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Application of Statistical Method for Screening of Factors Influencing the Production of B-cyclodextrin from Sago Starch Using Combination of Pullulanase and CGTase Enzymes

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Abstract: The production of β-cyclodextrin (β-CD) from sago starch was carried out using combination of debranching enzyme and Cyclodextrin Glucanotransferase (CGTase). The contribution of each reaction parameters was evaluated and optimised using statistical modelling. A 2^{5-1} Fractional Factorial Design (FFD) was employed to screen the effect of substrate concentration, CGTase concentration, pullulanase concentration, pH and temperature for CGTase reaction on the production of β-cyclodextrin (β-CD) using combination of pullulanase and CGTase. The result of first-order factorial design showed that pH and pullulanase had significant positive effect ($p<0.05$) to the reaction. Substrate concentration, CGTase concentration and temperature exhibited insignificant effect in this reaction. In addition, interaction between CGTase and pH, substrate and pH, pullulanase and temperature, CGTase and temperature, substrate and CGTase gave significant effects ($p<0.05$) to the β-CD production.

Key words: Cyclodextrin, sago starch, combined debranching and CGTase, statistical model

INTRODUCTION

Cyclodextrins (CDs) are a family of cyclic oligosaccharides joined together by á-1, 4-bonds. These macrocyclic carbohydrates with apolar internal cavities can form complexes and solubilize many normally water-insoluble compounds. As a result of molecular complexation phenomenon, CDs are widely used in many industrial products, technologies and analytical methods. The negligible cytotoxic effects of CDs are an important attribute in applications such as drug carrier, food and flavors, cosmetics, packing, textiles, separation processes, environment protection, fermentation and catalysis[1,2].

CGTase (EC 2.4.1.19) catalyzes both intra- and inter-molecular transglycosylation resulting in the direct production of CDs from oligosaccharides and starches[3] . Starch consists of amylopectin and amylose. Both can serve as raw materials for CD formation, but amylopectin gives higher yield than amylose because the reaction with CGTase begins at the non-reducing end of the starch molecule. However, α-1, 6-glucosidic linkages at the branching positions in amylopectin block the effect of CGTase. If amylopectin is treated with debranching enzymes such as pullulanase or isoamylase before the addition of CGTase, the level of starch conversion into CDs is increased[4]. The simultaneous action of CGTase and pullulanase [from alkalophilic Bacillus species (no 202-1)] on potato starch results in a 52 % conversion of starch to CD[5] or even to 93% in the presence of suitable complexant[6].

Due to the complexity of the reaction, Fractional Factorial Design (FFD) was used to screening factors that influence β-CD production significantly and insignificant ones were eliminated in order to obtain a smaller, more manageable set of factors. By using this method, interactions between variables also can be identified and quantified.

MATERIALS AND METHODS

Materials: Sago starch (*Metroxylon* sp.) from Sarawak, Malaysia was supplied by Nee Seng Ngeng and Sons Sago Industries Inc. Cyclodextrin glucanotransferase, Toruzyme™ (from *Bacillus macerans*) and Promozyme™ (amylopectin 6-glucanohydrolase) was supplied by Novozyme Inc. All other chemicals were of analytical grade.

Cyclodextrin production: Individual starch solution (50 mL) in 0.1 M phosphate buffer (pH 5.5) was gelatinized in jacketed vessel at 85°C for 15 min. Then each solution was reacted with pullulanase (0.1-0.3% v/v;

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pH 5.5) for 2 h in incubator-shaker (130rpm at 60°C). After 2 h, the pullulanase reaction was stopped by heating (100°C, 15 minutes) and 1 mL sample was taken for amylose concentration determination. The pH of reaction mixture was adjusted to the required pH by addition of 2 M NaOH. CGTase (1-3% v/v) was then added and the reaction mixture was incubated (temperature, 60-70°C) for 5 h in 130 rpm. Inactivation of the enzyme in the reaction mixture was accomplished by immersing it in the water bath at 100°C for 15 min[7].

Determination of amylose content: This method was a modified method of McGrance *et al.*[8]. One milli liter of reaction mixture was dissolved in 1 mL DMSO by heating in a water bath (85°C) for 15 min. The solution was diluted to 25 mL in a volumetric flask with deionised water. An aliquot (1.0 mL) was taken and then diluted with 50 mL of water and 5 mL of iodine solution (0.0025 mol L^{-1}) in potassium iodide (0.0035 mol L^{-1}) was, thoroughly mixed and the absorbance of this solution (in a 1 cm path length quartz cell) was read at 600 nm using UV/Visible spectrophotometer. A standard curve was plotted using mixtures of standard amylose (Sigma Chemical Co.) and amylopectin (Sigma Chemical Co.) from potato containing 0,10, 20, 50, 75 and 100% amylose. The yield of amylose was based on the initial substrate concentration.

Determination of β-CD: β-CD were determined by HPLC using Shodex Asahipak NH2P-50 4E (4.6 x 250mm) eluted with acetonitrile : water (70:30) at 1.0 mL/min and detected by Refractive Index Detector (Waters 410). Mobile phase was filtered using Whatman® membrane filter (47 mm diameter, 0.45 μm pore) and then degassed using ultrasonic bath (Ultrasons-H, Selecta). The column temperature was set at 30°C. All samples were filtered with Whatman® nylon membrane filter (0.2 μm pore size, 13 mm diameter) before injection.

Statistical operation: The statistical modelling, testing and operation were conducted using statistical software, Design Expert™ Ver. 6.0.4 (Stat-Ease, Inc., Minneapolis, USA)

RESULTS AND DISCUSSIONS

Fractional Factorial Design (FFD): In this study, five main factors were identified as the determinants for CGTase reaction in the production of β-CD, namely, substrate concentration, pullulanase concentration, CGTase concentration, temperature and pH. The range of experimental parameters was based on the previous studies by other researchers[9-15]. The pH and temperature for pullulanase reaction were fixed at pH 5.5 and 60°C, respectively. In this study, ½ fractional factorial design (2^{5-1}) was chosen as the experimental design. Table 1 represents the factors and coded values of FFD while Table 2 represents the design of experiment with predicted results of the FFD. The 22 runs consisted of 16 factorial experiments with 6 center runs.

Table 3 represents the ANOVA table for the linear regression model of β-CD production for 2^{5-1} FFD. The p- value of the model (<0.001) indicates that the model is significant. In addition to this, the result of lack of fit test

Table 1: Factors and coded values of FFD

	Level of factors		
Factors	-1	0	+1
Substrate conc. (X_1, g L^{-1})	40.00	60.00	80.00
Pullulanase conc. (X_2, % v/v)	00.10	00.20	00.30
CGTase (X_3, % v/v)	01.00	02.00	03.00
Temperature (X_4 °C)	60.00	70.00	80.00
pH (X_5)	07.00	08.50	10.00

Table 2: Design of experiment with predicted results of based on the selected Fractional Factorial Design (FFD).

Run	X_1 Substrate	X_2 Pullulanase	X_3 CGTase	X_4 Temp	X_5 pH	Predicted response (β-CD production, g/L)(y_i)
1	-1	-1	-1	-1	1	5.26
2	1	-1	-1	-1	-1	8.84
3	-1	1	-1	-1	-1	4.17
4	1	1	-1	-1	1	3.28
5	-1	-1	1	-1	-1	1.30
6	1	-1	1	-1	1	10.96
7	-1	1	1	-1	1	11.59
8	1	1	1	-1	-1	5.74
9	-1	-1	-1	1	-1	4.07
10	1	-1	-1	1	1	-0.04
11	-1	1	-1	1	1	9.80
12	1	1	-1	1	-1	10.15
13	-1	-1	1	1	1	8.56
14	1	-1	1	1	-1	2.58
15	-1	1	1	1	-1	0.44
16	1	1	1	1	1	9.97
17[a]	0	0	0	0	0	13.67
18[a]	0	0	0	0	0	13.67
19[a]	0	0	0	0	0	13.67
20[a]	0	0	0	0	0	13.67
21[a]	0	0	0	0	0	13.67
22[a]	0	0	0	0	0	13.67

[a] Center point

Table 3: ANOVA Table for 2^{5-1} FFD

Source	Sum of square	DF	Min square	F-value	*p*-value
Model	468.11	13.00	36.01	20.79	< 0.0001*
Curvature	372.17	1.00	372.17	214.92	< 0.0001*
Residual	43.29	25.00	1.73		
Lack of Fit	0.04	2.00	0.02	0.01	0.9886
Pure error	43.25	23.00	1.88		
Total Correlation	883.57	39.00			

R^2=0.8713 * Significant values (p < 0.05)

Table 4: Table of coefficient values for each factors involved in FFD

Factors	Coefficient Estimate	p *Value*
Intercept	6.04	
X_1 (Substrate)	0.39	0.1036
X_2 (Pullulanase)	0.85	0.0012*
X_3 (CGTase)	0.35	0.1446
X_4 (Temperature)	-0.35	0.1450
X_5 (pH)	1.38	< 0.0001*
$X_1 X_3$	0.53	0.0324*
$X_1 X_4$	-0.42	0.0834
$X_1 X_5$	-1.77	< 0.0001*
$X_2 X_3$	-0.31	0.1970
$X_2 X_4$	1.05	0.0001*
$X_2 X_5$	0.39	0.1091
$X_3 X_4$	-0.66	0.0093*
$X_3 X_5$	2.50	< 0.0001*
Center point	7.63	0.1036

* Significant values ($p<0.05$)

is not significant indicating that the first-order model is adequate in approximating the response surface of the experimental design. This statement is further supported by the satisfactory value of adjusted R^2=0.8713 indicating that 87.13% of the variation of response are accounted for by this model. The model adequacy checking using residual analysis also indicated that this model is adequate. The curvature's sum of square (372.17) implies there is significant curvature (as measured by difference between the average of the center points and the average of the factorial points) in the design space (Table 3).

Based on the data obtained, the next step was to proceed to second-order model to estimate the location of the curvature in order to obtain the optimum condition. From Table 4, it can be seen that only two factors are affecting β-CD production significantly ($p<0.05$). They are concentration of pullulanase (X_2) and pH of CGTase reaction (X_5). Both coefficients of the significant factors were positive, indicating that increment of these factors would give positive effect to the reaction. On the other hand, substrate concentration (X_1), CGTase (X_3) and temperature (X_4) were found to be not significant. Thus, these factors will be fixed at a constant level in the central composite design. The result also shows that the interaction among variables $X_1 X_3$, $X_1 X_5$, $X_2 X_4$, $X_3 X_4$ and $X_3 X_5$ were significant ($p<0.05$). Based on the above results, the model that fit with coded variables can be written as follows:

$$Y_{(\beta\text{-CD g/L})} = 6.04 + 0.39\ X_1 + 0.85\ X_2 + 0.35 X_3 - 0.35\ X_4 + 1.38\ X_5 + 0.53\ X_1 X_3 - 1.77\ X_1 X_5 - 1.05\ X_2 X_4 + 0.39\ X_2 X_5 - 0.66\ X_3 X_4 + 2.50\ X_3 X_5$$

From Table 4, it shows that the mean of centre point (7.63) exceeded the mean of factorial points (6.04), which indicate that the reaction is approaching the optimum point. According to Tang *et al.*[16], if the mean of the centre points exceeds the mean of factorial points, the optimum would be near or within the experimental design space. Fig 1 shows the high degree of accuracy (R^2=0.9828) between the predicted and the actual experimental values obtained from the design. By applying the statistical method, smaller number of experiments need to performed, experiments can be conducted more systematically, without sacrificing the accuracy of data.

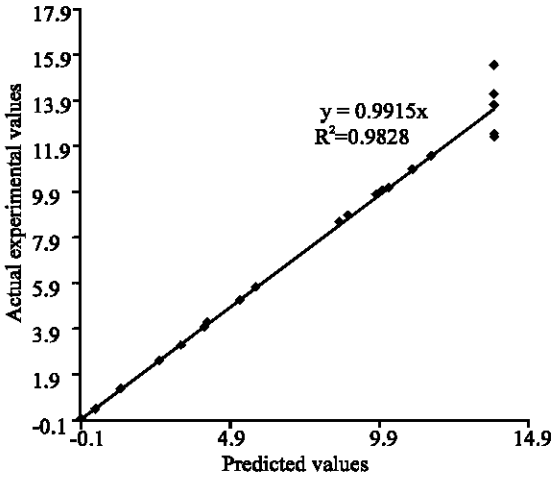

Fig. 1: Relationship of the predicted values to actual experimental values each point is average ± SD (n=3)

CONCLUSIONS

By using the FFD, pH and pullulanase concentration were successfully determined as the significant factors ($p<0.05$) affecting the production β-CD from the combined reaction of pullulanase and CGTase on sago starch.

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