



## **Kinetics of Enzymatic Hydrolysis of Southeast Sulawesi Sago Starch**

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### **Authors' contributions**

*This work was carried out in collaboration of both authors. Author AA designed the study performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author MN managed the analyses, particularly the enzymatic and chemical analysis. Both authors read and approved the final manuscript.*

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### **ABSTRACT**

The aims of this study were to characterize the kinetics of enzymatic hydrolysis of sago starch, obtained from Southeast Sulawesi Indonesia. The enzyme used for hydrolysis was bacterial  $\alpha$ -amylase (Termamyl 120L from *Bacillus licheniformis*, E. C. 3.2.1.1). The method to determine the initial velocity ( $V_o$ ) of the hydrolysis was developed by differentiation a nonlinear equation (NLE). The  $V_o$  of the hydrolysis was measured at various pH (6.0, 6.5, and 7.0), temperatures (40, 60, 75 and 95°C), enzyme concentrations (0.5, 1.0, 1.5 and 2.0  $\mu\text{g}$  per mL) and in the presence of 70 ppm  $\text{Ca}^{++}$ . The optimum conditions of this experiment were found to be at pH 6.5 – 7.0 and 75°C, and the  $V_o$  increased with increasing enzyme concentration. The  $V_o$  values at various substrate concentrations were also determined, which were then used to calculate the enzymes kinetics constant of the hydrolysis, including Michaelis-Menten constant ( $K_m$ ) and maximum velocity ( $V_{max}$ ) using a Hanes plot.  $K_m$  and  $V_{max}$  values were found to be higher in the measurement at pH 7.0 and 75°C. The  $K_m$  values at four different combinations of pH and temperatures (pH 6.5, 40°C; pH 6.5, 75°C; pH 7.0, 40°C; pH 7.0, 75°C) were found to be 0.86, 3.23, 0.77 and 3.83 mg/mL, respectively; and  $V_{max}$  values were 17.5, 54.3, 20.3 and 57.1  $\mu\text{g}/\text{mL}/\text{min}$ , respectively. The results obtained showed that hydrolysis rate of this starch was somewhat low.

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## 1. INTRODUCTION

The hydrolyzed sago starch is required in many purposes, such as for production of enzyme, ethanol, bio-hydrogen [1,2,3], and lactic acid which is used as raw materials in pharmaceutical and packaging application [4]. Hydrolysis of sago starch may be carried out by acidic or enzymatic catalysis or their combination. The enzymatic method has several advantages over acidic catalysis, including the production of fewer by products, less purification, more specific hydrolysis and higher yield [5].

The use of bacterial  $\alpha$ -amylase in hydrolyzing various starches, as well as kinetic data has been reported [6,7,8,9]. Commercial food grade enzymes, such as Termamyl 120L (E.C.3.2.1.1 from *Bacillus licheniformis*, NOVO Nordisk), is a bacterial  $\alpha$ -amylase which hydrolyzes the  $\alpha$ -1,4-glucosidic linkages located at the inner regions of the molecules of amylose and amylopectin.

Sago starch, which is derived from palm of *Metroxylon sago* is one of the typical underutilized indigenous food starch in Southeast Asia and the Pacific Region [10]. Southeast Sulawesi is one of the main sago producers in Indonesia. Modifying sago starch enzymatically may produce such products as maltodextrin, glucose, maltose, etc., which are required in many food and industrial application. In this regard, the information on the kinetic data of sago starch hydrolysis is important in deciding the suitability of this starch for manufacturing such starch hydrolysis products. Therefore, this study is intended to characterize the kinetics of enzymatic hydrolysis of sago starch, obtained from Southeast Sulawesi Indonesia. The present study was aimed to characterize the enzymatic hydrolysis kinetics of sago starch in terms of variation in pH, temperature and enzyme concentration.

## 2. MATERIALS AND METHODS

### 2.1 Materials

The sample was native sago starch from *Metroxylon* sp., obtained from Southeast Sulawesi Indonesia. The sago starch was self-prepared by obtaining the fresh starch from the palm pith, separating the fibers and foreign matters, suspending it in the cold water and subsequently sun-dried.

Termamyl 120L was a thermostable bacterial  $\alpha$ -amylase (E.C.3.2.1.1) from *Bacillus licheniformis*, with activity of 120 KNU/g. One KNU (Kilo Novo  $\alpha$ -amylase Unit) was equivalent to the amount of enzyme which broken down 5.26 g of amylopectin soluble starch (Merck) per hour at a temperature of 37°C and pH 5.6 (NOVO Enzyme Product Sheet). All reagents used in this experiment were of analytical grade unless otherwise specified.

### 2.2 Sample Preparation

The substrate solution was treated with borohydride to reduce the effects of the possible presence of reducing sugar compounds in the sample, which would contribute to a high zero-time blank. The method of Strumeyer, as described in [11], was used to prepare the stock solution by suspending about 2.5 g of the starch samples in 10 mL of distilled water, boiling for 1 min, then diluting to 45 mL (to make a 5% solution). It was then cooled in an ice bath; and a cold solution of 75 mg NaBH<sub>4</sub> in 5 mL water was stirred in. The mixture was held at room temperature for 1 hour, then stored in a refrigerator.

For use, 0.2 mL acetone was added drop-wise to 10 mL of the stock solution with good shaking to destroy excess borohydride. After 20 min at room temperature the solution was neutralized with 1 N acetic acid, 70 ppm Ca<sup>++</sup> added and diluted to the volume of 50 mL with potassium dihydrogen phosphate buffer of the desired pH (to give a 1% solution of reduced starch). This 1% solution was then used to make different initial substrate concentrations of 0.413, 0.625, 1.250, 2.500, and 5.000 mg/mL

### 2.3 Stock and Working Solution of Enzyme

Stock solution of the enzyme was prepared by diluting the supplied enzyme (thermostable  $\alpha$ -amylase, Termamyl 120L) in the potassium dihydrogen phosphate buffer of the desired pH. Working solution was prepared daily by adjusting/diluting the enzyme stock solution to 0.5, 1.0, 1.5 and 2.0  $\mu$ g per mL.

### 2.4 Enzymatic Hydrolysis of Sago Starch

An assay was firstly conducted to determine the optimum conditions (i.e. time, temperature and pH) of the sago starch hydrolysis. Hydrolysis was

done by combining 0.5 mL of the 1%-reduced starch solution with 0.5 mL of enzyme solution. The parameters of temperature and pH were selected based on a preliminary experiment. The temperatures of the reaction were 40, 60, 75 and 90°C, the pH was adjusted to 6.0, 6.5, and 7.0; and the times for hydrolysis extended from 5 up to 90 min. The reaction was halted by adding 1 mL of Somogyi-copper reagent, then boiling in a water bath for 20 min. The reaction mixture was then cooled to room temperature and 1 mL of Nelson-arsenomolybdate reagent added. The tubes were shaken on a vortex mixer, allowed to stand for 5 min, and then diluted by adding 7 mL of distilled water. The absorbance of the solution was read at 510 nm and the reducing sugar was measured against the blank in which the enzyme was excluded. A calibration curve was made with anhydrous-glucose of 0.2 to 1.0 mg in 2 mL of distilled water. Since the hydrolysis of the starch resulted in the formation of glucose, maltose, and oligosaccharides, the assay was performed by measuring the concentration of the reducing sugars by Nelson-Somogyi method.

The kinetic data of  $K_m$  and  $V_{max}$  was determined by measuring the hydrolysis rate at various substrate concentrations, and subsequently calculating them using the Hanes plot.

## 2.5 Enzymatic Hydrolysis of Various Starch Samples

To compare the hydrolysis rate of various starch samples, the enzymatic hydrolysis of arenga, corn, tapioca, and pure soluble starches were also carried out by the same manner as described earlier. The temperature of the reaction was kept constant at 40°C, and the pH was adjusted to 6.5.

## 2.6 Preparation of Somogyi-Nelson Reagents

The Somogyi-Nelson method, as detailed in [11], was used to measure the reducing sugars content. To prepare Somogyi-copper reagent, about 24 g anhydrous  $\text{Na}_2\text{CO}_3$  and about 12 g Rochelle salt (sodium potassium tartate,  $\text{NaKC}_4\text{H}_4\text{O}_6 \cdot 4\text{H}_2\text{O}$ ) were dissolved in 250 mL of distilled water. Then, 40 mL of a 10% solution of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  were dissolved in 500 mL water and boiled briefly to degas. It was cooled and added to the first solution, and the whole was made up to 1 L volume with water.

To prepare Nelson-arsenomolybdate reagent, about 25 g ammonium molybdate ( $(\text{NH}_4)_2\text{MoO}_4$ )

was dissolved in 450 mL water. Then, 21 mL of concentrated  $\text{H}_2\text{SO}_4$  was added, followed by 25 mL solution containing 3 g sodium arsenate ( $\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$ ). The reagent was mixed, held at 37°C for 24 to 48 h, then stored in a brown bottle.

## 2.7 Statistical Analysis

The parameters of the nonlinear equation, used in  $V_o$  determination, were estimated using the Minitab® 18.1 statistical package. Completely random design was employed in this study, and the results reported were the means of at least two replicates. Analysis of variance was also performed, and Duncan multiple range test was utilized for comparison among means.

## 3. RESULTS AND DISCUSSION

### 3.1 Initial Velocity ( $V_o$ ) Measurement

The extent of the hydrolysis was recorded by measuring the amount of reducing sugar released in  $\mu\text{g/mL}$ , denoted as  $[P]$ , and expressed as a function of the duration of the reaction. The  $[P]$  versus time graphs typically indicated a non-linear relationship, as shown in Fig. 1.

In an enzymatic kinetic study of sago starch, Lai et al. [12] employed a linearized graphical method to measure the  $V_o$ . The initial velocity of the hydrolysis was determined by drawing a straight line on the hydrolysis curve. The tangent of the curve was then calculated, and it was obtained based on the differentiation of polynomial equation as a function of the time course appearance of product data (reducing sugars). An attempt was made to measure the kinetics of sago starch hydrolysis by using a non linear equation (NLE) method. The NLE model was constructed to model the relationship of the rectangular hyperbolic graphs of  $[P]$  versus time for sago starch hydrolysis, as shown in Equation 1.

$$[P] = \alpha - \beta e^{(-kt)} \quad (1)$$

Where,

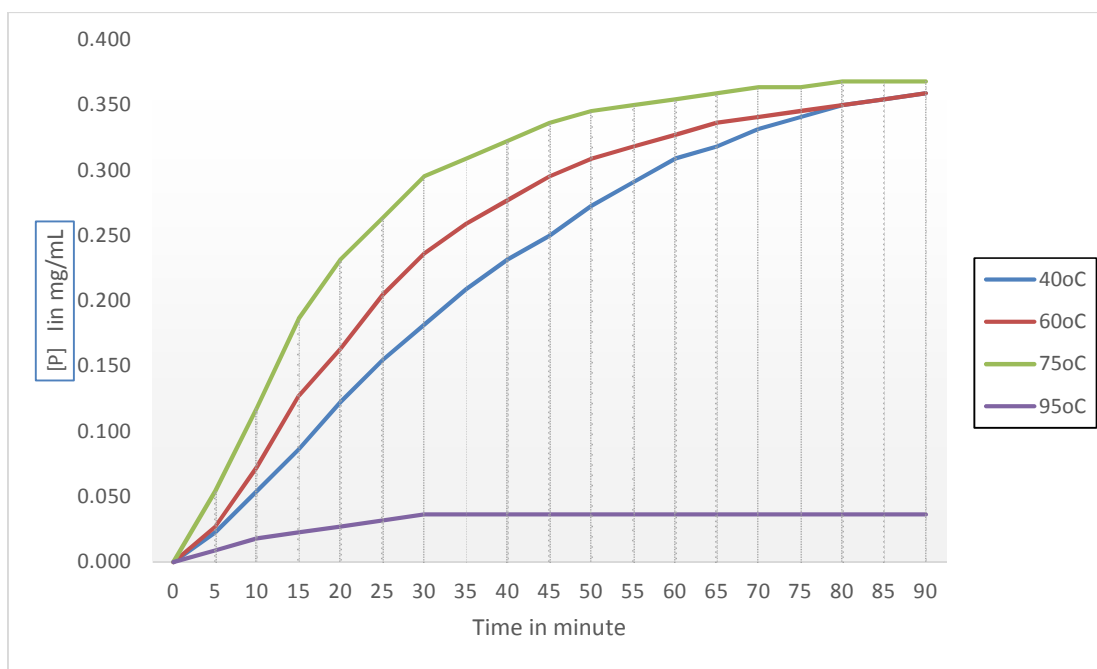
$[P]$  was the amount of reducing sugars released during hydrolysis in  $\mu\text{g/mL}$

$\alpha$  estimated the maximum  $[P]$  produced ( $\mu\text{g/mL}$ )

$\beta$  estimated the maximum change in  $[P]$  ( $\mu\text{g/mL}$ )

$k$  estimated the relative change in curve function ( $\text{min}^{-1}$ )

$t$  was the elapsed time of hydrolysis (min)



**Fig. 1. The amount of reducing sugar produced, [P], by the hydrolysis of sago starch by Termamyl 120L at various temperature as a function of incubation time**

The values of  $R^2$  of this relationship, which was found to be more than 0.99 for most of the data, indicated that most of the variations of the experimental data were explained by the model. The adequacy of the model was further evaluated by analyzing the residuals, which were the difference between the observed values and the predicted values. It was observed that the residuals were randomly distributed, suggesting that the model was adequate under this experimental condition [13].

The first derivative of this NLE equation would be  $\beta k e^{-kt}$ . This would then express the change in [P] per unit time, which apparently measured the velocity of the hydrolysis. At the initial reaction ( $t \approx 0$ ), it would become  $\beta k$  and would give an estimate of the initial reaction velocity ( $V_0$ ). By estimating the parameters of  $\alpha$ ,  $\beta$ , and  $k$  using the Minitab 18 computer package, the values of  $V_0$  may be obtained.

The  $V_0$  might also be determined using a simple approach by calculating the slope of the [P] versus time graph at a certain point of the initial reaction [12]. However, this method has a drawback in accurately deciding which point of the reaction duration should be used due to the progressive slope changes at different points. As shown in Fig. 1, the slope at 5 min, for example,

was different from that at 20 min, resulting in a different value of  $V_0$ . Therefore, it appeared that determination of  $V_0$  would be more accurate with the NLE method because the model was constructed over a greater proportion of the course of the reaction.

### 3.2 Factors Affecting Velocity of Hydrolysis

Factors affecting  $V_0$  of the hydrolysis of sago starch sample, including temperatures, enzyme concentration and pH were explored. A review by Stauffer [11] revealed that the increase of temperature had two disproportionate effects on enzyme reaction: (1) accelerating the rate of the reaction, and (2) at the same time increasing the rate of enzyme inactivation. This was most clearly demonstrated by the [P] versus time graph, as shown in Fig. 1. With increasing the temperatures from 40 to 75°C, the initial slope was steeper, suggesting the increase of the enzyme hydrolysis rate. On the other hand, the curvature of the graph was also increased, suggesting a progressive denaturation of the enzyme at the higher temperature.

The hydrolysis at 95°C had a lower velocity than those at 75 and 60°C. As the concentration of the enzyme used was low (2  $\mu\text{g}$  per mL), the lower

rate of hydrolysis at 95°C was apparently due to the severe inactivation of the enzyme in this high temperature.

The initial velocity increased progressively with the increasing enzyme concentrations, as shown in Fig. 2. The slopes of the lines were increased with an increasing temperature from 40 to 75°C, suggesting an increasing activity of the enzyme.

The effects of different pH on the enzymatic hydrolysis at two different temperatures (40 and 75°C) and their  $V_o$  values are given in Table 1. At both temperatures, the amount of reducing sugar released was significantly lower at pH 6.0 ( $p < 0.01$ ). Of the three pH values studied, the hydrolysis reached a virtual plateau after 40 min at 75°C. But at 40°C, the hydrolysis did not reach a plateau even after 70 min, except at pH 7.0.

This phenomenon might be due to a similar effect of the temperature, which was explained above. At 75°C, the enzyme was apparently more active, indicated by a higher  $V_o$  value, but less stable indicated by an earlier occurrence of the graph curvature. It seems that an earlier inactivation of enzymes has taken place at this condition. Conversely, at 40°C, the reaction velocity was less, but the enzyme reaction

appeared to be in a less stable condition, indicated by a lower activity.

### 3.3 Determination of $K_m$ and $V_{max}$

Based on the optimum conditions, as discussed earlier, the kinetic constants of  $K_m$  (Michaelis-Menten constant) and  $V_{max}$  (maximum velocity) were determined at pH 6.5 and 7.0; and at temperatures of 40 and 75°C. To calculate  $K_m$  and  $V_{max}$  the assay was carried out several substrate concentrations. A typical hyperbolic relationship of Michaelis-Menten kinetic was observed, with higher substrate concentration resulting in a higher initial velocity of the hydrolysis. This relationship might be described as in Equation 2 [14].

$$V_o = \frac{V_{max} [S_o]}{K_m + [S_o]} \quad (2)$$

The double reciprocal plot or Lineweaver-Burk plot was more commonly applied to estimate the kinetic constants of  $K_m$  and  $V_{max}$ . This method employed a linearization approach of the Michaelis-Menten equation [11], as given in Equation 3.

$$\frac{1}{V_o} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} \frac{1}{[S_o]} \quad (3)$$

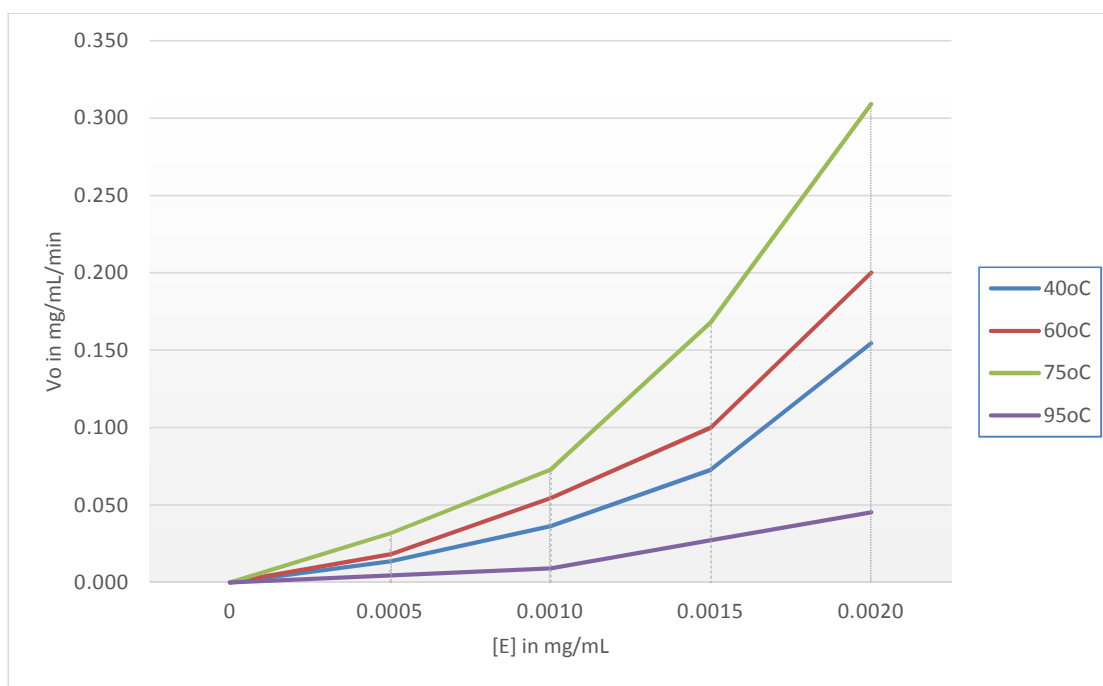


Fig. 2. The effect of enzyme concentration, [E], on the maximum velocity ( $V_{max}$ ) of the hydrolysis of sago starch at various temperatures

However, this method had a drawback of its tendency to overemphasize errors in velocity measurements at low substrate concentration. Considering that in practice, an experimental error was more likely to occur in the lower substrate concentration, these less accurate points would have a larger influence in determining the slope and the intercept, and consequently the accurate values of  $K_m$  and  $V_{max}$ .

Another approach, the Hanes plot, which was less susceptible to this disadvantage might be used. This plot, as given in Equation 4, was also a linearization of Michaelis-Menten plot. This method was claimed to give more accurate estimations in determining  $K_m$  and  $V_{max}$  [11].

$$\frac{[S_o]}{V_o} = \frac{K_m}{V_{max}} + \frac{1}{V_{max}} [S_o] \quad (4)$$

An attempt was made to convert the data into this Hanes form. As given in Table 2, the values of  $V_o$  at each initial substrate concentration  $[S_o]$  were converted to the form of  $[S_o]/V_o$  and  $[S_o]$ , which was used to construct the Hanes plot. From this equation  $K_m$  and  $V_{max}$  were calculated using linear regression analysis. Because of the heterogeneous nature of the starch molecules, molecular weight of the substrate could not be determined. Therefore  $K_m$  and  $V_{max}$  values were reported in mg/mL and  $\mu\text{g/mL/min}$ , respectively, instead of molar values.

As shown in Table 2, the values of  $K_m$  and  $V_{max}$  were found to be significantly higher at 75°C ( $p < 0.001$ ), but there was no significant difference between the values at pH 6.5 and 7.0. The higher  $K_m$  values suggested a lower affinity of the enzyme molecules to the substrates.

**Table 1. The Initial velocity ( $V_o$ ) at various pH and temperatures. Initial substrate concentration,  $[S_o]$ , was 5.000 mg/mL, and enzyme concentration was 2.0  $\mu\text{g/mL}$**

pH	$V_o \mu\text{(g/mL/min)}$ at <sup>1,2</sup>	
	40°C	75°C
6.0	9.8±0.3 <sup>a</sup>	27.9±0.9 <sup>a</sup>
6.5	15.1±0.9 <sup>b</sup>	32.0±0.7 <sup>b</sup>
7.0	18.0±0.7 <sup>c</sup>	33.2±0.4 <sup>b</sup>

<sup>1</sup>Values were means of duplicates

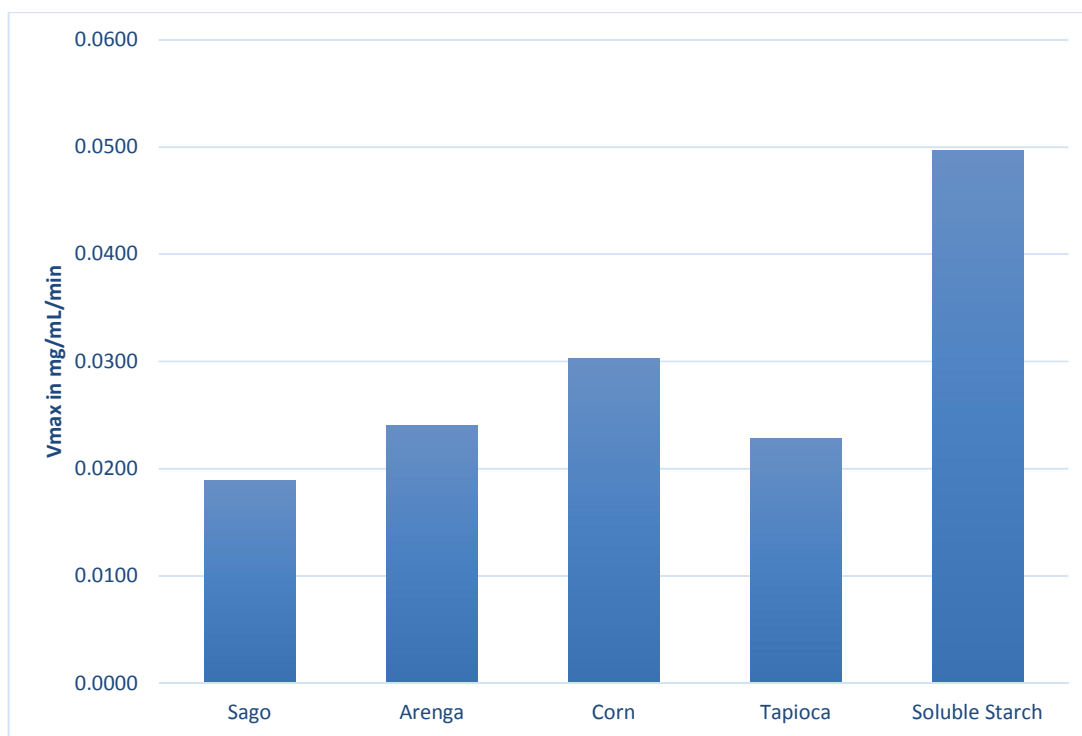
<sup>2</sup>Mean values in the column not followed by the same superscript were significantly different ( $p < 0.01$ )

**Table 2. The values of  $[S_o]$ ,  $V_o$ ,  $[S_o]/V_o$ ,  $K_m$  and  $V_{max}$  at temperatures of 40 and 75°C, and at 75°C, and at pH 6.5 and 7.0. The concentration of enzyme was kept constant at 2  $\mu\text{g/mL}$**

Temperature (°C)	pH	$[S_o]$ (mg/mL)	$V_o$ ( $\mu\text{g/mL/min}$ )	$[S_o]/V_o$ ( $\text{min}^{-1}$ )	$K_m$ <sup>1,2</sup> (mg/mL)	$V_{max}$ <sup>1,2</sup> ( $\mu\text{g/mL/min}$ )
40	6.5	0.413	3.7	111.62	0.86 <sup>a</sup>	17.5 <sup>a</sup>
		0.625	9.2	67.94		
		1.250	13.1	95.42		
		2.500	13.5	185.19		
		5.000	14.6	342.47		
40	7.0	0.413	5.8	71.21	0.77 <sup>a</sup>	20.3 <sup>a</sup>
		0.625	9.1	68.68		
		1.250	13.7	91.24		
		2.500	16.8	148.81		
		5.000	17.1	292.40		
75	6.5	0.413	6.6	62.58	3.22 <sup>b</sup>	54.3 <sup>b</sup>
		0.625	7.0	89.29		
		1.250	15.4	81.17		
		2.500	30.6	81.70		
		5.000	31.0	161.29		
75	7.0	0.413	6.1	67.71	3.82 <sup>c</sup>	57.1 <sup>b</sup>
		0.625	7.2	86.81		
		1.250	14.8	84.46		
		2.500	21.9	114.16		
		5.000	33.1	150.06		

<sup>1</sup>Values were means of duplicates

<sup>2</sup>Mean values in the column not followed by the same superscript were significantly different ( $p < 0.001$ )



**Fig. 3. The maximum velocity ( $V_{max}$ ) of the enzymatic hydrolysis of various starches**

The  $K_m$  and  $V_{max}$  values obtained from this study were comparable to the results of the similar starch and enzyme reported by Lai et al. [12], with the  $K_m$  and  $V_{max}$  of 13.52 mM and 2.35 mmoles/L.min, respectively. The difference in the values obtained might be due to the differences in the conditions of hydrolysis, including the use of substrate which was not reportedly treated with  $\text{NaBH}_4$ . In such a case, the reducing sugar content of this untreated substrate solution would be magnified resulting in an apparently higher rate of hydrolysis [11]. The other difference was that the latter employed about 100-fold higher enzyme concentration in the assay. Since each of these potential errors would be removed by appropriate blanks and controls, the source of the difference in values was not clear.

### 3.4 Rate of Hydrolysis of Various Starches

The results obtained indicated that the hydrolysis rates of sago starch samples were lower than those of other starch samples, as shown in Fig. 3. Similar findings were also observed by Yang et al. [15], Yang and Liu [16], who reported that sago starch was slightly resistant to the  $\alpha$ -amylase attacks.

This resistance might be due to a more compact physical structure of the sago starch granules. Okazaki [17] revealed that sago starch had a C type (a mixture of A type and B type). This was probably the cause why the sago starch granules were more resistant to  $\alpha$ -amylase attack. The presence of substances, such as polyphenols, might also retard the enzyme action, and consequently lower the hydrolysis rate. A study was carried out to identify the latter factor. A review by Karim et al. [18] concluded that sago starch was resistant to both microbial and enzyme digestion.

## 4. CONCLUSION

The results of this study indicated that the rate of sago starch hydrolysis by bacterial  $\alpha$ -amylase was somewhat lower. This confirmed the findings of other researchers who found that sago starch was a relatively poorer substrate for  $\alpha$ -amylase action. This resistance may be a benefit if the sago starch was applied, for instance, in the production of biodegradable packaging material. Since its digestibility was low, sago starch would not be degrading during the use of the materials formulated from the blend of sago starch with plastic resins.

This study also suggested that the possible presence of the polyphenols might have retarded the enzymatic reaction. The oxidation of these polyphenols was also known to cause browning of the sago starch. Therefore, in order to improve the quality of the starch and to utilize it for industrial or commercial use, the presence of these substances must be minimized.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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