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Full Length Research Paper

Optimization of the fermentation medium for *Paecilomyces tenuipes* N45 using statistical approach

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In the present study, a sequential statistical approach was applied to optimize the medium in submerged cultivation of *Paecilomyces tenuipes* N45. Desirability value was used as response to simultaneously enhance the yields of mycelium, adenosine, polysaccharide and cordyceps acid. Based on single-factor optimization strategy, the suitable carbon sources, nitrogen sources and inorganic salts were obtained. Then key medium components were identified by Plackett-Burman design (PBD) and further optimized by Box-Behnken design (BBD). Finally, response surface methodology (RSM) and artificial neural network – genetic algorithm (ANN-GA) were used to model and optimize the experimental results obtained from BBD. The optimum components of nutrient medium comprised (g/L): glucose 40, beef extract 10, soy peptone 10, KH_2PO_4 0.688, $MgSO_4 \cdot 7\ H_2O$ 1, NaCl 0.500, VB_1 0.201, VB_{12} 0.130. In a word, a mean value of desirability values *D*v = 0.493 was obtained, which was 20.540% higher than the value achieved by the basal medium. The biomass, the production of adenosine, the polysaccharide and the cordyceps acid yields were enhanced by 8.200, 3.580, 23.170 and 31.510% respectively.

Key words: Artificial neural network, optimization, *Paecilomyces tenuipes*, response surface methodology, genetic algorithm.

INTRODUCTION

Paecilomyces tenuipes (also referred to as *Isaria japonica*), is a fungus parasitic to moth larvae and pupae, which belongs to the subphylum *Ascomycotina,* class *Pyrenomycetes*, order *Clavicipitales* (Kikuchi et al., 2004; Xu and Yun, 2004). *P. tenuipes* has long been consumed as traditional health food and folk medicine in Japan, Korea and China as it offers various types of biological and pharmacological activities, such as immunomodulatory (Chen et al., 2008), anti-tumor (Kim et al., 2011), antidepressant (Kan et al., 2010) and hypoglycemic action (Park et al., 2011). Some valuable components, such as polysaccharide (Lu et al., 2007), nucleoside, protein (Xu et al., 2003), cordyceps acid (In-Pyo et al., 2007), cordycepin, tenuipesine, sterol, trichothecanes (Isaka et al., 2007) and cyclopeptide (Sapkota et al., 2011) have been found from *P. tenuipes* (Takano et al., 2005). The utilization of *P. tenuipes* is a topic of growing interest. Particularly, submerged fermentation is one of the most popular methods to produce mycelia and its bioactive metabolites of *P. tenuipes* in a large scale. However, there are some limitations and one major is low yields. Several efforts were underway in our previous studies for obtaining desired products with high yields. Mutation breeding approach is one of them, which is an efficient method used in microbiology (Ma et al., 2011). Nitrosoguanidine mutagenesis of wild strain *P. tenuipes* RCEF4339 was previously initiated with the objective of enhancing the mycelia, polysaccharide, adenosine and cordyceps acid production. An improved mutant, designated as

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N45, was obtained. The mutant showed higher production of four components than that of the wild strain. Alternatively, the production of active compounds in microorganism fermentation is under the influence of various factors. For all fungal fermentation, the right balance of nutrients is essential. Thereby, the medium optimization attracted a wide spread attention in recent years (Sivapathasekaran et al., 2010). This makes optimization of the culture medium an important part for studies.

Several literatures have been reported previously on optimization of fermentation media for elevating the fermentation levels (Wang et al., 2008; Lim et al., 2004). Most of them just only focused on enhancing one or two of the response values. Thus, the quality of *P. tenuipes* fermentation cannot be elevated comprehnsively. In order to realize simultaneous optimization of four desired products (mycelia, polysaccharide, adenosine and cordyceps acid), desirability function was used in this study. The desirability concept is used to combine the multi-response into a single response recently which was designed by Harrington (Sait et al., 2009). This method has been widely utilized by a number of researchers for overall multiple response optimization (Mourabet et al., 2012). In addition, to simplify the optimization process, an effective experimental design method is needed (Hanchinal et al., 2008).

There are a large number of techniques available to optimize the culture medium of microorganism. Response surface methodology (RSM) is a collection of statistical and mathematical techniques and has been proved to be an effective mean (Ren et al., 2011). The optimization process of this methodology includes studying the response of the statistically designed combinations, estimating the coefficients by fitting it in a mathematical model which fits best the experimental conditions, predicting the response of the fitted model and checking the adequacy of the model (Dong et al., 2009). This method has been widely applied in the optimization of different processes in food chemistry, material science, chemical engineering and biotechnology (Wang et al., 2011).

Recently, an alternative modeling technique, artificial neural network coupled with genetic algorithm (ANN-GA), has also been paid extensive attention in the field of predictive microbiology modeling (Hajmeer et al., 2002). The artificial neural network is an effective computational model. It imitates the structure and behavior of neurons in the human brain: the structure of the nodes, the topology of the network and the learning algorithm (Wang et al., 2010; Chen et al., 2004). This technology offers several advantages such as non-linearity, flexible, speed, simplicity, and high accuracy. The user does not require an explicit description of the nature of the underlying process in a mathematical form (Moustra et al., 2011; Poirazi et al., 2007; Arslan and Yetik, 2011).

On the other hand, GA is a random search technique based on the concept of natural selection and genetics, which presents a robust method for solving complex optimization problems (Ran et al., 2010). Compared with conventional optimization methods, genetic algorithm has three advantages: (i) less susceptibility to be stuck at local minima; (ii) requiring little knowledge of the process being optimized; (iii) capability to find the optimum conditions when the search space is very large (Fathi et al., 2011). Thus, GA is an effective tool for optimizing complex-structured problems with many variables in various scientific and technological fields (Yang et al., 2011).

In the current study, we optimized the best nutrient medium for maximizing the production of mycelia, polysaccharide, adenosine and cordyceps acid yields simultaneously in submerged cultivation of the mutant strain *P. tenuipes* N45 at the same time using sequential statistical methods combined with desirability function. In the first step, single factor experiment was employed to search the suitable carbon sources, nitrogen sources and inorganic salts. In the second step, Plackett-Burman design was used to identify the key factors influencing the fermentation of *P. tenuipes* N45. A response surface methodology and artificial neural network - genetic algorithm combined with a Box-Behnken design was then used to further optimize key variables, respectively.

MATERIALS AND METHODS

Microorganism and medium

The strain *P. tenuipes* N45 used in this study was obtained by treating *P. tenuipes* RCEF 4339 (Anhui Agricultural University) with nitrosoguanidine. The strain was maintained on basal medium agar slants (glucose 40 g/L, yeast extract 10 g/L, peptone 10 g/L). Slants were incubated at 26℃ for 4 days and stored at 4℃.

Inoculum and flask cultures preparation

The maintained strain, previously grown on agar slants, was inoculated into 50 mL Erlenmeyer flask containing 20 mL basal medium and cultured under aerobic conditions at 26℃ for 4 days with continuous shaking at 150 rpm. Then, 5 mL of primary culture was transferred into 250 mL Erlenmeyer flask containing 100 mL of the same liquid medium. After incubation at 26℃ for 3 days on a rotary shaker (150 rpm), these secondary cultures were transferred into 250 mL Erlenmeyer flask containing 100 mL fermentation medium which prepared depending on the experimental design to evaluate the influence of medium composition on *P. tenuipes* N45 fermentation. The other fermentation conditions were: inoculation amount 5% (v/v), temperature 26℃, rotate speed 150 rpm, natural pH and fermentation time 4 days.

Sample preparation

After submerged culture of this mutant strain at various conditions, the mycelium was harvested by centrifugation for 8 min at 3500 × g. The dry weight of mycelium was accurately measured after repeated washing of the mycelium with distilled water and

Table 1. The parameters of the desirability function in current study.

Parameter	Y_A (g·L^{-1})	Y_B (g·L^{-1})	Y_C (g·L^{-1})	Y_D (g·L^{-1})
y_i	5.00	0.01	0.10	0.40
L_i	30.00	0.15	1.50	3.00
w_i	0.10	0.35	0.30	0.25

Y_A: Yield of biomass; Y_B: Yield of adenosine; Y_C: Yield of polysaccharide; Y_D: Yield of cordyceps acid.

lyophilized. After the freeze-drying, the dried mycelium was pulverized. The mycelium samples were used for quantitative analysis of polysaccharide, adenosine and cordyceps acid.

The adenosine and cordyceps acid were extracted from the powder of lyophilized mycelium by warm water at 45℃, with extracting time 3 h and solid-liquid ratio 1:50 (w/v). After this extraction, the solution was centrifuged at 4297×g for 10 min to obtain the crude sample solution. The powder of lyophilized mycelium was processed by water extraction and ethanol precipitation technique in order to obtain the crude polysaccharide. The extracting conditions were: extracting time 3 h, extracting temperature 80℃, solid-liquid ratio 1:50 (w/v). After centrifugation (4297×g, 10 min), the water extraction solution was separated from insoluble residue and then 95% (volume fraction) ethanol was added to it with a final concentration of 80% (v/v). The precipitate was the crude polysaccharide.

Analytical methods

The polysaccharide concentrations were measured by anthrone-sulfuric acid colorimetry method (Leung et al., 2009). The adenosine contents in mycelium were determined by using a Shimadzu high performance liquid chromatography (HPLC) system with two LC-6AD pumps and SPD-A UV-vis detector (Shimadzu, Kyoto Japan). 20 μL of adenosine extract was injected into the separation column (3.9×250 mm; Agilent ZORBAX SB C-18, 4 μm) in a mobile phase of 85% methanol and 15% PBS (pH 6.5). The column temperature was 35℃ and the detection wavele ngth was 260 nm. The contents of cordyceps acid were determined using spectrophotometry (Dong and Yao, 2008).

Desirability function development

In this work, the desirability function approach was used for simultaneously enhancing the yields of biomass, adenosine, polysaccharide and cordyceps acid in *P. tenuipes* N45 fermentation. Desirability function involved the transformation of each predicted response to a dimensionless partial desirability function (Liu and Tang, 2010). In the present study, the four responses were all “the-higher-the-better” responses, that is, a higher biomass, adenosine, polysaccharide and cordyceps acid production were the desired outcome. For goal maximum, the desirability function of the-higher-the-better is defined as Equation 1:

$$d_i = \begin{cases} 0 & Y_i \le y_i \\ \dfrac{Y_i - y_i}{L_i - y_i} & y_i < Y_i \le L_i \\ 1 & Y_i > L_i \end{cases} \quad (1)$$

where Y_i is the ith response value. y_i is the lower tolerance limit of ith response. L_i is the upper tolerance limit of the ith response. d_i is the desirability value of the ith response. As a result, the obtained d_i ranges from nearly 0 (undesirable) to nearly 1 (most desirable). After these individual d_i functions are defined for all responses, they are combined into a single composite response Dv, representing the overall desirability, which is defined as follow:

$$Dv = d_1^{w_1} \cdot d_2^{w_2} \cdot d_3^{w_3} \cdots d_n^{w_n} \quad (2)$$

$$\begin{cases} 0 < w_i < 1 \\ w_1 + w_2 + w_3 + \cdots w_n = 1 \end{cases} \quad i = 1, \quad 2, \quad 3, \cdots n \quad (3)$$

In Equation 3, w_i is the weight of the ith response values which reflects the difference in the importance of various response, while n is the number of measured response variables. Obviously, the more important the response is, the higher the w_i is. The higher y_i is, the larger the Dv is. Considering the importance of biomass, adenosine, polysaccharide and cordyceps acid, the y_i, L_i and w_i of these responses are listed in Table 1.

Experimental design

To optimize the composition of medium, a three-step optimization strategy, including the single-factor test design, PBD and BBD, was employed. All the following experiments were implemented in triplicate and the average data was used as experimental data.

Single-factor test design experiments

On the basis of single variable at a time experiments, suitable carbon sources, nitrogen sources, and inorganic salts were firstly tested depending on Dv. Six different carbon sources namely glucose, lactose, maltose, sucrose, fructose and glycerol were chosen to supplement the basal medium for fermentation of *P. tenuipes* N45. The concentration of carbon sources was set at 40 g/L, while that of tested nitrogen sources (peptone, beef extract, yeast extract, yeast extract paste and soy peptone) was fixed at 20 g/L. The effects of different inorganic salts ($CaCl_2$, $CuSO_4$, $ZnCl_2$, $MnCl_2$, $FeCl_3$, NaCl, KH_2PO_4, $MgSO_4 \cdot 7H_2O$) on the *P. tenuipes* N45 fermentation were also studied. There were three concentrations of inorganic salt including 0.001 g/100 mL, 0.05 g/100 mL and 0.1 g/100 mL.

Plackett-Burman design experiments

In the second step, a study of the variables which might affect the *P. tenuipes* N45 fermentation was carried out using a two level screening PBD for nine variables in 10 treatments. Each variable was represented at two levels, high and low, which were represented by “+1” and “-1”, respectively. The levels of variables for PBD experiments were selected according to the results of

Table 2. The levels of variables tested in BBD.

Factors	Coded levels		
	-1	0	1
$KH_2PO_4(X_1)$/ g·100mL^{-1}	0.000	0.070	0.150
$VB_1(X_2)$/g·L^{-1}	0.194	0.200	0.206
$VB_{12}(X_3)$/g·L^{-1}	0.100	0.200	0.300

single-factor test design experiments. The linear regression model base on the data of PBD is:

$$Y = \beta_0 + \sum_{i=1}^{10} \beta_i X_i \quad (4)$$

Where Y is the response or dependent variable, β_0 is the model intercept, X_i is the independent variable, and β_i is the linear regression coefficient. *F*-test method was employed to exam the significances of each coefficient in the linear model. The significant components selection depended on corresponding significant coefficients. A Pareto chart analysis was performed using to understand the significance of variables on the response value.

Box-Behnken design experiments

After determining the key factors, the medium was further optimized using a three-factor-three-level BBD. The ranges of these variables are given in Table 2. There were two optimizing methods namely response surface methodology and artificial neural network coupled with genetic algorithm to analyze the data from BBD in this work.

One approach was tested in order to find the optimal medium by fitting a polynomial model through the response surface methodology. This methodology can be applied for determination of maximum response value and evaluation of the main effects, interaction effects and quadratic effects. After the experiments had been performed, the response variable (D_V) was fitted with a multi-quadratic regression model as follows (Guo et al., 2010):

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^{\,2} + \sum \beta_{ij} X_i X_j \quad (5)$$

Where Y is the desirability value; β_0 is the intercept term; β_i is linear coefficients; β_{ij} is interaction coefficients; β_{ii} is the quadratic coefficient, and X_i and X_j are the coded factors computed by Equation 6:

$$X_i = (x_i - X_0)/\triangle X_i \quad (6)$$

where X_i is the coded value of the variable, x_i is the independent variable real value, X_0 is the independent variable real value at the center point, and $\triangle X_i$ is the step length.

Analysis of the experiment results and calculation of predicted data were performed using SAS V8.02 software to estimate the response of the independent variables. Artificial neural network based modeling paradigm was also used. In this paper, a three layers neural network model was developed to optimize the effects of medium components on the fermentation of *P. tenuipes* N45. The design matrix of BBD was used as the input. The output was the data obtained from the design experiments. All the data was divided into three subsets such as training (70% of data), testing (15% of data) and validating (15% of data) by randomly. The network was trained with Levenberg-Marquardt back propagation algorithm. Sigmoid function was used for the hidden layer and linear function was used for the output layer. Besides, although the network will reach a great modeling ability when the number of neurons in the hidden layers is too many, the ability of generalizing and predicting will be lose. This phenomenon is called as overfitting (Liu et al., 2008). The suitable number of neurons in hidden layers had to be selected to obtain a network with satisfactory quality and predictive ability. To avoid this problem, one function named as degree of approaching (*D*a) which was defined as follows was used in present study to find the suitable number of hidden nodes:

$$D_a = \frac{c}{\frac{n_c}{n} \times RMSE_c + \frac{n_t}{n} \times RMSE_t + \left| RMSE_c - RMSE_t \right|} \quad (7)$$

where n_c and n_t represent the number of calibration set and test set; n is the sum number of calibration set and test set; $RMSE_c$ and $RMSE_t$ are the root mean square error (*RMSE*) of calibration set and test set, respectively; and c is a constant by which *D*a is adjusted to obtain a good graph. In present study, c is 0.08. The best ANN model was selected according to their *D*a. Obviously, the larger *D*a was, the more the ANN models approached the experimental data. The goodness of fit was determined by the determination of coefficient (R^2).

After establishing the relationship of response and variables by using ANN model, GA was employed to search the optimum culture medium in the test regions. Different parameters of GA used were: population type as double vector, population size as 20, the initial population as given randomly, selection function as stochastic uniform, elite count as 2, crossover fraction as 0.8, crossover function as scattered, migration fraction as 0.2, migration interval as 20, and penalty factor as 100. Optimum medium was selected after evaluation of GA for 100 generations to achieve optimum fermentation in the given range of input parameters. For the optimization using GA, a fitness function which was well-known to play an important role in the genetic evolution must be provided. This function was crucial to maximize the GA' s performance (Da Silva et al., 2011). The special fitness function equation used in this work was presented as follow:

$$Fitness = -Dv \quad (8)$$

Validation experiments

The optimal fermentation medium obtained in previous sections was investigated in quintuplicate to validate the feasibility of this method.

Data analysis and software

SAS Version 8.02 (SAS Institute Inc., USA) was used for the

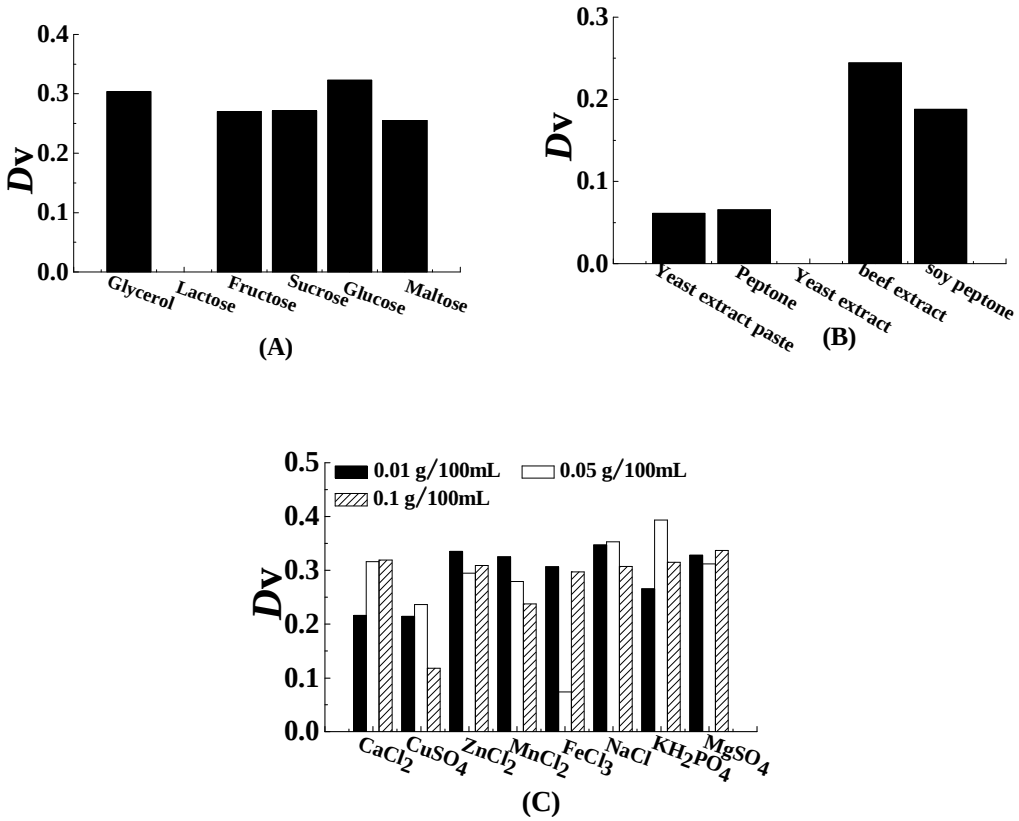

Figure 1. Effects of different carbon sources (A), nitrogen sources (B) and inorganic salts (C) on desirability value; cultures were carried out at 26℃, 250 rpm, and lasted for 4 days.

experiment design and regression analysis of the experimental data. Three-dimensional surface plots were constructed using Origin Pro 7.5 (OriginLab Inc, USA). Software Minitab 15 was used for constructing Pareto charts for the effects of the variables on the desirability value. Matlab version 7.6.0.324 (R2008a) (The Mathworks, Inc., USA) was used for establishing the ANN model and implementing GA.

RESULTS AND DISCUSSION

Selecting carbon sources, nitrogen sources and inorganic salts

To find a suitable carbon source for fermentation of *P. tenuipes* N45, various carbon sources were supplemented in the basal medium as a first step. The data are shown in Figure 1. Among carbon sources tested, maximum desirability value was obtained in glucose medium. Therefore, glucose was used as the carbon source in the following experiments. With the objective of increasing four active components production, not only the carbon sources but also the nitrogen sources which were essential for fungus growth and metabolism should be determined. Nitrogen is an essential element as it is required for protein synthesis and for the production of nucleotides and enzymes. In our case, mutant *P. tenuipes* N45 was cultivated in the medium to which the nitrogen source was varied. It also showed that the fermentation was performed better in the medium containing beef extract. However, considering the contents of four active components, we choosed two nitrogen sources (beef extract and soy peptone) in the medium. In order to identify a suitable ratio of two nitrogen sources, experiments with different ratios (3:1, 2:1, 1:1, 1:2 and 1:3) were performed and suitable ratio of soy peptone to beef extract was found to be 1:1 (data not shown). Similarly, the optimal inorganic salt was KH_2PO_4, the concentrate of which was 0.05 g/100 ml.

Screening of significant components in fermentation medium using a Plackett-Burman design

This experiment performed an attempt of screening the factors significantly affecting the fermentation of *P. tenuipes* N45 by the PBD. The design matrix and results of PBD are presented in Table 3. The *D*v value was found to vary from 0.2286 to 0.4032 in 12 experiments,

Table 3. The design matrix and results of PBD.

Run	Glucose X_1(g/L)	Beef extract X_2(g/L)	Soy peptone X_3(g/L)	KH_2PO_4 X_4(g/100 mL)	$MgSO_4·7H_2O$ X_5(g/100 mL)	NaCl X_6(g/100 mL)	VB_6 X_7(g/L)	VB_1 X_8(g/L)	VB_{12} X_9(g/L)	X_{10}	Dv
1	55	7	13	0.03	0.05	0.03	0.20	0.20	0.20	-1	0.3122
2	55	13	7	0.07	0.05	0.03	0.00	0.20	0.20	1	0.4032
3	25	13	13	0.03	0.15	0.03	0.00	0.00	0.20	1	0.3032
4	55	7	13	0.07	0.05	0.07	0.00	0.00	0.00	1	0.2576
5	55	13	7	0.07	0.15	0.03	0.20	0.00	0.00	-1	0.2777
6	55	13	13	0.03	0.15	0.07	0.00	0.20	0.00	-1	0.2696
7	25	13	13	0.07	0.05	0.07	0.20	0.00	0.20	-1	0.3066
8	25	7	13	0.07	0.15	0.03	0.20	0.20	0.00	1	0.3000
9	25	7	7	0.07	0.15	0.07	0.00	0.20	0.20	-1	0.3799
10	55	7	7	0.03	0.15	0.07	0.20	0.00	0.20	1	0.2676
11	25	13	7	0.03	0.05	0.07	0.20	0.20	0.00	1	0.2331
12	25	7	7	0.03	0.05	0.03	0.00	0.00	0.00	-1	0.2286

*D*v refers to the average desirability value of triplicate experiments.

Table 4. Results of regression analysis of PBD.

Source	*DF*	*SS*	*MS*	*F*	*Pr > F*
X_1	1	0.0001	0.0001	9.4079	0.2006
X_2	1	0.0002	0.0002	15.9328	0.1563
X_3	1	0.0001	0.0001	11.8128	0.1803
X_4	1	0.0080	0.0080	681.693	0.0244
X_5	1	0.0003	0.0003	22.7024	0.1317
X_6	1	0.0010	0.0010	86.2245	0.0683
X_7	1	0.0018	0.0018	148.2664	0.0522
X_8	1	0.0055	0.0055	465.3265	0.0295
X_9	1	0.0137	0.0137	1164.5870	0.0187
X_{10}	1	8.17E-06	8.17E-06	0.6921	0.5582
Model	10	0.0308	0.0031	260.6645	0.0482
Error	1	0.000012	0.000012		
Total	11	0.0308			

DF refers to degrees of freedom, SS refers to sum of squares, MS refers to mean square, *F* and *Pr>F* refer to *F* and *P* values, respectively.

which may be due to the strong influence of medium components on production. Regression analysis was performed to fit the response function with the experimental data and the regression analysis results of PBD are shown in Table 4. The linear regression equation which can be applied for screening key variables was as follows:

$$Y = 0.2949 + 0.0030\times X_1 + 0.0040\times X_2 - 0.0034\times X_3 + 0.0259\times X_4 + 0.0047\times X_5 - 0.0092\times X_6 - 0.0121\times X_7 + 0.0214\times X_8 + 0.0338\times X_9 - 0.0008\times X_{10} \quad (9)$$

The model presented a high determination coefficient (R^2 = 0.9996). The *P* value of linear model was 0.0482 which demonstrated that the fit of the linear model was satisfied. The *F*-test was used to determine the significances of the coefficients in the linear model. The corresponding *P*-values were used to check the significance of each coefficient. The smaller the *P*-value, the more significant is the corresponding coefficient (Liu et al., 2004). If the *P*-value less than 0.05 indicated that the corresponding coefficient was statistically significance. According to Table 4, the relative importance of the variables was found as follows: X_9>X_4>X_8> X_7> X_6> X_5 >X_2> X_3> X_1> X_{10}. The analyses of effect estimates also suggested that KH_2PO_4 (X_4), VB_1 (X_8) and VB_{12} (X_9) effects were significant contributor to response (P<0.05). Other variables below 95% confidence levels were considered insignificant (P>0.05). From the positive values of these three factors, a higher content of KH_2PO_4, VB_1 and VB_{12} would result in a higher

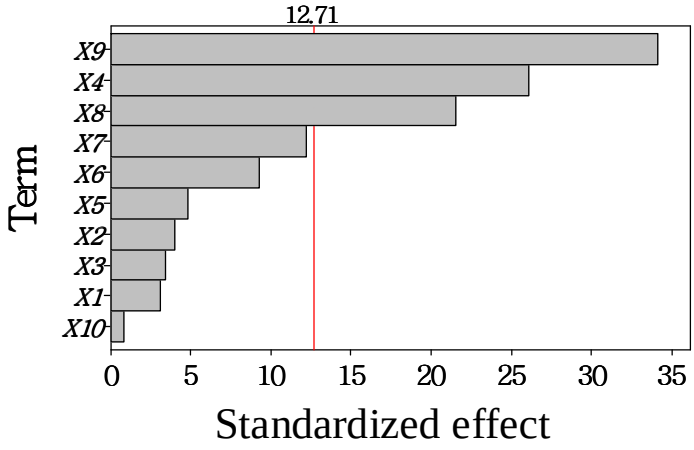

Figure 2. Pareto chart describes the effect of media components on the desirability value.

Table 5. The design matrix and the results of BBD.

Run	X_1	X_2	X_3	*D*v	Run	X_1	X_2	X_3	*D*v	Run	X_1	X_2	X_3	*D*v
1	-1	-1	0	0.1782	6	0	-1	1	0.2586	11	-1	0	1	0.2371
2	-1	1	0	0.1122	7	0	1	-1	0.2006	12	1	0	1	0.3234
3	1	-1	0	0.2937	8	0	1	1	0.1745	13	0	0	0	0.4204
4	1	1	0	0.2276	9	-1	0	-1	0.1693	14	0	0	0	0.4222
5	0	-1	-1	0.0946	10	1	0	-1	0.2486	15	0	0	0	0.4008

*D*v refers to the average desirability value of triplicate experiments.

desirability value.

The effects of the variables on the desirability value were also investigated by preparing a Pareto chart (Figure 2). The length of each bar in the chart indicated the standardized effect of that factor on the response. The vertical line on this plot represented 95% of the confidence interval. Effects that cross this reference line were significant values with respect to the response (Bingol et al., 2010). From this data, we can see the variables X_4, X_8, X_9 have significant contribution on the response. Hence, the three factors KH_2PO_4 (X_4), VB_1 (X_8) and VB_{12} (X_9), which had higher significant effects on D_V were taken into consideration for further study.

Further optimizing with RSM and ANN-GA

To determine the optimum concentrations of KH_2PO_4 (X_4), VB_1 (X_8) and VB_{12} (X_9), RSM and ANN-GA were used in the third step. This phase of the study involved the application of BBD. The design matrix of BBD and results of *Y* (response) for *D*v are listed in Table 5.

Response surface methodology was applied to study the individual effects and mutual interaction effects of candidate variables on *D*v. The BBD data were analyzed by multiple regression analysis using the SAS V8.02 and a multivariate quadratic regression model Equation 10 was developed based for determining the individual effects and mutual interaction effects of candidate variables.

$$Y = 0.4145 + 0.0496\times X_1 - 0.0138\times X_2 + 0.0351\times X_3 - 0.0745\times X_1\times X_1 - 0.000032\times X_1\times X_2 + 0.0017\times X_1\times X_3 - 0.1370\times X_2\times X_2 - 0.0475\times X_2\times X_3 - 0.0954\times X_3\times X_3 \quad (10)$$

Where *Y* is the desirability value and X_1, X_2, X_3 are the coded values of the test variables KH_2PO_4, VB_1 and VB_{12}, respectively.

Checking the goodness of fit of the model needed the information on the determination coefficient (R^2), which is always between 0 and 1 (Liu et al., 2004). The closer the R^2 value is to 1, the stronger the model is and the better is to predict the response. In this case, a value of determination coefficient (R^2 = 0.9752) was calculated, indicated that more than 97.52% of variability in the response could be explained by the second-order polynomial predicted equation given above. The value of the adjusted determination coefficient (R_{adj}^2 = 0.9305),

Table 6. The statistical results of MQR model.

Source	*DF*	*SS*	*MS*	*F*	*Pr > F*
X_1	1	0.0197	0.0197	25.9707	0.0038
X_2	1	0.0015	0.0015	2.0016	0.2163
X_3	1	0.0098	0.0098	12.9948	0.0155
$X_1 \times X_1$	1	0.0205	0.0205	27.0749	0.0035
$X_1 \times X_2$	1	2.5E-9	2.5E-9	3.305E-6	0.9983
$X_1 \times X_3$	1	0.00001	0.00001	0.0161	0.9040
$X_2 \times X_2$	1	0.0693	0.0693	91.5683	0.0002
$X_2 \times X_3$	1	0.0090	0.0090	11.9332	0.0182
$X_3 \times X_3$	1	0.0336	0.0336	44.3603	0.0012
Model	9	0.1486	0.0165	21.8141	0.0017
(Linear)	3	0.0310	0.0103	13.6557	0.0076
(Quadratic)	3	0.1086	0.0362	47.8036	0.0004
(Cross product)	3	0.0090	0.0030	3.9831	0.0855
Error	5	0.0038	0.0008		
(Lack of fit)	3	0.0035	0.0012	8.2769	0.1097
(Pure error)	2	0.0003	0.0001		
Total	14	0.1524			

DF refers to degrees of freedom, SS refers to sum of squares, MS refers to mean square, *F* and *Pr>F* refer to *F* and *P* values, respectively.

which was very close to the R^2 value, also confirmed that the model was highly significant. In addition, these results indicated that the quadratic regression model was highly significant (P <0.01). Information on lack of fit was needed for checking the adequacy of the model (Yetilmezsoy et al., 2009). The *F*-value and *P*-value of 'lack of fit' were 8.2769 and 0.1097, respectively, which indicated that 'lack of fit' was insignificant. The effects of individual variables and the mutual effects between the variables are shown in Table 6. In this case, the effects of linear coefficients (X_1, X_3), quadratic term coefficients (X_1^2, X_2^2, X_3^2), and interaction term coefficients (X_2X_3) were extremely significant, with very small *P*-values. The results also indicated that the individual factors, X_1 and X_3 had positive effects on D_V. This significant interaction between the factors X_2 and X_3 means that the effect of VB_1 concentration on yield was dependent on the level of VB_{12} used. In addition, the interactions between X_1 and X_2 and that between X_1 and X_3 were insignificant, indicating that there was no significant correlation between X_1 and other two variables, and that they did not help much in increasing the desirability value. Three-dimensional response surface plots and two-dimensional contour plots which are graphical representations of the regression equation were generated to gain a better understanding of the interactive effects of the two variables on the responses (Figure 3). Each 3D plot represented a number of combinations of two test variables. The response surface with circular contour plot indicates the negligible interaction between the corresponding variables, whereas elliptical or saddle nature of the contour plots indicates the significant interactions between the corresponding variables (Patil et al., 2011). It can be seen that, the term of X_1X_2, X_1X_3 was not significant, while X_2X_3 was significant which confirmed the statistical results of MQR model. These results indicated that the relationship between the variables and the response were not simply linear. The maximum value of *D*v (0.427) was recorded at 0.97 g/L KH_2PO_4, 0.1999 g/L VB_1, and 0.22 g/L VB_{12}. Therefore, the optimal culture medium composition obtained by RSM was (g/L): glucose 40, beef extract 10, peptone 10, KH_2PO_4 0.97, $MgSO_4 \cdot 7H_2O$ 1, NaCl 0.50, VB1 0.1999 and VB12 0.22.

Many reports have shown that ANN is superior to the method of RSM (Zahedi and Abbas, 2011). In the present work, an ANN with three layers was also used to determine the optimal levels of medium constituents for maximizing the production of desired products. One of the data of BBD experiments was randomly chosen and used as prediction set. Another one was randomly chosen and used as test set. The other experimental data were used as calibration set. The suitable number of hidden nodes which was set to 12 was selected depend on *D*a (Figure 4). The determination coefficient (R^2) of the optimized ANN model was 0.9945, indicating that the fitting of the ANN was satisfied. The root mean square

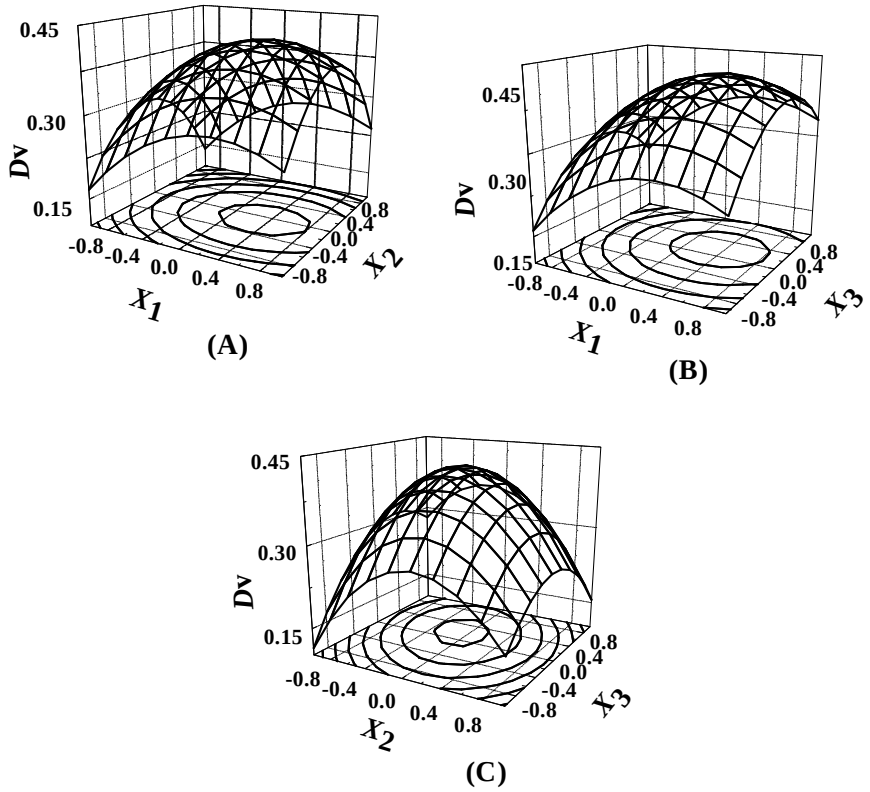

Figure 3. Response surface and contour plots showing the mutual effects between candidate variables and desirability value. (A) interaction between X_1 and X_2 for Y; (B) interaction between X_1 and X_3 for Y; (C) interaction between X_2 and X_3 for Y; Response Y: desirability value; Variables X_1: the code of KH_2PO_4 concentration; X_2: the code of VB_1 concentration; X_3: the code of VB_{12} concentration.

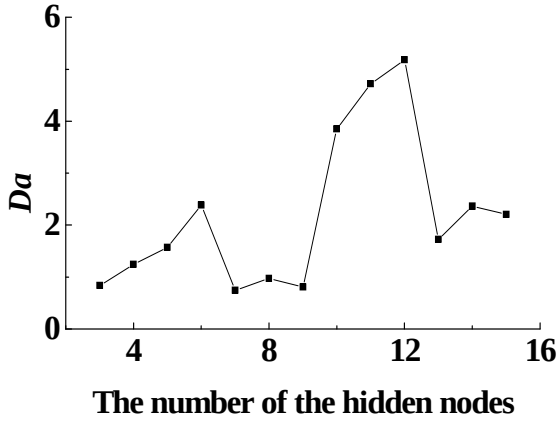

Figure 4. Effect of the number of hidden nodes on *D*a.

error of calibration set ($RMSE_c$), the root mean square error of test set ($RMSE_t$) and the root mean square error of prediction set ($RMSE_p$) were 0.009, 0.017 and 0.012, respectively. After the ANN model was established, GA was implemented to optimize the fermentation medium for maximum *D*v. The best fitness plot for the GA (Figure 5) maps the gradual convergence of the best fitness values of successive generations towards the final optimum value. The coded values of the optimum culture conditions obtained by GA were: X_1 = -0.015; X_2 = 0.020 and X_3 = -0.704 and the corresponding actual values were: x_1 = 0.688, x_2 = 0.201 and x_3 = 0.130. The expected *D*v was 0.502. In order to confirm the predicted results of the model, five further experiments using the aforementioned optimized parameters were performed and the average value of 0.493 was obtained with relative errors of 1.79% between expected value and experimental value. These results reflected the accuracy and applicability of the combined BBD and ANN-GA to optimize the fermentation medium of *P. tenuipes* N45. The *D*v under the optimal medium was 0.493, which was 0.427, the *D*v under the conditions recommended by RSM. Therefore, the method of ANN-GA performed better than the method of RSM in this optimization study.

For many years, interest has concentrated on utilization of *P. tenuipes*. Liquid fermentation obviously is a good choice fo the production of mycelial biomass and

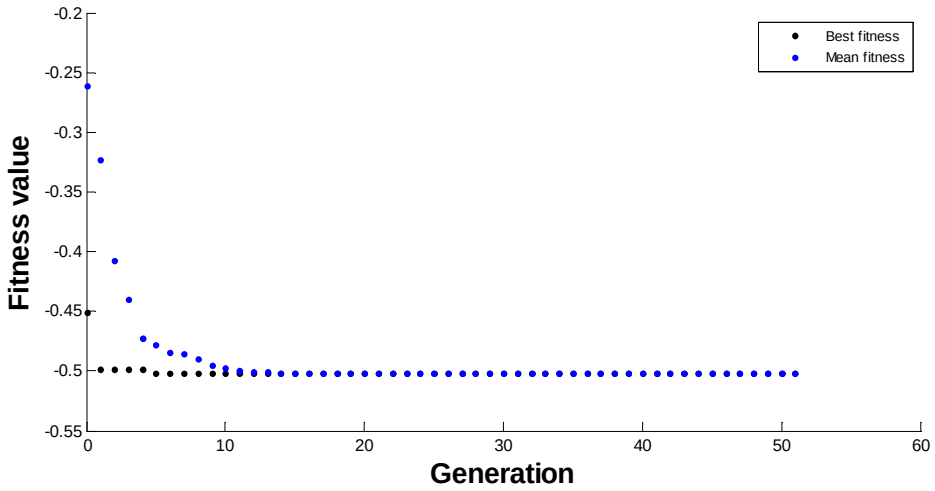

Figure 5. The effect of the number of generation on fitness.

bioactive compounds in large scale. Each strain has its specific nutritional parameter requirements for fermentation. Thus, it is most important to obtain optimal culture medium of *P. tenuipes* for further development. However, the classical method of optimization such as one-factor-at-a-time approach not only is laborious and time consuming but also has the limitation of ingnoring the explanation of interaction effects among the variables (Rajendran and Thangavelu, 2012; Khedher et al., 2011). To tackle this problem, application of more efficient statistical experimental design is increasingly accepted in biotechnology to optimize the culture medium components and conditions on account of its applicability and aaptness. Statistical methods were successfully used to determine the optimum media and conditions for exo-biopolymer and mycelial production by *P. tenuipes* (Xu et al., 2003, 2006). In the present study, sequential statistical methods including PBD, BBD, ANN and GA coupled with desirability function were successfully applied in screening the significant variables influencing *P. tenuipes* N45 fermentation and predicting the optimal level of significant variables. The yield of mycelial biomass and three bioactive compounds (polysaccharide, adenosine and cordyceps acid) were enhanced simultaneously via this approach. This work provided valuable information for further utilization of *P. tenuipes* N45.

Conclusions

In this paper, response surface methodology and artificial neural network coupled with genetic algorithm were used to optimize the medium constituents of *P. tenuipes* N45. It can be seen from the present study that ANN-GA provided more reliable results than RSM for defining the formulation of an improved medium for submerged fermentation of *P. tenuipes* N45. Optimum medium (glucose 40 g/L, beef extract 10 g/L, peptone 10 g/L, KH_2PO_4 0.688 g/L, $MgSO_4 \cdot 7H_2O$ 1 g/L, NaCl 0.5 g/L, VB_1 0.201 g/L and VB_{12} 0.130 g/L) were identified at last. The desirability value under this condition could reach the highest value of 0.493. Compared with the basal medium, the biomass 16.230 g/L, the production of adenosine 0.0724 g/L, polysaccharide 0.723 g/L and cordyceps acid 2.137 g/L were enhanced by 8.20, 3.58, 23.17 and 31.51%, respectively. These results clearly confirmed that RSM and ANN-GA are effective tools for mathematical modeling and factor analysis of medium optimization process.

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