Determination of Kinetic Parameters and The Effect of Ion Mg²⁺ InhibitionIntoPectinase Activities

¹Dian Puspita Anggraini, ¹Devita Sulistiana, ¹Dwi Kameluh Agustina, ²*Almira Ulimaz

¹Biology Education Department, Faculty of Teacher Training and Education, FKIP Islamic University of Blitar, Jl. Majapahit No. 04, Blitar East Java Province, 66137, Indonesia
²Agro-Industry Study Program, Department of Agricultural Industrial Technology, Politeknik Negeri Tanah Laut. Jl. A. Yani Km. 06, Panggung Village, Pelaihari District, Tanah Laut Regency, South Kalimantan Province, 70815, Indonesia

*Corresponding Author e-mail: almiraulimaz@politala.ac.id

Abstract

The purpose of study was to determine kinetic parameters and inhibitory effect of Mg²⁺ ions on pectinase. Activity test performed at pH 4,5 temperature 55°C for 50' with 10 mm concentrations of each ion 0-10mm. Galacturonic acid content, as product of pectin substrate hydrolysis, was used as basis for determining activity and analyzed by visible spectrophotometry. At concentrations 2 and 4 mm Mg²⁺ act as an activator, but at concentrations 6, 8, and 10 mM as inhibitors. The value of K_M with and without inhibitors is almost same, that is 0.3145% and 0.3105% but value of V_m from both are different, that is 80,645 µg.ml⁻¹.minute⁻¹ and 62,112 µg.ml⁻¹.minute⁻¹. The conclusion that type of inhibitory pectinase with Mg²⁺ is a non-competitive inhibition. The inhibition constant value (K_i) is 26,84.

Keywords: Bacillus firmus, Mg²⁺, Pectinase

INTRODUCTION

In nature, microorganisms have great potential in producing many types of enzymes that can be used commercially. Pectinase is one of the important enzymes in biotechnology that can be applied in extraction and purification of fruit essence (Rai et al, 2004). Pectinase is able tocatalyze the hydrolysis reaction of pectin compounds to produce galacturonic acid (Jayaniet al, 2005). Sources of pectinase are microbes, such as *Aspergillus niger* and *Bacillus* sp. (Wuet al, 2013). Pectinase isolation from *Bacillus firmus* derived from Blitar cow milk in East Java has optimum conditions at pH 7, temperature 40–50 °C, and fermentation time of 18 hours.The resulting enzyme shows the presence of pectinase activity (Roosdiana et al, 2013). In this study *Bacillus firmus* was used.

Enzyme activity be affected by several factors such as temperature, pH,incubation time and substrate concentration (Winarno, 2002) so that optimization is needed for each of these variables. In the process of fruit essence purification, metal ions contained in the fruit affect the activity of pectinase. Metal ions contained

in guava fruit include magnesium, calcium and zinc. Guava has magnesium contents 22 mg/100g. Calcium and zinc contents are 18 mg/100g and 0.23 mg/100g (Surahman, 2004).

The presence of metal ions can affect the active side of the enzyme and the stability of protein molecules, so that at high concentrations metal ions can affect bonds and substrates. Thus metal ions can act as activators or inhibitors. At concentrations of 0-30 mm alkali metals such as K^+, Na^+, Ca^{2+} and Mg^{2+} can increase pectinase activity (Banu et al, 2010). However, in the study of Banuetal (Adejuwon & Olutiola, 2007),states that metal ions can affect the catalytic work of enzymes. The addition of 5 mM Mg^{2+} metal ions are added to the pectinase activity produced by *Penicillium chrysogenum* can obstruct the activity of pectinase by 21.2%.

Enzyme kinetics are determined at optimum conditions. Enzyme kinetic, in the form of parameters K_M and V_{max}. By knowing the value, then the inhibitory constant (K_i) can be determined against the enzyme activity in the presence of metal ions which act as inhibitors. Based on the characteristics of the enzyme, it is necessary to conduct research to determine the enzyme kinetics parameters, that is the value of V_{max}, K_M and K_I, and can be applied in purifying guava fruit essence.

METHOD

Pure *Bacillus firmus* culture was obtained from the Medical Microbiology Laboratory, Brawijaya University, Malang. Observations were made on the pectinase enzyme with the addition of Mg^{2+} ions to determine its effect on pectinase activity. Pectinase activity was obtained from the calculation of reducing sugar levels using the DNS method. Then the values of V_m and $K_M$were determinedby varying the substrate concentration from 0.1–0, 5 %. Meanwhile, for the determination of the value of K_I, it is done by adding Mg^{2+} 6mM metal ions. The reducing sugar content of fruit essence purification was measured by spectrophotometer using the DNS method.

Determination of pectinase activity

Pectinase activity was determined by testing the pectin substrate solution 0,5% (b/v), a solution of pH 0.6 acetate buffer and pectinase added to tube no 1. This mixture was incubated at 55° C for 50 minutes, then added 2 ml of the DNS reagent then heated for 15 minutes, cooled to room temperature.The absorbance was measured using a UV–Vis spectrophotometer at a wavelength of 540 nm. Blanks prepared with the same treatment without the addition of the pectinase enzyme.

The magnitude of one unit enzyme activity can beseen in the following equation:

$$AE = \frac{x \cdot V \cdot fp}{p \cdot q}$$

AE = enzyme activity ($\mu g.ml^{-1} .menit^{-1)}$
X = sugar reducing concentration ($\mu g.ml^{-1}$)
V = total volume of experimental samples per tube (ml)
q = reaction time (minutes)
p = volume of crude extract pectinase (ml)
fp = dilution factor

Effect of addition of Mg^{2+} ions to pectinase activity

The effect of Mg^{2+} ions addition on pectinase activity can be identified by the addition of 1 ml $MgCl_2$ with varying concentrations of 0–10 mm.

Determination of V_m, K_M and K_i

To determine the value of V_{max} and K_M is done by testing the activity of pectinase by varying the concentration of pectin 0.1–0.5% (b/v). Whereas to determine the value of inhibition constants (K_I) determined by testing the activity of pectinase by varying the concentration of pectin substrate 0.1–0.5% (b/v) by adding Mg^{2+} 6mm metal ions.

RESULTS AND DISCUSSION

The 10 ml inoculum was added to the sterile media aseptically, incubated in conditions inside the shaker at a speed of 125 rpm, until it reached the end of the logarithmic phase. The bacteria condition is very active in synthesizing enzymes for life fulfillment. Followed by an isolation process, that is the release of enzymes from cells by destroying the cell wall or membrane chemically, mechanically, physically and enzymatically.

Isolation and Rough Pectinase Extract Production

The centrifugation method was chosen to isolate pectinase. Centrifugation is a separation method based on the difference in sedimentation velocity of the particles of molecules caused by the centrifugal force. Enzyme isolation is the release of enzymes from cells by destroying the cell wall or membrane chemically, mechanically, physically and enzymatically. To get a crude extract of the enzyme, centrifugation needs to be done in separating the enzyme from the rest of the cells that have been disintegrated (Murrayetal, 2003). The centrifugation method is the main operation for the separation of particles from the solution in the process of enzyme isolation, separation of cells from the culture medium, separation or destruction of cells and precipitates (Judomidjojo et al, 1992). To maintain enzyme stability during isolation, it is necessary to add a buffer solution and this needs to be done at low temperatures (Nicole et al, 1995)

In this study, the function of the addition of pH 0,6 citrate buffer is to increase solubility and also facilitate the separation from other molecules that are insoluble like the residual of *Bacillus firmus* texture. Besides that, pH 0,6 buffer was used because this pH was the optimum pH for the production of pectinase from *Bacillus firmus.* Then the mixture solution is coolly centrifuged at a temperature of 4^0C. The centrifugation process is carried out at low temperatures to maintain the stability of the enzyme. Deposits and supernatants are produced from this centrifugation process which are crude extracts of pectinase.

Determination of Pectinase Activity

Pectinase is an enzyme that can hydrolyze pectin to become galacturonic acid which is reducing sugar. Activity of pectinase can be determined based on the amount of reducing sugar produced by pectinase. In this study the determination of pectinase activity was determined by a spectrophotometric method using DNS reagents or 3.5-dinitrosalicylic acids. The reducing sugar produced from the hydrolysis reaction will reduce dinitrosalicylic acid so that it can form a brownish red complex. After that it was measured by a Uv-Vis spectrophotometer with $\lambda = 540$ nm (Chaplin, 2008).

The mechanism of the enzymatic reaction that the pectinase has an active group which can hydrolyze pectin, the carboxyl group which is the side chain of the aspartic type amino acid (Santen et al, 1999). The functional cluster on the side chain in the form of a carboxylic ion from the side chain of the amino acid type aspartic acid (Asp

180) binds to the H atom of water with hydrogen bonds, while the O atom from the water attacks the pectin polymer on the C_1 atom which binds the O atom to glycosidic bond. This attack will be easier with the presence of H^+ ions from other aspartic acids (Asp 201) which are able to attract free electron pairs of O atoms used to form glycosidic bonds. Then the hydrolysis process occurs which causes the termination of the enzyme bond with the substrate and reducing sugar monomers.

The Effect of Mg^{2+} Addition on Pectinase Activity

In determining the activity of pectinase with the addition of Mg^{2+} with variations in concentrations of 0, 2, 4, 6, 8, and 10 mm. Picture no 1. showed that the addition of Mg^{2+} ions affected the pectinase activity. On this curve it can be concluded that Mg^{2+} ions can act as activators or inhibitors.
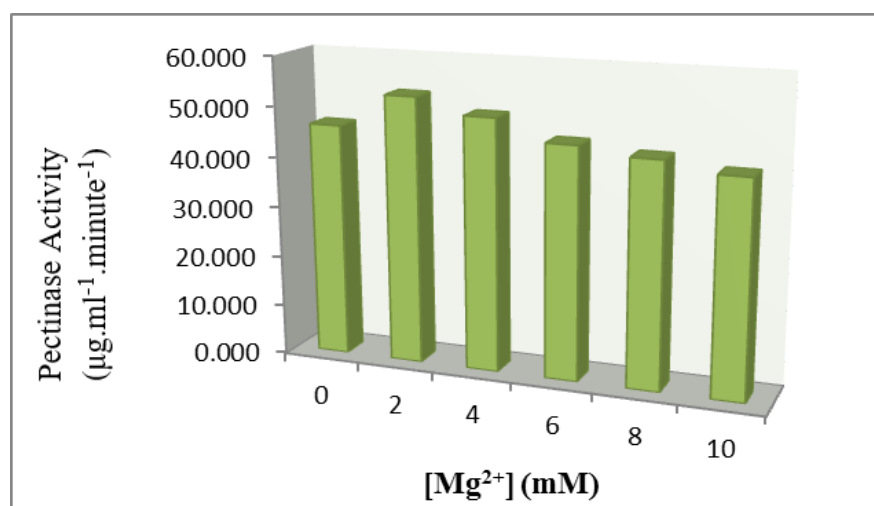

Figure 1. Pectinase activity curve with the addition of variations in the concentration of Mg^{2+} ions

From the above curve it can be seen that at concentrations of 2 and 4 mM Mg^{2+} ions function to increase the activity of pectinase (activator) which plays a role in the formation of enzyme complexes with substrate. Mg^{2+} ion is a lewic acid which has the same role as H^+ ion in aspartic acid, where Mg^{2+} ions attract free electron pairs from O atoms that are used to form glycoside bonds, the hydrolysis process occurs by the presence of H_2O which causes the termination of the enzyme bond with the substrate and the formation of reducing sugar monomers.

In this study, the addition of a concentration of 2 mM Mg^{2+} ions have the same number with the number of free electrons capable being tied with pectin pectinase enzymatic reaction, so the activity generated the highest activity. In addition, Mg^{2+} ions have properties as heat stabilizers which stabilize the transition state in reacting enzymes with the substrate (Lehninger, 2004).

The concentrations of 6,8 and 10 mM Mg^{2+} ions have decreased pectinase activity. This is because the Mg^{2+} ions are no longer attract the free pair on pectinase enzymatic reaction with pectin but binding the active side of pectinase and block pectin binds to pectinase and caused pectinase activity consequently decreased.

Furthermore, a statistical test with RAL was conducted to determine the effect of adding Mg^{2+} ions to pectinase activity. From the data obtained it was shown that the addition of Mg^{2+} ions had an effect on pectinase activity, this was indicated by F count

>F table while for BNT test 1% showed that at concentrations of 0, 2 and 4 mM had a significant effect on pectinase activity.

Determination of the kinetics parameter values of enzymatic reactions

One Factor that can affect enzyme activity is substrate concentration. The effect of substrate concentration on the activity of crude extract of pectinase was carried out by varying substrate concentration by 0.1; 0.2; 0.3; 0.4; 0.5; 0.6; 0.7; 0.8% (b/v) reacted at optimum conditions.

The values of K_M and V_m can be determined using the Lineweaver-Burk equation as follows (Lehninger., 2004):

$$\frac{1}{V_0} = \frac{K_M}{V_m} \cdot \frac{1}{[S]} + \frac{1}{V_m}$$

Based on the curve of the relationship between $1/V_O$ with $1/[S]$ explained in Figure 2 for the curve of the relationship between $1/V_O$ with $1/[S]$ without the addition of Mg^{2+} obtained intercept 0.0124 and slope of 0.0039 to obtain the value of V_m is 80.64 $\mu g.ml^{-1}.minute^{-1}$ and the value of K_m is 0.31%. while for the curve the relationship between $1/V_O$ and $1/[S]$ with the addition of Mg^{2+} is obtained intercept 0.0161 and slope 0.0050 in order to obtain a value V_m of 62.112 $\mu g.ml^{-1}.minute^{-1}$ and the value of K_m 0.31%.

In addition to substrate concentration, enzymes are also influenced by the presence of other substances in the form of activators and inhibitors. The addition of Mg^{2+} ions at concentrations of 6, 8, and 10 mm caused the enzyme activity to decrease, so that the type of inhibition needed to be known, but also to determine its inhibitory constant (Ki).

The inhibition constant (Ki) is a constant that shows the dissociation equilibrium of the enzyme inhibitor complex (EI). If the Ki value is large, then the enzyme has a low affinity for the inhibitor so that the enzyme-inhibition complex is unstable and tends to break down into enzymes and inhibitors. Conversely, if the Ki value is small, then the enzyme has a large affinity for the inhibitor so that the inhibitor will be strongly bound the enzyme that causes enzyme conformation changes, not in accordance with the conformation of the substrate so that the enzyme-substrate complex is not formed to be used to form reducing sugars namely galacturonic acid(Lehninger, 2004).
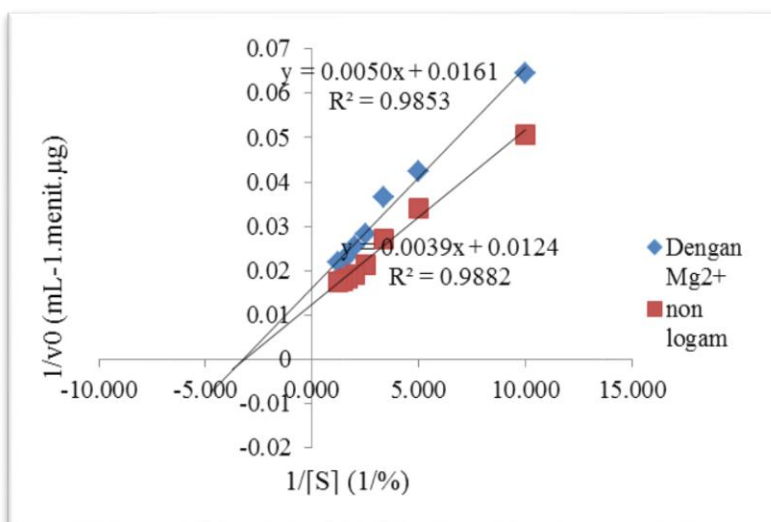
Figure 2. The relationship curve $1/V_0$ with $1/[S]$

The calculation result of V_m and K_m when compared to both have the same value of K_M, in contrast to the value of V_m pectinase without the addition of Mg^{2+} is greater than the value of V_m with the addition of Mg^{2+}. it can be seen on figure 2 that the type of inhibition of Mg^{2+} is non–competitive inhibition. Mg^{2+} ions in this condition do not play a role in helping the active side of the enzyme as an electron receptor because the ability of the active side of the enzyme to bind to Mg^{2+} ions is limited. Mg^{2+} ions bind to other than the enzyme active side (allosteric) to form an enzyme–inhibitor complex (EI), causing the enzyme conformational changes (EI) thereby reducing the capacity of the active enzymes that will bind to the substrate to form the enzyme-substrate complex.

The addition of the concentration of Mg^{2+} ions which can increase the enzyme conformation and substrate to be not optimal, this is because the enzyme is saturated with Mg^{2+} ions so that Mg^{2+} ions are not bound by the active side of the enzyme, but Mg^{2+} ions will be bound by the side other than the active side which is on the allosteric side. According to the theory of enzyme flexibility, the presence of enzyme inhibitors will be bound to the allosteric side and the enzyme will suppress the presence of active enzyme cluster so that the enzyme conformation does not match the substrate. As a result, the enzyme cannot form an enzyme-substrate complex and cannot inhibit the formation of reducing sugars.

As a non–competitive inhibitor (Mg^{2+}) in addition to being bound to the allosteric side, it can also be bound when the enzyme binds to the substrate to form an enzyme substrate inhibitor (ESI)(Lehninger, 2004). In this case, both the enzyme–substrate complex and the enzyme inhibitor complex enzyme are not active, because the inhibitors cannot increase the substrate concentration so that the formation of galacturonic acid becoming slower and the reaction speed changes. But because the substrate still binds to the enzyme then the K_M value remains.

CONCLUSION

Based on the results of research that has been done it can be concluded that the addition of variations in the concentration of Mg^{2+} can affect the activity of pectinase. At concentrations of 2 and 4 mm Mg^{2+} acts as an activator, but at concentrations of 6, 8, and 10 mm the pectinase activity decreases, this means that Mg^{2+} acts as an inhibitor.

The value of K_M with and without almost the same, that is 0.3145% and 0.3105% but the value of the V_m from both is different, that is, 80.645 µg.ml^{-1}.minute^{-1} and 62.112 µg.ml^{-1}.minute^{-1}. Thus it can be concluded that the type of inhibition between pectinases and Mg^{2+} is non–competitive inhibition, and for the inhibition constant value (K_i) in this study is 26,84.

RECOMMENDATION

Pectinase can be used for clarification and bleaching. For this purpose, pectinase must be purified first. Purification was carried out to obtain Pectinase purity in various fractions. From the existing purity level, it can be used for various fields such as food, health, environment and so on. Therefore, it is necessary to do further research on the characteristics of the waste generated during the pectinase production process such as levels of Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), and Total Soluble Solid (TSS) with the same treatment to determine the appropriate treatment can minimize the waste generated.

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REFERENCES

Adejuwon, A. O., & P. O. Olutiola. (2007). Pectin Lyase Activity In Culture Of Penicillium species. *Journal of Plant Sciences 2* (3): 347–352

Banu, A. R., M. K. Devi, G. R. Gnanaprabhal, B. V. Pradeep, & M. Palaniswamy.(2010). Production and Characterization of Pectinase Enzyme from *Penicillium chrysogenum*. *Indian Journal of Science and Technology*, 3(4).

Chaplin, M. (2008). *Enzymes and Enzyme Technology*. Retrieved 08 Maret 2020 fromwww.1sbu.ac.uk/biology/enzyme/practical1.html

Jayani, R. S., S. Saxena, & R. Gupta. (2005). *Microbial Pectinolytic Enzymes: A Review*. Process Biochemistry 40 2931-2944

Judoamidjojo, R.M., Darwis, A.A., &Sa'id E.G.(1992).*Teknologi Fermentasi*. Rajawali Press, Jakarta

Lehninger, L.A. (2004). *Dasar–dasar Biokimia*. Jil.1, Alih Bahasa: Maggy Thenawidjaja. Erlangga. Jakarta

Murray, R.K,.Granner, D.K., Mayes, P.A., & Rodwell, V.W. (2003). *Harper's Biochemistry 25thed*.Appleton and Lange, America

Nicole, M., Roy, M., Daniel, & Helen, H.P. (1995). The Effect Of Low Temperatures On Enzyme Activity. *Biochem. Journal*. 305: 17-20

Rai P., Majumdar G. C., Gupta S. D. & De S. (2004). Optimizing pectinase usage in pretreatment of mosambi juice for clarification by response surface methodology. *J. Food Engenering*. 64:397–403.

Roosdiana A., Prasetyawan S., Mahdi C., &Sutrisno. (2013). Production and Characterization of Bacillus firmus Pectinase.*J. Pure App. Chem*. Res. 2, 1, pp35–41.

Santen,Y. V., Jacques A. E. B., Klaus-Hasso, Kor, Sylvie, Jaap, &Bauke.(1999).1.68-Å Crystal structure of Endopolygalacturonase II from Aspergillusnigerand Identification of Activ Site Residues by Site-directed Mutagenesis. *The Journal Of Biological Chemistry*.

Surahman, D. N. (2004). *Kajian Analisa Kandungan Vitamin dan Mineral Pada Buah-buahan Tropis dan Sayuran di Toyama Prefecture Japan*. Fakultas Teknik Universitas Diponegoro. Semarang

Winarno, F.G. (2002). *Enzim Pangan*. Gramedia Pustaka Utama. Jakarta.

Wu R., He B., Zhao G., Qian L., & Li X. (2013). *Imobilization of Pectinase Oxidized Pilp Fiberand Its Application White Water Treatment*. Southchina University of Technology, Guangzhou, China, *Carbohydrate Poymers*. pp523-529.