Carbohydrate Polymers 77 (2009) 677–680

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Short communication

Optimization of process parameters of the *Pholiota squarrosa* extracellular polysaccharide by Box–Behnken statistical design

Haizhen Zhao, Juan Wang, Zhaoxin Lu *

College of Food Science and Technology, Nanjing Agricultural University, Nanjing, Jiangsu 210095, PR China

ARTICLE INFO

Article history:
Received 8 October 2008
Received in revised form 9 January 2009
Accepted 18 February 2009
Available online 24 February 2009

Keywords:
Extracellular polysaccharide
Pholiota squarrosa (Pers. *ex* Fr.) Quel.
Statistical optimization
Box–Behnken design

ABSTRACT

The quantitative effects of fermentation temperature, fermentation time and inoculum volume on the yield of *Pholiota squarrosa* extracellular polysaccharide were investigated using response surface methodology (RSM). The experimental data obtained were fitted to a second-order polynomial equation using multiple regression analysis and also analyzed by appropriate statistical methods. RSM analysis showed good correspondence between experimental and predicted values. It was found that three parameters represented significant effect. The coefficient of determination (R^2) for the model was 98.5%. Probability value ($P < .0001$) demonstrated a very high significance for the regression model. By solving the regression equation and also by analyzing the response surface contour plots, the optimal process parameters were determined: fermentation temperature 28.57 °C, fermentation time 7.82 d and inoculum volume 12.57 ml. Under the optimal conditions the corresponding response value predicted for extracellular polysaccharide production was 853.73 μg per milliliter of fermentation liquor, which was confirmed by validation experiments.

1. Introduction

Nowadays, following nutritional investigations, edible mushrooms are well known as a very rich food supplement, due to their favorable protein, carbohydrate and dietary fiber contents (Carbonero et al., 2006; Hou, Zhang, Xiong, Li, & Yang, 2008; Kalač, 2009; Ng, 1998; Wang & Li, 2001).

These basidiomycetes have caused an emerging interest in the biomedical area, mainly due to their contents of bioactive substances, especially various biologically active polysaccharides. Among these, edible fungi from the genera *Ganoderma*, *Agaricus* and *Lentinus* have been widely investigated (Kües & Liu, 2000; Wasser, 2002), and have also demonstrated some interesting biological properties, such as anti-inflammatory, immunomodulating and antitumor activity (Delmanto et al., 2001; Lin & Zhang, 2004; Yu, Yin, Yang, & Liu, 2009).

Also studied has been another genus of edible mushrooms, that of *Pholiota* Kummer, which, a genus of macrofungi, often endowed with attractive colors, belongs to the family Strophariaceae, order Agaricales, class Basidiomycetes. More than 30 species are known, distributed worldwide. These mushrooms are frequently active parasites and wood destroyers, attacking forest trees as well as park trees, and their mycelium continues the destruction after the tree is cut. A few Pholiotas are edible and no species have been reported as toxic so far.

Pholiota Kummer are rich in vitamins, amino acids, trace elements, lipid and polysaccharides (Tian, 2006) and many of them, represent significantly pharmacologic activity, such as *Pholiota nameko, Pholiota aurivella, Pholiota adiposa, Pholiota flammans, Pholiota lubrica* (Xu,1997). *Pholiota squarrosa* (Pers. *ex* Fr.) Quel. AS 5.245, a brown edible fungus is extensively distributed in China. However, little information about its polysaccharides is available compared with those of other *Pholiota* types, such as *P. adiposa*, *P. nameko* (Cui & Li, 2004; Dulger, 2004; Huang, Chang, & Xing, 2008; Li, Zhang, & Zhang, 2008; Wang et al., 2006).

Our previous studies have shown that the extracellular polysaccharide components of *P. squarrosa* exhibit the strong antitumor activity (data not shown). However, cultivation of *P. squarrosa* is fairly difficult and efficient production of *P. squarrosa* polysaccharides has been a bottleneck in all their promising applications. The objective of this present study is to optimize the process for production of *P. squarrosa* extracellular polysaccharides, using response surface methodology (RSM), employing a three-level and three-variable Box–Behnken design.

2. Materials and methods

2.1. Microorganism and culture

Pholiota squarrosa (Pers. *ex* Fr.) Quel. AS 5.245, obtained from China Center for Type Culture Collection, China, was grown on synthetic potato–dextrose–agar slant for fortnight at 25 °C and subcultured every 3 months.

* Corresponding author. Tel./fax: +86 25 84396583.
E-mail address: fmb@niau.edu.cn (Z. Lu).

0144-8617/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2009.02.013

The medium for fungal slant is synthetic potato–dextrose–agar. The medium for inoculum and shake flask cultivation are synthetic potato–dextrose without agar.

2.2. Shake flask cultivation

The optimization study experiments were carried out in 250 ml Erlenmeyer flasks, covered with 12 layers of cotton gauze, containing 50 ml cultivation medium, they were inoculated with designed volume of seed inoculum and then the flasks were incubated on a rotary shaker at 140 rpm and designed temperature for designed time.

2.3. Estimation of extracellular polysaccharide concentration (EPC)

After cultivation, 10 ml of the fermented filtrate obtained from the cultivation medium by filtration through Whatman no. 1 filter-paper was precipitated with 85% alcohol, centrifugated at 3000 r/min for 5 min and the supernatant was removed. The sediment was dissolved with distilled water to certain volume in which the extracellular polysaccharide concentration was determined according to the classical method of Dubois, Gilles, Hamilton, Rebers, and Smith (1956) using glucose solution as a standard reference.

2.4. Experimental design for response surface methodology

The levels of the significant parameters and the interaction between variables, which influence the extracellular polysaccharide concentration, were analyzed and optimized by Box–Behnken methodology (Box & Behnken, 1960). In this study, the experimental plan consisted of 17 trials and the independent variables are studied at three different levels, low (1), medium (0) and high (+1). The variables and their coded levels used for the study are shown in Table 1. All the experiments were done in triplicate and the average of extracellular polysaccharide concentration obtained was taken as the dependent variable or response ($\hat{Y}_{EPC}$). The second-order polynomial coefficients were calculated and analysed using the "Design Expert" software (Version 6.0.5, Stat-Ease Inc., Minneapolis, USA) statistical package. The general form of the second degree polynomial Eq. (1) is

$$\hat{Y}_{EPC} = c_0 + \sum_{i=1}^{n} a_i x_i + \sum_{j \le i}^{n} a_{ij} x_i x_j \quad (1)$$

where $\hat{Y}_{EPC}$ is the predicted response, c_0 the intercept term, a_i values are linear coefficient, a_{ij} values the cross-product coefficient, x_i and x_j the level of the independent variables, subscripts i and j takes values from 1 to the number of variables. In the present study, three variables are involved and hence n takes the value 3. Thus, by substituting the value 3 for n, and the coded variables for natural ones, Eq. (1) becomes the following form:

$$\hat{Y}_{EPC} = c_0 + a_1 A + a_2 B + a_3 C + a_{11} A^2 + a_{22} B^2 + a_{33} C^2 + +a_{12} AB + a_{13} AC + a_{23} BC \quad (2)$$

Table 1
Level and code of variables chosen for Box–Behnken design.

Variable	Symbols		Coded levels		
	Uncoded	Coded	−1	0	1
Fermentation temperature (°C)	x_1	A	25	28	31
Fermentation time (d)	x_2	B	6	8	10
Inoculum volume (ml)	x_3	C	5	10	15

The goodness of fit of the model was evaluated by the coefficient of determination (R^2) and the analysis of variance (ANOVA). Quadratic polynomial equations were attained by holding one of the independent variances at a constant value and changing the level of the other variables.

3. Results and discussion

3.1. RSM model fitting

Response surface optimization is more advantageous than the traditional single parameter optimization in that it saves time, space and raw material. All 17 of the designed experiments were conducted for optimizing the three individual parameters in the current Box–Behnken design. The results were analyzed by multiple regression analysis. Table 2 shows the experimental conditions and the results of extracellular polysaccharides yield according to the factorial design. Maximum yield of polysaccharides (848.1 μg per milliliter of fermentation liquor) was recorded under the experimental conditions of fermentation temperature 28 °C, fermentation time 8 d, and inoculum volume 10 ml.

In order to determine whether or not the quadratic model is significant, it is necessary to conduct ANOVA analysis. The P-values were used as a tool to check the significance of each coefficient, which also indicated the interaction strength of each parameter. The smaller the P-values are, the bigger the significance of the corresponding coefficient (Murthy, Swaminathan, Rakshit, & Kosugi, 2000). Here, the P-value of the model was smaller than .0001, which indicated that the model was suitable for use in this experiment. The P-value of "lack of fit" was .1771 ($P > .01$), which indicated that "lack of fit" was insignificant relative to the pure error. The coefficient of determination (R^2) and adjust coefficient of determination (Adj R^2) were also shown in Table 3. The values indicated that the accuracy and general availability of the polynomial model were adequate.

The regression coefficients and the corresponding P-values were presented in Table 4. From the P-values of each model term, it could be concluded that three linear coefficients (A, B, C), three quadratic coefficients (A^2, B^2, C^2) and three cross-product coefficients (AB, AC, BC) were significant.

Using the designed experimental data (Table 2), the polynomial model for the EPC was regressed and shown as below (in term of coded factors):

Table 2
Box–Behnken design matrix of the three variables in coded units along with the experimental and predicted values of EPC.

Trial no.	A	B	C	EPC (μg per milliliter)	
				Experimental	Predicted
1	−1	−1	0	612.21	623.58
2	1	−1	0	682.41	694.32
3	−1	1	0	720.38	708.47
4	1	1	0	711.03	699.66
5	−1	0	−1	650.69	649.34
6	1	0	−1	637.93	636.03
7	−1	0	1	709.31	711.21
8	1	0	1	785.10	786.45
9	0	−1	−1	651.10	641.09
10	0	1	−1	726.23	739.50
11	0	−1	1	813.79	800.53
12	0	1	1	782.33	792.35
13	0	0	0	838.10	836.30
14	0	0	0	833.10	836.30
15	0	0	0	841.10	836.30
16	0	0	0	848.10	836.30
17	0	0	0	821.10	836.30

Table 3
Analysis of variance for the fitted quadratic polynomial model of EPC by *P. squarrosa* (Pers. *ex* Fr.) Quel. AS 5.245.

Source	Sum of squares	*df*	Mean square	*F*-Value	Probability (*P*) > *F*
Model	102418.8	9	11379.87	52.67	<.0001
Lack of fit	1105.64	3	368.55	3.62	.1228
Pure error	406.8	4	101.7		
Correlation total	103931.3	16			

R^2 = .9854; Adj R^2 = .9667.

$$\hat{Y}_{EPC} = 836.30 + 15.49A + 22.56B + 53.07C - 19.89AB + 22.13AC - 26.6BC - 101.20A^2 - 53.59B^2 - 39.34C^2 \quad (3)$$

Many parameters can influence the *P. squarrosa* extracellular polysaccharides concentration. Eq. (2) shows that the yield of *P. squarrosa* extracellular polysaccharides has a complex relationship with independent variables that encompass both first and second-order polynomials and may have more than one maximum point.

Fig. 1 represents the effects of fermentation time, fermentation temperature and their reciprocal interactions on EPC at a constant inoculum volume of 12.57 ml. At the designed range of fermentation temperature from 25 °C to 31 °C, the augmentation of EPC resulted in a linear increase in fermentation time, then reduced. An increase of fermentation temperature increased the EPC at a constant fermentation time within a fermentation temperature of 28.57 °C. The appropriate maximal EPC was determined at a fermentation temperature of 28.57 °C and a short fermentation time.

Fig. 2 shows the effects of inoculum volume, fermentation temperature and their reciprocal interactions on EPC at a fermentation time of 7.82 d. At the designed range of fermentation temperature from 25 °C to 31 °C, the yield of extracellular polysaccharide increased with inoculum volume increasing. At a low temperature, the yield of extracellular polysaccharide increased with temperature increasing, then reduced when temperature was above 28 °C. EPC was affected significantly by fermentation temperature.

The effect of inoculum volume and fermentation time on the synthesis of EPC at a reaction temperature of 28.57 °C is provided in Fig. 3. An increase in EPC was observed with the increasing of inoculum volume and fermentation time at first. But the trend was reversed when the inoculum volume and fermentation time reached a certain value. It could be seen from Fig. 3 that the optimum inoculum volume and fermentation time for the maximum EPC was around 13 ml and 8 d, respectively.

3.2. Validation of the model

The optimal values of the selected variables were obtained by solving the regression equation (Eq. (3)) using Design-Expert 6 software. The optimal conditions for EPC synthesis estimated by the model equation were as follows: A = 28.57 °C, B = 7.82 d and C = 12.57 ml. The theoretical EPC predicted under the above conditions was 853.727 μg per milliliter. In order to verify the prediction of the model, the optimal reaction conditions were applied to three independent replicates for EPC synthesis. The average EPC was 847.68 ± 1.67 μg per milliliter (N = 3), a figure well within the estimated value of the model equation. This demonstrated the validation of the RSM model. The good correlation between these results confirmed that the response model was adequate for reflecting the expected optimization. The results also suggested that the models of Eq. (3) are satisfactory and accurate.

Table 4
Regression coefficients and their significance of the quadratic model of EPC by *P. squarrosa* (Pers. *ex* Fr.) Quel. AS 5.245.

Model term	Coefficient estimate	*df*	Standard error	Probability (*P*) > *F*
Intercept	836.3	1	6.57	–
A	15.49	1	5.20	.0205
B	22.56	1	5.20	.0034
C	53.07	1	5.20	<.0001
A^2	−101.20	1	7.16	<.0001
B^2	−53.59	1	7.16	.0001
C^2	−39.34	1	7.16	.0009
AB	−19.89	1	7.35	.0304
AC	22.14	1	7.35	.0196
BC	−26.65	1	7.35	.0084

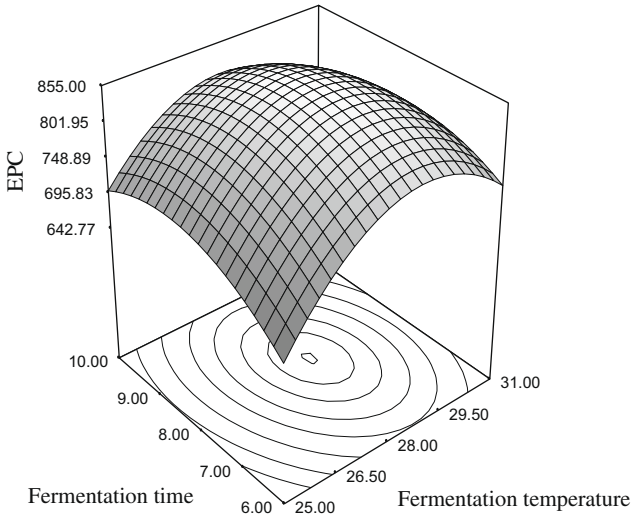

Fig. 1. Response surface plots representing the effect of fermentation temperature, fermentation time and their reciprocal interaction on EPC synthesis. Inoculum volume is constant at 12.57 ml.

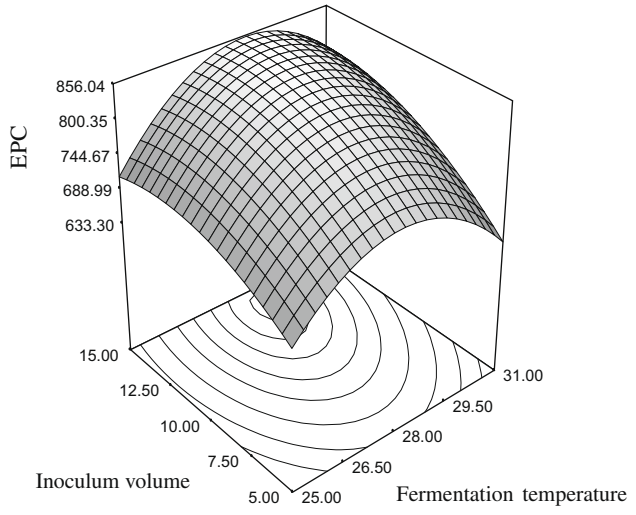

Fig. 2. Response surface plots representing the effect of inoculum volume, fermentation temperature and their reciprocal interaction on EPC synthesis. Fermentation time is constant at 7.82 d.

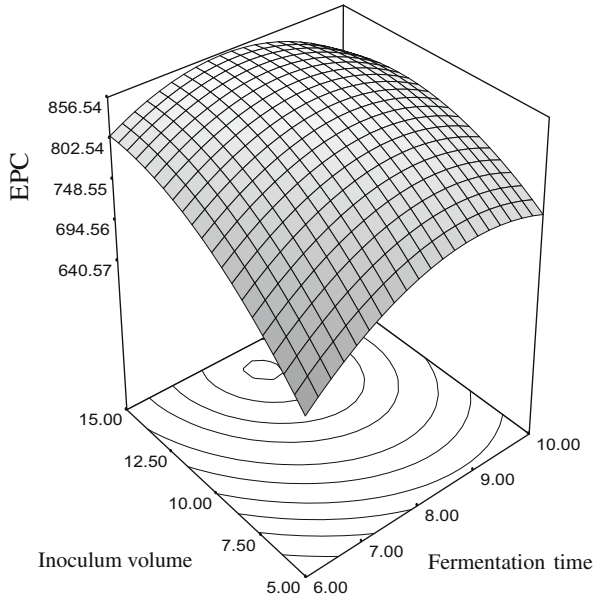

Fig. 3. Response surface plots representing the effect of inoculum volume, fermentation time and their reciprocal interaction on EPC synthesis. Fermentation temperature is constant at 28.57 °C.

4. Conclusions

Optimization of the process parameters for maximal EPC was carried out. RSM was used to optimize the production of *P. squarrosa* extracellular polysaccharide. The coefficient of determination (R^2) for the model is 98.5%. Probability value ($P < .0001$) demonstrates a very high significance for the regression model. EPC of 847.68 μg per milliliter of fermentation liquor was obtained when optimum conditions of the process parameters for maximal production of extracellular polysaccharide using *P. squarrosa* (Pers. *ex* Fr.) Quel. AS 5.245 were fermentation temperature 28.57 °C, fermentation time 7.82 d and inoculum volume 12.57 ml. Validation experiments verified the availability and the accuracy of the model. The predicted value was in agreement with the experimental value. The study proved the response surface method to be useful for optimization of process parameters for extracellular polysaccharide production using *P. squarrosa* (Pers. *ex* Fr.) Quel. AS 5.245 and statistical analysis is proved to be a useful and powerful tool in developing optimal production conditions.

References

Box, G. E. P., & Behnken, D. W. (1960). Some new three level designs for the study of quantitative variables. *Technometrics, 2*, 455–475.

Carbonero, E. R., Gracher, A. H. P., Smiderle, F. R., Rosado, F. R., Sassaki, G. L., Gorin, P. A. J., et al. (2006). A β-glucan from the fruit bodies of edible mushrooms *Pleurotus eryngii* and *Pleurotus ostreatoroseus*. *Carbohydrate Polymers, 66*(2), 252–257.

Cui, Y. J., & Li, Q. Z. (2004). Immune effect of *pholiota nameko* polysaccharide on the aging model mice in the different period. *Journal of Northeast Agricultural University, 35*(2), 159–161.

Delmanto, R. D., Alves de Lima, P. L., Sugui, M. M., Ferreira da Eira, A., Salvadori, D. M. F., Speit, G., et al. (2001). Antimutagenic effect of *Agaricus blazei* Murrill mushroom on the genotoxicity induced by cyclophosphamide. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 496*(1–2), 15–21.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry, 28*, 350–356.

Dulger, B. (2004). Antimicrobial activity of the macrofungus *Pholiota adiposa*. *Fitoterapia, 75*, 395–397.

Hou, X. J., Zhang, N., Xiong, S. Y., Li, S. G., & Yang, B. Q. (2008). Extraction of BaChu mushroom polysaccharides and preparation of a compound beverage. *Carbohydrate Polymers, 73*(2), 289–294.

Huang, Q. R., Chang, L. R., & Xing, Z. G. (2008). Research on several kinds of growth factors on mycelia and exopolysaccharide of *pholiota adipose*. *Food Research and Development, 4*, 49–52.

Kalač, P. (2009). Chemical composition and nutritional value of European species of wild growing mushrooms: A review. *Food Chemistry, 113*(1), 9–16.

Kües, U., & Liu, Y. (2000). Fruiting body production in basidiomycetes. *Applied Microbiology and Biotechnology, 54*, 141–152.

Li, H. P., Zhang, S. H., & Zhang, K. S. (2008). Study on antioxidant activity of *pholiota nameko* polysaccharides. *Food Research and Development, 4*, 54–60.

Lin, Z. B., & Zhang, H. N. (2004). Anti-tumor and immunoregulatory activities of *Ganoderma lucidum* and its possible mechanisms. *Acta Pharmacologica Sinica, 25*, 1387–1395 [China].

Murthy, M. S. R. C., Swaminathan, T., Rakshit, S. K., & Kosugi, Y. (2000). Statistical optimization of lipase catalyzed hydrolysis of methyloleate by response surface methodology. *Bioprocess Engineering, 22*, 35–39.

Ng, T. B. (1998). A review of research on the protein-bound polysaccharide (polysaccharopeptide, PSP) from the mushroom *Coriolus versicolor* (Basidiomycetes: Polyporaceae). *General Pharmacology, 30*, 1–4.

Tian, E. J. (2006). *Studies on taxonomy of the genus Pholiota from China*. Jinlin: Jilin Agricultural University dissertation for master.

Wang, Q., Zhang, J. G., Wang, S. K., Zhao, Q. Z., Liu, H. X., Liu, Y. X., et al. (2006). The effect study of ferment extract of *Pholiota adiposa* on adjusting blood lipids. *Journal of Hebei University (Natural Science Edition), 1*, 101–103.

Wang, Y. X., & Li, P. (2001). Research advances of the nutrient composition and pharmacologic effect of edible mushrooms. *Journal of Dali Medical College, 10*(4), 63–65.

Wasser, S. P. (2002). Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. *Applied Microbiology and Biotechnology, 60*, 258–274.

Xu, J. T. (1997). Chinese medical mycology. Beijing: China Medical University, Peking Union Medical Joint Press, 49–50.

Yu, Z. H., Yin, L. H., Yang, Q., & Liu, Yan (2009). Effect of *Lentinus edodes* polysaccharide on oxidative stress, immunity activity and oral ulceration of rats stimulated by phenol. *Carbohydrate Polymers, 75*(1), 115–118.