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Process modeling and optimization of high yielding L-methioninase from a newly isolated *Trichoderma harzianum* using response surface methodology and artificial neural network coupled genetic algorithm

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ABSTRACT

A high yielding L-methioninase producing fungus was isolated from soil samples and was identified as *Trichoderma harzianum*. The enzyme was purified using chromatographic techniques and the purified enzyme had an apparent molecular mass of 48 kDa on SDS PAGE. The purified L-methioninase showed a specific activity of 74.4 U/mg, which is the highest among other L-methioninase, reported and can be a potential candidate as an anticancer agent. The media components such as lactose, L-methionine, KH_2PO_4, K_2HPO_4 and Zinc chloride was found to have a significant effect on enzyme production by classical optimization method. Response surface methodology and artificial neural network linked genetic algorithm was employed to develop an optimized medium for L-methioninase production. Maximum enzyme production obtained using RSM was 30.2 U/ml at medium composition of 12.5 g/l of lactose, 10 g/l of L-methionine, 2 g/l of KH_2PO_4, 4 g/l of K_2HPO_4 and 0.0125 g/l of zinc chloride. ANN model was found to be superior to RSM with a higher coefficient of determination (R^2) of 0.995, lower RSME (0.306) and MSE (0.093). A higher enzyme production of 33.32 U/ml was achieved using ANN-GA optimized medium; 13.9 g/l of lactose, 11.37 g/l of L-methionine, 1.58 g/l of KH_2PO_4, 3.98 g/l of K_2HPO_4 and 0.01 g/l of zinc chloride, which is in agreement with the predicted value of 33.76 U/ml.

1. Introduction

L-Methioninase (EC 4.4.1.11), is a pyridoxal-L-phosphate dependent enzyme that catalyzes the oxidative deamination and demethiolation of L-methionine to methanethiol, ammonia and α-ketobutyrate (Tanaka et al., 1977). L-Methioninase cause selective destruction of methionine dependent tumor cells by depriving these cells of L-methionine. L-Methionine is essential for the metabolism of sulphur containing compounds. While normal cells are methionine independent due to their ability to synthesize methionine using methionine synthase from homocysteine (Mecham et al., 1983), cancer cells are methionine dependent. Methionine dependency of cancer cells is due to enhanced requirements of methionine for high protein synthesis and regulation of DNA expression in cancer cells (Kreis and Hession, 1973). The enzyme has received significant attention due to its anticancer properties against various tumor cell lines including breast, colon kidney, lung and glioblastoma (Tan et al., 1998). L-Methioninase is also involved in the regulation of obesity by depriving dietary methionine in the rats (Prada et al., 1995).

L-Methioninase has been isolated and characterized from various bacterial species including *Pseudomonas putida* (Ito et al., 1976), *Clostridium sporogenes* (Kreis and Hession, 1973), *Aeromonas sp.* (Nakayama et al., 1984), *Citrobacter intermedius* (Faleev et al., 1996), *and Brevibacterium linens* (Amarita et al., 2004), from fungus *Aspergillus flavipes* (Khalaf and El-Sayed, 2009), and *candida tropicalis* (Selim et al., 2015a). Bacterial L-methioninase is an intracellular enzyme whereas fungal L-methioninase is extracellular in nature (Tanaka et al., 1977; Khalaf and El-Sayed, 2009). In recent years much attention has been given for identification of L-methioninase enzyme from fungal sources due to its lower immunogenicity towards the human immune system and high substrate specificity for L- methionine which enhances the anticancer efficiency of the enzyme (Hawkins et al., 2004).

Modeling and optimization of biological process is crucial for the development of an efficient and economic bioprocess. Response surface methodology (RSM) is a modeling technique used to establish the relationship between the responses and the independent variables and can analyze the effects of the variables alone or in combination by regression analysis (Desai et al., 2008). RSM using central composite design has been extensively used to optimize the production of enzymes such as glutaminase (Singh et al., 2013) and α-amylase (Nithya et al.,

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2017).

Machine learning techniques such as artificial neural network (ANN) and genetic algorithm (GA) mimics different aspects of the biological system. These methods have been successfully applied as data analysis tools. ANN is an efficient nonlinear multivariate modeling tool with great flexibility and the ability to generalize the behavior of complex systems, which make it a promising tool for optimization (Livinstone, 2008). GA is a powerful stochastic search and heuristic optimization technique which is inspired by "Survival of Fittest" concept of Darwinian Evolution (Goldberg, 1989). It can be used to optimize fermentation conditions without the requirement of statistical designs and empirical models due to its flexibility in selection of objective function and constraints. The successful utilization of ANN integration with GA for enzyme production has been applied for optimization of fermentations like L-asparaginase production from *Aspergillus niger* (Gurunathan and Sahadevan, 2011) and alkaline protease from *Bacillus circulans* (Rao et al., 2008).

In the present investigation, an attempt was made to isolate and identify a highly active L-methioninase from fungus and to purify this enzyme. Central composite design and ANN linked GA were used to optimize media components for L-methioninase production from *Trichoderma harzianum*. The optimized media compositions for L-methioninase production obtained from both models were experimentally verified.

2. Materials and methods

2.1. Isolation of fungi

Soil samples collected from dumps of poultry places and vegetable garbage sites (Kozhikode, Kerala) were used as the source for L-methioninase producing fungi. The fungal culture was isolated by serial dilution of soil samples and was plated on to basal medium, containing L-methionine (5 g/l), glucose (10 g/l), K_2HPO_4 (1 g/l), KH_2PO_4 (1 g/l), $MgCl_2{\cdot}6H_2O$ (0.5 g/l), $CaCl_2{\cdot}2H_2O$ (0.1 g/l), $FeCl_3{\cdot}6H_2O$ (0.02 g/l), $ZnCl_2$ (0.02 g/l), and agar (20 g/l) (Ruiz- Herrera and Starkey, 1969). The pH of the medium was adjusted to 7.0 using 1 N sodium hydroxide solution. The plates were incubated at 28 ± 1 °C for 10 days and the fungal isolates were sub cultured and maintained on the same basal medium. For the screening of high yielding L-methioninase producers, the fungal isolates were inoculated on to basal agar medium supplemented with 0.007% phenol red. The pH of the medium was adjusted to 6.5 using 1 N sodium hydroxide solution and the plates were incubated for 7 days at 28 °C. The fungal cultures with significant L-methioninase production were identified by morphological and molecular methods.

2.2. Production of methioninase

The production of L-methioninase was carried out in Erlenmeyer flasks containing 50 ml of basal medium and incubated at 28 °C, 150 rpm for 7 days. After incubation, the fungal biomass was separated by centrifugation at 8000 × g for 20 min at 4 °C and the culture filtrate was used as the source of crude enzyme.

2.3. Determination of methioninase activity

L-Methioninase activity was quantified by demethiolation assay (Laakso and Nurmikko, 1976). The reaction mixture composed of 20 mM L-methionine in potassium phosphate buffer (pH 7.2), 0.1 mM pyridoxal phosphate, and 0.25 mM 5, 5-dithio-bis-2-nitrobenzoic acid (DTNB) in a final volume of 1.0 ml. After 30 min of incubation at 37 °C the absorbance was measured at 420 nm. One unit (U) of L-methioninase activity was expressed as the amount of enzyme that releases 1 μmol of free thiol per minute under optimal assay conditions.

2.4. Protein concentration

The enzyme protein content was determined by Lowry's method (Lowry et al., 1951), using bovine serum albumin as the standard.

2.5. Purification of L-methioninase

The culture supernatant was fractionated using ammonium sulphate precipitation. The precipitated protein was collected by centrifugation at 10,000 × *g* for 30 min, dissolved and further dialyzed against 25 mM potassium phosphate buffer (pH 7.2) at 4 °C. The sample was then applied to a DEAE Sephadex ion exchange column and the bound protein was eluted with a linear gradient of NaCl (0.1–1.0 M) in 25 mM potassium phosphate buffer at a flow rate of 1 ml/min. Fractions of 5 ml were collected and analyzed for L-methioninase activity. The fractions with L-methioninase activity were pooled, dialyzed and concentrated by freeze drying and loaded on to Sephacryl S-300HR gel filtration column. The column was eluted using 25 mM potassium phosphate buffer (pH 7.2) at the flow rate of 0.5 ml/min. Fractions of 2 ml were collected and analyzed for L-methioninase activity. The active fractions were pooled and concentrated.

2.5.1. SDS PAGE

SDS–PAGE was carried out as described by Laemmli (1970) on 5% (w/v) stacking and 10% (w/v) separating gel. The electrophoresis was performed at room temperature at 80 V. Protein molecular mass standard was used to determine the molecular weight of the purified protein. The protein bands were visualized by Coomassie Brilliant Blue G-250.

2.6. Optimization studies

2.6.1. Effect of various parameters on L-methioninase production

The effect of various cultural parameters and media components on L-methioninase production was screened by one factor at a time approach, keeping other media constituents constant. The effect of incubation period on enzyme production was analyzed. The samples were collected at regular time intervals and enzyme activity was measured. The effect of medium pH and incubation temperature for L-Methioninase production was determined at a range of 2.0–10.0 and 25–50 °C respectively. The influence of different carbon sources such as glucose, maltose, sucrose, lactose and galactose on L-methioninase production was studied at 1% (w/v) concentration. Effect of nitrogen supplements (0.5% w/v) such as ammonium sulphate, ammonium chloride, ammonium acetate, peptone, yeast extract, beef extract and malt extract on L-methioninase production was also analyzed. To study the effect of different mineral ions on L-methioninase production, the medium was supplemented with 0.05% of KH_2PO_4, K_2HPO_4, NaH_2PO_4, Na_2HPO_4, $MgCl_2$, $CaCl_2$, $FeCl_3$ and $ZnCl_2$. All experiments were conducted in triplicates and the mean values were used for further analysis.

2.6.2. Optimization of L-methioninase production using RSM

A central composite design was employed to evaluate the influence of various concentrations of media components which showed a significant effect on L-methioninase production. Five most influential media components were selected based on one factor at a time method. They were tested at five levels ($-\alpha$, -1, 0, $+1$, $+\alpha$) and the designed matrix consists of 52 experiments, comprising of 32 factorial points, 10 axial points and 10 replicates. All experimental runs were performed in triplicates and mean values were taken. The respective low, middle and high levels of each variable are presented in Table 1. The experiments were run in random order for the modeling of quadratic effects as well as main effects and their interactions. The experimental response was analyzed and fitted into second-degree polynomial equation

$$Yi = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad (1)$$

Table 1
Variables and their levels for Central composite design.

Variables	Variable levels (g/l)				
	- α level	-1 level	0 level	+1 level	+ α level
Lactose (X_1)	4.085	7.5	10	12.5	15.946
L-methionine (X_2)	3.107	10	15	20	26.892
$KH_2PO_4(X_3)$	0.621	2	3	4	5.378
$K_2HPO_4(X_4)$	0.621	2	3	4	5.378
$ZnCl_2(X_5)$	0.00405	0.0075	0.01	0.0125	0.0125

Table 2
Fungal isolates producing L-methioninase.

Fungal isolate	Enzyme activity (U/ml)	Specific activity (U/mg)	Morphological identification
K2	12.24	10.20	*Trichoderma harzianum*
K4	5.37	5.9	*Aspergillus niger*
Kr3	9.21	7.67	*Aspergillus terrus*
Va5	3.72	2.48	*Aspergillus flavus*
Va2	3.83	3.22	*Aspergillus acculeatus*

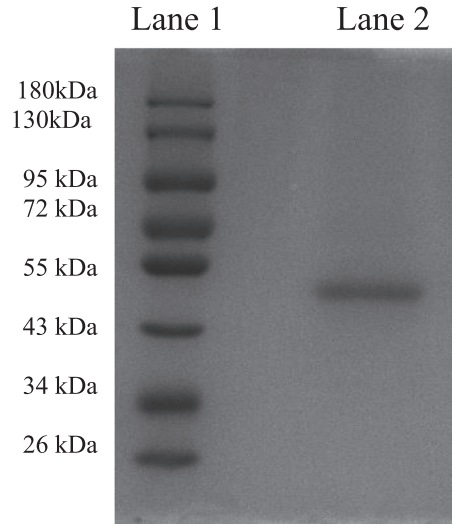

Fig. 1. SDS-PAGE of purified L-methioninase. Lane 1 marker, Lane 2 Purified L -methioninase.

Where i, j represents linear and quadratic coefficients, β_0 the interception coefficient, β_i the coefficient of linear effect, β_{ii} the coefficient of quadratic effect and β_{ij} coefficient of interaction effect and Y depicts the response for L-methioninase production. The model terms were analyzed by analysis of variance (ANOVA). The effect of variables individually and in combination was depicted using contour plots. Minitab 14.12.0 software was used to generate the design and analyze the variables.

2.6.3. The prediction and optimization of L-methioninase by artificial neural network coupled genetic algorithm

Artificial Neural Network is a highly interconnected structures comprising of adaptive simple processing elements called neurons that can learn the complex relationship between independent and dependent variables. Multi-layered feed-forward neural network architecture, using back-propagation algorithm was used for nonlinear mapping between input and output variables. A multi-layer neural architecture consists of input, output and hidden layer. The input layer consisting of five neurons represents the variables lactose, methionine, KH_2PO_4, K_2HPO_4 and Zinc chloride and the output layer with one neuron represents L-methioninase activity. The number of neurons in the hidden layer was iteratively determined based on the minimum value of mean squared error (MSE).

The input data was presented by the input layer to the hidden layer through weights. The hidden layer sums up the weighted inputs along with the biases as

$$\text{Sum} = \sum_{i=1}^{n} x_i w_i + \theta \tag{2}$$

Where, w_i (i = 1, n) represents the weight connections between the neurons of the input and the hidden layer, θ is defined as the bias and x_i signifies the input parameter. All pre-processing was done by the hidden layer and the sum of the weighted values modified by transfer functions were transferred to the output. The final response predicted by the ANN model was generated by the output layer. Sigmoid function and linear function are the transfer functions used for the hidden layer and output layer respectively. Weights and biases were adjusted to reduce the error between the predicted values from the desired values according to the Levenberg–Marquardt algorithm. All the variables of input and output layer were scaled in the range [-1, 1] using the equation.

$$\text{Normalized} = \left[\frac{2^* \ (X_{AC} - X_{min})}{(X_{max} - X_{min})}\right] - 1 \tag{3}$$

Where X_{min}, X_{max} and X_{AC} are minimum, maximum and actual data respectively.

The prediction capabilities of RSM and ANN were tested using statistical parameters. The estimated response of L-methioninase activity obtained from RSM and ANN model was further compared with actual response in terms of coefficient of determination (R^2), mean square error (MSE), Root mean square error (RMSE) using the equation:

$$R^2 = 1 - \frac{\sum_{i=1}^{n} (y_i - y_{di})^2}{\sum_{i=1}^{n} (y_i - y_a)^2} \tag{4}$$

$$\text{MSE} = \frac{1}{n} \sum_{i=1}^{n} (y_i - y_{di})^2 \tag{5}$$

$$\text{RMSE} = \sqrt{\frac{1}{n} \sum_{i=1}^{n} (y_i - y_{di})^2} \tag{6}$$

Where n represents the number of variables, y_i, the predicted value, y_{di} the experimental value and y_a is the average of experimental value.

GA is a global optimization tool which is employed to perform an extensive search over a range of the variable combinations to maximize enzyme production. For maximum L-methioninase production, the concentrations of media components were optimized by GA using ANN as its fitness function. Initially a random population of individuals was generated known as chromosomes. These individuals were evaluated based on the fitness function. GA repeatedly modifies the population of individuals using selection, crossover and mutation until the termination criterion is procured (Renner and Ekárt, 2003). Modeling and data simulations were performed using ANN and GA optimization toolboxes of MATLAB R 2014b software.

3. Results and discussion

3.1. Isolation and identification of methioninase producing fungi

A total of 45 fungal cultures were isolated from soil samples. Screening for efficient L-methioninase producers was done using phenol red supplemented basal agar media. Five fungal isolates were selected as significant L-methioninase producers based on the presence of pink color in the medium around the fungal growth, which is due to the release of ammonia by the action of L-methioninase on L-methionine. Similar results were also observed during the screening of L-

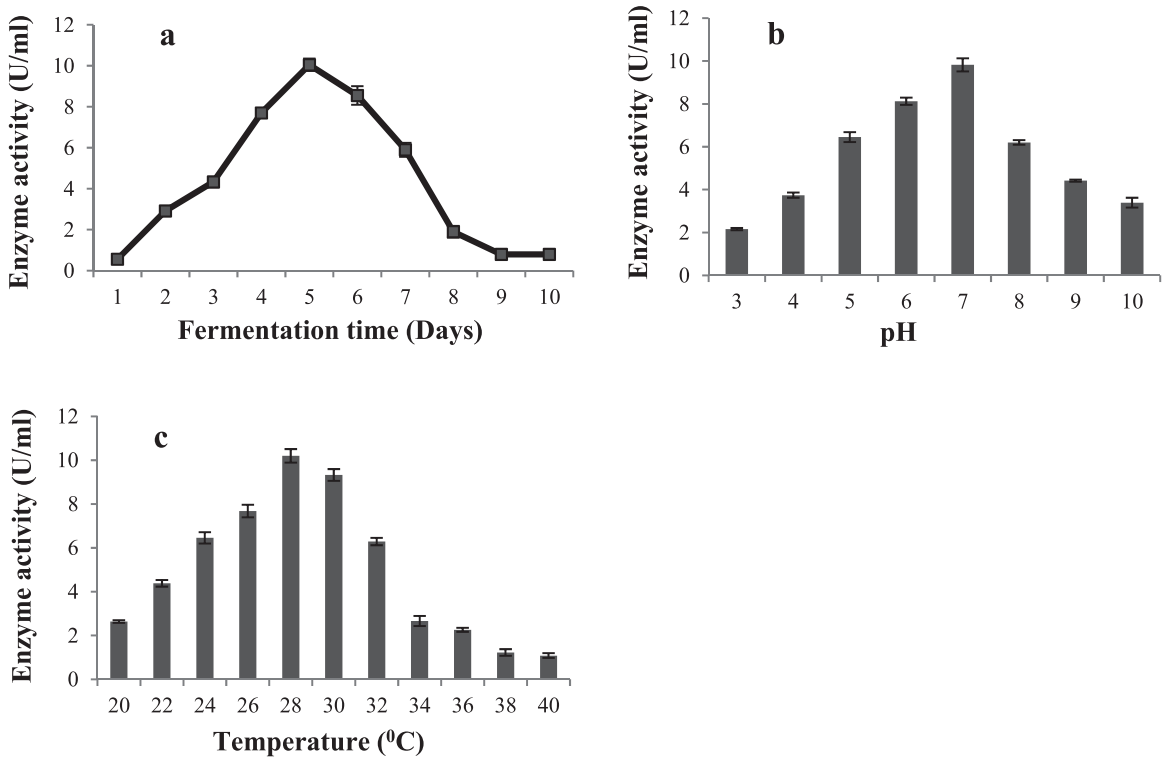

Fig. 2. Effect of various physical parameters on L-methioninase production a. Fermentation time, b. Medium pH, c. temperature.

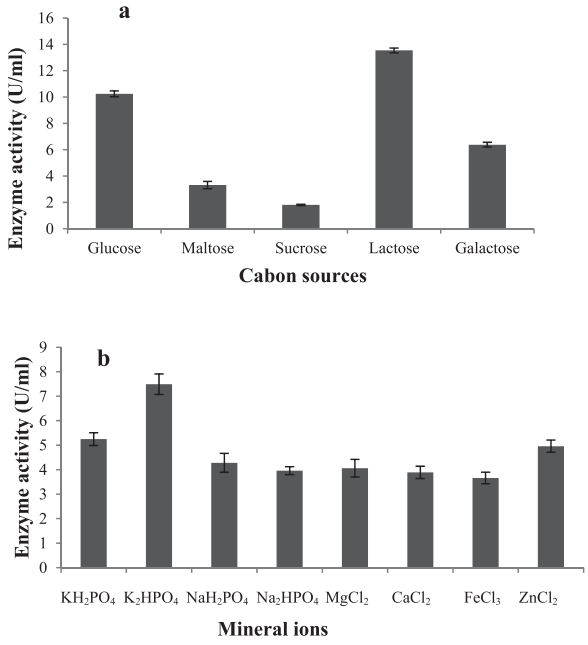

Fig. 3. Effect of various media components on L-methioninase production a. carbon sources b. mineral ions.

methioninase producing *Streptomyces* isolates (Selim et al., 2015a). L-methioninase production and morphological identification of these fungal isolates were carried out. The cultures K2 and Kr3 which showed highest L-methioninase production (Table 2) was selected and further identified using molecular methods. The rDNA region of fungi was amplified using ITS 4 and ITS 5 primers. The amplified rDNA was sequenced and aligned with other sequence available in the National center for Biotechnology information (NCBI) databases. The fungal culture K2 was identified as *Trichoderma harzianum* with 100% sequence similarity and Kr3 as *Aspergillus terrus* with 99% sequence similarity. The rDNA sequence of *Trichoderma harzianum* and *Aspergillus terrus* were deposited to Genbank under accession number MH828332.1 and MH892079.1. *Trichoderma harzianum* being the better producer of L-methioninase was selected for further studies.

3.2. L-methioninase purification

The extracellular L-methioninase produced by *Trichoderma harzianum* was purified to homogeneity using ammonium sulphate precipitation, ion exchange and gel filtration chromatography. The molecular weight of purified L-methioninase was analyzed using SDS-polyacrylamide electrophoresis, which revealed a single band of apparent molecular weight 48 kDa (Fig. 1). L-methioninase was reported to be a multi subunit enzyme comprising four identical subunits (El-Sayed, 2010) and L-methioninase purified from different sources possessed molecular weight in the range of 43–48 kDa per subunit (Ito et al., 1976; El-Sayed, 2010), which is in accordance with our results. The purified L-methioninase showed a specific activity of 74.4 U/mg which is the highest reported activity for fungal L-methioninase. The L-methioninase activity reported for *Aspergillus flavipes* is 14.6 U/mg (El-Sayed, 2011) and for *candida tropicalis* is 64.78 U/mg (Selim et al., 2015b).

3.3. Screening of nutrients and optimization of physical parameters using classical method

To determine the effect of incubation period, L-methioninase production was analyzed on samples withdrawn every 24 h for a period of 10 days. Maximum L-methioninase production of 12.24 U/ml was obtained on the fifth day (120 h) of production (Fig. 2a). To study the effect of medium pH, experiments were performed at different pH and the enzyme activity was determined. The enzyme production was enhanced when pH was increased from 5 to 7 and a further increase in pH caused a decline in enzyme production (Fig. 2b). Optimum enzyme production was observed at pH 7. The optimum temperature for enzyme production was found to be 28 °C (Fig. 2c).

Table 3
Central composite design of experimental variables and their corresponding experimental and RSM and ANN predicted values.

Runs	Lactose	Methionine	KH_2PO_4	K_2HPO_4	$ZnCl_2$	Activity U/ml	RSM predicted	ANN predicted
1	12.5	20	4	2	0.0075	20.54	19.68	20.45
2	7.5	10	4	4	0.0075	22.6	21.74	22.23
3	10	15	3	0.621	0.01	15	15.87	15.02
4	7.5	20	4	2	0.0075	20.5	21.25	20.4
5	12.5	20	4	4	0.0125	20.9	21.04	20.49
6	10	15	3	3	0.01	27.6	26.55	25.57
7	12.5	20	4	2	0.0125	20.69	19.43	20.31
8	7.5	20	4	4	0.0125	19.3	17.89	19.25
9	10	15	3	3	0.01	26.9	26.55	–
10	10	15	3	3	0.00405	21.5	22.25	21.01
11	12.5	20	2	4	0.0075	19.08	19.86	19.09
12	10	15	3	3	0.0159	22.7	24.14	22.66
13	10	15	3	3	0.01	26.1	26.55	–
14	12.5	10	4	2	0.0075	20.4	20.97	20.34
15	10	15	3	3	0.01	26.8	26.55	–
16	12.5	20	2	2	0.0125	23.6	22.70	23.99
17	7.5	10	2	2	0.0125	25.8	25.19	26.752
18	7.5	20	2	2	0.0125	26.1	26.03	26.02
19	7.5	10	2	4	0.0075	21.13	22.19	21.24
20	12.5	10	2	2	0.0075	22.3	22.02	22.34
21	12.5	10	2	4	0.0075	28.5	27.28	28.23
22	10	15	3	3	0.01	25.9	26.55	–
23	12.5	10	2	2	0.0125	23.6	24.20	23.55
24	7.5	20	2	4	0.0125	21.4	20.56	21.36
25	12.5	20	2	4	0.0125	22.4	22.63	22.33
26	7.5	20	2	4	0.0075	18.6	17.12	18.54
27	10	15	3	3	0.01	26.8	26.55	–
28	12.5	10	2	4	0.0125	30.2	28.44	30.17
29	4.054	15	3	3	0.01	19.3	20.36	19.53
30	12.5	10	4	4	0.0075	28.1	27.91	28.63
31	7.5	10	4	2	0.0075	21.3	20.21	21.27
32	7.5	10	4	2	0.0125	20.16	19.02	19.96
33	10	15	3	5.378	0.01	16.3	17.62	16.98
34	10	15	3	3	0.01	25.6	26.55	–
35	7.5	10	2	2	0.0075	22.8	22.33	22.92
36	10	3.107	3	3	0.01	23.6	24.47	23.78
37	12.5	10	4	4	0.0125	25.5	25.03	25.38
38	10	15	3	3	0.01	26.4	26.55	–
39	12.5	20	4	4	0.0075	23.3	22.31	23.17
40	10	15	5.378	3	0.01	19.4	20.53	19.46
41	7.5	20	2	2	0.0075	22.04	21.56	22.61
42	7.5	10	4	4	0.0125	19.1	19.53	19.15
43	10	15	0.621	3	0.01	23.9	24.95	24.79
44	12.5	10	4	2	0.0125	19.3	19.11	19.34
45	15.946	15	3	3	0.01	22.6	23.73	22.29
46	7.5	10	2	4	0.0125	24.19	24.03	24.14
47	7.5	20	4	4	0.0075	18.6	18.48	18.66
48	10	15	3	3	0.01	27.1	26.55	–
49	7.5	20	4	2	0.0125	21.4	21.68	21.43
50	12.5	20	2	2	0.0075	19.5	18.90	19.47
51	10	26.892	3	3	0.01	17.5	18.81	17.5
52	10	15	3	3	0.01	24.9	26.55	–

Among different carbon sources tested, lactose was found to be the best carbon source that showed an enzyme production of 13.5 U/ml followed by glucose (10.24 U/ml) (Fig. 3a). The capability of the fungus to degrade lactose to glucose and galactose may be attributed to the presence of lactase enzyme in *Trichoderma harzianum* as reported by Seyis and Aksoz (2004) which leads to an increase in L-methioninase activity when lactose was used as substrate. In the absence of any carbon source, fungal growth and enzyme production was significantly inhibited. This indicates the requirement of a carbon source as co-dissilmilator for L-methioninase production from *Trichoderma harzianum* similar to *Aspergillus flavipes* (Khalaf and El-Sayed, 2009) and *Acromobacter starkeyi* (Ruiz-Herrera and Starkey, 1970).

The effect of various organic and inorganic nitrogen sources on methioninase production was studied. It was observed that L-methioninase production was L-methionine dependent. In the absence of L-methionine, none of the nitrogen sources were able to produce L-methioninase. This L-methionine dependency was also reported for L-methioninase production by *Aspergillus sp. RS-1a* and *Aspergillus flavipes* (Khalaf and El-Sayed, 2009; Ruiz-Herrera and Starkey, 1969). The effect of essential mineral ions was studied along with the best carbon and nitrogen source in the medium. Among the tested phosphorous sources, KH_2PO_4 and K_2HPO_4 enhanced L-methioninase production than $NaHPO_4$ and Na_2HPO_4 (Fig. 3b). Phosphorous has a pivotal role in maintaining integral structure and controlling cellular reactions and the importance of phosphorous on L-methioninase production was also reported for *Aspergillus flavipes* (Khalaf and El-Sayed, 2009) and *Yarrowia lipolytica* (Bondar et al., 2005). Apart from the phosphorous sources, the effect of essential mineral ions on L-methioninase production was evaluated. Among the different mineral ions tested, Zinc chloride showed significant effect on L-methioninase production (Fig. 3b).

Table 4
Coefficient estimates by the regression model for optimization of methioninase production.

Term	Coefficient	*t*-value	*p*-value
Constant	− 26.7929	− 3.076	0.004
X_1	1.8610	2.609	0.014
X_2	1.3215	3.918	< 0.001
X_3	3.4501	2.046	0.049
X_4	8.3675	4.961	< 0.001
X_5	3363.25	4.715	< 0.001
X_1^2	− 0.1274	− 5.360	< 0.001
X_2^2	− 0.03469	− 5.836	< 0.001
X_3^2	− 0.6728	− 4.527	< 0.001
X_4^2	− 1.7334	− 11.664	< 0.001
X_5^2	− 94925	− 3.992	< 0.001
X_1X_2	− 0.04687	− 2.915	0.007
X_1X_3	0.1081	1.345	0.188
X_1X_4	0.5403	6.722	< 0.001
X_1X_5	− 27.0500	− 0.841	0.407
X_2X_3	0.09106	2.265	0.031
X_2X_4	− 0.2153	− 5.357	< 0.001
X_2X_5	32.2750	2.007	0.053
X_3X_4	0.4171	2.076	0.046
X_3X_5	− 404.125	− 5.027	< 0.001
X_4X_5	− 102.375	− 1.273	0.212

3.4. Optimization by response surface methodology

Based on the preliminary experiments, the five significant nutrients, lactose, L- methionine, KH_2PO_4, K_2HPO_4 and Zinc chloride were selected for statistical optimization. To evaluate the individual and combined effect of these medium components on L-methioninase production, a central composite design was developed. The design and experimental response obtained for L-methioninase production are given in Table 3 along with the predicted data. The coefficients, *t*-value and *p*-value for linear, quadratic and interaction terms of variables are given in Table 4. A *p*-value less than 0.05 indicate that the variable is significant (Lazic, 2004). All the linear and quadratic terms of the model were found to be significant, where as the interactive effect of lactose - KH_2PO_4, lactose - zinc chloride, Methionine - zinc chloride and K_2HPO_4- zinc chloride showed no significant impact on L-methioninase production. By applying multiple regression analysis on the experimental data, the following second-order polynomial equation was obtained to explain L-methioninase production:

$$\begin{aligned} Y = {} & -26.7929 + 1.8610\,X_1 + 1.32152\,X_2 + 3.45015\,X_3 + 8.36759\,X_4 \\ & + 3363.25\,X_5 - 0.127452\,X_1^2 \\ & - 0.0346914\,X_2^2 - 0.672831\,X_3^2 - 1.73349\,X_4^2 - 94925\,X_5^2 - 0.0468750\,X_1X_2 \\ & + 0.108125\,X_1X_3 \\ & + 0.540375\,X_1X_4 - 27.050\,X_1X_5 + 0.0910625\,X_2X_3 - 0.215312\,X_2X_4 \\ & + 32.2750\,X_2X_5 \\ & + 0.417188\,X_3X_4 - 404.125\,X_3X_5 - 102.375\,X_4X_5 \end{aligned} \quad (7)$$

Where X_1 represents the coded value of lactose and X_2 of L-methionine, X_3 of KH_2PO_4, X_4 of K_2HPO_4 and X_5 of zinc chloride

ANOVA analysis was performed to measure the statistical significance of the model and is depicted in Table 5. Significant F value (20.85) and *p*-value (< 0.001) with non significant lack of fit (0.083) confirmed that the model equation was adequate to predict enzyme production. The coefficient of determination (R^2) was 0.931 for L-methioninase production, which explains a higher degree of correlation between experimental and predicted responses. The closer the R^2 value to 1.0, the stronger the model and better it predicts the response.

The regression equation was graphically represented as contour plots to understand the interactions of medium components and the optimum concentration of each component required for L-methioninase production. The shape of the response surface indicates the significance of the interactions. The contour plots of factors with significant interactive effect has elliptical surface and the six significant interactions given in Fig. 4 emphasize the role played by these variables in L-methioninase production. Plots were generated for the pair-wise combination of factors, while the other factors are kept at their constant middle level (Lactose 10 g/l; L-methionine 15 g/l; KH_2PO_4 3 g/l; K_2HPO_4 3 g/l and zinc chloride 0.01 g/l). From contour plots, it is evident that L-methionine concentration has a significant effect on L-methioninase activity. Higher levels of L-methionine concentration resulted in a lower L-methioninase production which may be due to the down regulation of GATA gene transcription that hampers the gene expression of L-methioninase (Caddick et al., 1994; Mitchell and Magasanik, 1984), methionine catabolic repression or the trans inhibition (Pall, 1971).

By applying regression analysis, the optimum concentration of media components obtained for L-methioninase production were lactose 12.5 g/l, methionine 10 g/l, KH_2PO_4 2 g/l, K_2HPO_4 4 g/l and zinc chloride 0.0125 g/l. The predicted and experimental L-methioninase activity for optimized media was 28.44 U/ml and 30.2 U/ml. Optimization of medium constituents using RSM resulted in 2.4 times increase in L-methioninase production.

3.5. Prediction and optimization by ANN-GA model

ANN is a valuable tool for modeling, simulation and prediction of the behavior of nonlinear multivariate systems. The selection of an optimum neural network and topology is crucial for the estimation and prediction of a model. ANN model selected for the present work is multilayered feed forward neural network with Levenberg-Marqardt back propagation algorithm. The experimental design generated by central composite design was used for simulating ANN excluding the replicated data obtained at the center point, as the replicates do not improve the predictive ability of ANN network (Bas and Boyaci, 2007). The inputs and the targets are normalized between 1 and −1 to improve the generalization potentiality. The experimental data were divided into training and testing data to evaluate the generalizing ability of the neural network. For training neural network, 60% data was used for training, 20% for testing and 20% for validation.

The performance of the network was evaluated based on mean square error (MSE) and coefficient of determination (R^2). To avoid over training of network, the epoch was fixed at 1000 and validation check was done 6 times. To determine optimal ANN structure for prediction, the number of neurons in the hidden layer was iteratively determined

Table 5
Analysis of variance (ANOVA) for the parameters of response surface methodology fitted to second-order polynomial equation.

Source	DF	Sequential sum of squares	Adjacent sum of squares	Adjacent Mean square	F	*p*
Model	20	539.034	539.034	26.9517	20.85	< 0.001
Residual Error	31	40.070	40.070	1.2926		
Lack-of-Fit	22	34.341	34.341	1.5610	2.45	0.083
Pure Error	9	5.729	5.729	0.6366		
Total	51	579.105				

R^2: 0.931; R^2 (adj): 0.88.

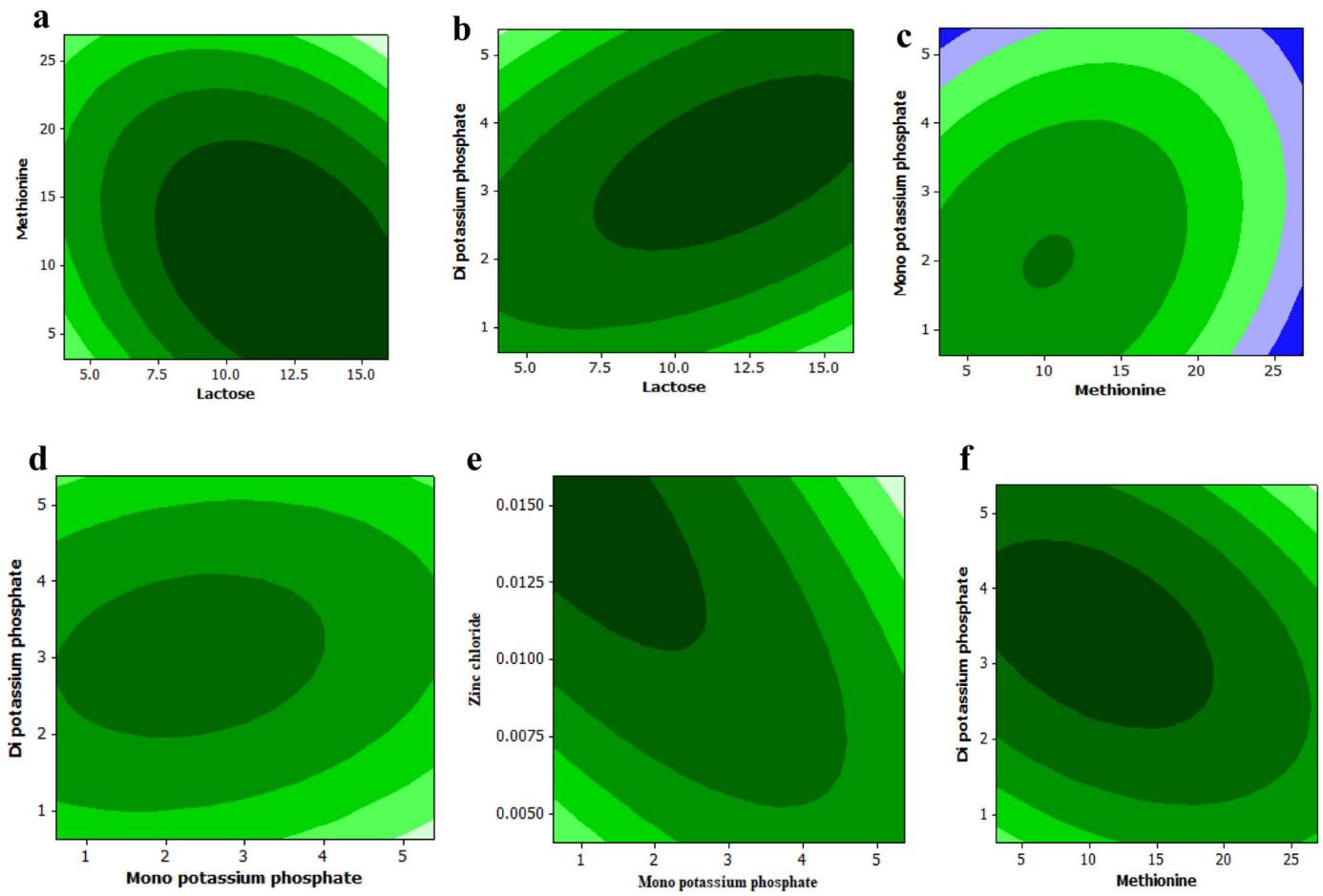

Fig. 4. Contour plots for L-methioninase production a. lactose and methionine, b. lactose and K_2HPO_4 (Dipotassium phosphate), c. Methionine and KH_2PO_4 (Mono potassium phosphate), d. Methionine and K_2HPO_4 (Dipotassium phosphate), e. KH_2PO_4 and K_2HPO_4, f. KH_2PO_4 and ($ZnCl_2$) Zinc chloride.

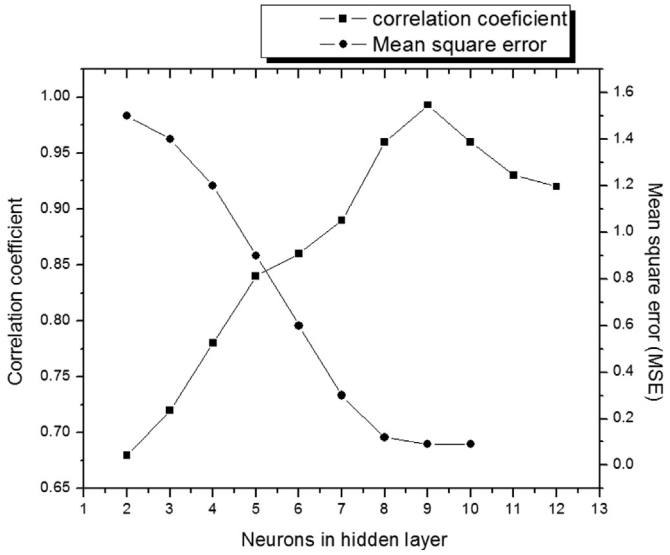

Fig. 5. Effect of number of neurons in hidden layer on mean square error and correlation coefficient during the training of ANN model.

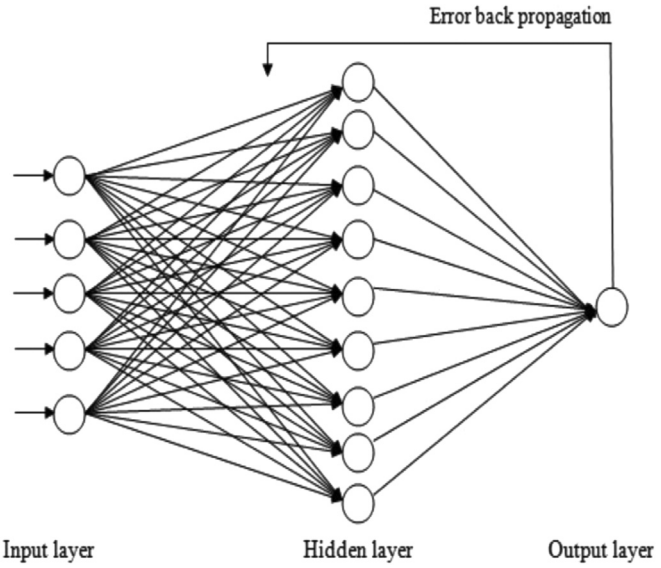

Fig. 6. Back propagation neural network for five input variables, nine hidden neurons and one output layer.

by varying the number from 2 to 12. After training the neural network using training data, the network was evaluated using testing and validation data to examine the performance of the developed network. From Fig. 5 it is evident that the neural network with 9 hidden neurons was found to have least MSE value and a good prediction of outputs for training and validation. The MSE is close to zero (0.09) when the number of neurons in the hidden layers is optimized for the neural network. The network topology developed for ANN was depicted in Fig. 6. The predicted values of response from ANN are shown in Table 3. The overall R^2 of the model was found to be 0.993 which

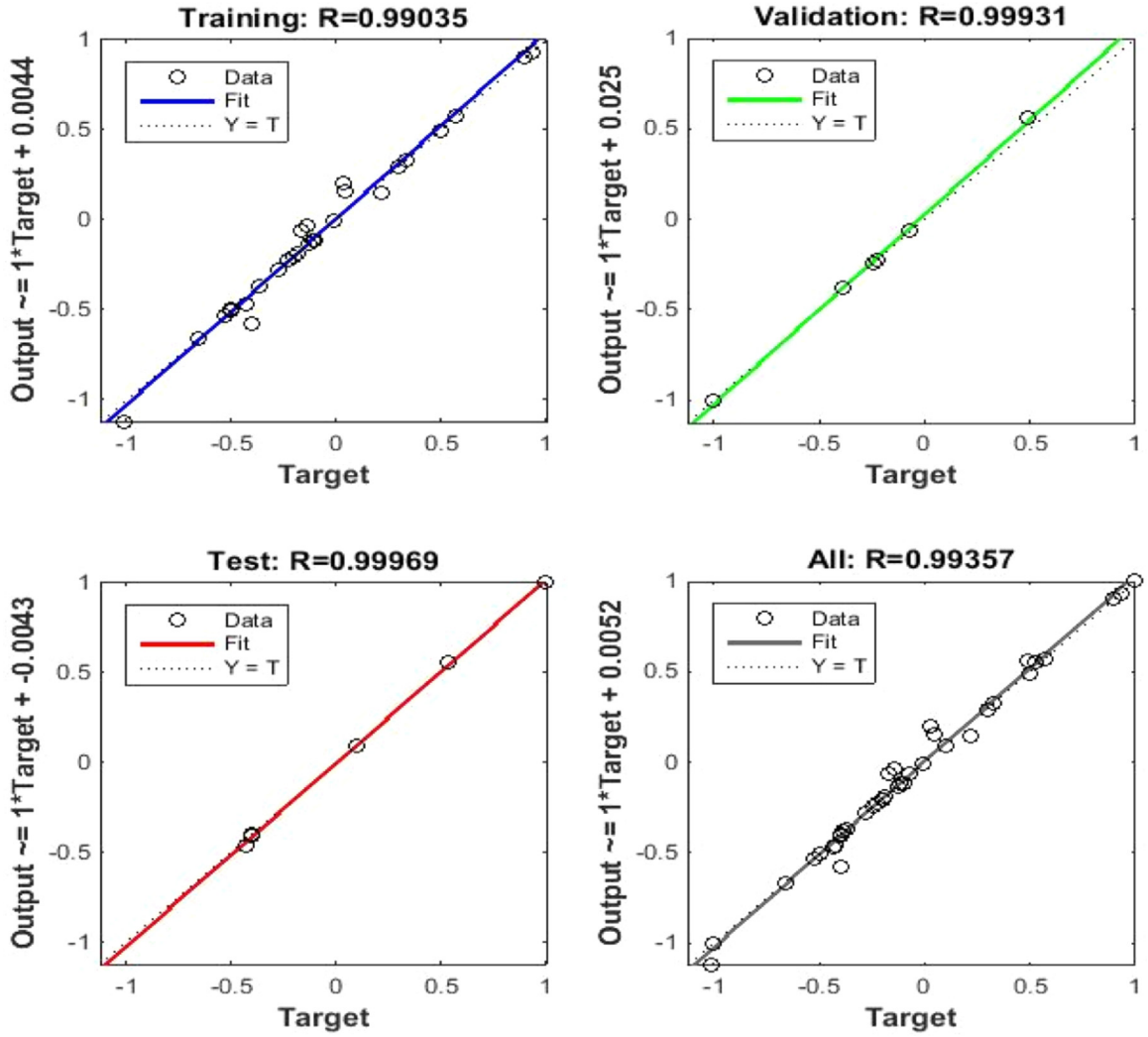

Fig. 7. Training testing and validation of the ANN model.

Table 6
Comparisons of RSM and ANN.

Parameters	RSM	ANN
R^2	0.931	0.995
MSE	0.770	0.093
RMSE	0.877	0.306

indicated the fitness of the model. The R^2 value for training, testing and validation are depicted in Fig. 7. The maximum L-methioninase activity predicted using ANN model was observed to be 30.17 U/ml which is close to the experimental value of 30.2 U/ml.

The effectiveness of ANN and RSM models was statistically evaluated in terms of R^2 and MSE and RMSE values between actual and estimated responses. The estimated values of MSE and RMSE represented in Table 6 confirmed the higher accuracy of ANN prediction compared to RSM. Fig. 8 showed the comparative plot of experimental responses to predictions made by both RSM and ANN for L-methioninase production. Compared to the regression plot of RSM, the data points of ANN are very close to the y = x-line indicating excellent fit. The higher predictability of ANN model may be attributed to its ability to learn and generalize the behavior of any complex non-linear systems (Geyikci et al., 2012).

The input space of the ANN model was optimized using genetic algorithm. The predictive model developed by ANN was used as a fitness function for optimization with GA. A population size of 50, a uniform crossover rate of 0.8, and a mutation probability of 0.1 were set as the parameters for GA optimization. The optimal medium concentrations of 13.9 g/l lactose, 11.37 g/l L-methionine, 1.58 g/l KH_2PO_4, 3.98 g/l $K_2HPO_4$and 0.01 g/l of zinc chloride was procured after evaluation of operators for 100 generations. The maximum L-methioninase production predicted by ANN-GA was 33.76 U/ml theoretically.

3.6. Validation of the RSM and ANN models

For validation of models, experiments were conducted in triplicates using the predicted optimum concentration of media components determined by both RSM and ANN-GA models. Experimental L-methioninase activity of 30.2 U/ml was attained using the predicted optimum concentration determined by RSM model. Validation of ANN-GA model resulted in L-methioninase production of 33.32 U/ml, which is close to the predicted activity of 33.76 U/ml, indicating a higher accuracy of this model. From Table 7, it is evident that the model developed by ANN linked GA resulted in optimized media with nearly 9.36% higher production compared to RSM model. Both predicted and experimental values for production of L-methioninase by ANN-GA were found to be more effective than RSM.

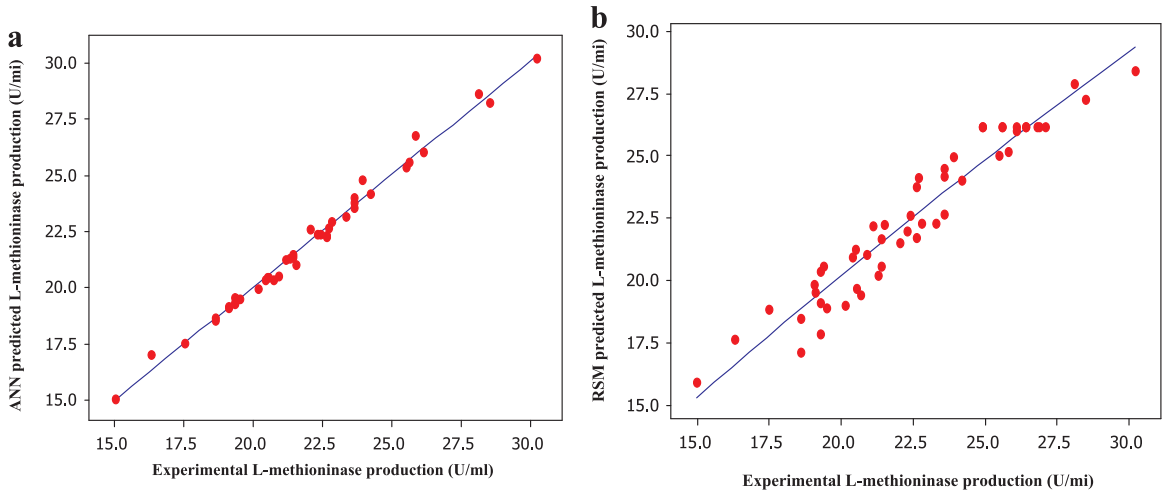

Fig. 8. Regression plot for ANN and RSM model, a Regression plot for ANN, b Regression plot for RSM.

Table 7
Optimum media composition for L-methioninase production.

Input variables	RSM model	ANN-GA model
Lactose	12.5 g/l	13.9 g/l
L-methionine	10 g/l	11.37 g/l
KH_2PO_4	2 g/l	1.58 g/l
K_2HPO_4	4 g/l	3.98 g/l
$ZnCl_2$	0.0125 g/l	0.01 g/l
Experimental enzyme activity	30.2 U/ml	33.32 U/ml
Predicted enzyme activity	28.44 U/ml	33.76 U/ml

4. Conclusion

The present study reports the production of L-methioninase from *Trichoderma harzianum,* which was isolated and identified from soil samples. The purified L-methioninase showed a specific activity of 74.4 U/mg which is the highest among fungal L-methioninase reports available in the literature. The optimization of media was carried out using RSM and ANN-GA model. ANN coupled GA has higher accuracy in modeling and prediction of L-methioninase production and was able to enhance the L-methioninase production from 12.24 U/ml to 33.32 U/ml. A 2.7 fold increase in L-methioninase activity was observed using an optimized medium. The individual and interactive effect of media components were best explained by RSM, whereas modeling using ANN showed more accuracy in prediction of the production of L-methioninase. To the best of our knowledge, this is the first report on isolation and production of L-methioninase from *Trichoderma harzianum* and also of modeling and optimization of L-methioninase production using ANN linked GA. Owing to its high specific activity, the L-methioninase can be a potential candidate as an anticancer enzyme.

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Conflict of interest

None.

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