Pectinase Production of *Aspergillus* sp. VTM5 Through Solid State Fermentation Using Coffee Pulp Substrate and Its Purification

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Abstract. Hydrolytic Pectinase is an important enzyme and is widely used in several industries. In the previous investigation, this enzyme could be produced by *Aspergillus* sp. VTM5 under solid-state fermentation and the coffee pulp waste substrates were used. The specific activity of about 0.4 U/ml of pectinase was detected when 40gr coffee pulp water-saturated inoculated with $1.2x10^7$ spores of *Aspergillus* sp. VTM5 was incubated at 30 °C for 168 h, and without any nutrients added. Against 0.5% alkaline extract of pomelo pulp substrate in 20 Mm acetate buffer pH 5, pectinase activity increases up to 1.03 U/ml, and the purity 3.5 times, after dialyzed using 12–14 kDa, pores cellulose membrane tube higher than previously step. Open column purification using Fractogel EMD SO3-(S) cation exchange chromatography, pectinase was eluted at 0.45 M NaCl. The pectinase activity significantly increased to 0.295 U/ml with 12.37-fold and 17.04% yield. Based on these results, it can be proved that coffee pulp waste is suitable as a substrate for pectinase production by *Aspergillus* sp. VTM5. Further research to analyze the properties and characterize the stability of pectinase against temperature, pH, the effect of heavy metals, and molecular analysis is needed.

Keywords: Coffee pulp · Pectinase · Production · Purification

1 Introduction

Aspergillus was one of the fungi that have been known as enzyme producers. These fungi are characterized by an extraordinary capability to produce and secrete large amounts of proteins, metabolites, and organic acid into their growth medium [1]. *Aspergillus* species have a high ability to secrete pectinase [2], such as *Aspergillus niger* V22 B35 can produce pectinase with an activity of 4.77 U/mL in coffee pulp under solid state fermentation [3]. *Aspergillus* sp. VTM5 is one of the fungi isolated from the waste of Palm Oil Empty Fruit Bunch (POEFB). This fungus has been known to hydrolyze POEFB and produce a reducing sugar of 5.57 μg/ml [4]. Other than that, this fungus

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was also known to be able to produce pectinase using coffee pulp waste as a substrate through solid-state fermentation [5].

Coffee pulp is one of the main by-products of coffee humid processing because the amount produced is quite large (approximately 40% f.w) [6]. However, the content of coffee pulp as waste is categorized as still high, especially for the growth of microorganisms such as fungi. Based on Frometa et al. [7], The content in coffee pulp includes reducing sugars (5.4% of dry pulp), proteins (9.4% of dry pulp), pectins (20.5% of dry pulp after Alcohol Insoluble Solid (AIS) fractionation), hemicellulose (9.2% of dry pulp after AIS fractionation), cellulose (35.6% of dry pulp after AIS fractionation) and caffeine (1.4% of dry pulp). This high content of pectin in coffee pulp is an inducer for the production of pectinase by fungi.

Pectinase is the enzyme that breaks down polygalacturonic acid by breaking glycosidic bonds [8]. Pectinase is one of the enzymes that have an important role in the industrial sectors, which is about 10% of the total enzyme production in the world [9]. These enzymes are used as a facilitator for the extraction process, filtration, and clarification to increase yield in the production of fruit juices and beverages. Pectinase is extensively used in the food processing industry, souring of cotton, degumming of plant fibers, wastewater treatment, vegetable oil extraction, tea and coffee fermentation, bleaching of paper, in the alcoholic beverage [10]. According to the cleavage site, pectinase is divided into three groups (1) pectin hydrolase; (2) pectin lyase, and (3) pectin esterase [11, 12]. Pectinase is naturally found in plants and produced by microorganisms with the main source of industrial enzymes being microorganisms. Fungi produce 50% of the enzymes from the total enzymes produced by industry [13]. One of the fungi that have been known to produce pectinase is *Aspergillus* sp. VTM5.

Aspergillus sp. VTM5 based on Hidayah et al. [5] can produce pectinase with a reducing sugar concentration of 85.9 μg/ml after 72 h incubation using coffee pulp as a substrate through solid-state fermentation. Crude pectinase has a stable pH range of 3–5, while the optimum pH is 4.5. However, in Hidayah et al. [5] research, large-scale production process and purification of pectinase have not been carried on. Therefore, this research aims to produce and purify pectinase from *Aspergillus* sp. VTM5 is based on coffee pulp through solid-state fermentation.

2 Materials and Methods

2.1 Inoculum Preparation

Pure culture of *Aspergillus* sp. VTM5 was isolated from POEFB [4] and obtained from a previous researcher. This isolate can grow in Potato Dextrose Agar (PDA) medium at a temperature of 30 °C and incubation time of 72 h. The culture was then maintained in the PDA slant medium and sub-culture after 30 days.

Aspergillus sp. VTM5 was inoculated in a slant of Potato Dextrose Agar and incubated for 72 h at 30 °C as pre-culture. After 72 h, spore suspension of *Aspergillus* sp. VTM5 was counted using a hemocytometer by harvesting the spores after adding 5 ml sterile distilled water to get 10^6 spores/ml [14].

2.2 Preparation of Substrate Pomelo Pulp Alkali Extract

Pomelo pulp was used to obtain pectinase substrate. This substrate was used to assay pectinase activity. Pomelo pulp was dried and sieved into powder. 50 g pomelo pulp powder was delignified using 20 g of NaOH and 500 ml distilled water, then homogenized with a magnetic stirrer for 24 h. The mixture was filtered to get the filtrate. The filtrate was adjusted to pH 7 using acetic acid. The filtrate was precipitated using 97% ethanol and centrifuged at 8.000 rpm for 10 min to obtain the pellets. This pellet was dried at 50 °C. The dry matter as pomelo pulp alkali (PPA) substrate [15, 16].

2.3 Crude Pectinase Production

Coffee pulp was used as a substrate for pectinase production. 5 g dried coffee pulp in the Erlenmeyer flask was added with distilled water until water content reaches 60%. Then sterilized using an autoclave for 25 min at 121 °C, 1 atm [17, 18]. This substrate was used as Solid-State Fermentation (SSF) medium. Optimization of pectinase production was done by inoculating *Aspergillus* sp. VTM5 with 10^7 spores/ml (10% spores of inoculum) in 5 g of SSF medium and incubated for 0–168 h at 30 °C. Crude of pectinase was harvested every 24 h by shaking at room temperature for 12 h. In this step, 10 ml H_2O contains 1% NaCl and 0.01% NaN_3. The supernatant as a crude enzyme source was obtained by centrifugation at 8.000 rpm for 5 min [14, 19] Large-scale production of pectinase was carried out using 40 g of the coffee pulp as SSF medium and the same method as an optimization process.

2.4 Enzyme Assays

Reducing sugars were measured by the Somogyi-Nelson method [20]. 50 μl crude pectinase was added to 500 μl of 0.5% PPA 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. One ml supernatant was measured using a spectrophotometer at a wavelength of 500 nm [14, 21]. One unit of enzyme activity was defined as the amount of enzyme that produced reducing sugar at a rate of 1 μmol per minute [15, 22].

2.5 Partial Purification of Crude Pectinase

Purification steps were carried out using 20 mM acetate buffer pH 5. The initial stages in the purification process, namely the dialysis process use a 12–14 kDa pore cellulose membrane tube to remove the remaining sugar and polysaccharides dissolved. 45 ml crude pectinase was put into 2 cellulose membrane tubes (25 and 20 ml). Crude pectinase in the membrane tube was dialyzed in 500 mL of 20 mM acetate buffer pH 5 and stirred for 24 h. The buffer was changed every 12 h [23, 24].

The next step in purification was using Fractogel EMD SO3-(S) cation exchange chromatography. 1.5 ml of the dialyzed enzyme was applied to Fractogel EMD SO3-(S) matrix which was equilibrated with 20 mM acetate buffer pH 5. Purification was done by eluting the protein using a gradient concentration of 0.25 to 0.5 M NaCl, and for each

fraction, the volume was 0.7 ml [25]. The enzyme activity of each step was measured using the Somogyi-Nelson method. Calculation of specific activity (1), yield (%) (2), and purification fold (3) of pectinase produced from each step were calculated based on the following equation [26]:

$$\text{Specific activity (U/mg)} = \frac{\text{Total activity (U/mL)}}{\text{Total Protein (mg/mL)}} \quad (1)$$

$$\text{Yield (\%)} = \frac{\text{Total activity (U/mL)}}{\text{Total activity of crude (U/mL)}} \text{x } 100 \quad (2)$$

$$\text{Purification fold} = \frac{\text{Specific activity (U/mg)}}{\text{Specific activity of crude (U/mg)}} \quad (3)$$

Protein value (4) was measured using a spectrophotometer with a wavelength of 280 nm. The protein value was divided by the path length cuvette [27] and counted using the following formula:

$$\text{Protein value (mg/ml)} = \frac{\text{The absorbance of 280 nm}}{\text{Path length cuvette (1 cm)}} \quad (4)$$

3 Results and Discussion

3.1 Pectinase Production by *Aspergillus* sp. VTM5

Optimization of enzyme production was done to determine the optimum incubation time of *Aspergillus* sp. VTM5 produces pectinase. Optimum incubation time was known by counting the reducing sugar level produced by *Aspergillus* sp. VTM5 [5]. Optimization of pectinase production by *Aspergillus* sp. VTM5 used coffee pulp as a substrate with a 60% water level. The water level corresponds to the growth of the fungi. A good water level for SSF is in the range of 12–18% [28]. Based on Darah et al. [29], the optimum water level for pectinase production was 60%. The water level that is high or too low will inhibit the growth of microorganisms so it will affect enzyme production.

The inoculum used in the pectinase production by *Aspergillus* sp. VTM5 has spore density $1.2\text{x}10^7$ spores/ml. The pectinase maximum activity was obtained with an inoculum amount of $1\text{x}10^7$ spores/ml [30]. The most optimum number of spore densities for pectinase production by *Aspergillus niger* LFP-1 was $1\text{x}10^7$ spores/ml. Inoculum with higher spore density will cause a decrease in pectinase production because the inoculum concentration is too high affected by competition between cells for nutrients [29]. The optimization of pectinase production by *Aspergillus* sp. For 168 h can be seen in Fig. 1.

The result of *Aspergillus* sp. VTM5 pectinase activity in the optimization process is shown in Fig. 1. The pectinase activity of *Aspergillus* sp. VTM5 continued to increase along with the length of fermentation time and reached the highest and optimum at 168 h with the activity of 0.624 U/ml. The optimum incubation time of polygalacturonase production by *Aspergillus niger* was 168 h in a mixed medium of wheat bran and apple pulp [31] Akhter et al. [32] reported that the most optimum incubation time of pectinase

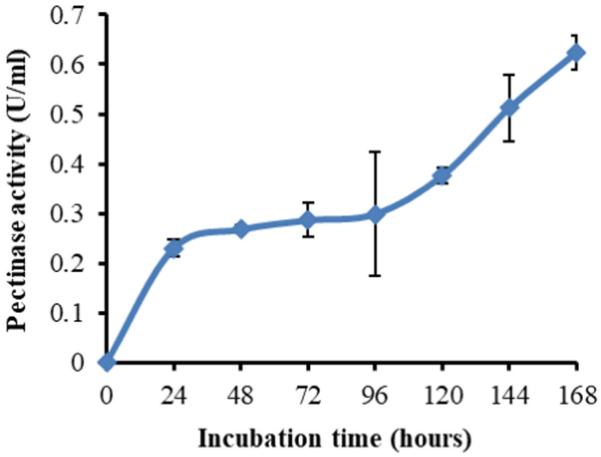

Fig. 1. Optimization of pectinase production by *Aspergillus* sp. VTM5 at 0–168 h incubation time.

produced by *Aspergillus niger* was found at 168 h in a mixed medium of wheat bran and rice husk.

The result of pectinase production optimization was used to determine the incubation time for large-scale production of pectinase by *Aspergillus* sp. VTM5. Pectinase activity of large-scale production by *Aspergillus* sp. VTM5 after 168 h of incubation was about 0.4 U/ml.

3.2 Partial Purification of Pectinase

Purification of crude pectinase from *Aspergillus* sp. VTM 5 was carried out through 2 steps, namely dialysis and Fractogel EMD SO3-(S) cation exchange chromatography. Dialysis was carried out using a 12–14 kDa pores cellulose membrane tube. Therefore, pectinase which has a size of 35–80 kDa will be retained on the membrane tube. Meanwhile, molecules, that are smaller than the pores, will diffuse out of the membrane [33, 34].

The pectinase activity of the crude after dialysis was 1.03 U/ml with 0.514 U/mg of specific activity, 102% yield, and the purification 3.5 times more than crude pectinase (Table 1). This result indicates that pectinase activity after dialysis increased more than before. During the dialysis process, there is a molecule transfer other than protein from the dialysis tube to the buffer solution through the membrane pores. This movement causes the protein inside the tube to become purer so that their activity increases [24, 35].

Further step purification of pectinase was done by cation exchange chromatography which aims to separate proteins based on their charge [36]. Dialyzed pectinase was put into the Fractogel EMD SO3-(S) chromatography column which was pre-equilibrium using 20 mM acetic buffer pH 5. Protein was eluted using a gradient concentration of 0.25 to 0.5 NaCl (Fig. 2).

Based on the result, it was found that there was only one peak as an active fraction (5^{th} fraction) with 0.45 M of NaCl. The pectinase activity in this fraction was 0.295 U/mL and 1.821 U/mg of the specific activity. This indicates that the pectinase of *Aspergillus*

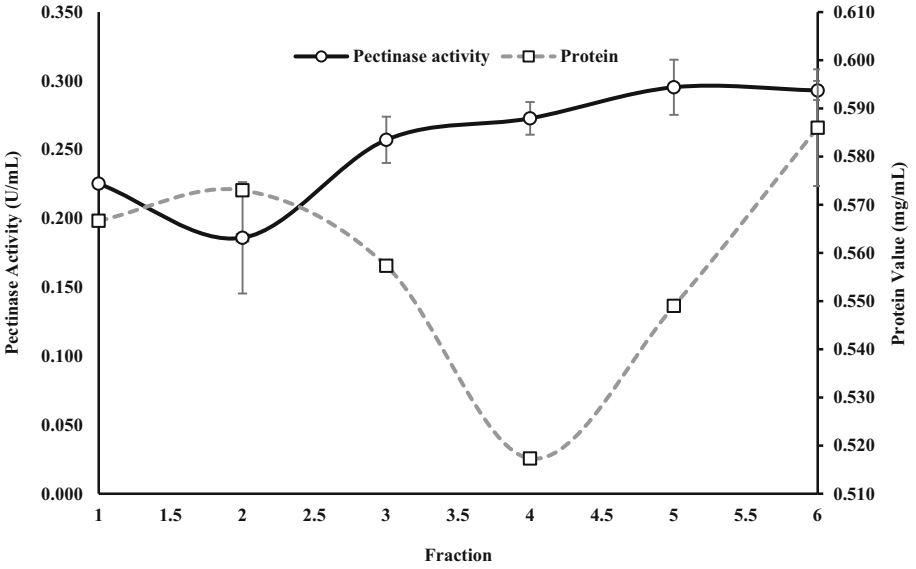

Fig. 2. *Aspergillus* sp. VTM5 pectinase activity and protein value after purification using Fractogel EMD SO3-(S) (x-axis from left to the right based on numerical order: 0.25; 0.3; 0.35; 0.4; 0.45; 0.5 M NaCl).

Table 1. Comparison of pectinase activity at various stages of purification

Purification steps	Volume (mL)	Activity (U/mL)	Total Activity (U/mL)	Protein (mg/mL)	Total protein (mg/mL)	Specific activity (U/mg)	*Yield* (%)	Purification fold
Crude pectinase	132	0.41	54.12	2.79	367.62	0.15	100	1
Dialysis	135	1.03	138.98	2.00	270.14	0.514	102	3.5
Fractogel cation chromatography	22.5	0.295	6.63	0.55	3.64	1.821	17.04	12.37

sp. VTM5 could be eluted by 0.45 M NaCl. The level of purity of pectinase in this fraction reached 12,37 times compared to the purity level of the crude pectinase before purification (Table 1) with 17.04% of yield.

Based on Table 1 it can be known that specific activity and purification fold of pectinase at each purification step increased. These results indicated that the purification process carried out in this study succeeded in separating the target enzyme, pectinase, from other proteins so that pectinase became purer. By a study by Septiani [37], the increased specific activity value indicates an increase in the purity of the enzyme.

Based on the results, it can be concluded that *Aspergillus* sp. VTM5 can produce pectinase using coffee pulp as substrate through solid-state fermentation. The best incubation time for producing pectinase by *Aspergillus* sp. VTM5 was 168 h. The purification process of pectinase from *Aspergillus* sp. Was successfully carried out, indicated by the

increase in the purity of pectinase reaching 12.37 times compared with the crude pectinase. Further research to analyze the properties and characterize the stability of pectinase against temperature, pH, the effect of heavy metals, and molecular analysis is needed.

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