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ARTICLE

Optimization for the Enhanced Production of Avermectin B1b from *Streptomyces avermitilis* DSM 41445 Using Artificial Neural Network

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Abstract Avermectin is an environment friendly bio-insecticide. Optimization of the culture conditions for avermectin B1b production has not been carried out before using Artificial Neural Network (ANN) method. The present work is therefore conducted to optimize some important factors including yeast extract, $MgSO_4.7H_2O$, and temperature for the avermectin B1b production using ANN methodology from *Streptomyces avermitilis* DSM 41445. The optimum levels for the yeast extract, $MgSO_4.7H_2O$, and temperature were 16.0 (g/L), 5.0 (g/L) and 32°C respectively. Maximum effect was observed by yeast extract. Avermectin B1b yield was increased up to 150% after optimization. ANN was found to be a powerful technique for the optimization and prediction of avermectin B1b production from *Streptomyces avermitilis* DSM 41445.

Keywords artificial Neural Network · avermectin · cultural conditions · culture condition optimization · medium optimization · *Streptomyces avermitilis* DSM 41445

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Introduction

Avermectins are anthelmintic and insecticidal agents that have activity against nematode and arthropod parasites with low level of toxicity (Ikeda and Omura, 1997) and have been produced from *Streptomyces avermitilis* by fermentation (Burg et al., 1979). Medium composition affectsthe product formation significantly, therefore there is a need to address the design of medium composition during fermentation process. Conventional optimization procedures are unable to determine the optimal conditions for a bioprocess, because they involved the sequential manipulation of each parameter and correlation between different parameters are neglected (Xiong et al., 2008). Combined effects of all factors involved in medium composition cannot be determined during single factor conventional optimization (Rao et al., 2000). Furthermore the process is laborious, time consuming, and requires a large number of experiments to be performed to determine the optimum levels of all factors (Dey et al., 2001). Statistical approaches such asRSM and Artificial neural network (ANN) are therefore utilized for optimization and modeling of fermentation processes (Azaman et al., 2010; Nelofer et al., 2011; 2012).

ANN is an alternative method to the quadratic polynomial for the data obtained from statistically designed experiments (Hornik et al., 1989). The artificial neural networks have been used as empirical models due to their ability to work as universal function approximators under definite circumstances (Cheema et al., 2002). Neural networks evaluate the control factors as inputs and responses as outputs during the first step, followed by the unimpeded desirability functions that are pooled with penalty functions. Third step is the use of genetic algorithm as potent optimization contrivance during any optimization process (Ortiz et al., 2004). Only vector forms are processed, and therefore, area of vague sets is divided to “n” equal intervals and in “n+1” result points; the degrees of membership are represented by an “n+1” element vector. The “n+1” element vector is an estimation of the membership function. Increasing the value of “n” consequently results in improved

estimation. ANN, being flexible, can provide the input/output relationships through training (Noorossana et al., 2009).

ANN has been found to articulate the non-linearity in a simpler and better way and has been considered as a suitable practice for optimization (Dutta et al., 2004). In ANN, screening experiments are not required before the application and could be applied to both the statistically designed and statistically non-designed data. For applying ANN, a data set must contain all the operating conditions and then the model may be formed according to the variable behavior (Dasari et al., 2009). In ANN model, if one of the elements fails to perform, it begins to execute through other elements (Hill and Lewicki, 2006).

The optimization of avermectin production was conducted by one parameter at a time technique (Curdova et al., 1989; Zhinan and Peilin, 1999) and RSM (Hong et al., 2009). However, no studies have found the optimization of avermectin production using ANN. The present study was, therefore, conducted for the optimization of avermectin B1b production from *Streptomyces avermitilis*DSM 41445 using ANN method.

Materials and Methods

Microorganism and inoculum preparation. Microbial culture of *Streptomyces avermitilis*DSM41445, provided by "Deutsche Sammlung von Mikroorganismen (DSM) and Zelkulturen GmbH", was used throughout the study. Culture was maintained on yeast-malt-glucose (YMG) medium 65 (glucose 4.0 g/L, yeast extract 4.0 g/L, malt extract 10.0 g/L, $CaCO_3$ 2.0 g/L, and agar 12 g/L) as specified by DSM. Loopful culture of the strain was scraped from the nutrient agar slant and inoculated into 50 mL YMG medium in a 250-mL flask and was incubated in orbital shaker at 150 rpm for 16-8 h at 28°C.

Fermentation conditions. All fermentations were carried out in 250-mL Erlenmeyer flasks with 50 mL of fermentation medium containing soluble corn starch (140.0 g/L), $CaCO_3$ (6.0 g/L), α-amylase (0.1 g/L), KCl (4.0 g/L), NaCl (4.0 g/L), Yeast extract (five levels 2.0, 7.6, 10.4, 13.2, and 16.0 g/L), and $MgSO_4.7H_2O$ (five levels 0.1, 0.26, 0.34, 0.42, and 0.5 g/L).5% inoculum was inoculated in each fermentation flask. The media pH was adjusted at 7.0±0.2. Fermentation studies were carried out in orbital shaker at 150 rpm for 10 days. Different temperatures (ranging from 28-35°C) were studied for optimum temperature. Variations in the levels of different factors are listed in Table 1.

Experimental design. Total 20 experiments with yeast extract, $MgSO_4.7H_2O$, and temperatures (28, 31,33, 35, 37°C) were conducted according to the Box-Wilson (BW) 2^3 full factorial central composite design (CCD). Each variable was set at five different levels of variations (Table 1). First 8 experiments ($2^3 = 8$, factorial CCD) were at factorial points, 6 at axial points ($\alpha = 2$), and 6 replicates were at central points.

Artificial neural network. ANN was selected based on the

Table 1 Box-Wilson 2^3 factorial central composite design for the optimization of avermectin B1b production from *Streptomyces avermitilis* DSM 41445 by ANN

Exp. No.	Yeast extract g/L X1	$MgSO_4.7H_2O$ g/L X2	Temp. °C X3	Observed avermectin B1b production (mg/L)	Avermectin B1b production Predicted by ANN (% difference[a])
1	**7.6**	**0.26**	**31**	**672.3673**	**666.1472 (0.9)**
2	7.6	0.26	35	411.174	550.6528 (-33)
3	*7.6*	*0.42*	*31*	*579.3333*	*642.5023 (-10)*
4	*7.6*	*0.42*	*35*	*400.2587*	*516.0763 (-28)*
5	*13.2*	*0.26*	*31*	*765.1336*	*765.4970 (-0.04)*
6	**13.2**	**0.26**	**35**	**598.26995**	**584.0858 (2.3)**
7	**13.2**	**0.42**	**31**	**732.85255**	**872.1135 (-19)**
8	**13.2**	**0.42**	**35**	**639.04**	**805.2171 (-26)**
9	**16.0**	**0.34**	**33**	**1124.71165**	**973.7062 (13.4)**
10	*2.0*	*0.34*	*33*	*696.86375*	*745.9921 (-7)*
11	**10.4**	**0.5**	**33**	**596.5527**	**783.8915 (-31)**
12	**10.4**	**0.1**	**33**	**1135.70615**	**783.8915 (30)**
13	10.4	0.34	37	353.25425	436.1454 (-23)
14	**10.4**	**0.34**	**28**	**618.6425**	**627.1866 (-1.3)**
15	10.4	0.34	33	639.05065	640.2415 (-0.1)
16	*10.4*	*0.34*	*33*	*624.41515*	*640.2415 (-2.5)*
17	**10.4**	**0.34**	**33**	**635.86405**	**640.2415 (-0.6)**
18	10.4	0.34	33	625.914	640.2415 (-2.2)
19	**10.4**	**0.34**	**33**	**620.3575**	**640.2415 (-3.2)**
20	10.4	0.34	33	810.5221	640.2415 (21)

Italic = select, bold = training, normal = testing.
[a]% difference was calculated as the % difference between observed value and the corresponding predicted value over the observed value.

highest coefficient of correlation and lowest selection error from forty different trained networks. The selected ANN for this study is a multilayer perception network.The number of experiments used for the training, selection, and testing were 10, 5, and 5, respectively by the ANN model (Table 1). Topology of network constructed consisted of three layers including one input, one hidden and one output layer.

Comparison of different ANNs. All 40 ANNs were compared by using the static values including adjusted R^2, AAD, and RMSE, as well as R^2 for their optimization and prediction capabilities. The formulas used for calculating R^2, Adjusted R^2, AAD, and RMSE are given in Eqs. (1), (2), (3), and (4), respectively.

$$R^2 = \frac{\Sigma_{i=i-n}(X_i - Y_i)^2}{\Sigma_{i=i-n}(\overline{Y}_i - Y_i)^2} \quad (1)$$

Where X is the ANN predicted avermectinB1b concentration, Y is the observed B1b concentration and **Y** is the average observed B1b concentration.

$$Adjusted\ R^2 = 1 - \left[(1 - R^2) \times \frac{N-1}{N-K-1}\right] \quad (2)$$

Where N is the total number of observations and K is the number of input variables.

$$AAD = \{[\Sigma_{i=1}^{p}(|y_{i,exp} - y_{i,cal}|/y_{i,exp})]/P\} \times 100 \quad (3)$$

Where $y_{i,exp}$ and $y_{i,cal}$ are the experimental and calculated responses, respectively, and P is the number of experiments.

$$RMSE = \sqrt{\frac{\Sigma(y_{i,exp} - y_{i,cal})^2}{n}} \quad (4)$$

$y_{i,exp}$ and $y_{i,cal}$ are the experimental and calculated responses, respectively, and n is the number of experiments.

Extraction of Avermectin B1b. Fermentation broth from each fermentation flask was centrifuged at 4°C for 20 min at 8,000 rpm (H-1500FR Japan). As avermectin is an intracellular molecule, the cell biomass was taken, and supernatant was discarded. The cell biomass in the form of pallet was mixed with appropriate amount of methanol to completely dissolve the avermectin produced. The mixture was centrifuged again, and the supernatant was collected for the analysis of avermectin by high performance liquid chromatography (HPLC).

HPLC analysis of avermectin B1b. The concentration of avermectin was determined quantitatively by reverse phase HPLC (LC-2080 Shimadzu, Japan). Each sample (20 μL) was injected to the C18 column (SMA C18) and detected by UV Variable Wavelength Detector (STD-M20A Shimadzu) at 246 nm. The samples were eluted by methanol:acetonitrile (98:2 v/v) at a flow rate of 0.5 mL/min (Chen et al., 2007). The standard compound used for the quantification of avermectin B1b was abamectin, which is a mixture of more than 80%avermectin B1a and less than 20% avermectin B1b. From the mixture, the pure form of avermectin B1bwas obtained through column chromatography. Standard curve was drawn between different concentrations of pure avermectin B1b and the area of peak obtained through HPLC.

Statistical analysis. STATISTICA software version 7 was employed for the statistical analysis. The neural networks were constructed using intelligent problem solver of STATISTICA. The best obtained network was then selected for prediction and optimization. Input variables were subsequently ranked using the sensitivity analysis of selected ANN network.

Results and Discussion

The present study was conducted for the optimization of process parameters and medium constituents using artificial neural network methodology. It was observed from the preliminary studies that the major effects on optimization conditions were from three factors that include yeast extract, $MgSO_4 \cdot 7H_2O$, and temperature on the avermectin B1b production system. These three factors were used for subsequent statistical optimization through ANN.

Comparison of different ANN. Total 40 ANNs were constructed and tested for the optimal model, based on the best statistical values. The comparison of ANNs is given in Table 2. Value of R^2 greater than 0.9 showed the accuracy of ANN model and reflected that there is a very good fit between the observed values and the predicted values. The model's significance can also be observed by higher value of *adjusted* R^2 (Elibol, 2004). The integrity and accuracy of selected model is supported by R^2 and *adjusted* R^2 in the present studies. The values of R^2 higher than 0.9 for a regression model represent the high correlation (Chen et al., 2009). The ANNs numbered 12, 13, 23, 31, 32, and 38 have R^2 value higher than 0.9, and the best value is exhibited by ANN 38. *Adjusted* R^2 value is higher than 0.9 only for the ANN 38. Values of ADD and RMSE are also important parameters for the model validation. For an optimal model, the value of ADD should be as small as possible, and the value of RSME should be close to zero. In the present study, the ANN 38 gave best calculated values for ADD and RSME (-29.85 and 1.076, respectively). Therefore, ANN 38 was selected as best ANN from all constructed ANNs for the data.

The selected ANN. Value of $R^2 = 0.989$ was optimum for the selected ANN model, showing that only 1.1% total variations are not explained by the given model. In the study, the precision of the selected model used for optimization purpose is well exhibited as shown from the small difference between the values of R^2 and *adjusted* R^2 (Table 2). High value of R^2 obtained through selected network supported the model accuracy. The production values predicted by this model are statistically very close to the observed values.

Designing of network topology is the main objective when applying the ANN modeling. Different designing parameters including the choice of activation function, training algorithms, training parameters, number of hidden layers, number of neurons

in each hidden layer, initial weights, and training durations also affected the optimization process appreciably. Normally one hidden layer with large number of hidden neurons imparts precise estimations to any continuous nonlinear function (Hornik et al., 1989). Topology of the selected network in the present study consisted of three layers (3:7:1), an input layer consisted of three fermentation variables, a middle hidden layer of seven neurons, and one output layer of avermectin B1b production. Activation levels of neurons are represented by different shades (Fig. 1).

Table 2 Comparison of prediction capabilities of different ANNs for the avermectin B1b production from *Streptomyces avermitilis* DSM 41445

Sr. No.	R^2	Adjusted R^2	AAD	RMSE
1	0.741	0.5079	-16.30	4.45
2	0.657	0.3426	-15.10	4.80
3	0.525	0.0975	-12.30	5.50
4	0.712	0.4528	-15.10	3.69
5	0.765	0.5535	-18.58	4.30
6	0.562	0.1678	-12.35	5.40
7	0.867	0.7473	-20.78	2.75
8	0.763	0.5497	-18.60	4.25
9	0.825	0.6675	-18.35	2.68
10	0.651	0.3369	-15.30	3.80
11	0.824	0.6656	-18.30	2.69
12	0.900	0.8100	-20.22	2.01
13	0.915	0.8385	-20.38	2.05
14	0.781	0.5839	-18.30	4.40
15	0.849	0.7131	-19.45	2.99
16	0.682	0.3958	-16.84	3.98
17	0.799	0. 6181	-17.99	4.96
18	0.763	0.5397	-18.60	4.25
19	0.852	0.7188	-19.44	3.01
20	0.863	0.7397	-19.40	3.98
21	0.841	0.6979	-19.35	2.47
22	0.876	0.7644	-18.55	3.98
23	0.911	0.8309	-20.57	2.045
24	0.769	0.5611	-18.40	4.28
25	0.872	0.7568	-18.50	3.75
26	0.736	0.4984	-15.90	3.65
27	0.791	0.6029	-18.66	4.50
28	0.842	0.6998	-19.33	3.46
29	0.798	0.6162	-18.69	4.45
30	0.832	0.6808	-19.12	3.58
31	0.939	0.8845	-20.76	2.26
32	0.936	0.8784	-20.98	2.33
33	0.845	0.7055	-19.35	2.01
34	0.793	0.6067	-17.91	4.80
35	0.775	0.5725	-16.65	4.00
36	0.842	0.6998	-19.30	2.01
37	0.693	0.4167	-15.60	3.35
38	**0.9752**	**0.95288**	**-29.85**	**1.076**
39	0.753	0.5307	-15.60	3.30
40	0.735	0.4965	-15.98	3.35

Optimization of fermentation variable using the selected ANN. The optimum levels of yeast extract, $MgSO_4 \cdot 7H_2O$, and temperature predicted by the selected ANN were 16.0, 0.5 g/L, and 32°C respectively. The ANN predicted optimum avermectin B1b production was 1095.556 mg/L at predicted optimum levels of three factors. The verification experiments were carried out using the predicted optimum levels of three variables while keeping the other variables constant. Avermectin B1b production obtained at optimum conditions was 1050.756±0.789 mg/L, which was in good agreement with the predicted value; thus showing the prediction capability of the selected ANN model (Table 3).

In most fermentations, process optimization leads to a prominent increase in production (Curdova et al., 1989; Zhinan and Peilin, 1999; Hong et al., 2009). In the present study, about 1.5 fold increase (150%) in avermectinB1b yield was observed in ANN-optimized medium as compared to the non-optimized SM2 medium from *Streptomyces avermitilis* DSM 41445 (Table 3). Zhinan and Peilin (1999) reported 22.6% increase in the production of

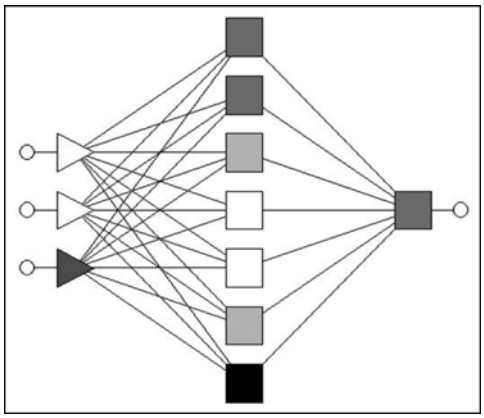

Fig. 1 Topology of neural networks for avermectin B1b production. Triangles represent the input (neurons added for ANN processing): yeast extract, $MgSO_4 \cdot 7H_2O$, and temperature. Squares represent the hidden and the output layers (neurons generated during ANN processing). Small open circles represent the input and output layers (the neurons that can be observed in the form of numerical values).

Table 3 The predicted optimal levels and avermectin B1b production obtained from *Streptomyces avermitilis* DSM 41445through optimization using ANN

Sr. no.	Method	Yeast extract X1 g/L	$MgSO_4.7H_20$ X2 g/L	Temp. X3 °C	Avermectin B1b Production Predicted by ANN (% diff.[a])	Avermectin B1b obtained from Experimental data
1	ANN predicted optima	16.0	0.5	32	1095.556	1050.756±0.789
2	Optima before optimization	2.0	0.34	33	745.9921 (-7)	696.86375

avermectinB1a (380 mg/L) from *Streptomyces avermitilis* IP 842 in an optimized medium as compared the non-optimized CSYC medium. About 1.45 fold increase was observed (Hong et al., 2009) in the production of avermectin B1a from *Streptomyces avermitilis* 14-12A in RSM optimized medium as compared to the non-optimized medium. In the present study, the increase in yield is better than the other earlier studies after optimization.

The interaction effects of factors are mentioned by 3D response surface (contour graphs). The interaction plots for avermectin B1b are represented in Figs. 2, 3, and 4. From the Fig. 2, it is observed that yeast extract and $MgSO_4 \cdot 7H_2O$ have a positive interaction effect on avermectin yield. With increasing the concentration of yeast extract and $MgSO_4 \cdot 7H_2O$, the avermectin yield is increased, and optimum is achieved close to their maximum concentrations. It was also observed that Yeast extract has a more prominent effect on the production of avermectin B1b. From the surface plot of yeast extract and temperature, it is observed that yeast extract has positive effect on yield, and temperature up to 32°C has a positive effect and above this temperature negative effect is observed. Therefore, the optimum avermectin B1b yield is located near the maximum levels of yeast extract and at the middle level of temperature. Similar effects are observed for temperature and $MgSO_4 \cdot 7H_2O$ on the avermectin B1b yield.

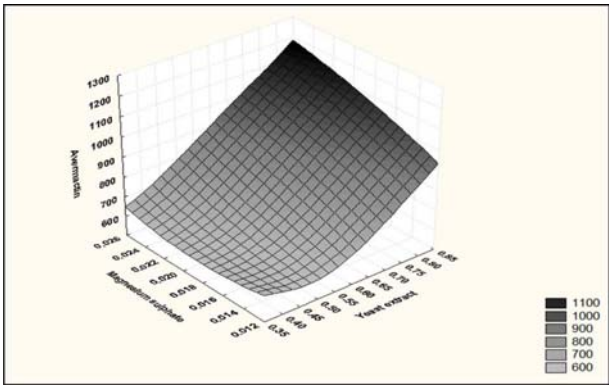

Fig. 2 Surface plot obtained optimization using ANN for the combined effect of yeast extract and $MgSO_4 \cdot 7H_2O$ on avermectin B1b production by keeping all other variables constant.

Sensitivity analysis. Ratio and ranking of each variable was calculated from ANN sensitivity analysis showing the network sensitivity for a particular variable. The variables with ratio equal to one or less than one were not considered as significant (Lou and Nakai, 2001). Predictive error ratio for each input variable was calculated according to their respective degree of validity, representing the contributions of different variables in predicting the outcome. In the present study, ANN sensitivity analysis showed that yeast extract (Ratio = 1.322824) has maximum significant effect on the production of avermectin B1b from *S. avermitilis* DSM 41445, followed by temperature (Ratio = 1.280772), and $MgSO_4 \cdot 7H_2O$ (Ratio = 1.072251) was found the least significant factor (Table 4).

Maximum effect was observed from yeast extract on the production of avermectin B1b from *S. avermitilis* DSM 41445. Yeast extract was used as a nitrogen source in the medium. Nitrogen source plays a vital role in the growth of microorganisms, as it provides nitrogen for proteins, which are the major part of all

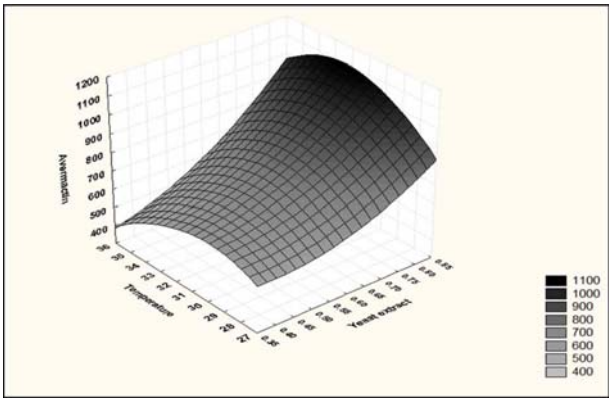

Fig. 3 Surface plot obtained optimization using ANN for the combined effect of yeast extract and temperature on avermectin B1b production by keeping all other variables constant.

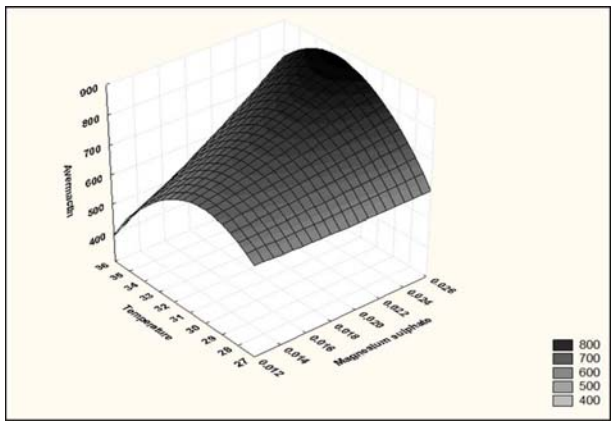

Fig. 4 Surface plot obtained optimization using ANN for the combined effect of $MgSO_4 \cdot 7H_2O$ and temperature on avermectin B1b production by keeping all other variables constant.

Table 4 ANN Sensitivity analysis for optimization of avermectin B1b production from *Streptomyces avermitilis* DSM 41445

Parameter	Yeast extract (X1) g/100 mL	$MgSO_4 \cdot 7H_2O$ (X2) g/100 mL	Temperature (X3) °C
Ratio	1.322824	1.072251	1.280772
Rank	1.000000	3.000000	2.000000

Ratios are values given by ANN as a result of sensitivity analysis to input variables. Rank is the order according to the ratios.

living organisms. In addition, it becomes more important when the target product is a protein as in the present case. Suutari et al. reported (2002) that in fermentation medium the nitrogen source, especially the yeast extract, directly affects the mycelium growth. It was also observed (Tuncer et al., 2004; Lazim et al., 2009) that for *Streptomyces* strains, nitrogen source is crucial for production of secondary metabolites. Whereas Hong et al. (2009) reported the most prominent effect from yeast extract on avermectin B1a production by *Streptomyces avermitilis* 14-12A was found using RSM.

The second important variable affecting the production of avermectin B1b from *S. avermitilis* DSM 41445 was the fermentation temperature with 32°C optimum level, as predicted by ANN. Da-silva et al. (2012) reported that during the production of secondary metabolites from *Streptomyces*, temperature, incubation period and culture medium play a vital role. Furthermore, Viana et al. (2010) revealed that during the production of clavulanic acid from *Streptomyces* strain No. 325, temperature played the most significant role. Significant effect of temperature for biosynthesis of validamycin-A from *Streptomyces hygroscopicus* 5008 between 35 and 37°C was observed by Liao et al. (2009). In another study (Oskay, 2011), 30°C was found as the optimum temperature for the avermectin production using *Streptomyces* sp. The optimum level of temperature in the present work using ANN method, is close to the optimum level of temperature observed in other studies.

The last factor effecting the production of avermectin B1b from *Streptomyces avermitilis* 41445 in the present study was the $MgSO_4 \cdot 7H_2O$ with 0.5 g/L, as predicted by ANN. Stanbury (2000) reported that production of secondary metabolites is greatly affected by addition of different metals specially the manganese, cobalt, iron, and zinc in the fermentation medium. The effect of different metals on avermectin B1a production from *S. avermitilis* IP 842 has been studied (Zhinan and Peilin, 1999). In the investigations, Mg^{+2} has been found to inhibit the production of avermectin B1a. In the present work, Mg was found to have appositive effect on avermectin production. However, the concentration used was much lower than that of the former study. This might be the possible reason for the different effects observed by the presence of Mg on avermectin production in the former studies (Zhinan and Peilin, 1999).

The present study showed that ANN predicted values are very close to the experimental values. ANN predicted the yeast extract to be the most important factor affecting the production of avermectin B1b from *S. avermitilis* DSM 41445. About 1.5 fold increase in avermectin B1b yield was observed in ANN-optimized medium as compared to the non-optimized medium. Therefore, ANN is a good technique for the optimization of avermectin B1b production from *S. avermitilis* DSM 41445.

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