Research Article

Statistical Modeling and Optimization for L-Methionine Production by *Corynebacterium glutamicumn* Using Plackett-Burman Design, Response Surface Methodology and Artificial Neural Networks

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ABSTRACT

The present study is concerned with the optimization of fermentation parameters through statistical approach for improvement of L-Methionine production by *Corynebacterium glutamicum* (MTCC 2745) from agricultural products in submerged shake flask fermentation. The medium components influencing the L-Methionine production were identified using Plackett-Burman design. Among the eight variables screened, four variables such as plantain, groundnut, $MgSO_4.7H_2O$ and inoculums size were showed significant effect on L-Methionine production. The optimum levels of these variables were determined using RSM based on CCD. A RSM involving 4 variables and 5 levels was adopted for acquiring the best medium for the production of L-Methionine. The second order polynomial equation was developed to correlate the relationship between four significant variables and L-Methionine production. The optimum values of most significant variables were determined by RSMas follows: plantain (21.19 g/l), groundnut (11.20 g/l), $MgSO_4.7H_2O$ (1.21 g/l) and inoculum size (3.30 ml). By using optimal medium components, the experimental L-Methionine production was 5.30 g/l as compared to 4.6 g/l with one factor at a time optimization method and 2.8 g/l with basal medium after 96 hrs of fermentation whereas the predicted response by the RSM statistical model was 5.424 g/l. The close agreement of RSM predicted response with that of experimental value showed that statistical design model can be used in order to improve the L-Methionine production to meet increasing demand as feed additive for food and pharmaceutical industries. This study also showed that RSM and ANN models provided desired predictions. However, compared with RSM (R^2=0.9886), the ANN model (R^2=0.9914) provided a better prediction for L-Methionine production.

Keywords: L-Methionine, *Corenybacterium glutamicum*, Statistical optimization, Plackett-Burman, RSM and ANN.

INTRODUCTION

Methionine, alpha-L-amino-gamma-methylthio-n-butyric acid is nutritionally essential for mammals and fowls. It can't be synthesized internally, but may be added to food and feed materials to improve the protein quality[1]. Methionine is generally being produced by chemical and enzymatic methods, both are expensive, chemical method requires hazardous chemicals and enzymatic method requires expensive enzymes. Methionine can be produced economically by using fermentation, because many fermentation processes have been developed to produce many other amino acids inexpensively [1,2,3] using agricultural products. The importance of using agriculture media is the low cost, rich in nutrients and free of toxins. It has been reported that India has a large production of corn, millet, plantain, rice, wheat, potato, cowpea, pigeon pea and ground nut. Due to huge production of these agricultural products in India, L-Methionine production is likely to be more economical.

The history of species *Corynebacterium* as amino acid producer started in the 1950s when Dr. Kinoshita was the first to discover that *Corynebacteria glutamicum* is a superior amino acid producer [4,5, 6]. Now a day's L-glutamic acid, L-lysine, L-isoleucine, L-threonine, L-aspartic acid and L-alanine are produced by *Corynebacteria* in terms of high production rate and economical value. Generally, optimization of medium composition is done, so as to obtain maximum response from minimum inputs. The conventional method of optimization (one factor at a time) involves varying one factor at a time and keeping others at fixed level is extremely time consuming and expensive when a large number of variables are evaluating at different levels and also often misses interactions among medium constituents. Response surface optimization of four significant components screened by Plackett-Burman fairly reduces the total number of experiments required (only 30) and also manifests any possible interaction between the medium constituents.

RSM may be summarized as a collection of experimental strategies, mathematical models and statistical interferences for constructing and exploring functional relationship between a response variable and a set of input design variables [7]. RSM has already been successfully applied for the optimization of media and culture conditions in many fermentation processes for the production of amino acids, enzymes and ethanol [8,9,10].

Artificial neural network (ANN) model is a well established and fashionable tool in the analysis and also used to understand the biotechnology applications such as expression function function, functional analysis of genomics and proteomics. ANN is an extremely connected network structure consists of many processing

elements which are capable of performing parallel computation for data processing[11].

In the present work, statistical approach has been employed for which a Plackett-Burman design is used for identifying significant variables influencing L-Methionine production under submerged fermentation by *C.glutamicum* using agricultural products as substrates. The levels of significant variables were further optimized by using response surface methodology and artificial neural networks to enhance the production of L-Methionine by *C.glutamicum* using agricultural products as substrate.

MATERIALS AND METHODS

Microorganism and Culture conditions

L-Methionine producing strain of *C.glutamicum* MTCC 2745 obtained from the microbial type collection centre, Chandigarh, India was used throughout this study. It was maintained on nutrient agar slants (Beef extract 1 g/l; Yeast extract, 2 g/l; Peptone, 5 g/l; Nacl, 5 g/l; Agar, 15 g/l and pH was adjusted to 7.2 with 1N NaOH) and stored at refrigeration temperature 4°C for further analysis.3 ml of 24 hour slant culture was used to inoculate a 100 ml Erlenmeyer flask containing 30 ml of seed medium.

Carbon sources (Preparation of starches)

Agriculture products utilized here for the preparation of starches are corn, millet, plantain, rice, wheat and potato. Starches were prepared according to the method portrayed by[12]. Potato and plantain samples were brought from Guntur (Andhra Pradesh, India) local market were first peeled, washed and cut into little pieces before being homogenized with water in Moulinex blender. Corn, millet, rice and wheat were drenched for 48 hours to soften the seeds and then homogenized with water. Every homogenate blended with excess water was tied in cheese cloth and placed on tripod stand overnight, to take into account extraction of starch into a clean plastic bowl. The supernatant was emptied and the sedimented starch dried at 50^0C for 48 hours. The resultant chips were grounded into powder and utilized as starches.

Saccharification of starch

Saccharification of starches took after the method illustrated by [13]. A 500 ml flask containing a mixture of 30 g of starch and 100 ml of water was heated for 15 min at 95^0C in a water bath to gelatinize starch. The beaker was covered with aluminium foil after adding 1 ml of α-amylase and again heated in water bath for 10 min at 95^0C to impact liquefaction. After cooling liquefied starch to 60^0C, 1 ml amyloglucosidase enzyme was added before replacing the beaker in the water bath at 60^0C for 48 hr for saccharification to takes place.

Nitrogen sources: Preparation of defatted proteins

The proteins utilized here from agricultural products as nitrogen sources are cowpea, pigeon pea and groundnut. For preparation of defatted proteins took after the strategy explained by study[13]. Cowpea, pigeon pea and groundnut were crushed in a blender and then some division of homogenized proteins was defatted by soxhlet extraction method using diethyl ether. The meals obtained after extraction were oven dried at 34-35^{0}C for 20 hr and afterward ground into fine powder.

Fermentation Experiments

Fermentation experiments were conducted based on the method described by [14]. The medium used for fermentation composed of the following composition: Plantain (starchy material as carbon source, 20 g/l; Groundnut (defatted protein as nitrogen source), 10 g/l; KH_2PO_4, 0.5 g/l; K_2HPO_4 0.5 g/l; $MgSO_4.7H_2O$ 1 g/l; $FeSO_4.7H_2O$ 0.01 g/l; $MnSO_4.4H_2O$ 0.01 g/l; biotin 100 µg/l; $CaCO_3$ 20 g/l; water 1 liter; pH was adjusted to 7.2 with 1N NaOH. 30 ml of medium in 100 ml Erlenmeyer flask was sterilized in an autoclave at 121^0C for 20 minutes and then cooled and inoculated with 3 ml of seed inoculums. After 96 hr of incubation on rotary shaker at 170 rpm and 30^0C, growth and methionine accumulation were determined from culture broth. Duplicate flasks were used and uninoculated flasks served as control. All the measurements were taken thrice and average values were reported.

L-Methionine Assay

Quantitative determination of L-methionine in the culture broth without purification was carried out by the modified calorimetric method of [15]. A 5ml volume of the culture broth was centrifuged at 5,000xg for 20 minutes and the cell free supernatant was assayed for L-methionine. 1 ml of 5N NaOH was added to a test tube followed by the addition of 0.1ml of 10% sodium nitroprusside solution with thorough mixing. The mixture was allowed to stand for 10 min. Then two milliliters of 3% aqueous solution of glycine was added to the reaction mixture with frequent shaking over a period of 10 min. After an additional 10 min interval, 2ml of concentrated *ortho*-phosphoric acid was added drop wise to the mixture with shaking. Colour development was allowed to proceed for 5 min and the colour intensity measured at 540nm in a spectrometer. A blank containing distilled water and all other reagent served as the 100% transference standard. Results obtained with the test samples were interpolated on a standard methionine curve.

Estimation of Reducing Sugar

The reducing sugar (glucose) in the time-course fermentation broth was estimated by the modified method described by [16]. A 1ml volume of dinitrosalicylic acid was added to 1ml of the supernatant in a test tube and the mixture heated in boiling water for 10 minutes. The test tube was cooled rapidly under tap water. 1ml of 4% potassium sodium tartarate was added and the volume was adjusted to 12 ml with distilled water. A blank containing 1 litre of distilled water and 1 ml of dinitrosalicylic acid was similarly prepared. The optical

density of the sample was read against the blank in a spectrophotometer at 540nm. The concentration of the reducing sugar in the supernatant was estimated from a standard glucose curve.

Screening of significant variables using Plackett-Burman experimental design

The purpose of PBD was to screen significant components in the medium with respect to their main effects. Plackett-Burman design is a statistical design proposed by previous study[17]. It is a two factorial design, that is, low level (-) and high level (+). It was based on the first order model with no interactions among the factors. The variables chosen for present study were plantain (X_1), groundnut (X_2), $CaCO_3$(X_3), K_2HPO_4 (X_4), KH_2PO_4 (X_5), biotin (X_6), $MgSO_4.7H_2O$ (X_7), inoculum size (X_8), dummy1 (D_1), dummy2 (D_2) and dummy3 (D_3). The effect of individual components on L-Methionine production was calculated by following equation.

$$E = \frac{2\left(\sum H^{+} - \sum H^{-}\right)}{N}$$

Where E is the effect of parameters and H^+ and H^- are responses of trails in which the parameter high and low levels respectively and N is the number of trails. Three dummy variables were incorporated to increase the reliability of results.

Optimization by using Response surface methodology

The second step in the medium formulation was to identify the optimum levels of significant variables for the production of L-Methionine. For this purpose, RSM using a CCD was adopted for the production of L-Mehtionine. The significant variables screened by Plackett-Burman design were as follows: plantain as carbon source (X_1), groundnut as nitrogen source (X_2), $MgSO_4.7H_2O$ (X_7) and inoculum size (X_8) and each of these variables were examined at five coded levels (-2,-1, 0, +1, +2) and a total of 30 experiments were conducted having six replicates at centre points. Response value in each experiment was the average of triplicates. The four variables used in RSM were coded according to the following equation

$$Z = \frac{\left(X - X_0\right)}{\Delta X}$$

here Z is the coded value of independent variable, X is the respective real value, X_0 is the centre point of real value, ΔX is the step change of real value.

The data obtained from RSM on L-Methionine production were subjected to analysis of variance (ANOVA). The experimental results of RSM were fitted to the following second order polynomial equation through response surface regression method.

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j$$

Where Y is the predicted response, β_0 is the offset term, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} and is the interaction effect. The statistical software package Design-Expert 7.0.0 version was used for the design of experiments and regression analysis of experimental data and also to plot the response surface graphs to understand the effects of interactions on the response. The significance of model and model terms are evaluated via the Fisher's test. The quality of fit of quadratic equation was expressed through the coefficient of determination (R^2) and adjusted R^2. The fitted polynomial equation was then expressed in the form of three dimensional surface plots in order to explain relationship between the responses and levels of each variables used in this study. The point optimization method was used in order to optimize the levels of each variable for maximum response.

Modeling using artificial neural networks

Artificial neural network (ANN) modeling is an alternative tool to RSM for regression analysis of polynomial non-linear systems. ANN architecture is an interlinked complex with elements such as neurons and the connections between the neurons were described by weights (w) and bias (b). The neurons were controlled by transfer and summing functions and general transfer functions include purelin, log sig, and tan sig [18]. In the present study, the predictive model was developed using plantain (g/l), groundnut (g/l), $MgSO_4.7H_2O$ (g/l) and inoculums size (ml) as input variables and L-Methionine production (g/l) as the output for the model. The input layer function is to pass the scaled input values to hidden layer through weights. A back-Propagation algorithm is used with one hidden layer enhanced with Levenberg–Marquardt optimization method [19].

RESULTS AND DISCUSSIONS

L-Methionine Assay (Nitroprusside method)

L-Methionine in the culture broth without purification was carried out by the modified calorimetric method of reference[15]. The liberated methionine gives yellow colour with nitroprusside solution under alkaline condition and turns red on acidification.

Identification of significant variables using Plackett-Burman design

Twelve experiments were conducted from eight variables and three dummy variables (plantain (X_1), groundnut (X_2), $CaCO_3$ (X_3), K_2HPO_4 (X_4), KH_2PO_4 (X_5), biotin (X_6), $MgSO_4.7H_2O$ (X_7) and inoculum size (X_8), Dummy1 (D_1), Dummy2 (D_2) and Dummy3 (D_3). The variables with a confidence level greater than 95% ($p<0.05$) were considered to have a significant effect on L-Methionine production. The high and low levels of each variable used in PBD experiments as shown in Table.1. Plackett-But man design matrix of the 12 experiments with eight independent variables and 3 dummy variables along with

observed and predicted values of L-Methionine production by *C.glutamicum* as shown in Table.2. * Indicates significant model terms for which p-values<0.05. From analysis of variance (ANOVA) of Plackett-Burman experimental design as shown in Table.3, plantain (P=0.0227) and $MgSO_4.7H_2O$ (P=0.0227) were the most influential variables on L-Methionine production followed by groundnut (P=0.0318) and inoculums size (P=0.0335). ANOVA also explains further significance by putting coded data in linear equation as follows

$$\text{Methionine concentration} = 3.10 + 0.46667X1 + 0.3333X2 + 0.11667X3 + 0.05X4 + 0.0001X5 + 0.0333X6 + 0.46667X7 + 0.31667X8 - 0.08333D1 - 0.05D2 - 0.066667D3$$

ANOVA of Plackett-Burman design showed that R^2 was 0.9996 which proved that model is significant where as predicted R^2 of 0.9410 is in reasonable agreement with adjusted $R^2$of 0.995. The Model F-value of 244.10 also implies the model is significant. There is only a 4.98% chance that a "Model F-Value" this large could occur due to noise. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Adequate precision of 52.463 indicated that an adequate signal for soundness of the model. This model and significant variables can be used for further optimization by RSM.

Plantain has been converted to starch powder according to method described by [12] and saccharification of starches carried out using procedure given by [13] and then estimated glucose concentration by using DNS method, 100 g/l initial glucose was obtained from 20 g/l of plantain starch powder. In Plackett-Burman screening plantain and ground nut were two of most influential variables on L-methionine production and in the previous works plantain and groundnut were reported by [20] using *Bacillus cereus* S8 from agricultural products obtained 2.05 mg/ml of methionine in submerged fermentation. In 2008, Adoki used plantain waste as carbon source for yeast growth and protein production by *Candida* species [21].

$MgSO_4.7H_2O$ had a positive effect on L-Methionine production, this is mainly due to methionine is sulphur containing amino acid addition of $MgSO_4.7H_2O$ to the medium enhanced the growth and L-Methionine production and many researchers have been used $MgSO_4.7H_2O$in their medium for production of L-Methionine[22, 23, 24]. The amount of inoculum added into fermentation broth affects metabolite production and results showed that 3% inoculum size was the optimal for maximum production of L-Methionine. The effect of inoculum size on metabolite production has been reported by [25, 26, 27].There are many number of reports in which PBD has been used to screen the variables in a fermentation medium and to be optimized in further experiments [9, 28, 29].After finding the significant variable, next step was to optimize the concentrations of these variables for maximal production of L-Methionine. For this purpose, a RSM using CCD was used.

Table 1: Nutrient concentrations at low and high levels in plackett-Burman design for L-Methionine production by *C.glutamicum*

Variable No.	Variables with designate	Low Level (-1)	High Level (+1)	Units
1	Plantain (X1)	15	25	g/l
2	Groundnut (X2)	5	15	g/l
3	$CaCO_3$ (X3)	15	25	g/l
4	K_2HPO_4 (X4)	0	1	g/l
5	KH_2PO_4 (X5)	0	1	g/l
6	Biotin (X6)	75	125	µg/l
7	$MgSO_4.7H_2O$ (X7)	0	2	g/l
8	Inoculum size (X8)	1	5	ml
9	Dummy1 (D1)	-	-	-
10	Dummy2 (D2)	-	-	-
11	Dummy3 (D3)	-	-	-

Optimization of significant variables by Response surface methodology

Ranges, levels, units and coded values of four significant variables (Plantain (X_1), Groundnut (X_2), MgSO4.7H2O (X_7) and Inoculum size (X_8)) screened by PBD for RSM experiments as shown in Table.4. In the present study 30 experiments were conducted to determine optimum levels and interaction of four significant variables on L-Methionine production as shown in Table.5 along with observed and predicted values.

Table 2: Plackett-Burman design of 8 independent variables and 3 dummy variables for L-Methionine production by *C.glutamicum.*

Run No.	X1	X2	X3	X4	X5	X6	X7	X8	D1	D2	D3	L-Methionine concentration (g/l)	
												Observed	Predicted
1	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	3.20	3.18
2	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	+1	3.30	3.28
3	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	-1	4.40	4.42
4	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	-1	3.70	3.68
5	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1	-1	2.20	2.18
6	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	+1	2.20	2.22
7	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	+1	3.80	3.78
8	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1	3.50	3.52
9	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	4.00	4.02
10	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	2.90	2.92
11	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	2.50	2.48
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	1.50	1.52

Table 3: Effect estimate from ANOVA of Plackett-Burman design for L-Methionine production.

Variables	Sum of squares	df	Mean square	Main effect	Standard Error	Coefficient	t-value	F-value	p-value (Prob>F)
Model	8.14	1	0.81	--	0.017	3.10	--	244.10	0.0498*
X1	2.61	1	2.61	0.93	0.017	0.47	32.10	784.00	0.0227*
X2	1.33	1	1.33	0.67	0.017	0.33	16.38	400.00	0.0318*
X3	0.16	1	0.16	0.23	0.017	0.12	2.01	49.00	0.0903
X4	0.03	1	0.03	0.10	0.017	0.05	0.37	9.00	0.2048
X5	3.333E-003	1	3.333E-003	0.033	0.017	0.01	0.041	0.55	0.4534
X6	0.013	1	0.013	0.067	0.017	0.033	0.16	4.00	0.2952
X7	2.61	1	2.61	0.93	0.017	0.47	32.10	784.00	0.0227*
X8	1.20	1	1.2	0.63	0.017	0.32	14.78	361.00	0.0335*
D1	0.083	1	0.083	-0.17	0.017	-0.083	1.02	25.00	0.1257
D2	0.03	1	0.03	-0.1	0.017	-0.05	0.37	9.00	0.2048
D3	0.053	1	0.053	-0.13	0.017	-0.067	0.66	16.00	0.1560

Further the ANOVA of RSM showed (Table.6) the model F-value of 93.22 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case X_1, X_2, X_7, X_1X_2, X_1X_8, X_2X_7, X_1^2, X_2^2, X_7^2, X_8^2 are significant model terms and X_8, X_1X_7, X_2X_8, $X_7X_8$are non significant (p>0.05). Among the significant model terms X_1X_2, X_1X_8, X_2X_7, X_1^2, X_2^2, X_7^2, X_8^2 are showed negative effect on response. The non significant model terms and model terms which showed negative impact has been discarded. The "Lack of Fit F-value" of 3.30 implies the Lack of Fit is not significant relative to the pure error. There is a 10.00% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit. The regression analysis data was fitted with second order polynomial equation in terms of coded terms as follows

$$\text{Methionine concentration} = 5.35 + 0.18X_1 + 0.27X_2 + 0.26X_7 + 0.046X_8 - 0.11X_1X_2 + 6.250E^{-003}X_1X_7 - 0.081X_1X_8 - 0.0.81X_2X_7 + 0.031X_2X_8 + 0.069X_7X_8 - 0.67X_1^2 - 0.52X_2^2 - 0.32X_7^2 - 0.28X_8^2$$

Regression analysis showed that R^2 was 0.9886 which showed that model can explain 98.86% of the variation in response which indicates the fitness of model. The predicted R^2 of 0.9410 is in reasonable agreement with the adjusted R^2 of 0.9780 for good fitness of model. The low value of C.V (3.39%) and adequate precision of 32.179 were significantly explains the goodness of model. Optimum values of four significant variables for maximum L-Methionine production were predict with the help of point prediction and contour surface plots generation. 3D response surface graphs were developed for the pair wise combination of the four variables, keeping the other two variables at centre points to understand the interaction effects of significant variables on L-Methionine production as shown in Figs. 1A-1F. In all Figs. 1A-1F an initial increase in one variable with simultaneous increase in other variable resulted in an increasing L-Methionine production until they reached optimum values, beyond this limit decreased L-Methionine production. The optimal process parameters were identified from the point prediction and central point of contour plots as shown in Figs.2A-2F. Optimal values of four significant variables were determined as plantain (21.19 g/l), groundnut as nitrogen source (11.20 g/l), $MgSO_4.7H_2O$ (1.21 g/l) and inoculum size (3.30 ml) for maximum L-Methionine production of 5.424 g/l after which further increase in amounts of variables decreased L-Methionine production. In previous reports, Banik and Majumdar (1975) reported 4.5 g/l methionine in the medium containing maltose, ammonium nitrate and biotin at pH 7.0 [30] and Anike and Okafor (2008) reported up to 3.5 g/l methionine produced by *Lactobacillus plantarum* isolated from cassava pulp[31]. From all the published data so far, not more than 5 g/l methionine could be obtained. However, the present work revealed L-Methionine concentration of 5.424 g/l by RSM and 5.30 g/l with experiment at optimized conditions in shake flask experiments using agricultural products as substrates as shown in Table.7.

Table 4: Ranges and levels of screened independent variables used for RSM experiments

Variable	Code	Units	Levels				
			-2	-1	0	+1	+2
Plantain	X_1	g/l	10	15	20	25	30
Groundnut	X_2	g/l	0	5	10	15	20
$MgSO_4.7H_2O$	X_7	g/l	0	0.5	1	1.5	2
Inoculum size	X_8	ml	1	2	3	4	5

Development of neural network model and analysis of results

Training, testing, and validation of neural networks were performed with four input variables and one output variable using tools, namely feed forward back-propagation network and TRAINLM in MATLAB R2013a version. The elevated regression value of 0.9914 was attained from the ANN model. The performance curve was developed using MATLAB R2013a for training, testing, and validation of the data. The regression plot of the output versus target was developed with ten hidden nodes and R^2 value 0.99 was accomplished with validation of the model as shown in Fig.3.

RSM- and ANN-predicted values vs. experimental data

The RSM and ANN models predicted data were compared with experimental data (Table 5 and Fig.4). It was observed that the RSM and ANN prediction is almost similar to experimental data as shown in Fig.5.

Model Validation

The model was validated by further fitting the optimized values of plantain as carbon source (21.19 g/l), groundnut as nitrogen source (11.20 g/l), $MgSO_4.7H_2O$ (1.21 g/l) and inoculum size (3.30 ml) into the second order polynomial equation. The predicted response based on the model for L-Methionine production was 5.424 g/l and experimental response was 5.30 g/l, thus proved validity of the model.

CONCLUSION

Total of eight variables (plantain, groundnut, $CaCO_3$, K_2HPO_4, KH_2PO_4, biotin, $MgSO_4.7H_2O$, and inoculum size) have been screened by using Plackett-Burman design to determine significant variables which shows maximum effect on L-Methionine production. In the next step, optimum levels of significant variables were found based on RSM and CCD. Optimized values for maximal production of L-methionine (5.424 g/l) based on RSM were found as: carbon source (21.19 g/l), groundnut as nitrogen source (11.20 g/l), $MgSO_4.7H_2O$ (1.21 g/l) and inoculum size (3.30 ml). R^2 value of RSM model improved with artificial neural networks. Based on this study, RSM and ANN predicted data is almost simillar to experimental data and theses models can be used to enhance the production of L-Methionine by using agricultural products as substrates.

Table 5: Central composite design for L-Methionine production by *C.glutamicum* with its observed and predicted values.

Run No.	X_1: Plantain (g/l)	X_2:Groundnut (g/l)	X_7:$MgSO_4.7H_2O$ (g/l)	X_8:Inoculum size (ml)	L-Methionine concentration (g/l)		
					Exp	RSM	ANN
1	-1	-1	-1	-1	2.60	2.65	2.58
2	+1	-1	-1	-1	3.40	3.37	3.44
3	-1	+1	-1	-1	3.50	3.50	3.50
4	+1	+1	-1	-1	3.80	3.80	3.79
5	-1	-1	+1	-1	3.10	3.18	3.42
6	+1	-1	+1	-1	3.80	3.93	3.76
7	-1	+1	+1	-1	3.80	3.71	3.83
8	+1	+1	+1	-1	3.90	4.03	4.21
9	-1	-1	-1	+1	2.80	2.70	2.82
10	+1	-1	-1	+1	2.90	3.10	2.94
11	-1	+1	-1	+1	3.70	3.68	3.71
12	+1	+1	-1	+1	3.70	3.65	3.71
13	-1	-1	+1	+1	3.40	3.51	3.49
14	+1	-1	+1	+1	3.90	3.93	3.88
15	-1	+1	+1	+1	4.10	4.17	4.09
16	+1	+1	+1	+1	4.10	4.16	4.25
17	-2	0	0	0	2.30	2.32	2.34
18	+2	0	0	0	3.20	3.04	3.09
19	0	-2	0	0	2.90	2.74	2.88
20	0	+2	0	0	3.80	3.82	3.83
21	0	0	-2	0	3.50	3.55	3.20
22	0	0	+2	0	4.80	4.60	5.00
23	0	0	0	-2	4.20	4.14	4.19
24	0	0	0	+2	4.40	4.32	4.42
25	0	0	0	0	5.40	5.35	5.34
26	0	0	0	0	5.40	5.35	5.34
27	0	0	0	0	5.20	5.35	5.34
28	0	0	0	0	5.40	5.35	5.34
29	0	0	0	0	5.40	5.35	5.34
30	0	0	0	0	5.30	5.35	5.34

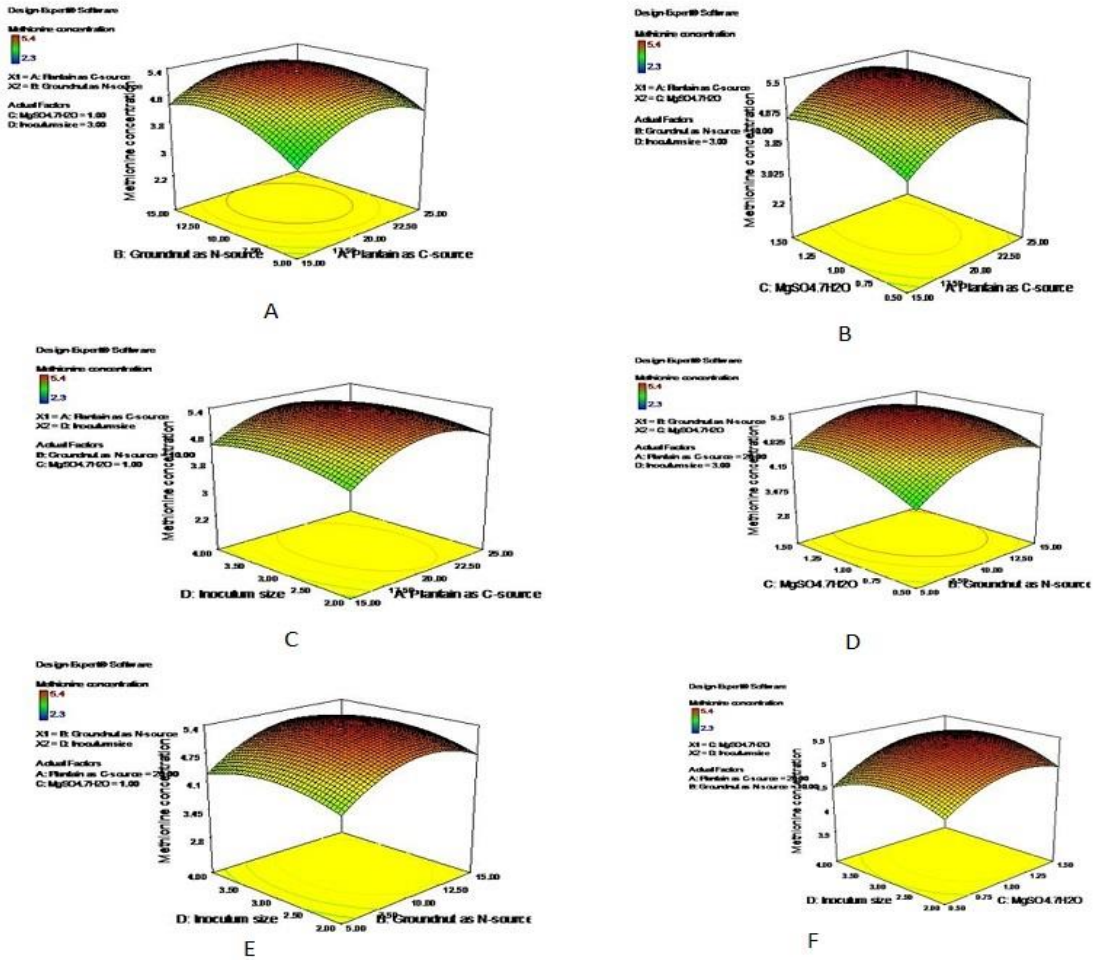
Table 6: Analysis of variance (ANOVA) for the experimental results of the central composite design response surface quadratic model

Source	Sum of squares	df	Mean square	Coefficient Effect	Standard Error	F-value	p-value (Prob>F)
Model	23.13	14	1.65	5.35	0.054	93.22	<0.0001*
X_1-Plantain as carbon source	0.77	1	0.77	0.18	0.027	43.47	<0.0001*
X_2-Groundnut as nitrogen source	1.76	1	1.76	0.27	0.027	99.33	<0.0001*
$X_7MgSO_4.7H_2O$	1.65	1	1.65	0.26	0.027	93.32	<0.0001*
X_8-Inoculum size	0.050	1	0.050	0.046	0.027	2.84	0.1123
X_1X_2	0.18	1	0.18	-0.11	0.033	10.19	0.0061*
X_1X_7	6.250E-004	1	6.250E-004	6.250E-003	0.033	0.035	0.8536

X_1X_8	0.11	1	0.11	-0.081	0.033	5.96	0.0275*
X_2X_7	0.11	1	0.11	-0.081	0.033	5.96	0.0275*
X_2X_8	0.016	1	0.016	0.031	0.033	0.88	0.3626
X_7X_8	0.076	1	0.076	0.069	0.033	4.27	0.0566
X_1^2	12.23	1	12.23	-0.67	0.025	690.02	<0.0001*
X_2^2	7.35	1	7.35	-0.52	0.025	414.82	<0.0001*
X_7^2	2.77	1	2.77	-0.32	0.025	156.22	<0.0001*
X_8^2	2.15	1	2.15	-0.28	0.025	121.52	<0.0001*
Residual	0.27	15	0.018				
Lack of Fit	0.23	10	0.023			3.30	0.1 Not significant
Pure Error	0.035	5	7.000E-003				
Cor Total	23.39	29					

Table 7: RSM optimized process variables for L-Methionine production

Plantain (g/l)	Groundnut (g/l)	$MgSO_4.7H_2O$ (g/l)	Inoculum size (ml)	L-Methionine concentration (g/l)	
X_1	X_2	X_7	X_8	Predicted by RSM	Experimental
21.19	11.20	1.21	3.30	5.424	5.30



Figsure 1: 3D response surface plots for the interaction effect of two variables on L-Methionine production by *C.glutamicum*by keeping other two variables at constant center levels.

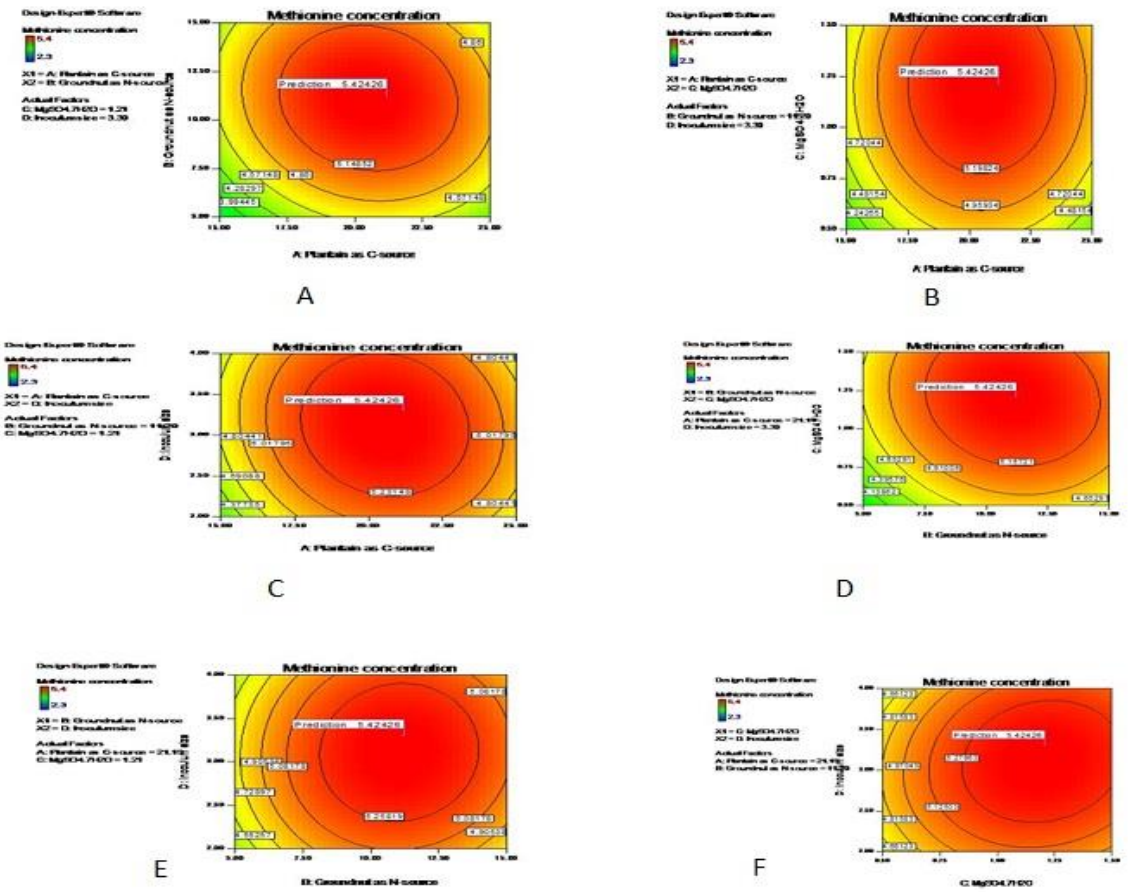


Figure 2: Contour plots showing maximum L-Methionine production of 5.424 g/l under optimized conditions, beyond which L-Methionine production decreased

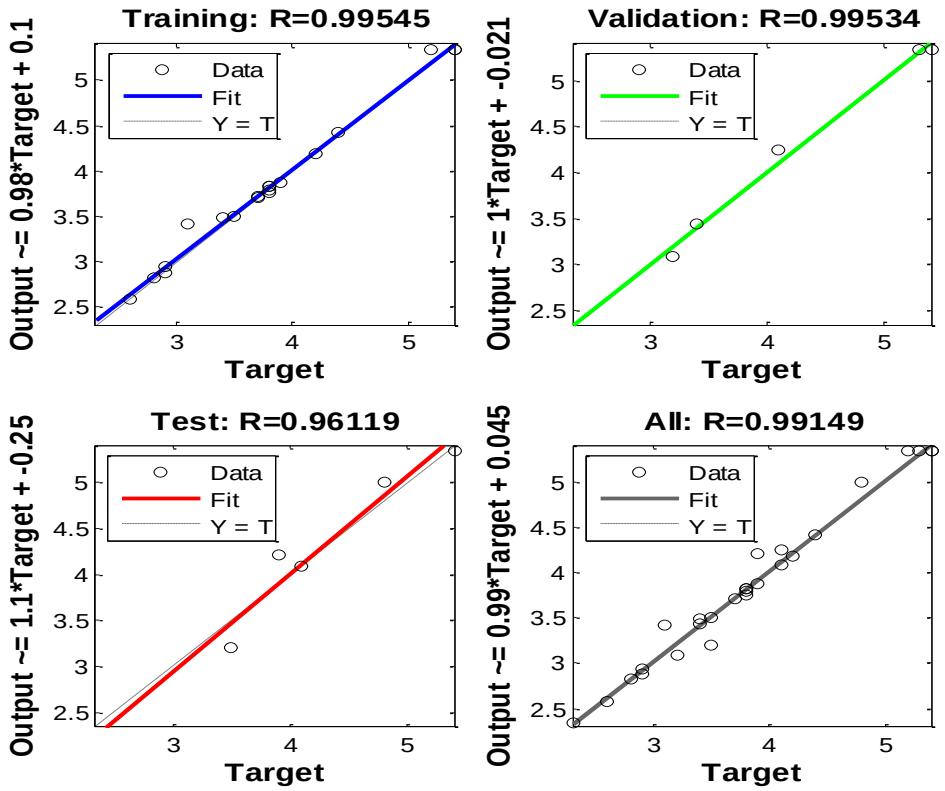


Figure 3: Output vs. target regression plot of artificial neural network

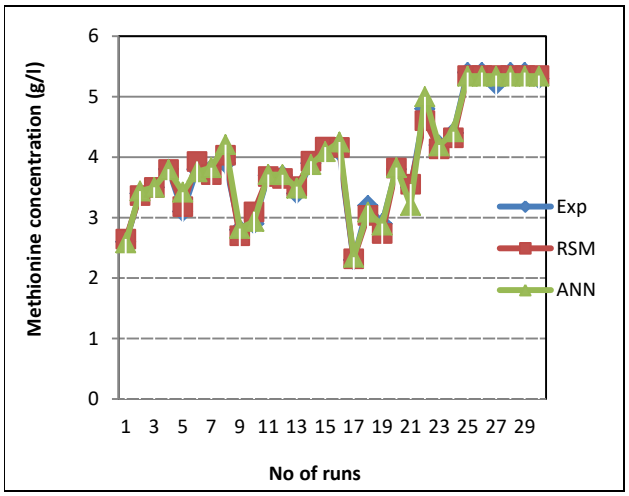


Figure 4: Comparison of experimental data with RSM and ANN predicted values

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