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Optimization of culture parameters of selenium-enriched *Ophiocordyceps sinensis* biomass by response surface methodology

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Abstract The medium composition and culture conditions of 25 mg/L selenium supplementing *Ophiocordyceps sinensis* was optimized by response surface methodology. The results showed that the optimum medium for Se enrichment of *O. sinensis* found to be 53.48 g/L saccharose, 11.17 g/L peptone, 1.05 g/L KH_2PO_4. The biomass and selenium content was 24,93 g/L, 960,31 µg/g, respectively. The obtained optimum conditions for *O. sinensis* consisted of daylight, temperature 20 ℃, initial pH value 6.1. The maximum biomass and total Se yield reached 26,45 g/L, 1068 µg/g, respectively. The result indicated that response surface methodology is a promising method for the optimization of selenium-enriched *O. sinensis* fermentation process, and which is the basis for further studies of the fungal bioactivity.

Keywords: Biomass, *Ophiocordyceps sinensis*, Optimization, Selenium, Response surface methodology

Introduction

Selenium (Se) is an essential trace element for human–and animal, selenium as a constituent of selenoproteins and some important enzymes such as GSH-Px and some dehydrogenase has been reported to have crucial physiological functions as antioxidation, anticancer, immunity stimulation, inhibiting HIV (Brown and Arthur, 2001, Rayman, 2012). Beside, Se deficiency is linked to several chronic diseases such as Keshan disease, and

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heart disorders (Fairweather-Tait *et al.,* 2011). In order to reduce the risk of these diseases, Se supplementation in the diet is a good option in most case, hence Se-enriched fungi have attracted much attention in recent years due to its high bioavailability and valuable in supplement food.

Ophiocordyceps sinensis (syn. *Cordyceps sinensis* (Berk) Sacc.) an Ascomycetes fungus, is a parasite on larvae of *Thitarodes* (*Hepialus*) moths (Yu *et al.,* 2011). It is one of the most valued traditional Chinese medicines. It is used commonly in the treatment of kidney and lung problems in traditional Chinese medicines. Recent studies have shown its multiple pharmacological effects, including immunomodulating activity, hypocholesterolaemic, antioxidation activity (Yu *et al.,* 2011; Fung *et al.,* 2017; Belwal *et al.,* 2019). Growth in liquid cultures has the potential advantage of high biomass production. Although the nutritional and conditional requirements of some *Cordyceps* (Fr.) species growth in liquid culture media have been determined, they have not been demonstrated in selenium-enriched *O. sinensis* fungi (Singh *et al.,* 2014; Xiao *et al.,* 2004; Kang *et al.,* 2014).

Response surface methodology (RSM) is a collection of mathematical and statistical techniques useful for designing experiments, building models and analyzing the effects of several independent factors. The advantage of RSM is not only the reduced number of an experimental run but also evaluate their multiple interactions between factors (Ba-Abbad *et al.,* 2013; Jiapeng *et al.,* 2014).

In the present work, RSM was applied to optimize the medium composition and culture conditions and find the optimization formula for the highest biomass production and total Se yield.

Materials and methods

Microorganism, inoculum preparation and flask culture

Ophiocordyceps sinensis strain was provided by Dr. Truong Binh Nguyen, University of Da Lat, Vietnam. It was maintained on potato glucose agar (PGA) slants supplemented with 25 mg/L Na_2SeO_4 (Se-PGA) at 4 ℃. The culture slants were incubated at 25 ℃ for 7 days, then stored at 4 ℃ before use, and transferred once every 3 months. Briefly, *O. sinensis* was first activated on the Se-PGA medium in a petri dish at 25 ℃ for 15 days and then two agar pieces (Φ, 8mm) from the petri dish were transferred to 200 mL seed culture in a 500 ml Erlenmeyer flask. The liquid seed culture was prepared in a 500 ml flask containing 200 ml PS (50 g/L saccharose, 6 g/L peptone, 4 g/L yeast extract) medium at 25 ℃ for 6 days. The culture medium was inoculated with

10% (v/v) the seed culture and fermented in 500ml round plastic container for 40 days. To find optimal culture requirements, the medium factor (carbon source, nitrogen source, mineral source) and condition factor (pH, temperature, light) were investigated using the RSM.

Optimized medium composition

The Box-Behnken was conducted to find the optimum level of the most significant variables (Saccharose - X1, peptone - X2, KH_2PO_4 - X3) (Ferreira *et al.*, 2007). Each independent factors were observed at three levels (-1, 0, and +1), as is shown in Table 1. Biomass production (Y1) was used as dependent output variable. A set of 17 experiments was carried out and the whole experimental design with 12 factorial points and 5 center points is given in Table 2. The model was explained by the following second-order polynomial equation:

$$Y = \beta_o + \sum_i \beta_i X_i + \sum_{ii} \beta_{ii} X_i^2 + \sum_{ij} \beta_{ij} X_{ij}$$

Where Y (Y1, Y2) represents the predicted response, Xi and Xj are independent variables which influence the response variable Y, β_o is intercept, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, and β_{ij} is the interaction coefficient.

Optimized culture conditions

D-optimal design was used to determine the optimum conditions for fungal growth and Se accumulation (Yin *et al.*, 2009). The experimental design and statistical analysis were performed using the software of DE. Temperature (A1), pH (A2), Light (A3) were chosen as the independent variables shown in Table.3 The response values were biomass yield and total Se yield. The design consisted of 19 combinations including four replicates (Table 4). The correlation of fit for the polynomial model was expressed using the values from the normal regression (R^2) and the adjusted regression (adj-R^2) coefficients.

Table 1. Coded and actual values of independent factors for Box-Behnken

Factors	**Units**	**Symbol**	**Level**		
			-1	**0**	**+1**
Saccharose	g/L	X_1	-1	0	+1
Peptone	g/L	X_2	-1	0	+1
KH_2PO_4	g/L	X_3	-1	0	+1

Table 2. Design matrix and experimental results of Box-Behnken

No.	Saccharose (g/L)	Peptone (g/L)	KH_2PO_4 (g/L)	Biomass production (g/L)		Selenium (μg/g) observed
				Observed	Predicted	
1	40	10	1.5	19.00	18.88	559.32
2	50	10	1	25.31	26.19	841.13
3	50	10	1	26.09	26.19	700.89
4	50	15	0.5	22.53	22.51	1100.98
5	60	10	0.5	22.10	22.22	548.19
6	50	10	1	26.76	26.19	771.83
7	50	10	1	26.57	26.19	862.47
8	40	15	1	22.11	21.99	759.83
9	40	10	0.5	17.64	17.77	1169.25
10	60	10	1.5	23.29	23.16	835.85
11	50	10	1	26.20	26.19	867.22
12	50	5	1.5	22.58	22.60	950.65
13	50	5	0.5	22.50	22.27	800.00
14	50	15	1.5	24.00	24.23	716.51
15	60	15	1	24.80	24.70	971.81
16	40	5	1	19.31	19.41	741.11
17	60	5	1	25.31	25.42	538.22

Table 3. Independent variables and their levels for D-optimal design of the saponification reaction

Factors	Symbol	Variable levels		
		-1	0	+1
Temperature	A_1	20	25	30
pH	A_2	6	7	8
Light	A_3	Red	White	Blue

Analytic methods

The biomass of *O. sinensis* dry weight was measured after repeated washing of the biomass with distilled water and left drying at 50 ℃ overnight at a constant weight. The Se determination was carried out according to the inductively coupled plasma mass spectrometry (ICP-MS; Agilent 7700 Series ICP-MS, Agilent, US) method (Pacquette *et al.,* 2011). Using isotope Se^{78}, internal Ge^{74} with standard Se concentration from 50ppb - 1000 ppb. The sample concentration was–calculated according to the first-order regression equation.

Table 4. Experimental runs of D-optimal design and response for biomass and Se accumulation

Run no.	Temperature	pH	Light	Biomass production (g/L)		Selenium (µg/g)	
				Observed	Predicted	Observed	Predicted
1	20	6	Red	24.00	24.45	1098	989.133
2	30	6	Red	21.34	21.77	1105	1080.28
3	20	8	Red	22.14	23.05	1042	1044.8
4	30	8	Red	18.06	18.03	1283	1146.61
5	20	7	Red	24.7	23.75	1072	1016.97
6	25	7	Red	22.64	21.83	743	1065.21
7	30	7	Daylight	21.22	22.34	1415	1254.97
8	25	6	Daylight	24.73	24.08	1226	1204.67
9	25	8	Daylight	22.61	23.14	1255	1102.25
10	20	6	Daylight	23.42	24.77	1173	1105.83
11	30	6	Daylight	24.65	23.39	1135	1303.51
12	20	8	Daylight	25.94	24.99	802	998.08
13	30	8	Daylight	20.72	21.28	1086	1206.43
14	25	8	Daylight	23.84	23.14	1186	1102.25
15	20	6	Blue	20.97	20.41	1016	1143.57
16	30	6	Blue	20.38	21.41	1252	1112.23
17	20	8	Blue	21.82	22.85	1159	1019.23
18	30	8	Blue	22.08	21.51	871	998.567
19	25	7	Blue	22.45	21.54	1044	1068.4

Statistical analysis

All assays were conducted in triplicate and significantly differed from tests at $p < 0.05$. Design-Expert V7.0 was used for the experimental designs and regression analysis of the experimental data.

Results

Optimization of Se-enriched O. sinensis biomass by Box – Behnken

Based on the result of the previous screening significant variables by Plackett-Burman design (Le Quoc Phong *et al.*, 2017), the three-factor for biomass growth was optimized using a Box – Behnken. A total of 17 runs were performed which shown in Table 2. Regression analysis of the experimental data, the polynomial model for biomass yield was expressed by Eq. 1:

$$Y\ (g/L) = -80{,}854 + 3{,}342Y_1 + 1{,}166Y_2 + 22{,}476Y_3 - 0{,}0166Y_1Y_2 - 0{,}008Y_1Y_3 + 0{,}058Y_2Y_3 - 0{,}029Y_1^2 - 0{,}014Y_2^2 - 10{,}909Y_3^2 \quad (1)$$

Where Y represents the response variable, and X_1, X_2, and X_3 represent the coded values of saccharose (g/L), peptone (g/L), KH_2PO_4 (g/L), respectively. Statistical analysis of this model revealed significantly differed at the 99% level. The variance analysis of the equation showed that R^2 0.9879, which indicated that 98.79% data variability can be explained by the model and Table 5 was listed the estimates of coefficients and associated F-values and significance levels. The three-dimensional response surface curve and the corresponding contour plot by the statistically significant model was shown in Figure 1. Each response was plotted as the function of saccharose and peptone, keeping KH_2PO_4 constant (Figures 1A); as a function of KH_2PO_4 and peptone, the saccharose was kept constant (Figures 1B); and as a function of KH_2PO_4 and saccharose, the peptone was kept constant (Figures 1C). After Se analysis, total selenium yielded from 17 experiments from 538.22 to 1169.25 μg/g.

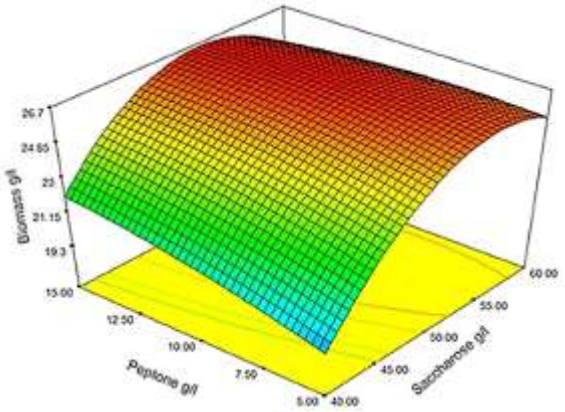
Based on the equation (1) and the response surface plots, a maximum response of 25.35 g/L biomass production and 960.31 μg/g total selenium yielded at levels of 53.48 g/L saccharose, 11.17 g/L peptone, 1.05 g/L KH_2PO_4 as optimized medium components.

Table 5. ANOVA for response surface quadratic polynomial model of Box-Behnken design

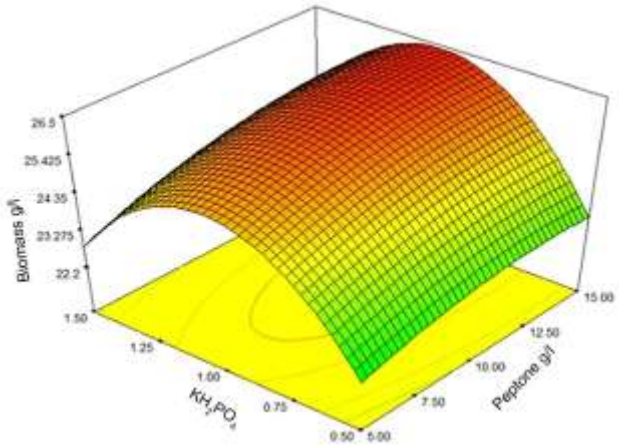
Source	Source	Degrees of freedom (DF)	Mean square (MS)	Standard error	F-value	p-value
Model	119.22	9	13.25		63.26	< 0.0001
Y_1	38.00	1	38.00	0.16	181.46	< 0.0001
Y_2	1.74	1	1.74	0.16	8.31	0.0235
Y_3	2.11	1	2.11	0.16	10.05	0.0157
Y_1Y_2	2.74	1	2.74	0.23	13.08	0.0085
Y_1Y_3	0.01	1	0.01	0.23	0.03	0.8616
Y_2Y_3	0.48	1	0.48	0.23	2.31	0.1722
Y_1^2	34.16	1	34.16	0.22	163.14	< 0.0001
Y_2^2	0.87	1	0.87	0.22	4.16	0.0807
Y_3^2	33.70	1	33.70	0.22	160.90	< 0.0001

R^2 = 0.9879; CV = 1,96%; Adj-R^2 = 0.9722; Pre-R^2- = 0.9556; C.V = 1,96;Adeq Precision = 23,965

A



B



C

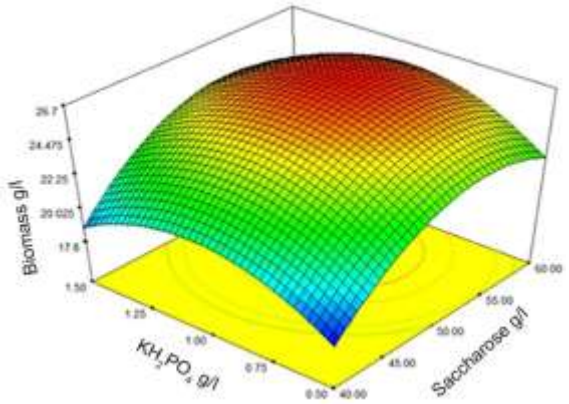


Figure 1. Response surface of biomass production by *O. sinensis*. A: the interaction of saccharose and peptone at constant levels of KH_2PO_4 (1.05 g/L); B: the interaction of peptone and KH_2PO_4 at constant levels of saccharose (53.48 g/L); C: the interaction of KH_2PO_4 and saccharose at constant levels of peptone (11.17 g/L)

Effect of culture conditions on Se accumulation in O. sinensis biomass by D-optimal model

Based on the optimal medium, the culture conditions were optimized with two output variables as biomass production and selenium content. The results of 19 experiments showed that total Se accumulation in biomass from 743 to 1415 μg/g (Table 4). In experiment, the model was not significantly differed ($p > 0.05$) according to ANOVA given in Table 6, therefore, condition factors were not affected to selenium content.

Table 6. ANOVA results of the value of total selenium yield from D-optimal design

Factor	p-value	Factor	p-value
Model	0.901	Temperature * Light (Red)	0.972
Temperature	0.418	Temperature * Light (Daylight)	0.472
pH	0.626	Temperature * Light (Blue)	0.474
Light (Red)	0.658	pH* Light (Red)	0.481
Light (Daylight)	0.379	pH* Light (Daylight)	0.729
Light (Blue)	0.703	pH* Light (Blue)	0.684
Temperature*pH	0.964		

$R^2 = 0.29$, Adj $R^2 = -0.420$

The influence of culture conditions on O. sinensis biomass by D-optimal model

According to the ANOVA in Table 7, the obtained model was significantly differed ($p < 0.05$). The coefficient of determination (R^2) for the model was 0.793, which could explain the 79.30% variability in the data of the model. In addition, the model's lack of fit with $p > 0.05$, which also proves adequate fit of the model.

Table 7. ANOVA results of the value of biomass from D-optimal design

Factor	p-value	Coefficients	Factor	p-value	Coefficients
Model	0.029		Temperature*Light (Red)	0.105	-0.829
Temperature	0.009	-1.095	Temperature*Light (Daylight)	0.705	-0.180
pH	0.283	-0.373	Temperature*Light (Blue)	0.066	1.009
Light (Red)	0.256	-0.498	pH*Light (Red)	0.089	-0.912
Light (Daylight)	0.0087	1.283	pH*Light (Daylight)	0.825	-0.096
Light (Blue)	0.099	-0.78503	pH*Light (Blue)	0.065	1.009
Temperature*pH	0.132	-0.584			

$R^2 = 0.793$, Adj-$R^2 = 0.585$

The effect of temperatures, pH and light on biomass yield were investigated and the results were represented in Table 7, namely, temperature and daylight were two significantly factors to biomass ($p < 0.05$) and other factors were non-significantly differed.

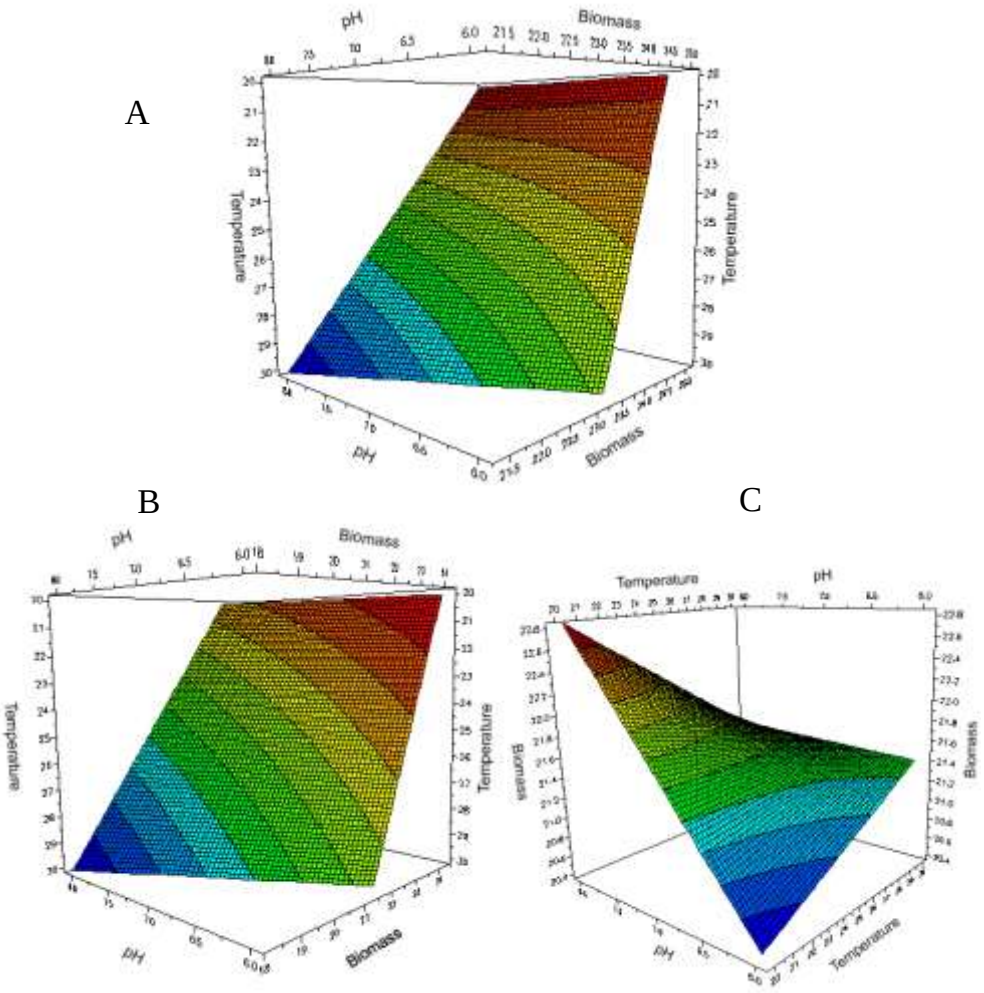


Figure 2. Response surface for the effect of the pH and temperature on the biomass; A: Daylight; B: Red light; C. Blue light

Based on the response surface plots, the optimum levels of three variables were 20 ℃, daylight, pH 6.1 and the predicted largest biomass yield and total Se yield were 24.79 g/L and 1097 μg/g, respectively.

Discussion

In this previous study, medium component did not affect selenium accumulation, thus, in this experiment, we only assessed the effect of medium composition on biomass of *O. sinensis* (Le Quoc Phong *et al.,* 2017). In order to validate the predicted results of the statistical model, experiments of the optimum medium composition were used to perform the highest point of the model and the obtained value of biomass and Se yield were 24,93 g/L and 1059 µg/g, respectively. Similarly, the predicted optimal conditions were conducted to evaluate fitted of model. The maximum of biomass and selenium content were 26,45 g/L and 1068 µg/g, respectively. It showed that all model was valid to simulate and in predict the output value.

Saccharose is important sources of carbon, which is favorable to the mycelium growth (about 30-50 g/L for *O. sinensis*, (Dong and Yao, 2005), (Singh *et al.*, 2014). On the other hand, a too high carbon concentration also made too high carbon/nitrogen ratio, which will inhibit the mycelium growth (Hoa and Wang, 2015). Beside, KH_2PO_4 provides phosphate and potassium, they also have an important role in balancing the pH environment, because selenate in the culture medium will produce selenic acid causing environmental acidification affecting the growth of *O. sinensis*.

White light has the strongest influence on the growth of mycelium ($p < 0.05$), while red and blue light do not affect significantly biomass ($p > 0.05$). Our study is different from previous publications, showing that red light stimulates biomass growth more strongly than blue and white light. Our study is different from previous publications, showing that red light stimulates biomass growth more strongly than blue and white light (Dong *et al.,* 2012). The pH does not affect significantly the growth of mushroom, possibly due to the mechanism of pH self-balancing of mushrooms or mineral in medium.

The biomass production of *O. sinensis* in this work was obviously higher than 22.15 g/L of *O. sinensis* (Dong and Yao 2005), 17.81 g/L of *O. sinensis* (Zheng *et al.,* 2014), 14.5 g/L of *Cordyceps jiangxiensis* JXPJ 0109 (Xiao *et al.,* 2004), 12.7 g/L *Cordyceps militaris* C738 (Kim *et al.,* 2003). The Se content of *O. sinensis* was also higher than 938.9 µg/g *Pleurotus ostreatus*, 1200-1400 µg/g *Saccharomyces cerevisiae* (Esmaeili and Khosravi-Darani 2014), 680.2 µg/g *C. militaris* (Zhang *et al.,* 2014), 748.0 µg/g *Lentinula edodes* (Milovanović *et al.,* 2014).

These data indicated that *O. sinensis* has potential capacities of Se-enrichment in liquid culture. Selenium-enriched culture cannot only increase the quality of *O. sinensis* but can also be a good selenium supplementation food.

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