International Journal of Scientific Research in Science and Technology
Print ISSN: 2395-6011 | Online ISSN: 2395-602X (www.ijsrst.com)
doi : https://doi.org/10.32628/IJSRST21841

Optimization of Technical Parameters on the Medium Formulation and Culture Conditions in Production of *Pleurotus eryngii* Liquid Spawn

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Article Info
Volume 8, Issue 3
Page Number : 1019-1036

Publication Issue
May-June-2021

Article History
Accepted : 20 June 2021
Published : 30 June 2021

ABSTRACT

Liquid spawn, an important technical support for industrial production of edible fungi, has some advantages, such as fast growth, strong activity, uniform quality and easy inoculation. However，the study of the fermentation parameters of Pleurotus eryngii liquid spawn is less, which cannot effectively guide the production practice. Therefore, in order to provide a theoretical basis for the production of liquid spawn and industrial production of Pleurotus eryngii, response surface analysis was used to optimize the liquid spawn formula and conditions. The best carbon and nitrogen source of liquid medium was glucose and yeast extract, respectively. The optimum formula of medium was obtained by response surface analysis. Combined with the practical operation, the formula was modified to 29.00 g/L glucose, 2.90g/L yeast extract, 0.90g/L KH2PO4 and 1.00g/L MgSO4. The effects of various factors on the dry weight of mycelia were analyzed, the results indicated that the influence follow the sequence: glucose, KH2PO4, yeast extract and MgSO4, among them, the first two factors had significant effect. The interaction between glucose and KH2PO4 had obviously effects on the dry weight of mycelia. The interaction between glucose and yeast extract had a certain effect. The optimum culture conditions of liquid spawn were obtained by response surface analysis. Combined with the actual situation, the conditions were modified to liquid volume 106.00mL/250mL, rotating speed 165.00r/min, temperature 23.60℃, initial pH 6.70. The effects of various factors on the dry weight of mycelia was analyzed, the results indicated that the influence follow the sequence: initial pH, liquid volume, temperature and rotating speed, among them, the first three factors had extremely significant effect. The interaction of liquid volume and rotating speed, temperature and initial pH had obviously effects on mycelial dry weight.

Keywords : Pleurotus Eryngii, Liquid Spawn, Technical Parameters, Response Surface Methodology, Edible Fungi

I. INTRODUCTION

In the process of liquid cultivation of edible fungi, the quality of liquid cultivation is particularly important. Many researches have been carried out on the formulation of culture medium. The medium formula mainly includes carbon source, nitrogen source and inorganic salt. Li Chao [1] used single factor test and L9 $(3)^4$ orthogonal optimization test to optimize the liquid culture medium of Auricularia auricula, and found that the best formula was 2% glucose, 1% yeast extract, 0.3% KH_2PO_4 and 0.15% $MgSO_4$. Qin Baoshan [2] also adopted orthogonal test for liquid medium formulation of Lentinus edodes, and found that the most suitable carbon and nitrogen source, inorganic salt and its concentration were 2% glucose, 0.1% yeast extract and 0.1% $MgSO_4$. Yu Changxia et al [3] found that the best liquid medium was 2.5% glucose, 0.4% yeast extract powder, 0.1% KH_2PO_4 and 0.1% $MgSO_4$ through single factor and orthogonal experiment in the study on the factory production of straw mushroom. Guo Xinxin [4] found through orthogonal test that the optimal formula of *Flammulina velutipes* liquid medium was 4% soluble starch, 5% soybean powder, 0.05% KH_2PO_4 and 0.0075% vitamin B_2. Zhao Na [5] adopted single factor and orthogonal test methods to optimize the liquid culture medium formula of *Eryngoides eryngoides,* and found that the best formula for liquid fermentation was 30g/L glucose, 5g/L peptone, 0.8g/L KH_2PO_4, 1.2g/L $MgSO_4$ and 10mg/L vitamin B_1. Mao Dong [6] adopted the same optimization method and found that the optimal formula was 4% glucose, 0.4% yeast powder, 0.25% KH_2PO_4, 0.06% $MgSO_4 \cdot 7H_2O$, and 0.004% vitamin B_1. Umesh Singh et al [7] adopted the response surface analysis method to optimize the four factors, and found that the optimal ratio was 26.87g/L sucrose, 1.42g/L peptone, 0.85g/L KH_2PO_4, and 1.05g/L $MgSO_4$. In addition, it was found that the order of influence of the four factors on the dry weight of mycelia was sucrose, KH_2PO_4, $MgSO_4$ and peptone. The interaction between sucrose and KH_2PO_4 had a significant effect on the results, and the interaction between sucrose and peptone was more significant. In conclusion, it can be inferred that the nutrient components required by liquid culture of different edible fungi are different, and the nutrient requirements of different varieties of the same edible fungus may also be different. Orthogonal test method is used in the study of liquid culture medium formulation, and response surface analysis method is rarely used. The results of orthogonal experiment optimize the optimal results, but cannot further explore the interaction between the factors. With the rapid development of automation equipment such as fermentation, scholars began to improve and optimize the culture conditions of liquid strains. Rui Yingzhang et al [8] found through single factor experiment that the optimal temperature for liquid culture of Auricularia auricula was 25°C and the initial pH was 7.0, and optimized different *Lentinus edodes* varieties through orthogonal test, and found that the optimal loading volume was 100 mL/250 mL, the temperature was 25°C, the rotation speed of the shaking table was 150r/min, and the initial pH was 4.5. Valentin ZĂGREAN et al [9] found through L9 $(3)^4$ orthogonal optimization experiment that the optimal initial pH of *Flammulina velutipes* for liquid culture was 6.5 and the rotation speed was 140 r/min. Meng Lijun et al [10] optimized the liquid culture conditions of *Flammulina velutipes* through response surface test design, and found the optimal initial pH of 6.9, temperature of 22.3°C, and speed of 114r/min. Meanwhile, the study found that the order of influence on the dry weight of mycelia was temperature, speed, and pH. In the study on the optimization of liquid culture conditions of *Eryngius eryngii,* Mai Thanh Nguyen [11] adopted the orthogonal test method and found that the optimal results existed when the natural pH, temperature was 25°C and the rotation speed of the shaking table was 140r/min. Shoji Ohga [12] adopted the same method and found the optimal pH 6.0, rotating speed 150r/min, and liquid loading volume

100mL/250mL. In conclusion, the liquid culture conditions of different edible fungi were different, and the culture conditions of different varieties of the same edible fungus were different. In the condition optimization method, most scholars use single factor or orthogonal experimental design method to explore the optimal value. In the optimization of culture conditions of *Flammulina velutipes* liquid strains, some scholars adopted response surface analysis. However, there are few reports in the study of *Pleurotus eryngii*. This research result provides a convenient and quick method for the rapid detection of early strain activity.

II. MATERIALS AND METHOD

2.1 The experimental materials

2.1.1 The selected strains

Pleurotus eryngii (Xing 528), purchased from Zehai Edible Fungi Research Institute, Pingyi County, Shandong Province. Fruiting body gray to dark gray, cylindrical, solid mushroom, strong disease resistance, yield temperature 5°C-29°C, biological conversion rate of 80%-100%, suitable for factory production.

2.1.2 Medium

Plate medium: 20% potato (boil for 20 min, extract the juice), 2% glucose, 0.2% KH_2PO_4, 0.1% $MgSO_4$, 20% Agar;

Seed medium in shaking flask: the same as flat medium except without adding Agar;

Liquid fermentation medium: 20g/L glucose, 2g/L peptone, 0.2% KH_2PO_4, 0.1% $MgSO_4$.

2.2 Preparation of strains

2.2.1 Preparation of plate strains

The medium was prepared according to the plate medium formula and then transferred to a conical flask. The sealing film was sealed and sterilized at 115°C for 30 min. Before the medium solidified, the sterilized medium was poured into sterile petri dishes in a clean table according to the aseptic operation method, each plate was about 10mL. After cooling completely, the test strains were inoculated into plate petri dishes. Then it was transferred to a 25°C constant temperature biochemical incubator for activation, and waited for its growth for reserve use.

2.2.2 Preparation of liquid strains

The corresponding medium was prepared according to the medium formula and divided into 250mL conical flask. The sealing film was sealed and sterilized at 115°C for 30min. After cooling completely, take 3 piece $2mm^2$ activated strains with a hole punch in a clean table (avoid the inoculation point and the edge area). The strains were inoculated into liquid medium, stood at 25°C for 24h, and then transferred to a constant temperature shaker at 25°C for culture.

2.3 Determination of mycelial activity index

2.3.1 Determination of mycelia dry weight

The liquid medium was sifted through an 80mesh screen, the filtrate was discarded and mycelia were collected. The mycelia were rinsed with deionized water repeatedly, and then placed in a constant temperature air blower dryer at 80°C to dry. When the difference between two adjacent weights is less than 2mg, it is considered as constant weight. Three replicates were set for each liquid medium, and the dry weight of mycelia in the liquid medium was determined by the mean value [13].

2.3.2 Determination of germination time of mycelium pellet tieback plate

Mycelium pellets with a diameter of about 1mm were selected from the liquid medium to be tested and placed in the center of PDA plate medium. The PDA plate medium with placed mycelium pellets was transferred to a constant temperature biochemical incubator at 25°C for culture. The observation time interval after starting culture was 1h/time. After germination of existing mycelium pellets, the observation interval was changed to 0.5h/time. Then

the germination time of mycelium pellets in each group was recorded. Three replicates were determined, and the germination time of the mycelium pellets after connecting the plate was determined by the mean value.

2.3.3 Determination of the growth rate of mycelia

The growth distance of mycelia was measured on the third day after inoculation on the flat medium of 2.3.2, and the average daily growth rate of mycelia was calculated. Three replicates were set, and the growth rate of mycelia in plate medium was determined by average value [14].

2.4 Optimization of technical parameters of liquid strain production

2.4.1 Screening of carbon sources

Selection: glucose, fructose, maltose, sucrose, lactose, starch. The culture conditions were 100mL/250 mL, 160r/min, 25℃, natural pH, and the optimal carbon source was selected according to the dry weight of the mycelium, the germination time of the tieback plate, and the growth rate.

2.4.2 Screening of nitrogen sources

Selection: peptone, beef extract, yeast extract, KNO_3, NH_4NO_3, $(NH_4)_2SO_4$. The culture conditions and screening indexes were the same as 2.4.1, and the optimal nitrogen source was screened.

2.4.3 Optimization of medium formulation by single factor method

In liquid fermentation medium formula, not only carbon and nitrogen sources are added, but also inorganic salts: KH_2PO_4 and $MgSO_4$ are often added. Therefore, in this study, the dry weight of mycelia was used as an indicator, and the screened carbon sources, nitrogen sources, KH_2PO_4 and $MgSO_4$ were used for single factor experiments.

Carbon sources: 15 g/L, 20 g/L, 25 g/L, 30 g/L, 35 g/L;

Nitrogen sources: 1 g/L, 2 g/L, 3 g/L, 4 g/L, 5 g/L;

KH_2PO_4: 0.5 g/L, 1.0 g/L, 1.5 g/L, 2.0 g/L, 2.5 g/L;

$MgSO_4$: 0.5 g/L, 1.0 g/L, 1.5 g/L, 2.0 g/L, 2.5 g/L.

2.4.4 Optimization of medium formulation by response surface methodology

The Design Expert V11.0.2 software was used to design the experiment using Box-Behnken response surface method. The dry weight of mycelium was taken as the response value, and glucose, yeast extract, KH_2PO_4 and $MgSO_4$ were taken as four factors, and three levels were set respectively, as shown in the table below (Table 1).

Table1 Experimental factors and levels of Box-Behnken formula optimization

Factor	Level		
	-1	0	1
X_1：Glucose g/L	25	30	35
X_2：Yeast extract g/L	2	3	4
X_3：KH_2PO_4 g/L	0.5	1	1.5
X_4：$MgSO_4$ g/L	0.5	1	1.5

2.4.5 Optimization of culture conditions by single factor method

With the dry weight of mycelium as the index, the culture conditions were set at different levels, and the single factor experiment was carried out.

Liquid loading volume: 80 mL, 100 mL, 120 mL, 140 mL, 160 mL;

Rotating speed: 120 r/min, 140 r/min, 160 r/min, 180 r/min, 200 r/min;

Temperature: 20℃, 22℃, 24℃, 26℃, 28℃;

Initial pH: 4, 5, 6, 7, 8.

2.4.6 Optimization of culture conditions by response surface methodology

The Design Expert V11.0.2 software was used to Design the test using Box-Behnken response surface method. The dry weight of the mycelium was taken as the response value, and the liquid loading volume,

rotating speed, temperature and initial pH were taken as four factors, and three levels were set respectively, as shown in the table below (Table 2).

Table 2. Experimental factors and levels of Box-Behnken condition optimization

Factor	Level		
	-1	0	1
X_1 : Liquid loading volume, mL	80	100	120
X_2 : Rotating speed, r/min	140	160	180
X_3 : Temperature, ℃	23	24	25
X_4: Initial pH	6	7	8

2.5 Data analysis

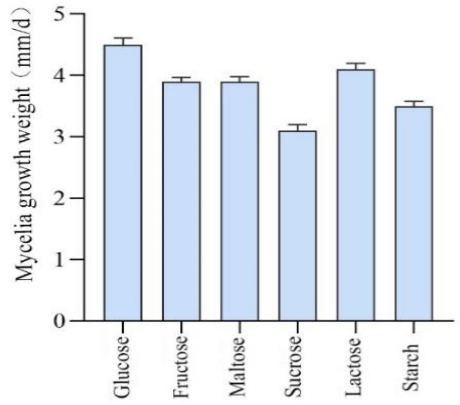
Design-Expert V11.0.2 and SPSS Statistics 26 were used to process and analyze the data and establish the model.

III. RESULTS

3.1 Optimization of technical parameters of liquid strain production

3.1.1 Screening of carbon sources

As shown in Figure 1, due to different carbon sources, the dry weight of mycelia, the germination time of the tie-back plate and the growth rate of mycelia were significantly different. In terms of dry weight of mycelia, the dry weight of mycelia with glucose, sucrose and lactose as carbon sources was significantly higher than that of the other three kinds. Among them, glucose was the best carbon source, followed by sucrose and lactose.



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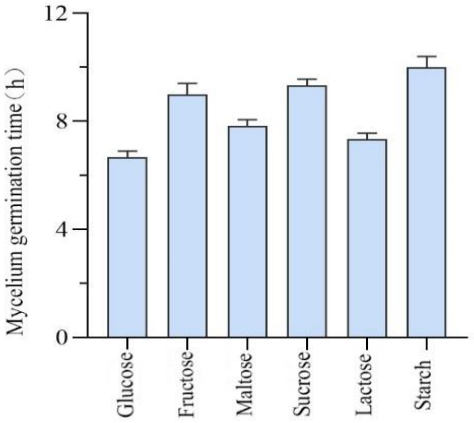

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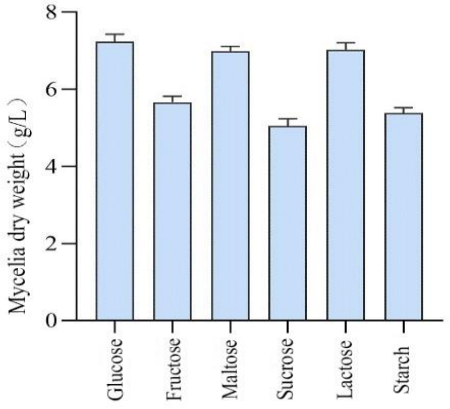

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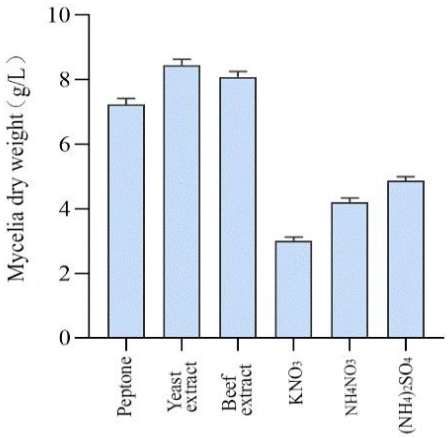
Figure1 Effects of different carbon sources on dry weight, germination time and growth rate

In terms of the germination time of mycelium tie-back plate, the mycelium germination time with glucose, sucrose and lactose as carbon sources was slightly faster than the other three. Among them, glucose is the fastest carbon source, sucrose is the next,

lactose is the slowest. In terms of mycelium growth rate, the mycelium with glucose and sucrose as carbon sources grew faster than the other four kinds. Glucose was the fastest carbon source followed by sucrose. In the process of liquid culture, it was observed that the diameter of mycelium with glucose as carbon source was the best, followed by lactose and sucrose. Therefore, glucose was selected as the most suitable carbon source to carry out the next experiment.

3.1.2 Screening of nitrogen sources

As shown in Figure 2 below, due to different nitrogen sources, the dry weight, the germination time of the tie-back plate and the growth rate of mycelia showed great differences. In terms of mycelium dry weight, the mycelium dry weight with yeast extract and beef extract as nitrogen source was heavier than the other four kinds.



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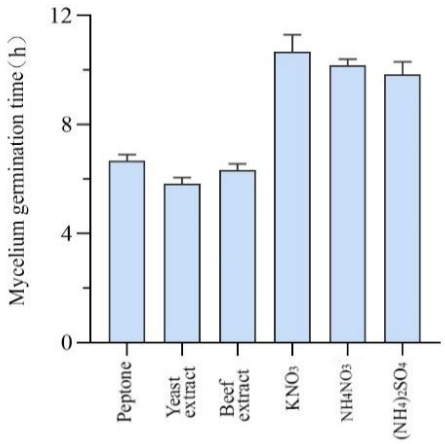

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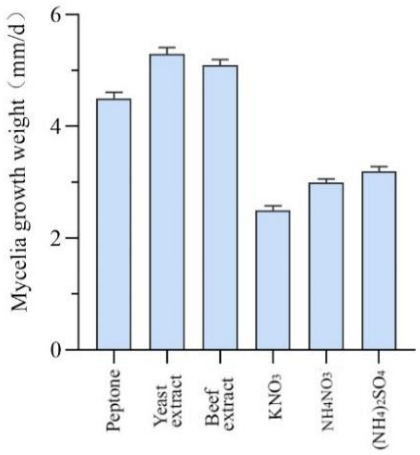


(c)

Figure 2 Effects of different nitrogen sources on dry weight, germination time and growth rate

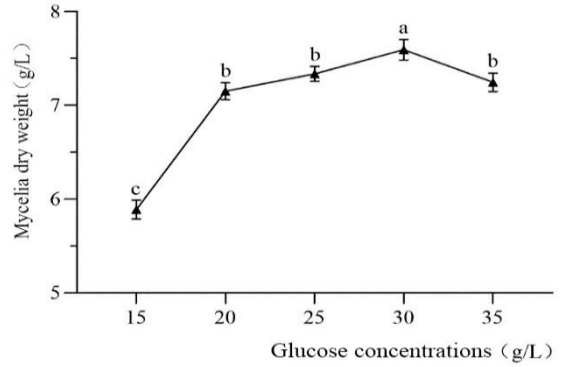
Yeast extract was the best nitrogen source and beef extract was the second. In terms of the germination time of mycelium tie-back plate, the mycelium germination time using yeast extract, beef extract and peptone as nitrogen sources was significantly faster than the other three kinds. Among them, yeast extract was the fastest nitrogen source, followed by beef extract and peptone. In terms of mycelial growth rate, the mycelial growth rate using yeast extract and beef extract as nitrogen sources was significantly faster than that of the other four kinds. Yeast extract was the fastest nitrogen source, followed by beef extract. In the process of liquid culture, it was observed that the diameters of mycelia using yeast extract, beef extract and peptone as nitrogen sources were obviously better than those of the other three kinds. Therefore, yeast extract was selected as the optimal nitrogen source to carry out the next experiment.

3.1.3 Optimization of medium formulation based on single factor method

Carbon source is an important nutrient source for the growth of mycelia, and the lack of carbon source will

lead to the inability of cells to synthesize cell materials normally and maintain life activities well. As shown in Figure 3 a), with the increase of glucose concentration, the dry weight of mycelium increased significantly. When the concentration reached 30g/L, the dry weight of mycelia reached the maximum.

When the concentration was too high, the dry weight of mycelium showed a downward trend. Therefore, the optimal concentration of glucose is 30g/L.



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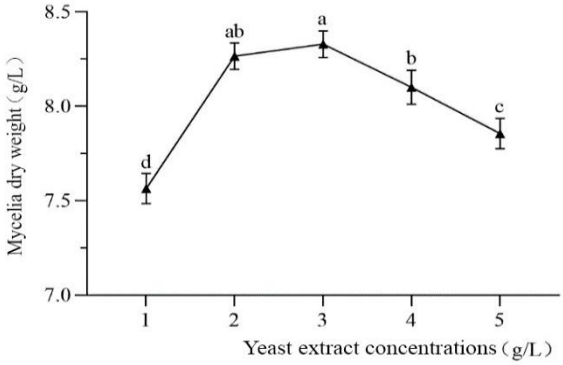


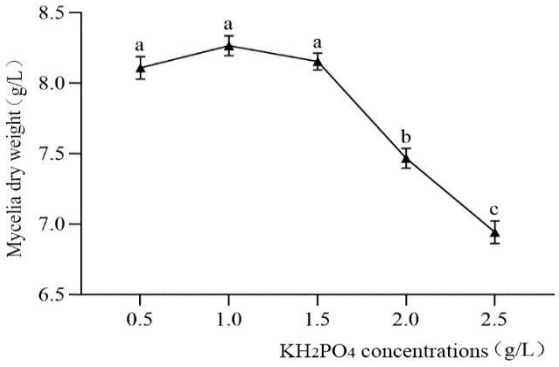
(b)

Figure 3 Effects of different concentrations of glucose and yeast extract on dry weight of mycelium

Note: Lower case letters indicate that different concentration of medium formula is significantly different

by Tukey test ($p < 0.05$), the same below.

Nitrogen source is an essential nutrient source for protein and nucleic acid synthesis and can promote the growth of mycelia. Insufficient nitrogen source will affect the growth of mycelia, and too high concentration of nitrogen source will lead to too fast growth of mycelia and aging. As shown in Figure 3 b), when the yeast extract concentration increased from 1g/L to 2g/L, the dry weight of mycelia increased significantly. When the concentration reached 3g/L, the dry weight of mycelia reached the maximum. When the concentration was too high, the dry weight of mycelium showed a significant downward trend. Therefore, the optimal concentration of yeast extract is 3g/L. KH_2PO_4 can increase the mycelial density and enhance the activity of mycelia. Excessive KH_2PO_4 will affect the body's absorption of Ca^{2+} and Mg^{2+} plasma, and affect the growth of mycelia.



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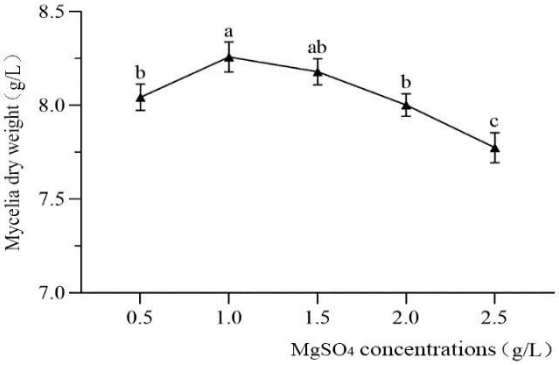


(b)

Figure 4 Effects of different concentrations of KH_2PO_4 and $MgSO_4$ on dry weight of mycelium

As shown in Figure 4 a), when the concentration of KH_2PO_4 was 1 g/L, the dry weight of mycelia reached the maximum. With the increase of KH_2PO_4

concentration, the dry weight of mycelia decreased significantly. Therefore, the optimal concentration of KH_2PO_4 is 1 g/L. Mg^{2+} in $MgSO_4$ is the activator of various enzymes, which can effectively enhance the activity of mycelia. However, excessive Mg^{2+} will antagonize other ions and inhibit the growth of mycelia. As shown in Figure 4 b), when the concentration of $MgSO_4$ was 1g/L, the dry weight of mycelia reached the maximum. With the increase of $MgSO_4$ concentration, the dry weight of mycelia decreased slowly. Therefore, the optimal concentration of $MgSO_4$ is 1g/L.

3.2 Optimization of medium formulation based on response surface methodology

3.2.1 Experimental design and results

In this experiment, a Box-Behnken test design was used, and the dry weight of mycelium was taken as the response value. There were 29 groups of experiments with four factors and three levels. The first 24 groups were factorial tests, and the last 5 groups were central tests.

Table 3 Box Behnken Formulation Optimization-Response surface test design and results

Test Number	Factors				Mycelia dry weight (g/L)
	X_1 Glucose (g/L)	X_2 Yeast Extract (g/L)	X_3 KH_2PO_4 (g/L)	X_4 $MgSO_4$ (g/L)	
1	-1	-1	0	0	8.372
2	1	-1	0	0	8.584
3	-1	1	0	0	8.676
4	1	1	0	0	7.291
5	0	0	-1	-1	8.848
6	0	0	1	-1	7.921
7	0	0	-1	1	8.664
8	0	0	1	1	7.452
9	-1	0	0	-1	8.694
10	1	0	0	-1	8.318
11	-1	0	0	1	8.799
12	1	0	0	1	8.201
13	0	-1	-1	0	8.633
14	0	1	-1	0	8.798
15	0	-1	1	0	8.425
16	0	1	1	0	8.235
17	-1	0	-1	0	8.271
18	1	0	-1	0	8.059
19	-1	0	1	0	8.776
20	1	0	1	0	6.187
21	0	-1	0	-1	8.548
22	0	1	0	-1	8.041
23	0	-1	0	1	8.146

24	0	1	0	1	7.073
25	0	0	0	0	10.135
26	0	0	0	0	9.854
27	0	0	0	0	10.799
28	0	0	0	0	9.345
29	0	0	0	0	9.121

3.2.2 Model establishment and analysis of variance

Through Design-Expert V11.0.2 software, quadratic multinomial regression fitting was carried out for the obtained data, and the regression equation was obtained as follows:

$$Y=-47.05132+2.47074X_1+7.32873X_2+14.04053X_3+7.82970X_4-0.07985X_1X_2-0.2377X_1X_3-0.0222X_1X_4-0.1775X_2X_3-0.283X_2X_4-0.285X_3X_4-0.034229X_1^2-0.781483X_2^2-3.40243X_3^2-3.18443X_4^2 \quad (1)$$

As can be seen from Table 4, the regression model $P < 0.01$ is extremely significant, while the missing item > 0.05 is not significant. This indicates that the error between the predicted value and the actual value in this model is small, and the unknown factors have little interference on the dry weight of mycelia. The R^2 of this model was 0.9355, which could explain 93.55% of the variation of response value. The results show that the model has a good fitting degree.

The influences of the four factors on the dry weight of mycelium from the largest to the smallest were X_1, X_3, X_2 and X_4. Among them, X_1 and X_3 had a significant effect on the dry weight of mycelia. The interaction of each factor was not significant, and X_1^2, X_2^2, X_3^2 and X_4^2 had extremely significant effects on the dry weight of mycelia. The results showed that the regression model could well reflect the relationship between the dry weight of mycelia and glucose, yeast extract, KH_2PO_4 and $MgSO_4$, and could predict the variation rule.

Table 4 Box Behnken Formulation Optimization-Variance analysis of response surface model

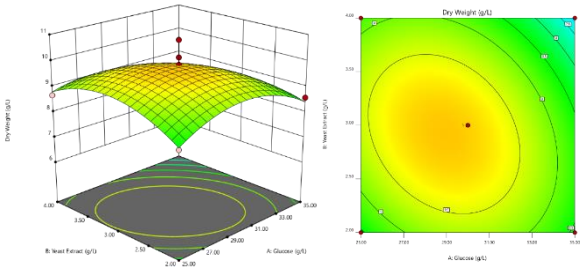
Source	Sum of Squares	df	Mean Square	F-value	P-value	Significance
Model	17.86	14	1.28	3.87	0.0081	**
X_1-Glucose	2.04	1	2.04	6.19	0.0260	*
X_2-Yeast extract	0.56	1	0.56	1.7	0.2130	
X_3- KH_2PO_4	1.52	1	1.52	4.63	0.0494	*
X_4- $MgSO_4$	0.35	1	0.35	1.05	0.3234	
X_1X_2	0.64	1	0.64	1.94	0.1859	
X_1X_3	1.41	1	1.41	4.29	0.0574	
X_1X_4	0.012	1	0.012	0.037	0.8494	
X_2X_3	0.032	1	0.032	0.096	0.7617	
X_2X_4	0.08	1	0.08	0.24	0.6296	
X_3X_4	0.02	1	0.02	0.062	0.8075	
X_1^2	4.75	1	4.75	14.42	0.0020	**
X_2^2	3.96	1	3.96	12.03	0.0038	**
X_3^2	4.69	1	4.69	14.25	0.0021	**
X_4^2	4.11	1	4.11	12.48	0.0033	**
Residual	4.61	14	0.33			
Lack of Fit	2.84	10	0.28	0.64	0.74	
Pure Error	1.77	4	0.44			
Cor Total	22.47	28				

Note: R^2=0.9355, Adj-R^2=0.8453; * means significant difference, $p < 0.05$, ** means extremely significant difference, $p < 0.01$.

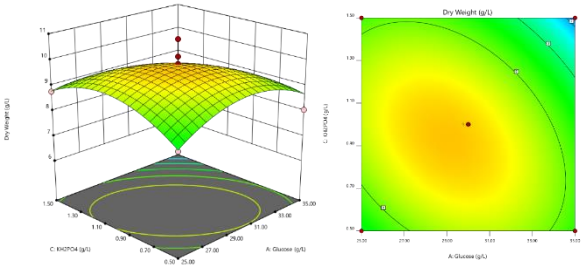
3.2.3 Response surface analysis

As shown in Figure 5 a), the three-dimensional response surface of glucose and yeast extract was steeper and the contour map showed an ellipse, indicating that glucose and yeast extract had a certain interaction.

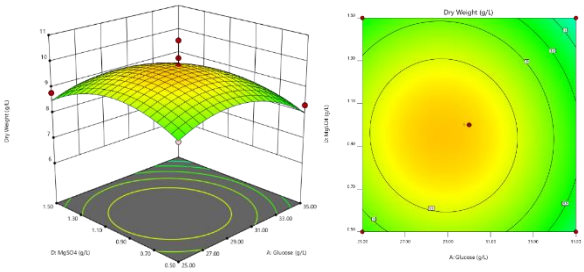
When the concentration of KH_2PO_4 and $MgSO_4$ was constant, glucose and yeast extract had a certain effect on the dry weight of mycelia. When the concentration of glucose is constant, the increase of yeast extract concentration will make the dry weight of mycelium increase rapidly to a certain extent, reaching the maximum and then decreasing.



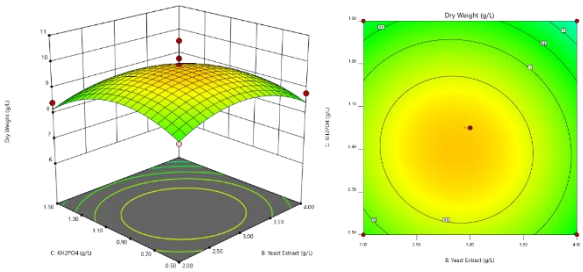
a) Glucose and Yeast extract



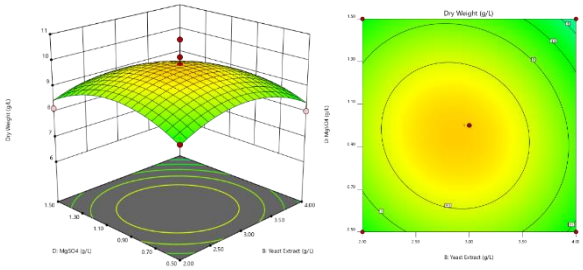
b) Glucose and KH_2PO_4



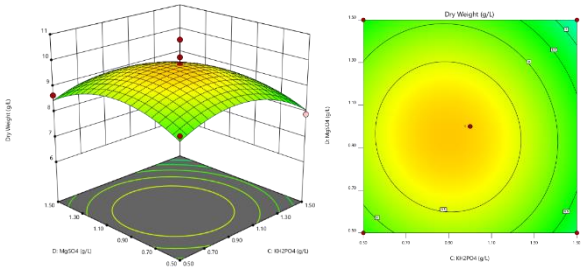
c) Glucose and $MgSO_4$



d) Yeast extract and KH_2PO_4



e) Yeast extract and $MgSO_4$



f) KH_2PO_4 and $MgSO_4$

Figure 5 3D response surface and contour for the interactive effect of various factors on dry weight of mycelium (Formula optimization)

According to the three-dimensional response surface and contour lines in Figure 5 b), it can be seen that the interaction between glucose and KH_2PO_4 is obvious. When the concentration of yeast extract and $MgSO_4$ was constant, glucose and KH_2PO_4 could significantly affect the dry weight of mycelia. In a certain range, when the concentration of KH_2PO_4 is constant, the increase of glucose concentration will significantly increase the dry weight of mycelia. The three-dimensional response surface of Figure 5 c) is relatively gentle compared with Figure 5 a), and the contour line is nearly circular compared with it, indicating that the interaction between glucose and $MgSO_4$ is not significant. Similarly, as shown in Figure 5 d), the interaction between yeast extract and KH_2PO_4 was not significant. The 3D response surface

of Figure 5 e) is not significantly different from that of Figure 5 c) and d). As can be seen from the density of contour line, yeast extract and $MgSO_4$ had some interaction, but it was still not significant. Figure 5 f) is similar to Figure 5 c), indicating that the interaction between KH_2PO_4 and $MgSO_4$ is not significant.

According to the analysis of regression model, the optimal formula of liquid medium was predicted as follows: glucose 29.15g/L, yeast extract 2.92g/L, KH_2PO_4 0.93g/L, $MgSO_4$ 0.96g/L. According to this formula, the maximum dry weight of mycelium was predicted to be 9.9272g/L. (Table 5)

3.2.4 Verification test analysis

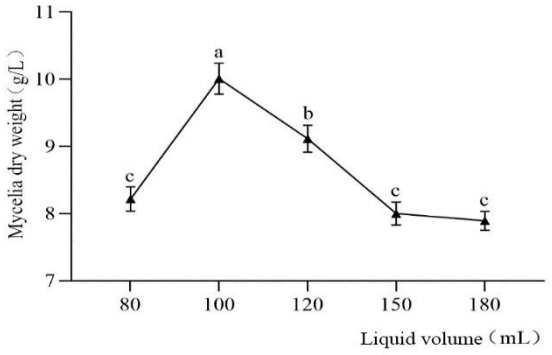
Table 5 The best prediction condition and verification test (Formula optimization)

Index	X_1	X_2	X_3	X_4	Mycelia dry weight g/L
Optimum formula (Prediction)	29.15	2.92	0.93	0.96	9.9272
Modified formula (Test)	29.00	2.90	0.90	1.00	9.8933

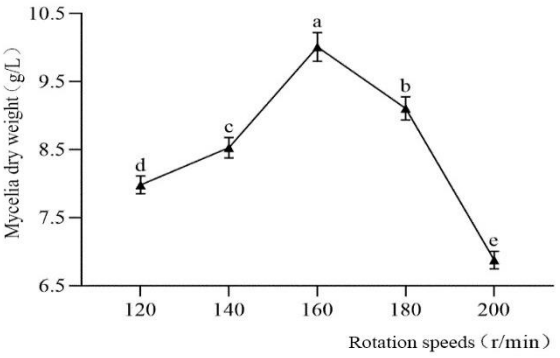
Combined with the feasibility of the actual test operation, the optimal formula was revised as follows: glucose 29.00g/L, yeast extract 2.90g/L, KH_2PO_4 0.90g/L, $MgSO_4$ 1.00g/L. The average dry weight of mycelia was 9.8933 g/L after three repeated tests according to the modified formula. The difference between the predicted value and the predicted value is small, which indicates that the model has high reliability and can be used to optimize the liquid medium formulation.

3.3 Optimization of culture conditions based on single factor method

When the liquid loading amount was small, the force of mycelium pellets was large, which affected the growth of mycelia. When the volume of liquid was large, the dissolved oxygen in the medium was less, which could not meet the oxygen consumption required by the normal growth and reproduction of mycelia. As shown in Figure 6 a), the liquid loading amount has a significant effect on the dry weight of mycelia. When the filling volume was 100mL/250mL, the dry weight of mycelia was the maximum. In the process of liquid culture, it was observed that when the liquid loading volume was greater than 150 mL/250mL, the diameters of mycelium pellets were different, which was not conducive to liquid inoculation. Therefore, the optimal loading volume is 100mL/250mL.



(a)

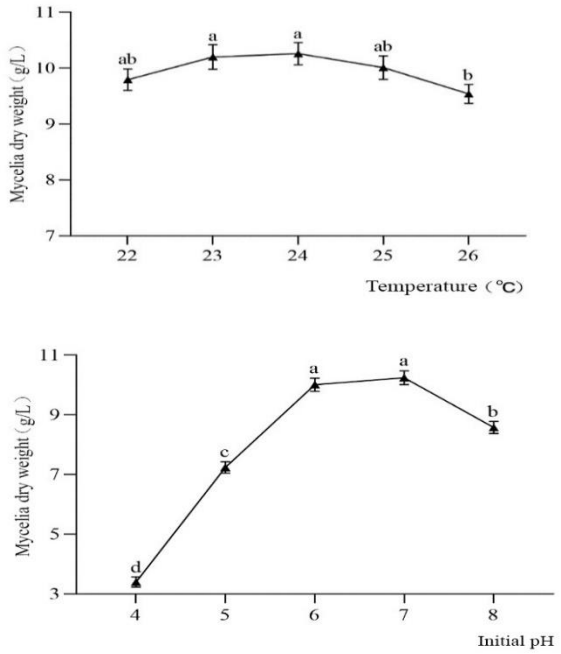


(b)

Figure 6 Effects of different liquid volume and rotation speeds on dry weight of mycelium

Note: The lowercase letters indicate that different culture conditions is significantly different by Tukey test
($p < 0.05$), the same below.

When the rotation speed was low, the amount of dissolved oxygen in the medium was less, and the mycelia could not grow normally. When the rotating speed is high, the shear force and mechanical stimulation of mycelia are large, and mycelia are easy to be damaged. As shown in Figure 6 b), the speed has a significant effect on the dry weight of mycelia. With the increase of rotating speed, the dry weight of mycelium increased significantly, and reached the maximum value at 160 r/min. Therefore, the optimal speed is 160 r/min. Temperature is closely related to the metabolic rate and enzyme activity of mycelia, which is one of the important conditions affecting the growth of mycelia.



(a)

(b)

Figure 7 Effects of different temperature and initial pH on dry weight of mycelium

Pleurotus eryngii belongs to middle and low temperature bacteria, and the relatively low temperature is suitable for the growth of mycelia. As shown in Figure 7 a), between 22℃ and 25℃, the dry weight of mycelia did not change significantly with temperature. The dry weight of mycelia was the largest in the range of 23℃ to 24℃. When the temperature was higher than 24℃, the dry weight of mycelium showed a downward trend. Therefore, the optimal temperature range is between 23℃ and 24℃. As shown in Figure 7 b), the initial pH had a significant effect on the dry weight of mycelia. When pH increased from 4.0 to 6.0, the dry weight of mycelia increased rapidly. When pH was 7.0, the dry weight of mycelia reached the maximum. When pH was greater than 7.0, the dry weight of mycelia began to decline. Therefore, the optimal initial pH is 7.0.

3.4 Optimization of culture conditions based on response surface methodology

3.4.1 Experimental design and results

In this experiment, a Box-Behnken test design was used, and the dry weight of mycelium was taken as the response value. There were 29 groups of experiments with four factors and three levels. The first 24 groups were factorial tests, and the last 5 groups were central tests. (Table 6)

Table 6 Box Behnken Condition Optimization-Response surface test design and results

Test Number	Factors				Mycelia dry weight (g/L)
	X_1 Liquid loading volume (mL)	X_2 Rotating speed (r/min)	X_3 Temperature (℃)	X_4 Initial pH	
1	-1	-1	0	0	7.535
2	1	-1	0	0	9.169
3	-1	1	0	0	6.733
4	1	1	0	0	11.433
5	0	0	-1	-1	9.202
6	0	0	1	-1	8.857
7	0	0	-1	1	9.483
8	0	0	1	1	5.879
9	-1	0	0	-1	8.578
10	1	0	0	-1	11.121
11	-1	0	0	1	6.154
12	1	0	0	1	7.308
13	0	-1	-1	0	9.785
14	0	1	-1	0	11.037
15	0	-1	1	0	9.081
16	0	1	1	0	9.001
17	-1	0	-1	0	9.488
18	1	0	-1	0	9.986
19	-1	0	1	0	7.254
20	1	0	1	0	9.058
21	0	-1	0	-1	9.802
22	0	1	0	-1	11.340
23	0	-1	0	1	8.026
24	0	1	0	1	8.227
25	0	0	0	0	12.959
26	0	0	0	0	13.423
27	0	0	0	0	12.611
28	0	0	0	0	11.281
29	0	0	0	0	11.579

3.4.2 Model establishment and analysis of variance

Through Design-Expert V11.0.2 software, quadratic multinomial regression fitting was carried out for the obtained data, and the regression equation was obtained as follows:

$$Y=-515.47492+0.7232X_1+1.09262X_2+21.78252X_3+42.23303X_4+0.001916X_1X_2+0.008163X_1X_3-0.017362X_1X_4-0.008325X_2X_3-0.016712X_2X_4-0.407375X_3X_4-0.005264X_1^2-0.002966X_2^2-0.3922X_3^2-2.08555X_4^2 \quad (2)$$

It can be seen from Table 7 that the regression model *P<0.01* is extremely significant, while the missing item > 0.05 is not significant. Moreover, $R^2=0.9182$ could explain 91.82% of the variation in response values.

Table 7 Box Behnken Condition Optimization-Variance analysis of response surface model

Source	Sum of Squares	df	Mean Square	F-value	P-value	Significance
Model	99.71	14	7.12	11.22	< 0.0001	**
X_1- Liquid loading volume	12.68	1	12.68	19.97	0.0005	**
X_2- Rotating speed	1.59	1	1.59	2.51	0.1354	
X_3- Temperature	8.09	1	8.09	12.74	0.0031	**
X_4- Initial pH	15.92	1	15.92	25.08	0.0002	**
X_1X_2	2.35	1	2.35	3.7	0.0749	
X_1X_3	0.43	1	0.43	0.67	0.4262	
X_1X_4	0.48	1	0.48	0.76	0.3981	
X_2X_3	0.44	1	0.44	0.7	0.4173	
X_2X_4	0.45	1	0.45	0.7	0.4156	
X_3X_4	2.66	1	2.66	4.18	0.0601	
X_1^2	28.76	1	28.76	45.3	< 0.0001	**
X_2^2	9.13	1	9.13	14.38	0.0020	**
X_3^2	15.96	1	15.96	25.15	0.0002	**
X_4^2	28.21	1	28.21	44.44	< 0.0001	**
Residual	8.89	14	0.63			
Lack of Fit	5.56	10	0.56	0.67	0.7243	
Pure Error	3.33	4	0.83			
Cor Total	108.6	28				

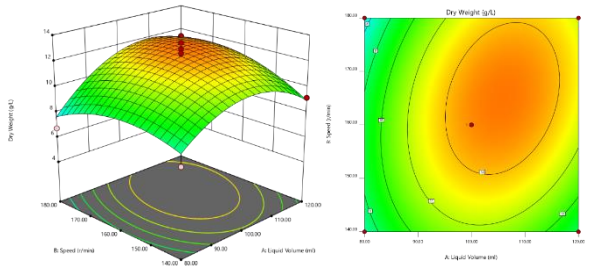
Note：$R^2=0.9182$, Adj-$R^2=0.8363$; * means significant difference, $p<0.05$, ** means extremely significant difference, $p<0.01$.

The results show that the model has a good fitting degree. The influences of the four factors on the dry weight of mycelia were X_4, X_1, X_3 and X_2 in descending order. Among them, X_1, X_3 and X_4 had extremely significant effects on the dry weight of mycelia. The interaction of each factor was not significant, and X_1^2, X_2^2, X_3^2 and X_4^2 had extremely significant effects on the dry weight of mycelia. It is shown that the regression model can reflect the relationship between the dry weight of mycelium and the liquid loading volume, rotating speed, temperature and initial pH, and can predict the change rule.

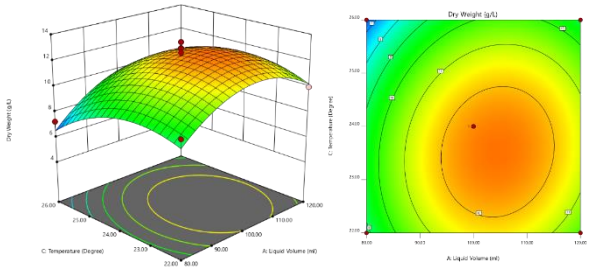
3.4.3 Response surface analysis

As shown in Figure 8 a), it can be seen from the three-dimensional response surface and contour map that the interaction between liquid loading volume and rotational speed is significant. When the temperature and initial pH were constant, the loading volume and rotation speed had obvious effects on the dry weight of mycelia. When the loading volume is constant, the dry weight of mycelium will increase significantly with the increase of rotating speed, and

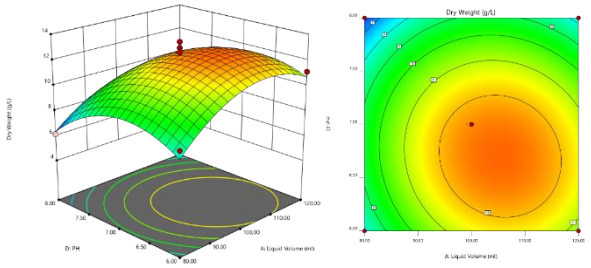
begin to decrease after reaching the maximum value. As can be seen from the 3D response surface and contour plot of Figure 8 b), there is no obvious interaction between liquid loading volume and temperature. The dry weight of mycelium did not increase significantly and rapidly with the increase of temperature when the loading volume was constant.



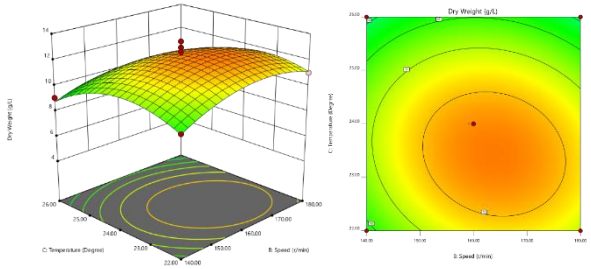
(a) Liquid loading volume and Rotating speed



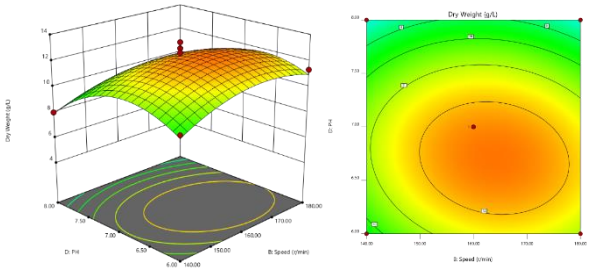
(b) Liquid loading volume and Temperature



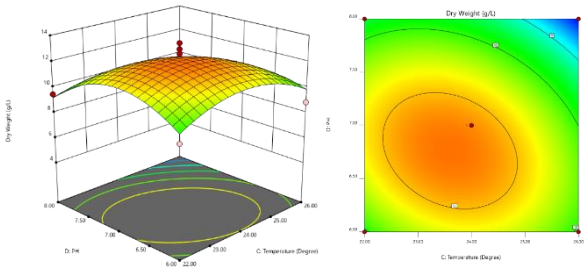
(c) Liquid loading volume and Initial pH



(d) Rotating speed and Temperature



(e) Rotating speed and Initial pH



(f) Temperature and Initial pH

Figure 8 3D response surface and contour for the interactive effect of various factors on dry weight of mycelium (Condition optimization)

It is concluded that the interaction of liquid volume and temperature has no significant effect on the dry weight of mycelia. As shown in Figure 8 c), the three-dimensional response surface between liquid loading and initial pH is steeper, indicating that the interaction between liquid loading and initial pH has a certain influence on the dry weight of mycelia within a certain range. However, the contour plot is approximately circular, indicating that the interaction between liquid loading volume and pH is not significant. Compared with the interaction of other factors, the three-dimensional response surface of Figure 8 d) is gentle and the contour plot is nearly circular, indicating that the interaction between rotation speed and temperature has no significant influence on the dry weight of mycelia. Similarly, the 3D response surface of Figure 8 e) is relatively flat. Although the contour line is slightly elliptical, when the initial pH is constant, the increase of rotation speed will not make the dry weight of mycelium increase significantly and rapidly. Therefore, the interaction between rotational speed and initial pH is not significant. As shown in Figure 8 f), the three-dimensional response surface of temperature and initial pH is steeper, and the contour plot is elliptical. Combined with the density of the contour line, it can be seen that the interaction between temperature and initial pH has obvious influence. The temperature and initial pH had obvious effects on the dry weight of mycelia when

the liquid loading volume and rotation speed were constant. In conclusion, in the interaction among the four factors of liquid loading volume, rotating speed, temperature and initial pH optimized by conditions, the interaction between liquid loading volume and rotating speed, temperature and initial pH has a significant influence on the dry weight of mycelia. The interaction of other factors had no significant effect on the dry weight of mycelia.

3.4.4 Verification test analysis

According to the regression model analysis, the optimal conditions of liquid culture medium were predicted as follows: liquid loading 106.23mL/250 mL, rotating speed 166.43r/min, temperature 23.63℃, initial pH 6.71. The maximum dry weight of mycelia was predicted to be 12.8344g/L according to the culture conditions. (Table 8)

Table 8 The best prediction condition and verification test (Condition optimization)

Index	X_1	X_2	X_3	X_4	Mycelia dry weight g/L
Optimum formula (Prediction)	106.23	166.43	23.63	6.71	12.8344
Modified formula (Test)	106.00	165.00	23.60	6.70	12.7166

Combined with the feasibility of actual test conditions, the optimal culture conditions were modified as follows: liquid loading 106.00 mL/250 mL, rotating speed 165.00 r/min, temperature 23.60℃, initial pH 6.70. The average dry weight of mycelia was 12.7166g /L after three repeated tests according to the modified conditions. The difference between the predicted value and the predicted value is small, which indicates that the model has high reliability and can be used to optimize the liquid culture conditions.

IV. DISCUSSION

In the formulation of edible fungus culture medium, carbon and nitrogen sources are important nutrient sources for the growth of mycelia. In this study, through the screening test of carbon and nitrogen sources, it was concluded that the most suitable carbon source was glucose and the most suitable nitrogen source was yeast extract for liquid culture of *Pleurotus eryngii.* (Figure 1 a), b), c), Figure 2 a), b), c)). In the previous studies on the screening of carbon and nitrogen sources for liquid strains, Li Chao [1] screened out glucose and yeast extract as the most suitable carbon and nitrogen sources for liquid strains of Auricularia auricula, which was consistent with the study in this paper. However, Che Xingxing [15] found that the most suitable carbon and nitrogen sources were potatoes and bran in the study of liquid strain formulation of *Auricularia auricula*. In the liquid strains of Lentinus edodes, Qin Baoshan [2] found that the most suitable carbon and nitrogen sources were glucose and yeast extract, which was consistent with this study. In the liquid culture process of *Flammulina velutiformis*, Guo Xinxin [4] found that the most suitable carbon and nitrogen sources were soluble starch and soybean powder. In conclusion, different types of carbon and nitrogen sources required by different edible fungi may be different, and the optimal carbon and nitrogen sources of the same kind of edible fungi may be different due to different varieties. Optimization of medium formulation and conditions is an effective means to improve the yield of mycelia. In the process of formulation optimization of liquid medium, Li Chao [1], Qin Baoshan [2], Yu Changxia [3], Guo Xinxin [4] et al. adopted single factor and orthogonal experimental design method in the formulation optimization of *Auricularia auricula*, *Lentinus edodes, Straw edodes* and *Flammulina* mushroom. In the process of optimization of liquid medium conditions,

Che Xingxing [15] et al. also adopted this method in the optimization of conditions of *Auricularia auriculata, Lentinus edodes* and *Flammulina velutiformis.* The single factor method is simple but only considers the influence of a single factor, which is susceptible to the influence of interaction among various factors, leading to deviation of the optimal value. Orthogonal experimental design method can solve the shortcomings of single factor method, but its optimal result is limited in the selected level range. Therefore, Box-Behnken response surface analysis (RSM) was used in this study (Table 4, Table 7), which enabled it to find the optimal values in the continuous region through more experimental combinations (Figure 5, Figure 8). Therefore, this method can get rid of the limitation that the optimal result cannot go beyond the selected level range. In addition, the regression equation of response surface analysis method is of high accuracy, and the interaction between various factors can be explored while analyzing the optimal results [16]. In addition, this method has high applicability and wide application in all kinds of tests. In the in-depth study of the production technology of liquid strains, the production technology can be further improved according to the influence of various factors on the results and the correlation between the factors. In the process of large-scale production of edible fungi, this method can not only be applied in the production of strains, but also in the cultivation, mushroom production, harvesting and other links. Using this method in the whole process can improve the accuracy of the test, maximize the production efficiency, and then improve the production technology level, increase revenue, which is conducive to promoting the rapid development of the entire edible fungus industry.

V. CONCLUSION

The optimal formula of liquid medium was glucose 29.00 g/L, yeast extract 2.90 g/L, KH_2PO_4 0.90 g/L and $MgSO_4$ 1.00 g/L. Glucose and KH_2PO_4 had significant effects on the dry weight of mycelia. The interaction between glucose and KH_2PO_4 had a significant effect on the dry weight of mycelia, and the interaction between glucose and yeast extract had a certain effect. The optimal conditions of liquid culture were as follows: loading volume 106.00 mL/250 mL, rotating speed 165.00 r/min, temperature 23.60°C, initial pH 6.70. The influences of initial pH, filling volume and temperature on the dry weight of mycelia were extremely significant. The interaction of liquid loading and rotation speed, temperature and initial pH had obvious effects on the dry weight of mycelia.

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Cite this article as :

Chol Jong, MyongIl Jin, YongChol Ju, DeTian Li, HaiFeng Zhu, TaeYun Jo, GyongChol Kim, " Optimization of Technical Parameters on the Medium Formulation and Culture Conditions in Production of Pleurotus eryngii Liquid Spawn ", International Journal of Scientific Research in Science and Technology(IJSRST), Print ISSN : 2395-6011, Online ISSN : 2395-602X, Volume 8, Issue 3, pp. 1019-1036, May-June-2021. Available at doi : https://doi.org/10.32628/IJSRST21841
Journal URL : https://ijsrst.com/IJSRST21841