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Identification, aflatoxin content, and antagonistic test of spoilage fungi in bread to *Aspergillus niger*

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Abstract. *Fendiyanto MH, Satrio RD. 2020. Identification, aflatoxin content, and antagonistic test of spoilage fungi in bread to Aspergillus niger. Bioteknologi 17: 60-66.* The major problem in shelf storage life in bread is contamination by molds, fungi and mycotoxins. The growth of spoilage fungi indicates that the bread has structural damage. Infection from this fungus will affect the shelf life of the bread to be shorter. Contaminated bread may influenced human health in the future. One of the many solutions to overcome the fungi-contamination of bread is the use of biological agents. *Aspergillus niger* has the ability, as a biological agent, to suppress food-destroying pathogens in many foods, including bread. However, there are few reports about the antagonistic test in bread, particularly between *A.niger* as biological control agent to spoilage fungi. Therefore, this study aimed to analyze the growth antagonistic test of *A. niger* against food spoilage fungi, expecting the storage life of bread could be extended and mycotoxin contamination avoided. The antagonistic test was in the form of a test that utilizes the properties of microorganisms that grow faster than pathogens or produce antibiotic compounds. The methods used in this study included isolation and identification of fungi, water content analysis, aflatoxin test, and antagonistic test. Interestingly, the antagonist test results showed *A. niger* can inhibit the growth of spoilage fungi on bread. In conclusion, the species of fungus with the highest inhibition value was *Hyphopichia burtonii*, while the lowest was *Saccharomyces cerevisiae*. These findings indicate that *A.niger* can be used as a biological control in extending bread shelf storage in the future.

Keywords: Antagonist test, *Aspergillus niger*, spoilage fungi

INTRODUCTION

The success of global development is determined by the availability of quality human resources (HR) which includes strong minds and excellent health, in addition to mastery of science and technology. The quality of a country's human resources will be low if there is malnutrition; as people whose health is disturbed, their intelligence decreases. Therefore, the main step in creating superior human resources is by improving the quality of nutrition. The continued impact of the decline in the nutritional status or health of these nutrient vulnerable groups in the long term will reduce the quality of Indonesia's human resources (Syarief 1997). FAO classifies Indonesia as a low income and food shortage country. The number of poor people in Indonesia is close to 50 million, of which, 32.7 million live in rural areas (BPS 2001). Fulfilling food needs for energy sources in activities. Food provides energy and stamina. Consuming food will increase vitality so that it is possible to live longer, not tire easily, increase immunity and help keep the body fit and healthy (Marshal 2005).

Carbohydrates are one of the most common macronutrients and the cheapest source of calories (Handewi and Sallem 2002). These nutrients are the main source of calories for much of the world's population, especially residents of developing countries. Carbohydrates have an important role in determining the characteristics of food ingredients such as taste, color, and texture. Whereas in the body, carbohydrates are useful for preventing ketosis, breaking down excessive body protein, losing minerals, and for helping metabolize fat and protein (Winarno 2008). It is recommended that carbohydrate foods be consumed by 6-11 servings per day. Example food sources of carbohydrates include bread, rice, cereals, and pasta (Astawan and Kasih 2008).

Bread is a group of carbohydrate foods that should be consumed in about one-third of the diet. Bread, like pasta, potatoes, rice, and other cereals, is the best food source of energy. These foods also contain complex B vitamins to release energy from food and maintain a healthy digestive and nervous system. The recommended amount of carbohydrate foods is 6 or more servings per day (Marshal 2005). Bread is made from wheat flour that is spread with yeast and baked. Bread has become one of the staple foods for Indonesians, even among teenagers and children. The position of this food is starting to shift from rice as the main source of carbohydrates, because of its nutritional content compared to rice or noodles.

Bread is a flour product that is easily damaged, especially due to fungi attack. The types of fungi that often contaminate food are mold and yeast. Fungi are microbes consisting of more than one cell in the form of fine threads called hyphae, a collection of hyphae is called mycelium, reproducing by spores, or dividing (SNI 7388; 2009). As a result of growing fungi on bread, that bread cannot be consumed. This is unfortunate considering that bread is a food that contains carbohydrates, and can be the main source of energy. In addition, the fast growth of fungi on bread often causes bakers to experience losses due to the low storage capacity of bread (BSN 2009).

The major problem for the shelf life of bread is mold fungal infection. Commonly, bread is contaminated by mold spoilage during storage, especially being contaminated after spreading of the fungi spores during storage (El Sheikha 2016). The mold will grow rapidly in the bakery products as well, particularly *Aspergillus*, *Fusarium*, and *Penicillium*. The environment sterility in the bakery is low, as usually the room contains mold spores and the dust containing spores spreads easily in the air (El Sheikha 2016). According to the report by El Sheikha (2016), there are 8000 mold spores per 1 gram of flour. Therefore, the crucial problem in the bakery industry is the presence of fungi spores and its potentially dangerous mycotoxins (El Sheikha 2016).

Microorganisms that cause food spoilage are bacteria, molds, and yeasts (Aryulina et al. 2005; Shehata et al. 2008; Pundir and Jain 2011). They are always present in specifically in the air and water in bread. They can activate many enzymes and furthermore, cause changes in color, flavor, and texture of the bread. Mold fungi can attack many loaves of bread, spread mycotoxins, and influence human health (El Sheikha and Mahmoud 2016). Mycotoxins of *Aspergillus*, *Fusarium*, and *Penicillium* are generated during the growth of molds on loaves of bread. Some of the mycotoxins are only present in the mold tissues, while most of them are excreted into the environment like loaves of bread (El Sheikha and Mahmoud 2016). Mold growth can be prevented by storing bread in low humidity conditions. The first molds that can colonize are of genus *Eurotium*, followed by *Aspergillus*, *Penicillium*, and *Fusarium*. The mold will produce mycotoxins and change the bread's texture. Filamentous molds are more easily recognized than other microbes like yeast or bacteria (El Sheikha and Mahmoud 2016).

Control of fungal contamination in bread can use several methods, i.e., reformulation to reduce bread Aw, freezing, preservatives, and the effect of bio preservatives. The use of biological controls in bread was never performed; this method is necessary to avoid spoilage fungi and to explore novel compounds against spoilage fungi in bread (El Sheikha and Mahmoud 2016). Nowadays, to detect the mycotoxigenic fungi in food has progressed, i.e., the use of molecular markers (Fendiyanto et al. 2019). The molecular markers to detect the toxins in the broad application are PCR-DGGE (El Sheikha 2019), RAPD, SSR, ISSR (Pratami et al. 2020), and ITS (El Sheikha et al. 2018). All the methods are able to detect many spoilage fungi species in food, including bread (El Sheikha et al. 2018). Besides molecular markers, there are many other markers, i.e., morphological markers (Pratami et al. 2019), metabolomics markers (Fendiyanto et al. 2020), and EST markers (Satrio et al. 2019).

According to El Sheikha (2015), food is safe if it is properly handled at all steps of production, processing, distribution, from retail and foodservice business through consumption. Safe food is unlikely to cause illness or injury. A major challenge in the food industry is food supply chains (El Sheika 2015). Therefore, this study aimed to analyze the growth of *Aspergillus niger* against spoilage fungi on bread as an effort to increase the shelf life of bread.

MATERIALS AND METHODS

Fungi isolation

Isolation media is a medium that contains nutrients for fungal growth. The isolation medium used to isolate postharvest fungi that were commonly used is DG18 media. Dichloran 18% Glycerol Agar (DG18) media is a medium for isolating xerophilic fungi. Xerophilic fungi are fungi that grow and develop on substrates with low water content, such as cereals, nuts, flour, nutmeg, and other spices. The selective or differential isolation media used to isolate *Aspergillus flavus*, *A.parasiticus*, and *A.nomius* were *Aspergillus flavus* and *Parasiticus* Agar (AFPA) media (Pitt et al. 1983; Zummo and Scott 1990). DG18 media is suitable for the growth of fungi *Eurotium* sp., *Aspergillus restrictus*, *A. penicillioides*, *Wallemia sebi*, *Trichoderma*, and *Endomyces*. DG18 media contained chloramphenicol and dichloran. Chloramphenicol was used to avoid contamination by bacteria, while dichloran was used to limit the growth of fast-growing fungi such as *Mucor* and *Rhizopus*. The composition of DG18 media included glucose 10 g, peptone 5 g, KH_2PO_4 1 g, $MgSO_4.7H_2O$ 0.5 g, glycerol A.R. 220 g, 15 g agar, 2 mg dicloran, 100 mg chloramphenicol, and 1 liter of distilled water. All the ingredients were mixed simultaneously and cooked and sterilized using an autoclave.

Fungi identification

Fungi identification was performed by two methods, namely the method of identification media and the method of fungi morphology on the microscope. The media used to identify were CYA, CY20S, MEA, and G25N media. The fungi isolated on DG18 media were transferred to identification media. Fungi were transferred using an inoculation loop and the fungi transferred had to be pure cultures. The pure cultures obtained were then grown on standard media, namely CYA, MEA, and G25N with an incubation temperature of 25°C and 37°C for seven days (Pitt et al. 1992). Fungi identification was carried out after seven days of incubation. The observations included: colony diameter, colony character, and making wet preparations. The colony diameter was determined using a ruler in mm. The diameter of the fungus was calculated on the reverse side of the Petri dish. Observations of colony characteristics included colony color, surface texture, and color of exudate produced by fungi (Pitt and Hocking 2009).

Water content test

The method of analyzing moisture content was performed by using the oven or distillation method (BSI 1980). In this study, the analysis of water content in bread used the distillation method. The principle of this method was the difference in the boiling point of water and xylol. The equipment used for the analysis of moisture content included a 500 ml boiling flask, Aufhauser device, electric bath, and analytical balance. A sample of 5-10 grams was weighed and put in a boiling flask, with an addition of 300 ml of xylol and a boiling stone. The boiling flask was connected to the Aufhauser appliance and heated over an electric bath for one hour from the time it started to boil. After just one hour, the electric bath was turned off and the Aufhauser appliance was allowed to cool. The coolant was rinsed using pure xylol or toluene. The amount of water volume in the instrument was then read. The calculation of water content was done using the following formula adopted from Satrio et al. (2019):

Water content = w/v x 100%
Where:
w: weight of sample in grams
v : volume of water read on the Aufhauser device in ml

Aflatoxin test

The aflatoxin analysis method used was high-performance liquid chromatography (HPLC). Bread samples that were already in the form of a solution mixed with xylol, were homogenized using a vortex device and then injected into the HPLC device. The principle of separation of materials by HPLC was to determine the presence of aflatoxins based on the retention time and the area of the curve produced by the standard aflatoxin curve. If there was a curve that has the same retention time as the standard, then the sample contains aflatoxin. The retention time and curve width was detected using a computer that was integrated with the HPLC tool (Ambarwati et al. 2011).

Antagonistic test

The antagonist test was carried out by growing the *Aspergillus niger* fungus and other types of fungi that had been isolated in one plate. Inhibition diameter was calculated by measuring the diameter of the inhibition zone on the isolated fungi based on the results of the isolation of the fungus on bread (Nakkeeran and Zhang 2005; Khalimi and Wirya 2008). The antagonistic test was carried out by comparing the results of the inhibition zone between one fungus and another. Calculations were made by calculating the diameter of hyphae that did not grow on fungi (Pitt et al. 1992). The calculation of the inhibition of fungi was done by using the following formula:

Inhibition (%) = (a-b)/a x 100%

Where:

a: length from the center of the fungus to the edge of the fungus on the side opposite the side that is blocked.

b: length from the center of the fungus to the edge of the fungus on the inhibited side

RESULTS AND DISCUSSION

Isolation and identification of fungi on bread

Bread that has been stored for a long time, or has passed its shelf life, will have many microorganisms that appear on it. Fungi and bacteria can grow on bread that has passed its expiration date. Variable *Trichosporon*, *Saccharomyces*, *Pichia*, and *Zygosaccharomyces* are fungi that are usually found in bread. Contamination of *Saccharomyces* sp. will cause the bread to have white spots, this is called chalk bread (Saranraj 2011; Amadi and Adeniyi 2009). Similar to Saranraj (2011), *Saccharomyces cerevisiae* was found in this fungal isolation of our bread samples (Figure 1). According to dominantly fungi identification, we simultaneously found *Aspergillus tamarii* on sample RT1, *Saccharomyces cerevisiae* on sample RT2, *Cladosporium sphaerospermum* on sample RT3, *Aspergillus flavus* on sample RT4, and *Aspergillus flavus* on sample RT5 (Figure 1). Many species of *Aspergillus* were found in many foods, particularly rice, sorghum, and wheat. Traditionally, bread was made from wheat. Therefore, on seven-day shelf life of bread in this study, spoilage fungi were commonly found in samples RT1, RT4, and RT5. Surprisingly, we successfully identified *Aspergillus flavus* on sample RT4, indicating bread in seven-day shelf life has potentially mycotoxins, particularly aflatoxin. This toxin was reported to periodically affect vital organs, i.e., liver, lung, heart attack (Probst et al. 2007; Ambarwati et al. 2011). Therefore, it is necessary to study mycotoxin activity on bread to avoid the health risk of the toxin (Amadi and Adeniyi 2009). Regarding fungi identification, *Aspergillus tamarii* had white abaxial hyphae and green-yellowish spores. According to this study, *Saccharomyces cerevisiae* had a texture resembling bacteria, as it was white and did not have hyphae. *Cladosporium sphaerospermum* had greenish-white spores and grew in many colonies in RT3 samples (Figure 1). *Aspergillus flavus* had a light-yellow texture with white abaxial hyphae. The colony diameter of *Aspergillus flavus* in RT5 was relatively larger compared to RT4 (Figure 1).

In general, the fungi that appeared on the identification results of the fungi at the seven-day shelf life were *Saccharomyces cerevisiae*, *Cladosporium sphaerospermum*, *Hyphopichia burtonii*, and *Aspergillus tamarii* (Table 1). *Cladosporium sphaerospermum*, *Hyphopichia burtonii*, and *Aspergillus tamarii* included postharvest destructive fungi (Amadi and Adeniyi 2009). Postharvest destructive fungi, based on Saranraj (2011) and Ambarwati et al. (2011), was called as spoilage fungi.

Saccharomyces cerevisiae is not a destructive fungus, so there are no disadvantages with the presence of this fungus in the bread sample (Ahmad 2005). In bread RT3, the fungus that grew was more varied. The most dominant fungus in the bread RT3 was *Saccharomyces cerevisiae*. In bread RT5, the dominant growing fungus was *Saccharomyces cerevisiae*.

Many of the post-harvest destructive eukaryotes-fungi (spoilage fungi) grew in all samples, except for RT5. It was periodically caused by the differences in water content among samples (Saranraj 2011; Ambarwati et al. 2011). Therefore, it is important to check the water content in each sample.

The dominant spoilage fungi in the seven-day bread sample was *Cladosporium sphaerospermum*. In our hypothesize, the growth of the fungus can be inhibited with *Aspergillus niger*. Therefore, it is necessary to have an antagonist test to determine the inhibitory level between *Aspergillus niger* and the postharvest fungal *Cladosporium sphaerospermum*, and other fungal spoilage from bread samples stored for seven days after expiration.

In general, the most common fungi that appear in the results of the experiment to identify fungi at a shelf-life of thirty days are *Saccharomyces cerevisiae*, *Cladosporium sphaerospermum, Aspergillus flavus, Eurotium chevalieri*, and *Aspergillus tamarii*. The most important thing that was obtained from the experimental results on the bread with a shelf life of thirty days, was the appearance of the *Aspergillus flavus*. This fungus is an aflatoxin-producing fungus which is very dangerous for the body and can cause chronic disease (Probst et al. 2007; Ambarwati et al. 2011).

The species of fungi were isolated and identified from bread stored for seven days with thirty different days. The diversity of destroying fungi was more dominant in the bread samples that were stored for seven days. This could have happened because of the moisture of the bread that was seven days old was higher. However, the type of fungi isolated from bread in thirty days old had more potential to produce aflatoxins. It was indicated by the growth of *Aspergillus flavus* in thirty-day-old bread samples. In addition, bread RT3, RT4, RT5 had a wider variety of spoilage fungi than bread accession RT1, and RT2 (Table 1,2). This can be demonstrated in either a seven-day or thirty-day sample of bread (Table 1, 2).

Water content analysis

The highest water content in the samples stored for seven days belonged to bread RT2. While the lowest water content was owned by bread accession RT5. In general, the water content of RT1 and RT2 was higher than that of bread accessions RT3, RT4, and RT5 (Table 3).

Table 1. Fungi identification of bread with a shelf life of seven days.

Sample code	Sample weight (g)	Species	Number of colonies – Replication 1	2	3	1/dilution factor	Fungi population (colony / g wet weight)
RT 1	25.02	*Aspergillus tamarii*	4	1	2	10^4	1.30×10^4
		Hyphopichia burtonii	6	7	7	10^4	6.67×10^4
		Saccharomyces cerevisiae	70	69	-	10^5	6.95×10^6
RT 2	25.00	*Hyphopichia burtonii*	14	21	17	10^5	1.73×10^6
		Paecilomyces variotii	4	4	6	10^5	4.67×10^5
		Saccharomyces cerevisiae	508	644	-	10^5	5.76×10^7
RT 3	25.03	*Cladosporium sphaerospermum*	132	263	206	10^5	2.00×10^7
		Hyphopichia burtonii	9	6	6	10^5	7.00×10^5
		Paecilomyces variotii	14	10	7	10^2	1.03×10^3
		Saccharomyces cerevisiae	184	240	-	10^5	2.12×10^7
RT 4	25.03	*Hyphopichia burtonii*	16	18	24	10^3	1.93×10^4
		Saccharomyces cerevisiae	400	460	-	10^5	4.30×10^7
RT 5	25.00	*Saccharomyces cerevisiae*	-	448	416	10^5	4.32×10^7

Table 2. Fungi identification of bread with a shelf life of thirty days.

Sample code	Sample weight (g)	Species	Number of colonies – Replication 1	2	3	1/dilution factor	Fungi population (colony / g wet weight)
RT 1	19.00	*Aspergillus tamarii*	10	4	9	10^4	7.67×10^4
		Cladosporium sphaerospermum	20	22	23	10^4	2.17×10^4
		Saccharomyces cerevisiae	4	2	-	10^4	3.00×10^4
RT 2	19.00	*Saccharomyces cerevisiae*	20	11	11	10^5	1.40×10^6
RT 3	25.00	*Aspergillus flavus*	3	5	6	10^2	4.67×10^2
		Cladosporium sphaerospermum	30	28	22	10^5	2.67×10^6
		Eurotium chevalieri	14	10	12	10^4	1.20×10^5
		Saccharomyces cerevisiae	41	41	-	10^6	4.10×10^7
RT 4	25.03	*Aspergillus flavus*	1	5	3	10^5	3.00×10^5
RT 5	25.01	*Aspergillus flavus*	2	3	4	10^3	3.00×10^3
		Saccharomyces cerevisiae	324	392	-	10^3	3.58×10^5

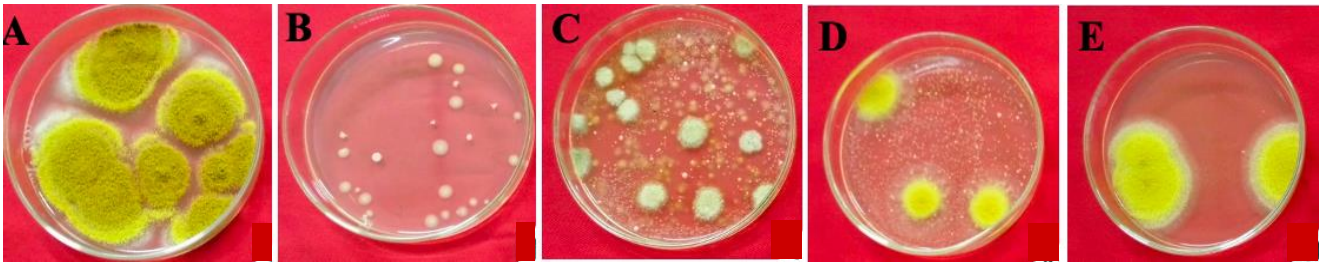

Figure 1. Isolation of the bread's fungus; *Aspergillus tamarii* on plain bread 1 (RT1) (A); *Saccharomyces cerevisiae* on plain bread 2 (RT2) (B); *Cladosporium sphaerospermum* on plain bread 3 (RT3) (C); *Aspergillus flavus* on plain bread 4 (RT4) (D); *Aspergillus flavus* on plain bread 5 (RT5) (E).

Table 3. Water content analysis of bread samples at seven and thirty days after storage.

Number	Bread Samples	Shelf life (days)	Water Content (%)	Method
1	RT 1	7	39,86	Gravimetry
2	RT 2	7	40,77	Gravimetry
3	RT 3	7	25,13	Gravimetry
4	RT 4	7	29,20	Gravimetry
5	RT 5	7	21,21	Gravimetry
6	RT 1	30	37,44	Gravimetry
7	RT 2	30	37,29	Gravimetry
8	RT 3	30	21,15	Gravimetry
9	RT 4	30	21,59	Gravimetry
10	RT 5	30	23,15	Gravimetry

Table 4. Biotoxicology test of bread samples based on aflatoxin B1, B2, G1, and G2.

Number	Bread's Samples	Aflatoxin content (ppb)			
		B1	B2	G1	G2
1	RT 1	< 1	< 2	< 1	< 2
2	RT 2	< 1	< 2	< 1	< 2
3	RT 3	< 1	< 2	< 1	< 2
4	RT 4	< 1	< 2	< 1	< 2
5	RT 5	< 1	< 2	< 1	< 2

The highest water content in the samples stored for seven days was bread RT2. While the lowest water content was in bread RT5. In general, the water content of fresh bread was higher than that of sandwiches. The moisture content between samples stored for seven days and samples stored for thirty days was relatively different. The moisture content of the samples at the shelf life of thirty days was lower than the moisture content of the samples at the shelf life of seven days. This is because the water vapor pressure in bread food is higher than the water vapor pressure in the environment, so the water in food will move outside the environment so that the water content will decrease further. The longer the storage period, the moisture content in the bread will decrease until at a certain point the water vapor pressure in the bread is equal to the water vapor pressure in the outer environment (Gibson et al. 1994; Addy 2007).

The water content suitable for the growth of *Aspergillus flavus* in the samples at the shelf life of seven days and the samples at the shelf life of thirty days, is owned by the sandwich samples. However, the fungus *Aspergillus flavus* only grew on samples stored for thirty days. This difference is caused by the growth period of the *Aspergillus flavus* fungus which takes a long time (Hedayati et al. 2007; Ambarwati et al. 2011).

Aflatoxin and antagonistic test of spoilage fungi to *A.niger*

The results of the aflatoxin test research on bread had the same levels for different types of aflatoxins. For aflatoxin B1 and G1 toxins, the aflatoxin content is lower than 1 ppb. Whereas aflatoxin B2 and G2, the aflatoxin content is lower than 2 ppb. The aflatoxin content was the same for all bread samples. The aflatoxin content in the sample bread was still below the recommended standard. Low aflatoxin content has shown to not cause toxins for humans (Brown et al. 1999; Ambarwati et al. 2011).

The results of the antagonist test showed that fungus was inhibited by *Aspergillus niger*. The type of fungus with the highest inhibition value was *Hyphopichia burtonii*, while the lowest was *Saccharomyces cerevisiae* (Table 6). Thus, *Aspergillus niger* has the potential to be used to inhibit destructive fungi in bread food.

A mycotoxin is a toxin that comes from fungi, one of which can be in the form of aflatoxins. Aflatoxins synthesized by *A. flavus* have four types of toxins, namely aflatoxins B1, B2, G1, and G2. The four types of aflatoxins are reported to be found in several foods such as '*gado-gado'*, *'karedok'*, *'ketoprak'* and in nutmeg and rice (Ambarwati et al. 2011). In this study, we confirmed that *A. flavus* can also be found in expired bread. On the other hand, the use of *A. niger* as a biocontrol agent can have a significant effect on some staple foods such as grain and nut foods. Therefore, antagonistic testing is necessary to understand the mechanism of biocontrol agent in various fungi species. According to the antagonistic test, *A.niger* effectively inhibits *Hyphopichia burtonii*, *Paecilomyces variotii*, *Aspergillus tamari*, *Aspergillus flavus*, and *Eurotium chevalieri*.

Table 5. Antagonistic analysis of spoilage fungi to *A.niger* as biocontrol.

Number	Species of spoilage fungi	*A. niger* inhibition (%)	Spoilage fungi inhibition (%)	Total inhibition (%)
1	*Aspergillus flavus*	0.00	51.23	51.23
2	*Aspergillus tamari*	7.96	59.44	51.48
3	*Paecilomyces variotii*	0.00	60.00	60.00
4	*Saccharomyces cerevisiae*	0.00	34.72	34.72
5	*Hyphopichia burtonii*	0.00	70.02	70.02
6	*Eurotium chevalieri*	0.00	45.15	45.15
7	*Cladosporium sphaeospermum*	0.00	nd	nd

Note: nd was similar to "not determined"

In conclusion, *A. niger* can be used as a biocontrol agent against various spoilage fungi with broad species inhibition on bread. The species of fungus with the highest inhibition value was *Hyphopichia burtonii*, while the lowest was *Saccharomyces cerevisiae*. In future studies, this finding can provide preliminary information for the metabolomics approach to understand many novel compounds in *A. niger* against spoilage fungi in the cell level in various foods.

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