Production and Partial Purification of Cellulase from *Aspergillus* sp. VT12 by Solid-State Fermentation Using Coffee Pulp

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Abstract. Cellulase has been widely used in many applications of industries and is commonly produced by a cellulase-producing-fungus using production substrates from agricultural wastes biomass. In the wet processing of the coffee, up to 63% of coffee pulp rich-cellulose has been released. This potential waste allows being used as a substrate for enzyme production. This study aims to produce cellulase using coffee pulp by Solid-State Fermentation from isolates of *Aspergillus* sp. VT12. A solid-state fermentation medium of coffee pulp was inoculated with *Aspergillus* sp. VT12 and incubated at 37 °C for 5 days, the culture produce a maximum cellulase activity of 0.52 U/ml against 0.5% carboxymethyl cellulose substrate. For the purification step, the crude cellulase obtained was dialyzed on a cellulose tube membrane 12–14 kDa and then loaded into open column anion exchanger DEAE Cellulose DE-52 chromatography with NaCl gradient 0–0.6M used. In this step, the activity of purified cellulase obtained was 1.00 U/ml with a yield of 51.86%. For all steps of dialysis and cellulase measurement activity, including purification steps, the 20 mM acetate buffer pH 5 was used. This research revealed that the potential coffee pulp waste can be used as a substrate for cellulase production. However, in an economical view, production efficiency still needs to be optimized and the purity of the enzyme as well.

Keywords: Aspergillus sp. VT12 · Cellulase · Coffee pulp · Solid-state fermentation · Partial purification

1 Introduction

Cellulase is an enzyme complex consisting of three enzymes namely endocellulase, exocellulase, and β-glucosidase [1]. The enzymes that make up the cellulase complex work synergistically and have different roles in the process of breaking down the polymer chain of cellulose into glucose. This glucose is used as a source of carbon for the life cycle

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needs and microorganism growth [2, 3]. Cellulase is an extracellular enzyme produced by fungi, bacteria, protozoa, and actinomycetes [4, 5].

Currently, cellulase enzyme is in third place after amylase and protease in the world market enzyme industry. Cellulase is often used in the pulp and paper industry, textile industry, and additives in detergents and animal food [5]. This causes an increase in the demand for cellulase, so large-scale production in the industry is required. But, high costs and low yields are the main obstacles to cellulase production on large scale. Therefore nowadays much research is performed to produce cellulase efficiently and economically.

One alternative to reduce the cost of cellulase production on a large scale is by utilizing agricultural or plantation waste. A plantation plant that is found a lot in nature and produces abundant waste is coffee. In addition, Indonesia is the 4th largest country that produced coffee after Brazil, Vietnam, and Columbia, i.e. 685.980 tons [6]. Five tons of coffee processing will produce about three tons of by-products [7]. One of the coffee wet processing products includes coffee pulp [8].

The main organic content of the coffee pulp is cellulose (63%), protein, and also contains tannins, caffeine, and polyphenols in sufficient quantity [9]. The high content of cellulose has the potential to be utilized in the bioconversion sector, one of which is as a substrate in the production of cellulase [10]. The most commonly used method in cellulase production is Solid State Fermentation (SSF). This method is best applied to fermentation processes involving organisms that require low moisture such as fungi (40–60%) [11].

The filamentous fungi have a high ability in the production and secretion of proteins so that they can be used for the production of cellulase [4, 12]. One of the fungi that have been known as cellulase producer is *Aspergillus* sp VT12. Almost all of the genus *Aspergillus* produce cellulase and are known to be efficient in cellulase production [13]. *Aspergillus* sp. VT12 is isolated from the vermicomposting process of oil palm empty fruit bunches (OPEFB). According to Yuniar, *Aspergillus* sp. VT12 can produce the enzyme cellulase with an activity of 0.076 U/ ml on the OPEFB substrate [14]. Therefore, this study aims to produce cellulase with *Aspergillus* sp. VT12 using high-cellulose coffee pulp as substrate, so that cellulase production can run efficiently and environmentally friendly.

2 Material and Methods

2.1 Inoculum Preparation

Aspergillus sp. VT12 is a fungus that is isolated from the vermicomposting process of oil palm empty fruit bunches (OPEFB). This isolate was inoculated on Potato dextrose agar (PDA) medium and incubated at 30 °C for 3 × 24 h [15]. After that, the cultures were suspended with 5 ml of aquadest. The spores that have been released and suspended with aquadest, were collected and homogenized in an Erlenmeyer flask. The spores density of *Aspergillus* sp. VT12 was counted by haemocytometer. The spores density of *Aspergillus* sp. VT12 reached 10^8 on the third day and it can be used as ideal inoculum for solid-state fermentation [16].

2.2 Solid-State Fermentation (SSF)

In this study, solid-state fermentation was used for cellulase production with coffee pulp as substrate. SSF was carried out by adding 5 g of coffee pulp powder with moisture content (1:2 w/v) [15, 17] in a 250 ml Erlenmeyer flask. The SSF mediums were sterilized with an autoclave at 121 °C and 1 atm for 25 min. The inoculum volume that has reached spores density 10^8, was inoculated in SSF medium as much as 1 ml per 5 g of substrate [13].

2.3 Optimization of Crude Cellulase Production

Incubation time optimization of crude cellulase production was carried out for 0–168 h at 37 °C [13]. The cellulase produced by VT12 is stable at pH 5 [18], so in this study, acetate buffer pH 5 was used. Then, the large-scale cellulase production was carried out based on the optimum incubation time for further purification.

2.4 Extraction of Crude Cellulase

The extraction of enzymes was begun by adding aquadest solution containing 0.01% Natrium azide and 1% NaCl into SSF cultures. Then, it was incubated on a rotary shaker for 12 h. The samples were filtered and centrifuged at 8000 rpm for 5 min. The supernatant obtained was a crude extracellular enzyme. The crude enzyme was stored at 4 °C [19].

2.5 Cellulase Activity Assay

The method used for reducing sugar assay in this study was Somogy-Nelson with some modifications. The substrate that was used to verify cellulase is carboxymethyl cellulose (CMC). 500 μl of 0.5% CMC in 20 mM acetate buffer pH 5 was incubated in the water bath at 37 °C for 20 min. Then, the substrate was added with 50 μl of crude enzyme. 500 μl of Somogy reagent was added to it and boiled for 15 min. 50 μl of the crude enzyme was added when boiled as a control. After the samples were cold, 500 μl of Nelson reagent and 2.5 ml of aquadest were added to them. The samples were centrifuged at 8000 rpm for 10 min. The absorbances of reducing sugar were measured using a spectrophotometer with a 500 nm wavelength. The absorbance value obtained is calculated using the glucose standard curve formula so it obtained the value of reducing sugar. Cellulase activity of *Aspergillus* sp. VT12 was obtained from the conversion of reducing sugar value to enzyme activity formula. The activity of cellulase was expressed as U/ml. One unit of cellulase activity was defined as the amount of enzyme which released 1 μM glucose per minute [15, 20].

2.6 Partial Purification of Crude Cellulase

Partial purification in this study was begun with dialysis using a cellulose membrane of 12–14 kDa pores. The crude enzyme was put into the membrane and soaked in 20 mM acetate buffer pH 5 solutions while stirring for 24 h. Every 12 h, the buffer solution was

replaced with new ones [6]. The dialysis enzyme is further purified by Chromatography using DEAE Cellulose DE-52 which has been equilibrated with the addition of 20 mM acetate buffer pH 5. Enzymes were applied to the DEAE Cellulose DE-52 matrix. The bounding protein in the matrix was eluted by stepwise method on a gradient mixer using various concentrations of 0M; 0.1M; 0.2M; 0.3M; 0.4M; 0.5M, and 0.6M NaCl solution in 20 mM acetate buffer pH 5. The eluent speed was set at 0.5 ml/min. The eluate from each concentration was collected as much as 5 ml of each fraction. The protein quantity of each fraction was measured at a wavelength of 280 nm [21].

3 Results and Discussions

3.1 Optimization of Crude Cellulase Production

Optimization of cellulase production aims to determine the fastest incubation time for *Aspergillus* sp. VT12 in producing cellulase with the highest enzyme activity. Figure 1 shows that the optimum incubation time occurred at 120 h with cellulase activity of 0.57 U/ml. It was supported by the research of Muzakhar et al. that the optimum incubation time of *Aspergillus niger* occurs at 120 h in solid fermentation media using Oil Palm Empty Fruit Bunches (OPEFB) substrate [22]. The spore density of *Aspergillus* sp. VT12 inoculated in solid fermentation media reached 2.06×10^8 spores/ml, so it becomes an ideal inoculum for cellulase production [16]. According to Das et al. enzyme secretion depends on the number of cells present. High cellulase secretion will increase cellulase activity [23, 24].

Based on Fig. 1, it can be seen that there was an increase in cellulase activity from the 24th hour to the 168th hour with a significant increase at the 120th hour. Cellulase activity was stable from the 120th hour to the 168th hour. When the incubation time gets longer, the process of hydrolysis of the coffee pulp substrate into glucose by cellulase secreted by *Aspergillus* sp. VT12 is becoming more and more increased as well. The glucose concentration increased with increasing incubation time, but the nutrients in the solid fermentation medium were running out. The lack of nutrients in the coffee pulp substrate in solid fermentation media can cause a decrease in enzyme activity [25]. Another factor that affects the decrease in enzyme activity is the crude extract enzyme which becomes unstable if left too long in the fermentation medium [24, 26]. So the

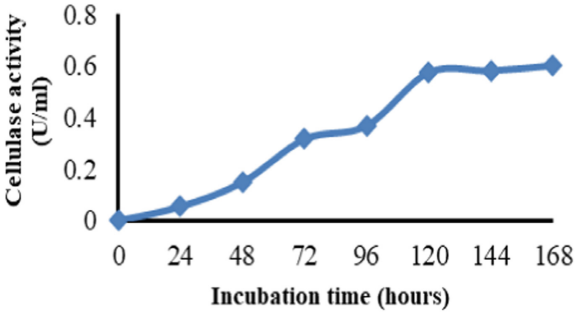

Fig. 1. Optimization of cellulase production of *Aspergillus* sp. VT12 at 0–168 h incubation time.

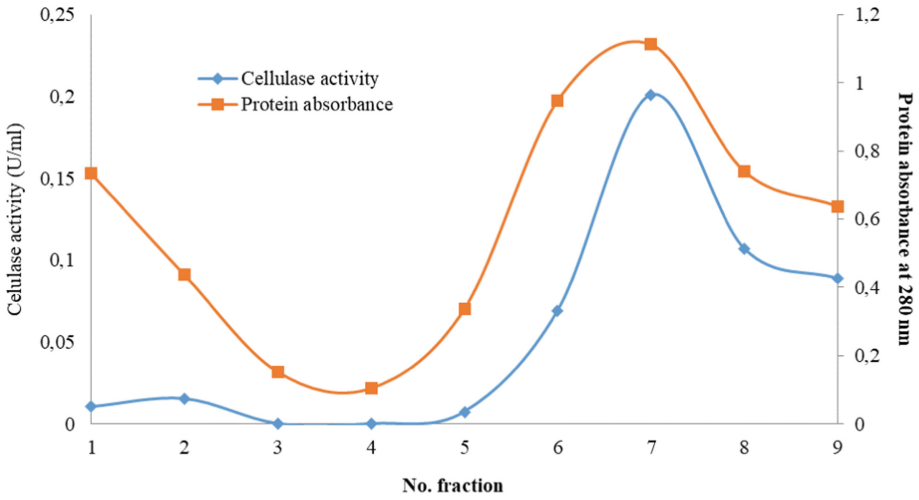

Fig. 2. Elution profile of 0–0,6 M NaCl on cellulase activity and protein absorbance 280 nm after purification using DEAE Cellulose DE-52 (x-axis from left to the right based on numerical order: 0; 0; 0; 0.1; 0.2; 0.3; 0.4; 0.5; 0.6M NaCl).

120th hour is the optimum incubation time for cellulase extraction by *Aspergillus* sp. VT12 in this study.

3.2 Cellulase Fraction of DEAE Cellulose DE-52 Chromatography

There was only one peak containing protein and cellulase activity on the elution profile (Fig. 2) [27]. The highest cellulase activity of 0.2 U/ml and absorbance value of 280 nm of 1.1 was found in the 0.4M NaCl fraction. It indicates that the cellulase enzyme successfully eluted well at a concentration of 0.4M NaCl. The absorbance value of 280 nm protein at a concentration of 0M NaCl (fraction 1) was also high but the cellulase activity was low (0.01 U/ml). These results indicate that the eluted molecules are impurities or non-target proteins other than cellulase. According to Nooralabettu, when the salt concentration increases, salt ions such as Na^+ or Cl^- compete with proteins to bind with the charge on the surface of the DEAE matrix and the protein will begin to be released and eluted to the bottom of the column [28].

3.3 Partial Purification of Cellulase

Table 1 showed that the specific activity of each purification stage is increasing. The higher the specific activity of the enzyme, the purity of the enzyme also increases. The specific activity of the enzyme is influenced by the protein content. The total quantity of protein at each stage of purification was reduced to 489.50 mg. This indicates that the purification process carried out succeeded in separating the enzyme from other proteins, so the purity increased by 25.49 times after being purified using the DEAE Cellulose DE-52 matrix with a yield of 51.86% [29].

Table 1. Partial purification of cellulase

Purification steps	Total volume (ml)	Enzyme activity (U/ml)	Total activity (U)	Protein content (mg/ml)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude enzyme	165.00	0.52	85.80	169.20	27,918.00	0.003	100.00	1.00
Cellulose membrane dialysis	178.00	0.35	61.45	96.80	17,230.40	0.004	71.62	1.16
DEAE cellulose DE-52 chromatography	44.50	1.00	44.50	11.00	489.50	0.091	51.86	25.49

The best incubation period for cellulase production of *Aspergillus* sp. VT12 during solid-state fermentation on coffee pulp was 120th hour of incubation time with the optimum activity of 0.52 U/ml. The specific activity of cellulase increased by 0.091 U/mg after purification using cellulose membrane dialysis and Cellulose DEAE DE-52 chromatography. These purification steps also increased the purification fold up to 25.49 with a yield of 51.86%. Therefore, the purification carried out succeeded in separating the cellulase from other enzymes and proteins.

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