Carbohydrate Polymers 78 (2009) 409–415

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

Optimization of medium composition for the production of exopolysaccharides from *Phellinus baumii* Pilát in submerged culture and the immuno-stimulating activity of exopolysaccharides

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ARTICLE INFO

Article history:
Received 12 February 2009
Received in revised form 17 April 2009
Accepted 27 April 2009
Available online 12 May 2009

Keywords:
Phellinus baumii Pilát
Exopolysaccharide
Medium composition
Statistical optimization design
Immuno-stimulating activity

ABSTRACT

Optimization of medium composition for the production of exopolysaccharides (EPS) from *Phellinus baumii* Pilát in submerged culture and the immuno-stimulating activity of EPS were carried out. Firstly, the medium components having significant effect on EPS production were screened out to be glucose, yeast extract and diammonium oxalate monohydrate by using a $2^{(7-3)}$ fractional factorial design. Secondly, the concentrations of the three factors were optimized using central composite design in response surface methodology. As results, a quadratic model was found to fit for EPS production, and the optimal medium composition was determined as following (g/l): 34.12 glucose, 4 peptone, 5.01 yeast extract, 0.88 diammonium oxalate monohydrate, 0.75 $MgSO_4$ and 1 KH_2PO_4 and 0.0075 thiamine (VB_1). A yield of 2.363 ± 0.04 g/l for EPS was observed in verification experiment. Finally, EPS from *P. baumii* Pilát was found to have direct immuno-stimulating activity *in vitro* on splenocyte proliferative response and acid phosphatase activity in peritoneal macrophages in a dose-dependent manner.

1. Introduction

During the past decades, deep interest has been generated in the polysaccharides from medicinal mushroom due to their diverse and potentially significant pharmacological activities, such as hypoglycemic, antioxidant, immuno-stimulating, anti-tumor, anti-inflammatory, and free radical scavenging properties (Carbonero et al., 2006; Han et al., 1999; Kim et al., 1996; Shon, Kim, & Sung, 2003; Wong, Wong, Chiu, & Cheung, 2007; Yu et al., 2007). As results, a number of polysaccharides with immuno-modulating or/and anti-tumor activity have been isolated from medicinal mushroom, for example, polysaccharidepeptide and Krestin from cultured mycelia of *Coriolus versicolor*, lentinan from fruiting body of *Lentinus edodes*, schizophyllan from fermented broth of *Schizophyllum commune*, and polysaccharide produced by the liquid culture of *Ganoderma lucidum* (Ikekawa, 2001; Moradali, Mostafavi, Ghods, & Hedjaroude, 2007; Wasser, 2002).

Phellinus baumii Pilát is a well-known fungus of the genus *Phellinus* in the family of *Hymenochaetaceae*. Its fruiting body is called "Sanghuang" in Chinese, which has been used as a traditional Chinese medicine in China for a long time (Zeng, Wang, & Su, 2008). Recently, it has been reported that the extracts from the fruiting body of *P. baumii* Pilát possess antioxidant and free radical scavenging activities (Shon et al., 2003), as well as anti-inflammatory property (Chang et al., 2007; Jang et al., 2004). In addition, the anti-diabetic effect of crude exopolysaccharides (EPS) from submerged mycelial culture of *P. baumii* Pilát has been demonstrated by Hwang et al. (2005). As a result of its perceived health benefits, *P. baumii* Pilát has gained wide popularity as an effective medicine and has become one of the valuable mushrooms in China.

Up to now, polysaccharides, one of the main active ingredients of *P. baumii* Pilát, are mainly extracted from the fruiting body growing on the solid culture. However, the time for the growth of fruiting body is too long and its product quality is difficult to control when *P. baumii* Pilát is traditionally cultivated in solid culture. Therefore, it deserves investigation to produce EPS from *P. baumii* Pilát by submerged culture. As submerged culture provides a number of potential advantages, for instance a greater mycelial and EPS production in a more-compact space over a shorter time with a lower chance of contamination, availability of convenient control and easy downstream processing (Cho, Hwang, Kim, Song, & Yun, 2002; Lee et al., 2004; Park et al., 2002; Sinha et al., 2001). To achieve higher yield in a submerged culture, it is a prerequisite to design an optimal production medium and a set of optimal process operating conditions. Single factor at a time, the most widely used optimization method, does not account for the combined effects of all the influential factors since other factors are maintained arbitrarily at a constant level. In addition, it is time consuming and requires a large number of experiments to determine the optimum levels of the production medium. However, such drawback of the single factor optimization method can be

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doi:10.1016/j.carbpol.2009.04.038

overcome by statistical optimization techniques (Chen, Zhao, Chen, & Li, 2008). Factorial design and response surface techniques are important statistical optimization methods which many factors can be optimized simultaneously and much quantitative information can be extracted by only a few experimental trials (Houng, Hsu, Liu, & Wu, 2003). They have been successfully applied to the improvement of culture media or the production of primary and secondary metabolites in the cultivation process of many edible and medicinal mushrooms (Chang, Tsai, & Houng, 2006; Chen et al., 2008; Cui et al., 2006; Mao, Eksriwong, Chauvatcharin, & Zhong, 2005). However, there is still lack of knowledge concerning submerged culture conditions for EPS production from *P. baumii* Pilát by statistical optimization techniques.

Here, we report in detail the optimization of medium composition for production of EPS and the investigation of immuno-stimulating activity *in vitro* of EPS from *P. baumii* Pilát. An optimal medium composition was achieved by $2^{(7-3)}$ fractional factorial design (FFD) and central composite design (CCD). To the best of our knowledge, optimization of nutritional requirements for EPS production from *P. baumii* in submerged culture and its immuno-stimulating activity has not been demonstrated.

2. Materials and methods

2.1. Chemicals

Concanavalin A (ConA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lipopolysaccharide (LPS) were purchased from Sigma Chemical Co. (USA). Fetal bovine serum and RPMI-1640 media were obtained from Gibco (USA). All other reagents were of the highest available quality.

2.2. Mice

Male BALB/c mice and male ICR mice (7-week-old, body weight 20 ± 2 g), were obtained from the Experimental Animal Center of Academy of the Military Medical Sciences (Beijing, China). Mice were housed in an air-conditioned animal room with constant temperature (23 ± 0.5 °C) and humidity in a 12 h light/dark cycles, and fed laboratory chows and water *ad libitum*. Experiments were in strict accordance with the Chinese legislation on the use and care of laboratory animals.

2.3. Microorganism

Phellinus baumii Pilát used in this study was obtained from China Forestry Culture Collection Center (CFCC, Beijing, China). The strain *P. baumii* Pilát was maintained on potato dextrose agar (PDA) slant. The slant was incubated at 25 °C for 10 days, then stored at 4 °C, and sub-cultured every 2 months.

2.4. Inoculum preparation and flask cultures

The seed culture was grown in a 250 ml flask containing 50 ml of basal medium (glucose 20 g/l, peptone 2 g/l, yeast extract 1 g/l, KH_2PO_4 1 g/l, $MgSO_4$ 0.5 g/l, thiamine (VB_1) 0.01 g/l, distilled water, pH 6.5) at 28 °C on a rotary incubator at 150 rpm for 7 days. Flask culture experiments were performed in 500 ml flasks containing 100 ml medium after inoculating with 10 ml seed culture at 28 °C on a rotary shaker at 150 rpm for 6 days.

2.5. Determination of EPS yield

Samples collected at various culture conditions from shake flasks were centrifuged at 5000 rpm for 20 min. The resulting supernatant was filtered through a Whatman filter paper and condensed to one-third of its total volume with a rotary evaporator under reduced pressure at 50 °C. The resulting residual was mixed with three times volume of absolute ethanol, stirred vigorously and kept overnight at 4 °C. The precipitate was collected by centrifugation, washed twice with acetone and ether, respectively, and dried to afford crude EPS. The weight of EPS was estimated and expressed as g/l dry weight (DW).

2.6. Optimization procedure and experimental design

Statistical experimental designs were applied in two steps. The first step is to identify the significant nutrients for production of EPS using $2^{(7-3)}$ FFD, the second is to optimize the significant nutrients resulted from FFD by using a CCD in response surface methodology (RSM). The experimental design and statistical analysis of the data were done by using Stat-Ease Design-Expert 7.1.3 (Stat-Ease Corporation, USA).

2.6.1. Screening of factors significantly affecting EPS production

A $2^{(7-3)}$ FFD was employed to determine the key ingredients significantly affecting EPS production from *P. baumii* Pilát. Seven nutrient factors considered for the design were glucose, peptone, yeast extract, diammonium oxalate monohydrate, KH_2PO_4, $MgSO_4$ and thiamine (VB_1). Each factor was examined at a high level (coded +1) and a low level (coded −1). The centre points were the trials with the basal level conditions (coded 0). Table 1 shows the variables and levels in detail. A 1/8 fraction of the full factorial design was adopted and consequently the experiment included 16 (2^{7-3}) combinations plus four replicates at the centre point. Each trial was performed in duplicate and EPS production was measured after 144 h of fermentation.

2.6.2. Optimization of significant nutrients using central composite design

The medium components that significantly affected the production of EPS were optimized by CCD. The variables were coded according to Eq. (1):

$$x_i = (X_i - X_0)/\Delta X_i \quad (1)$$

where X_i, the real value of variable; X_0, the real value of the X_i at the center point; ΔX_i, step change in X_i; x_i, the coded value of the variable; $i = 1, 2, 3$. The experimental levels for these variables were selected from our preliminary work, which indicated that an optimum could be found within the level of parameters studied.

The response Y (EPS yield) was analyzed by using a second-order polynomial equation in three independent variables and the data were fitted into the equation by multiple regression procedure. The model equation for analysis is given below (2):

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

where β_0, β_i, β_{ii}, and β_{ij} represent the constant process effect in total, the linear, quadratic effect of X_i and the interaction effect between X_i and X_j on EPS production, respectively.

Table 1
The coded and real values of variables in $2^{(7-3)}$ fractional factorial design.

Variables (g/l)	Levels of variables		
	−1	0	+1
A: glucose	20	30	40
B: peptone	2	4	6
C: yeast extract	1	3	5
D: KH_2PO_4	0.5	1	1.5
E: $MgSO_4$	0.5	0.75	1
F: thiamine (VB_1)	0.005	0.0075	0.01
G: diammonium oxalate monohydrate	0.1	0.3	0.5

2.7. Evaluation of immuno-stimulating activity in vitro of EPS

2.7.1. Measurement of splenocyte proliferation

The assay of cell proliferation was manipulated as described by Mosmann (1983) using MTT-based colorimetric method. Briefly, mice were killed by cervical dislocation and spleens were removed aseptically. A single spleen cells suspension was prepared by homogenization in RPMI 1640 medium. The suspension was centrifuged to obtain cell pellet. The erythrocytes were lysed with lysing buffer (0.15 M NH_4Cl, 0.1 mM Na_2EDTA, 10 mM $KHCO_3$) for 5 min. After two times washing, the cells were resuspended in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 units/ml) and streptomycin (100 μg/ml) with a concentration of 1×10^7 cells/ml. The purity and viability of splenocytes (tested by Trypan blue dye exclusion) was always over 95%. The spleen cell suspension was pipetted into 96-well flat-bottom plate (50 μl/well) and treated with EPS in the presence or absence of mitogen, LPS (10 μg/ml) or Con A (2.5 μg/ml). After incubation at 37 °C in a 5% CO_2 incubator for 72 h, 10 μl of MTT solution was added and the plate was further incubated for 4 h. To each well, 100 μl of 10% SDS in 0.01 M HCl was added and kept overnight for the dissolution of formazan crystals. The absorbance of each well was then read at 570 nm using an ELISA plate reader (TECAN Infinite F200, Switzerland).

2.7.2. Measurement of acid phosphatase in murine macrophages

The activity of acid phosphatase in murine macrophages was determined according to the reported method by Suzuki et al. (1990). In brief, one milliliter of sterile 3% Brewer's thioglycollate medium was injected intraperitoneally into ICR male mice as a stimulant to elicit peritoneal macrophages. Three days later, peritoneal exudates cells were harvested by a lavage of the peritoneal cavity with 5 ml of ice-cold RPMI 1640 medium. The resulting cells were washed twice, and resuspended in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 unit/ml) and streptomycin (100 μg/ml). The cell number was counted using a hematocytometer, and cell viability was examined by the Trypan blue exclusion method. A 100 μl of the cell suspension (5×10^5 cells/well), was placed in a 96-well flat-bottom plate. The cells were allowed to adhere at 37 °C in a humidified 5% CO_2 incubator for 2 h. Non-adherent cells were removed by washing twice with RPMI 1640 medium. Then, fresh medium and test sample were added to each well and the plate was incubated with macrophages at 37 °C for 24 h. The culture medium was removed by rapid inversion and flicking of the plate. And the macrophage monolayer in each well was solubilized by addition of 1% Triton X-100 (25 μl). Thereafter, 150 μl freshly prepared *p*-nitrophenyl phosphate (1 mg/ml) in 0.1 M citrate buffer (pH 5.0) was added as a substrate for acid phosphatase, and the plate was incubated at 37 °C for 1 h. The reaction was stopped by addition 50 μl of 3 M NaOH solution, and the absorbance (Abs) of the culture wells was measured at 405 nm using an ELISA plate reader. The activity of acid phosphatase was calculated by the following equation:

$$\text{Acid phosphatase activity } (\%) = (\text{Abs}_{\text{sample}} - \text{Abs}_{\text{control}})/\text{Abs}_{\text{control}} \times 100\%$$

Table 2
Results of two-level fractional factorial design of exopolysaccharide production from *P. baumii* Pilát in shake flask culture.

Run	A	B	C	D	E	F	G	EPS (g/l)
1	−1	−1	−1	−1	−1	−1	−1	1.06 ± 0.15
2	+1	+1	−1	−1	−1	+1	+1	0.96 ± 0.08
3	−1	−1	−1	+1	−1	+1	+1	1.13 ± 0.27
4	0	0	0	0	0	0	0	1.31 ± 0.11
5	+1	−1	+1	+1	−1	−1	+1	0.91 ± 0.07
6	0	0	0	0	0	0	0	1.42 ± 0.06
7	+1	−1	−1	+1	+1	+1	−1	0.59 ± 0.07
8	+1	−1	−1	−1	+1	−1	+1	1.14 ± 0. 04
9	−1	−1	+1	−1	+1	+1	+1	1.32 ± 0.19
10	−1	+1	+1	+1	−1	+1	−1	2.38 ± 0.04
11	+1	+1	+1	+1	+1	+1	+1	1.33 ± 0.12
12	−1	+1	+1	−1	−1	−1	+1	1.27 ± 0.01
13	−1	−1	+1	+1	+1	−1	−1	2.14 ± 0.35
14	−1	+1	−1	+1	+1	−1	+1	0.88 ± 0.11
15	0	0	0	0	0	0	0	1.28 ± 0.16
16	−1	+1	−1	−1	+1	+1	−1	2.05 ± 0.03
17	0	0	0	0	0	0	0	1.60 ± 0.12
18	+1	+1	+1	−1	+1	−1	−1	0.80 ± 0.08
19	+1	−1	+1	−1	−1	+1	−1	1.47 ± 0.21
20	+1	+1	−1	+1	−1	−1	−1	1.72 ± 0.31

2.8. Statistical analysis

Unless otherwise stated, data were expressed as mean ± standard deviation (SD) and analyzed statistically by ANOVA method. *P*-values below 0.05 were regarded as statistically significant.

3. Results and discussion

3.1. Factors significantly affecting EPS production

The medium components have played an important role for EPS production in submerged culture. In order to find out the key ingredients significantly affecting the production of EPS, the relative significance of seven nutrition factors, glucose, peptone, yeast extract, diammonium oxalate monohydrate, KH_2PO_4, $MgSO_4$ and VB_1, were investigated by the 2^{7-3} FFD plus four central points. Table 2 shows the experimental design and the experimental results. Apparently, the EPS yields varied greatly in a range of 0.59 to 2.38 g/l with the different combinations of the medium components.

Statistical analysis of the responses and the main effects of the selected variables are shown in Table 3. In present study, the values of Model *F* and Model *p*-value were found to be 12.15 and 0.0314, respectively, which implied that the model was significant. The curvature *p*-value of 0.3594 implied that the curvature in the design space was not significant relative to the noise. Among the variables screened, the concentration of glucose, yeast extract and diammonium oxalate monohydrate were determined as the most significant variables influencing EPS production. Peptone, KH_2PO_4, $MgSO_4$ and VB_1 in EPS production did not result in significant variation at 5% level. From the *p*-values of each model term, it could be concluded that three interactive terms (AC, AG, and ABD) were also significant at 5% level. Thus, the three variables, glucose (X_1), yeast extract (X_2) and diammonium oxalate monohydrate (X_3), were selected and used for further optimization by RSM. Peptone, KH_2PO_4, $MgSO_4$ and VB_1 in the following CCD experiments were set at their middle levels of 4 g/l, 1 g/l, 0.75 g/l, and 0.0075 g/l, respectively.

3.2. Optimization of significant medium components using central composite design

CCD in RSM was employed to investigate the interactions among the significant factors (glucose, yeast extract and diammonium oxalate monohydrate) and determine the exact optimal values of the individual factors. The design matrix and the corresponding results of RSM experiments to determine the effects of three independent variables are shown in Table 4. Multiple regression analysis was used to analyze the data obtained as shown in Table 5, and a second-order polynomial equation was derived from regression analysis as follows:

Table 3
Statistical analysis of fractional factorial design for screening the significant variables.

Source	Sum of squares	DF	Mean square	*F* value	*p*-Value
Model	3.94	15	0.26	12.15	0.0314[a]
A	0.68	1	0.68	31.68	0.0111[a]
B	0.17	1	0.17	7.88	0.0675
C	0.27	1	0.27	12.60	0.0381[a]
D	0.063	1	0.063	2.92	0.1859
E	0.028	1	0.028	1.28	0.3406
F	0.10	1	0.10	4.81	0.1159
G	0.67	1	0.67	30.92	0.0115[a]
AB	3.460*E*-003	1	3.460*E*-003	0.16	0.7158
AC	0.22	1	0.22	10.16	0.0498[a]
AD	0.025	1	0.025	1.17	0.3592
AE	0.20	1	0.20	9.03	0.0575
AF	0.19	1	0.19	8.91	0.0584
AG	0.48	1	0.48	22.36	0.0179[a]
BD	0.13	1	0.13	5.98	0.0921
ABD	0.70	1	0.70	32.44	0.0107[a]
Curvature	0.021	1	0.021	0.98	0.3954
Pure error	0.065	3	0.022		
Cor Total	4.02	19			

R^2 (predict) = 98.38%; R^2 (adjust) = 90.28%.
[a] 5% significance level.

$$Y = -2.64 + 0.13X_1 + 0.54X_2 + 2.74X_3 + 1.04E-003X_1X_2 - 0.03X_1X_3 + 0.07X_2X_3 - 1.47E-003X_1^2 - 0.06X_2^2 - 1.14X_3^2 \quad (3)$$

In the equation, Y represents EPS production (g/l); X_1, X_2 and X_3 represent the concentrations of glucose, yeast extract and diammonium oxalate monohydrate, respectively.

The adequacy of the model was checked using analysis of variance (ANOVA) which was tested using Fisher's statistical analysis. The model *F* value of 7.19 (Table 5) implied the model was significant and also showed that there was only a 0.24% chance that the model *F* value could occur due to noise. The fitness of the model was examined by determination coefficient (R^2 = 0.8662), which indicated that the sample variation of more than 86% was attributed to the variables. In addition, value of lack of fit *F* value and lack of fit *p*-value were found to be 1.44 and 0.3507, respectively, which implied that the lack of fit was insignificant relative to the pure error due to noise. Insignificant lack of fit made the model fit. The results suggested that the proposed experimental design was suitable for the simulation of EPS production from *P. baumii* Pilát within the range of variables employed.

Table 5
Variance analysis of response surface quadratic model for exopolysaccharide production from *P. baumii* Pilát.

Source	Sum of squares	DF	Mean square	*F* value	*p*-Value
Model	2.60	9	0.29	7.19	0.0024[a]
X_1	0.24	1	0.24	5.89	0.0357[a]
X_2	0.36	1	0.36	8.93	0.0136[a]
X_3	0.24	1	0.24	6.07	0.0335[a]
X_1X_2	1.962*E*-003	1	1.962*E*-003	0.049	0.8296
X_1X_3	0.23	1	0.23	5.73	0.0378[a]
X_2X_3	0.019	1	0.019	0.48	0.5024
X_1^2	0.31	1	0.31	7.74	0.0194[a]
X_2^2	0.26	1	0.26	6.35	0.0303[a]
X_3^2	1.18	1	1.18	29.29	0.0003[a]
Residual	0.40	10	0.040		
Lack of Fit	0.24	5	0.047	1.44	0.3507
Pure error	0.17	5	0.033		
Cor Total	3.01	19			

R^2 (predict) = 0.8662; R^2 (adjust) = 0.7458.
[a] 5% significance level.

The interactions and optimal levels of the variables were determined by plotting the response surface curves. The shapes of the contour plots indicate whether the mutual interactions between the variables are significant or not. The circular contour plot of response surfaces suggests that the interaction is negligible between the corresponding variables, while an elliptical or saddle nature of the contour plot indicates that the interactions between the corresponding variables are significant (Muralidhar, Chirumamila, Marchant, & Nigam, 2001). The response surface curves were generated as shown in Fig. 1, which depicted the interactions between two variables by keeping the other variables at their zero levels for EPS production. These 3D plots and their respective contour plots provided a visual interpretation of the interaction between two factors and facilitated the location of optimum experimental conditions. From the Eq. (3), the optimal values of X_1, X_2 and X_3 were estimated to be 0.41, 0.34 and −0.23, respectively. Correspondingly, their actual values were 34.12, 5.01 and 0.88 g/l for glucose, yeast extract and diammonium oxalate monohydrate, respectively. The predicted maximum yield of EPS was 2.342 g/l under the optimum condition.

Table 4
Central composition design with three independent variables and the response values for the yield of exopolysaccharide from *P. baumii* Pilát in shake flask culture.

Run	Glucose (g/l)		Yeast extract (g/l)		Diammonium oxalate monohydrate (g/l)		EPS (g/l)
	x_1	Code x_1	x_2	Code x_2	x_3	Code x_3	
1	30	0	4.5	0	0.16	−1.68	1.66 ± 0.01
2	20	−1	6.0	+1	1.5	+1	1.92 ± 0.09
3	30	0	4.5	0	1.0	0	1.87 ± 0.21
4	20	−1	3.0	−1	1.5	+1	1.50 ± 0.32
5	30	0	1.98	−1.68	1.0	0	1.62 ± 0.05
6	30	0	4.5	0	1.0	0	2.24 ± 0.34
7	40	+1	3.0	−1	1.5	+1	1.15 ± 0.15
8	46.82	+1.68	4.5	0	1.0	0	2.23 ± 0.02
9	13.18	−1.68	4.5	0	1.0	0	1.32 ± 0.02
10	30	0	4.5	0	1.0	0	2.25 ± 0.11
11	40	+1	3.0	−1	0.5	−1	1.85 ± 0.28
12	30	0	7.02	+1.68	1.0	0	2.01 ± 0.22
13	20	−1	3.0	0	0.5	−1	1.44 ± 0.18
14	30	0	4.5	0	1.84	+1.68	1.10 ± 0.07
15	30	0	4.5	0	1.0	0	2.34 ± 0.38
16	30	0	4.5	0	1.0	0	2.35 ± 0.07
17	40	+1	6.0	+1	1.5	+1	1.71 ± 0.05
18	20	−1	6.0	+1	0.5	−1	1.74 ± 0.24
19	30	0	4.5	0	1.0	0	2.31 ± 0.11
20	40	+1	6.0	+1	0.5	−1	2.13 ± 0.26

3.3. Experimental validation of the optimized conditions

In order to confirm the model adequacy and the results from an analysis of the response surface, four additional experiments were repeated randomly under the optimal medium composition. As a result, the mean value of EPS yields was 2.363 ± 0.04 g/l, which was close to the theoretical predicted value (2.342 g/l). The excellent correlation between predicted and measured values verifies the model validation and existence of an optimal point, indicating that the model was adequate for obtaining the optimum value in the range of studied parameters.

3.4. Immuno-stimulating activities in vitro of EPS

The ability of lymphocytes to respond to mitogen reflects the immune potential of the organism (Singh, Haldar, & Rai 2006). Therefore, we investigated the effect of EPS from *P. baumii* Pilát on the splenocyte proliferative response. Fig. 2 shows the effects of different concentrations of EPS on splenocytes proliferation in the absence and presence of ConA or LPS. In the absence of mitogen, EPS at 100 μg/ml enhanced the proliferation with the highest splenocyte proliferation index (1.18 ± 0.09, $p < 0.05$) compared to the control. In the presence of Con A, which is commonly used as T cells mitogen (Chang et al. 2007), the EPS elicited an increase of splenocyte proliferation index by 1.13 ± 0.02 ($p < 0.05$), 1.46 ± 0.05 ($p < 0.01$) and 1.62 ± 0.05 ($p < 0.01$) at concentration of 25, 50 and 100 μg/ml, respectively. In the presence of LPS, which is commonly used as B cells mitogen (Chang et al., 2007), the EPS at 50 and 100 μg/ml enhanced the proliferation index by 1.1 ± 0.05 ($p < 0.05$) and 1.19 ± 0.04 ($p < 0.01$) to the control, respectively. These results indicated that EPS from *P. baumii* Pilát could enhance both the LPS-induced B cells and Con A-induced T cells proliferation.

Macrophages play a significant role in the host defense mechanism. Activated macrophages are considered as the pivotal immunocytes of host defense which inhibit or kill invading pathogen or cancer cells in immune response (Jeong, Jeong, Yang, & Song, 2006). Polysaccharides are good stimulators of macrophages owing to the presence of various receptors on the macrophage membrane (Moradali et al., 2007). In present study, the ability of EPS from *P. baumii* Pilát to stimulate acid phosphatase activity in peritoneal macrophages was investigated. We found that the activities of acid phosphatase in macrophages increased significantly by EPS in a dose-dependent manner (25–200 μg/ml, Fig. 3), compared to the control. Therefore, it was evident that the EPS from *P. baumii* Pilát strongly induced the activation of macrophage and possessed higher immunomodulatory potential.

4. Conclusions

Statistically based experimental designs proved to be effective tools to optimize the medium components for maximal EPS production under submerged culture. Using FFD and CCD, an optimized medium for EPS production from *P. baumii* Pilát was obtained, which was composed of 34.12 (g/l) glucose, 4 (g/l) peptone, 5.01 (g/l) yeast extract, 0.88 (g/l) diammonium oxalate monohydrate, 0.75 (g/l) $MgSO_4$, 1 (g/l) KH_2PO_4 and 0.0075 (g/l) VB_1. In the experimental validation of the optimized conditions, a mean EPS yield of 2.363 ± 0.04 g/l was obtained. In addition, EPS from *P. baumii* Pilát exhibited direct immuno-stimulating activity *in vitro* on splenocyte proliferative response and acid phosphatase activity in peritoneal macrophages in a dose-dependent manner. Further works on the isolation, purification, characterization and functional effects of EPS from *P. baumii* Pilát is in progress.

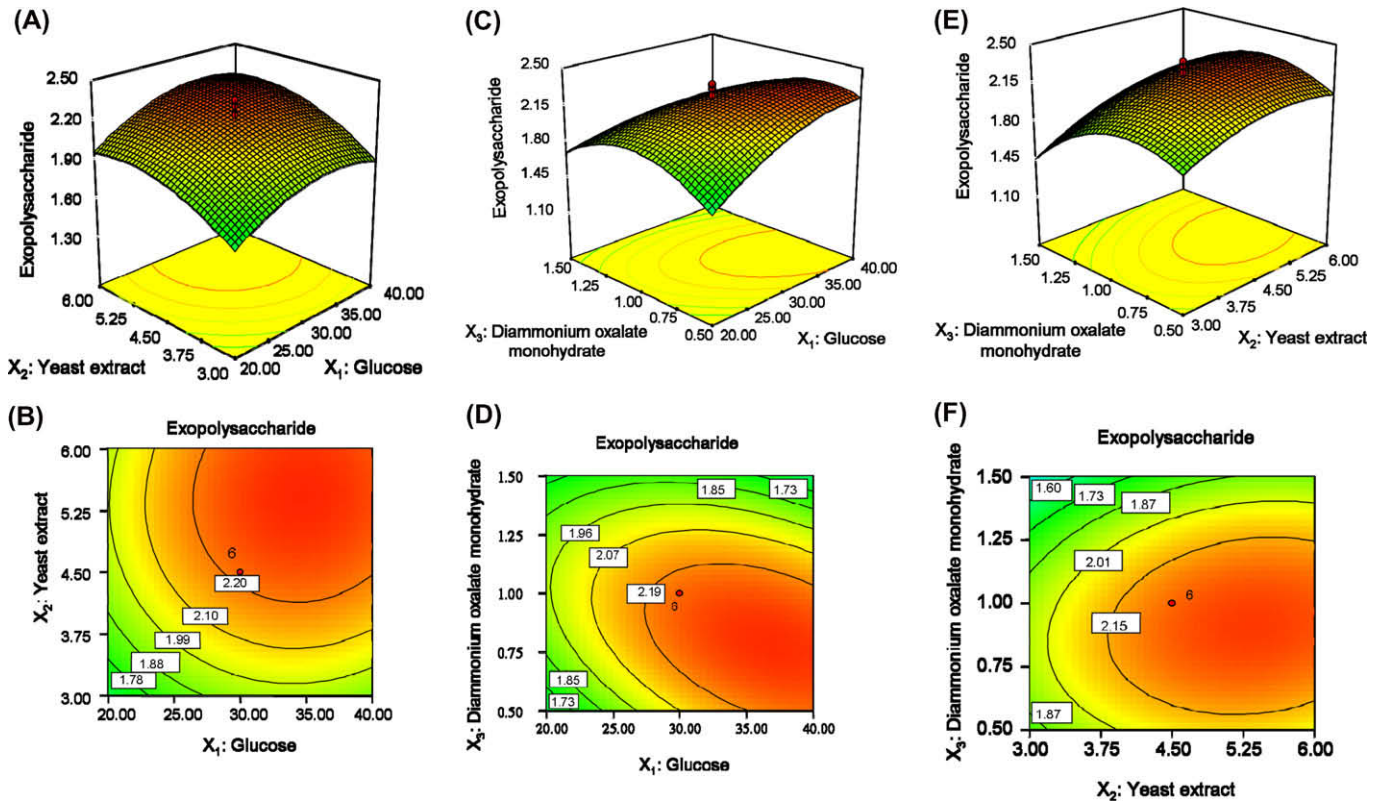

Fig. 1. Response surface plots (A, C and E) and contour plots (B, D and F) showing the effects of glucose, yeast extract and diammonium oxalate monohydrate on the yield of exopolysaccharide.

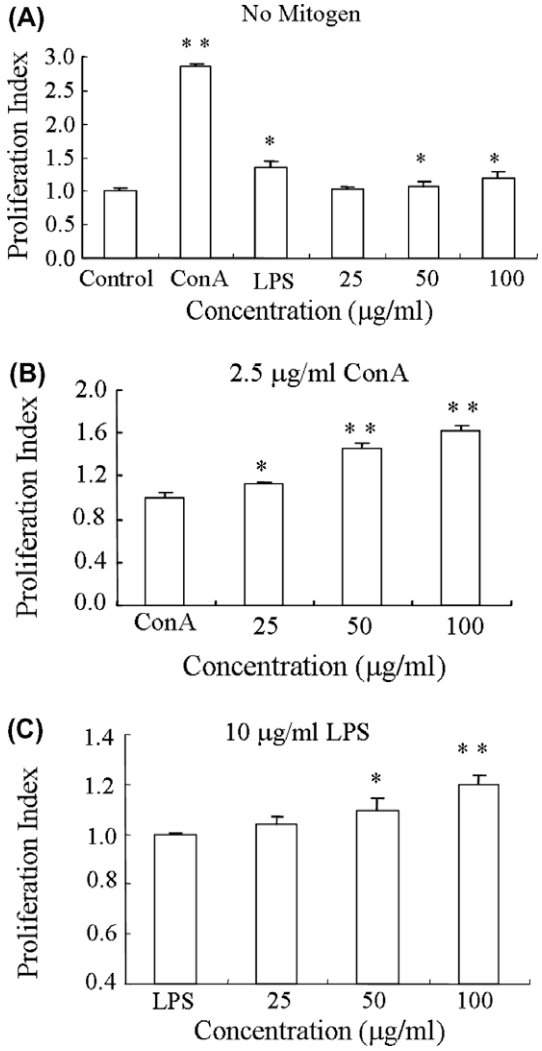

Fig. 2. Effects of exopolysaccharide (EPS) from *P. baumii* Pilát on murine splenocyte proliferation *in vitro* without or with mitogen (2.5 μg/ml ConA or 10 μg/ml LPS). (A) EPS without mitogen, (B) EPS with ConA, (C) EPS with LPS. Each value represents the mean ± SD of triplicates, comparing to the control or mitogen alone: $^{*}p < 0.05$, $^{**}p < 0.01$.

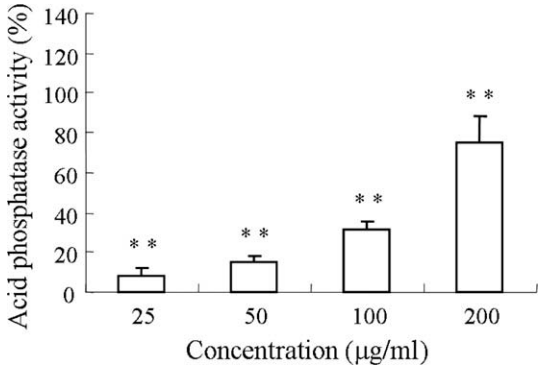

Fig. 3. Effect of exopolysaccharide from *P. baumii* Pilát on acid phosphatase activity in peritoneal macrophages. Each value is expressed as the mean ± SD, significantly different from the control: $^{*}p < 0.05$, $^{**}p < 0.01$.

Acknowledgments

This work was partly supported by a grant-in-aid for scientific research from the National Natural Science Foundation of China (No. 30870547) and a grant-in-aid from Nanjing Agricultural University for the Introduction of Outstanding Scholars (804066).

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