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SIMULTANEOUS MULTIRESPONSE OPTIMIZATION OF THE MEDIUM FOR SUBMERGED FERMENTING IRPEX LACTEUS FR. MYCELIA USING DESIRABILITY FUNCTION

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ABSTRACT

The aid of this paper was to optimize the fermentation medium of Irpex lacteus Mycelia for simultaneously enhancing the yields of mycelium, adenosine, intracellular polysaccharide, Cordyceps acid and protein. A sequential statistical strategy was investigated during this optimization process, which consisted of desirability function (DF), Plackett-Burman design (PBD), Box-Behnken design (BBD), Multi-quadratic regression (MQR), artificial neuron networks (ANN) and genetic algorithm (GA). Desirability value (Dv) developed by DF was used as criterion. Suitable carbon sources and nitrogen sources were chosen by single-factor test firstly. PBD combined with linear modeling method was used for identifying the significant component, BBD was used for further optimization. MQR and ANN were used for modeling the BBD data. Specially, MQR model was used for determining the individual effects and mutual interaction effects of the tested variables on *Dv*, ANN model was used for *Dv* prediction. While the ANN model was developed, genetic algorithm (GA) was employed to search for the optimum medium which was as follow (g/L): lactose 29.81, yeast extract powder 15, beef extract 15, $KH_2PO_4 \cdot 5H_2O$ 0.65, $MgSO_4 \cdot 7H_2O$ 0.6, NaCl 0.0078 and VB1 0.281, with expected maximum *Dv* of 0.6302. The validation experiments with the optimum fermentation media were implemented in triplicate and the average *Dv* was 0.6245 which was twice as that without optimization.

KEYWORDS

statistical methods; desirability function; Irpex lacteus, fermentation

1. INTRODUCTION

Irpex lacteus is a wood-decay fungi which belongs to Aphyllophorales species, Polyporaceae family and Irpex Fr. genus. Based on a study, it is a medicinal fungus which is used as a crude drug or invigorant [1,2]. Study showed irpex lacteus possesses many biological and pharmacological activities such as immunological inhibition, anti-tumor, anti-inflammatory, diuretic activities and so on [3-6].

According to research, there are several literatures about optimization of fermentation media for elevating the fermentation levels [7-10]. Most of them just only focused on enhancing one or two of the respond values such as the yields of mycelium, protein, adenosine, intracellular polysaccharide or Cordyceps acid. The quality of Irpex lacteus fermentation can not be elevated comprehensively.

Based on a research, desirability function (DF) is the most popular approaches for multi-response optimization [11,12]. It is widely used in biomedical processes and chromatography separation. However, there are a few literatures about application of DF to optimize the fermentation processes. Research showed the sequential experimental design methods including Plackket-Burmen design (PBD) and Box-behnken design (BBD) were widely used for optimization of fermentation processes and pharmaceutical processes [13,14]. Specially, linear model combined with T test method or F test method is usually applied to select the significant candidate factors. BBD is very suitable for developing multi-quadratic regression (MQR) model. It can be used for determining the individual effects and mutual interaction effects of candidate variables on the response values. And then it is used for finding out the optimum solutions depending on its predictive capability.

Artificial neural network (ANN) is a non-linear computational model based on biological neural networks [15-17]. It simulates the human brain learning process by mathematically modeling the network structure of interconnected node cells. ANN possesses good predictive capability for complex and non-linear systems. There were several literatures demonstrated that the predictive accuracy of ANN models were superior to RSM model using the same experiment design. However, ANN is known as a black box modeling approach. The relationships between the variables and responses are not described by specification of suitable fitting function in ANN models. Based on a study, the effects of factors on response values and the interaction effects among the factors cannot be studied by ANN model [18]. Genetic algorithm (GA) is usually suggested to search for optimum solutions in non-linear systems. According to research, it mimics the principles of biological evolution, namely "survival-of-the-fittest" and "random exchange of data during propagation" followed by biologically evolving species [19].

In present study, sequential statistical methods including PBD, BBD, MQR, ANN and GA combined with DF were successfully applied to simultaneously enhance the yields of mycelium, adenosine, intracellular polysaccharide, Cordyceps acid and protein. The predictive capability of ANN model was much more satisfied than MQR model in this complex system. The quality of the Irpex lacteus Mycelia fermentation was greatly improved by this method. This method will be popular in optimizing the fermentation processes.

2. MATERIALS AND METHODS

2.1 Microorganism and seed culture preparation

The strain of Irpex lacteus Mycelia was given by Jilin Tonghua Yongcang Pharmaceutical Co., Ltd. The Irpex lacteus Mycelia was maintained in cuvette with 20% glycerol modified PDA medium at -80 ℃. The modified PDA medium composed of (g·L -1): glucose 20, peptone 20, KH_2PO_4·5H $_2$O 3.0, $MgSO_4$·7H $_2$O 3.0 and was autoclaved at 121 ℃ for 30 minutes. The maintained strain was inoculated to 100 mL sterile modified PDA medium in Erlenmeyer flask (250 mL) incubated at 26 ℃ in rotary shaker at 150r/min for 3 d. Then, the culture was inoculated to Erlenmeyer flask (250 mL) containing 100 mL sterile modified PDA medium, which was incubated at 26 ℃ in rotary shaker at 150 r/m for 3 d, and the seed culture had been prepared.

2.2 Fermentation

The seed culture was inoculated to Erlenmeyer flask (250 mL) containing 100 mL fermentation media which were prepared depended on the experimental design. The inoculum volume was 5 mL. The fermentation conditions were listed as follow: temperature 26 ℃, shake speed 150 r/min and fermentation time 5 d.

2.3 Analysis method

The biomasses of the samples were determined by gravity weight method. The fermented broths were centrifuging at 4500 r/min for 8 min. We did not need the supernatants So, the precipitations which were treated with lyophilization method were used as the mycelium samples for quantitative analysis of adenosine, intracellular polymer, Cordyceps acid and Protein. The adenosine was extracted with warm water at 45 ℃. The ratio of solid to liquid was 1 : 50 (g : mL). The intracellular polysaccharide and Cordyceps acid were extracted with warm water at 53 ℃. According to a study, the concentrations of intracellular polysaccharide were determined by anthrone-sulfuric acid colorimetry method [20]. The adenosine contents in mycelium were determined by using a Shimadzu HPLC system with tow LC-6AD pumps and SPD-A UV-vis detector (Shimadzu, Kyoto Japan). 20 μL of each sample 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 wavelength was 260 nm [21]. Based on a study, the contents of Cordyceps acid were determined using spectrophotometry [22]. The contents of protein were determined using the Kjeldahl instrument (Buchi, Switzerland).

2.4 Desirability function development

In present study, a desirability function was developed for simultaneously enhancing the yields of biomass, adenosine, intracellular polysaccharide, Cordyceps acid and protein in Irpex lacteus Mycelia fermentation. The general desirability function is defined as follow:

$$d_i = \begin{cases} d_i = 0 & y_i < y_{il} \\ \dfrac{y_i - y_{li}}{y_{ih} - y_{il}} & y_{il} < y_i < y_{ih} \\ d_i = 1 & y_i > y_{ih} \end{cases} \quad (1)$$

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

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

where yi was the ith response value, yil was the low threshold of ith response. It was expected that yi was not lower than yil. yih was the high threshold of the ith response. di was the desirability values of the ith response. wi was the weight of the ith response values which reflects the difference in the importance of the different response. Obviously, the larger the wi is, the more important the response is. The higher yi is, the larger the Dv is. The yil, yih and wi of biomass, adenosine, intracellular polysaccharide, Cordyceps acid and protein were listed in table 1.

Parameters	Y_B (g·l^{-1})	Y_A (g·l^{-1})	Y_I (g·l^{-1})	Y_C (g·l^{-1})	Y_P (g·l^{-1})
y_{il}	2	0.01	0.2	0.06	1.5
y_{ih}	20	0.15	2	0.8	9
w_i	0.25	0.25	0.25	0.15	0.1

Y_B: Yield of biomass; Y_A: Yield of adenosine; Y_I: Yield of intracellular polysaccharide; Y_C: Yield of *Cordyceps* acid; Y_P: Yield of protein

2.5 Experimental design

All of the following experiments were implemented in twice and the average data was used as experimental data.

2.5.1 Single-factor test design

Single-factor test design was applied to select suitable carbon source, nitrogen source and inorganic salt sequentially. A modified PDA medium composed of (g·L -1): glucose 20, peptone 20, KH_2PO_4·5H 2O 3.0, $MgSO_4$·7H_2O 3.0 was used as the initial medium. The initial Dv which obtained by fermenting with initial medium was 0.1751. The carbon source of the modified PDA medium was replaced with glycerol, mannitol, maltose, fructose, lactose, glucose and sucrose at the same concentration respectively. The suitable carbon sources were selected depending on Dv. And then, the nitrogen source in modified PDA medium was replaced with beef extract, yeast extract, yeast extractive powder, peptone, ammonium acetate and $(NH_4)_2SO_4$ at the same concentration respectively. Suitable nitrogen source were selected depend on Dv [23]. The inorganic salts in modified PDA medium was replaced with CuSO4·5H 2O, ZnSO4·7H 2O, $MnCl_2$·4 H_2O, $FeCl_3$·6H $_2$O, KCl, $CaSO_4$·2H $_2$O and NaCl. Also we set the concentration of every inorganic salt two levels with 0.01 and 0.05 mmol/l, at the same concentration respectively. Suitable inorganic salts were selected depend on *Dv*.

2.5.2 Plackket-burman design

The concentrations of the components in the modified PDA medium with suitable carbon and nitrogen sources were used as the candidate factors in the PBD [24]. A linear model was developed base on the data of PBD. F-test method was employed to test the significances of each coefficient in the linear model. The significant components were selected out depended on corresponding significant coefficients.

2.5.3 Box-behnken design

The concentrations of the significant components obtained by PBD were used as the candidate variables in BBD. A MQR model was developed based on BBD data for determining the individual effects and mutual interaction effects of candidate variables on ***Dv***. An ANN model was also developed based on BBD data for predicting ***Dv***. To avoid the over-fit phenomenon, a criterion named degree of approaching (*Da*) was employed to select the suitable number of hidden nodes. *Da* was defined as follows:

$$D_a = c / \left(\frac{n_c}{n} \times RMSE_c + \frac{n_t}{n} \times RMSE_t + RMSE_c - RMSE_t \right) \quad (4)$$

where $RMSE_c$ and $RMSE_t$ were the root mean-square-errors (*RMSE*) of calibration set and test set, respectively; nc and nt were the number of calibration set and test set; n was the sum number of calibration set and test set; and c was a constant number (in present work c was 1000) by which *Da* was adjusted to get a good chart. According to the above equation, it was obvious that the larger *Da* was, the more the ANN models approached the experimental data. Since *Da* achieved the maximum value when there were 10 hidden nodes, the number of hidden nodes was set to 10.

GA was employed to search for the optimum concentrations of the significant components in the test regions based on BBD data. The parameters of GA were as follow: 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, penalty factor as 100 and number of generation over which GA evolved as 100. The fitness function was defined as follows:

$$Fitness = -Dv \quad (5)$$

Besides, the GA-based optimization simulations were repeated by using each time a different randomly initialized population of the candidate solutions [25]. Dissimilar initial populations ensured that each time the GA began its search for the optimal solution from a different search sub-space, which helped in locating the lowest local or the global minimum on the fitness function surface.

2.5.4 Validation experiments

The optimum fermentation medium obtained using ANN-GA was investigated triply to validate the feasibility of this method.

3. RESULTS AND DISCUSSIONS

3.1 Selecting carbon sources, nitrogen sources and inorganic salts

The effects of carbon sources, nitrogen sources and inorganic salts were investigated as section 2.5.1. The results were shown in figure 1. As can be seen, lactose with the highest *Dv* was the most suitable carbon source. The effects of nitrogen sources on *Dv* were much more significant than those of carbon sources. Yeast extractive powder with the highest *Dv* was the most suitable nitrogen source. Also, NaCl(0.01 mmol/L) with the highest *Dv* was the most suitable inorganic salts.

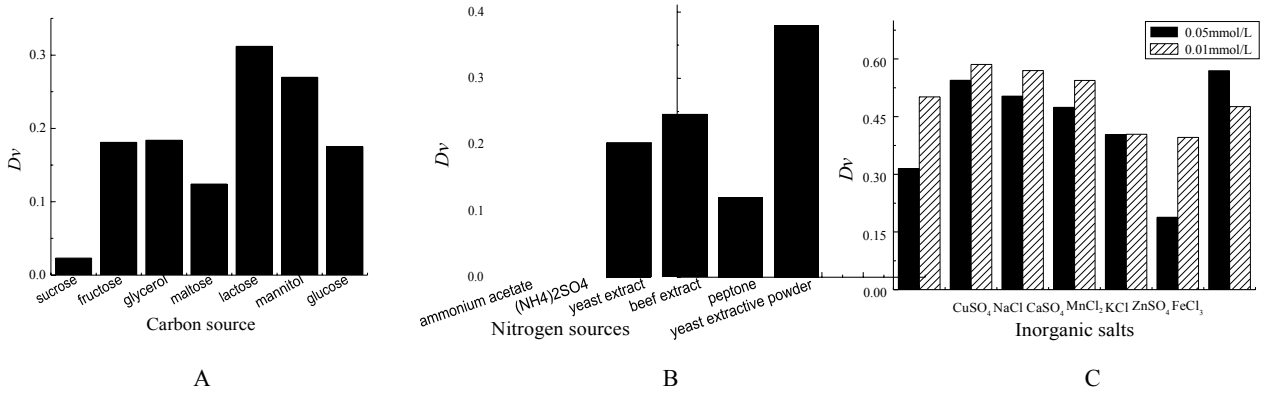

Figure 1: The effects of carbon source(A), nitrogen source(B) and inorganic salts(C) on *Dv*

Table 2: The design matrix and the results of PBD

Runs	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}
1	1	-1	1	-1	-1	-1	1	1	1	-1
2	1	1	-1	1	-1	-1	-1	1	1	1
3	-1	1	1	-1	1	-1	-1	-1	1	1
4	1	-1	1	1	-1	1	-1	-1	-1	1
5	1	1	-1	1	1	-1	1	-1	-1	-1
6	1	1	1	-1	1	1	-1	1	-1	-1
7	-1	1	1	1	-1	1	1	-1	1	-1
8	-1	-1	1	1	1	-1	1	1	-1	1
9	-1	-1	-1	1	1	1	-1	1	1	-1
10	1	-1	-1	-1	1	1	1	-1	1	1
11	-1	1	-1	-1	-1	1	1	1	-1	1
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1

Runs	Y_B(g·l^{-1})	Y_A(g·l^{-1})	Y_C(g·l^{-1})	Y_I(g·l^{-1})	Y_P(g·l^{-1})	*Dv*	*Sources*	$P_{0.10}$
1	9.83	0.0465	0.3909	1.4127	4.4554	0.4345	X_1	0.0474
2	11.04	0.0776	0.3797	1.5732	5.3420	0.5553	X_2	0.1397
3	4.51	0.0339	0.1580	0.3747	2.3756	0.1158	X_3	0.2066
4	10.36	0.0751	0.4234	1.3991	4.1246	0.5087	X_4	0.5590
5	9.64	0.0636	0.2708	1.1893	4.0741	0.4196	X_5	0.1085
6	9.77	0.0668	0.3777	1.3863	4.4881	0.4795	X_6	0.0945
7	7.38	0.0622	0.3121	0.7618	3.9326	0.3291	X_7	0.0998
8	7.08	0.0593	0.2011	0.6196	4.0329	0.2742	X_8	0.1385
9	6.35	0.0229	0.1393	0.5352	3.0726	0.1577	X_9	0.1833
10	12.25	0.0983	0.5199	1.7111	6.0775	0.5301	X_{10}	0.1009
11	16.64	0.0788	0.4832	1.6573	8.3574	0.6003	Model	0.1215
12	5.92	0.0272	0.1177	0.5431	3.2634	0.1642	*RMSE*	0.0277

Table 3 : The design matrix and the results of BBD

Runs	U_1	U_2	U_3	Y_B(g·l^{-1})	Y_A(g·l^{-1})	Y_C(g·l^{-1})	Y_I(g·l^{-1})	Y_P(g·l^{-1})	*Dv*
1	-1	-1	0	4.83	0.0265	0.1008	0.3686	2.2544	0.0981
2	-1	1	0	4.04	0.0176	0.0976	0.4732	2.3420	0.0943
3	1	-1	0	9.51	0.0703	0.5198	1.4147	4.3756	0.5075
4	1	1	0	10.16	0.0771	0.4934	1.1991	4.4575	0.5018
5	0	-1	-1	8.64	0.0536	0.2108	1.0893	3.7410	0.3547
6	0	-1	1	9.70	0.0623	0.3686	1.3292	4.0937	0.4541
7	0	1	-1	8.47	0.0622	0.3669	0.8813	4.3262	0.3797
8	0	1	1	9.11	0.0658	0.4250	0.9141	4.9450	0.4179
9	-1	0	-1	5.22	0.0247	0.1938	0.4325	2.0625	0.1241
10	1	0	-1	10.14	0.0804	0.4164	1.2129	5.0125	0.5053
11	-1	0	1	6.13	0.0502	0.3060	0.5384	3.2454	0.2360
12	1	0	1	13.42	0.0705	0.4700	1.4753	5.5134	0.5821
13	0	0	0	11.59	0.0655	0.5311	1.3201	4.9544	0.5281
14	0	0	0	9.12	0.0694	0.3455	0.9553	4.7210	0.4156
15	0	0	0	10.94	0.0681	0.5217	1.3357	4.4260	0.5169

3.2 Identifying the significant components in fermentation medium

PBD experiments had 10 factors and 2 levels, employed to identify the significant components in fermentation medium. Among the 10 factors, 7 were the components in the modified initial fermentation medium with selected carbon sources and nitrogen source. The high (+1) level and the low (-1) level of the five components were lactose (X_1) 30.0 and 10.0 g/L; yeast extractive powder (X_2) 20 and 10 g/L; beef extract (X_3) 20 and 10 g/L; $KH_2PO_4{\cdot}5H_2O$ (X_4) 1.0 and 0.02 g/L; MgSO4·7H 2O (X_5) 1.0 and 0.02 g/L; NaCl (X_6) 0.30 and 0 mmolg/L; VB1 (X7) 0.30 and 0.10 g/L. The other 3 factors were dummy variables (X_8, X_9, X_{10}), used for estimating the experimental error and checking the adequacy of the first-order model. Twelve experimental runs were carried out in present work. The experimental data were shown in table 2. A first-order model in coded variables was developed using SAS (version 8.02). The linear model was presented as follow:

$$Dv=0.3807+0.1072X_1+0.0358X_2-0.0238X_3-0.0066X_4-0.0513X_5+0.0535X_6+0.0506X_7+0.0362X_8-0.0270X_9+0.0500X_{10} \quad (6)$$

The determination coefficient (R^2) of the linear model was 0.9975 which demonstrated that the fit of the linear model was satisfied. F-test method was employed to determine the significances of the coefficients in the linear model and the results were shown in table 2. The smaller P value is, the more significant the coefficient is which indicating that the more significant the effect of the corresponding variable is. The P value is smaller than 0.1 indicates that the effect of corresponding variable was significant. The P value is smaller than 0.05 indicates that the effect of corresponding variable is very significant. As can be seen, the P value of linear model was 0.1215 which indicated that the effects of candidate variables on the response values were non-significant and the initial setting levels of the candidate variables were suitable. It also can be concluded that the effects of the concentrations of lactose (X_1), NaCl (X_6) and VB1 (X_7) were significant since the p values of their coefficients in linear model were smaller than 0.10. Therefore, lactose, NaCl and VB1 were the significant components for further optimization

3.3 Further optimizing with MQR and ANN-GA

Lactcose (U_1), NaCl (U_2) and VB1 (U_3) were selected out by PBD and used as the candidate variables for BBD. The concentrations of the other components in the fermentation medium keep in middle levels of PBD. The low levels (-1), middle levels (0) and the high levels (+1) of the three variables in BBD were 10, 20, 30 g/L, 0, 0.15, 0.30 mmol/L and 0.10, 0.20, 0.30 g/L respectively. The BBD design matrix and the corresponding experimental results were shown in table 3. A MQR model was developed by using BBD data. The MQR model was presented as Eq. (7).

$$Dv=0.4868+0.1930*U_1-0.0026*U_2+0.0408*U_3-0.1131*U_1^2-0.0005*U_1*U_2-0.0088*U_1*U_3-0.0734*U_2^2-0.0153*U_2*U_3-0.0119*U_3^2 \quad (7)$$

The R^2 of the MQR model was 0.9765 and the root mean square error (*RMSE*) was 0.0425. These results demonstrated that the fit of the model was satisfied. F-test method was employed to test the significances of the coefficients in the MQR model. The results were shown in table 4 and the effects of individual variables and the mutual effects between the variables were learnt from these results. Partial derivative method was employed to search for the extreme *Dv* in MQR model. The optimum fermentation medium obtained by the MQR model was (g/L): lactose 29.41, yeast extract powder 15, beef extract 15, $KH_2PO_4{\cdot}5H_2O$ 0.65, $MgSO_4{\cdot}7H_2O$ 0.6, NaCl 0.008, VB1 0.289. And the corresponding *Dv* was 0.5952. ANN a nonlinear modeling method was also employed to model the data of BBD for more accurate prediction and pursuing the true maximum *Dv* in the test regions.

The BBD data were divided into three sets. 2 of them were used as prediction set, 2 of them were used as test set and 11 of them were used as calibration set. *Dv* was used as output data. The coded variables were used as input data. And then, the ANN model was developed. The ANN model was optimized by selecting the suitable number of hidden nodes. The most suitable number of hidden nodes with the maximum Da was 9. The R2 of the optimized ANN model was 0.9829 which indicating that the fit of this model was very satisfied. The root mean square error of test set (*RMSET*) and the root mean square error of prediction set (*RMSEP*) were 0.0089 and 0.0154. These results indicated that the predictive capability of the ANN model was satisfied.

Table 4: The statistical results of MQR model

Sources	*DF*	*SS*	*MS*	*F*	*P>F*
U_1	1	0.2981	0.2981	165.2577	0.0001
U_2	1	5.4E-5	5.4E-5	0.0298	0.8696
U_3	1	0.0133	0.0133	7.3778	0.0420
U_1^2	1	0.0472	0.0472	26.1745	0.0037
U_1U_2	1	1.043E-6	1.043E-6	0.0006	0.9817
U_1U_3	1	0.0003	0.0003	0.1705	0.6977
U_2^2	1	0.0199	0.0199	11.0177	0.0210
U_2U_3	1	0.0009	0.0009	0.5200	0.5031
U_3^2	1	0.0005	0.0005	0.2887	0.6141
Model	9	0.3754	0.0417	23.1274	0.0015
Error	5	0.0090	0.0018		
Total	14	0.3844		R^2	0.9765

Table 5: The optimal fermentation media obtained by GA with different initial population

NO	U_1	U_2	U_3	Predictive *Dv*	Experimental *Dv*	Relative error (%)
1	0.981	-0.112	0.815	0.6302	0.6233	1.09
2	0.981	-0.112	0.815	0.6302	0.6257	0.71
3	0.981	-0.112	0.815	0.6302	0.6244	0.92

After the optimum ANN model was developed, GA was employed to search for the optimum fermentation medium in test regions. The parameters of GA were shown in section 2.5.3. While the ANN model was developed, genetic algorithm (GA) was employed to search for the optimum medium which was as follow (g/L): lactose 29.81, yeast extract powder 15, beef extract 15, $KH_2PO_4{\cdot}5H_2O$ 0.65, $MgSO_4{\cdot}7H_2O$ 0.6, NaCl 0.0078, VB1 0.281 in table 5, as can be seen. The maximum predictive *Dv* with the optimum fermentation medium was 0.6302 which increased 5.88% from that obtained by MQR model and increased 259.91% from that without optimization.

3.4 Validation experiments

The optimal fermentation medium listed in table 5 was used for Irpex lacteus Mycelia fermentation in triplicate. The results were shown in table 5. The relative errors between predictive values and experimental values were lower than 3.10 %. These results demonstrated that the predictive capability of the ANN model was good and this method is feasible. The optimization process in this paper has greatly enhanced the productions of biomass, adenosine, intracellular polysaccharide, Cordyceps acid and protein simultaneously. It should be popular in multi-response optimization for complex systems.

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