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The Study of Amylase's Reaction Kinetics From Soybean Sprouts (*Glycine max L.*) in Hydrolyzing Starch

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ABSTRACT
Amylase is an enzyme that has a role in hydrolyzing starch. This enzyme can be obtained from germinated seeds, one of them is soybean seed sprouts. The research aimed at studying the reaction kinetics in hydrolyzing the substrate, which is called starch, has been done. The research was conducted in three stages: 1) preparation of amylase which included germination of soybean seeds, isolation of amylase, purification with ammonium sulfate 35% (w/v); 2) optimization of amylase activity (germination time, enzyme concentration, temperature, and pH), optimization of germination time, pH, temperature, enzyme concentration, substrate concentration (starch); and 3) determination reaction kinetics of amylase in starch hydrolysis. The reaction kinetics study included the values of V_{max} (maximum reaction velocity) and K_M (Michaelis-Menten constant). Determination of amylase activity using the DNS (Dinitrosalicylate) method. The data that has been collected were analyzed by a descriptive quantitative method. The result showed that: 1) the optimum concentration of the amylase enzyme is 2.5% (v/v), the optimum temperature in hydrolyzing starch is 30 °C, and the optimum pH is 7; 2) value of V_{max} is 6.869 Units/minutess; and 3) value of K_M is 11.87 Units/mL. This information is very important to increase the economical value and efficiency of amylase in the industry.

Keywords: *Analysis optimization, Soybean, Reaction kinetics*

1. INTRODUCTION

Amylase is an enzyme that can hydrolyze starch molecules, a polymer made up of glucose units, which consist of glucose units. This enzyme is one of the main enzymes used in the industry. The need for α-amylase is very large, about 30% of the world's total enzyme production. The demand for amylase reaches at least 25% of the total enzyme requirement [1]. Amylase has a potential application in various industrial processes such as food, fermentation and the pharmaceutical industry [2] paper, pharmaceutical and detergent industries, clinical, medicinal and analytical chemistry, and their wide application in starch saccharification and the textile, food, brewing and refining industries [3][4]. This enzyme has been used for the hydrolysis of starch to fructose and glucose syrup [5]. This high amylase requirement has not been supported by its availability.

Amylase enzyme can be obtained by utilizing materials that are abundantly available in nature, there are: from plants, animals and microorganisms [6][2]. Amylase comes from plants that are easily obtained, namely from germinated seeds [7], one of which is sprouts from soybeans [8]. By utilizing soybean seeds as a source of amylase, it is a breakthrough in technological innovation that will support the achievement of a prog to meet the needs of local and global enzymes that utilize biological sources.

Utilization in industry involving enzymes in general and amylase in particular needs to consider efficiency, which is determined by the product of the hydrolysis reaction. The velocity of enzymatic reactions in hydrolyzing or breaking down substrates/materials is determined by many factors, including temperature, enzyme concentration, pH, and substrate concentration. The substrate concentration affects the velocity of the reaction catalyzed by the enzyme. At very low substrate concentrations, the reaction rate is also very low, but this velocity will increase with escalating the substrate concentration. At the maximum velocity limit (V_{max}), the enzyme becomes saturated with its substrate, and cannot function fast [9]. The use of substrates or raw materials to be hydrolyzed excessively is less efficient in terms of its economical value. Therefore, the enzymatic reaction

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kinetics of amylase needs to be observed, especially the value of V_{maks} dan K_M (Michaelis-Menten constant).

2. METHOD

2.1. Materials

This study used germinated commercial soybean seeds as a source of amylase and starch as amylase substrate. Starch in this study was obtained from the market

2.2. Soybean Amylase Extraction

This stage begins with sprouting soybeans. A small amount of 50 g soybean seeds washed thoroughly, soaked in water for 12 hours, then drained. Seeds are germinated under wet and dark environment conditions for 4 days. The sprouts that have grown are ready to be extracted and the amylase enzyme is isolated by blending with adding a little distilled water, the ratio between seeds and distilled water is 50 g of seeds: 400 mL of distilled water. This step will create a slurry. The slurry was centrifuged at 1500 rpm for 10 minutes. The formed-filtrate is a crude amylase extract and ready to be tested for its activity.

2.3. Preparation of the DNSA (3,5-dinitrosalicylic acid) reagent

The preparation of the DNSA reagent was carried out by dissolving 1 g of 3,5-dinitrosalicylic acid in 20 mL of distilled water, then put it into 100 mL volumetric flask and homogenize. Furthermore, 1 g of NaOH is added to the volumetric flask; 0.2 g of phenol; 0.05 g of Na_2SO_3; and 1 mL of Na-K-tartaric 40%, then add distilled water to the limit of the miniscus, then homogenize.

2.4. Determination of amylase activity

The determination of amylase activity begins with the production of a standard glucose curve. The first stage was making a standard solution of glucose with a vary concentration of 50 ppm, 100 ppm, 150 ppm, 200 ppm, and 250 ppm. Each standard solution of 1 mL was put into a separate test tube. For glucose solution blank replaced with distilled water. Each 0.5 mL of standard solution, blank or hydrolyzate sample was put into the test tube, then added with 0.5 mL of distilled water and 2 mL of DNS reagent and vortexed. Each tube was placed in boiling water for 10 minutes, then cooled on a room temperature. Next, measuring the absorbance using UV-VIS-spectrophotometer at a wavelength of 550 nm. Making a standard glucose curve by mapping the concentration of the standard solution versus the absorbance, then the linear regression equation is determined, namely Y = AX + B. By using the linear regression equation, the mapping result of glucose standard concentration vs absorbance can be known that the glucose ration is a result of amylase activity in hydrolyzing the substrate. This catalytic activity is expressed in IU/mL. One international unit is expressed as the total amount of enzyme capable of acting as a catalyst to convert 1 μM of substrate/minutes under standard conditions. Calculation of enzyme catalytic activity:

$$\text{Unit/ml} = \text{Glucose formed x P x } \frac{1}{T}$$

Information:
P: dilution
Q: incubation time (minutes)

2.5. Determination of the optimum amylase temperature

Soybean amylase activity was determined using the DNSA test. Six test tubes were filled with 1 mL of 1% starch solution (in phosphate buffer pH 7). Each tube was added with an amylase enzyme solution that was obtained from the extraction of soybean sprouts in the optimum germination time. The next step, each tube containing amylase and the substrate was reacted in temperature variations, there were 25, 30, 35, 40, and 45°C for 10 minutes. Each hydrolyzate that has been obtained is then tested for glucose concentration which was formed by adding 0.5 mL of distilled water and 2 mL of DNSA reagent and vortex. Each tube was placed in boiling water for 10 minutes, cooled at room temperature. Then, measuring the absorbance using a UV-VIS spectrophotometer at a wavelength of 550 nm. By entering the absorbance value into the glucose standard regression equation, it will be known that the glucose level is formed as amylase activity. The temperature that shows the highest glucose yield indicates the optimum temperature.

2.6. Determination of the optimum pH of amylase

Six test tubes were filled with 1 mL of 1% starch solution (in buffers of pH 5, 6, 7, 8, and 9). Each tube was added with amylase enzyme solution and reacted at optimum temperature for 10 minutes. Each hydrolyzate that has been obtained was then tested for glucose concentration which was formed by adding 0.5 mL of distilled water and 2 mL of DNS reagent and vortexed. Each tube was placed in boiling water for 10 minutes, then cooled to room temperature. Finally, measuring the absorbance using a UV-VIS-spectrophotometer at a wavelength of 550 nm. By entering the absorbance value into the glucose standard regression equation, it will be known that the glucose level is formed as an amylase activity. The pH that shows the highest glucose yield shows the optimum pH.

2.7. Determination of the optimum substrate concentration

Six test tubes filled with 1 mL of starch solution of varies concentration 0 (control); 0.5; 1; 1.5; 2; and 2.5 ppm (in the optimum pH buffer). Each tube was added with amylase enzyme solution and reacted at an optimum temperature for 10 minutes. Each hydrolyzate that has been obtained was then tested for glucose concentration, which was formed by adding 0.5 mL of distilled water and 2 mL of DNSA reagent and vortex. Each tube was placed in boiling water for 10 minutes, cooled at room temperature. After that, measuring the absorbance using a UV-VIS-spectrophotometer at a wavelength of 550 nm. By entering the absorbance value into the glucose standard regression equation, it will be known that the glucose level is formed as amylase activity. The substrate concentration that showed the highest glucose yield showed the optimum substrate concentration

2.8. Enzymatic Reaction kinetics of amylase

The reaction kineticss that have been tested were V_{max} and K_M. V_{max} and K_M were determined using the Lineweaver-Burg equation. The result of 1/[substrate] vs 1/[reaction velocity] mapping results obtained by the straight-line equation y = ax + b. The intercept with the Y axis where the value of X = 0, the value 1/Vmax is obtained, and the intersection point on the X axis (Y = 0) will give the value $-1/K_M$.

3. MATH AND EQUATIONS

3.1. Glucose Standard Curve

Table 1 shows the absorbance results of the standard solution of glucose in the concentration variations of 50 ppm, 100 ppm, 150 ppm, 200 ppm and 250 ppm.

Table 1. The absorbance of the glucose standard solution in various concentration

Glucose standard	Concentration (ppm)	Absorbance
Standard 1	50	0.183
Standard 2	100	0.354
Standard 3	150	0.507
Standard 4	200	0.646
Standard 5	250	0.803

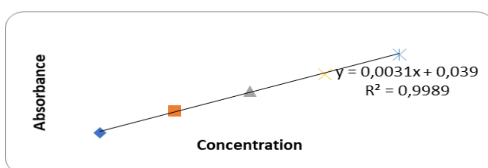

Figure 1 Glucose standard curve

Based on the result of glucose concentration mapping versus absorbance, a linear regression equation is obtained, Y = 0.003 X + 0.039 with a value of $R^2 = 0.998$. This equation is used to determine the glucose concentration in a sample as a result of amylase activity.

3.2. Determination of the Optimum Enzyme Concentration for Amylase

Data Table 2 shows the results of testing the amylase activity of various enzyme concentrations. The measurement result of mobile amylase are presented in Table 2.

Table 2. Amylase activity ad data in various enzyme concentration

Enzyme concentration (%)	Glucose yield (ppm)	Amylase activity (Unit / 2mL)	Amylase activity (Units / mL)
0	34.44	34.44	0
2.5	52	52	8. 78
5	71. 56	71. 56	18. 56
7.5	87.11	87.11	26. 33
10	145. 22	145. 22	55.39
15	158. 56	158. 56	62.06

Information: Measurements were made at a temperature of 37°C, pH 7 and an incubation time of 10 minutes

Table 2 shows that at 0% enzyme concentration the enzyme has no activity, at 2.5% enzyme concentration is 8.78 U/mL and increases within elevation of enzyme concentration. This proves that the enzyme concentration has an effect on amylase activity.

3.3. The Determination of the Optimum Temperature

The determination of optimum temperature was done by reacting amylase with 1% starch substrate in temperature variations of 25, 30, 35, 40, and 45°C, pH 7 for 15 minutes. Then measured the level of glucose formed, and the highest glucose level states the optimum temperature. The result of varied tests in incubation temperature are shown in Table 3.

Table 3 Amylase activity with temperature variations

Temperature (°C)	Glucose yield (ppm)	Amylase activity (Unit / 2mL)	Amylase activity (Units / mL)
25	101.00	101.00	50.5
30	105.00	105.00	52.5
35	100.33	100.33	50.2
40	97.67	97.67	48.8
45	87.00	87.00	43.5
50	71.33	71.33	35.7

The activity of crude amylase extract from soybean seeds at temperature variations is shown in Table 3. At a temperature of 25 °C, the amylase activity was 50.5 U/ mL and increased at 30 °C, which was 52.5 U/mL. Temperature 30 °C is the optimum temperature for mobile amylase in soybean seed sprouts, because it has the highest activity so that the amylase works optimally in degrading starch to glucose. After reaching the optimum condition, the amylase activity will decline again, namely at temperatures of 35 °C, 40 °C, 45 °C, and 50 °C, which is 50.2 U/mL, 48.8 U/mL, 43.5 U/mL, and 35.7 U/mL. The optimum temperature of the amylase enzyme is 30 °C with amylase activity of 52.5 U /mL.

3.4. The Determination of Amylase Catalytic Activity in Various Incubation Time

The optimum incubation time was carried out by reacting amylase with starch substrate in 0, 5, 10, 15, 20 minutes and then measuring the levels of sugar formed from each treatment using the DNSA method. The results of varied tests in incubation time are shown in Table 4.

Table 4. Amylase activity in various incubation times

Incubation Time (minutes)	Glucose yield /ppm)	Amylase Activity U/mL
5	87.83	43.92
10	88.67	44.33
15	78.17	39.08
20	72.67	36.33
25	0.26	0.13

The activity of crude mobile amylase extract from soybean seeds at various incubation times is shown in Table 4. At 5 minutes the amylase activity is 43.92 U/mL, at 10 minutes it is 44.33 U/mL, at 15 minutes the amylase activity is 39.08 U/mL. at 20 minutes the amylase activity was 36.33 U/mL, and at 25 minutes it was 0.13 U/mL. Based on the data, the results of amylase activity have been decreased with increasing incubation time. The optimum incubation time was 10 minutes/

3.5. Determination of amylase catalytic activity in various pH

The optimum pH had been done by reacting amylase with starch substrate at various pH values 5, 6, 7, 8 and 9. Then measuring the sugar-formed content from each treatment with the DNSA method. The reaction was carried out at the optimum temperature and enzyme concentration. The results of pH test variations are shown in Table 5.

Table 5. Amylase activity in various pH

pH	Glucose yield/ppm)	Amylase Activity U/mL
5	0,19	0,09
6	0,19	0,10
7	0,25	0,12
8	0,18	0,09
9	0,18	0,09

The activity of crude amylase extract from soybean seeds at various pH is shown in Table 5. At pH 5 the amylase activity is 0.09 U/mL and increasing within elevation of pH used, namely 0.10 U/mL at pH 6, and 0.12 U/mL at pH 7. At pH 7 the amylase activity shows the optimum activity because it has the highest activity, where amylase works optimally in degrading starch to glucose. After being at the optimum condition, the amylase activity will decrease again, namely at pH 8 and 9 of 0.09 U/mL. The optimum pH of the mobile amylase enzyme is pH 7 with amylase activity of 0.12 U/mL.

3.6. Determination of Amylase Catalytic Activity in Various Substrate

The substrate concentration used in this study was 0 (control); 0.5; 1; 1.5; 2; and 2 mL. The enzymes were used 10% (optimization result item c), incubation temperature 37°C, pH 7, and incubation time of 15 minutes. The results of the substrate concentration test are shown in Table 6.

Table 6. Amylase activity in various substrate concentrations

Substrate Concentration (%)	Glucose yield (ppm)	Amylase Activity (Unit/mL)
0	0,00	0,00
0.5	4,02	4,02
1	5,06	5,06
1.5	5,51	5,51
2	5,66	5,66
2.5	6,21	6,21

The activity of crude amylase extract from soybean seeds on substrate variations is shown in Table 6, the concentration of 0% substrate indicates that the relative amylase activity of amylase is 0 U/mL, at a substrate concentration of 0.5% of 4.02 U/mL and increases with respect to the increase in substrate concentration, namely, at 1% substrate concentration of 5.06 U/mL, at a substrate concentration of 1.5% of 5.51 U/mL, at a 2% substrate concentration of 5.66 U/mL, and at a substrate concentration 2.5% of 6.21 U/mL. This process that the substrate concentration affects amylase activity

3.7. Enzymatic Reaction Kinetics of Amylase

Based on the data in TABLE VI, it can be studied the kinetic of the enzymatic reaction of soybean amylase in hydrolyzing starch, namely V_{max} and K_M, using the Line-Weaver Burg equation.

Table 7. Correlation between starch substrate concentration and amylase activity

Substrate Concentration (ppm)	Amylase Concentratio n (Unit/1mL)	1/ [Substrate]	1/ [Rate]
0,5	4.02	2.00	0.249
1	5.06	1.00	0.198
1,5	5.51	0.67	0.181
2	5.66	0.50	0.177
2,5	6.21	0.40	0.161

Based on Table 7, a linear regression equation is made and the results are shown in Figure 2.

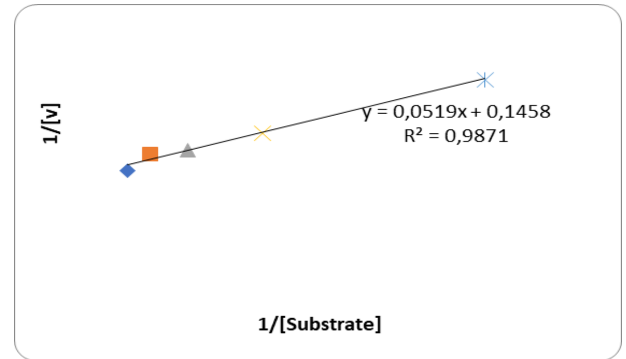

Figure 2 Line Weaver-Burg equation

The mapping result of 1/[substrate] vs 1/[v] obtained a linear regression equation y = 0.052x + 0.146 with a value of R^2 = 0.987. The intersection point with the Y axis where the value of X = 0, the value of Y = 0.145 ($1/V_{max}$) is obtained, then V_{max} = 6.869. The intersection point with the X axis (Y = 0) will get the value of X = 2.843, then K_M = 11.87 U/mL. Thus it can be said that the maximum activity for amylase is 6.869 U/mL. The V_{max} value of the soybean sprouts amylase in this study (6.869 U/minutes) was greater than that of amylase enzyme in sweet orange (*Citrus sinensis*) juice clarification (0.0134 - 0.0325 U/mL [10].

4. CONCLUSION

Based on the research results, it can be concluded that: 1) the optimum concentration of the amylase enzyme is 2.5% (v/v), the optimum temperature in hydrolyzing starch is 30 °C, optimum pH is 7; 2) the value of V_{max} is 6,869 U/mL; and 3) value of K_M is 11.87 U/mL. This information is very important to increase the economic value and efficiency of amylase in the industry

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