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The Effect of *Bacillus subtilis* Bacteria Concentration on Production and Characterization of Pektinase Enzymes from Pineapple Peel (*Ananas comosus* L. Merr) Waste

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ABSTRACT

This study aims to determine the effect of the concentration of Bacillus subtilis isolates on the production of pectinase enzymes from pineapple peel waste and the characteristics of the pectinase enzymes produced, including enzyme activity, optimum temperature, optimum pH, the value of K_m and V_{max}. The highest crude enzyme extract production yield was produced in the 10% concentration treatment of 1,59 g and the lowest at the 4% concentration treatment of 1,53 g. In the results of enzyme characterization, the highest enzyme activity was found at a concentration of 20% at 1,883, and the pectinase enzyme produced was able to work optimally in environmental conditions with a temperature of 40 °C and at pH 4 at all concentrations. The results of the highest enzyme activity at variations in temperature and pH were found in enzymes treated with 20% bacterial concentration, namely 0,680 at 40 °C and 0,650 at pH 4. Analysis of enzyme kinetics obtained from the pectinase enzyme produced showed the highest K_m value of 0,021 at a concentration of 4 %, while the lowest value is 0,0095 at a concentration of 20%. Then, for the enzyme's V_{max}, the highest value was found in the 20% concentration treatment of 0,671 and the lowest value in the 4% treatment of 0,481. Based on data analysis using 5% honestly significant difference (HSD) test, the bacterial concentration treatment showed differences between treatments, but the enzymes produced in the 20% treatment were not significantly different from the 16% treatment enzymes.

1. INTRODUCTION

Enzymes are compounds produced by a living cell and are also called biocatalysts. Pectinase is a group of enzymes that acts on pectin polymer hydrolysis or degradation so that numerous food and beverages industries use pectin to increase the product's quality. Pectinase enzyme can be obtained naturally from plant tissue with the help of microbes. One of the pectin enzyme sources is pineapple peel waste (*Ananas comosus* L.

Merr). Pineapple peel waste has the potential to produce pectinase enzyme based on its contents, considering pectinase enzyme has been imported all along to fulfill domestic needs.

Nowadays, microbes have become the main source of pectinase production because they reproduce easily, have high productivity, easiness, and rapid enzyme separation process from the media. Pectinase enzymes can be found naturally in a living organism. Moreover, the enzymes have already been isolated by a bacterium such as *Erwina cartoropora, E. crysanthemi* (Sittidilokratna *et al*., 2007), and *Bacillus sphaericus* (Jayani *et al*., 2005). Based on a study, it is stated that commercial pectinase enzymes with high enzyme activity are produced from productive microbial strain *Aspergillus* and *Rhizopus* (Fawole & Odunfa, 2003). However, the production of pectinase enzyme using mold microorganisms will take time for fermentation and its vulnerability to environmental conditions. Nevertheless, based on Banik and Ghosh's (2008) study, mold-produced enzymes have a lower yield with a time-consuming process and vulnerability to environmental conditions. Hence enzyme production method with maximized yield and shorter time is needed.

One of the enzyme production methods can be used by using *Bacillus subtilis*. Study by Kasyap *et al*. (2000) can produce an enzyme with high enzyme activity, survive the extreme environment, and produce external protein. Besides, Bacillus subtilis is an easily produced bacteria with reasonable price media and is non-toxic. Banik & Gosh (2008) stated that bacterial growth from Bacillus and Streptomyces species are higher, that more pectinase enzyme is produced than using *Aspergillus* sp. and *Penicillium* sp. fungus. Moreover, Bacillus subtilis isolation time is faster than mold's isolation time. This current study related to the effect of *Bacillus subtilis* bacteria concentration on production and characterization of pectinase enzyme from pineapple peel waste (*Ananas comosus* L. Merr) is expected to produce pectin enzyme that potentially can degrade pectin in beverages products. Therefore this study aims to determine the effect of Bacillus subtilis bacteria concentration on enzyme products and determine pectinase enzyme characteristics produced from the process.

2. MATERIALS AND METHODS

This research was conducted in June – September 2021 at Faculty of Agriculture and Business Laboratorium, Universitas Kristen Satya Wacana.

2.1. Materials

The materials used were ginger lemon drink, water, sugar, pineapple peel waste, tissue, alumunium foil, aquadest, NA media, *Bacillus subtilis* bacterial isolate, apple's pectin, acetate buffer (4; 4.5; 5.0; 5.5), bacto pepton, yeast extract, K_2HPO_4, $CaCl_2$, $MgSO_4$, Na_2CO_3, DNS reagent, 80% tween solution.

2.2. Equipment

The equipment used in this study included stove, Teflon, knife, pot, strainer, spoon, scale, incubator, stopwatch/smartphone, beaker glass, Erlenmeyer, stirring rod, stationery, spectrophotometer, cuvette, measuring flask, 100 ml Florence flask, test tube, measuring pipet and filler, water bath, funnel, filter paper, dropper, burette and static, magnetic stirrer, needle loop, 1000 ml Erlenmeyer flask, sterile cotton, oven, analytical balance, pH meter, autoclave, shaker, centrifuge, refrigerator, LAF, petri dish, and hand refractometer.

2.3. Procedures

The experimental design used is a Randomized Block Design (RBD) with bacterial suspension concentration to produce pectinase enzyme treatment. This research used pectinolytic bacteria, namely *Bacillus subtilis,* in the pectinase enzyme production. the bacterial suspension concentration treatment were divided into 7 treatments of varied concentration: 0%, 4%, 8%, 10%, 13%, 16%, and 20% in 50 ml of liquid media. Then the results were analyzed using Analysis of Variance (ANOVA) and continued with honestly significant difference (HSD) at a 5% significance level if there was an effect on the result.

2.3.1. Pectinase Enzyme Production Phase

Pineapple peel waste is washed with flowing water and then soaked in water for 2 hours. Pineapple peel is cut into 1x1 cm and baked at 45 °C for 24 hours. After drying, blend until smooth in the form of a powder. The production medium was made by mixing pineapple peel powder with a mineral solution consisting of bacto peptone, yeast extract, K_2HPO_4, $CaCl_2$, $MgSO_4$, Na_2CO_3. All ingredients were put in 50 ml Erlenmeyer, added aquadest, stirred until homogeneous, and then heated aseptically.

The production process of the pectinase enzyme begins with the suspension of *Bacillus subtilis* bacteria into sterile distilled water using a needle loop according to the treatment, namely 0%; 4%; 8%; 10; 13%; 16; and 20% of the production medium is 50 ml. Furthermore, the mixture is fermented at a temperature of 35 °C for 48 hours.

The following process is harvesting the crude extract of the pectinase enzyme, namely by stopping the bacterial fermentation process in the production media. The harvesting process begins with adding 100 ml of 0,1% tween 80 solutions into the production medium, then shakes using a shaker at a speed of 150 rpm for 15 minutes for further filtering. The filtrate from the filtering process was then centrifuged for 10 minutes at a speed of 3000 rpm. The precipitated filtrate containing enzymes was then separated and stored in a cooler at 4 °C in a closed bottle.

2.3.2. Pectinase Enzyme Characterization Phase

a) Enzyme Activity

Determination of reducing sugar content in enzyme was done by taking 0,05 g pectinase enzyme from each treatment. One millimeter of 0,5% apple's pectin was mixed with acetate buffer pH 5, then heated on a water bath at 50^0C for 50 minutes. One millimeter of aquadest and 2 ml of DNS reagent were added hereafter; then, the solution was reheated at 100 °C for 15 minutes on a water bath. The solutions were put in a 25 ml Florence flask, and aquadest was added until the flask limit. After determining reducing sugar content with a spectrophotometer on 495 wavelengths was done. The absorbance result obtained is then calculated using the following formula to determine the enzyme activity value.

$$EA = \frac{RS \; x \; DF \; x \; 1000}{t \; x \; MW} \qquad (1)$$

where *EA* is crude pectinase enzyme activity (U/mL), *RS* is reducing sugar concentration from the standard curve (mg/mL), 1000 is the conversion factor, *DF* is dilution factor, *t* is incubation time (minute), and *MW* is galacturonic acid molecular weight 212 g/mol.

b) Enzyme Optimum Temperature
Enzyme optimum temperature was examined on 35 °C – 60 °C with 5 °C and at pH 7 intervals (Dixit *et al.*, 2013). Then 0.1 ml of enzyme was taken and added with 0,9 ml of reactor media (0.7% (w/v) citrus's pectin and 0,025 M pH 4,8 sodium acetate buffer solution) on incubation temperature range 35; 45; 50; 55; and 60^{0}C for 30 minutes. Samples were analyzed with the DNS method.

c) Enzyme Optimum pH
Enzyme optimum pH was determined on the range of pH 4,0 – 6,0 with pH 0,5 interval (Frittrang *et al*., 1992). Then 0,1 ml of enzyme was taken and added with 0,9 ml of reactor media (0,5% (w/v) apple's pectin and 0,025 M acetate buffer solution) using pH range 4,0; 4,5; 5,0; 5,5; and 6,0. The solutions were then incubated at 37 °C for 30 minutes. Samples were analyzed with DNS method.

d) Enzyme Kinetics
Enzyme kinetics are determined by determining [S], Km, and V_{max} values. Enzymes from each treatment were taken by 0.05 g and added with 10 ml of citrus's pectin reactor media as substrate [S] with pectin substrate concentration varied to 5%, 6%, 7%, 8%, 9%, 10% and 0.05 M acetate buffer solution; 0,15 M NaCl (pH 4,5). the solutions was incubated at 37^{0}C for 30 minutes. Samples were analyzed with the DNS method. The K_m and V_{max} value was determined by 1/[S] of 1/enzyme activity curve, so a linear line was obtained with correlation:

$$\frac{1}{V} = \frac{1}{V_{max}} + \left\{\frac{K_m}{V_{max}} \times \frac{1}{[S]}\right\} \qquad (1)$$

That can be presented as a simple linear function as:

$$y = a + bx \qquad (2)$$

where:

$$y = \frac{1}{V}; \qquad x = \frac{1}{[S]}; \qquad a = \frac{1}{V_{max}}; \qquad b = \frac{K_m}{V_{max}}$$

The value of *a* and *b* can be obtained from the regression equation of the graph 1/V vs. 1/[S]. The value is then used to determine V_{max} and K_m as the following:

$$V_{max} = 1/a \quad \text{and} \quad K_m = b.V_{max}$$

3. RESULT AND DISCUSSION

In this study, four parameters were used to characterize pectinase enzyme obtained from *Bacillus subtilis* bacterial isolate concentration treatments: enzyme activity, optimum temperature, pH, and enzyme kinetics.

3.1. Pectinase Enzyme Production

In this research, pectinase enzyme production was done by using bacterial suspension concentration treatments varied to 4%, 8%, 10%, 13%, 16%, 20% from all the liquid media used, after bacterial suspension was inoculated, pectinase enzyme production media was incubated and shaken with shaker for 48 hours with no light or sealed. After incubation process, bacterial growth was stopped to harvest crude extract enzyme.

Therefore, the yield of crude extract pectinase enzyme from each bacterial concentration treatments were obtained as can be seen in Table 1.

Based on the data in Table 1, the treatment of bacterial inoculum concentration of *Bacillus subtilis* did not show a significant difference to the yield of crude enzyme extract. The treatment of bacterial inoculum concentration of 4% resulted in an average yield of crude pectinase enzyme as much as 1,53 g and was the lowest yield. The 10% treatment produced an average crude enzyme of 1,59 g and was the highest yield among other treatments. The second-highest yield of crude pectinase enzyme was produced in the 20% and 16% bacterial concentration treatment, which was 1,58 g.

Table 1. Pectinase Enzyme Analysis Result

Concentration (%)	Analysis Parameters				
	Yield (gr)	Reducing Sugar (Mg/ml)	Enzyme Activity (U/ml)	K_m	V_{max}
Control	$1,4_{B}$	$0,055_{B}$	$0,175_{C}$	$0,025_{A}$	$0,065_{D}$
4%	$1,53_{AB}$	$0,314_{B}$	$1,283_{B}$	$0,021_{AB}$	$0,481_{C}$
8%	$1,57_{AB}$	$0,337_{B}$	$1,323_{B}$	$0,018_{BC}$	$0,529_{BC}$
10%	$1,59_{A}$	$0,354_{B}$	$1,373_{B}$	$0,016_{BCD}$	$0,536_{BC}$
13%	$1,55_{AB}$	$0,390_{B}$	$1,485_{B}$	$0,013_{CD}$	$0,552_{B}$
16%	$1,58_{A}$	$0,495_{A}$	$1,823_{A}$	$0,0091_{D}$	$0,667_{A}$
20%	$1,58_{A}$	$0,503_{A}$	$1,833_{A}$	$0,0095_{D}$	$0,671_{A}$
	KV = 4,9%	KV = 6,4%	KV = 6,3%	KV = 16%	KV = 4,9%

Description: Different letters on the same column represent significant differences between treatments, while the same letters on the same column represent no significant difference between treatments based on data analysis.

Concentration or microbial inoculum is inoculated in fermentation media for enzyme production. Microorganism enzyme production is easily influenced by inoculum dose and incubation time. According to Marlida *et al*. (2002), the proportions of inoculum dose will affect the microorganism log phase on enzyme production, while incubation time will affect the amount of enzyme produced.

Hence this research showed that the highest *Bacillus subtilis* bacterial concentration (20%) could not increase the crude pectinase enzyme produced and have no significant difference with 14% inoculum concentration treatment. It is possible that competition among bacteria individuals to fulfill nutritional needs increased along with the increase of bacterial concentration inoculated on fermentation media for pectinase enzyme production. More bacterial inoculants cannot increase the crude enzyme biomass produced. After that, enzymes from 4%, 8%, and 13% inoculum concentration had not significantly different yields from the control treatment. This is probably caused by the low bacterial inoculum dose that requires more incubation time to maximize the pectinase enzyme produced. In contrast, the fermentation incubation time was equally treated for each treatment in this research.

3.2. Enzyme Activity

Pectinase enzyme was examined to know the polygalacturonase activity on polygalacturonic acid substrate (esterified pectin) by determining the reducing sugar content. The high reducing sugar contained in nitrite groups is due to reducing sugar due to pectinase enzyme hydrolysis substrate, reducing sugar measured with DNS

method using 498 nm wavelength with UV-Vis spectrophotometer.

One unit of pectinase enzyme activity is interpreted as the amount of enzyme that produces 1 µmol galacturonic acid per unit time for each milliliter of enzyme on research condition. Then the absorbance value is measured with the formula to determine enzyme activity value. The absorbance value from the spectrophotometer then correlated with the reducing sugar content or assumed as formed glucose based on incubation time. The calculation result and data analysis of enzyme activity can be seen in Table 1.

Based on Table 1, the calculation result of pectinase enzyme activity, it is known that the highest pectinase enzyme activity was produced at 20% bacterial isolate concentration, which was 1,473 U/ml as the average of 4 repetitions, which is not significantly different from 16% concentration which was 1,468 U/ml, whereas the lowest enzyme activity was produced at 4% bacterial isolate concentration which was 0,965.

Based on the results, the more bacterial concentration was inoculated to the substrate of pectinase enzyme production media, the more *Bacillus subtilis* increased its metabolism to degrade pectin and compounds contained in the substrate pectinase enzyme as the result of metabolism was also increased. However, suppose *Bacillus subtilis* bacterial inoculum concentration that was added to the substrate media is too high. In that case, it will lead to catabolic repression, specifically suppressing pectinase enzyme production, because of excess dissolved sugars in cell growth (Rangarajan *et al*., 2010). Therefore, the difference between 20% and 16% concentration treatment yields is insignificant.

3.3. Enzyme Optimum Temperature

Optimum temperature is when the enzyme reaches the highest reaction rate to process a substrate and creates a balance between the increasing activity rate and enzyme destruction rate (Bintang. 2010). In this study, enzyme optimum temperature characterization was examined at 30 °C – 45 °C, which is graphically seen in Figure 1.

Based on the optimum temperature graphic, all enzymes from each treatment of *Bacillus subtilis* bacterial concentration had the lowest enzyme activity at 30 °C; the activity increased to the highest at 40 °C and slightly decreased at 45 °C. Also, the graphic showed that the highest enzyme activity was produced with a 20% bacterial concentration at 40 °C, which was 0,680 U/ml. Therefore, pectinase enzymes for industrial needs are effectively used at 40 °C.

It is shown that enzyme isolates can work optimally at thermophile temperature for juice clarification purposes. Pectinase enzyme Optimum temperature ranged at 40 °C-75 °C (Sieiro *et al*., 2012). High temperature caused the increase of kinetic energy, leading to primary structure loss, which is connected to a covalent bond, so that enzyme activity decreased. Buga (2010) also engaged a similar case that the pectinase enzyme had maximum activity at a certain temperature. The temperature allows collisions between active molecular sites without denaturating enzyme, besides enzyme reaction rate increase as the temperature rise, and pectinase enzyme optimum temperature, the one produced from fungus and bacteria isolate was ranged at 37 °C. Pectinase enzyme characterization study by Widiowati *et al*. (2014) stated that the enzyme produced from pectinolytic bacteria works for orange juice clarification with pH 4,0 and stabilized at 30 °C-50 °C, enzyme activity decreased at 60 °C and inactivated at 100 °C. According to the study, the enzyme produced can be applied to the industrial scale of orange juice clarification because of the stabilized temperature at 30 °C-50 °C.

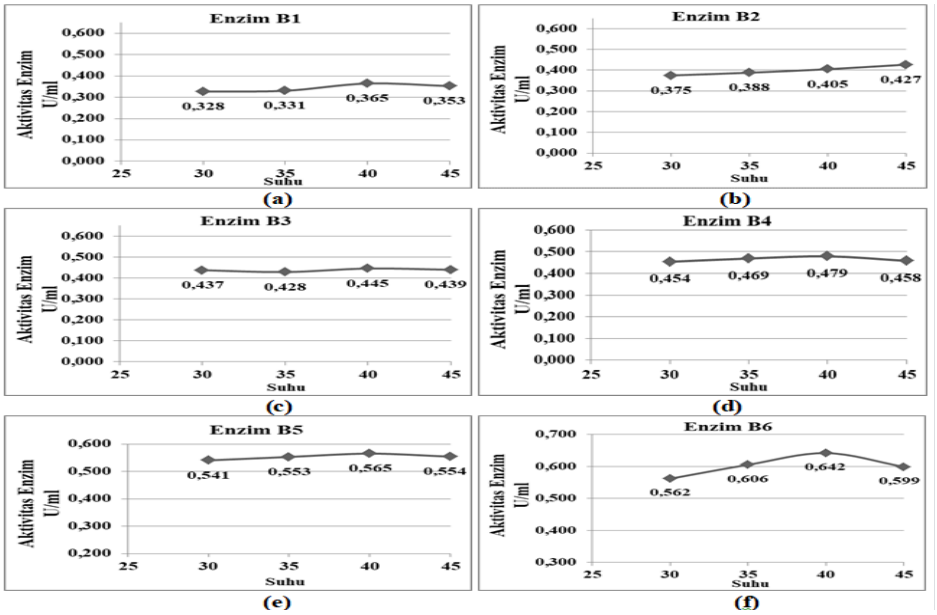

Figure 1. Enzyme Activity (a) 4%; (b) 8%; (c); 10%; (d) 13%; (e) 16%; (f) 20% under temperature variation

3.4. Enzyme Optimum pH

The optimum pH value is the pH that allowed the enzyme to reach its highest reaction rate. This research examined optimum enzyme pH at pH 4-5,5 and graphically shown in Figure 2.

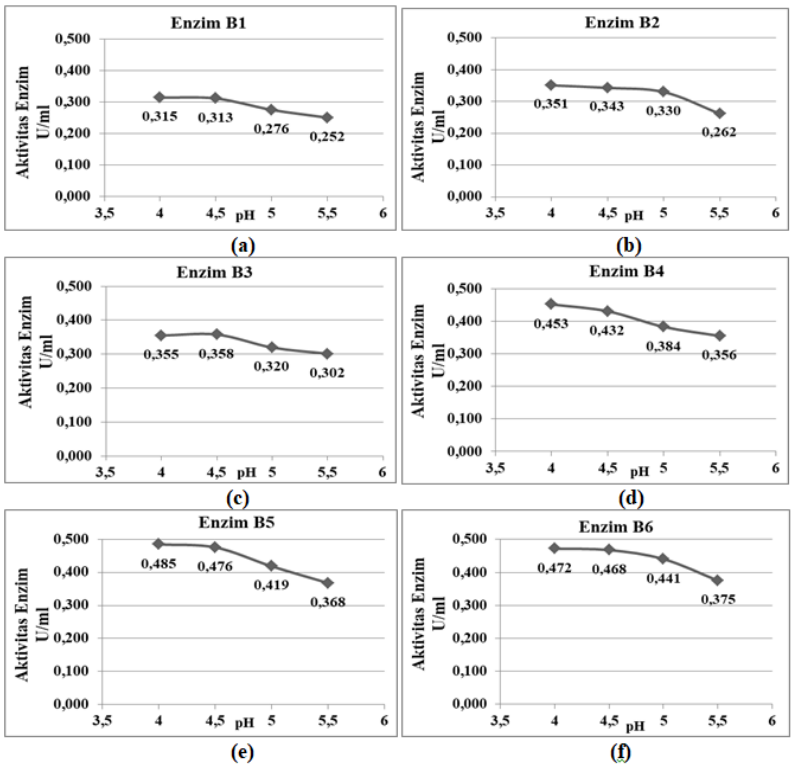

Figure 2. Enzyme Activity (a) 4%; (b) 8%; (c); 10%; (d) 13%; (e) 16%; (f) 20% under temperature variation

Based on Figure 2, all enzymes from each *Bacillus subtilis* bacterial concentration treatment reached their highest activity at pH 4 and started to decrease along with the increasing pH, whereas the highest enzyme activity was produced on 20% bacterial concentration, which was 0,650 U/ml at pH 4. Consequently, this pectinase enzyme from *Bacillus subtilis* is the potential for lemon or orange product clarification.

Alteration of pH happened on low standard deviation value will be caused enzyme activity to decrease because of the ionization changes of functional groups. In contrast, an enzyme is a protein compound consisting of amino acids that can bind and lose proton or hydrogen ions on amino, carboxyl, and other functional groups. Nonetheless, suppose the standard deviation is high. In that case, the pH alteration will cause enzyme denaturation associated with interference with non-covalent interaction, which supposedly maintains enzyme 3-dimensional structure and has an important role in maintaining enzyme active site conformation used to bind and change substrate into a product (Hames & Hooper, 2000).

Furthermore, suitable media pH affects the productivity of the fermentation process on pectinase enzyme production. This study works optimally at pH 4 media because pectinase from bacteria works well in an acidic environment. Polygalacturonic activity from fungi and bacteria isolates has optimized activity at pH 3,8-5 (Buga, 2010).

3.5. Enzyme Kinetics

Enzyme kinetics is related to the rate of the enzyme's reaction. Michaelis Menten constant (Km) is the substrate concentration to achieve half of the maximum enzyme activity rate, while V_{max} is the maximum activity rate of an enzyme. This particular research required pectinase enzyme produced examination using apple's pectin as substrate solution with ranged 5 – 10% concentration from reactant solution with 1% interval concentration substrate into reactant media which was acetate buffer solution pH 5 and 0,15 M NaCl, enzyme activity procedurally examined afterward. The results are then tabulated in table V and [S] and converted to 1/V and 1/[S], which are then plotted into a relation graph between 1/V and 1/[S]. From the graph, Km and V_{max} values were determined with the Lineweaver-Burk curve equation (Whitaker, 1996).

Based on Km and V_{max} values analysis data in Table 1, the highest Km value achieved at 4% bacterial concentration is 0,021, and the lowest Km value at 20% bacterial concentration is 0,0095, Km values decreased along with the increasing concentration of bacteria. As it is known that enzyme Km value determined the enzyme-substrate (E-S) affinity level, which is an equilibrium constant or as an indicator of E-S complex strength to dissociate into E and S. In addition, small Km value showed the solid E-S complex and high affinity towards enzyme. In contrast, a large Km value showed the affinity if low (Fox, 1991).

Thus, in this research, the enzyme from a 20% concentration has the largest affinity because of its lowest Km value, while the enzyme from a 4% concentration has the lowest enzyme affinity. Then enzyme from 20% concentration also has the highest V_{max} value, which is 0,671, and enzyme form 4% concentration has the lowest V_{max} value, which is 0,481, but enzyme from 20% was not significantly different from 16% concentration which is 0,667. It is due to the effect on the fermentation process of enzyme production. As known, fermentation is strongly influenced by microorganism inoculum dose and time. The dose level is related to the size of the microorganism population and can determine the microbial regeneration time to remodel substrate, which affects the final product (Fardiaz, 1992). A nonproportional amount of inoculum and substrate will lead the microorganism to a stationary phase (the growth rate is the

same as the death rate). Enzyme activity will decrease when microorganisms enter the stationary phase (Cai *et al*., 2008). Thus, the 20% bacterial concentration quality enzyme is not significantly different from the 14% bacterial concentration.

Enzyme V_{max} value is inversely proportional to enzyme Km value, which was caused by V value of an enzymatic reaction correlated to enzyme catalyst ability so that V value will increase along with the increasing substrate [S], but after further increased of [S], the rate will stay constant. At a constant (certain) enzyme concentration, the V value is almost linear with [S] (Wiesman, 1989). In a condition where V value cannot increase with the increasing of [S] called maximum rate (V_{max}), thus the higher V_{max} value indicates, the higher catalyst ability that can be achieved by an enzyme, vice versa, the lower V_{max} value, the lower catalyst ability of an enzyme can be achieved.

4. CONCLUSION

Bacillus subtilis bacterial isolate concentration treatment did not significantly affect crude extract pectinase enzyme produced biomass, namely crude enzyme which was mostly produced from 10% bacterial concentration as much as 1,59 g then five other treatments. Based on 5% Honestly Significant Difference (HSD), an enzyme from 20% bacterial concentration reached the highest activity at 1,833 U/ml, but not significantly different from an enzyme produced by 16% bacterial concentration at 1,823 U/ml. In comparison, the lowest enzyme activity was found in an enzyme produced by 4% bacterial concentration a 1,293 U/ml. Pectinase enzyme produced works optimally at 40 °C and decreased at 45 °C. Also, the enzyme works optimally at pH 4 and gradually decreases along with the increasing pH. As for Km and V_{max} value, the lowest Km value found in an enzyme produced by 20% concentration which is 0,0095, indicates it has the greatest enzyme affinity, the highest V_{max} value achieved from 20% bacterial concentration enzyme at 0,0671, however it is not significantly different from 16% bacterial concentration enzyme at 0,667. Meanwhile, the lowest V_{max} value was found in the 4% bacterial concentration enzyme at 0,481.

Overall, the pectinase enzyme produced by *Bacillus subtilis* is optimal at 40 °C and pH 4, then based on bacterial inoculum concentration treatment, enzymes from 20% and 16% bacterial concentration treatments were not significantly different. Thus, the 16% bacterial concentration enzyme was more effective than other concentrations.

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