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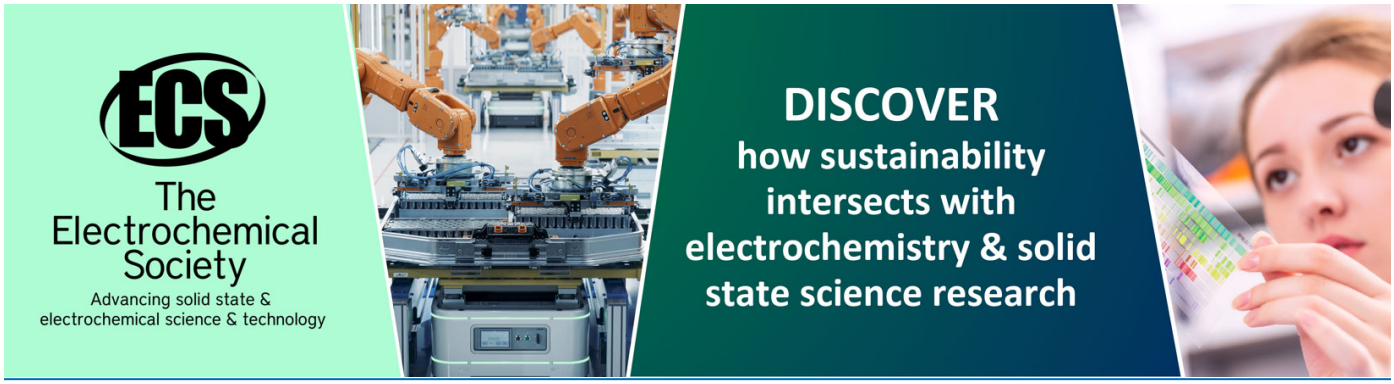

Immobilization of cyclodextrin glucanotransferase on hollow fiber membrane: optimization of the immobilization parameters by response surface methodology

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Abstract. Cyclodextrin glucanotransferase (CGTase) is a multifunctional industrial enzyme which undergoes cyclization reaction to converts starch into cyclodextrin. Due to their potential properties, cyclodextrin has been discovered to have numerous application in food industries, pharmaceuticals, agriculture and environmental engineering. However, the instability of the enzyme during the reaction process result in the low production of cyclodextrin. Thus, enzyme immobilization process has been used to improve the enzyme stability in order to achieve high production of cyclodextrin. In this study, CGTase from *Bacillus licheniformis* was immobilized on polyvinylidene difluoride hollow fiber membrane via physical adsorption. The optimization of the immobilization parameters and the performance of the immobilized CGTase were investigated. The adsorption of CGTase on hollow fiber membrane was evaluated by fourier transform infrared spectroscopy. Response surface methodology was employed to optimize enzyme immobilization by manipulating the immobilization parameters of contact time (15-33 h), immobilization pH (pH 6-8) and immobilization temperature (20-30 °C) on the immobilization yield. The optimized immobilization conditions were 24 °C of immobilization temperature, pH 6.7 and 24 h of contact time, with 88.25% of immobilization yield. Immobilization of CGTase on the hollow fiber membrane was successfully optimized and about 4.6-fold increment of immobilization yield was achieved after the optimization process. The kinetic parameters of the immobilized CGTase were 9.42 $mgml^{-1}$ h^{-1} and 9.99 mg ml^{-1} for V_{max} and K_m value, respectively. The kinetic studies revealed that the catalytic efficiency of the immobilized CGTase was similar to the free CGTase, demonstrated that upon the immobilization process, adsorption of CGTase on hollow fiber membrane does not cause structural changes to the enzyme. Hence, immobilization of CGTase on the hollow fiber membrane substantially improved the production of cyclodextrin and suggesting that the hollow fiber membrane appeared as a suitable support for the enzyme immobilization system.

1. Introduction

Cyclodextrin (CD) also known as cycloamyloses is a non-reducing maltoologosaccharides with a unique structure of hydrophobic and hydrophilic surface [1]. Because of these features, inclusion complexes can be formed when the interaction of cyclodextrin and hydrophobic molecules occur [1]. Application of CD in various industries especially in food, cosmetic, pharmaceutical and agrochemical industries have increased from year to year, due to their capabilities in improving physicochemical properties of organic molecules such as higher solubility and chemical resistance and reduced volatility [2]. CD is

produced by enzymatic reaction of cyclodextrin glucanotransferase (CGTase) with starch. The market demand for CDs had increased 15-20% annually, since there are numerous application of CD in various industries [3]. However, commercialization of CGTase for industrial purposes are highly challenging due to the instability of the enzyme, sensitivity to the process conditions and high cost of isolation and purification of enzyme [4,5]. Therefore, the enzyme immobilization technique has been chosen as a promising method to increase the enzymatic properties by improving the stability, activity and specificity of the enzyme [6]. More importantly, the application of immobilization system also benefits to the enzyme catalytic reaction by providing easy separation of the enzyme from the reaction medium, allows continuous operation of enzymatic processes as well as producing reusable enzyme that was beneficial for industries especially in term of cost reduction. There are numerous studies of enzyme immobilization by using various technique such as entrapment, adsorption, encapsulation and covalent attachment [7]. Nevertheless, adsorption method remain as the most attractive method due to its simplicity, inexpensive and does not involve chemical alteration [8]. Hollow fiber membrane has been mainly employed in biotechnology as filter-aids in biomass recycle fermenters, microfiltration, pervaporation and distillation. To date, the use of hollow fiber membrane has been expanded as a support for enzyme immobilization [9]. There are several benefits of using hollow fiber membrane for the development of immobilized enzyme such as the high surface-to-volume ratio, lower mass transfer resistance, lack of toxicity, cost effective and high mechanical strength [9-11].

The immobilization system where the enzyme was attached to the matrices has stabilized the structure of the enzyme and become more resistant to the environmental changes [12]. However, efficiency of the enzyme immobilization appears to be affected by several factors of contact time, agitation rate, pH and temperature. These factors can affect the process of the immobilization, the attachment of enzyme to the matrix and the production of desired product. Thus, the influence of process parameters in the process of immobilization was investigated in order to achieve the desired immobilized enzyme. The conventional method in determining the optimum conditions by varying one parameter at one time is a laborious and time-consuming method [13]. To overcome these problems, the immobilization parameters of CGTase was optimized by response surface methodology (RSM). This statistical experimental design protocol where several factors were simultaneously varied has reduce the number of experiments and time consumed for the analysis [13]. Other than that, this approach has also improved the statistical interpretation by reducing numerical noise and allow the use of derivative-based algorithms [14].

The aim of this study was to optimize the immobilization parameters of CGTase on PVDF membrane and to access the kinetic performance of the CGTase. Therefore, CGTase from *Bacillus licheniformis* was immobilized on the hollow fiber membrane via adsorption technique. Immobilization parameters of contact time, pH and temperature that affected in the immobilization of CGTase on hollow fiber membrane were optimized using response surface methodology (RSM).

2. Materials and method

2.1. Materials

The commercial CGTase from *Bacillus licheniformis* (Toruzyme, 3.0L) was supplied by Novozymes A/S (Bagsvaerd, Denmark). The concentration of enzyme used in this study was 161.37 U/ml. The polyvinylidene difluoride (PVDF) hollow fiber membrane was procured from Separation and Membrane Cluster, Faculty of Chemical and Natural Resource Engineering, Universiti Malaysia Pahang (UMP, Malaysia). Standard of α-CD (98%) was purchased from Sigma-Aldrich (St. Louis, USA). Acetonitrile HPLC grade and nylon membrane filter were purchased from Merck Sdn Bhd (Selangor, Malaysia).

2.2. Immobilization of CGTase

The immobilization process was performed as described by Schöffer et al. [6] with minor modifications. CGTase (100 U) was added into 0.05 M sodium phosphate buffer at different pH ranging (pH 6 to 8), before adding 3 cm of PVDF hollow fiber membrane. The immobilization process was carried out at

different temperatures (20 to 30 °C) under gentle orbital shaking of 100 rpm. The immobilization mixture was incubated at different contact time (15 to 33 h) according to the experimental design. The CGTase activity in the remaining buffer was further analyzed under CGTase assay. The immobilization yield was calculated as in equation (1).

$$\text{Immobilization yield (\%)} = \frac{W_0 - W_1}{W_0} \times 100 \quad (1)$$

where W_0 is the specific activity of CGTase before immobilization and W_1 is the specific activity of CGTase in the remaining buffer after the immobilization.

2.3. Experimental design by central composite design

Design Expert Software version 7.1.6 (Stat-Ease Inc., MN, US) was used in this study. The optimization was designed by using Central Composite Design (CCD) with a total of 17 experimental trials contain 6 star points and 3 central points, with the immobilization yield collected as a response. Table 1 shows the immobilization conditions of contact time (X_1), pH (X_2) and temperature (X_3) with the details of lower and upper limit values.

Table 1. Design variables for the optimization process.

Factors	Notation	Unit	Low star point	Low level	Centre point	High level	High star point
			-α	-1	0	+1	+α
Contact Time	X_1	h	9.00	15.00	24.00	33.00	39.00
pH	X_2	-	5.30	6.00	7.00	8.00	8.70
Temperature	X_3	℃	16.00	20.00	25.00	30.00	34.00

2.4. Kinetic study of the immobilized and free CGTase

The immobilized and free CGTase was suspended into 10 ml of different concentration of starch [1% - 3% (w/v)] in 0.05 M phosphate buffer (pH 6.0) before incubated at 40 °C under shaking condition of 100 rpm. After 6 h, the production of CD was further analyzed by HPLC. The K_m and V_{max} were estimated using the equation of Michaelis-Menten model and Lineweaver-Burk plot as shown in equation (2) and (3), respectively.

$$V_0 = \frac{V_{\max} S}{K_m + S} \quad (2)$$

$$\frac{1}{V_0} = \frac{K_m}{V_{\max}} \frac{1}{S} + \frac{1}{V_{\max}} \quad (3)$$

where V_{max} is the maximum reaction rate (mgml^{-1} h^{-1}), K_m is the Michealis-Menten constant (mgml^{-1}) and S is the substrate concentration (mgml^{-1}). The α-CD production initial reaction rate (V_0, mgml^{-1} h^{-1}) was calculated by the slope of α-CD production vs. time.

2.5. α-CGTase assay

Methyl orange assay was applied to determine the activity of α-CGTase. This assay was conducted according to the method by Jamil et al. [15]. All experiments were conducted in triplicate.

2.6. Fourier transform infrared spectroscopy (FTIR)

Samples of hollow fiber membrane, free and immobilized CGTase were analyzed using fourier transform infrared spectroscopy (FTIR) (ThermoFisher Nicolet iS5, USA) with diamond attenuated total

reflectance (ATR) system. The spectra obtained in the wavelength range of 400 to 4000 cm^{-1} was analyzed by using OMINIC software.

2.7. High performance liquid chromatography (HPLC)

The α-CD was determined by using the High Performance Liquid Chromatography (HPLC) with quaternary pump (Agilent 1260 Infinity Quaternary LC, California, USA). The column used was Zorbax Eclipse Plus C18, 150 mm x 4.6 mm (Agilent Technologies, California, USA). Mobile phase comprises of HPLC grade acetonitrile and ultrapure water with the ratio of 60:40 was filtered with 0.2 µm nylon membrane filter. Detection was performed using a refraction index detector (RID) at 1.540 min of retention time and analyzed by Agilent ChemStation 4.0.

3. Results and discussion

3.1. Immobilization of CGTase

The immobilization of CGTase on the PVDF membrane via adsorption was evaluated by FTIR analysis. Figure 1 shows the FTIR spectra of PVDF hollow fiber membrane, free and immobilized CGTase. The characteristic peaks of CGTase (figure 1b) were observed at 1400 cm^{-1} for carboxylic (COO) bonds, 3400 cm^{-1} for OH bonds and at 1640 cm^{-1} which corresponds to NH bonds [16]. Figure 1a shows FTIR spectrum of PVDF hollow fiber membrane without the immobilized enzyme. The stretching of C-H bonds at 1454 cm^{-1} and the bending of CF2 at 1120 to 1280 cm^{-1} were found to be the characteristic peaks of PVDF hollow fiber membrane [17,18]. Figure 1c confirmed the binding of CGTase on the hollow fiber membrane by the appearance of the characteristic peaks of CGTase together with those characteristic peaks of PVDF hollow fiber membrane in the immobilized CGTase spectra.

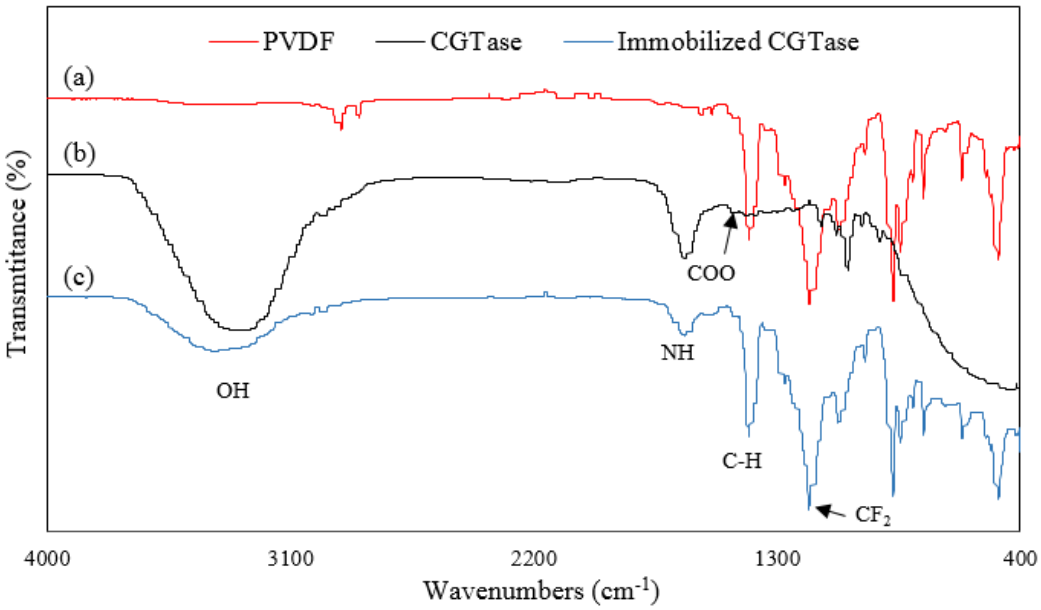

Figure 1. Comparison of FTIR spectra of (a) PVDF membrane; (b) Free CGTase and (c) Immobilized CGTase on PVDF membrane.

3.2. Optimization of immobilization parameters

Central composite design (CCD) was applied to optimize the immobilization parameters in the immobilization of CGTase on hollow fiber membrane. Immobilization conditions of contact time (X_1), pH (X_2) and temperature (X_3) were optimized with the immobilization yield collected as the response. The immobilization yield from the experiments were collected from range of 13.09% to 89.02% (Supp.

data). Immobilization of CGTase on hollow fiber membrane was described with quadratic polynomial model. The coefficient of determination (R^2) of 0.9970 and adjusted coefficient of determination (R^2_{adj}) of 0.9932 were recorded by the model indicated that both observed and predicted value of CGTase immobilization yield were in a good range. Moreover, 0.9787 of predicted R^2 showed that the model obtained was applicable to predict the optimum immobilization condition to maximize the immobilization of CGTase on hollow fiber membrane. The regression equation was used to predict the response by factors in terms of coded level as shown in the equation (4).

$$Immobilization\ yield = 87.423 + 0.7225\,X_1 - 0.4761\,X_2 - 0.2025\,X_3 + 0.0676\,X_1X_2 - 0.0841\,X_1X_3 + 0.00518\,X_2X_3 - 1.188\,X_1^2 - 0.7744\,X_2^2 - 3.0276\,X_3^2 \quad (4)$$

Analysis of variance (ANOVA) in table 2 showed that the confidence level of the model was greater than 95% with the *P*-value of 0.0001. Based on the calculated *P*-value in table 2, the main effects of contact time (X_1), pH (X_2), temperature (X_3) and interaction effects of X_1X_2, X_1X_3, X_1^2, X_2^2 and X_3^2 were significant factors that influenced the immobilization yield.

Table 2. ANOVA for optimization of the immobilization parameters.

Source	Sum of squares	Degree of freedom	Mean Square	*P*-value
Model	59.25	9	6.58	0.0001
X_1 (Contact time)	9.93	1	9.93	0.0001
X_2 (pH)	6.42	1	6.42	0.0001
X_3 (Temperature)	2.73	1	2.73	0.0001
X_1 X_2	0.52	1	0.52	0.0027
X_1 X_3	0.67	1	0.67	0.0013
X_2 X_3	0.042	1	0.042	0.2406
X_1^2	13.35	1	13.35	0.0001
X_2^2	8.80	1	8.80	0.0001
X_3^2	34.13	1	34.13	0.0001
Lack of Fit	0.16	5	0.031	0.2621

3.3. Optimization of immobilization parameters by RSM

Figure 2a illustrated the interaction of temperature and contact time at a constant pH value of 7.0. The immobilization yield was increase with the increased of temperature from 20 to 24 °C. The highest immobilization yield was achieved at 24 °C with 89.02%. As the temperature raised from 20 to 24 °C, the immobilization yield was increase because an increase of temperature would increase the rate of motion of the molecules. This has contributed more to the interactions between enzyme and the support. However, as the temperature was further increased to 25 and 30 °C, the amount of CGTase immobilized on the hollow fiber membrane was decreased, indicating that the immobilization system more effective at lower temperature. Physical adsorption of CGTase on the hollow fiber membrane that involved electrostatic interaction between the charges located on the surface of the enzyme and the support was a spontaneous and direct process. This spontaneous and direct adsorption (physical adsorption) was known to be exothermic reaction, whereby the heat energy was released to the surrounding [19]. Therefore, the adsorption process preferable to take place at low temperature. This argument was supported by Langmuir adsorption isotherm, whereby adsorption process that involved direct and spontaneous reaction was affording an exothermic process [20,21]. A study conducted by Liu et al. [21] revealed that the adsorption of lysozyme on polyacrylic nano-absorbents was decreased as the temperature increased from 20 to 30 °C. The study was further clarified by the negative enthalpy (-28.60 kJ mol^{-1}) of the adsorption isotherm which proved that the physical adsorption involved an exothermic reaction. A similar trend was observed by Zeng et al. [22] on the study of immobilization of lipase on dendritic composite magnetic particles, whereas the highest lipase immobilization yield was recorded at

25 °C and as the immobilization temperature further increased from 25 to 40 °C, the lipase immobilization yield was decreased up to 20%.

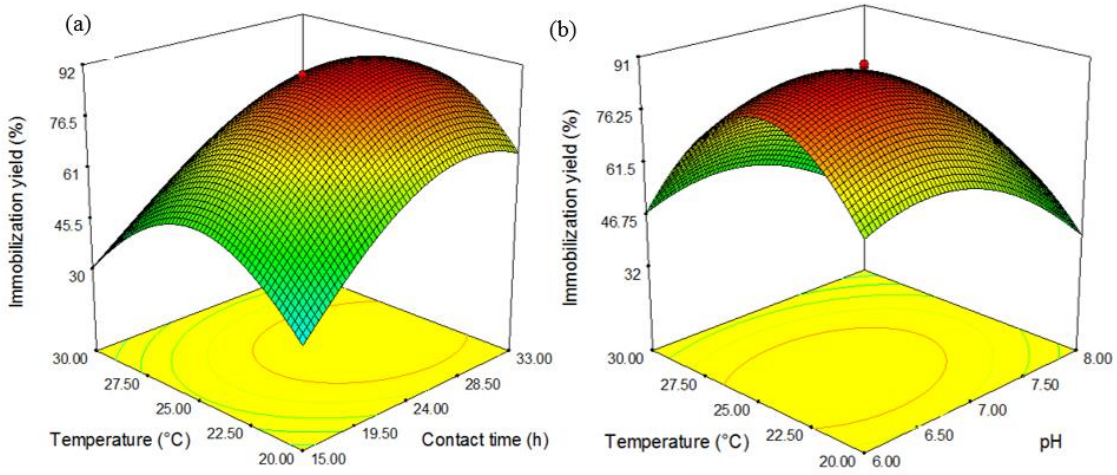

Figure 2. Response surface of plot of immobilization yield as a function of: (a) contact time and temperature at fixed level of pH (pH 7); (b) pH and temperature at fixed level of contact time (24 h).

Apart from that, figure 2a showed that the immobilization yield was increased with the increased of contact time from 15 h to 27 h indicating that the increase of contact time had enhanced the immobilization process by providing sufficient contact time between enzyme and the support thus increase the amount of enzyme interacted to the support. As the contact time was further increased from 27 to 33 h, the immobilization yield was decreased. At this phase, the desorption of the enzyme might be occurred due to the saturation of immobilization capacity on the surface of the hollow fiber membrane. These results were supported by Fang et al. [23] on the immobilization of α-amylase on chitosan, whereby the immobilization efficiency was increase as the contact time increased up to 12 h and decrease when the contact time was further increase.

The response surface plot in figure 2b illustrated the effect of pH and temperature on the CGTase immobilization yield at fixed contact time of 24 h. The result illustrated that, the highest immobilization yield was achieved at pH 6.5. However, when incubated at more acidic (pH 6.0) and alkaline pH (pH 8.0), low immobilization yield was obtained. The reduction of the immobilization yield at acidic (pH 6.0) and alkaline (pH 8.0) as shown in figure 2b, could be explained by the repulsion of the charges located on the surface of the enzyme and support. The variation of pH of the immobilization solution could affect the adsorption process due to the alteration of the net surface charges of the enzyme [24]. This phenomenon was also being observed by Sun et al. [25] on immobilization of lipase on microporous resin via physical adsorption whereby the reduction of immobilization yield was observed when incubation at acidic pH (pH 5.0) and alkaline pH (pH 8.0 and 9.0).

3.4. Validation of empirical model

The immobilization parameters for the adsorption of CGTase on hollow fiber membrane were analyzed and the optimum conditions for each factor were predicted as shown in table 3. Under the optimized immobilization parameters, the maximum CGTase immobilization yield of 89.61% was predicted when the temperature, pH and contact time were 24 °C, pH 6.7 and 24 h, respectively. To validate the accuracy of the model, experiment with the proposed immobilization conditions by the model was performed. The experimental result showed that the recorded optimal value of immobilization yield was 88.25%, which is suggested that both experimental result and predicted result of the immobilization yield were in a good agreement. The experimental result of the immobilization conditions before and after the optimization procedure were compared and tabulated in table 3. The results revealed that the

immobilization of CGTase was successfully optimized and improved by 4.6-fold after the optimization process.

Table 3. Summary of the optimized CGTase immobilization process parameters.

	Before optimization	**After optimization**
Culture condition:		
Temperature (°C)	25	24
pH	4	6.7
Contact time (h)	24	24
Response:		
Immobilization yield (predicted)		89.61%
Immobilization yield (actual)	19.21%	88.25%

3.5. Kinetic studies of the free and immobilized CGTase

The kinetic constants of K_m and V_{max} were calculated by Lineweaver-Burk plot (Supp. data). Both free and immobilized CGTase have a similar V_{max} value of 9.42 mgml^{-1} h^{-1}. The V_{max} value was defined as the maximum reaction rate when all the enzyme was saturated with the substrate, which reflect the intrinsic characteristic (catalytic efficiency) of the enzyme [26,27]. As there were no changes in the V_{max} value of the free and immobilized CGTase, this indicates that there were no changes in the intrinsic characteristic (catalytic efficiency) of the CGTase upon the immobilization process. These results indicated that the immobilized CGTase was able to achieve the similar reaction rate as the free CGTase. According to Lei and Bi [26], K_m value was defined as the substrate concentration when the enzyme achieved half of the V_{max} value. In this study, the increased of K_m value from 7.39 mgml^{-1} of free CGTase to 9.99 mgml^{-1} of immobilized CGTase was observed. The results indicated that low affinity of the immobilized enzyme towards the substrate. Decreased of affinity by the immobilized enzyme upon the immobilization process may be because of the diffusional limitation of substrate due to the interaction of enzyme and the support. Generally, immobilization process does not capable in controlling the orientation of the immobilized enzyme on the support surface. Therefore, improper fixation of enzyme onto support surface may hinder the active sites of the immobilized CGTase. This finding was supported by Fortes et al. [24], whereby the increased of the K_m value for the immobilized enzyme might be due to the fixation of enzyme to the support whereby had caused low accessibility of substrate to the active sites. The increased of K_m value after the immobilization process was observed in other immobilization techniques. For example, the immobilization of CGTase on chitin via covalent attachment has led to the increased of K_m value from 14.28 mgml^{-1} of free enzyme to 20.0 mgml^{-1} of immobilized enzyme [28]. An increment of K_m value from 2.5 gml^{-1} of free enzyme to 4.5 gml^{-1} of immobilized enzyme was also observed in the immobilization of CGTase in sodium alginate beads [29].

4. Conclusion

Immobilization of CGTase on the hollow fiber membrane was successfully optimized by using central composite design and the optimum conditions for each parameter was determined. The result showed that low immobilization temperature was favorable for the adsorption of CGTase on the hollow fiber membrane due to direct and spontaneous reaction of physical adsorption that affording an exothermic reaction. The effect of contact time on the immobilization yield can be observed by two phases whereby at first phase, the immobilization yield was increased with the increased of contact time and in the second phase, the immobilization yield was decreased as the contact time was further increase. This shows that at the second phase, the desorption of the enzyme had occurred due to the saturation of immobilization capacity on the surface of the hollow fiber membrane. In addition, low acidic pH (pH 6.7) of the immobilization mixture had promote a better adsorption of CGTase on the hollow fiber membrane due to the effective electrostatic interaction. Under the optimized conditions, the immobilization yield had increase 4.6-fold compare to the before optimization process. Performance of the immobilized CGTase was further studied in terms of their kinetic performance. The kinetic study of the free and immobilized

CGTase showed that the immobilization of CGTase on hollow fiber membrane via adsorption technique does not relatively changed the intrinsic characteristic of the enzyme. Hence, the present study shows that the immobilization of CGTase on hollow fiber membrane by adsorption did not cause structural changes to the enzyme and suitable for the production of cyclodextrin.

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