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Optimization of Culture Conditions for L-Glutamic Acid Overproduction by Mutant *Corynebacterium glutamicum* X680 Using Response Surface Methodology

Subhadeep Ganguly[1]* and Smaranika Pattnaik[2]
[1]Department of Physiology, Vidyasagar College, Kolkata, India
[2]Department of Biotechnology and Bioinformatics, Sambalpur University, India

ABSTRACT

L-glutamic acid is a non-essential, proteinogenic amino acid. Its large-scale industrial production using fermentation strategies was initiated in 1957. L-glutamic acid has a huge industrial demand which accounts for about more than fifty lakhs tons /annum throughout the globe. Japan, United States, South Korea, China and Europe are some major producer countries. Several trials have been made to scale-up the process by optimization of different parameters by one factor at a time. But recent studies revealed that Response Surface Methodology (RSM) is the most effective tool for process optimization as it involves multiple factors at a time. For this paper, Response Surface Methodology (RSM) was used for statistical optimization of fermentation conditions that influence the yield of L-glutamic acid by a biotin auxotrophic mutant *Corynebacterium glutamicum* X680. Response surface optimization of Box-Behnken design (BBD) was applied to evaluate the effects of culture conditions including some medium composition components. Among the variable screened, inoculum age, temperature, glucose concentration and urea concentration had significant effects on product yield. Production was significantly ($p<0.05$) increased. Inoculum age, 48.92 h; temperature, 29.99oC; glucose,11.56% and urea, 1% could result in highest L-glutamic acid production of 25.61 mg/ml by *Corynebacterium glutamicum* X680 as predicted by RSM which is very close to the actual value 25.7mg/ml that confirmed its validity. RSM appeared as a very effective tool for optimization of L-glutamic acid production by this mutant. This research has been conducted to bolster future researches with credible information and updated references. This paper can act as a primary source for future studies to begin their research from.

KEY WORDS: CORYNEBACTERIUM GLUTAMICUM X680, FERMENTATION, L-GLUTAMIC ACID, RESPONSE SURFACE METHODOLOGY, STATISTICAL OPTIMIZATION

INTRODUCTION

L-glutamic acid is a proteinogenic, non-essential amino acid. It was first discovered by Ritthausen in (1866) on hydrolysis of gliadin (Ritthausen 1866). In (1908), a Japanese scientist Kikunae Ikeda observed that glutamate was essential for a meal to taste good (Ikeda 1908). He isolated glutamic acid from kelp like sea weed broth, locally called 'konbu' (*Laminaria japonica*) in Japan. Later it was also found in asparagus, hydrolysate of wheat gluten, tomato, cheese etc. He identified L-glutamic acid as a separate food additive with a distinct taste which was a century later identified as the fifth basic taste modality 'umami' (Lindemann et al. 2002). Next, in the year (1909), monosodium salt

of glutamic acid was commercialized as monosodium glutamate (MSG) by Ajinomoto co. and gained the popular trade name Ajinomoto. Since then, it was used in the wide range of products within processed industry, as a flavoring agent to influence market economy (Ault 2004; Alharbi et al. 2020).

Several trials have been made to improve the commercial production of L-glutamic acid. Initially hydrolysis of protein was used but it turned out to be a cumbersome process. Later, its chemical synthesis was also tried, however, during the course of its synthesis, racemic mixture of DL isomers was generated from which separation of metabolically active L-isomer of glutamic acid was very difficult (Sano 2009; Alharbi et al. 2020). Over and above its flavor enhancing capacity, it has several commercial values. It is a precursor of multiple amino acids such as proline and arginine. It is also a neurotransmitter. So, excessive use of MSG as

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flavor enhancer may lead to development of neurotoxicity (Xiong et al. 2003). It has got several pharmaceutical and therapeutic uses. In cosmetic industry, glutamic acid and its derivatives are widely used (Hermann 2003; Lee et al. 2014; Alharbi et al. 2020).

A new dimension has opened through the discovery of L-stereo-specific glutamic acid over producer microorganism from Japanese soil in 1955-56 by a group of scientists of Kyowas Hakko Co. Inc headed by Dr. Shigezo Udaka (Kinoshita 1987). Multiple efforts led to development of several L-glutamic acid over producers through a series of successful research through several decades. But the major constrain of its industrial production lies upon its huge production cost. So, reduction of its production cost is another motto (Joseph and Rao 1973; Kishimoto et al. 1980; Nanninga and Gottschal 1985; Tsuchida et al. 1987; Neubeck et al. 1993; Amin 1994; Das et al. 1995; Bona and Moser 1997; Gourdon and Lindley 1999; Kumagai 2000; Delaunay et al. 2002; Choi et al. 2004; Takeno et al. 2007; Engels et al. 2008; Niaz et al. 2009; Tarek and Mostafa 2010; Nadeem et al. 2011; Zareian et al. 2012; Nishio et al. 2013; Shyamkumar et al. 2014; Vuoristo et al. 2015; Wendisch et al. 2016; Hirasawa and Wachi 2017; Sgobba et al. 2018; Wen and Bao 2019; Liu 2019).

Considering its huge industrial demands (which accounts for about more than fifty lakhs tons /annum throughout the globe), Japan, United States, South Korea, China and Europe are the major the major producer countries (Liu 2019). However, the huge market demand for L-glutamic acid in the fast-food industries in India is almost totally dependent on import. Each microorganism grows well within a definite range of environmental consortium which imparts the necessity for optimization of its culture conditions. Response surface methodology (RSM) is a strong statistical tool for analyzing the effects of multiple parameters on the process (Baş and Boyaci 2007; Mansouri et al. 2012; Zafar and Mahmood 2015). Empirical optimization is a single dimensional study which requires a long time to conduct, whereas RSM reduces number of experimental trials and makes the whole process more compact and quicker. Moreover, it calculates complex interactions between the variables depending on the experimental trials which give more accuracy in the experimental results (Fahimitabar et al. 2021).

Alharbi et al. (2020) used RSM for optimization of L-glutamic acid production by *Corynebacterium glutamicum* and obtained 16.499g/L L-glutamic acid. Very recently, Fahimitabar et al. (2021) applied RSM for optimization of L-glutamic acid production using *Corynebacterium glutamicum* in batch culture (Alharbi et al. 2020; Fahimitabar et al. 2021). Present study was undertaken to investigate the influence of independent variables and their interactions on L-glutamic acid production by a biotin-auxotrophic mutant *Corynebacterium glutamicum* X680 in sub-merged fermentation process.

MATERIAL AND METHODS

Isolation, characterization and development of a biotin

dependent L-glutamic acid overproducing strain *Corynebacterium glutamicum* X680 was done in our previous study (Ganguly 2019). This mutant was used throughout the present study. The growth medium for the mutant was composed of glucose, 2%; peptone, 0.05%; yeast extract, 0.1%; beef extract, 0.3%; K_2HPO_4, 0.1%; KH_2PO_4, 0.1%; $MgSO_4.7H_2O$, 0.025% and water, 1L. L-glutamic acid production was carried out in a medium composed of: glucose, 10%; urea, yeast extract, 0.2%; 0.8%; K_2HPO, 0.1%; K_2HPO, 0.1%; $MgSO_4.7H_2O$, 0.025% and water, 1L. The experiments were carried out at 30 °C for 72h incubation at pH7.0. L-glutamic acid accumulated in the fermentation broth was analyzed by Thin Layer Chromatography (TLC) method using silica gel (Merk, Germany) using n-butanol: methanol: water as mobile phase in a ratio of 5:4:3 (v/v/v) and the spots were visualized using ninhydrin (0.2%) in alcohol followed by heating. The product was confirmed as L-glutamic acid by FT-IR (Fig 2). L-glutamic acid recovered from the fermentation broth was analyzed further by agilent 1200 Infinity HPLC (using a phenyl-hexyl column and 50mM N-methylmorpholine/acetate buffer with pH 7.4 containing 12% acetonitrile as elute solvent and detected by UV absorption at 636nm with a flow rate of 1ml/min for 2.5 min and 2ml/min for 1.5 min). After filtration through a membrane filter (0.22µm), the sample was analyzed by HPLC to measure the amount of L-glutamic acid relating to the standard calibration curve.

The first step in this study was to identify the levels of process parameters which have high influence on enzyme production (response). After preliminary screening, a Box–Behnken factorial design (BBD) was employed for further optimization of the effective level of four most influencing factors for production of L-glutamic acid. An experimental design comprising 29 experimental run was created using the four parameters at three equidistance level (−1, 0 and +1) on Design Expert 12 software (Stat ease Corp, USA). The response was tannase activity (U/ml). A polynomial quadratic equation was adopted to evaluate the contribution of each independent variable in this process.

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j$$

Where, Y is the predicted response; xi and xj are the independent variables in coded values that influence the response, βo the offset term; βi represent the linier effect of xi; βij represent the interaction effect between xi and xj; βii represent the quadratic effect of xi. Regression analysis and estimation of the coefficients were performed using Design Expert software (Stat ease Corp, USA).

RESULTS AND DISCUSSION

Overproduction of desired metabolite by submerged fermentation is not only dependent on the selection of suitable strain, but also optimization of culture conditions and downstream purification technology exert significant impact (Alharbi et al. 2020). In this present investigation, after preliminary OVAT analysis it was found that four factors viz. inoculum age, temperature, glucose

concentration and urea concentration play pivotal role in L-glutamic acid production by the *Corynebacterium glutamicum* X680. Subsequently, the effects of these four factors were optimized through RSM according to BBD. A total of 29 run with different combination of the four variables (in three equi-distance levels) along with the actual and predicted responses were presented in Table 1. ANOVA of the quadratic regression models suggested that the models were significant with a computed F-value of 183.93 for L-glutamic acid production and P-value (Prob> F) lower than 0.0001 (Table 2). A lower value for the coefficient of variance (P < 0.0001) suggests higher reliability of the experiment. The fitness of the model was also examined by the coefficient of determination (R^2).

Table 1. RSM for optimizing L-glutamic acid production by BBD

Run	A: Inoculum age	B: Temperature	C: Glucose	D: Urea	Actual	Predicted
1	48	32	14	1	16.2	16.42083
2	48	28	14	1	19.6	19.9375
3	48	30	12	1	25.4	25.48
4	48	30	12	1	25.6	25.48
5	48	30	14	0.7	18.9	18.73333
6	60	30	10	1	22.1	21.97917
7	36	32	12	1	18.7	18.38333
8	48	32	10	1	21.9	21.52083
9	48	30	12	1	25.4	25.48
10	36	30	14	1	19.2	19.32917
11	36	30	12	0.7	20.9	20.77083
12	48	30	14	1.3	16.7	16.5
13	36	30	10	1	18.6	18.92917
14	48	30	12	1	25.7	25.48
15	60	30	12	0.7	16.4	16.22083
16	36	30	12	1.3	15.1	15.2375
17	60	28	12	1	19.1	19.45
18	48	28	12	1.3	18.1	17.84583
19	48	28	10	1	19.6	19.3375
20	36	28	12	1	18.9	18.75
21	48	30	10	1.3	20.2	20.4
22	48	28	12	0.7	19.2	19.17917
23	48	30	12	1	25.3	25.48
24	48	32	12	1.3	17.9	17.92917
25	60	30	14	1	17.4	17.07917
26	60	32	12	1	18.3	18.48333
27	48	32	12	0.7	17.5	17.7625
28	48	30	10	0.7	19.1	19.33333
29	60	30	12	1.3	20.5	20.5875

In the present study, R^2 values were found to be 0.9946 that indicated the response models can explain 99.46% of total variations for L-glutamic acid production and the rest may occur due to chance. Analysis of the quadratic effect indicated that all variables significantly contributed to these responses (P < 0.05). The models also showed statistically insignificant lack-of-fit of 4.70 for L-glutamic acid. All the statistical indices suggested that the models were suitable to represent the real relationship among the selected factors for L-glutamic acid production by *Corynebacterium glutamicum* X680. The mutual effects of any two factors on L-glutamic acid were also found encouraging. The most significant (P < 0.05) mutual interactions for L-glutamic acid production was in between inoculum age and urea concentration followed by temperature and glucose concentration which are revealed by AVOVA data (Table 2) and response surface-contour plots (Fig. 1). Using Design Expert 12 numerical optimization subroutine design space was explored with the fitted quadratic model to arrive at an optimum fermentation condition. The optimized variables were found using a desirability objective function that assigns relative importance to the responses.

Table 2. Statistical analysis of the BBD model

Source	Sum of squares	df	Mean square	F-value	p-value	Comments
Model	253.3	14	18.09	183.93	< 0.0001	Significant
A-Inoculum age	0.48	1	0.48	4.88	0.0443	
B-Temperature	1.33	1	1.33	13.55	0.0025	
C-Glucose	15.19	1	15.19	154.39	< 0.0001	
D-Urea	1.02	1	1.02	10.38	0.0062	
AB	0.09	1	0.09	0.9149	0.355	
AC	7.02	1	7.02	71.39	< 0.0001	
AD	24.5	1	24.5	249.09	< 0.0001	
BC	8.12	1	8.12	82.57	< 0.0001	
BD	0.5625	1	0.5625	5.72	0.0314	
CD	2.72	1	2.72	27.68	0.0001	
A²	72.54	1	72.54	737.44	< 0.0001	
B²	73.63	1	73.63	748.51	< 0.0001	
C²	51.1	1	51.1	519.44	< 0.0001	
D²	100.27	1	100.27	1019.31	< 0.0001	
Residual	1.38	14	0.0984			
Lack of Fit	1.27	10	0.1269	4.7	0.0746	not significant
Pure Error	0.108	4	0.027			
Cor Total	254.67	28				

Factor coding is Coded.

Sum of squares is Type III – Partial

Figure 1: Three-dimensional Response Surface and contour plots showing most effective interactions among selected variables influencing L-glutamic acid production by Corynebacterium glutamicum X680 (a)Temperature vs inoculum age; (b) glucose vs inoculum age; (c) (c) Urea vs inoculum age; (d) Glucose vs temperature; (e) Urea vs temperature; (f) Urea vs glucose
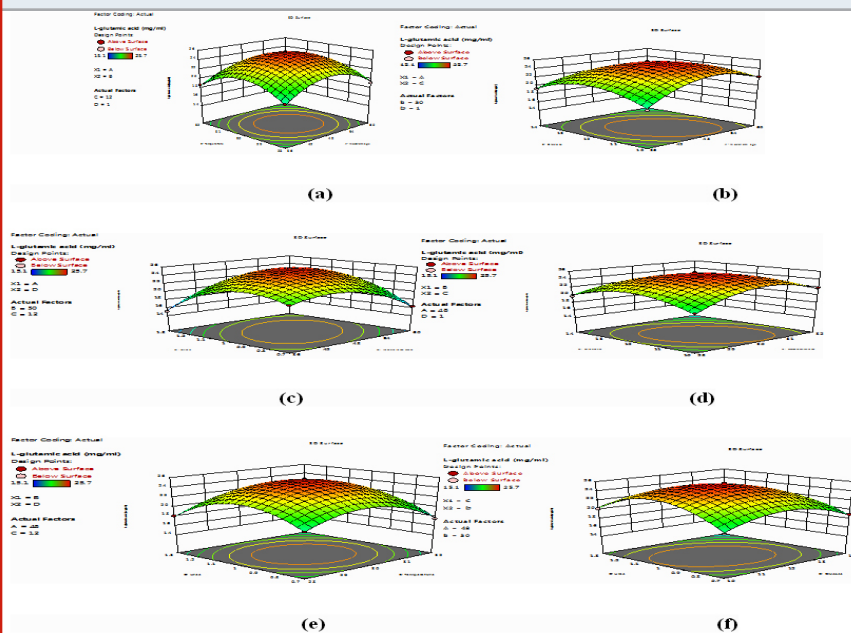
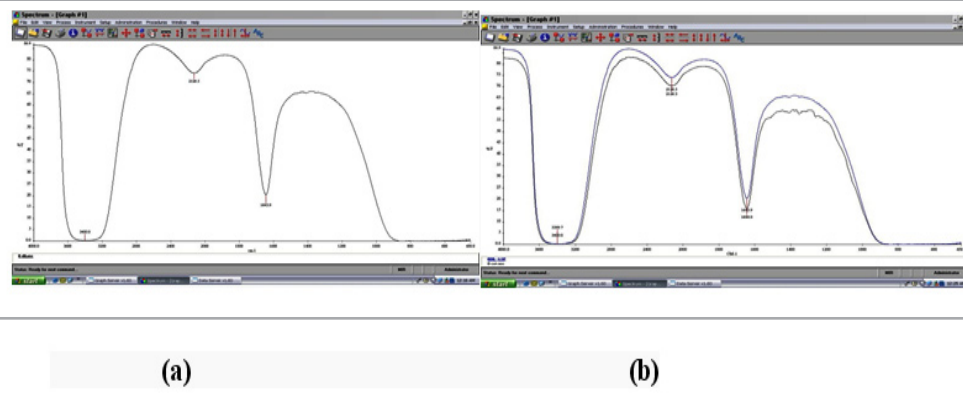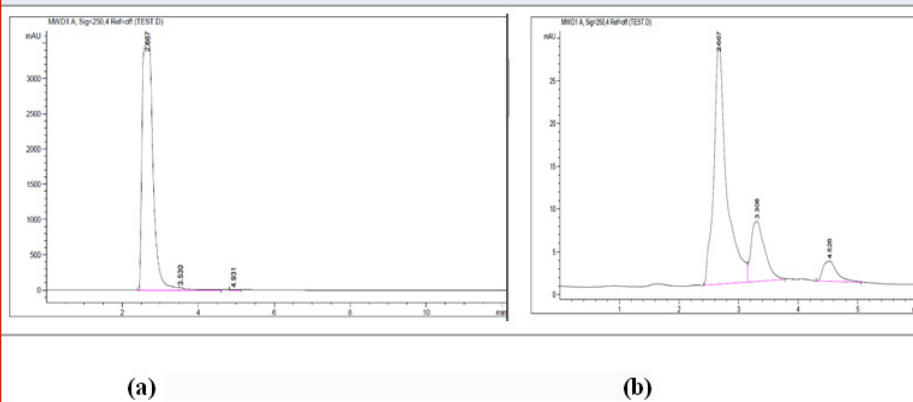**Figure 2: FT-IR for L-glutamic acid (a) Control; (b) Sample**

(a)   (b)

Figure 3: HPLC for L-glutamic acid. (a) Control, (b) Sample

(a)   (b)

Solutions with higher desirability suggested inoculum age of 48.92 h, temperature of 29.99oC, glucose concentration of 11.56% and urea concentration of 1% could resulted in highest L-glutamic acid production of 25.25.61 mg/ml by *Corynebacterium glutamicum* X680. Under these conditions, confirmation experiments were conducted in three replicates and the result have good agreement with the predicted model which validate the suitability of the model (Guo et al. 2017; Fahimitabar et al. 2021).

The Model F-value of 183.93 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case A, B, C, D, AC, AD, BC, BD, CD, A², B², C², D² are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

The Lack of Fit F-value of 4.70 implies there is a 7.46% chance that a Lack of Fit F-value this large could occur due to noise. Lack of fit is bad -- we want the model to fit. This relatively low probability (<10%) is troubling. Yang et al. (2014) optimized fermentation conditions using Streptomyces albus Y07 as a high yielding strain through RSM. Nor et al. (2017) made a comparative analysis on medium optimization for L-lysine-methionine production by a newly isolated strain Pendiococcus pentosaceus RF1 using one factor at a time, Response Surface Methodology and artificial neural network (Nor et al. 2017).

RSM appeared as a very effective tool for this experiment. Medium optimization for ε-poly-L-lysine production by *Streptomyces diastatochromogenes* was carried out by Guo et al. (2017) using RSM. Very recently, Alharbi et al. (2020) noted RSM as a very effective statistical tool for optimizing culture conditions for L-glutamic acid production by *Corynebacterium glutamicum* NCIM2168, however the production was 16.499g/L. Very recently, Fahimitabar et al. (2021) also applied RSM for optimization of L-glutamic acid by *Corynebacterium glutamicum* in batch fermentation. In our present study, it has been found that RSM appeared to be a very effective tool so far as the optimization of L-glutamic acid production by the mutant was concerned considering multifactorial interactions at a time (Fahimitabar et al. 2021).

CONCLUSION

The findings of the present study targets RSM that helps to point out the causal relationship between different factors and response using a reliable and robust mathematical model. For the validation of this model, statistical and

regression analysis was done. It was found that among different variables involved, most effective variables are inoculum age, 48.92h; temperature, 29.990C; glucose, 11.6% and urea, 1%. Under theses optimum conditions, the predicted response for L-glutamic acid was to be 25.61mg/ml and the actual value was found 25.7mg/ml under the same conditions. These results implied the validity of the predicted value by RSM and experimental value was quite close.

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