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Combination of Statistical Techniques for Submerged Fermentation for Extracellular Polysaccharide and Biomass of *Ganoderma tsugae*

[1]Nukrob Narkprasom, [2]Jia-Hsin Guo, [2,3]Tzou-Chi Huang, [2]Yuan-Kuang Guu

[1]Faculty of Engineering and Agro-Industry, Maejo University, Chiang Mai, Thailand
[2]Department of Food Science, National Pingtung University of Science and Technology, Pingtung, Taiwan
[3]Department of Biological Science and Technology, National Pingtung University of Science and Technology, Taiwan

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ABSTRACT

Biomass and extracellular polysaccharide of *Ganoderma tsugae* have various biological activity including anti-inflamatory activity, antioxidant activity and antitumor activity. However, the growth rate of *G. tsugae* in nature is very slow. Therefore, many studies have attempted to develop mass culture systems for *G. tsugae* using laboratory techniques. Many parameters of submerged fermentation for *G. tsugae* were studies to determine the optimization of process by combination of statistical techniques. Ten parameters from preliminary results and literature reviews (maltose, skim milk, KH_2PO_4+K_2HPO_4, $MgSO_4$-$7H_2O$, $CaCO_3$, vitamin B_5+B_6, olive oil, ethanol, pH and shaking speed) were screened by Packett Berman design. The significant parameters were determined the optimal ranges by path of steepest ascent method. The optimal condition of process was performed by response surface method. Maltose, skim milk and pH are significant parameters for *G. tsugae* cultivation. The conditions of 31.031 g L^{-1} maltose, 14.055 g L^{-1} skim milk and an initial pH of 7.12 resulted in the maximum extracellular polysaccharide content of 415 mg L^{-1} and the same fermentation broth at an initial pH of 6.46 exhibited the most biomass at 15.776 g L^{-1}. Finally, the optimal condition was compared with un-optimal condition which result indicates that the combination of statistical techniques enhance the productions of biomass and extracellular polysaccharide (13X and 1.5X of the control, respectively). Therefore, these strategies are useful for improvement of submerged fermentation of *G. tsugae* which it can apply in pharmaceutical industry.

Keywords: *Ganoderma Tsugae*, Plackett Burman Design, Steepest Ascent Method, Response Surface Method

1. INTRODUCTION

Song San Ling Chih or *Ganoderma tsugae* has been as a traditional medicine which it is used to promote of health and longevity in Asian countries (Lin *et al*., 2013). The polysaccharides produced by *G. tsugae* have been studied and used for pharmaceutical purposes as treatment for various diseases (Hsu *et al*., 2009) and have been applied to add in the food and medicinal industries (Rusell and Paterson, 2006). The cultivation of *G. tsugae* for fruiting body takes a long period and high risk of contamination from nature. Therefore, technique of submerged fermentation was interested from bio-pharmaceutical industry, because it has short time cultivation, easy to control condition for contaminated protection and obtain best product in quality and quantity (Narkprasom *et al*., 2012). The production yield of *G. tsugae* cultured using submerged fermentation is affected by many parameters, such as agitation, aeration, temperature, pH, dissolved oxygen, nutrient composition and fermentation time (Chang *et al*., 2006; Hsieh *et al*., 2006; Yang *et al*., 2004; Lee *et al*., 2003) and these parameters are critical for the production of *G. tsugae*. The optimization method for many parameters in fermentation process of *G. tsugae* has attracted significant interest from pharmaceutical industry. The

Corresponding Author: Nukrob Narkprasom, Faculty of Engineering and Agro-Industry, Maejo University, Chiang Mai, Thailand

combination of statistical techniques including screening by Plackett Burman Design (PBD), determining optimal variable ranges by steepest ascent method and performing optimal searching by Response Surface Method (RSM) have been applied to optimize biochemical and physical processes in many studies (Omar *et al*., 2004; Xu *et al*., 2008; Cansee *et al*., 2008; Xiao *et al*., 2010; Zhang *et al*., 2010) because of reasonable study design and excellent outcomes. Therefore, the aims of this report were to determine the optimal condition of submerged fermentation for Extracellular Polysaccharide (EPS) and mass cultivation of *Ganoderma tsugae* by combination of statistical techniques.

2. MATERIALS AND METHODS

2.1. Inoculums Preparation and Submerged Culture

G. tsugue BCRC 36203 in stock was culture with potato dextrose with agar in a Petri dish at 28°C for 7 days. After that, the mycelium was transfer to Erlenmeyer flask with 200 mL of potato dextrose broth at 30°C for 7 days. The mycelia pellet was crushed with sterilized bender and then transferred to various flasks with different experimental conditions (10 mL per flask) at 30°C for 5 days. The cultured products were separated between mycelia pellets and supernatant by centrifugation at 15,000×*g* for 20 min. The supernatant and precipitate were used to determine the EPS and dry cell weight of biomass, respectively. The precipitate was dried by freeze dry and EPS was determined by phenol-sulfuric acid method with a spectrophotometer reading at 490 nm.

2.2. Statistical Experimental Design for Optimization of Submerged Fermentation

Statistical experimental design procedure used here to optimize medicinal fungus production can be subdivided into four steps: (i) Identification of the most important media components (screening), (ii) identification of the optimal variable ranges (narrowing), (iii) identification of the optimum conditions (optimum search) and (iv) experimental verification of the identified optimum (verification).

2.3. Screening for Important Parameters

PBD is generally used to identify the most important media components. A two-level fractional factorial design for studying n-1 variables in n runs is an effective method to rapidly identify the most significant parameters. One-variable-at-a-time experiments identified many principal parameters affecting the EPS production of *G. tsugae*: Maltose, skim milk, KH_2PO_4, K_2HPO_4, $MgSO_4 \cdot 7H_2O$, $CaCO_3$, vitamin B_5+B_6, olive oil, ethanol, initial pH and shaking speed. The combined effects of these variables were evaluated using a PBD with a first-order polynomial equation. Each independent variable was tested at low (-1) and high (+1) levels. Different combinations of variables were tested in 12 experimental runs. The fitted first-order model is Equation 1:

$$Y = \beta_0 + \sum \beta_i x_i \quad (1)$$

where, Y is the predicted response, β_0 and β_i are constant coefficients and x_i represents the coded independent variables. Each experiment was carried out in triplicate and the average value was taken as the response. Variables with confidence levels above 95% were considered to have significant effects on the EPS production and therefore were used for further optimization.

2.4. Narrowing Optimal Variable Ranges

Method of steepest ascent is often used to identify the optimal variable range. To approach the maximum response region of a variable, the key variables screened by the PBD were further optimized via a path of steepest ascent. This procedure allows for sequential movement towards the maximum response. The path of steepest ascent was initiated from the mid-point values of each major factor identified by the PBD.

2.5. Optimum Search

Because the location of the optimum within the experimental range is unknown prior to RSM, a rotational design can be employed that allows for estimation with equal precision in all directions. Based on the approximate central point of a RSM, the optimum region was obtained through a path of steepest ascent. RSM was employed here to screen for the optimum levels of the three most significant factors identified by the PBD where it was assumed that the estimated response surface Y could be described with the aid of a second-order polynomial Equation 2:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ij} x_i x_j + \sum \beta_{ii} x_i^2 \quad (2)$$

To allow for an unconfounded estimation of the regression coefficients β, each parameter must be examined on at least three levels. To determine the regression coefficients of the second-order polynomial

using the fewest possible experiments, different experimental designs were proposed that differed in the number of minimum necessary experiments and in the range of application.

2.6. Verification of Model

High production of *G. tsugae* calculated from the quadratic equations of the RSM must be demonstrated experimentally to verify the optimal condition to confirm the results and also compare the yields of the optimized and unoptimized conditions.

2.7. Statistical Analysis and Software

In combinatorial experimental designs, the all regression analysis of the experimental data were calculated using Microsoft Excel 2007. Sigma Plot 10 was used to create the 3D graphs to present the results of RSM experiment.

3. RESULTS

Base on preliminary results, carbon source as maltose, nitrogen source as skim milk and vitamin B_5+B_6 were nutrients used during fermentation screening by PBD, whereas the other nutrients and fermenting conditions were set base on literature reviews.

3.1. Fermentation Condition Screening

Twelve runs by PBD were carried out to determine the effects of these ten variables on EPS production. As shown in **Table 1**, high EPS yields ranging from 93.045-167.009 m g L^{-1} were obtained. A first-order model was generated as follows Equation 3:

$$Y_{EPS} = 127. + 14.825x_1 + 10.560x_2 + 5.419x_3 - 3.140x_4 + 4.689x_5 + 2.293x_6 - 7.464x_7 - 2.994x_8 + 10.356x_9 + 3.286x_{10} \quad (3)$$

The fitness of model was evaluated from the coefficient of determination R^2. The R^2 value from Eq. 3 shows that 98.04% of the total variance of the system could be explained by the model (**Table 2**).

3.2. Determination of Optimal Ranges

As shown in **Table 2**, maltose, skim milk and pH were found to correlate positively with EPS production. The path of steepest ascent was determined, starting from the midpoint established from PBD, by increasing pH and the concentrations of maltose and skim milk. The results of this experiment are shown in **Table 3**. The maximum EPS yield of 338.249 mg L^{-1} with a biomass of 9.430 g L^{-1} was obtained under the following conditions: 30 g L^{-1} of maltose, 11 g L^{-1} skim milk and pH 6.70. These experimental results were used to determine the optimal variable ranges, which were then used to design a search for optimum by response surface method.

3.3. Optimization Searching

Central composite design (CCD) by RSM is a very useful tool to determine the optimal level of medium constituents and their interactions. The results of CCD experiments with different combinations of maltose (x_1), skim milk (x_2) and initial pH (x_3) are presented along with the mean predicted and experimental values in **Table 4**.

3.4. Optimization of EPS Production of G. Tsugae

Changing fermentation conditions led to considerable variation in the yield of EPS. Using the multiple regression analysis method, the predicted response for the EPS can be described by Equation 4:

$$Y_{EPS} = 374.199 + 28.950x_1 + 21.644x_2 + 43.411x_3 - 22.763x_1x_2 + 2.476x_1x_3 + 19.652x_2x_3 - 21.422x_1^2 - 20.307x_1^2 - 25.729x_3^2 \quad (4)$$

where, Y_{EPS} is the EPS yield and x_1, x_2 and x_3 are the coded factors of maltose, skim milk and initial pH, respectively. The quadratic regression model for EPS was demonstrated to be highly significant (F-test < 0.0001). The adjusted R^2 value suggested that the total variation of 96% for the yield of EPS was attributed to the independent variables and only about 4% of the total variation could not be explained by the model.

R^2 value closer to 1 indicate a better correlation between the experimental and predicted values. The R^2 value of 0.9917 indicated good agreement between the experimental and predicted EPS values. To better understand the effects of EPS of *G. tsugae*, the model was calculated using RSM.

The 3D response surface plots were presented in **Fig. 1** to visualise interactions between the variables and rapidly estimate the optimal value of each variable for EPS production. **Fig. 1** shows the combined effects of two variables, while the remaining variables were maintained at constant levels. By solving Eq. 4, the model predicted a maximum EPS yield of 415.178 mg L^{-1} when the coded levels of the three most significant variables were x_1 = 0.2064, x_2 = 1.0185 and x_3 = 1.243, representing maltose, skim milk and initial pH values of 31.0319 g L^{-1}, 14.0555 g L^{-1} and 7.12.

Central Composite Design (CCD) by RSM is a very useful tool to determine the optimal level of medium constituents and their interactions. The results of CCD experiments with different combinations of maltose (x_1), skim milk (x_2) and initial pH (x_3) are presented along with the mean predicted and experimental values in **Table 4**.

3.5. Optimization of biomass production of G. Tsugae

By applying multiple regression analysis on the experimental data reported in **Table 4**, the second-order polynomial equation for mycelium biomass was written as follows:

$$Y_{Biomass} = 13.506 + 0.697x_1 + 2.971x_2 - 1.253x_3 - 0.832x_1x_2 + 0.110x_1x_3 + 0.723x_2x_3 - 1.812x_1^2 - 0.994x_2^2 - 0.931x_3^2 \quad (5)$$

where, $Y_{Biomass}$ is the predicted biomass yield of *G. tsugae* in submerged fermentation and x_1,x_2 and x_3 are the coded factors of maltose, skim milk and initial pH, respectively. The results of the regression analysis for biomass production are shown in **Table 4**. The adjusted R^2 of the second-order polynomial Equation 5 was 0.9470. The high values of multiple R^2 (0.9859) and determinant coefficient R^2 (0.9721) show that the values predicted by model are close to the experimental data. To study effects of variables on biomass of *G. tsugae*, the predicted model was further assessed using CCD response surface analysis. The different input levels of maltose, skim milk and pH on the subsequent biomass production of *G. tsugae* were shown on **Fig. 2**. All 3D graphs indicate a quadratic function rather than a linear function. Solving for the variables of Eq. 5, the optimal values of test variables in actual units were 29.189 g L^{-1} maltose, 15.603 g L^{-1} skim milk and an initial pH of 6.46. This condition was associated with the maximum predicted amount of mycelium biomass from *G. tsugae* in submerged fermentation, 15.776 g L^{-1}.

3.6. Verification of Model

The model was confirmed by, comparing optimal and non-optimal conditions. **Table 5** shows that the EPS and biomass production were found to be very close to the predicted values, supporting the accuracy of the model. Moreover, the optimal experimental conditions enhanced the biomass and EPS production of *G. tsugae* 13-fold and 1.5-fold, respectively, when compared with a non-optimized condition.

Table 1. Plackett Burman design for screening of significant factors affecting EPS of *G. tsugae*

	Nitrogen source		Mineral salt						Shaking speed		
Carbon source	X_2	Potassium	----------								
	Skim milk	X_3	X_4		Vitamin	Oil	Alcohol		X_{10}		
X_1	g/l	KH_2PO_4	$MgSO_4$	X_5	X_6	X_7	X_8		rpm		
Maltose	Preliminary	$+K_2HPO_4$	$\cdot 7H_2O$	$CaCO_3$	B_5+B_6	Olive oil	Ethanol		Chang *et al*.	EPS	
g/l	result and	g/l	g/l	g/l	g/l	g/l	(%v/v)		(2006) and	mg/l	
Preliminary	Chang *et al*.	Hsieh *et al*.	Hsieh *et al*.	Chang *et al*.	Preliminary	Chang *et al*.	Yang *et al*.	pH	Lee *et al*.	Chang *et al*.	Biomass
result	(2006)	(2006)	(2006)	(2006)	result	(2006)	(2004)	X_9	(2007)	(2006)	g/l
1 (25)	1 (10)	-1 (1)	1 (1.5)	1 (1)	1 (0.03)	-1 (0.5)	-1 (0.4)	-1 (5.5)	1 (150)	158.071	5.312
-1 (15)	1	1 (2)	-1 (0.5)	1	1	1 (2.5)	-1	-1	-1 (120)	117.233	7.319
1	-1 (5)	1	1	-1 (0.2)	1	1	1 (2)	-1	-1	110.923	6.992
-1	1	-1	1	1	-1 (0.01)	1	1	1 (6.5)	-1	117.057	6.944
-1	-1	1	-1	1	1	-1	1	1	1	139.141	4.390
-1	-1	-1	1	-1	1	1	-1	1	1	96.901	3.881
1	-1	-1	-1	1	-1	1	1	-1	1	110.923	6.410
1	1	-1	-1	-1	1	-1	1	1	-1	156.318	5.245
1	1	1	-1	-1	-1	1	-1	1	1	167.009	5.425
-1	1	1	1	-1	-1	-1	1	-1	1	112.500	4.215
1	-1	1	1	1	-1	-1	-1	1	-1	150.534	5.169
-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	93.045	3.712

Table 2. Plackett Burman design for screening variables of EPS from *G. tsugae*

Variables	Low level (-1)	High level (+1)	Coefficients	P-value	Confidence level
X_1	15.00	25.00	14.825	4.356	85.635*
X_2	5.00	10.00	10.560	3.103	80.153*
X_3	1.00	2.00	5.419	1.592	64.300
X_4	0.50	1.50	-3.140	-0.923	47.444
X_5	0.20	1.00	4.689	1.378	60.029
X_6	0.01	0.03	2.293	0.674	37.748
X_7	0.50	2.50	-7.464	-2.193	72.765
X_8	1.40	2.00	-2.994	-0.880	45.936
X_9	5.50	6.50	10.356	3.043	79.787*
X_{10}	120.00	150.00	3.286	0.966	48.888

Multiple R = 0.9902, R2 = 0.9804, Adjusted R = 0.7848, Standard Error = 3.403

Table 3. Experimental design and results along the steepest ascent for EPS production of *G. tsugae*

Step	Maltose (g/l),x_1	Skim milk (g/l),x_2	pH,x_9	EPS (mg/l)	Biomass (g/l)
1	20	7.500	6.00	238.169	3.099
2	25	9.281	6.35	245.921	4.495
3	30	11.062	6.70	338.249	9.430
4	35	12.842	7.05	284.266	5.124
5	40	14.623	7.40	254.135	2.369

Table 4. Experimental design and results of the central composite design for optimization of the EPS production and biomass of *G. tsugae*

	Maltose (g/l)	Skim milk (g/l)	pH	EPS (mg/l)		Biomass (g/l)	
Run	X_1	X_2	X_3	Measured	Predicted	Measured	Predicted
1	-1(25)	-1(8)	-1(6)	211.879	212.100	7.413	7.339
2	1(35)	-1	-1	315.464	310.575	9.660	10.175
3	-1	1(14)	-1	275.327	261.611	12.867	13.496
4	1	1	-1	270.945	269.033	12.140	13.006
5	-1	-1	1(7)	261.655	254.666	3.707	3.201
6	1	-1	1	358.230	363.044	6.747	6.478
7	-1	1	1	386.799	382.786	12.407	12.252
8	1	1	1	409.233	400.111	11.767	12.202
9	-1.682(21.6)	0(11)	0(6.5)	254.645	264.919	6.973	7.210
10	1.682(38.4)	0	0	359.982	362.296	10.300	9.553
11	0(30)	-1.682(6.8)	0	280.585	280.361	5.327	5.699
12	0	1.682(15.2)	0	340.352	353.164	16.573	15.691
13	0	0	-1.682(5.66)	220.642	228.418	13.927	12.949
14	0	0	1.682(7.34)	369.622	374.434	8.327	8.794
15	0	0	0	372.426	374.199	13.533	13.506
16	0	0	0	370.148	374.199	13.847	13.506
17	0	0	0	387.149	374.199	14.253	13.506
18	0	0	0	360.333	374.199	12.760	13.506
19	0	0	0	378.911	374.199	12.853	13.506
20	0	0	0	378.386	374.199	13.700	13.506

Table 5. Comparison between G. tsugae production in non-optimized and optimise conditions

Non-optimized conditions from the initial basal medium[a]	EPS (mg/l)	274.904±67.452	
Optimal condition[b]	Biomass (g/l)	1.183±0.276	
	EPS (mg/l)	Measured	416.244±14.801
		Predicted	415.178
	Biomass (g/l)	Measured	15.701±0.323
		Predicted	15.534

[a]Glucose 20 g L^{-1}, Peptone 7.5 g L^{-1}, KH2PO4 and K2HPO4 1.5 g L^{-1}, MgSO4•7H2O 1 g L^{-1} and 150 rpm
[b]Maltose 30 g L^{-1}, Skim milk14 g L^{-1}, KH2PO4 and K2HPO41.5 g L^{-1}, MgSO4•7H2O 1 g L^{-1}, CaCO3 0.6 g L^{-1}, Vitamins B5 and B6 0.02 g L^{-1}, Olive oil 1.5 g L^{-1}, Ethanol 1.2 g L^{-1}, pH 7 and 135 rpm

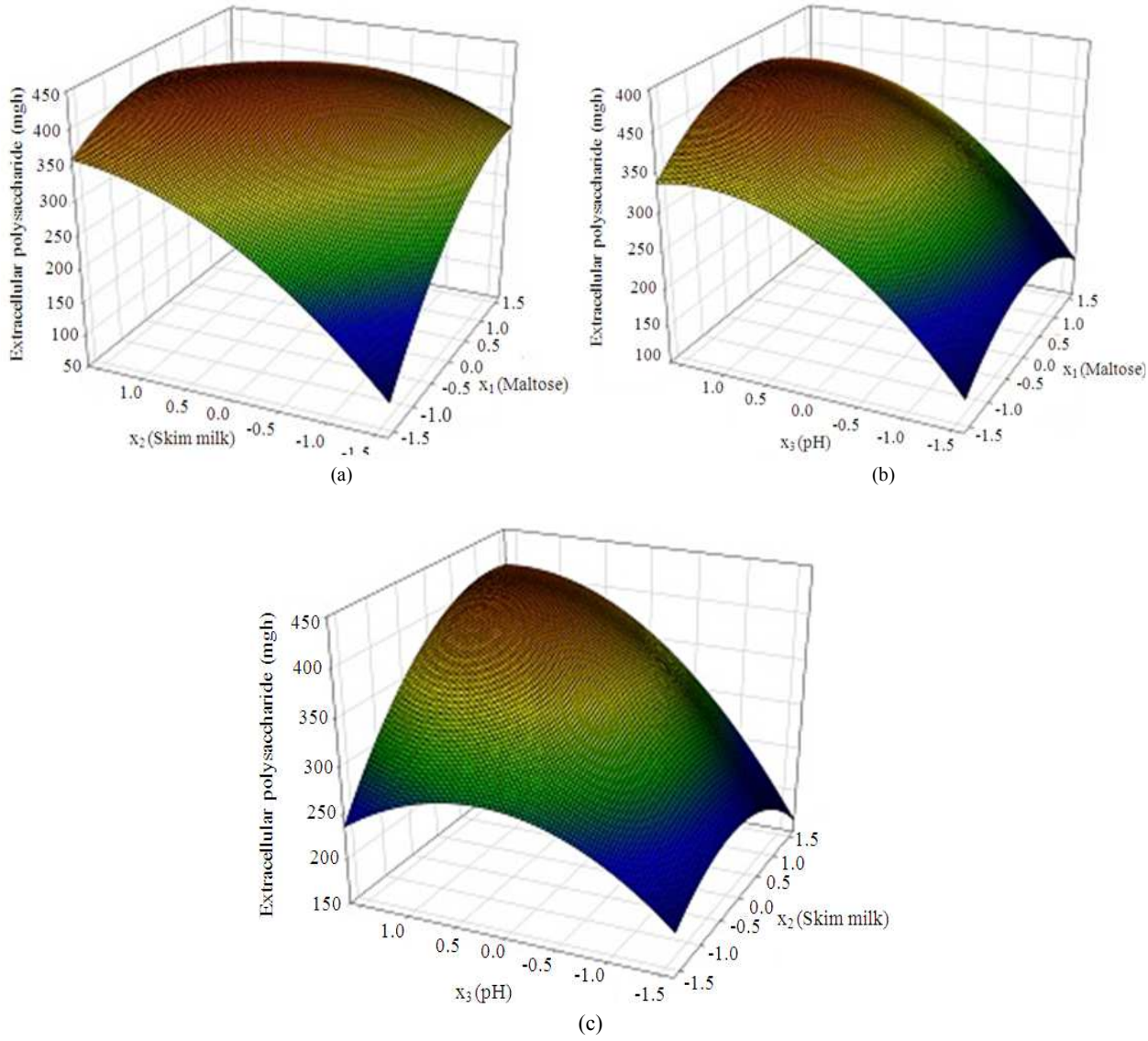

Fig. 1. Response surface 3D plot for EPS (mg/l) production of *G. tsugae*

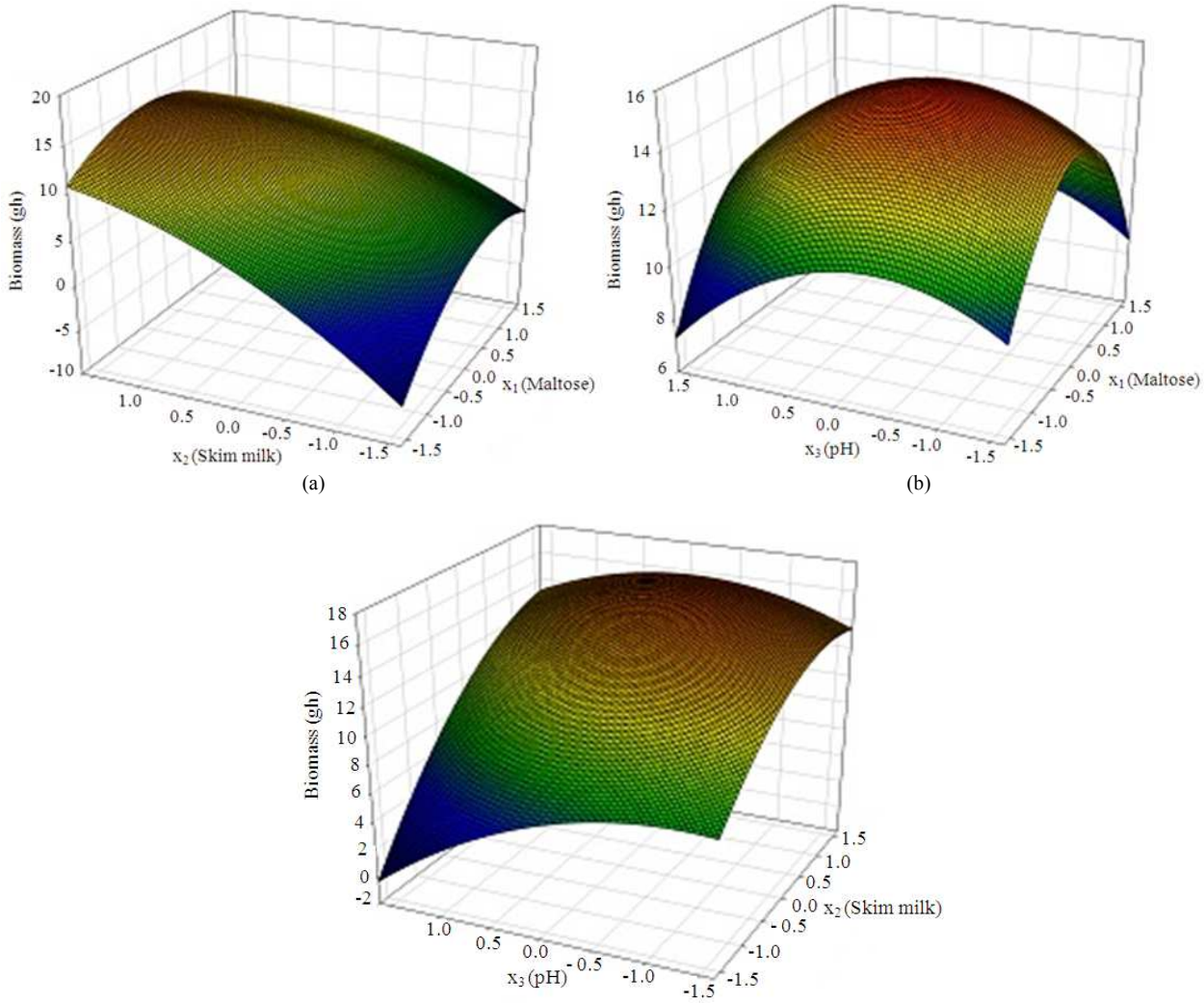

Fig. 2. Response surface 3D plot for biomass (g/l) of *G. tsugae*

4. DISCUSSION

Combination of statistical techniques consisted of screening by PBD, determination of optimal ranges by path of steepest ascent method and optimal searching by RSM was applied for optimization of submerged fermentation for EPS and biomass of *G. tsugae*. Combination of statistical techniques offers a strategy for studying many variables including nutrient requirements and culture conditions in fewer experimental runs, leading this method to be widely used to screen multiple variables of fermentation processes. Literature reviews of many studies on nutritional requirements for *Ganoderma* fungus has shown that a carbon source of brown sugar, a nitrogen source of skim milk, plant olive oil, $CaCO_3$ (Chang *et al*., 2006), $MgSO_4 \cdot 7H_2O$, K_2HPO_4, KH_2PO_4 (Hsieh *et al*., 2006) and ethanol (Yang *et al*., 2004) effectively promoted biomass and EPS. Moreover, cheese whey (Lee *et al*., 2003), brown sugar, skim milk (Chang *et al*., 2006) and molasses, increased production because these components contain various vitamins and other nutrients. Chang *et al*. (2006) showed that setting the pH and incubation temperature to 6.5 and 34°C, respectively, increased the biomass and EPS of *G. lucidum*. In contrast, (Lee *et al*., 2003) reported that the optimal conditions for biomass by *G. lucidum* were pH 4.2 and 28.3°C. Therefore, differences in the culture environment might have led to these different results. The data on *G. tsugae* production were subjected to multiple linear regression analysis and the coefficients, t-

values, p-values and confidence levels were estimated. The first-order model (Equation 3) predicted that increasing the concentrations of maltose, skim milk, KH_2PO_4+K_2HPO_4, $MgSO_4 \cdot 7H_2O$, $CaCO_3$, B_5+B_6, pH and shaking speed and decreasing the concentrations of other variables including $MgSO_4 \cdot 7H_2O$, olive oil and ethanol could further enhance EPS. Furthermore, the effect value of each factor tested was assessed by a t-test, with the values and confidence levels presented in **Table 2** indicating with high confidence levels that maltose, skim milk and pH are linked to EPS production. These three factors were selected as key factors for the next optimization step. While, the other factors including KH_2PO_4, K_2HPO_4, $MgSO_4 \cdot 7H_2O$, $CaCO_3$, vitamin B_5+B_6, olive oil, ethanol and shaking speed were not the primary factors controlling EPS production. They did affect biomass and the yields of triterpeniod compounds with high confidence levels (data not shown), which could be used in the future to enhance the production of these compounds and study the pathway of *G. tsugae* production. Carbon and nitrogen sources play a significant role in determining the production level of submerged fermentation because these nutrients are directly linked with cell proliferation and metabolite biosynthesis (Kim *et al*., 2003; Zou, 2005). Maltose has been found to be best carbon source for the cell mass and EPS production in *G. applanatum* (Lee *et al*., 2007) and *G. lucidum* (Tang and Song, 2002), respectively, because it can be easily used for biosynthesis of biomass and EPS, as glucose is the main sugar component of the cellular energy production system and EPS. Skim milk is substrate for protease enzymes, such as fibriolytic enzymes (Silva *et al*., 2003; Korat and Rizvi, 2004; Albillos *et al*., 2007; Merheb *et al*., 2007), which *Ganoderma* species have the ability to (Jo *et al*., 2011; Kumaran *et al*., 2011) in order to digest complex protein molecules into simple polypeptide chains and amino acids (Sharma *et al*., 2011) for the growth and metabolic activities. Moreover, pH in submerged fermentation is important because enzymes involved fermentation operate in optimum pH environment (Hashemi *et al*., 2013; Zaman *et al*., 2009).

5. CONCLUSION

This study combined PBD, steepest ascent and CCD strategies to enhance *G. tsugae* yields. These methods not only helped to obtain the optimal combination of factors with fewer experiments, materials and time but also proved to be useful and satisfactory in studying the fermentation process. In submerged cultivation, factors like carbon source, nitrogen source and pH were shown to be highly influential, so the optimal ranges of these variables for maximum biomass and EPS were calculated and subsequently verified. The optimal condition, (31.031 g L^{-1} maltose, 14.055 g L^{-1} skim milk and an initial pH of 7.12) yields approximately 13 and 1.5 times more biomass and EPS, respectively, when compared with the non-optimized condition. This optimal condition will apply to culture *G. tsugae* in large scale of bioreactor in bio-industry. The fundamental information obtained in this study will be highly useful for efforts to optimize the fermentation process of *G. tsugae* for further basic scientific studies as well as for medicinal purposes.

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