Coffee Pulp Waste Substrate Based in Cellulase Production by *Penicillium* sp. VT11 Under Solid-State Fermentation

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Abstract. Coffee pulp is an agro-industrial waste that is produced in huge amounts in Indonesia. The coffee pulp contains hemicellulose, with 63% of the total cellulose. In our previous study, the cellulase activity of *Penicillium* sp VT11 from coffee pulp waste was assayed under the solid-state fermentation (SSF) method. In this study, we increased the production of the enzyme using the SSF method and purified it using dialysis and ion-exchange chromatography. The optimum cellulase activity was 0.8 U/ml after four days of incubation at 30 °C. The enzyme after dialyzed using 12–14 kDa pores cellulose membrane resulted in 2.25 times increase in activity of 0.78 U/ml. The results proved that *Penicillium* sp. VT11 can utilize the coffee pulp waste as carbon and nitrogen for its growth and produce cellulose. The dialyzed enzyme was loaded into open column DEAE Cellulose DE-52 chromatography with gradient 0–0.6 of NaCl which two peaks of cellulase activity were detected. The peaks were eluted in 0.2 M of NaCl with cellulase activity of 0.14 U/ml and 0.5 M of NaCl with 0.156 U/ml cellulose activity respectively. The 20 mM acetate buffer pH 5 was applied for the dialysis, cellulase assay, and purification processes stated above. There were two peaks found, indicating that two types of cellulases were produced during solid-state fermentation. Molecular analysis is required to distinguish between these two forms of cellulases. This article details all of the research methods.

Keywords: Cellulase · Coffee pulp · *Penicillium* sp. VT11 · Solid-state fermentation

1 Introduction

Cellulases are a complex group of enzymes consisting of endo-glucanase, exo-glucanase, and glucosidase [1]–[4]. In recent years, many research groups have focused on cellulase because 8% of the total global enzyme market is cellulase [5]–[7]. However, due to the high price of cellulase, many studies have focused on cellulase production using cellulosic biomass [8]–[12], such as coffee pulp.

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The high cellulose content in the coffee pulp provides an opportunity for its use as a medium for cellulase production. Indonesia was the 4th largest coffee-producing country and the coffee pulp waste produced was very large. According to the Central Statistics Agency for East Java, coffee production in Jember Regency in 2017 reached 11,863 tons. The component of coffee pulp waste is hemicellulose with 63% of which is cellulose. However, this waste has low economic value and is generally only used as animal feed, or fertilizer or disposed of in a landfill until it is burned [13, 14]. It has been discovered that coffee pulp waste may be utilized as a substrate for the synthesis of enzymes such as cellulase by *Aspergillus* sp. VT12 [15]

Fungi are known as the main cellulase-producing microorganism because it contributes to the decomposition of 80% of cellulose biomass in nature [3]. *Penicillium* sp. VT11 isolated from vermicomposting of Palm Oil Empty Fruit Bunches (POEFB) had a cellulolytic activity index of 1.0 on 1% CMC media [16]. The cellulolytic potential of the *Penicillium* sp. VT11 prompted research on the production of cellulase by this fungus using coffee pulp waste.

Cellulase production can carried out using the solid fermentation method [1, 8, 17, 18], or liquid fermentation [8, 19]. Cellulase production by fungi with solid fermentation method produces cellulase filtrate with relatively high concentration and simpler technique compared to liquid fermentation [1]. Cellulase production by solid fermentation method using agro-industrial residue substrates and by-products can increase the economic value of the waste or by-products [1, 3, 20]–[22]. Therfore, the purpose of this study was to determine the produce and purify cellulose from *Penicillium* sp. VT11 using coffee pulp under solid-state fermentation.

2 Materials and Methods

2.1 Production of Cellulase Crude Extract

A total of 5 g of coffee pulp powder from the Jember plantation was added aquadest until reached 9% water content. This substrate was used as solid-state fermentation (SSF) media. 1 ml of *Penicillium* sp. VT11 (72 days old) culture suspension was inoculated in the substrate with a concentration of 10% (v/v). Extraction of cellulase crude extract was carried out at 24-h intervals starting from 0 to 168 h. Before harvesting the enzyme, the SSF media was added with 10 ml of H_2O containing 0.01% NaN_3 and 0.1% NaCl and then incubated by shaking for 12 h at 120 rpm and room temperature. The filtrate was collected using synthetic gauze [23, 24]. The supernatant as crude cellulose enzyme was obtained by centrifugation at 8.000 rpm for 10 min. The supernatant was stored at 4 °C until reached the optimum incubation time for cellulase production based on the cellulase activity test on 0.5% (w/v) CMC substrate in 20 mM acetate buffer pH 5 by the Somogyi-Nelson method [25]. Large-scale production of cellulase was carried out using 40 g of the coffee pulp as an SSF media and the same method as an optimization process with the optimum incubation time.

2.2 Enzyme Assay

The enzyme activity test was determined based on the analysis of reducing sugars on 0.5% (w/v) CMC substrate in 20 mM acetate buffer pH 5 [14] using the Somogyi-Nelson

method. 50 µl crude cellulase was added to 500 µl of 0.5% CMC substrate in 20 mM acetate buffer pH 5. The mixture was added with 500 µl Somogyi reagent to stop the enzyme reaction and then boiled for 15 min. After the mixture was cooled, added 500 µl Nelson and 2.5 ml H_2O. The sample was centrifuged for 10 min at a speed of 8,000 rpm at a temperature of 25 ± 2 ºC and the absorbance of the supernatant was measured using a spectrophotometer with a wavelength of 500 nm. Glucose level is determined by converting the absorbance value obtained to a linear equation of the glucose standard curve [26]. Cellulase activity was calculated based on the value of reducing sugar content using the following formula (1) [27]:

$$\text{Enzyme activity (U/ml)} = \frac{\text{reducing sugar content} \times \text{df}}{\text{v} \times \text{t} \times \text{MW}} \quad (1)$$

Note: df = dilution factor
v = volume of the enzyme (50 µl)
t = time of incubation assay (120 min)
MW = molecular weight of glucose (180 g/mol).

2.3 Measurement of Protein Content

Protein content was measured using a spectrophotometer at a wavelength of 280 nm. The blank solution was measured using water containing 0.01% NaN_3 and 0.1% NaCl for large-scale production and 20 mM acetate buffer pH 5 for enzymes after purification. If the absorbance value of the sample exceeds the linear range of the instrument, which is 3.0, then a dilution is carried out [28].

2.4 Partial Purification of Cellulase Crude Extract

Cellulase dialysis was conducted using a cellulose membrane with a size of ± 12–14 kDa (12,000–14,000 cut-off) [29, 30]. A cellulose membrane containing 25 ml of crude extract of cellulase was immersed in 20 mM acetate buffer pH 5. Dialysis was carried out on a magnetic stirrer for 1x24 hours with buffer replacement at 12 h.

Two grams of DEAE Cellulose DE-52 matrix were dissolved in 100 ml of distilled water. The matrix was rinsed by applying 100 ml of 0.1 M NaOH, 100 ml of 0.5 M NaCl, 100 ml of filtered distilled water, and equilibrated using 60 ml of 20 mM of pH 5 acetate buffer. The sample was then loaded into the chromatographic column and eluted using a concentration of NaCl solution of 0–0.6 M [26].

3 Result and Discussions

3.1 Optimization of Production of Cellulase Crude Extract of *Penicillium* sp. VT11

The results of the density calculation of *Penicillium* sp. VT11 spores aged 3 days on PDA media were 5.33 x 10^6 spores/ml for the optimization of cellulase production and 5.51 x 10^6 spores/ml for large-scale cellulase production. Optimal spore density for enzyme

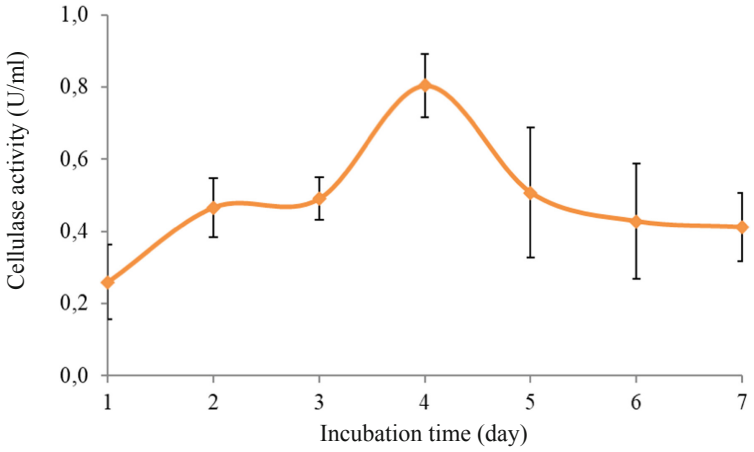

Fig. 1. Optimization of cellulase production by *Penicillium* sp. VT11 using coffee pulp under SSF method at 1–7 days incubation time.

production ranges from 10^6 to 10^8 spores/ml [31]. Enzyme activity was obtained from the measurement of reducing sugar in crude enzymes that had not been purified. Cellulase activity was obtained using 0.5% (w/v) Carboxymethyl Cellulosa (CMC) as a substrate (Fig. 1).

The optimization of cellulase production showed the optimum incubation time for cellulase production by *Penicillium* sp. VT11 was on day 4 with an enzyme activity value of 0.804 U/ml. After day 4 the enzyme activity continued to decrease. The decreased enzyme activity from day 5 to day 7. Enzyme activity with optimum incubation time on day 4 according to research by Gautam et al. [32] who reported cellulase production using *Aspergillus niger* and *Trichoderma* sp. on urban solid waste media with 1% CMC test substrate showed optimum incubation time on day 4. Khan et al. [33] Also, report Cellulase production on straw media with CMC test substrate showed optimum incubation time on day 4 using *Trichoderma harzianum, Trichoderma* spp., and *Phanerochaete chrysoportum.*

3.2 Cellulase Crude Partial Purification

Cellulase crude extract activity in large-scale production was 0.755 U/ml. This enzyme was dialyzed using cellulose membrane and then the activity was increased to 0.779 U/ml. The partial purification of the dialysis stage was also able to increase the purity by 2.249 times with the resulting yield of 114.5% (Table 1). The increased purity indicated that the purification of the dialysis stage was quite successful in separating the target enzyme from impurity molecules.

The next stage of purification is using ion-exchange chromatography. The ion-exchange chromatography technique was used to purify cellulase produced by *Penicillium* sp. VT11 after dialysis. Cellulase was applied to DEAE Cellulose DE-52 equilibrated with 20 mM acetate buffer pH 5. The bound negatively charged protein was eluted using NaCl with a concentration gradient of 0.1–0.6 M [34].

Table 1. Cellulase purification of *Penicillium* sp. VT11

Purification Stage	Volume (ml)	Activity (U/ml)	Total Activity (U/ml)	Protein content (mg/ml)	Total protein content (mg)	Specific Activity (U/mg)	*Yield* (%)	Purity fold
Crude	157	0.755 ± 0.09	118.6 ± 14.54	318.6 ± 0.1	50027.2 ± 3048.16	0.002 ± 0.0	100	1
Dialysis	170	0.779 ± 0.09	135.8 ± 14.91	177.6 ± 0.04	30192.0 ± 1360.9	0.004 ± 0.0	114.5	2.25
DEAE Cellulose								
Peak 1	43	0.138 ± 0.01	5.9 ± 0.43	18.5 ± 0.0	786.7 ± 2.87	0.008 ± 0.0	5.01	4.00
Peak 2	43	0.156 ± 0.02	6.7 ± 1.03	16.5 ± 0.0	699.9 ± 0.14	0.010 ± 0.0	4.93	5.00

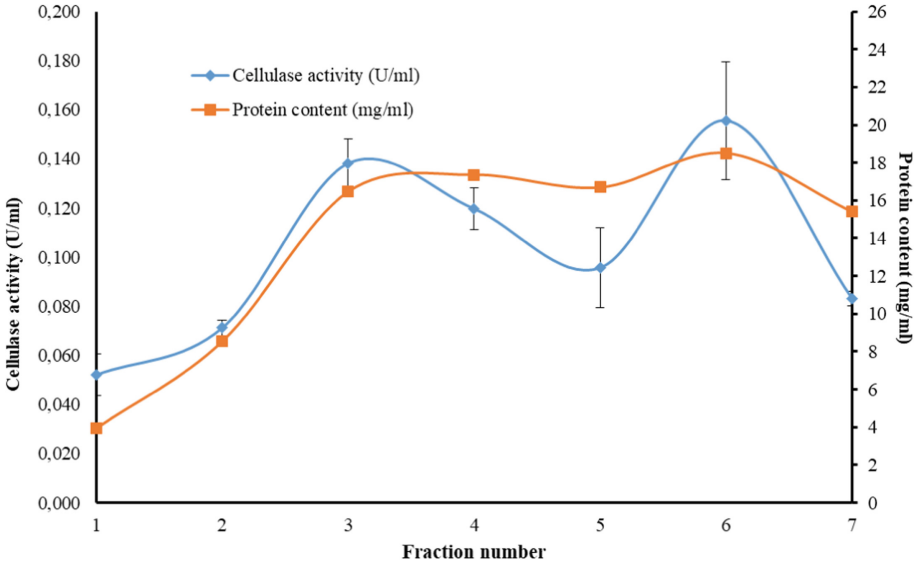

Fig. 2. *Penicillium* sp. VT11 cellulase activity and protein content after eluting using 0–0.6 M NaCl on DEAE-Cellulose DE-52.

The results of DEAE Cellulose DE-52 showed that 2 peaks emerged from elution with a gradient of NaCl concentration (Fig. 2). Peak 1 was found in fraction number 3 with 0.2 M NaCl concentration, while peak 2 was in fraction number 6 with 0.5 M NaCl concentration. This result was the same as Hamdan and Hameed's research which showed that there were two peaks from cellulase purification using DEAE Cellulose from *Trichoderma longibrachiatum* [34]. Meanwhile, Zeng et al. research [35] also showed that there were two peaks from the results of cellulase purification of *Trichoderma viriens* using Sephadex G-75 Chromatography.

Peaks 1 and 2 indicate that the cellulase activity is higher than the protein content. This indicates that the elution of the target protein due to higher cellulase activity than protein content will increase the specific activity. Cellulase produced by *Penicillium*

sp. VT11 was negatively charged because it was bound to the ionic matrix of DEAE-Cellulose which was eluted by NaCl at concentrations of 0.2 M and 0.5 M NaCl. Fraction number 6 had the highest activity of 0.156 U/ml with a purity level of 5 times. And a total yield of 4.93%. Meanwhile, fraction number 3 has an activity of 0.138 U/ml with a purity level of 4 times and a total yield of 5.01%. The specific activity value increased from the dialysis stage of 0.004 U/ml to 0.008 U/ml at peak 1 and 0.010 U/ml at peak 2. The specific activity of the enzyme indicates the purity of an enzyme, thus the greater the specific activity indicates that the purity of the enzyme is high [36, 37].

Based on the research, it can be concluded that *Penicillium* sp. VT11 can utilize the coffee pulp as carbon and nitrogen resources for their growth and produce cellulase with the optimum incubation time for cellulase production on day 4 with an enzyme activity value of 0.804 U/ml. The purification process of cellulase from *Penicillium* sp. VT11 was successfully carried out, indicated by increased cellulase purity levels of 4 and 5 times compared to cellulase crude extract. There were 2 peaks of cellulase that have been emerged in the purification process, suggesting that two kinds of cellulases were produced during solid-state fermentation. To clarify these two types of cellulases, molecular analysis is needed.

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