CHAPTER 4

Optimization of Cultural Conditions and Media Components for L-Glutaminase Production

4.1. Introduction

Glutaminase was reported to be present in a diverse group of microorganisms like bacteria, yeast and fungi (Wade et al., 1971; Imada et al., 1973; Ramkrishna and Prakasham, 1999; Renu and chandrasekaran, 1992; Nandakumar et al., 2003). The metabolic pathway in microorganism generally depends upon the utilization of different types of media components. The fermentation medium contains carbon sources, nitrogen sources, macronutrients and some micronutrients which are essential for the growth of microorganism. The optimization of media components is considered as an important parameter to enhance production of enzyme. The growth of microorganism is also affected by varying cultural conditions and hence it is necessary to optimize them to enhance the production of enzyme.

To design a medium for maximum production of L-glutaminase, optimization of medium components plays a key role. Nutritional requirement can be manipulated using classical method and statistical methods. Classical method involves changing one independent variable at a time, while keeping others at a constant level (Rathi et al., 2001). The determination of optimum concentration of media components by classical method does not depict the net effect of total interaction among various media components (Iyer and Singhal, 2008). Statistical methods account the interaction among nutrient components at various concentrations and reduce the total number of experiments (Rathi et al., 2001). Response surface methodology (RSM) was successfully used as a statistical tool for optimization of bioprocess in which predicted response was determined using quadratic function. RSM using central composite design was applied to optimize the media components and physiochemical

parameters of fermentation for production of various enzymes viz., alkaline phosphatase (Pandey et al., 2011), L-asparaginase (Kumar et al., 2009), phytase (Singh and Satyanarayana, 2008), alpha-amylase (Gangadharan et al., 2008), lipase (Gaur et al., 2008), glutaminase (Iyer and Singhal, 2008), alkaline protease (Reddy et al., 2008). Artificial neural network (ANN) methodology was recently being used as new optimization strategy because of having ability to deal high degree of non linearity in comparison to RSM (Bingol et al., 2012; Bas and Boyaci, 2007; Dutta et al., 2004; Lou and Nakai, 2001; Sim and Kamaruddin, 2008). ANN with genetic algorithm was employed to optimize fermentation condition of glutaminase from *Bacillus subtilis* RSP-GLU (Sathish and Prakasham, 2010) and alkaline protease from *Bacillus circulans* (Rao et al., 2009).

The effect of different carbon sources, nitrogen sources, L-glutamine and other amino acids on the production of L-glutaminase from *Bacillus cereus* MTCC 1305 was studied. Statistical tools of response surface methodology (RSM) and artificial neural network (ANN) were employed to optimize media components and cultural conditions to achieve maximum production of L-glutaminase.

4.2. Materials and Methods

4.2.1. Media components

Media components and chemicals like L-glutamine, L-asparagine, lysine, adenine, metheonine, glycine, Nessler's reagent, peptone, tryptone, glycerol, glucose, fructose, sucrose, maltose, lactose, succinate, pyruvate, starch, agar, yeast extract, beef extract, TRIS-HCl buffer, Tri-chloro actetic acid and NaCl were procured from Hi Media,

Bombay, India. Other chemicals like $CaCl_2$, $MgSO_4$, $NaH_2PO_4.2H_2O$, $Na_2HPO_4.2H_2O$, KH_2PO_4 , NH_4Cl, NH_4NO_3, $(NH_4)_2SO_4$, Tri-ammonia-citrate, urea, $MnSO_4$, $CaCO_3$, $MgSO_4$, $FeCl_3$, KNO_3, $ZnSO_4$, $NaNO_3$, $MgCl_2$, Na_2SO_4, Na_2SO_4, sodium citrate were procured from Qualigens, Bombay, India. All the chemicals used were of AR grade.

4.2.2. Optimization of incubation parameters for production of L-glutaminase

In order to determine effect of initial pH on the production of L-glutaminase, pH of media was varied from 4-11 using 0.1N HCl and 0.1N NaOH. Effect of temperature on the production of L-glutaminase was studied by incubating media (pH-7.5) in orbital shaker at different temperatures (24-45˚C).The optimum fermentation time was determined by incubating inoculated media for different incubation period from 4-55hrs. The effect of inoculum age on L-glutaminase production was determined using 2% of cell suspension of different ages (5-14hrs). Effect of Inoculum concentration on production of L-glutaminase was studied with addition of 1-5% (v/v) inoculum of 12hrs old. The effect of agitation speed on production of L-glutaminase was studied by incubating the flasks containing production media at different agitation speeds (100-400rpm). All the experiments were carried out in triplicate.

4.2.3. Effect of carbon and nitrogen sources on production of L-glutaminase

Bacillus cereus MTCC 1305 was grown in nutrient media (pH-7.0) containing beef extract (1g/l), yeast extract (2g/l), peptone (5g/l), and agar (15g/l). Inoculated slants were incubated at 35˚C for 24hr for microbial growth and stored at 4±1˚C in refrigerator. The production of L-glutaminase was further studied in 100ml

production media (pH 7.0) containing 0.1% glucose, 0.3% L-glutamine,0.3% $Na_2HPO_4 \cdot 2H_2O$, 0.2% KH_2PO_4, 0.05% NaCl, 0.05% $MgSO_4 \cdot 7H_2O$, 0.0015% $CaCl_2 \cdot 2H_2O$ and 0.5% yeast extract as reported in many research papers (Nelson et al., 1975; Davidson et al., 1977; Harayama and Yasuhira, 1991; Klein, et al., 2002; Wakayama et al., 2005; Sathish and Prakasham, 2010). The fermentation conditions for the production of L-glutaminase were already mentioned in previous chapter3.

Effect of different carbon sources (0.1%) like glucose, fructose, maltose, sucrose, starch, lactose, galactose, xylose, mannitol and sorbitol on cell growth and production of L-glutaminase were studied. Effect of different nitrogen (N) sources on cell growth and production of L-glutaminase was also investigated using inorganic N sources like ammonium chloride, ammonium nitrate, ammonium sulfate, ammonium citrate, potassium nitrate and complex N source like beef extract, peptone, yeast extract by maintaining total nitrogen content up to 0.5% in media.

4.2.4. Estimation of cell biomass

Cell biomass in the fermentation broth was quantified by dry-cell weight analysis. The fermented broth was centrifuged at 4˚C, and 10,000×g for 10 minutes and pellet was dissolved in minimal amount of Tris-HCl buffer (pH-7.5). The recovered cells were washed twice with distilled water and dried to constant weight in an oven at 80˚C for 24 hrs.

4.2.5. Optimization of cultural conditions and media components using RSM and ANN methods

Cultural parameters like incubation temperature, fermentation period, media pH, inoculum size, inoculum age, agitation speed were optimized using RSM and ANN methodologies. The statistical optimization strategies applied for these parameters are summarized in Table4.1.

Table4.1. Optimization of cultural conditions using statistical tools like response surface methodology and artificial neural network

Optimization of Cultural Conditions		
Cultural parameters	**RSM based optimization using Minitab-15 version statistical software**	**ANN based optimization using Neurosolutions-6 version, neural builder software**
Incubation temperature (20-50˚C), Fermentation period (20-60 hr), pH of media (5-10), Inoculum size (1-3%), Inoculum age (8-16hr), Agitation speed (120-240rpm)	• Central Composite Design with 2^3 level (α=2.37841) designed fifty three experiments with nine replicates • Experimental data was further analyzed using regression analysis, analysis of variance (ANOVA) • The interaction of the variables was studied using contour plots	• Forty six experimental data were divided into three sets: training (34), testing (6) and validation (6) sets. • Optimum number of neurons in hidden layer was selected as 3. • Back propagation network with Multi-layer perceptron (MLP) based on Levenberg-Marquardt algorithm and sigmoid transfer function was used • MLP network architecture was obtained as 6-3-1 with six input neurons, three neurons in hidden layer and one output neuron

The optimization strategies used to optimize media components using RSM and ANN are listed in Table4.2. In RSM, Plackett burman design (PBD) was first applied to screen significant media components and central composite design (CCD) was used to determine optimum levels of significant media components. Artificial neural network (ANN) methodology was further used to optimize experimental data obtained from CCD.

Table4.2. Optimization of media components using response surface methodology and artificial neural network statistical tools

Optimization of Media components		
Media Components	**RSM based optimization using Minitab-15 version statistical software**	**ANN based optimization using Neurosolutions-6 version, neural builder software**
• Parameters for **Plackett Burman design (PBD)**: Sucrose (1-2g/l), L-Glutamine (3-5g/l), Peptone (1-2g/l), Na_2HPO_4 (4-6g/l), KH_2PO_4 (1-3g/l), NaCl (0.2-0.5g/l), $MgSO_4$ (0.2-0.5g/l), $CaCl_2$ (0.01-0.02g/l) • Parameters for **Box-Behnken Design (**BBD): Sucrose (1-4g/l), Peptone (1-4g/l), L-Glutamine (2.5-7.5g/l), Na_2HPO_4 (4-8g/l), $MgSO_4$ (0.4-0.6g/l), NaCl (0.4-0.6g/l)	• Twelve experiments were designed using PBD and significant madia components were selected on basis of Pareto chart and ANOVA analysis. • Fifty four experiments with four replicates at centre points were designed using BBD and interaction between variables was further analyzed on basis of regression analysis, ANOVA and contour plots	• Forty nine experimental data were divided into three sets: training (35), testing (7) and validation (7) sets. • Optimum number of neurons in hidden layer was selected as 3. • Back propagation network with Multi-layer perceptron (MLP) based on Levenberg-Marquardt algorithm and sigmoid transfer function was used • MLP network architecture was obtained as "6-3-1" with six input neurons, three neurons in hidden layer and one output neuron

All the experiments designed by RSM for estimation of L-glutaminase activity were performed in triplicates. Multiple regression and ANOVA were further employed to investigate contribution of each parameter and their interaction. The interactive effect of each variable on response (L-glutaminase activity) was represented by contour plots.

Artificial neural network (ANN) was further applied on experiments designed by CCD and categorized the experimental data into three sets: Training, Testing and Validation. This network consisted of a large number of simple processing elements (neurons) which were connected with connection link. Each link had a weight that multiplied with transmitted signal in network and each neuron had an activation function to determine the output (Desai et al., 2008). Neurons were using sigmoid transfer function and a multilayer perceptron (MLP) together with back propagation to approximate nonlinear function to desired accuracy (Lou and Nakai, 2001). The performance of the RSM and ANN methodologies was statistically measured in terms of coefficient of determination (R^2), root mean squared error (RMSE) and the absolute average deviation (AAD).

4.3. Results and Discussion

4.3.1. Optimization of Cultural Conditions

The cultural conditions reported for L-glutaminase production were different for different micro-organisms (Sathish and Prakasham, 2010; Iyer and Singhal, 2008; Kumar and Chandrasekaran, 2003; Klein et al., 2002; Tachiki et al., 1996; Sato et al., 1999; Keerthi et al., 1999; Roberts et al., 1970; Roberts et al., 1972). RSM and ANN methodologies were employed to study the interactive effect of the cultural parameters on production of L-glutaminase and maximize its production. The level of cultural conditions and the design matrix used for CCD and ANN are shown in Table4.3.

Table4.3. Central composite design for six variables and experimentally determined actual data, predicted data by RSM and ANN for L-glutaminase production

Run Order	pH	Fermentation period (hr)	Temperature (˚C)	Inoculum size (%)	Inoculum age (hr)	Agitation Speed (rpm)	Actual Activity (U/L)	Predicted activity (U/L)	
								RSM	ANN
1	7.50	40.00	34.00	-0.38	10.00	175.00	89.02	158.39	89.26 TRD
2	7.50	40.00	34.00	2.00	10.00	-3.381	259.23	319.58	256.33TRD
3	7.50	40.00	34.00	2.00	14.76	175.00	612.23	651.44	612.26 TRD
4	7.50	40.00	34.00	2.00	10.00	175.00	667.23	666.47	666.97 TRD
5	6.00	55.00	38.00	3.00	12.00	100.00	237.34	255.97	238.53 TRD
6	9.00	25.00	38.00	3.00	8.00	250.00	373.12	428.49	373.64 TRD
7	3.93	40.00	34.00	2.00	10.00	175.00	12.09	46.15	14.23 TRD
8	6.00	55.00	38.00	1.00	12.00	250.00	219.39	242.39	220.74 TRD
9	7.50	40.00	34.00	2.00	10.00	175.00	667.23	666.47	-
10	6.00	55.00	38.00	1.00	8.00	100.00	158.92	133.29	158.67 TRD
11	7.50	40.00	34.00	2.00	10.00	175.00	667.23	666.47	-
12	7.50	40.00	34.00	2.00	10.00	353.381	523.98	498.68	524.39 TRD
13	9.00	55.00	30.00	3.00	12.00	100.00	252.13	249.76	254.27 TRD
14	6.00	25.00	38.00	3.00	12.00	250.00	289.12	253.34	287.81 TRD
15	9.00	25.00	38.00	1.00	8.00	100.00	219.89	179.47	221.37 TRD
16	6.00	25.00	30.00	3.00	12.00	100.00	198.28	182.39	196.41 TRD
17	7.50	40.00	34.00	2.00	10.00	175.00	667.23	666.47	-
18	9.00	25.00	30.00	1.00	8.00	250.00	298.23	257.76	298.38 TRD
19	6.00	55.00	30.00	3.00	8.00	100.00	229.38	220.42	231.53 TRD
20	6.00	25.00	30.00	3.00	8.00	250.00	212.12	220.16	211.14 TRD
21	9.00	55.00	38.00	1.00	12.00	100.00	278.21	248.33	279.32 TRD
22	9.00	55.00	38.00	3.00	8.00	100.00	298.12	299.00	297.63 TRD
23	7.50	4.32	34.00	2.00	10.00	175.00	13.00	48.35	12.60 TRD
24	9.00	55.00	38.00	1.00	8.00	250.00	312.12	319.48	310.94 TRD
25	7.50	40.00	34.00	2.00	10.00	175.00	667.23	666.47	-

26	9.00	25.00	30.00	3.00	8.00	100.00	265.12	233.59	266.12 TRD
27	6.00	25.00	38.00	3.00	8.00	100.00	239.12	268.38	239.14 TRD
28	7.50	40.00	34.00	2.00	5.24	175.00	598.12	601.87	598.36 TRD
29	6.00	55.00	30.00	3.00	12.00	250.00	248.23	266.81	249.28 TRD
30	6.00	55.00	30.00	1.00	12.00	100.00	289.13	225.22	288.43 TRD
31	9.00	55.00	30.00	1.00	12.00	250.00	436.78	385.68	436.87 TRD
32	9.00	25.00	30.00	3.00	12.00	250.00	312.98	316.77	312.98 TRD
33	7.50	75.68	34.00	2.00	10.00	175.00	128.54	136.14	125.45 TRD
34	9.00	55.00	30.00	3.00	8.00	250.00	456.18	453.04	455.35 TRD
35	11.07	40.00	34.00	2.00	10.00	175.00	239.38	248.28	239.54 TRD
36	7.50	40.00	34.00	2.00	10.00	175.00	667.23	666.47	-
37	9.00	25.00	38.00	1.00	12.00	250.00	324.95	325.37	324.26 TRD
38	7.50	40.00	34.00	2.00	10.00	175.00	667.23	666.47	-
39	9.00	55.00	30.00	1.00	8.00	100.00	151.12	178.36	151.18 TRD
40	7.50	40.00	34.00	4.378	10.00	175.00	351.29	324.88	369.53 TTD
41	9.00	25.00	30.00	1.00	12.00	100.00	234.35	258.46	236.21 TTD
42	7.50	40.00	34.00	2.00	10.00	175.00	667.23	666.47	-
43	9.00	55.00	38.00	3.00	12.00	250.00	523.09	544.81	535.28 TTD
44	6.00	25.00	38.00	1.00	8.00	250.00	149.23	143.06	142.11 TTD
45	7.50	40.00	43.51	2.00	10.00	175.00	329.38	327.58	317.81 TTD
46	6.00	25.00	30.00	1.00	12.00	250.00	195.23	172.51	192.92 TTD
47	6.00	25.00	38.00	1.00	12.00	100.00	256.12	237.42	256.12 VD
48	6.00	55.00	38.00	3.00	8.00	250.00	398.23	365.58	398.201VD
49	7.50	40.00	34.00	2.00	10.00	175.00	667.23	666.47	-
50	6.00	25.00	30.00	1.00	8.00	100.00	256.28	226.03	256.380 VD
51	9.00	25.00	38.00	3.00	12.00	100.00	501.78	468.21	501.755 VD
52	6.00	55.00	30.00	1.00	8.00	250.00	362.12	373.85	362.148 VD
53	7.50	40.00	24.49	2.00	10.00	250.00	212.28	257.03	212.389 VD

In ANN predicted activity column, TRD-training data, TTD-testing data, VD-validation data

Fifty four experiments were designed for selected six cultural parameters using CCD with six axial points (α=2.378), eight factorial (2^3) and nine center points. Experiments were performed in random order to obtain the amount of L-glutaminase produced as actual response. The experimental results were further analyzed by using multiple regressions and significance of each individual factors and their interaction on the production of L-glutaminase are presented in Table4.4.

Table4.4. Regression analysis of central composite design showing model coefficients and significance of regression coefficient

Term	Coef	SE Coef	Test value (t)	Probability (P)
Constant	-6267.92	748.990	-8.369	0.000
X_1	454.35	62.171	7.308	0.000
X_2	42.57	5.821	7.314	0.000
X_3	241.37	28.213	8.555	0.000
X_4	13.23	86.240	0.153	0.879
X_5	-44.94	46.628	-0.964	0.344
X_6	2.13	1.132	1.879	0.072
X_1^2	-40.80	2.347	-17.382	0.000
X_2^2	-0.45	0.023	-19.222	0.000
X_3^2	-4.15	0.339	-12.225	0.000
X_4^2	-75.10	5.281	-14.221	0.000
X_5^2	-1.76	1.320	-1.333	0.195
X_6^2	-0.01	0.001	-8.557	0.000
$X_1 X_2$	-0.24	0.319	-0.739	0.467
$X_1 X_3$	2.44	1.197	2.035	0.053
$X_1 X_4$	11.70	4.788	2.443	0.022
$X_1 X_5$	5.86	2.394	2.449	0.022
$X_1 X_6$	0.17	0.064	2.731	0.011
$X_2 X_3$	-0.20	0.120	-1.655	0.110

$X_2 X_4$	-0.05	0.479	-0.098	0.923
$X_2 X_5$	-0.19	0.239	-0.790	0.437
$X_2 X_6$	0.03	0.006	4.690	0.000
$X_3 X_4$	7.73	1.795	4.307	0.000
$X_3 X_5$	2.13	0.898	2.370	0.026
$X_3 X_6$	-0.01	0.023	-0.632	0.533
$X_4 X_5$	-3.67	3.591	-1.021	0.317
$X_4 X_6$	0.06	0.096	0.599	0.554
$X_5 X_6$	-0.09	0.048	-1.919	0.066

R^2 = 97.78%, R^2(pred) = 85.34%, R^2(adj) = 95.39%, X_1=Media pH, X_2= Fermentation time, X_3= Temperature, X_4 = Inoculum size, X_5=Inoculum age and X_6 =Agitation speed

The propability value (P) was used to check the significance of each of the coefficients to understand the pattern of the mutual interactions between the test variables. The factors having P value less than 0.05 are considered as significant parameters for response variables (Nathiya et al., 2011). Media pH and temperature as individual factors had high coefficient value which indicates their high linear significant effect on the production of L-glutaminase. Effect of individual parameters of inoculum age, inoculum size and agitation speed on production of L-glutaminase was observed as insignificant, while on interaction of these parameters with pH showed linear positive significant effect. Coefficient of determination (R^2) after regression analysis was obtained as 97.78 which indicate that the sample variation of only 2.22% of the total variation is not explained by the model. The following second-order regression equation was obtained to explain the production of L-glutaminase in terms of initial values of cultural conditions:

$$Y=(-6267.92)+454.35X_1+42.57X_2+241.37X_3+13.23X_4-44.94X_5+2.13X_6-40.80X_1^2-0.45X_2^2-4.15X_3^2-75.10X_4^2-1.76X_5^2-0.01X_6^2-0.24X_1X_2+2.44X_1X_3+11.70X_1X_4+5.86X_1X_5+0.17X_1X_6-0.20X_2X_3-0.05X_2X_4-0.19X_2X_5+0.03X_2X_6+7.73X_3X_4+2.13X_3X_5-0.01X_3X_6-3.67X_4X_5+0.06X_4X_6-0.09X_5X_6 \quad (4.1)$$

Where, Y=L-glutaminase activity as response and X_1=Media pH, X_2=Fermentation time, X_3=Incubation temperature, X_4=Inoculum size, X_5=Inoculum age and X_6 =Agitation speed

Results of analysis of variance (ANOVA) are summarized in Table4.5. These results indicate the statistical testing of the model in term of Fisher's F test. A highly significant quadratic regression model was obtained with high value of F and very low p-value. This indicates that the combined effects of all the independent variables significantly contributed to maximize the production of L -glutaminase.

Table4.5. Analysis of Variance (ANOVA) for analyzing model fitness for L-glutaminase production at optimum cultural parameters

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Model	27	1819997	1819997	67407	40.84	0.000
Linear	6	216872	256885	42814	25.94	0.000
Square	6	1472513	1473048	245508	148.75	0.000
Interaction	15	130613	130613	8708	5.28	0.000
Residual Error	25	41263	41263	1651	-	-
Lack-of-Fit	17	41263	41263	2427	*	*
Pure Error	8	0	0	0	-	-
Total	52	1861260	-	-	-	-

DF= Degrees of freedom, SS=Sum of squares, MS= Mean square

Contour plots were further used to analyze the interaction of these variables and predict their optimum conditions for L-glutaminase production. Interactions of pH

with agitation speed, pH with inoculum age and pH with inoculum size were shown in Fig4.1a, 4.1b, and 4.1c respectively. These plots indicate that the production of L-glutaminase was highly affected by change of media pH as compared to the other parameters such as agitation speed, inoculum size and inoculum age. The production of L glutaminase was increased with increase of media pH up to optimum point (7.5) and further increase of media pH resulted decrease in its production. The interaction effect of fermentation period and agitation speed on the production of L-glutaminase is shown in Fig4.1d. The interaction of fermentation period and agitation speed had positive significant effect on the production of L-glutaminase as shown in Table4.4. The simultaneous increase of agitation speed and fermentation period resulted increase in production of L-glutaminase. The interactive effect of temperature with inoculum size and inoculum age had positive significant effect as shown in Contour plots in Fig4.1e, and Fig4.1f respectively. These plots revealed that L-glutaminase activity decreased sharply with increase of temperature beyond the optimum temperature (34˚C). The optimal levels for cultural conditions were obtained as media pH (7.5), inoculum size (2%), inoculum age (10hr), incubation temperature (34˚C) fermentation period (40h) and agitation speed (175rpm). Graphical analysis was combined with the numerical optimization and production of L-glutaminase was obtained as 633.315U/l under these optimum cultural conditions.

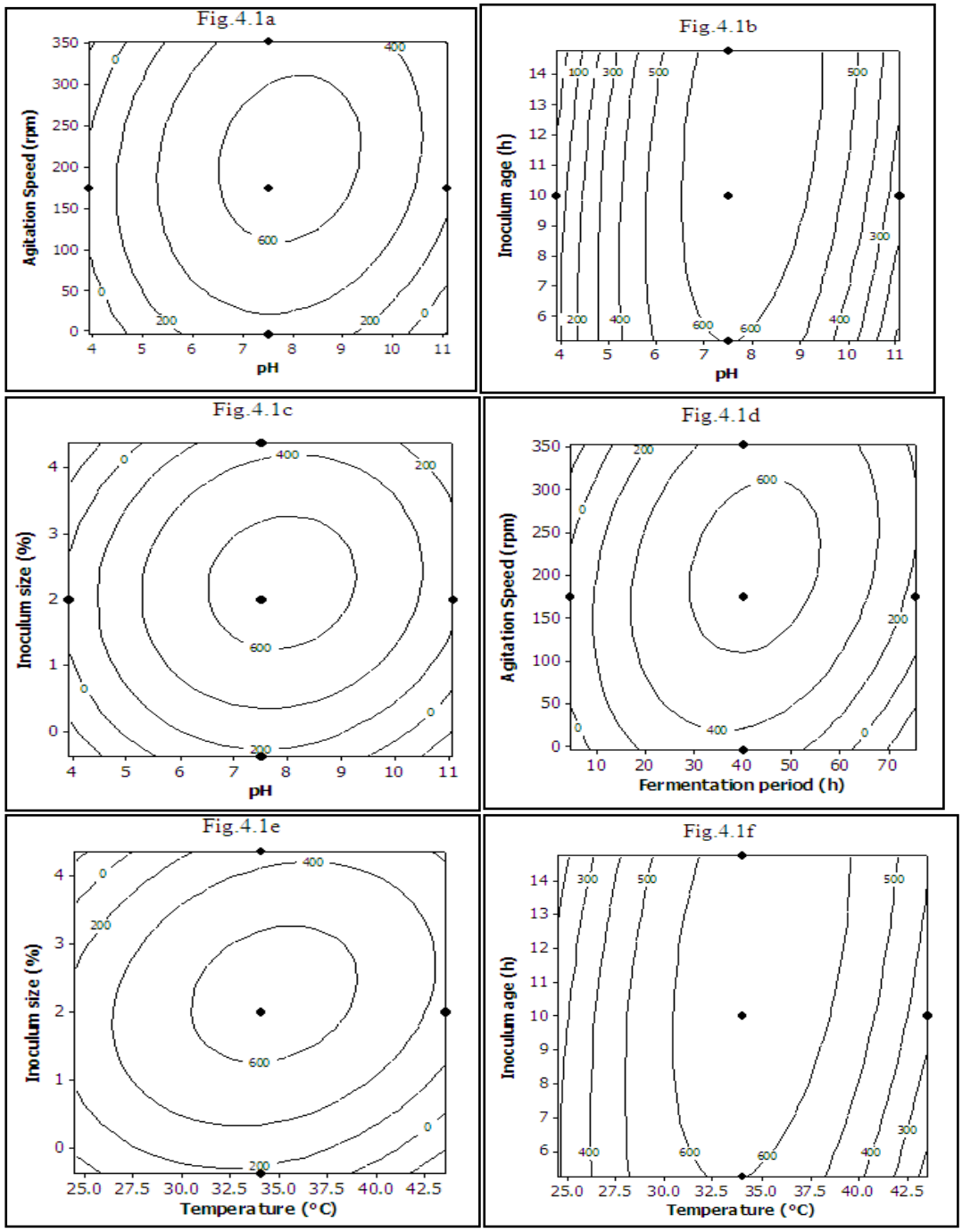


Fig.4.1. Contour plots showing interactive effect of selected variables on activity of L-glutaminase (a) pH and inoculum size (b) pH and inoculums age (c) pH and agitation speed (d) Fermentation period and agitation speed (e) Temperature and inoculum size (f) Temperature and inoculum age

Further, ANN methodology was applied which provide a non linear mapping between the input variables (pH of media, fermentation time, incubation temperature, inoculum concentration, inoculum age and agitation speed) and output variables (L-glutaminase production). The type of ANN selected for this study was back propagation network having a multilayer perceptron structure. The simulated predicted values of the response (activity of L-gutaminase) for different range of cultural conditions were listed in the last column of Table4.2. The response data obtained was analyzed using Levenberg-Marquardt algorithm with sigmoid transfer function. The minimum value of mean squared errors (MSE) was obtained for three neurons and hence was selected as optimum number of neurons in hidden layer for this neural network as shown in Fig4.2.The configuration of the neural network developed in this study was 6-3-1 with six input neurons-three neurons in hidden layer-one output neuron as shown in Fig4.3. Neurons of successive layers in MLP network were connected to each other by connection weight (W) and threshold for activation of these neurons was introduced in term of bias (θ_j). The values of weight and bias values for nonlinear function of input variables are shown in Table4.6. Input data was passed through input layer to hidden layer along with the weights and added to bias term (θ_j) to produce neuron input in the output layer using equation3.2. This neuron input was then passed through an activation function and transformed to output neuron using sigmoid transform function in equation 3.3. The activity of L-glutaminase was predicted as 666.97U/l using equation 3.4.

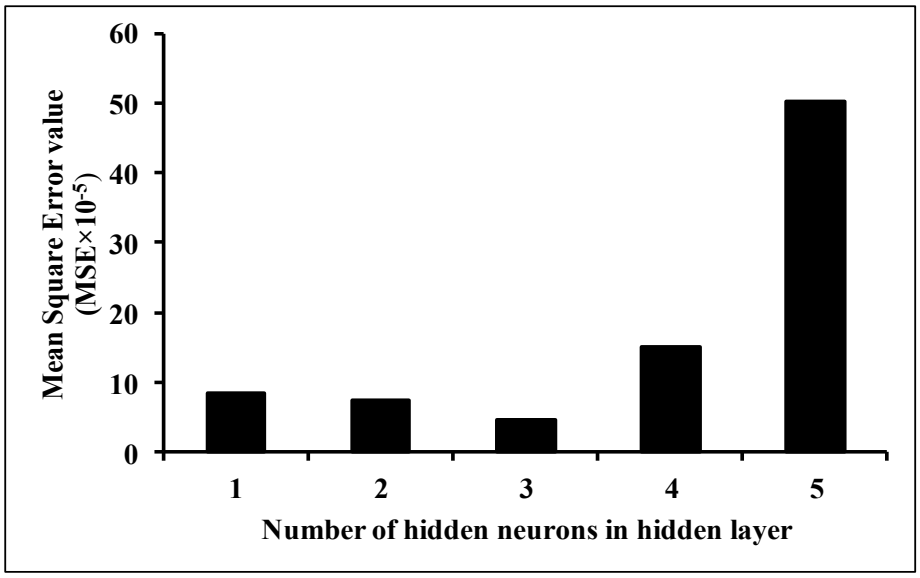


Fig4.2. Determination of number of hidden layer's neurons for artificial neural network designed for optimization of cultural parameters for L-glutaminase production

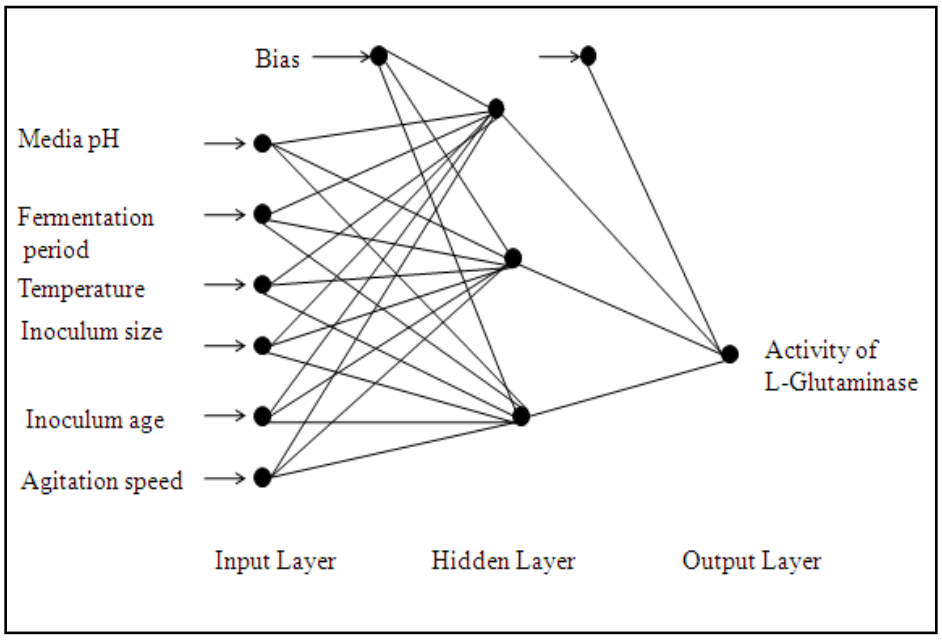


Fig.4.3. Multilayer perceptron neural network architecture used for optimization of culture parameters for L-glutaminase production

Table4.6. Weight and bias values of nonlinear function of cultural parameters for prediction of glutaminase activity using ANN methodology

Weight on connection between input and hidden nodes						
Media pH	Fermentation period	Temperature	Inoculum size	Inoculum age	Agitation speed	Bias term (b_1)
-0.2120	-0.03744	-5.9800	0.2317	0.0645	6.866	-0.0555
-0.1634	0.02195	1.1868	-0.0796	0.4126	5.856	0.1999
-0.0544	0.00142	0.0164	0.00752	0.0448	-4.0852	-0.0080
Weight on connection between hidden and output nodes 19.034, -73.663, Bias term (b_2=9.787)						

The comparison of RSM and ANN methodologies for predicted experimental results was done in terms of coefficient of determination (R^2), root mean square error (RMSE) and average absolute deviation (AAD). The value of coefficient of determination for RSM and ANN model was determined as 0.9547 and 0.9992 respectively. This indicates that both models are well fitted to experimental data but still ANN methodology was found to be better than RSM. The value of RMSE was determined as 4.0623 and 30.6213 for ANN and RSM model respectively, which indicates more closeness of predicted data to experimental data in case of ANN model. AAD values for RSM and ANN model were determined as 18.47% and 1.17% respectively, which showed greater deviation in RSM prediction than ANN. The methodology of calculating above mentioned three criteria are different and in ANN methodology, AAD showed much high degree of closeness of predicted data to experimental data in comparison to other two criteria. Results of all three criteria indicate that ANN methodology is superior to RSM for prediction of experimental

data. The validation of model was done by conducting the experiments in triplicates under the optimal level of cultural conditions and the amount of the enzyme obtained was 667.23U/l which is 1.58 times higher than the amount of L-glutaminase produced before optimizing the cultural conditions (423U/l). ANN with genetic algorithm approach has been employed for optimizing fermentation process parameters of L-glutaminase production from *Bacillus subtilis* RSP-GLU where its production has been enhanced by 1.64 times (Satish and Prakasham, 2010). The improved production of other biomolecules like poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (Zafar et al., 2012), L-asparaginase (Baskar et al., 2011), alkaline protease (Rao et al., 2009) has been also reported after employing RSM and ANN methodologies.

4.3.2. Optimization of media components

4.3.2.1. Effect of different amino acids on L-glutaminase production

Amino acids as major nitrogen source are utilized as common growth factor by microorganism. The production of L-glutaminase was also found to be varied on using different type of amino acid in media as shown in Fig4.4. The production of L-glutaminase was found to be maximum (429U/l) in presence of L-glutamine. The production of L-glutaminase was found to be very low in presence of L-asparagine, L-glutamic acid, proline, glycine, L-aspartic acid and lysine. The optimum production of L-glutaminase has been also reported in presence of L-glutamine for *Serratia marcescens* (Kumar et al., 2013), *Beauveria* sp. (Sabu et al., 2000), *Aspergillus* sp. (Kumar et al., 2012), *Pseudomonas* sp. (Kumar and Chandrasekaran, 2003). As L-glutamine is used as substrate for production of L-glutaminase, hence its addition to

fermentation medium might stimulate enzyme production. L-glutamine also serves as source of energy and carbon (Kumar et al., 2013).

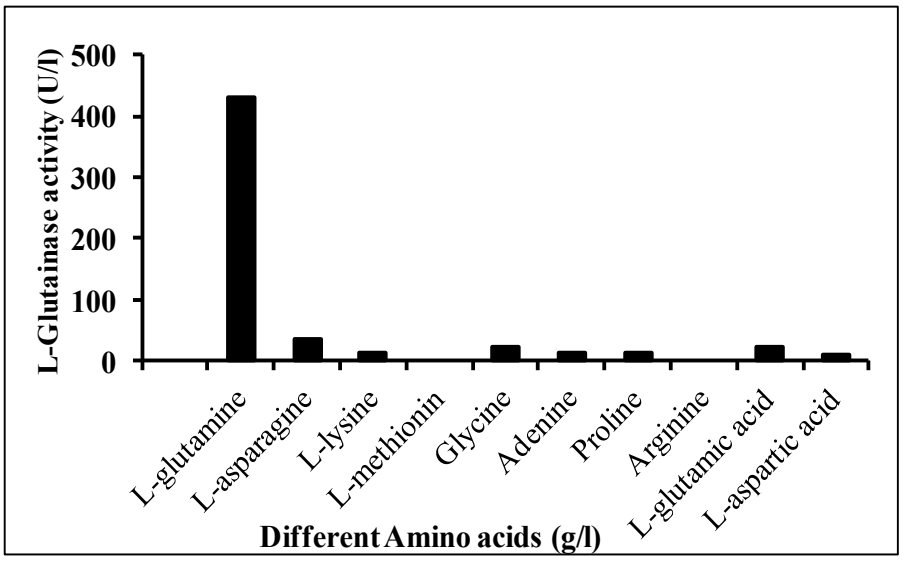


Fig.4.4. Effect of different amino acids on L-glutaminase production from *Bacillus cereus* MTCC 1305

4.3.2.2. Effect of different carbon and nitrogen sources on L-glutaminase production

Carbon and nitrogen sources generally play a significant role for microbial growth because these nutrients are directly linked with cell proliferation and metabolite biosynthesis (Mao et al., 2005). Effects of various carbon sources on gluaminase production were reported and glucose was generally used as carbon source in *Cryptococcus nodaensis* (Sato et al., 1999), *Pseudomonas* sp. (Kumar and Chandrasekaran, 2003), *Beauveria* sp. (Keerthi et al., 1999), *Achromobacteraceae* (Roberts et al., 1972), *Stenotrophomonas maltophilia* (Wakayama et al., 2005). The enhancement in production of L-glutaminase with addition of sucrose in media was reported in *Zygosaccaharomyces rouxii* (Iyer and Singhal, 2008). In order to identify

a suitable carbon source for L-glutaminase production by submerged cultivation of *Bacillus cereus* MTCC 1305, different carbon sources, i.e. lactose, sucrose, glucose, fructose, galactose, maltose, mannitol, sorbitol, starch and xylose were used. The organism showed better cell growth (Fig4.5a) with maximum cell biomass (1.332g/l) and higher L-glutaminase production (1043U/l) in media supplemented with sucrose (Fig4.5b).

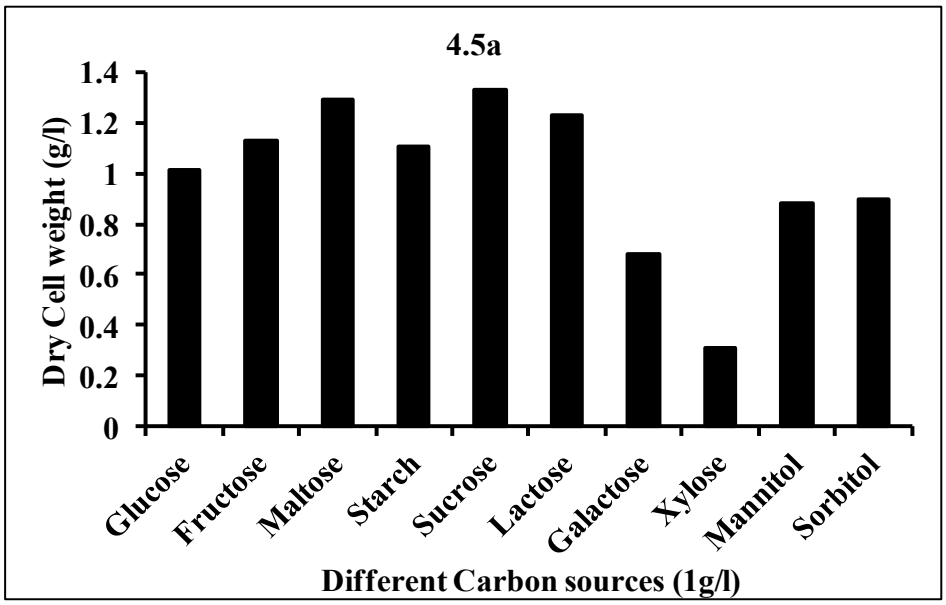


Fig.4.5a. Effect of different carbon sources on cell growth of *Bacillus cereus* MTCC 1305

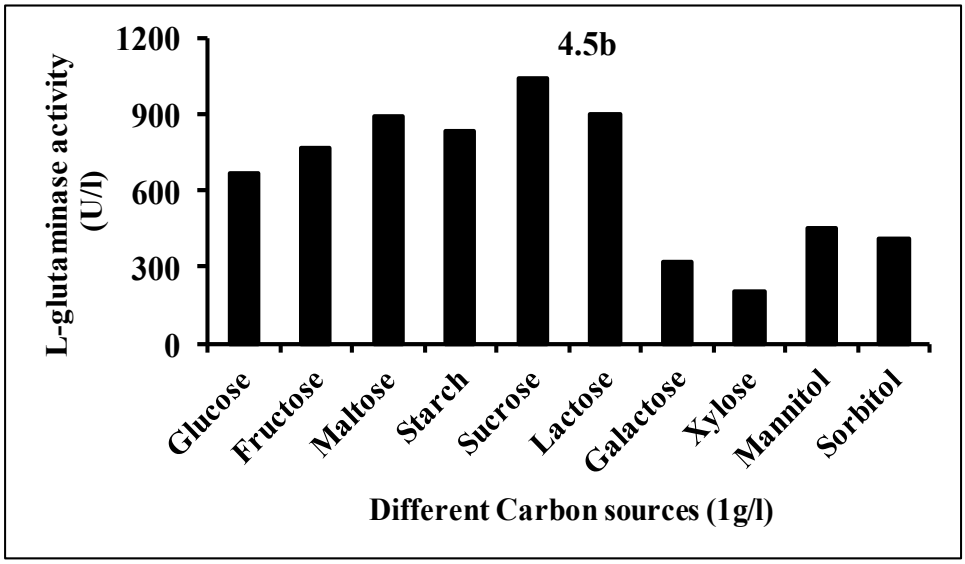
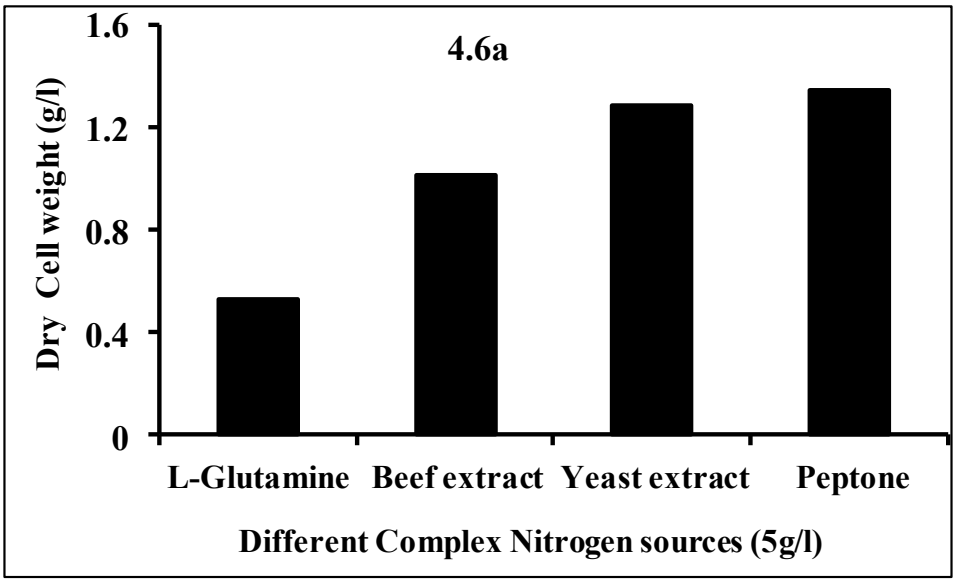


Fig.4.5b. Effect of different carbon sources on production of L-glutaminase from *Bacillus cereus* MTCC 1305

Most of the microorganisms utilize the complex organic nitrogen sources rather than inorganic sources for effective enzyme production. The enhancement in production of L-glutaminase with addition of yeast extract was reported for *Cryptococcus nodaensis* (Sato et al., 1999), *Beauveria bassiana* (Keerthi et al., 1999) and *Zygosaccharomyces rouxii* (Iyer and Singhal, 2009). The effect of different N sources on the production of L-glutaminase from *Bacillus cereus* MTCC 1305 was also studied. Inorganic N source (ammonium chloride, ammonium nitrate, ammonium sulfate, ammonium citrate and potassium nitrate) did not support cell growth and hence showed very low production of L–glutaminase. Complex organic nitrogen sources like beef extract, yeast extract and peptone showed better cell growth as shown in Fig4.6a. The production profile of L-glutaminase in presence of different complex nitrogen sources is shown in Fig4.6b and its maximum activity (1113U/l) was obtained in presence of peptone as N-source.

Media supplemented with L-glutamine as N-source also showed production of L-glutaminase with 346U/l showing utilization of this L-glutamine as nitrogen source.



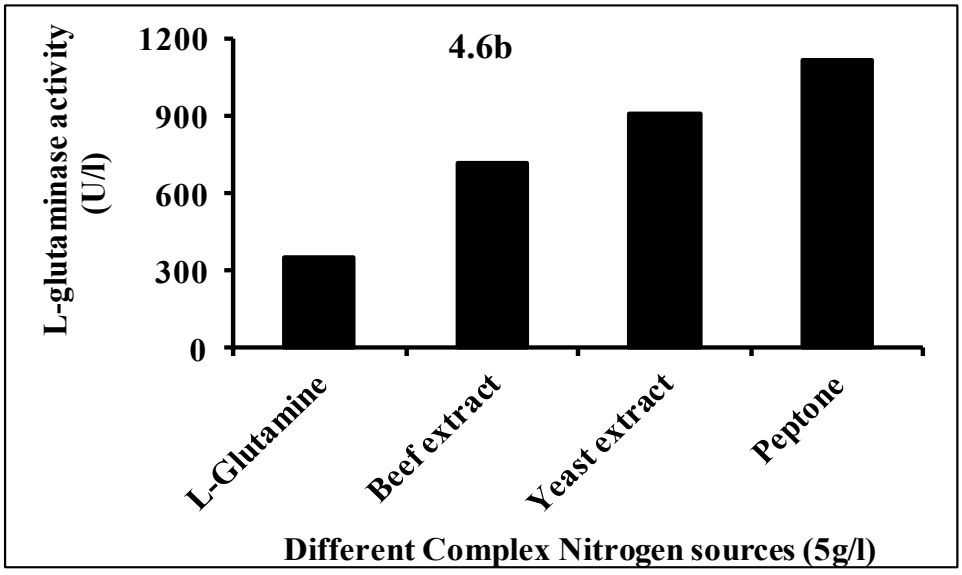


Fig.4.6. Effect of different nitrogen sources on (a) cell growth and (b) L-Glutaminase production in *Bacillus cereus* MTCC 1305

4.3.2.3. Statistical optimization of media components

It is very difficult to evaluate the significance of each medium component and interaction among them on the production of L-glutaminase by adopting the classical approach (one variable at a time). The significant variable was screened among different variables using PBD design proposed by Plackett and Burman (1944). Hence, experiments were carried out to screen the significant medium components and optimize their levels using Plackett-Burman Design and Box Benhken Design respectively. Both Plackett Burman and central composite designs have been applied in several studies to optimize medium components for production of L-asparaginase (Kumar et al., 2009), phytase (Singh and Satyanarayana, 2008), alpha-amylase (Gangadharan et al., 2008), lipase (Gaur et al., 2008), pectinase (Sharma and Satyanarayana, 2006), alkaline protease (Reddy et al., 2008), chitosanase (Sun et al., 2007). To the best of our knowledge, we could not find any report in the literature on the screening and optimization of significantly influencing medium components for L-glutaminase production from *Bacillus cereus* MTCC1305 in submerged fermentation. Twelve experiments were designed for selected eight media component (sucrose, L-glutamine, peptone, Na_2HPO_4, KH_2PO_4, NaCl and $MgSO_4$) by Plackett-Burman Design (Table4.7).

Table 4.7. Plackett Burman design matrix for eight factors with the experimental and predicted activity of L- Glutaminase

Run Order	Sucrose (g/l)	Peptone (g/l)	Glutamine (g/l)	Na_2HPO_4 (g/l)	KH_2PO_4 (g/l)	NaCl (g/l)	$MgSO_4$ (g/l)	$CaCl_2$ (g/l)	Exp Activity (U/l)	Predicted activity (U/l)
1	1	2	5	6	1	0.5	0.5	0.01	891	878.83
2	1	1	3	6	3	0.5	0.2	0.02	438	441.50
3	2	1	5	4	1	0.2	0.5	0.02	1078	1081.50
4	1	1	3	4	1	0.2	0.2	0.01	549	558.17
5	2	1	5	6	1	0.5	0.2	0.01	1213	1225.17
6	2	1	3	4	3	0.5	0.5	0.01	998	973.17
7	2	2	5	4	3	0.5	0.2	0.02	1312	1321.17
8	2	2	3	6	3	0.2	0.5	0.01	1023	1047.83
9	1	2	5	4	3	0.2	0.2	0.01	721	711.83
10	2	2	3	6	1	0.2	0.2	0.02	1267	1242.17
11	1	1	5	6	3	0.2	0.5	0.02	359	355.50
12	1	2	3	4	1	0.5	0.5	0.02	809	821.17

Where, experimental values of L-Glutaminase activity were mean values of triplicates with standard deviation (mean ± standard deviation)

The values of student's t distribution (T) and probability (P) were used to identify the effect of each factor on production of L-glutaminase. The factors having P values less than 0.05 were considered as significant factors. The media components like sucrose, L-glutamine, peptone, Na_2HPO_4, NaCl and $MgSO_4$ were obtained as significant factors as shown in Table4.6. Other media components had small effects with P>0.05 and were considered as insignificant factors. It is evident from Table4.8 that significant media components viz., sucrose, peptone, L-glutamine and NaCl had positive coefficient value, whereas Na_2HPO_4 and $MgSO_4$ had negative value of coefficient. It is inferred that the production of L-glutaminase enhanced with increase concentration of sucrose, L-glutamine, peptone, NaCl and decreased with increase concentration of Na_2HPO_4 and $MgSO_4$. The fitness of the model was obtained with the value of coefficient of determination R^2 =99.16%.

Table4.8. Statistical analysis of the Plackett Burman design showing coefficient, test value (T), and Probability (P) for each variable

Term	Effect	Coef	SE Coef	T	P
Constant		888.17	8.471	104.85	0.000
Sucrose	520.67	260.33	8.471	30.73	0.000[b]
Peptone	231.33	115.67	8.471	13.66	0.001[b]
L-Glutamine	81.67	40.83	8.471	4.82	0.017[b]
Na_2HPO_4	-46.00	-23.00	8.471	-2.72	0.003[b]
KH_2PO_4	-159.33	-79.67	8.471	-9.41	0.073[a]
NaCl	110.67	55.33	8.471	6.53	0.007[b]
$MgSO_4$	-57.00	-28.50	8.471	-3.36	0.044[b]
$CaCl_2$	-22.00	-11.00	8.471	-1.30	0.285[a]

R^2 = 99.77% , R^2 (Pred)=96.33%, R^2 (adj) = 99.16%, [a]Nonsignificant at P>0.05,[b]Significant at P<0.05

Box Benhken Design was used to determine the optimum levels of screened six factors (sucrose, L-glutamine, peptone, Na_2HPO_4, NaCl and $MgSO_4$) and 54 experiments were designed for these variables (Table4.9).

Table4.9. Experimental design matrix for five significant media components with actual and predicted activity of L-glutaminase using BBD and ANN tools

Run Order	Sucrose (g/l)	Peptone (g/l)	Glutamine (g/l)	Na_2HPO_4 (g/l)	NaCl (g/l)	MgSO4 (g/l)	Actual activity (U/l)	Predicted activity (U/l)	
								BBD	ANN
1	1.0	2.5	5.0	8	0.6	0.5	872	881.58	871.97TRD
2	4.0	2.5	5.0	8	0.6	0.5	1006	1025.33	1005.96TRD
3	2.5	1.0	7.5	6	0.4	0.5	632	609.23	632.23TRD
4	4.0	2.5	7.5	6	0.5	0.6	684	727.08	684.09TRD
5	2.5	2.5	2.5	8	0.5	0.6	632	603.17	632.01TRD
6	2.5	2.5	2.5	8	0.5	0.4	861	815.17	861.17TRD
7	1.0	4.0	5.0	8	0.5	0.5	153	114.58	153.01TRD
8	4.0	2.5	5.0	8	0.4	0.5	1021	955.92	1020.91TRD
9	2.5	2.5	5.0	6	0.5	0.5	1630	1630.00	1629.99TRD
10	4.0	1.0	5.0	8	0.5	0.5	723	796.42	722.90TRD
11	4.0	2.5	2.5	6	0.5	0.6	991	938.67	991.02TRD
12	2.5	2.5	5.0	6	0.5	0.5	1630	1630.00	-
13	4.0	4.0	5.0	4	0.5	0.5	1210	1136.08	1210.09TRD
14	2.5	2.5	2.5	4	0.5	0.6	998	931.17	998.01TRD
15	2.5	2.5	5.0	6	0.5	0.5	1630	1630.00	-1002.86TRD
16	2.5	4.0	5.0	6	0.4	0.4	1003	830.19	1011.96TRD
17	2.5	2.5	2.5	4	0.5	0.4	1012	999.17	764.90TRD
18	2.5	1.0	2.5	6	0.6	0.5	765	876.35	758.98TRD
19	2.5	4.0	2.5	6	0.6	0.5	759	665.90	761.88TRD
20	1.0	1.0	5.0	4	0.5	0.5	762	797.92	532.07TRD
21	2.5	4.0	7.5	6	0.4	0.5	532	536.52	550.09TRD
22	2.5	4.0	5.0	6	0.6	0.6	550	622.23	1041.95TRD
23	1.0	2.5	5.0	4	0.6	0.5	1042	1087.33	789.02TRD
24	2.5	2.5	7.5	4	0.5	0.4	789	832.08	722.99TRD
25	2.5	2.5	7.5	4	0.5	0.6	723	754.58	912.05TRD

26	2.5	1.0	5.0	6	0.6	0.6	912	968.94	912.05TRD
27	2.5	1.0	5.0	6	0.6	0.4	793	798.69	792.96TRD
28	2.5	1.0	7.5	6	0.6	0.5	1021	688.02	1021.15TRD
29	1.0	1.0	5.0	8	0.5	0.5	932	986.17	932.05TRD
30	4.0	1.0	5.0	4	0.5	0.5	529	547.67	529.01TRD
31	2.5	2.5	7.5	8	0.5	0.4	798	879.08	797.98TRD
32	2.5	4.0	5.0	6	0.6	0.4	865	932.48	865.15TRD
33	4.0	2.5	5.0	4	0.4	0.5	1021	1031.17	1020.99TRD
34	1.0	2.5	7.5	6	0.5	0.4	838	876.08	837.99TRD
35	1.0	2.5	5.0	4	0.4	0.5	912	872.92	912.07TRD
36	2.5	2.5	5.0	6	0.5	0.5	1630	1630.00	-
37	2.5	1.0	5.0	6	0.4	0.4	723	766.65	722.95TRD
38	1.0	2.5	2.5	6	0.5	0.6	513	445.67	513.06TRD
39	1.0	2.5	7.5	6	0.5	0.6	449	535.08	448.94TRD
40	2.5	2.5	7.5	8	0.5	0.6	659	657.58	659.07TRD
41	2.5	2.5	5.0	6	0.5	0.5	1630	1630.00	-
42	2.5	1.0	2.5	6	0.4	0.5	834	741.56	834.07TRD
43	1.0	2.5	5.0	8	0.4	0.5	779	737.17	778.89TRD
44	4.0	2.5	2.5	6	0.5	0.4	959	887.17	959.17TTD
45	2.5	4.0	5.0	6	0.4	0.6	492	370.44	491.91TTD
46	4.0	4.0	5.0	8	0.5	0.5	683	666.83	682.94TTD
47	2.5	4.0	7.5	6	0.6	0.5	709	685.56	708.93TTD
48	2.5	1.0	5.0	6	0.4	0.6	739	787.40	738.93TTD
49	4.0	2.5	7.5	6	0.5	0.4	632	685.08	631.93VD
50	1.0	4.0	5.0	4	0.5	0.5	698	644.33	698.02VD
51	1.0	2.5	2.5	6	0.5	0.4	806	777.17	806.02VD
52	2.5	2.5	5.0	6	0.5	0.5	1630	1630.00	-
53	2.5	4.0	2.5	6	0.4	0.5	623	624.85	623.02VD
54	4.0	2.5	5.0	4	0.6	0.5	1109	1170.58	1108.83VD

Where Experimental values of L-Glutaminase activity were mean values of triplicate with standard deviation (mean ±Standard Deviation)

The effects of all independent factors and the interaction of sucrose-peptone, sucrose-glutamine, sucrose-$MgSO_4$, peptone-Na_2HPO_4, peptone-$MgSO_4$, glutamine-Na_2HPO_4 and glutamine-NaCl were found as highly significant for the production of L-glutaminase. The following second order polynomial equation was found to explain the production of L-glutaminase by applying the multiple regression analysis on the experimental data obtained in Table4.10:

$$Y=(-14488.9)+296.4X_1+1555.1X_2+362.7X_3+815.2X_4+14506.1X_5+28106.5X_6-127.8X_1^2-186.2X_2^2-50.2X_3^2-53.1X_4^2-15986.1X_5^2-29165.3X_6^2+82.4X_1X_2-20.1X_1X_3+5.0X_1X_4-125X_1X_5+638X_1X_6-6.5X_2X_3-59.8X_2X_4-137.5X_2X_5-800.8X_2X_6+11.6X_3X_4+249.5X_3X_5-9.5X_3X_6-87.5X_4X_5-180.5X_4X_6+3737.5X_5X_6 \quad \text{.....(4.2)}$$

Where, Y=L-glutaminase activity as response, X_1= Sucrose, X_2= Peptone, X_3=L-glutamine, X_4 = Na_2HPO_4, X_5=NaCl and X_6= $MgSO_4$

Table4.10. Model coefficients estimated by multiple linear regressions for L-glutaminase production

Term	Coef	SE Coef	T	P
Constant	-14488.9	1153.34	-12.563	0.000
X_1	296.4	121.03	2.449	0.021 [b]
X_2	1555.1	113.93	13.650	0.000 [b]
X_3	362.7	69.76	5.200	0.000 [b]
X_4	815.2	100.72	8.094	0.000 [b]
X_5	14506.1	2375.35	6.107	0.000 [b]
X_6	28106.5	2369.31	11.863	0.000 [b]
$(X_1)^2$	-127.8	8.49	-15.048	0.000 [b]
$(X_2)^2$	-186.2	8.49	-21.928	0.000 [b]

$(X_3)^2$	-50.7	3.06	-16.591	0.000 [b]
$(X_4)^2$	-53.1	4.78	-11.122	0.000 [b]
$(X_5)^2$	-15986.1	1910.13	-8.369	0.000 [b]
$(X_6)^2$	-29165.3	1910.13	-15.269	0.000 [b]
$X_1 X_2$	82.4	9.63	8.565	0.000 [b]
$X_1 X_3$	-20.1	5.78	-3.474	0.002 [b]
$X_1 X_4$	5.0	5.11	0.988	0.332 [a]
$X_1 X_5$	-125.0	144.39	-0.866	0.395 [a]
$X_1 X_6$	638.3	144.39	4.421	0.000 [b]
$X_2 X_3$	-6.5	5.78	-1.125	0.271 [a]
$X_2 X_4$	-59.8	7.22	-8.288	0.000 [b]
$X_2 X_5$	-137.5	102.10	-1.347	0.190 [a]
X_2* X_6	-800.8	144.39	-5.546	0.000 [b]
$X_3 X_4$	11.6	4.33	2.666	0.013 [b]
$X_3 X_5$	249.5	86.64	2.880	0.008 [b]
$X_3 X_6$	-9.5	61.26	-0.155	0.878 [a]
$X_4 X_5$	-87.5	108.29	-0.808	0.426 [a]
$X_4 X_6$	-180.0	108.29	-1.662	0.108 [a]
$X_5 X_6$	3737.5	2165.88	1.726	0.096 [a]

R^2=98.28%, R^2(pred)=91.02%, R^2(adj)=96.50%, [a]Nonsignificant at P>0.05, [b]Significant at P<0.05

Sucrose (X_1), Peptone (X_2), L-glutamine (X_3), Na_2HPO_4 (X_4), NaCl (X_5) and $MgSO_4$ (X_6)

According to ANOVA of quadratic regression (Table4.11), model is highly significant with very low probability value. This indicates that the interactive effects of all the independent variables significantly contributed to maximize the production of L-glutaminase. The fitness of the model was checked by coefficient of determination, R^2, which implies that sample variation of 98.28% for L-glutaminase production is attributed to the media components and also only 1.72% of the total variation is not explained by the model. The value of the adjusted determination coefficient (R^2_{adj}) was found as 96.50%, which suggested that the experimental data are in good agreement with the predicted values.

Table4.11.ANOVA analysis for model fitness for production of L-glutaminase at optimum media components

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	27	5580439	5580439	206683	55.07	0.000
Linear	6	548987	1251163	208527	55.57	0.000
Square	6	4164402	4164402	694067	184.94	0.000
Interaction	15	867050	867050	57803	15.40	0.000
Residual-Error	26	97574	97574	3753	-	-
Lack of Fit	21	97574	97574	4646	*	*
Pure Error	5	0	0	0	-	-
Total	53	5678013				

DF-Degree of Freedom, MS-Mean Square, SS-Sum of square

The interaction of these variables on the activity of L-glutaminase was graphically analyzed using contour plots. The significant effect of interaction of variables like sucrose-peptone, sucrose-glutamine, sucrose-$MgSO_4$, peptone-Na_2HPO_4, peptone-$MgSO_4$, glutamine-Na_2HPO_4 and glutamine-NaCl on activity of L-glutaminase is shown in Fig4.7a, 4.7b, 4.7c, 4.7d, 4.7e, 7.7f, and 4.7g respectively. The positive coefficient values was obtained for all individual variables and some interactive variables like sucrose-peptone, sucrose-$MgSO_4$, glutamine-Na_2HPO_4 and glutamine-NaCl had positive which is shown in Table4.9. The contour plots for these interactive variables in Fig4.7a, 4.7c, 4.7f, and 4.7g showed that activity of L-glutaminase was increased with simultaneous increase of values of these variables up to an optimum point. The optimal levels for media components were obtained as sucrose (2.5g/l), peptone (2.5g/l), L-glutamine (5.0g/l), Na_2HPO_4 (6.0g/l), NaCl (0.5g/l) and $MgSO_4$ (0.5g/l). Model predicted L-glutaminase activity was determined as 1016.90 U/l by using these optimum values in second order equation.

Fig.4.7a

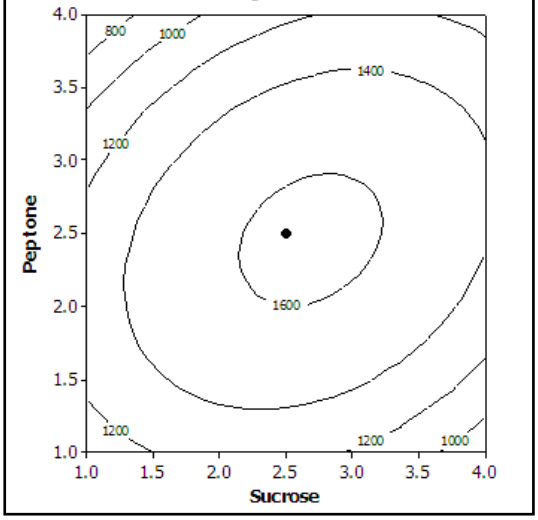


Fig.4.7b

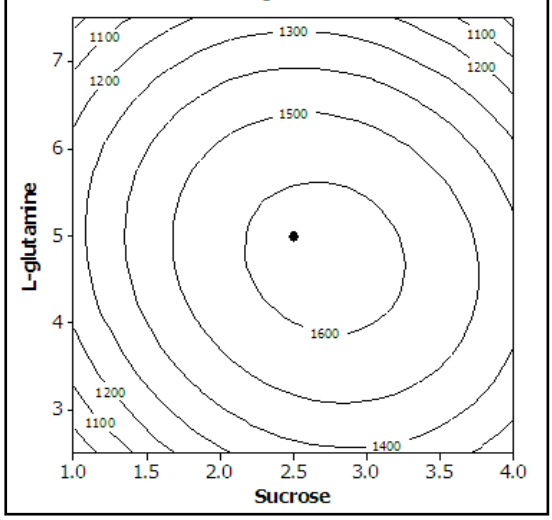


Fig.4.7c

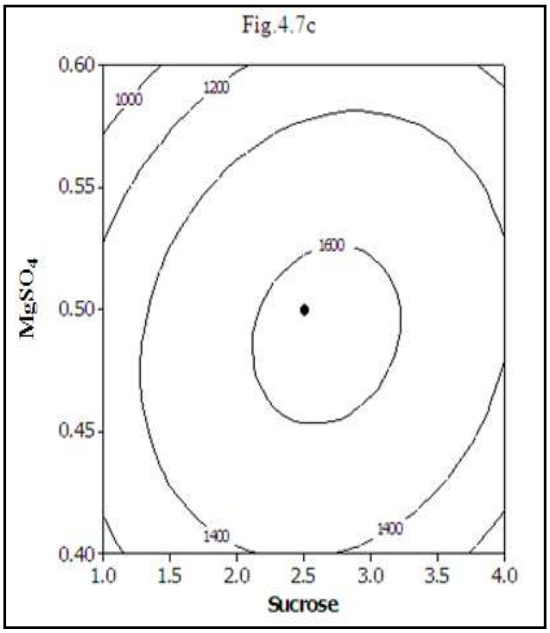
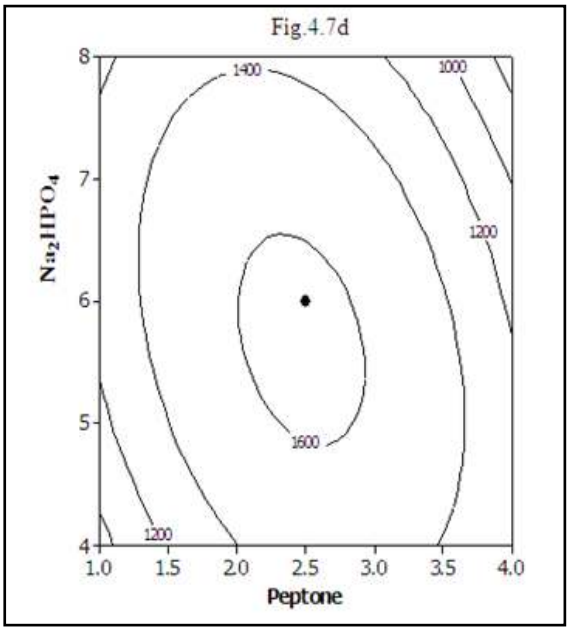


Fig.4.7d



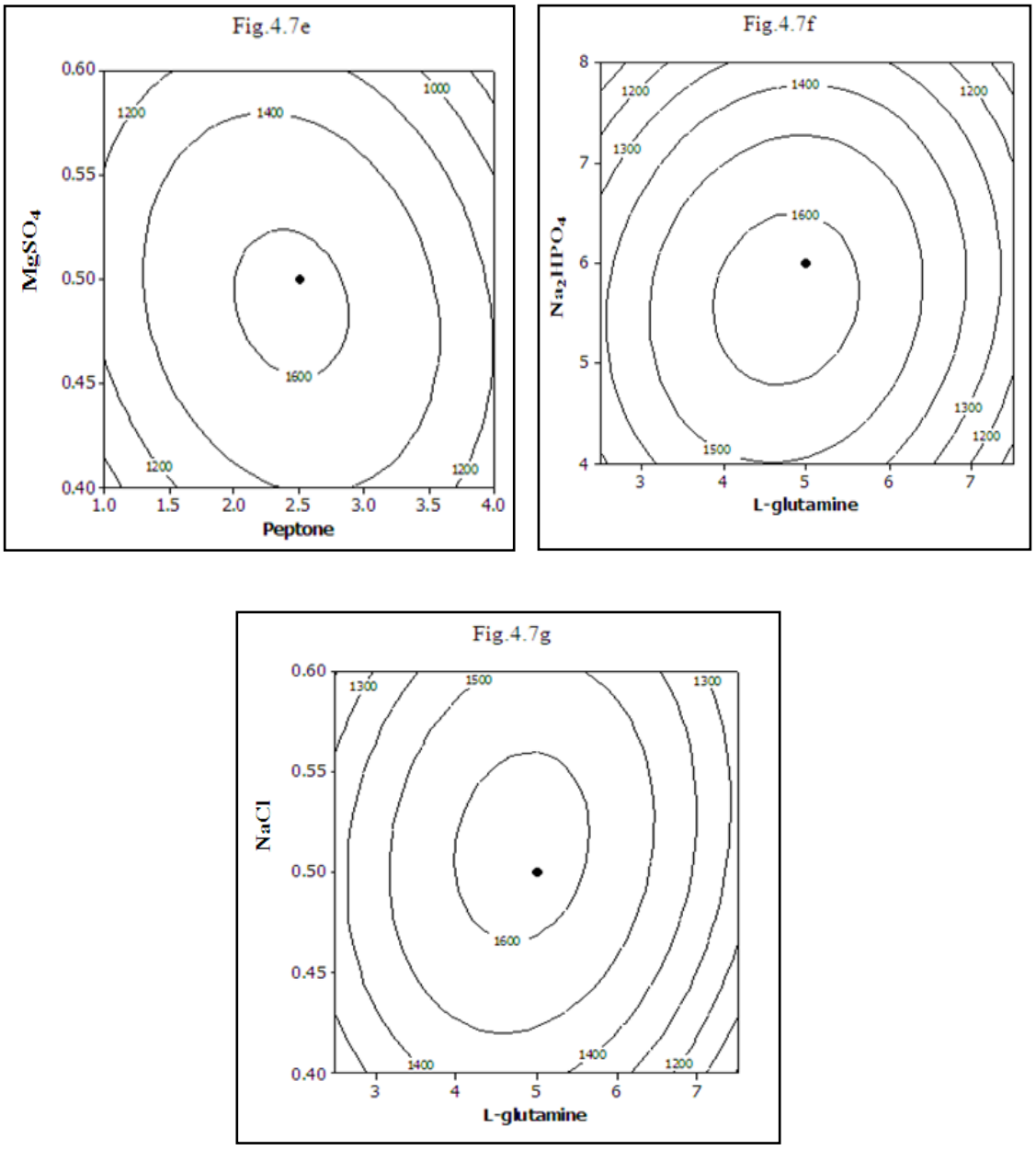


Fig.4.7. Contour plots showing interactive effect of selected variables on activity of L-glutaminase (a) sucrose and peptone (b) sucrose and L-glutamine (c) sucrose and $MgSO_4$ (d) peptone and Na_2HPO_4 (e) peptone and $MgSO_4$ (f) L-glutamine and Na_2HPO_4 (g) L-glutamine and NaCl

The high degree of non linearity between input variables (sucrose, peptone, L-glutamine, Na_2HPO_4, NaCl and $MgSO_4$) and output variable (L-glutaminase activity) was analyzed using Artificial Neural Network. Multilayer perceptron with Levenberg-Marquardt algorithm and sigmoid transfer function was used in this study further applied to analyze response. The enzyme production data obtained was analyzed using. The determination of suitable number of neurons in hidden layers is very important as it affects the training time and generalization property of neural networks. There is no general rule for selecting the number of neurons in a hidden layer. The most popular approach to find the optimal number of neurons in hidden layer is by trial and error. In this study, trial and error approach was used to determine the optimum number of neurons in hidden layer of the network (examined from 1 to 5 neurons) and minimum value of mean squared errors (MSE) was obtained for three neurons as shown in Fig.4.8. The scaled data were passed into the input layer and then is propagated from input layer to hidden layer and finally to output layer of the network. The "sigmoid" transfer function was used for non linear relationship of prediction L-glutaminase activity. The best architecture obtained for optimization of media components was "6-3-1" with six input neurons, three hidden neurons and one output neuron which is shown in Fig4.9.

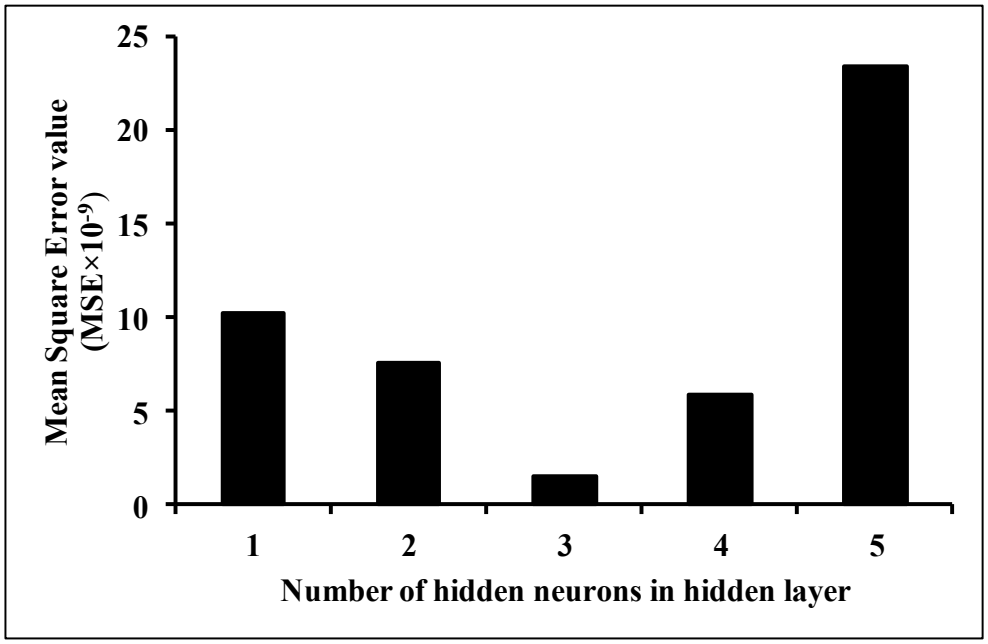


Fig.4.8. Determination of number of hidden layer's neurons for artificial neural network designed for optimization of media components for production of L-glutaminase.

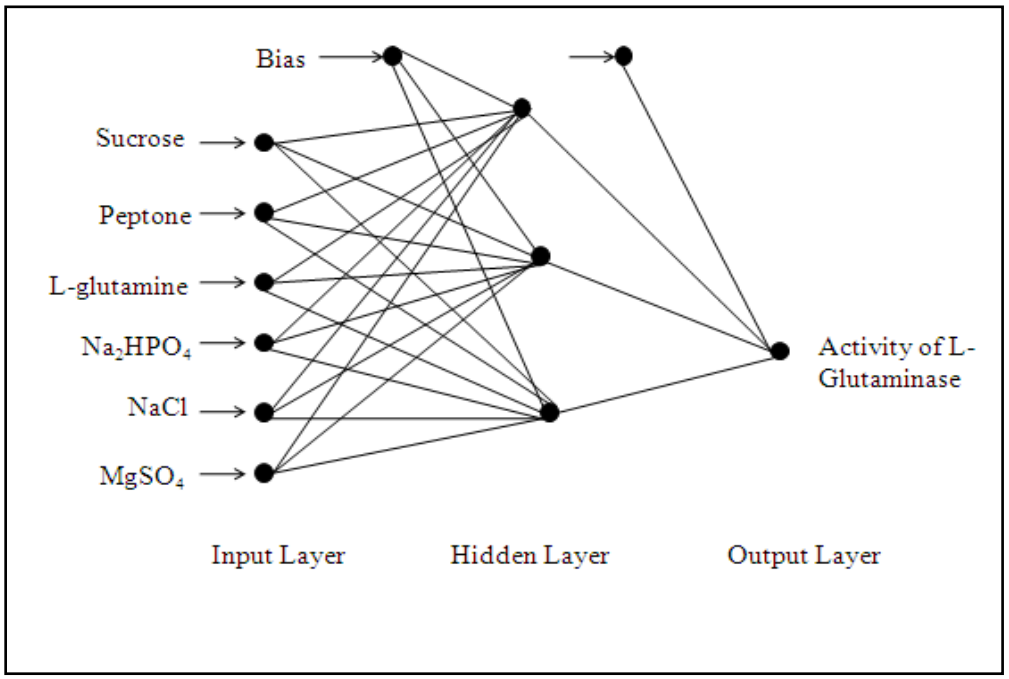


Fig.4.9. Multilayer perceptron neural network architecture used for optimization of media components for L-glutaminase production

The values of each weight in input, hidden and output node were presented in Table4.12. The predicted activity of L-glutaminase was simulated as 1629.9943U/l after substituting weight values of input data and bias term in equation 3.4.

Table4.12. Weight and bias values of nonlinear function at optimum media components

Weight on connection between input and hidden nodes						
Sucrose	Peptone	L-glutamine	Na_2HPO_4	NaCl	$MgSO_4$	Bias term (b_1)
-0.02768	0.01654	-0.00709	0.00090	0.00089	0.02089	-0.0120
0.01814	-0.02433	0.01704	0.00915	0.009147	-0.04316	0.01854
0.00029	-0.00026	0.00007	-0.000008	-0.000008	-0.00039	0.0026
Weight on connection between hidden and output nodes 14.465, -13.9971, Bias term (b_2=13.34145)						

ANN was found better predictor than RSM with higher value of coefficient of determination (0.9999_{ANN}>0.8209_{RSM}), lower value of RMSE (0.1068_{ANN}>96.309_{RSM}) and lower value of absolute average deviation ($0.0006\%_{ANN}$<$1.4180\%_{RSM}$).

The predicted condition was experimentally verified in triplicate by conducting experiment using optimum media components viz., sucrose (2.5g/l), peptone (2.5g/l), L-glutamine (5.0g/l), Na_2HPO_4 (6.0g/l), NaCl (0.5g/l) and $MgSO_4$ (0.5g/l). The experimental L-glutaminase activity was obtained as 1630.00 U/l at predicted optimal conditions, which was found to be enhanced by 3.83 fold than the activity of L-glutaminase obtained under unoptimized media component (425U/l).

4.4 Conclusion

ANN model was found better predictor than RSM for optimization of, cultural conditions and media components. ANN Predicted activity of L-glutaminase under optimum cultural conditions were predicted as pH (7.5), fermentation time (40h), temperature (34˚C), inoculum size (2%), inoculum age (10h) and agitation speed (175rpm) was obtained as 666.97U/l, which was very much closer to experimental activity 667.23U/l. Sucrose and peptone were selected as best carbon and nitrogen source for L-glutaminase production. Plackett Burmn design selected sucrose, L-glutamine, peptone, Na_2HPO_4, NaCl and $MgSO_4$ as significant factors. RSM based on Box-Benhken design and ANN model was further employed to get optimum concentration of these significant variables. Optimal levels for media components were obtained as sucrose (2.5g/l), peptone (2.5g/l), L-glutamine (5.0g/l), Na_2HPO_4 (6.0g/l), NaCl (0.5g/l) and $MgSO_4$ (0.5g/l). ANN predicted activity under these optimum conditions was obtained as 1629.9943U/l, which was very much closer to experimental activity (1630U/l).