J. Microbiol. Biotechnol. (2012), **22**(7), 923–929
http://dx.doi.org/10.4014/jmb.1112.12002
First published online April 1, 2012
pISSN 1017-7825 eISSN 1738-8872

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Optimization of Culture Conditions and Bench-Scale Production of L-Asparaginase by Submerged Fermentation of *Aspergillus terreus* MTCC 1782

Gurunathan, Baskar[1]* and Renganathan Sahadevan[2]

[1]*Department of Biotechnology, St. Joseph's College of Engineering, Chennai - 600 119, India*
[2]*Department of Chemical Engineering, Alagappa College of Technology, Anna University, Chennai - 600 025, India*

Received: December 1, 2011 / Revised: January 8, 2012 / Accepted: February 27, 2012

Optimization of culture conditions for L-asparaginase production by submerged fermentation of *Aspergillus terreus* MTCC 1782 was studied using a 3-level central composite design of response surface methodology and artificial neural network linked genetic algorithm. The artificial neural network linked genetic algorithm was found to be more efficient than response surface methodology. The experimental L-asparaginase activity of 43.29 IU/ml was obtained at the optimum culture conditions of temperature 35°C, initial pH 6.3, inoculum size 1% (v/v), agitation rate 140 rpm, and incubation time 58.5 h of the artificial neural network linked genetic algorithm, which was close to the predicted activity of 44.38 IU/ml. Characteristics of L-asparaginase production by *A. terreus* MTCC 1782 were studied in a 3 L bench-scale bioreactor.

Keywords: *Aspergillus terreus*, asparaginase, response surface methodology, artificial neural network, bench-scale bioreactor

L-Asparaginase catalyzes the deamidation of L-asparagine to L-aspartic acid and ammonia. Tsuji [24] in 1957 reported the deamidation of L-asparagine by extracts of *E. coli*. The regression of lymphosarcoma transplants in mice treated with guinea-pig serum has proved the nutritional dependence of the malignant cells on exogenous L-asparagine and provided evidence that L-asparaginase in the serum is the antitumor factor [5]. L-Asparaginase is used as a chemotherapeutic agent for acute lymphocytic leukemia and less frequently for acute myeloblastic leukemia [13]. Bacterial L-asparaginase causes hypersensitivity in the long-term use and leads to allergic reactions. Eukaryotic microorganisms like yeast and filamentous fungi have been reported to produce a substantial amount of extracellular L-asparaginase with less adverse effects [1, 19].

L-Asparaginase produced by fungal sources, such as *Aspergillus nidulans* and *A. terreus* isolated from decomposing vegetables, was not toxic and appeared to have immunosuppressive activity [20]. *A. tamari* [19], *A. terreus* [2], *A. niger* using agro-wastes from three leguminous crops [15], *Aspergillus* sp. [22], and *Bipolaris* sp. BR438 [12] were also reported to produce L-asparaginase. L-Asparaginase demand will increase several fold in the coming years owing to its potential applications in food industries besides its clinical applications. L-Asparaginase cleaves the L-asparagine in fresh starchy foods and ultimately reduces the levels of acrylamide formation in fried potato chips and french fries [10, 17].

Production of L-asparaginase is greatly influenced by fermentation media composition and culture condition factors such as temperature, pH, inoculum size, agitation rate, and incubation time [9, 25]. Although many researchers have reported on optimization of carbon and nitrogen sources for L-asparaginase production, very less research work is found on the influence of culture conditions [11]. Hence, the present work was focused on optimization of culture conditions, namely temperature, initial pH, inoculum size, agitation rate, and incubation time, using RSM and ANN linked GA and L-asparaginase production by submerged batch fermentation of *A. terreus* MTCC 1782 using a 3 L bench-scale bioreactor.

Materials and Methods

Microorganism

The filamentous fungus *Aspergillus terreus* MTCC 1782 used in the present study was obtained from Institute of Microbial Technology, Chandigarh, India.

*Corresponding author
Phone: +91 9443678571;
E-mail: basg2004@gmail.com

Reagents

L-Asparagine and L-proline were purchased from HiMedia Laboratories Pvt Ltd., Mumbai, India. All other chemicals were of analytical grade and purchased from Qualigens Fine Chemicals, Mumbai, India.

Stock and Inoculum Culture Conditions

A. terreus MTCC 1782 was grown in Czapek–Dox agar slants at 37°C for 4 days. Czapek–Dox agar medium contains 50 ml of solution A, 50 ml of solution B, 1 ml of solution C, 1 ml of solution D, 900 ml of distilled water, 30 g of glucose, and 20 g of agar. Solution A: 10.0 g L-asparagine, 40.0 g $NaNO_3$, 10.0 g KCl, 0.52 g $MgSO_4 \cdot 7H_2O$, and 0.2 g $FeSO_4 \cdot 7H_2O$ were dissolved in 1 L of distilled water and refrigerated. Solution B: 20.0 g K_2HPO_4 was dissolved in 1 L of distilled water and refrigerated. Solution C: 1.0 g $ZnSO_4 \cdot 7H_2O$ was dissolved in 100 ml of distilled water. Solution D: 0.5 g $CuSO_4.H_2O$ was dissolved in 100 ml of distilled water. Spores were harvested with spore count of 2×10^7 to 10^8 per ml of spore suspension.

Production Culture for Optimization of Culture Conditions

The submerged batch fermentation for optimization of culture conditions was conducted in 250 ml Erlenmeyer flasks with 50 ml of modified Czapek–Dox medium with the following composition: 1.7% (w/v) L-proline, 1.99% $NaNO_3$, 1.38% L-asparagine, 0.65% glucose, 0.0152% K_2HPO_4, 0.052% $MgSO_4 \cdot 7H_2O$, 0.052% KCl, 0.001% $ZnSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, and 0.001% $CuSO_4 \cdot H_2O$ [3]. The initial pH of the medium was adjusted, kept in an orbital shaker, inoculated using the spore suspension with spore count of 2×10^7 to 10^8 per ml, and incubated at a temperature as per the CCD given in Table 1. Culture samples were filtered through Whattman #2 filter paper and the cell-free filtrate was used as crude enzyme solution.

Optimization of Media Components Using Response Surface Methodology

RSM is an efficient statistical technique for optimization of multiple variables in order to predict the best performance conditions with a minimum number of experiments. CCD is a type of response surface methodology, usually utilized to obtain data that fit a full second-order polynomial model. Input variables are tested at three coded levels as −1, 0, and +1 for low, middle, and high value, respectively. Experimental response was analyzed to find the optimum level of the variables. The effect of the input variables on response was fit into a second-order polynomial model according to Eq. (1).

$$Y = b_0 + \sum b_i X_i + \sum b_{ii} X_i^2 + \sum b_{ij} X_i X_j \quad (1)$$

where Y is the response variable, X_i and X_j are independent variables in coded units, and b_i, b_{ii}, b_{ij} are the measures of the X_i, X_j, X_i^2, and X_iX_j of linear, quadratic, and interaction effects, respectively [9, 18]. The second-order polynomial model was solved by a constraint search procedure using the MATLAB 7.0 program for maximum L-asparaginase activity to find the optimal levels of the variables.

Optimization of Media Components Using Artificial Neural Network Linked Genetic Algorithm

The development of an accurate model is still a critical challenge in microbial bioprocesses mainly due to the nonlinear nature of the biochemical interactions. Artificial Neural Networks (ANNs) are nonlinear statistical data modeling tools, mimic the different aspects of biological information processing, and proved to be useful in media optimization for fermentation processes [6, 8, 16]. ANN is capable of predicting the output when any input similar to the pattern that it has learnt is fed. The ANN with five neurons in input layer, four in hidden layer, and one in output layer with "Tanh" as the neuron activation function was used in the present study. A multilayer feed-forward ANN was used to train and evaluate the system performance by adaptive gradient learning rule using Neural power 2.5. The thump-rules considered while selecting number of neurons in hidden layer are (i) number of hidden neurons should be between the size of the input layer and the size of the output layer, and (ii) the number of hidden neurons should be 2/3 the size of the input layer plus the size of the output layer. The "Tanh" returns the hyperbolic tangent of sum of the input patterns and connection weights [7, 8]. Genetic algorithms (GAs) are global optimization techniques that optimize the complex models developed by ANN. GAs are general purpose search algorithms inspired by Charles Darwin's principle of the "survival of the fittest" to solve complex optimization problems. GA starts with an initial population of randomly generated chromosomes. During successive iterations, the initial chromosomes advance towards stronger chromosomes by reproduction among members of the previous generation. New generations are created by three genetic operators such as selection, crossover, and mutation. Selection of the best chromosomes makes sure that only the best chromosomes can crossover or mutate by rating the individual chromosomes by their adaptation [4, 21]. Once the ANN model was developed, GA was used to optimize the media components for maximum L-asparaginase activity.

L-Asparaginase Production in a Bench-Scale Bioreactor

The submerged batch fermentation of *A. terreus* for L-asparaginase production was conducted in a 5 L bench-scale bioreactor (Scigenics Pvt. Ltd. Chennai, India) with working volume of 3 L, using modified Czapek–Dox media as mentioned above. The initial pH of the medium was adjusted to 6.2 and inoculated by 2% of 16 h grown pre-inoculum culture prepared in modified Czapek–Dox media using 1% (v/v) spore inoculum with spore count of 2×10^7 to 10^8 per ml. The bioreactor was operated at 35°C with an aeration rate of 0.4 vvm and an agitation rate varying from 140 to 180 rpm. Samples were collected periodically and filtered through preweighed Whatman #2 filter paper. Culture filtrates were used as crude enzyme solution for estimation of L-asparaginase activity and residual glucose concentration.

Assay of L-Asparaginase Activity

Nesslarization is the most commonly used method for estimation of L-asparaginase activity. The quantity of ammonia formed during the hydrolysis of 0.04 M L-asparagine by crude enzyme solution was estimated using Nessler's Reagent in spectrometric analysis at 480 nm. One unit (IU) of L-asparaginase activity is defined as the amount of enzyme that liberates 1 μM of ammonia per minute under the standard assay [26].

Estimation of Biomass and Residual Glucose

The fungal growth was measured in terms of biomass concentration using the dry cell weight (DCW) method. The culture sample of 10 ml was filtered through preweighed Whatman #2 filter paper and dried at 80°C until it reached a constant dry weight. The difference

Table 1. Randomized CCD with experimental, RSM, and ANN predicted L-asparaginase activity.

Run order	X_1 °C	X_2	X_3 % (v/v)	X_4 rpm	X_5 H	L-Asparaginase activity, IU/ml		
						Experimental	RSM Predicted	ANN Predicted
1	38	6.3	2	150	72	33.48	34.58	33.38
2	33	6.3	2	150	96	35.41	38.01	35.29
3	28	5.8	3	120	48	21.17	20.06	21.35
4	33	6.3	3	150	72	31.57	38.52	31.56
5	28	6.8	3	180	48	18.93	18.08	18.78
6	33	6.8	2	150	72	26.72	30.51	26.80
7	28	6.8	3	120	96	19.46	17.89	19.24
8	38	6.8	3	120	48	21.86	20.67	21.85
9	28	6.8	1	120	48	19.36	19.04	19.63
10	28	6.8	1	180	96	17.27	17.52	16.99
11	33	6.3	2	120	72	34.71	39.53	34.70
12	33	6.3	2	150	72	43.99	40.36	43.53
13	28	5.8	1	180	48	20.21	20.92	20.16
14	38	6.8	1	120	96	27.88	27.78	27.99
15	28	5.8	1	120	96	16.26	16.25	16.42
16	28	5.8	3	180	96	21.86	21.31	21.86
17	33	6.3	2	180	72	39.67	39.39	39.64
18	33	6.3	2	150	72	43.51	40.35	43.54
19	33	6.3	2	150	48	38.50	40.43	38.35
20	28	6.3	2	150	72	27.99	31.43	28.04
21	33	6.3	1	150	72	42.50	40.07	42.37
22	33	5.8	2	150	72	30.55	31.29	30.37
23	33	6.3	2	150	72	42.71	40.35	43.57
24	38	5.8	3	120	96	17.97	17.07	14.74
25	38	5.8	1	180	96	18.87	19.79	19.04
26	38	6.8	1	180	48	22.55	23.17	22.55
27	38	5.8	1	120	48	25.06	25.43	25.01
28	38	6.8	3	180	96	17.01	16.37	17.23
29	33	6.3	2	150	72	42.97	40.35	43.54
30	33	6.3	2	150	72	43.99	40.35	43.52
31	33	6.3	2	150	72	43.08	40.35	43.53
32	38	5.8	3	180	48	26.13	25.95	26.13

in filter paper weight was reported as DCW [23]. The residual glucose concentration in the culture filtrate was analyzed using the dinitrosalicylic acid method [14].

RESULTS AND DISCUSSION

Statistical Optimization of Culture Conditions Using Response Surface Methodology

The experimental L-asparaginase activity reported in Table 1 was statistically analyzed using Student's t-test and Fisher's F-test at 95% confidence level (p=0.05). The coefficients, t-value, and p-value for linear, quadratic, and interaction effects of the variables are given in Table 2, at 95% significance level. It was observed that the overall effect of the variables on L-asparaginase production was highly significant ($p<0.001$). Significant quadratic effect ($p<0.05$) was observed for pH on L-asparaginase production. Insignificant quadratic effect ($p>0.05$) was observed for all other variables. The independent and interactive effects of all the variables on L-asparaginase production were also found to be insignificant. Contour plots were used to study the general shape of the response surface fitted by the regression model. The interactive effect between any two variables on L-asparaginase activity was illustrated graphically in the contour plot shown in Fig. 1, with other variables kept constant at their middle level. The regular and near-parallel lines of the response surface curves among all the variables indicate that there was less or no interactive effect of the variables on L-asparaginase production.

Statistical testing of the regression model was done in the form of analysis of variance (ANOVA), which is required to test the significance and adequacy of the model. The

Table 2. Estimated regression coefficients for optimization of L-asparaginase activity using RSM.

Variable	Estimated coefficients	t-Value	p-Value
Constant	40.358	34.762	<0.001
X_1	1.573	1.643	0.129
X_2	-0.391	-0.408	0.691
X_3	-0.779	-0.814	0.433
X_4	-0.068	-0.071	0.945
X_5	-1.208	-1.262	0.233
X_1*X_1	-7.349	-2.838	0.016
X_2*X_2	-9.456	-3.651	0.004
X_3*X_3	-1.057	-0.408	0.691
X_4*X_4	-0.897	-0.347	0.735
X_5*X_5	-1.137	-0.439	0.669
X_1*X_2	0.359	0.354	0.354
X_1*X_3	-1.233	-1.214	0.250
X_1*X_4	-0.639	-0.630	0.630
X_1*X_5	-0.566	-0.558	0.588
X_2*X_3	-1.033	-1.017	0.331
X_2*X_4	-1.213	-1.194	0.257
X_2*X_5	1.033	1.017	0.331
X_3*X_4	0.819	0.807	0.437
X_3*X_5	-0.306	-0.302	0.768
X_4*X_5	-0.433	-0.427	0.678

ANOVA results of the RSM regression model is given in Table 3, at 95% confidence level. The ANOVA of the RSM regression model demonstrates that the model was highly significant ($p<0.001$). Hence, the RSM regression model given in Eq. (2) is the good prediction of the experimental results. The high value of coefficient of determination ($R^2 = 0.939$) implies a high degree of correlation between the experimental and predicted L-asparaginase activity. Hence, the RSM regression model was fitted well to represent the effect of the operating conditions on L-asparaginase production using the central composite design.

$$Y_{LA} = 40.36 + 1.57X_1 - 0.39X_2 - 0.78X_3 - 0.07X_4 - 1.21X_5 - 7.35X_1^2 - 9.45X_2^2 - 1.06X_3^2 - 0.89X_4^2 - 1.14X_5^2 - 0.36X_1X_2 - 1.23X_1X_3 - 0.64X_1X_4 - 0.57X_1X_5 - 1.03X_2X_3 - 1.21X_2X_4 + 1.03X_2X_5 + 0.82X_3X_4 - 0.31X_3X_5 - 0.43X_4X_5 \quad (2)$$

where Y_{LA} is the L-asparaginase activity (IU/ml), and X_i and X_j are independent variables in coded units. Optimum culture conditions were found by solving the regression model [Eq. (2)] for maximum L-asparaginase production. Temperature of 33.85°C, pH of 6.29, inoculum size of 1.52% (v/v), agitation rate of 144 rpm, and incubation time of 60.61 h were obtained as the optimum values with a maximum predicted L-asparaginase activity of 40.97 IU/ml.

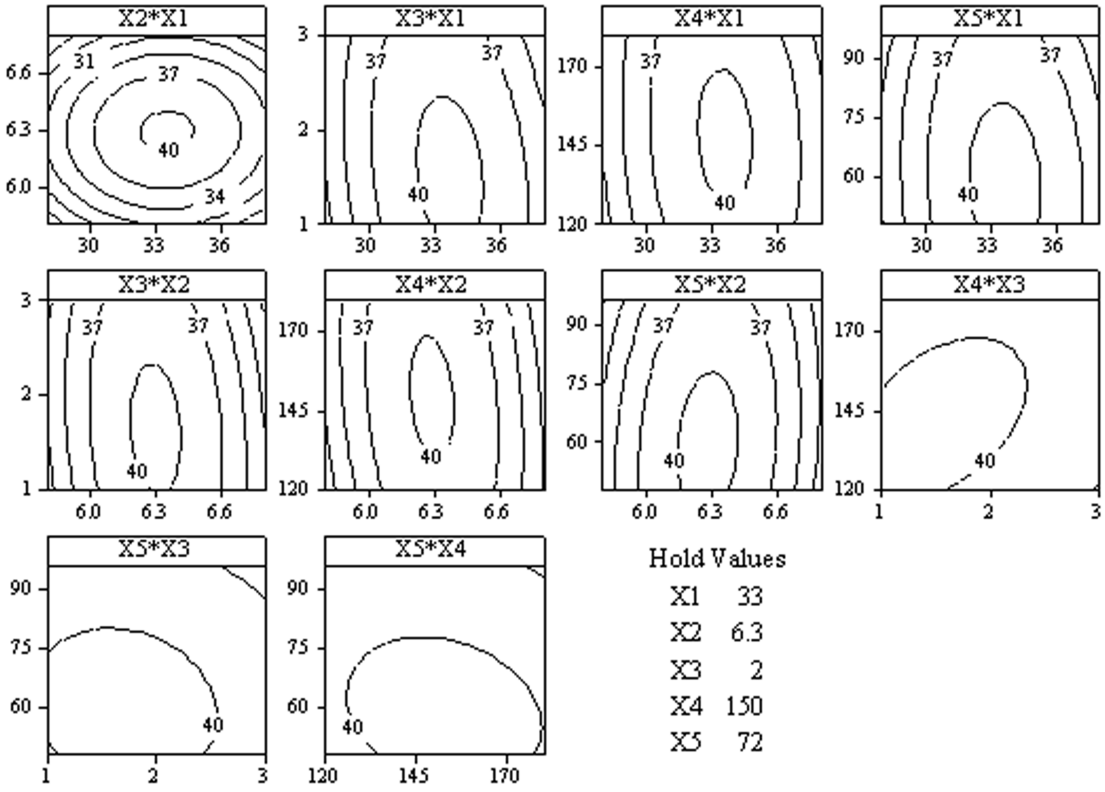

Fig. 1. Interaction effect of variables on L-asparaginase production in contour plot (X_1, Temperature, °C; X_2, initial pH; X_3, Inoculum size, % (v/v); X_4, Agitation rate, rpm; X_5, Incubation time, h).

Table 3. Analysis of variance for optimization of L-asparaginase activity using RSM.

Source	Degree of freedom	Sum of Squares (SS)	Mean Square (MS)	F-Value	p-Value
Regression	20	2,815.03	140.751	8.53	<0.001
Linear	5	84.60	16.921	1.03	0.449
Quadratic	5	2,619.37	523.873	31.74	<0.001
Interaction	10	111.06	11.106	0.67	0.730
Residual error	11	181.54	16.504		
Pure error	5	1.46	0.292		
Total	31	2,996.57			

Table 4. Optimal values of the independent variables, experimental, and RSM and ANN predicted L-asparaginase activity.

Method	Optimum conditions					L-Asparaginase activity, IU/ml		Cal.R^2-value	Pred.R^2-value
	X_1,°C	X_2	X_3,%	X_4, rpm	X_5, h	Predicted	Experimental		
RSM model	33.85	6.29	1.52	144.24	60.61	40.97	40.56	0.939	0.935
ANN model	35.06	6.25	1.00	140.18	58.45	44.38	43.29	0.999	0.995

Evolutionary Optimization of Culture Conditions Using Artificial Neural Network Linked Genetic Algorithm

The experimental L-asparaginase activity given in Table 1 was used to train the ANN to understand the dependence of L-asparaginase production on operating conditions such as temperature, pH, inoculum size, agitation rate, and incubation time, using neural power software. A multilayer feed-forward ANN using incremental back propagation algorithm and adaptive gradient learning rule with learning rate of 0.8 and momentum of 0.8 using was used. The ANN was trained using randomly selected 25 experimental runs in Table 1, and ANN performance was tested using 7 other experimental runs. Then ANN was used to predict the L-asparaginase activity for all data sets on operating conditions in Table 1. The high coefficient of determination ($R^2 = 0.999$) for L-asparaginase activity predicted by the ANN model indicates that it was highly accurate in successful prediction of L-asparaginase activity. Hence, the ANN can be adequately used to represent the relationship between the operating conditions and L-asparaginase activity. The ANN predicted L-asparaginase activity is given in Table 1. Although both RSM and ANN provided accurate predictions, ANN showed better and accurate correlation with the experimental L-asparaginase activity than RSM.

GA, on the other hand, is a commonly used global optimization technique that optimizes the given ANN over a particular range, and is based on the evolutionary methods of natural selection of the best individuals in a population [7, 21]. GA with a population size of 30, mutation rate of 0.1, and uniform cross-overrate of 0.8 was run for 94,501 iterations to find the predicted optimum operating conditions for maximum L-asparaginase activity. The percentage importance of the operating conditions on L-asparaginase production was found to be Temperature 25.28%, initial pH 24.81%, agitation rate 18.87%, incubation time 18.1%, and inoculum size 13.15%, as shown in Fig. 2. It was predicted that a temperature of 35°C, pH of 6.25, inoculum size of 1%, agitation rate of 140.18 rpm, and incubation time of 58.45 h favors the maximum production of this enzyme. The maximum predicted L-asparaginase production at ANN predicted condition was 44.38 IU/ml (Table 4). Fig. 3 shows the closeness of the experimental L-asparaginase activity with the RSM and ANN predicted L-asparaginase activity. The higher predicted R^2-value of 0.995 for ANN than RSM ($R^2 = 0.935$) indicates the high degree of accuracy of the ANN. Hence, Fig. 3 shows a good agreement between experimental and ANN predicted L-asparaginase activity.

Experimental Confirmation of Predicted Culture Conditions

The confirmation experiment was conducted in triplicate at predicted optimum operating conditions of RSM and ANN linked GA for validation. All other fermentation conditions and medium composition were fixed as CCD experiment for optimization. The experimental L-asparaginase activity

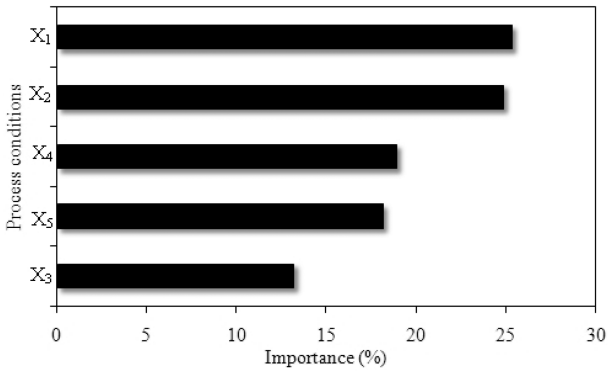

Fig. 2. Effect of operating conditions on L-asparaginase production.

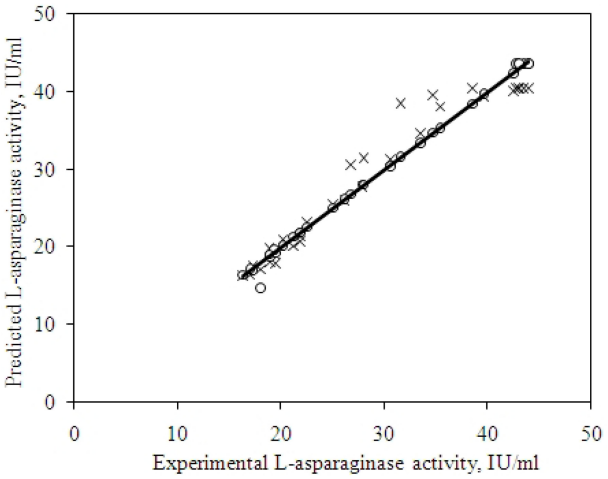

Fig. 3. Predicted distribution coefficient of RSM and ANN predicted L-asparaginase activity (×, RSM predicted; ○, ANN predicted).

of 40.56 IU/ml was obtained at the predicted optimal conditions of RSM regression model (Table 4). The experimental L-asparaginase activity of 43.29 IU/ml was obtained at the predicted optimal conditions of ANN linked GA, which was higher than RSM and other reports in the literature [9, 11, 12, 22]. The optimization operating conditions for L-asparaginase production using ANN linked GA was found to be more accurate.

Bench-Scale Production of L-Asparaginase

The profile of L-asparaginase activity, biomass, and residual glucose concentration in time course of fermentation was studied. L-Asparaginase production starts to increase significantly after the eighth hour of fermentation, when the growth of the microorganism reaches the mid-exponential phase. The maximum L-asparaginase activity of 44.58 IU/ml was obtained in the post-exponential growth phase and remained constant after 72 h. The biomass yield coefficient was found to be 0.97 ($Y_{X/S}$) g of biomass/g of glucose and the product yield coefficients were found to be 8,770 ($Y_{P/S}$) IU/g of glucose and 8,916 ($Y_{P/X}$) IU/g of biomass. The rate of L-asparaginase formation and growth were high in the exponential phase, low in the post-exponential phase, and constant in the stationary phase. The glucose utilization rate was low in the early growth phase until 8 h and high in the exponential phase until 48 h of fermentation, and it was low in the late exponential phase (after 56 to 64 h) and very low in the stationary phase (after 64 h). Almost 94% of glucose was depleted in 56 h of fermentation. The exponential phase of the fungal growth was observed from 8 to 48 h. The biomass concentration reached a maximum of 5.29 g/l in 64 h and there was no further increase in biomass until 72 h. Later, the biomass concentration decreased slowly. L-Asparaginase production was noted in the exponential growth and early stationary phases. Fungal L-asparaginase production characteristics in a bench-scale fermentor evident the possible industrial-scale production.

References

1. Ali, S. S., V. Rai, K. Soni, P. Kulshrestha, and S. K. Lai. 1994. A fungal L-asparaginase with potential antitumor activity. *Ind. J. Microbiol.* **34:** 73–76.
2. Baskar, G. and S. Renganathan. 2009. Production of L-asparaginase from natural substrates by *Aspergillus terreus* MTCC 1782: Effect of substrate, supplementary nitrogen source and L-asparagine. *Int. J. Chem. React. Eng.* **7:** A41.
3. Baskar, G. and S. Renganathan. 2010. Optimization of L-asparaginase production by *Aspergillus terreus* MTCC 1782 using response surface methodology and artificial neural network linked genetic algorithm. *Asia Pac. J. Chem. Eng.* DOI: 10.1002/apj.520.
4. Baskar, G. and S. Renganathan. 2011. Design of experiments and artificial neural network linked genetic algorithm for modeling and optimization of L-asparaginase production by *Aspergillus terreus* MTCC 1782. *Biotechnol. Bioproc. Eng.* **16:** 50–58.
5. Broome, J. D. 1963. Evidence that the L-asparaginase of guinea pig serum is responsible for its antilymphoma effects. I. Properties of the L-asparaginase of guinea pig serum in relation to those of the antilymphoma substance. *J. Exp. Med.* **118:** 99–120.
6. Ebrahimpour, A., R. Rahman, D. Ean Chng, M. Basri, and A. Salleh. 2008. A modeling study by response surface methodology and artificial neural network on culture parameters optimization for thermostable lipase production from a newly isolated thermophilic *Geobacillus* sp. strain ARM. *BMC Biotechnol.* **8:** 96.
7. Goldberg, D. 1989. *Genetic Algorithms in Search, Optimization, and Machine Learning*. Addison-Wesley.
8. Holland, J. 1975. *Adaptation in Natural and Artificial Systems*. University of Michigan Press, Ann Arbor.
9. Hymavathi, M., T. Sathish, Ch. Subba Rao, and R. S. Prakasham. 2009. Enhancement of L-asparaginase production by isolated *Bacillus circulans* (MTCC 8574) using response surface methodology. *Appl. Biochem. Biotechnol*. **159:** 191–198.
10. JECFA (Joint FAO/WHO Expert Committee on Food Additives). 2001. Compendium of food additive specifications. General specifications and considerations for enzyme preparations used in food processing. *FAO Food Nutr.* **52:** 37–39.
11. Kumar, S., V. V. Dasu, and K. Pakshirajan. 2010. Localization and production of novel L-asparaginase from *Pectobacterium carotovorum* MTCC 1428. *Process Biochem*. **45:** 223–229.
12. Lapmak, K., S. Lumyong, S. Thongkuntha, P. Wongputtisin, and U. Sardsud. 2010. L-Asparaginase production by *Bipolaris* sp. BR438 isolated from brown rice in Thailand. *Chiang Mai J. Sci*. **37:** 160–164.
13. Mashburn, L. and J. C. Jr. Wriston. 1964. Tumor inhibitory effect of L-asparaginase from *Escherichia coli*. *Arch. Biochem. Biophys*. **105:** 450–452.
14. Miller, G. L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem*. **31:** 426–428.

15. Mishra, A. 2006. Production of L-asparaginase, an anticancer agent, from *Aspergillus niger* using agricultural waste in solid state fermentation. *Appl. Biochem. Biotechnol*. **135:** 33–42.
16. Pal, M. P., B. K. Vaidya, K. M. Desai, R. M. Joshi, S. N. Nene, and B. D. Kulkarni. 2009. Medium optimization for biosurfactant production by *Rhodococcus erythropolis* MTCC 2794: Artificial intelligence verses a statistical approach. *J. Ind. Microbiol. Biotechnol*. **36:** 747–756.
17. Pedreschi, F., K. Kaack, and K. Granby. 2008. The effect of asparaginase on acrylamide formation in French fries. *Food Chem.* **109:** 386–392.
18. Rai, S. K. and A. K. Mukherjee. 2010. Statistical optimization of production, purification and industrial application of a laundry detergent and organic solvent-stable subtilisin-like serine protease (Alzwiprase) from *Bacillus subtilis* DM-04. *Biochem. Eng. J*. **48:** 173–180.
19. Sarquis, M. I. M., E. M. M. Oliveira, A. S. Santos, and G. L. Costa. 2004. Production of L-asparaginase by filamentous fungi. *Mem. Inst. Oswaldo Cruz* **99:** 489–492.
20. Shaffer, P. M., H. N. Jr. Arst, L. Estberg, L. Fernando, T. Ly, and M. Sitter. 1988. An asparaginase of *Aspergillus nidulans* is subject to oxygen repression in addition to nitrogen metabolite repression. *Mol. Gen. Genet*. **212:** 337–341.
21. Sivapathasekaran, C., S. Mukherjee, A. Ray, A. Gupta, and R. Sen. 2010. Artificial neural network modeling and genetic algorithm based medium optimization for the improved production of marine biosurfactant. *Bioresour. Technol*. **101:** 2884–2887.
22. Sreenivasulu, V., K. N. Jayaveera, and P. M. Rao. 2009. Optimization of process parameters for the production of L-asparaginase from an isolated fungus. *Res. J. Pharmacogn. Phytochem.* **1:** 30–34.
23. Tang, Y. J. and J. J. Zhong. 2004. Modeling the kinetics of cell growth and ganoderic acid production in liquid static cultures of the medicinal mushroom *Ganoderma lucidum*. *Biochem. Eng. J*. **21:** 259–264.
24. Tsuji, Y. 1957. Studies on the amidase. IV. Supplemental studies on the amidase action of the bacteria. *Japan Arch. Int. Med*. **4:** 222–224.
25. Wei, D. Z. and H. Liu. 1998. Promotion of L-asparaginase production by using *n*-dodecane. *Biotechnol. Tech*. **12:** 129–131.
26. Wriston, J. C. Jr. and T. O. Yellin. 1973. L-Asparaginase – A Review. *Adv. Enzymol. Relat. Areas Mol. Biol.* **39:** 185–248.