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Statistical Optimization of Fermentation Conditions for L-Glutamic Acid Production by Free Cells of Corynebacterium Glutamicum ATCC13032

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ABSTRACT :*Response surface methodology (RSM) was used to optimize the fermentation variables for enhancing L-Glutamic acid production by free cells of Corynebacteriunglutamicum.The effects of four independent variables such as pH, Temperature, Agitation rate and Glucose concentration were found to be significantly affecting the percentage yield of L-Glutamic acid. Further 2^4 factorial Central Composite Design (CCD) was used to determine the optimal levels of significant variables .The predicted optimum values were obtained as pH: 5.90, Temperature : 29.97^{0}C, Agitation rate: 158.58 rpm and glucose concentration: 101.04g/L. Under optimal conditions, the percentage yield of L-Glutamic acid production was 0.3518.The determination coefficient (R^2) was 0.9957, which ensures adequate credibility of the model.*

KEYWORDS : *L-Glutamic acid, free cells, Corynebacterium glutamicum, Process variables, Response surface methodology (RSM), Optimization*

I. INTRODUCTION

In early days of 1900s, MSG was extracted from natural protein rich foods, such as seaweed and it was also prepared by the acid hydrolysis of wheat gluten or soybean protein which were expensive materials (Jyothi. et.al, 2005). MSG is used in foods as a taste enhancer because of its own unique flavour called"Umami"in Japanese. Prepared foods usually contain 0.1-0.8%MSG but especially in east Asian dishes a higher supplementation is common(Herman.T,2003). Monosodium glutamate (MSG), the sodium salt of L-glutamic acid is a popular flavour enhancer (Calik.G. et.al 2001) and additivefor foods. It was used primarily in Asian foods but its use is now wide spread (Jyothi. et.al, 2005). L-Glutamic acid is widely used flavor enhancer, feed supplements, food additives& therapeutic compound. L-Glutamic acid is mainly produced through microbial method (Amin.G. et.al,2007).Mono Sodium Glutamate (MSG) is largest product out of all amino acids and recent survey indicates that the production is about 1.5 million tons and the market is growing by about 6% every year (Taoro.P. et.al.1963). L-Glutamic acid is produced mainly through microbial means because chemical method produces a racemic mixture of DL-Glutamic acid. This production of Glutamic acid from sugar is thought to proceed predominantly through the Embden-Meyerhof-Paranas (EMP) pathway and the early steps of the tricarboxylic acid cycle, with oxygen acting as terminal electron acceptor **(**Jerome.b.et.al, 1969). Various fermentation techniques have been reported for the production of L-Glutamic acid (Amin.G.et.al, 1993), but with wide variation in sugar conversion efficiency into L-Glutamic acid (Yoshioka.T. et.al, 1999). In all systems and among the other parameters, excretion of L-Glutamic acid by bacterial cells was the rate limiting factor (Sunuk.C.et.al, 2004). The demand for amino acid as food supplements and in pharmaceutical industries is fast expanding.

II. MATERIALS AND METHODS

Microorganism

The organism employed throughout in this experimentation was *Corynebacterium glutamicum ATCC 13032* obtained from Institute of Microbial Technology, Chandigarh (India) .The culture was maintained on the agar slants of PASB medium containing composition (g/L) of peptone- 5,Agar -20, Sodium chloride-5 and Beef extract-3.The pH of the medium adjusted to 7 and incubated at 37^{0}C for 24 hours.

Fermentation technique

A Completely grown slant of 24 hours old *Corynebacterium glutamicum* and were scrapped off and suspended in 0.01 M citrate buffer (pH-7.0). The cell suspension was shaken thoroughly to break up the cell aggregates. The cell count was determined by plating each mL of the cell suspension, on solid agar medium. The cell counts were adjusted in the range of 10^{-5} to 10^{-9} cells per mL. The cells were grown for 24 h at 30°C in 250 mL Erlenmeyer flasks containing 50 mL of inoculation medium on a rotary shaker at 160 rpm. The cells were separated from the inoculation medium by centrifugation and washed thoroughly with 0.01 M citrate buffer (pH 7.0) Fermentations conditions were maintained at Temperature - 30°C, pH-6.0, Agitation rate - 160rpm, Glucose concentration-100g/L, Aeration rate -1.0 vvm Biotin Concentration -1.0g/L and Fermentation time – 96 hours. Among these parameters only significant parameters has been taken out for the optimization of the production L-Glutamic acid.

III. RESULTS AND DISCUSSION

Optimization for free cells using Response Surface Methodology (RSM)

The effects of four independent variables (pH, Temperature, Agitation rate and glucose concentration) on % yield of Glutamic acid are analyzed using Central Composite Design (CCD). The optimum conditions for the four independent variables on the extent of Glutamic acid yield are formed within the quadratic model. Levels of different process variables for percentage yield are shown in table–3.1

Table 3.1: Levels of different process variables in coded and un-coded form for % yield of Glutamic acid using free cells

Variable	Name	Range and levels				
		-2	-1	0	1	2
X_1	pH	5	5.5	6	6.5	7
X_2	Temperature, ^oC	26	28	30	32	34
X_3	Agitation rate, rpm	80	120	160	200	240
X_4	Glucose concentration, g/L	80	90	100	110	120

Regression equation for the optimization of % yield by using STATISTICA Software 6.0 Version is:

% Yield of Glutamic acid (Y) is function of pH (X_1), Temperature (X_2), Agitation rate (X_3) and Glucose concentration (X_4).

The multiple regression analysis of the experimental data has yield the following equation:

$$\mathbf{Y = -5.76515 + 0.64447\ X_1 + 0.17170\ X_2 + 0.00347\ X_3 + 0.02704\ X_4 - 0.055\ X_1^2 - 0.00289 X_2^2 - 0.00001\ X_3^2 - 0.00014\ X_4^2 + 0.00013\ X_1X_2 - 0.00004\ X_1X_3 + 0.00007 X_1X_4 - 0.00001\ X_2X_3 + 0.00002\ X_2X_4 - 0.0000\ X_3X_4 \quad \text{----- (3.1)}}$$

Table-3.2 represents the results obtained in CCD. The response obtained in the form of analysis of variance (ANOVA) from regression eq.3.1 is put together in table–3.3. Fischer's '*F*-statistics' value is defined as MS_{model}/MS_{error}, where MS is mean square. Fischer's '*F*-statistics' value, having a low probability 'p' value, indicates high significance.

Table 3.2: Results from CCD for % yield of Glutamic acid by free cells

Run No.	X_1, pH	X_2, T	X_3, A.R	X_4, G.C	% yield of Glutamic acid	
					Experimental	Predicted
1	-1 (5.5)	-1 (28)	-1 (120)	-1 (90)	0.2998	0.300475
2	-1 (5.5)	-1 (28)	-1 (120)	1 (110)	0.3068	0.306308
3	-1 (5.5)	-1 (28)	1 (200)	-1 (90)	0.3018	0.302642
4	-1 (5.5)	-1 (28)	1 (200)	1 (110)	0.3052	0.305275
5	-1 (5.5)	1 (32)	-1 (120)	-1 (90)	0.3002	0.299858
6	-1 (5.5)	1 (32)	-1 (120)	1 (110)	0.3066	0.307492
7	-1 (5.5)	1 (32)	1 (200)	-1 (90)	0.2998	0.299525
8	-1 (5.5)	1 (32)	1 (200)	1 (110)	0.3038	0.303958
9	1 (6.5)	-1 (28)	-1 (120)	-1 (90)	0.2906	0.290392
10	1 (6.5)	-1 (28)	-1 (120)	1 (110)	0.2966	0.297625

11	1 (6.5)	-1 (28)	1 (200)	-1 (90)	0.2898	0.289658
12	1 (6.5)	-1 (28)	1 (200)	1 (110)	0.2934	0.293692
13	1 (6.5)	1 (32)	-1 (120)	-1 (90)	0.2896	0.290275
14	1 (6.5)	1 (32)	-1 (120)	1 (110)	0.3002	0.299308
15	1 (6.5)	1 (32)	1 (200)	-1 (90)	0.2866	0.287042
16	1 (6.5)	1 (32)	1 (200)	1 (110)	0.2928	0.292875
17	-2 (5.0)	0 (30)	0 (160)	0 (100)	0.3072	0.306783
18	2 (7.0)	0 (30)	0 (160)	0 (100)	0.2859	0.285617
19	0 (6.0)	-2 (26)	0 (160)	0 (100)	0.3063	0.305617
20	0 (6.0)	2 (34)	0 (160)	0 (100)	0.3042	0.304183
21	0 (6.0)	0 (30)	-2 (80)	0 (100)	0.2968	0.296483
22	0 (6.0)	0 (30)	2 (240)	0 (100)	0.2926	0.292217
23	0 (6.0)	0 (30)	0 (160)	-2 (80)	0.2908	0.290317
24	0 (6.0)	0 (30)	0 (160)	2 (120)	0.3022	0.301983
25	0 (6.0)	0 (30)	0 (160)	0 (100)	0.3512	0.351200
26	0 (6.0)	0 (30)	0 (160)	0 (100)	0.3512	0.351200
27	0 (6.0)	0 (30)	0 (160)	0 (100)	0.3512	0.351200
28	0 (6.0)	0 (30)	0 (160)	0 (100)	0.3512	0.351200
29	0 (6.0)	0 (30)	0 (160)	0 (100)	0.3512	0.351200
30	0 (6.0)	0 (30)	0 (160)	0 (100)	0.3512	0.351200

Experimental conditions [Coded Values] and observed response values of central composite design with 2^4 factorial runs, 6- central points and 8- axial points.

Table 3.3: ANOVA of % yield of Glutamic acid for entire quadratic model

Source of variation	SS	df	Mean square(MS)	F-value	*P* > F
Model	0.014709	14	0.00105064	2626.6	0.00000
Error	0.000006	15	0.0000004		
Total	0.014715				

Df- degree of freedom; SS- sum of squares; F- factor F; *P*- probability.
R^2=0.99957; R^2 (adj):0.99917

Table 3.4: Estimated regression coefficients for the % yield of Glutamic acid

Terms	Regression coefficient	Standard error of the coefficient	t-value	*P*-value
Mean/Interc.	-5.76515	0.060056	-95.996	0.000000
(1)pH (L)	0.64447	0.008451	76.260	0.000000
pH (Q)	-0.05500	0.000496	-110.912	0.000000
(2)Tempertaure, ^oC (L)	0.17170	0.002275	75.475	0.000000
Tempertaure, ^oC (Q)	-0.00289	0.000031	-93.367	0.000000
(3)Agitation Rate, rpm(L)	0.00347	0.000091	37.952	0.000000
Agitation Rate, rpm(Q)	-0.00001	0.000000	-114.642	0.000000
(4)Glucose Concentration, g/L(L)	0.02704	0.000404	66.961	0.000000
Glucose Concentration, g/L(Q)	-0.00014	0.000001	-111.012	0.000000
1L by 2L	0.00013	0.000162	0.770	0.453204[a]
1L by 3L	-0.00004	0.000008	-4.467	0.000453
1L by 4L	0.00007	0.000032	2.156	0.047705
2L by 3L	-0.00001	0.000002	-3.850	0.001572
2L by 4L	0.00002	0.000008	2.772	0.014232
3L by 4L	-0.00000	0.000000	-4.929	0.000182

[a]insignificant ($P \geq 0.05$)

The ANOVA of the regression model is sufficiently great, as proven from the Fisher's *F*-test (F_{model} = 2626.6) and has a very low probability value ($P_{model} > F$=0.000000). Besides, the computed *F*-value [$F_{0.05\ (14.15)} = MS_{model}/MS_{error}$ = 2626.6] is much higher compared to *F*-value ($F_{0.05\ (14.15)\ tabulars}$ = 2.42) at 5% level, suggesting that the treatment differences are sufficiently great. Student's *t*-test can implicate regression coefficient of the parameter, while pattern of interactions amidst all the factors can be entailed by 'p' values. It is noted from table-3.4 that more significant corresponding coefficient term can be possessed by having high't' value and low 'P' value. By analyzing't' and 'p' values from table-5.7, X_3, X_4, X_1^2, X_2^2, X_3^2, X_4^2, X_1X_3, X_1X_4, X_2X_3, X_2X_4 and X_4X_3 have high importance to explain the individual and interaction effects of independent variables on the % yield of glutamic acid to anticipate the response. Rest of the terms (X_1X_2) are undistinguished in eq.3.1. The model is reduced to the following form by excluding undistinguished terms in eq.3.1.

$$\mathbf{Y = -5.76515 + 0.64447\ X_1 + 0.17170\ X_2 + 0.00347\ X_3 + 0.02704\ X_4 - 0.055\ X_1^2 - 0.00289 X_2^2 - 0.00001\ X_3^2 - 0.00014\ X_4^2 - 0.00004\ X_1X_3 + 0.00007 X_1X_4 - 0.00001\ X_2X_3 + 0.00002\ X_2X_4 - 0.0000\ X_3X_4} \quad \text{------- (3.2)}$$

Measure of the model's variability to the responses indicated is presented by correlation coefficient (R^2). As R^2 —> 1, model is inviolable and the response is estimated better. In our study, R^2 = 0.99957 suggests that 0.043 % of the total variations are not adequately explained by the model. Statistical relevance of the ratio of mean due to regression and mean square due to residual error is tested with the help of ANOVA. F-values implicate that % yield of glutamic acid can be sufficiently explained by the model equation. If '*P*' *value* is lower than 0.05, the model is considered to be statistically significant at the 95 % confidence level. All square terms of all variables ($P < 0.05$) are in good agreement (table 3.4) along with in linear terms also. All the interaction terms ($P < 0.05$) are highly influential on the yield of Glutamic acid except X_1X_2.

Interpretation of residual graphs:

Normal probability plot (NPP) is a graphical technique used for analyzing whether or not a data set is normally distributed to greater extent. The difference between the observed and predicted values from the regression is termed as residual. Fig. 3.1 exhibits normal probability plot for the present data. It is evident that the experimental data are reasonably aligned implying normal distribution.

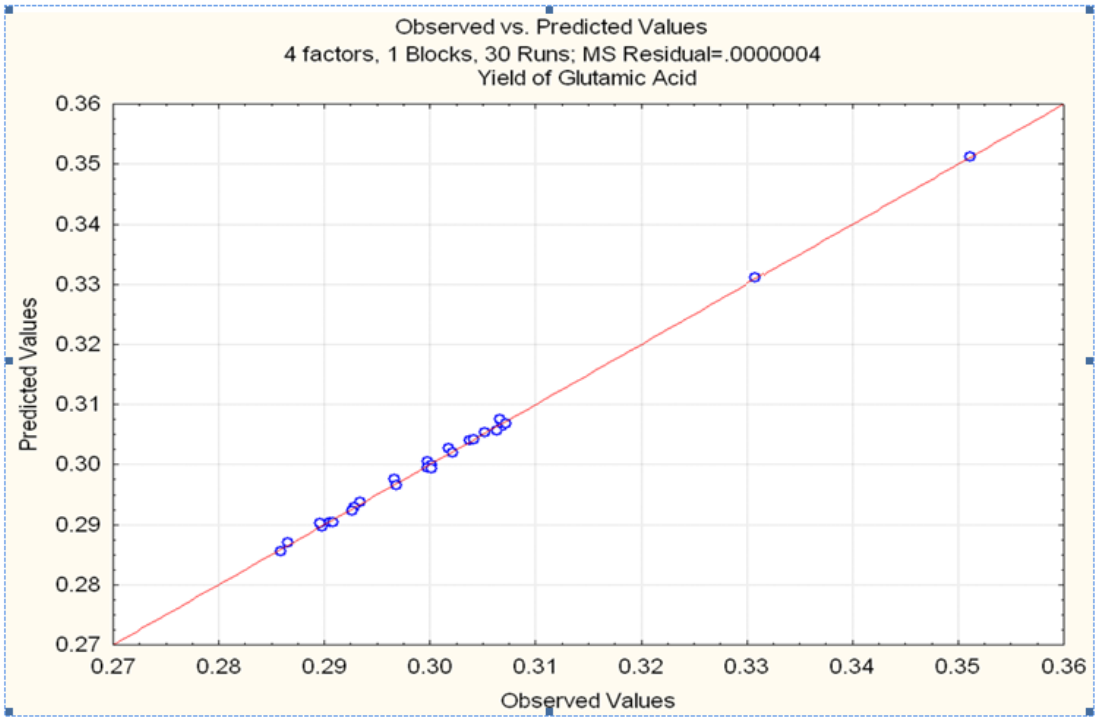

Fig. 3.1 Normal probability plot for % yield of Glutamic acid

Interaction effects of variables: Three-dimensional view of response surface contour plots [Fig. 3.2 (a) to 3.2 (f)] exhibit % yield of Glutamic acid using free cells for different combinations of dependent variables. All the plots are delineated as a function of two factors at a time, imposing other factors fixed at zero level. It is evident from response surface contour plots that the % yield of Glutamic acid is minimal at low and high levels

of the variables. This behavior conforms that there is a presence of optimum for the input variables in order to maximize % yield. The role played by all the variables is so vital in % yield of Glutamic acid and seen clearly from the plots. The predicted optimal sets of conditions for maximum % yield are:

pH	=	5.9049
Temperature	=	29.9730 ^{o}C
Agitation rate	=	158.5875 rpm
Glucose Concentration	=	101.0435 g/L
% Yield of Glutamic acid	=	0.3518767

The experimental optimum values are compared in table-3.5. The experimental values are in close agreement with those from CCD.

Table 3.5: Comparison between optimum values from Experimentation and CCD

Variable	Experimental	CCD
pH	6	5.90
Temperature, ^{o}C	30	29.97
Agitation rate, rpm	160	158.58
Glucose Concentration, g/L	100	101.04
% Yield of Glutamic acid	0.3313	0.3518

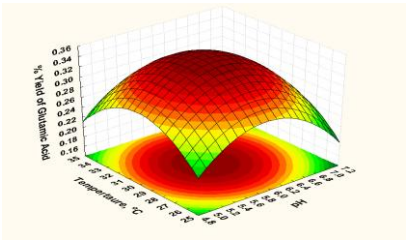

Fig. 3.2 (a) Surface contour plot for the effects of pH and temperature on % yield of Glutamic acid

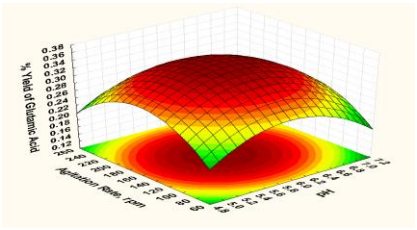

Fig. 3.2 (b) Surface contour plot for the effects of pH and agitation rate on % yield of Glutamic acid

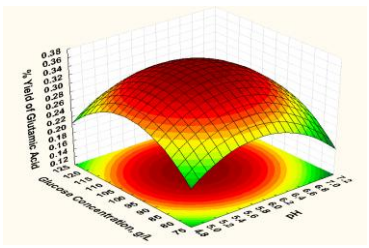

Fig. 3.2 (c) Surface contour plot for the effects of pH and glucose concentration on % yield of Glutamic acid

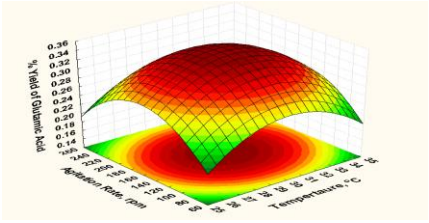

Fig. 3.2 (d) Surface contour plot for the effects of temperature and agitation rate on % yield of Glutamic acid

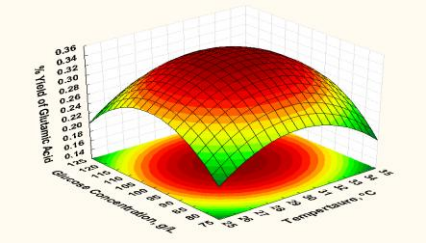

Fig 3.2 (e) Surface contour plot for the effects of temperature and glucose concentration on % yield of Glutamic acid

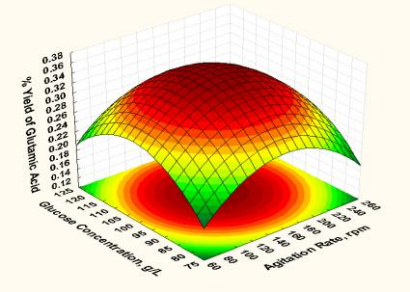

Fig. 3.2 (f) Surface contour plot for the effects of agitation rate and glucose concentration on % yield of Glutamic acid

IV .CONCLUSION

Response Surface methodology was used to optimize Fermentation parameters for L-Glutamic acid production. It was found to be a very efficient method for optimization and fermentation conditions setting. We screened four significantly affecting variables from seven variables. Using optimal conditions, the percentage yield of L-Glutamic acid obtained by Central Composite Design was 0.3518 approximately equal to the experimental yield 0.3313 percentage of L-Glutamic acid. These results are encouraging for optimization under the pilot scale or industrial scale.

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