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POWDER FORMULATION OF YEAST TO CONTROL OCHRATOXIN A PRODUCING FUNGUS IN ARABICA COFFEE

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ABSTRACT

The research related on formulation of yeast powder to control ochratoxin A (OTA) producing fungus in Arabica coffee beans was conducted since March until November 2020. This research aimed 1) to test some carrier and additives materials on the survival of yeast *Issatchenkia orientalis* BIO 211287, and 2) to test the effect of powder formulation in combination yeast, carrier and additives materials on OTA production and the taste of Arabica coffee processed using semi-wet method. Methodology of these research were 3 stages, i.e. *screening* of additive materials that could increase the activity of antagonistic yeast using well method, testing of additive and carrier materials combinations for yeast survival, and biocontrol of OTA producing fungus (*Aspergillus ochraceus* BIO 37310) using formulation powder of *I. orientalis* BIO 211287 *in vivo*. Result showed that the highest yeast survival was found in 0.5% chitosan shrimp shell powder additive (80.9%) and could inhibit 100% of *A. ochraceus*. It means that 0.5% chitosan shrimp shell powder was the best of additive materials to keep yeast survival and to increase the activity of antagonistic yeast on OTA producing fungus. Tapioca powder was the best of carrier materials to keep yeast survival for 3 months of storage. *Issatchenkia orientalis* BIO 211287 could inhibit OTA producing fungal growth *in vitro,* and also increased the taste of Arabica coffee beans processed by semi-wet method.

Key words **:** *Arabica coffee beans, Issatchenkia orientalis, OTA producing fungus, yeast powder formulation*

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% (GEKI 2018).

In Indonesia, coffee fruits are fermented after harvesting. There are three methods of coffee bean processing, i.e. dry, wet and semi-wet processing (Kementerian Pertanian Republik Indonesia 2012). 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 to obtain green coffee. After shelling, the beans were further dried using sun-drying for 5 days until the moisture content attained ± 10%. Dharmaputra *et al.* (2018) reported that the population of *Issatchenkia orientalis* BIO 211287 and BIO 211288 in Arabica coffee inoculated with *Aspergillus ochraceus* BIO 37310 were higher in coffee processed using semi-wet method compared to that processed using wet method.

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. 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.

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 discoloration, decreases in physical quality and nutritional contents, and mycotoxin contamination.

Ochratoxin A (OTA) contamination in coffee beans have been becoming an important as some consumer countries have imposed their Maximum Tolerable Limits (MTL) of OTA. OTA is a potent nephrotoxic mycotoxin that has been linked to kidney problems in both livestock and human populations (Clark and Snedeker 2006). BPOM (2018) has determined MTL of OTA in coffee powder and *kopi sangrai* (roasted coffee) are 5 ppb, respectively, while that of in instant coffee is 10 ppb. In Brazil the maximum limit allowed for OTA in coffee is 10 ppb (Brazil, 2011). In tropical regions OTA is mainly produced by *Aspergillus carbonarius*, *A. niger* and *A. ochraceus*, while in sub-tropical regions it is produced by *Penicillium nordicum* (Pitt *et al*. 2000).

Nine of ten samples of green coffee bean samples collected from Argopuro mountain areas, Jembar, East Java Province, Indonesia during coffee year were detected OTA with the highest concentration was 0.4319 ppm and the lowest concentration was 0.0146 ppm (Rosavani and Harada 2019). Dharmaputra *et al.* (2019) reported 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.

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 different 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.

Dharmaputra *et al.* (2018) reported that three isolates of *Issatchenkia orientalis* (BIO 211287, BIO 211288, 211289) were able to grow either in coffee beans processed using wet or semi-wet methods inoculated with *A. ochraceus* BIO 37310. Total score specialty grades of coffee beans inoculated with the yeasts were higher than those uninoculated and inoculated with commercial yeast. The three yeast isolates can be used as biocontrol agents of *A. ochraceus* BIO 37310 and increased the sensorial quality of coffee beverages.

After obtaining a yeast isolate *Issatchenkia orientalis* BIO 211287 as an antagonist of *A. ochraceus* BIO 37310, technology for mass production and formulation is required to develop the yeast on commercial scale. Since the yeast is relatively easy to be massed cultured in a cheap medium (Potato Dextrose Broth), formulation technology is a critical step. Appropriate formulation technology will facilitate storage, transportation, application technique and also bio-performance of the yeast. Solid formulation is chosen to be developed for easier transportation and handling.

The effect of carrier materials (talc, bentonite and tapioca) and additives (crab shell powder and $MnSO_4$) which is able to keep the bioperformance, including antibiosis activity to *Phytophthora capsici* and *Colletotrichum acutatum* and plant growth promoting effect, of two antagonistic bacteria *Pseudomonas fluorescens* PG 01 and *Bacillus polymixa* BG 25 in biofungicide powder formulations was studied by Widodo and Wiyono (2012). Talc and bentonite formulations were effective after 3 months of storage, while tapioca was only effective to *B. polymixa* up to 3 months of storage. Additive materials that can enhance the antibiosis activity of the bacteria, keeping up the growth and no toxicity effect to chili seedlings were crab shell powder 0.25% and MnSO4 1 to 2%. After eight months of storage with 20% moisture content, the bacteria population survived in powder formulation developed in this study was still suitable for seed treatment and/or after transplanting through soil drenching with water. In this period of storage, population of the two bacteria was 10^6 cfu/g formulation.

According to Wiyono and Widodo (2013) best carrier materials in powder formulation of *Cryptococcus terreus* was talc, able to maintain yeast survival for four months of storage without contamination of other microorganisms. Talc powder provided best survival of *C. albidus*, with survival more than 5 months of storage. Crab shell at concentration of 1.25% can be used as additive in powder formulation containing a mixture of *C. terreus* ans *C. albidus*, based on its enhancement of antagonistic activity on *C. terreus* and survival of both antagonistics yeasts.

The research which was conducted in 2020 is the continuation of the research conducted in 2018. The research is also related to the 2nd Programme Thrust SEAMEO BIOTROP for 10th Five Year Development Plan (2018–2023), i.e. Sustainable Management of Intensively Used Ecosystems/Landscapes. It consists among others Food and Feed Security and Safety.

1.2. Objectives

- To test some carrier materials and additives on the antagonistic of yeast *Issatchenkia orientalis* BIO 211287
- To test the effect of powder formulation in combination of carrier materials and additives on the survival of yeast, OTA producing fungus, OTA production and the taste of Arabica coffee processed using semi-wet method.

1.3. Expected Output

It is expected, that the research result could obtain:

- cheap material as carrier which ensure long time survival and bioperformance of *Issatchenkia orientalis* BIO 211287
- powder formulation in combination of carrier materials and additives that can control OTA producing fungus, OTA production and increase the taste of Arabica coffee processed using semi-wet method.

2. BENEFIT AND IMPORTANCE OF RESEARCH

This research is useful to be conducted, because the use of powder formulation with a cheap carrier and additive materials combinations to inhibit OTA producing fungal growth, OTA production and also to increase the taste of coffee needs to be developed as an alternative formulation in liquid form. Therefore, it will make effective and efficient in transportation, storage, and packaging.

3. METHODOLOGY

3.1. Time and location of research

Preparation and research activity was conducted from March up to November 2020 at SEAMEO BIOTROP, Bogor.

3.2. Yeast and OTA producing fungus isolates

Issatchenkia orientalis BIO 211287 was used as antagonistic yeast. OTA producing fungus *Aspergillus ochraceus* BIO 37310 was used for bioassay of the yeast. The yeast and OTA producing fungus were obtained from Culture Collection of Plant Pathology Laboratory, SEAMEO BIOTROP, Bogor, Indonesia.

Issatchenkia orientalis BIO 211287 and *A. ochraceus* BIO 37310 were cultured on Potato Dextrose Agar (PDA) in petridish with diameter 90 mm and incubated for 4 and 7

days at room temperature (28±2)^{o}C. These isolates of yeast and fungus were used in the next steps.

3.3. Preparation additive and carrier materials

Materials tested for additives to increase the activity of antagonistic yeast were clam shell powder, chitosan (shrimp shell powder) and calcium chlorida ($CaCl_2$ p.a). Each material was tested with acetic acid 0.1% suspension/solution (for clam shell powder and chitosan), and destillation water (for $CaCl_2$) with the concentrations of 0.5, 1.0 and 2% (w/v). Carrier material that was used for selecting of carrier and additive materials for yeast survival were talc and tapioca powder.

3.4. *Screening* additive materials that could increase activity of antagonistic yeast

Each additive materials was soluted using 0.1% acetic acid solution to obtain stock solution with concentration 2.4% (2.4 g additive material + 100 mL acetic acid solution), it was then sterilized at temperature of 121^{o}C for 15 minutes.

As much as 1 mL yeast suspension (5 x 3.8 x 10^2 cells/mL) was added into 5 mL of each additive materials suspension with concentration 0.5; 1.0 and 2.0% included control (distillation water). They were then shaked using Vortex mixer Corning/LSE for 30 seconds, and they were left for 1 minute. Each mixed suspensions was isolated using dillution method followed by pour plate method on Yeast Extract 10 g, Peptone 20 g, Dextrose or Glucose 20 g, distillation water 1000 mL and Bacto agar 18 g (YPDA) media and incubated for 48 hours at room temperature (28 ± 2) ^{o}C (Yu *et al.* 2012). Three replicates were used for each treatments and control. *Screening* observation was conducted on yeast population for each treatments and control.

3.5. *Screening* of additive materials that increase activity of antagonistic yeast on *A. ochraceus* BIO 37310 using well method

Additive material that was used did not inhibit yeast growth at the previous stage, i.e. chitosan (shrimp shell powder) with concentration 0.5 and 1.0% and $CaCl_2$ with concentration 0.5, 1.0 and 2.0%.

Each PDA media that contained additive materials with various concentration was perforated in diameter 5 mm of the center. It was then inoculated by 20 µL of yeast (1 x 10^7 cells/mL), and it was also inoculated by 20 µL of *A. ochraceus* (1 x 10^4 conidia/mL) after 1 hour. As controls, PDA media contained of additive material and inoculated by *A. ochraceus* , PDA media without additive material and *A. ochraceus*, and PDA media without additive material but inoculated by yeast and *A. ochraceus*. *Screening* determination was conducted on diameter of yeast and *A. ochraceus* colonies after 5 days incubation.

3.6. Combinations of carriers and additive materials for yeast survival

Best additive materials for growing of the yeast on *A. ochraceus* as result of previous stage were chitosan (shrimp shell powder) with 0.5% concentration combined

with each carrier material (talc, tapioca powder, and mix talc and tapioca powder 1:1). Each carrier and additive materials was sterilized in an autoclave at temperature of 121^{o}C for 15 minutes.

As much as 250 g of sterile carrier materials were combined with 40 mL of additive materials solution until homogenous, they were then added by 60 mL of yeast suspension (0.6 x 10^{9} cells/mL) and stirred until homogenous. Those materials were packed in heat resistant polyethylene plastic bags and were sealed by a sealer. As control, sterile carrier materials were not inoculated by yeast, but inoculated by distillation water as yeast substitution. Five replicates were used for each treatment. They were then stored at room temperature. The temperature and relative humidity were noted.

The yeast survival in each combination of material was determined at the beginning of storage, subsequently after 30, 60 and 90 days of incubation using dilution method, followed by pour plate method on PDA media. The moisture content of each combination of material will be determined. The moisture content of each combination of material was determined by oven method at temperature 130 ± 3^{o}C for 1 hour (INS 2009).

3.7. Biocontrol of ochratoxin A producing fungus using powder formulation of *Issatchenkia orientalis* BIO 211287 in vivo

3.7.1 Making of yeast powder (*Issatchenkia orientalis* BIO 211287) for *in vivo* treatment

As much as 200 g of sterile tapioca powder was inoculated by 48 mL yeast suspension with concentration 2.1 x 10^{8} cells/mL and 32 mL of 0.5% chitosan solution. Chitosan solution 0.5% was obtained from 2 L of 0.1% acetic acid solution + 62.5 g chitosan, it was then sterilized at temperature 121^{o}C for 15 minutes.

3.7.2 Inoculation of yeast powder and *Aspergillus ochraceus* in coffee beans

Coffee beans were collected from harvested coffee cherries in Pangalengan, Bandung. The husk of coffee cherries were shelled by a machine. As much as 2 kg of coffee beans were inoculated by 200 g yeast powder, they were then stirred until homogenous and were left for 3 hours. After that, as much as 10 mL of *Aspergillus ochraceus* BIO 37310 suspension (2.6 x 10^{6} conidia/ mL) was inoculated into those samples. As controls : coffee beans were inoculated by yeast powder, coffee beans were inoculated by *A. ochraceus,* and coffee beans without yeast powder and *A. ochraceus*.

3.7.3 Drying and shelling of coffee beans

Drying of coffee beans were conducted using sun-drying until the moisture content was 10 - 11%. Moisture content of coffee beans was determined using moisture analyzer merck Delmhorst during drying process. Shelling of hull of coffee beans was conducted using by hand manually.

3.7.4 Packaging and storing of coffee beans

As much as 500 g of coffee beans with moisture content 10 - 11% were packed in hermetic plastic bags (SEMAR) with double packed in gunny bags. Four

replicates for each treatment. Those samples were stored at room temperature. Temperature and relative humidity were noted.

Yeast survival in coffee beans and bioperformance of yeast to control ochratoxin A producing fungus (*A. ochraceus* BIO 37310) were determined by calculating of yeast population and OTA producing fungus at the beginning of storage, subsequently after 30, 60 days of storage using dillutiong method followed by pour plate method on PDA media. The moisture content of coffee beans treatments and controls were determined by oven method at temperature 105 ± 1^{o}C for 16 hours (INS 2008).

3.8. Cupping test

Cupping test of the samples was conducted Standard Cupping Protocol given by Coffee Quality Institute and Specialty Coffee Association of America (SCAA 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.

3.9. Statistical analyses

The data of the screening for additives was analyzed using Completely Randomized Design One Factor (kind of additive materials), selection of combination of yeast powder formulations was analyzed using Completely Randomized Design Factorials (carrier materials and duration of storage), and biocontrol of ochratoxin A producing fungus using *I. orientalis* BIO 211287 powder formulation *in vivo* was analyzed using Completely Randomized Design Factorials (inoculation yeast treatment with or without OTA producing fungus and duration of storage) (Mattjik and Sumertajaya 2002).

4. RESULTS AND DISCUSSION

4.1 *Screening* additive materials that increase the activity of antagonistic yeast

4.1.1 Yeast survival of *Issatchenkia orientalis* BIO 211287 in additive materials

Yeast *I. orientalis* BIO 211287 survival in chitosan shrimp shell powder on controls (destillation water) decreased with increasing of the concentrations (Table 1). Claim shell powder inbited yeast growth in the high concentration (2.0%) and low concentration (0.5%), while the yeast growth in $CaCl_2$ at low and high concentrations had similar value. The highest yeast survival was found in 0.5% chitosan shrimp shell powder additive (80.9%). Yu *et al.* (2012) reported that the yeast *Cryptococcus laurentii* survival in chitosan crab shell powder additive decreased with increasing of concentrations. Yeast colony growth in various additive materials and concentrations were shown in Figure 1.

Table 1 Yeast survival of *Issatchenkia orientalis* BIO 211287 in various additive materials on controls

Treatment	Concentration (%)	Yeast survival in various additive materials on controls (%)
Chitosan (shrimp shell powder)	0.5	80.9 a
	1	18.9 b
	2	1.4 b
Claim shell powder	0.5	1.6 b
	1	0.6 b
	2	4.5 b
$CaCl_2$	0.5	74.5 a
	1	74.5 a
	2	75.3 a

Number followed by the same letter, are not significantly different based on Duncan's test at 95% of confidence level.

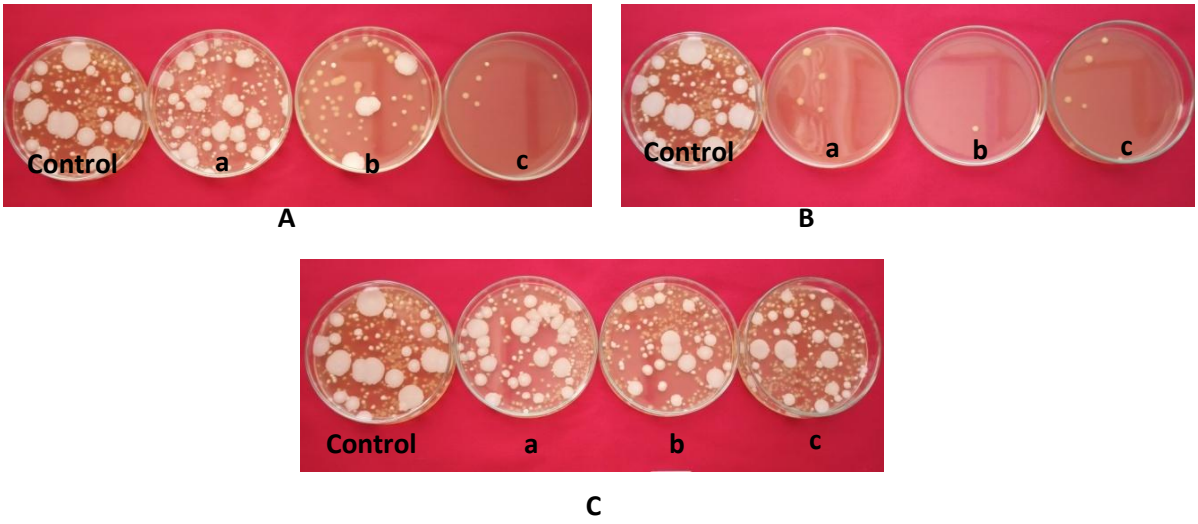

Figure 1. Isolation result of *Issatchenkia orientalis* BIO 211287 in samples with various additive materials and concentrations.
A = chitosan shrimp shell powder; B= claim shell powder; C= $CaCl_2$;
a= 0.5%; b= 1.0%; c= 2.0%

4.1.2 Additive materials that increased activity of antagonistic yeast on *A. ochraceus* BIO 37310

Some additive materials that could keep the yeast growth in previous stage, i.e. chitosan 0.5%, CaCl2 0.5, 0.1 and 2.0%. These additive materials were then tested to

determine their effect on activity of antagonistic yeast on OTA producing fungus (*A. ochraceus* BIO 37310).

The highest diameter of yeast colony were found in 1.0% chitosan was inoculated by *I. orientalis* and *A. ochraceus* (79.9 mm) and 0.5% chitosan (77.6 mm). These treatments also could inhibit *A. ochraceus* BIO 37310 (100%) (Table 2 and Figure 2). If we compared to previous result on percentage of yeast survival, 0.5% chitosan was more available than 1.0% chitosan to be used as additive materials. It means that 0.5% chitosan additive could increased the antagonistic yeast on *A. ochraceus* BIO 37310.

Table 2 Diameter of *Issatchenkia orientalis* BIO 211287 and *Aspergillus ochraceus* BIO 37310 colonies in various concentrations of chitosan shrimp shell powder and $CaCl_2$

Treatment	**Mean of diameter colony (mm)**	
	***Issatchenkia orientalis* BIO 211287 (Io)**	***Aspergillus ochraceus* BIO 37310 (Ao)**
Chitosan 0.5-inoculated by Io and Ao	77.6 a	0 c
Chitosan 1.0-inoculated by Io and Ao	79.9 a	0 c
Chitosan 0.5-inoculated by Ao	-	36.2 a
Chitosan 1.0-inoculated by Ao	-	35.5 a
$CaCl_2$ 0..5- inoculated by Io and Ao	45.5 d	0 c
$CaCl_2$ 1.0- inoculated by Io and Ao	50.1 cd	0 c
$CaCl_2$ 2.0- inoculated by Io and Ao	54.7 c	0 c
$CaCl_2$ 0.5- inoculated by Ao	-	35.9 a
$CaCl_2$ 1.0- inoculated by Ao	-	36.3 a
$CaCl_2$ 2.0- inoculated by Ao	-	37.3 a
Inoculated by Io and Ao-without additives	70.0 b	8.5 b
Inoculated by Ao-without additives	-	36.7 a

Number followed by the same letter, are not significantly different based on Duncan's test at 95% of confidence level.

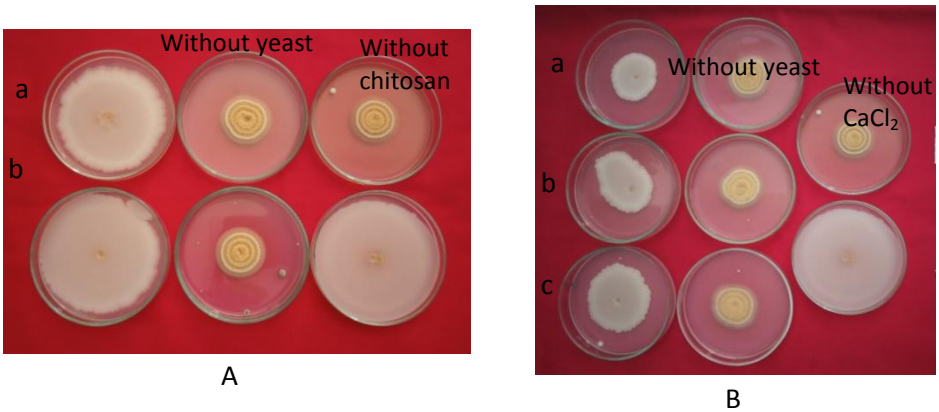

Figure 2 *Issatchenkia orientalis* BIO 211287 and *Aspergillus ochraceus* BIO 37310 colonies in samples treatment :
A) chitosan shrimp shell powder: a. 0.5%; b. 1.0% and

B) $CaCl_2$: a. 0.5%; b. 1.0%; c. 2.0%

4.2 Combinations of carriers and additives materials for yeast survival

Carrier materials are important component of formulations to keep life force and activity of antagonistic microbes. Tapioca powder was the best of three carrier materials, because it could keep life force yeast until 3 months of storage (Table 3). According to Wiyono *et al.*(2013), tapioca powder could keep the growth of yeast *Cryptococcus terreus* for 4 months of storage.

Table 3 Population of yeast *Issatchenkia orientalis* BIO 211287 in various formulations of yeast powder during storage

Kind of carrier material	Duration of storage (month)	Yeast population (*cfu*/g w.b)	Moisture content (%)
Tapioca	0	$3.8x10^7$ a	36.4 b
	1	$1.4x10^5$ b	36.6 b
	2	$1.6x10^5$ b	36.8 ab
	3	$4.3x10^4$ b	37.1 ab
Talc	0	$4.2x10^6$ b	26.5 d
	1	$9.4x10^4$ b	23.5 ef
	2	$5.4x10^2$ b	23.0 f
	3	6.7x10 b	21.2 g
Mix talc-tapioca	0	$1.1x10^6$ b	33.2 c
	1	$1.8x10^5$ b	32.6 c
	2	$9.3x10^4$ b	33.9 c
	3	$4.2x10^3$ b	32.9 c

Number followed by the same letter, are not significantly different based on Duncan's test at 95% of confidence level.

4.3. Biocontrol of ochratoxin A producing fungus using formulation powder of *Issatchenkia orientalis* BIO 211287 *in vivo*

Yeast survival in coffee beans with or without *A. ochraceus* BIO 37310 could survive for 1 month (Table 4). The highest population of yeast (4.6×10^2 cfu/g w.b.) and total microbes (7.5×10^3 cfu/g w.b.) was found in control was only inoculated by yeast *I. orientalis* BIO 211287.

Table 4 Population of yeast *Issatchenkia orientalis* BIO 211287 and total microbes in coffee beans in samples treatment and controls during storage

Treatment and control	Duration od storage (month)	Population (*cfu*/g w.b.)	
		Yeast	Total microbes
Treatment : coffee beans were inoculated by yeast powder and *A. ochraceus* BIO 37310	0	$4.4x10^2$	$4.0x10^3$
	1	$4.2x10^2$	$3.0x10^3$
Control: coffee beans without yeast powder and *A.ochraceus* BIO 37310)	0	0	$2.4x10^5$
	1	0	$3.5x10^4$
Control: coffee beans without yeast powder, but they were inoculated by *A.ochraceus* BIO 37310)	0	0	$8.5x10^3$
	1	0	$9.1x10^2$
Control: coffee beans without *A.ochraceus* BIO 37310, but they were inoculated by yeast powder	0	$1.9x10^3$	$1.4x10^4$
	1	$4.6x10^2$	$7.5x10^3$

4.4. Result of cupping test

Final score of cupping test of coffee beans was inoculated by *I. orientalis* (83.7%) was higher than the coffee beans was not inoculated by it (82.2%) (Table 5). It means that yeast *I. orientalis* BIO 211287 could increased the taste of Arabica coffee beans processed using semi-wet method. Result of cupping test of coffee beans were inoculated by *I. orientalis* BIO 211287 was shown in Table 5, while the explanation of criteria attributes in cupping test was shown in Appendix 6.

Table 5 Result of cupping test of coffee beans were and were not inoculated by yeast *I. orientalis* at the beginning of storage

Treatment of coffee beans	Final score
Coffee beans were inoculated by yeast	83.7
Coffee beans were not inoculated by yeast	82.2

5. CONCLUSIONS

Chitosan shrimp shell powder with concentration 0.5% was an effective additive material to keep yeast *Issatchenkia orientalis* BIO 211287 survival and activity of the antagonistic yeast. Tapioca powder was the best of carrier materials to keep yeast survival for 3 months of storage. Yeast *I. orientalis* BIO 211287 could inhibit OTA producing fungus (*Aspergillus ochraceus* BIO 37310) *in vitro*, and also could increased the taste of Arabica coffee beans processed by semi-wet method.

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APPENDICES

Appendix 1 Analysis of variance of yeast *Issatchenkia orientalis* BIO 211287 survival in various additive materials on controls

Source	DF	Sum of squares	Mean square	F value	Pr > F
Treatments	8	34287.39185	4285.92398	23.16	<0.0001
Error	18	3330.32667	185.01815		
Corrected total	26	37617.71852			

Appendix 2 Analysis of variance of yeast *Issatchenkia orientalis* BIO 211287 and *Aspergillus ochraceus* BIO 37310 in various concentrations of chitosan shrimp shell powder and $CaCl_2$

Source	DF	Sum of squares	Mean square	F value	Pr > F
Treatments	5	3308.836111	661.767222	62.91	<0.0001
Error	12	126.240000	10.520000		
Corrected total	17	3435.076111			

Appendix 3 Analysis of variance of *Issatchenkia orientalis* BIO 211287 population in various formulations of yeast powder during storage

Source	DF	Sum of squares	Mean square	F value	Pr > F
Powder	2	1.0254634E15	5.1273172E14	11.85	<0.0001
Time	3	2.2677483E15	7.559161E14	17.47	<0.0001
Powder*Time	6	3.0431876E15	5.0719793E14	11.72	<0.0001
Error	48	2.0766309E15	4.3263144E13		
Corrected total	59	8.4130302E15			

Appendix 4 Analysis of variance of *Issatchenkia orientalis* BIO 211287 population in various formulations of yeast powder during storage

Source	DF	Sum of squares	Mean square	F value	Pr > F
Powder	4	3222.067000	805.516750	575.80	<0.0001
Time	3	30.502800	10.167600	7.27	0.0002
Powder*Time	12	79.544200	6.628683	4.74	<0.0001
Error	80	111.916000	1.398950		
Corrected total	99	3444.030000			

Appendix 5 Result of cupping test of coffee beans were inoculated by yeast *Issatchenkia orientalis* BIO 211287

Attribute*)	Score*	
	Issatchenkia orientalis	Tidak diinokulasi khamir
Fragrance	7.7	7.5
Flavor	7.9	7.38
Aftertaste	7.4	7.25
Acidity	7.8	7.5
Body	7.8	7.5
Balance	7.8	7.5
Uniformity	10	10
Clean Cup	10	10
Sweetness	10	10
Overall	7.5	7.5
Total score	83.9	82.20
Defect	0	0
Final score	83.9	82.20

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

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.

Source: Specialty Coffee Association of America (2015)

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.

Source: Specialty Coffee Association of America (2015)

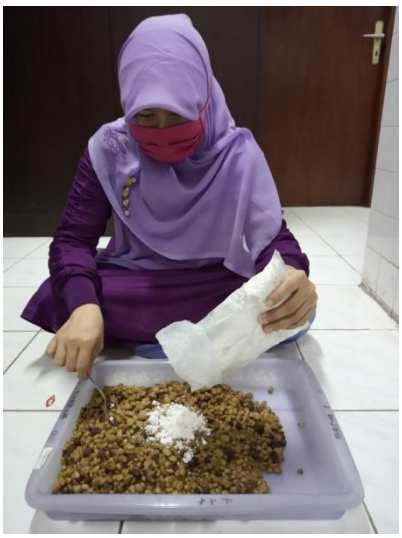
Appendix 6. Explanation of attributes criteria in cupping test of coffee beans (Continued)

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.

Source: Specialty Coffee Association of America (2015)

Appendix 7 Test tube filled with additive material and yeast *Issatchenkia orientalis* BIO 211287

Appendix 8 Storing of yeast powder in various formulations of carrier and additive materials at room temperature (28±2)^{o}C



Appendix 9 Inoculation of yeast powder into coffee beans

Appendix 10 Drying of coffee beans using sun-drying : A. coffee beans with hulls; B. green coffee

Appendix 11 Storing of coffee beans were packed in hermetic plastic bags(SEMAR) with double packed in gunny bags at room temperature (28±2)^{o}C