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Cheap Cellulase Production by *Aspergillus* sp. VTM1 Through Solid State Fermentation of Coffee Pulp Waste

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Abstract. Coffee pulp biomass waste can easily be found anywhere in Indonesia, considering it is the fourth world's largest coffee exporter. The utilization of coffee pulp is very limited and is categorized as a source of pollutants in water bodies and soils. In contrast, coffee pulp waste is very potential because 63% of the main compound is cellulose. Microbial utilization of this waste for enzyme production purposes, especially cellulase, is a breakthrough that may lead to reduce production costs. Initial investigations showed that *Aspergillus* sp. VTM1 through solid-state fermentation (SSF) could produce cellulases. Optimal cellulase could be produced if 10 g coffee pulp with 10% moisture is inoculated using 10^8 spores/mL of *Aspergillus* sp. VTM1 for 48 hours at 30 °C. Hydrolysis of 1% carboxymethyl cellulose (CMC) substrate in 50 mM acetate buffer pH 5 by this cellulase showed that the enzyme activity reached up to 1.18 U/mL. The optimum pH of the enzyme was 5 and stable at 3-3.5 and 4-7. The success of the first step of this investigation will be a cheap way of producing cellulases.

Introduction

Cellulase is an enzyme that hydrolyzes the β-1,4 glycosidic linkage of cellulose and has a wide application, especially in industries. Microbial cellulase is applied in the pulp and paper industry, food processing, detergent, textile industry, bioethanol industry, animal feed industry, and agricultural industry [1]. The high cost of production and low yield of cellulase become a complex problem for commercial production [2]. The high cost of pure cellulose substrate and some chemicals is the major factor. Therefore the use of a cheap cellulose source is needed [3].

Cellulase has been produced from various microorganisms, especially fungi and bacteria [3, 4]. The enzyme activity produced by fungi is higher than obtained by yeast and bacteria, so commercial enzyme production by fungi is preferred [5]. Nowadays, the concern about cellulase production is directed into filamentous fungi utilization. In recent years, *Aspergillus* have been extensively studied because of their ability to synthesize cellulase. Previous research reported that almost all fungi of genus *Aspergillus* synthesize cellulase, so it has the potential to be applied in the industrial enzyme [6, 7]. *Aspergillus* sp. VTM1 is filamentous fungi that were isolated from oil palm empty fruit bunch waste [8]; these fungi were reported to produce cellulases with reducing sugar products of 8.00 µg/L on the carboxymethyl cellulose substrate.

Solid-state fermentation is a state of fermentation with low water content using a solid substrate. Solid-state fermentation is the most common method used for filamentous fungi because they can grow on solid materials with low water content. Besides that, the solid-state fermentation (SSF) technique can improve the yield and reduce the enzyme production costs. The use of agricultural waste as a solid-state fermentation substrate will greatly minimize the waste and production costs [9].

Coffee pulp is an agro-industrial residue that has potential as a substrate for cellulase production, considering that Indonesia is the fourth largest coffee producing country in the world with a total production of 637 thousand tons per year [10]. Each processing of 2 tons of coffee had 1 ton of

coffee pulp [11]. Coffee pulp compounds are about 63% cellulose and other organic components, supporting microorganism growth [12]. This research is expected to reduce the cost of cellulase production and get the optimum incubation time for *Aspergillus* sp. in producing cellulase in solid fermentation. Other than that, optimum pH and stability are very important for commercial production and further purification of the enzymes.

Material and Method

Inoculum Preparation. *Aspergillus* sp. VTM1 was inoculated on slant Potato Dextrose Agar and incubated at 37 °C for 3 days. This pre-cultured was inoculated on slant alkali-pretreated coffee pulp media containing nutrients element and agar, then incubated at 37 °C for 4 days to obtain 10^8 spore density. Spore density was observed using the Petroff Hauser counting chamber. A total of 10^8 spores/mL were inoculated in the coffee pulp solid-state substrate for enzyme production [13].

Solid-State Fermentation. Solid-state fermentation contains 10 g of coffee pulp substrate in 250 mL Erlenmeyer flasks. The added water content is based on the calculation of coffee pulp water content, which is 20 mL. The flasks were sterilized at 121 °C and cooled at room temperature. About 1 mL of inoculum that contains 10^8 spores/mL was added and incubated at 30 °C for 0-168 hours [6].

Enzyme Extraction. Extraction was carried out in 24 hours with the addition of 20 mL distilled water containing sodium chloride and sodium azide [14]. Erlenmeyer flask was shaken for 12 hours and the filtrate was obtained after squeezing. Extracts were centrifuged at 8000 rpm for 5 min and the collected supernatant was the crude enzyme [6].

Enzyme Assay. Enzyme activity assay was carried out by reducing sugar analysis using the Somogyi-Nelson method. As much as 500 µL 0.5% CMC substrate in a 20 mM acetate buffer pH 5 was incubated at 37 °C for 20 minutes. Then 50 µl crude enzyme was added to the test tube and incubated at 37 °C. Somogyi reagents were added after 2 hours of incubation and boiled for 15 minutes. Five hundred microliter of the crude enzyme was added to the control tube in the boiling state. Next 500 µL of Nelson reagent and 2.5 mL of distilled water were added after the mixture was cooled down. The sample of 1.3 mL was centrifuged for 10 minutes at 8000 rpm and its absorbance was measured using a spectrophotometer with a wavelength of 500 nm. The glucose level is determined by converting the absorbance value obtained to the standard glucose curve [15].

Optimization and Stability of pH. The optimal pH of the crude enzymes was evaluated by dissolved the crude enzyme in different pH buffer. The buffer used was 20 mM acetate buffer for pH 3-5 and phosphate buffer for pH 5.5-8. Then the activity was measured by the Somogyi-Nelson method. The stability pH was tested after incubating the mixture for 4 hours at 37 °C, then the activity was measured by the same method [16].

Result and Discussion

Isolate Density. Spore density determination aims to get the optimum time in obtaining *Aspergillus* sp. VTM1 spores will be inoculated on coffee pulp solid-state substrate. Spore density calculations were performed on *Aspergillus* sp.VTM1 was grown in 5 mL slant media containing 0.1% coffee pulp extract, Na_2HPO_4, KH_2PO_4 NaCl, NH_4Cl, $MgSO_4$, and agar. The calculation curve of the spore density of *Aspergillus* sp. VTM1 could be seen in Fig.1.

Fig. 1 shows a significant increase in the spore density of *Aspergillus* sp. VTM1 from 0.2×10^8 spores/mL at 48 hours to 1.78×10^8 spores/mL at 72 hours and decreased at 96, 144 to 168 hours. The maximum spore density achieved at 120 hours was 1.79×10^8 spores/mL. Previous studies stated that the density of 10^8 spores/mL was the optimum spore density in cellulase production through solid-state fermentation [17]. So the optimum incubation time used in producing *Aspergillus* sp. VTM1 spores are at 72 hours, considering this time is the fastest time to produce 10^8 spore density. A total of 12.5 mL of spore suspension with a density of 10^8 was inoculated into 125 g of coffee pulp solid-state substrate to achieve 10^7 spores/g density in a solid-state medium. This refers to the research of Raimbault and alazard, which uses spore density 2×10^7 spores/g to

produce cellulase from *Aspergillus niger* on cassava meal solid substrates [18]. Another study was conducted using 10^7 spores/g spore density in producing cellulases from *Trichoderma harzianum* using a mixture of wheat straw (80%) and bran (20%) substrate [19].

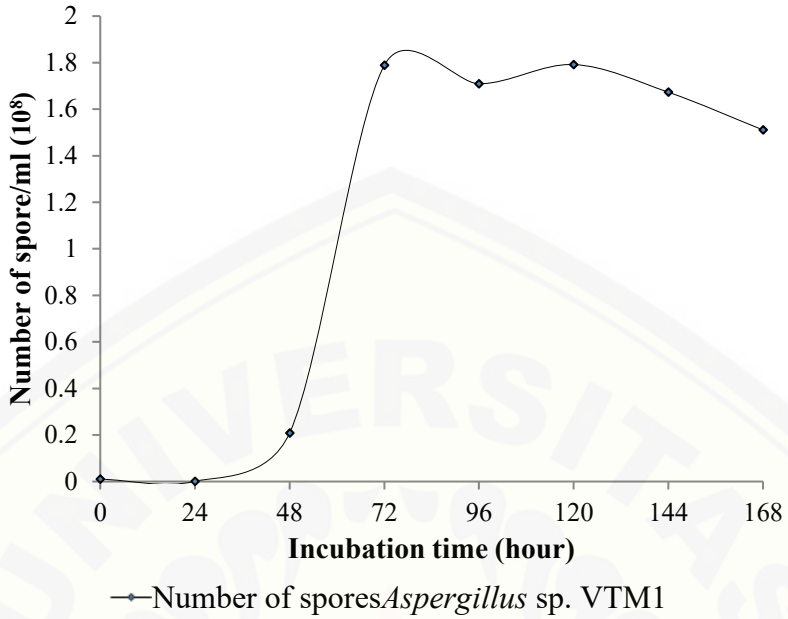

Figure 1. The number of spores (10^8)/ml of *Aspergillus* sp. VTM1

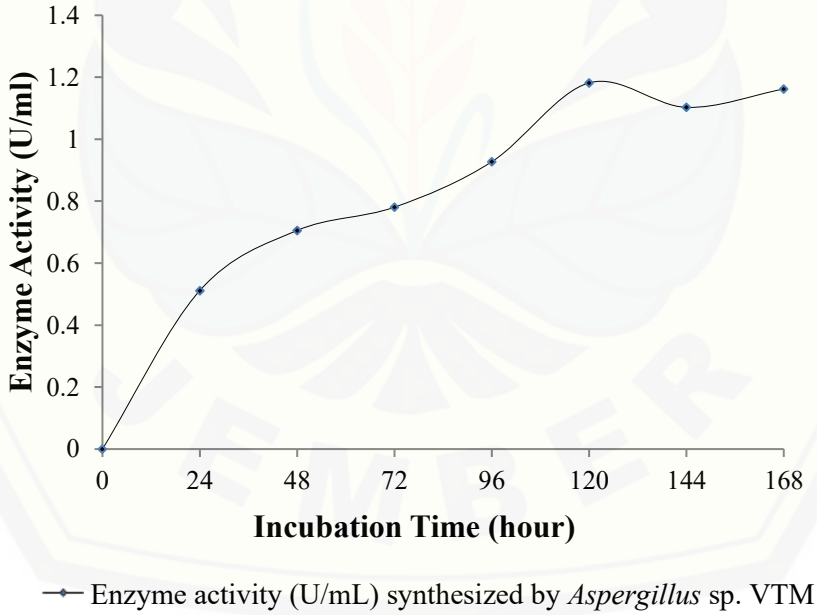

Figure 2. Effect of incubation period on cellulase activity (U/mL) by *Aspergillus* sp. VTM1

Optimization of cellulase production by *Aspergillus* sp. VTM1 under SSF. This optimization aims to know the optimum incubation period of *Aspergillus* sp. VTM1 in produce cellulase under solid-state fermentation. This information is important in large scale cellulase production. *Aspergillus* sp. VTM1 showed the ability to grow on coffee pulp media under SSF conditions; these organisms use cellulose from the coffee pulp as a primary carbon source [7,20]. The data showed that *Aspergillus* sp. VTM1 produce cellulase with different levels in different incubation periods. After measuring the enzyme activity of each crude enzyme, the results showed that the highest activity was obtained from the crude enzyme with 120 hours incubation periods. Fig. 2. shows that the activity increased steadily and reached a maximum at 120 hours of incubation that produce

activity up to 1.18 U/mL. This activity value was higher than the enzyme activity produced by *Aspergillus* sp. VTM1 on the oil palm empty fruit bunch substrate. So, it indicates that the optimization is successful.

Optimum and Stability of pH. The optimum pH for cellulase activity was found at pH 5 with the higher activity that is 0.97 U/mL. Cellulases tend to prefer acidic conditions. When it is in a strong alkaline condition, there will be a stretching of protein molecules, which causes damage and degeneration of the enzyme active center, causing a loss of cellulase enzymatic activity [21]. While its relative activity is above 80% in the range of pH 3 to 3.5 and 4.5 to 7. It was also noted that the enzyme activity was stable at a pH range of 3-3.5 and 4.5-7.0. This data is supported by the research of cellulase production from *Aspergillus fumigatus* is optimum at pH 5 and stable in the pH range 4-8 [22]. In comparison, the optimum pH of cellulase produced by *Aspergillus niger* is in the pH range 6-7 and stable at pH 5-8 [23]. Other studies have also reported that the crystalline style of cellulase enzyme is stable at acidic pH (at pH range = 3.0-5.0) [21].

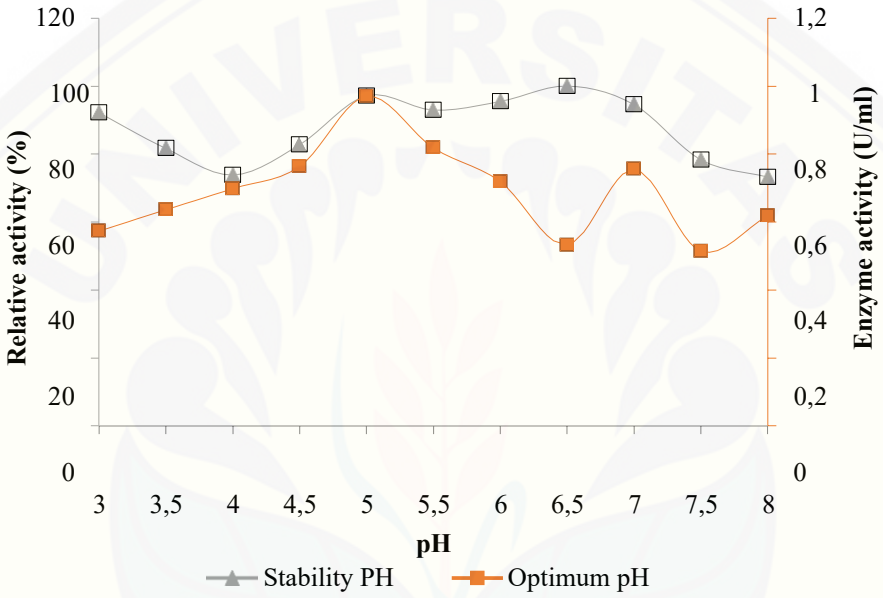

Figure 3. Effect of pH on cellulase activity (U/mL) by *Aspergillus* sp. VTM1

Summary

In this present study, we investigated that *Aspergillus* sp. VTM1 produced the highest amount of cellulase enzyme in 120 hours incubation with 10^8 inoculum density. This isolate effectively utilized the coffee pulp as a carbon source and produced cellulase up to 1.18 U/mL in 120 hours incubation. The optimum pH for cellulase activity was pH 5, and the enzyme activity was stable between pH 3-3.5 and 4.5-7. This result was suggesting that the coffee pulp has great potential for cheap substrate in the cellulase industry by *Aspergillus* sp. VTM1. However, further investigations are required to get the optimum condition for enhancing the yield, one of them is doing substrate pretreatment and enzyme purification.

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