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Enzyme and Microbial Technology 35 (2004) 467–473
ENZYME and MICROBIAL TECHNOLOGY
www.elsevier.com/locate/enzmictec

Production of cyclodextrin glucanotransferase (CGTase) from alkalophilic *Bacillus* sp. TS1-1: media optimization using experimental design

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Received 29 April 2004; accepted 9 July 2004

Abstract

Cyclodextrin glucanotransferase (CGTase) was produced when the *Bacillus* sp. TS1-1 was grown in a medium containing sago starch, yeast extract, phosphorus and mineral salt sources, using shake flask mode at 37 °C for 24 h. Response surface methodology (RSM) was applied to optimize the medium constituents with respect to CGTase production and activity. A 2^4 full factorial design (first order model) was carried out to identify the significant effect of medium components towards CGTase production. The variables involved in this initial screening study were sago starch, yeast extract, K_2HPO_4 and $MgSO_4{\cdot}7H_2O$. Statistical analysis of results have shown that only sago starch and yeast extract have a significant effect on CGTase production. A second-order model was proposed by using 2^2 central composite design to represent the production CGTase activity as a function of sago starch and yeast extract. The optimized values of 1.48% and 1.89% of sago starch and yeast extract was obtained, respectively. Under these proposed optimized conditions, the model predicted a CGTase activity of 79.66 U/ml and via experimental rechecking the model, an activity of 84 U/ml was attained.

Keywords: Cyclodextrin; Cyclodextrin glucanotransferase; Medium optimization; Response surface methodology

1. Introduction

Cyclodextrins (CDs) are cyclic homogeneous oligosaccharides of 6–12 α-1,4-D linked glucose residues. CDs are synthesized from starch by the cyclization reaction of cyclodextrin glucanotransferase (E.C. 2.4.1.19) enzyme. The most common available CDs to be synthesized are composed of 6, 7 and 8 glucose units named α-, β- and γ-cyclodextrins, respectively [1].

The interior of CDs is relatively apolar compared to water, and thereby CDs can easily form inclusion complex with many organic substances which can change the physicochemical properties of the guest molecule; thus increasing their water solubility and stability [2–5]. These properties made CDs became increasingly important as molecular encapsulator for industrial application particularly in food, pharmaceutical, dairy and cosmetics industry [4].

The β-form of cyclodextrin is reported to be more suitable for industrial use since inclusion complexes can be prepared easily which are very stable due to the low solubility of β-CD in water. In addition, the yield of β-CD

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0141-0229/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.enzmictec.2004.07.008

from starch is usually higher than that of other cyclodextrins [6].

Cyclodextrin glucanotransferase (CGTase,1 EC 2.4.1.19), can be found in several bacterial species, catalyzes the inter- and intramolecular transglycosylation of α-1,4-glucan. The reaction will produce cyclodextrins with 6, 7, and 8 glucosyl residues and maltooligosaccharides of various degrees of polymerizations. Besides cyclization (the conversion of starch and related α-1,4-glucans into CDs through an intramolecular transglycosylation reaction), the enzyme also catalyzes a coupling reaction (opening of the rings of CDs and transfer of the linear maltooligosaccharides to acceptor) through intermolecular transglycosylation reactions [7]. CGTase also possesses a weak hydrolyzing activity [8].

Major producers of CGTases are belongs to *Bacillus* sp. especially aerobic alkalophilic types. Other psychrophilics, mesophilic and thermophilic microorganims that have been reported able to produce CGTase enzymes are *Bacillus stearothermophilus*, *Klebsiella pneumoniae* [9], *Klebsiella oxytoca* 19-1 [10,11], *Brevibacterium* sp. [12] and hyperthermophilic archaea-bacteria [13]. To date, all known CGTases will produce α-, β- and γ-CDs from starch in different ratios depending on the reaction conditions and the nature of the CGTase.

Production of CGTase can be optimized by manipulating physio-environmental factors such as the nutrient concentrations and compositions of the production media. Media optimization using statistic experimental design has been cited by Casas et al. [14], Lee and Chen [15], Pujari and Chandra [16], Dey et al. [17], Liu et al. [18], Cockshott and Sullivan [19], Hujanen et al. [20], Chen et al. [21] and Li et al. [22] in optimizing either biomass growth, enzymes, certain extracellular proteins and bioactive metabolites.

Typically modes of CGTase production will be conducted initially using shake flask culture and submerge fermentation utilizing selective. Various types of medium composition mainly carbon and nitrogen source concentration, inoculums size, pH and temperature for fermentation production of CGTase had been studied in several published paper [4,9,23].

In this study, full factorial design was used as an initial screening process to identify the critical, crucial and significant nutrient and also the interaction between two or more nutrients in relatively few experiments as compared to the one-factor at a time techniques. A second level design study was conducted with central composite design experiment to develop a mathematical correlation model between the significant nutrients for the optimum production of CGTase from *Bacillus* sp. TS1-1.

2. Materials and methods

2.1. Preparation of bacterial inoculum

The bacterium was isolated from the soil and has been identified as *Bacillus* sp. TS1-1 [24]. Bacteria innocula were grown in 20 ml seed medium containing 2% (w/v) soluble starch, 5% (w/v) yeast extract, 5% (w/v) peptone, 0.1% (w/v) K_2HPO_4, 0.02% (w/v) $MgSO_4{\cdot}7H_2O$ and 1% (w/v) Na_2CO_3 (autoclave separately) in a conical flask. The culture was incubated at 37 °C with shaking at 200 rpm for 18 h. Cells were then harvested by centrifugation at 5000 rpm for 5 min and washed once with normal saline solution to give an optical density (OD) reading of 0.5 at 600 nm.

2.2. Preparation of crude enzyme

10% (v/v) of *Bacillus* sp. TS1-1 inoculum was used to cultivate the production media at 37 °C for 24 h with continuous shaking in conical flask containing 100 ml of medium. The production medium containing 2% (w/v) soluble starch, 5% (w/v) yeast extract, 5% (w/v) peptone, 0.1% (w/v) K_2HPO_4, 0.02% (w/v) $MgSO_4{\cdot}7H_2O$ and 1% (w/v) Na_2CO_3 (autoclave separately). Experimental studies were carried out and after cultivation cells were removed by centrifugation at 5000 rpm for 2 min. The supernatant was used as crude enzyme solution for assaying enzyme activity.

2.3. CGTase activity assay

CGTase activity was determined using phenolphthalein method assay with slightly modification [25]. Reaction mixture containing 40 mg of soluble starch in 1.0 ml of 0.1 M sodium phosphate buffer (pH 6.0) and 0.1 ml of crude enzyme was incubated at 60 °C for 10 min. Reaction was stopped by an addition of 3.5 ml of 30 mM NaOH and 0.5 ml of 0.02% (w/v) phenolphthalein in 5 mM Na_2CO_3 solution. The colour intensity was then measured at 550 nm. One unit of the enzyme activity was defined as the amount of enzyme that forms 1 μmol of β-cyclodextrin per minute. Standard curve was plotted with β-cyclodextrin concentrations.

2.4. Experimental designs and optimization

Experimental designs [26,27] were carried out using Design Expert Software (Stat-Ease Inc., Statistic made easy, Minneapolis, MN, USA, Version 6.0.4). A 2^4 full factorial design was used to show the statistical significance of the composition of sago starch (carbon source), yeast extract (nitrogen source), K_2HPO_4 (phosphorus source) and $MgSO_4{\cdot}7H_2O$ (mineral source) on the production of CGTase. A total of 16 sets of experiments were employed in this study to determine the significant factors affecting the CGTase activity (Table 1). Two level factorial designs is a statistically based method that involves simultaneous adjustment of experimental factors at only two levels: high and low. The range and the levels of the variables investigated in this study were given in Table 2. The settings of range for factors were bases primarily on the investigation of single factors (screening process) and literature.

The design was further expanded to a central composite design with CGTase activity as the dependent variable and

Table 1
Experimental design and results (experimental and predicted values) of 2^4 full factorial design

Runs	Code levels				CGTase activity (U/ml) (experiment)	CGTase activity (U/ml) (predicted)
	x_1	x_2	x_3	x_4		
1	−1.00	−1.00	−1.00	−1.00	0.00	2.35
2	1.00	−1.00	−1.00	−1.00	11.18	8.62
3	−1.00	1.00	−1.00	−1.00	65.18	64.43
4	1.00	1.00	−1.00	−1.00	76.71	77.68
5	−1.00	−1.00	1.00	−1.00	1.48	−0.37
6	1.00	−1.00	1.00	−1.00	0.00	2.07
7	−1.00	1.00	1.00	−1.00	65.64	65.89
8	1.00	1.00	1.00	−1.00	75.79	75.32
9	−1.00	−1.00	−1.00	−1.00	0.00	−1.10
10	1.00	−1.00	−1.00	1.00	0.00	1.31
11	−1.00	1.00	−1.00	1.00	65.64	64.79
12	1.00	1.00	−1.00	1.00	75.79	74.19
13	−1.00	−1.00	1.00	1.00	0.00	0.60
14	1.00	−1.00	1.00	1.00	0.00	−0.81
15	−1.00	1.00	1.00	1.00	69.68	70.68
16	1.00	1.00	1.00	1.00	77.03	76.25

the significant term from first design as the independent variables. In this study, a full central composite design with three times replications, eight star points and five replicates at the center point were chosen. The quadratic model for predicting the optimal point was expressed according to Eq. (1).

$$Y = b_0 + \sum b_i x_i + \sum b_{ii} x_i^2 + \sum b_{ij} x_i x_j \quad (1)$$

where y is the response variables, b is the regression coefficients of the model, and x is the coded levels of the independent variables. The regression equation above was optimized for optimal values also using *Design Expert* software. The statistical significance of the second-order model equation was determined by F-value and the proportion of variance explained by the model obtained was given by the multiple coefficient of determination, R^2.

3. Results and discussion

The effects of sago starch, yeast extract, K_2HPO_4 and $MgSO_4{\cdot}7H_2O$, were initially screened using 2^4 full factorial design. The experimental design and the results (observed and predicted values) of were shown in Table 1. ANOVA was employed for the determination of significant variables. On the basis of these experimental values (Table 1), statistical testing was carried out using Fisher's statistical test for ANOVA. The F-value was the ratio of mean square due to regression to the mean square due to residual and indicates the influence (significant or not) of each controlled factor on tested models. Generally, the calculated F-value should be several times the tabulated value, if the model was good predictor of the experimental results. In addition, the P-value corresponding to the F-value indicated the probability that differences between calculated and tabulated statistics was due only to random experimental error.

Table 2
Independent variables and the concentration levels studied in the screening design

Variable	Unit	Low level (−1)	High level (+1)
$A(x_1)$: sago starch	% w/v	0.50	3.00
$B(x_2)$: yeast extract	% w/v	0.10	2.50
$C(x_3)$: K_2HPO_4	% w/v	0.02	0.20
$D(x_4)$: $MgSO_4{\cdot}7H_2O$	% w/v	0.002	0.03

The regression models can be applied in screening crucial and critical medium components. The regression equation (Eq. (2)) was obtained from analysis of variance and all terms regardless of their significance was included in the following equation:

$$\begin{aligned} Y\,(\mathrm{U/ml}) &= 36.37 + 2.96x_1 + 34.78x_2 - 0.17x_3 - 0.63x_4 \\ &\quad + 1.75x_1x_2 - 0.96x_1x_3 - 0.96x_1x_4 + 1.05x_2x_3 \\ &\quad + 0.95x_2x_4 + 1.11x_3x_4 \end{aligned} \quad (2)$$

If the coefficient of model was relatively large, it had more significant effects on the production of CGTase. Furthermore, the variable with positive fitted constant has an enhancer effects towards CGTase production than the one with negative coefficient, which had inhibitory effects. From ANOVA analysis the confidence level was greater than 95% ($P < 0.05$) in CGTase production, and the F-value and P-value of the model were 357.21 and 0.0001, respectively (as shown in Table 3). Thus, the estimated models fit the experimental data adequately. The coefficient of determination R^2 of the model was calculated to be 0.9986 indicating that the model able to comprehend a 99% of the data variability.

The CGTase activity varied markedly with the conditions tested, in the range of 0–77.03 U/ml (Table 1). The lowest and the highest values of CGTase activity were obtained when minimal and maximal levels of yeast extract (x_2) were used, respectively. These results suggested that this variable significantly affect the CGTase production and hence its activity.

Table 3
Regression analysis of the 2^4 full factorial design

Factors	Mean square	*F*-value	*P*-value
x_1 (sago starch)	140.07	25.48	0.0039
x_2 (yeast extract)	19359.94	3521.76	<0.0001
x_3 (K_2HPO_4)	0.44	0.079	0.7896
x_4 ($MgSO_4{\cdot}7H_2O$)	6.35	1.16	0.3316
x_1x_2	48.79	8.88	0.0308
x_1x_3	14.63	2.66	0.1637
x_1x_4	14.86	2.70	0.1611
x_2x_3	17.56	3.19	0.1340
x_2x_4	14.52	2.64	0.1651
x_3x_4	19.54	3.55	0.1181
Model	1963.67	357.21	<0.0001

R^2 = 0.9986.

The high level of yeast extract concentration (1.0 in coded value or 2.5%, w/v) allowed the strain to produce a higher CGTase activity than the low level of yeast extract concentration (−1.0 in coded value 0.1%, w/v) as showed in Fig. 1. As can be seen from Table 3, the *F*-test values for factors sago starch concentration (x_1), yeast extract concentration (x_2) and interaction between sago starch and yeast extract concentration (x_1x_2) were above the 5% level of significance.

From the ANOVA analysis results (Table 3), both sago starch (x_1) and yeast extract (x_2) has been proven to be the two most important variables for the production of CGTase, especially the concentration of yeast extract (the largest effect of the coefficient, +34.78). Gawande et al. [4] reported that yeast extract was able to improve CGTase production by *Bacillus firmus*. This is presumably due to the presence of some essential nutrients or inducers to stimulate CGTase production. CGTase production was also affected by the level of sago starch, increasing with the elevated concentration of sago starch (Fig. 2). As shown in the half normal plot (Fig. 3), the significant effect of yeast extract and sago starch was compared to the others. Other components in the media such as K_2HPO_4 (x_3) and $MgSO_4{\cdot}7H_2O$ (x_4) and interaction terms of x_1x_3, x_1x_4, x_2x_3, x_2x_4 and x_3x_4 did not significantly influence CGTase production.

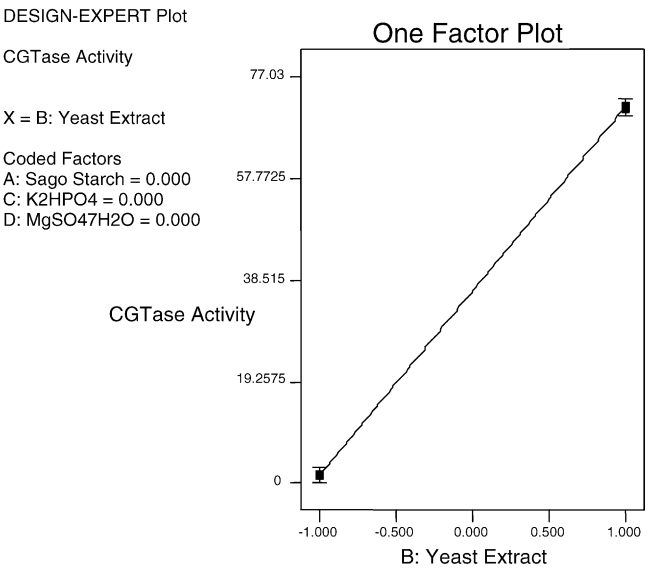

Fig. 1. Plot of effect of yeast extract concentration on CGTase production.

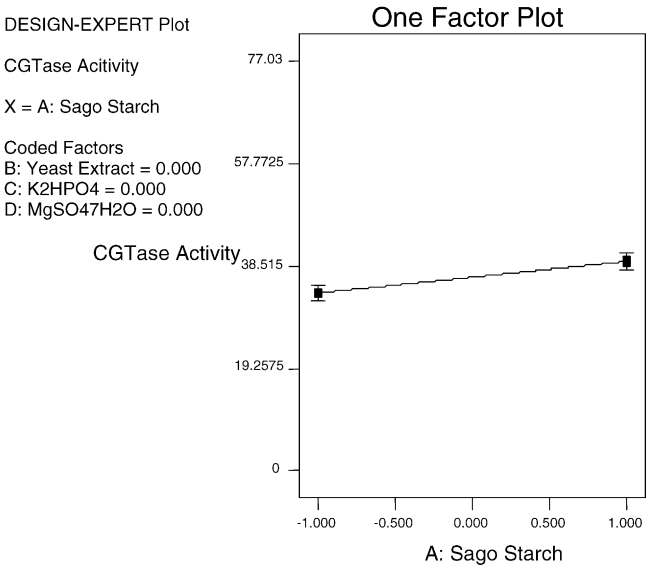

Fig. 2. Plot of effect of sago starch concentration on CGTase production.

The two significant variables, sago starch (x_1) and yeast extract (x_2) were further optimized using a central composite design. In this design, concentration of K_2HPO_4 and $MgSO_4{\cdot}7H_2O$ were set at their center point settings due to their insignificancies for CGTase production (0.1%, w/v and 0.02%, w/v, respectively). Table 4 summarised the central composite design matrices along with the experimental and predicted response for each individual experiment. The multiple regression equation for the CGTase activity after 24 h of cultivation by *Bacillus* sp. TS1-1 using sago starch (x_1) and

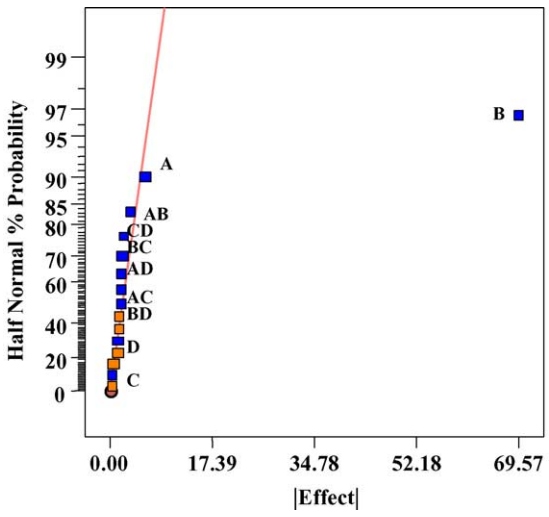

Fig. 3. The half normal plot for 2^4 full factorial design (initial screening for significant values).

Table 4
Experimental design and results (experiment and predicted value) of the 2^2 full factorial central composite design

Run	Factor				*Y*-value (CGTase activity, U/ml)	
	x_1		x_2		Experiment	Predicted
	Coded value	Real value (% w/v)	Coded value	Real value (% w/v)		
1	−1.00	0.6	−1.00	1.0	54.9	48.43
2	−1.00	0.6	−1.00	1.0	54.53	48.43
3	−1.00	0.6	−1.00	1.0	52.09	48.43
4	1.00	1.6	−1.00	1.0	47.4	43.87
5	1.00	1.6	−1.00	1.0	38.25	43.87
6	1.00	1.6	−1.00	1.0	40.06	43.87
7	−1.00	0.6	1.00	2.0	68.57	66.77
8	−1.00	0.6	1.00	2.0	67.02	66.77
9	−1.00	0.6	1.00	2.0	68.03	66.77
10	1.00	1.6	1.00	2.0	75.23	78.79
11	1.00	1.6	1.00	2.0	71.71	78.79
12	1.00	1.6	1.00	2.0	70.6	78.79
13	−1.565	0.37	0.00	1.5	58.1	65.28
14	−1.565	0.37	0.00	1.5	59.37	65.28
15	1.565	1.63	0.00	1.5	80.05	71.11
16	1.565	1.63	0.00	1.5	77.37	71.11
17	0.00	1.0	−1.565	0.72	19.77	23.63
18	0.00	1.0	−1.565	0.72	20.3	23.63
19	0.00	1.0	1.565	2.28	70.74	65.30
20	0.00	1.0	1.565	2.28	69.17	65.30
21	0.00	1.0	0.00	1.5	71.17	73.41
22	0.00	1.0	0.00	1.5	72.2	73.41
23	0.00	1.0	0.00	1.5	74.37	73.41
24	0.00	1.0	0.00	1.5	75.31	73.41
25	0.00	1.0	0.00	1.5	74.97	73.41

yeast extract (x_2) as the main variables was as follows:

$$Y = 73.41 + 1.86x_1 + 13.31x_2 - 2.13x_1^2 - 11.82x_2^2 + 4.14x_1x_2 \quad (3)$$

Based on the result, a quadratic model for final CGTase activity was calculated. The model adequacy was checked by *F* test and the determination coefficient R^2. In here, the computed *F*-value (41.25) was greater than the tabulated *F*-value, $F_{5,19}$ = 4.17 at level of significance of 1%, so that the null hypothesis (H_0) was rejected. Having rejected the null hypothesis, it can be inferred that medium optimization were highly significant. Also, the high *F*-value and a very low probability ($P > F = 0.0001$) indicated that the present model was in good prediction of the experimental results. Therefore, the obtained mathematical model was adequate. The goodness of fit of the model was expressed by the coefficient of determination R^2, which was calculated to be 0.9157, indicating that 91.57% of the variability in the response could be explained by the model or only about 8.43% of the total variation were not explained by the model. As closer the value of *R* (multiple correlation coefficient) to 1, a better correlation existed between the experimental and predicted values. The value of *R* (=0.9569) indicating a good agreement existed between the experimental and predicted values of CGTase activity. Table 5 shows the ANOVA for selected quadratic model of 2^2 full factorial central composite design.

The effect of concentration of yeast extract and sago starch on CGTase production by *Bacillus* sp. TS1-1 was further analyzed using the contour plot and a response surface plot was simulated according to the model (Figs. 4 and 5).

Results from the response surface plot, have shown that optimal concentration of sago starch and yeast extract for CGTase production were calculated to be 1.48%, w/v and 1.89%, w/v, respectively. The maximum value of enzyme activity predicted from the model was 79.66 U/ml. To confirm the predicted optimization conditions, experimental rechecking was performed using conditions proposed by the optimization mode. The highest CGTase activity of 84 U/ml could be obtained after 24 h of fermentation.

Table 5
ANOVA for a 2^2 full factorial central composite design

Source	Sum of squares	Degree of freedom	Mean square	*F*-value	$P > F$
Model	6242.71	5	1248.54	41.25	<0.0001
Residual (error)	575.03	19	30.26		

R^2 = 0.9157, *R* = 0.9569.

Table 6
Summary of medium optimization using experimental design for CGTase production from *Bacillus* sp. TS1-1

Factor	Concentration before optimize (% w/v)	Concentration after optimize (% w/v)	CGTase activity (U/ml)		
			Before optimize	After optimize	
				$Y_{predicted}$	$Y_{experiment}$
Sago starch	2	1.48	72	79.66	84.32
Yeast extract	1	1.89			
K_2HPO_4	0.1	0.1			
$MgSO_4{\cdot}7H_2O$	0.02	0.02			
Na_2CO_3	1	1			

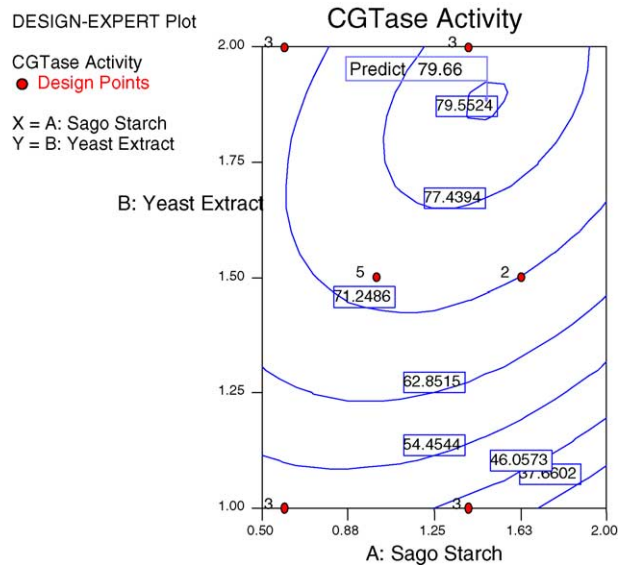

Fig. 4. Contour plot of the CGTase activity from the model equation: effect of sago starch and yeast extract.

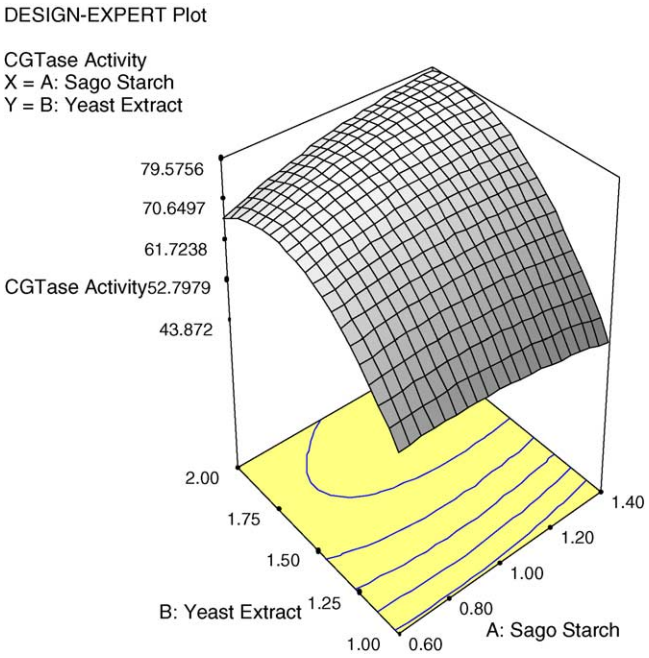

Fig. 5. Response surface plot of the CGTase activity from the model equation: effect of sago starch and yeast extract.

4. Conclusion

Full factorial design and central composite design can be used to determine the significant variables and optimum condition for CGTase production, respectively. Two components medium, sago starch and yeast extract has been identified to optimize the production of CGTase by nearly 15%, which was 72 U/ml before optimization and increased to 84.32 U/ml after optimization (Table 6).

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