Isolation and screening caffeine-degrading bacteria

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Abstract. Naturally, the coffee pulp contains caffeine. Caffeine-degrading bacteria can use caffeine for their growth. The purpose of this research was to obtain high potency of caffeine-degrading bacterial isolates. These bacteria were isolated from naturally-fermented pulp waste of Coffea arabica. The bacteria were isolated by using minimal medium M9 contain 1 g/L caffeine and was screened based on their activity to degrade caffeine. There 13 isolates that successfully isolated by this medium. The research found five isolates had high potency to degrade caffeine. They were KAFS 33, KAFS 34, KAFS 16, KAFS 47, and KAFS 35 respectively. Those isolates were the potential to be further analysis as an agent of decaffeinating coffee.

Keywords: caffeine-degrading bacteria, isolation, screening

1. Introduction
Indonesia has ranked fourth as the world’s biggest coffee producer [1]. This country contributes 7.3% of the world's coffee with an average production rate of 35 thousand tons each year [2]. Sempol, Bondowoso is one of the coffee production centers in East Java, Indonesia. The species that produced in this area are Coffea arabica and Coffea robusta.

There are two processing steps of commercial coffee beans are produced after the coffee beans pass through. They are the wet process and the dry process. The fruit is removed using a demucilaging machine in the wet process. This process generates a lot of pulp waste which amounted to 50%. The coffee pulp of C. arabica contains 0.26% of caffeine [3,4].

The secondary metabolite produced by some coffee plants is caffeine (C8H10N4O2). This molecule formed by purine nucleotides [5]. The existence of caffeine in the fermented coffee pulp makes it possible to obtain caffeine-degrading bacteria from those substrates. This bacteria degrade caffeine from their growth. The existence of caffeine in the fermented coffee pulp makes it possible to obtain caffeine-degrading bacteria from those substrates.

Caffeine-degrading bacteria produce demethylase or oxidative enzymes to degrade caffeine as carbon and nitrogen source for their metabolism and produce CO2 and NH3 as the final product. Those bacteria were isolated from tea plants [6] and coffee pulp [7,8]. The previous study stated that
caffeine-degrading bacteria belong to Genus of *Alcaligenes, Brevibacterium, Klebsiella, Pseudomonas, Rhodococcus, Serratia, and Stenotrophomonas* [6,7,8,9,10]. The purpose of this research to get high potency of caffeine-degrading bacteria isolates from natural fermented *Coffea arabica* pulps in Sempol, Bondowoso. Those bacteria can be utilized as a decaffeinating agent of coffee.

2. Materials and Methods

2.1 Sampling Area

The fermented coffee pulp of *Coffea arabica* were collected in Sempol, Bondowoso at 07°58’35”S 114°02’11.9”E and 901 m above the sea level. The sample 1 Kg was collected from sampling point then stored in cooling boxes and immediately brought to the laboratory for further analysis [11].

2.2 Isolation of Caffeine-Degrading Bacteria

The sample 25 was suspended into 225 mL of sterile isotonic salt solution (0.85 % NaCl) in a 500 mL Erlenmeyer flask and diluted until $10^{-5}$. The suspension was homogenized using a shaker for 15 minutes at room temperature and left for several minutes until the solid phase precipitated. The liquid phase from $10^{-3}$, $10^{-4}$, and $10^{-5}$ as much as 100 μL was spread on M9 medium (Na₂HPO₄·12H₂O 15 g/L, KH₂PO₄ 3 g/L, NaCl 0.5 g/L, MgSO₄ 0.25 g/L, NH₄Cl 1 g/L, and bacto agar 15 g/L) (Na₂HPO₄·12H₂O 15 g/L, KH₂PO₄ 3 g/L, NaCl 0.5 g/L, MgSO₄ 0.25 g/L, NH₄Cl 1 g/L, and bacto agar 15 g/L) supplied 1 g/L caffeine. It was incubated at 30°C for 24 hours [12]. The bacteria isolates that grow on this medium were isolated and were analyzed using Gram staining [13].

2.3 Screening of Caffeine-Degrading Bacteria

The screening of caffeine degrading bacteria based on caffeine degradation activity of these bacteria on M9 medium added 1 g/L caffeine for 3 days. The caffeine concentration on medium was analyzed by spectrophotometer at an absorbance of 273 nm. The caffeine degradation activity measured using the equation 1. The standard curve of caffeine concentration was made at range 0.2-2.0 g/L [14]. The data were analyzed using the Kruskal-Wallis variance and Dunn-Bonferroni with a 0.05.

$$\text{activity} = \frac{\text{initial coffee concentration} - \text{caffeine concentration after incubated}}{\text{initial coffee concentration}} \times 100\%$$  \hspace{1cm} (1)

2.4 The Pattern of Cell Numbers and Caffeine Degradation activity of Selected Bacteria

The selected bacteria isolates were inoculated into 50 mL M9 medium supplied 1 g/L caffeine as the starter culture. The bacteria culture was incubated in the shaker incubator at 120 rpm, at room temperature for 24 hours. The culture was diluted on minimal medium to obtain OD 0.4 at λ 600 nm using a spectrophotometer. Ten percent of the starter culture was inoculated into 200 mL medium and it incubated in the shaker incubator at 120 rpm, at room temperature for 60 hours. The experiment was replicated three times. Samples were taken from the culture at 12 hours intervals. An analysis was conducted on cell numbers and caffeine concentration.

The optical density (OD) of the culture suspension was measured by a spectrophotometer at the optimum wavelength (λ) of 600 nm. The cell numbers was calculated based on the previously determined value and plotted against time to conclude the growth pattern. The cell numbers (CFU/mL) were counted by a plate count at various dilutions of the culture. The generation time of each isolate was measured in the exponential phase. Caffeine degradation activity of bacteria was measured by spectrophotometer at 273 nm wavelength. The correlation between cell numbers and caffeine-degradation ability of each isolate did by bivariate correlation analysis.
3. Result and Discussion

The coffee pulp samples had been degraded for approximately 6 months. In an open space, those coffee pulp samples were stacked and left alone for months which led to naturally fermentation. Afterwards, the fermented pulp will be put back to the coffee plantation as organic fertilizer.

Based on the caffeine content from the pulp waste, caffeine-degrading bacterial isolates were obtained. Caffeine-degrading bacteria utilize caffeine as carbon and nitrogen source. This research showed that 13 bacterial isolates could grow on M9 medium added 1g/L caffeine (Table 1). There were 12 Gram-negative isolates and one isolate was Gram-positive. In some bacteria, caffeine is as an antibacterial by lysing bacterial cell walls [15]. Gram-negative bacteria are more resistant than Gram-positive [16]. The Gram-negative bacteria having a double membrane system and having a thick cell wall containing peptidoglycan, which is located between the inner membrane and the outer membrane. In this study, it was shown that the bacterial isolates obtained were not only resistant to caffeine but were also able to grow on the M9 medium added caffeine.

| No | Isolate | Gram |
|----|---------|------|
| 1  | KAFS 16 | negative |
| 2  | KAFS 21a | negative |
| 3  | KAFS 21b | negative |
| 4  | KAFS 24 | negative |
| 5  | KAFS 33 | negative |
| 6  | KAFS 34 | negative |
| 7  | KAFS 35 | negative |
| 8  | KAFS 37 | negative |
| 9  | KAFS 39 | positive |
| 10 | KAFS 41 | negative |
| 11 | KAFS 44 | negative |
| 12 | KAFS 47 | negative |
| 13 | KAFS 65 | negative |

The bacterial isolates showed a varies of caffeine degradation abilities. The result of the research showed that five isolates KAFS 16, KAFS 33, KAFS 34, KAFS 35, KAFS 47 had high potency to degradation of caffeine more than 95% for 3 days incubation in the M9 medium contain 1 g/L caffeine (Figure 1). Differences in stain from caffeine degrading bacteria isolate obtained affect the difference in caffeine degradation activity. Based on these data, then the five isolates were analyzed growth patterns and patterns of caffeine degradation. The differences in stain of isolates affect to the difference of caffeine-degradation activity. Based on these data, then the five isolates were analyzed growth patterns and patterns of caffeine degradation.

Figure 2a shows the growth curve of bacterial isolates, and Figure 2b shows the pattern of caffeine degradation on minimal media M9 added 1 g/L caffeine. Based on the figure 2a, using the same
number of bacteria cell on initial incubation, KAFS 16 isolate was grown fastest among isolates. This isolate showed an exponential phase from 0-hour to 36-hour with generation times 18.48 hour, followed by stationary phase until 60-hour. This pattern also appeared on KAFS 47, but it had generation times (19.92 our) lower than KAFS16. Isolates of KAFS 33, KAFS 34, and KAFS 35 were exhibited similar growth pattern which low growth rate until 60 hours incubation (generation time of these bacteria 136.46, 55.88, 105.93 hour respectively).

\[ \text{Figure 1. The potency of Caffeine-Degrading Bacteria on M9 medium contains 1 g/L caffeine at 3 days incubation} \]

\[ \text{Figure 2. The pattern of bacterial growth (a) and degradation of caffeine (b) of caffeine degrading bacteria in the minimum media M9 broth + 1 g/L caffeine for 60 hours incubation} \]

The growth of bacteria was followed by decreasing coffee concentration on the culture media. Decreasing of coffee concentration due to the caffeine was utilized by those bacteria to provide carbon and nitrogen for their growth. An isolate of KAFS 16 was able to degrade the caffeine on the culture
media fastest during 36 hours incubation, with followed by KAFS 47, KAFS 34, KAFS 33, and KAFS 35. Whole isolates able to degrade of caffeine in the culture media more than 90% after 48-hour and 60-hours incubation (Figure 2b). Correlation values between decreasing of coffee concentration (%) and cell numbers of KAFS 16 (0.98), KAFS 33 (0.90), KAFS 34 (0.94), KAFS 35 (0.89), and KAFS 47 (0.98). The degradation ability of those isolates were lower than KRM9 isolate, which able to degrade 99% of caffeine in the culture media M9 containing 1 g/L caffeine during 30 minutes [8], but it higher than P. putida C3024 which was able to degrade 50% of caffeine during 30 hours in the media containing 5 g/L of caffeine. Isolate of P. putida NCIM 5235 able to degrade 59.9% - 21.5% of caffeine in the media containing 7.5 - 10 g/L caffeine, and it degraded 100% of caffeine in 18 hours when grown on media containing 6.4 g/L of caffeine with 700 and 800 rpm aeration [17]. Those isolates were able to degrade 100% of caffeine in the media containing 10 g/L of caffeine during less than 45 minutes incubation [18].

4. Conclusion

Five caffeine-degrading bacterial isolates were KAFS 16, KAFS 33, KAFS 34, KAFS 35, and KAFS 47 from C. arabica coffee pulp waste was potential as the coffee decaffeinating agent. Isolate KAFS 16 has the highest decreasing of caffeine concentration in the media during 36 hours incubation, followed by KAFS 47, KAFS 34, KAFS 33, and KAFS 35 respectively. Whole isolates were able to degrade more than 90% of caffeine after 48 to 60 hours of incubation.

5. References

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