BPE-P01: OPTIMIZATION OF MEDIA FOR MASS PRODUCTION OF *SCHIZOPHYLLUM COMMUNE* USING RESPONSE SURFACE METHODOLOGY (RSM)

Irnada Syuhaidah Mohd Kamal [1], Khairul Hazwat Abdul Halim [1], Noraswati Mohd Nor Rashid [2], and Maizirwan Mel [1]

[1]International Islamic University Malaysia, Faculty of Engineering,
Department of Biotechnology Engineering, IIUM Gombak, 53100 Kuala Lumpur
[2]Agro-Biotechnology Institute Malaysia, P.O. Box 341, UPM Serdang, 43400 Serdang, Selangor, Malaysia.
Email: irnadasyuhaidah@yahoo.com

Abstract

Schizophyllum commune (SC) is an edible mushroom which can be found worldwide except Antactica area. This type of mushroom has attracted attention because of its medicinal important. Optimization of the media composition is required for the higher production of SC mycelium mass in order to meet the demand particularly in pharmaceutical industries. Glucose and yeast extract was selected as carbon and nitrogen sources for the growth of this fungus and were optimised firstly using OFAT (one factor at a time) method. This media composition were then optimized using a response surface methods (RSM) to find the best composition of factors such as inoculum (seed), carbon, nitrogen concentration to produce a maximum mycelium mass as a response. Thirty set of different range medium composition were conducted by RSM design using Design Expert software. The statistical analysis showed that optimum media containing 11.7 % (v/v) of inoculum, 27g glucose and 1.2g yeast extract has given maximum production of mycelium mass where the yields of mycelium mass increased from 6.148g/l (unoptimized) to 15.68g/l in medium (optimized).

Key word: *Design Expert, Glucose, Mycelium, OFAT, RSM, Yeast extract*

INTRODUCTION

The demand of *Schizophyllum commune* (*S. commune*) or split gill fungus were related to the formation of schizophyllan which has been discovered by researchers worldwide to have medicinal effect. Schizophyllan consist of 1,3-β-D-linked backbone of glucose residues with 1,6-β-D-glucosyl side groups and chemically related to curdlan with a typical molecular weight range from 6 to 12 x 10^6 g mol^{-1} [1]. This neutral extracellular polysaccharide recently has attracted attention in pharmaceutical industries. Okamura [2] reported that the tumor-reducing effect of schizophyllan was significant in patients diagnose with stage II and III cervical cancer. In another researched by Matsumoto [3] has clarified that chemically modified schizophyllan can act as a new potential candidate for antisense-oligonucleotide carrier. The higher mass production of *S. commune* was required to meet the demand of schizophyllan. Optimization of media constituents is needed for the improvement of *S. commune* mass production economically. The optimal design of the culture medium is a very important aspect as medium composition can significantly affect product yield.

The first step one factor-at-a-time (OFAT) method was required to screen whether these factors (inoculum, carbon and nitrogen) has any effect to the media constituents. Optimization of all these parameters would be crucial to maximize *S. commne* mass production. The objective of OFAT is to reduce the list of variables to a small number so that subsequent experiments can be more efficient before further analysis using statistical method.

Response Surface method (RSM) is a statistical technique for designing experiments, building models, evaluating the effects of several factors, and searching optimum conditions for desirable responses [4]. The main advantage of this method is the number of experiments trials to evaluate multiple parameters can be reduced. This is one of the major importance in industry, where experiments can be very expensive and time consuming. Results can continually receive from each run rather than having to wait until the entire experiment is completed. RSM has successfully been demonstrated for its efficiency in evaluating and optimizing the interactions between various physicochemical parameters and process variables in fermentation. In the second step, the concentrations of these three factors (inoculums, glucose and nitrogen) were optimized using RSM.

MATERIAL AND METHODS

Materials

Strain of Malaysian *S. commune* from Batu Gajah Perak (S06), was selected for this experiment as the strain shows higher growth rate in previous experiment [5]. The strain were inoculated into 50 ml flask containing basic media reported by Rau (yeast extract 1.0g/l, glucose 30g/l, $MgSO_4.7H_2O$ 0.5g/l, KH_2PO_4 1.0g/l) [1] and incubated for 11 days and agitated for 150 rpm using orbital shaker.

Then, glucose in basic media were replaced with six different type of other carbon sources namely sucrose, fructose, lactose maltose, dextrin and soluble starch to determine which carbon sources gives the best results for the production of mycelium mass.

Yeast extract was replaced with other different nitrogen sources such as peptone, beef extract, sodium nitrate, ammonium chloride, ammonium sulphate, ammonium nitrate and urea.

Methods

1. Optimization of culture medium using One-factor-at-a-time methods.

Fifty mililiter of autoclaved medium (pH 5.5) was inoculated with 5 ml inoculum culture of *S. commune*. Fermentation was carried out 28ºC and 150 rpm for 11 days.

1.1 Effect of different carbon sources

The basic media 50 ml (yeast extract 1.0g/l, glucose 30g/l, $MgSO_4.7H_2O$ 0.5g/l, KH_2PO_4 1.0g/l) [1] were prepared in 250ml Erlenmayer flask. Glucose was substituted with six different carbon sources namely sucrose, fructose, lactose maltose, dextrin and soluble starch. Each carbon sources were replicated into three flask to obtain the average of mycelium mass. The pH of each flask were adjust to 5.5±0.2. Fermentation was carried out at 28ºC and 150 rpm for 14 days [6]

1.2 Effect of nitrogen sources

Seven different nitrogen sources namely peptone, beef extract, sodium nitrate, ammonium chloride, ammonium sulphate, ammonium nitrate and urea which consist of organic and inorganic nitrogen sources were replace with yeast extract (1.0g/l) from basic media. The pH of each flask were adjust to 5.5±0.2. Fermentation was carried out at 28ºC and 150 rpm for 14 days [6]

2. Optimization of culture medium component using RSM

The first step of optimization was identification of the significance of the media constituent for the production of mycelium biomass. The central composite design (CCD) under the response surface methodology (RSM) was employed in order to obtain the combination of value that optimize the response within the region of three dimensional observation spaces. The experiment were designed using the software, Design Expert Version 6.0.8. Three major variables namely inoculum, carbon and nitrogen concentration were include in this model. Other factors such as pH, temperature, and time were fixed from the result of

one-factor-of –a-time study. All of the different variables were prepared at two levels and these were designated as -1 for the low level and +1 for the high level. According to the CDD for the three variables, 30 flask of *S. commune* were cultivated in 250ml erlenmeyer flasks containing 50mL of medium of which the composition was specified according to the experimental design as shown in table 2 and their observations were fitted to the following second order polynomial model [7]

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC \quad (1)$$

`Y is the dependent variable (mycelium biomass); A, B and C are the independent variable (inoculum, carbon and nitrogen concentration); β_0 is the regression coefficient at center point; β_1, β_2 and β_3 are the linear coefficients; β_{11}, β_{22} and β_{33} are the quadratic coefficients and β_{12}, β_{13}, β_{23} are the second order interaction coefficient. Analysis of variance (ANOVA) was used to estimate the statistical parameters.

RESULTS AND DISCUSSION

Optimization using one factor at a time

As shown in Fig 1 and 2, the effect of different carbon and nitrogen sources has given different mycelium biomass concentrations. Dextrin was found to be the best carbon source to produce 8.26 g/L mycelium biomass and beef extract was found to be the best nitrogen source to produce 6.5 g/L mycelium biomass. Previous research reported that the best *S. commune* mycelial biomass yield was achieved by glucose (88.32±1.50 mg/ml) and urea as a nitrogen sources [8]. Futhermore sucrose also have been reported to gave maximum *S. commune* biomass while dextrin gave the lowest [9]. This is due to the different type of strain that have been used in the experiment, respectively. However, by considering the cost of these two sources, glucose (6.15 g/L mycelium biomass) and yeast extract (6.1 g/L mycelium biomass) were selected.

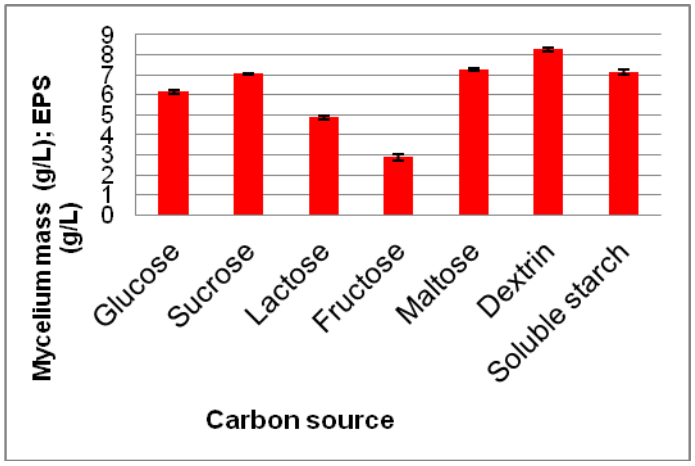


Fig.1: Effect of different carbon sources on mycelium mass production by *S.commune*.

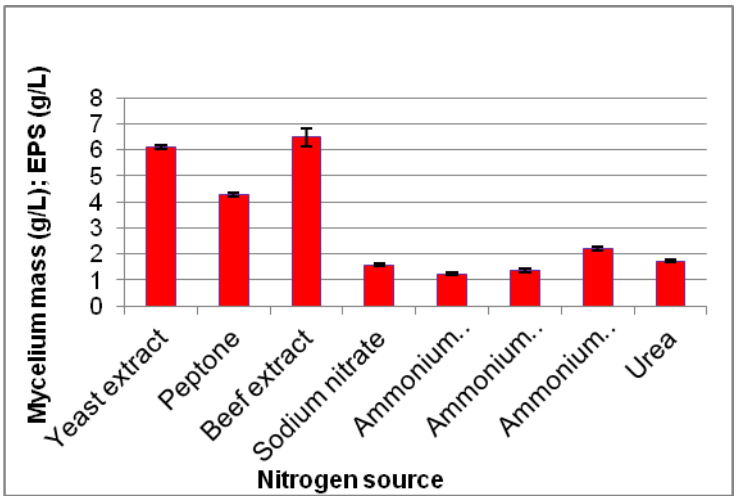


Fig.2: Effect of different nitrogen sources on mycelium mass production by *S. commune*

Optimization using RSM

The media constituents (inoculum, carbon and nitrogen sources) which are independent variables were optimized for the maximum production of mycelium biomass *from S. commune*. The full experimental design (CCD), with respect to the real values of the independent variables and attained values for the response, is presented in Table 1.

Table 1: Experimental design using CCD showing coded and actual values along with the experiment and predicted value of mycelium biomass.

Run	Inoculum (g/L) A		Glucose(g/L) B		Yeast (g/L) C		Mycelium mass production	
							Experimental	Predicted
1	140	(1)	20	(-1)	0.5	(-1)	5.58	6.54
2	100	(0)	30	(0)	1.0	(0)	13.1	16.2
3	140	(1)	20	(-1)	1.5	(1)	8.93	9.37
4	60	(-1)	40	(1)	0.5	(-1)	5.93	7.71
5	60	(-1)	40	(1)	1.5	(1)	7.87	13.8
6	100	(0)	50	(2)	1.0	(0)	7.64	7.53
7	140	(1)	20	(-1)	0.5	(-1)	5.96	6.54
8	60	(-1)	40	(1)	1.5	(1)	19.2	13.8
9	140	(1)	40	(1)	1.5	(1)	12.2	13.2
10	140	(1)	20	(-1)	1.5	(1)	7.41	9.37
11	100	(0)	30	(0)	1.0	(0)	18.4	16.2
12	180	(2)	30	(0)	1.0	(0)	13.9	11.9
13	100	(0)	30	(0)	1.0	(0)	12.2	16.2
14	100	(0)	30	(0)	2.0	(2)	6.53	7.51
15	60	(-1)	20	(-1)	0.5	(-1)	5.64	6.87
16	140	(1)	40	(1)	0.5	(-1)	0.75	6.78
17	100	(0)	10	(-2)	1.0	(0)	4.78	2.80
18	100	(0)	30	(0)	1.0	(0)	17.9	16.2
19	100	(0)	30	(0)	1.0	(0)	18.7	16.2
20	100	(0)	30	(0)	1.0	(0)	19.9	16.2
21	60	(-1)	20	(-1)	1.5	(1)	8.17	9.36
22	60	(-1)	20	(-1)	1.5	(1)	11.9	9.36
23	100	(0)	30	(0)	1.0	(0)	11.9	16.2
24	100	(0)	30	(0)	0.0	(-2)	1.59	-1.47
25	60	(-1)	20	(-1)	0.5	(-1)	4.58	6.87
26	140	(1)	40	(1)	1.5	(1)	15.7	13.2
27	20	(-2)	30	(0)	1.0	(0)	13.0	12.8
28	40	(1)	40	(1)	0.5	(-1)	9.49	6.78
29	40	(-1)	40	(1)	0.5	(-1)	9.80	7.71
30	30	(0)	30	(0)	1.0	(0)	19.8	16.2

For predicting the optimal value of mycelium biomass yield in the experiment, a second order polynomial model was fitted to the experiment result by the software. The model develop is as follow [7]

Final equation in terms of Actual Factors

$$\begin{aligned} \boldsymbol{Y} \text{ (Mycelium Dry weight)} = \quad & -30.66450 + 0.12169\boldsymbol{A} + 1.63817\boldsymbol{B} \\ & + 25.08148\boldsymbol{C} - 6.02433E\text{-}004\boldsymbol{A^2} \\ & - 0.027751\boldsymbol{B^2} - 13.25057\boldsymbol{C^2} \\ & - 3.74375E\text{-}004\boldsymbol{AB} \\ & + 4.30000E\text{-}003\boldsymbol{AC} \\ & + 0.18270\boldsymbol{BC} \end{aligned} \qquad (2)$$

Where, the mycelium biomass as yield *(Y)* is a function of inoculum *(A),* carbon *(B)* and nitrogen *(C).*

The fit of the model was also expressed by the coefficient of determination.The correlation between the experimental and predicted values is better when the value of correlation coefficient, R is closer to 1. In this experiment, the value of R and the determination coefficient R^2 were 0.7199 and 0.8485, respectively for the mycelium mass production. These values indicate a moderate degree of correlation between the experimental and the predicted values. The value of R^2 indicates that 85% of the variables inoculums, glucose and yeast extract contribute very positively to the response. It can be mentioned that only about 15% of the total variations were not explained by the *S .commune* mycelium mass production yield. However Kumari [9] from his research found that 0.98 of the variability in the response could be explained by the model involving the three same factors for *S. commune* mass production indicates a high significance of the model.

The results were than analyzed using analysis of variance suitable for the experimental design, ANOVA (Table 2). The ANOVA of the quadratic regression model and F-value indicates the model to be significant.

Table 2: Analysis of variance (ANOVA) for response surface quadratic model

Source	**Sum of square**	**F-value**	**p-value > F**
Model	637.94	5.71	0.0006
Inoculum, A	1.26	0.10	0.7534
Glucose, B	33.62	2.71	0.1154
Yeast extract , C	121.03	9.75	0.0054
A^2	26.01	2.10	0.1632
B^2	215.64	17.38	0.0005
C^2	307.26	24.76	<0.0001
AB	0.36	0.029	0.8667
AC	0.12	9.536E-003	0.9232
BC	13.35	1.08	0.3120

* $P < 0.05$ indicate the model term are significant.

**$p< 0.01$ indicate the model term is highly significant.

It was observed that the highly significant variables were the yeast extract (C), the square term of glucose and yeast extract B^2 and C^2. ($p< 0.01$). Inoculum, Glucose and the square term of inoculum shown in the analysis were not significant ($p>0.05$). All of the interactive term between inoculum and glucose, inoculum and yeast extract and glucose and yeast extract were also not significant ($p >0.05$). Linear and quadratic effect of parameters were significant meaning that the Model F-value of 5.71 implies the model is significant. There is only a 0.06% chance that a "Model F-Value" this large could occur due to noise.

The 3D response surface plots and their 2D contour plots describe by the second order polynomial equation are shown in Fig 3-5 to determine the optimum values of the variables within the ranges considered to illustrate the effects of the independent variables, and interactive effects of each independent variable on the response variables. The shape of the corresponding contour plots indicates whether the mutual

interactions between the independent variables are significant or not. The results showed the mycelium mass production was considerably affected by varying the concentration of inoculum, glucose and yeast extract. The maximum production was obtained at the point of intersection of the major and minor axes of the ellips. An elliptical nature of the contour plots indicates that the interactions between the independent variables are significant [10].

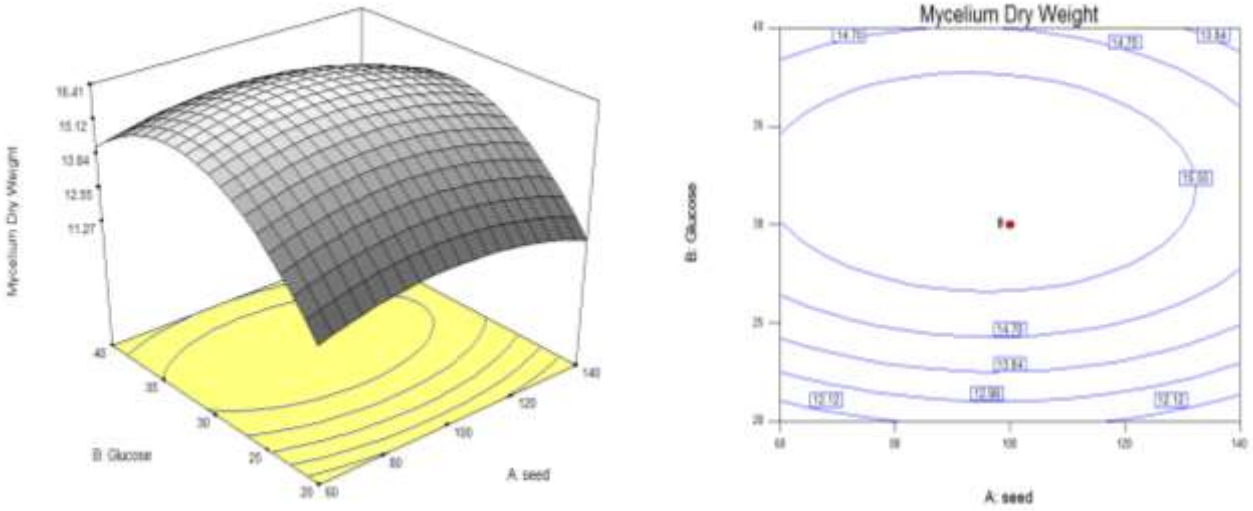


Fig. 3: 3D response surface and 2D contour plots shows the effect of glucose concentration (g/l) and inoculum concentration (g/l) on the production of mycelium biomass (g/l)

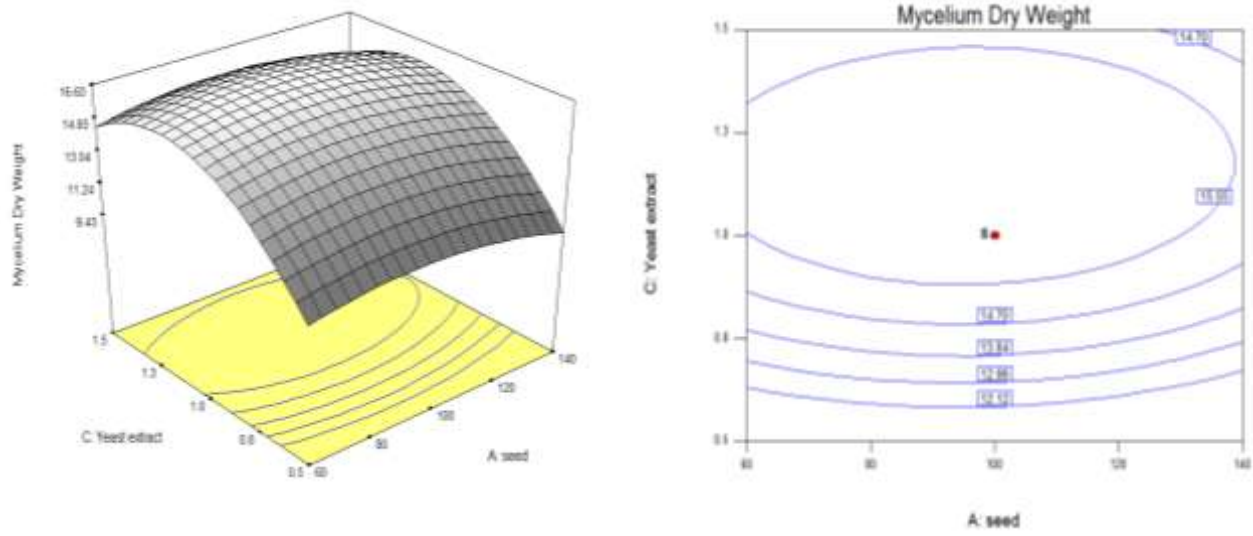
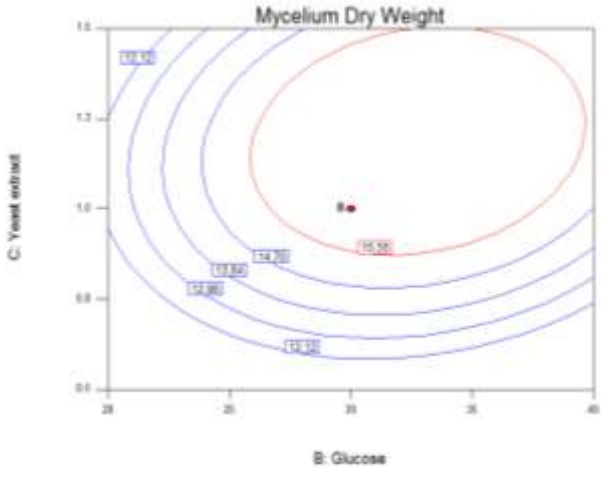


Fig. 4: 3D response surface and 2D contour plots shows the effect of yeast extract concentration (g/l) and inoculum concentration (g/l) on the production of mycelium biomass (g/l)



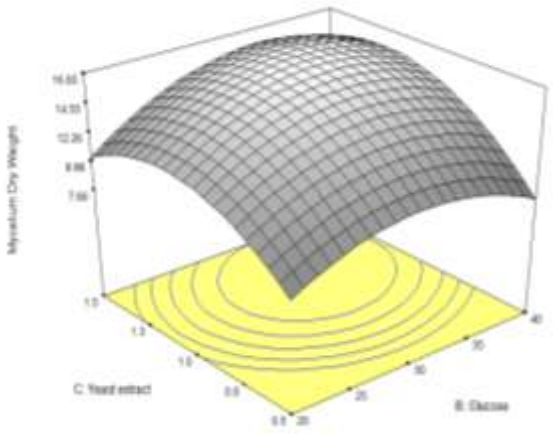


Fig. 5: 3D response surface and 2D contour plots shows the effect of yeast extract concentration (g/l) and glucose concentration (g/l) on the production of mycelium biomass (g/l)

CONCLUSION

It was possible to determine optimal media composition using one factor at a time method and RSM to maximize the production of mycelium biomass. The results from initial value of 6.148g/l to 15.68g/l confirming that the RSM could be effectively used to optimize the process parameters in complex process using the statistical design of experiments. The results of this study provided useful information and reference for the optimization of medium composition for the other submerged mushroom fermentation processes and on the other hand, maximum yield can be achived at the minimum production cost.

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