Application of yeasts isolated from fermented cocoa beans for biocontrol of pathogenic mold in chocolate fruit

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Abstract. Contamination by pathogenic mold in postharvest cocoa beans becomes a significant concern by most Indonesian farmers. Pathogenic mold can cause damage to cocoa beans by such as rotting diseases in fruit. One alternative that can be used to control pathogenic mold is using biological agents such as yeasts. Some group of yeasts can produce cellulase enzyme that can degrade cellulose, and it can possibly break the cell wall with of mold which composed of semi-crystalline chitin, β-need, and cellulose. This study aims to determine the yeast originated from fermented cocoa beans which can produce cellulase enzymes and their potential ability as a biocontrol for pathogenic molds in chocolate fruit. This study includes yeast isolation from fermented beans, screening of yeast isolates that produce cellulase enzymes, and in-vitro antagonistic testing against pathogenic molds on chocolate fruit. The results showed that there were 21 yeast isolates from fermented cocoa beans, and among all, there were five isolates which can produce cellulase enzymes, namely isolate C4.-3.3, C4.-3.13, C4.-4.9, C4.-4.10, and C4.-5.9. Yeast isolate C4.-4.10 can produce cellulase enzymes with an index of 0.32 U/mL. This research showed that the 5 yeast isolates have the low category of cellulase enzyme, and further study is needed to be done to confirm their ability to act as a biocontrol agent.

1. Introduction

Indonesia is the third-largest producer of cocoa beans (*Theobroma cacao* L.) in the world. Data from the United Nations Food and Agriculture Organization (FAO) showed that Indonesia produced 574,000 tons of cocoa in 2010 [1]. Cocoa bean fermentation is a vital processing step to ensure the production of good chocolate, which has competitive flavor and texture. Cocoa bean fermentation also plays an essential role in the development of aromas and characters, as well as to reduce the bitter and unwanted taste in chocolate [2]. The process of cocoa bean fermentation generally takes place naturally by endophytic microorganisms with the intervention of environmental microbes found in soil, water, and air [3].

Yeasts are known to play an important role in producing alcohol under limited oxygen conditions and high sugar levels during cocoa bean fermentation. Yeasts can also produce enzymes like pectinase that give effects to the pulp shape. Seed death will begin a series of chemical reactions that form the color, taste, and aroma of chocolate in the cotyledons [4]. Besides, yeasts can produce amylase [5], invertase and cellulase enzymes using different substrates [6-9]. Yeasts have been also found in a variety of fermented palm wine substrates [10]; yeast from *Paphiopedilum* sp. produce amylase enzymes [11]. Yeast can improve the quality of a substrate, especially in the process of fermentation and control of other microorganisms that cause disease.

The types of damage found in cocoa beans include physical, biological, microbiological, and chemical contamination [12]. Damage to the production of cocoa beans is the presence of pathogenic fungi such as *Phytophthora palmivora* fungi [13,14]. *Phytophthora palmivora* mold, Fusarium sp, Aspergillus niger causes damage to cocoa [15]. Biological control can be an alternative to reduce the contamination by pathogenic mold in postharvest fruit. As antagonistic agents, microorganisms can control pathogenic by producing semi-crystalline chitin, β-glucan, cellulose [16], and also cellulase enzymes.

Cellulase enzymes are extracellular enzymes produced in cells and released into the growth medium. Cellulase works by hydrolyzing β-1,4-glycosidic bonds in cellulose. Cellulase enzymes are classified into three groups, which are endo-1,4-β-D-glucanase, exo-1,4-β-D-glucanase, and β-D-glucosidase. These three types of enzymes work together to hydrolyze unsoluble cellulose into glucose [17]. These enzymes degrade insoluble cellulose molecules into simpler molecules that will be used by microbes as an energy source. Cellulose degradation is the result of the synergistic action of three enzyme components [18]. The type of yeasts belonging to the genus *Cryptococcus* plays a role in cellulose metabolism because it can produce β-glycosidase [19]. Some species of molds such as *Aspergillus*, *Chaetomium*, *Fusarium*, *Penicillium*, *Rhizoctonia*, *Rhizopus,* and *Trichoderma*, have been reported having the ability to produce cellulase enzymes. *Aureobasidium pullulans*, *Aureobasidium melanogenum* and *Rhodotorula taiwanensis* from *Morinaga oleifera* leaves can control the pathogenic microbe, *Aspergillus flavus* which are capable of producing aflatoxin [20]. Therefore, this study was aimed to screen and identify yeasts isolated from fermented cocoa beans for biocontrol of pathogenic mold in chocolate fruit.

2. Methods

2.1. Yeast isolation from fermented cocoa beans

Yeast isolation was carried out using the enrichment culture method with Potato Dextrose Broth (PDB) medium [8]. A total of 20 gr of cocoa beans was put into 200 mL of PDB media, followed by homogenization using rotatory shaker at 100 rpm for 1 hour. A serial dilution of yeasts suspension was made in 10^{-3}, 10^{-4}, 10^{-5} and 0.1ml of each was inoculated into Malt Extract Agar (MEA) medium. Each dilution was done in duplicate and was incubated for 48-72 h at 30^{o}C, parallel with purification using quadrant streak method on Yeast Malt Agar (YMA) medium.

2.2. Screening of cellulase-producing yeasts from fermented cocoa beans

A total of 21 yeast isolates has been successfully isolated from fermented cocoa beans. These isolates were then tested their ability to produce cellulase using the agar diffusion method on Carboxyl Methyl Cellulose (CMC) medium. 0.1 ml of 48 h-incubated yeast suspension with a cell density of 10^8 CFU/ml was inoculated into well in the CMC medium. Incubation was carried out for 72 h at 30°C. Positive isolates were shown by the presence of a clear zone around the yeast colony after staining with Congo red solution. The measurement of the clear zone was done to determine the potential ability of each isolate to produce cellulase enzymes [21].

2.3. In-vitro antagonistic test of cellulose-producing yeast against mold pathogen collected from Universitas Negeri Jakarta Culture Collection (UNJCC)

In-vitro antagonistic test of cellulose-producing yeast against mold pathogen was performed [22]. Pathogen mold was collected from Universitas Negeri Jakarta Culture Collection (UNJCC). This mold was isolated from chocolate fruit. A total of 0.1 ml of 48 h-old yeast suspension with a cell density of 10^8 CFU/ml and 3 days-old of the tested mold with a cell density of 10^7 CFU/ml were used in this study. The test using Potato Dextrose Agar (PDA) medium with 7-days incubation time [8-9]. Measurement of the ability was done by measuring the percentage of relative inhibition rates based on [23]. The study design used in this test is Completely Randomized Design (CRD). Data analysis was run ANOVA with Duncan test with 5% error.

3. Result and discussions

3.1. Cellulase-producing yeast isolated from fermented cocoa beans

A total of 21 yeast isolates has been isolated from fermented cocoa beans. The screening and selection were carried out using Carboxyl Methyl Cellulose (CMC) medium. Carboxyl Methyl Cellulose (CMC) solid medium contains cellulose which will be used by yeasts as the substrate for growth. Yeast that produces cellulase enzymes can react with the Carboxyl Methyl Cellulose (CMC) medium, which is characterized by a clear zone. 5 out of 21 yeast isolates were shown positive results to produce cellulase enzymes, namely isolate C4.-3.3; C4.-3.13; C4.-4.9; C4.-4.10; and C4, -5.9. Isolate C4.-4.9 showed the highest cellulolytic activity, with a value of 0.46 U/mL (Table 1).

Table 1. Calculation of cellulolytic activity index of yeast isolated from fermented cocoa beans.

Yeast Isolates	Cellulolytic Index (U/mL)
C4.-3.3	0,35 ± 0,01[a]
C4.-3.13	0,38 ± 0,01[a]
C4.-4.9	**0,46 ± 0,12[a]**
C4.-4.10	0,36 ± 0,02[a]
C4.-5.9	0,40 ± 0,01[a]

The presence of clear zone after incubation and staining of 0.3% Congo red solution to the yeast isolates will be shown if the yeast isolates can produce cellulase enzymes. The principle of this coloring is that the dye will diffuse into the agar medium and will only be absorbed by a long chain of polysaccharides that have β-D-glucan bonds [24]. The presence of a clear zone indicates the presence of cellulase enzymes. The clear zone shows the zone where the β-1,4-glycosidic that connects the D-glucose monomer at the CMC link was broken Celullase enzymes are extracellular enzymes in which microorganisms release these enzymes into the media in response to cellulose substrates existing in the medium. Extracellular enzymes have several advantages over intracellular enzymes, including being able to hydrolyze high molecular weight substrates, relatively stable, can be produced with higher purity, and are easier to be extracted. Based on the results, cellulolytic activity index by yeast isolates C4.-3.3;

C4.-3.13; C4.-4.9; C4.-4.10; and C4,-5.9 are not significantly different. Based on the value, it showed that the cellulase enzymes produced by the yeast isolates were categorized as low activity. Cellulose degradation rate based on cellulolytic index (CI) values: category low if CI ≤1; category moderate if 1≤ CI ≤ 2; and category high if CI≥2 [25].

3.2. Results of in-vitro antagonistic test of cellulose-producing yeast against mold pathogen collected from Universitas Negeri Jakarta Culture Collection (UNJCC)

Five yeast isolates with positive results were tested their ability to act against pathogenic molds. The five isolates were shown having the ability to inhibit mold pathogen in chocolate fruit (Table 2).

Table 2. Measurement of inhibition zone using dual culture diffusion method.

Isolate codes	Rate of Inhibition Zone (%)				
	Day 1	Day 2	Day 3	Day 4	Day 5
C4.-3.3	25.43	4.9	8.34	32.58	1.48
C4.-3.13	35.48	14.52	10.14	4.55	10.78
C4.-4.9	1.21	5.67	2.71	1.33	0.73
C4.-4.10	10.57	23.29	33.4	9.42	9.38
C4.-5.9	4.63	0.35	1.23	19.35	2.6

Isolate C4.-3.3 has an inhibitory rate of 25.43% on the second day; 4.9% on the 4th day; 8.34% on the 6th day; 32.58% on the 8th day; and 1.48% on the 10th day. Isolate C4.-3.13 has an inhibitory value of 35.48% on the 2nd day; 14.52% on the 4th day; 10.14% on the 6th day; 4.55% on the 8th day; and 10.78% on the 10th day. Isolate C4.-4.9 has an inhibitory value of 1.21% on the 2nd day; 5.67% on the 4th day; 2.71% on the 6th day; 1.33% on the 8th day; and 0.73% on the 10th day. Isolate C4.-4.10 has an inhibitory value of 10.57% on the 2nd day; 23.29% on the 4th day; 33.4% on the 6th day; 9.42% on the 8th day; and 9.38% on the 10th day. Isolate C4-5.9 has an inhibitory value of 4.63% on the 2nd day; 0.35% on the 4th day; 1.23% on the 6th day; 19.35% on the 8th day; and 2.6% on the 10th day.

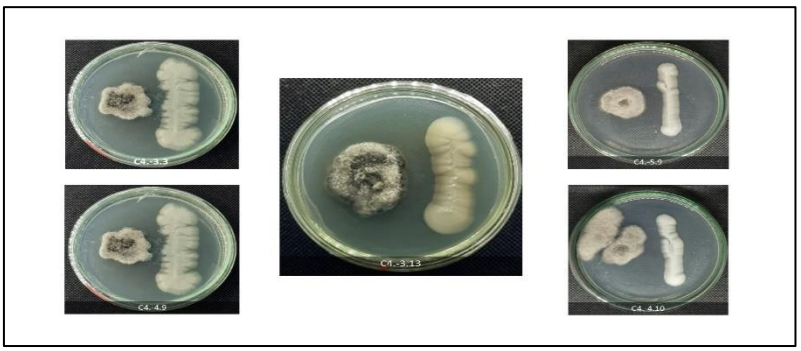

Figure 1. The figure shows the ability of yeast from fermented cocoa beans to inhibit the growth of pathogenic mold from chocolate fruit with an incubation time of 10 days at 30 ºC.

The antagonistic test of the five yeast isolates, namely C4.-3.3, C4.-3.13, C4.-4.9, C4.-4.10, and C4.-5.9 to pathogenic molds in chocolate fruit was aimed to explore the ability of yeast isolate with cellulolytic activity applied in vitro. Antagonistic ability is the ability of a microorganism to inhibit the growth of other microorganisms [26]. Meanwhile, the interactions that occur between antagonists and other organisms are called antagonisms [27].

Isolate C4.-3.13 has the highest inhibitory rate of 10.78%, which indicates that there is a competition between yeasts and molds through in substrate and the ability to form biofilm. The mechanism of the antibiosis involved secondary metabolite produced by yeasts to inhibit mold growth [28]. Space and

nutrient competition occurs when yeast and mold pathogens are grown in the medium simultaneously [29]. The ability of yeast to inhibit the growth of pathogenic molds can be used as biological agents to control the growth of pathogens. The types of biological agents that are widely developed are natural microbes [30]. Natural microbes can be found in soil, water and organic matter, as well as those that live in plant tissues (endophytes) have the property of inhibiting growth and competing in space and nutrients with pathogens target.

The low inhibition rate of yeast isolates against pathogenic molds possibly caused by the low production of cellulase enzymes produced by yeast isolates. Cellulase enzyme is one of the enzymes that can degrade the cell wall of the mold pathogen. The antagonistic mechanism between yeast and mold pathogens can happen through the production of enzymes or toxins that play a role in cell wall breaking [31]. The activity of exo-β, 1,3-glucanase can increase the toxin produced by *Pichia anomala* [32]. In the yeast antagonist test of *Pichia guilliermondii* with the pathogenic *Penicillium diginatum*, it is showed that competition occurred due to nutrition and the production of cell wall degrading enzymes [33]. The exo-β-1,3-glucanase is one of cellulase derivatives that can breakdown the cell wall of mold pathogen. With the low production of cellulase enzymes, the inhibitory rate will also be low. This is proven by the research conducted by Ezziyyani that the inhibition of pathogenic molds can be caused by the production of extracellular enzymes such as 1-3-glucanase, chitinase, protease, and cellulose which are categorized as hydrolytic enzymes [34]. The enzyme is a key tto degrade pathogen mold cell walls. This will result in the formation of holes in hypha of mold pathogens [35].

4. Conclusion

Form this study, 21 yeasts were successfully isolated from fermented cocoa beans and among all, 5 isolates namely C4.-3.3; C4.-3.13; C4.-4.9; C4.-4.10; and C4, -5.9 showed cellulolytic activity, even though categorized as low. Isolate C4.-4.9 showed the highest cellulolytic activity index of 0,46 ± 0,12 U/ml. Yeast with the highest inhibition rate is isolate C4.-3.13 on day 2 incubation with a value of 35.48%, followed by isolate C4.-4.10 on the day 6 with a value of 33.4%, isolate C4.-3.3 on the day 8 with a value of 32.58%, and isolate C4.-4.9 with a value of 0.73%.

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