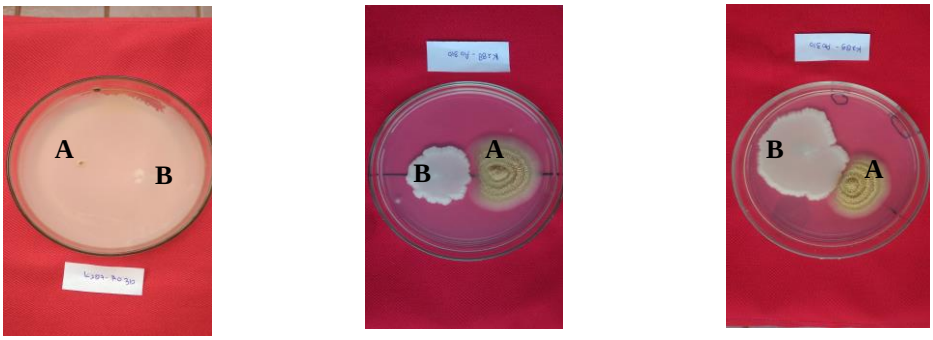
Figure 3. Test of antagonisms between yeast isolates and *A. niger* using well (dip) method on PDA media containing 15% Arabica coffee beans juice extract after 7 days of incubation at temperature 28±2 °C. (1) test of antagonism between yeast BIO 211287 and (a) *A. niger* BIO 35117, (b) *A. niger* BIO 35409; (2) control of *A. niger* (a) BIO 35117 and (b) BIO 35409; (3) control of yeast BIO 211287.
A = *A.ochraceus*, B = yeast isolate



(1a) (2a) (3)

(1b) (2b)

Figure 4. Test of antagonisms between yeast isolates and *A. ochraceus* using well (dip) method on PDA media containing 15% Arabica coffee beans juice extract after 7 days of incubation at temperature 28±2 °C. (1) test of antagonim between yeast BIO 211288 and (a) *A. ochraceus* BIO 37128, (b) *A. ochraceus* BIO 37310; (2) control of *A. ochraceus* (a) BIO 37128 and (b) BIO 37310; (3) control of yeast BIO 211288.



(a) (b) (c)

Figure 5. Mechanism of antagonisms between yeast (a) BIO 211287, (b) BIO 211288, (c) BIO 211289) and *A. ochraceus* BIO 37310 with E type of interaction on PDA media after 7 days of incubation at temperature 28±2°C.

A = *A.ochraceus*, B = yeast isolate