Cellulase-producing yeast isolated from fermented cocoa beans as biocontrol for pathogenic mold chocolate fruit collected from Sentul, Jawa Barat, Indonesia

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Abstract. Mold is one of the microorganisms that can cause damage in chocolate fruit by producing phytopathogenic toxins. Until now, pesticides have been widely used for controlling postharvest loss in fruit. Yeasts can produce secondary metabolites, which can inhibit the growth of pathogenic mold. In this study, we isolate, identify, and apply yeasts isolated from fermented cocoa beans to control the growth of pathogenic mold in chocolate fruit collected from Sentul, Indonesia. This research includes yeast isolation using the dilution method with YMA medium, mold isolation using direct planting method on PDA medium, screening ability to produce cellulase using diffusion method on CMC medium, and in-vitro antagonist testing using dual culture method on PDA medium. The results showed that 128 yeast isolates and 37 mold isolates were obtained in this study. The results of screening representative cellulase capability of 77 yeast obtained 6 positive yeast isolates produced cellulase with the highest cellulolytic index of 0.23-0.30. The 6-yeast antagonism test with the highest cellulolytic index showed that C3.3.1 isolate had the best ability to inhibit pathogenic molds with 37.36% inhibitory power.

1. Introduction

Cocoa bean fermentation is an important process in releasing seed flesh and developing chocolate flavor in chocolate production [1]. Glucose and polysaccharides in cocoa beans are fermented by microorganisms and produce metabolites that can initiate biochemical reactions as precursors of chocolate flavor [2]. Microorganisms that play a role in the process of chocolate fermentation are complex microorganisms and from various species such as yeasts, lactic acid bacteria, and molds [1].

Yeast *Hanseniaspora guilliermondii* or *Hanseniaspora opuntiae* is one of the dominant yeast in the process of chocolate fermentation besides *Saccharomyces cerevisiae, Kluyveromyces marxianus, Pichia membranifaciens, Pichia kudriavzevii*, and *Candida* sp. [3]. Research conducted by De Vuyst et al., showed that yeast initiates alcoholic fermentation in glucose in seed meat that produces ethanol [4].

The microbial concentration and fermentation time differ in many countries, according to several aspects, including the type of chocolate, social-culture, standard of procedures, technology, etc. During fermentation, the first microorganisms to appear are yeasts, followed by lactic acid bacteria (LAB), acetic acid bacteria [5]. Yeast produces secondary metabolites that can provide benefits in controlling mold pathogens [6,7]. Yeast can be found from various substrates of fermented drinks [8]; and yeast *Paphiopedilum* sp. are known to have the ability to produce amylase enzymes [9].

In cacao fruit planting, it is found that rot disease caused by pathogen *Phythopthora palmivora* categorized as a very threatening disease that can cause damage in chocolate fruit [10]. In Southeast Sulawesi, Indonesia, this pathogen is reported to reduce cocoa production by 52.99% [11], while in Java it reduced the production by 50% [12]. In addition to *P. palmivora*, there are other mold pathogens that can cause damage in post-harvest chocolate fruit such as *Aspergillus*, *Penicillium, Fusarium, Rhizopus*, and *Mucor* [13].

Currently, control of pathogenic molds in chocolate fruit uses pesticides. However, the use of pesticides is harmful to humans, animals, and ecosystems if applied in the long-term [14]. Therefore, we need an alternative agent that can control the pathogenic mold in chocolate production. Microorganisms such as yeasts are reported to have the ability to act as biocontrol agents to control pathogenic molds. Yeast can produce secondary metabolites like cellulase and other enzymes so that it can be used as a control for pathogenic molds [15]. Besides, yeasts can produce amylase, invertase and cellulase enzymes using different substrates [16].

Cellulase is a straight-chain glucose polymer with a β-1.4 glucoside bond from cellobiose (dimer from glucose) [17]. The straight chain in the glucose polymer is connected through hydrogen bonds and the Van Dew Waals force [18]. The β-1.4 glucoside bond can be broken down into glucose monomers through an enzymatic process or by means of acid hydrolysis [19]. Cellulase can be degraded by cellulase enzymes produced by microorganisms.

Apart from its cellulolytic ability, yeast is able to grow quickly and adapt well to a variety of substrates [20]. Yeast has antagonistic activity against mold pathogen and is often used as a biopreservation agent in post-harvest fruit [21]. The ability possessed by yeasts produces a barrier that inhibits the growth of other microorganisms [22]. Yeast has potential as an antagonistic agent that reduces the growth of mold pathogen by the mechanism of nutrition competition and living space [23]. Research conducted by Puspitasari et al., showed that the yeast *Rhodotorula* sp. potentially inhibit anthracnose disease caused by *Colletotrichum* spp. on chili and strawberries and yeast *Metschnikowia* sp. has the potential ability to inhibit anthracosis in beans [24]. This study was aimed to select, identify, and apply the cellulase-producing yeast from fermented cocoa beans to inhibit the growth of pathogenic mold in chocolate fruit collected from Sentul, Indonesia.

2. Methods

2.1. Yeasts isolateion from fermented cocoa beans

Yeast is isolated from cocoa bean fermentation. Isolation was carried out based on Jamili et al., with modifications using the spread plate method and dilution technique with two repetitions [25]. Brown beans are taken 25 grams and homogenized for 1 hour in PDB (Potato Dextrose Broth) media. Yeast

was isolated in the YMA (Yeast Malt Agar) medium. Yeast purification was carried out by the quadrant streak method in MEA (Malt Extract Agar) media based on Cappuccino and Sherman [26]. Yeast from the dilution results is carried out by the colony library using the YMA medium and then purified. Purification results were incubated for 48 hours at 29°C. Pure isolates were transferred in the YMA slant medium and used as stock culture.

2.2. Cellulase activity test

Cellulase test was carried out using Carboxyl Methyl Cellulose (CMC) cellulose media with the composition (g / L): 10 g yeast extract, 5 g peptone, 25 g agar and 18 g CMC. The cellulase activity test used in this study is the agar diffusion method by inoculating 20 µl of yeast suspension into the well. Incubation was carried out for 72 hours at a temperature of 300. Yeast incubation results for 72 hours at a temperature of 300 were stained by pouring congored 0.2% until the colony sank and waited for 15 minutes. The congored solution is removed slowly, then the clear zone is observed. The cellulolytic index value is determined based on Anagnostakis and Hankin [27] by calculating the ratio between the clear zone and the diameter of the colony.

2.3. In-vitro antagonistic test to mold pathogen by cellulase-producing yeasts

The yeast used in the antagonism test is the yeast cellulase test results, which have the highest index of cellulase enzyme activity, amounting to 6 pieces. The antagonism test was carried out by the dual culture method based on Hartati [28] with modifications. Pathogenic mold used is the result of pathogenicity test which has the highest severity. Pathogenic mold are placed ± 6 mm in diameter and 5 cm in yeast on PDA media at the edges of different petri dishes at a distance of 3 cm, then incubated at 28 ° C for 7 days. Media inoculated with pathogenic mold isolates without antagonistic mold act as controls. Observations include the percentage of inhibition [29]. The study design used in in vitro antagonist testing used a Completely Randomized Design (CRD). Data analysis used Analysis of Variance Analysis (ANOVA) with Duncan test with an error level of 5%.

2.4. Morphological observation of cellulase-producing yeasts and mold pathogens

Macroscopic observation of molds was carried out based on Samson et al [30]. Mold was grown on PDA media for 120 hours. Macroscopic observations of molds include colony surface (granular, such as flour, mountainous, smooth), colony texture, zoning, growing area, radial, and concentric lines, reverse colony color, and exudate drops. Macroscopic observations of the yeast are based on Kurtzman and Fell [31]. Yeast is grown in a solid medium for 48 hours. Macroscopic observations of macroscopic morphology are texture, color, surface, profile and edge of the colony.

3. Results and discussion

3.1. Yeast isolation

Based on the research results obtained 128 yeast isolates from cocoa beans fermentation. Mold and yeast are found in various substrates that contain nutrients for growth. Research conducted by Sukmawati et al., [6] found that amylolytic yeasts can be isolated from Artocarpus heterophyllus Lam., Pathogenic fungi from cloves [32], and pathogenic fungi from fruit origin [33]. The presence of mold and yeast on the cocoa is due to the presence of cellulose, hemicellulose, and lignin content in chocolate [34].

3.2. Cellulolytic activity test

A total of 77 representative isolates from 128 yeast isolates from positive brown fermentation contained cellulases. The indicator used is the presence of clear zones of the medium after being dropped by congored solutions. Six isolates had the highest enzyme activity index marked by the formation of clear zones around the isolates (Table 1). Isolates with the C code indicate the isolates were taken from the chocolate fermentation process. The three numbers behind the letter C are fermentation days, dilution numbers, and isolate numbers.

Congo red solutions can interact with polysaccharides containing β-D-glucan units in cellulase. The principle of congo red coloring is that the coloring reagents will diffuse into the agar and are absorbed by the polysaccharide chain. The clear zone produced by each cellulolytic yeast varies because each yeast produces different cellulase enzyme complexes [35].

Table 1. Measurement of cellulolytic activity index by yeast isolates.

Yeast isolates	Cellulolytic activity index (a/b)
C1.0.4	0.40±0.04[a]
C1.1.3	0.43±0.03 [a]
C2.3.10	0.31±0.02 [a]
C2.3.14	0.38±0.03 [a]
C3.5.11	0.31±0.03 [a]
C3.3.1	0.33±0.10 [a]

Cellulolytic yeast isolates (A) C1.0.4, (B) C1.1.3, (C) C2.3.10, (D) C2.3.14, (E) C3.5.11, (F) C3.3.

The research results showed that yeasts with cellulolytic activity were successfully isolated and are shown by the presence of clear zone after staining with congo red solution. The calculation results showed that the cellulase enzymes produced by six yeast isolates were not significantly different (Table 1). Classify cellulase degradation rate based on cellulolytic index values, which are low if ≤ 1, moderate between 1 and 2 and high if ≥ 2 [36]. Based on classification [36], six yeast isolates belong to the low category.

Yeast has the ability to carry out the process of breaking down cellulase into simpler structures, namely glucose. If this glucose reacts with 3,5 dinitrosalicylic acid it will show a change in color from yellow to reddish-brown. The difference in cellulase activity in each of these can be caused by the specific nature of microbes in decomposing various substrate components. This decomposition mechanism has been explained by Sadhu and Maiti that the mechanism of cellulase action in the bacterial system is through several possibilities, namely (1) [37]. Adhesion through complexes that resemble cellulosomes, (2). Adhesion through fibrils or villi, (3). Adhesion through epitope of carbohydrate glycocalyx bacteria, (4). Adhesion through the cellulose-binding domain in cellulolytic enzymes. Finally arrived at the stage of glucose release. Increased cellulase activity is offset by factors of the nature of microorganisms on the environment, nutrient content, temperature, pH, and the number of substrates [38]. Besides, due to the microorganisms contained in the sample are able to degrade the substrate optimally by using cellulose as the primary nutrient [39].

3.3. In-vitro antagonistic testing by yeasts isolates against mold pathogens

Biological control is an action to reduce the activity of pathogens so that they do not cause symptoms in plants, by using one or more biological control agents through environmental manipulation, host or antagonistic [40]. The results of observations of mold control on the PDA medium for 8 days showed mold growth (Table 2; Figure 1). Morphological characteristics of the control mold were cottony texture, whitish-pink colony, reverse color, brownish-white colony, and fine sporulation. The medium used for antagonism testing is PDA (Potato Dextrose Agar). The composition of PDAs contain starch, protein, and potassium. Starch will be degraded into simple sugars by yeast and mold [41,42].

Table 2. Measurement of the inhibition rate of yeast isolates against mold pathogens.

Isolate codes	Inhibition rate (%)			
	Day 2	Day 4	Day 6	Day 8
C1.1.3	-3,88	12,28	8,03	15,34
C3.3.1	18,54	21,70	16,79	10,19
C1.0.4	-15,99	-14,85	6,73	13,50
C2.3.10	-0,50	-6,67	6,09	16,92
C3.5.11	4,94	-13,74	20,08	12,85
C2.3.14	-22,42	-5,45	12,27	15,98

Based on antagonistic tests using dual culture method, it was found that six yeast isolates were able to inhibit mold growth on PDA media for 8 days. The percentage of inhibition is shown in Table 2. The highest and lowest inhibition rate are shown by isolates C3.3.1 and C1.0.4 with inhibition percentage of 18.06% and 5%, respectively (Figure 1).Observation of inhibition seen from yeast colonies on vertical lines cannot be passed by mold isolates found on each side of petridish. The growth of mold colonies filled the PDA media, which was not inoculated with yeast. Therefore the distance between the yeast colony and the unified mold looks like an obstacle zone. Based on the graph above it can be seen that the highest average inhibition of yeast occurs on the 8th day. This is because on the 8th day the mold has grown close to yeast so that there is direct contact between the yeast cells and hypha from the mold. On day 2, the inhibition activity of yeast is still very low because molds still grow quite far from the yeast cells. Biocontrol agents have four antagonistic mechanisms in the form of hyperparasitism, antibiosis, competition and resistance induction [43]. The antagonistic mechanism of yeast is in the form of space and nutrition competition, tolerance to high ethanol concentration, and the secretion of compounds such as toxins [44].

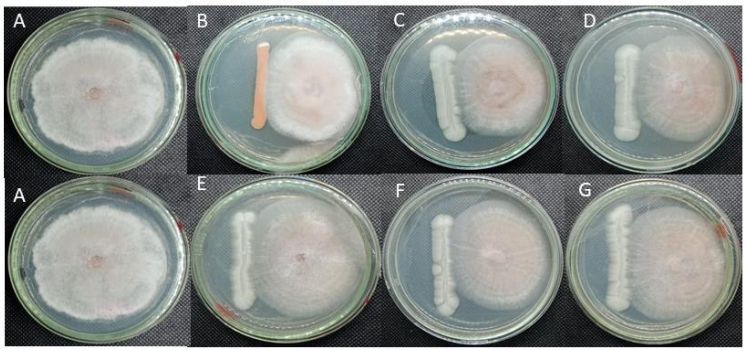

Figure 1. Results of yeast antagonists test on mold day 8. Yeast isolate (A) Control, (B) C3.5.11, (C) C2.3.10, (D) C1.1.3, (E) C1.0.4 (F) C2.3.14, (G) C3.3.1.

The mechanism of hyper parasitism occurs when yeast cells come into direct contact with mold hyphae. Yeast cells will utilize mold hyphae as hosts. The test results showed five yeast isolates can hyper parasitism, namely C1.0.4, C1.1.3, C2.3.10, C2.3.14, and C3.3.1 (Fig. 2).

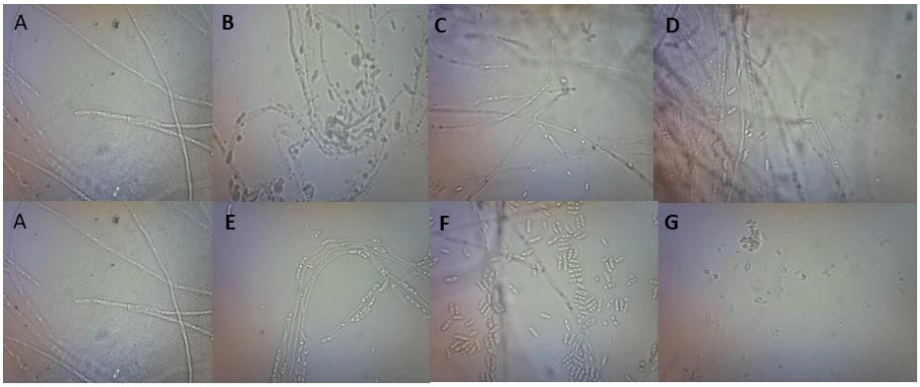

Figure 2. Morphology of hyphae of the 8th day of 400x magnification. Isolates (A) Control, (B) C1.0.4, (C) C1.1.3, (D) C2.3.10, (E) C2.3.14, (F) C3.5.11, (G) C3.3.1.

Based on morphological observations of mold hyphae, it is seen that yeast cells attach to mold hyphae, causing hyphae to become short due to fractures and smaller size (Fig. 2 (B), (C), (D), (E), (G)). The attachment of yeast cells will block space for hyphae to absorb nutrients. Research conducted by Widyastuti found that the attachment of *P. Guilliermondii* to the hypha of *Pennicillium expansum* can inhibit the secretion of hydrolytic enzymes used by molds to degrade the medium [45]. Hyperparasitism

mechanism involves the process of adhesion between biological agents and pathogens [28]. The adhesion process involves functional proteins of antagonistic agents and pathogens in their antagonistic activity [46].

The mechanism of hyperparasitism can also be caused by cellulolytic activity. Hydrolytic enzyme secretion of an antagonistic agent can degrade mold cell walls [47]. Five yeast isolates (C1.0.4, C1.1.3, C2.3.10, C2.3.14, and C3.3.1) have the ability to hyperparasitism and cellulolytic activity. Yeast cells will produce enzymes to assist in up-taking of nutrients during the adhesion process. After secreting the enzyme, yeast cells will absorb nutrients from mold.

3.4. Morphological characteristics of cellulase-producing yeasts and mold pathogens

Based on morphological observations, pathogenic mold isolates have a morphological texture of cottony, brownish colony color in the middle with white edges, reverse brownish color colonies in the middle with white edges, and fine sporulation. Yeast isolates obtained having smooth surface characteristics (33%), rough (67%), butyrous texture (83%), mucoid (17%), white color (83%), pink (17%), edge of wave, flat, and filaments (33%) and convex elevation (50%), arise (33%), flat (17%).

4. Conclusion

From this study, 128 yeast isolates were obtained from fermented cocoa beans. After screening of cellulase enzyme, 77 yeast isolates showed the presence of clear zone around the mold pathogen colony indicating that the isolates can produce cellulase enzyme. Yeast isolate with the highest cellulolytic activity index are 6 isolates (C1.0.4, C1.1.3, C2.3.10, C2.3.14, C3.5.11, C3.3.1). The results of the antagonistic test showed that isolate C3.3.1 has the most significant inhibition rate of pathogenic molds which was 37.36%.

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