Optimization of reaction conditions for the production of cyclodextrin (CD) using cyclodextrin glucanotransferase (CGTase) immobilized on hollow fiber membrane

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Optimization of reaction conditions for the production of cyclodextrin (CD) using cyclodextrin glucanotransferase (CGTase) immobilized on hollow fiber membrane

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Abstract. Cyclodextrin glucanotransferase (CGTase) is an enzyme that convert starch into cyclodextrin (CD) by transglycosylation reaction. The CD has been used in various industries due to the unique characteristics. However, the production of CD is usually restricted due to the instability of the enzyme which easily denatured during the reaction process. Thus, enzyme immobilization technique was applied and optimization of reaction conditions was conducted to enhance the amount of CD production. In this study, CGTase was immobilized on polyvinylidiene difluoride (PVDF) hollow fiber membrane by adsorption. The optimization of the reaction conditions using response surface methodology (RSM) on the production of CD was studied. Under optimized conditions (2.8% w/v of soluble potato starch concentration, 45.2°C of reaction temperature and pH 5.6), the production of CD was 5.65 mg/mL, about 2-fold compared to the value before optimization process. Therefore, the immobilized CGTase on hollow fiber membrane proved to be valuable for the enhancement of CD production.

1. Introduction

Cyclodextrin (CD) or also known as cycloamylose, cyclomaltose and schardinger dextrin is a cyclic oligosaccharide that has a doughnut-liked shape molecule. The CD can be divided into three types which are α, β and γ-CD consisting of six, seven and eight glucose units, respectively [1]. The CD is produced by the enzymatic action of cyclodextrin glucanotransferase (CGTase) on starch by intramolecular transglycosylation process [2]. The CD is able to enhance the physical and chemical properties of organic molecules and drugs due to the distinctive structure of the CD. The CD is also capable of solubilizing hydrophobic substances and entrap volatile molecules by forming inclusion complexes with the guest molecules [3]. Due to this properties, the market demand for the CD has been increasing year to year and has been applied in various industries such as in plastic, cosmetic, food and agricultural industries. However, the use of CGTase for industrial application is often limited. This is because, the enzyme is normally unstable and highly sensitive to the reaction process which lead to the low CD production [4].

The technique of enzyme immobilization for the product formation has several advantages compared to the conventional reaction method using a free enzyme. Other than the enhancement of the enzyme stability, this technique also provides better enzyme kinetic characteristics. The immobilized enzyme

also often shows higher enzyme affinity towards substrate than the free enzyme. Hence, the enzyme immobilization technique is a promising strategy to increase the amount of product formation and improving the enzyme properties [5].

Numerous techniques of enzyme immobilization have been explored including adsorption, covalent binding, entrapment and cross-linking. Nevertheless, adsorption is preferred due to the simplicity and does not require any addition of chemical. Nanofiber [6], silica microsphere [7] and alginate [8] are some of the immobilization support for CGTase that have been explored by the researchers previously. The choice of support is important and greatly affect the amount of enzyme attach to the support. In this study, CGTase was immobilized on polyvinylidiene difluoride (PVDF) hollow fiber membrane. The polymer membrane has been widely used in biotechnology field such as in microfiltration and ultrafiltration processes [9]. The hollow fiber membrane is known to has high mechanical stability and lack of toxicity which make the membrane suitable to be used as the support for the enzyme immobilization [4].

Many factors including substrate concentration, temperature and pH can greatly influence the enzymatic reaction process. Nevertheless, in order to ensure a stable and efficient enzymatic system for the reaction process, optimization method is needed. The use of the classical method for optimization which is "one-factor-at-a-time" (OFAT) approach is normally unable to detect the interaction between variables [10]. This has led to the application of response surface methodology (RSM) that able to analyze the effect of several variables and also consider the interactions with less number of experiments [11]. The RSM have been extensively used for biochemical process such as enzymatic synthesis of fructo-oligosaccharides [12] and synthesis of glucose from immobilized invertase [13].

The aims of this study were to optimize the reaction parameters of the immobilized CGTase on the hollow fiber membrane for the CD production.

2. Materials and Methods

2.1. Materials

Cyclodextrin glucanotransferase (CGTase) from *Bacillus lincheniformis* was purchased from Novozymes A/S (Bagsvaerd, Denmark). Polyvinylidiene difluoride (PVDF) hollow fiber membrane was procured from Separation and Membrane Cluster Faculty of Chemical and Natural Resources Engineering, Universiti Malaysia Pahang. An α-cyclodextrin standard with 98% purity was purchased from Sigma-Aldrich (St. Louis, USA). Hydrochloric acid with 37% purity, methyl orange, acetonitrile High Performance Liquid Chromatography (HPLC) grade were purchased from Merck Sdn Bhd (Darmstadt, Germany). Glycine and soluble potato starch were purchased from Friendemann Schmidt (Parkwood, Australia).

2.2. Immobilization of CGTase

Hollow fiber membrane was cut into 3 cm length and was immersed in a conical flask containing 1 mL of CGTase solution and 9 mL of 0.05M sodium phosphate buffer (pH 6.0). The sample was then incubated in 25°C with 100 rpm of agitation for 24 h. The membrane was rinsed with fresh sodium phosphate buffer to remove any non-immobilized enzyme on the hollow fiber membrane. Then, the immobilized CGTase was transferred in a conical flask containing 20 mL of starch solution for the enzymatic reaction.

2.3. Optimization of reaction parameters of immobilized CGTase on CD production

Response surface methodology (RSM) was performed to optimize the reaction parameters for the production of CD using the immobilized CGTase on the hollow fiber membrane. A central composite design (CCD) was applied using Design Expert 7.1.6 (Stat-East, Inc., USA) to analyse the optimum reaction conditions and the interactions among the individual conditions. Table 1 shows the reaction conditions with the details of lower and upper limit values. In total, 17 sets of experiments including 3

centre points were generated by the design from three reaction conditions [starch concentration (X_1), temperature (X_2) and pH (X_3)].

Table 1. Actual coded value of the design variables for the optimization process.

Factor	Unit	Low-level star point (-α)	Low-level factorial (-1)	Centre point (0)	High level factorial (+1)	High level star point (+α)
X_1: Concentration	% w/v	0.48	1.5	3	4.5	5.52
X_2: Temperature	°C	23.18	30	40	50	56.82
X_3: pH	-	4.32	5	6	7	7.68

2.4. Analytical analysis

2.4.1. High Performance Liquid Chromatography.
The amount of α-CD produced was identified by using High Performance Liquid Chromatography (HPLC). Samples were prepared by diluting with 3 volumes of acetonitrile and centrifuged for 10 min at 5000 rpm prior to the analysis. Agilent Eclipse Plus C18 column with mobile phase of 60% acetonitrile and 40% of ultrapure water were used. Reflective index detector (RID) was used to detect the CD in the sample.

3. Results and Discussion

3.1. Optimization of reaction conditions of immobilized CGTase on CD production
Central composite design (CCD) under response surface methodology (RSM) was employed to determine the optimum reaction conditions of the immobilized CGTase and the interactions among the individual factors. From Table 2, the lowest concentration of CD (2.05 mg/mL) was determined at run number 8 with reaction conditions of 4.5% w/v of starch concentration, pH 7 and temperature at 30°C. Meanwhile, the highest concentration of CD produced was obtained at run number 6 (5.55 mg/mL) with reaction conditions of 3% w/v of starch concentration, pH 6 and at temperature 40°C.

Table 2. Experimental design and results for central composite design (CCD).

Run	Factors			CD production (mg/mL)	
	X_1: Starch concentration (% w/v)	**X_2: pH**	**X_3: Temperature (°C)**	**Actual value**	**Predicted value**
1	3.00	6.00	23.18	2.57	2.64
2	5.52	6.00	40.00	2.33	2.39
3	1.50	5.00	30.00	2.35	2.41
4	3.00	6.00	40.00	5.53	5.52
5	1.50	7.00	30.00	2.21	2.14
6	3.00	6.00	40.00	5.55	5.52
7	1.50	7.00	50.00	3.31	3.37
8	4.50	7.00	30.00	2.05	2.01
9	3.00	7.68	40.00	2.54	2.60
10	3.00	6.00	56.82	4.60	4.54
11	3.00	6.00	40.00	5.47	5.52
12	1.50	5.00	50.00	4.09	4.13
13	4.50	5.00	30.00	3.21	3.15
14	3.00	4.32	40.00	4.25	4.19
15	4.50	5.00	50.00	4.11	4.17
16	4.50	7.00	50.00	2.61	2.55
17	0.48	6.00	40.00	2.51	2.46

The fitness of the model for the CD production was expressed by the coefficient of determination (R^2) value which was 0.997 indicating that only 0.003 of the total variation in the response could not be explained by the model. Meanwhile, the adjusted R^2 was calculated to be 0.995 indicating that only 0.5% of the total variation was not included in the model. A good predictability of the model was confirmed with the agreement between predicted R^2 (0.984), whereby the difference of the predicted R^2 and the adjusted R^2 were less than 0.01.

The quadratic regression analysis using the analysis of variance (ANOVA) was determined to estimate the significant factors in the model design. The significant factors were determined by the P-value less than 0.05 which also reflect the interaction strength between each factors. The P-value for the model was less than 0.0001 which implied that the model was significant. Other than that, the lack of fit was found to be insignificant with P-value of 0.16 denoted that the model was desirably fit with the experimental data.

Besides, the quadratic model obtained in this study can be expressed as a second-order polynomial equation for the production of CD. All terms regardless of their significance were included in the Equation 1.

$$CD\ production = 5.66 + 0.23x_1 - 0.19x_2 + 0.99x_3 + 0.123x_1x_2 + 0.033x_1x_3 + 0.16x_2x_3 - 0.97{x_1}^2 - 1.13{x_2}^2 - 0.80{x_3}^2 \quad (1)$$

The interactions between two independent factors were shown in three-dimensional (3D) surface plots based on the model Equation 1. The plots represent the CD concentration as a function of two independent factors while another factor was at fixed central level. The graphical representations allow easier determination of the optimum points of the process conditions within the considered experimental ranges.

Figure 1 shows the response and contour curve of the starch concentration and temperature while the pH was kept constant at pH 5.6. Based on the surface contour plot, 3% (w/v) was selected as the optimum starch concentration for the highest amount of CD produced with the concentration about 5.5 mg/mL. The moderate concentration of starch influences the enzymatic reaction of the immobilized enzyme. This behavior was due to the low viscosity of the starch, resulting in easier stirring of the reaction mixture and better contact between the starch and the active site of the immobilized enzyme [12-13]. In addition, the high amount of amylopectin contained in the soluble potato starch also contributed to the high production of CD. The branched structure of the amylopectin greatly assisted in the enzymatic reaction with the immobilized CGTase to produce CD. This is because, the present of the branched structure of the amylopectin allows the reaction to start at various points of the starch [16].

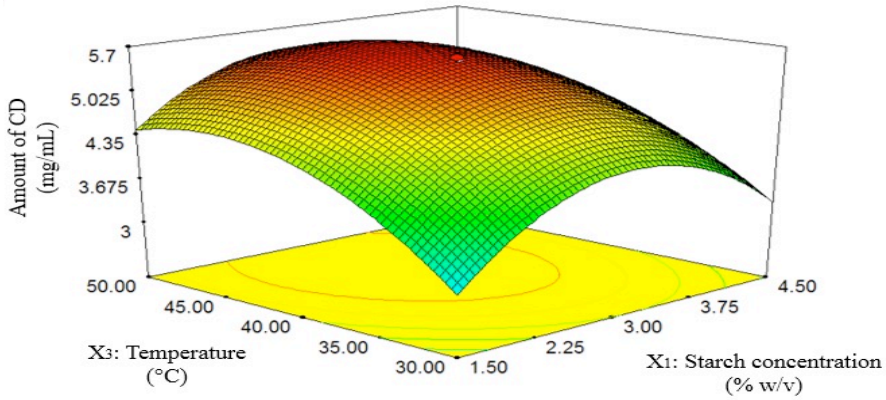

Figure 1. Response surface plot for CD production as a function of temperature and starch concentration at pH 5.6.

A research conducted by Muria et al. [17] who studied the production of CD by free CGTase from *Bacillus* sp. found that the high concentration of sago starch (7.5% w/v) was required to achieve the maximum CD production. The higher starch concentration needed was probably due to the lower percentage of amylopectin contained in the sago starch. According to Srichuwong et al. [18], the amylopectin content in sago starch was 72%, while in soluble potato starch was 82%. Thus, the present of high amount of amylopectin content resulted in low concentration of starch needed to produce high CD amount. The present study demonstrated that the maximum CD production can be achieved by using the low concentration of soluble potato starch due to high amount of amylopectin reacted with the enzyme active site.

The reaction temperature is an important factor that could severely affect the enzymatic reaction. Enzyme denaturation is the effect of thermal stress in enzymatic reaction which consequently resulted in low product formation. According to Daniel and Danson [19], the enzymatic activity increases as the temperature increases until at a certain point called optimum point. The enzyme denatures and losses enzymatic activity irreversibly at temperature higher than the optimum point [19] .

In the present study, the reaction temperature ranging from 30 to 50°C was investigated. Based on Figure 1, temperature of 42°C was the best reaction temperature for the production of CD (5.6 mg/mL). The moderately high temperature (42°C) most likely to be associated with the high kinetic energy during the reaction to produce CD. It is suggested that the high kinetic energy provides collision more frequently between the enzyme and substrate for the enzymatic reaction. However, in a study conducted by Muria et al. [17], the high temperature (65°C) was needed to produce the high amount of CD. Besides, Rajput et al. [20] also obtained high CD at high reaction temperature (60°C). A high temperature may disrupt the hydrogen bonds and non-polar hydrophobic interactions in the enzyme. This is due to the vibration of the enzyme occurs rapidly and violently that the bonds are disrupted. Hence, permanently alter the shape and size of the active site of the enzyme. Therefore, it is suggested that lower reaction temperature is favorable for the production of CD by the immobilized enzyme.

Based on Figure 2, pH 6 was found to be the optimum pH for the highest production of CD with concentration of 5.2 mg/mL. The acidic environment influenced the binding of enzyme and substrate during the enzymatic reaction to produce CD [21]. Besides, the production of CD after the enzyme immobilized on the hollow fiber membrane promoted stability of the enzyme due to the static position during the reaction process. In a study conducted by Abdel-naby [22] showed that, the CD production decreased as the pH increased from pH 6 (acidic environment) to pH 9 (alkaline environment) by the immobilized CGTase on polyvinyl chloride (PVC). It was reported that the positively charged support affected the attachment and reaction of the enzyme in more acidic condition [23]. Therefore, it can be concluded that the acidic condition of the reaction mixture was the most suitable in the present study in order to increase the production of CD by the immobilized enzyme.

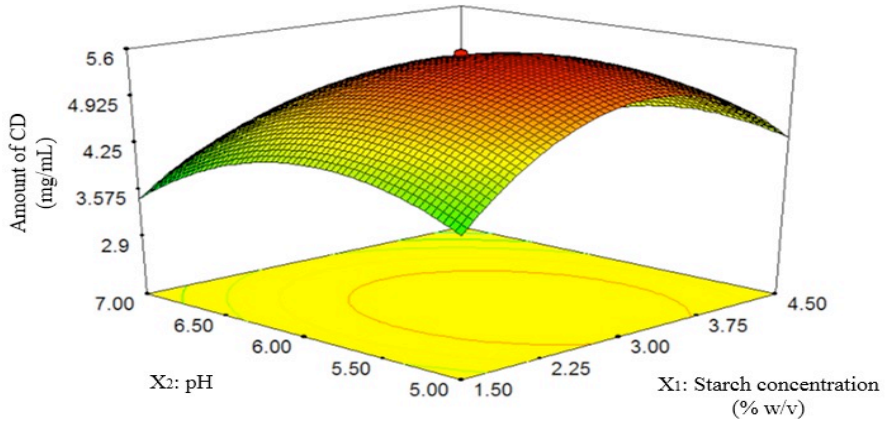

Figure 2. Response surface plot for CD production as a function of pH and starch concentration at reaction temperature of 45°C.

3.2. Validation of the empirical model

The validation of experiment was conducted using the conditions proposed by the RSM in order to verify the adequacy of the designed model. The substrate concentration of 2.8 % (w/v), pH 5.6 and reaction temperature of 45.2°C were the conditions proposed by the model. Based on Table 2, 5.65 mg/mL of CD was successfully produced by the immobilized enzyme and the value had a good agreement with the predicted value of CD (5.71 mg/mL). The deviation of the data was less than 5% which confirmed that the model was well fitted with the experimental data and was successfully validated.

Additionally, the CD production after optimization (5.65 mg/mL) showed 2-fold higher than the value before optimization (2.66 mg/mL) process. Besides, the production of CD by the immobilized enzyme after the optimization process also recorded 2-fold higher than the free enzyme (2.71 mg/mL). Therefore, the high production of CD by the immobilized CGTase can be achieved using RSM.

4. Conclusion

The optimization of reaction conditions for the production of CD using immobilization technique has been successfully evaluated via CCD. The result revealed that low soluble potato starch concentration and low reaction temperature as well as acidic conditions were preferred for the CD production.

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