Türk Biyokimya Dergisi [Turkish Journal of Biochemistry–Turk J Biochem] 2008; 33 (2) ; 50–57.

Araştırma Makalesi [Research Article] Yayın tarihi 23 Temmuz, 2008 © TurkJBiochem.com

[Published online 23 July, 2008]

Statistical Optimization of Process Variables for L-lysine Production by *Corynebacterium Glutamicum*

[*Corynebacterium Glutamicum* Tarafından Üretilen L-lizin için İşlem Değişkenlerinin İstatistiksel Optimizasyonu]

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ABSTRACT

Objectives: Response Surface Methodology was used to optimize the fermentation variables for enhancing the L-lysine production by a mutant strain of *Corynebacterium glutamicum* AEC-2.

Methods: Among the eight independent variables studied by using Plackett-Burman design, only four variables like, the level of sugar in molasses, ammonium sulphate, incubation temperature and inoculum size were found to be significantly affecting variable for L-lysine production. Further, 2^4 factorial central composite design was used to determine the optimal levels of significant variables. A second order polynomial regression model was applied on the experimental data.

Results: The predicted optimal levels of these variables were as follows, sugar concentration: 95.777 g/L, ammonium sulphate concentration: 24.223 g/L, temperature 30.317 °C and inoculum size: 6 % with 18.689 g/L predicted lysine value. There was 2.6 fold increase in L-lysine production as compared with lysine value obtained at original levels of these components.

Key Words: L-lysine, fermentation, Corynebacterium glutamicum, process variables, optimization, response surface methodology

ÖZET

Amaç: *Corynebacterium glutamicum* AEC-2 mutant suşu tarafından L-lizin üretiminin arttırılması amacıyla fermentasyon değişkenlerinin optimize edilmesi için yanıt yüzey yöntemi kullanılmıştır.

Gereç ve Yöntemler: Plackett-Burman tasarımı kullanılarak çalışılan sekiz bağımsız değişken arasında sadece melastaki şeker düzeyi, amonyum sülfat miktarı, inkübasyon sıcaklığı ve aşılama büyüklüğü gibi dört değişkenin L-lizin üretimini belirgin bir şekilde etkilediği görülmüştür. Bu değişkenlerin en uygun düzeylerini belirlemek amacıyla 2^4 faktöryel merkezi karma tasarım kullanılmıştır. Deneysel verilere ikinci derece polinomik regresyon modeli uygulanmıştır.

Bulgular: Bu değişkenlerin en uygun düzeyleri 18.689 g/L lizin miktarı için aşağıdaki şekilde belirlenmiştir. Şeker derişimi 95.777 g/L, amonyum sülfat derişimi 24.223 g/L, sıcaklık 30.317 °C ve aşılama büyüklüğü % 6. L-lizin üretiminde 2.6 kat artış saptanmıştır.

Sonuç: Belirleme katsayısının (R^2) 0.841734 olarak bulunması seçilen modelin güvenilirliğini göstermektedir.

Kayıt tarihi: 09 Mayıs 2008, Kabul tarihi: 10 Haziran 2008

[Received: 09 May 2008, Accepted: 10 June 2008]

Anahtar Kelimeler: L-Lizin, Corynebacterium glutamicum, işlem değişkenleri, optimizasyon, yanıt yüzey yöntemi

Introduction

Amino acids are the basic biomolecules of proteins, which are the most important macromolecules for the functions of human and animal biochemical systems. L-lysine is one of the 9 amino acids which are essential for human and animal nutrition. L-lysine is useful as medicament, chemical agent, food material and feed additive (1). The steriospecificity of amino acids makes the fermentation advantageous as compared with synthetic processes. Mutant strains of so called coryneform bacteria are generally used, including the genera of *Brevibacterium* and *Corynebacterium* united to the genus (1). Because of the L-lysine's great importance, efforts are constantly being made in order to improve the fermentation processes, comprising strain and process development (e.g. effect of stirring and oxygen supply, temperature, pH and CO_2) as well as media optimization (e.g. influence of initial and operational sugar concentration, important nutrients and additives).

Physical conditions such as pH, temperature, agitation or shaking speed, inoculum size, inoculum age and fermentation time play a vital role in a fermentation process. A wide range of optimum and operational temperatures have been disclosed in the international patent bibliography for L-lysine fermentation. In addition to physical parameters, medium composition is also very strongly influenced the fermentation process. Mutants of *Corynebacterium* and related microorganisms enable the inexpensive production of amino acids from cheap carbon sources by direct fermentation. Various sources of nitrogen are utilized individually or as mixtures for the commercial and pilot scale production of lysine, including inorganic compounds and natural nitrogen containing organic materials. Therefore the fermentation conditions and medium optimization studies are very important.

Response surface is an optimization methodology, mainly based on statistical techniques. Response surface methodology has been successfully used to modal and optimizes biochemical and biotechnological processes (2-6). The application of response surface methodology in fermentation process can result in improved product yield, reduced process variability and development time and over all costs (7). The objectives of a statistically designed optimization study are to (i) confirm previous effects and interactions, (ii) estimate specific curvature or quadratic effects, and (iii) determine optimal settings of the critical factors (8). Screening should be done when the investigator is faced with a large number of factors. Once a list of variables has been made, the settings for each variable must be determined. In a screening experiment, two settings are chosen for each variable. When the critical variables have been identified via screening, the investigator can proceed to the optimization stage of the experimental design.

In general, optimization by traditional one variable at a time technique was used (9). This technique is not only laborious and time consuming but also often leads to an incomplete understanding of the system's behavior, resulting in confusion and a lack of predictive ability. Response surface methodology (RSM) is a powerful and efficient mathematical approach applied in the optimization of fermentation processes, e.g. media components on enzyme production (10-12), production of other metabolites (13-16), spore production (17) and biomass production optimization (18). It gave information necessary for design and process optimization and also helpful in the analysis of multiple responses at the same time (19-21).

In this study, statistical optimization of physical conditions and media components was investigated for L-lysine production using a strain of *Corynebacterium glutamicum* by response surface methodology (RSM). In the first step, screening of different media components like carbon source (sugar concentration), nitrogen source ($(NH_4)_2SO_4$), $CaCO_3$ and dissolved oxygen and physical conditions like temperature, pH, inoculum size and fermentation time was carried out to determine the significance of these parameters in a molasses based medium. In the second step the significant factors were optimized using a central composit design (CCD). CCD is an efficient method to calculate the significance of different parameters, interaction between parameters and the optimal level for each parameter.

Materials and Methods

Microorganism

A mutant strain of *Corynebacterium glutamicum* (AEC-2) was used in these studies (22). This strain was homoserine auxotroph and S-2-(aminoethyl)-L cystein resistant. The culture was maintained on the agar slants of the TYG medium with following composition g/L; Tryptic soy digest 10, yeast extract 5, glucose 5, NaCl 5 and agar 20. The initial pH was adjusted to 7.2-7.4.

Fermentation technique

Inoculum was developed in a rotary shaker operating at 150 rpm shaking speed and in 500 ml Erlenmeyer flasks with 100 ml of the TYG medium without agar. The incubation temperature was 30 °C for 24 hours.

Molasses based medium was used for fermentation. Cane molasses was clarified by the method of Panda (23). After neutralization with calcium hydroxide (1.55 %), the sugar concentration was maintained at required level. Fermentation medium had following composition g/l; sugar concentration 40-120, $(NH_4)_2SO_4$ 10-80, $CaCO_3$ 10-50, NaCl 2, $MgSO_4$ 0.4, KH_2PO_4 0.5, K_2HPO_4 1 and 0.7 mg/ml homoserine. A 2 L fermenter (Eyla, Japan) equipped automatic control of DO and pH was used. Fermentation process was carried out at 500 rpm, pH 6-8, DO 2-10 ppm, Inoculum size 1-9 % for 24 to 120 hours. The DO level was maintained by pure oxygen enriched air. After fermentation, the broth was centrifuged

at 10,000 rpm for 10 minutes and supernatant was used for lysine analysis.

Analysis

Fermented broth was centrifuged at 10,000 rpm for 10 minutes. L-lysine was determined in supernatant using Hitachi's computerized amino acid analyzer of L-8500 modal (24).

Statistical analysis

Process variables for lysine production were optimized by using response surface methodology. In this methodology, Plackett and Burman design (PB design) (25,26) was used to screen eight variables; sugar (X1), ammonium sulphate (X2), calcium carbonate (X3), temperature (X4), pH (X5), Inoculum size (X6), dissolved oxygen concentration (X7) and fermentation time (X8) in 12 different experiments (Table 1 and 2). This design was used to evaluate the significant variables for lysine production. However it does not consider the interaction effects among the variables. Each variable was set at its high and low levels coded as 1 and -1 respectively.

After identification of the significant variables using PB design, Box-Wilson 2^4 factorial central composite design (CCD) was used to optimize these variables. Five levels of variation were selected for each variable (Table 3). To simplify the calculations, the independent variables will be coded as;

$$Z = (X - X^o)/\Delta X \qquad \textbf{(Equation 1)}$$

Where Z is the coded value, X is the corresponding natural value, X^o is the natural value in the centre of domain, and ΔX is the increment of X corresponding to one unit of Z. Box-Wilson 2^4 factorial central composite design (CCD) with 8 axial points (α=2) and seven replications at the centre points leading to a total number of 31 experiments was employed (Table 4). Second order modal of response surface was used to calculate the predicted response.

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \qquad \textbf{(Equation 2)}$$

Where Y represents response variable, β_o is the interception coefficient, β_i is coefficient of the linear effect, β_{ii} is the coefficient of quadratic effect and β_{ij} is the coefficient of interaction effect. STATISTICA software (99^{th} edition) was used for multiple regression analysis and to construct the plots of the obtained data. F-test was employed to evaluate the statistical significance of the quadratic polynomial modal (equation 2). The coefficient

Table 1. Fermentation conditions for lysine production used in screening of variables using a PB design

X8	Fermentation time (hours)	24	120

Variables	Factor	Low Level (-1)	High Level (1)
X1	Sugar conc. (g/L)	20	100
X2	NH_2SO_4 conc. (g/L)	20	100
X3	$CaCO_3$ (g/L)	10	50
X4	Temperature (°C)	27	39
X5	pH	6	8
X6	Inoculum size (%)	2	10
X7	Dissolved oxygen conc. (ppm)	2	10

Table 2. The experimental design using the PB method for screening of lysine fermentation conditions

Run No.	X1	X2	X3	X4	X5	X6	X7	X8	Lysine yield (g/L)
1	1	-1	1	-1	-1	-1	1	1	4.025 ± 0.054
2	1	1	-1	1	-1	-1	-1	1	5.924 ± 0.021
3	-1	1	1	-1	1	-1	-1	-1	4.321 ± 0.046
4	1	-1	1	1	-1	1	-1	-1	4.102 ± 0.057
5	1	1	-1	1	1	-1	1	-1	7.462 ± 0.016
6	1	1	1	-1	1	1	-1	1	3.307 ± 0.081
7	-1	1	1	1	-1	1	1	-1	4.210 ± 0.062
8	-1	-1	1	1	1	-1	1	1	4.192 ± 0.037
9	-1	-1	-1	1	1	1	-1	1	3.012 ± 0.029
10	1	-1	-1	-1	1	1	1	-1	3.972 ± 0.073
11	-1	1	-1	-1	-1	1	1	1	2.519 ± 0.072
12	-1	-1	-1	-1	-1	-1	-1	-1	1.726 ± 0.016

Table 3. Levels of the variables tested for optimization in CCD

Coded values (Z)	Actual values			
	X1 (Sugar conc. g/L)	X2 (NH_2SO_4 conc. g/L)	X4 (Temperature °C)	X6 (Inoculum size %)
-2	20	20	27	2
-1	40	40	30	4
0	60	60	33	6
1	80	80	36	8
2	100	100	39	10

Table 4. Results of Box-Wilson central composite design for lysine yield optimization

Trial	Factors				Lysine yield (g/L) ± SD	Predicted values	Residual values
	X1	**X2**	**X4**	**X6**			
01	-1	-1	-1	-1	12.581 ± 0.034	9.68742	2.89358
02	-1	-1	-1	1	11.543 ± 0.051	9.96913	1.57387
03	-1	-1	1	-1	5.026 ± 0.028	5.36246	-0.33646
04	-1	-1	1	1	5.978 ± 0.094	5.85942	.11858
05	-1	1	-1	-1	6.457 ± 0.064	7.81296	-1.35596
06	-1	1	-1	1	8.749 ± 0.024	8.99542	-0.24642
07	-1	1	1	-1	9.072 ± 0.100	6.64625	2.42575
08	-1	1	1	1	10.518 ± 0.057	8.04396	2.47404
09	1	-1	-1	-1	16.742 ± 0.075	17.25246	-0.51046
10	1	-1	-1	1	16.450 ± 0.019	16.88192	-0.43192
11	1	-1	1	-1	14.725 ± 0.082	12.48475	2.24025
12	1	-1	1	1	15.649 ± 0.039	12.32946	3.31954
13	1	1	-1	-1	8.592 ± 0.062	6.71675	1.87525
14	1	1	-1	1	9.547 ± 0.092	7.24696	2.30004
15	1	1	1	-1	5.497 ± 0.083	5.10729	0.38971
16	1	1	1	1	4.953 ± 0.051	5.85275	-0.89975
17	-2	0	0	0	3.451 ± 0.097	5.24579	-1.79479
18	2	0	0	0	8.457 ± 0.056	10.61962	-2.16262
19	0	-2	0	0	9.549 ± 0.043	12.00379	-2.45479
20	0	2	0	0	2.150 ± 0.094	3.65263	-1.50263
21	0	0	-2	0	9.704 ± 0.028	10.77429	-1.07029
22	0	0	2	0	2.168 ± 0.017	5.05513	-2.88713
23	0	0	0	-2	10.541 ± 0.064	12.37312	-1.83212
24	0	0	0	2	11.275 ± 0.038	13.40029	-2.12529
25	0	0	0	0	14.021 ± 0.069	14.00529	0.01571
26	0	0	0	0	13.976 ± 0.052	14.00529	-0.02929
27	0	0	0	0	13.794 ± 0.047	14.00529	-0.21129
28	0	0	0	0	14.106 ± 0.053	14.00529	0.10071
29	0	0	0	0	13.947 ± 0.083	14.00529	-0.05829
30	0	0	0	0	14.12 ± 0.091	14.00529	0.11471
31	0	0	0	0	14.073 ± 0.054	14.00529	0.06771

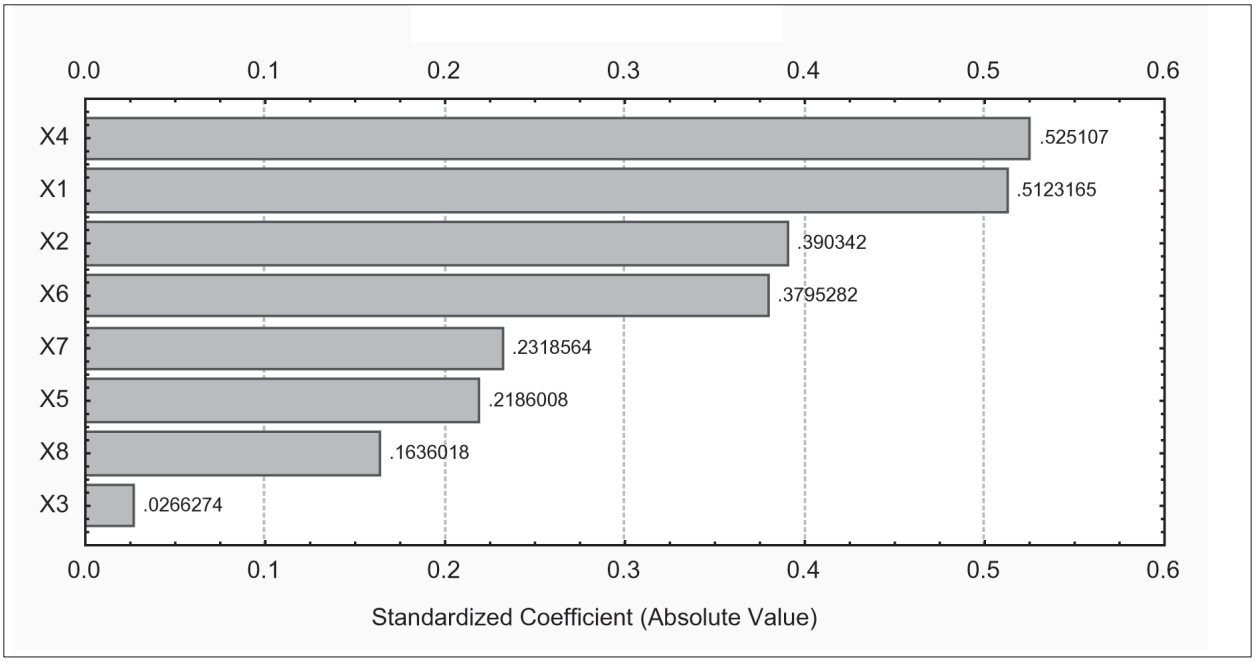

Figure 1. Pareto chart of fermentation conditions for lysine production

of multiple correlation R and the coefficient of determination of correlation R^2 were calculated to evaluate the performance of the regression equation. The optimum levels of the selected variables were obtained by solving the regression equation and by desirability charts. All the experiments were conducted in triplicate and their mean values were used for analysis.

Results and Discussion

In the present work response surface methodology was applied to optimize the process variables for the production of L-lysine, in two steps. In the first optimization step (screening step) 12-run PB design was used to identify the significant variables for lysine production by *Corynebacterium glutamicum*. Eight variables, X1, X2, X3, X4, X5, X6, X7 and X8 were used for screening (Table 1). Each variable was set at two levels that are high level and low level coded as 1 and -1 respectively. The high level of each variable was set far enough from the low level to identify which ingredients of the medium have significant influence on lysine production. Lysine yield of the 12 experiments was given in Table 2. Each variable was tested an equal number of times at its low and high levels. Because of this equal allocation, the resulting data provided a fair and efficient estimate of the linear effect of each factor.

Data obtained from the trials was statistically analyzed using STATISTICA software (99^{th} edition) to evaluate and rank variables by their degree of impact on the fermentation process. It is obvious from the results (Table 5) that X1, X2, X4 and X6 has significant effect on lysine production as they have large t values and p values less than 0.05. It can be seen that X1, X2 and X4 have positive effect on response (lysine yield), while X6 has a negative effect. The positive effects of X1 and X2 were probably caused by the requirement of a large quantity of substrate to synthesize cells and lysine product.

The ranking of the variables in respect of their effect was shown in pareto chart (Figure 1). X4 (incubation temperature) had the most significant effect as compared to the other three variables. The objective of the screening was minimization in number of variables for optimization studies, which was successfully gained. If we optimized eight variables, then we have to do 281 experiments. However we have carried out only 31 experiments for optimization. This tremendous decrease in experiments was only due to screening of variables before optimization. Therefore PB design was proved to be a powerful tool to rapidly determine the effects of different variables on lysine production by *Corynebacterium glutamicum*. However the optimum levels of significantly effecting variables could not be obtained. Further work need to be done to find out this information.

Box-Wilson central composite design with five settings and α=2 was used for the optimization of significantly effecting variables such as; sugar concentration (X1), ammonium sulphate concentration (X2), temperature (X4) and inoculum size (X6). Other variables tested in the BP design were set at their centre point. The variables and their settings are described in Table 3. Total 31 experiments in triplicate were conducted in which 16 experiments were for the estimation of main effects and two-factor interactions, 7 experiments of central points were to estimate the pure process variability reassess gross curvature and 8 experiments (two for each vari-

able) were to estimate the quadratic effects of each variable (Table 4). The explanatory modal obtained from the data is:

$$Y = -177.873 + 0.933X1 + 0.216X2 + 9.957X4 + 0.578X6 - 0.004X1^2 - 0.004X2^2 - 0.169X4^2 - 0.070X6^2 - 0.005X1 \times X2 - 0.002X1 \times X4 - 0.004X1 \times X6 + 0.013X2 \times X4 + 0.006X2 \times X4 + 0.009X4 \times X6 \quad \textbf{(Equation 3)}$$

Where Y is the response value, which is the lysine yield in these studies. The statistical analysis of the quadratic regression modal demonstrated that the equation 3 was a highly significant modal. It was evident from the Fisher's F-test (F calculated = 6.078240) with a very low probability value (0.000478). The model's goodness of fit was also checked by coefficient of determination (R^2). The value of the coefficient of determination ($R^2 = 0.841734$) was indicated that only 15.82 % of the total variations were not explained by the modal. The value of the adjusted coefficient of determination (Adj $R^2 = 0.70325$) also supported the high significance of the modal. The observed response and the predicted values are in close agreement as exhibited by the low values of residuals (Table 4). The analysis of variance indicated that the main effects of X1 (sugar concentration) and X4 (Incubation temperature) were significant, the quadratic effects of X1 (sugar concentration), X2 (ammonium sulphate concentration) and X4 (incubation temperature) were significant and two-factor interactions of X1 and X2 were significant (Table 6).

In this way we have estimated the main effects, quadratic effects, interaction effects between two variables and optimum levels of each variable, at a time, using Box-Wilson CCD. The interaction effect of X1 and X2 on the response was represented in Figure 2 (Surface plot of X1 and X2 while other variables were kept at their optimal levels).

Maximum lysine yield (16.742 g/L) obtained from experiment number nine at 80 g/L (coded value 1) sugar concentration, 40 g/L (-1 coded value) ammonium sulphate concentration, 30 °C (-1 coded value) incubation temperature and 4 % (-1 coded value) inoculum size. The

Table 5. Parameter estimates and p values for Plackett-Burman study of lysine fermentation

Variables	Estimates	t values	p vales
X1	0.01836	4.65309	0.018723*
X2	0.01599	3.54526	0.038220*
X3	-0.00191	-0.24184	0.824491
X4	0.15053	4.76926	0.017511*
X5	0.31333	1.98543	0.141310
X6	-0.13600	-3.44705	0.041025*
X7	0.08308	2.10582	0.125866
X8	-0.00489	-1.48591	0.233994

Table 6. Analysis of variance for lysine optimization study using Box-Wilson central composite design

Effect	SS	Degree of freedom	MS	F	p
Intercept	41.53560	1	41.53560	7.84165	0.012834*
X1	34.84243	1	34.84243	6.57802	0.020773*
$X1^2$	65.90639	1	65.90639	12.44269	0.002797*
X2	1.86242	1	1.86242	0.35161	0.561490
$X2^2$	68.19421	1	68.19421	12.87462	0.002460*
X4	47.82887	1	47.82887	9.02977	0.008392*
$X4^2$	66.29768	1	66.29768	12.51656	0.002736*
X6	0.13387	1	0.13387	0.02527	0.875675
$X6^2$	2.23621	1	2.23621	0.42218	0.525070
X1 X2	75.01725	1	75.01725	14.16276	0.001699*
X1 X4	0.19603	1	0.196.03	0.03701	0.849868
X1 X6	0.42543	1	0.42543	0.08032	0.188912
X2 X4	9.97454	1	9.97454	1.88313	0.780501
X2 X6	0.81135	1	0.81135	0.15318	0.700680
X4 X6	0.04633	1	0.04633	0.00875	0.926646
Error	84.74874	16	5.29680		

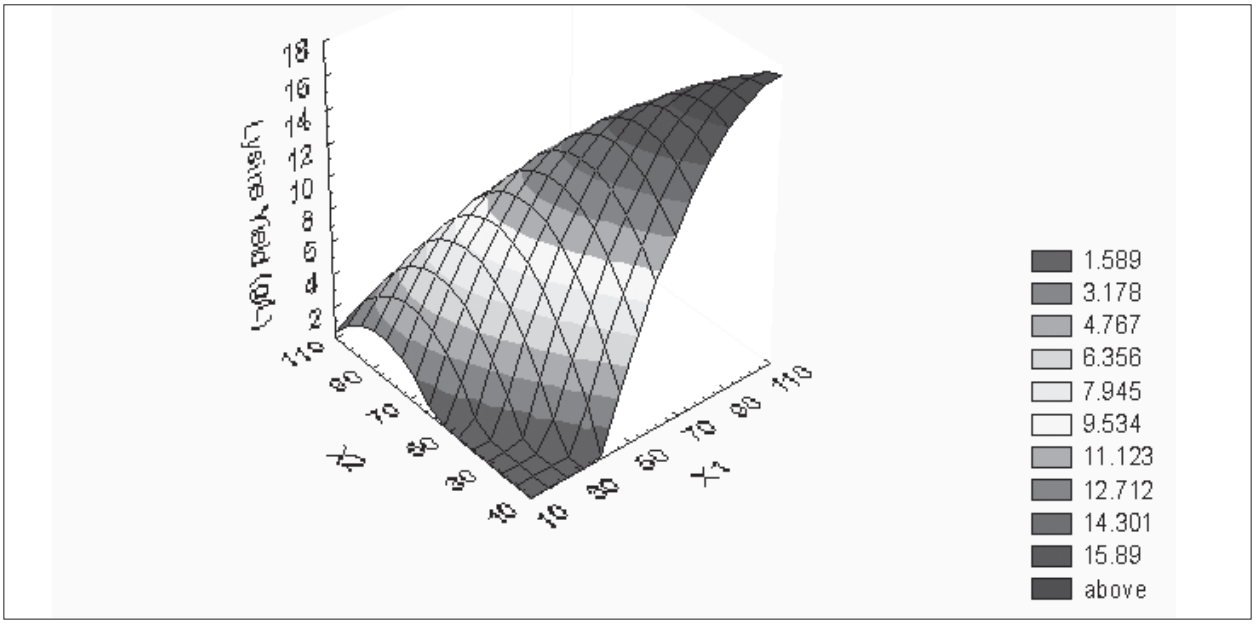

Figure 2. Surface plot X1 and X2 for lysine production

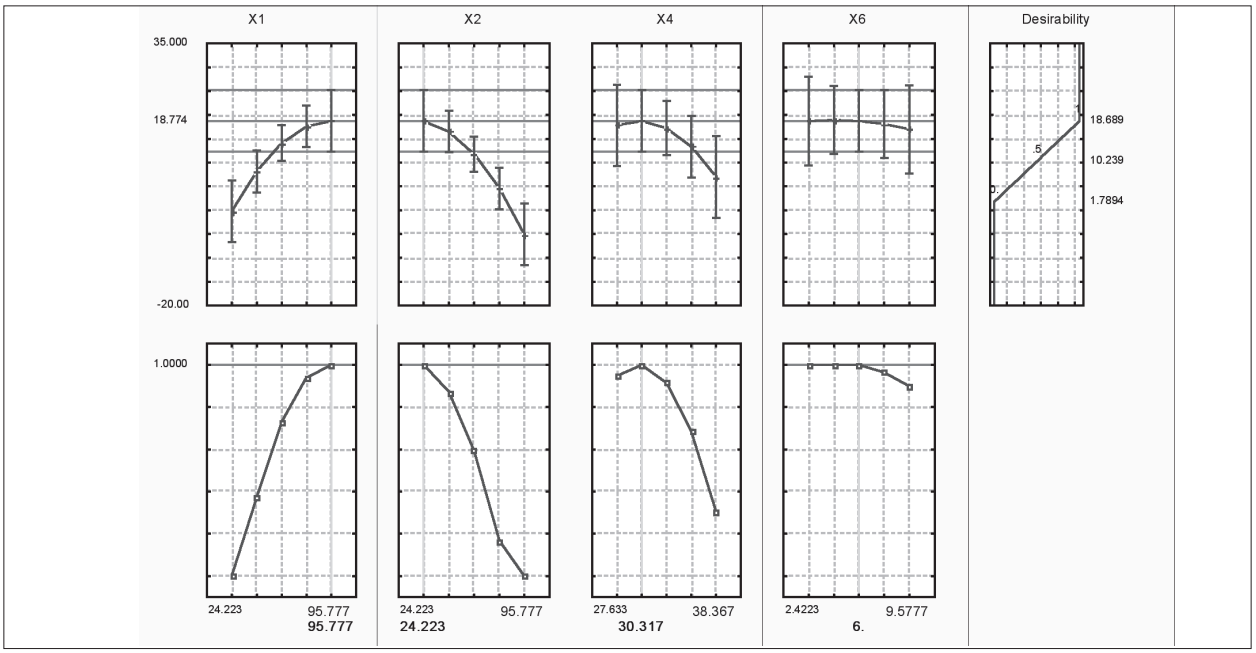

Figure 3. Desirability charts of variables for maximum response (lysine yield)

volumetric productivity and Lysine yield ($Y_{p/s}$) obtained at these levels were 0.998g/L/h and 0.226 g/g respectively. The predicted response at these variable values was found 17.252 g/L, which is close to the observed values.

The optimum levels of the tested variables were represented in desirability charts (Figure 3), constructed using Response Surface Regression in STATISTICA software (99th edition). These levels were as follows: Sugar concentration: 95.777 g/L, ammonium sulphate concentration: 24.223 g/L, temperature 30.317 °C and inoculum size 6 % with 18.689 g/L predicted lysine value. Experiments were also conducted at these predicted optimum levels. Results obtained confirmed the predicted level of lysine yield. L-lysine produced using original medium components and conditions (Sugar concentration: 60 g/L, ammonium sulphate concentration: 40 g/L, temperature 30 °C and inoculum size 4 %) was 7.24 g/L. Production of lysine with optimized medium increased 2.58 times as compared to the original medium. Coello (27) was reported 2.6 times increase in lysine yield with an optimized medium using response surface methodology.

In the present studies significant variables were determined only in 12 experiments by using PB design. In this way variables were reduced to half for optimization and a lot of time was saved. Similarly in optimization

studies, all the factors were studied at a time which not only saves time but also helped us to determine the interactions between factors that was not possible if we use one factor at a time technique. Prediction for maximum yield was also being possible due to this methodology. Our observed value (16.742 g/L) of lysine yield was not much different from predicted value (18.689 g/L) at calculated optimum levels. This little difference indicated that the range setting of variables was almost appropriate. The results obtained from the studies may help us in further scale up studies of lysine.

CONCLUSION

Response surface methodology was used to optimize cultural conditions for lysine production. It was found to be a very efficient method for optimal media and conditions setting. We screened 4 significantly effecting variables from 8 in the first step and the optimal levels of these four variables were sorted out in the second step. Using that optimal levels lysine yield was increased up to 2.58 folds than the yield obtained at their original levels.

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