

Optimization of medium constituents for *Cephalosporin C* production using response surface methodology and artificial neural networks

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Abstract

Artificial neural networks (ANN) and response surface methodology (RSM) were used to build a model to describe the effects of four independent variables (moisture content, concentrations of glucose, ammonium nitrate and methionine) on the yield of cephalosporin C (CPC) from *Acremonium chrysogenum* under solid state fermentation. The respective uses of RSM and ANN were found to be effective in locating the optimum conditions within the range fixed from the preliminary runs. When compared with the predictions given by RSM, ANN was found to be superior in describing the fermentation process for the production of CPC. When a global optimization routine was employed to optimize the equation resulted from the neural networks, the optimum predicted antibiotic yield was found to be 29.4 mg/g which is 14.8 % higher than the optimum value obtained from preliminary runs, and 9.2 % higher than value obtained from Box-Behnken design of RSM.

Keywords: Optimization, Cephalosporin C, *Acremonium chrysogenum*, Solid state fermentation, Artificial neural networks, Response surface methodology

Introduction

Cephalosporins are bactericidal agents that disrupt the synthesis of the peptidoglycan layer of bacterial cell walls, which eventually kills the bacteria. Cephalosporins are used to treat a wide variety of bacterial infections, such as respiratory tract infections (pneumonia, strep throat, tonsillitis, and bronchitis), skin infections and urinary tract infections. Cephalosporin-C (CPC), an antibacterial compound, was first isolated by Newton and Abraham (1955). CPC, the starting material used for the synthesis of various cephalosporins is

produced by aerobic fermentation using different strains of *Cephalosporium acremonium* and by streptomyces species like *Streptomyces clavuligerus*. CPC has a weak antibacterial activity, but the modification of its side chain generates semi-synthetic cephalosporins having diversified antibacterial activity. The mold is renamed as *Acremonium chrysogenum* (Brakhage 1998; Demain and Zhang 1998; Silva et al. 1998; Schmitt et al. 2004; Tollnick et al. 2004).

The traditional one-factor-at-a-time technique used for optimizing a variable system is not only time consuming but also often easily confuses the alternative effect between the components and also it requires a larger number of experiments to determine the optimum levels. The drawbacks can be eliminated by optimizing all the effecting parameters collectively by response surface methodology (RSM) which includes factorial design and regression analysis. Recently, a number of statistical experimental designs with RSM have been employed for optimizing production from microorganisms (Adinarayana et al. 2005; Veera et al. 2006; Sangok and Makoto 2005).

An artificial neural network (ANN) is a superior and more accurate modeling technique when compared to the RSM method, as it represents the non linearities in a much better way (Jayathi et al. 2004). However, surface and contour plots provide a good way to visualize the interactions between the independent and dependent variables. Therefore, both techniques are often used in unison for predicting optimum conditions for the production of various microbial products.

Owing to the importance of this particular industrial fermentation, more efforts are still going on to improve the CPC fermentation process especially from the stand point of savings and production cost. It is known from the preliminary studies that CPC production by microorganisms is greatly influenced by medium constituents. We report here the optimization of CPC production under solid state fermentation from *Acremonium chrysogenum* as a result of interactive effects of four variables (i.e., moisture content, concentrations of glucose, ammonium nitrate and methionine) using RSM and ANN.

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Materials and Methods

Microorganism

The CPC producing *Acremonium chrysogenum* NCIM 893 obtained from National Collection of Industrial Microorganisms, Pune, was used in the present study.

Seed inoculum

Inocula were prepared by transferring 5 ml of suspension prepared from a 72-hour-old slant culture into 250 ml Erlenmeyer flasks containing 45 ml sterile inoculum medium. The composition of the inoculum medium was (g/l): soluble starch 15.0, yeast extract 4.0, dipotassium hydrogen phosphate 1.0, magnesium sulphate 1.0 and pH 7.0. The flasks were incubated on a rotary shaker at 220 rpm at 30°C for 2 days.

Solid state fermentation

10 g of substrate was placed in 250 ml Erlenmeyer conical flasks with 1 ml of salt solution, after which distilled water was added to adjust the final moisture content to 60 %, mixed thoroughly and autoclaved at 121°C for 15 min. The composition of salt solution was (g/l): dipotassium hydrogen orthophosphate 0.5, magnesium sulphate 0.5, ferrous sulphate 0.01 and sodium chloride 0.5. After cooling the flasks to room temperature, the flasks were inoculated with 5% inoculum level (10^8 spores/ml). The contents were mixed thoroughly and incubated at 30° C in a slanting position to provide maximum surface area (Adinarayana et al. 2003).

Substrate

Sugarcane bagasse was collected from the local market (Visakhapatnam, India) and dried in the open air sunlight for 2 days. Then the bagasse was ground to obtain a mean particle size and stored for further use in an airtight container.

Experimental design and CPC production

The production of CPC antibiotic using *Acremonium chrysogenum* NCIM 893 under solid state fermentation was studied with temperature and pH being fixed at 30°C and 6.5 respectively. The preliminary studies have been carried out with the following variables: selection of substrate, incubation period, salt solution concentration, inoculum level, moisture content, various carbon and nitrogen sources, and methionine. The optimum levels for CPC production by *Acremonium chrysogenum* strain in solid state fermentation were obtained by single factor optimization by conducting the experiments in an 250 ml Erlenmeyer flask / 250 ml Erlenmeyer flasks (as described earlier).

In the next stage, both RSM and ANN were used to study the interactive effects of those variables which were selected from the data obtained in the preliminary experiments for improving total CPC production. Experiments were conducted in triplicate and results were the average of the three default trails.

Response Surface Methodology (RSM)

RSM combines statistical experimental designs and empirical model building by regression for the purpose of process optimization. The relationships among the variables were expressed mathematically in the form of a quadratic polynomial model, which gave the response as a function of relevant variables. This work was based on the Box-

Behnken design utilized to obtain the experimental data, which would fit an empirical, full second-order polynomial model representing the response surface over a relatively broad range of parameters. RSM had not only been used for optimization of culture parameters in the fermentation process but also for studying the combined effects of media components. The production of CPC was optimized using Box-Behnken design (Box and Behnken 1960), when CPC production is related to independent variables by a response equation

$$Y = f(x_1, x_2, x_3, \dots, x_k) \quad (1)$$

The true relationship between Y and x_k may be complicated and, in most cases, it is unknown; however a second-degree quadratic polynomial can be used to represent the function in the range of interest (Annadurai and Sheeja 1998),

$$Y = R_0 + \sum_{i=1}^k R_i X_i + \sum_{i=1}^k R_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=2}^k R_{ij} X_i X_j + \epsilon \quad (2)$$

where $X_1, X_2, X_3 \dots X_k$ are the independent variables which affect the response Y, R_0, R_i, R_{ii} and R_{ij} ($i=1-k, j=1-k$) are the known parameters, ϵ is the random error. A second-order model is designed such that variance of Y is constant for all points equidistant from the center of the design.

The Box-Behnken design helps in investigating linear, quadratic and cross-product effects of these factors each varied at these levels and also includes three center points for replication. The design is performed because relations for experimental combination of the variables are adequate to estimate potentially complex response functions. The 'STATISTICA' software was used for regression and graphical analysis of the data obtained. The optimum values of the selected variables were obtained by solving the regression equation and also by analyzing the response surface plots.

Artificial Neural Networks (ANN)

An ANN is a biologically inspired computational model formed from hundreds of single units, artificial neurons, connected with coefficients (weights) which constitute the neural structure. They are also known as processing elements (PE) as they process information. Each PE has weighed inputs, transfer function / transfer functions and one output. ANN analysis is quite flexible as regards to the amount and form of the training (experimental) data which makes it possible to use more informal experimental designs than with statistical approaches. Also, neural network models might generalize better than regression models since regression analyses are dependent on predetermined statistically significant levels. This means less significant terms are not included in the model. ANN uses all the data potentially, making the models more accurate. ANN along with RSM has been used to optimize the culture parameters for protease production from an isolated *Pseudomonas* sp. (Jayathi et al. 2004).

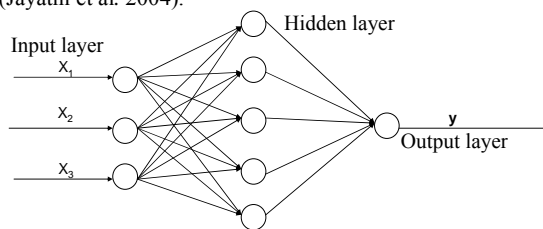


Figure 1: The neural network topology with single hidden layer

Usually a neural network in its basic form is composed of several layers of neurons, there being one input layer, one output layer and at least one hidden layer (Fig. 1). The use of at least one hidden layer enables the ANN to describe nonlinear systems. A problem in constructing ANN is to find the optimal number of hidden neurons. W_{ij} is the weight-connection to neuron j from neuron i , x_i denotes the input values and $bias_j$ is the bias of neuron j . The activation of the j th neuron (Net_j) is defined as the sum of the weighted input signal to that neuron:

$$Net_j = \sum_i W_{ij}x_i + Bias_j \quad (3)$$

This activation is transformed to the neuron output by a transform function. Different ANN classes use different definitions of the activation function. The most common transform function in back-propagation neural networks (BNNs) is a sigmoidal function:

$$y_j = \frac{2}{1 + e^{-Net_j}} - 1 \quad (4)$$

Each neuron in the input layer is connected to each neuron in the hidden layer and each neuron in the hidden layer is connected to each neuron in the output layer to produce the output vector. Information in BNNs is stored as weights, which are connections between neurons in successive layers and as bias values (neuron activation threshold). The neural network used in this work is the feed-forward, back-propagation neural network type, most often used in analytical applications. Information from various sets of inputs is fed forward through the BNNs to optimize the weight between neurons, or to 'train' it. The error, or bias, in prediction is then propagated through the system and the inter-unit connections are changed to minimize the error in the prediction. This process is continued with multiple training sets until the error is minimized across many sets.

During training, neural techniques need to have some way of evaluating their own performance. Since they are learning to associate the inputs with outputs, evaluating the performance of the network on the training data may not produce the best results. If a network is left to train for too long, it will over train and will lose the ability to generalize. Thus, two types of data sets are used - training data: used to train network and test data: used to monitor the neural network performance during training. The MATLAB version 7.0 was used for neural network program.

Antibiotic extraction

At the end of fermentation, the biomass was treated with 50 ml of distilled water and agitated thoroughly on a magnetic stirrer for 30 min. The whole contents were filtered through muslin cloth. The residue was again treated with another 50 ml of distilled water, in the same way and filtered. The filtrates were pooled, centrifuged and the clear supernatant was used as the antibiotic source.

Antibiotic assay

The supernatant was used for the estimation of CPC content by microbiological assay using *Alcaligenes faecalis* as test organism (Grove and Randall 1955; Pharmacopoeia of India 1985). The standard cephalosporin C Zinc salt (Sigma Chemicals, USA) was used to construct the calibration curve. All experiments were conducted in triplicate and the mean of the three is represented as number of units of antibiotic produced per gram of dry sugarcane bagasse.

Estimation of moisture content

The moisture content of the sugarcane bagasse was estimated by drying 10 g of sugarcane bagasse to constant weight at 105°C and the dry weight was recorded. To fix the initial moisture content of the solid medium, sugarcane bagasse was soaked with the desired quantity of water. After soaking, the sample was again dried as described above and percent moisture content was calculated as follows,

Percent of moisture content (initial) of solid medium = (wt. of the sugarcane bagasse - dry wt.) x 100 / dry wt.

Results and discussion

A submerged culture was used for the production of CPC from *Acremonium chrysogenum*. Preliminary experiments on CPC from the above strain indicated that the most important factors for the production were moisture content, the concentrations of glucose, ammonium nitrate and methionine. Hence these factors were considered as the independent variables and their effects on CPC production were studied using a Box- Behnken design of RSM and back propagation of ANN.

Table 1: Range of independent variables in the experimental design

Variables	Coded levels		
	-1	0	+1
Moisture Content (% v/w) X_1	60	80	100
Glucose (%w/w) X_2	1.0	3.0	5.0
Ammonium nitrate (% w/w) X_3	0.5	1.0	1.5
Methionine (% w/w) X_4	0.5	1.0	1.5

The range and levels of experimental variables investigated in this study were presented in Table 1. The central values (zero level) chosen for experimental design were: moisture content (80 %v/w) - (X_1), glucose (3.0 %w/w) - (X_2), ammonium nitrate (1.0%w/w) - (X_3) and methionine (1.0 %w/w) - (X_4). The results of Box- Behnken design experiments and ANN for studying the effects of four independent variables, viz., moisture content, concentrations of glucose, ammonium nitrate and methionine, on CPC production are presented in Table 2 along with the predicted and observed responses. The application of RSM (Box *et al.* 1978; Khuri and Cornell 1987) yielded the following regression equation, which is an empirical relation ship between the antibiotic yield and test variables in coded units.

Table 2: The Box-Behnken design matrix employed for four independent variables in coded units along with observed values and predicted values of both RSM and ANN

Run No.	X_1	X_2	X_3	X_4	CPC yield (mg/ml)		
					Observed values	Predicted by RSM	Predicted by ANN
1	-1	-1	0	0	12.40	12.92	12.40
2	-1	-1	0	0	18.46	19.03	18.46
3	-1	1	0	0	21.47	21.31	21.47
4	1	1	0	0	23.90	23.79	23.90
5	0	0	-1	-1	15.10	14.27	15.10
6	0	0	1	-1	22.2	22.75	22.20
7	0	0	-1	1	22.00	21.86	22.00
8	0	0	1	1	22.40	23.64	22.40
9	0	0	0	0	25.50	25.58	25.50
10	-1	0	0	-1	14.20	14.83	14.20
11	1	0	0	-1	20.40	21.28	20.40
12	-1	0	0	1	21.40	21.22	21.40
13	1	0	0	1	23.30	23.37	23.30
14	0	-1	-1	0	11.20	11.99	10.03
15	0	1	-1	0	21.60	22.32	21.60
16	0	-1	1	0	20.90	20.88	19.77
17	0	1	1	0	23.80	23.70	23.80
18	0	0	0	0	25.60	25.58	25.58
19	-1	0	-1	0	15.10	14.96	15.10
20	1	0	-1	0	23.70	23.26	23.70
21	-1	0	1	0	24.80	24.10	24.80
22	1	0	1	0	25.40	24.40	24.91
23	0	-1	0	-1	12.85	11.85	12.85
24	0	1	0	-1	20.60	20.35	20.60
25	0	-1	0	1	18.90	18.01	18.90
26	0	1	0	1	22.80	22.66	22.80
27	0	0	0	0	25.65	25.58	25.58

$$Y = 18.07 + 2.14 X_1 + 3.28 X_2 + 2.56 X_3 + 2.12 X_4 + 1.08 X_1X_1 + 2.06 X_2X_2 + 0.86 X_3X_3 + 1.61 X_4X_4 - 2.00 X_1X_3 - 1.07X_1X_4 - 1.87 X_2X_3 - 0.96 X_2X_4 - 1.67 X_3X_4 \quad (5)$$

Where Y= CPC yield, X_1 , X_2 , X_3 and X_4 are the coded values of the moisture content, concentrations of glucose, ammonium nitrate and methionine, respectively.

Table 3: Analysis of variance (ANOVA) for the quadratic model

Source of variation	Sum of squares	Degrees of freedom	Mean square	F-value	p-value
$X_1 + X_1X_1$	80.7	2	40.4	52.9	0.000001
$X_2 + X_2X_2$	220.9	2	110.5	144.7	0.000000
$X_3 + X_3X_3$	94.8	2	47.4	62.1	0.000000
$X_4 + X_4X_4$	109.5	2	54.8	71.7	0.000000
X_1X_2	3.3	1	3.3	4.3	0.059910
X_1X_3	16.0	1	16.0	20.9	0.000635
X_1X_4	4.6	1	4.6	6.05	0.030000
X_2X_3	14.1	1	14.1	18.42	0.001047
X_2X_4	3.7	1	3.7	4.85	0.047862
X_3X_4	11.2	1	11.2	14.7	0.002378
Error	9.2	12	0.76		
Total SS	492.9	26			

$R^2 = 0.9814$

Statistical testing of the model was done by Fisher's statistical test for analysis of variance (ANOVA) and the results are shown in Table 3. The calculation of regression analysis gives the value of the determination coefficient ($R^2 = 0.9814$) which indicates that only 1.86 % of the total variations are not explained by the model and the F-value of 400.68 indicates that CPC production from *Acremonium chrysogenum* has a good model fit due to the high values of R^2 and F. The p-values are used as a tool to check the significance of each coefficient, which also indicate the interaction strength between each independent variable. The smaller the p-values, the more evidence indicates that support for rejecting the null hypothesis (Cui et al. 2006).

The model equation (5) indicated that glucose concentration (X_2) had a significant effect ($p < 0.01$) on Y and it had the largest coefficient followed by ammonium nitrate concentration (X_3), moisture content (X_1) and methionine concentration (X_4). The statistical analysis of the design shows a high precision of the

polynomial model that reflects the high degree of fitting between the predicted and the experimental data. This great similarity between the predicted and the observed results reflects the accuracy and applicability of the Box- Behnken model in the optimization process / the optimization processes.

The relationship between coded variables and responses can be better understood by examining the series of surface plots (Fig. 2). These response surfaces display the variation of two factors while the third is kept at the optimum level.

Table 4: Weights and biases of the neural networks

Training Function	Weights and biases of the neural networks						
	w1			w2		b1	b2
trainlm	-3.8	-3.16	-3.88	-3.31	-5.27	-0.50	16
	0.93	-2.24	-0.904	0.086	-3.93	-2.15	
	7.64	2.06	-10.0	4.845	-0.62	-0.045	
	-3.9	2.483	2.823	2.461	-0.65	3.132	
	-6.0	-3.84	-6.821	0.497	2.056	3.137	
	-6.7	8.261	4.412	-7.66	0.671	-5.93	
	-0.1	-2.93	0.48	-5.81	-1.56	-1.97	
	-1.7	-5.62	-4.59	5.304	0.252	-4.24	
	9.18	-0.14	-3.943	-4.597	1.353	2.036	

The analysis revealed a maximum CPC yield of 26.92 mg/g which was 5.15 % more than the value (25.6 mg/g) obtained with the initial experiments, at the points where moisture content, concentrations of glucose, ammonium nitrate and methionine are 84.32 (%w/w), 3.53 (%w/w), 1.9 (%w/w) and 1.64 (%w/w), respectively. The final experiment repeated at the optimal settings of the process variables produced antibiotic activity of 26.5 mg/g which was quite close to the optimal value predicted by the Box-Behnken design (Table 5).

Table 5: The predicted and experimental CPC production values at preliminary and optimized conditions

S.No.		X_1	X_2	X_3	X_4	CPC Yield (mg/ml)	
						Prec	Exp
1	Prelim. Studies	80.00	3.00	1.50	1.50	-	25.6
2	RSM	84.32	3.53	1.90	1.64	26.9	26.5
3	ANN	77.14	4.30	1.53	1.32	29.4	29.18

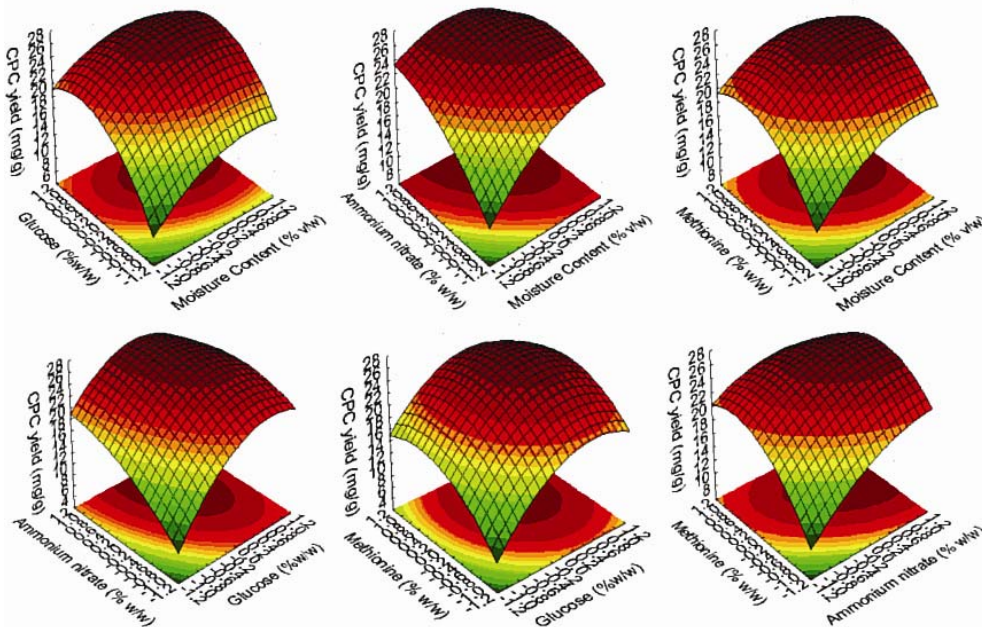


Fig 2: The surface plots describing the effect of four independent variables on CPC yield

Further, ANN methodology was applied to provide a non-linear mapping between input variables (moisture content, concentrations of glucose, ammonium nitrate and methionine) and the output variable (CPC yield) for the runs reported in Table 2. The type of ANN chosen was the back propagation network having a feed-forward structure. The simulated values of the response (CPC yield) are listed in the last column of Table 2.

The configuration of the neural network developed in this work (a 4-9-1 structure: four input neurons- nine neurons in hidden layer- one output neuron) was determined by trail and error, and the topology of network is shown in Fig. 3.

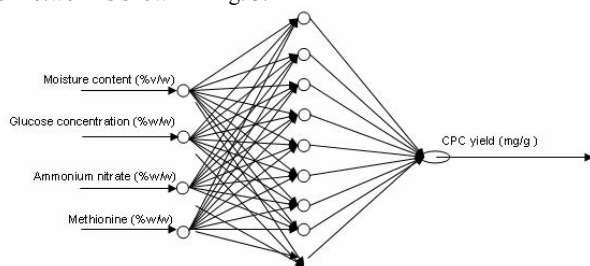


Figure 3: The neural network topology with single hidden layer having nine neurons.

The transfer functions used in the neural networks are 'transig' and 'purelin' at the hidden layer and output layer respectively. 'Newff' function is used for the training of the neural networks. The training function 'trainlm' is used in this work. The following equation is the outcome of the neural network training, relating the input variables (x_1, x_2, x_3 and x_4) to the output variable, y , in terms of weights and biases.

$$y = w_2 * \left(\frac{2}{1.0 + e^{-2/(w_1 * x^v + b_1)}} - 1 \right) + b_2 \quad (6)$$

Where w_1 and w_2 are the weights, b_1 and b_2 are the biases (Table 4). ' y ' is the predicted value from the neural network and x^v is the row vector of 4 independent variables (x_1, x_2, x_3 and x_4), while x^v represent the transpose of the vector with a dimension of (4×1) ; x_1, x_2, x_3 and x_4 represent moisture content, glucose concentration, ammonium nitrate concentration and methionine concentration respectively. For any given set of x_1, x_2, x_3 and x_4 , CPC yield (y value) can be predicted using the above equation.

Equation (6) represents the output, y (CPC yield), for the given set of independent variables represented in ' x^v ' when 'transig' was used as the transfer function in the hidden layer and 'purelin' was used as the transfer function in the output layer. The input data of the independent variables were transformed between -1 and +1 using the built-in function 'premnf' prior to neural network training while 'postmmx' was used to transform back the optimized set of independent variables into the original scale, after the global optimization method was applied. However, the output data was used without any transformation. The simulated values of CPC yield as predicted by equation (6) are in close agreement with those of the experimental values as evident from the values in the Table 2.

The optimum CPC yield (29.4 mg/g) predicted by ANN is higher than the value predicted by Box-Behnken design; however, it is 14.8 % higher than the value obtained from preliminary runs (25.6 mg/g), at the points where moisture content, concentrations of glucose, ammonium nitrate and methionine are 77.14 (%v/w), 4.3 (%w/w), 1.53 (%w/w) and 1.32 (%w/w), respectively. The final experiment repeated at the optimal settings of the process variables produced antibiotic activity of 29.18 mg/g which was closer to the optimal

value predicted by back propagation of ANN (Table 5). This demonstrates the superiority of ANN represented by equation (6) over the RSM represented by equation (5) in the fermentation medium. We also report for the first time the potential use and superiority of the artificial neural networks in combination with global optimization technique for optimizing the process variables for CPC production using sugarcane bagasse as the potential substrate.

Conclusions

This study compared the performance of the Box- Behnken design of RSM and back propagation of ANN in the estimation of fermentation performance parameters (moisture content, concentrations of glucose, ammonium nitrate and methionine) for CPC production from *Acremonium chrysogenum*. Both models provide quality predictions for the above four independent variables in terms of CPC production with ANN showing more accuracy in estimation. The superiority of ANN over multi-factorial approaches would make the estimation technique a very helpful tool for fermentation monitoring and control.

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