Pectinase Production by *Aspergillus* VTM4 Induced by Pomelo Pulp (*C. maxima* Merr.) As Substrate

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Abstract. Pectinase is a heterogeneous enzyme that hydrolyzes pectin, which is currently in high demand in the industry, so a strategy is needed to produce it economically. *Aspergillus* VTM4 is a mold that can grow on pomelo pulp and produce pectinase. Under solid-state fermentation, the crude pectinase was produced with the activity of 1.6 U/ml when 10 gr of dried pomelo pulp was inoculated by 10^7 spores of *Aspergillus* sp. VTM4 and incubated for 48 h at 30 °C. After dialysis using a cellulose membrane tube 12–14 kDa, the pectinase activity was increased by 0.182 U/ml. Further purification step on the DEAE-Cellulose DE-52 open column with a gradient of NaCl 0–0,6M in 20 mM acetate buffer pH 5 was carried out. The peak fraction as pectinase activity increased to 0.369 U/ml with 1,516 purification fold and 5,3% yield. Based on these results, it can be proved that pomelo pulp can be used as a substrate for pectinase production by *Aspergillus* VTM4. 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. Complete steps and results of this investigation are discussed in this paper.

Keywords: *Aspergillus* sp. VTM4 · Pectin-rich pomelo pulp · Pectinase

1 Introduction

Orange peel is the outermost structure of citrus fruits that are often not utilized. Orange peel is composed of epicarp (flavedo) and mesocarp (albedo). Pomelo (*C. maxima*) is a type of orange that has the thickest albedo [1]. One method of processing orange waste that has been developed is fermentation. Fermented orange peel is used for the manufacture of ethanol, methane [2], and citric acid [3].

Pectinase is a heterogeneous group of enzymes that hydrolyze pectin [4]. Pectin is a type of heteropolysaccharide with the main chain of galacturonic acid polymers bound to α-1,4-glycosidic [5]. Pectin is one of the organic polymers that are widespread in

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pomelo fruit (*Citrus maxima*). The content of pomelo pectin is quite large, which is 42.5 [6]. The enzyme pectinase hydrolyzes pectin in the bonds of α-1.4 glycosidic to galacturonic acid and galacturonic methoxyl. Pectinase decomposes the substance of pectin through depolymerization and de-esterification reactions [7].

The need for pectinase enzymes is increasing in the industrial sector including the food and animal feed industry, textile industry, wastewater treatment, and paper industry [4]. Pectinase can be produced by microorganisms such as bacteria, squash, and yeast. Fungi have been widely developed in the production of enzymes [8]. Extracellular enzymes are secreted by the fungi through an apical part of hyphae that spreads widely to the substrate [9]. The use of whenever in producing enzymes have the advantages of other cheap growth media [10], is not susceptible to genetic manipulation, and is environmentally friendly [11].

The production of pectinase by fungi through solid fermentation can be used as an alternative to the production of environmentally friendly enzymes. The ability of the fungi to produce enzymes is widely developed to increase the useful value of organic waste by making it a growth substrate [8]. One of the fungi successfully isolated [12] from the substrate of POEFB (Palm Oil Empty Fruit Bunch) is VTM4 isolate. Preliminary test results showed that the VTM4 isolate positively breaks down the pectin medium so that it indicates the isolate can secrete pectinase. It is been observed that the isolate VTM4 can produce pectinase from coffee pulp waste [13].

An orange peel substrate containing abundant amounts of pectin will induce the production of pectinase by fungi [14]. The pectinase activity produced by the VTM4 isolate as a selected isolate of the POEFB substrate has never been studied, so research is needed. The goal of the study was to identify VTM4 isolates up to the genus level and measure the pectinase activity of VTM4 isolates in the solid-state fermentation of pomelo pulp substrate (*C. maxima* Merr).

2 Materials and Methods

2.1 Rejuvenation of Aspergillus VTM4

Fungi isolate *Aspergillus* VTM4 is rejuvenated on PDA media (Potato Dextrose Agar) using the scratch method, then incubated at 30 °C for 3 days.

Pure culture of *Aspergillus* sp. VTM5 was isolated from POEFB [12] and obtained from a previous researcher. *Aspergillus* VTM4 is rejuvenated on PDA media (Potato Dextrose Agar). *Aspergillus* VTM4 were inoculated using the streak method. These Isolate were then incubated at 30 °C for 3 days.

2.2 Production of Pectin Extract

The albedo portion of pomelo pulp (*C. maxima*) is cut then dried and mashed. Pomelo pulp powder as much as 50 g mixed with NaOH 1 M in 500 ml of water for the delignification process. The mixture is stirred until homogeneous, then filtered. Filtrate plus CH3COOH 1M solution until it reaches pH 7. Furthermore, the filtrate is precipitated using 97% alcohol (4:6), then concentrated at 8000 rpm for 10 min. The pellets are dried in a 57 °C oven for 7 days. Pectin extract is mashed using mortar [15].

2.3 Qualitative Assay of Aspergillus VTM4 Pectinolytic Activity

A qualitative assay was used to know the pectinolytic activity of *Aspergillus* VTM4 based on a clear zone on the growth medium. *Aspergillus* VTM4 (3 days old) was injected as much as 1 loop into 10 ml of M9 + pectin extract 0.1% medium and incubated at 30 °C for 3 days. Furthermore, the isolate is tested using iodine 0.33% to find out the absence of clear zones. The presence of clear zones indicates the secretion of pectinase by this isolate, resulting in a hydrolysis process in the areas that form clear zones [16].

2.4 Determination of Saturated Water Content of Pomelo Pulp Substrate (*C. maxima*)

As much as 2 g of pomelo peel powder is packaged in a used tea bag. Then soaked in distilled water for 2 h, after that hang it until no water drips. The sample was weighed as a wet weight value and then dried using an oven at 57 °C. Every 7-h interval the dry weight of the sample was measured.

The water saturation content of pomelo pulp was obtained from the difference between the wet weight and dry weight of the sample

$$\text{Water saturation level}(\%) = \frac{\text{wet weight - dry weight}}{\text{Tdry weight}} \times 100\% \quad (1)$$

2.5 Microscopic and Macroscopic Characterization of Aspergillus VTM4

Microscopic characteristic of *Aspergillus* VTM4 was observed using the slide-culture method. The observations were done using a microscope and an optilab camera.

Macroscopic characteristics were observed by taking 1 loop of VTM4 isolate and inoculating it on a PDA medium plate using the dot method. Then incubated at 30 °C for 3 × 24 h.

2.6 Crude Pectinase Production

The SSF medium used 1 g of pomelo peel powder that has been added with 5.6 ml distilled water (according to the results of the measurement of water content). Optimization of pectinase production was done by inoculating 500 μl of *Aspergillus* VTM4 with a spores density of 10^6 spores/ml and incubated for 0–168 h at 30 °C. Optimization of pectinase production was done to obtain the best incubation time of *Aspergillus* VTM4 which could produce pectinase with optimum activity.

Crude of pectinase was harvested every 24 h by shaking at room temperature for 12 h. Harvesting crude extracts of enzymes using a solution of NaCl 1% and NaN_3. A 2 ml NaN_3 solution is added to a solid fermentation culture medium at the harvesting age. The next culture is shaken and filtered [17, 18]. Crude extract of enzymes is concentrated at a speed of 8000 rpm for 10 min [19]. Large-scale production of pectinase was carried out using 10 g of the pomelo pulp as an SSF medium, 10^7 spores/ml of inoculum's spore, and the same method as an optimization process.

2.7 Enzyme Activity Assay

A total of 500 µl of 0.5% pectin substrate in a 20 mM pH 5 acetate buffer was incubated at 37 °C for 20 min. The test sample after 20 min added 50 µl of the crude pectinase and then incubated. Furthermore, Somogyi reagents are added to as much as 0.5 ml to stop the reaction between the substrate and the crude pectinase. The sample was boiled for 15 min [20]. The control sample added crude pectinase as much as 50 µl when the sample was boiled. 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 [21].

2.8 Partial Purification

The purification process was carried out through two processes, dialysis and anion exchange chromatography. The first process, dialysis, aimed to separate protein based on the size of the molecule [22]. The dialysis used a 12–14 kDa pore cellulose membrane tube. A total of 50 ml of the crude enzyme is put into a cellulose membrane tube. Crude pectinase was dialyzed in 500 mL of 20 mM acetate buffer pH 5 (changed every 12 h) and stirred for 24 h at 25 °C.

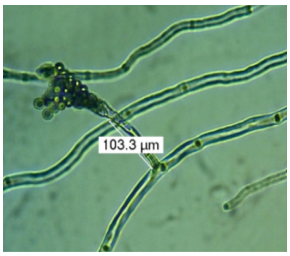
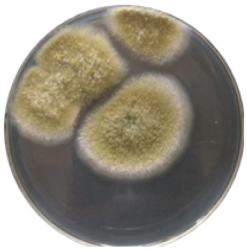
The second process was purification using DEAE Cellulose DE-52 anion exchange chromatography. A total of 40 ml of the dialyzed enzyme was applied to the DEAE Cellulose DE-52 matrix which has been regenerated using 100 ml of distilled water, 100 ml NaOH 0.1 M, and 100 mL of NaCl in 20 Mm acetate buffer pH 5. Purification was done by eluting the protein using a gradient concentration of 0 to 0.6 M NaCl, and for each fraction, the volume was 5 ml [23]. The enzyme activity of each step was measured using the Somogyi-Nelson method and the protein value was measured using a spectrophotometer with a wavelength of 280 nm.

3 Results and Discussions

3.1 Macroscopic and Microscopic Characteristics of VTM4 Isolates

VTM4 isolate was isolated from empty bunches of palm oil by Yuniar [12] in his research on cellulolytic fungi in the process of vermicomposting POEFB at the University of Jember. Microscopic and macroscopic observations of VTM4 isolates on PDA media showed the colony of VTM4 isolates was yellowish-green. While microscopic insulated VTM4 has the characteristics of septa, round-shaped vesicles, and stipe measuring approximately 104 µm. Stipe grows to form metulae measuring approximately 54 µm round and becomes oblong (columnar) as it ages. Fungi isolated VTM4 has a cylindrical phialide, conidia (spores) shaped round green. Based on the observed characteristics VTM4 isolates have many similarities with the characteristics of *Aspergillus* [24, 25]. Fungi and Food Spoilage are two characteristics typical of Aspergillus namely stipe formed from short cells infertile hyphae and stipe usually without septa so that vesicles stipe and foot-cell form a single cell that is very large (Table 1).

Table 1. Characteristics of Fungi Isolate VTM4

Picture of observation	Characteristic
	Hyphae: hyalin septa Stipe: elongate Vesicle: circular Metula: biseriate Phialide: cylinder Conidia: circular
	Colony color: yellowish green Surface: convex Reserve: white Shape: compact Edge: entire Exudate drop: exist

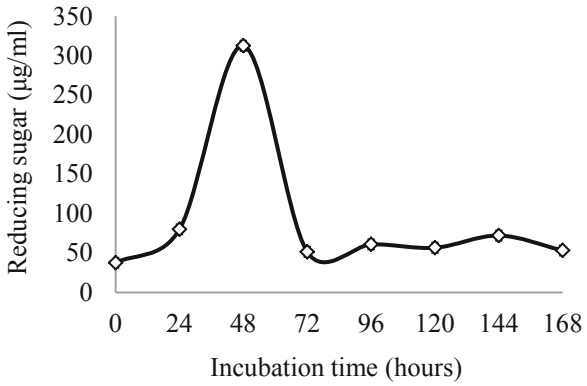


Fig. 1. The production optimization curve of crude extract pectinase when isolates VTM4.

3.2 Optimization and Production of Crude Extracts of Enzymes

Optimization of the production of crude extract enzymes is done to find out the incubation time of VTM4 isolates that can produce a rough extract of pectinase with high activity. The results of optimization of rough extract production of pectinase showed the highest activity occurred on the 2nd day of incubation with activity reaching 2.315 U/ml (Fig. 1). The results of the optimization curve show instability of enzyme activity after optimum enzyme activity on the second day.

The substrate of the fermentation media is an orange peel measuring 30 mesh. The saturated water content of the substrate is 85% or about 5.65 ml/g. The water content is following research conducted by Rahman *et al*. [26] which examined the saturated content of water produced by orange peels which are about 80–90%. The saturated condition of the water is condensed as its original condition (fresh orange) as a VTM4 insulated substrate in solid fermentation. Large-Scale Production of Enzyme Crude Extracts.

Data optimization of the production of crude extracts of enzymes is used as a reference for the production of large-scale enzyme crude extracts. Large-scale production results showed the value of pectinase activity reached 1.600 U/ml after 48 h. The production activity value of large-scale pectinase crude extract is lower than the value of pectinase activity when optimization. The decrease in value is due to the difference in the amount of spore density between optimization and large-scale production. The density of spores that is calculated when large-scale production is 1×10^7 spores/ml. This amount is greater than the density of spores calculated when optimizing (6×10^6 spores/ml). According to Mukhtar *et al*. [27], the overly high number of inoculums and toxins produced during fermentation by cell death results in low enzyme yields. Another possibility is because during the solid fermentation process also produced proteases (proteinases) that break down proteins.

3.3 Partial Purification of Pectinase

The production of large-scale pectinase crude extracts is partially purified using dialysis and anion exchanger chromatography. Dialysis of enzyme crude extract is done using a dialysis membrane tube (cellulose) measuring 12–14 kDa resulting in decreased enzyme activity to reach 0.18 U / ml. The decrease in activity is due to proteins in large-scale production that non-target proteins react with test substrates, so the reaction is expressed in sugar reduction and enzyme activity. Udenwobeleetal's [28] purification process allows the selection of non-target proteins and increases enzyme-specific activity.

Further purification used DEAE Cellulose DE-52. The DEAE Cellulose DE-52 matrix removes non-target proteins through its ion bonds. The stages of the purification process using this matrix are, equilibration, binding, elution, and regeneration. This stage was done using 20 Mm acetate buffer pH 5. The results of purification showed the highest enzyme activity in the fraction produced from the NaCl 0.2 M eluate reached 0.369 U/ml (Fig. 2). That indicates that at the concentration of NaCl 0.2 M, the target protein can be released most than at other concentrations. In anion-exchange chromatography, Cl^- competes with negatively charged analytes to bind with the positively charged stationary phase, thereby eluting the analytes [29].

The results of enzyme activity tests from each fraction showed there was still pectinase activity in fractions 1–3. The high activity in the free fraction indicates that there are still many target proteins that are not bound by the membrane so the purity of the enzyme is significantly increased. But this purification can be said to be successful because the specific activity produced is increasing.

Table 2 showed that the specific activity and purification fold of pectinase in each purification step increased although the increase was small. Several factors influence the enzyme purification process including column packing material. particle size and pore diameter of the support. column length. mobile phase. and temperature. especially the

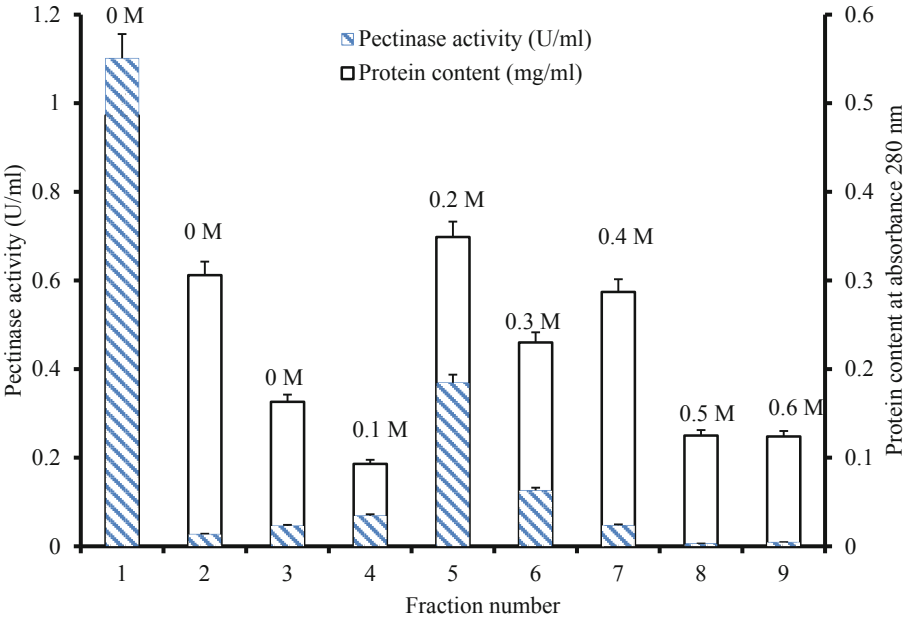


Fig. 2. The purification curve uses DEAE Cellulose DE-52.

Table 2. Purification of pectinase from VTM4 isolate

Purification step	Volume total (ml)	Enzyme activity (U/ml)	Activity total (U)	Protein content (mg/ml)	Protein total (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude enzyme	320	1.60	512.00	42.07	13461.33	0.038	100.00	1.00
Dialysis membrane cellulose	294	0.18	53.58	4.60	1354.24	0.040	10.465	1.04
DEAE cellulose DE-52	74	0.37	27.16	6.15	452.64	0.060	5.304	1.52

isoelectric point of the enzyme to be separated. This is because the choice of the appropriate ion exchange resin for a particular enzyme. separation depends on the isoelectric point of the enzyme to be separated [30].

Based on research it can be concluded that the characteristics of isolate VTM4 belong to the genus *Aspergillus*. *Aspergillus* VTM4 can produce pectinase using pomelo pulp under solid-state fermentation with the optimum was found on the 2nd day with enzyme activity reaching 1.600 U/ml. The purification process of pectinase from *Aspergillus* VTM4 was successfully carried out. indicated by the increase in the purity of pectinase

although the increase was small. 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 to increase the activity of pectinase.

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Authors' Contributions. ONG carried out all the experiments. analyzed data. and drafted and wrote the manuscript. TB participated in the writing of the manuscript. KM participated in the design of the study. and manuscript revision. and become the corresponding author. All authors read and approved the final manuscript.

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