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Nutrients interaction investigation to improve *Monascus purpureus* FTC5391 growth rate using Response Surface Methodology and Artificial Neural Network

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ABSTRACT

Aims: Two vital factors, certain environmental conditions and nutrients as a source of energy are entailed for successful growth and reproduction of microorganisms. Manipulation of nutritional requirement is the simplest and most effectual strategy to stimulate and enhance the activity of microorganisms.
Methodology and Results: In this study, response surface methodology (RSM) and artificial neural network (ANN) were employed to optimize the carbon and nitrogen sources in order to improve growth rate of *Monascus purpureus* FTC5391, a new local isolate. The best models for optimization of growth rate were a multilayer full feed-forward incremental back propagation network, and a modified response surface model using backward elimination. The optimum condition for cell mass production was: sucrose 2.5%, yeast extract 0.045%, casamino acid 0.275%, sodium nitrate 0.48%, potato starch 0.045%, dextrose 1%, potassium nitrate 0.57%. The experimental cell mass production using this optimal condition was 21 mg/plate/12days, which was 2.2-fold higher than the standard condition (sucrose 5%, yeast extract 0.15%, casamino acid 0.25%, sodium nitrate 0.3%, potato starch 0.2%, dextrose 1%, potassium nitrate 0.3%).
Conclusion, significance and impact of study: The results of RSM and ANN showed that all carbon and nitrogen sources tested had significant effect on growth rate (*P*-value < 0.05). In addition the use of RSM and ANN alongside each other provided a proper growth prediction model.

Keywords: Growth rate, Monascus purpureus FTC5391, media optimization, response surface methodology, artificial neural network

INTRODUCTION

Certain environmental conditions and nutrients as a source of energy are essential for microorganisms to grow and reproduce. Microorganisms have adapted to the habitats most suitable for their requirements in the natural environment. The simplest and most efficacious strategy to increase the yield and productivity is manipulation of nutritional requirements.

Monascus spp. a nontoxic fungi was employed to produce fermented product for thousand of year in China (Wang and Lin, 2007; Yeh *et al.*, 2012). *Monascus* fermented product (MFP) as a natural nutraceutical supplement contains a wide range of useful compound such as polyunsaturated fatty acids, sterols, B-complex vitamins, flavonoids, pyrolinic compounds and monacolins with antioxidant properties (Kalaivani *et al.*, 2009; Wang and Lin, 2007). In addition, since solid MFP can be consumed directly after sterilization, down- streaming producer is not required and this can produce multiple therapeutic benefits with low cost. On the other hand, most of the beneficial secondary metabolite that is produced by microorganisms is intracellular. In order to produce such fermented product, enhancement of cultivation method for mass production of *Monascus* spp. cells should be taken into consideration.

Modeling and optimization are important aspects in the microorganisms development (Gougouli and

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Koutsoumanis, 2012; Seraman *et al.*, 2010). Conventional optimization method (single variable optimization) is not only time-consuming and tiresome but also unable to describe the complete effects of the parameters in the process, and ignores the interactions between physicochemical parameters. In addition, the conventional method may lead to misinterpretation of results (Bas and Boyaci, 2007a; Seraman *et al.*, 2010). Statistical methods, such as, response surface methodology (RSM) and artificial neural networks (ANN) are rapid and reliable methods, which may be use to overcome the problem in conventional methods via decreasing the total number of experiments, preparing short lists significant factors and process by regarding the reciprocal interactions among the variables and to give an estimate of the united effects of these variables.

RSM includes a comprehensive of mathematical and statistical techniques, which can be applied to explain the relationships between the response and the independent variables alone or in combination, in the processes. Hence, it is very useful not only for optimization but also for developing, processes improving, designing, evaluation and formulation of new products, as well as improvement of existing product designs (Myers and Montgomery, 1995). RSM has been successfully utilized to optimize the medium composition for secondary metabolite production (Seraman *et al.*, 2010) and to improve enzyme production (Ebrahimpour *et al.*, 2008). Although RSM is widely used for many processes, it may not be successful to be used in optimization and modeling of certain bioprocess (Bas and Boyaci, 2007b).

ANNs are methods that apply artificial learning tool for optimization (Basri *et al.*, 2007). ANN is inspired by the way biological nervous systems generate process information.

Pervious study revealed that the Hiroi-PDA, a modified medium was the most favorable for growth, in the case of *M. purpureus* strains (Ajdari *et al.*, 2011). In this study both RSM and ANN were employed to optimize the carbon and nitrogen sources of Hiroi-PDA medium in order to find the best combination of nutrients for high mass production and radial growth rate of locally isolated red pigment producer, *M. purpureus* FTC5391 (Musaalbakri *et al.*, 2005).

MATERIALS AND METHODS

Materials

Yeast extract, casamino acid, agar, and potato starch were purchased from Difco (Detroit, Mich). Other chemicals were purchased from Merck KGaA (Darmstadt, Germany).

Microorganism

The fungus, *M. purpureus* FTC5391, was isolated from local sources and maintained at the culture collection in Malaysian Agricultural Research and Development Institute. The culture was maintained on the PDA slants at 4 °C, and subcultured monthly.

Growth experiments

Small pieces of mycelium (2 mm^2) obtained from 7-day old PDA slant were located in the center of Petri dishes containing Hiroi-PDA medium that was used in the experimental design. This medium consisted of sucrose 100 (g/L), yeast extract 3 (g/L), casamino acid 5 (g/L), $NaNO_3$ 2 (g/L), KH_2PO_4 1 (g/L), $MgSO_4.7\ H_2O$ 0.5 (g/L), KCl 0.5 (g/L), $FeSO_4$ 0.01 (g/L), potato starch 4 (g/L), dextrose 20 (g/L), and agar 15 (g/L). All the experiments were performed in triplicate.

Analytical procedures

The growth of fungi was estimated by the determination of dry cell weight and the radial growth. For determination of dry cell weight, the method as described by Shin et al. (Shin *et al.*, 1998) was employed. In this method, the whole agar of the cultivation plate was mixed with 100 ml distilled water and boiled to dissolve the agar. The agar solution containing the fungal biomass was filtered through the dry Whatman filter paper No. 5 and the filter paper with the retained fungal cells was then dried in an oven at 90 °C for 24 h, until a constant dry weight was attained. Radial growth was estimated according to the method as described by Carvalho et al. (2005). The radius of each colony was measured by a ruler, from the center of the Petri dish, along two perpendicular axes (four measurements per dish).

Experimental design

A five-level-seven-factor CCRD was employed in this study, requiring 41 experiments. The fractional factorial design consisted of; 22 factorial, 14 axial and 5 central points (Table 1). The variables and their levels selected for the optimizations were: sucrose (0-10 g/100); yeast extract (0- 0.3 g/100); casamino acid (0- 0.5 g/100); sodium nitrate (0- 0.6 g/100); potassium nitrate (0- 0.6 g/100) ; dextrose (0- 2 g/100); and, potato starch (0- 0.4 g/100). The responses were cell mass production and radial growth. The experimental data [41 points include CCRD design (Table 1) and optimization data (Tables 2 and 3) were divided into three sets: the training set, testing set and validating set. All tests were performed in triplicate.

Response surface methodology analysis

Three steps have been defined to RSM performance: statistical experimental designed, estimating the coefficients in a mathematical model, and forecasting the response and checking the fitness of the model (Xin *et al.*, 2005). Design Expert version 6.06 (Stat Ease Inc. Minneapolis, USA) was applied to analyze experimental response data and followed by interpretation. CCRD design experimental data was employed for model fitting in RSM to find the best polynomial equation. Analysis of variance (ANOVA), a regression analysis and the plotting of response surface as three major analytical steps were performed to establish an optimum point for cell mass production and radial growth. For model testing, the RSM

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Table 1 : Experimental design used in the optimization of medium composition for of *M. purpureus* using RSM and ANN studies with seven independent variables

Sucrose	Yeast extract	Casamino acid	Sodium nitrate	Potato starch	Dextrose	Potasium nitrate	Radial growth (mm)	Dry cell weight (mg/plate)
7.31	0.22	0.37	0.16	0.29	1.46	0.44	37.5	0.11
7.31	0.22	0.13	0.44	0.11	1.46	0.16	39.5	0.109
7.31	0.22	0.13	0.44	0.29	0.54	0.44	38.5	0.078
7.31	0.08	0.37	0.44	0.11	1.46	0.44	39.5	0.062
2.69	0.22	0.37	0.16	0.11	1.46	0.16	36.5	0.125
7.31	0.08	0.13	0.16	0.29	0.54	0.16	37.5	0.102
2.69	0.22	0.13	0.44	0.29	0.54	0.44	38.5	0.111
7.31	**0.22**	**0.37**	**0.44**	**0.11**	**1.46**	**0.16**	**39**	0.098
7.31	0.08	0.37	0.16	0.11	0.54	0.44	39.7	0.09
2.69	0.08	0.13	0.16	0.29	1.46	0.16	36.5	0.034
2.69	**0.22**	**0.13**	**0.44**	**0.11**	**0.54**	**0.16**	**37**	0.09
7.31	0.22	0.37	0.16	0.29	0.54	0.16	39	0.074
7.31	**0.08**	**0.13**	**0.44**	**0.11**	**0.54**	**0.16**	37.5	**0.115**
2.69	0.08	0.37	0.16	0.11	0.54	0.44	39.5	0.044
2.69	0.22	0.13	0.16	0.11	1.46	0.44	38	0.063
7.31	**0.08**	**0.13**	**0.16**	**0.11**	**1.46**	**0.44**	**32**	**0.178**
2.69	**0.22**	**0.13**	**0.16**	**0.29**	**1.46**	**0.44**	**35**	0.055
7.31	0.08	0.13	0.44	0.29	1.46	0.44	37.5	0.084
2.69	**0.08**	**0.37**	**0.44**	**0.29**	**0.54**	**0.44**	38.5	**0.038**
2.69	0.08	0.37	0.44	0.29	1.46	0.16	39	0.04
2.69	**0.22**	**0.37**	**0.44**	**0.11**	**0.54**	**0.44**	37.5	**0.092**
2.69	0.08	0.13	0.16	0.11	0.54	0.16	38	0.068
0	0.15	0.25	0.3	0.2	1	0.3	38	0.084
10	0.15	0.25	0.3	0.2	1	0.3	38.5	0.111
5	0	0.25	0.3	0.2	1	0.3	37	0.162
5	0.3	0.25	0.3	0.2	1	0.3	38.5	0.118
5	0.15	0	0.3	0.2	1	0.3	38	0.15
5	0.15	0.5	0.3	0.2	1	0.3	39.5	0.11
5	0.15	0.25	0	0.2	1	0.3	37	0.118
5	0.15	0.25	0.6	0.2	1	0.3	38.5	0.092
5	0.15	0.25	0.3	0	1	0.3	34	0.081
5	0.15	0.25	0.3	0.4	1	0.3	39	0.18
5	0.15	0.25	0.3	0.2	0	0.3	38.5	0.135
5	0.15	0.25	0.3	0.2	2	0.3	39.5	0.112
5	0.15	0.25	0.3	0.2	1	0	35	0.142
5	0.15	0.25	0.3	0.2	1	0.6	38.5	0.116
5	*0.15*	*0.25*	*0.3*	*0.2*	*1*	*0.3*	*39.5*	*0.105*
5	*0.15*	*0.25*	*0.3*	*0.2*	*1*	*0.3*	*39.5*	*0.105*
5	*0.15*	*0.25*	*0.3*	*0.2*	*1*	*0.3*	*39.5*	*0.105*
5	*0.15*	*0.25*	*0.3*	*0.2*	*1*	*0.3*	*39.5*	*0.105*
5	*0.15*	*0.25*	*0.3*	*0.2*	*1*	*0.3*	*39.5*	*0.105*

ANN training set: normal, and italic (center points) numbers
ANN testing set: bold numbers

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Table 2 : Solution of optimum condition for cell mass production.

Sucrose	Yeast extract	Casamino acid	Sodium nitrate	Potato starch	Dextrose	Potassium nitrate	Actual production	ANN predicted production	RSM predicted production
2.5	0.045	0.275	0.48	0.045	1	0.57	0.21	0.20	0.2
5	0.11	0.47	0.12	0.24	0.933	0.57	0.165	0.161	0.162
3.5	0.02	0.5	0.52	0.36	0.8	0.12	0.199	0.198	0.2
7	0.15	0.5	0.15	0.32	1	0.12	0.195	0.198	0.198
4.5	0.28	0.375	0.27	0.0267	1.9	0.1	0.183	0.182	0.18

ANN R^2 = 0.89 RSM R^2 = 0.89
ANN AAD =1.95% RSM AAD = 2.05%

Table 3 : Solution of optimum condition for radial growth rate.

Sucrose	Yeast extract	Casamino acid	Sodium nitrate	Potato starch	Dextrose	Potassium nitrate	Actual production	ANN predicted production	RSM predicted production
5	0.24	0.05	0.1	0.06	0.2	0.06	41	40	41.2
6	0.195	0.175	0.24	0.14	1.33	0.15	39.8	39.6	39
5.5	0.2	0.275	0.45	0.187	1.3	0.09	39.5	39.7	42
6.19	0.16	0.23	0.33	0.24	0.8	0.27	41	42	39.5
3.5	0.09	0.15	0.51	0.16	2	0.08	40	39.6	39

ANN R^2 = 0.47 RSM R^2 = 0.73
ANN AAD =1.38% RSM AAD = 2.99%

model predictor values were compared with actual values obtained experimentally (Tables 4 and 5). Finally, the experimental values of predicted optimal conditions, which were taken based on RSM and ANN, were used as validating set and were compared with predicted values (Tables 2 and 3).

Artificial neural network analysis

A commercial ANN software, NeuralPower version 2.5 (CPC-X Software), was used to investigate the interaction effect of medium parameters effects on cell mass production and radial growth of *M. purpureus* FTC5391. The data achieved from the experimental design of CCRD was employed in ANN for modeling analysis. The experimental data based on the CCRD design were divided into training and testing sets. In ANN modeling, the replicates at the center point do not improve the prediction capability of the network with regards to the similar inputs (Bas and Boyaci, 2007). Therefore, the accuracy of the model was improved by using the mean of center points instead of 5 center points (Table 1, italic numbers). Among different combinations of parameters, 33 combinations were used for training of the network, and 4 remaining combinations were employed for the network testing (Basri *et al.*, 2007; Ebrahimpour *et al.*, 2008) (Table 1, bold numbers).

Technically, testing data should not be at the extreme levels. Multilayer full and normal feed forward connection types were used to predict the responses. Networks were trained by different learning algorithms including; incremental back propagation (IBP), batch back propagation (BBP), quickprob (QP), genetic algorithm (GA) and Levenberg-Marquardt algorithm (LM). The network architecture consisted of an input layer with seven neurons, an output layer with one neuron, and a hidden layer. To determine the optimal network topology, only one hidden layer was used and the number of neurons in this layer and the transfer functions of hidden and output layers (sigmoid, hyperbolic tangent function, Gaussian, linear, threshold linear and bipolar linear) were iteratively determined by developing different networks. Each ANN was trained until the network root of mean square error (RMSE) was lower than 0.001, and the average correlation coefficient (R) and average determination coefficient (DC) were equal to 1. Other ANN parameters were chosen as the default values of the software. Based on the IBP algorithm, first, weights values were chosen randomly, and then adjusted through a training process in order to minimize network error. Finally, in order to test the reliability of model achieved, experimental values of predicted optimal points were used as a validating set (Tables 2 and 3).

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Table 4 : Actual and predicted cell mass production by ANN and RSM models along with absolute deviation, coefficient of determination, R^2 and absolute average deviation, AAD

Actual production	ANN predicted production	ANN absolute deviation	RSM predicted production	RSM absolute deviation
0.11	0.102	0.072727	0.109	0.009091
0.109	0.108	0.009174	0.116	0.06422
0.078	0.089	0.141026	0.09	0.153846
0.062	0.071	0.145161	0.066	0.064516
0.125	0.123	0.016	0.125	0
0.102	0.115	0.127451	0.095	0.068627
0.111	0.121	0.09009	0.108	0.027027
0.098	0.099	0.010204	0.097	0.010204
0.09	0.094	0.044444	0.091	0.011111
0.034	0.039	0.147059	0.028	0.176471
0.09	0.088	0.022222	0.087	0.033333
0.074	0.092	0.243243	0.075	0.013514
0.044	0.051	0.159091	0.036	0.181818
0.063	0.081	0.285714	0.052	0.174603
0.055	0.043	0.218182	0.049	0.109091
0.084	0.084	0	0.077	0.083333
0.04	0.052	0.3	0.026	0.35
0.068	0.073	0.073529	0.072	0.058824
0.084	0.082	0.02381	0.089	0.059524
0.111	0.11	0.009009	0.114	0.027027
0.162	0.15	0.074074	0.161	0.006173
0.118	0.11	0.067797	0.118	0
0.15	0.15	0	0.15	0
0.11	0.107	0.027273	0.109	0.009091
0.118	0.118	0	0.115	0.025424
0.092	0.083	0.097826	0.088	0.043478
0.081	0.074	0.08642	0.081	0
0.18	0.164	0.088889	0.18	0
0.135	0.124	0.081481	0.134	0.007407
0.112	0.12	0.071429	0.112	0
0.142	0.128	0.098592	0.142	0
0.116	0.107	0.077586	0.115	0.008621
0.105	*0.12*	*0.142857*	*0.101*	*0.038095*
0.115	0.12	0.043478	0.115	0
0.178	0.175	0.016854	0.175	0.016854
0.038	0.031	0.184211	0.021	0.447368
0.092	0.089	0.032609	0.092	0

ANN training set R^2 =0.92
ANN training set AAD (%) =8.25
RSM R^2 = 0.99
RSM AAD (%) = 6.1
ANN testing set AAD = 6.92%

ANN training set: normal and italic (center points) numbers
ANN testing set: bold numbers
ANN testing set R^2 = 0.99

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Table 5 : Actual and predicted radial growth rate by ANN and RSM models along with absolute deviation, coefficient of determination, R^2 and absolute average deviation, AAD.

Actual growth rate	ANN predicted growth rate	ANN absolute deviation	RSM predicted growth rate	RSM absolute deviation
37.5	37.5	0	37	0.013333
39.5	39.5	0	39.5	0
38.5	38.5	0	38	0.012987
39.5	39.5	0	39.8	0.007595
36.5	36.5	0	36	0.013699
37.5	37.5	0	37.6	0.002667
38.5	38.5	0	38.5	0
39.7	39.7	0	39.4	0.007557
36.5	36.5	0	36.7	0.005479
39	39	0	38.8	0.005128
37.5	37.5	0	37.7	0.005333
39.5	39.5	0	39.6	0.002532
38	38	0	38	0
37.5	37.5	0	37.4	0.002667
38.5	38.5	0	38.7	0.005195
39	39	0	39.2	0.005128
37.5	37.5	0	37.6	0.002667
38	38	0	38.1	0.002632
38	38	0	38	0
38.5	38.5	0	38.6	0.002597
37	37	0	37	0
38.5	38.5	0	38.5	0
38	38	0	38.3	0.007895
39.5	39.5	0	39.6	0.002532
37	37	0	37	0
38.5	38.5	0	38.5	0
34	34	0	34.1	0.002941
39	39	0	38.9	0.002564
38.5	38.5	0	38.5	0
39.5	39.5	0	39.3	0.005063
35	35	0	35	0
38.5	38.5	0	38.5	0
39.5	*39*	*0.012658*	*39*	*0.012658*
39	39	0	39	0
37	38	0.027027	37	0
32	33	0.03125	32	0
35	36	0.057143	35	0

ANN training set R^2 =0.99
ANN training set AAD (%) = 0.00034
RSM R^2 = 0.99
RSM AAD (%) = 0.0035

ANN training set: normal and italic (center points) numbers
ANN testing set: bold numbers
ANN testing set AAD (%) =2.17 %
ANN testing set R^2 = 0.88

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Validation of the optimized condition

An analysis of the residuals ($y_{i\ exp}$ - $y_{i\ cal}$) to evaluate the predicted model relevance, is the key factor. This equation is used to estimate the capabilities of the techniques by the calculation of coefficient of determination (R^2), and absolute average deviation (AAD). R^2 and AAD were calculated by equations 1 and 2, respectively.

$$R^2 = 1 - \frac{\sum_{i=1-n}(model\ prediction_i - experimental\ value_i)^2}{\sum_{i=1-n}(model\ prediction_i - experimental\ value_i)^2} \quad \textbf{(1)}$$

$$\boldsymbol{AAD} = \left\{ \frac{(\sum_{i=1}^{p}(\frac{\left|y_{i,exp} - y_{i,cal}\right|}{y_{i,exp}}))}{p} \right\} \times \mathbf{100} \quad \textbf{(2)}$$

where $y_{i,exp}$ and $y_{i,cal}$ are the experimental and calculated responses, respectively. *p* and *n* is the number of the experimental run and experimental data, respectively. Comparison of the effectiveness of ANNs and RSM were performed using R^2 and ADD values. R^2 is a factor that showed the reduction amount in the variability of response obtained by using the repressor variables in the model. It is necessary to use AAD analysis as a direct method for describing the deviations, because R^2 alone is not a measure of the model's accuracy. Hence, the accuracy of the model would be better to check with evaluation of R^2 and AAD values together. Where R^2 must be closed to 1.0 and the AAD between the predicted and observed data must be as small as possible. The acceptable values of R^2 and AAD indicates that the model equation defines the true behavior of the system, and it can be used to interpolate the experimental domain (Bas and Boyaci, 2007).

RESULTS

Cell mass production and radial growth rate modeling using RSM

The experimental responses with the central composite rotary design (CCRD) are shown in Table 1. In the beginning, the 41 points and their achieved responses data of CCRD design were fitted to various models (linear, two factorial, quadratic and cubic) but their subsequent ANOVA showed that all models were unable to explain the effects of nutritional factors on the cell mass production and radial growth rate. Therefore, the backward elimination strategy was used followed by hierarchical terms addition to find the best model and solve this problem. In fact, backward elimination method verifies all of the predictors in the model, then the variable which is least significant (with the largest P-value) is removed and the model is refitted. Each subsequent step removes the least significant variable in the model until all remaining variables have individual P-values smaller than 0.05 (Basri *et al.*, 2007; Ebrahimpour *et al.*, 2008). Prediction of model equation is one of the steps involves in RSM optimization procedure that explains the influences of independent variables. In this study, finally modification turned the cubic equations (equations 3 and 4) to quadratic as normally used by several researchers (Bas and Boyaci, 2007; Lou and Nakai, 2001).

In the case of cell mass, the modified model was a quadratic model with four eliminated terms (Suc.dex, YE.PN, CA.Dex and Suc^2),and one additional (Suc.Ye.Ca) terms (equations 3).

Cell mass (mg/plate) = 0.75 - 0.2 Suc - 10.37 YE - 4.67 CA + 2.67 SN + 3.19 PS - 1.2 Dex + 2.2 PN + 1.71 YE2 + 0.45 CA2 + 0.72 PS2 + 0.02 Dex2 + 0.30 PN2 + 2.57 Suc.YE + 1.19 Suc.CA - 0.18 Suc.SN - 0.27 Suc.PS + 0.0367 Suc.Dex - 0.36 Suc.PN + 43.21 YE.CA - 0.59 YE.SN - 10.61 YE.PS - 4.8 YE.PN - 3.29 CA.SN - 7.27 CA.PS + 1.4 CA.Dex - 2.93 CA.PN + 6.22 SN.PS - 1.14 SN.Dex - 3.36 SN.PN + 0.84 PS.Dex - 3.94 PS.PN + 2.63 Dex.PN - 8.17 Suc.YE.CA **(3)**

Where Suc is sucrose, YE is yeast extract, CA is casamino acid, SN is sodium nitrate, PS is potato starch, Dex is dextrose and PN is potassium nitrate.

Based on ANOVA analysis results of RSM, with very small "model P-value" (< 0.0001) and acceptable "lack of fit P-value" (0.20) from the analysis of ANOVA and a suitable coefficient of determination (R^2 = 0.99) and adjusted coefficient of determination (R^2 adjusted = 0.99), the modified cubic polynomial model was highly significant and sufficient to represent the actual relationship between the response and the significant variables (Table 6).

In the case of radial growth rate, indeed, the modified model was a quadratic model with three eliminated terms (YE.Sn, YE.PN, and Ca^2), and one additional (Suc.YE.Ca) terms (equation 4).

Radial growth rate (mm) = 33.60 + 2.42 Suc + 193.03 YE - 67.26 CA - 64.76 SN - 88.71 PS + 22.88 Dex + 35.43 PN - 0.03 Suc2 - 52.14 YE2 - 13.03 SN2 - 60.58 PS2 - 24.15 PN2 - 39.30 Suc.YE - 9.54 Suc.CA + 5.92 Suc.SN + 8.33 Suc.PS - 1.02 Suc.Dex + 1.23 Suc.PN - 395.45 YE.CA + 189.32 YE.PS - 28.33 YE.Dex + 61.33 CA.SN + 249.14 CA.PS + 135.69 CA.PN - 35.51 SN.PS + 37.24 SN.Dex - 17.73 PS.Dex + 70.06 PS.PN - 69.166 Dex.PN + 90.77 Suc.YE.CA **(4)**

Where Suc is sucrose, YE is yeast extract, CA is casamino acid, SN is sodium nitrate, PS is potato starch, Dex is dextrose and PN is potassium nitrate.

Based on ANOVA analysis of RSM, with very small "model P-value" (< 0.0001) and a large "lack of fit P-value" (0.87) from the analysis of ANOVA and a suitable coefficient of determination (R^2 = 0.99) and adjusted coefficient of determination (R^2 adjusted = 0.97), the modified cubic polynomial model was highly significant and sufficient to represent the actual relationship between the response and the significant variables (Table 7).

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Table 6 : ANOVA for joint test (cell mass)

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	0.047	33	1.435E-003	107.40	< 0.0001	significant
A	4.062E-004	1	4.062E-004	30.40	0.0009	
B	9.646E-004	1	9.646E-004	72.20	< 0.0001	
C	8.124E-004	1	8.124E-004	60.80	0.0001	
D	4.854E-004	1	4.854E-004	36.33	0.0005	
E	4.947E-003	1	4.947E-003	370.25	< 0.0001	
F	2.653E-004	1	2.653E-004	19.86	0.0029	
G	3.792E-004	1	3.792E-004	28.38	0.0011	
B^2	2.417E-003	1	2.417E-003	180.94	< 0.0001	
C^2	1.323E-003	1	1.323E-003	99.03	< 0.0001	
E^2	1.370E-003	1	1.370E-003	102.55	< 0.0001	
F^2	7.873E-004	1	7.873E-004	58.93	0.0001	
G^2	1.232E-003	1	1.232E-003	92.19	< 0.0001	
AB	4.365E-003	1	4.365E-003	326.74	< 0.0001	
AC	1.059E-003	1	1.059E-003	79.25	< 0.0001	
AD	7.458E-003	1	7.458E-003	558.21	< 0.0001	
AE	4.180E-003	1	4.180E-003	312.88	< 0.0001	
AF	7.888E-003	1	7.888E-003	590.42	< 0.0001	
AG	0.010	1	0.010	749.07	< 0.0001	
BC	2.692E-003	1	2.692E-003	201.52	< 0.0001	
BD	1.451E-004	1	1.451E-004	10.86	0.0132	
BE	5.656E-003	1	5.656E-003	423.37	< 0.0001	
BG	5.188E-003	1	5.188E-003	388.29	< 0.0001	
CD	5.685E-003	1	5.685E-003	425.54	< 0.0001	
CE	4.898E-003	1	4.898E-003	366.61	< 0.0001	
CF	9.115E-003	1	9.115E-003	682.25	< 0.0001	
CG	8.154E-003	1	8.154E-003	610.34	< 0.0001	
DE	6.533E-003	1	6.533E-003	488.99	< 0.0001	
DF	8.808E-003	1	8.808E-003	659.31	< 0.0001	
DG	0.012	1	0.012	913.74	< 0.0001	
EF	2.453E-003	1	2.453E-003	183.59	< 0.0001	
EG	6.407E-003	1	6.407E-003	479.57	< 0.0001	
FG	6.509E-003	1	6.509E-003	487.23	< 0.0001	
ABC	0.016	1	0.016	1161.95	< 0.0001	
Residual	9.352E-005	7	1.336E-005			
Lack of Fit	6.072E-005	3	2.024E-005	2.47	0.2016	not significant
Pure Error	3.280E-005	4	8.200E-006			
Cor Total	0.047	40				

A: Sucrose
B: Yeast extract
C: Casamino acid
D: Sodium nitrate
E: Potato starch
F: Dextrose
G: Potassium nitrate

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Table 7 : ANOVA for joint test (radial growth rate)

Source	Sum of Square	DF	Mean Square	F Value	Prob > F	
Model	103.48	30	3.45	44.44	< 0.0001	significant
A	0.32	1	0.32	4.16	0.0686	
B	1.15	1	1.15	14.75	0.0033	
C	1.86	1	1.86	23.98	0.0006	
D	1.34	1	1.34	17.23	0.0020	
E	13.42	1	13.42	172.90	< 0.0001	
F	0.38	1	0.38	4.89	0.0515	
G	7.57	1	7.57	97.47	< 0.0001	
A^2	0.82	1	0.82	10.60	0.0086	
B^2	2.50	1	2.50	32.19	0.0002	
D^2	2.50	1	2.50	32.19	0.0002	
E^2	10.66	1	10.66	137.36	< 0.0001	
G^2	8.58	1	8.58	110.48	< 0.0001	
AB	11.19	1	11.19	144.16	< 0.0001	
AC	12.45	1	12.45	160.40	< 0.0001	
AD	14.40	1	14.40	185.46	< 0.0001	
AE	9.22	1	9.22	118.80	< 0.0001	
AF	5.63	1	5.63	72.52	< 0.0001	
AG	0.66	1	0.66	8.56	0.0151	
BC	1.98	1	1.98	25.57	0.0005	
BE	5.44	1	5.44	70.11	< 0.0001	
BF	2.54	1	2.54	32.75	0.0002	
CD	3.73	1	3.73	48.01	< 0.0001	
CE	13.23	1	13.23	170.47	< 0.0001	
CG	11.20	1	11.20	144.23	< 0.0001	
DE	0.58	1	0.58	7.41	0.0215	
DF	22.54	1	22.54	290.34	< 0.0001	
EF	5.31	1	5.31	68.39	< 0.0001	
EG	2.41	1	2.41	31.08	0.0002	
FG	12.84	1	12.84	165.46	< 0.0001	
ABC	7.76	1	7.76	99.93	< 0.0001	
Residual	0.78	10	0.078			
Lack of Fit	0.28	6	0.046	0.37	0.8679	not significant
Pure Error	0.50	4	0.13			
Cor Total	104.26	40				

A: Sucrose
B: Yeast extract
C: Casamino acid
D: Sodium nitrate
E: Potato starch
F: Dextrose
G: Potassium nitrate

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Cell mass production and radial growth rate analysis by using ANN

ANNs are adaptable computational systems, which the parameters under the study change during operation (adjusting input weights in the training/learning phase). After fixation of the system parameters, the developed ANN should be able to solve the problem at hand (the validation/testing phase). In this study herein, CCRD (Table 1) was used as a statistical experimental design to reduce the number of experiments to apply in the ANN method (Ebrahimpour *et al.*, 2008). Capability of ANN for the model prediction depends on the selection of processing model that is done in the learning stage of ANN. This stage includes: the number of hidden neurons, connection types, learning algorithms, and transfer functions of input and hidden layers. Although, the meticulous selections of the optimal number of hidden neurons are important to select the optimal number of hidden neurons, they would be depending on the model and complexity of the task that they usually have to be done by trial and error. Basically, increasing the number of hidden neurons up to a point results a better learning performance. In contrast, too few hidden neurons limit the ability of the neural network to model the process, and too many may allow too much freedom for the weights to adjust, which result in learning the noise present in the database used in training (Linco *et al.*, 1999). In this study, the effect of the number of hidden neurons on the goodness of fit was tested. In three cases tested, the optimum numbers of hidden neurons were 15 (Figure 1), with an obvious over fitting when too many hidden neurons were used. Training process for an ANN essentially means selecting one network from the set of allowed networks that minimizes the cost criterion. Different learning algorithms for training neural network models were tested. All accepted models (RMSE < 0.0001, R = 1 and DC = 1) have shown that incremental back propagation (IBP) was the most suitable learning algorithm for prediction of cell mass production and radial growth rate.

The type of transfer function employed affects the neural network learning rate and is instrumental in its performance. Among all employed transfer functions for hidden and output layers, accepted models were produced by linear function for output layer and Gaussian function or hyperbolic tangent (Tanh) for the hidden layer. The best models have been obtained by a Gaussian function. Several neural-network architectures and topologies were tested for the estimation and prediction of cell mass production and radial growth rate. A multilayer full feed-forward incremental back propagation network with Gaussian transfer function consisted of a 7-15-1 topology was chosen as the best ANN, after good prediction of testing data for cell mass production and radial growth rate (Figure 1).

The optimized values of network for learning rate and momentum were 0.15 and 0.8, respectively. The learning for cell mass production and radial growth rate was completed in RMSE< 0.001, R = 1 and DC = 1.

In the case of cell mass production, training data set R^2 and AAD were 0.92 and 8.25, respectively, whereas for the testing data set, R^2 was 0.99 and AAD was 6.9% (Table 4), while for validating data set R^2 and AAD were 0.89 and 1.9%, respectively (Table 2).

In the case of radial growth rate, training data set R^2 and AAD were 0.92 and 8.25, respectively. The testing data set R^2 was 0.99 and AAD was 6.9% (Table 5) while for validating data set R^2 and AAD were 0.89 and 1.9%, respectively (Table 3).

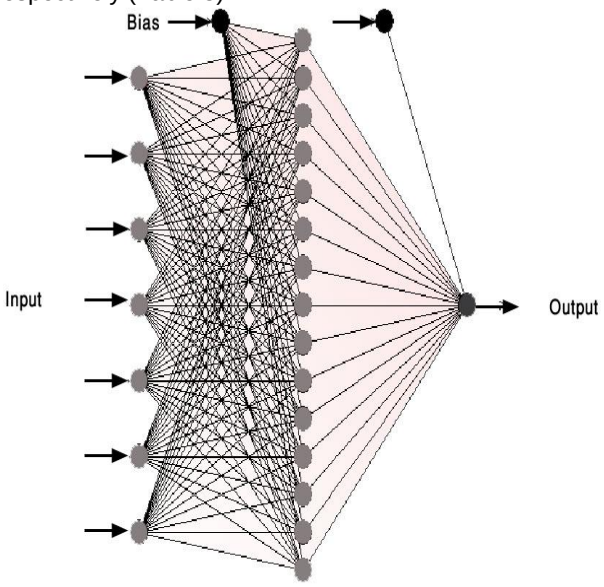

Figure 1: Topology of multilayer full feedforward neural network for the estimation of cell mass production and radial growth.

Comparison of RSM and ANN predicted values

RSM and ANN are alternative methods for the classical approach, one-variable-at-a-time, as modeling and optimization techniques for various biological processes. The predicted output values of RSM and ANN are shown in **Tables 4** and **5**. Both models obtained performed well, and offered stable responses.

Main effects and interaction between parameters

The optimum level of each variable and the effect of their interactions on cell mass production, and radial growth rate as a function of two variables were studied by plotting three dimensional response surface curves (while keeping the other variables at optimum point). ANOVA analysis (Tables 6 and 7), and three dimensional plots (Figures 2 and 3) reveal that sucrose, yeast extract, casamino acid, sodium nitrate, dextrose, and potato starch had significant effects on cell mass production and radial growth rate.

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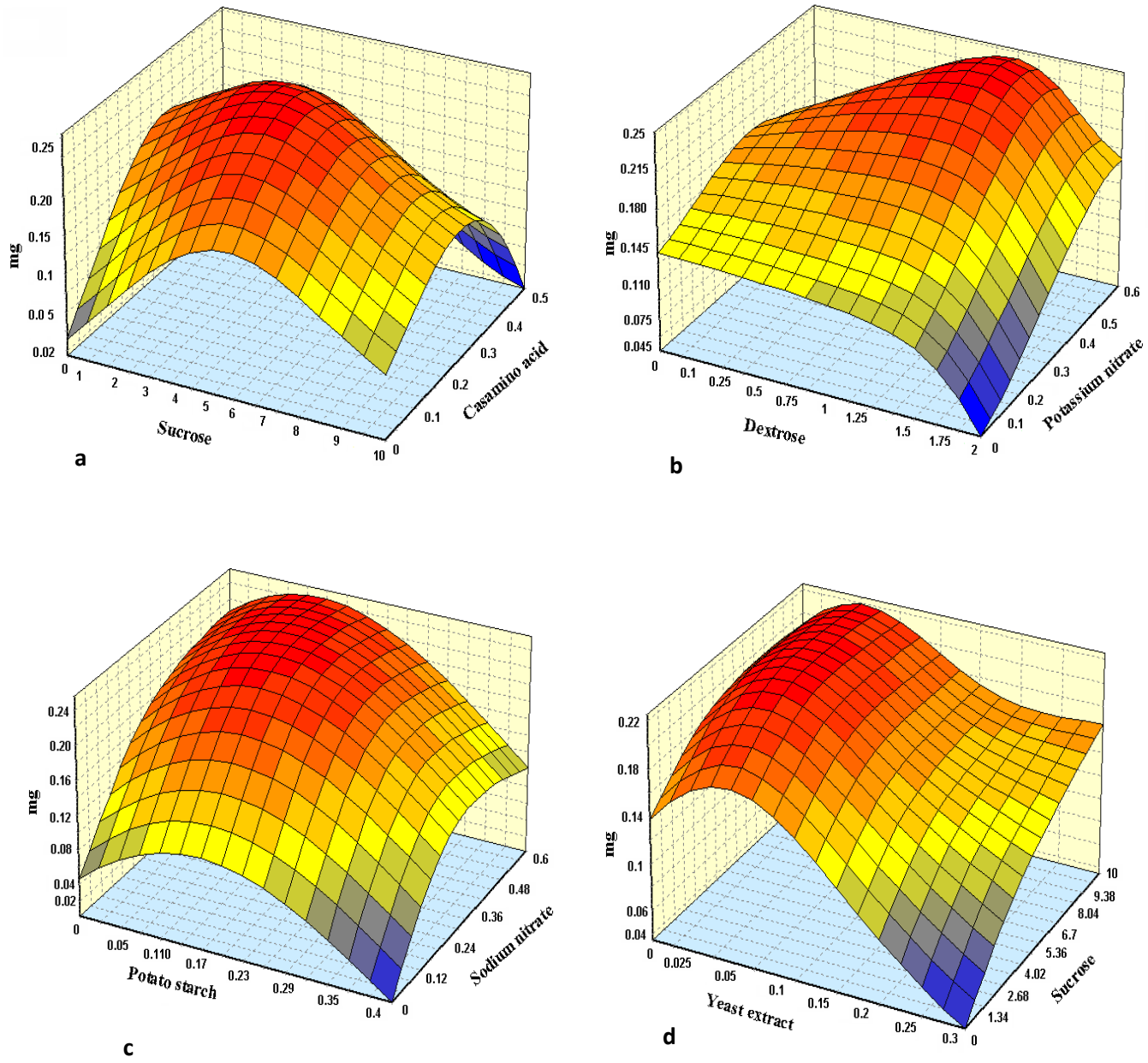

Figure 2 : Three dimensional plots showing the effect of: (a) sucrose, casamino acid; (b) potassium nitrate, dextrose; (c) potato starch, sodium nitrate; and (d) yeast extract, sucrose, and their mutual effect on the cell mass production by *Monascus purpureus* FTC5391. Other variables are constant: sucrose (2.5%), yeast extract (0.045%), casamino acid (0.275%), sodium nitrate (0.48%), potato starch (0.045%), dextrose (1%), and potassium nitrate (0.57%).

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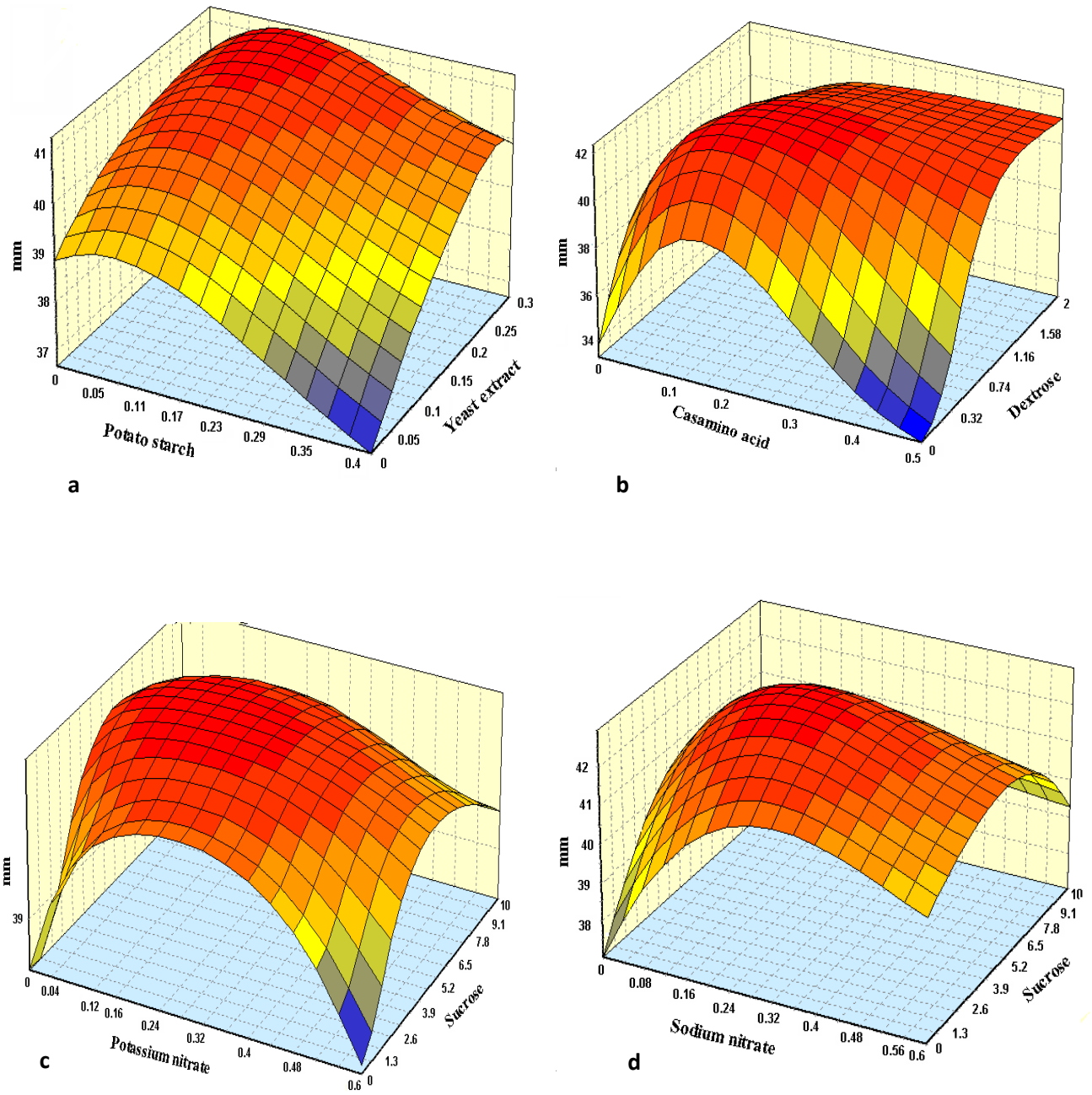

Figure 3: Three dimensional plots showing the effect of: (a) sucrose, casamino acid; (b) potassium nitrate, dextrose; (c) potato starch, sodium nitrate; and (d) yeast extract, sucrose, and their mutual effect on the radial growth rate by *Monascus purpureus* FTC5391. Other variables are constant: sucrose (5%), yeast extract (0.24%), casamino acid (0.05%), sodium nitrate (0.1%), potato starch (0.06%), dextrose (0.2%), and potassium nitrate (0.06%).

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Importance of parameters and optimization of reaction

The importance of effective parameters on the cell mass production and radial growth rate are shown in **Figure 4** and these can be summarized of follows.
Cell mass production: sucrose (23.37%) > sodium nitrate (14.33%) > potato starch (13.95%) > dextrose (13.93%) > casamino acid (12.95%) > yeast extract (11.76%) > potassium nitrate (9.71%)
Radial growth rate: potato starch (19.96%) > potassium nitrate (18.82%) > casamino acid (14.32%) > yeast extract (13.53%) > sodium nitrate (11.93%) > sucrose (11.04%) > dextrose (10.4%)

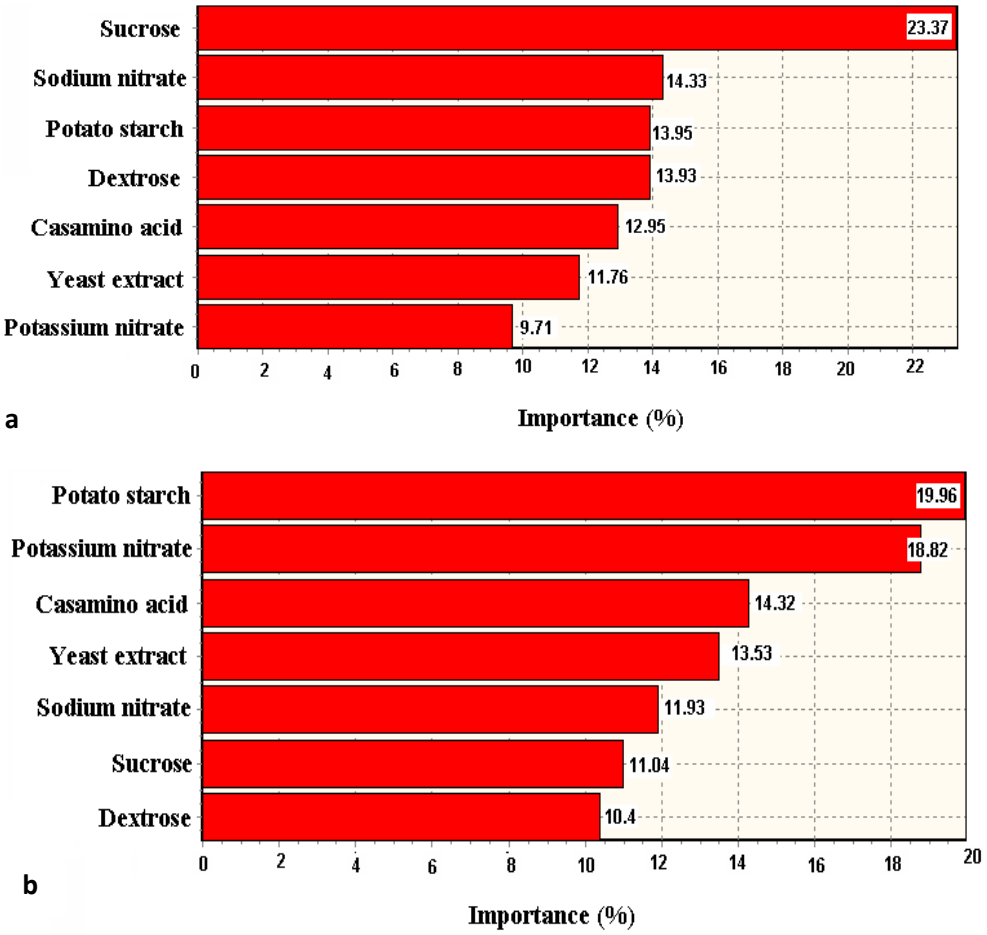

Figure 4: Importance of effective parameters on cell mass production (a) and radial growth rate (b).

DISCUSSION

RSM and ANN are alternative methods for the classical approach, one-variable-at-a-time, as modeling and optimization techniques for various biological processes. These both methods have some advantages and disadvantages. RSM has important applications in the design, development, and formulation of new products, as well as, in the developing, improving and optimizing processes. RSM generates a mathematical model to analyzing the effects of the independent factors that defines the biochemical process (Myers and Montgomery, 1995). However, RSM has some limitations. The co-linearity problems between factors may exist, and sensitivity analysis of input variables is hard to perform because of the presence of cross interactions (Lou and Nakai, 2001). In contrast, ANN has shown better predictability than RSM in the case of model nonlinearities (Bas and Boyaci, 2007a; Basri *et al.*, 2007; Ebrahimpour *et al.*, 2008; Lou and Nakai, 2001). ANN is a massively interacted network structure consisting of many simple processing elements (neurons) capable of performing parallel computation for data processing. ANNs are known as universal function approximations, and can be used to provide a tool that both programs learn on their own. Therefore, ANNs have elasticity so that they can be updated with new data (Bas and Boyaci, 2007a; Linko *et al.*, 1999). It is a fact that ANN has some limitations beside its advantages. ANN cannot explain its actions satisfactorily in typical situations, which has been reported as major disadvantages (Linko *et al.*, 1999). The ANN approach could not give a prediction equation (Bas

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and Boyaci, 2007b; Lou and Nakai, 2001). It needs large amounts of training data in comparison with RSM that offers a large amount of information from a small number of experiments (Basri *et al.*, 2007; Myers and Montgomery, 1995). We have shown in this study herein, that combined application of RSM and ANN could cover some of their individual disadvantages. On the other hand, in this study, analyses of experimental data using both RSM and ANN methods could provide some error points, and one can repeat these points until gaining an acceptable model with both RSM and ANN.

The first and more important step of modern optimization methods (ANN and RSM) is the selection of experimental design (Bas and Boyaci, 2007b). Different designs have been used in various research works based on the special criteria. Dutta et al. (2004) and Seraman et al. (2010) used central composite design (CCD) for extracellular protease production with 14 and lovastatin production with 16 different combinations, respectively. Face-centered design (FCD) and modified face-centered design (MFCD) were employed to investigate enzymatic reaction using RSM and ANN (Bas and Boyaci, 2007b). In the current study CCRD was used as experimental design.

Prediction of polynomial model is one of the steps involve in RSM optimization procedure that explains the influences of independent variables. In order to find the best fitted model, various models were tested (linear, two factorial, quadratic and cubic). In this study, modified cubic model by backward elimination strategy was used followed by hierarchical terms additionally, to find the best model. In fact, backward elimination method verifies all of the predictors in the model. It removes the variable which is least significant (with the largest P-value) and the model is refitted. Each subsequent step removes the least significant variable in the model until all remaining variables have individual P-values smaller than 0.05 (Ebrahimpour *et al.*, 2008).

The final modified cubic equations were almost quadratic models as have been reported by several researchers (Bas and Boyaci, 2007b; Lou and Nakai, 2001). ANOVA result of cell mass production showed three nominal elimination (Suc.Dex, YE.PN and CA.Dex), in the modeling equations of cell mass production. Although YE and PN as well as CA and Dex were important in the modeling equation, the interaction between each pair was not significant and elimination of them caused improvement of the model.

The results of ANN and RSM showed that all carbon and nitrogen sources tested in this study (sucrose, dextrose, potato starch as carbon sources and casamino acid, potassium nitrate, sodium nitrate and yeast extract as nitrogen sources) had significant effect (*P*-value < 0.05) on radial growth and cell mass production.

In the case of cell mass production, dextrose was at the middle of selected concentration range, whereas potato starch and sucrose were at the lowest concentration. Lactose and sucrose inhibited the biomass and pigment production in mould (Tseng *et al.*, 2000). Krasniewski et al. (2006) reported that fungal biomass production was positively correlated to glucose concentration in the culture medium for *Penicillium camemberti*. In this study, maximum cell mass production (0.21 mg/plate) was obtained when sucrose (2.5%) and cassamino acid (0.275%) were used. Further increase or decrease in these parameters led to the decrease in the cell mass production. The maximum cell mass production was obtained where dextrose was 1% and potassium nitrate was 0.57%. Low concentration of sodium nitrite (0.2 g/L) promoted mycelial growth and pigment production (Tseng *et al.*, 2000). In addition, the inclusion of yeast extract, caused an increase in cell yield based on glucose consumed ($Y_{x/s}$) as high as 40% (Pereira and Kilikian, 2001). As a summary, in this study, in the case of cell mass production the non-organic nitrogen sources were at the highest concentration (sodium and potassium nitrate) whereas yeast extract (organic nitrogen source) was in the lowest concentration range.

In the case of radial growth, ANOVA analysis showed that although sucrose and dextrose were not significant parameters (P value > 0.05), they had important and significant interactions with other parameters; hence they have been used to develop the model. Maximum radial growth rate of 41 mm/plate was obtained at yeast extract and potato starch concentration of 0.24% and 0.06%, respectively. Further increase or decrease in these parameters led to decrease in the radial growth rate. The maximum radial growth rate was obtained at casamino acid and dextrose concentration of 0.275% and 1%, respectively.

According to the plot, the medium with 0.06% potassium nitrate and 5% sucrose gave maximum radial growth rate after 12 days of cultivation. Potassium nitrate had a better effect on reducing the length of the germ tube in *Beauveria bassiana* (Bosch and Yantorno, 1999). Hence, low concentration of potassium nitrate seemed to have increased the length of the germ tube and radial growth rate. On the other hand, sodium nitrate at low amount (0.1%) of pointed was the maximum affect on radial growth rate. All parameters had significant interaction on radial growth rate in the optimal point. The radial growth decreased remarkably as the parameters changed.

The comparison of ANOVA results between cell mass production and radial growth rate showed that one nominal YE.PN was eliminated in these equations. Although YE and PN were important in the modeling equation, the interaction between these two was not significant and its elimination turned to improve the model.

In the case of cell mass production, the highest dry cell weight (0.21 mg/plate; 2.2-fold increase) was obtained at following condition: sucrose (2.5%), yeast extract (0.045%), casamino acid (0.275%), sodium nitrate (0.48%), potato starch (0.045%), dextrose (1%) and potassium nitrate (0.57%). In the case of radial growth rate, the highest radial growth (41mm; 1.1-fold increase) was obtained at the following condition: sucrose (5%), yeast extract (0.24%), casamino acid (0.05%), sodium nitrate (0.1%), potato starch (0.06%), dextrose (0.2%) and potassium nitrate (0.06%). Attention to R^2 and AAD values between actual and estimated responses

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demonstrated that the prediction accuracy of ANN and were very close together.

CONCLUSION

Results from this study have demonstrated that RSM and ANN have been successfully used for optimization of responses for the cultivation of *M. purpureus* FTC5391. All factors including sucrose, dextrose, and potato starch as carbon sources; and casamino acid, potassium nitrate, sodium nitrate, and yeast extract as nitrogen sources had significant (*P*-value < 0.05) effects on cell mass production and radial growth rate. The best models were achieved by a multilayer full feed-forward incremental back propagation network and a modified response surface model by using backward elimination.

Even though the modified response surface model and ANN have provided good quality predictions for the seven independent variables in terms of the cell mass production and radial growth rate, using RSM and ANN along together showed a clear superiority over using each of them alone. On the other hand, the use of of RSM and ANN results beside each other could cover some disadvantages of each method and highlight the error in experimental data.

The effects of amount and type of carbon and nitrogen sources in the optimum point on cell mass production and radial growth rate were greatly different. In the case of cell mass production dextrose was at the middle concentration, whereas potato starch and sucrose were at low concentration. On the other hand, organic nitrogen sources were at high concentration (sodium and potassium nitrate) whereas yeast extract was at low concentration. In the case of radial growth rate, sucrose was at the middle concentration whereas potato starch and dextrose were at low concentration. Moreover, yeast extract was at higher concentration than sodium and potassium nitrate.

In addition, results from optimization indicate that cell mass production and radial growth rate were influenced by a synergistic combination of effective nutritional parameter interactions. These parameters were in equilibrium, and the change of one parameter could be compensated by the changes of other parameters to give similar results.

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