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Optimization of vegetative growth conditions for submerged cultivation of edible medicinal mushroom *Hericium erinaceus* through resonance surface methodology

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ABSTRACT

Edible medicinal mushroom *Hericium erinaceus* commonly known as Lion's mane contains various bioactive metabolites associated with many health benefits and used as nutraceuticals. The objective of this study was to optimize vegetative growth conditions. In this study, conditions such as pH, temperature, and agitation of the fermenter were used to study the relative effect on biomass in the submerged medium. The experiments were designed using the Box Behnken Design (BBD). A total of 17 trial runs with varying ranges were carried out. Vegetative growth condition variables were found to play a decisive role in determining the quantity of the total biomass production in submerged conditions. The response surface methodology (RSM) was further used to analyze the results and finally to optimize the relationship of growth conditions. The results showed that maximum biomass production (34.44g/l) was at temperature 24.4 °C, pH 6.4, and 147.4 rpm agitation. The predicted maximal growth was within 0.13-1.49 percent error of the experimental data under these conditions. With R^2 value > 0.9, the model produced an acceptable outcome. Therefore, we concluded that these indicator parameters can be used for better production of mycelium biomass and metabolites, used for medicinal and pharmacological products.

Keyword: Medicinal mushrooms, *Hericium erinaceous*, growth conditions, response surface methodology

Hericium erinaceus, also known as Monkey Head mushroom is an medicinal mushroom widely distributed in China, Korea, Japan, Europe, and North America (Wang *et al*., 2019). It is also recognized as Hou Tou Gu (Chinese), Yamabu shitake (Japanese) and Lion's Mane mushroom (European and Malaysian). Since ancient times, *H. erinaceus* has been used in Asia in the cure of ulcers, cancer, diabetes, dyslipidemia, inflammatory bowel diseases and microbial infections (Diling *et al*., 2017; Hiwatashi *et al*., 2010; Jinn *et al*., 2005; Kawagishi, 2005; Li *et al*., 2014; Qin *et al*., 2016). *H. erinaceus* is rich in numerous bioactive compounds such as erinacines, polysaccharides, hericerins, glycoproteins, erinacerins and erinaceolactones (Friedman, 2015). These have been directly associated with various health diseases.

The fermentation technology for fast mycelial growth is gaining popularity in *H. erinaceus*. In fact, environmental factors like medium pH, temperature,

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and agitation speed have been demonstrated to affect mycelial growth rate (Giang *et al*., 2021). The pH of the growth medium had a crucial impact in the organism's morphological alterations. During the development of the organism, the change in pH affected the product in the medium (Huu-Nghi Nguyen, 2012; Teoh *et al*., 2012). In fact, adverse interactions in an alkaline or acidic environment might result in the loss of protein nutritional value and the creation of potentially poisonous compounds like lysinoalanine, which can inhibit fungal development (Liu *et al*., 2008). Furthermore, temperature is one of the most critical environmental factors influencing fungal proliferation. Most well-studied microorganisms flourish at temperatures between 25 and 40 °C, whereas others may grow best (although slowly) from 0 to 15 °C (Atila *et al*., 2021a). Another important variable that altered mixing and oxygen transfer rates in several fungal fermentations, and hence influenced mycelia growth shape, is agitation strength. According to Rahman *et al*. (2005), raising the agitation speed from 150 to 350 rpm resulted in a slight increase in enzyme activities, indicating that aeration is a significant element in the development of aerobic strains. It has also been proven that a faster agitation speed might be harmful to mycelia development (Huu-Nghi Nguyen, 2012). The traditional optimization strategy includes modifying one variable at a time while leaving the others constant. It took a long time and did not ensure that the best circumstances would be found. As a result, experimental design methodologies outperformed the one-factor-at-a-time strategy for fermentation enhancement. Response surface methodology is a mathematical and statistical technique used in the design of experiments (Pereira *et al*., 2021). The goal is to optimize a response that is influenced by a number of independent variables. Many researchers used response surface methodology to optimize process parameters and created a regression equation to predict a response (Breig & Luti, 2021).

Therefore, the goal of this research was to find the best vegetative growth conditions. Temperature, pH, and fermenter agitation were utilized to investigate the relative influence on biomass in the submerged medium in this study. Initially, the biomass experiments were designed using the Box Behnken Design (BBD). In submerged settings, vegetative growth condition factors were discovered to be crucial in affecting the quantity of total biomass output. The response surface methodology (RSM) was then utilized to evaluate the data and, lastly, to improve the growth conditions connection.

MATERIALS AND METHODS

Fungal strain

The Fungal strain of *Hericium erinaceus* used in present study was collected from Regional Research Centre, Murthal (Sonipat) of Maharana Pratap Horticultural University (MHU), Karnal (Haryana). The strain was transferred to potato dextrose agar (PDA) plates and stored at 4 °C for future use.

Mycelium suspension

Suspension culture of *Hericium erinaceus* mycelia was made by suspending 5 mm mycelia discs from 14 days-old pure agar culture using sterilized cork borer in liquid medium Potato dextrose Broth (PDB) in a sample vial. To make the mycelia suspensions homogenous, 10 discs were vortexed for 5 minutes for every 100 ml PDB.

Fermentation condition

Fifteen milliliter (10% v/v) of the mycelium suspension culture was inoculated to 135 ml medium in 300 ml culture bottle containing modified PDB with following composition: Potato dextrose broth 24g, Peptone 5g, Dipotassium Phosphate 0.5g,

Monopotassium phosphate 0.5g and Magnesium Sulphate 1g. The culture media was autoclaved at temperature 121°C for 15 minutes prior of adding the mycelia (Khurana *et al*., 2022a).

Experimental design using Response Surface Methodology

RSM is a combination of mathematical and statistical tools for modelling and analyzing the problems in which a desired response is impacted by numerous factors and the goal is to maximize that response (Breig & Luti, 2021). By evaluating the input parameters, a second order polynomial regression equation (Eq. 1) is employed to predict the answer.

Equation 1:

$$Y = \beta_o + \Sigma\beta_i x_i + \Sigma\beta_{ij} x_i x_j + \Sigma\beta_{ii} x_i^2$$

where Y is the expected response, β_0 is a constant, β_i is the linear coefficient, β_{ii} is the squared coefficient, and β_{ij} is the cross-product coefficient, and (first-order interaction between X_i and X_j). As indicated in Table 1, the RSM utilized in this work was a Box-Behnken Design (BBD) incorporating three separate factors: pH, temperature, and agitation (Pereira *et al*., 2021). Design Expert 13 software was used to evaluate the data with Analysis of Variance (ANOVA). Three-dimensional plots can be used to investigate the simultaneous interactions of the three elements. The ideal region was also found using the overlay plot's key parameters. The experiment was then performed five times, with each outcome being compared to the expected values to assess the model's validity (Ha *et al*., 2021).

RESULT AND DISCUSSION

Experiment design

In order to improve *Hericium erinaceus* mycelial growth, a Box-Behnken Design (BBD) was employed to construct a link between pH of the culture medium, temperature at which it incubated, and agitation speed rate (Lee *et al*., 2021). As indicated in Table 2, there was a significant difference in biomass depending on the fermentation conditions. It is noticeable that replication at the center point parameters produced more biomass than replication at other levels.

Tables 2 and 3 indicate the factor values as well as the experimental design. Standard analysis of variance (ANOVA) with square root transformation was used to assess the data, and the Box-Behnken design was fitted with the second-order polynomial equation. According to Table 3, the biomass's projected response Y is as follows:

Equation 2:

$$Y = 5.80 + 0.0665A + 0.1349B + 0.2397C + 0.1087AB + 0.0189AC + 0.0437BC - 0.5749A^2 - 0.2072B^2 - 0.4321C^2$$

Here A is temperature in °C; B is pH and C is agitation speed in RPM

According to the aforementioned equation, "the coefficients with one factor reflected the influence of that specific factor, whereas the coefficients with two factors and those with second-order terms represented the interaction between the two factors and the quadratic effect, respectively. Furthermore, the positive sign in front of the digit denoted synergistic impact, whereas the negative sign denoted antagonistic effect" (Du *et al*., 2022; Si *et al*., 2019).

Table 1. Variables and levels used for Box-Behnken design

Factors	Name	Units	Minimum	Maximum	Coded Low	Coded High	Mean coded as 0
A	Temperature	°C	20	28	-1 (20)	+1 (28)	0 (24)
B	pH		5	7	-1 (5)	+1 (7)	0 (6)
C	Agitation speed	RPM	50	200	-1 (50)	+1 (200)	0 (125)

ANOVA of experimental Design

The Analysis of Variance (ANOVA) of Response Surface Square Root Quadratic Model of Mycelia Growth was reported in Table 4. "The p-values were used to determine the relevance of each coefficient and the model projected; the lower the p value, the more significant the model and corresponding coefficient" (Breig & Luti, 2021). The ANOVA of the regression model was significant in this investigation, with an F-test of a very low probability value with F-value of 126.04 and p-value < 0.0001. Also, significant relations were present in between all three linear coefficients and quadratic coefficients (A, B, C, A^2, B^2, C^2), as well as interaction coefficients AB. Because it was a hierarchical model, the insignificant coefficients were not eliminated from Eq.2.

Table 2. Box-Behnken design matrix with biomass as response

	Factor 1	Factor 2	Factor 3	Response
Run	A (Temp. in °C)	B (pH)	C: Agitation speed (RPM)	Biomass (g/l)
1	0	1	-1	25.11
2	0	-1	-1	23.51
3	1	1	0	28.51
4	1	-1	0	23.35
5	-1	0	-1	20.27
6	-1	0	1	24.54
7	0	0	0	33.91
8	0	-1	1	27.36
9	0	0	0	33.72
10	1	0	1	26.31
11	1	0	-1	21.18
12	0	1	1	31.00
13	0	0	0	34.90
14	-1	1	0	24.94
15	0	0	0	32.89
16	-1	-1	0	24.22
17	0	0	0	33.02

In order to determine the optimum condition, "the 3D-surface plot could visually show the response over a region of interesting factor levels, the relationship between the response and experimental levels of each variable, and the type of interaction between test variables" (Gang *et al*., 2016; Lee *et al*., 2021; Teoh & Don, 2012).

Optimal parameter range

The relationship of pH, temperature, and agitation speed on mycelium biomass output is displayed in this design-expert graphic. Initial temperature of 24.4 °C, pH 6.4 and agitation speed of 147.4 rpm were the

Table 3. Coefficients in Terms of Coded Factors

Factor	Coefficient Estimate	Standard Error	95% CI Low	95% CI High	VIF
Intercept	5.800	0.024	5.75	5.86	
A-Temperature	0.066	0.019	0.021	0.111	1.000
B-pH	0.134	0.019	0.090	0.180	1.000
C-Agitation speed	0.239	0.019	0.195	0.285	1.000
AB	0.108	0.027	0.045	0.172	1.000
AC	0.019	0.027	-0.045	0.082	1.000
BC	0.044	0.027	-0.020	0.107	1.000
A^2	-0.575	0.026	-0.637	-0.513	1.010
B^2	-0.207	0.026	-0.269	-0.145	1.010
C^2	-0.432	0.026	-0.494	-0.370	1.010

Table 4. Analysis of variance (ANOVA) of response surface quadratic model of mycelia growth

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	3.28	9	0.365	126.04	< 0.0001	significant
A-Temperature	0.035	1	0.035	12.21	0.0101	
B-pH	0.146	1	0.146	50.29	0.0002	
C-Agitation speed	0.460	1	0.460	158.75	< 0.0001	
AB	0.047	1	0.047	16.32	0.0049	
AC	0.001	1	0.001	0.4934	0.5051	
BC	0.008	1	0.008	2.64	0.1483	
A^2	1.390	1	1.390	480.62	< 0.0001	
B^2	0.181	1	0.181	62.44	< 0.0001	
C^2	0.786	1	0.786	271.52	< 0.0001	
Residual	0.020	7	0.003			
Lack of Fit	0.001	3	0.0004	0.0734	0.9712	not significant
Pure Error	0.019	4	0.005			
Cor Total	3.300	16				
Std. Dev.	0.054					
R^2	0.994					
Adjusted R^2	0.986					
Predicted R^2	0.986					

optimum conditions for maximum development of *Hericium erinaceus* (Fig. 1). The desirability value in this investigation was 0.972, demonstrating the applicability of this concept.

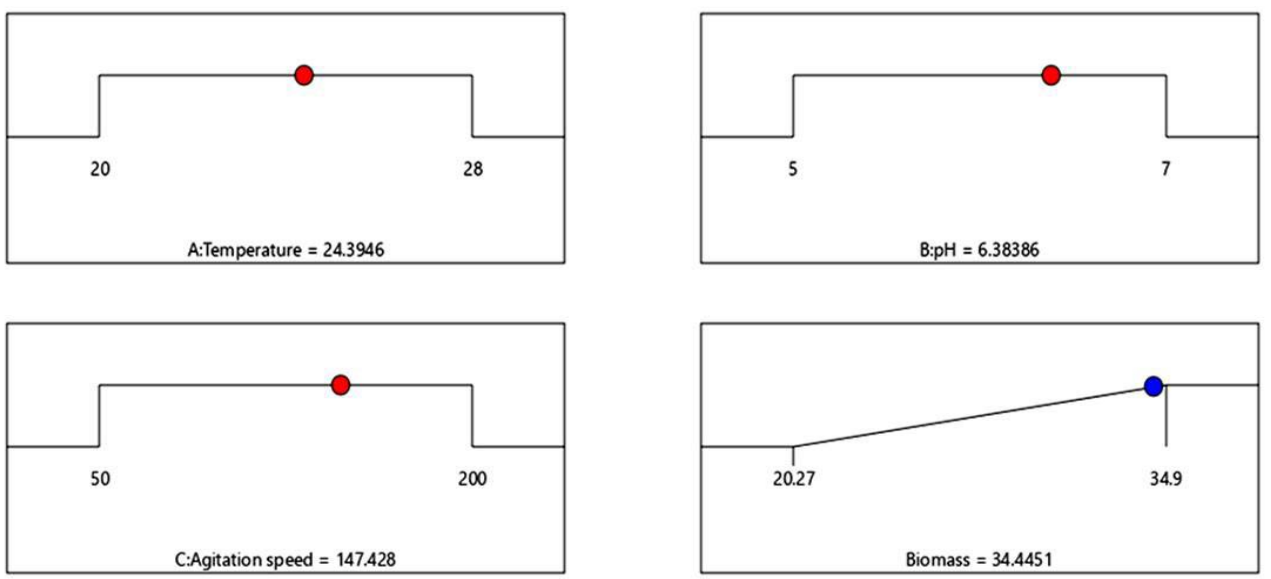

Fig. 1. Optimal condition for maximum development of *Hericium erinaceus*

The influence of factors

The impact of pH and temperature on the biomass is depicted in Figure 2(a), with the agitation rate set to 125 rpm. When the temperature was increased to around 24°C, the biomass output rose considerably. A substantial drop in trend was noted beyond this point. This event demonstrated that the influence of incubation temperature on growth was sensitive within the studied range, allowing for a maximum yield. The same pattern may be seen in Figs. 2 (b) and 2 (c) as well.

In the present study, foregoing findings are consistent with (Atila *et al*., 2021b; Khurana *et al*., 2022b). Atila *et al*. (2021) reported 25°C as the optimum temperature for the growth of *Hericium* isolates. Huang *et al*. (2007) observed 25°C temperature and pH 6.0 for luxurious growth and biomass of *Hericium erinaceus* strain CZ-2. Other researchers have also shown 25°C as the optimal temperature for *Hericium erinaceus* and *Hericium americanum* strains to develop and produce biomass (Grace & Mudge, 2015; Imtiaj *et al*., 2008; Park *et al*., 2001). The findings are also comparable

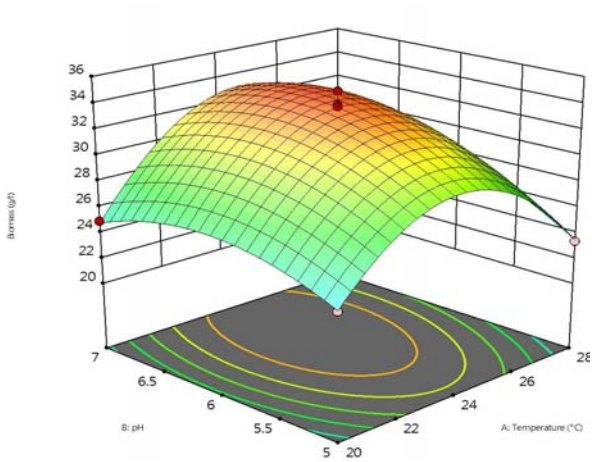

Fig. 2(a). The interaction between B: pH on biomass and A: temperature at 125 rpm

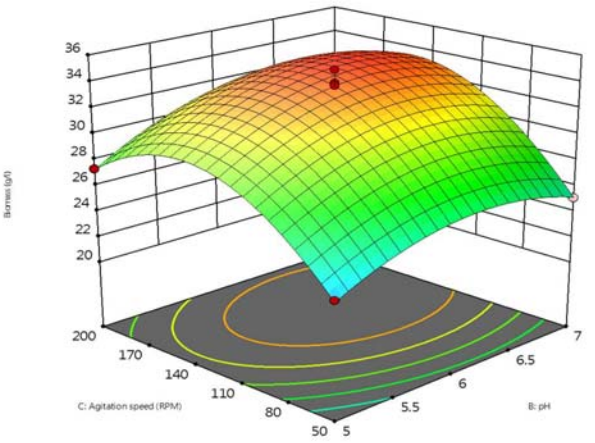

Fig. 2(b). The interaction between C: agitation speed and B: pH on biomass at 24 °C temperature

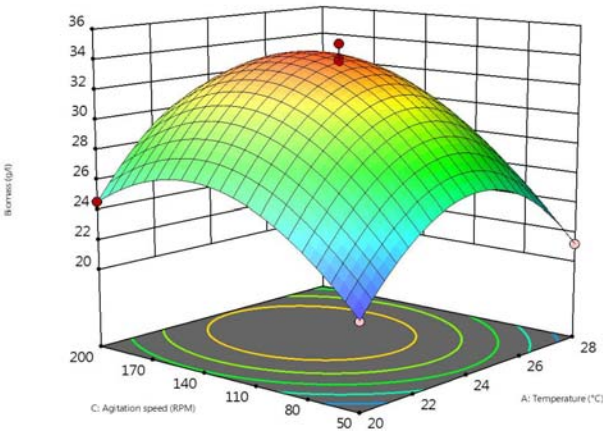

Fig. 2(c). The interaction between C: agitation speed and A: temperature on biomass at pH 6

to those of Imtiaj *et al*. (2008) where *Hericium erinaceus* required mild acidic conditions for growth and biomass production. Similarly, Pawlak *et al*. (2003) reported maximum biomass production at a pH 6.0. Agitation speed is also reported to have significant effect on growth during the submerged cultivation of fungi (Ibrahim *et al*., 2015; Mustafa *et al*., 2019; Teoh *et al*., 2012). Increased agitation rates may harm the pellets by shaving hair off the surface, resulting in denser pellets with a smaller diameter. High agitation rates also restrict pellet formation (Casas López *et al*., 2005; Huu-Nghi Nguyen, 2012; Yang *et al*., 2009). At high agitation rates (≥200 rpm), small and compact pellets were formed while tiny pellets of mycelia biomass formed at low agitation rates (≤125 rpm), may be owing to mycelial aggregation being prevented at low agitation speeds. The circumstances for fungal development were better at 147.5 rpm than at the other three speeds (50, 125, 200). The maximal biomass achieved at this speed was 34.45g/l (Fig 2). It was consistent with finding of (Huu-Nghi Nguyen, 2012; Mustafa *et al*., 2019).

Experiments for validation

Five sets of experiments were randomly performed at optimal conditions to acquire maximum growth by *Hericium erinaceus* in order to demonstrate the model's suitability. The percentage error in results of the experimental and projected values was in the range of 0.130-1.49 percent, as shown in Table 5. Since the variations between actual and anticipated responses have always been less than 1%, the validity of the model has been established.

CONCLUSION

RSM, a statistical technique, was utilized to infer conditions for maximum mycelial development in *Hericium erinaceus* using coded factors such as pH, temperature, and agitation speed. In shake flask culture, the greatest biomass (34.44 g /L) was predicted at pH 6.4, temperature 24.4 °C, and agitation speed 147.4 rpm. Meanwhile, the experimental data was within 0.130-1.49 percent error of the model's expected values. Thus, these indicator factors may be utilized to improve the production of mycelium biomass and metabolites, which are mostly employed as medicinal and pharmacological products.

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