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The optimization of *Marasmius androsaceus* submerged fermentation conditions in five-liter fermentor

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Abstract Using desirability function, four indexes including mycelium dry weight, intracellular polysaccharide, adenosine and mannitol yield were uniformed into one expected value (*Da*) which further served as the assessment criteria. In our present study, Plackett–Burman design was applied to evaluate the effects of eight variables including initial pH, rotating speed, culture temperature, inoculum size, ventilation volume, culture time, inoculum age and loading volume on *Da* value during *Marasmius androsaceus* submerged fermentation via a five-liter fermentor. Culture time, initial pH and rotating speed were found to influence *Da* value significantly and were further optimized by Box–Behnken design. Results obtained from Box–Behnken design were analyzed by both response surface regression (Design-Expert.V8.0.6.1 software) and artificial neural network combining the genetic algorithm method (Matlab2012a software). After comparison, the optimum *M. androsaceus* submerged fermentation conditions via a five-liter fermentor were obtained as follows: initial pH of 6.14, rotating speed of 289.3 rpm, culture time of 6.285 days, culture temperature of 26 °C, inoculum size of 5%, ventilation volume of 200 L/h, inoculum age of 4 days, and loading volume

of 3.5 L/5 L. The predicted *Da* value of the optimum model was 0.4884 and the average experimental *Da* value was 0.4760. The model possesses well fitness and predictive ability.

1. Introduction

As an efficient way to produce bioactive metabolites for fungus, submerged fermentation has been studied for years (Saha et al., 2014; Zhou et al., 2014). *Marasmius androsaceus*, a traditional Chinese medicine, possesses analgesic and antioxidant effects. In China, "An-Luo Tong", produced by fermentation mycelium of *M. androsaceus,* has been used as a painkiller for years. However, its large-scale application both as a medicine and asfood is limited by immature artificial cultivation technology. The optimum fermentation conditions of *M. androsaceus* in a fermentor has not been reported yet (Surhio et al., 2014; Naureen et al., 2014).In our previous study, by using chemometric methods, the optimum submerged fermentation medium of *M. androsaceus* was obtained. Moreover, in our group, desirability function combining Plackett–Burman design and Box–Behnken design was successfully applied to optimize submerged fermentation medium of *Paecilomyces tenuipes* N45 and *Saccharomyces cerevisiae* (Dong et al., 2012; Du et al., 2012). Similar to previous studies, desirability function is employed to unite several response data into one expected value which served as the assessment criteria (Dong et al., 2012; Du et al., 2012; Heidari et al., 2014). Generally, following with Plackett–Burman design which is used to identify the important process variables (Giordano et al., 2011), Box–Behnken design is further applied to optimize selected variables and quantify the functional relationship between measured response values and explanatory factors (Dalvand et al., 2014; Safi et al., 2015). As a statistical and mathematical techniques response surface methodology (RSM) is widely applied in the optimization of various processes in food chemistry, chemical engineering, material science and biotechnology (Bezerra et al., 2008; Kiyani et al., 2014; Khaskheli et al., 2015). Moreover, artificial neural network combined with genetic algorithm (ANN-GA) is considered as an alternative modeling technique used in the field of microbiology (Tripathi et al., 2012; Batool et al., 2015). Artificial neural network (ANN) imitates the behavior of neurons in the human brain and possesses advantages including non-linearity, flexible, speed, simplicity, and high accuracy (Manning et al., 2014). Based on the concept of natural selection and genetics, genetic algorithm (GA) is an effective tool to solve complex optimization problems in various scientific and technological fields (Wang and Li, 2014).

Our present study was conducted in an attempt to search an optimal submerged fermentation condition of *M. androsaceus* in a five-liter fermentor by using statistical and mathematical techniques including Plackett–Burman design and Box–Behnken design. Both RSM and ANN-GA were performed to analyze results from Box–Behnken design.

2. Materials and methods

2.1. Strains, agents and equipment

M. androsaceus (CCTCC M2013175) was deposited in China Center for Type Culture Collection, China.

M. androsaceus was cultured in a five-liter full-automatic fermentor (Baoxing Bioscience company, Shanghai, China) using a defined liquid medium containing: 20 g/L sucrose, 10 g/L peptone, 10 g/L yeast extract powder, 1 g/L $MgSO_4 \cdot 7H_2O$, 1 g/L $KH_2PO_4 \cdot 3H_2O$, and 0.1 g/L Vitamin B_1.

All the chemical reagents used in the present experiment for submerged fermentation and effective constituent determination were obtained from Sigma–Aldrich, USA.

2.2. The concentration of effective constituents determination

2.2.1. Measurement of polysaccharide

The amount of total saccharides was determined by anthrone–sulfuric acid method (Leyva et al., 2008). Monosaccharide concentration was measured by 3, 5-2 nitrate salicylic acid (DNS) colorimetry (Teixeira et al., 2012).

The concentration of polysaccharide = The concentration of total saccharides – the concentration of monosaccharide.

2.2.2. Measurement of adenosine

As it is recommended in the Chinese Pharmacopoeia that the adenosine concentration in *M. androsaceus* is detected via high performance liquid chromatography (HPLC) (Chinese Pharmacopoeia Commission, 2010).

2.2.3. Measurement of mannitol

The concentration of mannitol in *M. androsaceus* mycelium was measured using the colorimetric method as described previously (Dong and Yao, 2008).

2.3. The optimization of submerged fermentation conditions of M. androsaceus in a five-liter fermentor

2.3.1. Desirability function establishment

Based on desirability function (Kleijnen and Sargent, 2000), the mycelium yield, the concentration of intracellular adenosine, mannitol and polysaccharide were uniformed into one index—*Da* (ranged: 0–1) according to the following equations.

$$d_i = \left| \frac{\hat{y}_i - y_{\min}}{y_{\max} - y_{\min}} \right| \quad \hat{y} > y_{\max}, \quad d_i = 1; \quad \hat{y} < y_{\min}, \quad d_i = 0 \qquad (1)$$

where, $\hat{y}_i$ is the response value of an *i* analyzed factor.

$$Da = d_1^{w_1} \times d_2^{w_2} \times d_3^{w_3} \times \cdots \times d_i^{w_i} \ldots w_1 + w_2 + w_3 + \cdots w_i = 1 \qquad (2)$$

where, w_i is weighted value of index *i*.

Table 1 Parameters of the desirability function.

Parameters	Mycelium weight (g/L)	Adenosine (g/L)	Mannitol (g/L)	Polysaccharide (g/L)
y_{il}	2.00	0.01	0.00	0.10
y_{ih}	15.00	0.14	0.80	2.40
w_i	0.25	0.30	0.15	0.30

The parameters of desirability function used in the present study were displayed in Table 1. As shown in the equations, the more important index was weighted higher.

2.3.2. Plackett–Burman design

To evaluate the contribution of the following factors during *M. androsaceus* submerged fermentation in a five-liter full-automatic fermentor, Plackett–Burman design was applied (Giordano et al., 2011). According to the design matrix (Table 2), each independent variable was tested at a high (+1) and a low (−1) level. The general form of the linear regression model follows as Eq. (3). The significance of each variable was determined by a one-way variance analysis (ANOVA). Statistical significance was defined as $P < 0.05$.

$$Y = \beta_0 + \sum_{i=1}^{10} \beta_i X_i \qquad (3)$$

where, Y is the response or dependent variable; β_0 is the model intercept; X_i is the independent variable; β_i is the linear regression coefficient.

2.3.3. Box–Behnken design

Based on the results obtained from Plackett–Burman design, three variables and three levels Box–Behnken design were performed to optimize *M. androsaceus* submerged fermentation conditions in a five-liter automatic fermentor. According to the design matrix (Table 3), the experiments were performed. Using Design-Expert.V8.0.6.1 software, the response surface methodology was used to evaluate the three selected variables (Basri et al., 2007; Du et al., 2012; Fatiha et al., 2013). The quadratic regression model was used for studying the relationship between the chosen factors and Da. The general form of the quadratic regression model is as follows:

$$Y = \beta_0 \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \qquad (4)$$

where, Y is the predicted values of the response; x_i and x_j are chosen factors; β_0 is the intercept; β_i is linear coefficient; β_{ii} is the quadratic coefficient; β_{ij} is the interaction coefficient.

Matlab2012a software was applied to analyze the results obtained from Box–Behnken design via ANN-GA (Basri et al., 2007; Du et al., 2012; Fatiha et al., 2013). In our present study, a three layer neural network model was developed. 11 data from Box–Behnken design was randomly chosen to serve as calibration set. Another 3 data were randomly selected as test set and the left 3 data was served as prediction set. The network was trained with Levenberg–Marquardt back propagation algorithm. Sigmoid function and linear function were used for the hidden layer and output layer respectively. Approaching degree (Dv), defined as Eq. (5), was applied to search the optimal number of hidden nodes which strongly influence the quality and predictive ability of the developing network (Liu et al., 2008).

$$Dv = \frac{c}{\frac{n_c}{n} \times MSE_c + \frac{n_t}{n} \times MSE_t + |MSE_c - MSE_t|} \qquad (5)$$

where, n_c and n_t represent the number of calibration sets and test sets; n is the sum number of the calibration set and test set; MSE_c and MSE_t are the root mean square error (MSE) of the calibration set and test set, respectively; and c is a constant by which Dv is adjusted to obtain a good graph. In the present study, c is 0.08. The best ANN model was selected according to related Dv values.

GA was further employed to search optimal culture conditions in test regions. The used parameters of GA were as follows: 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

Table 2 The design matrix and results of the Plackett–Burman design.

	Culture Temperature Z_1/°C	Initial pH Z_3	Inoculum size Z_4/%	Inoculum age Z_5/d	Rotate speed Z_6/rpm	Ventilation volume Z_7/L*h^{-1}	Culture time Z_8/d	Loading volume Z_{10}/L*5L^{-1}	Da
1	28(1)	7(1)	3(−1)	3(−1)	150(−1)	250(1)	7(1)	3(−1)	0.2846
2	28(1)	5(−1)	5(1)	3(−1)	150(−1)	150(−1)	7(1)	4(1)	0.3763
3	22(−1)	7(1)	3(−1)	5(1)	150(−1)	150(−1)	5(−1)	4(1)	0.1875
4	28(1)	7(1)	5(1)	3(−1)	350(1)	150(−1)	5(−1)	4(1)	0.3003
5	28(1)	5(−1)	5(1)	5(1)	150(−1)	250(1)	5(−1)	3(−1)	0.3035
6	28(1)	7(1)	3(−1)	5(1)	350(1)	150(−1)	7(1)	3(−1)	0.3368
7	22(−1)	7(1)	5(1)	3(−1)	350(1)	250(1)	5(−1)	3(−1)	0.2458
8	22(−1)	7(1)	5(1)	5(1)	150(−1)	250(1)	7(1)	4(1)	0.3116
9	22(−1)	5(−1)	5(1)	5(1)	350(1)	150(−1)	7(1)	3(−1)	0.4354
10	28(1)	5(−1)	3(−1)	5(1)	350(1)	250(1)	5(−1)	4(1)	0.3349
11	22(−1)	5(−1)	3(−1)	3(−1)	350(1)	250(1)	7(1)	4(1)	0.3591
12	22(−1)	5(−1)	3(−1)	3(−1)	150(−1)	150(−1)	5(−1)	3(−1)	0.2776

Table 3 The design matrix and the results of the Box–Behnken design.

	V_1 (initial pH)	V_2 (rotate speed /rpm)	V_3 (culture time/d)	Da
1	7 (1)	350 (1)	6 (0)	0.4358
2	5 (−1)	350 (1)	6 (0)	0.4594
3	6 (0)	250 (0)	6 (0)	0.4603
4	6 (0)	250 (0)	6 (0)	0.4700
5	5(−1)	250 (0)	5 (−1)	0.4349
6	6 (0)	350 (1)	7 (1)	0.4686
7	6 (0)	150 (−1)	5 (−1)	0.4094
8	7 (1)	250 (0)	5 (−1)	0.4303
9	7 (1)	250 (0)	7 (1)	0.4705
10	6 (0)	250 (0)	6 (0)	0.4648
11	5 (−1)	150 (−1)	6 (0)	0.3941
12	5 (−1)	250 (0)	7 (1)	0.4156
13	7 (1)	150 (−1)	6 (0)	0.4579
14	6 (0)	350 (1)	5 (−1)	0.4256
15	6 (0)	250 (0)	6 (0)	0.4703
16	6 (0)	150 (−1)	7 (1)	0.4086
17	6 (0)	250 (0)	6 (0)	0.4733

0.8, crossover function as scattered, migration fraction as 0.2, migration interval as 20, and penalty factor as 100. Among them, a fitness function, which plays crucial to maximize the performance of GA, was specially presented as Eq. (6) (da Silva et al., 2011; Butt et al., 2015). After a 100 generation evaluation by GA, based on the given range of input parameters, optimal culture conditions were achieved.

$$Fitness = -Dv \qquad (6)$$

The statistical significances of the coefficients in each model were identified by a one-way ANOVA. Statistical significance was defined as $P < 0.05$.

2.3.4. Independent verification test

Based on the analysis via Design-Expert.V8.0.6.1 and Matlab2012a software, three parallel experiments for each analysis result were performed to test the selected fermentation conditions obtained from RSM and ANN-GA respectively. After comparing, the optimum submerged fermentation conditions of *M. androsaceus* in five-liter fermentor were achieved.

3. Results and discussion

3.1. Variables selection via Plackett–Burman design

During *M. androsaceus* submerged fermentation via a five-liter automatic fermentor, significant variables were screened via the Plackett–Burman design (Haddad et al., 2014). Through analysis via a one-way ANOVA, the low P-value corresponding to the high F-value for one factor indicates its high significance (Pulimi et al., 2012). The linear regression equation was obtained based on the experimental results (Table 2). Determinate coefficient (R^2) was used to evaluate the fitness of the established model. When R^2 is closer to 1, the model possesses better predictive ability. In our present experiment, R^2 is 0.9997 which suggests that nearly 99.97% changes of Da can be explained by the established model. According to
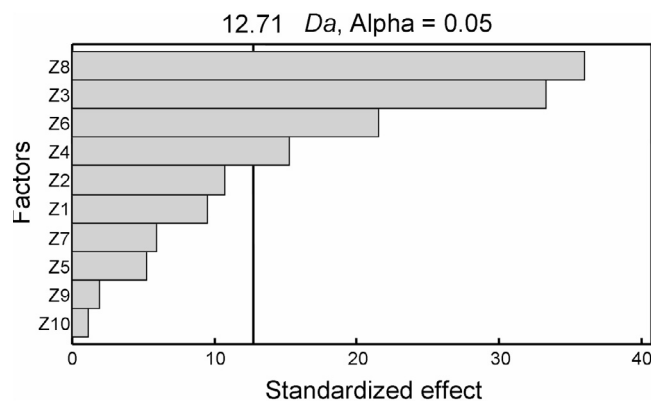
Figure 1 The effects of fermentation conditions on the desirability value.

Eq. (7), Da was influenced by the values of initial pH, rotating speed, culture temperature, inoculum size, ventilation volume, culture time, inoculum age and loading volume. By comparing F- and P-values, the order of each investigating factor was $Z_8 > Z_3 > Z_6 > Z_4 > Z_2 > Z_1 > Z_7 > Z_5 > Z_9 > Z_{10}$.

$$\begin{aligned}
Y(Da) = {} & 0.3128 + 0.0099^*Z_1 - 0.0113^*Z_2 - 0.00350^*Z_3 \\
& + 0.0160^*Z_4 + 0.0055^*Z_5 + 0.0226^*Z_6 - 0.0062^*Z_7 \\
& + 0.0378^*Z_8 - 0.0020^*Z_9 - 0.0012^*Z_{10}
\end{aligned} \qquad (7)$$

Culture time (Z_8), initial pH (Z_3), rotating speed (Z_6) and ventilation volume (Z_4) strikingly influence *M. androsaceus* submerged fermentation; comparatively, the confidence levels of other factors were below 95% which were not served as significant influence elements (Table 3; Fig. 1). According to Eq. (7), the positive coefficient of Z_8, Z_6, and Z_4, and the negative coefficient of Z_3 indicate that the enhancement of culture time, rotating speed and ventilation volume, and the reduction of initial pH benefits the increase in Da value.

Based on the Pareto principle, a Pareto chart was designed to select more important elements via rearranging the effects of each variable on response value (Bingol et al., 2010; Du et al., 2012). Minitab 15 software was used to draw a Pareto chart in our study and the vertical represents 95% confidence interval (Fig. 1). Although Z_8, Z_3, Z_6 and Z_4 were all served as significant influence factors based on results from the Plackett–Burman design, the significance of Z_4 was much smaller than that of the other three variables. Collectively, culture time (Z_8), initial pH (Z_3), and rotating speed (Z_6) were selected for further optimum.

3.2. Optimizing submerged fermentation conditions of M. androsaceus by RSM and ANN-GA

The selected variables including culture time, initial pH, and rotating speed were optimized by Box–Behnken design. The concentrations used in Plackett–Burman design served as central points, and the experiments were performed following with the design matrix (Table 4). The individual and mutual interaction effects of selected variables on Da were analyzed via response surface methodology (Dong et al., 2012). The quadratic regression model (Eq. (8)) was established to access the relationship between the candidate factors and Da values.

Table 4 Regression analysis of the Plackett–Burman design experiment.

Source	DF	SS	MS	F	Pr > F	Significance
Z_1	1	0.0012	0.0012	89.47	0.0670	Not significant
Z_2	1	0.0015	0.0015	115.40	0.0591	Not significant
Z_3	**1**	**0.0147**	**0.0147**	**1109.24**	**0.0191**	Significant
Z_4	**1**	**0.0031**	**0.0031**	**232.70**	**0.0417**	Significant
Z_5	1	0.0004	0.0004	27.36	0.1203	Not significant
Z_6	**1**	**0.0061**	**0.0061**	**462.24**	**0.0296**	Significant
Z_7	1	0.0005	0.0005	34.89	0.1068	Not significant
Z_8	**1**	**0.0172**	**0.0172**	**1296.22**	**0.0177**	Significant
Z_9	1	< 0.0001	< 0.0001	3.71	0.3049	Not significant
Z_{10}	1	< 0.0001	< 0.0001	1.22	0.4680	Not significant
Model	**9**	**0.0447**	**0.0045**	**337.24**	**0.0423**	Significant
Error	2	< 0.0001	< 0.0001			
Total	11	0.0447		R^2	0.9997	

$$Y(Da) = 0.47 + 0.011^*V_1 + 0.015^*V_2$$
$$+ 7.87E - 003^*V_3 - 0.022^*V_1^*V_2 + 0.015^*V_1^*V_3$$
$$+ 0.011^*V_2^*V_3 - 0.011^*V_1^2 - 0.020^*V_2^2 - 0.019^*V_3^2 \quad (8)$$

Significant *F*-value (26.73) and *P*-value (< 0.001), with non-significant lack of fit (*P* = 0.1921) suggest that the established model was well adapted to the response (Table 5). The value of adjustment coefficient (R^2 adj) was 0.9354 which is similar to R^2 (0.9718) indicating the well fitness of the quadratic regression model (Ren et al., 2008) and nearly 97.18% changes on *Da* value can be explained by Eq. (8). The once migration (V_1 (*P* = 0.001), V_2 (*P* = 0.0018) and V_3 (*P* = 0.0005)), interaction migration (V_1V_2 (*P* = 0.0019) and V_1V_3 (*P* = 0.0009)), and quadratic migration (V_1^2 (*P* = 0.0005), V_2^2 (*P* = 0.0017) and V_3^2 (*P* = 0.0016)) showed strong influence on Eq. (8) indicating that the relationship between the selected variables and *Da* values was rather a simple linear relation (Bas and Boyaci, 2007). Furthermore, the fitness and predicted ability of established model was estimated by Design-Expert V8.0.6.1 software. The normal distribution of studentized residuals of *Da* confirmed the well fitness of the quadratic regression model (Fig. 2A). The developing model achieved satisfactory predictive ability indicated by similar values obtained from experiments and model prediction

(Fig. 2B). All three independent variables significantly influence *Da* value which was displayed by linear-circular changing (Fig. 2C). After analyzing via RSM, the fermentation conditions of *M. androsaceus* in a five-liter fermentor was as follows: initial pH of 6.88, rotating speed of 254 rpm, culture time 6.56 days, culture temperature 26 °C, inoculum size of 5%, inoculum age of 4 days, ventilation volume of 200 L/h and a loading volume of 3.5 L/5 L.

ANN-GA is performed to search for the optimal fermentation conditions for maximizing the desired products production, which is considered to be superior to RSM (Zahedi and Abbas, 2011). During the establishment of the ANN model, *Dv* value served as evaluation index to optimize the number of hidden layer nodes which was finally chosen as 15 (Fig. 3A). The well fitness of ANN model was demonstrated by the satisfied determination coefficient (R^2) (0.9961). The values of $RMSE_c$, $RMSE_t$, and $RMSE_p$ were 0.0024, 0.0035 and 0.0079. Furthermore, GA was performed to search for the best conditions with maximum *Dv* based on experimental results. The best fitness plot revealed successive generations toward the final optimum value (Fig. 3B). After analyzing via ANN-GA, the fermentation conditions of *M. androsaceus* in a five-liter fermentor was as follows: initial pH 6.14, rotate speed of 289.3 rpm, culture time of 6.285 days, culture

Table 5 Variance analysis of response surface methodology.

| Variance source	SS	DF	MS	F	Pr > F	Significance
Model	0.0011	9	0.0012	26.76	0.0001	Significant
V_1	0.0010	1	0.0010	22.92	0.0020	significant
V_2	0.0018	1	0.0018	39.79	0.0004	Significant
V_3	0.0005	1	0.0005	11.08	0.0126	Significant
V_1V_2	0.0019	1	0.0019	42.69	0.0003	Significant
V_1V_3	0.0009	1	0.0009	19.78	0.0030	Significant
V_2V_3	0.0005	1	0.0005	10.76	0.0135	Significant
V_1^2	0.0005	1	0.0005	10.52	0.0142	Significant
V_2^2	0.0017	1	0.0017	39.03	0.0004	Significant
V_3^2	0.0016	1	0.0016	35.21	0.0006	Significant
Residual	0.0003	7	< 0.0001			
Lack of fit	0.0002	3	< 0.0001	2.57	0.1921	Not significant
Pure error	0.0001	4	< 0.0001			
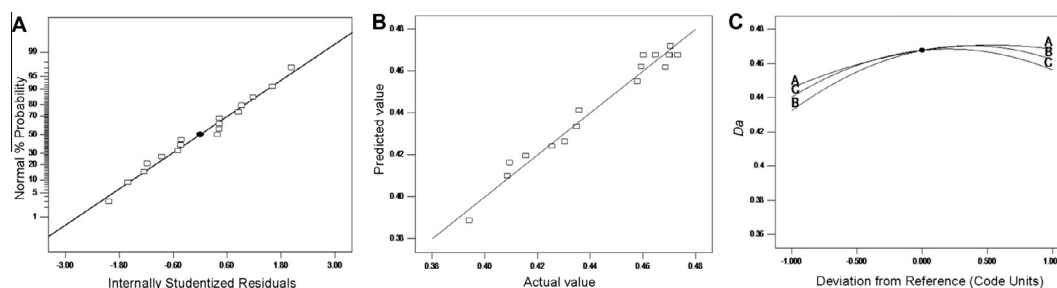
Figure 2   (A) Normal probability plot of the studentized residual for *Da*. (B) Predicted versus actual values plot for *Da*. (C) Perturbation plot for *Da*.
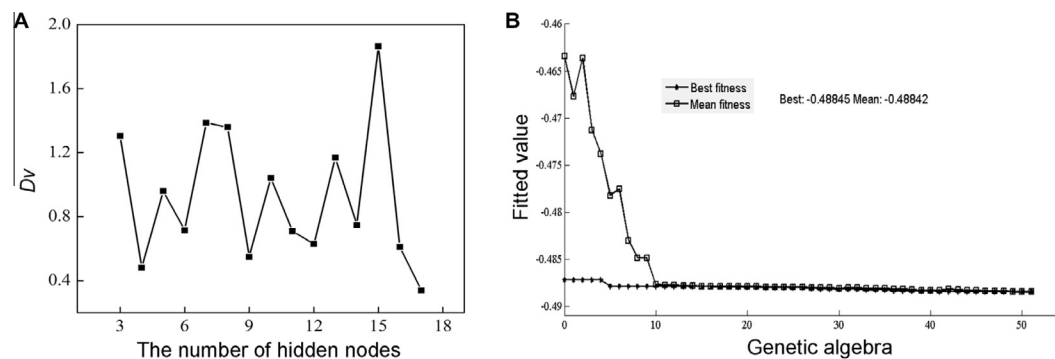

Figure 3   (A) The effects of the number of hidden nodes on *D*v. (B) The effects of genetic algebra on model fitness.

temperature of 26 °C, inoculum size of 5%, inoculum age of 4 days, ventilation volume of 200 L/h and a loading volume of 3.5 L/5 L.

3.3. Independent verification test

Based on the fermentation conditions obtained from RSM and ANN-GA, six independent verification tests (three for each result) were applied to investigate the similarity between experimental data and model predictive values. The predictive *Da* value from RSM via Design-Expert.V8.0.6.1 software was 0.4752, and the average experimental value was 0.4409. Comparatively, the predictive *Da* value from ANN-GA via Matlab2012a software was 0.4884, and the average experimental value was 0.4760. Finally, the optimum submerged fermentation condition of *M. androsaceus* in five-liter fermentor was obtained after ANN-GA optimization.

4. Conclusions

As a traditionally used painkiller, the large-scale application of *M. androsaceus* is limited by immature artificial cultivation technology. The fermentation conditions of *M. androsaceus* in a fermentor have not been yet determined. However, the classical optimal methods including a one-factor-at-a-time approach are not only laborious and time consuming, but also ignoring the explanation of interaction among selected variables (Din et al., 2014; Yazdani et al., 2014). In our present study, Plackett–Burman design and Box–Behnken design were applied to optimize submerged fermentation conditions as of *M. androsaceus* in a five-liter automatic fermentor. Both

RSM and ANN-GA were performed to analyze the data obtained from Box–Behnken design. After comparison, the optimum fermentation conditions were obtained as follows: initial pH of 6.14, rotating speed of 289.3 rpm, culture time of 6.285 days, culture temperature of 26 °C, inoculum size of 5%, inoculum age of 4 days, ventilation volume of 200 L/h and a loading volume of 3.five-liter/five-liter. Our finding reveals that Plackett–Burman design and Box–Behnken design are effective tools for mathematical modeling and factor analysis of the fermentation optimization process.

Conflict of interest

The authors have declared that there is no conflict of interest.

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