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DETERMINATION OF ENZYME KINETIC PARAMETERS ON SAGO STARCH HYDROLYSIS BY LINEARIZED GRAPHICAL METHODS

(Penentuan Enzim Parameter Kinetik pada Hidrolisis Kanji Sagu dengan Kaedah Grafik Lelurus)

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Abstract

Amyloglucosidase (E.C. 3.2.1.3) from *Aspergillus niger* was used to hydrolyze the sago (*Metroxylon sagu*) starch into reducing sugars. The experiment was conducted at constant temperature, 55 °C; pH, 4.5 and enzyme amount, 0.2 U/ml, respectively. In this investigation, the substrate concentration was varied ranging from 1.0 – 7.0 g/L. The obtained data were then fixed into linearized plots namely Lineweaver-Burk and Langmuir models to calculate enzyme kinetic parameters, K_m and V_{max}. Both of the K_m and V_{max} (mM, mol/min) values from each plot were: Lineweaver-Burk (26.53, 3.31) and Langmuir (13.52, 2.35). Among the linearized models, K_m and V_{max} values acquired from Langmuir plot was chosen.

Keywords: enzyme kinetic parameters, K_m and V_{max}, Langmuir, Lineweaver-Burk, sago starch hydrolysis

Abstrak

Enzim amyloglucosidase (EC 3.2.1.3) dari *Apergillus niger* digunakan untuk menghidrolisis kanji sagu (*Metroxylon sagu*) kepada gula penurun. Eksperimen ini dijalankan pada suhu 55°C; pH, 4.5 dan enzim 0.2U/ml. Kepekatan substrak yang diuji dalam kes ini adalah dalam lingkungan 1.0 – 7.0 g/L. Data yang diperolehi akan digunapakai terus ke dalam linear plot iaitu Lineweaver-Burk serta Langmuir bagi tujuan mengira enzim parameter kinetic: K_m dan V_{max}. Kedua-dua nilai K_m dan V_{max} (mM, mol/min) dari Lineweaver-Burk and Langmuir masing – masing adalah 26.53, 3.31; 13.52, 2.35. Nilai K_m and V_{max} dari Langmuir model dipilih dalam kajian ini.

Kata kunci: enzim parameter kinetik, K_m dan V_{max}, Langmuir, Lineweaver-Burk, hidrolisis kanji sagu

Introduction

Enzyme kinetic is the branch of enzymology that deals with the factors affecting the rate of enzyme-catalyzed reactions. In general, the most important factors are those of enzyme concentration, ligand concentrations (substrates, products, inhibitors and activators), pH, ionic strength and temperature. When all these factors are analyzed properly, it is possible to learn a great deal about the nature of the enzyme-catalyzed reaction. In addition, a kinetic analysis can lead to a model for an enzyme-catalyzed reaction and conversely, the principles of enzyme kinetic can be used to write the velocity equation for a model which can then be tested experimentally [1]. In general, the Michaelis and Menten [2] framework has proven to be simple yet powerful approach to describe the kinetic of most enzyme reactions (Eq. 1.0):

$$v = \frac{V_{max}[S]}{K_m + [S]} \quad \text{(Eq. 1.0)}$$

where v is the initial reaction velocity, [S] is the concentration of reactant, V_{max} is the maximum rate of reaction at infinite reactant concentration, K_m is the Michaelis constant for reactant. Typical unit for V_{max} and K_m is kg.mol/m^3.s and kg.mol/m^3, respectively. According to Segel [3], Equation (1.0) is valid only if v is measured over short enough time, so that [S] remains essentially constant. This requires that no more than 5% of substrate to be utilized over the assay period.

In examining the relationship between v and [S] in Equation (1.0), when [S] < 0.01 K_m, the [S] in the denominator can be ignored and the equation reduced to:

$$v = \frac{V_{max}[S]}{K_m} \quad \text{(Eq. 1.1)}$$

This is called first order kinetic. As stated by Segel [1], the zero order kinetic is obtained when [S] > 100 K_m. The K_m in the denominator of Equation (1.0) may be ignored and equation simplifies as shown as below:

$$v = \frac{V_{max}[S]}{K_m + [S]} \rightarrow = \frac{V_{max}[S]}{[S]}$$
$$\rightarrow v = V_{max} \quad \text{(Eq. 1.2)}$$

In general, the Michaelis-Menten equation leads the plot of v versus [S] in the form of a rectangular hyperbolic curve through origin, with asymptotes $v = V_{max}$ and [S] = – K_m. Since the v versus [S] gives a hyperbolic curve and thus it is rather difficult to determine V_{max} and the [S] that yields ½ V_{max} (i.e. K_m) [3]. In addition, the physical necessity restrict measurement of v to finite positive values of [S] and this consequently make the measurement of K_m and V_{max} become less accurate and precise from such plot. According to Eisenthal & Cornish-Bowden [4], it happened due to asymptotes cannot be approached closely enough. Therefore, the determination of K_m and V_{max} are usually performed by plotting several well-known linear graphs. By using these transformation linear methods, it is easier to estimate the values of kinetic constants. Table 1 depicts the model, equation, slope and intercept of these linearized plots.

Table 1. Common linear transformation methods to evaluate enzyme kinetic parameters [5, 6]

Model	Equation	Slope and Intercept
Lineweaver-Burk	$1/v = K_m/V_{max} + 1/V_{max}$	Slope: K_m/V_{max}
		Intercept: $1/V_{max}$
Langmuir	$[S]/v = K_m/V_{max} + s/V_{max}$	Slope: $1/V_{max}$
		Intercept: K_m/V_{max}

The basic idea of this research is to estimate the kinetic parameters, K_m and *Vmax* on sago starch hydrolysis by using amyloglucosidase (E.C. 3.2.1.3) derived from *Aspergillus niger*. This paper was aimed to demonstrate the determination of K_m and V_{max} values using simple linearized graphical methods particularly Lineweaver-Burk and Langmuir plot.

Materials and Methods

Chemicals

All chemical used in the experiment such as acetic acid, CH_3COOH; 3, 5–dinitrosalicylic acid, $C_7H_4N_2O_7$; phenol, C_6H_5OH; potassium sodium tartrate tetrahydrate, $C_4H_4KNaO_6.4H_2O$; sodium acetate anhydrous, CH_3COONa; sodium hydroxide, NaOH and sodium sulfite anhydrous, Na_2SO_3 were of analytical grade with highest purity.They were purchased from Merck (M) Sdn. Bhd.

Substrate

The sago starch (*Metroxylon sagu*) was purchased from local commercial producer, Wah Chang International, Malaysia. The chemical composition of sago starch (dry weight basis, %) is as follows: amylose 28; amylopectin 72; fat 0.18; protein 0.11; ash 0.12 and fiber 0.21 [7].This milky-white starch powder was dried at 60 °C to a constant weight prior to use [8].

The sago starch solution was prepared accordingly to response surface methodology (RSM) design as described by Wee *et al*. [9] i.e. in % (w/v) of 0.0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.7 in 0.05 M acetate buffer solution. (All the substrate concentration in % (w/v) will be expressed in g/L where 0.1 % (w/v) is equivalent to 1.0 g/L and so forth). The sago starch solution was preheated using stirring hotplate while carefully kept from boiling. This procedure was aimed to enhance the solubility of sago starch in buffer solution [10].

Enzyme

The commercial lyophilized amyloglucosidase (E.C. 3.2.1.3) from *Aspergillusniger*was purchased from Sigma-Aldrich (M) Sdn. Bhd. This enzyme has specific activity at 31.2 U/mg. One unit of enzyme could be defined as liberation of 1.0 mg glucose from starch in 3 min under standard assay conditions i.e. at pH 4.5 and temperature 55 °C. Enzyme solution was prepared by dilute enzyme powder in 0.05 M sodium acetate buffer. After preparation, it was kept at -20 °C before used [11]. The amyloglucosidase enzyme has also been claimed possess wide industrial applications particularly for large scale saccharification of malto-oligosaccharide into β-D-glucose units [12]. Hence it was selected as a biocatalyst to hydrolyze the sago starch in this study. A hydrolysis on starch by amyloglucosidase as followed (Equation 2.0):

$$\text{Starch} + H_2O \xrightarrow{\text{Amyloglucosidase}} \text{D} - \text{Glucose} \qquad \text{(Eq. 2.0)}$$

Enzyme hydrolysis reaction

In this investigation, the substrate concentrations ranging from 1.0 – 7.0 g/L were verified and tested in order to determine the enzyme kinetic parameters (K_m and V_{max}). Meanwhile, the pH (4.5), temperature (55 °C) and enzyme loading (0.2 U/ml) in sago starch hydrolysis were preset at its optimal conditions as described in previous study [9].

The 1.0 ml of amyloglucosidase enzyme (0.2 U/ml) was pipetted and then discharged into test tube which contained 1.0 ml of sago starch solution. Once the enzyme was discharged into test tube, time was recorded accordingly. All trials were performed in triplicate and errors were within ± 5 %. The amount of product formed i.e. reducing sugars (glucose), were determined using the dinitrosalicylic (DNS) acid method [13]. The enzyme hydrolysis reaction was ceased by adding DNS reagent into test tubes and followed by immediately brought to 100 °C in water bath for at least 15 min. The test tubes were then added with 40 % (w/v) Rochelle salt and vortex before left it to cool down to room temperature. The cooled samples were further measured using UV-visible spectrophotometer (Thermo, Genesys 20, USA) at 575 nm to determine the concentration of reducing sugars formed. The absorbance (Abs) was converted to concentration using a calibration curve as shown in Figure 1 and the reading was fitted the following equation (Eq. 3.0):

$$C_{rs} = 769 Abs_{575} \qquad \text{(Eq. 3.0)}$$

where C_{rs} the concentration of the reducing sugars in µg/ml, Abs_{575} the absorbance of the solution at 575 nm.

Equation (3.0) had a regression coefficient of 0.9818 and applied over a concentration range from 0 to 700 μg/ml glucose standard solution. A control blank containing an initial concentration of un-reacted starch and heat-deactivated amyloglucosidase was prepared to correct for the baseline reading in the sample measurement.

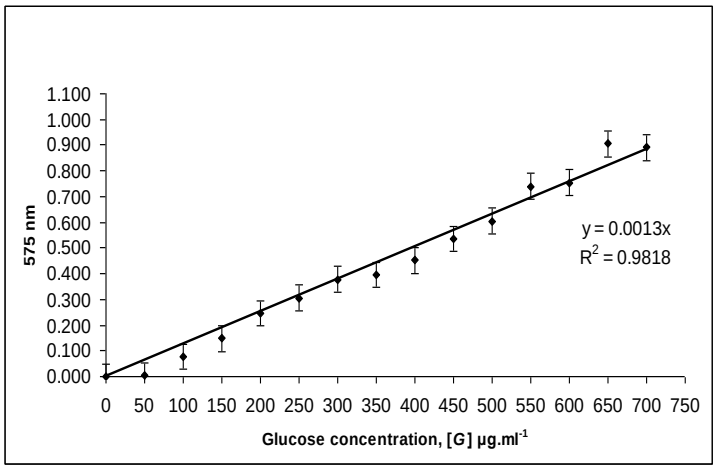

Figure 1. Glucose standard calibration plot

Calculation of initial reaction velocity, *v*

The initial reaction velocity, *v* can be determined by drawing a straight line on hydrolysis curve as shown in Figure 2. The tangent of the curve is calculated and value of *v* is recorded. The tangent was obtained based on the differentiation of polynomial equation as a function of the time course appearance of product data (reducing sugars). A suitable value of the independent variable i.e. time was then substituted into the differentiated polynomial to obtain the estimate of the tangent. Microsoft Excel® 2003 was used to perform the polynomial fitting. The rate of sago starch hydrolysis, *v* versus substrate concentration, [*S*] is plotted in Figure 3.

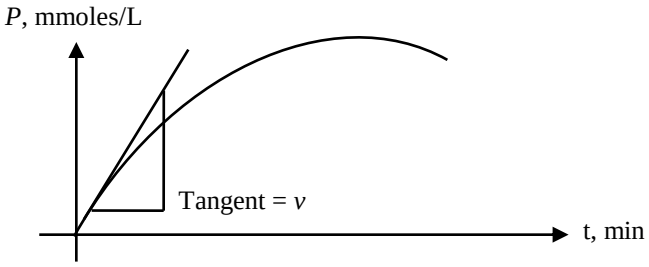

Figure 2. Determination of initial reaction velocity, *v* by drawing a straight line and tangent on hydrolysis curve

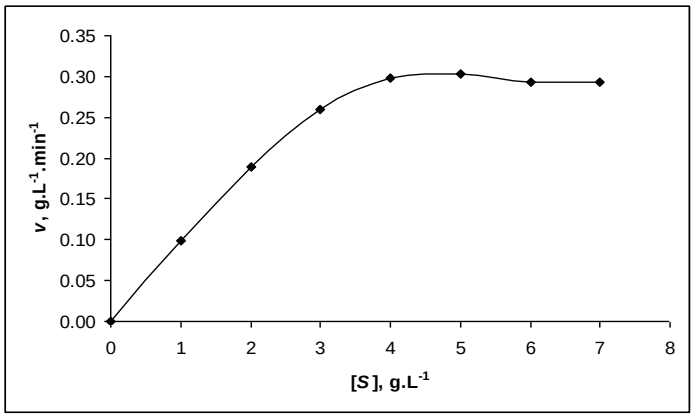

Figure 3. Michaelis-Menten plot

Results and Discussion

In order to understand the kinetic aspect of the reaction, an explicit understanding of its kinetic behavior is important. This can be done by determining the enzyme kinetic parameters using mathematical model. Hence, the linearization graphical methods such as Lineweaver-Burk and Langmuir plots were used to estimate the Michaelis constant, K_m and maximum reaction velocity (maximum reaction rate), V_{max}. The linearization methods used in this study were Lineweaver-Burk and Langmuir plot, respectively. Both methods provided the regression coefficient, R^2 = 0.9687 and 0.9260 as shown in Figures 4 and 5, respectively.

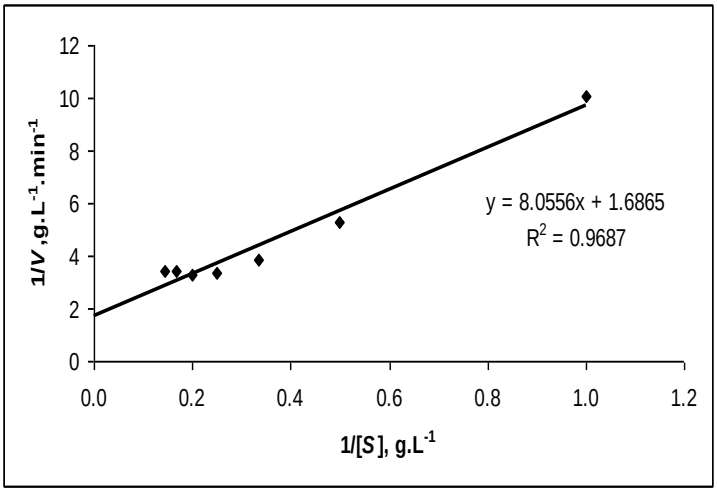

Figure 4. Lineweaver-Burk plot

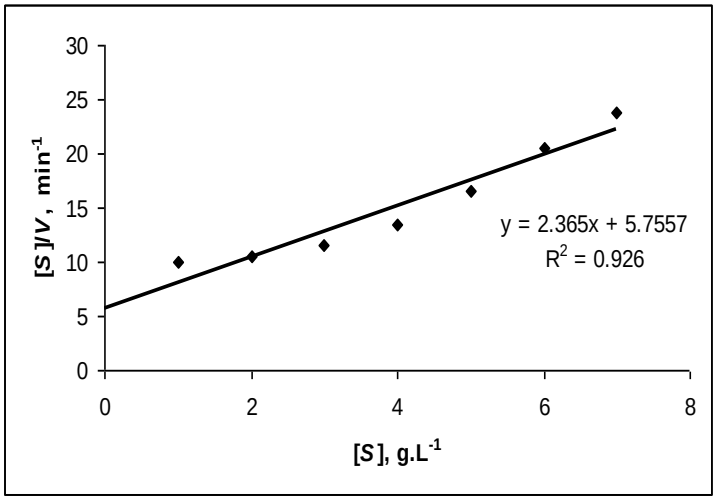

Figure 5. Langmuir plot

Both of K_m and V_{max} values were calculated based on linear equations with y = 8.0556x + 1.6865 (Lineweaver-Burk) and y = 2.365x + 5.7557 (Langmuir). The calculated values of K_m and V_{max} for Lineweaver-Burk and Langmuir models were 4.7762 and 2.4335 g/L; 0.5959 and 0.4228 g/L.min, respectively.

Table 2 shows the K_m and V_{max} values from Langmuir and Lineweaver-Burk plots. In general, the Langmuir plot appears to be more accurate than Lineweaver-Burk. This is because Lineweaver-Burk model was found to distort the experimental error in v and this error is amplified at low substrate concentration. Eventually it will give inaccurate results. Therefore this model is not recommended to evaluate both K_m and V_{max} even though the regression coefficient of Lineweaver-Bulk (R^2 = 0.9687) is higher than Langmuir (R^2 = 0.9260) [4]. This is also supported by Ranaldi and co-workers [14], i.e. they claimed that since the errors on v are most likely to be significant at low values of v, these measurements affect the slope of the plot in decisive manner.

Table 2. Enzyme kinetic values for $K_m$andV_{max} by using linearized model

Kinetic parameters	**Langmuir**	**Lineweaver-Burk**
K_m, g/L	2.43	4.78
V_{max}, g/L.min	0.43	0.60
Regression coefficient, R^2	0.9260	0.9687
Conversion of unit		
K_m, mM	13.52	26.53
V_{max}, mmoles/L.min	2.35	3.31

Moser has proposed that the Langmuir model plot is able to minimize distortions in experimental error and therefore it is recommended that Langmuir model should apply in evaluating both values of K_m and V_{max} [15]. The above

statement was also in close agreement with Wilkinson who stated that in the Langmuir plot, the points corresponding to low *v* values cluster on the left side and this only affects the slope of the plot in a very small degree [16]. Apart from that, Doran has also suggested that the Langmuir model is more suitable in the evaluation of K_m and V_{max} for linearization method [6].

Hence, based on these view points, the values of enzyme kinetic parameter determined from Langmuir model were selected. Both K_m and V_{max} values were determined at 13.52 mM and 2.35 mmoles/L.min, respectively. The regression coefficient, R^2 at 0.926 was also indicated that less than 7.4 % error was not explained by Langmuir model for the sago starch-amyloglucosidase hydrolysis.

Conclusion

The sago starch hydrolysis was successfully performed. The enzyme kinetic parameters for both K_m and V_{max} were determined at fix temperature, pH and enzyme amount with varied of substrate concentration. The obtained data were plotted in two difference linearization models to acquire K_m and V_{max}. In this case, kinetic parameters values obtained from Langmuir plot (K_m = 13.52 mM and V_{max} = 2.35 mmoles/L.min) were chosen as the best determined values.

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