Available online at BCREC website: https://bcrec.id

Bulletin of Chemical Reaction Engineering & Catalysis, 14 (3) 2019, 646-653

Research Article

Inhibition Effect of Ca^{2+} Ions on Sucrose Hydrolysis Using Invertase

H. Hargono*, Bakti Jos, A. Abdullah, Teguh Riyanto

Department of Chemical Engineering, Faculty of Engineering, Universitas Diponegoro, Semarang, 50275, Indonesia

Received: 4th March 2019; Revised: 15th July 2019; Accepted: 9th August 2019; Available online: 30th September 2019; Published regularly: December 2019

Abstract

Fermentable sugar for bioethanol production can be produced from molasses due to its high sucrose content but Ca^{2+} ions found in the molasses may affect the hydrolysis. Therefore, this paper was focused to study the effect of Ca^{2+} ions as CaO on sucrose hydrolysis using invertase and to obtain the kinetic parameters. The kinetic parameters (K_M and V_{max}) were obtained using a Lineweaver-Burk plot. The value of K_M and V_{max} parameters were 36.181 g/L and 21.322 g/L.h, respectively. The Ca^{2+} ions act as competitive inhibitor in sucrose hydrolysis using invertase. Therefore, the inhibition mechanism was followed the competitive inhibition mechanism. The value of inhibition constant was 0.833 g/g. These parameters were obtained from the non-substrate inhibition process because this study used the low substrate concentrations which means the fermentable sugar production was low. Hence, there were still more challenges to studying the simultaneous effect of substrate and Ca^{2+} on sucrose hydrolysis to produce high fermentable sugar.

Keywords: Sucrose; Invertase; Enzymatic Hydrolysis; Ca^{2+} Ions; Competitive Inhibition

How to Cite: Hargono, H., Jos, B., Abdullah, A., Riyanto, T. (2019). Inhibition Effect of Ca^{2+} Ions on Sucrose Hydrolysis Using Invertase. *Bulletin of Chemical Reaction Engineering & Catalysis*, 14(3): 646-653 (doi:10.9767/bcrec.14.3.4437.646-653)

Permalink/DOI: https://doi.org/10.9767/bcrec.14.3.4437.646-653

1. Introduction

One of the by-products of the sugar refinery process which is the most economical source of carbohydrate for ethanol and citric acid fermentation is molasses [1]. Molasses can be converted into fermentable sugar through the enzymatic hydrolysis due to its high reduced polymeric sugars content. Usually, molasses has a water content of 17-25% and high sugar content (sucrose 30-40%, glucose 5-9%, and fructose 5-12%) [1,2]. Some minerals are also found in the molasses such as potassium 1.5-6% and calcium 0.3-0.9% [3]. The appearance of calcium (Ca^{2+}) in molasses is a consequence of the clarification process. In the sugar refinery process, the sugarcane juice is acidic. For the clarification process reason, the sugarcane was mixed with milk of lime (MOL) as CaO, so that the pH of sugarcane juice changes to a range of 8.9 to 9. Besides the clarification process, the sulphitation process also occurs as a part of the sugar refinery process. The sulphitation process was done to maintain the pH of the process considered as one of the main reasons for the whitening of the sugar crystals. In the sulphitation process, SO_2 is added to the sugarcane juice so that the pH value becomes 7.4 to 7.5 [4].

As has been mentioned before, calcium was found in the final molasses. It mixed in the molasses which could interfere with the products produced in the next process, for example, the fermentation process for ethanol production.

* Corresponding Author.
E-mail: hargono@che.undip.ac.id (H. Hargono)

Ethanol production would decrease during the sucrose fermentation in the presence of calcium, possibly caused by the inhibition process on invertase by calcium [5]. Takeshige and Ouchi [6] reported that ethanol productivity was affected by yeasts, which were strain dependent. When the process used molasses containing metal ions such as copper, potassium, and calcium, the yeast strain with low ethanol showed a lower invertase activity which indicated an inhibition effect on invertase activity [6]. In this case, calcium in molasses influenced the invertase activity.

Several studies have investigated the enzymatic hydrolysis of sucrose using different enzyme and with or without the presence of metal ions which thought to affect the hydrolysis process. Ettalibi and Barati [7] investigated the sucrose hydrolysis using the thermostable inulinase. The enzyme activity depended on sucrose concentration. At high sucrose concentration, sucrose could inhibit the process. Onishi *et al.* [8] reported the influence of enzymatic prehydrolysis of sugarcane molasses with α-amylase and amyloglucosidase treated with the amylolytic enzymes and then submitted to alcoholic fermentation for 8 h at 32 °C, in the presence of baker's yeast (*Saccharomyces cerevisiae*) and nutrients. The best result for the treatment of molasses with the two enzymes yielded a 10.2% increase of sugars in comparison to molasses without pre-treatment [8]. Bhalla *et al.* [9] investigated the sucrose hydrolysis using invertase enzyme from *Saccharomyces cerevisiae*. They explained that the optimum temperature and pH for invertase acivity were 40 ºC and 6.0. The K_M parameter value was 11 mM. It can be concluded that the invertase potentially to be used for sucrose hydrolysis due to the lower K_M value.

The effect of metal ion on sucrose hydrolysis was investigated by several authors [10–15]. Bagal-Kestwal *et al.* [10] studied the effect of several metal ions such as Hg^{2+}, Pb^{2+}, Ag^{+} and Cd^{2+} on sucrose hydrolysis. Those metals ions acted as the inhibitor and classified as competitive inhibitors. Essel and Ossei [11] also investigated the effect of Ag^{+} ions as silver nitrate on sucrose hydrolysis and concluded that the Ag^{+} acted as an inhibitor on sucrose hydrolysis. Plascencia-Espinosa *et al.* [12] investigated the effect of several metal ions, such as: Cu^{2+}, Zn^{2+}, Hg^{2+}, Mn^{2+}, and Co^{2+}, on sucrose hydrolysis. Some metal ions (Cu^{2+}, Zn^{2+}, and Hg^{2+}) acted as the inhibitors, but interestingly, the presence of Mn^{2+} and Co^{2+} could increase the enzyme activity. de Gine´s *et al.* [13] has investigated that bivalent metal ions such as Ca^{2+}, Cu^{2+}, Cd^{2+}, and Hg^{2+} significantly inhibited the activity of invertase. Kaur and Sharma [14] investigated that all metal ions were well tolerated and did not adversely affect the activity of invertase except Na^{+}, Hg^{2+}, and Ca^{2+}. It means that the Na^{+}, Hg^{2+}, and Ca^{2+} was adversely affect invertase activity and could inhibit the invertase. Maruyama and Onodera [15] also investigated that invertase activity would decrease in the presence of Ca^{2+}, Zn^{2+}, and Cu^{2+}.

Studies on sucrose hydrolysis using invertase have not been widely conducted. Therefore, this research was conducted to investigate hydrolysis sucrose with and without added CaO. It has been clarified that Ca^{2+} ions on sucrose hydrolysis have the stronger effect than K^{+} ions [16] but the inhibition type of Ca^{2+} on invertase is still not clear. Due to this reason, this study aims to investigate the effect of Ca^{2+} ions as CaO during of hydrolysis of sucrose using invertase and to obtain the kinetic parameters. The kinetic parameters (K_M and V_{max}) obtained from this study is supposed to be used for the scale-up of the hydrolysis process into a pilot plant or even the commercial scale.

2. Materials and Method

2.1 Materials

The enzyme used in this study was invertase (EC.3.2.1.26) from *Saccharomyces cerevisiae* (purchased from BIO-CON^{R}) and its activity was determined by measurement the liberated reducing sugar (fructose and glucose) produced from sucrose [17]. The other chemicals were sucrose (99.5%, Sigma-Aldrich), sulfuric acid (98.5%, Merck), 3,5-Dinitrosalicylic acid (98%, Merck), potassium sodium tartrate tetrahydrate (99%, Merck), sodium acetate buffer (pH 5.2±0.1, Merck), sodium sulfite (98.5%, Merck), glucose (99.5%), and sodium hydroxide (98%, Merck).

2.2 Batch Hydrolysis Enzymatic of Sucrose

Sucrose solution (pH 7) with concentrations of 10-80 g/L was used in the present study. The pH of the solution was adjusted to 5 using 0.1 M of sodium acetate as a buffer. Then, the invertase was added to the sucrose solution with a concentration of 1% (w/w). A thermostatic water bath heater was used to maintain the temperature of the mixture so that the temperature can be adjusted at 50±1 ºC. During the incubation for 5 h, the mixture was continuously stirred at 100 rpm. In order to determine the reducing sugar concentration, initial velocity, and kinetic parameters, the samples were

collected at 1 h interval. In order to study the effect of Ca^{2+} ions inhibition, Ca^{2+} ions as CaO was added to the mixture with various concentrations of 0.25, 0.5, 0.75, 1, and 1.25% (w/w).

2.3 Analytical Methods for Reducing Sugar Determination

Dinitrosalycilic acid (DNS) method as described by Miller was used to measure the reducing sugar produced [18]. An aqueous solution containing 1 %wt of DNS, 20 %wt of potassium sodium tartrate tetrahydrate, 1 %wt of sodium hydroxide, and 0.05 %wt of sodium sulfite was added to the samples in the mass ratio of 3:1. During the incubation in a boiling water bath for 8 min, the solution was shaken. After that, it was cooled in an iced water bath for 5 min then its absorbance was measured at 540 nm using a UV/visible spectrophotometer (UV-160A, SHIMADZU, Kyoto, Japan). The total reducing sugars produced was determined using the procedure described by Somogyi [19] and Nelson [20] methods in which fructose (0 to 10 g/L) was used as the standard solution.

2.4 Determination of Kinetic Parameters

The enzymatic reaction mechanism follow the Michaelis-Menten mechanism [21]. First, the enzyme combined reversibly with the substrate to form an enzyme-substrate complex in a relatively fast reversible step. Then, the enzyme-substrate complex broken down in a slower step to yield the free enzyme and the reaction product.

$$E + S \leftrightarrow ES \rightarrow E + P$$

The general structure of Michaelis-Menten equation to determine the reaction velocity is presented in Equation 1.

$$V_0 = \frac{V_{\max}[S_0]}{K_M + [S_0]} \quad (1)$$

where V_0 noted as initial reaction velocity, $[S_0]$ noted as the initial substrate (sucrose) concentration, V_{max} represented at the maximum rate of reaction, and K_M noted as the Michaelis-Menten constant. This equation was used to measure the initial reaction velocity, noted as V_0, in the kinetic experiments. The initial reaction velocity at a particular initial substrate concentration was determined from the slope of the curve of the product concentration versus time at the beginning of the reaction [21]. Generally, the K_M and V_{max} values were determined using a Lineweaver-Burk plot [22]. Equation 2 was obtained by rearranging Equation 1. Plotting of $1/V_0$ versus $1/[S_0]$ would get a straight line. The y-intercept of the Cartesian diagram was used to determine the V_{max} value and the x-intercept of the Cartesian diagram was used to determine the K_M value [23,24].

$$\frac{1}{V_0} = \frac{K_M}{V_{\max}} \frac{1}{[S_0]} + \frac{1}{V_{\max}} \quad (2)$$

In the presence of a competitive inhibitor, the measured K_M will be higher than the K_M in the absence of the inhibitor [25]. Equation 3 represented the modification of Michaelis-Menten equation in the presence of a competitive inhibitor.

$$V_0 = \frac{V_{\max}[S_0]}{\alpha K_M + [S_0]} \quad (3)$$

The meaning of the α term was explained in Equation 4.

$$\alpha = 1 + \frac{[I]}{K_I} \quad (4)$$

The value of α can be obtained by a Lineweaver-Burk plot of Equation 5 which was obtained by rearranging Equation 3.

$$\frac{1}{V_0} = \alpha \frac{K_M}{V_{\max}} \frac{1}{[S_0]} + \frac{1}{V_{\max}} \quad (5)$$

The value of the inhibition constant (K_I) was determined by the plot of α versus inhibitor concentration, [I]. For this plot, the x-intercept of the Cartesian diagram was used to determine the K_I value.

3. Results and Discussion

3.1 Effect of Initial Substrate Concentration on Enzymatic Hydrolysis of Sucrose using Invertase

Based on Figure 1, the higher the substrate concentration, the greater the reducing sugar concentration obtained. In the range of sucrose concentrations of 10 - 80 g/L, the reducing sugar concentration produced was linear with the initial sucrose concentration. It indicates that in the range of substrate concentrations used in this study there was still no substrate inhibition. There were several studies reporting the substrate inhibition of sucrose hydrolysis begins to occur at sucrose concentrations of 0.4 M (137 g/L) [12] and 0.5 M (171 g/L) [26], and 50 g/L [27].

One of the factors influencing the enzymatic hydrolysis reaction was substrate concentration [9,28]. The higher the substrate concentration, the higher the product concentration because the enzyme activity increases with increasing substrate concentration [7,28]. En-

zyme activity will increase with increasing substrate concentration, but enzyme activity will decrease at certain substrate concentrations due to the inhibition of the substrate [29]. Substrate inhibition occurs at high substrate concentrations. The substrate viscosity will be higher along with the magnitude of substrate concentration. It will slow down the diffusion process so that the enzymatic reaction will be controlled by the diffusion process (diffusion limitation) [30]. Therefore, the substrate will be more difficult to be hydrolyzed by acid and enzymatic treatment [31]. At high substrate concentration, the degree of hydrolysis will decrease, possibly due to the high concentration of the substrate itself or caused by irreversible enzyme inhibitors found in the substrate [32]. Corazze *et al.* [33] states that at high substrate concentration, the substrate will compete each other to get the active site of the enzyme.

3.2 Effect of Ca^{2+} Ion on Hydrolysis of Sucrose Using Invertase

Effect of Ca^{2+} ion on hydrolysis of sucrose using invertase was studied and the result was shown in Figure 2. Figure 2 shows the hydrolysis of sucrose using invertase in presence of different Ca^{2+} ion concentration (0-1.25%) in 5 hours. It can be seen, that the reducing sugar concentration increase with the increase of hydrolysis time over 5 h of hydrolysis. During hydrolysis, the longer the hydrolysis time, the higher the possibility of collision between the enzyme and sucrose (substrate) [34]. Therefore, the enzyme-substrate complex will be more formed. Hence, reducing sugar produced also will be more.

As can be seen at Figure 2, the reducing sugar produced has same pattern and constant at 5 h even without Ca^{2+} (Ca^{2+} = 0%). It is caused by the time dependence of product. The product pattern follows the exponential pattern, therefore, it will constant at certain time [28]. At low substrate concentration, the product obtained is linear with the inhibitor concentration [35,36]. From Figure 2, we conclude that the reducing sugar concentration decreased with the increasing of Ca^{2+} ion concentration in the hydrolysis system. When the Ca^{2+} ion concentration increased from 0 to 1.25 %, reducing sugar concentration decreased from 9.03 g/L to 5.28 g/L. It indicated that Ca^{2+} ion could inhibit the hydrolysis of sucrose using invertase. The presence of Ca^{2+} ion reduced the enzyme activity. Therefore, the degree of hydrolysis decreased as well as reducing sugar concentrations obtained. The inhibition mechanism of Ca^{2+} will be provided in the following section.

Several studies have reported that several metal ions could inhibit the sucrose hydrolysis such as Hg^{2+}, Pb^{2+}, Ag^{+}, Cd^{2+} as their chloride salts [10] and Ag^{+} as silver nitrate [11]. Those metal ions were classified as competitive inhibitor [10,11] which can be indicated by the increasing of the apparent K_M value. Based on the study of Bagal-Kestwal *et al.* [10], Hg^{2+} and Pb^{2+} ions were classified as irreversible competitive inhibitor while Ag^{+} and Cd^{2+} were classified as reversible competitive inhibitor. The type of Ca^{2+} inhibition on sucrose hydrolysis using invertase will be determined in the following section.

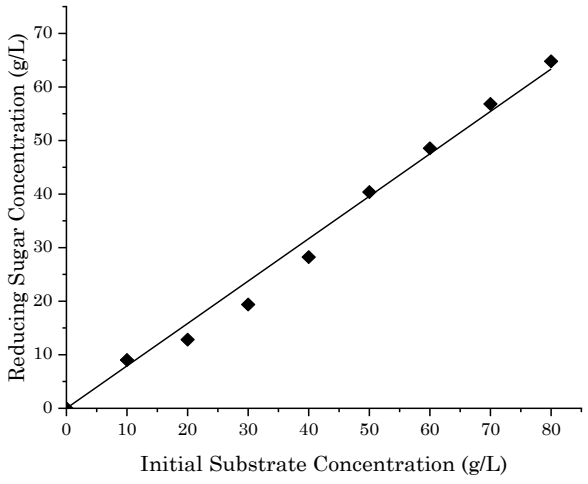


Figure 1. Reducing sugar concentration as the function of initial sucrose concentration at t = 5 h and Ca^{2+} = 0%.

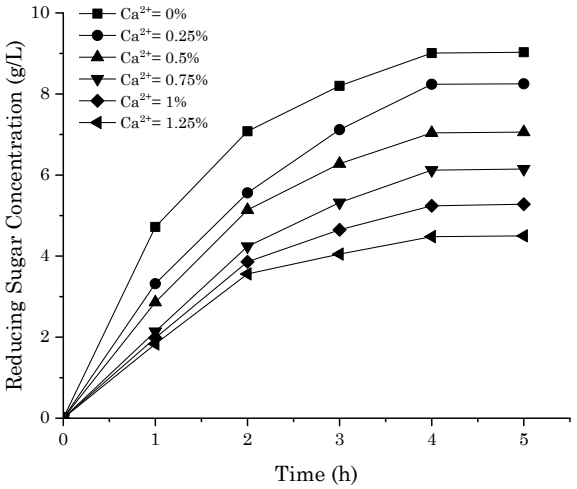


Figure 2. Effect of Ca^{2+} ion on hydrolysis of sucrose using invertase at [S_0] = 10 g/L.

3.3 Type of Ca^{2+} Inhibition on Sucrose Hydrolysis Using Invertase and Kinetic Parameters Determination

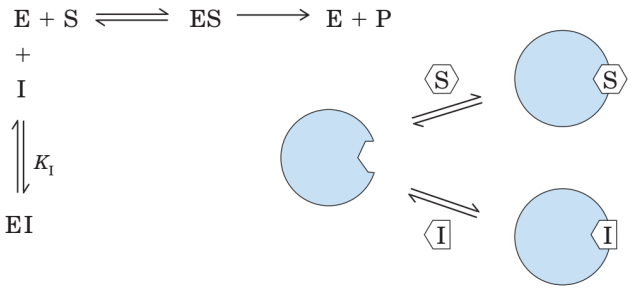
The effect of the presence of Ca^{2+} on sucrose hydrolysis has been described in the previous section. It has been observed that Ca^{2+} ion acted as an inhibitor on hydrolysis. Subsequently, it's important to determine the type of inhibition of Ca^{2+} on the hydrolysis of sucrose using invertase. The inhibition type of inhibitor can be determined by the Lineweaver-Burk plot. The inhibition type can be classified as competitive, non-competitive and un-competitive inhibition. Competitive inhibition can be noticed by the increasing of apparent K_M parameter with fixed V_{max} parameter. Non-competitive inhibition can be noticed by the decreasing of apparent V_{max} parameter with fixed K_M parameter while un-competitive inhibition can be noticed by the decreasing of the apparent K_M and V_{max} parameters.

It can be seen from Figure 3 and Table 1 that the K_M and V_{max} values for native (without Ca^{2+}) sucrose hydrolysis using invertase were 36.181 g/L (105.7 mM) and 21.322 g/L.h (1.973 mM/min), respectively. The value of K_M parameter in this study seemed different with the obtained value from several studies. Some studies calculated the value of K_M parameter on sucrose hydrolysis using invertase, such as: 24 mM [11], 45.2 mM [27], 227 mM [37], 24.5 mM [38], and 99 mM [39]. The different value between of K_M parameter in the present study with the previous study may be due to the different operating condition of hydrolysis and the different sources of invertase [37].

As can be seen from Figure 3 and Table 1, plots of $1/V_0$ versus $1/[S_0]$ and the value of apparent V_{max} and K_M for different Ca^{2+} ion concentrations showed a positive agreement that Ca^{2+} ion was a competitive inhibitor on sucrose hydrolysis using invertase. Several studies have shown that several metal ions acted as competitive inhibitors on sucrose hydrolysis using invertase [10,11]. The metal ions competed with substrate molecules to bind with the active sites of enzyme. For example, Ag^+ ions make the invertase inactive through the binding of Ag^+ ions to histidine side chains [11].

Table 1. Apparent kinetic parameters value

Ca^{2+} (%wt)	V_{max} (g/L.h)	K_M (g/L)	K_M/V_{max} (h)	α
0.00	21.322	36.181	1.697	1.000
0.25	21.413	43.171	2.016	1.188
0.50	21.231	50.601	2.383	1.405
0.75	21.186	61.752	2.915	1.718
1.00	21.505	78.103	3.632	2.140
1.25	21.368	100.058	4.683	2.760



Scheme 1. Competitive inhibition mechanism [21]

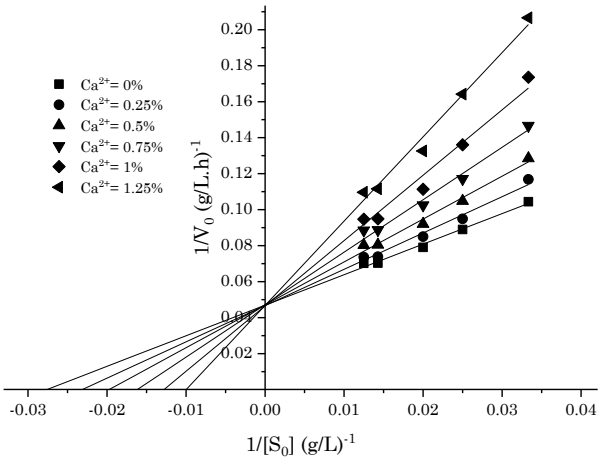


Figure 3. Lineweaver-Burk plot of sucrose hydrolysis using invertase in the presence of different Ca^{2+} ion concentration.

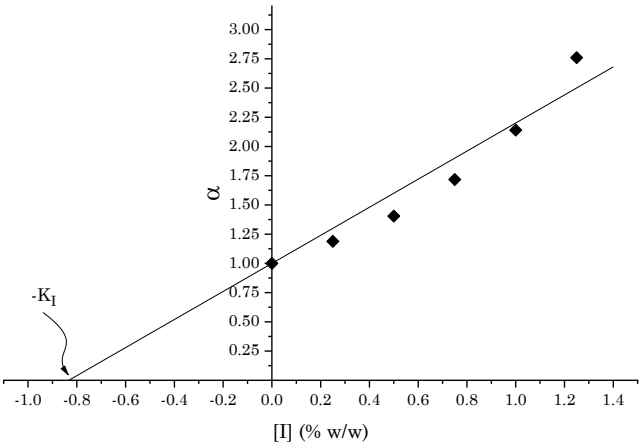


Figure 4. Plots for determination of inhibition constant of Ca^{2+} on sucrose hydrolysis using invertase

The mechanism of Ca^{2+} ion inhibition on invertase through sucrose hydrolysis follows the competitive inhibition. The inhibitor competes with the substrate for the active site of the enzyme [40]. The inhibitor will combine reversibly with the enzyme to form an enzyme-inhibitor complex which can reduce the amount of enzyme for interaction with the substrate, therefore, the reaction rate decreases. As known before, a competitive inhibitor combines reversibly with the enzyme, therefore, the effect of the inhibitor can be minimized by increasing the substrate concentration [41]. The mechanism of competitive inhibition is provided in Scheme 1.

The inhibition constant (K_I) can be determined from the plot of α versus Ca^{2+} ions concentration. As can be seen in Figure 4, the inhibition constant for Ca^{2+} inhibition on sucrose hydrolysis using invertase was 0.833 g/g. Equation 6 represents the general equation for sucrose hydrolysis using invertase in the presence of Ca^{2+} as a function of sucrose (substrate) concentration (in g/L) and Ca^{2+} ion (inhibitor) concentration (in g of Ca^{2+}/g of sucrose).

$$V_0 = \frac{21.322\left[S_0\right]}{\left[1+\frac{\left[I\right]}{0.833}\right]\times 36.181+\left[S_0\right]} \quad (6)$$

4. Conclusions

The presence of Ca^{2+} ions in the system of sucrose hydrolysis using invertase decreases reducing sugar production. The Ca^{2+} ions inhibit the invertase activity. It can be seen from the decrease of reducing sugar production over sucrose hydrolysis using invertase. The higher Ca^{2+} ions concentration in the solution, the lower reducing sugar produced. The inhibition process of Ca^{2+} was classified as competitive inhibition. The kinetic parameters (K_M and V_{max}) were obtained using a Lineweaver-Burk plot. The value of K_M and V_{max} parameters were 36.181 g/L and 21.322 g/L.h, respectively. The inhibition constant also was obtained; its value was 0.833 g/g. These parameters were obtained from the non-substrate inhibition process because this study used the low substrate concentrations which means the fermentable sugar production was low. Hence, there were still more challenges to study the simultaneous effect of substrate and Ca^{2+} on sucrose hydrolysis to produce more fermentable sugar.

Acknowledgment

The authors would like to thank Diponegoro University for the financial support.

References

[1] Najafpour, G.D., Shan, C.P. (2003). Enzymatic hydrolysis of molasses. *Bioresource Technology*, 86(1): 91–94.

[2] Chen, J.C.P., Chou, C.C. (1993). *Cane Sugar Handbook: A Manual for Cane Sugar Manufacturers and Their Chemists* (12th ed.). New York: John Wiley & Sons Inc.

[3] Higginbotham, J.D., Mc Carthy, J. (1998). Quality and storage of molasses. In P.W. van der Poel, T.K. Schwartz, & H.M. Schiweck (Eds.), *Sugar Technology Beet and Cane Manufacture* (pp. 973–992). Berlin: Verlag Dr Albert Bartens KG.

[4] Doherty, W.O.S., Greenwood, J., Pilaski, D., Wright, P.G. (2002). The Effect of Liming Conditions in Juice Clarification. In *Proceedings of the Australian Society of Sugar Cane Technology* (Vol. 24, pp. 1–12).

[5] Chotineeranat, S., Wansuksri, R., Piyachomkwan, K., Chatakanonda, P., Weerathaworn, P., Sriroth, K. (2010). Effect of calcium ions on ethanol production from molasses by *Saccharomyces cerevisiae*. *Sugar Tech*, 12(2): 120–124.

[6] Takeshige, K., Ouchi, K. (1995). Factors affecting the ethanol productivity of yeast in molasses. *Journal of Fermentation and Bioengineering*, 79(5): 449–452.

[7] Ettalibi, M., Baratti, J.C. (2001). Sucrose hydrolysis by thermostable immobilized inulinases from *Aspergillus ficuum*. *Enzyme and Microbial Technology*, 28(7–8): 596–601.

[8] Onishi, V.C., Olivo, J.E., Zanin, G.M., Moraes, F.F. (2014). Enzymatic hydrolysis of sugarcane molasses as pre-treatment for bioethanol production. *International Sugar Journal*, 16(1392): 906–910.

[9] Bhalla, T.C., Bansuli, B., Thakur, N., Savitri, S., Thakur, N. (2017). Invertase of *Saccharomyces cerevisiae* SAA-612: Production, characterization and application in synthesis of fructo-oligosaccharides. *LWT - Food Science and Technology*, 77: 178–185.

[10] Bagal-Kestwal, D., Karve, M.S., Kakade, B., & Pillai, V.K. (2008). Invertase inhibition based electrochemical sensor for the detection of heavy metal ions in aqueous system: Application of ultra-microelectrode to enhance sucrose biosensor's sensitivity. *Biosensors and Bioelectronics*, 24(4): 657–664.

[11] Essel, K.K., Osei, Y.D. (2014). Investigation of Some Kinetic Properties of Commercial Invertase from Yeast. *Natural Products Chemistry & Research*, 2(6): 152–156.

[12] Plascencia-Espinosa, M., Santiago-Hernández, A., Pavón-Orozco, P., Vallejo-Becerra, V., Trejo-Estrada, S., Sosa-Peinado, A., Benitez-Cardoza, C.G., Hidalgo-Lara, M.E. (2014). Effect of deglycosylation on the properties of thermophilic invertase purified from the yeast *Candida guilliermondii* MpIIIa. *Process Biochemistry*, 49(9): 1480–1487.

[13] de Ginés, S.C., Maldonado, M.C., de Valdez, G.F. (2000). Purification and Characterization of Invertase from Lactobacillus reuteri CRL 1100. *Current Microbiology*, 40(3): 181–184.

[14] Kaur, N., Sharma, A.D. (2005). Production, optimization and characterization of extracellular invertase by an actinomycete strain. *Journal of Scientific & Industrial Research*, 64(7): 515–5019.

[15] Maruyama, Y., Onodera, K. (1979). Production and Some Properties of Invertase Isozymes of *Fusarium oxyporum*. *The Journal of General and Applied Microbiology*, 25: 361–366.

[16] Yu, J., Chou, C.C., Saska, M. (2011). The Behavior of Invert Sugar in Sugar Processing. In *Technical Proceedings of the Sugar Industry Technologists* (Vol. 4, pp. 1–26). Montreal: Curran Associates, Inc.

[17] El-Sayed, E.-S. M., El-Sayed, S.T., Elmallah, M.I.Y., Shehata, A.N. (2015). Immobilization, Optimization and Properties of Pea Invertase within Sodium Alginate Gel. *Research Journal of Pharmaceutical Biological and Chemical Sciences*, 6(6): 1213–1222.

[18] Miller, G.L. (1959). Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Analytical Chemistry*, 31(3): 426–428.

[19] Somogyi, M. (1952). Notes on Sugar Determination. *Journal of Biological Chemistry*, 195: 19–23.

[20] Nelson, N. (1944). A photometric adaptation of the Somogyi method for the determination of glucose. *Journal of Biological Chemistry*, 153: 375–380.

[21] Nelson, D.L., Cox, M.M. (2005). *Lehninger - Principles of Biochemistry* (Fourth Edition.). New York: W.H. Freeman and Company.

[22] Lineweaver, H., Burk, D. (1934). The Determination of Enzyme Dissociation Constants. *Journal of The American Chemical Society*, 56(3): 658–666.

[23] Fange, D., Lovmar, M., Pavlov, M.Y., Ehrenberg, M. (2011). Identification of enzyme inhibitory mechanisms from steady-state kinetics. *Biochimie*, 93(9): 1623–1629.

[24] Yoshino, M., Murakami, K. (2009). A graphical method for determining inhibition constants. *Journal of Enzyme Inhibition and Medicinal Chemistry*, 24(6): 1288–1290.

[25] Pál, G. (2013). Enzyme kinetics. In G. Hegyi, J. Kardos, M. Kovács, A. Málnási-Csizmadia, L. Nyitray, G. Pál, L. Radnai, A. Reményi, I. Venekei (Eds.), *Introduction to Practical Biochemistry*. Budapest: Eötvös Loránd University.

[26] Szymańska, K., Pudło, W., Mrowiec-Białoń, J., Czardybon, A., Kocurek, J., Jarzębski, A. B. (2013). Immobilization of invertase on silica monoliths with hierarchical pore structure to obtain continuous flow enzymatic microreactors of high performance. *Microporous and Mesoporous Materials*, 170: 75–82.

[27] Marquez, L.D.S., Cabral, B.V., Freitas, F.F., Cardoso, V.L., Ribeiro, E.J. (2008). Optimization of invertase immobilization by adsorption in ionic exchange resin for sucrose hydrolysis. *Journal of Molecular Catalysis B: Enzymatic*, 51(3–4): 86–92.

[28] Copeland, R.A. (2000). *Enzymes: a practical introduction to structure, mechanism, and data analysis* (2nd ed.). New York: Wiley.

[29] German, D.P., Weintraub, M.N., Grandy, A.S., Lauber, C.L., Rinkes, Z.L., Allison, S.D. (2011). Optimization of hydrolytic and oxidative enzyme methods for ecosystem studies. *Soil Biology and Biochemistry*, 43(7):1387–1397.

[30] Baks, T., Bruins, M.E., Janssen, A.E.M., Boom, R.M. (2008). Effect of Pressure and Temperature on the Gelatinization of Starch at Various Starch Concentrations. *Biomacromolecules*, 9(1): 296–304.

[31] Permanasari, A.R., Yulistiani, F., Purnama, R.W., Widjaja, T., Gunawan, S. (2018). The effect of substrate and enzyme concentration on the glucose syrup production from red sorghum starch by enzymatic hydrolysis. *IOP Conference Series: Earth and Environmental Science*, 160: 1–6.

[32] Weber, B.A., Nielsen, S.S. (1991). Isolation and Partial Characterization of a Native Serine-Type Protease Inhibitor from Bovine Milk. *Journal of Dairy Science*, 74(3): 764–771.

[33] Corazza, F.C., Calsavara, L.P.V., Moraes, F.F., Zanin, G.M., Neitzel, I. (2005). Determination of inhibition in the enzymatic hydrolysis of cellobiose using hybrid neural modeling. *Brazilian Journal of Chemical Engineering*, 22(1): 19–29.

[34] Salwanee, S., Wan Aida, W.M., Mamot, S., Maskat, M.Y., Ibrahim, S. (2013). Effects of Enzyme Concentration, Temperature, pH and

Time on the Degree of Hydrolysis of Protein Ectract from Viscera of Tuna (*Euthynnus affinis*) by Using Alcalase. *Sains Malaysia*, 42(3): 279–287.

[35] Kharlamova, A.D., Lushchekina, S.V., Petrov, K.A., Kots, E.D., Nachon, F., Villard-Wandhammer, M., Zueva, I.V., Krejci, E., Reznik, V.S., Zobov, V.V., Nikolsky, E.E., Masson, P. (2016). Slow-binding inhibition of acetylcholinesterase by an alkylammonium derivative of 6-methyluracil: mechanism and possible advantages for myasthenia gravis treatment. *Biochemical Journal*, 473(9): 1225–1236.

[36] Rohatgi, N., Nielsen, T.K., Bjørn, S.P., Axelsson, I., Paglia, G., Voldborg, B.G., Palsson, B.O., Rolfsson, Ó. (2014). Biochemical Characterization of Human Gluconokinase and the Proposed Metabolic Impact of Gluconic Acid as Determined by Constraint Based Metabolic Network Analysis. *PLoS ONE*, 9(6): 1–9.

[37] Rubio, M.C., Runco, R., Navarro, A.R. (2002). Invertase from a strain of *Rhodotorula glutinis*. *Phytochemistry*, 61(6): 605–609.

[38] Azodi, M., Falamaki, C., Mohsenifar, A. (2011). Sucrose hydrolysis by invertase immobilized on functionalized porous silicon. *Journal of Molecular Catalysis B: Enzymatic*, 69(3–4): 154–160.

[39] Martínez, D., Menéndez, C., Echemendia, F.M., Pérez, E.R., Trujillo, L.E., Sobrino, A., Ramírez, R., Quintero, Y., Hernández, L. (2014). Complete sucrose hydrolysis by heat-killed recombinant Pichia pastoris cells entrapped in calcium alginate. *Microbial Cell Factories*, 13(1): 87–95.

[40] Whidden, M., Ho, A., Ivanova, M.I., Schnell, S. (2014). Competitive inhibition reaction mechanisms for the two-step model of protein aggregation. *Biophysical Chemistry*, 193–194: 9–19.

[41] Dutta, R. (2008). *Fundamentals of Biochemical Engineering*. Berlin, Heidelberg: Springer Berlin Heidelberg.