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Neural Network Modeling and Genetic Algorithm Optimization strategy for the production of L-asparaginase from Novel *Enterobacter sp.*

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Abstract

L-asparaginase is the widely used enzyme drug to treat Acute Lymphoblastic Leukemia (ALL). It also finds its applications in food industry to minimize the acrylamide formation in fried foods. The enzyme hydrolyses L-asparagine into Aspartic acid and Ammonia. The sequential modeling and optimization strategy was applied for submerged fermentation to produce L-asparaginase from *Enterobacter aerogenes MTCC 111*using Artificial Neural Networks (ANN) and Genetic Algorithm (GA) respectively. Effects of process variables i.e., incubation time (0-54 hours), pH (6-10), temperature (20-35˚C), substrate concentration (0.5-1.5 % w/v) and inoculum size (0.5-2 % v/v) were examined on enzyme activity. It is observed that process variables had a considerable effect on L-asparaginase production. Neural networks model was developed for the process and predicted values were compared with the values generated from statistical model. Then the process was optimized using Genetic Algorithm. It is found that ANN model prediction (R^2=0.9928) is more accurate than the statistical model prediction (R^2=0.9527). Optimal process variables were found to be incubation time (40h), pH (6), temperature (34˚C), substrate concentration (1.2% w/v) and inoculum size (2% v/v). Maximum enzyme activity was experimentally observed as 20.15 IU/ml which is 3 folds greater than the value before optimization.

Keywords: L-asparaginase; Bioprocess Optimization; Statistical Optimization methods; Artificial Neural Networks; Genetic Algorithm.

INTRODUCTION

L-asparaginase is a hydrolytic enzyme of choice used for *lymphoblastic leukemia* therapy [1]; is also a significant substance in food industry as it can diminish acrylamide formation due to Maillard reaction between the carbonyl group of reducing sugars and free amino acid asparagine. The enzyme drug is widely produced by the flora, fauna and microbes. Microbial cells are superior source of anti-cancer drugdue to ease of culture and convenient downstream processing steps like extraction and purification, facilitating the bulk manufacture of enzyme.

A range of bacteria like *Escherichia coli* [2], *Erwinia carotovora* [3,4], *Erwinia aroideae* [5], *Bacillus sp.* [6], *Zymomonas mobilis* [7], *Pseudomonas aeruginosa* [8], *Thermus thermophiles* [9], *Bacillus aryabhattai ITBHU02* [10], *Bacillus licheniformis* [11], *Enterobacter aerogenes* [12], *Bacillus subtilis B11−06* [13] and *Nocardiopsis alba NIOT-VKMA08* [14] have been found to produce L-asparaginase. Though *E. coli* and *Erwinia sp*. mainly produces L-asparaginase, anaphylactic reactions of *E.coli* L-asparaginase and less half-lifeof *Erwinia* asparaginase than *E. coli* [15] proposing the requirement to find new L-asparaginase that is serologically different but with analogous therapeutic property. Even though microbial production and purification of L-asparaginase are well established, yields of the enzyme have been low [16]. So, screening of physical and nutritional parameters and evaluation is the considerable phase in the progress of bioprocess. In this respect, studying the effect of one variable at a time which is a traditional optimization technique of bioprocess is time consuming, expensive and tedious. In contrast, statistical optimization methods are favored in general because of their advantages [17, 18] and statistical experiments shrink the error in defining the effect of variables in a reasonably priced way [19, 20]. The traditional One-Factor-at-A-Time (OFAT) technique does not reveal the interactions between different factors though a huge number of experiments have been conducted. Response Surface Methodology (RSM) [21] and Taguchi methodology [22] are some of statistical methods through which above said limitation can be overcome and are increasingly being used in process optimizations. RSM reveals the interaction effects among numerous process and response variables that can be quantified, and the tool is a commanding method for testing several factors of bioprocess and offers less number of experimental runs than OFAT method. Sometimes, quadratic polynomial generated in RSM model-building stage is incapable to symbolize a given relationship to the preferred degree of accuracy, so confirming the applicability of RSM to all modeling and optimization studies is difficult [23]. The alternative techniques in this perspective include artificial neural networks (ANNs) and genetic algorithms (GAs). An ANN model mimics the learning aptitude of brain that takes a whole ‘black box’ methodology to model the data. It is capable to model almost all types of nonlinear functions and a past knowledge of the system dynamics is not a requisite [24]. GAs are optimization algorithms which are unorthodox search based and help in the direct search for a elucidation to a problem by imitating part of the process of natural evolution. Through a given set of alternatives GA perform direct random searches to find the finest choice with regard to specified criterion for goodness of fit, which are expressed as a fitness function. Use of ANNs and GAs in biochemical engineering and environmental biotechnology is well established, with applications ranging from pattern recognition in chromatographic spectra, modeling of analytical

biochemistry signals, cancer research, expression profiles, to functional analyses of genomic and proteomic sequences, analyzing changes in soil microbial community composition in response to hydrocarbon pollution and bioremediation etc., [25]. In the present study, to trim down the experimental error a feed forward neural network (FFNN) with error back propagation is applied for non-linear modeling and subsequently GA optimization of L-asparaginase production from *Enterobacter aerogenes MTCC111* is performed.

MATERIALS AND METHODS

Microorganism and its Culture

Enterobacter aerogenes MTCC 111 (KCTC 2190) imported from IMTECH, Chandigarh, India was used in this work and the inoculum was cultured on growth media containing beef extract, 1 g; yeast extract, 2 g; peptone, 5 g; NaCl, 5 g and agar, 15 g in 1 litre of distilled water. The strain was maintained at 4°C with regular sub cultures after every 4 weeks.

Analytical Methods

Activity of crude L-asparaginase enzyme was done by quantifying ammonia development by spectroscopy. Standard Nesslerization technique was employed for assessment of L-asparaginase activity by quantifying the extent of ammonia liberated during L-asparagine hydrolysis spectroometrically at 480 nm (JASCO V 600). One unit (IU) of L-asparaginase activity is defined as the magnitude of enzyme which liberates 1 μmol of ammonia per minute under the typical assay conditions [26].

Experimental Design for Statistical Model

RSM can be used for experimental designing, model building, evaluationof influence of process variables and searching for best possible process conditions for pleasing responses. RSM has been extensively applied for bioprocess optimization. On the basis of previous experimentations among all the variables tested, five (incubation time, pH, temperature, substrate concentration, and inoculum size) were observed to havethe major influence on L-asparaginase activity. The actual levels of coded factors were shown in table 1. Successive optimization on these five factors was done through Full Factorial Central Composite Design (FFCCD) by putting enzyme activity as the response function of interest. The response function was approximated by a second degree polynomial of quadratic using the method of least squares. To find out the curvature and to balance for the lack of fit values experiments at central points were run, which specify the model significance. Incubation time, pH, temperature, substrate concentration and inoculum size were nominated as X_1, X_2, X_3 and X_4, X_5 respectively. The performance of the system was described by the second-degree polynomial as mentionedin equation (1):

$$Y = B_0+\Sigma B_iX_i+\Sigma B_{ii}X_i^2+\Sigma B_{ij}X_iX_j \quad \ldots\ldots(1)$$

Where Y is response; B_0=constant, B_i=linear coefficient, B_{ii}=quadratic coefficients and B_{ij}=second-order interaction. The variable, X_i is the non-coded independent variable. Thus equation 1 now turns into equation (2):

$$Y=B_0+B_1X_1+B_2X_2+B_3X_3+B_4X_4+B_5X_5+B_6X_1^2+B_7X_2^2+B_8X_3^2+B_9X_4^2+B_{10}X_5^2+B_{12}X_1X_2+B_{13}X_1X_3+B_{14}X_1X_4+B_{15}X_1X_5+B_{23}X_2X_3+B_{24}X_2X_4+B_{25}X_2X_5+B_{34}X_3X_4+B_{35}X_3X_5+B_{45}X_4X_5 \quad \ldots.(2)$$

Where Y=predicted response, and X_1, X_2, X_3, X_4 and X_5 are input variables. B_0=constant and B_1, B_2, B_3, B_4 and B_5 are linear coefficients. B_6, B_7, B_8, B_9 and B_{10} are nonlinear coefficients. B_{12}, B_{13}, B_{14}, B_{15}, B_{23}, B_{24}, B_{25}, B_{34}, B_{35} and B_{45} are cross-product coefficients [27].

Modeling using Artificial Neural Networks (ANNs)

ANN models imitate the role of a biological network, made up of neurons and are applied to decipher composite functions in diverse applications. Simple synchronous processing elements are included in NN which are motivated by the biological nerve systems. Neurons are the basic unit of ANN and they are linked to one another by synapses, and a weight factor is allied with every synapse [28]. Back-Propagation (BP) is one of the trendiest algorithms in ANN which is used in this study, with one hidden layer enhanced with numerical optimization technique named Levenberg-Marquardt (LM) [29].

Process Optimization by GA

A theoretical universal search and optimization technique called GA, copies the metaphor of natural biological evolution. GA works on a population of likely solutions implying the principle of survival of the fittest to produce sequentially superior estimations to a solution. A fresh set of estimation is produced at each generation by the process of individual selection as per their fitness level in the domain of problem and their replication using rented operators from natural genetics. This practice directs to the progression of individual populations that better suited for their environment compared to the individuals from which they were created, just as in normal adaptation process.

The GA optimization begins with initialization of the population of solutions P(t). The population size was 20 (4*No. of variables) and the initial population type chosen was double. In every chromosome the evaluation function computes the fitness value; in this study, the error between target output and current output was the fitness function. The choice of the individuals to generate the successive generation has a vital role in GA. The apparent choice begins from each individual's fitness which provides the error between the objective and actual outputs, so that smallest error generating individual has greater chance to be elected. Many methods like Rank selection, Geometric ranking method and Roulette wheel selection etc., are used for the process of the selection and Rank method was opted in the present optimization. Crossover and mutation offer the fundamental search mechanism of a GA. The operators build fresh solutions based on preceding solutions produced. Crossover accepts two individuals and generates two novel recombinant individuals, but mutation alters the individual by arbitrary adjustment in a gene to turn out a fresh solution. Use of these genetic operators and their derivatives depends on chromosome depiction. Scattered option was used as crossover operator and other constraints used for reproduction and mutations are 0.8 crossover rate and constraint dependent mutations function. Other

approximated parameters were forward migration direction, 0.2 migration fraction and 20 as migration interval. The stopping criterion usually advises the upper limit of iterations or verifies if the finest solution attained is acceptable. Values considered for stopping criteria includes maximum number of iterations equals to 500 (100* number of variables), infinite time limit, infinite fitness limit, 50 stall generations, infinite stall time limit, function tolerance and nonlinear constraint tolerance of 10^{-6} [30].

RESULTSAND DISCUSSION

Model Development and Optimization of bioprocess parameters by Full FFCCD

The use of RSM resulted in the quadratic regression equation for Asparaginase activity (Eq.3). A FFCCD with three coded levels for all five factors: Incubation time (X_1), pH (X_2), temperature (X_3), substrate concentration (X_4) and inoculum size (X_5) was used and the input variable levels for central composite design were considered as per the preliminary outcomes. Table2 describes the results attained for enzyme activity through design of experiments. The outcomes of present study illustrated that the final response was reliant on the blend of incubation time, pH, temperature, substrate concentration and inoculum size. The second-order polynomial equation fitted to the experimental data of the CCD (described as coded values) for enzyme activity prediction is given in equation (3).

$$Y=-16.30620-0.088620*X_1+4.66715*X_2+1.50271*X_3+8.04739*X_4-15.85249*X_5-0.00591667*X_1*X_2+0.00541111*X_1*X_3-0.0005*X_1*X_4-0.000333333*X_1*X_5-0.039417*X_2*X_3-0.22625*X_2*X_4-0.37417*X_2*X_5+0.10467*X_3*X_4+0.10444*X_3*X_5-0.04*X_4*X_5-0.000254406*(X_1^2)-0.25931*(X_2^2)-0.026973*(X_3^2)-4.06897*(X_4^2)+6.38713*(X_5^2) \quad \ldots\ldots\ldots(3)$$

Analysis of Variance (ANOVA) was performed to confirm the suitability of the model. A calculated F value of 29.21 for the quadratic regression model suggests that the model is significant. The present analysis attained the CV value of 6.77% that validates a higher consistency of the trials. The R^2 for response of L-asparaginase activity is 0.9527, signifying that the model can elucidate 95.27 % of inconsistency in the response and only 4.73 % of the variations for enzyme activity is not described by it. The value of Adj R^2 for L-asparaginase activity (0. 9201) is also convincing, supporting the significance of the model developed. The values of Prob>F smaller than 0.05 denote that the model terms are significant and in this case X_2, X_3,X_4, X_1X_3, X_2X_3, X_2X_5, X_3X_4, X_3X_5, X_3^2 and X_5^2 were found to be significant.

Developmentof Neural Network Model and Result Analysis

With five inputs and one output using feed forward back propagation network and TRAINLM training function training, testing and validation of NN were carried out. Table 2 describes the results. The outcomes found from the analysis were very pleasing, and an elevated regression value of 0.9928 was attained. The subsequent performance curve was gained on training, testing and validation of the data shown in the **Fig. 1** using MATLAB 2009a. Regression plot showing the output vs. target was attained with ten hidden nodes and 0.9928 regression value of was accomplished which shows the model validation. Table 2 shows the experimental and predicted data from statistical regression and ANN.

Genetic Algorithm based Process Optimization

The nonlinear statistical regression equation obtained from RSM was optimized using GA and the plausible results were described in table 3. Utmost response (enzyme activity) of 20.15 IU/ml was achieved at following optimum process conditions, i.e., incubation time-40 h, pH-6, temperature-34°C, substrate concentration-1.2% and inoculum size of 2%(v/v). At these conditions the predicted maximum enzyme activity is 19.96 IU/ml. **Fig.2** signifies that the incubation time, pH and temperature are showing foremost effect on response. The predicted and experimentally determined enzyme activity by *Enterobacter aerogenes MTCC111* are higher than the activity attained by *Aspergillus terreus, Escherichia coli* and *Pectobacterium carotovorum* [31, 32, 33]. This novel bacterium attained the maximum activity at 34°C unlike the other sources of L-asparaginase resulting greatest activity at higher temperatures [34, 35, 36, 37, 38, 39, 40]. The fermentation time is also very less compared to *Actinomycetales bacterium BkSoiiA* as reported by Chitrangada Dash et al. [41]. The results specify that highest L-asparaginase activity was obtained when incubation time was 40 h, pH was 6, temperature maintained at 34°C, substrate concentration of 1.2% and inoculum size at 2% (v/v). These optimized process parameters were validated by conducting an experiment, and the resulted enzyme activity of 20.15 IU/ml is quite closer to predicted activity of 19.96 IU/ml. Experimental values were compared with predicted responses by RSM and Neural Network (**Table 2** and **Fig. 3**). In the present study, Genetic Algorithm gave more accurate predicted values and the optimized response value compared to Neural Network prediction and RSM optimization **(Table 4)**.

Table 1 Variables Used in Experimental Design

Variables	Codes	Code Levels		
		-1	0	+1
Time(h)	A	10	25	40
pH	B	6	8	10
Temperature (°C)	C	20	27.5	35
Substrate Concentration (%)	D	0.5	1	1.5
Inoculum Size (%)	E	0.5	1.25	2

Table 2 Observed and Predicted Values of L-asparaginase Activity by RSM and ANN

S.No.	Variables					Response (Enzyme Activity IU/ml)		
	A	B	C	D	E	Experimental[a]	RSM Predicted	NN Predicted
1	+1	+1	+1	+1	+1	9.10±0.019	9.96	9.44
2	-1	-1	-1	-1	-1	13.92±0.011	13.66	13.99
3	-1	+1	+1	+1	-1	8.86±0.005	9.19	8.94
4	+1	-1	-1	-1	+1	12.30±0.022	12.68	12.39
5	0	0	0	0	+1	15.50±0.023	15.39	15.40
6	0	+1	0	0	0	8.00±0.011	7.99	8.02
7	+1	+1	+1	-1	-1	9.28±0.009	8.76	9.28
8	0	0	0	0	0	12.16±0.007	11.84	12.17
9	+1	-1	-1	+1	-1	12.68±0.006	13.4	12.79
10	-1	-1	-1	+1	+1	13.44±0.002	14.13	13.24
11	-1	-1	+1	+1	+1	17.28±0.008	17.17	17.27
12	0	0	0	0	0	12.16±0.011	11.84	12.17
13	-1	+1	-1	-1	+1	9.64±0.012	8.81	9.44
14	0	0	+1	0	0	10.00±0.006	10.88	10.05
15	+1	-1	+1	-1	+1	15.78±0.008	16.59	18.18
16	+1	-1	+1	-1	-1	15.22±0.014	14.35	15.20
17	+1	-1	+1	+1	+1	18.20±0.015	18.70	18.20
18	+1	+1	-1	+1	-1	9.98±0.010	9.27	10.13
19	0	0	0	0	0	12.16±0.008	11.84	12.17
20	-1	+1	-1	+1	-1	11.66±0.013	10.86	11.77
21	0	0	0	0	-1	14.72±0.015	15.47	14.60
22	+1	+1	+1	+1	-1	10.04±0.021	10.03	10.07
23	0	0	0	0	0	12.16±0.003	11.84	12.17
24	-1	-1	+1	-1	+1	14.54±0.013	15.04	14.26
25	+1	-1	+1	+1	-1	15.28±0.005	16.52	15.28
26	-1	+1	+1	-1	-1	8.24±0.002	7.90	8.23
27	-1	-1	+1	-1	-1	12.44±0.013	12.79	12.47
28	0	0	-1	0	0	10.00±0.020	9.76	10.03
29	0	0	0	0	0	12.16±0.005	11.84	12.17
30	-1	+1	+1	+1	+1	9.34±0.019	9.14	9.40
31	-1	-1	-1	+1	-1	14.58±0.011	14.29	13.67
32	-1	+1	-1	-1	-1	9.96±0.009	11.15	9.92
33	+1	+1	-1	-1	+1	7.00±0.003	7.21	7.00
34	0	0	0	0	0	12.16±0.006	11.84	12.17
35	-1	-1	-1	-1	+1	14.22±0.006	13.57	14.20
36	-1	+1	+1	-1	+1	8.32±0.002	7.91	8.42
37	+1	-1	-1	+1	+1	13.26±0.007	13.23	13.75
38	0	0	0	0	0	12.16±0.012	11.84	12.17
39	+1	0	0	0	0	12.00±0.004	11.77	12.00
40	0	0	0	+1	0	11.00±0.009	11.28	11.10
41	+1	+1	+1	-1	+1	8.72±0.008	8.75	8.92
42	-1	+1	-1	+1	+1	7.66±0.001	8.46	7.71
43	-1	0	0	0	0	10.92±0.004	11.79	10.90
44	0	0	0	-1	0	10.00±0.003	10.36	10.00
45	0	-1	0	0	0	12.96±0.006	13.62	12.97
46	+1	+1	-1	+1	+1	7.08±0.001	6.85	7.01
47	0	0	0	0	0	12.16±0.004	11.84	12.17
48	+1	+1	-1	-1	-1	8.90±0.008	9.57	8.86
49	+1	-1	-1	-1	-1	13.40±0.005	12.79	13.47
50	-1	-1	+1	+1	-1	15.80±0.002	14.98	15.79

[a] Values are mean±SD.

Table 3 Optimized Process Variables by GA for Maximum L-asparaginase Activity

Time (h)	pH	Temp (^{0}C)	Substrate Concentration (%)	Inoculum Size (%)	Experimental Enzyme Activity (IU/ml)	Predicted Enzyme Activity (IU/ml)
A	B	C	D	E	Y	Y
40	6	34	1.2	2	20.15±0.004	19.96

Table 4 Comparison of RSM and GA optimization for Maximum L-asparaginase Activity

S. No	Method of Optimization	Experimental Enzyme Activity (IU/ml)	Predicted Enzyme Activity (IU/ml)
1	RSM	18.35	18.70
2	GA	20.15	19.96

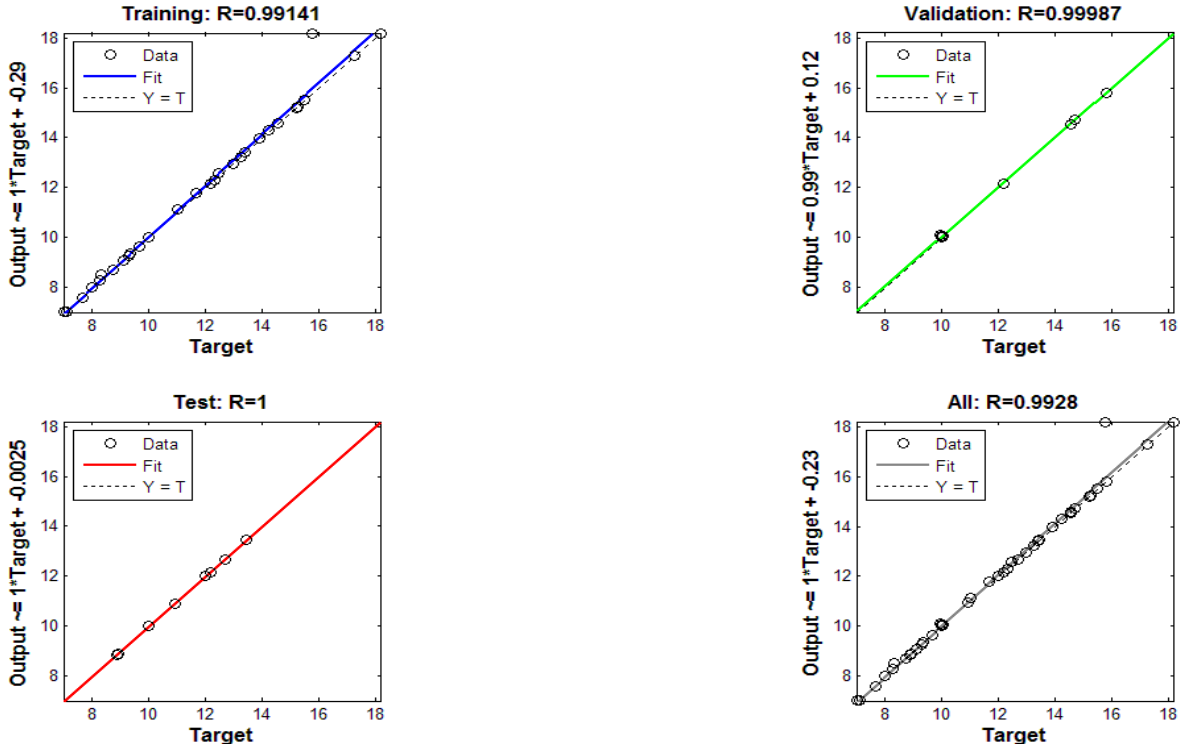

Fig. 1 Output vs. target regression plot

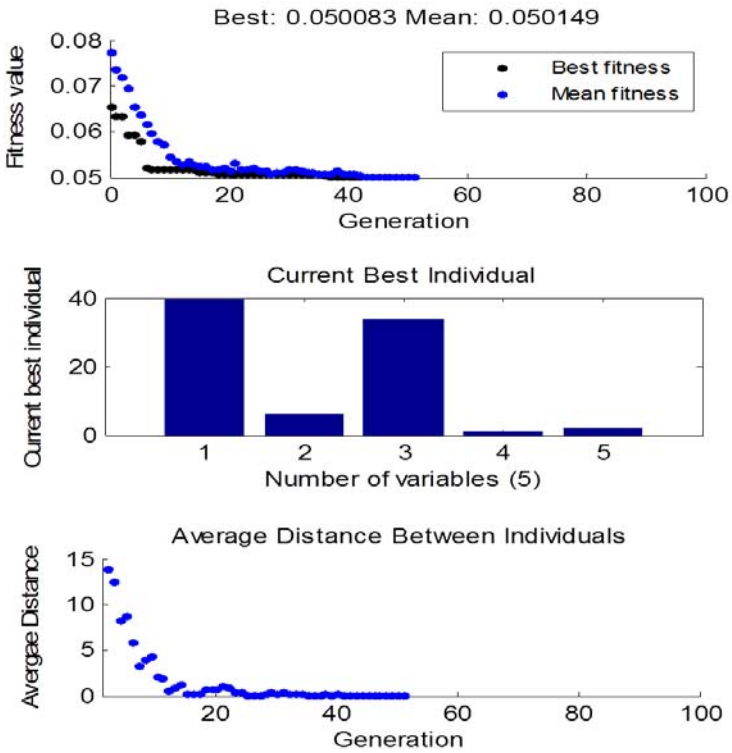

Fig. 2 GA Optimization results

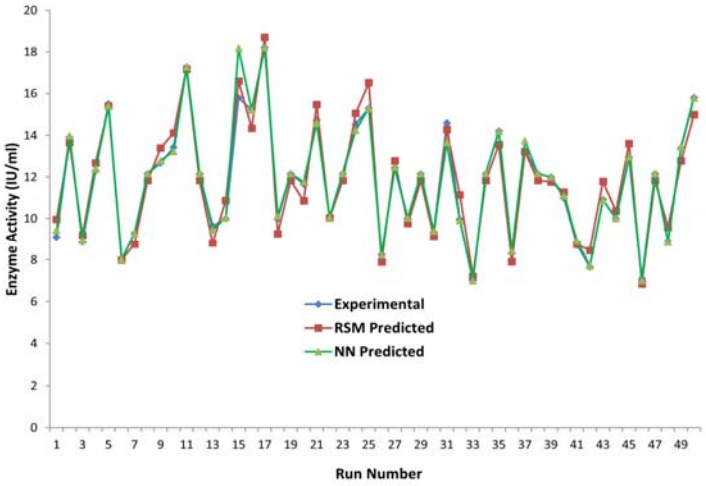

Fig. 3 Observation of predicted and actual values

CONCLUSION

L-asparaginase production was studied in shake flasks using novel *Enterobacter aerogenes MTCC111*. Based on preliminary results five diverse fermentation parameters were considered for further optimization of enzyme production. An effective correlation of 0.9928 was achieved for predicted enzyme activity values using ANN. This experimental study confirmed that ANN prediction is better than statistical regression prediction (RSM). It is observed that a significant increase of L-asparaginase activity by 3 fold after optimization of process variables using GA. Based on GA optimization greatest enzyme production was influenced by the Incubation time, pH and Temperature.

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