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Research Article

Antagonistic Activity of Cellulase-Producing Yeasts Isolated from Cocoa Fermentation Against Pathogenic Molds Collected from Damaged Cocoa Fruits

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Abstract.
The presence of pathogenic molds in post-harvested cocoa beans poses a significant problem to most Indonesian farmers. Pathogenic molds secrete phytopathogenic toxins which cause rotting, seriously damaging the cocoa fruits. The common pathogenic molds found in damaged cocoa beans are *Phytophthora palmivora* and *Phytophthora megakarya*. These molds are so far controlled by using chemical pesticides, whose long-term use is potentially harmful to human health and the environment. Thus, a healthier and safer alternative is needed, especially using biological agents such as yeasts. Some yeasts produce cellulose-degrading enzymes that can possibly break down the fungal cell wall which is composed mostly of the polysaccharide's chitin and glucan. In this study, yeasts were isolated during the cocoa fermentation process, followed by screening for cellulolytic activity using direct planting and paste method, and subsequent antagonistic testing by double culture technique. Out of 128 yeast isolates, 77 possessed cellulase activity, with 6 of them having the highest activity index. Antagonistic activity test of these 6 isolates (C1.0.4, C1.1.3, C2.3.10, C2.3.14, C3.5.11, and C3.3.1) against the pathogenic molds resulted in isolate C3.3.1 showing the highest inhibition percentage from the 2nd to 8th day of incubation.

Keywords: antagonistic, cellulase, yeast, cocoa fermentation, pathogenic molds, cocoa fruits

1. INTRODUCTION

Cocoa bean fermentation is a spontaneous process driven by an ordered microorganisms from a wide range of yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB), some aerobic spore forming bacteria and various species of filamentous fungi [1]. The process of cocoa beans fermentation is a very important step for developing chocolate flavor precursors which contributes to the metabolism of microorganisms involved. During the fermentation, microbes use glucoses and polysaccharides present in the seed flesh of cocoa fruits to produce secondary metabolites which are able to initiate biochemical reactions as precursors of chocolate flavor (Schwan and Wheals, 2004) [2]. Yeasts colonies first grow, followed by Lactic Acid Bacteria (LAB), Acetic Acid Bacteria (AAB) and finally if the fermentation continues, molds will appear [3, 4]. The microbial diversity and time consumption during fermentation differ according to the cocoa origin countries [4].

Yeasts are mainly responsible for the initiation of cocoa fermentation. Research by [5] showed that yeasts plays a role in ethanol production from glucose during the early stages of the fermentation, when the cocoa pulp-bean mass is anaerobic with low pH and temperature. Yeasts are also the major contributors to pectinolysis for pulp removal, enabling air ingress in the cocoa pulp-bean mass [2]. *Saccharomyces cerevisiae* is the most often detected species and also the most abundant species during cocoa bean fermentations, followed by *Kluyveromyces marxianus, Pichia kudriavzevii* and *Pichia membranifaciens*, *Candida* sp., commonly after an initial fermentation stage dominated frequently by *Hanseniaspora guilliermondii* [4].

The chocolate production, contamination by mold pathogen commonly occurs mostly during post-harvest operations: harvesting, pod-breaking, fermentation, drying, storage, and transportation. The most common types of mycotoxins found in cocoa beans are ochratoxin and aflatoxin produced by *Aspergillus* sp. and *Penicillium* sp. [6]. The consumption of these ochratoxin contaminated foods may lead to human and animal diseases. The previous studies have demonstrated that ochratoxin is a carcinogenic, nephrotoxic, teratogenic, immunotoxic, and hepatotoxic compound in various types of human and animal [4].

Phytophthora is one of the Plant Pest Organisms (OPT) which is dangerous and pathogenic for plantation and agricultural commodities in Indonesia [7]. Phytophthora mold is a type of mold that causes black pod disease (fruit rot) in cocoa (*T. cacao* L.) [8]. In Southeast Sulawesi, this pathogen was reported to reduce cocoa production by 52.99% [9], while in Java the present of this species can reduce the production by 50%

[10]. Apart from *P. palmivora* which causes fruit rot in cocoa, it is estimated that there are other types of fungi, namely *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp., *Rhizopus* sp., and *Mucor* sp. [11].

Currently, the control of pathogenic molds in chocolate production uses pesticides. However, the use of these pesticides is harmful to humans, animals, and ecosystems [12]. Therefore, a biocontrol agent is needed to control pathogenic molds during the chocolate production. One of the biocontrol agents that can be used to control fungal pathogens is yeast. Yeast is able to grow quickly and adapt well to a variety of substrates [13]. The research of Madigan proved that yeast has the antagonistic activity against mold pathogen and is often used as a biopreservation agent in post-harvest fruit [14]. Yeasts are able to produce a barrier that inhibits the growth of other microorganisms [15]. Yeasts has potential as antagonist agent that reduces the growth of the mycelium of molds by competition for nutrients and habitat [16]. Research by [17] stated that the yeast *Rhodotorula* sp. showed its potential to inhibit anthracnose disease by *Colletotrichum* spp. on chili and strawberry and yeast *Metschnikovia* sp. potentially inhibit intercrosses in chickpeas. Futhermore, [18] found that the yeast *Aureobasidium melanogenum* can inhibit the growth of *Aspergillus flavus*. The main objective of this study was to evaluate the antagonistic activity of cellulose-producing yeasts isolated from cocoa fermentation against mold pathogen collected from the damaged cocoa fruits in Sentul, West Java, Indonesia. Yeasts isolated from cocoa fermentation was tested to determine its potential to produce cellulose and its ability to control the growth of pathogenic molds in cocoa fruit.

2. MATERIAL AND METHODS

2.1. Sample Collection

This research was conducted at the Microbiology Laboratory of the Faculty of Mathematics and Natural Sciences, Jakarta State University from November 2019 to January 2020. The samples of pathogenic molds came from cocoa fruit from the Sentul Bogor cocoa plantation and yeast isolates from cocoa fermentation from the Sentul Bogor cocoa plantation. The cocoa fruit with rotten symptoms were collected from the Sentul cocoa plantation, Bogor. The mold was isolated using the direct and paste planting method based on [19] with modifications.

Cocoa fruit was first sterilized using a 0.5% sodium hypochlorite (NaOCl) solution for one minute, then soaked in 70% alcohol for one minute and rinsed with sterile distilled

water. Direct planting method is done by cutting the fruit on the damaged part at the tip and base of the fruit with a size of ± 0.5 cm. The paste method is done by taking pieces of PDA medium with a size of ± 0.5 cm and attaching them to the damaged cocoa fruit. Fruit pieces and the agar were grown on PDA medium. Each petri dish was covered with 2 pieces of fruit or agar and incubated for 24 hours at 29°C. The growing mold isolates were purified by the hyphal tips to obtain single colonies and transferred to PDA medium. The pure isolate was transferred to a PDA slant and was used as a stock culture.

Yeast isolates were obtained from the cocoa fermentation every 24h from the first day of fermentation to the sixth day. Isolation was carried out using the spread plate method and the dilution technique with two repetitions. Yeast was isolated in Yeast Malt Agar (YMA) medium. Yeast purification was carried out using the quadrant streak method in Malt Extract Agar (MEA) media based on [20]. The diluted yeast was carried out by the colony library using YMA medium and then purified. The purified products were incubated for 48 hours at 29°C. The pure isolate was transferred to YMA slant medium and used as stock culture.

2.2. Determination of Cellulose Activity by Yeast Isolates

Cellulose activity test was carried out using agar diffusion method by inoculating 20 μl of yeast suspension into the well of Carboxyl Methyl Cellulose (CMC) media with the composition of (g/L): 10 g yeast extract, 5 g peptone, 25 g agar and 18 g CMC. Incubation was carried out for 72 hours at a temperature of 30°C. At the end of the incubation period, the plates were flooded with 0.2% Congo red and waited for 15 minutes. The Congo red solution was removed slowly and a clear zone was observed. A clear halo zone around the colonies indicates the ability of an isolate to produce pectinase. The value of the enzyme activity index was determined based on [21] by calculating the ratio between the clear zone and the diameter of the colony.

2.3. Antagonistic Activity Tests by Yeast Isolates Against Phytophthora palmivora

6 yeast isolates with the highest index of cellulose enzyme activity were used in this study. The antagonistic activity test was carried out using the double culture method based on [22] with modifications. The mold isolates with the highest severity value (unpublished) were used. The mold isolates were cut into circle with a diameter of ±6

mm and the same treatment for yeast isolates with ±5 cm cut in length were placed on PDA media at the edges of different petri dishes with a distance of 3 cm. It is then incubated at 28°C for 7 days. Control treatment without antagonistic yeasts was also used in this study.

2.4. Observation of Antagonistic Activity of Yeasts Isolates Against Mold Pathogen

Macroscopic observations of molds were done based on [23]. The molds were grown on PDA media for 120 hours. Macroscopic observations of molds included: colony surface (granular, powdery, mountainous, and slippery), colony texture, zoning, growing area, radial and concentric stripes, reverse color of the colony, and exudate drops. Macroscopic observations were performed based on [24]. Yeast was grown in solid medium for 48 hours. The macroscopic observations of yeast morphology were texture, color, surface, profile, and colony edges.

3. RESULTS AND DISCUSSION

3.1. Results of Mold Isolation from the Damaged Cocoa Fruits and Yeast Isolation from the Cocoa Fermentation

Based on the results of the study, there were 36 isolates of mold isolated from the damaged cocoa fruit and 128 yeasts isolated from cocoa fermentation. Molds and yeasts are able to live in a variety of substrates that contain nutrients for growth. Previous studies demonstrated that amylolytic yeasts were found in *Artocarpus heterophyllus* Lam. [25], pathogenic molds in cloves [26], fruits [27] and fermented beverages. The presence of molds and yeasts in the cocoa fruit is due to the content of cellulose, hemicellulose, and lignin which can be used as nutrients.

3.2. Cellulolytic Activity Test Results by Yeast Isolates

A total of 77 representative isolates from 128 yeast isolates collected from cocoa fermentation were positive for cellulose. The indicator used is the presence of a clear zone of the medium after being dropped by the Congo red solution (Figure 1). Six isolates have the highest enzyme activity index which was indicated by the formation of a clear zone around the isolates (Table 1). Isolates with code C indicated that the isolates were taken from the cocoa fermentation process. The three numbers after the

letter C represent the day of fermentation, the dilution number, and the isolate number, respectively.

Congo red solutions are able to interact with polysaccharides containing β-D-glucan units in cellulose. The principle of Congo red staining is that the reagent will diffuse into the agar and be absorbed by the polysaccharide chain. The clear zone produced by each cellulolytic yeast varies because each yeast produces different cellulase enzyme complexes.

Table 1: Results of cellulose activity index value by six yeast isolates.

Yeast isolates	Diameter of yeast colony (mm) (a)	Diameter of clear zone (mm) (b)	Cellulose activity index value (a/b)
C1.0.4	6.38	2.58	0.40
C1.1.3	6.38	2.69	0.42
C2.3.10	6.38	1.90	0.30
C2.3.14	6.38	2.38	0.37
C3.5.11	6.38	1.98	0.31
C3.3.1	6.38	2.28	0.36

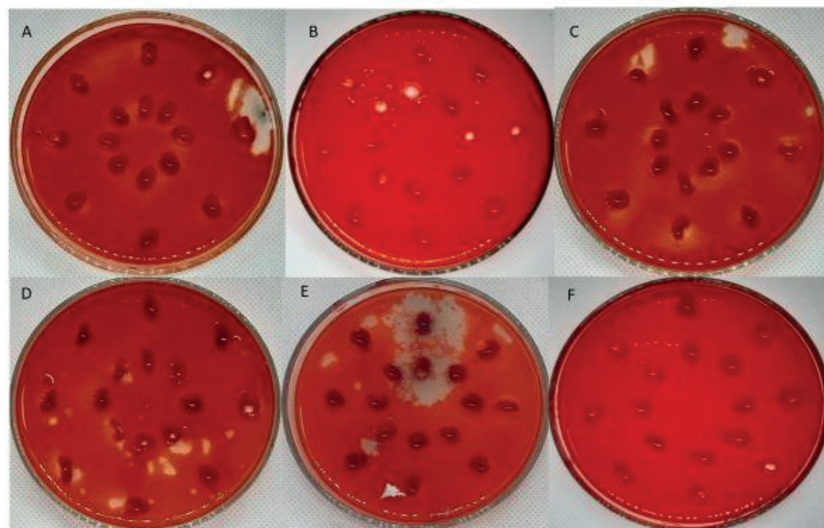

Figure 1: A clear zone was formed around the yeast colonies on CMC medium, incubated at 30°C for 72 hours. Cellulolytic yeast isolates (A) C1.0.4, (B) C1.1.3, (C) C2.3.10, (D) C2.3.14, (E) C3.5.11, and (F) C3.3.1.

The results of this study showed the presence of cellulase activity by the yeasts isolated from the cocoa fermentation. The yeast isolates have the ability to carry out the process of breaking down cellulose into a simpler structure, namely glucose. If this glucose reacts with 3,5-dinitrosalicylic acid, it will exhibit a color change from yellow to reddish brown. The difference in cellulase activity in each isolates might be caused by the specific nature of microbes in decomposing various substrate components.

The action mechanism of this decomposition has been described through several possibilities, which are (1) adhesion through cellulosome-like complexes, (2) adhesion through fibrils or villi, (3) adhesion via bacterial glycocalyx carbohydrate epitope, and (4) Adhesion via cellulose-binding domains on cellulosic enzymes. The increase in cellulase activity is balanced by the nature of microorganisms on the environment, nutrient content, temperature, pH, and the amount of substrate. In addition, the microorganisms contained in the sample were able to optimally degrade the substrate by using cellulose as the main nutrient.

3.3. Results of Antagonistic Activity by Yeast Isolates Against Mold Pathogen

Biological control is an action to reduce the activity of pathogens so that they do not cause symptoms in plants, using one or more biological control agents through environmental, host or antagonistic manipulation. The results of control showed mold growth on PDA medium containing no yeast isolates after 8 days incubation. The morphological characteristics of the control molds were cottony texture, whitish pink colony color, brownish white reverse color, and fine sporulation.

Based on the dual culture antagonistic test, it was found that six yeast isolates were able to inhibit mold growth on PDA medium for 8 days. The inhibition percentage are shown in Figure 2. The highest and lowest inhibition percentage were shown by isolates C3.3.1 and C1.0.4 with the value of 18.06% and 5%, respectively. Observations of inhibition were seen from yeast colonies on vertical lines that could not be passed by the mold isolates found on each side of the petridish. The growth of mold colonies filled the PDA media that were not inoculated with the yeast. Therefore, the distance between the yeast and mold colonies that did not unite was exhibited as the inhibition zone.

Based on the graph above, it can be seen that the highest average inhibition of yeast occurred on the eighth day. This might be caused by the extensive growth of mold in the media and that there was a direct contact between the yeast cells and the hyphae of the mold. On the second day, the inhibition of yeast was still very low because the mold was still growing quite far from the yeast cell. Isolate C3.3.1 exhibited higher inhibition rate on the fourth day of inhibition (21,1%), but right after that, the inhibition rate declined slightly on the sixth day (17,49%) and on the eight day (15,19%). Most of the yeast isolates showed their antagonistic activity on the fourth day of incubation. On the sixth day of incubation, isolate C3.3.1, C2.3.10 and C3.5.11 presented a slight

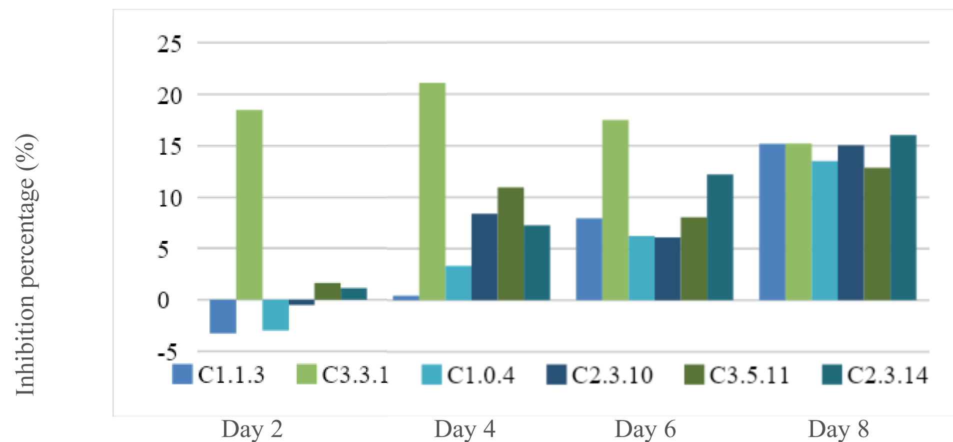

Figure 2: Results of inhibition percentage by yeast isolates against mold pathogen, observed every 2 days during 8 days of incubation on PDA medium.

decline in their antagonistic activity meanwhile the other three isolates (C1.1.3, C1.0.4, and C2.3.14) increased. This phenomenon could happen since the mold itself has a mechanism to secrete secondary metabolites in order to push its competitiveness with other microorganisms. Biocontrol agents have four mechanisms of antagonism, namely hyperparasitism, antibiosis, competition and induction of resistance. The antagonism mechanism of yeast is competition for space and nutrients, tolerance to high ethanol concentrations, and the secretion of compounds such as toxins [27].The mechanism of hyperparasitism occurs when yeast cells come into direct contact with fungal hyphae. Yeast cells will use mold hyphae as a host [29]. The test results showed that five yeast isolates had hyperparasitism abilities, namely C1.0.4, C1.1.3, C2.3.10, C2.3.14, and C3.3.1 (Figure 3).

Based on morphological observations of mold hyphae, it can be seen that yeast cells were attached to the mold hyphae, causing the hyphae to become shorter due to fractures and smaller in size. The attachment of yeast cells will block the space for the hyphae to absorb nutrients. Research by [4] stated that the attachment of the yeast *P. guilliermondii* to the hyphae of the *Penicillium expansum* can block the secretion of hydrolytic enzymes used by fungi to degrade the medium. The mechanism of hyperparasitism involves the adhesion process between biological agents and pathogens [6]. The adhesion process involves the functional proteins of antagonistic agents and pathogens in their antagonistic activity. The mechanism of hyperparasitism can also be caused by cellulolytic activity or the secretion of hydrolytic enzymes that can degrade fungal cell walls. From this study, five yeast isolates (C1.0.4, C1.1.3, C2.3.10, C2.3.14, and C3.3.1) showed hyperparasitism and cellulolytic activity. Yeast cells will produce enzymes to

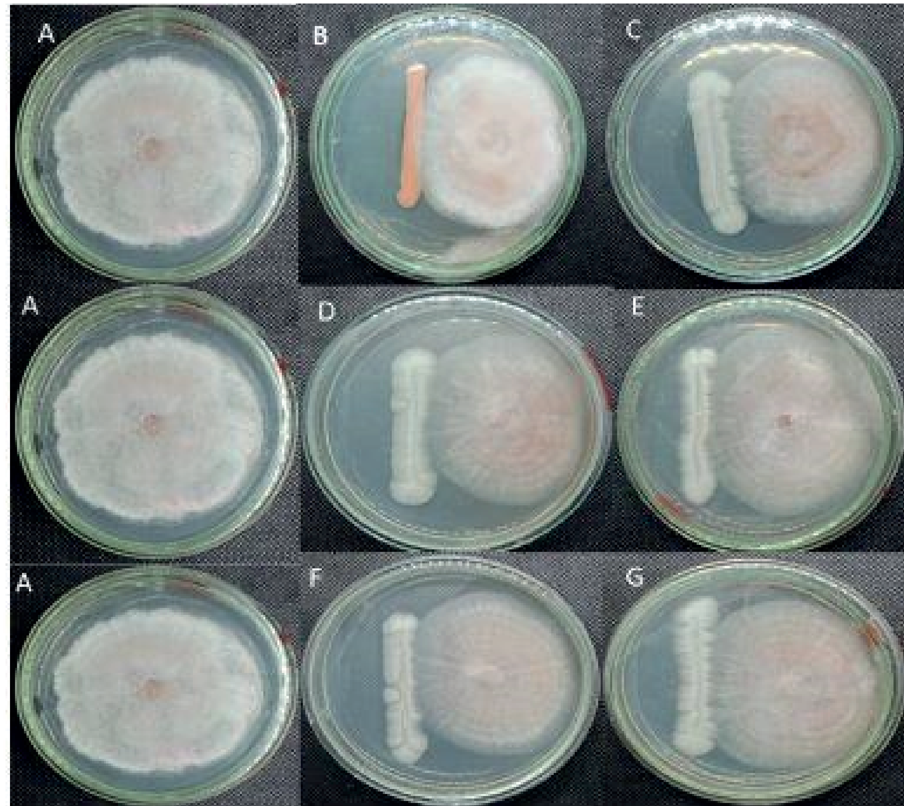

Figure 3: Results of yeast antagonistic test against mold after 8 days of incubation on PDA media. Yeast isolate (A) Control, (B) C3.5.11, (C) C2.3.10, (D) C1.1.3, (E) C1.0.4 (F) C2.3.14, (G) C3.3.1.

assist in the uptake of nutrients during the adhesion process. After secreting enzymes, yeast cells will absorb nutrients from the mold.

3.4. Morphological Characteristics of Yeast Isolates and Mold Pathogen

Based on the observations, the pathogenic mold isolates have a cottony texture morphology, brownish colony color in the middle with white edges, reverse brownish colony color in the middle with white edges and fine sporulation. The yeast isolates obtained have smooth surface characteristics (33%), rough (67%), butyrous texture (83%), mucoid (17%), white color (83%), pink (17%), wavy edges, flat, and filamentous (33%) and convex elevations (50%), raised (33%), average (17%).

4. CONCLUSION

From the isolation results obtained 128 isolates of yeast and 36 isolates of mold. After screening for cellulose enzymes activity, it was found that 77 yeast isolates were positive for cellulose activity. Six yeasts with the highest cellulose activity index namely C1.0.4, C1.1.3, C2.3.10, C2.3.14, C3.5.11, C3.3.1, were chosen as representative for antagonistic activity test against mold pathogen. The results showed that isolates C3.3.1 has the highest inhibition percentage against mold pathogen from the second day to the eighth day of incubation. Most of the isolates showed the declined after fourth day since there is a competition of nutrients between the yeast isolates and mold pathogen.

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