International Journal of Science and Research (IJSR)
ISSN: 2319-7064
SJIF (2022): 7.942

Kinetics of Hydrolysis of Cassava Starch (*Manihot esculanta*): Effect of Addition of K and Mg Ions on Reducing Sugar

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Abstract: ***Sweet cassava (Manihot esculanta) can produce reducing sugars through enzymatic hydrolysis. The addition of K and Mg ions can increase reducing sugars concentration. The objective of this study was effect the addition of K and Mg ions on reducing sugars concentration as well as to study the kinetics of hydrolysis. Experiments were carried out using100, 200, 300 g/L of starch; 1, 1.5% (w/v) of enzyme, and 100 ppm of K and Mg ions. The hydrolysis was conducted at 30°C with the Stargen™ 002 at pH 4 for 18 hours. The results showed that the presence of enzyme along with starch were able to significantly increase the production of reducing sugars. The addition of K and Mg ions increased the reducing sugar to 38.79% and 78.25%. The best reducing sugar concentration of 68.32 g/L was obtained at 200 g/L starch concentration, 1.5% (w/w) enzyme concentration with the addition of Mg ions for 12 hours. Kinetic studies show that this hydrolysis phenomenon follows the Michaelis-Menten equation with a Km value of 98.8, 107.32 and 106.77 g/L and a Vmax value of 7.69, 9.26 and 15.63g/L.h, respectively, so that the addition of K and Mg ions can increase enzyme activity.***

Keywords: cassava; enzymatic hydrolysis; K and of Mg ions; kinetic studies.

1. Introduction

Starch is a carbohydrate that can be converted into glucose through a hydrolysis process. The starch hydrolysis method can be carried out using acids or enzymes. Enzymatic hydrolysis produces more specific products than acid hydrolysis because it does not require neutralization [1]. Hydrolysis using α-amylase and glucoamylase can produce 95% more glucose than acid hydrolysis [2]. Conventional hydrolysis requires higher temperatures in the liquefaction process (90-125°C) and in the saccharification process (55-65°C) [3]. In terms of energy requirements. this method is no longer efficient than the hydrolysis method at low temperatures.

Enzymatic hydrolysis at low temperatures can reduce energy costs in an industry that converts starch to glucose. One enzyme that is effective at low temperatures is granular starch hydrolyzing enzyme (GSHE) because it does not require a heating process [4]. Several researchers have carried out starch hydrolysis using granular starch hydrolyzing enzyme (GSHE) [5]. Hargono et al. [6] conducted a starch hydrolysis study using GSHE on sweet cassava starch. bitter cassava starch. and gadung starch. Based on this research. the highest concentration of reducing sugar and the best enzyme activity were obtained from sweet cassava starch. GSHE simplifies the reducing sugar production process and saves energy consumption by up to 10-20% [7].

The presence of metal ions can affect the active site of the enzyme and the stability of protein molecules. so that at high concentrations metal ions can affect the bond and substrate. Thus, metal ions can act as activators or inhibitors [8]. In the enzymatic hydrolysis process. it is necessary to have a pre-treatment process to maximize enzyme activity. inhibitor activity. and hydrolysis kinetics. Pre-treatment methods for enzymatic hydrolysis have been developed by several previous researchers. Anggraini et al. [8] conducted a study on the addition of Mg^{2+} ions to the activity of the pectinase enzyme substrate concentration 0.1-0.5%. The results showed that at the enzyme concentration 2-4 mm. The addition of Mg^{2+} ion acted as an activator. Wang et al. [9] conducted research on the addition of metal ions Fe^{2+}, Fe^{3+}, and Co^{2+} on the hydrolysis of wheat straw. The addition of Fe^{3+} ions give the best product results. Lie et al. [10] carried out hydrolysis of rice straw. the results obtained showed that Fe^{2+} and Cu^{2+} were able to degrade rice straw. While Mg^{2+} and Fe^{3+} inhibited enzymatic hydrolysis on the same substrate. Presecki et al. [11] performed starch hydrolysis using a mixture of enzyme amylase and glucoamylase with the addition of Ca ions. The conversion of reducing sugar obtained is 100%. The addition of Ca ions significantly increased the production of reducing sugars and maintained the stability of the enzyme.

Hydrolysis kinetics data on the effect of adding Mg ions to cassava starch substrates are limited.so, it is necessary to investigate the hydrolysis kinetics. The objective of this study is to investigate the effectof K and Mg ions to the hydrolysis of sweet cassava starch substrates on the concentration of reducing sugars, also to study the hydrolysis kinetics of cassava starch substrates without addition and with the addition of K and Mg ions.

2. Materials and method

2.1 Sweet cassava

Ten months old of sweet cassava (*Manihot esculanta*) tuber was obtained from Wonogiri district in Indonesia.

2.2 Sweet cassava starch extraction

The procedure for extraction of starch from Suweg tuberused in this study was the same as that previously used by Hargono [6].

2.3 Chemical Reagents

Potassium sodium tartrate tetrahydrate (Merck. DNS 3.5-dinitrosalicylic acid), sodium hydroxide (Merck. 98%), sodium sulfite (Merck. 98.5%), sulfuric acid (Merck. 98.5%), buffer solution, 0.01 M sodium phosphate, citric acid, glucose (Merck. 99.5%).

2.4 Enzyme

The enzyme used is cocktail Stargen^{TM} 002.An enzyme mixture of -amylase derived from *Aspergillus kawachi* in *Trichoderma reseei* and glucoamylase in *Trichoderma reseei*. known as granular starch hydrolyzing enzyme (GSHE).This enzyme is a second-generation enzyme produced by Genencor International. BV (Genencor International in Palo Alto. CA. USA). The activities of endo -amylase and exo-gluco-amylase completely hydrolyze starch granules and work synergistically to hydrolyze starch substrates into glucose.

2.5 Effect of Cassava Starch Concentration on Reducing Sugar Concentration.

The prepared cassava starch was then used as a substrate for hydrolysis at concentrations of 100, 200 and 300 g/L. The addition of K^+ (KCl) and Mg^{2+} ($MgCl_2$) at each concentration of starch slurry was then incubated in a shaker at 100 rpm for 10 minutes at pH 4. The pH of slurry was controlled using 0.01 M sodium phosphate buffer solution citric. Furthermore, the sweet cassava starch slurry was transferred to a test jar for the hydrolysis process. Stargen^{TM}002of 1 and 1.5% (w/w) was then added and stirred at 30°C, pH 4for 18 hours [12]. Each period of 3, 6, 9, 12, 15 and 18 hours, samples were taken to centrifuged for 4 minutes at 100 Hz rotation. The filtrate was filtered using Whatman CAT 40 filter paper No.1440-125 mm to remove the residual solids. It was then analyzed to determine the concentration of reducing sugar.

2.6 Reducing Sugar Analysis Method.

The hydrolyzed filtrate was then analyzed for the concentration of reducing sugars using the dinitrosalicylic acid (DNS) method [13].A solution consists of 1% of 3.5-dinitrosalicylic acid, 0.05% of sodium sulfite, 20% of sodium-potassium tartrate, and 1% of sodium hydroxide was added at a ratio of 3:1 to the sample in a test tube. It was then shaken, incubated using boiling water for 8 minutes, and cooled using ice water for 5 minutes. Previously, the absorbance was measured at 570 nm using a UV/visible spectrophotometer (UV-160A) SHIMADZU Kyoto Japan. Pure glucose 0 to 10 g/L was used as the standard solution. Therefore, the measurement of reducing sugar concentration is written in g/L units.

2.7 Determination of Initial Rate of Reaction

The initial speed (Vo) of sweet cassava starch hydrolysis was determined by the slope which represent the relationship curve of reducing sugar concentration and hydrolysis time. Figure 1 shows the method of Vodetemination.

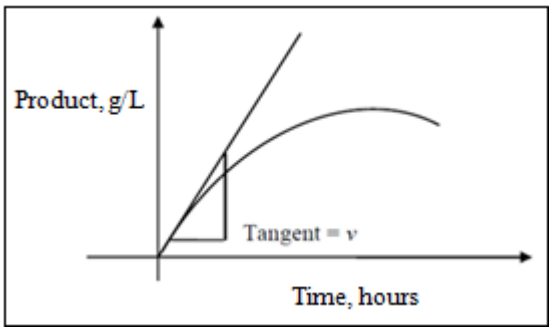

Figure 1: Determination of the initial reaction rate. Vo by drawing a straight line and tangent to the hydrolysis curve [12]

2.8 Determination of Kinetic Parameters

The characteristics of the constants (V_{max} and K_m) can be determined based on experimental data by incubating the enzyme at various concentrations of the initial substrate (S_o). Furthermore, a graph of the relationship between (V_o) and (S_o)was made. Linearization of the curve (straight line) is carried out by graphing the relationship between 1/Vo vs $1/S_o$. The slope value is K_m/V_{max}. The y-axis intercept is $1/V_{max}$ and the point of intersection of the curve with the negative x-axis is $-1/K_m$ [14]. The Michaelis-Menten equation (Eq. 1) is the rate equation for an enzymatic reaction of one substrate. This equation represents the quantitative relationship between the initial reaction rate (Vo),the maximum velocity (V_{max}, g/Lh), and the substrate concentration (S, gl/L), all related via the Michaelis-Menten constant (Km). This equation was then inverted by Lineweaver Burk to Eq. 2.

$$V_0 = \frac{V_{maks}[S]}{[S] + K_m} \quad (1)$$

$$\frac{1}{V} = \frac{K_m + [S]}{V_{max}[S]} = \frac{K_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}} \quad (2)$$

2.9. Determination of the Percentage Increase in Reducing Sugar Concentration.

The addition of K and Mg ions is expected to result in an increase in the concentration of reducing sugar to the hydrolysis product without the addition of K and Mg ions. The percentage increase is calculated using Eq 3.

$$\% \text{ increasing} = \frac{Ci-Co}{Ci} \times 100\% \quad (3)$$

C_i and C_0 are the concentrations of reducing sugars with the addition of K and Mg ions and the concentrations of reducing sugars without the addition of K or Mg ions. respectively.

3. Results and Discussion

3.1 Effect of Addition of K and Mg Ions on Various Concentrations of Starch on Reducing Sugar Concentration

Enzymatic hydrolysis of sweet cassava (*Manihot esculanta*) was carried out using various concentrations of cassava starch (100, 200, and 300 g/L) and various enzyme concentrations (1 and 1.5% w/w). At each concentration of starch and enzyme concentration was carried out without pre-treatment (original) and with the addition ofK^+ and Mg^{2+} ions. The effect of K^+ and Mg^{2+} ions addition on 1% (w/w) enzyme concentration is shown in Table 1.

Table 1: Effect of K^+ and Mg^{2+} ions addition on reducing sugar concentration at enzyme concentration **1%(w/w),** pH 4, and temperature of 30°C in varying concentrations of starch

Time (hours)	Reducing sugar concentration (g/L)								
	100 g/L of starch			200 g/L of starch			300 g/L of starch		
	Original	+ K^+	+ Mg^{2+}	Original	+ K^+	+ Mg^{2+}	Original	+ K^+	+ Mg^{2+}
0	0	0	0	0	0	0	0	0	0
3	7.58	8.87	12.12	10.32	12.56	18.09	11.06	13.28	19.61
6	11.90	13.17	15.41	15.64	16.97	23.23	16.78	17.96	23.56
9	15.65	16.36	19.74	20.87	22.87	27.63	21.90	23.88	28.46
12	**18.78**	**20.77**	**24.55**	**24.68**	**26.71**	**31.12**	**25.86**	**27.32**	**31.46**
15	18.82	20.81	24.58	24.78	26.81	31.16	25.90	27.34	31.48
18	18.84	20.78	24.62	24.85	26.89	31.18	25.92	27.38	31.54

Table 2: Effect of K^+ and Mg^{2+} ions addition on reducing sugar concentration atenzyme concentration **1.5% (w/w),** pH 4 and temperature 30°C in varying concentrations of starch

Time (hours)	Reducing Sugar Concentration, g/L								
	100 g/L of starch			200 g/L of starch			300 g/L of starch		
	Original	+ K^+	+ Mg^{2+}	Original	+ K^+	+ Mg^{2+}	Original	+ K^+	+ Mg^{2+}
0	0	0	0	0	0	0	0	0	0
3	16.34	17.45	21.10	19.45	23.12	26.12	18.12	20.86	23.12
6	24.16	29.08	32.88	29.20	35.90	48.92	24.65	29.82	39.45
9	28.24	34.64	38.65	34.12	44.20	59.07	32.34	40.12	52.84
12	**30.12**	**40.23**	**48.44**	**38.33**	**53.20**	**68.32**	32.65	44.47	53.60
15	30.34	40.45	48.56	38.89	53.82	68.76	32.70	44.49	53.70
18	30.56	40.54	48.60	38.94	53.84	68.79	32.72	44.53	53.78

As shown in Table 1, the same phenomenon from each variable that the concentration of reducing sugar increases with increasing hydrolysis time until 12 hours and then tends to be constant. This is due to the fact that the saturation point has been reached so that the enzyme is no longer able to convert starch into reducing sugars [15]. The highest reducing sugar concentration was obtained at a starch concentration of 100 g/L for no metal addition (original) and with the addition of K^+ ions and the addition of Mg^{2+} ions was 18.78; 20.77 and 24.55 g/L, respectively, whereas at a starch concentration of 200 was **24.68**, **26.71** and 31.12 g/L. Under the same conditions at the enzyme concentration of 1.5% the result of reducing sugar was shown in Table 2. The highest reducing sugar concentration was obtained at a starch concentration of 100 g/L for original and with the addition of K^+ and Mg^{2+} was**30.12**; **40.23** and **48.44** g/L, respectively, whereas at a starch concentration of 200 g/L was **38.33**;**53.20** and **68.32** g/L. For the same reason of based on these data in Table 1 the increase in the concentration of reducing sugar from a concentration of 200 g/L to 300 g/L did not show a significant difference. Based on these data the increase in reducing sugar concentration from starch concentration of 100 g/L to 200 g/L was quite significant. This is because the higher the starch concentration. the more substrate will be converted into reducing sugars. This indicates that the active site of the enzyme is still able to convert a larger amount of substrate [16]. Based on these data in Table 1and Table 2 the increase in the concentration of reducing sugar from a concentration of 200 g/L to 300 g/L did not show a significant difference. This is due to the increase in viscosity which inhibits the diffusion process. In addition. there is a possibility of inhibition due to high substrate concentrations [17].Previous study carried out hydrolysis with a substrate concentration of 1-10% (w/v) [16].A higher substrate concentration (up to 5% w/v) can increase the reducing sugar product. At a substrate concentration above 5% (w/v), it is not able to increase the concentration of reducing sugar or tends to be constant. The concentration of reducing sugars after being given the addition of K^+ and Ca^{2+} ions increased compared to those without metal ions (original) as presented in Table 3.

Table 3 The Percentage Increasing of Reducing Sugars Concentration to the Addition of K and Mg Ions at Enzyme Consentration 1.5% (w/w), 30°C

Increasing of reducing sugars concentration (%)						
Time, hours	Starch Concentration, 100 g/L		Starch Concentration, 200 g/L		Starch Concentration, 300 g/L	
	+K	+Mg	+K	+Mg	+K	+Mg
0	0	0	0	0	0	0
3	6.7930	29.1310	18.8689	34.2931	15.12141	27.5938
6	20.3642	36.0927	22.9452	67.5343	20.97688	60.0406
9	22.6629	36.8626	29.5428	73.1243	24.0569	63.3889
12	**33.5657**	**60.8234**	**38.7947**	**78.2416**	**36.21072**	**64.1654**
15	33.3224	60.0527	38.3903	76.8064	36.05872	64.2202
18	32.6571	59.0314	38.2640	76.6564	36.08802	64.3643

Compared to hydrolysis without the addition of metal ions, during the hydrolysis time of 12 hours at a starch concentration of 100 g/L, the addition of K and Mg resulted reducing sugar was 33.57 and 60.82%, and at a starch concentration of 200 g/L there was an increase in reducing sugar 38.79 and 78.24% while at starch concentration of 300 g/L the increase in reducing sugar was not significant. David et al. [18]studied K^+ is able to increase the concentration of reducing sugars and activate enzymes by binding water and appropriate protein residues. Zohra et al. [19] have conducted research on enzymatic hydrolysis with the addition of monovalent, divalent, and trivalent metal ions. The addition of Ca^{2+} ions resulted in the highest enzyme activity among other metal ions. This is because amylase is generally known as a metalloenzyme and contains at least one Ca^{2+} ion as an integral component to increase enzyme activity. In addition, to maintain stability and prolong the half-life of the enzyme, Ca^{2+} is the preferred cation. Thermostable -amylase describes more thermo stability in the presence of Ca^{2+} [19]. A similar study was also conducted by Wang et al. [9]have conducted hydrolysis of wheat straw with the addition of metal ions. The results obtained that the higher the ionic charge on the metal, the higher the enzyme activity produced. However, not all metals with a higher charge can act as activators. Anggraini et al. [8] have studied on hydrolysis at a concentration of 6-10 mM, Mg^{2+} ions can reduce enzyme activity because they are able to bind to the active site of the enzyme. Ezugwu et al. [20] studied the activity of the glucoamylase enzyme using several divalent metal ions, namely Ca^{2+}, Zn^{2+}, and Fe^{2+}. The use of metal ions is able to hold the substrate to interact with the enzyme using the nucleophilic hydroxide ion produced by the metal ion. The increase in enzyme activity with Ca^{2+}, Zn^{2+}, and Fe^{2+} ions can be based on the ability to interact with negatively charged amino acids, such as aspartic acid by stabilizing the negative charge formed at the active site [21].

3.2 Kinetic Parameters of Cassava Starch Hydrolysis to the Addition of K+ and Mg^{2+}

In this study, variations in substrate concentrations of 100, 150, 200, 250, 300, 350, and 400g/L were used. The initial reaction rate (V_o) was determined using the tangent of the polynomial equation of the relationship between time and the production of reducing sugars as shown in Figure 1. The relationship between substrate concentration (S_o) and the initial reaction rate (Vo) as presented in Figure 2.

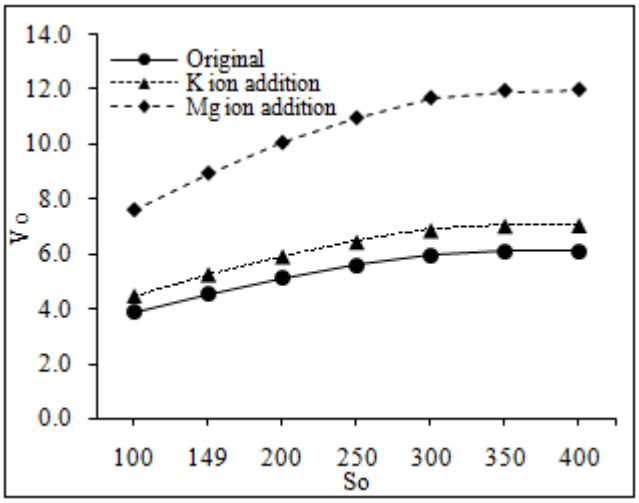

Figure 2: The relationship of initial substrate concentration (S_o) to the initial reaction rate (V_o) on the addition of K^+ and Ca^{2+}.

The addition of the substrate concentration increased the initial reaction rate and then the substrate concentration after 300 g/L resulted in the initial reaction rate which tended to be constant. This is in accordance with the Michaelis-Menten concept that the initial substrate concentration affects the reaction rate. The increase in substrate concentration will increase the reaction rate to a certain point then it will not increase even though more substrate is added. This is because the enzyme has reached the saturation point of the substrate and has reached the maximum reaction speed (V_{max}) so that the enzyme activity is not affected by the substrate concentration [22].The kinetic parameters of cassava hydrolysis can be determined using the help of the linearized equation from the Michaelis-Menten equation using the Lineweaver-Burk curve as shown in Figure 3. The relationship between $1/S_o$ vs $1/V_o$ will produce a line equation where the slope is K_m/V_{max} and the intercept is $1/V_{max}$. The kinetic data are presented in Table 3 and the relationship curve of $1/S_o$ vs $1/V_o$ is presented in Figure 3.

The calculation results of the Lineweaver-Burk curve show that the addition of K ions and Mg ions increases the value of V_{max}. The addition of Mg ions significantly increased V_{max} to 15.625 g/L.h, while the addition of K ions increased V_{max} to 9.2592 g/L.h. Based on this phenomenon, the presence of Mg and K ions act as activators because they can increase the value of V_{max}. In the Michaelis-Menten equation, the addition of an inhibitor can decrease the value of V_{max} and increase the value of Km while the addition of

an activator can decrease the value of K_m and increase the value of V_{max} [23].

Table 3: The kinetics parameter of cassava starch hydrolysis on the addition of K ions and Mg . ions

	Equation	V_{max} (g/L.h)	K_m (g/L)
original	Y = 12.33 X + 0,130	7.69	98.841
+ K	Y = 11.59 X + 0,108	9.2592	107.3148
+ Mg	Y = 6.833X + 0,064	15.625	106.7656

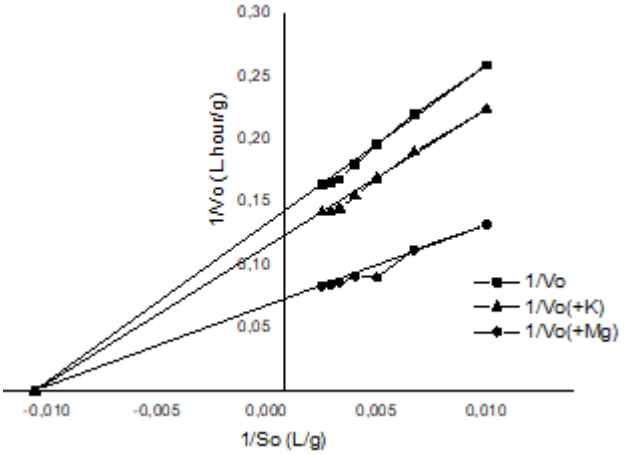

Figure 3: The relationship of $1/S_o$ to $1/V_o$ on the addition of K and Mg ions.

4. Conclusion

The addition of Mg ions resulted in an increase in the concentration of reducing sugars greater than the addition of K ions. The addition of K and Mg ions increased the reducing sugar to 38.79% and 78.25%. The best reducing sugar concentration was 68.32 g/L was obtained at 200 g/L starch concentration, 1.5% (w/w) enzyme concentration with the addition of Mg ions during 12 hours. Kinetic studies show that this hydrolysis phenomenon follows the Michaelis-Menten equation with a Km value of 98.8, 107.32 and 106.77 g/L and a V_{max} value of 7.69, 9.26 and 15.63g/L.h, respectively, so that the addition of K and Mg ions can increase enzyme activity.

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