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OPTIMIZATION OF NUTRIONAL FACTORS FOR THE PRODUCTION OF CYCLODEXTRIN GLYCOSYLTRANSFERASE BY A NOVEL MUTATED BACILLUS SP TPR71HNA6 USING PLACKETT-BURMANN DESIGN

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ABSTRACT

The cyclodextrin glycosyltransferase (CGTase) enzymes are the most important group of commercially produced enzymes. The production of CGTase was optimized using a newly isolated mutated *Bacillus* sp. TPR71HNA6. The fermentation variables were selected and optimized via Plackett–Burman design. Eleven nutrients belonging to three categories viz.; nitrogen sources, carbon sources and mineral sources were screened. This design screens *n* variables in *n*-1 number of experiments. The results showed that the CGTase production varies from 18 to 68U/mL indicating that the selected compounds showed significant effect on the enzyme production. From these results it was also observed that yeast extract has highest significant than all other variables followed by starch, peptone and urea. Glucose has the least effect on the CGTase production by mutated *Bacillus* sp. TPR71H NA6.

KEY WORDS: CGTase, Plackett-Burman Design, Optimization, *Bacillus* sp. TPR71HNA6.

INTRODUCTION

Process optimization became a central importance and undisputed component in industrial production processes especially with regards to biotechnological production processes, in which even small improvements can be decisive for commercial success. Optimization of different fermentation conditions can be achieved either by conventional or statistical

methods. Conventional methods are time consuming and do not provide any information on interactions influence on overall productivity. Whereas, statistical methods are rapid and reliable, short lists significant nutrients, helps understanding the interactions among the nutrients at various concentrations and reduces the total number of experiments tremendously. [1] Different types of statistical methods are developed for such optimization experiments. [2] Among all, Plackett–Burman Design (PBD) [3] helps in identification of the most significant compounds and their concentration from a large group of nutritional compounds for improving the further optimization.

The Plackett–Burman Design aims to select the most important variables in the system [3]. This Design is practical especially when the investigator is faced with large number of factors and is unsure which settings are likely to produce optimal or near optimum responses. The PB design allows evaluation of N - 1 variables using N number of experiments (N must be a multiple of four). Each variable is represented at two levels, namely, ‘‘high’’ (+) and ‘‘low’’ (-). These levels define the upper and lower limits of the range covered by each variable. If there are not enough ‘N-1’ variables, one can use the dummy variables. In some cases during the analysis of the results no degrees of freedom left to estimate the error variability so one may not get the standardized effects and other ANOVA factors like F & p Values; this situation is normally called as a saturated PBD. To avoid this situation, most of the authors use the dummy variables or perform the additional number of experiments with central or base values [4-8].

In the present study PBD was employed for screening of the various carbon and nitrogen sources for CGTase production from isolated mutated *Bacillus* sp TPR71HNA6.

MATERIALS AND METHODS

Microorganism and Culture Conditions

In the present study a mutated *Bacillus* sp. TPR71HNA6 (Gen Bank No: FN993946) was used. This culture was stored in a nutrient agar slants and subcultured periodically once a week. The production of CGTase experiments were conducted according to the PBD. The liquid samples are withdrawn and centrifuged at 10,000rpm 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 [9] 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 Na_2CO_3 was added and mixed well. After leaving the mixture to stand for 15min at room temperature, the reduction in colour intensity was measured at 550nm. 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.

Plackett–Burman Design (PBD)

Various carbon and nitrogen compounds required for the effective CGTase production by mutated *Bacillus* sp. TPR71H NA6 were selected based on Plackett-Burman design. Table 1 shows the selected compounds and their levels. All the compounds were chosen at three levels. In these levels the concentration of each compound was fixed based on the literature survey and our own experience gained. Table 2 depicts the experimental PB experimental plan. Analysis of the experimental results was performed based on the effect of each variable. The effect of the each selected variable on CGTase production was determined based on the first-order model

$$Y = \beta_0 + \sum \beta_i x_i - - - - - (1)$$

Where Y is the response (enzyme activity), β_0 is the model intercept β_i is the linear coefficient, and x_i is the level of the independent variable. The significance of each variable was determined based on ANOVA. All experiments were conducted in triplicates and averages of the results were taken as the response.

RESULTS AND DISCUSSION

The PBD allows for the screening of the main factors from a large number of process variables. These designs are quite useful in preliminary studies in which the principal objective is to select variables that can be fixed or eliminated in further optimization processes. PBD was employed in order to screen the significant factors from the selected 11 compounds. Experiments were performed according to the design matrix as shown in Table

3. The obtained CGTase activity at various trail conditions is depicted in table 3. The CGTase production varies from 18 to 68U/mL indicating that the selected compounds showed significant effect on the enzyme production. The obtained results were analyzed based on the regression model to screen the significant medium components. The following regression equation was obtained from the analysis of variance (ANOVA), only significant variables ($P<0.05$) were included in this equation.

$$Y = 49.6875 + 14.00\ Starch + 8.6667\ Xylose - 16.00\ Yeastextra\ ct + 13.00\ Peptone + 12.3333\ Urea + 10.6667\ A.S + 9.6667\ S.N \quad (1)$$

From the ANOVA results it was observed that yeast extract, starch, peptone and urea have highest significant effect on the CGTase production. Galactose, glucose, maltose and fructose were found to show insignificant effect on the CGTase production by mutated *Bacillus* sp. TPR71HNA6. The analysis of the data from the Plackett–Burman experiment was performed based on first-order (main effects) model. The adequacy of the model was calculated, and the variables evidencing statistically significant effects were screened via student's t-test from ANOVA. Table 4 represents the effects, values of coefficients, t, and p values of each component from the responses. The main effect of each variable upon CGTase production by mutated *Bacillus* sp. TPR71HNA6 was estimated as the difference between both averages of measurements made at the high level (+1) and at the low level (−1) of that variable.

On the analysis of the regression coefficients of the medium components except galactose, glucose, maltose and fructose remaining all compounds were found to be having a significant effect on the CGTase production by mutated *Bacillus* sp. TPR71HNA6. The components were screened based on their absolute value of effects (either positive or negative) and 'p' value below at 0.05. The absolute values of effect in table 4 were used to indicate the relative contribution of the variable on the response. A positive sign indicates that a higher-level variable setting results in a higher response than the lower-level variable setting. On the other hand, a negative sign indicates that the lower-level variable setting results in a higher response than the high-level variable setting. In the present experiment except yeast extract remaining all significant variables are significant at '+' level.

The Pareto chart (Fig 1) shows the ranking of variables according to the absolute values of standardized effect, is important in the design of the experiment for optimization and is a convenient way to view the results of a Plackett–Burman experimental design. The reference line indicates that effects were significant with the α value of 0.05. The effects of variables,

which extend past the line, were known to be significant at a particular α. The standardized effects were the't' statistics shown in Fig 1. From the Fig 1 it is observed that yeast extract has the highest significance than all other variables followed by starch, peptone and urea. Glucose has the least effect on the CGTase production by mutated *Bacillus* sp. TPR71HNA6. Further confirmation study was made by analysis of the probability plot (Fig 2). The significant factors were shown as outliers in the graph. From the Fig 2 it was observed that yeast extract and starch were found to be outliers, both were observed in different edges. Fig 3 indicates the levels plot, from this plot it was confirmed that yeast extract at '–' level has the highest effect on the CGTase production. From the scrutiny of ANOVA, pareto chart and probability plot it was observed that yeast extract at low concentration and starch at higher level were most significant components for effective CGTase production by mutated *Bacillus* sp. TPR71HNA6. These two components concentration was further optimized by response surface methodology.

In the literature reports Cavalitto and Mignone, 2007 [6] used the PBD with center points in order to screen 11 nutrient components for enhancement of protopectinase production. The obtained results are similar to the previous reports. Various authors worked on the different starches and found that differences in enzyme activity obtained in media with various starches may be related to their physical structure, a trait that seems to be discriminated by the Bacillus species. Thus, Gawande *et al*., 1998 [10] have observed maximum CGTase production with corn starch for *B. firmus*. Jin-Bong *et al*., 1990 [11] reported better yields with soluble starch for *B. stearothermophilus*, while Sreenivasan *et al*., 1991 [12] reported maximum CGTase activity when tapioca was used as carbon source for *B. macerans.* In this study, for mutated *Bacillus* sp.TPR71HNA6 increased CGTase yields were obtained with soluble starch.

These results are also contradicting with the other reports i.e Stefanova *et al*., 1999 [13] reported that in *B. stearothermophilus* glucose was found to be the most suitable substrate. Whereas xylose and glucose were best for *B. cereus*. [14] Thatai *et al*., 1999 [15] observed that addition of glucose and xylose to the starch medium enhanced the CGTase production. Table 5 depicts the various microorganisms and their suitable medium components for the effective CGTase production.

From these results it is observed that yeast extract has highest significant than all other variables followed by starch, peptone and urea. Glucose has the least effect on the CGTase production by mutated *Bacillus* sp. TPR71H NA6.

Table 1. Factors in coded and real values used for screening of significant components by PBD.

S. No	Compound	Levels (mg)		
		-1	0	1
1	Glucose	50	100	200
2	Maltose	50	100	200
3	Starch	50	100	200
4	Fructose	50	100	200
5	Galactose	50	100	200
6	Xylose	50	100	200
7	Yeast extract	100	150	200
8	Peptone	100	150	200
9	Urea	100	150	200
10	Ammonium sulphate	100	150	200

Table 2. Plackett–Burman design matrix used to screen the various compounds for CGTase production by mutated *Bacillus* sp. TPR71H NA6.

S. No	Glucose	Maltose	Starch	Fructose	Galactose	Xylose	Yeast extract	Peptone	Urea	A.S	S.N	CGTase activity (U/mL)
1	1	-1	1	-1	-1	-1	1	1	1	-1	1	
2	1	1	-1	1	-1	-1	-1	1	1	1	-1	
3	-1	1	1	-1	1	-1	-1	-1	1	1	1	
4	1	-1	1	1	-1	1	-1	-1	-1	1	1	
5	1	1	-1	1	1	-1	1	-1	-1	-1	1	
6	1	1	1	-1	1	1	-1	1	-1	-1	-1	
7	-1	1	1	1	-1	1	1	-1	1	-1	-1	
8	-1	-1	1	1	1	-1	1	1	-1	1	-1	
9	-1	-1	-1	1	1	1	-1	1	1	-1	1	
10	1	-1	-1	-1	1	1	1	-1	1	1	-1	
11	-1	1	-1	-1	-1	1	1	1	-1	1	1	
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	
13	0	0	0	0	0	0	0	0	0	0	0	
14	0	0	0	0	0	0	0	0	0	0	0	
15	0	0	0	0	0	0	0	0	0	0	0	
16	0	0	0	0	0	0	0	0	0	0	0	

A.S=Ammonium sulphate; S.N=Sodium nitrate

Table 3. Plackett–Burman Design matrix along with observed CGTase production by mutated *Bacillus* sp. TPR71H NA6

S.No	Glucose (mg)	Maltose (mg)	Starch (mg)	Fructose (mg)	Galactose (mg)	Xylose (mg)	Yeast extract (mg)	Peptone (mg)	Urea (mg)	A.S (mg)	S.N (mg)	CGTase activity (U/mL)
1	200	50	200	50	50	50	200	200	200	100	200	54
2	200	200	50	200	50	50	100	200	200	200	100	65
3	50	200	200	50	200	50	100	100	200	200	200	67
4	200	50	200	200	50	200	100	100	100	200	200	68
5	200	200	50	200	200	50	200	100	100	100	200	18
6	200	200	200	50	200	200	100	200	100	100	100	56
7	50	200	200	200	50	200	200	100	200	100	100	48
8	50	50	200	200	200	50	200	200	100	200	100	42
9	50	50	50	200	200	200	100	200	200	100	200	64
10	200	50	50	50	200	200	200	100	200	200	100	32
11	50	200	50	50	50	200	200	200	100	200	200	51
12	50	50	50	50	50	50	100	100	100	100	100	21
13	100	100	100	100	100	100	150	150	150	150	150	51
14	100	100	100	100	100	100	150	150	150	150	150	53
15	100	100	100	100	100	100	150	150	150	150	150	54
16	100	100	100	100	100	100	150	150	150	150	150	51

A.S=Ammonium sulphate; S.N=Sodium nitrate

Table 4. Effects and ANOVA Table

	Effect	Coefficients	SS	df	MS	F-value	t-value	p-value
Mean/ Intercept	49.6875	49.68750	--	--	--		61.50360	0.000000
Glucose	0.0000	0.00000	0.000	1	0.0000	0.00000	0.00000	1.000000
Maltose	4.0000	2.00000	48.000	1	48.0000	4.59651	2.14395	0.098650
***Starch**	14.0000	7.00000	588.000	1	588.0000	56.30723	7.50381	0.001688
Fructose	4.0000	2.00000	48.000	1	48.0000	4.59651	2.14395	0.098650
Galactose	-4.6667	-2.33333	65.333	1	65.3333	6.25636	-2.50127	0.066676
***Xylose**	8.6667	4.33333	225.333	1	225.3333	21.57805	4.64522	0.009696
***Yeast extract**	-16.0000	-8.00000	768.000	1	768.0000	73.54414	-8.57579	0.001015
***Peptone**	13.0000	6.50000	507.000	1	507.0000	48.55062	6.96783	0.002230
***Urea**	12.3333	6.16667	456.333	1	456.3333	43.69875	6.61050	0.002715
***A.S**	10.6667	5.33333	341.333	1	341.3333	32.68628	5.71719	0.004631
***S.N**	9.6667	4.83333	280.333	1	280.3333	26.84489	5.18121	0.006601
Error			41.771	4	10.4427			
Total SS			3369.438	15				

*Indicates significant factors

Table 5. Summary of CGTase production by various types of micro-organisms and fermentation medium in batch fermentation

Micro-organism	Carbon source	Nitrogen source	Minerals	Ref
B. amyloliquefaciens	Glucose & starch	Yeast extract, peptone & meat extract		[16]
B. amyloliquefaciens AL 35	Potato starch	Yeast extract, peptone	$MgSO_4$ & K_2HPO_4	[17]
B. halophilus INMIA-3849	Potato starch	Peptone & yeast extract	NaCl. KCl, $MgSO_4.7H_2O$ & natrium citrate	[18]
Klebsiella oxytoca 19-1	Soluble starch	Polypeptone	KH_2PO_4 & $MgSO_4$	[19]
B. sphaericus	β-CD	Peptone & yeast extract	NaCl	[20]
Alkalophilic *Bacillus* sp.	Soluble starch	Yeast extract & peptone	$MgSO_4$, K_2HPO_4 & Na_2CO_3	[21]
Alkalophilic bacteria	Soluble starch	Yeast extract & peptone	$MgSO_4$, K_2HPO_4 & Na_2CO_3	[22]
Bacillus sp.	Soluble starch	Yeast extract & peptone	$MgSO_4$, K_2HPO_4 & Na_2CO_3	[23]
B. firmus	Soluble starch	Yeast extract & peptone	$MgSO_4$, K_2HPO_4 & Na_2CO_3	[24]
B. circulans C31	Soluble starch	Meat extract, yeast extract & polypeptone	NaCl.	[25]
B. stearothermophillus HR1	Sago starch	Peptone & casein	K_2HPO_4, & $CaCl_2$	[26]
Bacillus sp. TS1-1	Sago starch	yeast extract	$MgSO_4.7H_2O$, K_2HPO_4 & Na2CO3	[27]
Bacillus sp. G1	Tapioca starch	yeast extract	$MgSO_4.7H_2O$, K_2HPO_4 & Na_2CO_3	[28]

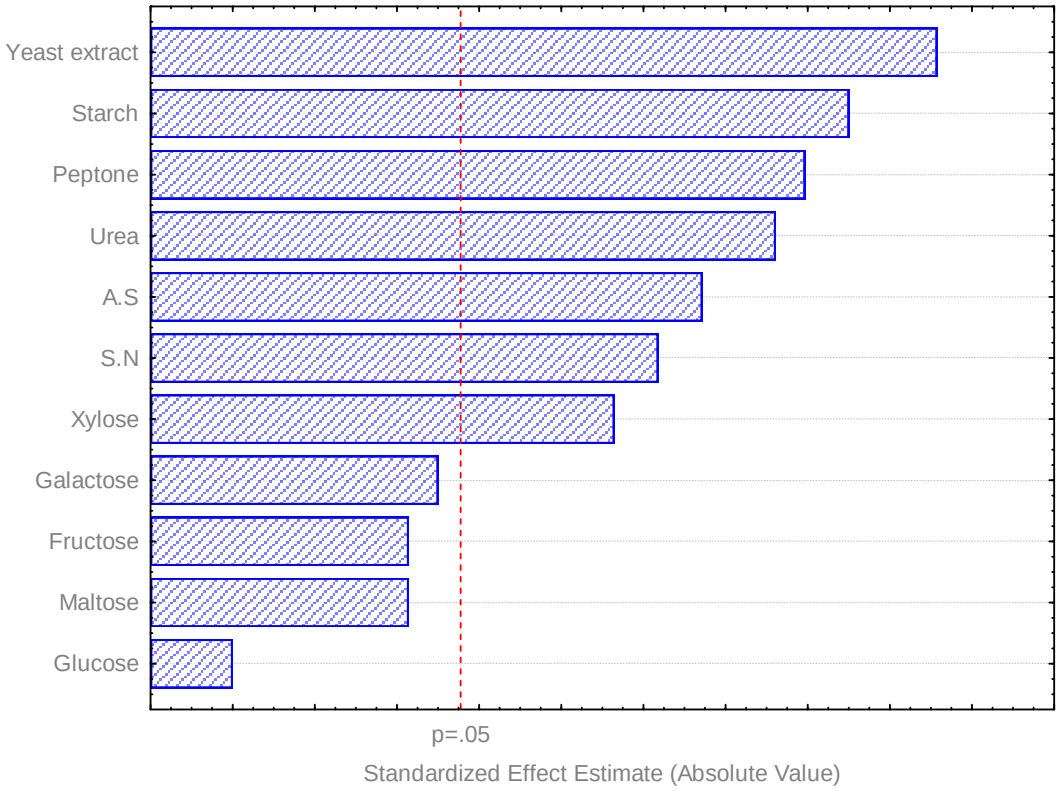

Fig 1. Pareto chart of standardized effects

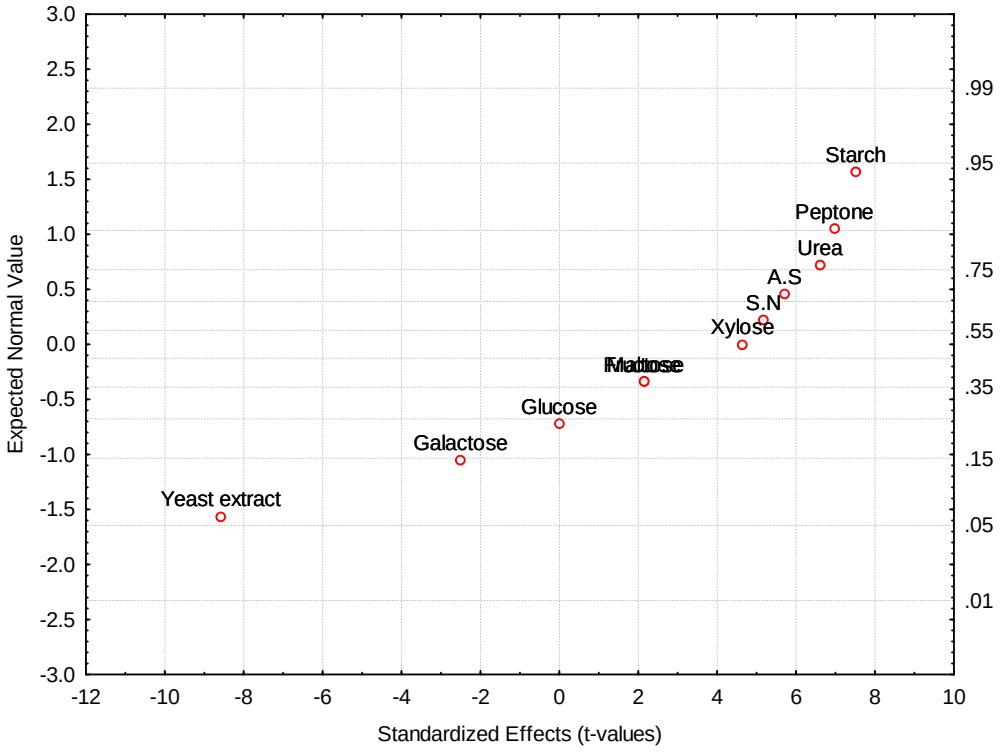

Fig 2. Probability plot of the variables used in the PBD for CGTase production by Mutated *Bacillus* sp. TPR71H NA6

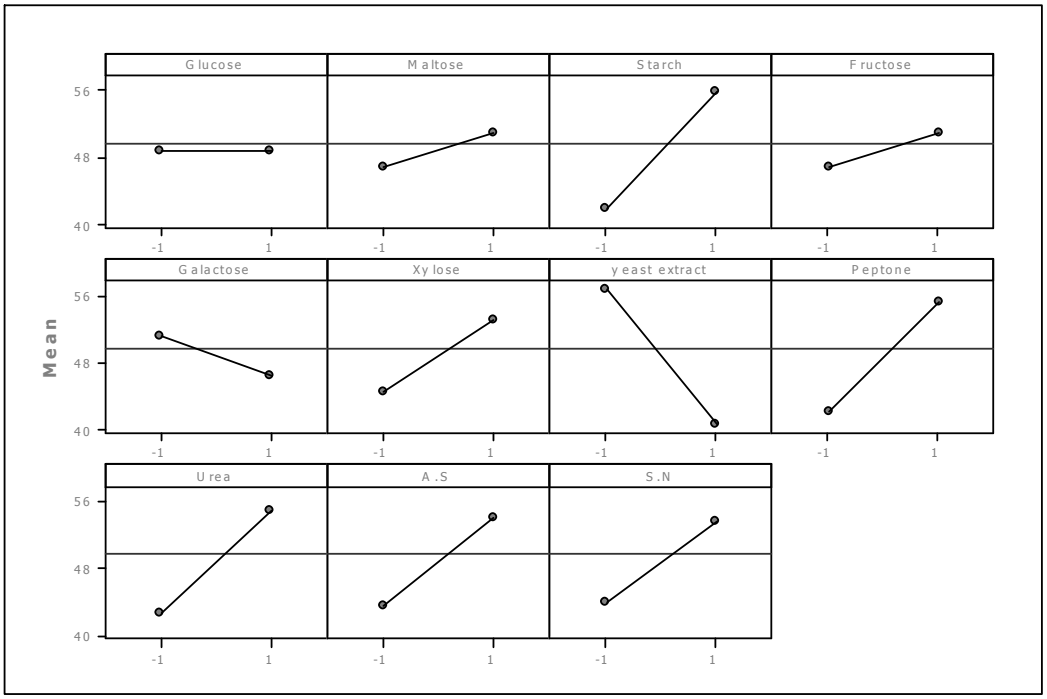

Fig 3. Levels plot for various components used in the PBD for CGTase production by mutated *Bacillus* sp. TPR71H NA6

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