Exploring cellulolytic microorganisms from coffee industry by-products and their enzyme properties

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Abstract. Cellulolytic microorganism has immense potential due to their cellualse production, enzyme complexity and widespread habitat of life. This study was conducted to obtain microbial cellulase with vast industrial applicability from the coffee industry by-product in East Java, Indonesia. Fifty-four isolates with significant clear zone formation were obtained by Congo red staining in CMC agar plates. Eighteen bacteria, two yeasts and two moulds with high cellulolytic index were subjected to protein content determination as well as reducing sugar analysis in various conditions such as pH, temperature, addition of metal ions, surfactant and inhibitor agent. The specific activity measurements of all the crude enzymes result in the highest value of cellulase activity produced by isolate C12 which was 0.401 ± 0.018 U/mg. This enzyme activity was known to be optimum at 50°C and pH 9. It was also stimulated by K⁺, Na⁺, Mg²⁺, Fe³⁺, and SDS. However, the enzyme activity was inhibited by EDTA at 10 mM concentration. The use of coffee industry by-products as the source of cellulolytic microorganisms offers a promising approach for its various types of indigenous microorganisms and their unique property of cellulase produced that is useful for industrial application.

1. Introduction
Cellulases (EC 3.2.1.4) have a wide range of industrial applications such as textile, laundry, pulp and paper, fruit juice extraction, and animal feed additives as well as in bioethanol production [1]. The use of cellulase enzymes in the industry continues to increase until it reaches 20% of global market demand nowadays [2]. In Indonesia, the use of cellulase also continues to increase by around 7% every year [3]. Unfortunately, 99% of the enzymes used are still imported, cost 187.5 billion USD approximately in 2015 [4]. As one of an effort to reduce dependence on imported enzymes in Indonesia, it is necessary to produce enzymes by utilizing local resources. Many studies have been carried out to obtain cellulase-producing microorganisms with high specific activity and efficiency when applied to industry [5]. One of the research focuses is the use of agricultural and agro-industrial wastes with high cellulose content as a source of isolation and nutrition for cellulolytic microorganisms. This is believed could reduce production costs, increase the economic value of waste while reducing environmental pollution [6]. Coffee industry by-products become one-of-a-kind waste with high cellulose content and have been widely used in biotechnology processes [7,8].

The wet processing of Arabica coffee produces nearly 43.2% (w/w) of coffee pulp and 4.2% (w/w) of coffee silverskin [9]. These residues are substrate rich in carbohydrates (57%), proteins (24%), and
minerals with potential for utilization in bioprocess. Thus, various types of microorganisms such as bacteria, molds and yeasts grow during the processing of Arabica coffee beans [10–12]. These microorganisms can produce cellulase enzymes with specific growth and enzyme production conditions [13]. Thus, it is necessary to do a specific identification process to determine the optimum conditions for enzyme production of each microorganism. This present study was to isolate cellulolytic microorganisms from coffee pulp and silver skin then characterize their enzyme in order to obtain robust cellulase for industrial and biotechnological usage.

2. Materials and methods

2.1. Isolation and screening of cellulose-degrading microorganisms

Coffee Arabica pulp and silver skin were obtained from Kalisat Jampit-Coffee Plantation area in Bondowoso, East Java, Indonesia. Coffee pulp consists of outer skin (exocarp), pectin layer, pulp (mesocarp) and parchment (endocarp) of the coffee cherries that obtained from the pulping process during the wet processing method of the Arabica coffee bean. Meanwhile, silver skin refers to testa or epidermis that obtained by the roasting process of coffee bean. The samples were placed in a sterile plastic bag and transported to the laboratory. The samples then incubated in CMC broth (CMC 8 g/l, KCl 1 g/l, yeast extract 1 g/l, glucose 1 g/l, MgSO₄·7H₂O 0.5 g/l, NH₄H₂PO₄ 1 g/l) for enrichment. It then serially diluted and spread on the Plate Count Agar (Mercks), Nutrient Agar (Mercks) and Potato Dextrose Agar (Mercks) plates and incubated for 48-72h. The grown cultures were obtained, then spread on to the CMC agar (CMC broth with 17 g/l agar) and incubated for 48h 37°C. Visualization of the hydrolysis zone was done by flooding the agar plate with an aqueous solution of 0.1% (w/v) Congo red for 15 min, followed by 1M NaCl solution. The cellulolytic activity of the cultures indicates as the cellulolytic index which is representing the ratio between the diameter of the clear zone and the diameter of the colony.

2.2. Cellulase enzyme production and assay

One loop of a positive isolate from the previous step was aseptically inoculated into 10 mL of CMC broth then incubated at 37°C, 24h as a culture starter. The enzyme production was done by adding 10 mL of culture starter into 90 mL CMC broth and incubated in a water bath shaker at 37°C for 24-72 hours at 100 rpm. The solutions were then centrifuged at 8000 rpm for 10 minutes at 4°C to obtain the supernatant which is a crude extract of the cellulase enzyme. The cellulase activity of each culture was measured by determining the amount of reducing sugars liberated by using a dinitrosalicylic acid (DNS) method [14]. One unit of cellulase was defined as the amount of enzyme which released μmole of reducing sugar measured as glucose per minutes under the assay conditions.

2.3. Protein content and enzyme specific activity determination

The total protein content of crude enzyme were measured by Bradford method [15]. The specific activities of enzyme were calculated by dividing the crude enzyme activity by its protein content. The highest values of enzyme-specific activity indicate the most potential cellulolytic microorganism.

2.4. Enzyme characterization

2.4.1. Effect of incubation time on enzyme activity. 10 mL of culture stock was added to 90 mL of CMC broth then incubated at 37°C in a shaker water bath at 100 rpm for 3 days. The enzyme activity was measured every day with the standard assay conditions mentioned above.

2.4.2. Effect of temperature and pH on enzyme activity. The influences of temperature and pH on the activity of cellulase were studied by incubating the reaction mixture at different temperatures (37, 50, 63°C) and pH range (5, 7, 9) for 1 h [16]. The enzyme activity was measured with standard assay conditions mentioned above.
2.4.3. **Effect of metal ions and other additives on enzyme activity and stability.** The effect of various metal ions and additives such as inhibitors and surfactant (10 mM) on enzyme activity was investigated by using KCl, NaCl, MgCl$_2$, FeCl$_3$, ethylene diamine tetra acetic acid (EDTA) and SDS. The enzyme was incubated with different metals and additives at optimum temperature for 1 h and assayed under standard assay conditions [17].

3. **Results and discussion**

3.1. **Isolation and screening of cellulose-degrading microorganisms**

The microbial diversity of Arabica coffee pulp and silver skin were observed by incubation of samples on four different media at room temperature (37°C). It was known that there was a microbial variability on coffee pulp and silver skin (Table 1). The differences in the number of microorganisms on coffee pulp and silver skin were caused by various things as stated on the references, such as the differences in the nutrient content of the samples as well as the effect of treatment during the coffee cherries processing process [18,19]. The difference in organic matter especially cellulose content in the pulp and silver skin causes differences in microorganisms that can live and utilize nutrients in the two samples [20]. Furthermore, it was influenced by the variety of coffee processing and environmental factors including temperature, humidity and soil conditions including microorganisms found in the soil [21].

|                          | Coffee pulp (CFU/g) | Coffee silver skin (CFU/g) |
|--------------------------|---------------------|---------------------------|
| Total microorganism      | 1.32 x 10$^8$       | 2.23 x 10$^6$             |
| Total bacteria           | 5.20 x 10$^6$       | 9.33 x 10$^5$             |
| Total molds              | 8.20 x 10$^5$       | 4.19 x 10$^5$             |
| Total yeast              | 4.53 x 10$^6$       | 1.30 x 10$^5$             |

Based on the results of this study, it can be seen that at the beginning of the natural decomposition process of coffee industry by-products there is a dominance of bacterial growth, followed by yeast and then mold [11,12]. On the other hand, the samples preparation and isolation process in this study were mostly carried out at room temperature (37°C). Thus, it was assumed that most of the microorganisms isolated are mesophilic correspond with the fact that at early stages of the decomposition process of organic matter, mesophilic bacteria will dominate the growth of microorganisms in the sample and raise the temperature of the material to trigger the growth of thermophilic microbes [22].

3.1.1. **Qualitative test of cellulose-degrading microorganisms.** A hundred isolates were inoculated on CMC agar plates with duplication and incubated at 37°C for 2 days to obtain the potential cellulose-degrading microorganism from coffee pulp and silver skin. The grown colonies were observed for the morphological characteristics such as shape, size colour and elevation to differentiate them visually. Furthermore, the cellulolytic index of each colony were measured by dividing diameter ratio of the colony with the formed clear zone (Figure 1) after staining process with 1% (w/v) Congo red solution and distaining with 1M NaCl solution.

**Figure 1.** Cellulolytic colony in CMC agar plate (left) and its clear zone formation (right).
Twenty-seven cellulolytic microorganisms were obtained from the coffee pulp samples with isolate A5 and A17 showed the highest cellulolytic index reached 2.333 for both. As in coffee silver skin, there were also 27 positive isolates with the highest cellulolytic index 2.000 showed by isolates G11, G20, G22 and K5. Other researchers found cellulolytic clear zone ranged from 1.5 to 4.7 cm [23]. The difference in the cellulolytic index can be caused by the different types of excreted cellulase by each isolates whether it was dominated by endoglucanase with or without cellulose-binding molecule (CBM), exoglucanase or β-glucanohydrolase. This will lead to a different mechanism of decomposing substrates in growth media [24].

3.1.2. Quantitative test of cellulose-degrading microorganism. Fifty-four cellulolytic microorganisms were obtained from qualitative tests then subjected to enzyme production and activity assay for 3 days in row to figure out the optimum incubation condition for each isolate. Based on the results of daily activity measurements (data not shown), it is known that each isolate has a different incubation period for enzyme production. On the first day, the highest crude cellulase enzyme extract activity was shown by isolate M3 with a value of 0.495 ± 0.083 U/ml, however, the activity decreased on the second day. The highest activity on the second day was indicated by the isolate C12 with a value of 0.432 ± 0.027 U/ml. In general, the crude extract activity of the cellulase enzyme decreased on the third day, however, isolate G13 showed the highest value of 0.337 ± 0.345 U/ml. The enzyme activity of obtained isolate are higher than cellulolytic microorganism from Kalibendo coffee plantation [25] but lower than Malangsari area [26]. The differences in enzyme activity are caused by the variability of cellulolytic microorganism that can be found in the coffee industry by-products.

3.2. Characterization of the crude enzyme

3.2.1. Effect of temperature on enzyme activity. Twenty-two isolates with the highest activity value then measured its enzyme activity against various temperatures to predict their optimum temperature. In general, as seen in Figure 2, the optimum temperature of enzyme activity was 50°C. The highest activity was shown by isolate C12 (0.311 ± 0.017 U/ml), which accordance with isolates from organic solid waste [27]. Temperature plays a very important role in enzymatic reactions, when the temperature increases until it reaches the optimum temperature, the enzyme reaction rate increases because the kinetic energy increases. The increase in kinetic energy will accelerate the vibrational, translational, and rotational motion of both enzymes and substrates. This will increase the opportunity for the enzyme and substrate to react so as to increase their activity [28]. In addition, the higher the incubation temperature, the lower the viscosity of CMC, this can increase the mobility of CMC molecules which can help substrate access to cellulase enzymes. Although the microorganisms were isolated at room temperature, the enzyme activity at 37°C was known to be lower than the activity at 50°C. It was because the viscosity of the substrate was the highest at 37°C so that it was difficult to be hydrolysed by the cellulase enzyme and gave the lowest activity value in some isolates. Thus, it was suggested to perform other methods of cellulase activity assay in future research [16]. On the other hand, temperatures above 50°C tend to damage the tertiary structure and cause denaturation and a sharp decrease in protein solubility [29]. Denaturation that occurs in the enzyme will cause a change in the active site of the enzyme so that the enzyme is unable to bind specifically to the substrate and then decrease its activity.
3.2.2. Effect of pH on enzyme activity. Cellulase enzyme activity was influenced by the pH value. It was seen (Figure 3) that crude cellulase enzyme from isolate C12 showed the highest activity among all, with the highest activity shown at pH 9 (0.322 ± 0.001 U/ml), similar with cellulase from Bacillus [30]. The changes in the pH of the medium affect the ionization of side groups in amino acids. This will affect the hydrogen bonding between functional groups as well as the conformation of the enzyme and substrate in maintaining the tertiary and quaternary structure of the active enzyme. At optimum pH conditions, the cellulase enzyme will show the highest activity in hydrolyzing the substrate. Changes in pH or pH that are not suitable will cause the catalytic region and the conformation of the enzyme to change. In addition, changes in pH also cause enzyme denaturation and result in loss of enzyme activity [31].

3.2.3. Protein content and specific activity determination. The specific activity determination of the enzyme is done by dividing enzyme activity (U/mL) with the protein content in (mg/mL) of the enzyme solution. Since the specific activity (U/mg) of the enzyme represents the purity of the enzyme towards its total protein content. Determination of protein content was carried out by the Bradford method. This is because the Bradford test has several advantages compared to other methods, including easy to perform, inexpensive, the reaction takes place quickly, the resulting color complex being more stable, and the minimum interference from other substances [15].
Table 2. Protein content and specific activity of crude cellulase.

| Isolate code | Protein content (mg/ml) | Specific activity (U/mg) | Isolate code | Protein content (mg/ml) | Specific activity (U/mg) |
|--------------|-------------------------|--------------------------|--------------|-------------------------|--------------------------|
| A3           | 0.736 ± 0.019           | 0.180 ± 0.005            | D7           | 0.437 ± 0.014           | 0.081 ± 0.003            |
| A4           | 0.487 ± 0.074           | 0.294 ± 0.045            | E6           | 0.343 ± 0.032           | 0.104 ± 0.010            |
| C12          | 0.552 ± 0.025           | 0.401 ± 0.018            | G7           | 0.474 ± 0.032           | 0.150 ± 0.010            |
| H8           | 0.343 ± 0.069           | 0.368 ± 0.074            | G11          | 0.890 ± 0.005           | 0.099 ± 0.001            |
| J6           | 0.382 ± 0.050           | 0.233 ± 0.031            | G13          | 0.465 ± 0.020           | 0.247 ± 0.011            |
| L5           | 0.539 ± 0.007           | 0.148 ± 0.002            | G14          | 1.327 ± 0.037           | 0.099 ± 0.003            |
| L6           | 0.853 ± 0.050           | 0.132 ± 0.008            | G20          | 0.618 ± 0.074           | 0.231 ± 0.028            |
| L10          | 0.409 ± 0.074           | 0.353 ± 0.011            | G22          | 0.526 ± 0.008           | 0.235 ± 0.004            |
| M3           | 0.683 ± 0.076           | 0.130 ± 0.014            | I5           | 0.461 ± 0.061           | 0.252 ± 0.033            |
| M6           | 0.539 ± 0.013           | 0.033 ± 0.001            | K8           | 0.551 ± 0.043           | 0.080 ± 0.006            |
| N3           | 0.644 ± 0.024           | 0.192 ± 0.007            |             |                         |                          |
| N14          | 0.317 ± 0.025           | 0.356 ± 0.028            |             |                         |                          |

Based on the data in Table 2, it is known that the highest specific activity was shown by the enzyme from isolate C12 was 0.401 ± 0.018 U/mg, hence it was lower than *Bacillus* species [17]. Isolate C12 was selected as the best cellulose-degrading microorganism from coffee industry by-products and its enzyme was subjected for further characterization.

3.2.4. Effect of additives on enzyme activity. The biochemical properties, that is, inhibitors and activators, are also important in the characterization of the enzyme. The enzyme was slightly stimulated by K⁺, Na⁺, Mg²⁺ ions and the surfactant agent SDS at 10 mM concentration (Figure 4). The surfactant may increase the concentration of the catalytic domain of enzymes on the surface of the substrate and, consequently, promoting the accessibility of substrate and high catalytic performance. This property will benefit for application of enzyme in the detergent industry. The enzyme was also observed to be strongly stimulated by trivalent ions Fe³⁺ until 1.9 folds its initial activity. However, the addition of EDTA decrease its residual activity to 90.92%, since it could chelate the metal ions that were probably present on the culture medium, thus it cannot bind with thiol groups at the catalytic site of cellulase and inhibit the overall performance of the enzyme [32].

![Figure 4](image)

Figure 4. The effect of additives on enzyme activity.

4. Conclusions
Culturable mesophilic (37°C) cellulolytic microorganisms were collected from Arabica coffee pulp and silver skin in Kalisat Jampit Plantation, East Java, Indonesia. The enzyme assay results in the highest specific activity of isolate C12. This unique property of thermostable-alkalophilic cellulase produced by this isolate proves the potency for biomass conversion into fuel and other industrial products.
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