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Characterization Of Pure and Immobilized Invertase Enzyme Using Activated Carbon

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Abstract
This study aims to investigate the optimal pH, temperature, and incubation time for both mobile and immobile forms of invertase enzyme, as well as determine their Km and Vmaks values. The research variables encompass the characteristics of the mobile and immobile invertase enzymes, including their optimal pH, temperature, incubation time, Km, Vmaks, and enzymatic activity. The spectronic UV-Vis instrument was employed for data collection in this research. The obtained results consisted of absorbance values, which were subsequently used to generate a regression curve to determine the concentration of invert sugar products. These concentration values were then utilized to assess enzymatic activities. The collected data were analyzed using descriptive methods, including correlation graphs to explore the relationship between enzymatic activity and the research variables. The findings indicate that the mobile form of invertase enzyme exhibits an optimal pH of 4.5, an optimal temperature of 55°C, an optimal incubation time of 25 minutes, a Km value of 1,279 mg/mL (4.737×10^{-6} mol/L), and a Vmaks value of 23,256 mg/mL/minute. On the other hand, the immobile form of invertase enzyme demonstrates an optimal pH of 4, an optimal temperature of 60°C, an optimal incubation time of 30 minutes, a Km value of 2,255 mg/mL (8.352×10^{-6} mol/L), and a Vmaks value of 19,608 mg/mL/minute.

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INTRODUCTION

Invertase or β-fructofuranosidase (E.C.3.2.1.26) is an enzyme which can hydrolyze sucrose disaccharides into glucose and fructose monosaccharides (Aranda et al, 2006). Invertase can be produced from several microorganisms, such as Aspergillus niger, Saccharomyces cerevisiae, Zymomonas mobilis and others. In Aspergillus niger, invertase can be produced intracellularly or extracellularly (Sirisansaneeyakul et al, 2000). Intact cells of Aspergillus sp. are known to produce invertase (β-fructofuranosidase). Invertase has hydrolysis and transfructosylation activities that can be controlled activity as reaction conditions (pH, temperature, and sucrose concentration) (Gibson et al, 2008).

Invertase is used in various industries, such as the food industry (chocolate, candy, jam, and artificial honey), soft drinks, the manufacture of invert syrup, lactic acid and ethanol (Aranda et al, 2006). Because it is widely applied in various industries, the demand and production of invertase increases (Triantarti et al, 2011).

Invertase can be used to hydrolyze sucrose, raffinose, or stakhiosa, but the highest activity in sucrose hydrolysis (Slominski, 2006). Hydrolysis of sucrose to invert syrup can be done with

acid as well as by inverse. Hydrolysis with invertase provides advantages, among others, the conversion coefficient of sucrose into invert syrup can reach almost 100% using low temperatures so that the cost is needed low, purer and produces quality products (Ensymm, 2007)

One unit of enzyme activity is an activity that can produce products with an increase in absorption of 0.001 per minute from control absorption under experimental conditions (Togu G and Eddy, 2006: 46). The activity of the invertase enzyme is measured from the levels of invert sugar products formed using the Nelson Somogyi method. This method is based on the reduced event of kupri oxide to kupro oxide due to the presence of reducing sugars.

The advancement of enzymes is increasingly rapid in several business fields, such as medicine, food factories, textile factories, leather factories, cosmetics, and so on. A good environment for one enzyme will be a barrier for another enzyme as a catalyst. That is why efforts are made to detect the optimization of the condition of each enzyme. There are many uses for enzymes in various industries, therefore higher enzyme stability is required. Until now it is still difficult to isolate enzymes and the results are also small. The use of enzymes in a chemical reaction is usually by reacting directly between the enzyme and its substrate. This method is of course considered uneconomical, once the enzyme is used up and discarded, it is very difficult to take more enzymes from the mixture of products that occur even though the price of enzymes is expensive. In addition, enzymes are easily damaged, so a way is needed so that enzymes can be used repeatedly and can protect them from damage, namely by mobilizing the enzymes.

Methods for enzyme immobilization can be classified in three basic categories: career bonding methods, crosslinking methods, and entanglement methods. This study used the physical adsorption method included in the career bonding method to mobilize the invertasew enzyme because of this method only a little or nothing causes changes in enzyme protein conformation (Togu Gultom, 2016).

Activated carbon has a high adsorption power, therefore activated carbon is often used for immobilization compared to other careers. Apart from being a matrix for mobilizing an enzyme, the chemical industry also uses activated carbon for solvent and gas purification, mixture separation, and others. The pharmaceutical industry uses it to remove color and odor from vitamins and purify vitamins (Murniagus, 2017). This study chose activated carbon as an invertase enzyme mobilizer because active kerbon has a high absorption capacity so that it can attract enzymes into the pores of activated carbon so that the enzyme will be protected from damage. Another reason to choose activated carbon is because activated carbon is easy to obtain.

METHOD

Tools and Materials

The tools used in this study were Genesis 20 Spectronics, water bath, type H 103N clinical centrifuge, incubator, analytical balance, pH meter, shaker, measuring flask, timing device, erlenmeyer, test tube, funnel, drip pipette, volume pipette, measuring pipette, and filter paper

The materials used by all pro-analyses are enzyme invertase (branded Sigma) with pH 4.5 and temperature 55ºC, NaOH, CuSO4.5H2O, Folin-Ciocalteu reagent, Bovine Serum Albumin (BSA), Na2CO3, anhydrous sucrose, glucose and fructose monohydrate, acetate

buffer, Nelson A reagent, Nelson B reagent, arsenomolybdate color reagent, pH 4 activated carbon, and aquades

Research Procedures

The carbon used as the imobilizer matrix has been activated. An enzyme invertase solution of 1 mg/mL concentration is prepared by dissolving 0.1 grams of pure invertase in powder form with aqueous up to a volume of 100 mL.

Reagents used are Lowry method for determination of enzyme protein levels and Nelson Somogyi's method for determining the content of invertase enzyme products (invert sugar)

Manufacture of Immobilized Invertase Enzyme

A total of 5 grams of activated carbon was mixed with 50 mL of enzyme invertase concentration of 1 mg/mL and stirred at a speed of 70 rpm for 45 minutes. Then filtered and rinsed with buffer solution as much as three times to liberate enzymes that are not bound to activated carbon. This immobilized enzyme is further used in characterization determination.

Characterization of Pure Invertase Enzymes

a. Determination of the optimum incubation time of pure invertase enzyme
b. Determination of optimum pH of pure invertase
c. Determination of the optimum temperature of pure invertase
d. Determination of Km and Vmax of pure invertase

Characterization of Immobilized Invertase Enzyme

a. Determination of the optimum incubation time of immobilezed invertase enzyme
b. Determination of optimum pH of immobilized invertase
c. Determination of the optimum temperature of immobilized invertase
d. Determination of Km and Vmax of immobilized invertase

Data Analysis

From the data obtained on variations in experimental conditions, each activity is sought. The activity of car and immobilized invertase enzymes is calculated in units of enzyme units. One enzyme unit is the activity of an enzyme that can produce a product with an increase in uptake of 0.001 per minute of control uptake under experimental conditions.

$$\text{Act} = \frac{\text{absorbance Te} - \text{absorbance To}}{0.001 \text{ x specific incubation time}}$$

By plugging the absorbance value into the linear regression equation of the standard solution curve of an invert sugar, the above equation would be:

$$\text{Act} = \frac{\text{concentration Te} - \text{concentration To}}{0.001 \text{ x specific incubation time}}$$

Information:
Act : enzyme activity
Te : experiment tube To : control tube
To : control tube

From these activities, it can be known the characteristics of car and immobilized invertase enzymes, which include optimal incubation time, optimal temperature, optimal pH, Km and Vmax. The data can be analyzed by graphing the relationship between enzyme activity (Y-

axis) and each of these experimental conditions (X-axis). From the graph obtained, it can be seen the optimum condition of the mobile and immobilized invertase enzymes, namely in conditions that produce the largest enzyme activity.

RESULTS AND DISCUSSION

To measure the amount of protein in a solution, a standard curve is needed that describes the relationship between protein concentration and absorbance at a wavelength of 540 nm. The standard solution commonly used is Bovine Serum albumin (BSA). Protein levels are determined from the standard curve. The operating time of the standard solution of albumin was obtained at minutes 35 to 45, the maximum wavelength at 540 nm and the regression equation the standard curve of albumin is Y= 2.052X + 0.027 with a regression coefficient r2= 0.991. As for the standard solution of invert sugar, the operating time is obtained in minutes 15th to 25th minute, maximum wavelength at 560 nm and the regression equation of the standard curve of invert sugar is Y= 5.560X + 0.015 with regression coefficient r2= 0.994.

Characterization of Pure Invertase and Immobilized Enzymes

Optimum Incubation Time of Pure and Immobilized Invertase Enzymes

The incubation time will give the enzyme a chance to react with the substrate. In this study, the optimum incubation time was determined with time variations of 15, 20, 25, 30, and 35 minutes. From the average activity of pure and immobilized invertase enzymes in various variations of incubation time can be seen in the following table:

Table 1. Average Activity of Pure and Immobilized Invertase Enzymes on Incubation Time Variation

Incubation Time (minutes)	Pure Invertase Enzyme Activity (mgmL-1min-1)	Immobilized Invertase Enzyme Activity (mgmL-1min-1)
15	2.28	3.89
20	2.54	4.12
25	2.65	4.74
30	2.87	4.15
35	2.31	3.55

Based on the table above, it can be seen that there is a shift in incubation time, namely 25 minutes for pure invertase enzyme and 30 minutes for immobilized invertase enzyme. This is due to the presence of activated carbon as a mobilizer in the immobilized invertase enzyme which can block the contact between the enzyme and the substrate, so that the immobilized invertase enzyme requires a longer incubation time than pure invertase enzymes (Crini, 2016)

Optimum pH Conditions of Pure and Immobilized Invertase

Enzymes have a typical optimum pH, which is the pH that causes maximum activity. The pH activity profile of an enzyme describes the pH at which the important proton-giving or receiving group on the catalytic side of the enzyme is within the desired ionization level (Lehninger, 1994). In this study, the optimal pH was determined with a pH variation of 3.5; 4,0; 4,5; 5,0; and 5.5. From the average activity of pure and immobilized invertase enzymes in various pH variations can be seen in Table 2 below.

The activity of pure invertase enzyme is greatest at pH 4.5 while for immobilized invertase enzyme the activity is greatest at pH 4.0. The optimal pH of the immobilized invertase enzyme obtained is lower than that of its pure enzyme. The shift in the optimum pH value of

immobilized enzymes depends on the surface charge of the matrix, causing changes in the charge of the enzyme proteins.

Table 2. Average Activity of Pure and Immobilized Invertase Enzymes at pH Variations

pH	Pure Invertase Enzyme Activity ($mgmL^{-1}min^{-1}$)	Immobilized Invertase Enzyme Activity ($mgmL^{-1}min^{-1}$)
3,5	2,27	3,78
4	2,89	4,06
4,5	2,19	4,67
5	1,95	4,48
5,5	1,79	3,03

The optimum pH shifts towards a more acidic direction due to the following: if the enzyme is mobilized on the polycationic matrix, then the positive charge of the enzyme protein will increase and the pH of the immobilized enzyme region becomes more alkaline than the outer solution. Because polycationics have the property of removing protons from around enzymes, they tend to lower the surrounding pH. Because there is a partitioning effect of protons, the pH of the outer solution is more acidic than the pH around the enzyme itself (Chibata, 1978). In this study using an activated carbon matrix containing a polycationic group so that the positive charge of the enzyme increases causing the optimum pH of immobilized invertase to shift to a more acidic area.

Optimum Temperature of Pure and Immobilized Invertase

The optimum temperature is the temperature that can provide the greatest enzyme catalytic activity. Reactions that use enzyme catalysts are strongly influenced by temperature. The speed of chemical reactions will generally be magnified by rising temperatures. However, because enzymes are proteins, a rise in temperature can cause the denaturation process. If there is a denaturation process, the active part of the enzyme will be disrupted and thus the effective concentration of the enzyme will decrease and the reaction speed will decrease (Anna Poedjiadi, 2019). In this study, the optimum temperature was determined with temperature variations of 45ºC, 50ºC, 55ºC, 60ºC, and 65ºC. From the average activity of pure and immobilized invertase enzymes in various temperature variations can be seen in Table 3.

Table 3. Average Activity of Pure and Immobilized Invertase at Temperature Variations

Temperature (ºC)	Pure Invertase Enzyme Activity ($mgmL^{-1}min^{-1}$)	Immobilized Invertase Enzyme Activity ($mgmL^{-1}min^{-1}$)
45	1,48	3,50
50	1,95	4,12
55	2,17	4,58
60	2,85	3,57
65	2,60	2,58

Based on the table above, it can be seen that the activity of pure invertase enzyme is largest at a temperature of 55ºC, while for immobilized invertase enzyme the activity is greatest at 60ºC. The optimum temperature of immobilized invertase enzyme obtained is higher than that of pure enzyme. This is because in immobilized invertase enzymes, enzymes are bound to activated carbon so that the enzymes are protected from external influences such as heat. According to Kennedy and Cabral (1982), the optimum temperature of immobilized invertase enzyme by physical adsorption method, ionic bonds, and covalent bonds will have a higher temperature than the temperature of the pure enzyme.

Km and Vmax values of pure and Immobilized Invertase

The determination of Km and Vmax values in this study was carried out at an incubation time of 25 minutes, pH 4.5 and a temperature of 55ºC for pure invertase enzymes. As for the immobilized invertase enzyme, it was carried out at an incubation time of 30 minutes, pH 4.0 and a temperature of 40ºC. In determining the value of Km and Vmax, it uses a variation in substrate concentration of 0.1; 0,2; 0,3; 0,4; and 0.5 mg/mL. From the average activity of pure and immobilized invertase enzymes in various variations in substrate concentrations can be seen in Table 4.

Table 4. Average Activity of Pure and Immobilized Invertase Enzymes on Substrate Concentration Variations

Substrate Concentration (mg/mL)	**Pure Invertase Enzyme Activity ($mgmL^{-1}min^{-1}$)**	**Immobilized Invertase Enzyme Activity ($mgmL^{-1}min^{-1}$)**
0,1	0,98	1,80
0,2	1,49	3,12
0,3	2,97	4,82
0,4	3,30	5,95
0,5	3,34	5,98

Based on the table above, it can be seen that at a small substrate concentration, the reaction activity is low, but the activity will increase with increasing substrate concentration until at some point there is a very small and almost constant increase, at which point saturation has occurred. To calculate the value of Km and Vmax, a graph of the relationship between 1/[S] and 1/v was made and obtained a linear regression equation, namely Y = 0.055X + 0.043, regression coefficient r^2 = 0.997 for pure invertase enzyme and Y = 0.115X + 0.051, regression coefficient r^2 = 0.958 for immobilized invertase enzyme. Based on this linear regression process, the value of pure Km invertase is 1.279 $mgmL^{-1}$ (4.737 x 10^{-6} mol/L) and immobilized invertase 2.255 $mgmL^{-1}$ (8.352 x 10^{-6} mol/L). As for the Vmax value of pure invertase enzyme is 23.256 $mgmL^{-1}min^{-1}$ and immobilized invertase enzyme is 19.608 $mgmL^{-1}min^{-1}$.

The shift in the Km value of immobilized enzymes can be influenced by the electrostatic interaction between the matrix and the substrate and the effect of diffusion of the substrate with the products in the matrix (Chibata, 1978). The diffusion of the substrate in free invertase is greater than that of invertase already bound to the matrix. Immobilization of enzymes on the matrix leads to conformational changes and an increase in the steric space. Changes in the conformation of enzyme protein molecules and steric disruption of their bases increase the Km value and decrease the activity between the enzyme and substrate (Allan et al, 2016).

The Vmax value of the mobile invertase enzyme is greater than that of the immobilized invertase enzyme. This is because the mobile enzyme is free to break down sucrose enzyme is not hindered by anything so that the reaction speed of pure invertase enzyme is greater when compared to immobilized invertase enzyme bound to activated carbon (Crini, 2016)

CONCLUSION

Based on the results of research and discussion, the following conclusions can be drawn that The findings indicate that the mobile form of invertase enzyme exhibits an optimal pH of 4.5, an optimal temperature of 55°C, an optimal incubation time of 25 minutes, a Km value of 1,279 mg/mL (4.737 x 10^{-6} mol/L), and a Vmaks value of 23,256 mg/mL/minute. On the

other hand, the immobile form of invertase enzyme demonstrates an optimal pH of 4, an optimal temperature of 60°C, an optimal incubation time of 30 minutes, a Km value of 2,255 mg/mL (8.352 x 10^{-6} mol/L), and a Vmaks value of 19,608 mg/mL/minute.

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