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Isolation of non-starch polysaccharides from bulb of tiger lily (*Lilium lancifolium* Thunb.) with fermentation of *Saccharomyces cerevisiae*

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ABSTRACT

This study involved the development of the method for removing proteins impurities from lily non-starch polysaccharides (NSP) with fermentation of *Saccharomyces cerevisiae*, which was different from Sevag and trichloroacetic acid methods. We optimized the extraction conditions of NSP; meanwhile, the optimal deproteinization conditions were determined. Firstly, a Plackett–Burman design was utilized to evaluate the effects of variables on the NSP yield and the ratio of protein removed (RPR). Lily bulb powder, temperature, pH and KH_2PO_4 exerted significant effects on NSP yield ($P < 0.05$), whereas lily bulb powder and pH had significant effects ($P < 0.01$) on RPR. Subsequently, these four factors were optimized using response surface analysis. The analysis revealed that the optimum conditions were 60.0 g/L lily bulb powder, 1.50 g/L KH_2PO_4, pH4.8 and temperature 26.9 °C. In the verification experiments, the experimental NSP yield of 8.81 ± 0.18% and RPR of 91.71 ± 0.08% perfectly matched with the predicted values (8.99% for NSP yield and 91.70% for RPR), which verified the practicability of this optimum strategy.

1. Introduction

Tiger lily, *Lilium lancifolium* Thunb. (*L. lancifolium*), belongs to the genus Lilium of the family Liliaceae, is widely distributed in China and cultivated in Taihu Lake Basin. Lily has been used in traditional Chinese medicine for many centuries, as its curative effects on tuberculosis, pertussis, chronic bronchitis, chronic gastritis, etc. Bulb of lily is not only a good source of nutrient substances including starch, protein and dietary fiber (Mullin, Peacock, Loewen, & Turner, 1997; Shin, Chakrabarty, & Paek, 2002), but also contains a variety of bioactive substances, such as polysaccharides, saponin, and colchicine (Satou, Mimaki, Kuroda, Sashida, & Hatakeyama, 1996). Wozniewski, Blaschek, and Franz (1989) reported that *Lilium testaceum* bulbs contained nearly 20% (dry weight) of a partially acetylated high molecular weight (MW = 230 kDa) β-1,4-glucomannan with a Man:Glu ratio of 7:3. Lily polysaccharides LP_1 (Man:Glu = 2.46:1; MW = 79.4 kDa) and LP_2 (MW = 18,150 Da) consisted of Glue:Man:Ara:GalA with a rate of 1:0.73:2.6:1.8:0.84 were shown to possess remarkable effects on lowering blood glucose levels in diabetic mice induced by alloxan (Liu, Fu, Tu, & Wan, 2002). The α-glucan from *Lilium brownii* bulbs with a molecular weight of 30,200 Da significantly inhibited the melanoma B16 cancer cell line and Lewis lung cancer line in mice (Zhao, Li, & Chen, 2002). Other studies demonstrated that lily polysaccharides had strong antioxidant activity and ability to regulate immune (Hu, Cai, Zhang, & Zhang, 2007; Li et al., 2008).

The bulb of tiger lily contains not only high content of starch (137 g/kg in fresh bulb) but also a considerable amount of proteins, 40.5 g/kg in fresh bulb. Because proteins impurities are similar with polysaccharide in solubility, the polysaccharides cannot be separated from proteins through extraction and precipitation with ethanol. Many methods have been carried out to remove the protein impurities, including Sevag method, trichloroacetic acid (TCA) method and enzymolysis. Sevag method is complicated and time-consuming, whereas TCA method involves violent reaction, which leads to degradation of polysaccharides. Compared to the methods mentioned, enzymolysis is the method with mild reaction conditions and relatively easy operation, which has been utilized for deproteinization of polysaccharides from fruit calyx of *Physalis alkekengi* var. *francheti* (Ge, Duan, Fang, Zhang, & Wang, 2009), brown alga *Sargassum pallidum* (Ye, Wang, Zhou, Liu, & Zeng, 2008) and *Hizikia fusiformis* (Choi, Hwang, Kim, & Nam 2009), *Lycium barbarum* Linnaeus (Lin, Wang, Chang, Stephen Inbaraj, & Chen 2009) and *Salicornia herbacea* (Im, Kim, & Lee, 2006), etc. Wang, Chang, and Chen (2009) reported that about 96.7% protein was removed by proteinase, which was much greater than that of Sevag method as only 19.41–21.99% of protein was removed.

Proteases used to hydrolyze proteins in crude polysaccharides are obtained through fermentation of microorganisms. Published data have demonstrated that yeast cells which subjected to stresses, such as oxidation and infiltration, not only synthesize intracellular stress proteins, but also secrete proteases or extracellular

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proteins as a kind of signal vector (Leisegang & Stahl, 2005; Lu, Wang, Bai, & Du, 2005; Mercier-Bonin, Ouazzani, Schmitz, & Lorthois, 2004). Cooper, Stewart, and Bryce (2000) reported that the higher levels of yeast extracellular proteinase were observed in the high gravity (20°P) fermentation because of the increased stresses on the yeast due to increased osmotic pressure and ethanol. Kondo et al. (1999) found that fermentation under low nitrogen conditions, especially in conjunction with low amino acid content, resulted in more proteases being excreted from the yeast cells than when the medium was rich in nitrogen. The kind of nitrogen source also affected the secretion of proteases. Yeast grown with casein as sole nitrogen source secreted proteolytic enzymes to a small extent into the medium, while with ammonium sulphate and casein hydrolysate there was no comparable secretion (Maddox & Hough, 1970).

The objective of this work was to isolate non-starch polysaccharides (NSP) by removal of starch with amylolytic enzymes and proteins impurities with extracellular protease secreted by *Saccharomyces cerevisiae*. The major nutrients and culture conditions for NSP yield and ratio of protein removed (RPR) were selected and optimized using statistical experiment design and response surface methodology (RSM).

2. Materials and methods

2.1. Microorganism and materials

The strain *Saccharomyces cerevisiae* (*S. cerevisiae*) was generously supplied by the Centre of Biological Technology in Nanjing Agriculture University, China. The fresh bulb of *L. lancifolium* Thunb. was purchased from Yixing city, Jiangsu Province, China. After cleaned and dried at 55 °C, lily bulb was pulverized into particles of approximately 0.45 mm by a disintegrator (FSD-100A, Taizhou city, Zhejiang Province, China). Heat-stable α-amylase (10^3 U/ml) from *Bacillus licheniformis* was provided by Jiangyin BSDZYME Bio-Engineering Co., Ltd. (Jiangsu Province, China), whose optimum temperature and pH are 95 °C and 6.0. The optimum temperature and pH for *Aspergillus niger* glucoamylase (10^3 IU/g), provided by Aidun Biology Engineering Co., Ltd. (Jiangsu Province, China), are 60 °C and 5.0, respectively. 1 U of α-amylase corresponds to the amount of enzyme which hydrolyzes 1 mg soluble starch per minute at pH 6.0 and 70 °C, while 1 IU of glucoamylase corresponds to the amount of enzyme which liberates 1 μmol of glucose per minute at pH 5.0 and 60 °C. All other reagents were of grade AR.

2.2. Maintenance and inoculum preparation

The strain *S. cerevisiae* was maintained on Potato Dextrose agar (PDA) slants, and sub-cultured once in a month and stored at 4 °C. One loopful of culture from the slant was transferred aseptically into an Erlenmeyer flash (250 ml) containing 100 ml of malt extract medium, whose soluble solid was adjusted to 10.0 °Brix, and incubated for 24 h on a rotary shaker operating at 160 rpm at 28 °C. The seed, whose cell count was 3.5×10^9/ml, was prepared by transferring 10 ml of 24 h culture to 100 ml of malt extract medium and incubating for 10 h on a rotary shaker operating at 160 rpm at 28 °C.

2.3. Pretreatment of lily bulb powder

Lily bulb powder preparations whose starch removed by amylolytic enzymes was prepared according to the method as described by Lai, Lu, He, and Chen (2006) with some modifications. Lily bulb powder was mixed with distilled water in an Erlenmeyer flash (250 ml) and gelatinized at 65 °C for 2 h. The mixture was then hydrolyzed with α-amylase of 50 U/g lily bulb powder at 95 °C for 15 min, followed by successively cooling to 60 °C, saccharification with glucoamylase of 100 IU/g lily bulb powder at 60 °C for 1 h. The starch-iodine assay (Iagher, Reicher, & Ganter, 2002) and the dinitrosalicylic acid (DNS) assay (Toma & Leung, 1987) were utilized to ensure that the removal of starch was complete. The concentration of lily bulb powder and the volume of distilled water were varied according to the detailed arrangement of the experiment design (Tables 1 and 2).

2.4. Medium and culture conditions

Nutrients were added into the pretreated lily bulb powder based on the experimental design. The mixture whose pH was adjusted to the designed value by adding 1 M hydrogen chloride solution or 1 M sodium hydroxide solution was sterilized at 121 °C for 20 min and then used as the fermentation medium.

The fermentation medium inoculated with 10 h inoculum was incubated on a rotary shaker operating at 160 rpm for 24 h, and then under static condition for 24 h. The temperature and the inoculum were varied based on the experimental design.

2.5. Analytical methods

2.5.1. Determination of NSP yield

After cultivation specified for each set of experiments, the culture broth was centrifuged at 2000*g* for 15 min. The supernatant, which was adjusted to 200 ml with distilled water, was centrifuged at 6000*g* for 15 min, and then precipitated with four volumes of ethanol at 4 °C overnight. The precipitates collected by centrifugation (2000*g* for 15 min) were washed with 80% ethanol and centrifuged at 2000*g* for 15 min for three times to remove adherent reducing sugars, and then solubilized in distilled water (50 ± 1 °C) to obtain the polysaccharides. The content of NSP was determined by the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) using glucose as a standard.

$$\text{NSP yield } (\%) = \text{polysaccharides weight (g)/dried raw material weight (g)} \times 100\%$$

2.5.2. Determination of RPR

After cultivation, the supernatant obtained by centrifugation (2000*g* for 15 min) of the culture broth was adjusted to 200 ml with distilled water and then centrifuged at 6000*g* for 15 min for protein determination. The concentration of total proteins was determined by the Bradford method (Bradford, 1976), using bovine serum albumin as the standard. The control was the pretreated lily bulb powder which sterilized at 121 °C for 20 min.

$$\text{RPR } (\%) = (1 - \text{protein content of culture broth (mg/ml)/protein content of control (mg/ml)}) \times 100\%$$

2.6. Experimental design and statistical analysis

2.6.1. Selection of significant variables by Plackett–Burman design

The Plackett–Burman (P–B) design, an effective technique for culture condition optimization (Bule & Singhal, 2009; Gao et al., 2009), was used to select factors that significantly influenced NSP yield and RPR. Based on P–B design, each independent variable was tested at two levels, high and low, which are denoted by (+) and (−), respectively. The experimental design with the name, symbol code, and actual level of the variables is shown in Table 1.

Two dummy variables were studied in 12 experiments to calculate the standard error. NSP yield and RPR were carried out in trip-

Table 1
P–B experimental design for evaluating factors influencing NSP yield and RPR.

Run	Variable levels											NSP yield[a] (%)	RPR[b] (%)
	A Lily bulb powder (g/L)	*B* Temperature (°C)	*C* Inoculum (%)	*D* pH	*E* Culture volume (ml)	*F* $MgSO_4{\cdot}7H_2O$ (g/L)	*G* KH_2PO_4 (g/L)	*H* $CaCl_2$ (g/L)	*J* VB_1 (mg/L)	*K* Dummy variable	*L* Dummy variable		
1	−1 (80)	−1 (20)	1 (10)	−1 (4.5)	1 (100)	1 (0.5)	−1 (0)	1 (0.1)	1 (5)	1	−1	7.56	89.6
2	−1 (80)	−1 (20)	−1 (5)	1 (6.0)	−1 (50)	1 (0.5)	1 (4.5)	−1(0)	1 (5)	1	1	7.35	86.2
3	1 (120)	−1 (20)	−1 (5)	−1 (4.5)	1 (100)	−1 (0)	1 (4.5)	1 (0.1)	−1 (0)	1	1	7.01	85
4	1 (120)	1 (28)	−1 (5)	−1 (4.5)	−1 (50)	1 (0.5)	−1 (0)	1 (0.1)	1 (5)	−1	1	7.1	86.1
5	1 (120)	−1 (20)	1 (10)	1 (6.0)	1 (100)	−1 (0)	−1 (0)	−1(0)	1 (5)	−1	1	6.56	79.8
6	−1 (80)	1 (28)	−1 (5)	1 (6.0)	1 (100)	−1 (0)	1 (4.5)	1 (0.1)	1 (5)	−1	−1	8.06	86
7	1 (120)	1 (28)	−1 (5)	1 (6.0)	1 (100)	1 (0.5)	−1 (0)	−1(0)	−1 (0)	1	−1	6.75	84.7
8	1 (120)	−1 (20)	1 (10)	1 (6.0)	−1 (50)	1 (0.5)	1 (4.5)	1 (0.1)	−1 (0)	−1	−1	6.54	81.1
9	−1 (80)	1 (28)	1 (10)	−1 (4.5)	1 (100)	1 (0.5)	1 (4.5)	−1(0)	−1 (0)	−1	1	8.12	88.3
10	−1 (80)	1 (28)	1 (10)	1 (6.0)	−1 (50)	−1 (0)	−1 (0)	1 (0.1)	−1 (0)	1	1	7.55	88.1
11	1 (120)	1 (28)	1 (10)	−1 (4.5)	−1 (50)	−1 (0)	1 (4.5)	−1(0)	1 (5)	1	−1	7.2	85.5
12	−1 (80)	−1 (20)	−1 (5)	−1 (4.5)	−1 (50)	−1 (0)	−1 (0)	−1(0)	−1 (0)	−1	−1	7.65	89.6

[a] NSP yield represented for average non-starch polysaccharides yield of triplicate experiments.
[b] RPR represented for average ratio of protein removed of triplicate experiments.

licate and the average values were taken as the response. The factors significant at 95% level ($P < 0.05$) were considered to have significant effect on NSP yield and RPR and thus used for further optimization by RSM.

2.6.2. Optimization by RSM

The significant variables identified from the screening experiment were optimized using central composite design (CCD) (for the experiments) and RSM (for identifying the optimal levels) (Guo et al., 2009), while the culture volume was fixed at the high level (100 ml) and the other culture conditions which had been tested as non-significant were fixed at the low level. Table 2 represents the design matrix and the corresponding experimental data.

The analysis of variance (ANOVA) for the experimental data and the model coefficients were analyzed with the "Design Expert" software (Version 7.0.0 Trial, Stat-Ease Inc., USA). In addition, two-dimensional contour plots were constructed for visual observation of the trend of maximum responses and the interactive effects of the significant variables on the responses.

3. Results and analysis

3.1. Screening of significant variables using P–B design

Tables 3 and 4 show the ANOVA for the experimental responses, NSP yield and RPR, respectively. Among the nine variables tested, four factors, namely, lily bulb powder, temperature, pH and KH_2PO_4 had significant effects ($P < 0.05$) on NSP yield, while lily bulb powder and pH had very significant effects ($P < 0.01$) on RPR. Therefore, these four factors were chosen and tested in the following optimization experiments.

3.2. Optimization of significant variables using CCD

The optimal levels of the significant factors (lily bulb powder, pH, temperature and KH_2PO_4) and their interaction effects on NSP yield and RPR were further explored by the CCD of RSM. By applying multiple regression analysis, the following two second-order polynomial equations were established to explain NSP yield (Eq. (1)) and RPR (Eq. (2)), respectively:

Table 2
The CCD matrix of independent variables with corresponding experimental value.

Run	*A* Lily bulb powder (g/L)	*B* pH	*C* Temperature (°C)	*D* KH_2PO_4 (g/L)	NSP yield[a] (%)	RPR[b] (%)
1	0 (100)	0 (5)	−2 (20)	0 (4.5)	7.46 ± 0.05	87.5 ± 0.38
2	0 (100)	0 (5)	0 (28)	0 (4.5)	7.68 ± 0.02	87.38 ± 0.57
3	0 (100)	−2 (3)	0 (28)	0 (4.5)	9.16 ± 0.18	62.93 ± 0.32
4	2 (140)	0 (5)	0 (28)	0 (4.5)	7.07 ± 0.02	84.38 ± 0.43
5	−1 (80)	−1 (4)	−1 (24)	−1 (3)	8.34 ± 0.25	84.45 ± 0.31
6	0 (100)	0 (5)	0 (28)	0 (4.5)	7.51 ± 0.07	86.93 ± 0.00
7	0 (100)	0 (5)	0 (28)	2 (7.5)	7.68 ± 0.18	87.19 ± 0.64
8	−1 (80)	−1 (4)	1 (32)	−1 (3)	8.55 ± 0.39	83.65 ± 0.18
9	0 (100)	0 (5)	0 (28)	0 (4.5)	7.53 ± 0.11	87.66 ± 0.61
10	−1 (80)	1 (6)	−1 (24)	1 (6)	7.97 ± 0.15	86.51 ± 0.26
11	−2 (60)	0 (5)	0 (28)	0 (4.5)	8.52 ± 0.16	91.29 ± 0.25
12	0 (100)	0 (5)	0 (28)	0 (4.5)	7.55 ± 0.16	87.68 ± 0.76
13	0 (100)	0 (5)	0 (28)	0 (4.5)	7.57 ± 0.22	88.75 ± 0.07
14	0 (100)	2 (7)	0 (28)	0 (4.5)	7.81 ± 0.28	71.97 ± 0.66
15	1 (120)	−1 (4)	1 (32)	1 (6)	8.27 ± 0.13	75.25 ± 0.07
16	−1 (80)	1 (6)	1 (32)	1 (6)	8.05 ± 0.07	85.72 ± 0.42
17	1 (120)	1 (6)	−1 (24)	−1 (3)	6.71 ± 0.05	82.66 ± 0.26
18	0 (100)	0 (5)	2 (36)	0 (4.5)	7.62 ± 0.1	82.01 ± 0.29
19	1 (120)	−1 (4)	−1 (24)	1 (6)	8.17 ± 0.12	77.16 ± 0.09
20	0 (100)	0 (5)	0 (28)	−2 (1.5)	7.5 ± 0.12	87.4 ± 0.3
21	1 (120)	1 (6)	1 (32)	−1 (3)	6.76 ± 0.09	81.45 ± 0.51

[a] NSP yield represented for average non-starch polysaccharides yield of triplicate experiments.
[b] RPR represented for average ratio of protein removed of triplicate experiments.

Table 3
ANOVA for NSP yield from P–B screening test.

Source	Sum of squares	df	Mean square	*F* value	*P* value
Model	2.95	4	0.74	43.25	<0.0001
A – Lily bulb powder	2.19	1	2.19	128.80	<0.0001
B – Temperature	0.37	1	0.37	21.79	0.0023
D – pH	0.28	1	0.28	16.39	0.0049
G – KH_2PO_4	0.10	1	0.10	6.03	0.0437
Residual	0.12	7	0.017		
Cor. Total	3.07	11			

R-squared = 0.9611; Adj *R*-squared = 0.9389.

Table 4
ANOVA for RPR from P–B screening test.

Source	Sum of squares	df	Mean square	*F* value	*P* value
Model	82.22	2	41.11	19.99	0.0005
A – Lily bulb powder	54.61	1	54.61	26.55	0.0006
D – pH	27.60	1	27.60	13.42	0.0052
Residual	18.51	9	2.06		
Cor. Total	100.73	11			

R-squared = 0.8162; Adj *R*-squared = 0.7754.

$$Y_1 = 14.4935 - 0.0139A - 1.5452B + 0.0119C - 0.445D - 0.0109AB + 0.0048AD + 0.00014A^2 + 0.2295B^2 \quad (1)$$

$$Y_2 = -26.0592 - 0.3098A + 44.1576B + 2.2016C - 4.1975D + 0.0447AB + 0.8325BD - 5.0253B^2 - 0.0437C^2 \quad (2)$$

where Y_1 is the predicted yield of NSP and Y_2 is the predicted RPR; *A*, *B*, *C* and *D* are lily bulb powder, pH, temperature and KH_2PO_4, respectively.

The ANOVA for the CCD experiments gave relatively high *F* values (360.65 for NSP yield and 235.54 for RPR), very low probability values (<0.0001 for both NSP yield and RPR), and fairly large coefficients of determination (R^2 = 0.9959 for NSP yield and 0.9937 for RPR) as shown in Tables 5 and 6. These indicated a good agreement between experimental and predicted values and implied that the mathematical model was very reliable for NSP yield and RPR in the present study.

The *P*-value is used as a tool to check significance of each variable, which also indicates the interaction strength between each independent variable. As shown in Tables 5 and 6, the linear effects of lily bulb powder (*A*), pH (*B*) and temperature (*C*) were significant ($P < 0.0001$) for both NSP yield and RPR, while KH_2PO_4 (*D*) was

Table 5
ANOVA for NSP yield from CCD experiment.

Source	Sum of squares	df	Mean square	*F* value	*P* value
Model	6.98	8	0.87	360.65	<0.0001
A – Lily bulb powder	2.18	1	2.18	899.38	<0.0001
B – pH	0.91	1	0.91	376.70	<0.0001
C – Temperature	0.036	1	0.036	14.92	0.0023
D – KH_2PO_4	0.016	1	0.016	6.70	0.0238
AB	0.19	1	0.19	78.22	<0.0001
AD	0.081	1	0.081	33.58	<0.0001
A^2	0.088	1	0.088	36.52	<0.0001
B^2	1.43	1	1.43	591.84	<0.0001
Residual	0.029	12	0.002		
Cor. Total	7.01	20			

R-squared = 0.9959; Adj *R*-squared = 0.9931.

Table 6
ANOVA for RPR from CCD experiment.

Source	Sum of squares	df	Mean square	*F* value	*P* value
Model	875.62	8	109.45	235.54	<0.0001
A – Lily bulb powder	23.87	1	23.87	51.38	<0.0001
B – pH	71.87	1	71.87	154.66	<0.0001
C – Temperature	15.39	1	15.39	33.11	<0.0001
D – KH_2PO_4	0.022	1	0.022	0.047	0.8312
AB	3.20	1	3.20	6.88	0.0223
BD	6.24	1	6.24	13.42	0.0032
B^2	686.35	1	686.35	1477.03	<0.0001
C^2	13.28	1	13.28	28.58	0.0002
Residual	5.58	12	0.46		
Cor. Total	881.20	20			

R-squared = 0.9937; Adj *R*-squared = 0.989.

significant ($P < 0.05$) for NSP yield. Furthermore, the square terms of lily bulb powder (A^2) and pH (B^2) played significant roles in the higher NSP yield ($P < 0.0001$), whereas B^2 and C^2 were significant at the level of $P < 0.01$ for RPR. The interactive terms between lily bulb powder and pH (*AB*) and lily bulb powder and KH_2PO_4 (*AD*) were highly significant model terms for NSP yield ($P < 0.0001$), while *AB* ($P < 0.05$) and *BD* ($P < 0.01$) were significant for RPR.

By Eqs. (1) and (2), the quadratic models predicted that the maximum yield of NSP was 8.99% and the maximum RPR was 91.70%, when the medium consisted of 60.0 g/L lily bulb powder and 1.50 g/L KH_2PO_4, and its initial pH and the temperature were 4.8 and 26.9 °C.

Figs. 1 and 2 showed the response surface plots for the present study and depicted the pair-wise interaction of the four variables which had a significant effect ($P < 0.05$) on NSP yield or RPR, while maintaining the other variables at their zero levels.

As shown in Fig. 1a, NSP yield decreased rapidly with the increase of the concentration of lily bulb powder when the pH value was higher than 4, and the influence of the concentration of lily bulb powder on NSP yield became slighter with reducing the pH value. When the concentration of lily bulb powder was higher than 100 g/L, the fluctuation of NSP yield caused by the pH value was similar with the trend that induced by the concentration of lily bulb powder. At the lowest level of lily bulb powder, NSP yield decreased firstly and then rose with pH increasing.

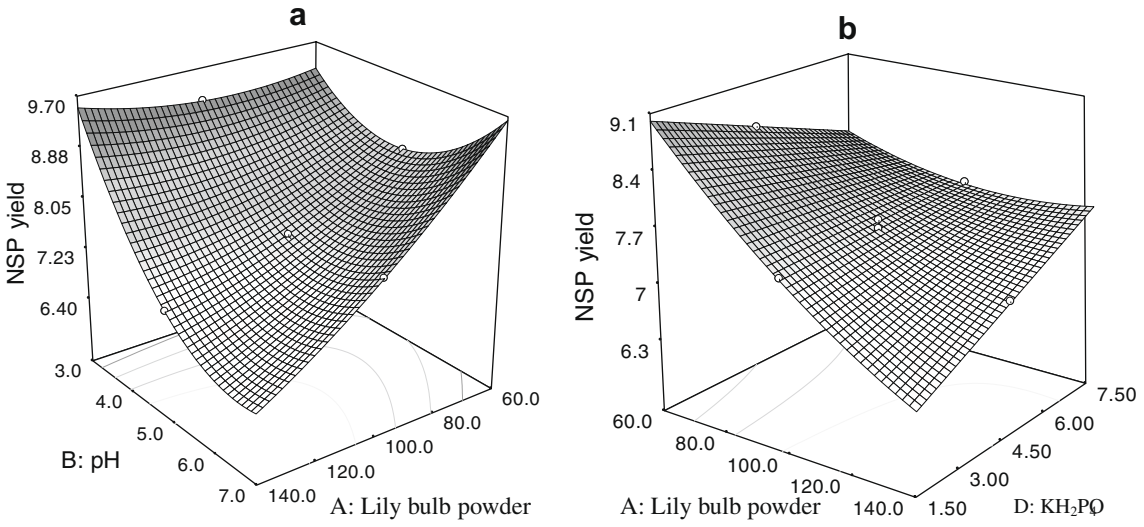
The effect of the concentration of lily bulb powder and KH_2PO_4 on NSP yield was shown in Fig. 1b. It was clear that the augment tendency of NSP yield with the decreasing concentration of lily bulb powder became slower with the addition of KH_2PO_4. The yield of NSP was found to increase with the elevation of KH_2PO_4 when the concentration of lily bulb powder was high, while it showed a downward trend when the concentration of lily bulb powder was low.

Fig. 2a showed the interaction effect of the concentration of lily bulb powder with pH on RPR. With pH increasing the RPR rose firstly and then decreased, while it showed an augment trend with the decrease of the concentration of lily bulb powder. And it was found that the fluctuation of RPR caused by the variation of concentration of lily bulb powder was slighter with the increase of pH.

The effects of pH and KH_2PO_4 on the RPR were shown in Fig. 2b. It was evident that the RPR increased with the elevation of KH_2PO_4 when the pH was higher than 5.0, while with lower pH it showed a downward trend.

3.3. Verification experiments

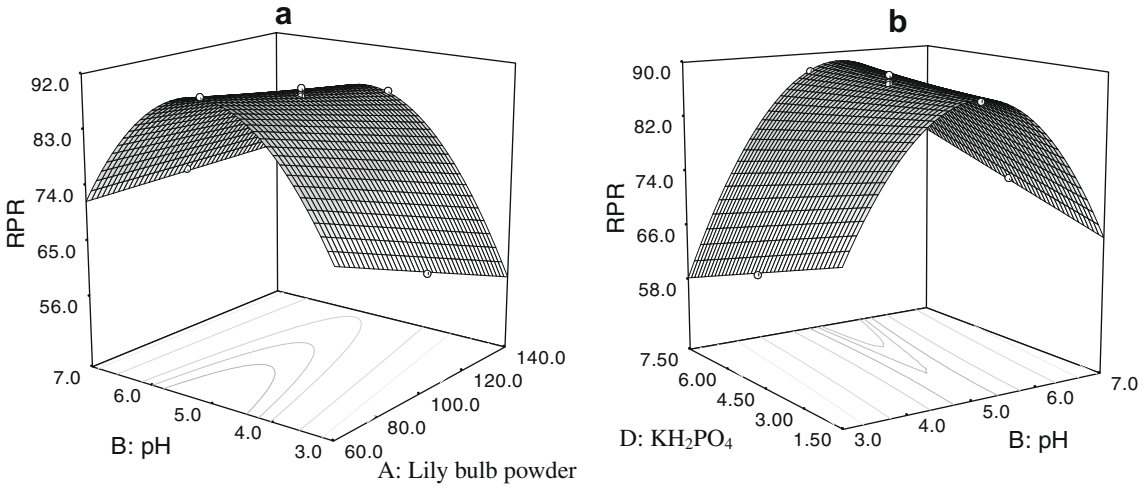
According to the results of the statistical design, the optimized medium was prepared as follows: 60.0 g/L lily bulb powder and



Fixed levels: C Temperature =28 °C, D KH_2PO_4 =4.5 g/L

Fixed levels: B pH=5.0, C Temperature =28 °C

Fig. 1. The response surface plots and the corresponding contour plots showing the effects of variables on NSP yield.



Fixed levels: C Temperature =28 °C, D KH_2PO_4 =4.5 g/L

Fixed levels: A Lily bulb powder=100.0 g/L, C Temperature =28 °C

Fig. 2. The response surface plots and the corresponding contour plots showing the effects of variables on RPR.

1.50 g/L KH_2PO_4. The initial pH and the temperature were 4.8 and 26.9 °C, respectively. Under the above optimized condition, the maximum yield of NSP and the maximum RPR were 8.81 ± 0.18% and 91.71 ± 0.08%, respectively, suggesting that experimental and predicted values (8.99% for NSP yield and 91.70% for RPR) were in good agreement.

4. Discussion

When the volume of distilled water is constant, the ratio of solvent to raw material is determined by the concentration of lily bulb powder. Ratio of solvent to raw material had important effect on extraction yield of polysaccharides (Liang, 2008; Qiao et al., 2009). The lower the concentration of lily bulb powder, the higher the ratio of solvent to raw material, which could increase solution concentration difference inside and outside plant cells, and consequently prompted diffusion rate of solute particles and made more polysaccharides molecules enter solution to get higher NSP yield. As shown in Table 6, the concentration of lily bulb powder also had significantly effect on RPR. Kondo et al. (1999) reported that fermentation under low nitrogen conditions, especially in conjunction with low amino acid content, resulted in more protease being excreted from the yeast cells than when the medium was rich in nitrogen, and protease activity increased dramatically after nutrient assimilation by the yeast cells. Bulb of lily provided not only a mixture of carbon energy sources but also nitrogenous components in this study. When the concentration of nitrogen source

and other nutrients decreased with the descending concentration of lily bulb powder, *S. cerevisiae* secreted more protease which hydrolyzed proteins in medium.

Acid and alkali could promote the release of intracellular polysaccharides by hydrolyzing cellulose and pectin of cell walls, but also degrade polysaccharides in extract. The fluctuation of NSP yield caused by the pH value represented the difference between the release and degradation of NSP. The RPR rose firstly and then decreased with pH increasing, and the maximum was obtained when the pH was approximately 5.0–5.5, which inconsistent with that for proteolytic activity reported by others, such as pH 4.0 (Maddox & Hough, 1970), pH 2.4 (Magni, Natalini, Santarelli, & Vita, 1982) and pH 4.3 (Kondo et al., 1999). The reason is that pH not only affected protease activity, but also influenced the vitality of yeast and the secretion of protease (Kondo et al., 1999). In addition, the difference of the substrate also had effect on the optimal pH (Magni et al., 1982).

Temperature was found to influence polysaccharides yield in present and previous studies (Qiao et al., 2009). The temperature optimal for NSP yield and RPR in this study was 26.9 °C different from that for proteolytic activity reported by Maddox and Hough (1970). This may be attributed the effects of temperature on the activity and excretion of yeast extracellular protease.

Phosphorus and potassium are the elements affect microbial growth and metabolism (Almagro et al., 2000). Phosphates regulate medium pH, while K^+ affects the activity of enzymes. Hien and Fleet (1983) and Inouhe, Sugo, Tohoyama, Joho, and Nevins (1997) found that the cell walls of yeast contained several enzymes, such as β-1,3-glucanase, β-1,6-glucanase, and mannanase. As lily NSP comprised glucose and mannose (Wozniewski et al., 1989), it is possible that lily NSP was hydrolyzed by β-glucanase systems in the culture medium secreted by yeast (Farkaš, Biely, & Bauer, 1973) and consequently influenced by KH_2PO_4 through affecting activities of β-glucanase systems.

5. Conclusions

In this study, we developed a new method for removing proteins from lily NSP with fermentation of *S. cerevisiae*, which was different from Sevag and TCA methods. Under the culture condition optimized using response surface analysis (60.0 g/L lily bulb powder, 1.50 g/L KH_2PO_4, pH4.8 and temperature 26.9 °C), the NSP yield and RPR reached 8.81 ± 0.18% and 91.71 ± 0.08%, respectively.

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