Research Article

The Effect of Yeast Antagonist Isolated from the Fermentation of Cocoa Beans (Theobroma cacao) from Lampung, Indonesia, on the Growth of Aspergillus flavus UNJCC F-55

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Abstract.

Mold is one of the microorganisms that causes damage to cocoa fruit because it produces mycotoxins as secondary metabolites that can decrease the crop yield. One type of pathogenic mold that destroys cocoa fruit is *Aspergillus flavus*. *A. flavus* produces aflatoxin B1 which is the most potent hepatotoxin and carcinogen to humans. Amongst the most effective ways to avoid alpha-toxin contamination in cocoa beans is to inhibit the growth of *A. flavus* in the beans. However, the use of pesticides and fungicides can increase the development of resistant strains of fungi and have a negative impact on the environment and human health. Therefore, we need other alternatives such as bio-control agents using antagonistic microorganisms, including yeasts. Thus, this study aims to characterize yeasts isolated from the fermented cocoa beans (*Theobroma cacao*) from Lampung, Indonesia, and evaluate their ability to inhibit the growth of *Aspergillus flavus* UNJCCF-55. The methods used were yeast screening, dual culture technique for antagonistic test, and morphological characterization. The 37° C-growth screening gave 89 out of 98 yeast isolates. The subsequent antagonist test resulted in 13 isolates with the highest inhibition zone against *A. flavus* UNJCCF-55. These yeast isolates were macroscopically of smooth surface, butyrous texture, milky white color, irregular edges, and convex elevation. Microscopic observation showed that the isolates have oval cell shape, asexual reproduction of budding, and non-hyphae structure.

OPEN ACCESS

Keywords: yeast antagonist, cocoa fermentation, aspergillus flavus

1. INTRODUCTION

Chocolate production in Indonesia is mostly done by smallholder plantations as a source of community income in several areas such as in Maluku, South Sulawesi, East Kalimantan, Irian Jaya, and Lampung. This business sector has become one of the export commodities to various countries including the Netherlands, Germany, the United States, and Singapore to increase the country's foreign exchange. Cocoa has been a featured crop that is used as a source of community income, especially in one of province in Indonesia, Lampung. Lampung ranks third as the largest area of cocoa plantations on the island of Sumatra [1].

Cocoa bean fermentation is a very important step to improve the quality of cocoa beans. Chocolate fermentation is basically a process of overhauling sugar and citric acid in fruit flesh into organic acids carried out by the microbes including yeast [2]. *Hanseniaspora guilliermondii, Pichia kudriavzevii, Kluyveromyces marxianus*, and *Saccharomyces cerevisiae*, are known to be found during the fermentation of cocoa [2]. Physical and chemical analysis showed that fermented cocoa beans without yeast have low ethanol content, sugar concentration, low pH, and limited oxygen [3]. In addition, yeast can produce secondary metabolites that can provide benefits in controlling fungal pathogens [4].

The decline in cocoa production in several parts of Indonesia such as in Southeast Sulawesi and in the island of Java was caused by the presence of pathogenic fungus *Phytoptora palmivora* [5]. In addition to this, there are other types of pathogenic molds that cause damage to post-harvest cocoa fruit such as Aspergillus, Penicillium, Fusarium, Mucor, and Rhizopus [6]. During its growth, this type of molds produce mycotoxins and other secondary metabolites that are harmful to the human body [7]. Aflatoxins are the most common group of mycotoxins referring to the four aflatoxins B1, B2, G1 and G2 produced by *Aspergillus flavus* in food and feed ingredients [8]. Among these aflatoxins, the most important and dangerous is aflatoxin B1 since it is produced in high concentrations and identified as the most potent hepatotoxin and carcinogen [9].

The most effective way to avoid aflatoxin contamination in cocoa beans is to inhibit the growth of *A. flavus* in the beans. However, the use of pesticides and fungicides can increase the development of fungal resistant strains and have a negative impact on the environment and human health [10]. Therefore, it is required other alternatives such as bio-control agents that can inhibit the growth of *A. flavus* in cocoa beans. Among the many antagonistic microorganisms, it is known that the most effective as a bio-control agent that can inhibit the growth of *A. flavus* is yeast [11].

Yeast can be isolated from various substrates including fruit [12] as well as fermented foods and beverages [13]. Yeast is known as a non-pathogenic microorganism that is able to colonize and has cell wall components that can bind various mycotoxins [14]. Yeasts also produce secondary metabolites in the form of various enzymes such as cellulase, amylase and other enzymes [14]. Research by [15] found that *S. cerevisiae* showed to reduce mycotoxins produced by pathogenic molds by adsorption on yeast cell walls. In this study, yeast isolated from fermented cocoa beans (*Theobroma cacao*) from Lampung, Indonesia was identified and examined for its inhibition activity against the growth of *Aspergillus flavus* UNJCC F-55 collected from Universitas Negeri Jakarta Collection (UNJCC).

2. RESEARCH METHOD

2.1. Yeast Isolation from Cocoa Beans Fermentation

Isolation was carried out using the spread plate method and the serial dilution technique (10^{-3}, 10^{-4}, 10^{-5}) with two repetitions. 25 g of cocoa beans were taken and homogenized for 1 hour in PDB (Potato Dextrose Broth) medium. Yeast was isolated in YMA (Yeast Malt Agar) media. Yeast purification was carried out using the quadrant streak method on YMA media. Yeast solution from the dilution was collected to make a colony library using YMA media and is followed by purification. The purified product was incubated for 48 h at 28 ^{o}C. The pure isolate was transferred to YMA slant media and used as stock culture.

2.2. Screening Test of Isolates at the Growth Temperature of 37 ^{o}C

98 yeast isolates were collected from the isolation step to continue screening test. The screening test at 37 ^{o}C was carried out using Yeast Malt Agar (YMA) media. This test was carried out by streaking 8 quadrants on YMA media and then incubating at 37^{o}C for 24-48 h.

2.3. Antagonistic Activity Test of Yeast Isolated from Fermentation of Cocoa Beans on the Growth of Aspergillus Flavus UNJCC F-55

The yeast isolates with highest resistance index of screening at 37 ^{o}C were chosen to carry out the antagonistic activity test. This test was carried out using Potato Dextrose

Agar (PDA) media. *A. flavus* UNJCC F-55 was used as pathogenic yeast and inoculated on PDA media with an incubation time of 96 h. This test was carried out using the well method [16]. After forming a well in the middle of the PDA medium, *A. flavus* was inoculated and the yeast isolates were streaked in 8 quadrants around the hole. It was then followed by for 7 days at 30 ^{o}C to see the ability of the yeast to inhibit the growth of *Aspergillus flavus*.

2.4. Morphological Observation of Yeast Isolates

Yeast isolates with the highest inhibition rate against the growth of *A. flavus* UNJCC F-55 were observed macroscopically and microscopically based on the method of [17]. Morphological observations were carried out by growing yeast isolates on Yeast Malt Agar (YMA) medium which has been incubated for 48 h at 30 ^{o}C. Macroscopic morphological characteristics include the color of colony, colony surface, texture, elevation, and colony edge. The observed microscopic characteristics include cell shape, hyphae type and type of asexual reproduction.

3. RESULTS AND DISCUSSION

3.1. Yeast Isolation from Fermented Cocoa Beans

In this study, 98 yeast isolates from cocoa beans fermentation have been successfully isolated. Molds and yeasts are able to grow on a variety of substrates that have nutrients for growth. Cocoa beans fermentation is basically a process of reshuffling sugar and citric acid in fruit flesh into organic acids carried out by fermenting microbes [2]. Fermentation of cocoa beans in this study was carried out by a conventional method, defining as undertaking the process on sterile styrofoam for 5-7 days and observing the temperature and pH every day. Yeasts commonly found in the fermentation process are *Hanseniaspora guilliermondii, Pichia kudriavzevii, Kluyveromyces marxianus,* and *Saccharomyces cerevisiae*. During natural fermentation, without any addition of microbial starter culture, the indigenous microbes will enhance the texture and flavour development of chocolate and therefore produces good quality chocolate.

3.2. Screening Growth of Yeast at 37 ^{o}C

Based on the results of the study, there were 81 isolates that survived at 37 ^{o}C. The screening test to 37 ^{o}C was carried out with the aim of knowing the ability to grow at internal body temperature conditions. The isolates were grown on YMA media which was divided into 8 quadrants and then incubated for 48 h at 37 ^{o}C. Yeasts in the group *Kluyveromyces, Yarrowia, Debaryomyces, Saccharomyces*, and *Candida* have been known to grow at 37 ^{o}C [18].

3.3. Antagonistic Activity Test Against Aspergillus Flavus UNJCC F-55

Based on the results, it was found that each yeast isolates showed different zones of inhibition (Table 1) and the inhibition rate ranges between 80.52%-85.50%.

TABLE 1: Inhibitory zone of yeast isolates against the growth of *a. flavus* UNJCC F-55 with the incubation time of 96 h at 37 ^{o}C on PDA medium.

Isolate Codes	Growth Observation	Radius Zone of Inhibition (mm)	Inhibition Rate (%)
C1.-4.4	++++	7.13	80.75
C1.-4.11	+++	7.11	80.52
C1.-4.17	++++	7.36	83.35
C2.-3.6	**++++**	**7.55**	**85.50**
C2. -3.11	++++	7.35	83.24
C3. -5.1	++++	7.33	83.01
C4.-3.1	**++++**	**7.52**	**85.16**
C4.-3.6	++++	7.13	80.75
C5.-2.4	++++	7.36	83.35
C5.-3.1	++++	7.46	84.48
C6.-3.1	++++	7.39	83.69
C6.-3.2	**++++**	**7.73**	**87.54**
C6.-3.5	++++	7.15	80.97

Screening was carried out to determine the antagonistic activity of the yeast isolated from fermented cocoa beans from Lampung on the growth of *Aspergillus flavus* UNJCC F-50 using Potato Dextrose Agar (PDA) media which is known to be suitable for optimal aflatoxin growth since the media contains glucose needed for the growth of *A. flavus*. There were 13 isolates known to inhibit the growth of *Aspergillus flavus* UNJCC F-50 (Table 1). The ability of yeast to inhibit the growth of *A. flavus* was indicated by the presence of a clear zone formed between the mold and the yeast. It is known that yeasts can also potentially inhibit *A. flavus* sporulation.

The yeast isolate with code C6.-3.2 showed the highest antagonistic activity, namely 87.54% based on the diameter of the clear zone formed. The ability of yeast to inhibit the growth of pathogenic microorganisms can be indicated by a decrease in mycelium growth, a decrease in the sporulation process, and by the presence of a clear zone formed. In addition, the clear zone is formed due to the presence of secondary metabolites produced by fungi and is known as an antibiosis mechanism.

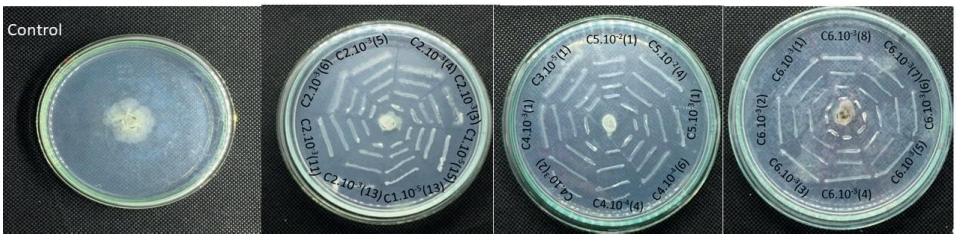

Figure 1: Antagonistic activity of yeast isolated from cocoa beans fermentation against the growth of *aspergillus flavus* UNJCC F-50 based on its inhibition zone, incubated at 30 ^{o}C, 96 h, on PDA medium.

Several studies noted the antagonistic effect of *S. cerevisiae* against *A. flavus* [19][20]. The antagonistic activity of *S. cerevisiae* may be related to the competition for nutrients and space, direct contact, production of hydrolytic enzymes [21]. Previous research has shown that yeast has antagonistic properties against the fungus *Aspergillus flavus* which has mycoparasite abilities [22]. The ability of mycoparasites occurs due to the stimulation of chemical compounds released by *A. flavus,* and yeasts have a chemotropic response to these stimuli. Previous research said that *A. flavus* experienced growth inhibition due to the presence of secondary metabolites, such as harzianolide and butenolide.

This compound can be produced by *Trichoderma harzianum* as well which inhibits almost 90% of the growth of *A. flavus* [22]. This mechanism has a degradation effect on dioctyl phthalate, methyl jasmonate, butabarbitol, and cyclopentanyone present in the mycelium of *A. flavus*. This degradation causes the release of the cyclopentane ring from aflatoxins [22]. Inhibition zones can also be formed by space and nutrient competition between molds and yeasts [23]. The ability of yeast to inhibit pathogenic molds indicates an antibiotic mechanism. Yeast produces organic metabolites that can inhibit the mycelium growth process [4].

Morphological characterization was carried out to determine the macroscopic and microscopic characteristics of the yeast which were potential inhibitors of *A. flavus*. The results showed that 3 yeast isolates has the highest inhibition rate, namely isolate C2.10^{-3}.6 (85.50%), C4.10^{-3}.1 (85.16%), and C6.10^{-3}.2 (87.54%).

Macroscopic observations on isolates C2.10^{-3}.6 are known to be milky white in color with a smooth surface and have a butyrous texture, while microscopic observations

Table 2: Macroscopic and microscopic morphological observation results of yeast isolates with the highest inhibition rate against the growth of *a. flavus* UNJCC F-50, incubated at 28^{o}C, 48 h, on YMA medium.

Isolate Codes	Surface	Texture	Colony color	Colony edge	Elevation	Cell Shape	Budding
C2.10^{-3}.6	Smooth	Butyrous	Milky White	Notched	Convex	Oval	Monopolar
C4.10^{-3}.1	Smooth	Butyrous	Milky White	Irregular	Convex	Cylinder	Monopolar
C6.10^{-3}.2	Smooth	Butyrous	Milky White	Irregular	Convex	Oval	Monopolar

showed that the edges of the colonies on isolates C2.10^{-3}.6 are grooved, have convex elevations and have oval-shaped cells with monopolar budding. Macroscopic observations of isolate C4.10^{-3}.1 has milky white colonies with a smooth surface and a butyrous texture, microscopic observations showed that the edges of the colonies were irregular, has convex elevations and has cylindrical cells, and monopolar budding. The results of macroscopic observations on isolate C6.10^{-3}.2 has a smooth surface, has a butyrous texture, and are milky white in color. Microscopic observations showed that the colony edges were irregular, has a convex elevation, has cells with an oval shape, and has monopolar budding.

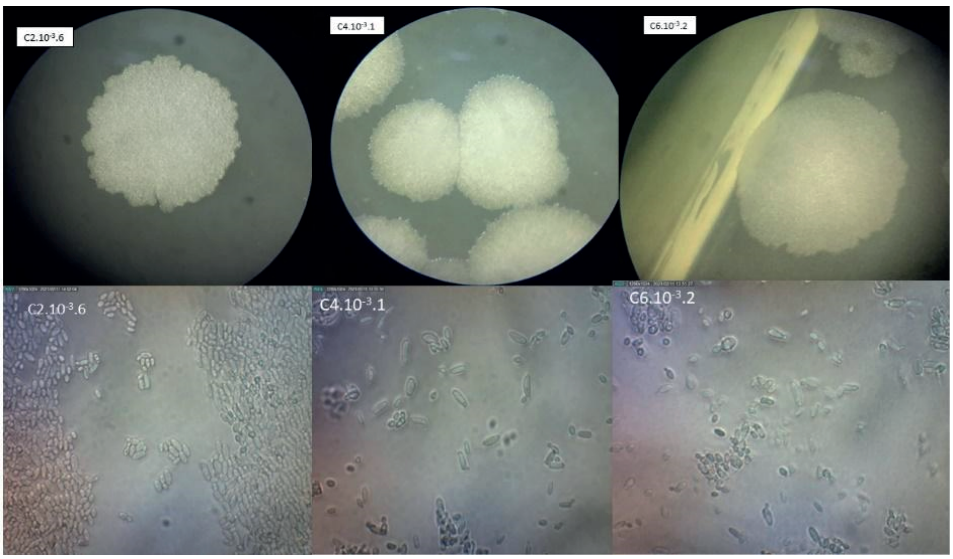

Figure 2: Macroscopic and microscopic morphological observation resulted from isolate C2.10^{-3}.6 (a). C4.10^{-3}.1 (b). and C6.10^{-3}.2 (c). incubated at 28^{o}C, 48 h, on YMA medium.

4. CONCLUSION

This study, it can be concluded that 98 isolates have been isolated from fermented cocoa beans from Lampung. There were 81 yeast isolates from fermented chocolate fruit that were able to survive and grow at 37 ^{o}C. The results showed that 3 isolates with the highest inhibition rate namely isolates C2.10^{-3}.6 (85.50%), C4.10^{-3}.1 (85.16%), and C6.10^{-3}.2 (87.54%). The results of macroscopic observations showed that isolates C2.-3.6, C4-3.1, and C6.-3.3 has the same colony color of milky white, with a smooth colony surface and butyrous texture. The microscopic observations showed different characteristics of each isolates, exhibiting the different types of yeast. The three isolates will be further investigated in the laboratory to determining the molecular characteristics. This will provide the latest information that yeast originating from cocoa beans fermentation can be used as a growth inhibitor of the mold *Aspergillus flavus*.

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