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The optimization study of α-amylase activity based on central composite design-response surface methodology by dinitrosalicylic acid method

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Abstract

The optimum conditions of an α-amylase activity were experimentally investigated in details comparing with those of using a response surface methodology (RSM). Its optimum activity was achieved with 0.5 concentration unit of α-amylase and 1.0% (w/v) starch concentration in phosphate buffer solution pH 6.9 at 37°C for 10 min. The α-amylase activity was monitored by dinitrosalicylic acid method based on the measurement of maltose. The enzymatic activity was then statistically optimized using RSM, alternatively to focus on the effects of pH buffer solution, incubation temperature and incubation time on the yield of maltose. A quadratic regression model that related the yield of maltose concentration (response) to three basic factors was developed using Design-Expert software. Regression analysis revealed that the maximum concentration of maltose (0.915 mg/mL) could be reached with the buffer solution pH 7.3 at 39°C for 10 min. The high value of the adjusted R-square of the regression (0.9255) also demonstrates the regression equation providing a good model to fit the data obtained.

Introduction

Amylases have been reported in microorganisms, although the enzymes are also found in plants and animals. Two major classes of the enzyme, namely α-amylase and β-glucoamylase, have been identified. α-Amylases (endo-1,4-α-D-glucan glucohydrolase) are extracellular enzymes that randomly cleave the 1,4-α-D-glucosidic linkages between adjacent glucose units in the linear amylose chain (Anto *et al*., 2006). The classification of starch digestive enzymes in malt α- and β-amylases according to the anomeric type of sugars produced by the enzyme reaction was reported (Nakakuki *et al*., 1984). α-Amylases of different origins have been extensively studied (Gogoi *et al*., 1987; Mountfort and Asher, 1988; Coronadoa *et al*., 2000; Gonzalez *et al*., 2002; Wanderley *et al*., 2004). Amylases can be divided into two categories, endoamylases and exoamylases. Endoamylases catalyse hydrolysis in a random manner in the interior of the starch molecule. This action causes the formation of linear and branched oligosaccharides of various chain lengths. Exoamylases hydrolyse from the non-reducing end, successively resulting in short end products. Today a large number of the enzymes are known which hydrolyse starch molecule into different products and a combined action of various enzymes is required to hydrolyze starch completely (Gupta *et al*., 2003). Endoamylases are able to cleave, 1-4 glycosidic bonds present in the inner part (endo-) of the amylose or amylopectin chain. α-Amylase is a well-known endoamylase one, since it is found in a wide variety of microorganisms (Pandey *et al.*, 2000).

Determination of reducing sugars was generally carried out by 3,5-dinitrosalicylic acid (DNS) method. It detects the presence of free carbonyl group of the reducing sugars. DNS is an aromatic compound that reacts with reducing sugars and other reducing molecules. The aldehyde group of the sugar was reduced with DNS to form 3-amino-5-nitrosalicylic acid (Miller, 1959). The concentration of the reducing sugars was determined at 540 nm spectrophotometrically. It is mainly used in the assay of α-amylase activity. This enzymatic method is preferred to DNS due to its specificity.

In the case of multi-parameter optimized study, the response surface methodology (RSM) based on a central composite design (CCD) is widely applicable for optimization conditions. Generally, RSM is a statistical and mathematical tool for designing experiments, building model, evaluating the effect of many variables, investigating the optimum conditions for desirable response, and reducing the number of required experiments. A total of 17 experiments were conducted according to the CCD in random order. The following second-order polynomial model was fitted to the response variable with the independent variables (Myers, 1971; Montgomery, 1991).

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Based on a CCD, the parameters (independent variables) were analyzed within a range of (-1.68, -1, 0, +1, +1.68), where -1 corresponds to the value encoded on the lower level of the parameters, 0 corresponds to the intermediate level, +1 at the top level and ±1.68 corresponds α-values ($\alpha = [2^3]^{1/4} = 1.68$).

The total number of experiments in a CCD could be calculated following Eq. (1)

$$N = 2^K + 2K + X_0 \qquad (1)$$

where, N is the number of experiment run, K is the number of variables and X_0 is the number of central points.

For statistical calculation, the variables were coded according to the following Eq. (2)

$$X_i = (A_i - A_0) / \Delta A_i \qquad (2)$$

where, X_i is the coded value of the independent variables. A_i is the actual values of the independent variable. A_0 is the actual value of A_i at the central point. ΔA_i is the step change of the independent variables.

The data obtained from the central composite design is subject to a second order multiple regression analysis to explain the behavior of the system using the least square regression methodology as Eq. (3)

$$Y_i = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC \qquad (3)$$

where, Y_i is the predicted response. β_0 is the intercept coefficient. β_1, β_2, β_3 are the linear coefficients. β_{11}, β_{22}, β_{33} are the quadratic coefficients. β_{12}, β_{13}, β_{23} are the cross-product coefficients. A, B, C are the independent variables studied.

The analyses of results are performed with statistical and graphical analysis software. The software is used for regression analysis of the data obtained and to estimate the coefficient of regression equation. ANOVA (analysis of variance) which is statistical testing of the model in the form of linear term, squared term and interaction term is also utilized to test the significance of each term in the equation and goodness of fit of the regression model obtained.

This research was aimed to investigate the optimum conditions for α-amylase activity. By the way, the reducing sugars obtained from the α-amylase activity are determined spectrophotometrically using DNS method. In the present study, the response surface methodology based on a central composite design is used to find mathematically these enzymatic optimal conditions compared with its conventional assay.

Materials and Methods

Chemicals and enzyme

All chemicals and reagents were mainly of analytical grade (AR). α-Amylase (from Bacillus subtilis) 50.5 U/mg, D-(+)-maltose monohydrate, sodium dihydrogen phosphate and sodium hydrogen phosphate were obtained from Sigma-Aldrich (USA). 3,5-Dinitrosalicylic acid and starch from potato were from Fluka (China) and Fluka (Switzerland), respectively. Potassium sodium tartrate was from Ajax Finechem (New Zealand). Sodium chloride was from BDH (UK). Sodium hydroxide was obtained from Carlo Erba (Italy).

Instruments

The instruments and some equipments used included analytical balance, BSA224S-CW (Sartorious, USA), centrifuge, EBA20 (Hettich Zentrifugan, Germany), hot plate stirrer, MR 3001 (Heidolph, Germany), UV-Visible spectrophotometer, Spectronic 15 (Thermo Scientific, USA), Vortex mixer, G-560E (Scientific Industries, USA), water bath, Isotemp 228 (Fisher Scientific, UK) and pH meter, model 251 (Denver Instrument, UK).

Determination of reducing sugars by DNS method

Determination of reducing sugars was carried out by dinitrosalicylic acid (DNS) method. The stock solution of standard maltose (2,000 mg/L) was prepared by dissolving 0.2 g of D-(+)-maltose monohydrate in 100 mL deionized water (DI) water. 20 mM sodium phosphate buffer solution with 6.7 mM sodium chloride, pH 6.9 was prepared in 250 mL DI water using sodium hydrogen phosphate 0.7148 g, sodium dihydrogen phosphate 0.6898 g and sodium chloride 0.0980 g, and then adjusted to pH 6.9 with 1M sodium hydroxide. The solution should be stored at 20°C and can be used for few days. 1% (w/v) starch solution was prepared by dissolving 0.25 g of potato starch in 25 mL of 20 mM sodium phosphate buffer solution (pH 6.9 plus 6.7 mM sodium chloride). To facilitate the solubility of starch solution, it was heated directly on a hot plate using constant stirring, bring to boil and maintain the solution at that temperature for 15 min. DNS reagent was prepared by dissolving 1 g of 3,5-dinitrosalicylic acid in 20 mL DI water. It was then mixed with sodium potassium tartrate tetrahydrate solution (30 g of potassium tartrate tetrahydrate in 2N sodium

hydroxide), and heated directly on a hot plate with constant stirring at 50-70°C and diluted to 100 mL with DI water. The solution should be stored in an amber bottle at room temperature and stable for 6 months. The stock solution of standard enzyme (10 activity units/mL) was prepared by dissolving 1.949 mg of the enzyme in 10 mL of the sodium phosphate buffer solution under cool situation.

The optimization conditions of α-amylase activity by conventional method

Generally, the optimization study of any enzymatic activity is carried out by a conventional assay. In this case, five parameters affecting the α-amylase activity including an enzyme and starch concentration, pH of the solution, and an incubation temperature and time were investigated in details. All assays were performed in triplicate.

Effects of enzyme and starch concentration

The enzyme concentration was studied between 0.25 and 1.0 activity unit. The test was performed with 500 µL of α-amylase solution and 500 µL of 0.02 M sodium phosphate buffer (pH 6.9 plus 0.006 M NaCl). The reaction mixture was incubated at 37°C in water bath for 10 min. Then 500 µL of 1.0% (w/v) starch in the phosphate buffer solution was added, and the mixture was re-incubated in water bath at 37°C for 10 min. The reaction was terminated with 1.0 mL of DNS reagent. Then, the mixture was incubated in boiling water for 10 min, 10 mL of water was added and cooled down to room temperature. The absorbance of the reaction mixture was measured at 540 nm.

The starch concentration was also studied between 0.25 and 1% (w/v). The test was performed using 500 µL of the obtained α-amylase solution in 500 µL of 0.02 M sodium phosphate buffer solution, and incubated at 37°C for 10 min. 500 µL of the starch solution was added, and the mixture was re-incubated at 37°C for 10 min. The later procedure was done in the same manner as mentioned above.

Effect of pH

The difference in pH buffer solution including pH values of 5.5, 6.0, 6.9, 7.5 and 8.0 was investigated. The test was performed with 500 µL of α-amylase solution in 500 µL of 0.02 M sodium phosphate buffer solution, and incubated at 37°C for 10 min. 500 µL of 1% starch solution was added, and the mixture was re-incubated at 37°C for 10 min. The later procedure was done in the same manner as mentioned above.

Effects of incubation temperature and time

The incubation temperature was certainly evaluated ranging from 25-60ºC. The test was performed with 500 µL of α-amylase solution in 500 µL of 0.02 M sodium phosphate buffer solution, and incubated for 10 min with varying the extraction temperatures at 25, 30, 37, 40, 45, 50, 55 and 60ºC. 500 µL of 1% starch solution was added, and the mixture was re-incubated 10 min. The later procedure was done in the same manner as mentioned above.

The incubation time was also evaluated ranging from 0-60 min. The test was performed with 500 µL of α-amylase solution in 500 µL of 0.02 M sodium phosphate buffer solution, and incubated at 37°C for 10 min, 500 µL of 1% starch solution was added, and the mixture was re-incubated 37°C by varying incubation times for 0, 5, 10, 15, 20, 25, 30, 40, 50 and 60 min. The later procedure was done in the same manner as mentioned above.

The RSM optimization conditions for α-amylase activity

Experimental design

The response surface methodology based on a central composite design (CCD) was used to optimize the activity conditions to obtain the high concentration of reducing sugar (maltose). In this case, the effects of pH buffer solution (A), incubation temperature (B) and incubation time (C) were chosen as the independent variables. The optimization experiments were designed using the Design-Expert 6.0.10 software package (Stat-Ease Inc., Minneapolis, MN. USA). The range and levels of the variables investigated are given in Table 1.

A three-level, three-factor factorial CCD consisting of 8 factorial points, 6 axial points and 3 replicates at the central point leading to 17 runs were employed for extraction, as shown in Table 2. For statistical calculation, the variables were coded according to the following Eq. (4):

$$X_i = (A_i - A_0) / \Delta A_i \quad (4)$$

where, X_i is the coded value of the independent variables. A_i is the actual values of the independent variable. A_0 is the actual value of A_i at the central point. ΔA_i is the step change of the independent variables.

For predicting the optimal point, a second-order polynomial function was fitted to correlate the relationship between the independent variables and the response. The quadratic model for predicting the optimal point was expressed as Eq. (5):

Table 1. Experimental range and levels of the independent process variables to study on α-amylase activity

Variable	Symbol	Coded level				
		- α	-1	0	+1	+ α
pH buffer solution	A	4.38	5.4	6.9	8.4	9.42
Incubation temperature (°C)	B	11.80	22	37	52	63.20
Incubation time (min)	C	1.60	5	10	15	18.40

*α = 1.68

Table 2. Experimental design with three independent variables and the results obtained from the α-amylase activity conditions

Run	Variable						Concentration of maltose (mg/mL)	
	Coded value			Actual value				
	A	B	C	A	B	C	Observed	Predicted
1	-1	-1	-1	5.4	22	5	0.58	0.57
2	+1	-1	-1	8.4	22	5	0.69	0.70
3	-1	+1	-1	5.4	52	5	0.64	0.67
4	+1	+1	-1	8.4	52	5	0.74	0.74
5	-1	-1	+1	5.4	22	15	0.77	0.77
6	+1	-1	+1	8.4	22	15	0.87	0.83
7	-1	+1	+1	5.4	52	15	0.82	0.80
8	+1	+1	+1	8.4	52	15	0.79	0.80
9	0	0	0	6.9	37	10	0.69	0.69
10	0	0	0	6.9	37	10	0.79	0.80
11	0	0	0	6.9	37	10	0.71	0.73
12	-1.68	0	0	4.38	37	10	0.80	0.78
13	+1.68	0	0	9.42	37	10	0.64	0.62
14	0	-1.68	0	6.9	11.8	10	0.82	0.84
15	0	+1.68	0	6.9	63.2	10	0.90	0.91
16	0	0	-1.68	6.9	37	1.60	0.92	0.91
17	0	0	+1.68	6.9	37	18.40	0.91	0.91

$$Y_i = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC \quad (5)$$

where, Y_i is the predicted response (extraction yield, mg/g). β_0 is the intercept coefficient. β_1, β_2, β_3 are the linear coefficients. β_{11}, β_{22}, β_{33} are the quadratic coefficients. β_{12}, β_{13}, β_{23} are the cross-product coefficients. A, B, C are the independent variables studied.

Model fitting and statistical analysis

All extraction experiments were carried out in triplicate and the results were expressed as mean values. The results obtained from CCD were used to determine the regression coefficients of the second-order multi regression model. The analysis of variance (ANOVA) was evaluated using Design-Expert 6.0.10. The quality of the fit of the polynomial model equation was assessed by determining the R^2 coefficient and the adjusted R^2 coefficient; its statistical and regression coefficient significance were checked with F-test and P-value, respectively. Three-dimensional (3D) surface plot and corresponding contour plots were drawn to illustrate the effect of the independent variables on the responses (reducing sugar yield). The optimum values for the selected variables were obtained by solving the regression equation.

Results and Discussion

The optimum conditions of α-amylase activity by conventional assay

The effectiveness of the enzyme concentration was investigated between 0.25 and 1.0 activity units depending on the ability to transform substrate into product (data not shown). It was found that the enzyme concentration of 0.5 units gave adequate amount of the maltose. The effect of starch concentration on α-amylase was the factor that must be studied to increase an amount of the maltose. The starch concentration was studied between 0.25 and 1.0% (w/v) (data not shown). It was found that the starch concentrations higher than 0.5% (w/v) gave no difference in the amounts of the maltose. Thus, the starch concentration was performed at 1.0 % (w/v) in excess.

The effect of pH buffer solution was also one of the important factors that must be studied to optimize the enzyme conditions. The difference in pH 5.5-8.0 was investigated (data not shown). It was found that the optimum conditions of the pH buffer solution ranging from pH 6 to pH 8 gave no difference in the amounts of the maltose. However, it preferred to choose the buffer solution of pH 6.9.

The effect of incubation temperature on the optimum enzyme condition was also investigated. In this study, the incubation temperature was evaluated ranging from 25 to 60ºC (data not shown). The amount of the maltose increased from 25 to 45°C and then decreased from 45 to 60°C. It was found that the optimum conditions of the incubation temperature ranging from 25 to 45°C gave linearly rather high amounts of the maltose. Thus, the normal temperature of 37°C was preferred.

The effect of an incubation time on the optimum enzyme conditions was concerned ranging from 0 to 60 min (data not shown). The amount of the maltose increased from 0 to 40 min and then decreased from 40 to 60 min. It was found that the optimum conditions of the incubation time ranging from 10 to 40 min gave rather higher amounts of the maltose. Thus, it preferred to fix the reaction about 10 min.

The RSM optimum conditions for α-amylase activity

The quantitative feature of the proposed method was studied under the optimum conditions. For the linear range of the method, a series of standard solutions were prepared covering a concentration range of 200-1200 µg/mL. The calibration curve was plotted between the absorbance at 540 nm and the concentrations of maltose. The regression coefficients (R^2) obtained were higher than 0.9900.

Statistical model analysis

The design matrix and the corresponding results of RSM experiments were used to determine the effect of the three independent variables including the pH buffer solution (A), incubation temperature (B) and incubation time (C) as shown in Table 2.

By employing a multi-regression analysis for the experimental data, the predicted response Y for the yield of reducing sugar could be obtained using the following second-order polynomial Eq. (6):

$$Y = 0.91 + 0.032A + 0.016B + 0.066C - 0.059A^2 - 0.055B^2 - 0.062C^2 - 0.018AB - 0.018AC - 0.018BC \quad (6)$$

where, Y is the predicted yield of reducing sugar (mg/mL of maltose), A, B and C are the code values of pH buffer solution, incubation temperature and incubation time, respectively.

The ANOVA that carried out to test the significance of the fit of the second-order polynomial equation for the yield of reducing sugar was presented in Table 4. ANOVA was used to estimate the statistical significant of the factors and interactions between term. A model F-value of 23.07 and a low probability value [(Prob > F) less than 0.05)] implying significant model fit of the model were investigated. The quality of the fit of the quadratic regression model equation was evaluated by the coefficient of determination (R^2). The value of R^2 was 0.9672, indicating that 96.72 % of the variability in the response could be explained by the statistical model. The model was stronger and the predicted response better as the R^2 value becomes closer to 1.0000. A regression model, with R^2 value greater than 0.9000, was considered to have a very high correlation (Jaya *et al*., 2010).

The adjusted R^2 value corrected the R2 value for the sample size and the number of terms. The value of the adjusted R^2 was 0.9255, which was also high. These results show that the regression model provides a good fit to the data. The coefficient of variation (CV) indicates the degree of precision with which the treatments were compared (Cao *et al*., 2009). A very low value of CV (3.59%) clearly indicates a high degree of precision and a good deal of reliability for the experimental values. The signal to noise ratio was measured by "Adequate Precision". A ratio greater than 4 was desirable. The ratio of 16.239 obtained indicates an adequate signal, which implies this model could be used to navigate the design space. Furthermore, the results of the error analysis indicated that the Lack of Fit of 8.45 imply the Lack of Fit was not significant [(Prob > F) more than 0.05)], which indicated the model was a good to fit. The model equation was adequate to predict the reducing sugar yield of α-amylase within the range of experimental variables.

The significance of each coefficient was determined by p-value (Table 3). Value of "Prob > F" less than 0.05 indicates that the model terms were significant whereas the value greater than 0.100 indicates that model terms were not significant (Diler and Ipek, 2012). In this case, linear terms (A, C), quadratic terms (A^2, B^2, C^2) were significant model terms ($P < 0.05$) whereas linear terms (B,) and two-way interaction terms (AB, AC, BC) were not significant model terms ($P > 0.05$). This suggests that pH buffer and incubation time have a significant effect on the reducing sugar yield. To determine the optimum levels of the variables for concentration of maltose yield, the 3D response surface and contour plots were determined using Eq. (6) and then an

Table 3. ANOVA for quadratic model for the yield of the reducing sugar

Source	Sum of square	Degree of freedom	Mean square	F-value	Prob > F
Model	0.16	9	0.018	23.07	0.0002
A	0.014	1	0.014	18.99	0.0034
B	3.705×10^{-3}	1	3.705×10^{-3}	4.87	0.0631
C	0.059	1	0.059	77.16	0.0001
A^2	0.039	1	0.039	51.74	0.0002
B^2	0.034	1	0.034	44.29	0.0003
C^2	0.044	1	0.044	57.46	0.0001
AB	2.450×10^{-3}	1	2.450×10^{-3}	3.22	0.1158
AC	2.521×10^{-3}	1	2.521×10^{-3}	3.31	0.1115
BC	2.521×10^{-3}	1	2.521×10^{-3}	3.31	0.1115
Residual	5.325×10^{-3}	7	7.607×10^{-3}		
Lack of Fit	5.084×10^{-3}	5	1.017×10^{-3}	8.45	0.1092
Pure Error	2.407×10^{-3}	2	1.203×10^{-3}		
Core Total	0.16	16			

CV = 3.59; R^2 = 0.9672; Adjusted R^2 = 0.9255; Adequate Precision = 16.239

Table 4. Confirmation for the optimum conditions of α-amylase activity by RSM

pH of buffer sol.	Incubation temperature (°C)	Incubation time (min)	Concentration of maltose (mg/mL)		
			Predicted	Observed	Error (%)
7.3	39	10	0.915	0.997	8.96

overlay contour plot was created to select the optimum α-amylase conditions.

The RSM was indeed a useful technique to simultaneously study and optimize the activity conditions of α-amylase. Normally, this methodology was important one to check the adequacy of the second degree polynomial model to ensure that it provided maximum approximation on the relationship between independent variables and dependent variable (response). The residuals from the least squares were important tool for evaluating the model adequacy (Li *et al.*, 2007; Qi *et al*., 2009).

Normal probability was checked by plotting the normal probability plot of residual (plotting of internally studentized residuals for α-amylase activity conditions). The normal assumption was satisfactory as normal residuals fall along a straight line (data not shown). The residual plot of model were randomly distributed without any trends, indicating that good predictions of maximum response along with constant variance and adequacy of the second degree polynomial model. Plotting of the observed yield of reducing sugar (the response) with respect to that from the quadratic model was also demonstrated (data not shown). It is demonstrated that the predicted data of the response from the quadratic model agree well with the observed results in the range of the operating variables.

Optimization for α-amylase activity

The effects of variables and their interactions on the concentration of maltose (reducing sugar) yield were described by the 3D response surface plots and 2D contour plots. These plots were obtained from plotting the response (concentration of maltose yield) on the Z-axis against any two variables while keeping the other variable constant at its '0' level.

Figure 1(a) shows the response surface for pH buffer solution and the incubation temperature on the concentration of maltose yield. The results revealed that the concentration of maltose yield increases from 0.761 to 0.885 mg/mL when pH buffer solution and incubation temperature increase. Then the value gradually decreases from 0.885 to 0.761 mg/mL with decrease in pH buffer solution (from pH 6.9 to pH 5.4) and incubation temperature (from 37 to 22 °C). The increasing trends in the reducing sugar yield at too high pH buffer solution and incubation temperature. The results revealed that the concentration of maltose yield increases from 0.761-0.885 mg/mL when pH buffer solution and incubation time increase as shown in Figure 1(b). The increasing trends in the concentration of the maltose yield at too high pH buffer solution and incubation time. It is implied the effect of the interactions between the incubation temperature and incubation time on the concentration of maltose yield. The 3D response and contour lines reveal that the concentration of maltose yield continuously increases as the incubation temperature and incubation time increase. To optimize α-amylase activity conditions, an overlay contour plot was created using Design-Expert software. The optimum conditions of α-amylase activity to achieve high concentration of the maltose yield were defined with

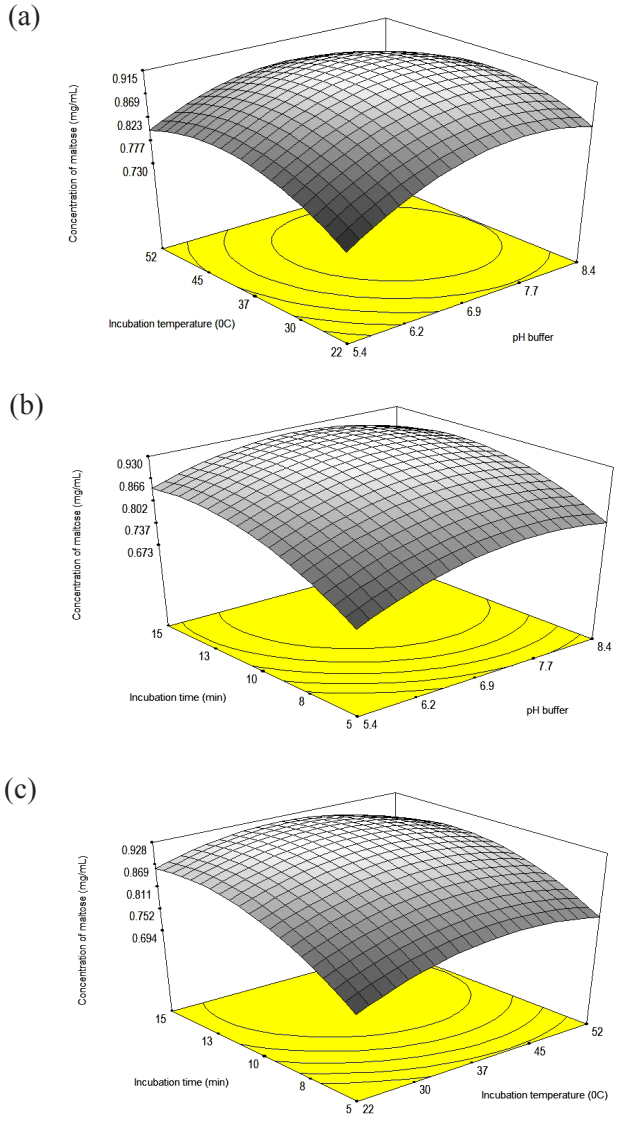

Figure 1. Response surface plots showing (a) the effect of pH buffer solution vs. incubation temperature, (b) the effect of pH buffer solution vs. incubation time, and (c) the effect of incubation temperature vs. incubation time on concentration of maltose

the following criterion: concentration of maltose yield > 0.800 mg/mL. The non-shaded area in the overlay plot in Figure 1(c) is the region that meets the proposed criterion. Based on the overlay plot, the optimum parameters were found to be at pH buffer solution of 7.3, 39°C incubation temperature and 10 min incubation time. The predicted maximum yield of the maltose concentration was 0.915 mg/mL.

Validation of the enzymatic activity model

The validity of the results predicted by the RSM model was confirmed by carrying out the repeated experiments under the optimum conditions. The results obtained from ten replications demonstrated that the maximum concentration of maltose yield (0.997 mg/mL) was closed to the predicted values (0.915 mg/g) with the error of 8.96% (Table 4). This result indicates that there is an excellent correlation between experimental and predicted results and in turn proves the validity of the model.

Conclusion

For α-amylase activity study, spectrophotometric determination of maltose is conducted based on the measurement of its absorbance. The optimum conditions for the measurement of the enzyme activity were experimentally investigated. The optimum conditions for the highest yield of the reducing sugar of α-amylase standard were also determined based on the three levels of three factorial CCD of RSM methods. A quadratic regression model equation for the reducing sugar yield was developed, with R^2 and adjusted R^2 determining to check the quality to fit the equation. Both high value of R^2 and adjusted R^2 greater than 0.9000 have revealed that the model provides a good fitting to the data. Under these optimum conditions, the predicted value from the model of maltose concentration gave excellent correlation with experimental value and in turn proved the validity of the extraction model because of the small deviation error in between them. Therefore, the model could be successfully used to identify the synchronous optimum conditions and to predict the maximum concentration of the maltose of the α-amylase activity.

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