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Kinetics of Tapioca Slurry Saccharification Process Using Immobilized Multi-Enzyme System Enhanced with Sg. Sayong Clay

Siti Noraida Abd Rahim
Alawi Sulaiman
Nurul Aini Edama
Tropical Agro-Biomass Research Group
Faculty of Plantation and Agrotechnology, Universiti Teknologi MARA
40450 Shah Alam, Selangor, Malaysia

ABSTRACT

In this paper, immobilization of multi-enzyme using calcium alginate-clay beads for tapioca slurry saccharification process was carried out. The kinetics parameter and immobilization yield for alpha-amylase, glucoamylase, cellulase and multi-enzyme system were compared. The Michaelis constant, K_m for free alpha-amylase, glucoamylase, cellulase and multi-enzyme were 2.0831, 1.8326, 7.8592 and 3.5367 mg/mL, respectively. As for the immobilized system, the K_m values were 3.1604, 2.1708, 9.2791 and 4.91176 mg/mL, respectively. The K_m value for the immobilized multi-enzyme system was 1.39 times higher than free multi-enzyme system. This suggests that the immobilized multi-enzyme is a potential system for tapioca saccharification process due to affinity of enzyme to the substrate. For the immobilization yield, the yield for alpha-amylase, glucoamylase, cellulase and multi-enzyme were 51.19, 50.21, 40.68 and 53.71 %, respectively. The result showed the immobilized multi-enzyme had a higher yield compared to the single enzyme which could be explained due to the synergistic action of alpha-amylase, glucoamylase and cellulose enzymes. Therefore, it can be concluded that the use of immobilized multi-enzyme system had a very good potential for starch bioconversion into glucose.

Keywords: *Immobilization, calcium alginate-clay beads, multi-enzyme, tapioca slurry, kinetic parameter, immobilization yield*

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Introduction

Enzyme immobilization technology has been a subject of interest to the scientific community for many decades and recently the momentum is getting stronger due to better development on stability and activity in organic solvent, less diffusion limitation problem and single enzyme nanoparticle. The immobilization technology provides a better platform over the free enzyme system because it can reduce the enzyme cost, increase stability, easier enzyme separation from the media and recyclable [1-3]. There are various methods that have been developed for enzyme immobilization such as adsorption, covalent binding, entrapment and encapsulation [4]. Encapsulation is an effective technique that can be defined as a process where enzymes are enclosed physically or chemically within a semi-permeable membrane layer [5].

Alginate is a commonly used encapsulation medium because of easier formulation, non-toxic, biocompatibility, lower material cost and larger large surface area. Despite all these advantages, the main drawback of using alginate is the enzyme leakage problem. The leakage of enzyme from the beads often occurred during the bioconversion process as reported in literatures [6, 7]. To rectify this problem, alginate can be blended with clay in order to produce more stable encapsulated enzyme medium [8, 9]. Kaolinite clay has been reported as a good supporting material candidate for encapsulation purposes because it is stable, relatively good pore aperture and possess good mechanical strength and thermostability [10].

The clay enhanced enzyme encapsulated beads can be used for many types of saccharification and fermentation processes. In the glucose production, cassava root is often used as the feedstock, the glucose can be later on fermented to produce bioethanol [11, 12]. Common enzymes which can be used for glucose saccharification are alpha-amylase, glucoamylase and celulase as reported in the previous study [13]. Alpha-amylase can hydrolyze starch by randomly cleaving α-(1→4) glycosidic bonds in starch to produce maltodextrin while glucoamylase is able to hydrolyze α-(1→4) and α-(1→6) glycosidic bonds in starch to produce glucose [14]. For the hydrolysis of fibre, cellulase is frequently used due to its ability to attack β-(1→4) linkages in fiber to produce glucose [15].

In this study, the indigenous kaolinite clay material obtained locally from Sg. Sayong, Perak, has been prepared and blended with alginate to improve the enzymes encapsulation process as described earlier [16]. The kinetic parameter of immobilized alpha-amylase, glucoamylase, cellulase and multi-enzyme within calcium alginate-clay beads was determined and compared with the free enzymes system. Besides, the immobilization yield for each enzymes system was also studied.

Materials and Methods

Materials

Alpha-amylase from *Bacillus subtilis*, glucoamylase from *Rhizopus niveus* Lyophilized and cellulase from *Aspergillus niger* were purchased from MP Biomedicals, United States. All other chemicals were of analytical of grade.

Preparation of tapioca slurry

Cassava root was purchased from local market in Shah Alam, Selangor, Malaysia. Then the root was washed free of dirt, hand peeled and sliced into smaller pieces. The chips were dried in oven at 65°C for 24 hrs and then ground into powder form. The powders were gelatinized using buffer solution at boiling water bath for 1 hr.

Preparation of clay powder

The raw kaolinite clay was obtained from Sg. Sayong, Perak, dried at 90°C for 24 hrs, ground into fine powder and lastly sieved through 150 μm mesh before further use [17].

Preparation of calcium alginate-clay beads

2.5 g of previously prepared kaolinite clay was dissolved in 100 mL of buffer solution and stirred for 1 hr at room temperature. Then, 2.5 g of alginate powder was added to the clay solution and stirred for another 4 hrs. Then, 2.5 mL of glycerol was added to the alginate-clay solution. For the enzymes encapsulation process, 3 mL of enzyme solution (1 mg solid/mL of alpha-amylase, glucoamylase, cellulase or multi-enzyme) was mixed with 12 mL of alginate-clay solution which produced the final clay concentration 2% w/v. The mixture was continuously stirred thoroughly to ensure complete mixing. For the beads preparation, the mixture solution was then dropped by using syringe into 0.2 M $CaCl_2$ solution under mild agitation. After 3 hrs of hardening, the beads were collected by simple filtration and then washed with buffer solution several times to remove any unbound enzymes. Finally, the beads were stored at 4°C until used as mentioned earlier [8].

Determination enzyme activity

The activity of enzymes was measured by using spectrophotometer at 540 nm with 3,5-dinitrosalicylic acid (DNS) as an indicator as proposed in the Bernfeld method [18]. Theoretically one unit of alpha-amylase activity is defined as the quantity of enzyme that releases 1 mg of reducing sugar as maltose per minute at pH 6.6 and 30°C. One unit of glucoamylase is expressed as the amount of enzyme releasing 10 mg of glucose per minute at pH 4.5 and 40°C. One unit of cellulase activity is liberated as the amount of enzyme that produces 1 μmole of glucose at pH 5 and 37°C in 1 min.

Kinetics analysis

The K_m and V_{max} of free and encapsulated alpha-amylase, glucoamylase, cellulase and multi-enzyme were determined by measuring the initial rates of the reaction at different substrate concentrations (1.0–5.0 mg/mL). The conditions for enzyme as described for the activity assays. Kinetic constants were calculated from the Lineweaver-Burk plot.

Results and Discussion

Kinetics constant

The kinetic parameters of tapioca slurry saccharification process were determined for both free and immobilized enzymes in order to determine the Michaelis constant and maximal enzymes activity that influence the immobilization process. The relationship between the substrate concentration and the rate of enzymatic reaction can be described by Michaelis-Menten equation:

$$V_0 = \frac{V_{max}[S]}{K_m + [S]} \tag{1}$$

where V_0 is the initial reaction rate (mg/mL.min), S is the substrate concentration (mg/mL), K_m is the Michaelis constant and V_{max} is the maximum reaction rate (mg/mL.min). By using Michaelis-Menten equation, the curve obtained was in the hyperbolic form and therefore difficult to obtain a reliable value of V_{max} because it did not reach the substrate concentration line and consequently unable to determine the K_m value of the enzymes [19]. Therefore, the Michaelis-Menten equation was linearized to form Lineweaver-Burk equation:

$$\frac{1}{V_0} = \frac{K_m}{V_{max}} ¥ \frac{1}{[S]} + \frac{1}{V_{max}} \tag{2}$$

where the intercept on the x-axis is equal to $-1/K_m$ while the intercept on the y-axis is equal to $1/V_{max}$. In this study, the Lineweaver-Burk plot for both free and immobilized alpha-amylase, glucoamylase, cellulase and multi-enzyme were able to be obtained and presented in Figure 1-4. The value of K_m is defined as measurement of the affinity of enzymes towards substrates and therefore, the lower K_m value emphasizes the higher affinity between enzymes and substrates [20].

Figure 1 shows the Lineweaver-Burk plot for both free and immobilized alpha-amylase that was carried out at pH 6.6 and 30°C. The K_m value for free and immobilized alpha-amylase was 2.0831 and 3.1604 mg/mL, respectively and the immobilized alpha-amylase was found to be 1.52 times higher than free alpha-amylase [16]. The result indicates that the immobilization reduces the affinity of enzyme to the substrate. This finding was consistent with another

study by Talekar and Chavare [2] where they found that the K_m value of free and immobilized alpha-amylase tested with alginate gel beads was 0.93 and 1.12 mg/mL, respectively (1.2 times higher than free system). Much higher K_m (2.48 times higher) value was reported in another study using chitosan-clay composite beads [21]. Therefore, it can be stated that the addition of clay into the alginate gel might have contributed to the blocking of active sites of the enzyme and therefore caused reduction of enzyme affinity towards the substrate.

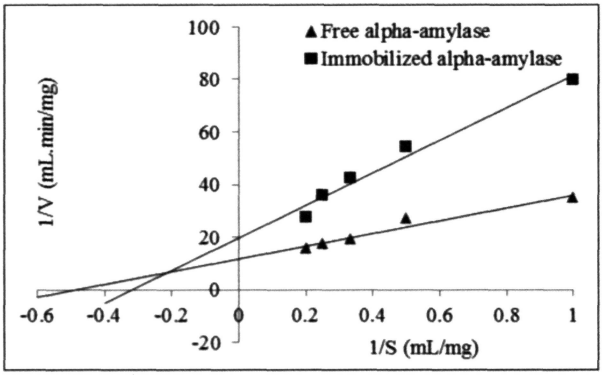

Figure 1: Lineweaver-Burk plot for free and immobilized alpha-amylase

The kinetic constant for both free and immobilized glucoamylase was determined using Lineweaver-Burk plot as given in Figure 2. The kinetic constant for glucoamylase system was performed at pH 4.5 and temperature 40°C. The calculated K_m value was 1.8326 mg/mL for free glucoamylase and 2.1708 mg/

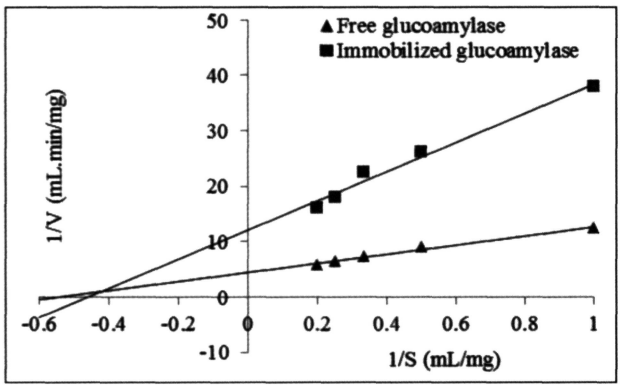

Figure 2: Lineweaver-Burk plot for free and immobilized glucoamylase

mL for immobilized glucoamylase (1.18 times higher than the free glucoamylase system) [16]. Rebros et al. [22] and Tanriseven et al. [23] reported the K_m value of glucoamylase after immobilized onto poly(vinylalcohol) hydrogel lens shaped capsules LentiKats® and immobilized by adsorption to gelatinized corn starch and subsequent alginate fiber entrapment were 1.4 and 1.7 times higher compared to the free enzyme system. This result shows that glucoamylase in alginate-clay beads retained more affinity of enzyme to its substrate after immobilization process compared to previous studies.

Figure 3 shows the Lineweaver-Burk plot of free and immobilized cellulase. The analysis of kinetic constant for both free and immobilized cellulase was carried out at pH 5.0 and 37°C. The result of K_m value for immobilized cellulase was 9.2791 mg/mL while the free cellulase was 7.8592 mg/mL. The calculated K_m value for immobilized cellulase shows similar result with glucoamylase which was 1.18 times higher after immobilization [16]. From work done by Wang et al. [24], the K_m value obtained was 0.26 mg/mL for free enzyme and 0.33 mg/mL for adsorbed cellulase onto polyamidoamine denrimer-grafted silica. The K_m value of adsorbed cellulase was determined to be 1.27 times higher than the free enzyme system. Therefore, the immobilized cellulase onto calcium alginate-clay beads by using encapsulation technique still had a lower affinity to the substrate due to higher diffusion limitation of the substrate getting into the active site of the enzyme similar with alpha-amylase and glucoamylase cases.

When all enzymes including alpha-amylase, glucoamylase and cellulase were combined together, the kinetic parameters for both free and immobilized multi-enzyme system was changed and the plot for this system is presented in Figure 4. This multi-enzyme system was carried out under optimum conditions (pH 6 and 50°C) as reported earlier [25]. It was found that the calculated K_m

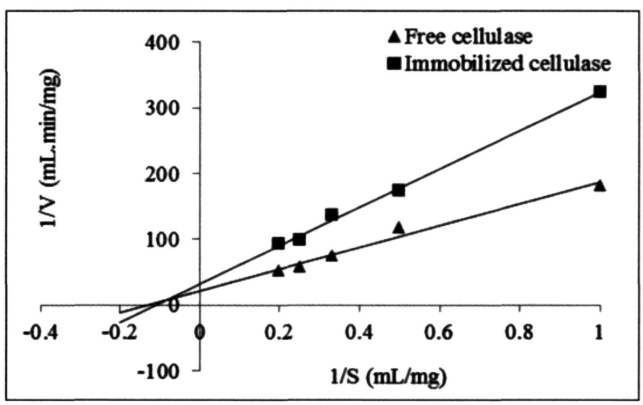

Figure 3: Lineweaver-Burk plot for free and immobilized cellulase

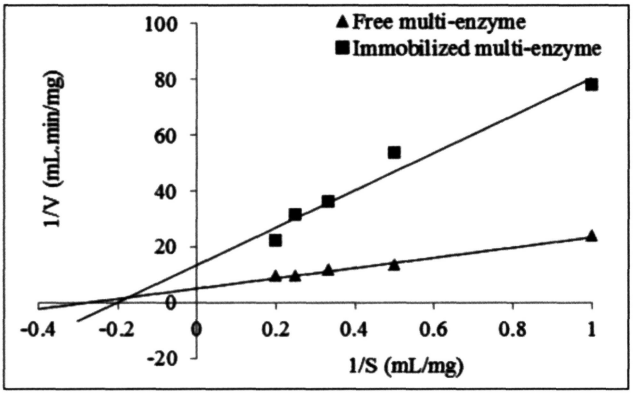

Figure 4: Lineweaver-Burk plot for free and immobilized multi-enzyme

value for free and immobilized multi-enzyme system was 3.5367 and 4.9176 mg/mL, respectively. The K_m value for the immobilized multi-enzyme was 1.39 times higher than free multi-enzyme system. Compared with immobilized single enzyme (alpha-amylase, glucoamylase and cellulase), the immobilized multi-enzyme still retained more affinity of enzymes to its substrate where the result of K_m value was closed to the immobilized single enzyme.

In general, the immobilized enzymes exhibit lower K_m and higher V_{max} values than free enzymes indicating an improved activity upon immobilization. However, Ghiaci et al. [26] had reported that this situation was very rare occurred for the inorganic supports. In this study, it was found that all the immobilized enzymes had a higher K_m value compared to free enzymes system. Thus, it can state that the affinity of enzymes for the substrate was reduced after immobilization process. The change in affinity of the enzymes to its substrate was probably caused by structural changes of the enzymes which lower the accessibility of the substrate to the active sites of the enzymes and therefore created diffusion barrier of the matrix of enzyme as reported in the literature [22]. This is in agreement with the previous study where it was reported that the kinetic parameter of enzymes in alginate-clay beads can be affected by external or internal diffusion corresponding to the transport of reactants and products from the bulk solution to the outer surface of the enzyme and to the transport of these species inside the porous system [27].

Immobilization Yield

The effect of enzyme types on the immobilization yield was also studied by using alpha-amylase, glucoamylase, cellulase and multi-enzyme system (Figure 5). The result revealed that the immobilization yield for immobilized alpha-amylase,

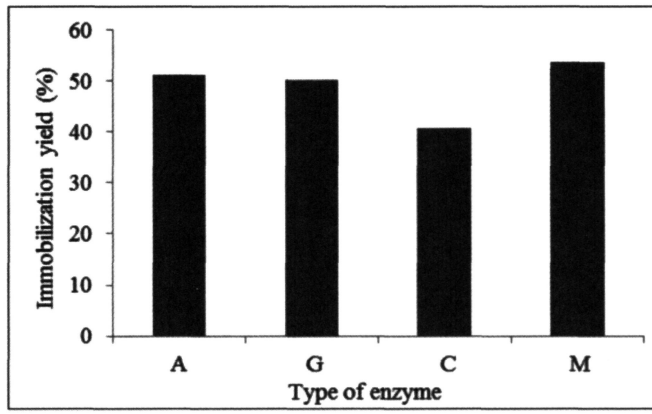

Figure 5: Immobilization yield for enzyme types (A: alpha-amylase; G: glucoamylase; C: cellulase; M: multi-enzyme)

glucoamylase, cellulase and multi-enzyme system was 51.19, 50.21, 40.68 and 53.71%, respectively. The immobilized multi-enzyme system had a higher immobilization yield compared to the immobilized single enzyme. Therefore, it can conclude that the use of multi-enzyme system in the immobilization was more efficient due to the synergistic action between the alpha-amylase, glucoamylase and cellulase on the tapioca slurry saccharification. This finding was also supported by Roy and Gupta [11] who mentioned that the use of immobilized glucoamylase and pullulanase together in the starch hydrolysis has resulted in higher amount of product compared to use enzyme separately.

Conclusion

Immobilization of multi-enzyme system onto calcium alginate-clay beads by using encapsulation method was successfully performed in the tapioca slurry saccharification process. The kinetic constant and immobilization yield for immobilized multi-enzyme were determined and compared with single enzyme such as alpha-amylase, glucoamylase and cellulase. The result of kinetic constant for immobilized multi-enzyme system had a lower affinity due to the diffusion limitation between the substrate and enzymes. However, the immobilized multi-enzyme system still had a good potential because the kinetic constant of immobilized multi-enzyme was very close to single enzyme. Besides, the immobilization yield for the multi-enzyme system was higher compared to the single enzyme. This situation happened because of the synergistic action between the alpha-amylase, glucoamylase and cellulase during the saccharification

process. Therefore, the use of immobilized multi-enzyme onto alginate-clay beads had good potential for tapioca bioconversion into glucose.

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