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Research Article

OPTIMIZATION OF PROCESS VARIABLES BY RESPONSE SURFACE METHODOLOGY

Ravinder Kashipeta*

Department of Biotechnology, College of Dry Land Agriculture and Natural Resources, Mekelle University, Mekelle, Tigray, Ethiopia.

*Email: RAVIKAMAL999@gmail.com, Phone: +251 919040957

Abstract

In the present study optimisation of the growth medium for the production of Cyclodextrin glucanotransferase (CGTase) was carried out using response surface methodology. Four important parameters namely starch, yeast extract, K2HPO4 and MgSO4 concentrations were selected as the independent variables and the enzyme activity (CGTase activity U/mL) was the dependent response variable. Each of these independent variables was studied at five different levels as per central composite design (CCD) in four variables with a total of 28 experimental runs. The optimal calculated values of tested variables for maximal production of CGTase were found to be comprised of: starch, 2.16 %; yeast extract, 0.6 %; K2HPO4, 0.62 %; MgSO4, 0.04 % with a predicted CGTase activity of 150 U/ml. These predicted optimal parameters were tested in the laboratory and the final CGTase activity obtained was very close to the predicted value at 148.2 U/ml.

Keywords: Response surface methodology; CGTase; Central composite design; TPR71H.

Introduction

Cyclodextrin glucanotransferase (CGTases; EC 2.4.1.19) is an enzyme which converts starch into the cyclodextrins (CDs). Based on the number of glucose moieties the CDs are classified as α-, β-, and γ-CDs. CDs have the capacity to encapsulate hydrophobic molecules within their hydrophobic cavity, based on this nature it is used in the various industries. Leemhuis *et al.*, (2010), Martin Del Valle (2009), Li *et al.*, (2007) and Biwer *et al.*, (2002) reviewed the numerous applications of the CDs in the pharmaceutical, cosmetics, and food and textile industry. CDs have a hydrophilic outside and hydrophobic inside due to this it is used in the encapsulation of hydrophobic molecules which is particularly advantageous as many drug molecules are poorly soluble in water (Loftsson and Duchene, 2007), or to protect guest molecules from light, heat, or oxidizing conditions (Astray *et al.*, 2009). Cyclodextrins are also used to lower the volatility of odour molecules in perfumes and room refreshers for controlled release of the odour. In the chemical industry, CDs are used in the separation of enantiomers to extract toxic chemicals from waste streams (Martin Del Valle, 2009) and in soil bioremediation (Fava and Ciccotosto, 2002). Various other applications of CDs include the suppression of undesirable (bitter) tastes and the extraction of compounds such as cholesterol from foods (Szente and Szejtli, 2004; Szejtli and Szente, 2005).

The composition and concentration of the medium plays a vital role in the growth and enzymes production by the microorganisms. The optimization of the media components and culture conditions are the primary task in a biological process. The traditional optimization approach used is one-at-a-time optimization. In this method one parameter is optimized by changing it at the same time other factors were maintained at a constant level (Suvarna Laxmi *et al.*, 2008). This method of optimization requires a large number of experiments, it is a tedious process and also consumes a lot of chemicals and resources leads to the process development which is cost ineffective. Apart from this, there is a chance for misconception of results because the interaction effects between different factors are unnoticed (Hymavathi *et al.*, 2009). Response surface methodology (RSM) is a useful tool for studying the effect of several factors influencing the responses by varying them simultaneously and carrying out a limited number of experiments. RSM in concise, is explained as a collection of experimental strategies, mathematical methods and statistical inference for constructing and exploring an approximate functional relationship between a response variable and a set of design variables. Very few authors have reported satisfactory optimization of CGTase production from microbial sources using a statistical approach (Gawande and Patkar, 1999; Rahman *et al.*, 2004; Ibrahim *et al.*, 2005).

During a screening program, a CGTase activity producing strain was mutated and identified as Bacillus sp. TPR71HNA6. With the help of Plackett–Burman design (PBD) four significant nutritional parameters which influence the CGTase production were selected. The objective of the present study was to optimize the levels of chosen significant nutritional parameters using central composite design (CCD).

In the preliminary studies and PBD it was observed that the parameters namely starch, yeast extract, K2HPO4, and MgSO4 concentrations were playing a vital role in the CGTase production. These four parameters were further optimized based on the Response surface methodology. RSM has been proved to be a powerful tool for optimization of fermentation parameters by many research groups (Hymavathi *et al.*, 2009). This method has been successfully applied in the optimization of fermentation medium components, conditions for enzymatic production as well as CDs production processes. It allows the calculation of maximum enzyme production based on few sets of experiments in which all the factors are varied within selected range and also to study interactive effects of various process parameters.

Materials and Methods

Microorganism and Culture Conditions

In the present study a mutated Bacillus sp. TPR71H (GenBank Accession No: FN993946) was used. This culture was stored in nutrient agar slants and subcultured periodically once ever week. The production of CGTase experiments were conducted according to the PBD. The liquid samples are withdrawn and centrifuged at 10,000 rpm for 10min to remove the biomass and other insoluble substrates from the culture. After centrifugation the supernatant liquid was collected and estimated for CGTase activity.

Estimation of CGTase Activity

Enzyme activity was measured by decrease of phenolphthalein colour intensity. Enzyme assay was carried out according to the Kaneko *et al.*, (1987) method. The reaction mixture containing 1mL of 40mg of soluble starch in 0.1M potassium phosphate buffer (pH 6.0) and 0.1mL of the crude enzyme from the culture was incubated in water bath at 60°C for 10min. The reaction was stopped with 3.5mL of 30mM NaOH. Finally, 0.5mL of 0.02% (w/v) phenolphthalein in 5mM Na2CO3 was added and mixed well. After leaving the mixture to stand for 15min at room temperature, the reduction in colour intensity was measured at 550 nm. A blank lacking the enzyme is tested simultaneously with each batch of samples. One unit of enzyme activity was defined as the amount of enzyme that forms 1µgm of β-CD from soluble starch in 1min.

Optimization by Response Surface Methodology (RSM)

Response surface methodology using Central composite design was applied for optimization of CGTase production from mutated Bacillus sp. TPR71HNA6. Four important parameters namely starch (X1), yeast extract (X2), K2HPO4 (X3), and MgSO4 (X4) concentrations were selected as the independent variables and the enzyme activity (CGTase activity U/mL) was the dependent response variable. Each of these independent variables was studied at five different levels as per CCD in four variables with a total of 28 experimental runs. CGTase activity (U/mL) corresponding to the combined effects of four variables was studied in their specified ranges as shown in Table 1. The process variables such as temperature, pH and agitation speed were kept constant throughout the experiment. All the flasks were analysed for CGTase activity at the end of the experiment. The plan of CCD in the coded levels of the four independent variables is shown in Table 2.

For statistical calculations the independent variables were coded as

$$xi = \frac{(X_i - X_0)}{\delta X_i} - - - -(1)$$

Where Xi is the experimental value of variable; X0 is the midpoint of Xi, δXi is the step change in Xi and xi is the coded value for Xi, i = 1–4.

This response surface methodology allows the modelling of a second order equation that describes the process. CGTase production data was analysed and response surface model given by Eq. (2) was fitted with multiple regressions through the least squares method.

$$Y_i = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i}^{ii} \sum_{j}^{j} \beta_{ij} x_i x_j \quad \text{--------2)}$$

Where Yi is the predicted response, in the present study CGTase production (Yi) taken as a response, xi xj are input variables which influence the response variable Y; □0 is the offset term; □i is the ith linear coefficient; □ii the ith quadratic coefficient and □ij is the interaction coefficients

Table 1: Experimental range and coded levels of process variables for CGTaseproduction

S. N.	Variables	Range and levels				
		-2	-1	0	1	2
1	Starch (X1)	1.00	1.50	2.00	2.50	3.00
2	Yeast extract (X2)	0.30	0.45	0.60	0.75	0.90
3	K_2HPO_4 (X3)	0.40	0.50	0.60	0.70	0.80
4	$MgSO_4$ (X4)	0.02	0.03	0.04	0.05	0.06

Table 2: Lay out of full factorial central composite design for CGTase production by mutated Bacillus sp. TPR71HNA6

S. N.	Starch (X1)	Yeast extract (X2)	K_2HPO_4 (X3)	$MgSO_4$ (X4)	CGTase activity (U/mL)
1	-1	-1	-1	-1	
2	-1	-1	-1	1	
3	-1	-1	1	-1	
4	-1	-1	1	1	
5	-1	1	-1	-1	
6	-1	1	-1	1	
7	-1	1	1	-1	
8	-1	1	1	1	
9	1	-1	-1	-1	
10	1	-1	-1	1	
11	1	-1	1	-1	
12	1	-1	1	1	
13	1	1	-1	-1	
14	1	1	-1	1	
15	1	1	1	-1	
16	1	1	1	1	
17	-2	0	0	0	
18	2	0	0	0	
19	0	-2	0	0	
20	0	2	0	0	
21	0	0	-2	0	
22	0	0	2	0	
23	0	0	0	-2	
24	0	0	0	2	
25	0	0	0	0	
26	0	0	0	0	
27	0	0	0	0	
28	0	0	0	0	

Data Analysis and Interpretation of the Results

The results of the experimental design were analysed and interpreted using the STATISTICA version 7.0 (StatSoft, USA) statistical software. Prediction of optimum fermentation parameters and shape of the curves generated by the model was also done by the same software.

Results and Discussion

Table 3 depicts the results of the 28 runs CCD in four selected variables at five levels for optimization of CGTase production. CGTase production varied markedly in the range of 97-148U/mL with the conditions tested. High CGTase activity was observed in experimental runs with the mid values of the parameters. It was observed from various experimental runs that CGTase production was quite high with higher starch concentration.

CGTase activity (U/mL), the response variable was transferred to natural log values in order to stabilize its variance. ANOVA (analysis of variance) was employed for the determination of significant effects of variables for CGTase production. The experimental results suggest that the variables selected for the fermentation process had strong effect on CGTase production. On the basis of these experimental values statistical testing was carried out using the Fisher's 'F'-test and students'-'t'-test. Analysis of variance for CGTase production shows that fitted second order response surface model is highly significant with F-test = 18.59 ($P < 0.0001$).

The coefficients for the linear effect of K2HPO4 and MgSO4 were highly significant while starch and yeast extract concentrations were statistically insignificant (Table 4). In the quadratic terms all variables were significant. The starch and yeast extract concentrations were insignificant at linear terms and significant in quadratic terms which indicate that these two factors are highly influential parameters on the CGTase production. With small variation in the concentration of these variables, a significant change in the production could be observed. The interactive effect between trace elements (K2HPO4 and MgSO4) were not significant, all other remaining interactions are significant. The interaction of the starch and K2HPO4 has the highest magnitude (8.125) when compared to the other interactions (Table 4). The fitted second order response surface model

as specified by Eq. (2) for CGTase activity (U/mL) in coded process variables is:

$$Y= 147.25 +0.7083\ X1 + 0.9583\ X2 + 4.2917\ X3+ 1.8750\ X4 -5.6563\ X1^2-6.4063\ X2^2-9.9063\ X3^2-4.4063\ X4^2+3.8125X1*X^2 +4.0625\ X1*X3 + 2.4375\ X1*X4 -3.0625\ X2*X3 -2.1875\ X2*X4 -1.1875\ X3*X4 - (3)$$

Table 3: Experimental design along with the observed and predicted CGTase production

S. N.	Starch (X1)		Yeast Extract (X2)		K_2HPO_4 (X3)		$MgSO_4$ (X4)		CGTase Activity (U/mL)		
	Coded	Real	Coded	Real	Coded	Real	Coded	Real	Observed	Predicted	Error
1	-1	1.50	-1	0.45	-1	0.50	-1	0.03	118.00	116.91	1.083
2	-1	1.50	-1	0.45	-1	0.50	1	0.05	119.00	122.54	-3.541
3	-1	1.50	-1	0.45	1	0.70	-1	0.03	125.00	125.87	-0.875
4	-1	1.50	-1	0.45	1	0.70	1	0.05	126.00	126.75	-0.750
5	-1	1.50	1	0.75	-1	0.50	-1	0.03	123.00	121.70	1.291
6	-1	1.50	1	0.75	-1	0.50	1	0.05	119.00	118.58	0.416
7	-1	1.50	1	0.75	1	0.70	-1	0.03	118.00	118.41	-0.416
8	-1	1.50	1	0.75	1	0.70	1	0.05	105.00	110.54	-5.541
9	1	2.50	-1	0.45	-1	0.50	-1	0.03	100.00	97.70	2.291
10	1	2.50	-1	0.45	-1	0.50	1	0.05	114.00	113.08	0.916
11	1	2.50	-1	0.45	1	0.70	-1	0.03	123.00	122.91	0.083
12	1	2.50	-1	0.45	1	0.70	1	0.05	129.00	133.54	-4.541
13	1	2.50	1	0.75	-1	0.50	-1	0.03	119.00	117.75	1.250
14	1	2.50	1	0.75	-1	0.50	1	0.05	122.00	124.37	-2.375
15	1	2.50	1	0.75	1	0.70	-1	0.03	131.00	130.70	0.291
16	1	2.50	1	0.75	1	0.70	1	0.05	132.00	132.58	-0.583
17	-2	1.00	0	0.60	0	0.60	0	0.04	126.00	123.20	2.791
18	2	3.00	0	0.60	0	0.60	0	0.04	126.00	126.04	-0.041
19	0	2.00	-2	0.30	0	0.60	0	0.04	121.00	119.70	1.291
20	0	2.00	2	0.90	0	0.60	0	0.04	125.00	123.54	1.458
21	0	2.00	0	0.60	-2	0.40	0	0.04	97.00	99.04	-2.041
22	0	2.00	0	0.60	2	0.80	0	0.04	121.00	116.20	4.791
23	0	2.00	0	0.60	0	0.60	-2	0.02	122.00	125.87	-3.875
24	0	2.00	0	0.60	0	0.60	2	0.06	140.00	133.37	6.625
25	0	2.00	0	0.60	0	0.60	0	0.04	146.00	147.25	-1.250
26	0	2.00	0	0.60	0	0.60	0	0.04	148.00	147.25	0.750
27	0	2.00	0	0.60	0	0.60	0	0.04	147.00	147.25	-0.250
28	0	2.00	0	0.60	0	0.60	0	0.04	148.00	147.25	0.750

Table 4: Regression coefficients and ANOVA

Model Term	Effect	Regression Coefficients	SS	df	MS	F-value	t-value	p-value
Mean/ Intercept.	147.2500	147.2500					78.6005	0.000000
X1	1.4167	0.7083	12.042	1	12.042	0.8578	0.9262	0.371240
X2	1.9167	0.9583	22.042	1	22.042	1.5701	1.2530	0.232264
X3	8.5833	4.2917	442.042	1	442.042	31.4879	5.6114	0.000085
X4	3.7500	1.8750	84.375	1	84.375	6.0103	2.4516	0.029126
X1*X1	-11.3125	-5.6563	767.836	1	767.836	54.6952	-7.3956	0.000005
X2*X2	-12.8125	-6.4063	984.961	1	984.961	70.1616	-8.3763	0.000001
X3*X3	-19.8125	-9.9063	2355.211	1	2355.211	167.7685	-12.952	0.000000
X4*X4	-8.8125	-4.4063	465.961	1	465.961	33.1917	-5.7612	0.000066
X1*X2	7.6250	3.8125	232.563	1	232.563	16.5661	4.0701	0.001325
X1*X3	8.1250	4.0625	264.063	1	264.063	18.8099	4.3370	0.000806
X1*X4	4.8750	2.4375	95.063	1	95.063	6.7716	2.6022	0.021913
X2*X3	-6.1250	-3.0625	150.063	1	150.063	10.6894	-3.2695	0.006096
X2*X4	-4.3750	-2.1875	76.562	1	76.562	5.4538	-2.3353	0.036202
X3*X4	-2.3750	-1.1875	22.563	1	22.563	1.6072	-1.2678	0.227124
Error			182.500	13	14.038			
Total SS			4502.429	27				

The coefficient of determination R2 for the above predicted Eq.(3) was 95.94. The correlation coefficient (R2 = 0.9594) was indicating that the statistical model can explain 95.94% of the variability in the response. Therefore this equation can be used for predicting the response at any combination of four variables in and around the experimental range. CGTase activity (U/mL) at specific combination of four variables can be predicted by substituting the corresponding coded values in Eq. (3). Figure 1 depicts the correlation between the observed and predicted values. From this figure it was observed that all of the data points are concentrated near the diagonal line, and no scattered points were observed, it indicates that there is a good correlation between the observed and predicted values.

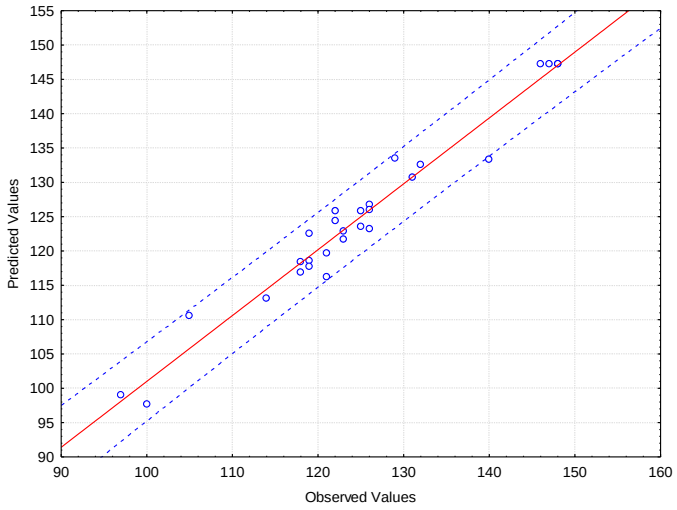

Fig 1: Correlation between the observed and predicted values of CGTase from mutated Bacillus sp. TPR71HNA6

The value of the adjusted determination coefficient is close to the R2 value (Adj R2 = 0.9158) is also very high to advocate for a high significance of the model (Box *et al.*, 1978; Cochran and Cox, 1957). If there are many terms in the model and the sample size is not very large, the adjusted R2 may be noticeably smaller than the R2. Here in this case the adjusted R2 value is 0.9158, which is lesser than the R2 value of 0.9594. The Predicted R2 of 0.7318 is in reasonable agreement with the adjusted R2 of 0.9158. At the same time, a relatively lower value of the coefficient of variation (CV = 3.31%) indicates a better precision and reliability of the experiments carried out (Myers and Montgomery, 1995; Khuri and Cornell, 1987).

The surface (3D) and contour (2D) plots based on Eq. (3) were prepared using STATISTICA 7.0 software. The surface plot (Fig 2–7) shows the behavioural change with respect to simultaneous change in two variables. Proper choice of fermentation parameters is desirable for maximum enzyme production and surface plots based on well fitted model provides these choices. Surface and contour plots were prepared for six pairs of variables which were having significant interaction effects in maximizing CGTase production at specific hold values.

The behaviour of CGTase production with respect to change in starch and yeast extract concentrations at specific hold values is shown in Fig 2. From the figure it was observed that the contour plot is slightly inclined towards the starch, indicating that the interaction between these two parameters is significant and starch has a high influence on CGTase production. It was observed that starch at 1.8-2.2% (Fig 2-4) and yeast extract at 0.5-0.65% (Fig 2, 5 & 6) concentrations were effective for enzyme production was noticed. Gawande and Patkar 1999 reported that the nature and concentration of the carbon source plays a vital role in

the CGTase production. Ai-Noi *et al.*, 2008 and Khairizal *et al.*, 2004 reported that increasing the sago starch concentration increased enzyme production. Gawande and Patkar 1999 also commented that above certain concentration of carbon source, when other nutrients are kept constant, catabolite repression may occur. It was noticed that the CGTase production with starch concentration above 20-30g/L, resulted in low enzyme production by Bacillus sp (Gawande *et al.*, 1998).

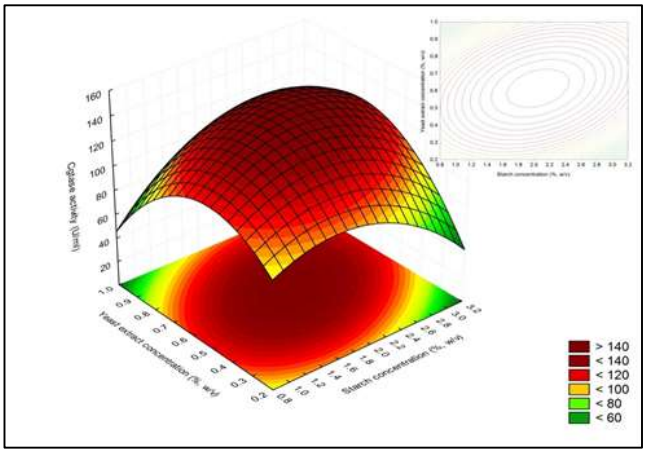

Fig 2: Interaction influence of starch and yeast extract on CGTase production by mutated Bacillus sp. TPR71HNA6

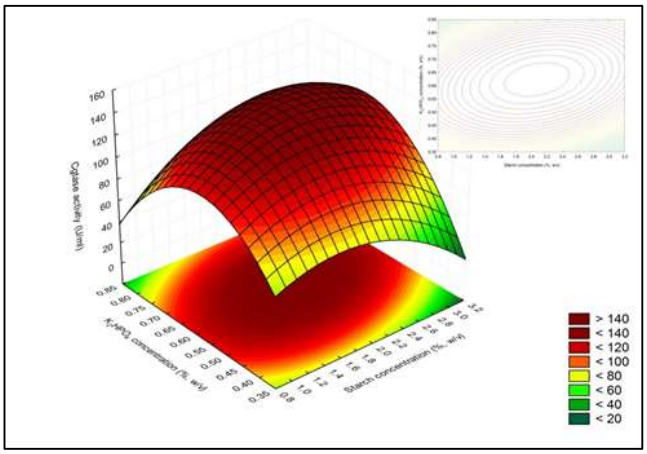

Fig 3: Interaction influence of starch and K_2HPO_4 on CGTase production by mutated Bacillus sp. TPR71HNA6

Generally, a phosphorus source is considered to be necessary for cells for the synthesis of nucleic acids and phospholipids (Madigan *et al.*, 1997). From the Fig. 3, 5 and 7 it was noticed the interaction behaviour of phosphorus with other variables. It was observed that concentration of phosphorus slightly depends on the starch concentration (Fig 3). A lower concentration of phosphorus is preferable for the effective CGTase production. Swinkels, 1985 reported that starch contains the trace metals, in that case when using the starch, lower concentrations of trace elements addition is preferable. It was observed that K2HPO4 concentration at 0.55-0.65% is optimum for CGTase production by the mutated Bacillus sp TPR71HNA6 (Fig 3, 5 & 7). It was noticed that the concentration of MgSO4 in the range of 0.035 - 0.05% (Fig 4, 6 & 7). The low concentrations of these salts were needed to increase the production of CGTase. A similar result was reported by Gawande and Patkar 1999 whereby using experimental design, they found that the concentration of mineral salts (magnesium sulphate in their case) at 0.5g/L can increase the CGTase production by *Klebsiella pneumoniae* AS-22.

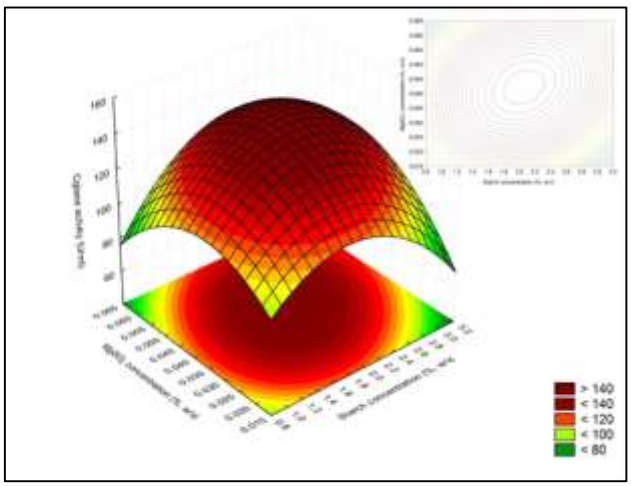

Fig 4: Interaction influence of starch and $MgSO_4$ on CGTase production by mutated Bacillus sp. TPR71HNA6

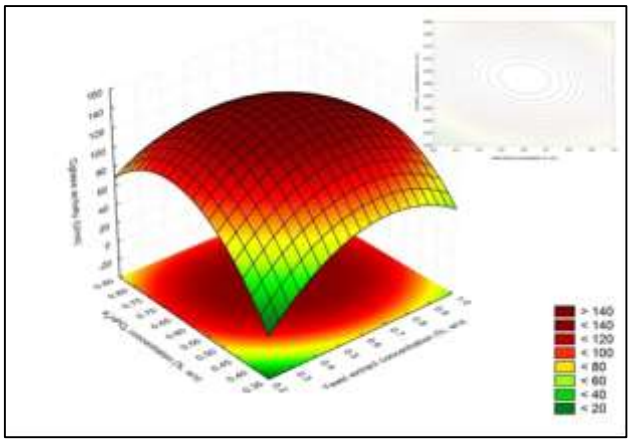

Fig 5: Interaction influence of yeast extract and K_2HPO_4 on CGTase production by mutated Bacillus sp. TPR71HNA6

Validation of the experimental model

A repeat fermentation for CGTase production by mutated Bacillus sp. TPR71HNA6 under optimal conditions was carried out for the validation of optimized parameters. The CGTase production under optimized parameters viz. starch 2.16%, yeast extract 0.6%, K2HPO4 0.62% and MgSO4 0.04% yielded CGTase activity of 150U/mL. The CGTase yield so obtained under optimized parameters was even higher than the predicted value (148.2U/mL) by the model. These validation studies indicate that the proposed model was adequate to predict the optimisation of CGTase

production from mutated Bacillus sp.TPR71HNA6. Similarly Rahman *et al.*, 2004 and Ibrahim *et al.*, 2005 used the statistical optimization techniques for improvement of the CGTase production.

The optimization of CGTase production by mutated Bacillus sp. TPR71HNA6 was conducted in batch culture. From central composite design the optimum concentration for starch, yeast extract, K2HPO4 and MgSO4 were observed to be 2.16%, 0.6%, 0.62% and 0.04% respectively. The predicted enzyme production was 148.2U/mL. While conducting the experiments at the predicted optimum conditions, the CGTase production obtained was 150U/mL. An overall increase of 55% in yield was achieved by applying statistical tools for the optimization of CGTast production.

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