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MODELING AND OPTIMIZATION OF GLUTAMIC ACID PRODUCTION USING MIXED CULTURE OF *Corynebacterium glutamicum* NCIM2168 AND *Pseudomonas reptilivora* NCIM2598

Rajaram Shyam Kumar,[1] Innasi Muthu Ganesh Moorthy,[1] and Rajoo Baskar[2]

[1]*Department of Biotechnology, Centre for Research, Bioprocess and Downstream Processing Laboratory, Kamaraj College of Engineering and Technology, Virudhunagar, Tamilnadu, India*
[2]*Department of Chemical Engineering, School of Chemical and Food Sciences, Kongu Engineering College, Perundurai, Erode, Tamilnadu, India*

□ *In this study, a hybrid system of response surface methodology followed by genetic algorithm has been adopted to optimize the production medium for L-glutamic acid fermentation with mixed cultures of* Corynebacterium glutamicum *and* Pseudomonas reptilovora. *The optimal combination of media components for maximal production of L-glutamic acid was found to be* $49.99\,g\,L^{-1}$ *of glucose,* $10\,g\,L^{-1}$ *of urea, 18.06% (v/v) of salt solution, and 4.99% (v/v) of inoculum size. The experimental glutamic acid yield at optimum condition was* $19.69\,g\,L^{-1}$*, which coincided well to the value predicted by the model (*$19.61\,g\,L^{-1}$*). Using this methodology, a nonlinear regression model was developed for the glutamic acid production. The model was validated statistically and the determination coefficient (*R^2*) was found to be 0.99.*

Keywords genetic algorithm, glutamic acid, mixed culture, optimization, response surface methodology

INTRODUCTION

Commercial use of and demand for amino acids are steadily increasing. Amino acids are being used as additives in foods, feed supplements, therapeutic agents, infusion compounds, cosmetics, pharmaceuticals, precursors for peptides synthesis, and agricultural chemicals.[1,2] Among all amino acids, L-glutamic acid is considered as the most important amino acid,

Address correspondence to Rajaram Shyam Kumar, Department of Biotechnology, Centre for Research, Bioprocess and Downstream Processing Laboratory, Kamaraj College of Engineering and Technology, S.P.G.C. Nagar, Virudhunagar, Tamilnadu, India–626001. E-mail: kingshyam2003@yahoo.co.in

because of its taste, acidic character, and flavor. L-Glutamic acid available in the form of monosodium L-glutamate (MSG) is being used as an aroma enhancer, and the total annual production is approximately 2.2 million tons with 6% growth rate per year.[3–6] Historically, Kikunae Ikeda discovered monosodium glutamate in 1908. Earlier, L-glutamic acid was produced by the acid hydrolysis of plant protein such as wheat gluten and soybeans. Recently industrial fermentation has been widely used in the production of L-glutamate and other amino acids.[5] Production of glutamic acid using several strains of bacteria with various substrates and nutrients has been reported elsewhere.[7–9] However, *Corynebacterium glutamicum* (formerly known as *Micrococus glutamicus*) and *Pseudomonas reptilivora* were generally recognized strains for the mass production of L-glutamic acid.[10–12] In industries the major challenge is the overall cost of production of amino acids. Therefore, process optimization is most important to the industrial production of amino acids. From a historical viewpoint, one-factor-at-a-time experimental design has been fairly popular with researchers, chiefly due to its ease of use. However, it is time-consuming, requires more experimental runs, and cannot examine the interaction between the independent factors. Factorial design can be used in order to defeat these limitations, in that two-level factorial design can detect interaction between factors, more practical and informative than the one-factor-at-a-time approach. Statistical approaches such as Plackett–Burman (PB) factorial design and response surface methodology (RSM) are generally preferred for achieving substantial improvement in terms of product yield and also reduction in the production cost of amino acid. Besides, artificial neural network (ANN) and stochastical approaches (genetic algorithm [GA]) have proven to be extremely suitable tools for optimization of problems associated with fermentation media development.[9,13,14] However, several attempts have been continuously made to find cheaper alternatives from microbial sources for the production of glutamic acid. With this background, a study was undertaken with a focus on the optimization of medium components toward the production of L-glutamic acid by mixed cultures of *Corynebacterium glutamicum* and *Pseudomonas reptilivora* through hybrid statistical and stochastical optimization approaches.

EXPERIMENTAL

Media and Chemicals

All media components of high purity were obtained from HiMedia Laboratories Private Limited, Mumbai, India. All remaining ingredients used were of analytical grade and purchased from Merck Limited, SD Fine

Chemicals Limited, Mumbai, India. All media and chemicals were used as supplied without any pretreatment.

Microorganisms

Stock culture *Corynebacterium glutamicum* NCIM2168 and *Pseudomonas reptilivora* NCIM2598 were obtained from National Collection of Industrial Microorganisms (NCIM), Pune, India. These cultures were growing at 30°C and preserved on nutrient agar slant at 4°C. Subculturing was done on every 10th day to maintain its viability.

Inoculum Preparation and Glutamic Acid Production

Five milliliters of a suspension of 24-hr-old slant culture of *C. glutamicum* and *P. reptilivora* was separately transferred into a 250-mL Erlenmeyer flask containing 45 mL of sterile nutrient broth medium. The flasks were kept in an orbital incubator shaker at 120 rpm and 30°C. A 1% (v/v) aliquot (equal volumes of each culture) in 100 mL of sterilized production medium (production medium content ($g\,L^{-1}$): glucose 50, urea 10, salt solution [K_2HPO_4 1, $MgSO_4 7 \cdot H_2O$ 2.5, $MnSO_4 7 \cdot H_2O$ 0.1, $CaCO_3$ 16] 20% [v/v] and biotin $0.002\,mg\,L^{-1}$) served as its inoculum, and this was kept in a rotary shaker at 120 rpm at 30°C for 48 hr. Then the cells and debris were removed by centrifugation at $10{,}000 \times g$ at 4°C for 10 min. Supernatants were used as the crude glutamic acid source for estimation. All the experiments were performed in triplicate and average values were used for further studies.

Glutamic Acid Estimation

Thin-layer chromatography (TLC) was employed for detecting L-glutamic acid in the clarified culture broth. The solvent system used in TLC includes *n*-butanol:acetic acid:water (2:1:1) (v/v). Ninhydrin at 0.02% in acetone solution was sprayed to visualize the spots, and colorimetric estimation was used for quantitative estimation of L-glutamic acid in the suspension.[15]

Design of Experiments

Design of experiments (DoE) is a statistical and systematic approach employed to estimate main effects, interaction effects, and quadratic effects of the independent parameters on the response. In the DoE, two values can be assigned to each factor, that is, −1 for low values and +1 for high values.

Transformed variables −1 and +1 are called coded variables and they have no unit of measure. The transformation used for coded values is as follows:

$$Z = \frac{x - [(x_{\min} + x_{\max})/2]}{[(x_{\max} - x_{\min})/2]} \tag{1}$$

Actual values or uncoded values can be calculated from the coded values by using the following formula:

$$x = Z\frac{(x_{\max} - x_{\min})}{2} + \frac{(x_{\max} + x_{\min})}{2} \tag{2}$$

In this study, face-centered central composite design (FCCCD) was used to investigate the glutamic acid yield. Parameters such as glucose, urea, salt solution, and inoculum size were investigated. Biotin concentration was fixed in the production medium since it was used in least quantity. The FCCCD consists of three parts with the first part being a two-level full factorial design, which is often chosen to be a resolution V design. This factorial portion contributes to estimate the linear and two-factor interactions terms. The number of runs at this portion is denoted by n_F. This portion has 2^k runs, where k is the number of independent parameters. The second part of the FCCCD is the center point portion. This portion consists of a number of replications at the center. The number of replications at the center, denoted by n_C, is often calculated as follows:

$$n_C = 4\sqrt{n_F + 1} - 2k \tag{3}$$

The center points provide information about the existence of curvature in the system. The third part is the axial portion, which comprises the axial points, sometimes referred to as the star points. It contains $2k$ experimental runs. The axial portion contributes to estimate the quadratic terms. In total, 30 runs are needed to analyze the data. The center values of all variables were coded as zero. Tables 1 and 2 show variable ranges and experimental

TABLE 1 Variables and Their Levels for RSM Experimental Design

		Levels				
Particulars	Variables	−α level	−1 level	0 level	+1 level	+α level
X_1	Glucose (g L^{-1})	5	5	27.5	50	50
X_2	Urea (g L^{-1})	5	5	7.5	10	10
X_3	Salt solution (% v/v)	5	5	12.5	20	20
X_4	Inoculum size (% v/v)	1	1	3	5	5

TABLE 2 RSM Experimental Design

	Coded Variables				Glutamic Acid Yield (g L^{-1})		
Run Number	X_1	X_2	X_3	X_4	Experimental	Predicted	Residual Value
1	−1	−1	−1	−1	8.98	8.97	0.01
2	1	−1	−1	−1	16.74	16.78	−0.04
3	−1	1	−1	−1	8.66	8.68	−0.02
4	1	1	−1	−1	16.63	16.61	0.02
5	−1	−1	1	−1	6.28	6.25	0.03
6	1	−1	1	−1	15.53	15.57	−0.04
7	−1	1	1	−1	8.24	8.27	−0.03
8	1	1	1	−1	17.74	17.72	0.02
9	−1	−1	−1	1	7.75	7.77	−0.02
10	1	−1	−1	1	16.21	16.19	0.02
11	−1	1	−1	1	8.92	8.89	0.03
12	1	1	−1	1	17.42	17.45	−0.03
13	−1	−1	1	1	6.02	6.05	−0.03
14	1	−1	1	1	16.02	16.00	0.02
15	−1	1	1	1	9.53	9.49	0.04
16	1	1	1	1	19.55	19.57	−0.02
17	−1	0	0	0	8.63	8.65	−0.02
18	1	0	0	0	17.63	17.59	0.04
19	0	−1	0	0	11.02	10.97	0.05
20	0	1	0	0	12.58	12.61	−0.03
21	0	0	−1	0	10.8	10.79	0.01
22	0	0	1	0	10.49	10.49	0.00
23	0	0	0	−1	10.88	10.84	0.04
24	0	0	0	1	11.14	11.16	−0.02
25	0	0	0	0	11.37	11.35	0.02
26	0	0	0	0	11.32	11.35	−0.03
27	0	0	0	0	11.33	11.35	−0.02
28	0	0	0	0	11.34	11.35	−0.01
29	0	0	0	0	11.30	11.35	−0.05
30	0	0	0	0	11.37	11.35	0.02

design, respectively. A second-order nonlinear polynomial equation was fitted for the experimental data:

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{ij} X_i X_j + \varepsilon \tag{4}$$

where Y is the response, that is, glutamic acid yield in g/L, n is the number of variables, β_0 is the intercept term for the model and β_i is the linear effect term, β_{ii} is the square effect term, β_{ij} is the interaction effect term, X_i and X_j are the level of the independent variables and ε is the random error. The experimental data were analyzed by Design-Expert 8.0.7.1, Stat-Ease, Inc., Minneapolis, MN.

Optimization Using Desired Function Methodology

In order to optimize the responses, a useful approach is Derringer and Suich's desirability function methodology.[16] This methodology is often used to optimize the multiple responses. However, this approach can be used for a single response. The response y_i was transformed into an individual desirability function d_i. The desirability function d_i varies over the range 0 to 1, with 0 being a completely undesirable and 1 being a completely desirable or ideal response value. Overall desirability function could be written by combining all the individual desirability, given by

$$D = \left(\prod_{i=1}^{n} d_i \right)^{\frac{1}{n}} \tag{5}$$

where D is the overall desirability and n is the number of responses. The following criterion has been adopted to predict the highest yield of glutamic acid:

$$d = \begin{cases} 0, & y < L \\ \left(\frac{y-L}{U-L}\right)^r, & L \leq y \leq U \\ 1, & y > U \end{cases} \tag{6}$$

where r is a weight factor, L is the lower response, and U is the higher response. In this study the r value was chosen as 1 and importance values of 3 and 5 were chosen, respectively, for independent variables and response.

Genetic Algorithm-Based Optimization

The genetic algorithm is an artificial intelligence-based approach that was used to optimize the RSM input space. The input vector consists of independent variables (glucose, urea, salt solution, and inoculum size). Initialization is of apopulation of independent variables called chromosomes, which were randomly formed. Then, according to the objective function, the fitness of each independent variable was evaluated. To create a new population of chromosomes, genetic operations like mutation followed by crossover were applied to the appropriate chromosomes. Until an optimal solution is found, this process continues. In this study, the RSM model equation was used as an objective function.

RESULTS AND DISCUSSION

Design of Experiments

According to the FCCCD, 31 experiments were carried out with various combinations of the four independent variables (Table 2). The data were fitted with a second-order polynomial equation represented by Eq. (7) in terms of actual values of independent variables:

$$Y = 13.66 - 0.037X_1 - 1.36X_2 - 0.049X_3 - 0.242X_4 + 3.51 \times 10^{-3}X_1^2 + 0.071X_2^2 - 0.013X_3^2 - 0.086X_4^2 + 5.78 \times 10^{-4}X_1X_2 + 2.25 \times 10^{-3}X_1X_3 + 3.47 \times 10^{-3}X_1X_4 + 0.031X_2X_3 + 0.071X_2X_4 + 0.017X_3X_4 \quad (7)$$

The significance of each individual, interaction, and quadratic term was determined by the *F*-test and probability *p*-value, shown in the analysis of variance (ANOVA) table (Table 3). All the effects of independent variables were statistically significant ($p < 0.05$). The *F*-value for the model was high (18859.42), and lack of fit was found to be not significant (0.1711), indicating that the predicted model was good. The model adequacy was confirmed by the correlation coefficient (R^2), adjusted R^2, predicted R^2, absolute

TABLE 3 Analysis of Variance for Glutamic Acid Yield (g L^{-1})

Source	Sum Square	Degree of Freedom	Mean Square	*F*-value	*p*-Value
Model	398.72	14	28.48	18859.42	<0.0001*
X_1	359.66	1	359.66	238161.4	<0.0001*
X_2	12.04	1	12.04	7971.26	<0.0001*
X_3	0.41	1	0.41	270.18	<0.0001*
X_4	0.46	1	0.46	305.14	<0.0001*
X_1X_2	0.017	1	0.017	11.19	0.004*
X_1X_3	2.31	1	2.31	1529.93	<0.0001*
X_1X_4	0.39	1	0.39	258.67	<0.0001*
X_2X_3	5.36	1	5.36	3548.84	<0.0001*
X_2X_4	2.02	1	2.02	1335.24	<0.0001*
X_3X_4	1.02	1	1.02	675.50	<0.0001*
X_1^2	8.17	1	8.17	5410.81	<0.0001*
X_2^2	0.52	1	0.52	341.09	<0.0001*
X_3^2	1.30	1	1.30	862.74	<0.0001*
X_4^2	0.31	1	0.31	2031.17	<0.0001*
Residual	0.023	15	0.0015		
Lack of fit	0.019	10	0.0019	2.42	0.1711**
Pure error	0.004	5	0.0008		
Cor total	398.75	29			

*Significant; **not significant.

average deviation (AAD), coefficient of variation (CV), and predicted error sum of squares (PRESS). The R^2 value was 0.9999, indicating that the model could explain 99.99% of the total variation. Besides, adjusted and predicted R^2 had good agreement with the determination coefficient, which indicated the aptness of the model.[17] The adequate precision value for the predicted model was found to be 491.859. The adequate precision value greater than 4, indicating that the signal-to-noise ratio is very low for the model. Therefore, the predicted model could be used to navigate the design space. AAD between experimental and predicted data was calculated to verify the model accuracy. AAD was calculated through the equation given here:

$$AAD = \left\{ \frac{\left[\sum_{i=1}^{n} \left(\left| y_{i,\exp} - y_{i,cal} \right| / y_{i,\exp} \right) \right]}{n} \right\} \times 100 \tag{8}$$

where $y_{i,\exp}$ and $y_{i,cal}$ are the experimental and predicted yield of glutamic acid, respectively, and n is the number of experimental runs. A small value of AAD (0.23%) indicated that the predicted model equation can be used for interpolation in the experimental region. The CV was 0.32% and a value less than 10% indicated that the conducted experiments were accurate and consistent.[17–19] The PRESS was found to be 0.13. The small value of PRESS was desirable. This result indicated that the predicted model was well fitted and could be used to calculate the response for a new experiment. Bias is used to find the statistical significance of the developed second-order nonlinear quadratic mathematical model. Bias is an estimator used to find out the normal distribution of errors between experimental and predicted value. Bias can be calculated as follows:

$$Bias = \exp\left[\frac{1}{n} \sum_{i=1}^{n} \ln \frac{y_{i,\exp}}{y_{i,cal}} \right] \tag{9}$$

In this study, bias value of 1 indicted that the errors are normally distributed and showed that the model was good fit. The plot between experimental and predicted glutamic acid yield is shown in Figure 1. It was clearly evident that the actual responses obtained were fairly close to the predicted responses, and the points of all actual and predicted responses fell very close to the 45° line. This result indicated that the developed model was successful in corroborating the correlation between the independent variables on the glutamic acid yield. The three-dimensional

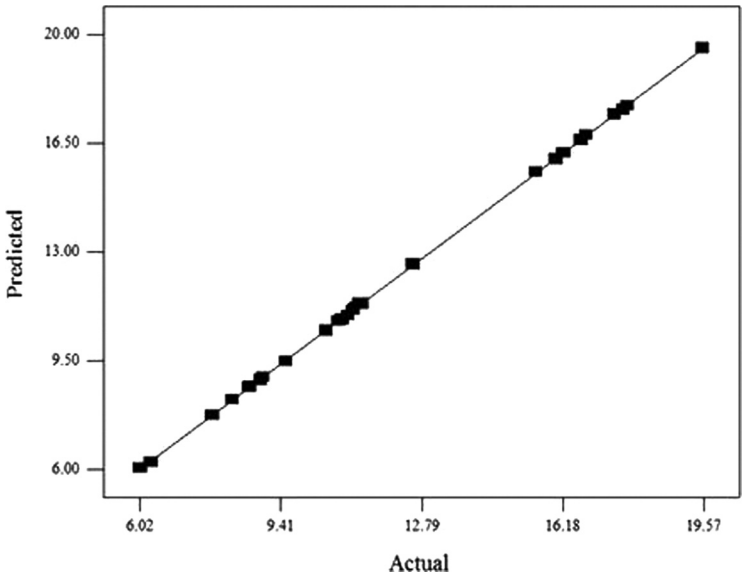

FIGURE 1 Plot of predicted vs. actual responses of glutamic acid production for model adequacy checking.

(3D) response surface graphs are shown in Figure 2, a–f. Based on the developed model, these graphs were plotted in order to determine the interaction between the variables. These plots were drawn by taking the glutamic yield on the *z*-axis against any two independent parameters on *x* and *y* axes, while all other remaining parameters were maintained at the center level. From these plots it was clearly evident that there was a strong interaction among the independent variables. Generally, productivity and growth of both the cultures were increased with the higher carbon source concentration.[20] The glutamic acid yield was increased at higher concentrations of glucose in the production medium. High yield of glutamic acid (17–19 $g\,L^{-1}$) was observed at glucose concentration was ranging from 45 to 50 $g\,L^{-1}$ in the production medium (Figures 2a–2c). Similar trend was observed and reported elsewhere.[21] Correspondingly, higher concentrations of urea and salts (Figures 2a, 2b, 2d, and 2e) in the production medium induced better yields of glutamic acid. Inoculum size plays vital role in the production of amino acid.[22] Remarkable glutamic acid production was observed (Figures 2c, 2e, and 2f) with higher inoculum density. This result was contrary to the result obtained by Tavakkoli et al.[21]

Optimization Using Desired Function Methodology

The desirability function value was found to be 1, indicating a completely desirable or ideal response value and the optimum combination

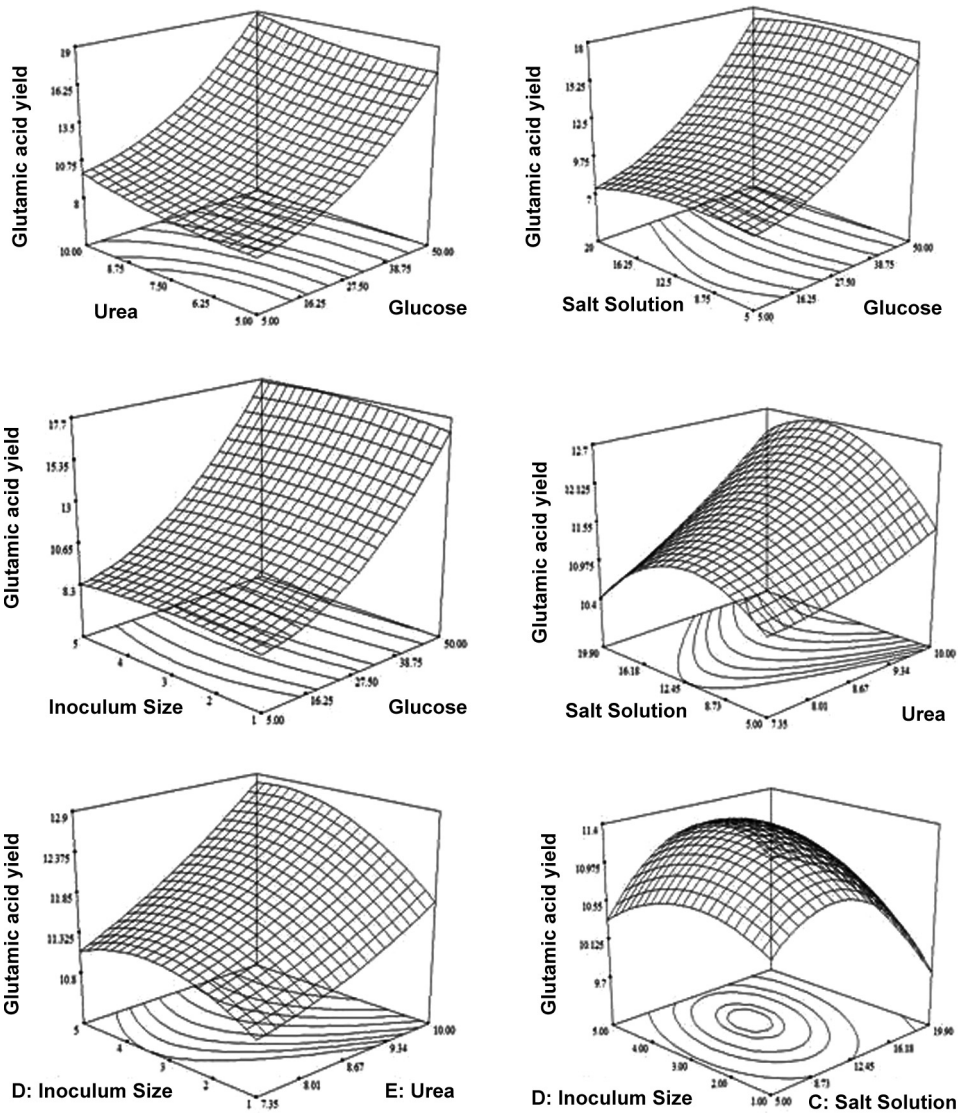

FIGURE 2 Three-dimensional response surface plot showing interaction effects of the selected fermentation factors on glutamic acid yield by mixed cultures of *Corynebacterium glutamicum* NCIM2168 and *Pseudomonas reptilivora* NCIM2598: (a) glucose vs. urea, (b) glucose vs. salt solution, (c) glucose vs. inoculum size, (d) urea vs. salt solution, (e) urea vs. inoculum size, and (f) salt solution vs. inoculum size. In these figures, only two factors mentioned on *x* and *y* axes were varied and the other two factors were kept at the center level.

of the different criteria. Optimal values of each variable and response found through desired function methodology were in very close agreement with the values obtained by experiments. The optimum conditions were found to be 49.99 g L^{-1} of glucose, 9.99 g L^{-1} of urea, 19.73% v/v of salt

concentration, and 4.91% of inoculum size. The maximum yield of glutamic acid was found to be $19.56\,g\,L^{-1}$ at the optimal conditions of the variables.

Genetic-Algorithm Based Optimization

In order to optimize the RSM model input space, a genetic algorithm (GA) approach was employed with an objective to maximize glutamic acid yield. This technique has been adopted to find the appropriate fermentation parameters for the glutamic acid production.

For the nonlinear optimization problems, GA technique is generally preferred rather than RSM, since the GA approach gives a global solution whereas the RSM model gives a local solution. The constraints applied in the GA technique were chromosome length = 30, population size = 30, crossover probability = 0.8, mutation probability = 0.2, and the number of generations over which GA is evolved = 50. The GA toolbox of MATLAB 7.0 (Mathworks, Inc., Natick, MA) was used in the optimization studies. The following objective function was used:

$$\text{Maximize } y = f(x);\ x_i^L \le x_i \le x_i^U, i = 1, 2, \ldots \ldots n \tag{10}$$

where $f(x)$ represents the objective function obtained from RSM model; x denotes the input vector; y denotes the glutamic acid yield; n refers to the number of independent variables; and x_i^L and x_i^U represent the lower and upper bounds of x_i. The GA approach was repeated several times to find the global solution. The GA converged similar solution was observed at various initial conditions, indicating that the solution was globally optimum. The predicted glutamic acid yield was found to be $19.61\,g\,L^{-1}$ at the appropriate conditions identified by the GA, which was comparatively higher than that of the glutamic acid yield predicted ($19.56\,g\,L^{-1}$) by the RSM model. This result indicated that the optimum solution predicted by RSM approach was not assured to be optimum, and a similar trend was reported in other studies.[23,24] Similar work has been carried out for enzymological studies, which increases the yield of enzymes[25] through RSM followed by ANN models, while another report[26] confirmed improved yield of exopolysaccharide through the PB-ANN-GA model. Many researchers have followed either the RSM-ANN-GA or RSM-GA approach [25–27] for the optimization process. However, in this study, the RSM model was verified with R^2, CV, AAD, PRESS, and so on, and the results revealed that the model was a good fit. However, the RSM gives only a local solution, where as the GA gives a global solution. Hence, the RSM model was further optimized by GA. It was found that the optimum solution predicted by GA

was better than that obtained by the RSM approach alone. The optimal conditions were found to be glucose 49.99 g L^{-1}, urea 10 g L^{-1}, salt solution 18.06% (v/v), and inoculum size 4.99% (v/v). Glutamic acid yield predicted (RSM-GA approach) at optimum conditions was 19.61 g L^{-1}.

Verification of the Predicted Model

Batch fermentation was carried out at optimized conditions predicted by RSM-GA design in a 3.7 L Bioreactor (Bioengineering KLF2000, Swiss) with a working volume of 2.5 L, and an intelligent frequency module was used to control various physicochemical parameters in this investigation. The maximum glutamic acid yield of 19.69 g L^{-1} was observed at 48 hr. A prolonged incubation time beyond 48 hr did not increase the glutamic acid yield. This result coincided with the RSM-GA predicted results and confirmed the validity of the model.

CONCLUSIONS

The studies on glutamic acid production using fermentation are now being increased because of tremendous applications in feed, pharmaceutical products, food, and so on. However, insight into bioprocess technology for the production of glutamic acid, the selection of optimal medium for the productivity, and growth of microorganism are difficult tasks. Hence, RSM analysis is a useful tool to make the design for various factors used for optimization of medium component for higher yield of glutamic acid production. In this study, the effects of glucose, urea, salt solution, and inoculum size were experimentally demonstrated for the production of glutamic acid. A second-order quadratic model has been developed through RSM and it was validated statistically. The genetic algorithm technique was adopted for the optimization of the RSM model. The production of glutamic acid obtained experimentally (19.69 g L^{-1}) coincides with the value predicted (19.61 g L^{-1}) by genetic algorithm, and the model was proven to be good and exhibits effectiveness. This approach has an advantage in producing a higher yield of glutamic acid in large-scale production.

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