An Extracellular Cellulase Production Under Solid-State Fermentation of Coffee Pulp Waste by *Aspergillus* sp. VTM1 and Its Purification

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Abstract. Microbial utilization of agricultural biomass wastes has been widely used to synthesize certain an enzyme. We previously investigated that cellulase can be produced under solid stated fermentation based on coffee pulp waste substrate using *Aspergillus* sp. VTM1. Crude cellulase production was optimized for a week of incubation at 30 °C, the crude was harvested daily with distilled water containing 1% NaCl and 0.1% NaN3**.** To measure cellulase activity, the reducing sugar Somogy-Nelson method was used. In the next step, crude cellulase was dialyzed using a 45μm cellulose membrane tube 12–14 kDa against acetate buffer pH 5. Further analysis found that the maximum activity of cellulase production was obtained after inoculating 10^8 spores/ml of *Aspergillus* sp. VTM1 to the 5g of sterile coffee pulp (67% humidity) at 30 °C for 96 h. Large-scale production of cellulase crude extract results in an activity of 0.70 U/ml with a total activity of 121.1 U/ml. After the dialysis, the enzyme activity (0.81 U/ml) and specific activity (0.01 U/mg) were increased compared to the crude extract. On an anion exchanger using DEAE cellulose DE-52, the specific activity was 0.1 U/mg, increased 25.03 times with a yield of 36,82%. The findings of this study lead to a green strategy for cellulase production under solid-state fermentation by *Aspergillus* sp. VTM1 and its purification.

Keywords: Aspergillus sp. VTM1 · Cellulase · Coffee pulp · Purification · Solid-state fermentation

1 Introduction

Cellulose, a linear polymer of glucose units connected by β-1,4-glycosidic linkages, comprised a huge proportion of plant biomass [1]. This insoluble substrate was catalyzed by elaborating a group of cellulase [2]. Animals and plants can produce cellulase, but microbes play a significant role on a larger scale of industry. Cellulase is a commercially

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available enzyme that is used in a variety of industries, including paper, bioethanol, detergents, textiles, and agriculture [3].

Microbes can synthesize cellulase when cellulose is available and hydrolyze it into simple sugars necessary for growth [2]. Coffee pulp is a waste product that could be used as a substrate containing cellulose [4]. Coffee processing produces a by-product in the form of coffee pulp, which accounts for about 29% of the dry weight of whole coffee cherries [5]**.** Each processing of 1 ton of coffee cherries is known to produce half a ton of coffee pulp [6]. The waste contains organic components including lignin, protein, tannin, pectin, hemicellulose, and cellulose [5]. The cellulose contained can be utilized to produce cellulase by potential microbes.

According to a prior study, *Aspergillus* sp. VTM1 can produce cellulase using coffee pulp as a substrate, Cellulase enzyme activity reached 1.18 U/ml in these isolates, with optimal activity at pH 5 and stability in the ranges of 3–3.5 and 4–7 [7]. A purification method is required since the crude extract of the cellulase enzyme is still contaminated with undesired proteins or metabolites.

Membrane dialysis and ion-exchange chromatography are some of the simple purification procedures that have been developed. Dialysis is a molecular separation process that uses diffusion to separate proteins based on their size using a semipermeable membrane [8]. Ion chromatography, on the other hand, is a protein separation process that uses a pH gradient to create a difference in charge on the protein surface. Partial purification using several methods such as dialysis and anion exchange chromatography can increase the enzyme's specific activity, resulting in greater purity [9]. In this study, we used coffee pulp waste as a substrate to partially purify the cellulase enzyme produced by *Aspergillus* VTM1.

2 Materials and Methods

2.1 Fungal Spores Preparation and Cellulase Enzyme Optimization

Aspergillus sp. VTM1 was recultured on Potato Dextrose Agar (PDA) for 3 days at 37 °C [10]. The inoculum was inoculated to the media containing alkali-pretreated coffee, nutrients element, and agar at 37 °C for 4 days to get 10^8 spores/ml [11]. Then added to the solid-state coffee pulp for the production of cellulase enzyme. The method of solid-state fermentation refers to Rusdianti et al. [7] work with little modification, we used 5 g of the coffee pulp as a substrate. Optimization was conducted by incubation of the mixture for 7 days at 30 °C with 120 rpm of agitation**.** While the production at a large scale was using 40g of coffee pulp for 4 days (maximum activity) of incubation.

2.2 Enzyme Extraction

Crude cellulase enzyme was obtained every day for a week. The extraction was carried out by adding 10 ml of distilled water containing 1% NaCl and 0.01% NaN_3 to the growth media [12]. The formed suspension was shaken for 12 h, squeezed using synthetic gauze, and the filtrate was centrifuged at 8000 rpm for 10 min [12].

2.3 Enzyme Assay

The Somogy-Nelson method was used to measure the activity of the cellulase enzyme, 500 µl of 0,5% CMC in 20 mM acetate buffer pH 5 was mixed with 50 µl cellulase crude extract and incubated at 37 °C for 2 h. The mixture was then added with 500 µl Somogyi reagent and boiled for 15 min, after cooling up, 500 µl Nelson reagent and 2,5 ml distilled water were added and homogenized using a vortex [12]. One milliliter of each treatment was centrifuged at 8000 rpm for 10 min and the supernatant was analyzed spectrophotometrically at 500 nm [13]. The cellulolytic activity was calculated using a standard curve of glucose [14]. One unit of enzyme activity was defined as the amount of enzyme releasing 1 µmol glucose each minute under the circumstance. Protein concentration was determined at 280 nm.

2.4 Purification of Cellulase

Dialysis membrane and chromatography ion were applied to purify the cellulase. The crude extract was dialyzed using a 45µ cellulose membrane tube (12–14 kDa) against 20 mM acetate buffer (pH 5) for 24 h [15]. Acetate buffer was substituted every 12 h. After dialysis, 20 ml of the enzyme was applied to a DEAE Cellulose DE-25 equilibrated by 20 mM acetate buffer pH 5 and eluted using 0.1–0.6 M of NaCl in the same buffer [16]. Each fraction (5 ml) showing cellulolytic activity was pooled and measured the protein concentration as mentioned above.

3 Results and Discussions

3.1 Optimization of Cellulase Enzyme

Optimization of cellulase production from isolates of *Aspergillus* sp. VTM1 aims to determine the optimum incubation time for producing cellulase with the highest activity. The activity value was obtained from the inoculum density of 1.4×10^8 spores/ml in 5 g of the solid-state substrate with 67% water content. The humidity level is suitable for mold growth. Generally, molds require humidity of around 40–60%. In some research, by using the SSF method, the optimum water content in cellulase production was 70% [17]. While in another study, 65% water content was enough to produce cellulase on several lignocellulose solid substrates (grass, corn cobs, and bagasse) [2].

The coffee pulp offered a good growing medium for *Aspergillus* sp. VTM1, the capacity to produce cellulase was shown by the existence of hydrolysis activity when tested using CMC as a substrate in the Somogyi-Nelson method. Production of this enzyme can occur due to the high content of cellulose and organic nutrition that serves as a source of carbon and nitrogen for mold development [4].

The cellulose content in the coffee pulp (63%) was higher than that of OPEFB (45.9%) so *Aspergillus* sp. VTM1 was able to produce higher reducing sugars than the OPEFB substrate. According to previous research, *Aspergillus* sp. VTM1 through solid fermentation on OPEFB substrate produced reducing sugar of 8 µg/ml after being tested on 0.5% CMC substrate. In this study, we were able to produce reducing sugar up to 109.3 µg/ml. This indicates that *Aspergillus* sp. VTM1 was able to grow and produce

Table 1. Cellulase activity from *Aspergillus* sp. VTM1 at each stage of partial purification

Purification Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purity fold
Crude extract	121.10	31006.8	0.004	100.00	1.00
Membrane cellulose	166.05	21361.0	0.01	137.11	1.99
DEAE-C DE-52	44.59	456.13	0.10	36.82	25.03

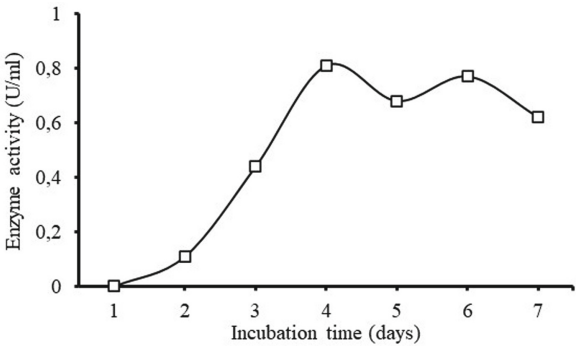

Fig. 1. Optimization of the production of crude cellulase extract of *Aspergillus* sp. VTM1 at various incubation times.

a higher cellulase enzyme in coffee pulp waste than OPEFB. Another research also shows the potential for the production of cellulase using coffee pulp waste after 96 h of incubation with 1.8 U/ml activity [18].

The activity of the crude extract of cellulase increased when it was produced at an incubation time of 2 days (0.11 U/ml), 3 days (0.44 U/ml), until it reached the highest activity at an incubation time of 4 days, which was 0.81 U/ml. Cellulase activity began to decrease at an incubation time of 5 days (0.68 U/ml), 6 days (0.66 U/ml) to 7 days (0.62 U/ml) (Fig. 1). The activity value indicates that the incubation period of 4 days is the optimal incubation time to produce crude extract cellulase with the highest activity. Similar results were found in another study that used *Aspergillus* isolates as a cellulase producer using coffee pulp waste as a substrate [18].

3.2 Purification of Cellulase

Crude extract cellulose at a large scale that was produced on solid-state fermentation was dialyzed using cellulose membrane yielding an activity of 0.81 U/ml, while the crude extract was 0.70 U/ml. The specific activity of the enzyme was increased after the dialysis and its purity was 1.99-fold compared to the crude extract (Table 1). Meaning there was a separation of undesiring molecules through the pores of the cellulose membrane. Small molecules will diffuse across the membrane, while the larger would retain [8]. It was

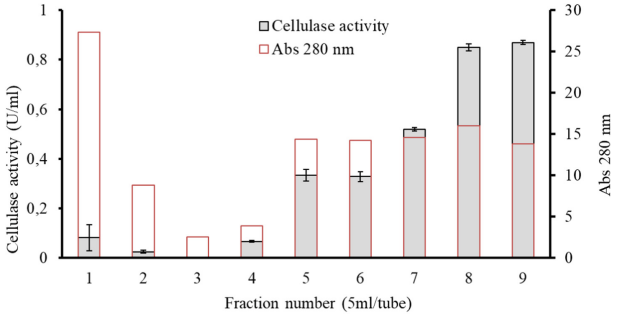

Fig. 2. Enzyme activity curve for each fraction resulting from purification using DEAE Cellulose DE-52.

also demonstrated in another study that using dialysis can increase the purity of cellulase crude extract produced from solid-state fermentation [19].

Cellulase from dialysis was purified again using DEAE Cellulose DE-52. The purification process using DEAE aims to separate proteins based on their charge so that a purer fraction is obtained [20]. This purification stage produces 9 fractions which are the result of elution using acetate buffer pH 5 20 mM and NaCl with a concentration of 0.1–0.6 M. Each fraction produced is measured for its cellulase activity.

Cellulase purification results by chromatography anion exchanger showed the presence of one peak at fractions number 8 and 9 (Fig. 2). Fractions number 8 and 9 had the highest cellulase activity (0.85 U/ml and 0.87 U/ml) compared to the other fractions. The high cellulase activity in this fraction indicated that the target protein was bound to the matrix and eluted [21] at a certain NaCl concentration (0.5–0.6 M). While the highest protein content was found in fraction no. 1 which was eluted by acetate buffer pH 5 20 mM. However, after the activity test, it is known that the activity value of fraction no. 1 is quite low at 0.082 U/ml. This indicates that the eluted protein in fraction no. 1 is a non-target protein. The elution of non-target proteins causes an increase in the purity of the cellulase enzyme up to 25.03 times that of the crude enzyme extract.

The highest specific activity of cellulase purified using DEAE Cellulose DE-52 was achieved in the 9th fraction with a specific activity of 0.1 U/mg. This specific activity value is 10 times higher than the dialysis enzyme, which is 0.01 U/mg. It was stated that the purer an enzyme, the higher the value of its specific activity [22]. At each stage of the purification process, there is an increase in a specific activity, indicating that the purification was successful to remove the contaminant.

Based on the results of this study, we found that cellulase production using coffee pulp waste as a substrate was optimal after incubation for 4 days. We demonstrated simple purification by dialysis (cellulose membrane) and ion exchange chromatography (DEAE cellulose DE-52). With this step, we can obtain cellulase with a purity of up to 25.03.

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