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Preliminary Investigation of Cellulase Producer Candidate Isolate VT11 Using Coffee Pulp Waste Under Solid-State Fermentation

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Abstract. Isolate VT11 is a fungal cellulolytic isolated from vermicomposting oil palm empty fruit bunch (OPEFB). Isolate VT11 has a cellulolytic activity index of 1.0 on 1% CMC, but this isolate has never been used to produce cellulase from coffee pulp waste. The coffee pulp consists of organic components with the highest cellulose content (63%) so that it can be used as a substrate for cellulase production by fungal cellulolytic under solid-state fermentation (SSF). This study aims to know the potential of isolate VT11 as a candidate for cellulase producer. The potential of isolate VT11 as a candidate for cellulase producer was known by the cellulase activity on coffee pulp waste under solid-state fermentation. After that, cellulase was characterized by pH optimization and stabilization. Cellulase production was done by inoculating isolate VT11 in 10 g solid substrate of coffee pulp. The result shows that the isolate VT11 can potentially produce cellulase with the highest enzyme activity of 1.857 U/mL after 96 hours of incubation at 30 °C. Cellulase from isolate VT11 is optimal at pH 4.5 and stable at pH 5-6.5. Based on this result, it is suggested that the isolate VT11 can be used for cellulase production using coffee pulp waste as substrate agro-industrial residues. Further investigation such as species identification of isolate VT11, purification, and characterization of cellulase produced by isolate VT11 was needed.

Introduction

Microorganisms that can be used for cellulase production must have the ability to hydrolyze cellulose. Microorganisms like bacteria, fungi and actinomycetes are used for cellulase production and they may be aerobic, anaerobic, thermophilic, or mesophilic [1]. Microorganisms are the main organisms in cellulose degradation with total cellulose biomass of 80% of which is naturally decomposed by fungi [2]. Cellulase in fungi is structurally simpler than in bacteria. Cellulase in fungi consists of two domains, namely the catalytic domain (CD) and cellulose-binding module (CBM) [1]. Divisions in fungi known to degrade cellulose include Ascomycota, Basidiomycota, and Chytridiomycota, found in some animals' rumen [2]. Filamentous fungi capable of producing cellulase to break down cellulose are commonly called cellulolytic fungi [3]. The genus of fungi that are often used in cellulase production is the genus *Trichoderma* and *Aspergillus* [4]. Isolate VT11 is a fungal cellulolytic isolated from vermicomposting oil palm empty fruit bunch (OPEFB). Isolate VT11 has a cellulolytic activity index of 1.0 on 1% CMC, but this isolate has never been used in the production of cellulase from coffee pulp waste [5].

Enzymes of the cellulase complex, namely, endoglucanases (EG, EC 3.2.1.4), cellobiohydrolases (CBH, EC 3.2.2.91), and β-glucosidase (BGL, EC 3.2.1.21) act synergistically to hydrolysis cellulose. Endoglucanases cleaving glycosidic bonds to produces nicks in the cellulose polymer, cellobiohydrolases cleaving cellobiosyl units from the ends of cellulose chains to produce cellobiose, and β-glucosidase cleaving cellobiose to produce glucose molecules [6]. Global Industrial Enzymes reveals that the global demand for industrial enzymes is increasing. Cellulase in 2021 is estimated to increase by 4% annually to $5.0 billion [7]. Cellulase is an industrial enzyme that focuses on biocatalyses, with a contribution reaching 8% of the total global enzyme market [8]

because of its important role in various industrial applications [3]. However, due to the high price of cellulase, many studies have focused on cellulase production from cellulose's conversion biomass.

Coffee pulp is one of the abundant cellulosic biomass as agro-industrial waste in Indonesia. According to the International Coffee Organization (ICO) (2019), Indonesia is ranked as the fourth largest coffee producer in the world, with total production in 2018, reaching 612000 tons [9]. A large amount of coffee production has the potential to produce 40-45% of coffee pulp from a total production [10]. Coffee pulp consists of organic components such as 63% cellulose, 17% lignin, 11.5% protein, 2.3% hemicellulose, 1.8-8.56% tannins, 6.5% pectin, 12.4% reducing sugar, and 2% non-reducing sugar, 6.5% caffeine, 2.6% chlorogenic acid and 1.6% caffeic acid [11]. Coffee pulp has the highest cellulose content, so that it can be used as a substrate for cellulase production by fungal cellulolytic. Cellulase production is carried out using solid-state fermentation [7] or submerged fermentation [12]. Solid-state fermentation (SSF) has been defined as the fermentation process in the absence or near-absence of free water. However, the substrate must possess enough moisture to support the microorganism's growth and metabolic activity [6]. Cellulase production using filamentous fungi under SSF produces cellulase filtrate with relatively high concentrations. The solid substrate using agro-industrial residues can add economic value to lower the required cost [2]. This study aims to know the potential of isolate VT11 as a candidate for cellulase producer. The potential of isolate VT11 as a candidate for cellulase producer was known by the cellulase activity on coffee pulp waste under solid-state fermentation.

Material and Methods

Preparation of isolate VT11. Isolate VT11 is a fungal isolated from vermicomposting oil palm empty fruit bunch (OPEFB), and it becomes a collection of microbiology laboratory, biology department, faculty of mathematics and natural science, Jember University. Isolate VT11 was pre-culture on 5 mL slant Potato Dextrose Agar medium and incubated for 72 hours at 30 ℃.

Preparation of coffee pulp. The coffee pulp is a robusta coffee (*Coffea canephora*) waste from a community plantation in Silo, Jember city. The coffee pulp is dried under the heat of the sun until all parts are evenly dry. After that, the coffee pulp was smoothed using a 1 mm coffee grinder.

Delignification processes of coffee pulp. One hundred fifty grams of coffee pulp powder was added 30 g NaOH in 500 mL of aquadest. Then, homogenized using a magnetic stirrer for 24 hours. The suspension is filtered using filter paper to get the filtrate. The pH of the filtrate was measured and neutralized using acetic acid (CH_3COOH) to pH 7. The filtrate was extracted with the addition of 97% alcohol with ratio filtrate: alcohol was 4:6. The filtrate was performed by centrifugation at 8000 rpm for 10 minutes. The pellet was dried in an oven of 50 ℃ until it reaches a constant weight.

Spore density. The spore suspension was prepared on a slant medium containing M9 + 0.1 (%w/v) coffee pulp extract and incubated for 0 to 168 hours at 30 ℃. These were added 5 mL sterilized aquadest. Spore density was calculated using Neubauer Hemocytometer in a series of cell-depths (100 microns). Spore density was calculated into 10^6 to 10^8 spores/mL [13]. This step aims to determine the optimum incubation for spore density. The calculation of spore density is carried out with the following equation:

$$S \text{ (Spores/mL)} = \frac{n}{v} \; x \; fp \qquad (1)$$

Information:
S = spore density (spores/mL)
n = the average of spores in the haemocytometer area
v = haemocytometer count field volume in medium box (0,004 mm^3)
fp = the dilution factor

Preparation of inoculum. Isolate VT11 was pre-culture on slant Potato Dextrose Agar medium and incubated for 72 hours at 30 ℃. Isolate VT11 on Potato Dextrose Agar medium was inoculated on medium containing M9 + 0.1 (%w/v) coffee pulp extract and incubated according to the optimum incubation for spore density in the previous step. Five mL of medium containing M9 + 0.1 (%w/v) coffee pulp extract aim to adapt cells before inoculated to the solid substrate.

Solid-state fermentation for cellulase production by Isolate VT11. Solid-state fermentation media made by 10 grams of coffee pulp with 20 mL of water content (1:2) (%w/v) [14]. The water content of coffee pulp is based on the previous result of coffee pulp water content calculation. The spore density for inoculum used ranges from 10^6 to 10^8 spores/mL because it's optimal spore density for enzyme production [13]. These were inoculated with 1 mL suspension of spores and incubated at 30 ℃ for 24 to 168 hours. The crude enzyme in solid substrate of coffee pulp was harvested through filtration every day [15] by adding 20 mL aquadest containing 0.01 % Na Azide + 0.1% NaCl (v/v) [16] for stop the fermentation process. After that, incubated on a shaker for 12 hours at 30 ℃. The crude enzyme was harvested by centrifugation to separate the medium from the fungi. The crude enzyme was centrifuged at 10000 rpm for 15 minutes.

Enzyme Activity. Enzyme activity was performed by reducing sugars assay using the Somogyi-Nelson method on 0.5 (%w/v) CMC containing acetate buffer pH 5 20 mM. Five hundred µL Somogyi reagent was entered to 50 µL crude enzyme. Then, it was boiled for 15 minutes. The mixture after cold added 500 µL Nelson reagent and 2.5 mL aquadest. The crude enzyme was performed by centrifugation at 8.000 rpm for 10 minutes. Reducing sugar assays are measured using a spectrophotometer at a wavelength of 500 nm for a 1 mL sample. The reducing sugar is determined by plotting to the standard glucose curve and enzyme activity calculated based on the value of reducing sugar using the following equation:

$$\text{Enzyme activity } \left(\frac{\text{U}}{\text{mL}}\right) = \frac{\text{reducing sugar x fp}}{\text{v x t x BM}} \quad (2)$$

Information :
fp = the dilution factor
v = volume (mL)
t = incubation time (second)
BM = molecular weight (g/mol)

Optimization and stabilization of pH. Optimization and stabilization of pH from 3 to 8 with a pH range of 0.5. The pH optimization was performed by incubating the mixture of 250 µL crude enzyme and 250 µL buffer at a certain pH for 4 hours at 37 ℃. The sample was added 500 µL 0.5 (%w/v) CMC containing acetate buffer pH 5 20 mM. Then, it was incubated for 2 hours at 37 ℃. pH stabilization is done by adding 500 µL 0.5 (%w/v) CMC containing acetate buffer pH 5 20 mM to a mixture of 250 µL crude enzyme and 250 µl buffer at a certain pH. Then, it was incubated for 2 hours at 37 ℃. 500 µL Somogyi reagent was added to the sample. Then, it was boiled for 15 minutes. Sample after cold added 500 µL Nelson reagent and 2.5 mL H_2O filtration. The sample was performed by centrifugation at 8000 rpm for 10 minutes. Reducing sugar assays are measured using a spectrophotometer with a wavelength of 500 nm for a 1 mL sample.

Results and Discussion

Spore density. Spore density calculation aims to determine the spores inoculums that will be inoculated on a solid substrate. The amount of inoculum is one of the important factors in the success of enzyme production [17]. The amount of inoculum can affect the metabolism of fungi [18]. The relation of spore density and incubation time (Fig. 1) to show increased spore on 96 hours incubation.

Isolate VT11 is growing and multiplication cell until it reaches its peak at 96 hours. The spore density curve was showed that the incubation from 0 hours until 72 hours was a lag phase. There was no significant increase in spore density because of fungi in the adaptation phase. The optimal

spore density for enzyme production ranges from 10^6 to 10^8 spores/mL [13]. The optimal spore density produced by isolate VT11 was 2.48 x 10^6 spores/mL after 96 hours of incubation at 30 ℃. These were an exponential phase, which is the multiplication phase of cells, which used inoculum for cellulase production. After 96 hours of incubation, spore density was decreased. The decrease of spore density because of limited nutrition in the medium, so that suppresses the physiological of the fungi.

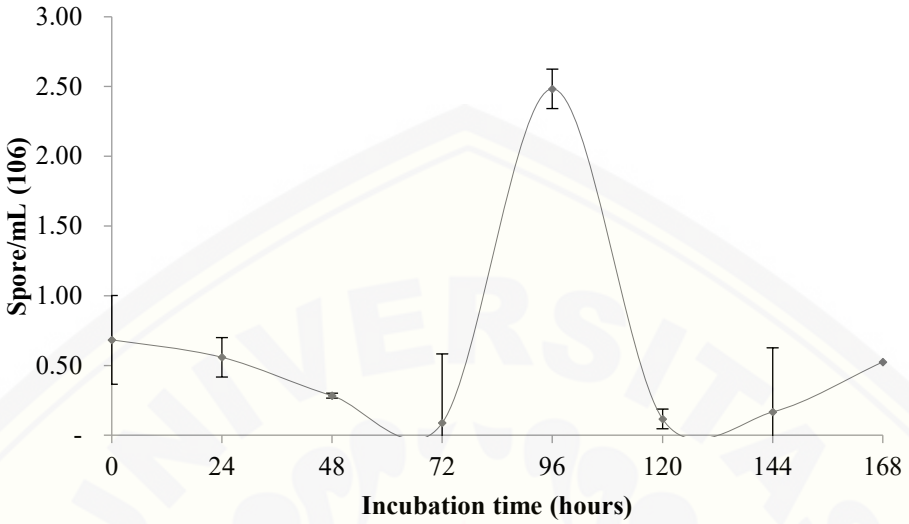

Figure 1. Spore density curve isolate VT11

Optimization of cellulase production using coffee pulp under SSF. Cellulase activity states how much the ability of the cellulase to hydrolysis cellulose to glucose. Cellulase activity obtained is the result of the measurement of reducing sugars assay from crude enzyme. The supernatant used for measuring sugar assay is a crude enzyme that has not been purified. The effect of incubation time towards cellulase activity was presented in Fig. 2.

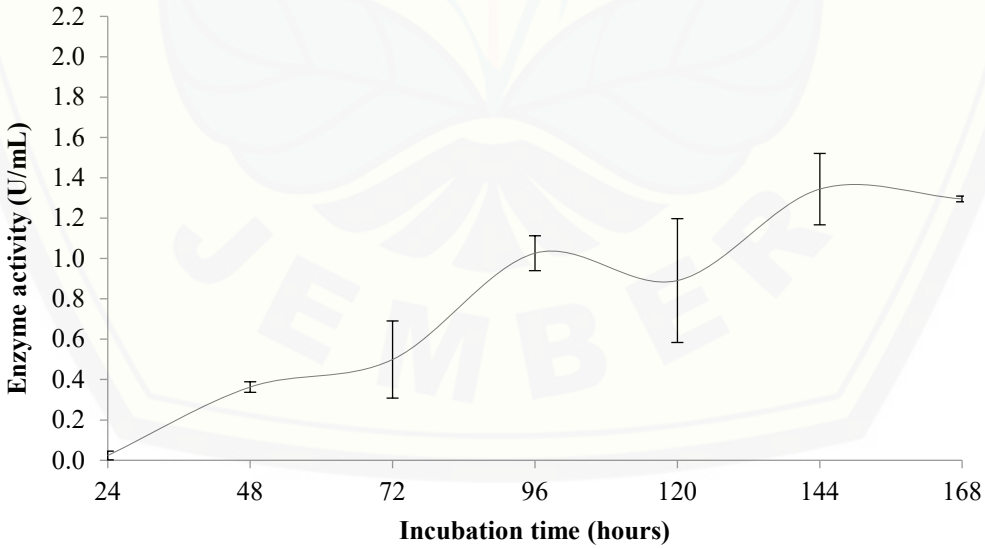

Figure 2. Effect of incubation time for cellulase activity isolates VT11 on coffee pulp waste under SSF

The optimization of cellulase production was achieved at an optimum incubation time of 96 hours, with an enzyme activity value of 1.026 U/mL (Fig. 2). The enzyme activity continued to increase after 24 hours until 72 hours of incubation and peaked at the highest activity value at 96 hours. It was the log phase, which is the multiplication phase of cells, so that the cellulase activity of isolate VT11 increase. Similarly, the highest cellulase activity from *Trichoderma viridae* is optimum after 96 hours of incubation [19]. Even though the result showed that the highest cellulase

activity after 144 hours of incubation. This is because the optimization of cellulase production looks for high cellulase activity in a relatively shorter incubation time. After 144 hours of incubation, cellulase activity is relatively stable because the decrease and increase are not significant.

Enzyme characterization. Cellulase production by fungal cellulolytic is affected by several environmental factors such as pH. Among physical parameters, the pH plays an important role in enzyme activity. The effect of pH on cellulase activity was investigated using 0.5 (%w/v) CMC in acetate buffer pH 5 20 mM (Fig. 3). The crude cellulase by isolate VT11 was active in a broad pH range between pH 3 and 8. The optimal pH varies with different microorganisms and enzymes. Cellulase enzyme from *Bacillus* sp. is also active in the range pH of 3-9 [20].

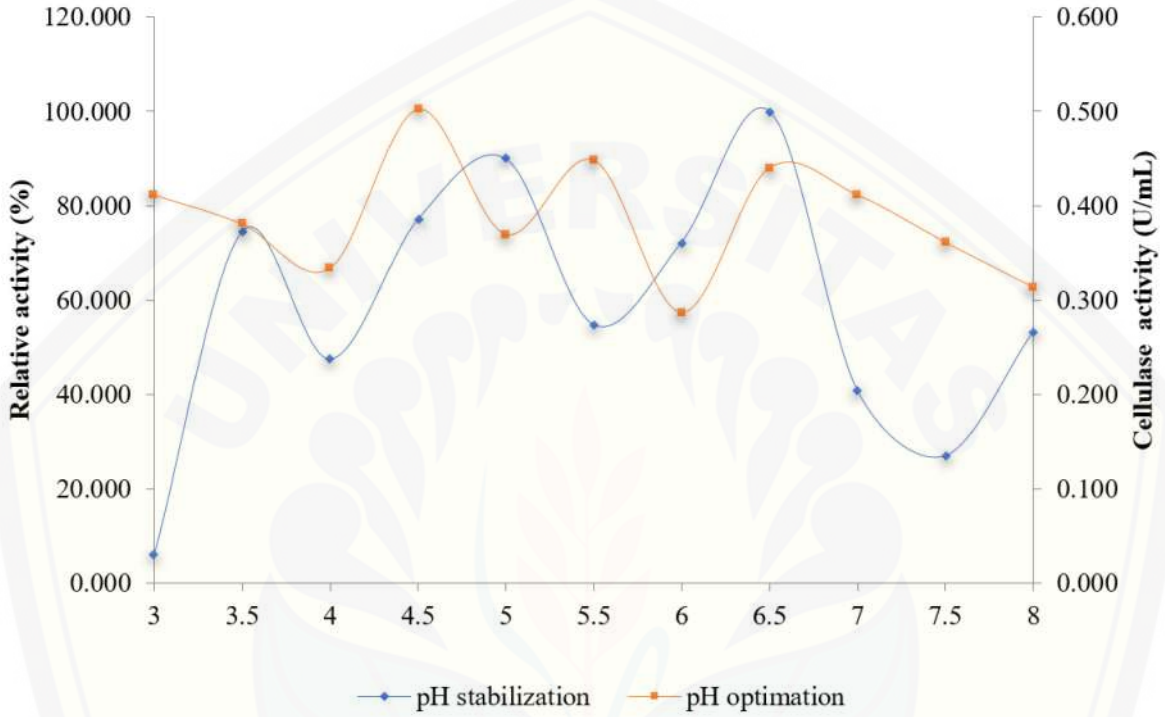

Figure 3. Effect of pH for cellulase activity by isolate VT11 on coffee pulp under SSF

The pH optimation was performed by cellulase activity (U/mL), while the pH stabilization was performed by relative activity (%) of cellulase. The curve showed that the production of cellulase by isolate VT11 is optimum at pH 4.5. This result is similar to a cellulase enzyme from Aspergillus sp, which is stable at pH 4.5 [12]. The maximum cellulase activity was found at pH 6.5, decreasing significantly at pH 7 to 8. The curve showed that the production of cellulase by isolate VT11 was stable at pH 5 to 6.5. This result was in accordance with the cellulase enzyme from *Trichoderma viridae*, which is stable at pH 5-6 [19] and cellulase enzyme from *Bacillus* sp. at pH 5 to 6.5 [21]. The cellulase activity was decreased when it was treated under pH 5 or above pH 6.5. Cellulase from bacteria is more stable than cellulase from fungi at alkaline conditions [22].

Summary

Isolate VT11 has the potential as cellulase producer using coffee pulp under SSF. The optimal spore densiry produced by isolate VT11 was 2.48×10^6 spores/mL after 96 hours of incubation. The highest cellulase activity produced by isolate VT11 was 1.026 U/mL after 96 hours of incubation at 30 °C. Cellulase by isolate VT11 optimal at pH 4.5 and stable at pH 5-6.5.

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