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Effect of *Penicillium chrysogenum* Concentration on The Production and Characteristic of Pectinase Enzyme from Pineapple (*Ananas comosus*) Peel Waste

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ABSTRACT

This study aimed to determine the effect of various concentrations of Penicillium chrysogenum *isolates on the production of pectinase enzymes from pineapple peel waste. The experiment was arranged in CRD (completely randomized design) with six different isolate concentrations (4%; 8%; 14%; 16%; 20%; and 24%). The experiment constituted of three stages, namely production of enzymes by using* Penicillium chrysogenum *isolates, enzyme harvesting with a goal to obtain a crude extract of the enzymes, and enzyme characterization. Parameters in this study included enzyme activity, optimum temperature, optimum pH, Km value, and Vmax value. Results showed that the highest yield (1.87 g) was obtained from the 24% treatment and the lowest in the 4% treatment at 1.63 g. The highest enzyme activity in the 24% treatment was 1.924 U/ml and the lowest was in the 4% treatment, namely 0.949 U/ml. The highest Vmax value was 0.690 U/ml from the 24% treatment and the lowest was 474 from the 4% treatment. Conversely, the highest Km (0.040 mg/ml) value was found in 4% treatment and the lowest (0.016 mg/ml) was observed in 24% treatment.*

1. INTRODUCTION

Pectinase enzyme is an important material used in degrading plant cells to improve the quality and yield of juice production. The pectinase enzyme is a biocatalyst that is safe to use and is utilized by juice drink industries. The use of pectinase enzymes will increase the clarity and quality of the juice because the pectinase enzyme functions as a beverage clarifier that can degrade pectin so that the resulting drink becomes clear (Sharma *et al.*, 2014).

Based on the nature of the catalyst, pectinase enzymes are classified into 2, namely pectinase depolymerase enzymes and pectinase esterase enzymes (Panji *et al.*, 2015). The pulp is destroyed by the pectiolytic depolymerase enzyme. The ongoing depolymerase of pectin indicates the activity of hydrolysis of pectin substrate by pectiolytic enzymes in the

intercellular space and the primary cell wall in the polysaccharide structure (Cornuault *et al*., 2018). Pectiolytic enzymes cause tissue damage, pulp disintegration due to hydrolysis of pectin, release of seed piles and formation of liquid. The performance of the pectinase enzyme in damaging the polysaccharide substrate is shown by the breakdown of polygalacturonic acid into monogalacturonic acid by releasing glycosidic bonds (Oyeletke *et al*., 2012).

The need for pectinase enzymes is currently fulfilled through imports from abroad. To reduce imports, pectinase enzymes can be produced naturally from plant tissues. The process of forming the enzyme pectinase can be conducted with the help of fungi or bacteria. Pineapple (*Ananas comosus*) peel waste has the potential as a source of pectinase enzyme production because it contains natural pectin which can be broken down by bacteria or fungi into pectinase enzymes (Anggraini, 2013). According to Respati (2016), pineapple production in 2015 reached 1.73 tons/year. With a large number of pineapple fruit production, the waste generated will be a new problem for the company. Problems caused by pineapple peel can be overcome by utilizing it as a source of pectin which has the potential to produce pectinase enzymes.

According to Banik & Ghosh (2008), pectinase enzymes can basically be produced naturally using bacteria or fungi. The pectinase enzyme can be produced through the isolation of *Bacillus sp* and *Streptomyces sp* bacteria and the isolation of *Aspergillus sp* and *Penicillium sp*. Based on research, filamentous fungi that can be used as starters to convert pectin into pectinase enzymes are *Aspergillus niger*, *Aspergillus layori*, *Penicillium chrysogenum*, *Penicillium restriksi*, *Trichoderma viride*, *Mucor piriformis*, and *Yarrowia lipolytica*. The advantage of this microbe is that it can be used in both wet (submerged) and dry (solid state) fermentations for the production of various important industrial products. The advantages of using *Penicillium chrysogenum* mold microbes are that they can be grown in a variety of wet and dry media conditions, are easy to breed, and have high enzyme production yields (Chowdhury *et al*., 2017). The fungi is often used to produce enzymes in solid media because it has the advantage of being easy to grow in liquid or solid media (Mufarrikha *et al*., 2014). Filamentous fungi are more profitable if used in solid media fermentation because using natural media that is singular will produce a low level of contamination, simpler inoculum preparation, incubation conditions resemble natural conditions, can produce higher concentrations, and optimum aeration between particles and media (Muhpidah, 2013). This study aims to determine the effect of the concentration of *Penicillium chrysogenum* bacteria on the enzyme product produced and to determine the characteristics of the pectinase enzyme produced.

2. MATERIALS AND METHODS

The research was carried out during June – September 2021 at the Agriculture and Business Laboratory, Universitas Kristen Satya Wacana, Salatiga, Indonesia.

2.1. Materials and Equipment

The materials used in this study were distilled water, PDA (potato dextrose agar) media, apple pectin, acetate buffer (pH 4.0; 4.5; 5.0; 5.5), honey pineapple peels, *Penicillium chrysogenum* isolate, $NaNO_3$, KH_2PO_4, $MgSO_4$, KCL, $CaCL_2.2H_20$, $FeSO_4.7H_2O$, and DNS (Dinitrosalicylate) reagent. The DNS method is used to analyze the enzyme activity, reducing sugar, pH, optimum temperature, *Km* and *Vmax* values. The equipment used in this research were analytical balance, oven, beaker glass, erlemeyer, spectrophotometer, cuvette, measuring cup, measuring flask, test tube,

funnel, burette and static filter paper, magnetic stirrer, needle loop, autoclave, pH meter, shaker, centrifuge, inkas, hand refracto meter.

2.2. Design of Experiment

This study used a randomized block design (RBD) with *Penicillium chrysogenum* suspension treatment to produce pectinase enzyme. The concentration of *Penicillium chrysogenum* suspension was 0%, 4%, 8%, 14%, 16%, 20% and 24%. The data from the analysis were analyzed using analysis of variance (ANOVA) and if there was a significant effect, then it was continued using the LSD test at the level of α = 5%.

2.3. Pectinase Enzyme Production

Pineapple peels that have been washed clean was cut into pieces with a size of 1x1 cm then dried using an oven at 45 °C until dry. Dried pineapple peels was pulverized using a grinder until it becomes powder. Preparation of the medium for the production of pectinase enzymes was was carried out by mixing pineapple peel powder with mineral solutions of $NaNO_3$, KH_2PO_4, $MgSO_4$, KCL, $CaCl_2.2H_2O$, and $FeSO_4.7H_2O$. All ingredients are put into a 1000 ml Erlemeyer and added with distilled water up to the tera limit.

Penicillium chrysogenum which was grown in PDA slant media was then suspended in 2 ml of sterile distilled water using an ose needle. The production of pectinase enzymes 1, 2, 4 and 6 ml of inoculum *Penicillium chrysogenum* was covered with sterile cotton and plastic wrap and then fermented at 40 °C for 96 hours. The fermentation termination process during enzyme harvesting was carried out by using 100 ml of 0.1% twin 80 solution into the media. The enzyme filtrate was centrifuged at 3000 RPM for 10 min. The resulting enzyme filtrate was stored at 4 °C.

2.4. Pectinase Enzyme Characterization

The characterization of the pectinase enzyme includes several stages. First, testing the enzyme activity. Enzyme activity was obtained from the calculation of the resulting reducing sugar. The second stage is the determination of the optimal temperature and pH. The optimal temperature is the temperature with the highest enzyme activity produced. While the optimal pH is the pH that produces the highest enzyme activity. The last step is determining the enzyme kinetics to obtain the values of *Km* and *Vmax*.

2.4.1. Pectinase Enzyme Activity

The enzyme produced from pineapple peel waste was taken as much as 0.05 g for testing reducing sugar content. One ml of 5% apple pectin was taken, and then added with acetate buffer pH 5. Apple pectin which had been added with acetate buffer was heated in a water bath for 50 minutes at a temperature of 50 °C. Add 1 ml of distilled water and 2 ml of DNS reagent to the solution. Reheat in a water bath for 15 minutes at a temperature of 100 °C. Put the solution into a 25 ml volumetric flask and add distilled water up to the measuring mark. Test the reducing sugar content of the solution with a wavelength of 495 nm on a spectrophotometer. The resulting sugar concentration is used to calculate the enzyme activity through Equation (1):

$$AE = \frac{[GP]\ x\ FP\ x\ 1000}{t\ x\ BM} \quad (1)$$

where *AE* is crude pectinase enzyme activity (U/ml), [*GP*] is concentration of reducing sugar which is obtained from the standard curve (mg/ml), 1000 is a conversion factor, *FP* is dilution factor, *t* is incubation time (min), and *BM* is molecular weight of galacturonic acid (212 g/mol).

2.4.2. Optimal Temperature

The pectinase enzyme from pineapple peel was taken as much as 0.1 ml and mixed with 0.9 ml of reagent media. The reagent media is a mixture of 0.7% (w/v) citrus pectin and sodium acetate buffer solution of 0.025 M pH 4.8 which has been incubated at 35, 45, 50, 55 and 60 °C for 30 min. DNS method was used for sample analysis.

2.4.3. Optimal pH

The pectinase enzyme from pineapple peel was taken as much as 0.1 ml and mixed with 0.9 ml of reagent media. The reagent medium was a mixture of 0.7% (w/v) citrus pectin and sodium acetate buffer solution of 0.025 M pH 4.8 which had been incubated at 37 °C for 30 minutes at pH 4.0, 4.5, 5.0, and 5.5. Samples were analyzed by using DNS method.

2.4.4. Enzyme Kinetics

Enzyme kinetics is determined by the graph obtained from the relationship between substrate concentration [*S*] and enzyme activity (*V*). Pectinase enzyme from pineapple peel was taken as much as 0.05 g and then mixed with 10 ml of reagent media. Citrus pectin reagent medium was used as a substrate with a concentration of 2%; 4%; 6%; 8%; 10%, 12% with 0.05 M buffer solution NaCl 0.15 (pH 4.5). The solution was incubated at 37 °C for 30 minutes. Experiment was performed with 4 repetitions. Samples were analyzed by using DNS method. The values of *Km* and *Vmax* were determined from a curve of 1/[*S*] vs. 1/*V* to get linear relationship in the form of:

$$\frac{1}{V} = \frac{1}{Vmax} + \frac{Km}{Vmax}\left(\frac{1}{[S]}\right) \quad (2)$$

which can be presented in a simple form as:

$$\boldsymbol{Y = a + bX} \quad (3)$$

with $Y = 1/V$ = 1/ enzyme activity = (enzyme activity)$^{-1}$ = (enzyme rate)$^{-1}$; $X = 1/[S]$ = 1/ substrate concentration = (substrate concentration)$^{-1}$; $a = 1/Vmax$; and $b = Km/Vmax$.

The values of *a* and *b* were got from linear regression of 1/*V* vs. [1/*S*]. The values of *Vmax* and *Km* (½ *Vmax*) were determined from the above relation as $Vmax = 1/a$ and $Km = b.Vmax$.

3. RESULTS AND DISCUSSION

The parameters used in the research on the production and characterization of the pectinase enzyme by the fungus *Penicillium chrysogenum* were temperature, pH, enzyme kinetics, and enzyme activity. Table 1 shows the experimental results.

3.1. Enzyme Production

The first step before carrying out enzyme production is to regenerate *Penicillium chrysogenum* isolates into PDA slant medium and stored in incubation for one week. and to get new, better ones. This regeneration is to get molds that are still active in cell division so that in the enzyme production process using good quality molds so that they can accelerate the enzyme production process (Muhpidah, 2013). Selection is made on molds that produce a lot of spores and high fungal growth. This phenomenon can be seen from the number of spores that grow on the surface of the PDA slant

media. Mold spores were suspended in sterile distilled water with concentration treatment of 0%; 4%; 8%; 14%; 16%; 20% and 24%. The treatment was applied to the substrate media in the form of pineapple peel powder which had been added with minerals. Fermentation is carried out for 48-96 hours so that the pectinase produced is more optimal. After 96 hours, the enzyme product produced will decrease (Abdullah *et al*., 2018). The pectinase enzyme is harvested by stopping the fermentation process. The crude extract of the pectinase enzyme was obtained from the extraction process in the form of a filtrate (Yulianti & Herawati, 2020).

Table 1. Pectinase yield from pineapple peels and its characteristic

Isolate concentration (%)	Pectinase enzyme parameters			
	Pectinase yield (gram)	Enzyme activity (U/ml)	*Km* (g/ml)	*Vmax* (U/ml)
0%	1.59 a	0.188 a	0.04 b	0.083 a
4%	1.63 a	0.949 b	0.035 b	0.474 b
8%	1.72 b	1.251 c	0.033 b	0.520 c
14%	1.77 c	1.432 c	0.029 b	0.579 d
16%	1.80 c	1.714 d	0.026 ab	0.605 d
20%	1.82 d	1.836 de	0.02 ab	0.684 e
24%	1.87 e	1.924 e	0.016 a	0.690 e
Coefficient of variance	2%	7%	21%	4%

Note: The numbers followed by different lowercase in the same column show a significant difference at the 5% level according to the LSD test.

In Table 1, it can be seen that the treatment inoculum concentration of *Penicillium chrysogenum* resulted in a significant difference in the yield of the enzyme produced. The highest yield of pectinase enzyme was obtained from a concentration of 24% with an average of 1.87 g and the lowest yield was produced from a concentration of *Penicillium chrysogenum* mold inoculum 4% as much as 1.59 g. This study showed that the higher the concentration of *Penicillium chrysogenum* mold, the higher the yield of enzyme or biomass. This is due to the higher the concentration of molds or microbes used, the higher the pectin in the substrate which can be degraded by microbes optimally. Chowdhury *et al*. (2017) stated that molds can grow optimally, faster and higher if they are at higher concentrations which are balanced with substrate requirements so as to produce high biomass. In addition, supporting factors such as temperature and pH also affect the amount of production produced. A temperature of 40 0C and a pH of 5.5-6 is the optimum temperature for the production of pectinase enzymes. This is in accordance with the opinion of Banu *et al*. (2010) who reported that the production of the enzyme pectinase by *Penicillium chrysogenum* was higher at pH 5 to 5.5 and with an optimal temperature of 35 to 40 °C.

3.2. Enzyme Activity

The activity of the pectinase enzyme was tested using the DNS method. The DNS method tested the pectin substrate which was esterified by polygalacturonase into galacturonic acid monomer. The results of enzyme activity are related to reducing sugars because enzyme activity is the amount of energy used to produce glucose during the process of forming the pectinase enzyme (Rohishoh, 2012). Enzyme activity was analyzed by calculating the value of enzyme activity (AE) at the end of harvesting

enzymes by the fungus *Penicillium chrysogenum*. The results of this study showed the highest enzyme activity at a concentration of 24%. This is in accordance with the statement of Yulianti & Herawati (2020) that with the addition of the concentration of mold inoculum can increase enzyme activity. Table 1 shows that the treatment of mold concentrations resulted in significantly different pectinase enzyme activities. At a mold concentration of 24%, the highest calculation of enzyme activity was 1,924 U/ml among other treatments. On the other hand, at a concentration of 4%, the calculation of the lowest enzyme activity was 0.949 U/ml. The enzyme activity shown in Table 1 shows that the higher the concentration, the higher the activity. This is consistent with the results of other studies where the optimum concentration of mold balanced with a suitable substrate will produce more pectinase enzymes (Barman *et al*., 2015).

3.3. Optimal Temperature

According to Sieiro *et al*. (2012) the optimum temperature for the reaction rate of the pectinase enzyme is 40 to 75 °C. Enzymes that are able to work at optimum temperatures at thermophilic temperatures can be used in the clarification process. High temperature causes kinetic energy to increase and enzyme activity to decrease due to loss of primary structure in covalent bonds.

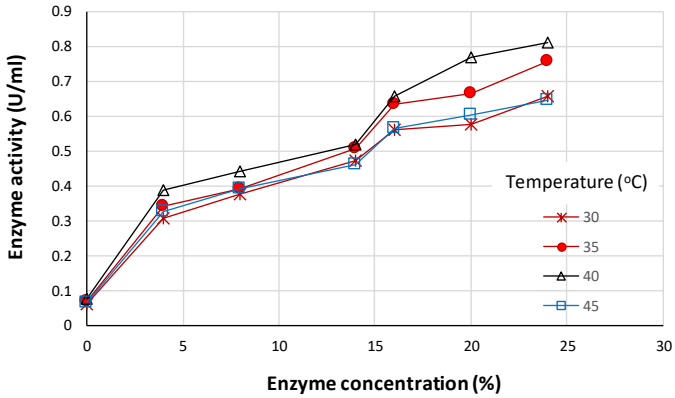

Figure 1. Relationship of enzyme activity and enzyme concentrations at various temperatures

The optimum temperature obtained in this study was 40 °C and the worst temperature was at 45 °C. This happens because at a temperature of 40 °C and above is a thermophilic temperature which causes damage to the enzyme structure from the loss of the primary structure and covalent bonds of enzyme activity. The breaking of the covalent bonds has no effect on the rate of the enzyme. This is because the higher the temperature, the faster the rate of the enzyme. According to Buga et al. (2010) (cited in Faizah, 2017) the activity of the pectinase enzyme is influenced by temperature and 40 °C is the optimal temperature. According to Khatri *et al*. (2015) the optimal temperature of the pectinase enzyme is in the range of 30-42 °C and is supported by Deshmukh et al. (2018) the optimal temperature is in the range of 35-40 °C. This condition results from the collision between the active sites of the molecule without causing damage to the enzyme. In addition, along with the increase in temperature, the speed of the enzyme is directly proportional to the increase in temperature. The optimal temperature for *Penicillium chrysogenum* isolates is in the range of 37 °C (Sudeep *et al*., 2020).

3.4. Optimum pH

pH is an important factor in the production of pectinase because it affects the type and content of enzymes produced by fungi. In our study the maximum activity of 0.675 U/ml was at a concentration of 24% with a pH of 5.5. Either an increase or decrease in pH beyond the optimum value indicates a decrease in enzyme production. This is in accordance with statement of Sarkar (2014) that the optimum pH of *Penicillium chrysogenum* is at pH 5.5. Increasing pH levels significantly affect enzyme production. In our case, enzyme activity increased from 0.311U/mL to 0.675 U/mL when the pH raise from 4.0 to 5.5. Banu et al. (2010) reported that the enzyme production by *Penicillium chrysogenum* was higher at pH 5.5. Similarly, Piccoli-Valle *et al*. (2001) observed that high polygalacturonase and pectin esterase activity were shown by *P. griseoroseum* at more acidic pH 4.5 and 5.

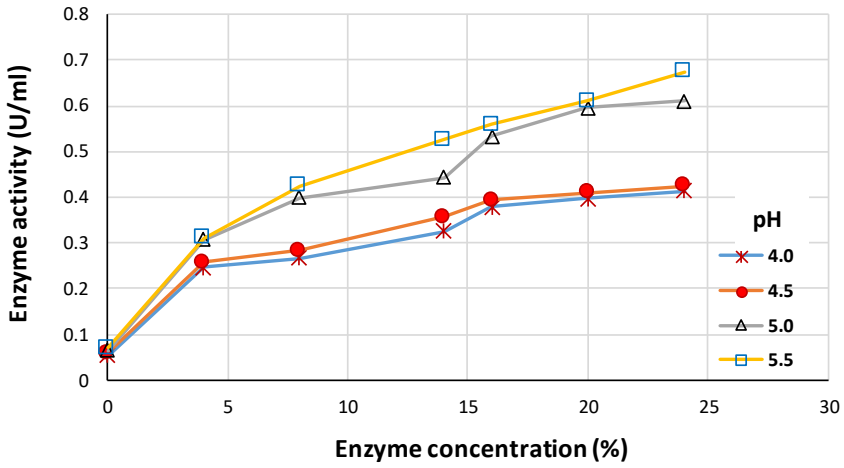

Figure 2. Relationship of enzyme activity and enzyme concentrations at various pH

3.5. Enzyme Kinetics

Enzyme kinetics is one of the important parameters in the study of enzyme characteristics. The Michaelis-Manten constant (*Km*) is the ability of the substrate concentration to have half the active site filled or the enzyme speed has reached half the maximum speed or the enzyme reaction rate has reached *Vmax*. The value of *Vmax* can be measured from the speed that can no longer change or increase. Enzyme affinity is high when the *Km* value is small, and conversely the affinity is low when the *Km* value is high. The affinity value is also influenced by various factors such as optimal temperature, the type of substrate [S] used, and the ionic strength (Putra, 2009).

Table 1 shows the results of the analysis of *Km* and *Vmax* values where the highest *Km* (0.040 g/ml) value was observed at treatment of 4% fungi concentration and the smallest *Km* value (0.016 g/ml) was arose from treatment of 24% concentration. The highest *Vmax* value at 24% mold concentration was 0.690 U/ml and the lowest *Vmax* value at 4% mold concentration was 0.474 U/ml. The results of this analysis show that the higher the concentration, the *Km* value will decrease and the smaller the concentration value, the greater the *Km* value. This small *Km* value indicates that the enzyme has a high affinity for the [S] substrate. The *Km* and *Vmax* values are inversely proportional meaning that if the *Km* value is high, the *Vmax* value will be low and if the *Km* value is low, the *Vmax* value will be high. The value of *Vmax* is related to the speed of the catalytic reaction or hydrolysis and other decomposition.

4. CONCLUSION

Treatment with *Penicillium chrysogenum* concentration significantly affected the yield of crude enzyme extract and the highest enzyme production of 1.87 g was gained at a treatment of 24% fungi concentration. The highest enzyme activity was detected at a concentration of 24% with a value of 1.924 U/ml and the lowest was at a concentration of 4% with 0.929 U/ml. The highest enzyme activity was observed at temperature of 40 °C and a drastic decrease occurred at 45 °C due to covalent bond damage due to thermophilic temperature. The most optimal pH is pH 5.5 because at this pH the enzymes produced by *Penicillium chrysogenum* have the highest activity. At pH 4 the enzyme activity reached the lowest value. The highest *Km* value (0.035 g/ml) was noticed in the treatment of 4% fungi concentration and the lowest value (0.016 g/ml) was in the treatment of 24% concentration. Meanwhile, the optimal value of *Vmax* (0.690 U/ml) was ensued at a concentration of 24% and the lowest *Vmax* (0.474 U/ml) occurred at a fungi concentration of 4%.

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