Optimization of Carbon and Nitrogen Sources for L-asparaginase Production by *Enterobacter aerogenes* using Response Surface Methodology

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A full factorial central composite design (CCD) was applied to study various effects of sodium citrate, diammonium hydrogen phosphate (DAHP) and L-asparagine to determine the optimal concentration (γ) of these compounds on L-asparaginase production by *Enterobacter aerogenes* MTCC 2823 under shake flask fermentation conditions. A second order polynomial model describing the relationship between the variables and the L-asparaginase activity was fitted in coded units of variables. The statistical reliability and significance of the model was validated by F-test for analysis of variance at higher R^2 value ($R^2 = 0.871$). The optimum estimated concentration of sodium citrate (X_1), DAHP (X_2) and L-asparagine (X_3) was 18.76, 5.72 and 8.58 g L^{-1} respectively with maximum L-asparaginase activity of 19.129 IU mL^{-1}. The composite desirability of 98.38 % reveals the validity of the model and predicted values. The L-asparaginase activity was increased by 5.96 % than predicted activity, after optimization of carbon and nitrogen sources for L-asparaginase production by *Enterobacter aerogenes* MTCC 2823 using CCD.

Key words:

Fermentation, optimization, polynomial model, response surface plots, carbon and nitrogen sources

Introduction

L-asparaginase (L-asparagine amidohydrolase; EC.3.5.1.1), catalyzing the deamidation of L-asparagine to L-aspartic acid and ammonia, is used as a chemotherapeutic agent for acute *lumphocytic leukeamia* and less frequently for acute *myeloblastic leukeamia*, chronic *lumphocytic leukeamia*, Hodgkin's disease, *melonosarcoma* and non-Hodgkin's lymphoma. Although Clementi in 1922 had reported its presence in guinea-pig serum, the anti-tumour properties of the enzyme were only recognized some time later.[1] Tsuji first reported deamidation of L-asparagine by extracts of *E. coli*.[2] Broome in 1961 discovered that the regression of *lymphosarcoma* transplants in mice treated with guinea-pig serum was due to the nutritional dependence of the malignant cells on exogenous L-asparagine.[3] Commercial production of L-asparaginase appeared desirable only after Mashburn and Wriston in 1973 showed that L-asparaginase from *E. coli* inhibits tumours in mice.

Various bacteria such as *Erwinia carotovora, Thermus thermophilus, Thermus aquaticus*, *Vibrio succinogenes, Citrobacter freundii*, *Streptomyces griseus, Escherichia coli*, *Erwinia aroideae, Proteus vulgaris, Enterobacter aerogenes, Zymomonas mobilis, Bacillus licheniformis* and *Pseudomonas aeruginosa* have been found to produce L-asparaginase.[4–10] The production of L-asparaginase by bacterial sources is mainly regulated by different degree of carbon catabolite and oxygen repression.[11,12] Variety of fungi, yeasts and algae also found to produce L-asparaginase.[13]

The optimization of nutritional requirements and operating conditions is an important step in any bioprocess development. In addition, traditional method of bioprocess development by studying the effect of one variable at a time is tedious, time consuming and expensive. Statistical experimental have been used in several steps of optimization strategy and it is better acknowledged than traditional one variable at a time method.[14] The response surface methodology (RSM) is an efficient statistical technique for optimization of multiple variables in order to predict the best performance conditions with a minimum number of experiments.

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These designs are used to find improved or optimal process settings, troubleshoot process problems and weak points and make a product or process more robust against external and non-controllable variables.[15]

RSM is suited for studying the main and interaction effects of factors on growth or metabolite formation during microbial fermentation. Compared to classical method of optimization, CCD was more effective in bioprocess optimization.[16–18] A full factorial CCD was applied to study various effect of sodium citrate, DAHP and L-asparagine to determine the optimal concentration of these compounds on L-asparaginase production by *Enterobacter aerogenes* MTCC 2823 under shake flask fermentation conditions.

Materials and methods

Microorganism

The bacteria *Enterobacter aerogenes* MTCC 2823 was obtained from the Institute of Microbial Technology, Chandigarh, India. It was grown on nutrient agar slants for 24 h at 35 °C and maintained at 4 °C.

Inoculum culture

Peptone 1 g, Yeast extract 0.5 g, L-asparagine 0.5 g, potassium chloride 0.05 g, $MgSO_4 \cdot 7H_2O$ 0.05 g, $FeSO_4 \cdot 7H_2O$, 0.001 g, K_2HPO_4 0.1 g, and 1 mL of glucose solution (20 g L^{-1}) per 100 mL of liquid media was prepared, inoculated with Stock culture of *Enterobacter aerogenes* MTCC 2823 and grown at pH 7 and temperature of 30 °C for 24 h.

Production and isolation of crude enzyme

Culture suspension of $\varphi = 5$ % inoculum size was transferred to Erlenmeyer flasks with 100 mL of liquid Czapek-Dox medium prepared with carbon and nitrogen sources based on experimental design (Table 2) at pH 6.7 with fixed concentration of other nutrients such as glucose 0.5 g L^{-1}, potassium chloride 0.05 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 0.05 g L^{-1}, $FeSO_4 \cdot 7H_2O$ 0.001 g L^{-1} and K_2HPO_4 0.1 g L^{-1}. The culture was kept in orbital shaker (186 rpm) at 35 °C. A culture sample of 2 mL was collected at maximum L-asparaginase production time (t = 6 h).[5]

Assay of L-asparaginase activity

The cells were separated from fermentation broth by centrifugation (10000 rpm) at 5 °C and cell mass was suspended and shaken vigorously with 2 mL phosphate buffer (pH 7.0) containing triton X-100 (0.01 g L^{-1}) for 5 min and centrifuged again. The cell mass was suspended in 1.5 mL sodium-borate buffer pH 8.65, and assayed for intracellular L-asparaginase activity by Nesslarization, the most common method for estimation of L-asparaginase activity.[5]

Optimization by central composite design

The important carbon and nitrogen sources such as sodium citrate (X_1), DAHP (X_2) and L-asparagine (X_3) for L-asparaginase production by *Enterobacter aerogenes* was derived from literature.[3] The variables were prescribed into three levels, −1, 0, + 1 for low, middle and high and the central composite experimental design was developed using Minitab15 *software* in coded units. Table 1 shows the coded and actual levels of variables and Table 2 shows the experimental design, experimental and predicted L-asparaginase activity. Experimental results were analyzed using RSM. The response variable was fitted into quadratic model to correlate the effect of the variables on L-asparaginase activity. At the model level, the closer the value of R^2 is to 1, the better the correlation between the observed and the predicted values.[15–18]

$$Y=\beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_{11}X_1^2+\beta_{22}X_2^2+ \\ +\beta_{33}X_3^2+\beta_{12}X_1X_2+\beta_{13}X_1X_3+\beta_{23}X_2X_3 \quad (1)$$

where Y is the predicted response, β_0 model constant; X_1, X_2 and X_3 are independent variables; β_1, β_2 and β_3 are linear coefficients; β_{12}, β_{13} and β_{23} are cross product coefficients; β_{11}, β_{22} and β_{33} are the quadratic coefficients.

Experiments were performed in duplicate and the average of observations was used. The statistical significance of second order polynomial model was determined by F-test for analysis of variance (ANOVA) and residuals analysis was performed to validate the model. The optimum levels of the selected variables were obtained by solving the regression equation and also by analyzing the response surface plot and optimization.

Table 1 – *Experimental levels in coded and actual unit of the variables*

Independent variable	Coded unit				
	−1.681	−1	0	1	1.681
Sodium citrate, X_1, g L^{-1}	3.19	10	20	30	36.81
DAHP, X_2, g L^{-1}	3.29	5	7.5	10	11.70
L-asparagine, X_3, g L^{-1}	3.29	5	7.5	10	11.70

Table 2 – *Central composite design in coded units of variables in L-asparaginase production by Enterobacter aerogenes MTCC 2823*

Std. Run	Sodium citrate (X_1)	DAHP (X_2)	L-asparagine (X_3)	Experimental L-asparaginase activity (IU mL^{-1})	Predicted L-asparaginase activity (IU mL^{-1})
1	−1.000	−1.000	−1.000	14.537	15.802
2	1.000	−1.000	−1.000	14.895	14.983
3	−1.000	1.000	−1.000	15.448	16.353
4	1.000	1.000	−1.000	14.635	15.287
5	−1.000	−1.000	1.000	18.417	18.660
6	1.000	−1.000	1.000	17.114	17.104
7	−1.000	1.000	1.000	14.014	14.821
8	1.000	1.000	1.000	13.388	13.018
9	−1.681	0.000	0.000	18.493	17.010
10	1.681	0.000	0.000	14.587	14.805
11	0.000	−1.681	0.000	18.172	17.661
12	0.000	1.681	0.000	15.442	14.688
13	0.000	0.000	−1.681	16.731	15.432
14	0.000	0.000	1.681	15.893	15.927
15	0.000	0.000	0.000	19.224	18.674
16	0.000	0.000	0.000	18.762	18.674
17	0.000	0.000	0.000	18.576	18.674
18	0.000	0.000	0.000	18.573	18.674
19	0.000	0.000	0.000	18.224	18.674
20	0.000	0.000	0.000	18.468	18.674

Results and discussion

In order to define the optimal response region of the L-asparaginase activity, experimental values of L-asparaginase activity in Table 2 were subjected to multiple linear regression analysis using *MINITAB 15* (Trail version). The effect of sodium citrate, DAHP and L-asparagine on L-asparaginase activity was described in the form eq. 2, a second order polynomial model in coded unit of the variables. Student's t-test was performed to determine the significance of regression coefficients. The regression coefficient, t and p values for linear, quadratic and combined effects is given in Table 3, with a 95 % significance level. It was observed that the coefficients for overall effect of the variables ($p \leq 0.0001$), individual effect of sodium citrate ($p = 0.029$) and DAHP ($p = 0.006$), quadratic effect of sodium citrate ($p = 0.003$), DAHP ($p = 0.005$) and L-asparagine ($p = 0.002$) and interaction effect of DAHP and L-asparagine ($p = 0.009$) were highly significant in increasing the L-asparaginase production.

$$Y_{\text{activity}} = 18.674 - 0.655\ X_1 - 0.883\ X_2 + \\ + 0.147\ X_3 - 0.978\ X_1^2 - 0.883\ X_2^2 - 1.058\ X_3^2 - \\ - 0.061\ X_1X_2 - 0.184\ X_1X_3 - 1.097\ X_2X_3 \quad (2)$$

Table 3 – *Estimated regression coefficients of second order polynomial model for optimization of L-asparaginase production by Enterobacter aerogenes MTCC 2823*

Factor	Coefficient	Estimated coefficient	Standard deviation	t-value	p-value
	β_0	18.674	0.387	48.157	0.000
X_1	β_1	−0.655	0.257	−2.548	0.029
X_2	β_2	−0.883	0.257	−3.435	0.006
X_3	β_3	0.147	0.257	0.572	0.580
X_1^2	β_{11}	−0.978	0.250	−3.906	0.003
X_2^2	β_{22}	−0.883	0.250	−3.529	0.005
X_3^2	β_{33}	−1.058	0.250	−4.227	0.002
X_1X_2	β_{12}	−0.061	0.336	−0.184	0.858
X_1X_3	β_{13}	−0.184	0.336	−0.548	0.596
X_2X_3	β_{23}	−1.097	0.336	−3.265	0.009

The statistical reliability and significance of the model was validated by F-test for ANOVA at higher R^2 value ($R^2 = 0.871$). A higher value of coefficient of correlation justified an excellent correlation between the sodium citrate, DAHP and L-asparagine and the model fitted well with the L-asparaginase activity, and hence the second order polynomial model (eq. 2) was highly significant and adequate to represent the effects of sodium citrate, DAHP and L-asparagine on L-asparaginase activity.

Response surface plot (Fig. 1) shows the interaction effect of varying concentration of sodium citrate and L-asparagine on L-asparaginase activity, at fixed DAHP concentration (7.5 g L^{-1}). Closer to the middle level of sodium citrate and L-asparagine, an increase (maximum 18 IU mL^{-1}) in L-asparaginase production was determined. Response surface plot (Fig. 2) shows the interaction effect of varying concentration of DAHP and L-asparagine on L-asparaginase activity at fixed sodium citrate concentration (20 g L^{-1}). It was observed that the L-asparaginase activity increased (maximum 17.5 IU mL^{-1}) at higher level of L-asparagine and closer to the middle level of DAHP. This is also evidence that the interaction between DAHP and L-asparagine increased the L-asparaginase production significantly ($p = 0.009$, Table 4). Similar response surface plot (Fig. 3) shows the interaction effect of varying mass concentration of sodium citrate and DAHP on L-asparaginase activity, at fixed L-asparagine concentration (7.5 g L^{-1}). It was observed that the L-asparaginase activity was higher at middle level of sodium citrate and DAHP concentration, L-asparaginase activity increased up to 17.5 IU mL^{-1}. The predicted optimal concentration of carbon and nitrogen sources are sodium citrate 18.76 g L^{-1}, DAHP 5.72 g L^{-1} and L-asparagine 8.58 g L^{-1}, constitutes for maximum L-asparaginase activity of 19.129 IU mL^{-1} (Fig. 4). After applying CCD,

Table 4 – *Analysis of variance (ANOVA) for second order polynomial model for optimization of L-asparaginase production by Enterobacter aerogenes MTCC 2823*

Factor	Degree of freedom (DF)	Sum of squares (SS)	Mean square (MS)	*F*-value	*p*-value
model	9	61.203	6.800	7.52	0.002
linear	3	16.831	5.610	6.21	0.012
square	3	34.433	11.478	12.70	0.001
interaction	3	9.938	3.312	3.66	0.051
residual error	10	9.039	0.904		
lack-of-fit	5	8.472	1.694	14.94	0.05
pure error	5	0.567	0.113		
total sum of squares	19	70.243			

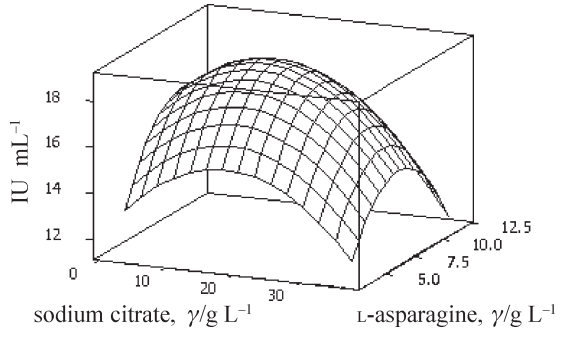

Fig. 1 – *Surface plot shows the effect of sodium citrate and L-asparagine on L-asparaginase activity*

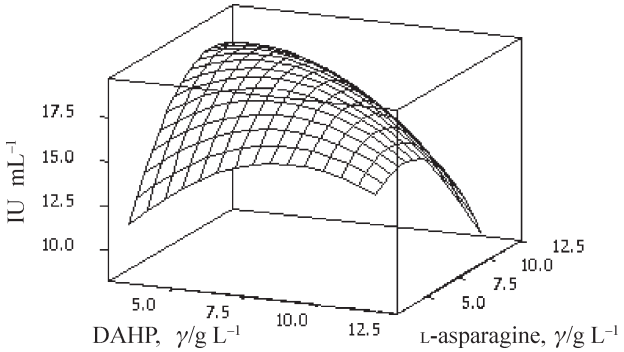

Fig. 2 – *Surface plot shows the effect of DAHP and L-asparagine on L-asparaginase activity*

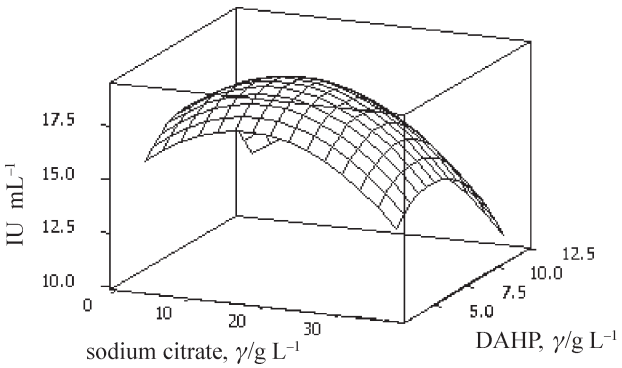

Fig. 3 – *Surface plot shows the effect of sodium citrate versus and DAHP on L-asparaginase activity*

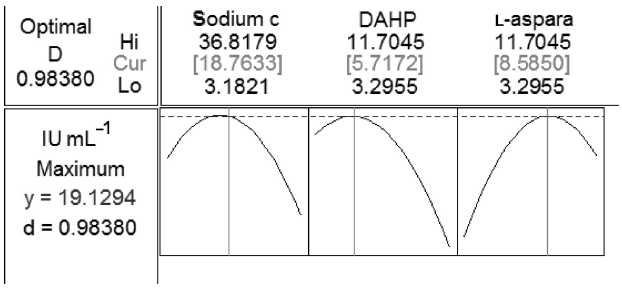

Fig. 4 – *Composite desirability and optimization plot for maximum L-asparaginase activity*

L-asparaginase activity was nearly 15 times that compared to media optimized by the classical method.[5] The composite desirability of 98.38 % at optimal condition reveals the high accuracy of the regression model in optimization.

Conclusion

The second order polynomial model was highly significant and adequate to represent the linear, quadratic and interaction effects of sodium citrate, DAHP and L-asparagine on L-asparaginase activity. The composite desirability of 98.38 % at optimal condition reveals the high accuracy of the regression model. The predicted maximum L-asparaginase activity was 19.129 IU mL^{-1} and the corresponding optimal concentration of sodium citrate, DAHP and L-asparagine were 18.76 g L^{-1}, 5.72 g L^{-1} and 8.58 g L^{-1} respectively. The composite desirability of 98.38 % reveals the validity of the model and predicted values. In confirmation experiment, L-asparaginase activity increased by 5.96 % than the predicted value.

List of symbols

Cur – current settings (optimum condition)
D (d) – composite desirability
F – Fishers's function
Hi – high level
Li – low level
m – mass, g
p – corresponding level of significance
R^2 – correlation coefficient
t – student's test
X_1 – sodium citrate
X_2 – DAHP
X_3 – L-asparagine
Y – predicted response
β – coefficient
γ – mass concentration, g L^{-1}
φ – volume fraction, %

Abbreviations

ANOVA – Analysis of variance
CCD – central composite design
DAHP – diammonium hydrogen phosphate
RSM – response surface methodology

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