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OPTIMIZATION OF BIOPROCESS PARAMETERS FOR THE PRODUCTION OF β-GALACTOSIDASE BY EMPLOYING STATISTICAL METHODS

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ABSTRACT

Optimization of medium components or process parameters by the classical method i.e., changing one independent variable (lactose, soya peptone, ammonium di-hydrogen phosphate and agitation) while fixing all other at a fixed level is extremely time consuming and expensive for a large number of variables. To overcome this difficulty, experimental factorial design and response surface methodology can be employed to optimize the medium components and cultural conditions. The marine derived fungus *Aspergillus flavus* MTCC*9349 has been studied for the production of β-galactosidase. The effect of medium, pH, temperature, carbon source, nitrogen source and incubation time on the production of β-galactosidase was studied by employing statistical methods. Central composite experimental design maximizes the amount of information that can be obtained, while limiting the number of individual experiments. By employing the statistical methods 1.95 times increase in the yield of the enzyme was obtained (499.63 U/gm dry cell weight).

KEYWORDS

Placket & Burman designs, Response surface method, Transgalactosylation, Dry cell weight, SmF Submerged fermentation, SSF Solid state fermentation.

INTRODUCTION

β-galactosidase (E C 3.2.1.2 3) is mainly used in food processing and dairy industry. The enzyme not only hydrolyses lactose in to its mono saccharides glucose and galactose, and also plays an important role in the production of galacto-oligosaccharides by a mechanism called transgalactosylation. Low activity of β-galactosidase causes digestive insufficiency, called lactose intolerance. Decreased β-galactosidase activity is rather frequent especially with seniors and infants. β-galactosidase activity was assayed by using ONPG as a substrate. The statistical methodologies are preferred because of various advantages in their use in terms of rapid and reliable short listing of nutrients. A substantial reduction in total number of experiments results in saving of valuable time, glassware, chemicals and good deal of manpower[1]. Selection of appropriate carbon, nitrogen and other nutrients is one of the most critical stages in the development of an

efficient and economic process. The use of these methodologies also results in saving of tremendous amount of time[2]. The statistical methodologies have been earlier applied only in a limited number of SmF and SSF processes for the production of microbial metabolites.

1. OBJECTIVE

Objective of the present work is to improve the yield of β-galactosidase activity by using statistical methods like Placket & Burman designs (PBD) and Response surface method (RSM).

2. MATERIALS AND METHODS

2.1 Chemicals: 2-nitrophenyl- β-D-galactopyranoside was procured from Carbosynth Limited, Berkshire, U.K. All other chemicals and medium constituents in this study were of analytical grade and procured from Sigma-Aldrich and s.d fine chemicals.

2.2 Microorganism: The *Aspergillus flavus* derived from the samples collected at Bay of Bengal near Vishakapatnam in August 2005, using dilution-plate method on PDA medium. It was identified according to its morphological characteristics and confirmed by Prof. Ananthpadnabhan (IMTECH, Chandigarh). Working stocks were prepared on Potato Dextrose Agar slants and stored at 4^0 C.

2.3 Fermentation and assay of samples: The experiments were conducted and galactosidase activity of the samples was estimated by using Ortho Nitrophenyl-β-galactoside (ONPG) substrate. Standard curve was constructed with Ortho nitro-phenol.

2.4 Methods: Selection of appropriate carbon and nitrogen sources including additional nutrients is one of the most critical stages in the development of an efficient fermentation process. The statistical methodology is used for understanding the interactions among the nutrients at varying concentrations and also results in a substantial reduction of total number of experiments. Thus, the statistical methods[3] are advantageous and allow rapid and reliable short listing of a few nutrients for further optimization and these methods were applied to a limited number of submerged fermentation processes[4] and Solid-state fermentation processes. The use of Placket & burman designs (PBD), a statistical methodology in the form of an orthogonal matrix, allows screening of up to 'n-1' variables in just 'n' experiments. In this design, generally a multiple of four, i. e, 4, 8, 12, 16, 20….4n experiments, are required to screen 3, 7, 11, 15, 19 … 4n-1, compounds respectively, where 'n' is a multiple of 4. The ingredients are taken at two levels (lower and higher). Lower level in the design is represented as "_" and higher level as "+". The contribution of an ingredient towards the growth of the organism or yield of the enzyme is determined based on the t- value (main effect) calculated from the experimental result. The value (main effect) of an ingredient is calculated as follows. Some medium components and process parameters have been predicted by Plackett-Burman Design (PBD), to play a very significant role in enhancing the production of enzyme. There are considered to be critical components during the production of enzyme. The statistical optimization of these components (lactose, soya peptone, ammonium di-hydrogen phosphate and agitation) was carried-out using the Response Surface Methodology (RSM).

Recently, the experimental factorial design[6] and response surface methodology[7] have already been successfully applied in optimization of the media and culture conditions for the production of various enzymes. RSM has been applied for optimization of the media and culture conditions in many fermentation processes[8] for the production of the primary and secondary metabolites and to study the interaction between the parameters.[9] A 2^4 full factorial central composite design and response surface methodology (RSM) was used in this study.[10]

2.4 Experimental design and optimization by response surface methodology: Response surface methodology consists of a group of empirical techniques devoted to the evaluation of

relations existing between a cluster of controlled experimental factors and the measured responses, according to one or more selected criteria[11]. A prior knowledge and understanding of the process variables under investigation are necessary for achieving a more realistic model[12]. Based on the results obtained in preliminary experiments, lactose, soya peptone, ammonium di-hydrogen phosphate and agitation were found to be major variables in the enzyme production. Hence these variables were selected to find the optimized conditions for higher enzyme productivity using central composite design and response surface methodology.

The range and the levels of the experimental variables investigated in this study are given in the Table 1.1. In the first step of optimization, with Plackett-Burman design, lactose, soya peptone, ammonium di hydrogen phosphate and agitation were found to be the important factors affecting □□galactosidase production significantly. The central values (zero level) chosen for experimental design[13] were lactose 1.25%, soya peptone 1.25%, ammonium di-hydrogen phosphate 0.75, and agitation at 150 rpm.

For this study, 2^{5-1} full factorial design consisting of 16 experiments with eight star points and seven replicates at the central points were employed to fit the second order polynomial model, which indicated that 31 experiments are required for this procedure. The 'Minitab' software was used for regression and graphical analysis of the data obtained. The optimum values of the selected variables were obtained by solving the regression equation and also by analyzing the response surface contour plots.[14] All the experiments were conducted in triplicate and the average values of □□galactosidase yield are presented in Table 1.2. The predicted values were calculated by using the mathematical model derived.

Table 1.1.

Experimental range and levels of the independent variables

Variable (%)	Range and levels				
	-2	**-1**	**0**	**+1**	**+2**
Lactose (%)	0.75	1	1.25	1.5	1.75
Soya peptone (%)	0.75	1	1.25	1.5	1.75
Ammonium di hydrogen phosphate (%)	0.25	0.5	0.75	1	1.25
Agitation (rpm)	130	140	150	160	170

Table 1.2.

Central composite design consisting of 31 experiments for the study of four experimental factors in coded units.

Run NO	L	S	A	E	Coefficients assessed by	Enzyme yield (U/gm)	
						Observed response	Predicted response
1	-1	-1	-1	-1	Full factorial Design	343.2	339.208
2	1	-1	-1	-1		363.3	359.825
3	-1	1	-1	-1		401.3	385.108
4	1	1	-1	-1		376.8	377.125
5	-1	-1	1	-1		365.4	356.092
6	1	-1	1	-1		354.0	342.558
7	-1	1	1	-1		423.4	402.642
8	1	1	1	-1		365.6	360.508
9	-1	-1	-1	1		302.1	295.375

10	1	-1	-1	1		412.0	400.842
11	-1	1	-1	1		365.5	366.725
12	1	1	-1	1		465.9	443.592
13	-1	-1	1	1		343.2	310.958
14	1	-1	1	1		375.4	382.275
15	-1	1	1	1		386.5	382.958
16	1	1	1	1		453.6	425.675
17	-2	0	0	0	Star points	289.9	324.950
18	2	0	0	0		359.8	388.283
19	0	-2	0	0	(8 Points)	354.0	391.017
20	0	2	0	0		453.8	480.317
21	0	0	-2	0		398.0	430.733
22	0	0	2	0		398.9	429.700
23	0	0	0	-2		212.2	249.700
24	0	0	0	2		245.0	271.033
25	0	0	0	0	Central	486.5	486.486
26	0	0	0	0	points	479.7	486.486
27	0	0	0	0		465.8	486.486
28	0	0	0	0	(7 points)	498.7	486.486
29	0	0	0	0		489.9	486.486
30	0	0	0	0		487.9	486.486
31	0	0	0	0		496.9	486.486

The variables are coded as shown below: Lactose: L, Soya peptone: S, Ammonium di hydrogen phosphate: A, Agitation E

Table 1.3.
Estimated Regression Coefficients for Yield of galactosidase

Term	Coef	SE coef	t- value	p-value
Constant	486.486	10.141	47.973	0.000
L	15.658	5.477	2.859	0.011
S	24.150	5.477	4.410	0.000
A	1.617	5.477	0.295	0.772
E	7.367	5.477	1.345	0.197
L*L	-33.388	5.017	-6.655	0.000
S*S	-13.626	5.017	-2.716	0.015
A*A	-14.988	5.017	-2.987	0.009
E*E	-57.451	5.017	-11.450	0.000
L*S	-4.100	6.708	-0.611	0.550
L*A	-10.987	6.708	-1.638	0.121
L*E	23.950	6.708	3.571	0.003
S*A	0.138	6.708	0.020	0.984
S*E	6.100	6.708	0.909	0.377
A*E	-0.663	6.708	-0.099	0.923

Table 1.4: Observed responses and predicted values

Run No	Enzyme yield (U/gm)		
	Observed response	Predicted response	Residual
1	343.2	339.208	3.992
2	363.3	359.825	3.475
3	401.3	385.108	16.192
4	376.8	377.125	-0.325
5	365.4	356.092	9.308
6	354	342.558	11.442
7	423.4	402.642	20.758
8	365.6	360.508	5.092
9	302.1	295.375	6.725
10	412	400.842	11.158
11	365.5	366.725	-1.225
12	465.9	443.592	22.308
13	343.2	310.958	32.242
14	375.4	382.275	-6.875
15	386.5	382.958	3.542
16	453.6	425.675	27.925
17	289.9	324.95	-35.05
18	359.8	388.283	-28.483
19	354	391.017	-37.017
20	453.8	480.317	-26.517
21	398	430.733	-32.733
22	398.9	429.7	-30.8
23	212.2	249.7	-37.5
24	245	271.033	-26.033
25	486.5	486.486	0.014
26	479.7	486.486	-6.786
27	465.8	486.486	-20.686
28	498.7	486.486	12.214
29	489.9	486.486	3.414
30	487.9	486.486	1.414
31	496.9	486.486	10.414

R^2 = 92.91 %, R^2 (pred) = 61.15%, R^2 (adj) = 86.70%, DF= Degrees of freedom, SS= Sum of squares, P = Probability

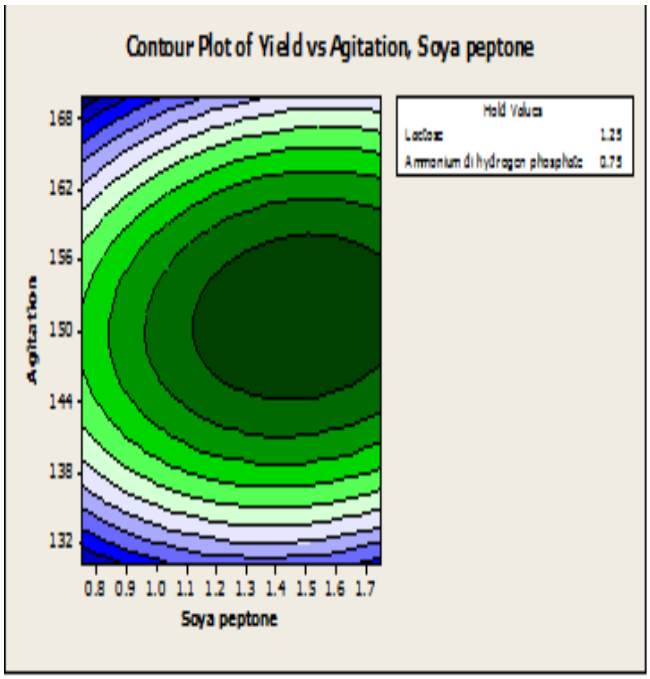

Figure 1.1: *Yield versus agitation and soya peptone (contour plot)*

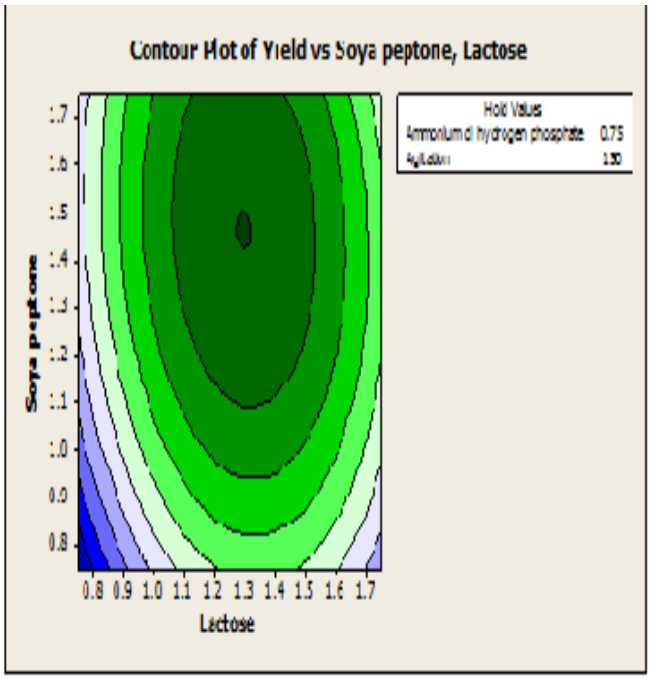

Figure 1.2: *Yield versus soya peptone and lactose (contour plot)*

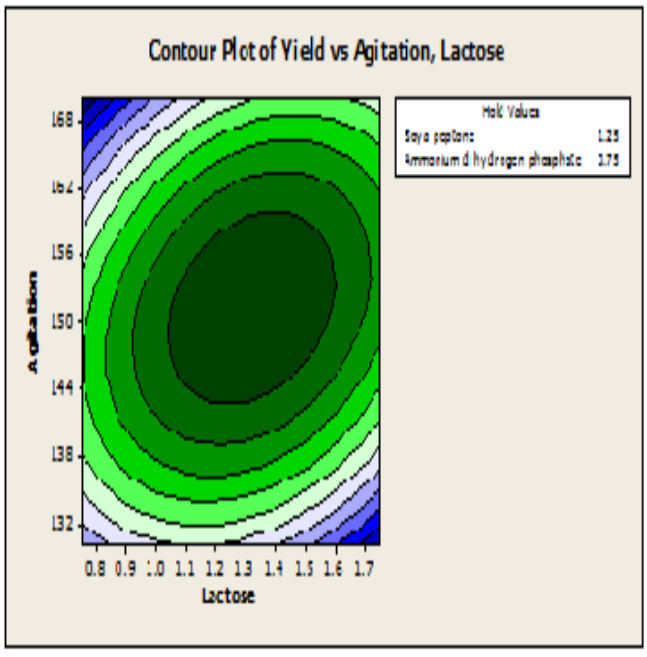

Figure 1.3: *Yield versus agitation and lactose (contour plot)*

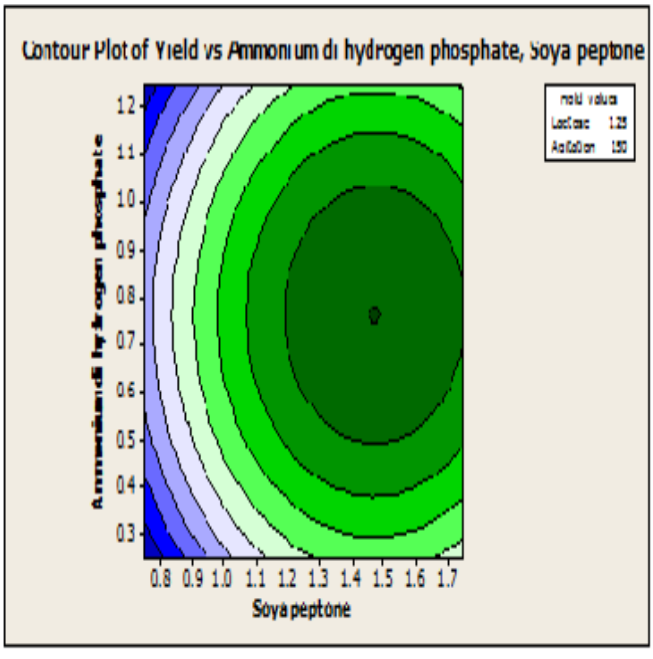

Figure 1.4: *Yield versus ammonium di hydrogen phosphate and soya peptone (contour plot)*

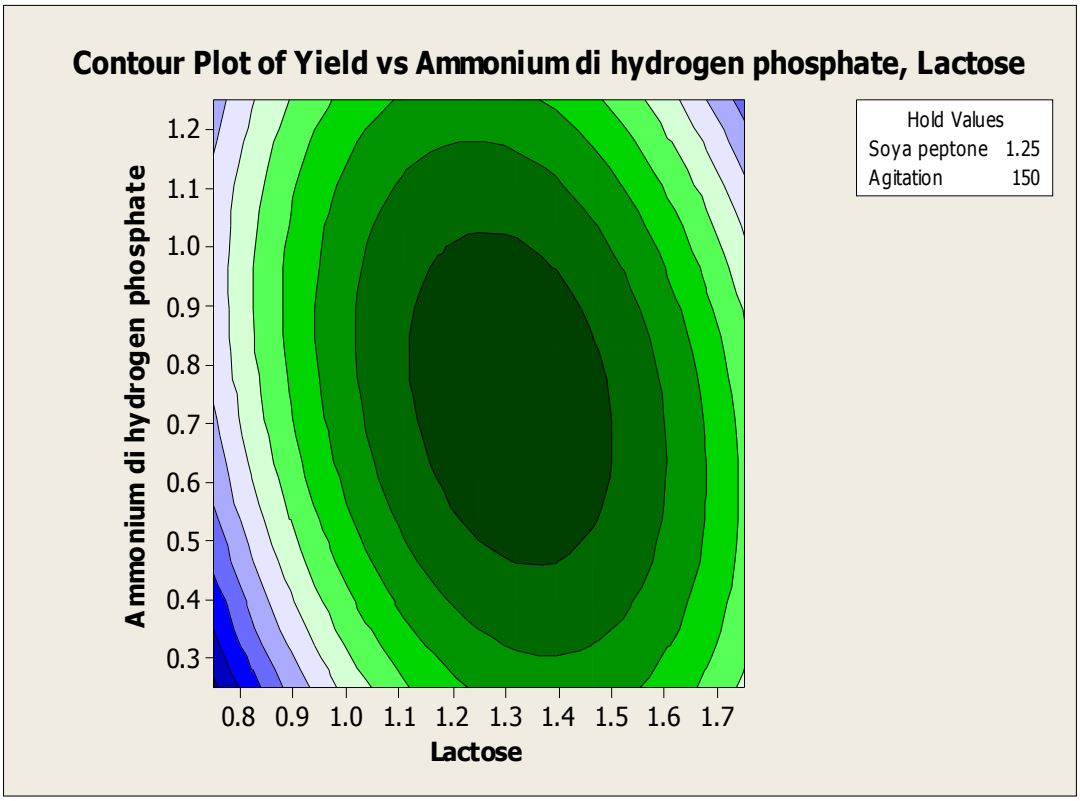

Figure 1.5: ***Yield versus ammonium di hydrogen phosphate and lactose (contour plot)***

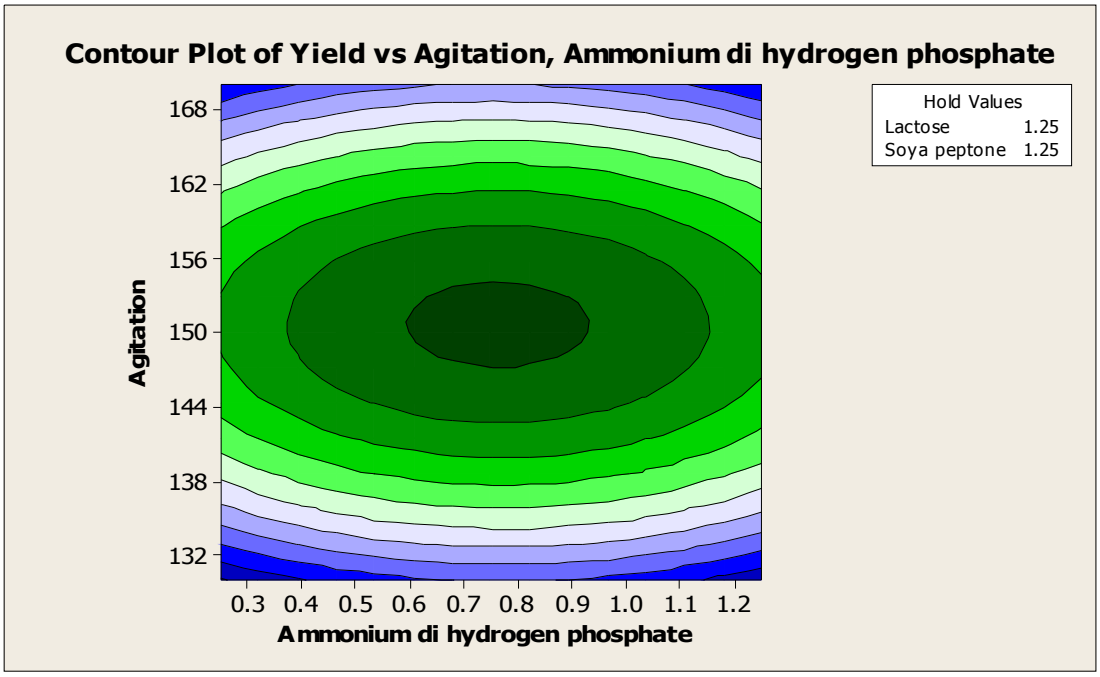

Figure 1.6: ***Yield versus agitation and ammonium di hydrogen phosphate (contour plot)***

RESULTS AND DISCUSSION

The results of the second order response surface model fitting in the form of analysis of variance (ANOVA) are given the Table 1.4 (Model coefficients). The fisher F-test [$F_{(20,15)}$ =7.38] with a very low probability value (P_{model} > F = 0.0001) demonstrates a very high significance for the regression model.[13]

The goodness of fit of the model was checked by the determination coefficient (R^2). In this case, the value of the determination coefficient (R^2 = 0. 9291) indicates that the model does not explain only 7 % of the total variations. The value of adjusted determination coefficient (Adj R^2 = 0.867) is also very high, which indicated a high significance of the model.

The application of response surface methodology yielded the following regression equation which is an empirical relationship between the logarithmic values of enzyme yields and test variables in coded unit.

Y = 486.486+ 15.658*L + 24.150*S + 1.617*A + 7.367*E - 33.388*L*L -13.626*S*S - 14.988 *A*A - 57.451*E*E - 4.100*L*S - 10.987*L*A + 23.950*L*E + 0.138*S*A + 6.100*S*E - 0.663*A*E.

Where Y is the response, i.e., the enzyme yield and the terms L, S, A and E are the coded values of the test variables lactose, soya peptone, ammonium di- hydrogen phosphate and agitation respectively. The significance of each coefficient was determined by student's t-test and p values. The larger the magnitude of the t-value and the smaller the p-value, the more significant is the corresponding coefficient[13]. This implies that the quadratic main effects of lactose (L*L < 0.0001, E*E < 0.0002) are more significant than their first order main effects (lactose <0.011, and agitation < 0.197). Whereas, the first order main effect of soya peptone is highly significant, as it is evident from the respective p-value (S<0.000) compared to the second order main effects of (S*S<0.15, L*S<0.550, S*A<0.984, S*E< 0.377)

Contour plots of the response surface as a function of two factors at a time, holding all other factors at fixed levels (Zero for instance), are more helpful in understanding both the main effect and the interaction of these two factors. These plots can be easily obtained by calculating from the model, the values taken by one factor where the second varies (from -2 to +2, step 1 for instance) with constrain of a given Y value. The yield values for different concentrations of the variables can also be predicted from the respective contour plots (Figures 1.1 to 1.6). The maximum predicted yield is indicated by the surface confined in the smallest ellipse in the contour diagram.

Figures 1.1, 1.2 and 1.4 showed that the relatively circular nature of the line and gives the optimum concentration of soya peptone is around 1.5%. From the Figures 1.2, 1.3 and 1.5 it is evident that the optimum concentration of lactose is around 1.3 %. From the Figures 1.4, 1.5 and 1.6 showed that the optimum concentration of ammonium di hydrogen phosphate is around 0.7%. From the Figures 1.1, 1.3 and 1.6, it is evident that the 150 rpm agitation was optimum for the production of β-galactosidase.

Contour plots are very helpful in visualizing the main effects and interaction of the factors. Thus, smaller and less time consuming experimental designs are generally sufficient for optimization of many fermentation processes. In the present study, the use of a central composite factorial design in β-galactosidase production has been demonstrated by determining the conditions leading to higher yields of the enzyme.

CONCLUSION

Statistical analysis proved to be a useful and powerful tool in developing optimum fermentation conditions. The optimum conditions established for the production of β-galactosidase were (%) lactose 1.31, Soya peptone 1.46, ammonium di hydrogen phosphate 0.734 and agitation 151 rpm. Employing the above optimal conditions, fermentation runs and assays were carried out (in triplicate), as described earlier and average values were calculated. An enzyme yield of 499.63

(~500) U/gm was obtained with the above optimal data.

Yield before optimization =256 U/gm DCW

Yield after optimization =499.63 U/gm DCW

Therefore employing the statistical methods 1.95 times increase in the yield of the enzyme was obtained. So the model predicts the maximum yield of the □□galactosidase can be obtained using the above optimum concentrations of the variables is 499 U/gm DCW. The verification of the results using the optimized conditions was accomplished by carrying out shake flask experiments, which showed a higher yield of □□galactosidase 511 U/gm. These experimental finding are in close agreement with the model predictions.

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