CHAPTER 3

Selection of Most Potent Culture for L-Glutaminase Production and Optimization of Assay Parameters

3.1. Introduction

Glutaminase is ubiquitously present in living organisms and plays an essential role in cellular metabolism (Sinsuwan et al., 2012). The hydrolytic property of glutaminase enables glutamine to donate its amide (γ nitrogen) group for the synthesis of other non essential amino acids (NEAAs) (Voet and Voet, 1995; Cowan et al., 2012). This enzyme has been reported to be present in diverse group of microorganism, plant and animal sources. Microbial glutaminases are generally preferred because of their applications in food industry as flavor enhancing agent (Yano et al., 1988; Moriguchi et al., 1994; Tachiki et al., 1996; Sabu et al., 2000; Kumar et al., 2012) and in pharmaceutical industry as antileukaemic agent (El-Asmar and Greenberg, 1996; Roberts et al., 1970).

Although production of glutaminases were studied in several bacterial strains, the best characterized were from members of *Enterobacteriaceae* family like, *Escherichia coli* (Prusiner et al., 1976), *Proteus morganni* (Wade et al., 1971), *Erwnia carotovora* (Imada et al., 1973), *Serratia marcescens* (Wade et al., 1971),*Vibrio costicola* (Renu and Chandrasekaran, 1992), *Micrococcus luteus* (Moriguchi et al., 1994), *Pseudomonas aeruginosa* (Ohshima, 1976), *Pseudomonas aurantica* (Lebedeva and Berezova, 1997), *Pseudomonas fluorescens* (Eremenko et al., 1975). Some of the bacterial strains like, *Pseudomonas* sp.(Lebedeva and Berezova, 1997; Eremenko et al., 1975; Ohshima, 1976), *Vibrio costicola*, (Renu and Chandrasekaran, 1992), *Bacillus subtilis* (Harayama and Yasuhira, 1991; Brown et al., 2008), *Bacillus licheniformis* (Cook et al., 1981), *Bacillus pasteurii* (Klein et al., 2002),

Debaryomyces sp. (Dura et al., 2002), and *Beauveria* sp. (Keerthi et al., 1999) were reported to produce extracellular L-glutaminase,

Most of the yeast strains like, *Hansenula, Rhodotorula, Candida scottii*, *Cryptococcus albidus* and *Candida utilis* (Imada et al., 1973; Fukushima and Motai, 1990) were reported to produce intracellular glutaminase. Glutaminase A of *Saccharomyces cerevisiae* was reported as membrane bound, while glutaminase B as cytoplasmic (Soberon and Gonzalez, 1987). As extracellular glutaminase is of much significance for its in vitro application, hence search of new microbial strain producing extracellular glutaminase is highly desired.

L-glutaminase hydrolyzes L-glutamine to L-glutamic acid and ammonia. Many methods were reported to estimate its activity either by estimating concentration of released products ammonia or glutamic acid. Nierlich and Magasanik (1965) estimated intracellular glutaminase activity by measuring concentration of glutamic acid after coupling its oxidation to α-ketoglutarate and formation of the reduced form of the 3-Acetyl Pyridine Adenine Dinucleotide (AcPyAD) through glutamate dehydrogenase. Imada et al. (1973) estimated glutaminase activity by measuring concentration of ammonia using Nessler's reagent. Brown et al. (2008) estimated glutaminase activity of purified proteins using the chromogenic substrate L-glutamyl-p-nitroanilide (Sigma) in a reaction mixture. Mongin et al. (2011) estimated activity of intracellular glutaminase by quantifying the intracellular conversion of L-[^{3}H] glutamine to L-[^{3}H] glutamate. Among all these methods, assay method proposed by Imada et al. (1973) was found to be simple, easy, and economical method to estimate glutaminase activity and was being reported in many recent papers. The optimization

of significant parameters (pH of reaction mixture, incubation temperature, reaction time, substrate concentration and enzyme volume) of enzyme reaction plays a vital role to get optimum activity of enzyme. The optimization of bioprocess parameters using statistical tools (RSM and ANN) are generally preferred over the conventional method of "one variable at one time" because of accounting interactive effects of the variables with screening and prediction of large experimental domain (Rathi et al., 2001; Sim and Kamaruddin, 2008; Bas and Boyaci, 2007; Dutta et al., 2004). ANN is found as more accurate modeling technique as compared to RSM for prediction and modeling of nonlinear relationships of variables (Bas and Boyaci, 2007; Haykin, 2009).

The objective of this study was to select a potent microbial strain producing high amount of L-glutaminase and develop an efficient estimation method for estimation of glutaminase. The assay parameters were optimized with respect to pH, temperature, reaction time, substrate concentration and enzyme volume. Further the statistical tools like ANN and RSM were employed to improve the production of L-glutaminase.

3.2. Materials and Methods

3.2.1. Materials

L-Glutamine, Nessler's Reagent, Peptone, Glucose, Agar, Yeast extract, Beef extract, Malt extract, TRIS-HCl buffer, Tri-chloro actetic acid, NaCl, CH_3COONa, Tween 80, ammonium citrate, were purchased from Hi Media, Bombay, India. $CaCl_2$, $MgSO_4$, $MnSO_4$, $Na_2HPO_4.2H_2O$, KH_2PO_4 were purchased from Qualigens, Bombay, India. All chemicals used were of AR grade.

3.2.2. Microorganisms and culture conditions

Microbial strains like *Staphylococcus aureus* MTCC 3160*, Streptococcus lactis* MTCC 460*, Streptococcus* sp. MTCC 389*, Escherichia coli* MTCC 2893*, Bacillus megaterium* MTCC 2412*, Bacillus subtilis* MTCC 1789*, Bacillus cereus* MTCC 1305*, Bacillus licheniformis* MTCC 1483*,* were obtained from Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India. *Bacillus stearhothermophilus* NCIM 2235, *Pseudomonas aeruginosa* NCIM 2948*, Pseudomonas Putida* NCIM 2650*, Pseudomonas fluorescens* NCIM 5096*, Lactobacillus casei* NCIM 2364*, Lactobacillus plantarum* NCIM 2373, and *Aeromonas formicans* NCIM 2319 were obtained from National Collection of Industrial Microorganisms (NCIM), Pune, India. The bacterial strains like *Bacillus* sp., *Pseudomona* sp., *Escherichia coli*, and *Aeromonas* sp. were grown in nutrient media (pH-7.0) containing beef extract (1g/l), yeast extract (2g/l), peptone (5g/l), and agar (15g/l) at 35˚C. *Lactobacillus* and *Streptococcus* were grown in MRS broth media (pH-7.0) containing glucose (20g/l), peptone (10g/l), beef Extract (10g/l), yeast extract (5g/l), CH_3COONa (5g/l), Tween 80 (1g/l), ammonium citrate (2g/l), $MgSO_4$ (0.1g/l) and $MnSO_4$ (0.05g/l). Inoculated slants were incubated at 35˚C for 24hr for microbial growth and stored at 4±1˚C in refrigerator for further use. The production of L-glutaminase from bacterial strains was studied in production media (pH-7.0) containing glucose (1g/l), yeast extract (5g/l), L-glutamine (3g/l), $Na_2HPO_4 \cdot 2H_2O$ (3g/l), KH_2PO_4 (2g/l), NaCl (0.5g/l), $MgSO_4 \cdot 7H_2O$ (0.5g/l), and $CaCl_2 \cdot 2H_2O$ (0.015g/l) (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

inoculum was prepared by adding a loop full of freshly prepared pure culture into 50ml growth media in 250ml Erlenmeyer flask. The production media (100ml) was inoculated with 2% inoculum (2.13×10^5cell/ml, 10hrs age) and incubated at 35˚C, 120rpm for 36hrs in an orbital shaker (Scigenics, India). The fermented sample was centrifuged at 10,000×*g* at 4±1˚C for 10 minutes and collected cell free broth was used as an enzyme source.

3.2.3. Estimation of glutaminase activity

The extracellular activity of L-glutaminase was determined using cell free broth by modified method of Imada et al. (1973), in which reaction mixture (pH of 7.5) containing 0.5ml of crude extract of enzyme, 0.5ml of 40mM L-glutamine solution, 1.0ml of 0.1M phosphate buffer was incubated at 37˚C for 30minutes. The reaction was terminated by the addition of 0.5ml of 1.5M Trichloro-acetic acid to reaction mixture. Reagent blank and substrate blank were also prepared simultaneously. 3.7 ml of distilled water was added to 0.1ml reaction mixture and then 0.2ml of Nessler's reagent was added. The absorbance of the blank and test sample was measured at 450nm. One unit of glutaminase activity was defined as enzyme required for deamination of 1.0µmole of glutamine per minute per ml of enzyme solution at pH of 7.5 and temperature 35˚C (Curthoys and Watford, 1995). A standard graph using NH_4Cl (12×10^{-4}M) was plotted for computation of the concentration of ammonia (Fig3.1).

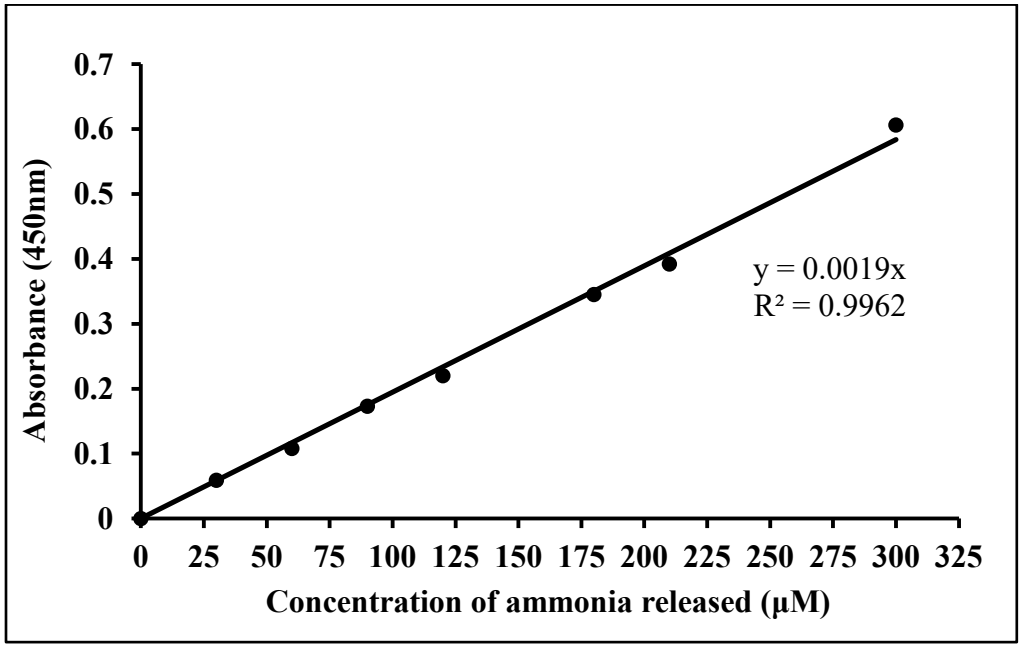


Fig.3.1. Standard curve for estimation of glutaminase activity

3.2.4. Protein estimation by Bradford method

Dye stock reagent was prepared by dissolving Coomassie Blue G (100mg) in 50ml of ethanol. Solution was then dissolved in 100ml of 85% H_3PO_4, and diluted to 200ml with water. This solution was of dark red color and stable indefinitely in a dark bottle at 4˚C. The assay reagent was prepared by diluting 1 volume of the dye stock with 4 volumes of distilled water. A convenient standard curve was obtained by using bovine serum albumin (BSA) with concentrations of 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.08, 0.1 and 0.2mg/ml (Bradford, 1976). Assay reagent of 2.0ml was added to above solution and absorbance at 595nm was taken after 5minutes (Fig3.2).

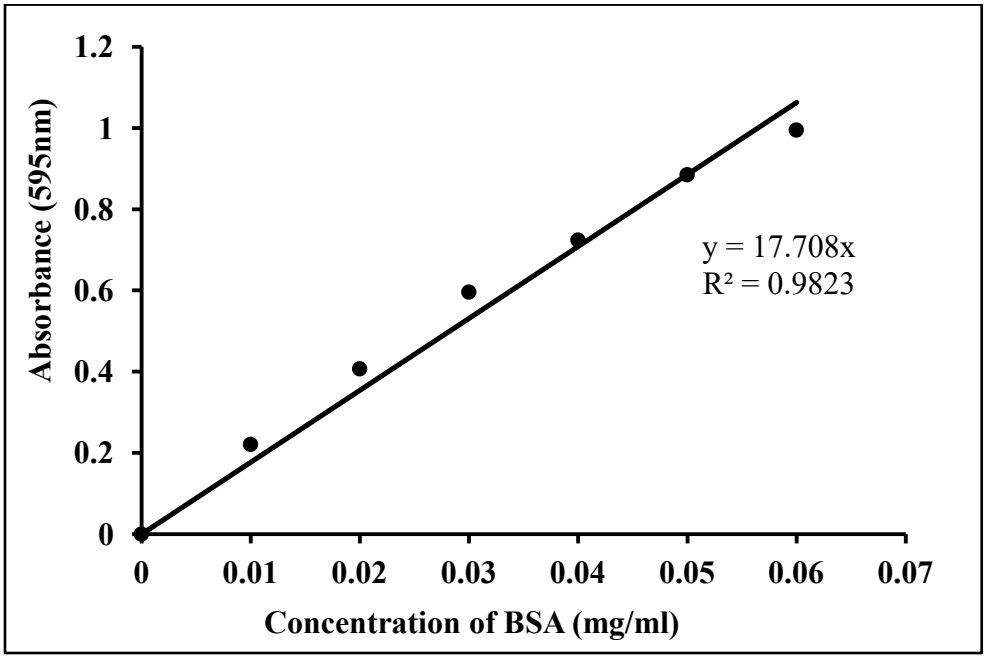


Fig.3.2. Protein standard curve for BSA by using Bradford reagent

3.2.5. Optimization of assay parameters using RSM and ANN methodologies

The statistical optimization strategy was applied for five assay parameters viz., pH, reaction time, temperature, substrate (L-glutamine) concentration, and volume of L-glutaminase enzyme. The assay parameters with upper and lower limits for experiments designed to estimate optimum activity of L-glutaminase using RSM and ANN methodologies are shown in Table3.1. All experiments designed by RSM for estimation of L-glutaminase activity were performed in triplicates. The experimental data was further analyzed on basis of multiple regressions and ANOVA. The following second order polynomial model was fitted for prediction of optimal levels (equation3.1):

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \ldots\ldots\ldots (3.1)$$

Where, Y=Predicted response, β_0=Intercept coefficient, β_i=Linear coefficient, β_{ii}=Quadratic coefficient and β_{ij}=Interaction coefficient

The effect of interaction of significant variables on activity of L-glutaminase was further studied using contour plots.

Table3.1. Optimization of assay parameters of L-glutaminase from *Bacillus cereus* MTCC 1305 using RSM and ANN methodologies

Assay parameters	**RSM based optimization using Minitab-15 version Statistical Software**	**ANN based optimization using Neurosolutions-6 version, neural builder software**
Reaction time (10-30 minutes), Reaction temperature (20-50ºC), pH of reaction mixture (5-10), concentration of L-glutamine (20-60mM), Enzyme volume (0.2-0.8ml)	• Central Composite Design with 2^3 level factorial, α=2.366, • 32 experiments were designed with eight replication at centre points • Regression Analysis • ANOVA • Response surface plots and contour plots	• 24 Experimental data were divided into three sets: training (16), testing (4) and validation (4) 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 5-3-1with five input neurons, three neurons in hidden layer and one output neuron.

ANN software (neurosolutions version 6) was further applied to provide a non linear mapping between the input variables and output variables. ANN was used for

simulating same set of experimental data used for RSM except the replicated data obtained at centre point. These replicates do not improve the prediction ability of ANN network (Bas and Boyaci, 2007). The experimental data fed in neural network was categorized into three sets: Training, Testing and Validation. A multi-layer perceptron (MLP) together with back propagation was used to approximate nonlinear function to desired accuracy by changing the number of layers and number of neuron in each layer (Lou and Nakai, 2001). The performance of the network was measured in terms of mean squared error (MSE), which is the difference between output variable and pre specified external desired signal. The optimum number of neurons in hidden layer was determined on the basis of minimum value of MSE. The MLP network architecture designed for analysis of data was categorized in input layer, hidden layer and an output layer. The input layer comprised of neurons for input variables, while the output layer of one neuron represents predicted response. Neurons of successive layers in MLP network are connected to each other by connection weight (W_{ij}) and threshold for activation of these neurons was introduced in term of bias (θ_j). Input data (X_i) was passed through input layer to hidden layer along with the weights. These weighted outputs ($X_i W_{ij}$) were then summed and added to bias term (θ_j) to produce neuron input (I_j) in the output layer according to equation 3.2:

$$I_j = \sum X_i W_{ij} + \theta_j \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (3.2)$$

This neuron input was then passed through an activation function f(I_j) and transformed to output neuron by using sigmoid transform function as shown in equation 3.3:

$$f(I_j) = \frac{1}{1 + e^{-I_j}} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (3.3)$$

The following equation was the outcome of the MLP neural network training, relating the input variables (X_1, X_2, X_3, X_4, X_5 and X_6) to the output variables (y) in terms of weight and biases (Prasanthi et al., 2008).

$$Y = w_2 \times \left(\frac{2}{1+e^{\left(-2 \times (w_1 \times xt^1 + b_1)\right)-1}}\right) + b_2 \ldots\ldots\ldots (3.4)$$

Where, w_1 and w_2=Weights, b_1 and b_2=Bias terms. Y=Predicted L-glutaminase activity, and xt=Row vector of six independent variables, xt^1=Transpose of the vector with a dimension of 3x1

The performance of the ANNs was statistically measured in terms of coefficient of determination (R^2), root mean squared error (RMSE) and the absolute average deviation (AAD) which were determined by using following formulae:

$$RMSE = \left(\frac{1}{n}\sum_{i=1}^{n}(y_i - y_{di})^2\right)^{\frac{1}{2}} \ldots\ldots\ldots\ldots\ldots\ldots\ldots (3.5)$$

$$R^2 = 1 - \frac{\sum_{i=1}^{n}(y_i - y_{di})^2}{\sum_{i=1}^{n}(y_{di} - y_m)^2} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (3.6)$$

$$AAD = \left\{\left[\sum_{i=1}^{n}(|y_i - y_{di}\ |/y_{di})\right]/n\right\} \times 100 \ldots\ldots\ldots (3.7)$$

Where, n =Number of points, y_i =Predicted value, y_{di} =Actual value, and y_m=Average of the actual values.

R^2 is a measure of the amount of the reduction in the variability of response obtained by using the repressor variables in the model. Since R^2 alone is not a measure of the model's accuracy, it is necessary to use RMSE and AAD analysis which are direct method for describing the deviations. Evaluation of R^2, RMSE and AAD values together would be better to check the accuracy of the model. The adequacy of model

would be considered good with values of R^2 close to 1.0 and small values of RMSE and AAD (Bas and Boyaci, 2007; Ebrahimpour et al., 2011).

3.3. Results and Discussion

3.3.1. Selection of potent microbe for production of L-glutaminase

Glutaminases produced from different microbial strains are generally preferred for their application in food industry and pharmaceutical purposes in comparison to those produced from plant and animal sources (Wade et al., 1971; Imada et al., 1973; Nandakumar et al., 2003; Brown et al., 2008). The extracellular nature of L-glutaminase is of much significance for considering its potential in vitro applications. Most of the bacterial strains (Cook et al., 1981; Lebedeva and Berezov, 1997; Harayama and Yasuhira, 1991; Renu and Chandrasekaran, 1992; Keerthi et al., 1999; Klein et al., 2002; Dura et al., 2002; Brown et al., 2008) have been reported to secrete extracellular L-glutaminase. In this study, the production of L-glutaminase from fifteen bacterial strains was estimated and results are summarized in Table3.2. The maximum activity was estimated for L-glutaminase from *Bacillus cereus* MTCC 1305 (425U/l) and hence was selected for further study.

Table3.2. Estimation of L-glutaminase activity among different bacterial strains

Bacterial strains	L-Glutaminase activity (U/l)
Pseudomonas aeruginosa NCIM 2948	220
Pseudomonas putida NCIM 2650	198
Pseudomonas fluorescens NCIM 5096	134
Lactobacillus casei NCIM 2364	000
Lactobacillus plantarum NCIM 2373	170
Bacillus megaterium MTCC2412	248
Bacillus subtilis MTCC1789	023
Bacillus cereus MTCC 1305	425
Bacillus licheniformis MTCC1483	013
Bacillus stearothermophilus NCIM 2235	043
Aeromonas formicans NCIM 2319	116
Streptococcus lactis MTCC 460	013
Streptococcus sp. MTCC 389	000
Staphylococcus aureus MTCC 3160	043
Escherichia coli MTCC 2893	389

3.3.2. Optimization of assay parameters

The activity of L-glutaminase was estimated using modified method of Imada et al. (1973). The activity of L-glutaminase from *Bacillus cereus* MTCC 1305 was estimated as 425U/l after conducting the experiments under these reported assay conditions viz., pH of reaction mixture (7.5), reaction time (30minutes), reaction temperature (37˚C), glutamine's concentration (40mM), and enzyme volume (0.5ml).

Response surface methodology (RSM) was employed to optimize these five significant assay parameters and thirty two experiments were designed using central composite design (CCD) as shown in Table3.3.

Table3.3. Central composite design for five variables with actual experimental and predicted activity of L-glutaminase using RSM and ANN methodologies

Run order	pH	Time (min)	Temp. (˚C)	Glutamine conc. (mM)	Enzyme volume (ml)	Exp activity (U/l)	CCD Predicted activity (U/l)	ANN predicted activity (U/l)
1	5.0	10	50	60	0.8	125	126.712	124.996 TRD
2	7.5	20	35	0	0.5	103	151.242	101.334 TRD
3	10.0	10	50	20	0.8	34	12.712	33.818 TRD
4	7.5	20	35	40	0.5	634	627.364	628.035 TRD
5	5.0	10	20	60	0.2	69	48.879	68.997 TRD
6	10.0	30	20	20	0.8	103	98.879	104.023 TRD
7	7.5	20	35	40	1.1	369	353.909	368.833 TRD
8	7.5	20	35	40	0.5	634	627.364	-
9	5.0	10	20	20	0.8	104	87.212	105.181 TRD
10	5.0	30	50	60	0.2	119	124.712	118.723 TRD
11	5.0	30	20	60	0.8	231	249.879	231.096 TRD
12	7.5	20	35	40	0.5	634	627.364	-
13	7.5	20	65	40	0.5	109	120.242	109.215 TRD
14	5.0	10	50	20	0.2	23	-6.955	23.787 TRD
15	7.5	20	35	40	0.5	634	627.364	-
16	5.0	30	50	20	0.8	189	198.045	188.733 TRD
17	7.5	20	5	40	0.5	64	92.576	62.714 TRD

18	7.5	0	35	40	0.5	267	329.909	266.994 TRD
19	2.5	20	35	40	0.5	34	36.242	32.894 TRD
20	7.5	40	35	40	0.5	538	514.909	538.358 TRD
21	5.0	30	20	20	0.2	28	15.212	29.5497 TRD
22	10.0	30	50	20	0.2	142	124.712	141.995 TRD
23	10.0	30	20	60	0.2	329	321.545	328.572 TTD
24	7.5	20	35	40	-0.1	98	152.909	98.321 TTD
25	7.5	20	35	40	0.5	634	627.364	-
26	10.0	10	20	20	0.2	39	-4.121	38.647 TTD
27	10.0	30	50	60	0.8	487	501.379	486.893 TTD
28	7.5	20	35	40	0.5	634	627.364	-
29	10.0	10	20	60	0.8	403	391.545	403.134 VD
30	7.5	20	35	80	0.5	529	520.576	528.935 VD
31	12.5	20	35	40	0.5	209	246.576	209.002 VD
32	10.0	10	50	60	0.2	263	238.379	263.474 VD

Where TRD=Training Data, TTD=Testing Data, VD=Validation Data

Regression analysis data in Table3.4 showed that all individual variables had linear positive significant effect ($p<0.05$) on L-glutaminase activity but pH of reaction mixture and enzyme volume as individual factors had high coefficient value which indicates their high degree of significant effect on the activity of L-glutaminase. The interaction effect of pH-glutamine concentration and time-temperature also showed linear positive significant effect with $p<0.05$, whereas interaction of other variables were found to be insignificant with $p>0.05$. Among these insignificant interactive variables, the interaction of pH-enzyme volume, temperature-glutamine concentration

and temperature-enzyme volume had negative coefficient value which showed that interaction of these variables decreased the activity of L-glutaminase.

Table3.4. Regression analysis of central composite design showing model coefficients and significance of regression coefficient for glutaminase activity

Term	Coef	SE Coef	Test value (T)	Probability (P)
Constant	-1965.07	189.261	-10.383	0.000
X_1	265.47	24.855	10.681	0.000
X_2	15.83	5.689	2.782	0.018
X_3	39.04	3.897	10.018	0.000
X_4	9.59	2.845	3.371	0.006
X_5	1184.87	185.107	6.401	0.000
$(X_1)^2$	-19.44	1.215	-15.999	0.000
$(X_2)^2$	-0.51	0.076	-6.748	0.000
$(X_3)^2$	-0.58	0.034	-17.151	0.000
$(X_4)^2$	-0.18	0.019	-9.596	0.000
$(X_5)^2$	-1038.76	84.372	-12.312	0.000
X_1 X_2	0.19	0.411	0.462	0.653
X_1 X_3	0.05	0.274	0.170	0.868
X_1 X_4	1.20	0.206	5.860	0.000
X_1 X_5	-13.00	13.709	-0.948	0.363
X_2 X_3	0.17	0.069	2.529	0.028
X_2 X_4	0.01	0.051	0.267	0.794
X_2 X_5	2.50	3.427	0.729	0.481
X_3 X_4	-0.03	0.034	-0.924	0.375
X_3 X_5	-1.22	2.285	-0.535	0.603
X_4 X_5	2.79	1.714	1.629	0.132
R^2 = 98.83% R^2(Predicted) = 70.05% R^2 (adjacent) = 96.70%				

Where, X_1= pH of reaction mixture, X_2=Reaction time, X_3=Reaction temperature, X_4=Glutamine concentration, X_5=Enzyme volume

The statistical testing of the model was done by Fisher's F test and probability (p) value for analysis of variance (ANOVA) and data is shown in Table3.5. A highly significant quadratic regression model was obtained with high value of F and very low value of P. This indicates that the combined effects of all the independent variables significantly contributed to maximize the production of L -glutaminase. Coefficient of determination (R^2) after regression analysis was obtained as 98.83 indicating that the sample variation of only 1.17% of the total variation is not explained by the model. The predicted activity of L-glutaminase (Y) was determined after substituting central values of variables in following second-order regression equation:

$$Y=(-1965.07)+265.47X_1+15.83X_2+39.04X_3+9.59X_4+1184.87X_5-19.44X_1^2-0.51X_2^2-0.58X_3^2-0.18X_4^2-1038.76X_5^2+0.19X_1X_2+0.05X_1X_3+1.20X_1X_4-13X_1X_5+0.17X_2X_3+0.01X_2X_4+2.50X_2X_5-0.03X_3X_4-1.22X_3X_5+2.79X_4X_5 \quad \text{(3.8)}$$

Table3.5. Results of Analysis of Variance (ANOVA) for analyzing model fitness of L-glutaminase activity

Source	DF	Seq SS	Adj SS	Adj MS	F-value	P-value
Regression	20	1570484	1570484	78524	46.43	0.000
Linear	5	384058	316919	63384	37.47	0.000
Square	5	1108160	1108160	221632	131.03	0.000
Interaction	10	78266	78266	7827	4.63	0.009
Residual Error	11	18605	18605	1691		
Lack-of-Fit	6	18605	18605	3101	*	*
Pure Error	5	0	0	0		
Total	31	1589089				

The interactive effect of these variables on L-glutaminase production with prediction of their optimum values was analyzed using contour plots. The contour plots for positive significant effect of interaction of variables like enzyme volume-glutamine concentration, enzyme volume-temperature, enzyme volume-time, enzyme volume-pH, glutamine concentration-temperature, glutamine concentration-time, glutamine concentration-pH, temperature-time, temperature-pH, time-pH on activity of L-glutaminase was shown in Fig 3.3a, 3.3b, 3.3c, 3.3d, 3.3e, 3.3f, 3.3g, 3.3h, 3.3i and 3.3j respectively. These plots showed increase of L-glutaminase activity with simultaneous increase of values of the variables up to an optimum point and decrease of its activity beyond this optimum level. The optimal levels for assay conditions were obtained as pH (7.5), time (20minutes), temperature (35˚C), enzyme volume (0.5ml), glutamine concentration (40mM) and predicted activity was determined as 626.67 U/l by using second order equation.

Fig.3.3a

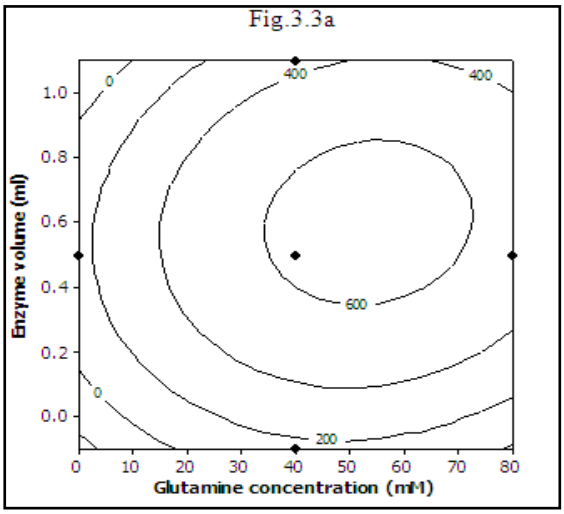


Fig.3.3b

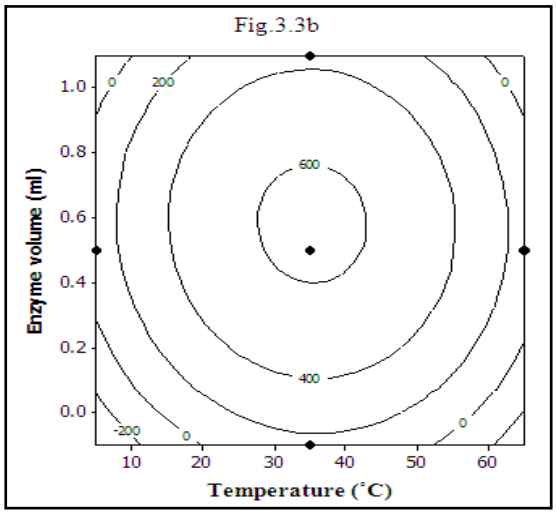


Fig.3.3c

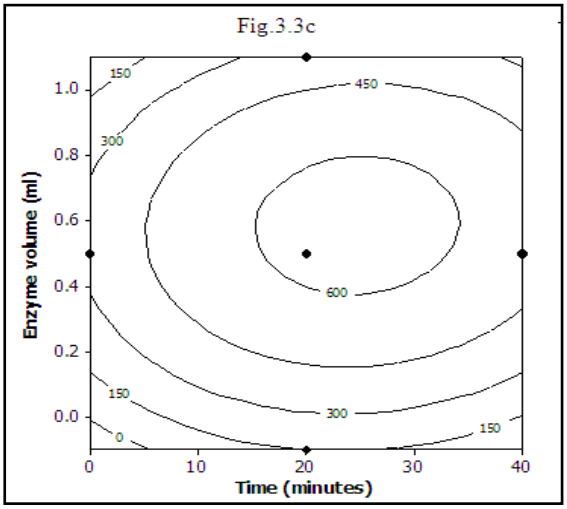


Fig.3.3d

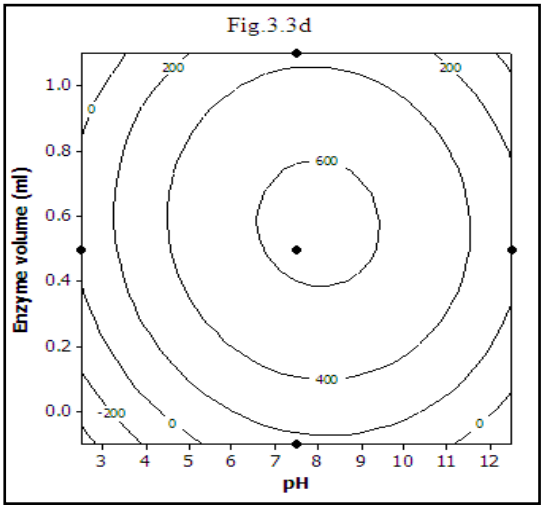


Fig.3.3e

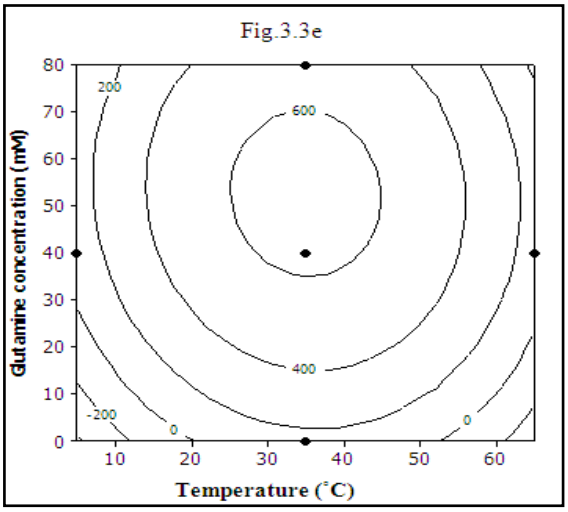
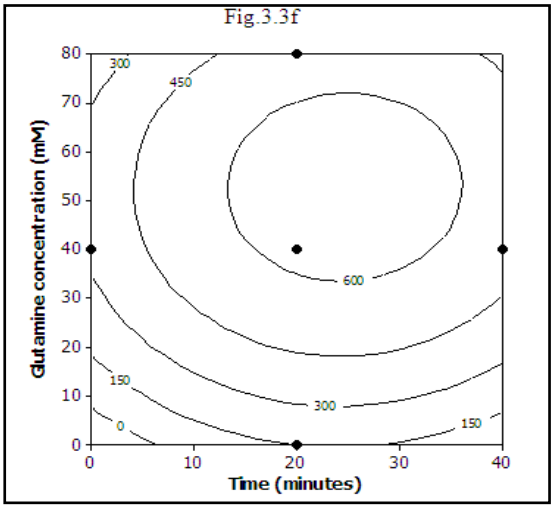


Fig.3.3f



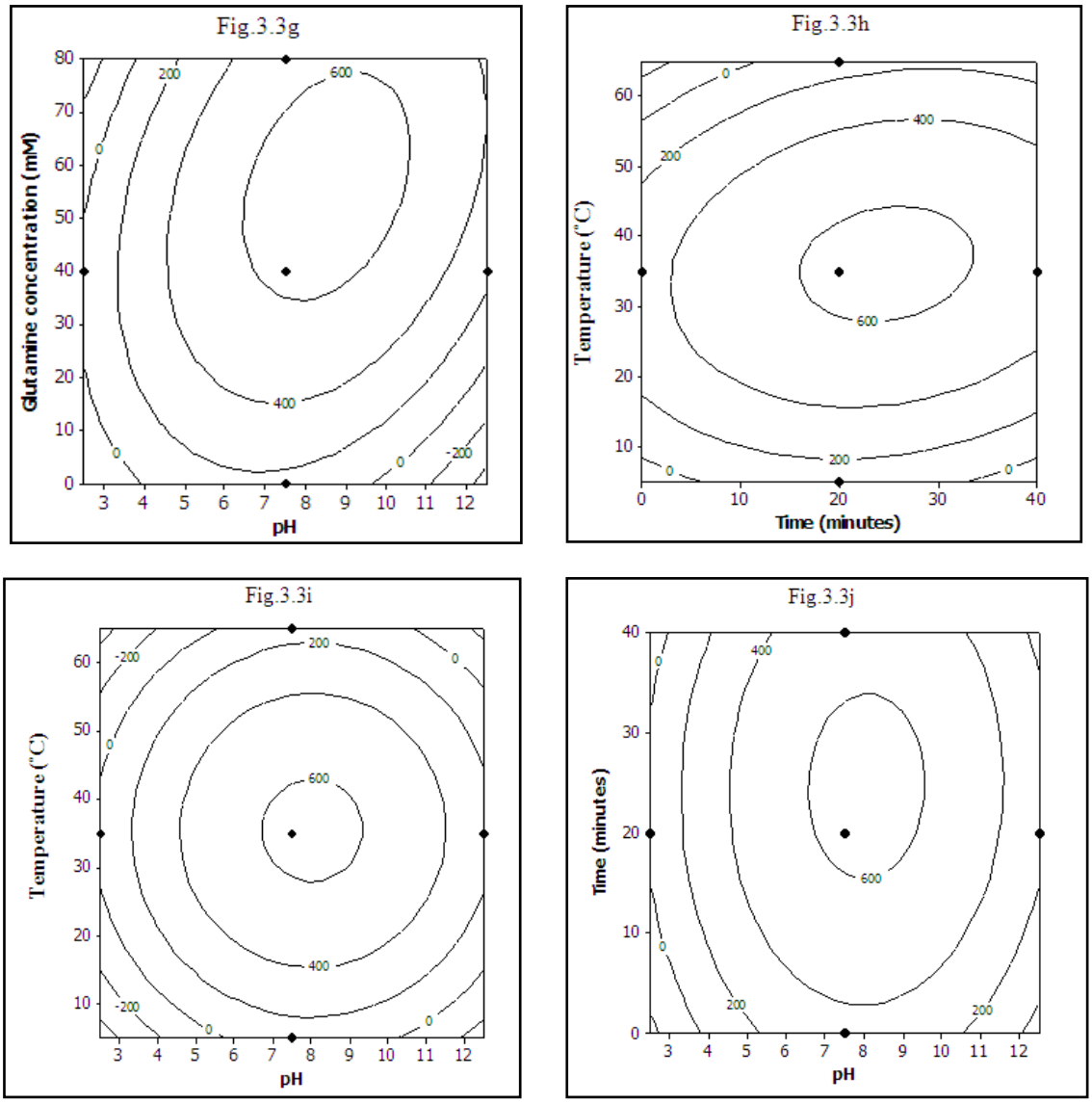


Fig.3.3. Contour plots showing interactive effect of selected variables on activity of L-glutaminase (a) Enzyme volume versus Glutamine concentration; (b) Enzyme volume versus Temperature; (c) Enzyme volume versus Time (d) Enzyme volume versus pH; (e) Glutamine concentration versus Temperature; (f) Glutamine concentration versus Time; (g) Glutamine concentration versus pH; (h) Temperature versus time; (i) Temperature versus pH; (j) Time versus pH

Artificial Neural Network was further applied to analyze response in term of high degree of non linearity between input variables (pH, reaction time, incubation temperature, substrate concentration, and enzyme volume) and output variable (L-glutaminase activity). Multilayer perceptron with Levenberg-Marquardt algorithm and sigmoid transfer function was used to determine predicted activity of L-glutaminase. The minimum value of mean squared errors (MSE) was obtained for three neurons as shown in Fig3.4 and hence was selected as optimum number of neurons in hidden layer for this study. The data of central composite design was passed into the input layer and then was propagated from input layer to hidden layer and finally to output layer of the network. The architecture of MLP network was obtained as "5-3-1" with five input neurons, three neurons in hidden layer and one output neuron as shown in Fig3.5. The predicted activity was determined as 628.035U/l after substituting values of weight on synaptic nodes variance and bias term (Table3.6) in equation 3.4.

Table3.6. Weight and bias values of nonlinear function at optimum assay parameters to estimate glutaminase activity in ANN methodology

Weight on synaptic connection between input and hidden nodes					
pH	Reaction Time	Temperature	L-glutamine concentration	Enzyme volume	Bias term (b_1)
-0.34400	-0.63407	-0.12757	0.10810	3.48091	-0.31476
-0.33867	-0.15598	-0.73089	-0.18507	-10.90464	-0.32896
-0.81767	-1.69041	2.28465	0.95756	3.49753	0.61193
Weight on synaptic connection between hidden and output nodes are -4.8728, -1.09035, Bias term (b_2=9.99078)					

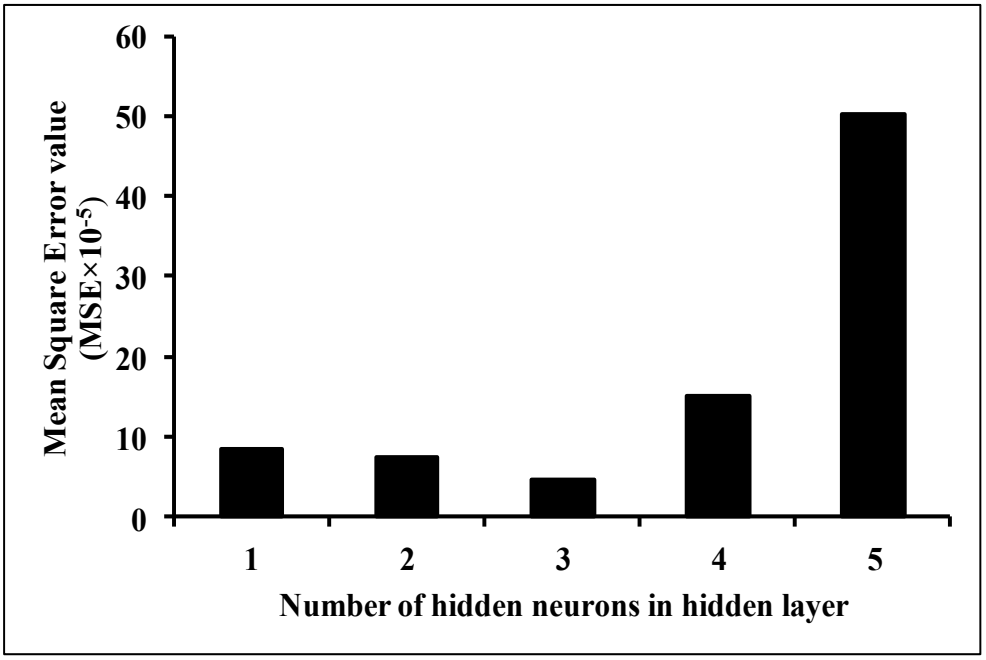


Fig3.4. Determination of number of hidden layer's neurons for artificial neural network designed for optimization of assay parameters for L-glutaminase activity

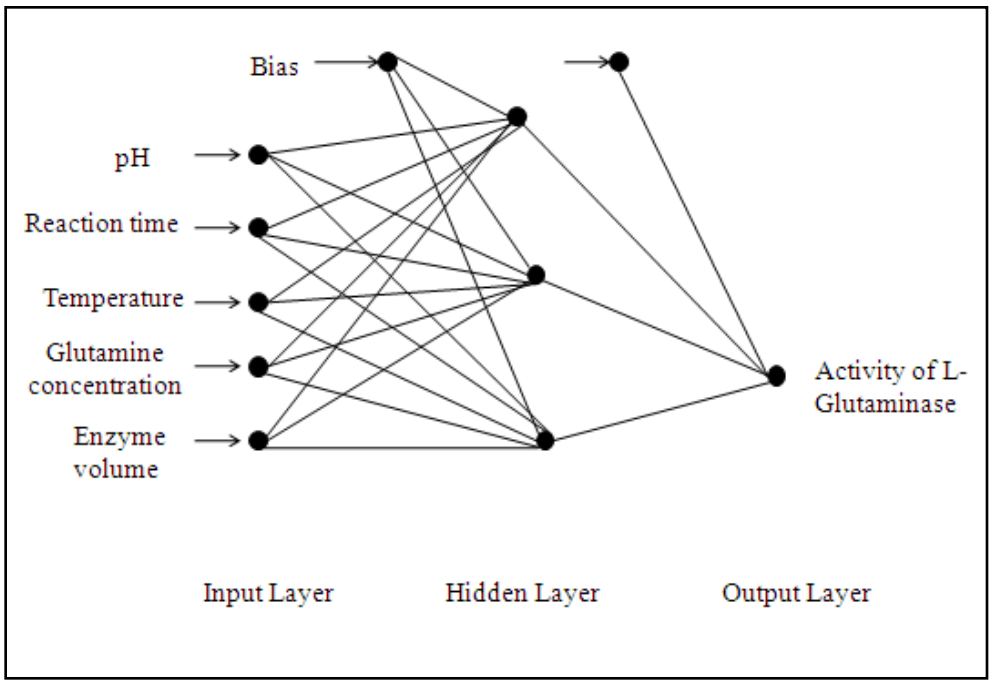


Fig3.5. Multilayer perceptron neural network architecture used for optimization of assay parameters of L-glutaminase activity

The value of coefficient of determination (R^2), root mean squared error (RMSE) and average absolute deviation (AAD) was determined as 0.98172, 24.606, 3.239% respectively for RSM and 0.9999, 0.6697, 0.086 % respectively for ANN. The high value of R^2, low value of RMSE and low value of AAD for ANN showed better predictor of this model in comparison to RSM. The predicted condition was experimentally verified in triplicate by conducting experiment at predicted optimum assay condition viz., pH of reaction mixture (7.5), reaction time (20min), incubation temperature (35˚C), L-glutamine concentration (40mM), and enzyme volume (0.5ml). The activity of L-glutaminase activity was obtained as 628.035U/l after conducting experiments at predicted optimum assay conditions, which was enhanced by 1.485 times than the activity of L-glutaminase obtained under un-optimized assay conditions (423U/l).

3.4. Conclusion

Bacillus cereus MTCC 1305 was selected as potent L-glutaminase producing bacteria. The activity of L-glutaminase was enhanced by 1.485 times after optimizing the reaction conditions, viz., pH of reaction mixture (7.5), reaction time (20minutes), incubation temperature (35˚C), substrate concentration (40mM), and enzyme volume (0.5ml). The statistical tools of RSM and ANN both were employed for optimization of reaction conditions. ANN model was obtained as better predictor than RSM with high value of coefficient of determination, low value of average absolute deviation.