Selection of Pectinolytic Yeast from Liberica Green Coffee Beans (*Coffea liberica*)

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Abstract. Liberica coffee has an inferior quality when compared to arabica and robusta coffee. This flavour can be improved through a fermentation process involving indigenous microbes, one of which is a group of pectinolytic yeasts. Therefore, this study aimed to obtain yeast isolates with pectinolytic activity. Green bean samples were used for yeast isolation using YEPD agar media. Selected yeast isolates were screened for their pectinolytic activity semi-quantitatively using the well-diffusion method and quantitatively using the *dinitrosalicylic acid* (DNS) method. A total of six yeast isolates with different colony morphological characters were obtained and referred to as isolates Y1, Y2, Y3, Y4, Y5, and Y6. The isolates suspected to produce pectinase enzyme due to a clear zone around the well are Y2, Y3, Y4, and Y5. Y5 had the highest pectinolytic index, 022 and 0,73 on the second and fifth days of incubation, respectively. The four selected isolates were then screened quantitatively and showed that Y2 had the highest pectinase enzyme activity with a value of 12,66 ± 0,17 U/mL. The results showed that four yeast isolates from Liberica green coffee beans had pectinolytic activity with the highest enzyme activity found in Y2.

1 Introduction

Liberica coffee (*Coffea liberica*) only contributes to a small portion of Indonesia's coffee production because of the taste inferiority compared to Arabica and Robusta coffee. Nevertheless, Liberica coffee has flavour uniqueness, including an aroma similar to Arabica, with a less bitter taste than Robusta coffee. Liberica coffee can also adapt to various environmental conditions, such as acidic environments, high humidity, and high-temperature climates [1]. Thus, Liberica coffee has the potential to be further explored so that its inferior taste can be overcome. The improvement of coffee quality can be achieved by choosing an appropriate post-harvest processing method through fermentation [2].

Coffee fermentation is carried out to degrade the mesocarp or mucilage layer to produce green coffee beans, which will be processed further [3]. The mucilage layer of coffee cherries is mainly composed of polysaccharides in the form of pectin that can be degraded with the

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help of the pectinase enzyme [4]. Various microorganisms known to have pectinolytic activity are lactic acid bacteria, acetic acid bacteria, filamentous fungi, and yeast [5]. *Saccharomyces*, *Pichia*, *Candida*, and *Wickerhamomyces* are the types of pectinolytic yeast often found to play a role in coffee fermentation [4,6]. Besides its pectinolytic activity, yeasts also determine the final quality of the coffee produced [7]. This is because yeasts can produce various volatile compounds that act as aroma precursors [8,9].

Even though the fermentation process will influence the final quality of the coffee, it should be noted that uncontrolled and prolonged fermentation can cause flavor defects [5]. However, this problem can be overcome by adding a starter culture [3]. One of the criteria for a coffee fermentation starter culture is the ability to produce pectinase enzyme during the fermentation process [10]. Information regarding the presence of pectinolytic yeasts from Liberica green coffee beans originating from Kalipuro, Banyuwangi, East Java is currently unknown. Therefore, this study was conducted using the culture-dependent method to obtain pectinolytic yeasts that can be developed as starter culture to create a more controlled fermentation.

2 Material and methods

2.1 Coffee fermentation

Liberica coffee cherries (26.090 kg) were harvested from the Ijen Geopark area, Kalipuro District, Banyuwangi, East Java, at the coordinate of S08º08.283'E114º19.702. Coffee cherries were washed, sorted, and pulped to separate the beans from the exo- and mesocarp layers. Pulped coffee beans (19.385 kg) were then fermented spontaneously on a container for 96 hours using the wet method by adding 9.5 L of water (2:1, m/v) and CO_2 carbonation (206 rpm). After fermentation, the coffee beans were washed and sun-dried for 14 days to reduce the water content. Afterwards, the dried coffee beans were hulled to remove the parchment layer so that the green coffee beans were obtained.

2.2 Yeast isolation

The yeast isolation was carried out by an enrichment process as an initial stage to increase yeast's density [11]. Green coffee beans (25 g) were added into 225 mL of yeast extract peptone dextrose (YEPD) medium pH 5.5 containing dextrose (20 g/L), yeast extract (5 g/L), peptone (10 g /L), agar (15 g/L), and ampicillin (0.1 g/L). The mixture was homogenized manually, then homogenized using a stomacher for 5 minutes. Then, serial dilution was carried out using 0.85% NaCl solution (9 mL) until 10^{-6}. The suspension (0.1 mL) was transferred to a petri dish, poured with the YEPD agar, and incubated at 37˚C for 72 hours. The growth of yeast colonies was observed, determined in colony-forming units (CFU), and morphologically characterized. Colonies with different morphological characters were then purified using the streak plate method and subcultured on an agar media for further use [12, 13].

2.3 Selection of pectinolytic yeast

The selection of pectinolytic yeast was carried out semi-quantitatively using a well-diffusion method. The pure isolated yeasts were inoculated into 10 mL of synthetic pectin media with the composition: MnSO4 (0.05 g/L), KH2PO4 (0.2 g/L), (NH4)2SO4 (1 g/L), CaCl2 (0.05

g/L), MgSO4 (0.8 g/L), yeast extract (1 g/L), and citrus pectin (10 g/L) [14]. Cultures were incubated for 24 hours with agitation (121 rpm) at room temperature. Each culture suspension (1.5 mL) was then centrifuged at 10.000 rpm, 4°C for 5 minutes at the end of incubation. The pellet containing yeast biomass was resuspended in a fresh medium. Pectin agar (same composition as synthetic pectin medium, but with 1.5% agar) was prepared and punched with the cork borer to make a 8 mm wells. One of the wells was filled with sterile media as a control and the others were filled with resuspension culture (0.06 mL) [15]. The plates were then incubated at 37°C for 48 and 120 hours. When the culture suspensions were completely absorbed, the pectin agar was flooded with 0.1% Lugol (potassium iodide-iodine) and rinsed with sterile distilled water. Subsequently, the clear zones that indicated an area of pectin hydrolysis were observed and measured to determine the pectinolytic index (Equation 1).

$$\textbf{Pectinolytic index} = \frac{\textbf{clear zone diameter (mm)} - \textbf{well diameter (mm)}}{\textbf{well diameter (mm)}} \qquad (1)$$

2.4 Enzyme activity assay using DNS method

Selected yeast isolates were inoculated into 10 mL of synthetic pectin medium and incubated for 72 hours with agitation (121 rpm). At the end of the incubation, the yeast cultures were centrifuged at 10.000 rpm, 4°C for 5 minutes. The supernatant containing a crude enzyme was collected and filtered using a 0.22 µm filter membrane [14].

Enzyme activity assay was carried out by preparing crude enzyme (0.4 mL) and diluted into 0.5% pectin substrate (0.8 mL) in 50 mM acetate buffer (pH 5), then kept in a water bath for 10 minutes at 55°C. Afterwards, DNS reagent (2.4 mL) was added to the reaction mixture and kept in boiling water for 10 minutes. Finally, the absorbance of the mixture that was initially cooled was measured using a UV-Vis spectrophotometer (λ= 540 nm) with D-galacturonic acid as a standard [16]. One unit of enzyme activity value (U) indicates the amount of enzyme needed to release 1 µmol of reducing sugar in every minute (Equation 2).

$$\text{Enzyme activity } \left(\frac{\text{U}}{\text{mL}}\right) = \frac{\text{C} \times \text{V1}}{\text{V2} \times 194.1 \times \text{t}} \qquad (2)$$

Where C is the concentration of *galacturonic acid* released, V1 is the total volume of solution used, V2 is the volume of the crude enzyme used, 194.1 is the molecular weight of D-galacturonic acid, and t is the reaction time in minutes.

2.5 Data analysis

Statistically significant differences in the enzymatic activities of yeast isolates were analyzed using one-way ANOVA followed by Tukey HSD test with $p<0.05$.

3 Results and discussion

3.1 Morphological characters of yeast isolates from Liberica green coffee beans

A total of six yeasts with different morphological characters were successfully isolated from Liberica green coffee beans and referred to as isolates Y1, Y2, Y3, Y4, Y5, and Y6 (Table 1). Most colony morphologies found were convex elevation, smooth consistency, opalescent optical characteristics, white pigmentation, circular or irregular shapes, and entire or

undulated margins. All yeast isolates had cells with an oval shape that appeared blue through microscopic observation with methylene blue dye.

Table 1. Morphological characters of yeast colonies obtained from Liberica green coffee beans

Morphological characters		Isolate codes					
		Y1	Y2	Y3	Y4	Y5	Y6
Shape	Circular	+	-	-	+	+	-
	Irregular	-	+	+	-	-	+
Margin	Entire	+	-	-	+	+	-
	Undulate	-	+	+	-	-	+
Elevation	Raised	-	-	-	-	+	-
	Convex	+	+	+	+	-	+
Texture	Smooth	+	+	+	+	+	+
Consistency	Viscous	+	+	+	+	+	+
Optical characteristic	Opalescence	-	+	+	+	+	-
	Glistening	+	-	-	-	-	+
Pigmentation	Cream	-	-	+	-	-	-
	White	+	+	-	+	+	+

Note : "+" = discovered, "-" = not discovered

3.2 Selection of pectinolytic yeast

Selection for the ability of yeast isolates to produce pectinase enzyme was carried out through the well-diffusion method using Lugol's solution on pectin agar media supplemented with citrus pectin as a carbon source. Lugol coloured the media brownish purple and will appear clear if there is any pectinolytic activity [17]. Among the yeast isolates, three showed pectinolytic activity after two days of incubation **(**Fig. 1). The pectinolytic index produced by isolates ranged from 0 – 0.22 and was classified as low according to Dewiyanti et al. [18]. The isolate that had the highest pectinolytic index was Y5, but there were no significant differences in value between isolates.

Fig. 1. The clear zones formed on pectin agar with Lugol's staining after two days (left) and five days of incubation (right). Note: 1) Control; 2) Y4; 3) Y5; 4) Y6

When the incubation period was extended to 5 days, 4 out of 6 isolates produced a clear zone. Besides, the pectinolytic index of these isolates tended to increase even though the values were not different significantly. The contrast increase of pectinolytic index was shown by the isolate Y5, which was initially 0.22 and then increased to 0.73. This pectinolytic index

value was the highest among other isolates but still classified as low. Daskaya-Dikmen also reported that 9 out of 16 yeast isolates produced the pectinase enzyme through the formation of a clear zone around the colony [14]. When the incubation period was extended to 12 days, the diameter of the clear zone observed increased, and the number of isolates producing the pectinase enzyme increased to 10. Therefore, incubation time is suspected to influence the formation of the clear zone. According to Haile & Kang, incubation time is one of the factors that influences pectinase enzyme production [12]. It is said that a higher pectinolytic index can be used as an indication of higher enzyme activity [18]. However, it requires further quantitative assay to exactly know the enzyme activity of each isolate.

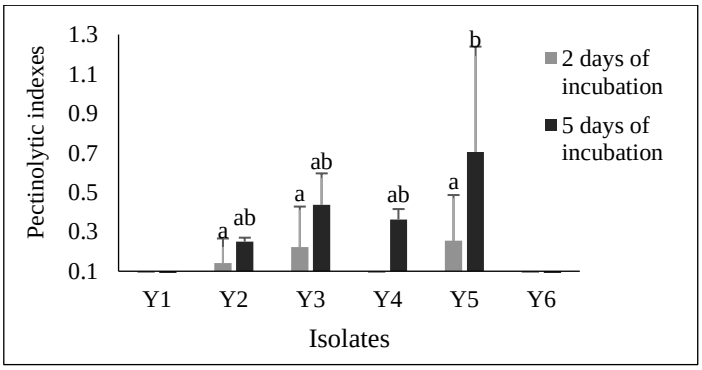

Fig. 2. Pectinolytic index of yeast isolates after two and five days of incubation

3.3 Pectinase activity of the yeast isolates

The pectinase enzyme activity assay was carried out using the DNS method to measure the concentration of reducing sugars, which are the pectin hydrolysis products of the pectinase enzyme [16]. The enzyme activity among isolates showed a value ranged from 11.77 – 12.66 U/mL. The isolate that had the highest pectinase enzyme activity was Y2, with an enzyme activity value of 12.66 ± 0.17 U/mL (Fig. 3). The differences in enzyme activity in each isolate indicate that the ability to produce the pectinase enzyme depends on the isolate type [12]. Besides being influenced by the isolate type, enzyme activity is also influenced by enzymatic reaction parameters, such as temperature and reaction time [14].

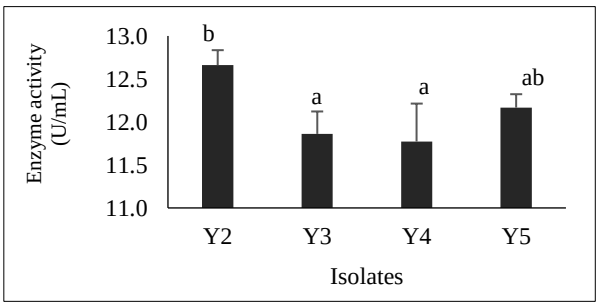

Fig. 3. Pectinase activity of yeast isolates from Liberica green coffee beans

The ability to produce pectinase enzyme during the coffee fermentation process is one of the criteria which must be fulfilled by a starter culture. The other starter culture criteria such as the microbes are able to synergize with endophytic species that already exist in the coffee substrate, able to adapt to low pH conditions, are easy to culture in large quantities for

commercial purposes, able to produce aroma precursors, and able to prevent toxin production, either by metabolite products production or nutrient competition [19]. The yeast isolates obtained in this study can be used as a coffee fermentation starter culture based on its pectinolytic activity. However, further tests still need to be carried out on the yeast isolates to fulfil other criteria for coffee fermentation starter culture.

4 Conclusion

There were six yeast isolates with different morphological characters found in Liberica green coffee beans and four of them had pectinolytic activity with the highest enzyme activity value of 12.66 ± 0.17 U/mL in isolate Y2.

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