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Optimization of ultrasound-assisted compound enzymatic extraction and characterization of polysaccharides from blackcurrant

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ABSTRACT

In the present study, an efficient procedure for ultrasound-assisted compound enzymatic extraction of polysaccharides from blackcurrant fruits was investigated using response surface methodology (RSM). The Box–Behnken design was applied to optimize the effects of enzyme concentration (X_1), pH (X_2) and ultrasonic time (X_3). The statistical analysis indicated that the independent variables (X_1) and the quadratic terms (X_1^2 and X_3^2) had significant effects on the yield of blackcurrant polysaccharides (BCP). The optimal conditions were: enzyme concentration 1.575%, pH 5.3, and ultrasonic time 25.6 min. The experimental yield of BCP was 14.28 ± 0.06%, which was closely matched with the predicted yield of 14.31%. After preliminary purification, BCP I was obtained and characterized by GC, HPLC, and IR. BCP I comprised rhamnose, arabinose, xylose, mannose, glucose, and galactose in a molar ratio of 1.818:1.362:0.377:0.501:1.581:1.722 and its molecular weight was 8146 kDa. BCP I showed notable α-amylase inhibitory activity.

1. Introduction

Polysaccharides are polymeric carbohydrate structures, formed of repeating units joined together by glycosidic bonds (Shao, Deng, Shen, Fang, & Zhao, 2011). In recent years, polysaccharides from fruits and vegetables have become a focus for research attention, because such isolated polysaccharides have been found to play an important biomedical role due to their antioxidant (Guo et al., 2010), immunostimulatory (Sun & Liu, 2009) and antitumor (Zhou, Song, Feng, & Tan, 2011) effects.

The blackcurrant (*Ribes nigrum* L.), originating from Northern Asia and Europe, is a small berry which is rich in many health-beneficial substances, such as polysaccharides, organic acids, different vitamins, flavonoids, and anthocyanins (McDougall, Gordon, Brennan, & Stewart, 2005). Blackcurrants have been used for centuries in Chinese folk medicine with diuretic, diaphoretic, and febrifuge effects. Furthermore, they are also well known as base-materials for the production of wines, juices, and jams in China and Europe (Matsumoto et al., 2005). Blackcurrant has received considerable attention because of its nutritional and health protective values. Various beneficial effects of blackcurrant polysaccharides have been demonstrated including immunostimulatory, antitumor, antioxidant and anti-inflammatory activities (Tabart et al., 2012; Takata, Yamamoto, Yanai, Konno, & Okubo, 2005).

Ultrasonic-assisted extraction (UAE) is a fully reproducible food process, completed in a shorter time with high reproducibility, reduced processing cost, simplified manipulation and work-up when compared to conventional extraction techniques (Sun, Liu, Chen, Ye, & Yu, 2011). It is reported that extraction efficiency is greatly enhanced by ultrasonic treatments. Extraction by UAE was, with regards polysaccharides, non-destructive, and benefitted from a shorter extraction time. Enzyme-assisted extraction (EAE), which is considered as a mild, efficient, environmentally friendly method, has been proved to be effective in improving the yield of the target component (Li, Smith, & Hossain, 2006). Enzymes can

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effectively catalyze the degradation of cell walls, helping the release of polysaccharides contained within.

UAE and EAE have advantages over conventional extraction techniques and are widely used in the extraction of polysaccharides. Moreover, previous studies have shown that ultrasound-assisted enzymatic extraction (UAEE) has been used to isolate polysaccharides from *Lycium barbarum* (Liu et al., 2014), corn silk (Chen et al., 2014), and so on. However, little attention is devoted to extracting polysaccharides by the method of ultrasound-assisted compound enzymatic extraction (UACEE) which would probably be more effective (Chen, Li, Liu, Yang, & Li, 2012). To the best of our knowledge, this method has not been reported in extraction of BCP previously.

RSM is a collection of statistical and mathematical techniques that have been successfully used for developing, improving, and optimizing biochemical processes in recent years (Zhu, Nie, Liang, & Wang, 2013). RSM is an effective tool for optimizing process, when many factors and interactions affect desired response (Box & Wilson, 1951).

In the present study, the ultrasound-assisted compound enzymatic extraction (UACEE) of BCP was first investigated. RSM was designed to systematically analyze the effects of extraction parameters (enzyme concentration, pH, and ultrasonic time) on the yield of BCP. The preliminary characterization of BCP I was estimated by GC, HPLC and IR. In addition, the α-amylase inhibitory activity of BCP I was tested to determine its anti-hyperglycemic activity.

2. Materials and methods

2.1. Materials

Blackcurrant fruits were collected at the horticulture station of Northeast Agricultural University in Harbin (Harbin, China) and identified there by Prof. Yu. The fruits, at their fully mature stage, were washed and stored at −20 °C until used for further analysis. Before extraction, the fruits were gently defrosted and crushed in a JJ-2 tissue homogenization machine (Changzhou Guohua Electric Appliance Co., Ltd., Jiangsu Province, China). Pectinase (1000 U/mg) was obtained from Shanghai Jinsui Biotechnology Co., Ltd. (Shanghai, China). Papain (500 U/mg) was purchased from Beijing Obo Star Biotechnology Co., Ltd. (Beijing, China). All other chemicals and solvents were of analytical grade.

2.2. Extraction of polysaccharides

The blackcurrant fruits (5.00 g) and certain concentration (1–2%) of enzymes (pectinase, papain, compound enzyme comprised the papain and pectinase which ratio was 2:1) were put into a 500 mL beaker, then aqueous solutions at different pH (3.5–5.5) were added at different liquid to solid ratios (10:1–50:1 mL/g). The polysaccharides were extracted at 40 °C over various periods (15–55 min) in an ultrasonic cell disintegrator (JY92-2D, Ningbo Xinzhi Biological Technology Co., Ltd., Ningbo, China). After the extraction process, the extract was centrifuged at 3500 rpm for 20 min and the supernatant was filtered through a 0.45 μm microporous membrane (Shanghai Wanzi Shiye Co., Ltd., Shanghai, China) under vacuum. The filtrate was dissolved in deionized water, then set aside in a 100 mL flask for determining the yield of polysaccharides. The yield of total polysaccharide was measured by the phenol-sulfuric acid method using D-glucose as a standard. The percentage polysaccharide yield (%) was calculated as follows:

$$Y(\%) = \frac{c \times v}{w} \times 100\% \quad (1)$$

where c is the concentration of polysaccharide solution, v is the volume of polysaccharide solution, and w is the dried sample mass.

2.3. Single-factor experiments of UACEE

When blackcurrant fruits were processed simultaneously by ultrasonic wave and enzymes, the effects of concentration and ratio of compound enzymes, pH, liquid to solid ratio, and ultrasonic time were studied by a single-factor design as follows: one factor was changed while the other factors were kept constant in each experiment. The effect of each factor was evaluated by determining the yield of BCP.

2.4. Experimental design of RSM

2.4.1. Optimization of UACEE by RSM

Box–Behnken design (BBD) with three independent variables was used for the optimization of the extraction of BCP. Experiments were established based on a BBD with three factors at three levels and each independent variable was coded at three levels: −1, 0, and +1. Three parameters including concentration of compound enzymes (%), pH, and ultrasonic time (min) were chosen as key variables based on the results of single-factor experiments and designated as X_1, X_2, and X_3, respectively. Extraction yield (Y) was taken as the response of the design experiments. Table 1 lists the ranges of independent variables and their levels. The coding of the variables was undertaken using Eq. (2) (Prakash Maran & Manikandan, 2012):

$$X_i = \frac{x_i - x_0}{\Delta x} \quad (i = 1, 2, 3) \quad (2)$$

Table 1
Box–Behnken design and response values for the yield of BCP.

Number	X_1 (enzyme concentration, %)	X_2 (pH)	X_3 (ultrasonic time, min)	Yield of BCP (%)
1	−1 (1.0)	0 (4.5)	−1 (15)	11.42
2	0 (1.5)	0 (4.5)	0 (25)	14.15
3	0 (1.5)	1 (5.5)	1 (35)	13.06
4	−1 (1.0)	1 (5.5)	0 (25)	13.01
5	1 (2.0)	−1 (3.5)	0 (25)	12.75
6	1 (2.0)	1 (5.5)	0 (25)	13.69
7	0 (1.5)	0 (4.5)	0 (25)	14.22
8	0 (1.5)	0 (4.5)	0 (25)	14.52
9	1 (2.0)	0 (4.5)	1 (35)	12.43
10	0 (1.5)	0 (4.5)	0 (25)	14.09
11	−1 (1.0)	−1 (3.5)	0 (25)	12.30
12	0 (1.5)	0 (4.5)	0 (25)	14.06
13	0 (1.5)	−1 (3.5)	1 (35)	13.18
14	0 (1.5)	1 (5.5)	−1 (15)	12.82
15	0 (1.5)	−1 (3.5)	−1 (15)	12.97
16	−1 (1.0)	0 (4.5)	1 (35)	11.83
17	1 (2.0)	0 (4.5)	−1 (15)	12.18

where X_i and x_i are the dimensionless and actual values of the independent variable i, respectively, x_0 is the actual value in the center of the domain, and Δx is the increment of x_i corresponding to a variation of one unit of X_i.

The response function (Y) was partitioned into linear, quadratic, and interactive components:

$$Y = \beta_0 + \sum_{i=1}^{3}\beta_i X_i + \sum_{i=1}^{3}\beta_{ii}X_i^2 + \sum_{i=1}^{2}\sum_{i=1}^{3}\beta_{ij}X_iX_j \quad (3)$$

where Y is the dependent variable, β_0 is the constant coefficient, β_i is the linear coefficient (main effect), β_{ii} is the quadratic coefficient, and β_{ij} is the two-factor interaction coefficient.

2.4.2. Total percentage contributions of process variables

According to the regression coefficients obtained from an analysis of variance (ANOVA), the total percentage contribution (P_i) from each individual process variable was calculated. The following equation was used to find out the P_i for individual process variables as the reference (Khataee, Fathinia, Aber, & Zarei, 2010)

$$P_i = \frac{\beta_i}{\sum\beta_i^2} \quad (i \neq 0) \quad (4)$$

where β_i is the regression coefficient of each individual process variable.

2.4.3. Verification of the predicted optimized conditions

Triplicate experiments were performed under optimal conditions to determine the validity of the developed mathematical model. The average value of the experiments was compared to the predicted value of the developed model to ascertain the accuracy and suitability of the optimized conditions.

2.5. Isolation and structural characterization of BCP I

2.5.1. Extraction and purification of BCP I under optimal conditions

The BCP obtained under optimal conditions was precipitated with EtOH to a final concentration of 80% (v/v) and lyophilized. The crude polysaccharide solution (4 mg/mL) was loaded into a column (2.0 cm × 30 cm) with macroporous resin (D4006). Then, the column was eluted with deionized water at a flow rate of 1.00 mL/min. The obtained elute (1 mL/tube) was collected automatically and the polysaccharides in each tube were measured by phenol-sulfuric acid method. The collected fraction was freeze-dried after being dialyzed (3500 Da). The fraction was designated as BCP I for further structural characterization and bioactivity assay. The content of uronic acid was determined according to the carbazole-sulfuric acid method by using D-galacturonic acid as the standard (Kintner & Buren, 1982). The protein content was determined by the method using Coomassie Brilliant Blue G-250 and bovine serum albumin was used as the standard (Lott, Stephan, & Pritchard, 1983).

2.5.2. Determination of molecular weight

The molecular weight of BCP I was determined by high performance liquid chromatography (HPLC, LC-10AVP, Shimadzu Corporation, Japan), which was performed on a waters ultra-hydrogel linear column (7.8 mm × 30 cm) and detected by evaporative light scattering detector (ELSD) at 35 °C. The pressure was held constant at 1.4 MPa. Sample BCP I was dissolved in distilled water (2 mg/mL) and passed through a 0.45 μm filter, applied to the gel-filtration column, and eluted with distilled water at a flow rate of 1.0 mL/min. Standard dextrans with different molecular weights (T-70, T-110, T-500, T-2000) were passed through the column, and a standard curve was plotted according to the retention time and the logarithm of their respective molecular weights. The molecular weight of BCP I was calculated by comparison to the standard curve.

2.5.3. Analysis of monosaccharide composition

The monosaccharide compositions were analyzed by gas chromatography (GC, GC-2010, Shimadzu Corporation, Japan). Briefly, polysaccharides sample BCP I (30 mg) was dissolved in 2.0 mL of 2 mol/mL trifluoroacetic acid solution (TFA) and incubated for 3 h at 120 °C. After removing the residual TFA with ethanol under reduced pressure, the hydrolyzates and six standard sugars (xylose, galactose, mannose, glucose, arabinose, and rhamnose) were acetylated following a published protocol (Zhu et al., 2014). Each derivative was then analyzed by GC equipped with a flame ionization detector (FID) and an RTX-1701 silica capillary column. The column temperature increased from 180 °C to 220 °C at 5 °C/min and was held at 220 °C for 5 min, then elevated to 280 °C at 10 °C/min and finally held at 280 °C for 20 min. The temperature of detector and injector were set to 280 °C. High-purity nitrogen gas (N_2) was used as the carrier.

2.5.4. UV and IR spectroscopy

The UV spectrum of BCP I was recorded with a double beam UV spectrophotometer (TU-1901, Beijing Purkinje General Instrument Co., Ltd., Beijing, China). The IR spectrum of the BCP I was determined by using a Fourier transform infrared spectrophotometer (FTS135, BID-BAD Co., USA). The sample was ground with spectroscopic grade potassium bromide (KBr) powder and then pressed into 1 mm pellets for FT-IR determination over the wave number range 4000–400 cm^{-1}.

2.6. Determination of α-amylase inhibitory activity

The α-amylase inhibitory activity was measured according to Chen et al. (2013) with slight modification. One milliliter of 2 mg/mL soluble starch solution was added to 1 mL of BCP I solution at different concentrations (0.2, 0.4, 0.6, 0.80, 1.0, 2.0, 3.0, and 4.0 mg/mL) and mixed with 1 mL of 0.6 mg/mL α-amylase solution at 37 °C for 20 min. The reaction was stopped by the addition of 5.0 mL of DNS reagent. Then the mixture was incubated in a boiling water bath for 5 min, and cooled to room temperature. The absorbance was measured at 540 nm. Acarbose used as a positive control group. The α-amylase inhibitory activity was calculated as follows:

$$\mathrm{IR} = \frac{A_2 - (A_x - A_3)}{A_2 - A_1} \times 100\% \quad (5)$$

where A_2, A_1, A_x, and A_3 are defined as the absorbance of 100% enzyme activity (only that solvent with the enzyme), 0% enzyme activity (only that solvent without the enzyme), a test sample (with the enzyme), and a blank (a test sample without the enzyme), respectively.

3. Results and discussion

3.1. Single-factor tests of UACCE

3.1.1. Effect of different ratios of papain to pectinase on extraction yield of BCP

Extractions were carried out at different ratios of papain to pectinase while the other extraction variables were set as follows: liquid to solid ratio 10:1 (mL/g), pH 5.5, enzyme concentration 2%, ultrasonic time 25 min, and ultrasonic power 400 W. As shown in Fig. 1A, the yield of BCP increased with increasing ratio of papain to pectinase and reached its maximum yield (13.61 ± 0.15%) when the ratio of papain to pectinase was 2:1. As the ratio of papain to pectinase

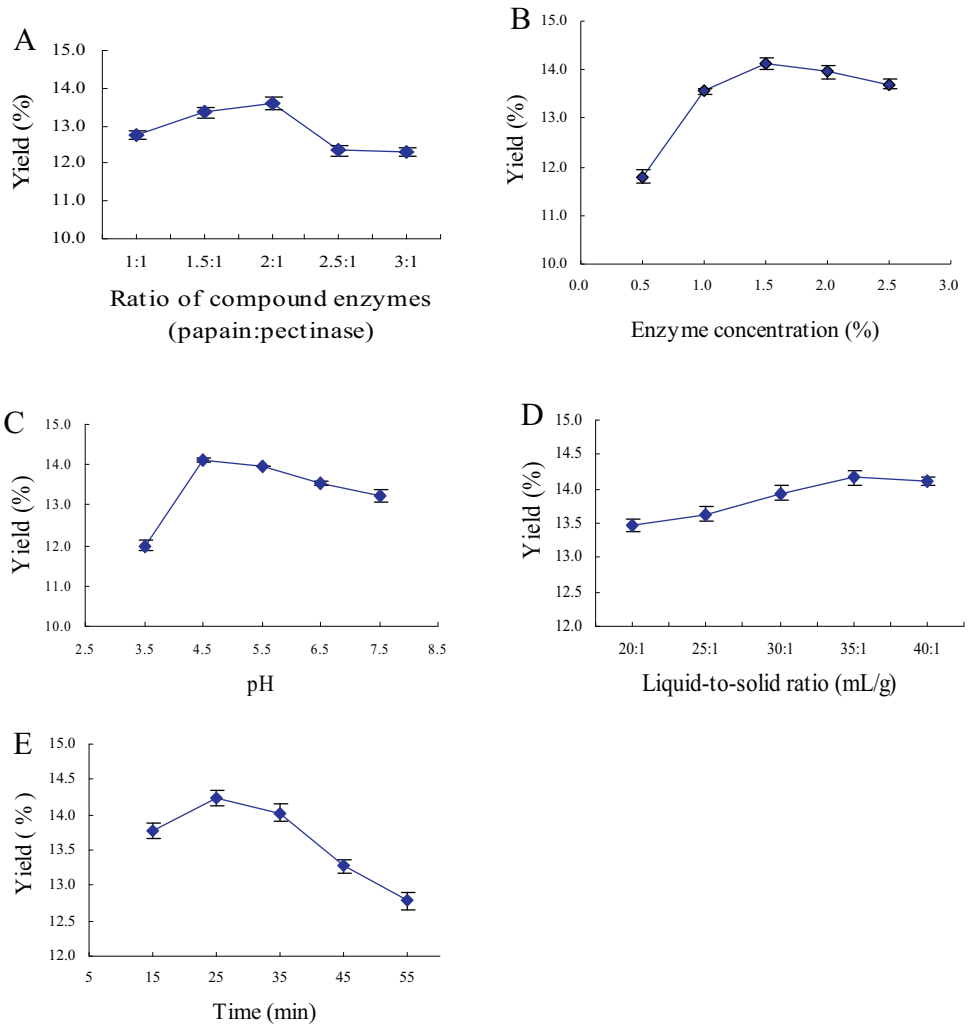

Fig. 1. Effects of different extraction parameters on the extraction yield of BCP (A: ratio of compound enzymes (papain:pectinase); B: enzyme concentration; C: pH; D: liquid-to-solid ratio; E: time).

continued increasing, the yield of BCP decreased a little and thereafter remained constant. However, the ratio of papain to pectinase had no significant influence ($P > 0.05$) on the yield of BCP. Therefore, enzyme ratio will not be considered when selecting factors in RSM.

3.1.2. *Effect of different concentrations of compound enzymes on extraction yield of BCP*

The effect of different concentrations of compound enzymes on the yield of BCP is shown in Fig. 1B for ratio of papain to pectinase of 2:1 (all other conditions fixed, as described above). The yield of BCP increased with increasing enzyme concentration until value of 1.5% was reached after which it began to decrease: the maximum extraction yield was 14.12 ± 0.12%. Hence, 1.5% was selected as the center point for further experiment.

3.1.3. *Effect of different pH on extraction yield of BCP*

The pH can affect enzyme activity since different enzymes have their own optimal pH (Yin, You, & Jiang, 2011). Extractions were carried out under different pH conditions at ratio of papain to pectinase of 2:1, enzyme concentration 1.5%, and all other extraction parameters constant. The effect of pH on the yield of BCP is shown in Fig. 1C: the yield of BCP increased with increasing pH, and the highest yield (14.18 ± 0.001%) was obtained when the pH was 4.5. Thereafter the yield began to decrease because enzyme was sensitive to pH. Therefore, pH 4.5 was considered to be optimal in the present experiment.

3.1.4. *Effect of different liquid to solid ratios on extraction yield of BCP*

The effect of different liquid to solid ratios on the extraction yield of BCP is shown in Fig. 1D, when the other three factors (ratio of papain to pectinase, enzyme concentration, and pH) were fixed at 2:1, 1.5%, and 4.5, respectively. The extraction yields of BCP increased from 13.48 ± 0.01% to 14.17 ± 0.03% as the ratios of liquid to solid increased from 20:1 to 35:1, then began to drop due to the reduction of substrate concentration: 35:1 (mL/g) was chosen as the optimal ratio. However, the effects of different ratios of liquid to solid on the extraction yield of BCP were not significant ($P > 0.05$) and therefore this factor was ignored in future experiments.

3.1.5. *Effect of different ultrasonic time on extraction yield of BCP*

Extraction time is another factor influencing the extraction efficiency and selectivity of the fluid (Ye & Jiang, 2011). The yield of BCP extracted over different time from 15 to 55 min is shown in Fig. 1E, when the other factors were as follows: ratio of papain to pectinase 2:1, ultrasonic power 400 W, enzyme concentration 1.5%, and pH 4.5. The results indicated that the maximum yield of BCP (14.23 ± 0.014%) was reached when ultrasonic time was 25 min, which began to decrease due to the degradation of polysaccharides

Table 2
Results of ANOVA of regression model for the extraction yield of BCP.

Source	Sum of squares	*DF*	Mean square	*F*-value	*P*-value
Model	12.62	9	1.40	16.10	0.0007**
X_1	0.78	1	0.78	8.90	0.0204*
X_2	0.24	1	0.24	2.73	0.1422
X_3	0.15	1	0.15	1.77	0.2253
X_1X_2	0.013	1	0.013	0.15	0.7083
X_1X_3	0.0064	1	0.0064	0.073	0.7941
X_2X_3	0.00023	1	0.00023	0.0026	0.9609
X_1^2	5.63	1	5.63	64.67	<0.0001**
X_2^2	0.055	1	0.055	0.63	0.4540
X_3^2	4.97	1	4.97	57.57	0.0001**
Residual	0.61	7	0.087		
Lack of fit	0.47	3	0.016	4.61	0.0896
Pure error	0.14	4	0.034		
Cor total	13.23	16			
C.V.%			2.25		
R^2			0.9539		
adj-R^2			0.8947		

* $P < 0.05$ significant.
** $P < 0.01$ extremely significant.

over longer ultrasonic time. Thus, the favored extraction time was 25 min.

According to the results of single-factor study, concentration of compound enzymes, pH, and ultrasonic time were adopted for RSM experiments.

3.2. Optimization of UACEE procedure by RSM

3.2.1. Statistical analysis and model fitting

The extraction yields of BCP under different conditions are listed in Table 1. The mathematical model describing the extraction yield of BCP (Y) as a function of the test independent variables over their selected ranges was given by Eq. (6):

$$Y = 14.208 + 0.31125X_1 - 1.1565X_1^2 - 1.0865X_3^2 \quad (6)$$

The results from the second-order response surface model in the form ANOVA are shown in Table 2. The statistical significances of the model equation were evaluated by *F*-test ANOVA. The significance of each coefficient was determined by *F*-values and *P*-values which demonstrated that the corresponding variables would be more significant if their *F*-value increased and their *P*-value decreased. Thus, the *F*-value ($F = 16.10$) and *P*-value ($P < 0.001$) implied that the model was significant. Significance of the model was also determined by lack-of-fit test. The lack-of-fit *F*-value of 4.61 and the associated *P*-value of 0.0896 were insignificant which meant that the model was sufficiently accurate for predicting the relevant response.

The goodness-of-fit of the model was also checked by multiple correlation coefficient (R^2) and adjusted determination coefficient (R^2_{adj}). The quadratic regression model showed that R^2 and R^2_{adj} were 0.9539 and 0.8947, which indicated that 95.39% of the variations could be illustrated by the fitted model and 89.47% of the total variations were explained by the model. If there are many terms in the models and the sample size is not large enough, R^2_{adj} may be noticeably smaller than R^2 (Yetilmezsoy, Demirel, & Vanderbei, 2009).

A relatively low value of *C.V.* (the coefficient of variation) indicates a better reliability of the experimental values (Li, Wang, Wang, Walid, & Zhang, 2012). In this study, the low *C.V.%* (2.25) indicated a high degree of precision in, and good reliability of, the experimental values.

It can be seen from Table 2 that the linear coefficients (X_1) and a quadratic term coefficient (X_1^2, X_3^2) were significant, with very small *P*-values ($P < 0.05$). The other term coefficients were not significant

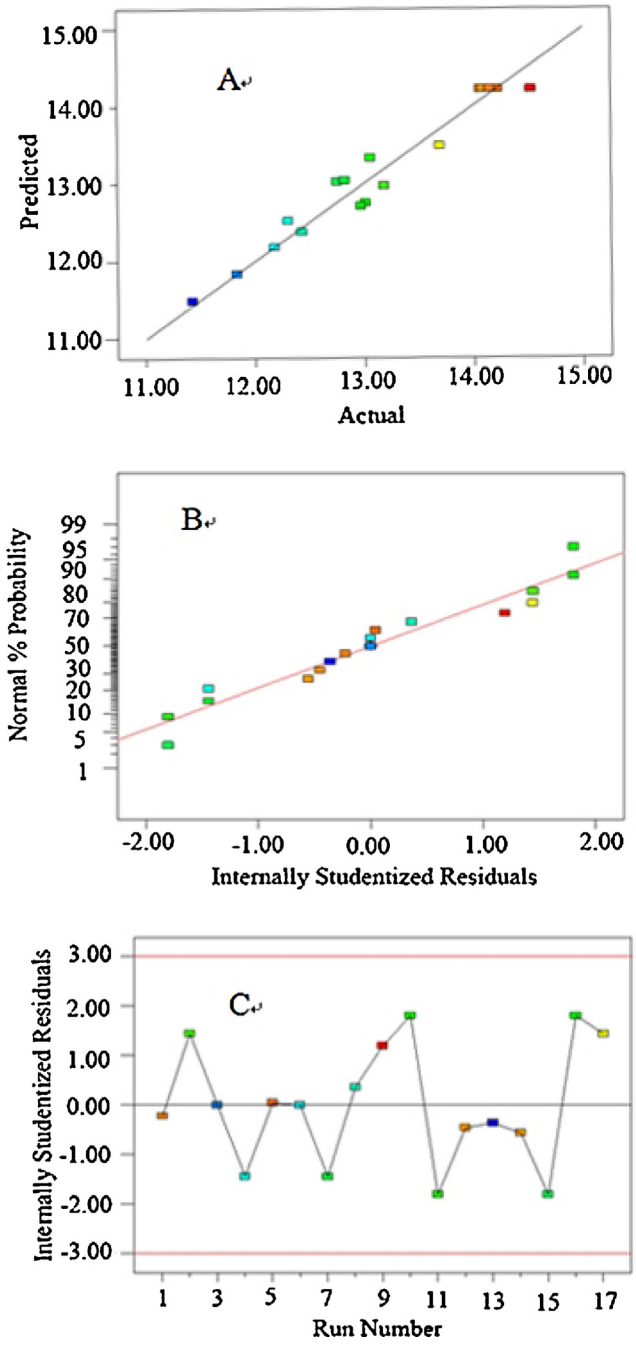

Fig. 2. Diagnostic plots for the model adequacy. (A: plot of predicted and actual values; B: the normal% probability plot; C: plot of internally studentized residuals *versus* experimental runs).

($P > 0.05$). Therefore, X_1, X_1^2, and X_3^2 were important factors in the UACEE process of BCP.

3.2.2. Diagnostics of model adequacy

Model adequacy checking is performed to determine whether or not the approximating model would give poor or misleading results (Prakash Maran, Manikandan, Thirugnanasambandham, Vigna Nivetha, & Dinesh, 2013). Fig. 2A shows the relationship between the predicted and actual values which were used to evaluate the suitability of the model. The data lay reasonably close to a straight line, which indicated adequate agreement between real, and predicted, data.

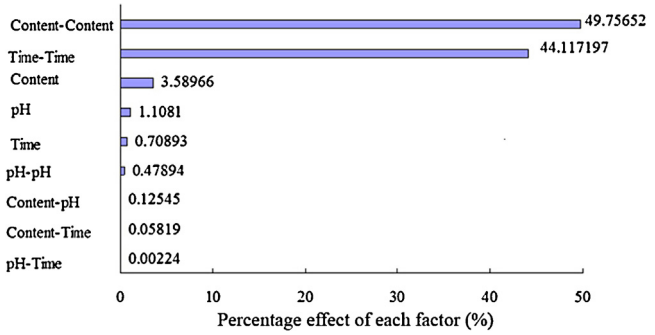

Fig. 3. Detailed schematic diagram showing the percentage contributions of individual process variables.

Fig. 2B shows that the normal percentage probability plot of residuals for the response was normally distributed which could confirm that the data were normally distributed. The data also lay reasonably close to a straight line and showed no deviation in variance.

Fig. 2C was used to analyze the experimental data to ascertain the most satisfactory fit of the developed models by constructing internally studentized residuals *versus* experimental runs. The result showed that all data points lay within acceptable limits.

All of the plots (Fig. 2) were satisfactory, so it was concluded that the empirical model was adequate with regards its description of the BCP extraction yield by response surface method.

3.2.3. Percentage contribution of process variables

The percentage contributions (P_i) for each individual process variables can help to visualize their principal effect on the extraction of polysaccharide and impart more significant information for interpretation of the results. Fig. 3 shows the percentage contributions of process variables to the response in which the quadratic effect of concentration of compound enzymes and ultrasonic time (X_1^2, X_3^2; 49.76%, 44.12%) produced the main effect on extraction of polysaccharide from blackcurrant. Hence, P_i proved that, the quadratic independent variables had a direct relationship with the dependent variable.

3.2.4. Interpretation of response surface model

The three-dimensional response surface provided a visual interpretation of interactions between two tested variables as well as the relationships between responses and experimental levels of each variable. It was also used to locate the optimum conditions.

As shown in Fig. 4A, the extraction yield of BCP was given as a function of enzyme concentration (X_1) and pH (X_2) when the extraction time was fixed at 25 min. When pH (X_2) was kept at lower levels, the yield initially increased and then decreased with increasing enzyme concentration (X_1) thereafter. Examination of the plots in Fig. 4A showed that there were optimal concentration levels (1.55–1.60%) for BCP. This was due to the quadratic effect of enzyme concentration (X_1) as supported by the results in Table 2 (Bachir bey, Meziant, Benchikh, & Louaileche, 2014). Although the quadratic effect of enzyme concentration (X_1) on the yield of BCP was significant, it had a negative effect on polysaccharide yield for its negative cross-product coefficient. The singular effect of enzyme concentration on polysaccharide yield was significant and positive in its experimental range, as it had a positively valued coefficient.

Fig. 4B shows the mutual interactions between enzyme concentration (X_1) and ultrasonic time (X_3) on the yield of BCP for fixed pH 4.5. When the enzyme concentration (X_1) was kept at a lower level, the yield initially increased and then decreased with increasing ultrasonic time (X_3). This phenomenon could be explained that, the cells will be completely cracked in the earlier period of extraction. However, longer extraction time with ultrasound treatment might induce the degradation of polysaccharide (Liu et al., 2014) and decreased the yield. The results are in agreement with Prakash Maran et al. (2013). Examination of the plots in Fig. 4B also showed that there was optimal ultrasonic time (25–26 min) for BCP yield. Ultrasonic time (X_3) demonstrated quadratic effects on the extraction yields but it had a negative effect on polysaccharide yield.

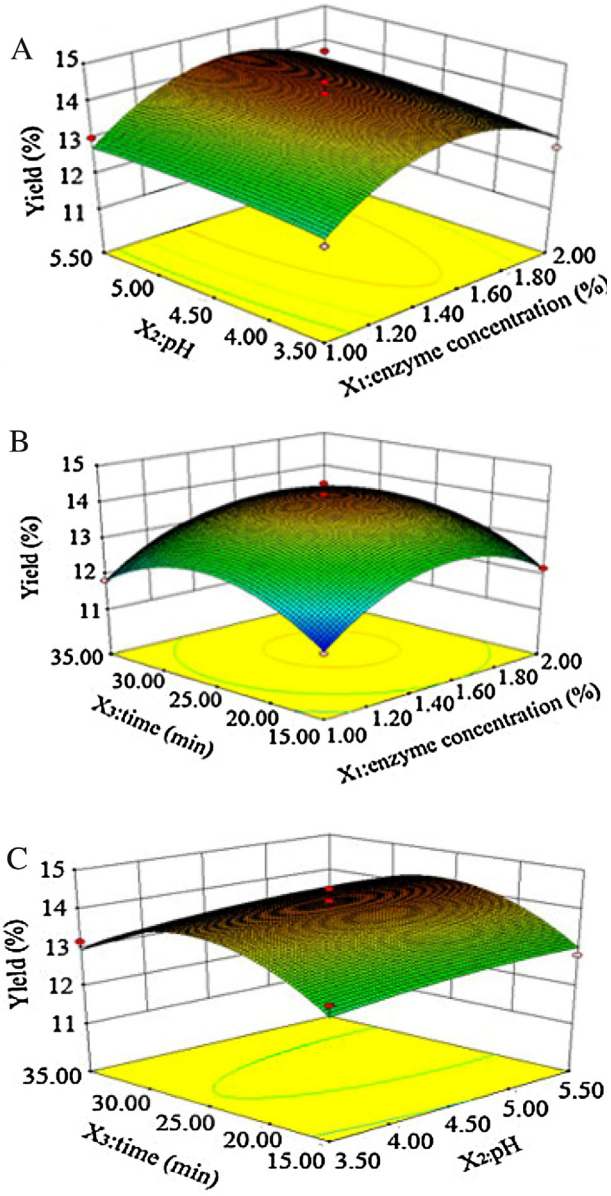

Fig. 4. Response surface plots showing the effect of enzyme concentration (X_1), pH (X_2) and ultrasonic time (X_3) on the yield of BCP.

Fig. 4C shows the relationship between the yield of BCP and two variables, pH (X_2) and ultrasonic time (X_3), for a fixed enzyme concentration of 1.5%. The variations of yields were insignificant with increased pH (X_2) and the mutual interaction between pH and ultrasonic time was also not significant ($P > 0.05$).

According to Fig. 4, the investigated extraction parameters for obtaining the maximum extraction yield of BCP were respectively calculated by the software Design Expert and the optimal UACEE conditions of BCP were as follows: enzyme concentration 1.575%, pH 5.3, and ultrasonic time 25.6 min. The predicted value of the maximum response was 14.31%.

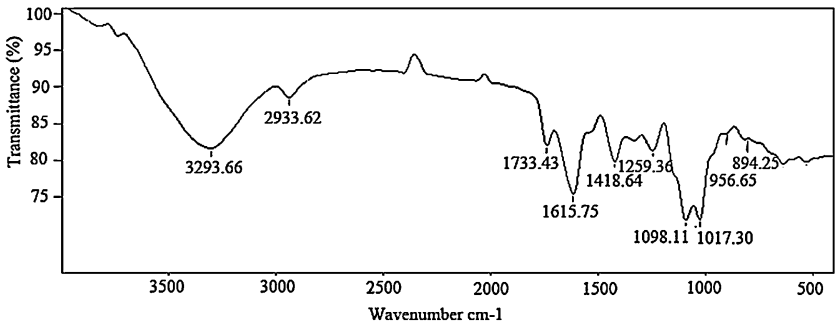

Fig. 5. FT-IR spectrum of BCP I.

3.2.5. Verification of predictive model

The optimum conditions obtained by RSM were used to validate the predictive model of BCP extraction: because the optimal values of enzyme concentration, pH, and ultrasonic time were difficult to control during actual extraction experiments, they were carried out with slight modifications and enzyme concentration 1.6%, pH 5.3, and ultrasonic time 26 min were used. Under these conditions, the experimental yield of BCP was 14.28 ± 0.06%, which closely agreed with the predicted value (14.31%). The results showed that there was no significant difference between experimental and predicted values, suggesting that the response model was adequate for reflecting the expected optimization and that the model could identify operating conditions for selective extraction of BCP.

3.3. Isolation and structural characterization of BCP I

3.3.1. Preliminary purification of BCP I

The content of uronic acid in BCP I was determined to be 4.36%. The yields of protein in BCP and BCP I were 5.01 and 0.07 μg/mL, respectively, illustrating that the rate of protein removal reached 98.6%. BCP I had no absorption at 260, 280, or 520 nm in the UV spectrum, indicating the absence of protein, nucleic acid, and pigment, respectively. Macroporous resin D4006 showed a significant purifying effect with regards the removal of protein and pigment.

3.3.2. Molecular weight and composition of polysaccharides

The HPLC system was used to determine the molecular weight of BCP I. The equation of the standard curve was: $\lg M_w = -1.8063t + 19.703$ (where M_w represents the molecular weight and t the retention time) with a correlation coefficient of 0.9975. The results showed that the retention time for BCP I was 7.08 min. According to the aforementioned regression equation, the weight-average molecular weight of BCP I was 8146 kDa.

The monosaccharide composition of BCP I was analyzed by GC. The monosaccharide composition was identified by comparing the retention time against standards. Composition analysis indicated that the BCP I was composed of rhamnose, arabinose, xylose, mannose, glucose, and galactose in a molar ratio of 1.818:1.362:0.377:0.501:1.581:1.722, respectively.

3.3.3. IR spectra analysis of BCP I

The IR spectrum was shown in Fig. 5. The bands around 3301 and 2937 cm^{-1} were attributed to the —OH stretching, and C—H stretching, vibrations, respectively. The peak at around 1259 cm^{-1} was asymmetrical carbonyl stretching. Two stretching peaks at about 1737 and 1614 cm^{-1} suggested the presence of C=O and COO^-. In addition, the absorption at around 1421 cm^{-1} in the IR spectrum indicated the presence of uronic acids. Two stretching peaks at about 1098 and 1017 cm^{-1} suggested the presence of C—O bonds (Yang et al., 2014). Moreover, the characteristic absorption bands at 894.25 and 956.65 cm^{-1} indicated that both α- and β-configurations of sugar units existed in BCP I. The spectra of BCP I and the polysaccharide from *Nephelium lappaceum* L. fruit peel (Prakash Maran & Priya, 2014) were rather similar. Overall, these results showed that BCP I exhibited the typical absorption peaks of a polysaccharide.

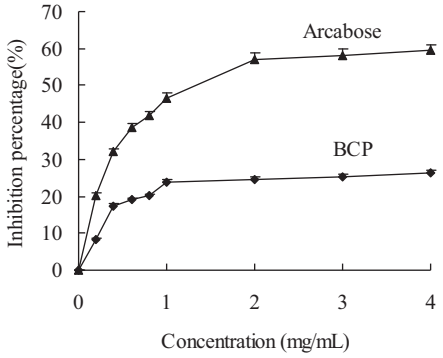

Fig. 6. Inhibition effects of BCP I and acarbose on α-amylase activity.

3.4. The inhibition effect of α-amylase activities by BCP I

The α-amylase inhibitors could retard the starch digestion rate of food and restrain the increase of post-meal blood sugar. Thus, α-amylase inhibitors have been research hotspot as oral hypoglycemic agents for diabetics (Wang, Yang, & Wei, 2010).

As shown in Fig. 6, BCP I and acarbose exhibited certain inhibitory effects. As the concentration (in the range of 0–4 mg/mL) increased, the inhibition effects increased accordingly. The inhibitory effect of BCP I was 26.44 ± 0.12% at a concentration of 4 mg/mL, which was weaker than the positive control arcabose. This might have been due to the different structure and molecular weight of arcabose compared to BCP I. It is reported that the unsaturated cyclohexene ring and the glycosidic nitrogen linkage were essential structural features for inhibition of arcabose for α-amylase (Kim et al., 1999). However, BCP I might have different structural features. Besides, the molecular weight of BCP I was relatively large compared to that of arcabose which might affect its combination with the active sites of α-amylase and the further formation of enzyme–inhibitor complexes (Bompard-Gilles, Rousseau, Rougé, & Payan, 1996).

4. Conclusion

Based on the single-factor experiments, RSM was used to estimate and optimize the experimental variables including enzyme concentration, pH, and ultrasonic time. Under a modified condition (enzyme concentration 1.6%, pH 5.3, and ultrasonic time 26 min), the experimental yield of BCP was 14.28 ± 0.06%, which closely

matched the predicted value (14.31%). Model summary statistics showed that, the developed model was adequate and precise when compared with experimental data. The BCP I was composed of rhamnose, arabinose, xylose, mannose, glucose, and galactose in a molar ratio of 1.818:1.362:0.377:0.501:1.581:1.722 and its molecular weight was 8146 kDa. The α-amylase inhibition effect of BCP I reached 26.44 ± 0.12% at a concentration of 4.0 mg/mL. Further studies on the precise chemical structures and biological functions of BCP I are underway.

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