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Statistical optimization of culture conditions for enhanced mycelial biomass production using *Ganoderma lucidum*

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ABSTRACT

The study aimed at optimizing the mycelial biomass production of *Ganoderma lucidum* by submerged fermentation. Plackett–Burman design was used to screen the important growth conditions coupled with central composite design to study the interaction of various variables with one another. Using Plackett–Burman design, temperature, yeast extract concentration, and glucose concentration were found to be significant variables contributing the most to biomass production. The R^2 value of the model was 0.9623 which indicated that the model is good. These three variables were used for further optimization studies by central composite design. Through central composite design, temperature and glucose concentration were found to be the most significant factors affecting the mycelial biomass of *G. lucidum*. The overall model was found to be statistically significant with a $P < 0.0001$. Statistical optimization was found to be an effective tool as it helped to increase the biomass production significantly.

1. INTRODUCTION

Ganoderma lucidum is a basidiomycete which has been used for over 2000 years in Japan, China, and Korea as a traditional medicine due to its properties associated with health and healing, long life, and happiness. The basidiocarp, mycelia, and spores of *G. lucidum* contain approximately 400 different bioactive compounds with polysaccharides, peptidoglycans, and triterpenes being the three major physiologically active constituents [1]. The specific reported attributes of *G. lucidum* include lowering the risk of cancer, heart disease, and infection; these health-promoting effects are believed to be mediated through the antioxidant, hypotensive, anti-inflammatory, and immunomodulatory properties of the mushroom [2]. Modern uses of the mushroom therefore include treatment of coronary heart diseases, arteriosclerosis, hepatitis, arthritis, nephritis, bronchitis, asthma, hypertension, cancer, and gastric ulcer [3].

Due to the above-mentioned reasons, there has always been an avid interest in exploring various media components and environmental factors, necessary for the growth of mycelial biomass of *G. lucidum*. The growth of mycelia has been found to be related with various environmental factors such as pH and temperature and the nutrients that are available to it. In general, culture conditions are optimized using a one factor-at-a-time approach, i.e., varying one factor while keeping all the others constant [4-6]. However, this method does not allow testing two factors simultaneously which when interacted together could improve the production. Also, analysing the results becomes difficult when using one-factor-at–a-time approach. Hence, this technique could be used for an initial screening process for studying the appropriateness of various culture conditions, thus making the optimization process more credible.

In contrast to a "one factor-at-a-time" study, statistical experimental designs such as Plackett–Burman, Taguchi orthogonal array designs, and Response Surface Methodologies including Central Composite Design and Box-Behnken allow the study of a very large number of factors in a very limited number of runs. It allows to focus on the factors that have a real effect and eliminate the ones that are not significant.

Although *G. lucidum* has been used for thousands of years and also widely studied, there are not many reports regarding the statistical optimization of various environmental and nutritional factors that affect its mycelial growth. Hence, the current study was carried out with an aim to achieve a higher biomass of *G. lucidum* by submerged fermentation using Plackett–Burman design to screen the important growth conditions, coupled with central composite design to study the interaction of various variables with one another using lesser trials and cutting back on time and chemicals.

2. MATERIALS AND METHODS

2.1. Microorganism

Mycelia of *G. lucidum* (Microbial Type Culture Collection [MTCC] 1039) were procured from the MTCC and Gene Bank, Institute of

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Microbial Technology, Chandigarh, India. It was maintained in potato dextrose agar (PDA) plates at 25°C for 9 days and was periodically transferred onto a new PDA medium. The strain was maintained at 4°C, and the growth was observed.

2.2. Media and Inoculum Preparation

Three pieces of 5 mm diameter of actively growing culture from agar plate (9 days old) were transferred with the help of a 5 mm cork borer into 250 mL Erlenmeyer flasks containing 100 mL of the seed culture at 25°C in an orbital shaker at 150 rpm for 10 days [7]. The seed culture media consisted of the following components dissolved in 100 mL double-distilled water (DDW): 1.5 g glucose, 0.2 g yeast powder, 0.1 g KH_2PO_4, 0.1 g K_2HPO_4, 0.15 g $MgSO_4.7H_2O$, and 0.25 g peptone.

2.3. Statistical Optimization for Biomass Production

2.3.1. Selection of significant variables by Plackett–Burman design

Plackett–Burman design [8] was used to screen the significant variables for biomass production of *G. lucidum* using submerged fermentation. Based on the results of one factor at a time studies, seven factors including temperature (°C), pH, inoculum size (% v/v), yeast extract (% w/v), incubation period (days), and carbon sources, i.e., glucose and maltose (% w/v) were selected. Four variables were kept as dummy, (D_1, D_2, D_3, and D_4) to satisfy the requirement of the design and to calculate the standard error. Each variable was tested at two levels, high (+) and low (-), and the effect of each variable on the biomass production was studied in 12 experimental runs. The experiments were carried out in 250 mL Erlenmeyer flasks containing 100 mL media combination prepared according to the design. All the experiments were conducted in triplicates, and the average of biomass production (mg/100 mL) was taken as a response. Further optimization was performed using the central composite design by including the factors that were found to be significant and showed a positive effect on the biomass production. Table 1 depicts the selected variables and the levels at which they were tested. Table 2 shows the detailed experimental design along with the response.

Table 1: Experimental variables at different levels used for the biomass production of G. lucidum using Plackett–Burman design. Each variable was tested at two levels, high (+) and low (-).

Symbol code	Variable	Units	Experimental levels	
			Lower (-)	Higher (+)
A	Temperature	°C	20	40
B	pH	-	4	6
C	Inoculum size	% v/v	5	7
D	Glucose	% w/v	1	2
E	Maltose	% w/v	1	2
F	Yeast extract	% w/v	0.2	0.3
G	Incubation period	Days	6	8
H-K	Dummy	-	-	-

2.3.2. Central composite design for optimizing the selected variables

Following Plackett–Burman design, the next step was to determine the optimum levels of the screened components. For this purpose, response surface methodology using central composite design was applied to study the effect of the significant variables on the biomass production (Y). The variables used were temperature, glucose, and yeast extract. The actual and coded values of each of these variables are represented in Table 3. The simultaneous interactions of the three factors are shown in the three-dimensional (3D) plots. The following second-order polynomial equation was used for the prediction of the optimum biomass production:

$$Y = \beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_{12}X_1X_2+\beta_{13}X_1X_3+\beta_{23}X_2X_3+\beta_{11}{}^2X_1{}^2+\beta_{22}{}^2X_2{}^2+\beta_{33}{}^2X_3{}^2+\varepsilon$$

Where Y is predicted response, β is the model's regression coefficient, and X the independent variables' coded levels. ε is the error term.

A total of 20 experimental runs were conducted [Table 4] in 250 mL Erlenmeyer flasks containing 100 mL media. The flasks were incubated in an orbital shaker at 150 rpm and the biomass harvested after 10 days. All the experiments were conducted in triplicates and the average of biomass production (mg/100 mL) was taken as response. Each result obtained was compared with the predicted values to determine the validity of the model [Table 4].

Table 2: Plackett–Burman experimental design for screening important variables for the biomass production of *G. lucidum*. Each variable was tested at two levels, low (-) and high (+) and the effect of each variable on the biomass production was studied in 12 experimental runs

Run	Coded levels											Biomass mg/100 mL
	A	B	C	D	E	F	G	H	I	J	K	
1	-	-	+	-	+	+	-	+	+	+	-	421±1.56
2	-	+	+	+	-	-	-	+	-	+	+	280±0.28
3	+	+	+	-	-	-	+	-	+	+	-	272±2.76
4	-	-	-	-	-	-	-	-	-	-	-	315±1.94
5	+	-	-	-	+	-	+	+	-	+	+	221±1.88
6	-	+	+	-	+	+	+	-	-	-	+	408±0.95
7	+	-	+	+	+	-	-	-	+	-	+	192±0.73
8	-	+	-	+	+	-	+	+	+	-	-	274±2.13
9	+	+	-	+	+	+	-	-	-	+	-	288±0.52
10	+	+	-	-	-	+	-	+	+	-	+	293±1.09
11	-	-	-	+	-	+	+	-	+	+	+	323±2.17
12	+	-	+	+	-	+	+	+	-	-	-	284±1.43

A: Temperature (°C), B: pH, C: Inoculum size (% v/v), D: Glucose (% w/v), E: Maltose (% w/v), F: Yeast extract (% w/v), G: Incubation period (days), H-K: Dummy variables

2.4. Harvesting

After the incubation period, the culture media containing the mycelia were decanted and each medium was separately filtered using Whatman #4 filter paper until a clear filtrate was obtained. The obtained mycelia were washed with DDW twice. The mycelia were oven dried overnight at 50°C until a constant dry weight was obtained [9]. The dry mycelia were weighed in mg/100 mL and recorded.

2.5. Statistical Analysis

All experiments were performed in triplicates to ensure reproducibility. The data obtained from Plackett–Burman design and response surface methodology were subjected to analysis of variance (ANOVA). ANOVA was carried out using Design Expert 7.0.0 statistical package (Stat Ease, Inc., Minneapolis, MN, USA) and was also used to plot the 3D response surface graphs. Any variable with $P < 0.0500$ was considered to be significant at 95% level of confidence.

3. RESULTS AND DISCUSSION

3.1. Plackett–Burman Design

The Plackett-Burman experimental design was used to screen the factors that could have a significant effect on the biomass production (mg/100 mL) of *G. lucidum.* This design was not only used to find the optimum combination of the variables that gave the maximum yield of biomass but was also used to determine the most potential variables using very few experimental runs. The effect of seven variables was evaluated using 12 experimental runs. The detailed experimental design for screening the significant variables along with the response is shown in Table 2. The variables were screened at a confidence level of 95%. Among all the seven variables evaluated, temperature, glucose, and yeast extract were found to be the most significant factors ($P = 0.0030$, 0.0167, and 0.0032, respectively) affecting the growth of mycelium of *G. lucidum* and were used for further optimization studies. Table 5 depicts the % contribution and *P* value of each variable. Among all the variables evaluated, it was seen that temperature was the most significant variable contributing the most (39.16%) in the biomass production, followed by yeast extract (37.85%) and glucose (14.75%). The overall model was found to be statistically significant at a $P = 0.0105$. The R^2 value of the model was 0.9623 which indicated that the model is good.

There have been very few studies reported on the use of Plackett–Burman design to screen the significant medium components and environmental factors for the growth of *G. lucidum* mycelia. One such study was done by Wei *et al*. [10], where statistical optimization of the fermentation medium for various *Ganoderma* strains was carried out. The Plackett–Burman design was used by them to study the effects of vitamins and microelements on mycelial biomass. In another study [11], the culture medium of *G. lucidum* was statistically optimized, wherein the Plackett–Burman Design was used to evaluate the importance of different carbon and nitrogen sources on *G. lucidum* biomass production in submerged culture. In the case of other basidiomycetes, there have again been few studies [9,12-16] where Plackett–Burman Design was used as a tool for statistical optimization.

3.2. Central Composite Design

Central Composite design was used to study the effect of the significant variables: Temperature, glucose, and yeast extract (which were screened using Plackett–Burman Design) on the biomass production by studying the interaction of these components with one another. Using a total of 20 experimental runs, the optimum levels of the screened components were also determined. The effects of the three independent variables (in coded form) on biomass production are shown in Table 4 along with the predicted values of effect. The results of ANOVA are summarized in Table 6. From Table 6, it is clear that the model terms A, D, AD, DF, A^2, D^2, and F^2 were statistically significant, all having a $P < 0.0500$. Thus, the independent variables, temperature and glucose were found to be the most significant variables of the three tested, having a $P = 0.0068$ and 0.0126, respectively, indicating that they had the maximum influence on the biomass production. Yeast extract was found to be non-significant ($P = 0.2117$) and indicated no effect on the biomass production. The interactions between temperature and glucose (AD) and glucose and yeast extract (DF) were also found to be statistically significant, having $P = 0.0001$ and 0.0417, respectively.

The *P*-value of the model was <0.0001 and that of lack of fit was 0.0524, indicating that the model was statistically significant (lack of fit having a $P > 0.0500$ indicates the model being statistically significant). The F-value of the model was 196.05 which was a high

Table 3: Experimental variables at different levels used for the central composite design experiment. The actual and coded values of each variable are represented.

Variable	Units	Experimental levels				
		−2	−1	0	+1	+2
Temperature (A)	°C	16.59	20	25	30	33.41
Glucose (D)	% w/v	0.66	1.0	1.5	2.0	2.34
Yeast extract (F)	% w/v	0.17	0.20	0.25	0.30	0.33

−2, −1,0, +1, +2- coded values of each variable.

Table 4: Central composite design showing obtained and predicted response values. Response here is biomass, represented by "Y".

Run	Variables at different levels			Biomass (Y) (mg/100 mL)	
	Temperature	Glucose	Yeast extract	Obtained	Predicted
1	0	0	0	565±1.18	562.27
2	0	0	0	571±0.88	562.27
3	+1	−1	−1	281±1.25	287.56
4	+1	−1	+1	313±2.07	310.98
5	0	+2	0	324±2.95	337.01
6	0	0	0	557±1.67	562.27
7	0	0	0	568±1.02	562.27
8	−2	0	0	320±2.34	337.88
9	−1	−1	+1	347±1.46	343.02
10	+1	+1	−1	381±1.92	375.19
11	0	0	0	553±2.74	562.27
12	−1	+1	−1	308±0.51	300.23
13	−1	+1	+1	310±0.39	293.65
14	0	0	+2	331±0.31	339.51
15	+1	+1	+1	360±1.47	360.11
16	−1	−1	−1	321±1.82	311.10
17	0	−2	0	304±2.26	304.84
18	0	0	0	562±1.83	562.27
19	+2	0	0	378±1.59	373.97
20	0	0	−2	320±1.63	325.35

enough value for the model to be significant and that of lack of fit was 4.93 again, indicating that the model is significant. since the *P*-value of the model was <0.0001 and that of lack of fit was 0.0524, hence there was a 5.24% chance that a lack of fit F-value this large could occur due to noise.

The predicted response for the biomass production (Y) can be expressed using the following second-order polynomial equation:

$$Y\ (mg/100\ mL) = 562.27+10.73A+9.57D+4.21F+24.63AD-2.12AF-9.62DF-72.95A^2-85.33D^2-81.26F^2$$

Where A is temperature, D is glucose, and F is yeast extract.

The models coefficient of determination (R^2) indicated a very high correlation between the experimentally obtained and predicted response values with a $R^2 = 0.9944$. This indicated that the model is good, as for a good model, the R^2 value should be close to 1.0. The predicted R^2 of 0.9625 was found to be in reasonable agreement with the adjusted R^2 of 0.9893. A maximum response (biomass production) of 571 mg/100 mL was obtained at the following levels of the variables: 25°C (temperature), 1.5% w/v (glucose), and 0.25% w/v (yeast extract). With such low levels of optimum concentrations obtained for glucose and yeast extract, it proved that the model was economical.

The 3D surface plots were made to study the interaction of the three variables with one another and their combined effect on the biomass production of *G. lucidum*. The 3D response surface plots were generated by plotting the response (biomass production) on the Z-axis and the two variables on the X and Y axis, whose interactions were to be studied. The 3D response plots for the interaction between the variables temperature (A) and glucose (D) are shown in Figure 1a and that between glucose (D) and yeast extract (F) are shown in Figure 1b. The 3D graph showing the interaction between temperature and yeast extract is not shown as the interaction (AF) was found to be non-significant ($P = 0.6175$).

To confirm the effectiveness of the model, the mycelial biomass was grown at the optimum levels of the variables obtained (25°C temperature, 1.5 % w/v glucose and 0.25% w/v yeast extract) and the experiments were performed in triplicates. The validity of the experimental run was determined by comparing the predicted values of biomass production (response) with the experimental values obtained. Using these optimized conditions, the experimental value of biomass production was found to be 571 mg/100 mL, which was 1.55% higher than the predicted value of 562.27 mg/100 mL [Table 4]. Thus, with just a difference of 1.55%, the experimental value agreed with the predicted value, confirming the effectiveness of the model.

There have been a few reports on the use of response surface methodology for optimizing different factors responsible for the growth of *G. lucidum*, but extremely few studies where central composite design in particular were used. One particular study on *G. lucidum* [11] used central composite design as a statistical tool for identifying optimum levels of the significant variables which were selected by Plackett–Burman design. Different concentrations of olive oil, sucrose, and yeast extract were optimized, and their combined effect on the biomass production was studied. Yeast extract and olive oil were found to be significant factors affecting the biomass production.

In another study done by Agudelo-Escobar *et al*. [17], different operational conditions affecting the cultivation of *G. lucidum* such as pH, aeration, and agitation were studied using a Box–Behnken

Table 5: ANOVA results for biomass production obtained from Plackett–Burman design.

Variable	Sum of squares (SS)	*P* value	% contribution
Temperature	18486.75	0.0030	39.16
pH	290.08	0.4647	0.61
Inoculum size	1704.08	0.1220	3.61
Glucose	6960.08	0.0167	14.75
Maltose	114.08	0.6392	0.24
Yeast extract	17864.08	0.0032	37.85
Incubation period	4.08	0.9283	3.78

% contribution indicates how much each variable has contributed in the biomass production of *G. lucidum*.

Table 6: ANOVA results for biomass production obtained from central composite design.

Independent variables	Sum of squares	F value	*P* value
Temperature (A)	1572.48	11.56	0.0068
Glucose (D)	1249.61	9.19	0.0126
Yeast extract (F)	242.09	1.78	0.2117
AD	4851.13	35.67	0.0001
AF	36.13	0.27	0.6175
DF	741.12	5.45	0.0417
A^2	76700.03	564.02	<0.0001
D^2	1.049E+005	771.58	<0.0001
F^2	95165.23	699.80	<0.0001
Lack of fit	1130.56	4.93	0.0524

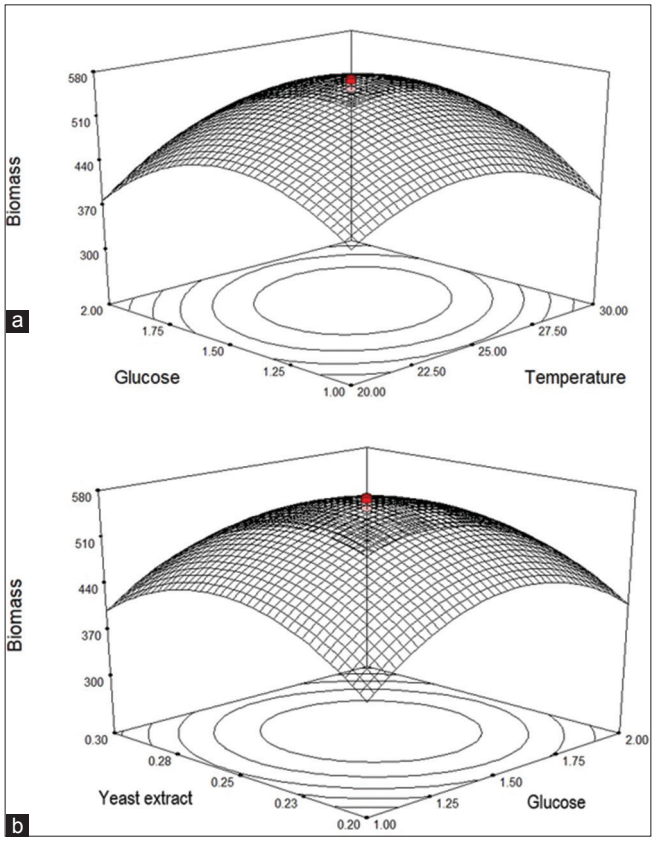

Figure 1: Three-dimensional response plots of *Ganoderma lucidum* dry mycelial biomass showing the interaction between the variables (a) temperature and glucose and (b) glucose and yeast extract.

experimental design. From the three factors studied, pH and agitation were found to be the most significant factors, giving a maximum biomass value of 6.73 g/L. Furthermore, in a previous study done by Wei *et al*. [10], Box–Behnken design was used to optimize the concentrations of three variables (glucose, yeast extract, and $Fe_2(SO_4)_3$) to study their effect on mycelia dry cell weight of *G. lucidum*. Glucose was found to be significant and yeast extract non-significant, which was similar to the results obtained in this study. In a similar study done by Mao *et al*. [18], on *Cordyceps militaris*, central composite design was conducted to locate the optimum concentrations of glucose and peptone for cordycepin production.

Different studies have reported the use of diverse factors and their combinations for the statistical optimization of biomass of *G. lucidum* [4,6,10,11,17,19,20]. Hence, it is not justified to exactly compare the biomass obtained in the present study with those obtained in other studies. The present study includes a combination of environmental and nutritional factors to optimize the biomass production. To the best of our knowledge, this is the only study, where such a combination of factors has been studied. Most of the studies include either nutritional factors or environmental factors exclusively.

4. CONCLUSIONS

In the present study, statistical optimization methods such as Plackett–Burman design and central composite design were used to optimize the biomass production of *G. lucidum* by submerged fermentation. Using statistical optimization, it was concluded that temperature and glucose concentration were found to be the most significant factors affecting the mycelial biomass of *G. lucidum.* The overall model was found to be statistically significant with a $P < 0.0001$. Furthermore, a non-significant lack of fit indicated the model to be significant. The highest biomass of 571 mg/100 mL was obtained at a temperature of 25°C, glucose concentration of 1.5% w/v, and yeast extract concentration of 0.25% w/v. These results proved that statistical optimization is an effective tool in increasing the biomass production of *G. lucidum* by a considerable amount.

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